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## A STUDY OF HYPOXIA INDUCIBLE FACTOR AND RELATED GENES IN DISEASE IN MAN

by

## KAREN LYNN SØNDERGAARD

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

# DOCTOR OF PHILOSOPHY

Plymouth Postgraduate Medical School Department of Molecular Medicine

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Mom og Far

with love

"I'll be home next year..."

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#### Karen Lynn SØNDERGAARD

#### A Study Of Hypoxia Inducible Factor And Related Genes In Disease In Man

#### ABSTRACT

In this study, the mRNA and protein levels of hypoxia inducible factor 1 (HIF-1 $\alpha$ ), and a number of genes regulated by hypoxia (VEGF, GLUT-1, p53), were determined in four breast carcinoma cell lines, peripheral blood mononuclear cells (PBMCs) of patients with breast cancer and Type 1 diabetes (T1DM), and in human breast and brain tumour tissue. Breast carcinoma cells and PMBCs from both patients and normal controls were exposed to hypoxia  $(\leq 1\% O_2)$  and/or high glucose. Both up-regulated and down-regulated HIF-1  $\alpha$ , GLUT-1 and p53 mRNA expression was observed in the breast carcinoma cell lines exposed to hypoxia and/or high glucose, and in controls for osmolarity, confirming that hypoxic regulation of HIF-1α, p53 and possibly GLUT-1 occurs post-transcriptionally. Conversely, up-regulation of HIF- $1\alpha$  and GLUT-1 mRNA was observed in patients with T1DM exposed to high glucose. The GLUT-1 mRNA up-regulation observed in patients without complications differed significantly from normal controls, where up to a 2 fold increase in expression was observed over that of patients with complications. This may indicate that the expression and function of glucose transporters differs in these patients, potentially leading to fewer complications. Investigation of breast and glial cell tumour tissue demonstrated that both HIF-1 $\alpha$  and GLUT-I mRNA expression levels increase with disease progression, indicating that up-regulation of HIF-1a is partly at the transcriptional level (Søndergaard et al, 2002). Follow-up survival studies in all patients with glial cell tumours showed that HIF-1 $\alpha$  protein expression is a significant prognostic factor in cumulative overall survival. An additional investigation of p53 or p73 polymorphisms in the development of carcinoma of the breast did not find that they were significant risk factors in the development of the disease in the British Caucasoid population. Further studies are required using larger sample populations investigating HIF-1 $\alpha$ protein to determine the precise role of HIF-1 in the response to hypoxia and angiogenesis in disease in man.

- iv -

## **TABLE OF CONTENTS**

Dedication	ii
Copyright	iii
Abstract	iv
Table of Contents	v
List of Tables	xviii
List of Figures	xxiii
Abbreviations	xxx
Units of Measurement	xxxiv
Acknowledgement	xxxv
Author's Declaration	xxxvi
Conferences	xxxvii
Publications	xxxviii
CHAPTER 1. Introduction	1
1.0 Introduction	2
1.01 Knudson Hypothesis	2
1.02 Multistage Model	3
1.1 Proto-Oncogenes and Oncogenes	4
1.2 Tumour Suppressor Genes	7
1.2.1 Genomic Imprinting	7
1.2.2 p53	9
1.2.2.1 The p53 Gene Family	10
1.2.2.2 p73	12
1.2.2.3 p63	15

1.2.2.4 p53 and Human Papilloma Virus (HPV) 18

1.2.2.5 p53 and p73	19
1.2.2.6 p53 and MDM-2	21
1.2.3 Apoptosis	21
1.3 Tumour Angiogenesis	24
1.3.1 Angiogenic Process	25
1.3.2 Regulators of Angiogenesis	26
1.3.3 Promoters of Angiogenesis	29
1.3.3.1 Vascular Endothelial Growth Factor	29
1.3.3.1.1 VEGF Receptor Family	32
1.3.3.1.2 VEGF Induction and Expression	34
1.3.3.1.3 VEGF and Angiogenesis	35
1.3.4 Inhibitors of Angiogenesis	36
1.4 Tumour Hypoxia	37
1.4.1 Hypoxia Inducible Factor-1 (HIF-1)	39
1.4.1.1 Structure of HIF-1	39
1.4.1.2 HIF-1 Transcriptional Activity	42
1.4.1.3 Mediators of HIF-1 Transcriptional Activity	43
1.4.1.4 HIF-1a Degradation	46
1.4.1.5 HIF Splice Variants and Related Proteins	49
1.5 HIF-1 and Human Disease	51
1.5.1 Cancer	51
1.5.1.1 Breast Cancer	53
1.5.1.1.1 Risk Factors	53
1.5.1.1.2 Genetic Factors	55
1.5.1.1.3 Classification	56
1.5.1.1.4 Staging	59

	1.5.2.3.9 Vascular Endothelial Growth Factor (VEGF)	91
	1.5.2.3.10 Retinopathy	92
	1.5.2.3.11 Nephropathy	94
	1.5.2.3.12 Neuropathy	96
	1.5.2.4 Glucose Transporter Gene Family	97
	1.5.2.4.1 Glucose Transporter 1 (GLUT-1)	98
	1.5.2.4.1.2 Glucose Metabolism	100
	1.5.2.4.1.3 Glucose Transport	102
	1.5.2.4.1.4 Glucose Transport and Cancer	102
	1.5.2.4.1.5 Glucose Transport and Hypoxia	103
	1.5.2.4.1.6 Neurons	105
1.6	HIF-1 Interactions	106
	1.6.1 Tumour Suppressor: p53	106
	1.6.2 Angiogenic Factor: VEGF	107
	1.6.3 Metabolite: GLUT-1	10 <b>8</b>
	1.6.4 Vasoactive Agent: Nitric Oxide	109
1.7	Aims of the Project	112
СН	APTER 2. Materials and Methods	114
2.1	Subjects	115
	2.1.1 Patients with Breast Cancer	115
	2.1.2 Patients with Type-1 Diabetes Mellitus (T1DM)	115
	2.1.3 Controls Subjects	118
	2.1.3.1 Normal Controls (NC)	118
	2.1.3.2 Diabetic Controls (DC)	118

2.2	Tumour Samples	119
	2.2.1 Breast Cancer	119
	2.2.2 Glioblastoma Multiforme	119
2.3	Materials	122
	2.3.1 Water	122
	2.3.2 Reagents	122
	2.3.2.1 Specialised Reagents and Materials	123
2.4	Autoclaving	125
2.5	Cell Culture	126
	2.5.1 Established Cell Lines	126
	2.5.2 Characteristics of Human Breast Carcinoma Cell Lines	129
	2.5.3 Peripheral Blood Mononuclear Blood Cells (PBMCs)	131
	2.5.4 Cell Passage	131
	2.5.5 Cell Counting	132
	2.5.6 Cryopreservation	132
	2.5.7 Cell Resuscitation	133
	2.5.8 Experimental Culture Conditions	133
	2.5.8.1 High Glucose	133
	2.5.8.2 Osmolarity	134
	2.5.8.2.1 Mannitol	134
	2.5.8.2.2 L-Glucose	134
	2.5.8.3 Hypoxic Exposure	135
	2.5.8.3.1 Cobalt Chloride	135
	2.5.8.3.2 CO <sub>2</sub> /O <sub>2</sub> Incubator	135
	2.5.8.3.3 Fyrite® Gas Analyser	136

2.6	Deox	yribonucleic Acid (DNA) Methodologies	137
	2.6.1	Extraction of Total DNA	137
	2.6.2	Polymerase Chain Reaction (PCR)	138
	2.6.3	Optimisation of PCR	138
	2.6.4	Custom Oligonucleotides (Amplimers)	139
	2.6.5	PCR Reactions	140
	2.6.6	Nested PCR	140
		2.6.6.1 Restriction Enzyme Digestion of PCR Products	141
	2.6.7	Agarose Gel Electrophoresis	145
	2.6.8	Purification of PCR Fragments	146
		2.6.8.1 Glass Wool	146
		2.6.8.2 Quick Precip <sup>™</sup> (Advanced Biotech Corp., USA)	146
		2.6.8.3 Wizard <sup>™</sup> PCR Preps DNA Purification System (Promega, Southampton, UK)	147
		2.6.8.4 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) From Whole Blood	148
2.7	Plasm	nid DNA Methodologies	149
	2.7.1	pcDNA3 Plasmid (Invitrogen Life Technologies, Paisley, UK)	149
	2.7.2	Preparation of Competent Cells	152
		2.7.2.1 JM109	152
		2.7.2.2 MC1061/P3	153
	2.7.3	Transformation of Competent Cells	154
		2.7.3.1 JM109	154
		2.7.3.2 MC1061/P3	155
	2.7.4	Isolation of Plasmid DNA	155
	2.7.5	Extraction of Plasmid DNA	156
		2.7.5.1 Small-Scale Preparation	156

2.7.5.2 Large-Scale Preparation	157
2.7.6 Purification of Plasmid DNA	157
2.7.7 Restriction Endonuclease Mapping of Inserts	158
2.7.8 Transfection of Saos-2 Cells	159
2.7.8.1 pcDNA3 (containing p53 Pro and p53 Arg DNA Inserts)	159
2.7.8.2 pCH110 Eukaryotic Assay Vector (Amersham Pharmacia Biotech, Little Chalfont, UK)	160
2.7.9 Selection of Transfected Saos-2 Cells	163
2.7.10 Storage of Plasmid Cultures	163
2.7.10.1 Short Term Storage of Bacteria	163
2.7.10.2 Long Term Storage of Bacteria	164
2.7.11 Recovery of Bacteria From Long Term Storage	164
2.8 Labelling of DNA	165
2.8.1 Radiolabelling	165
2.8.1.1 Random Primed Labelling of DNA	165
2.8.1.2 5' End Labelling of DNA	165
2.8.1.2.1 Removal of Unincorporated Radiolabelled Nucleotides	166
2.8.1.2.2 Labelling Efficiency	167
2.8.2 Digoxigenin (DIG) labelling	168
2.9 Sequencing of PCR products	170
2.9.1 Sequenase PCR Product Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, UK)	170
2.9.1.2 Enzymatic Pre-Treatment of PCR Products	170
2.9.1.3 Annealing	170
2.9.1.4 Termination Reaction	171
2.9.1.5 Denaturing Gel Electrophoresis for Manual Sequencing	172

2.9.2 Automated (ABI Prism 377 Automated Sequencer, Perkin Elmer, Chesire, UK)	174
2.10 Ribonucleic Acid (RNA) Methodologies	174
2.10.1 Ribonuclease Free Environment	174
2.10.2 Isolation of RNA	175
2.10.2.1 RNeasy <sup>TM</sup> Total RNA Purification Kit (QIAGEN Ltd., Crawley, UK)	175
2.10.2.2 RNA STAT-60 <sup>TM</sup> Total RNA Isolation Reagent (Biogenesis, Poole, UK)	176
2.10.3 Quantitation of DNA and RNA	1 <b>77</b>
2.10.4 Reverse Transcription of Total RNA	1 <b>77</b>
2.10.5 Northern Blotting	1 <b>78</b>
2.10.5.1 Separation of Total RNA by Agarose Formaldehyde Gel Electrophoresis	178
2.10.5.2 Transfer	179
2.10.5.3 Radiolabelled Probes	180
2.10.5.4 Hybridisation	180
2.10.5.4.1 Hybridisation Buffer (Sambrook et al, 1989)	1 <b>8</b> 0
2.10.5.4.2 Rapid-Hyb Buffer (Amersham Pharmacia Biotech, Little Chalfont, UK)	181
2.10.5.5 Autoradiography	182
2.10.5.6 Membrane Stripping	182
2.10.6 Ribonuclease Protection Assay (RPA)	183
2.10.6.1 RPA Probes	183
2.10.6.2 Transcription	187
2.10.6.3 Hybridisation	188
2.10.6.4 RNase Digestion	188
2.10.6.5 Denaturing Polyacrylamide Gel Electrophoresis	189

		2.10.6.6	Gel Purification of Probe	190
		2.10.6.7	Quantitation of mRNA Gene Expression	191
2.11	Proteir	n Methode	ologies	192
	2.11.1	Lactate A	Assay	192
		2.11.1.1	Deproteinisation	192
		2.11.1.2	Measurement of Lactic Acid	193
		2.11.1.3	Quantitation of Lactate	193
	2.11.2	Quantita	tion of Protein	195
	2.11.3	Electrop	horetic Mobility Shift Assay (EMSA)	197
		2.11.3.1	Preparation of Nuclear Extract	1 <b>97</b>
			2.11.3.1.1 Dignam, Lebovitz and Roeder, 1983	1 <b>98</b>
			2.11.3.1.2 Semenza and Wang, 1992	198
			2.11.3.1.2.1 Preparation of Dialysis Tubing	200
		2.11.3.2	Oligonucleotide Probe Labelling	200
		2.11.3.3	Binding Reaction	201
		2.11.3.4	Non-Denaturing Gel Electrophoresis	201
		2.11.3.5	Competition and Supershift Assays	203
	2.11.4	Western ]	Blotting	203
		2.11.4.1	Preparation of Protein	203
			2.11.4.1.1 Cytoplasmic Protein	203
			2.11.4.1.2 Whole Cell Lysate	204
		2.11.4.2	SDS-Polyacrylamide Gel Electrophoresis	204
		2.11.4.3	Transfer	207
		2.11.4.4	Immunodetection	208
		2.11.4.5	Membrane Stripping	210
		2.11.4.6	Gel Staining	210

2.11.5 Immunocytochemistry	211
2.11.5.1 Hypoxia Inducible Factor-1a (HIF-1a)	211
2.11.5.2 Vascular Endothelial Growth Factor (VEGF)	212
2.11.5.3 p53	213
2.12 Statistical Analysis	214
2.12.1 Allele and Genotype Frequencies	214
2.12.2 Hardy-Weinberg Equilibrium	214
2.12.3 Odds Ratio	215
2.12.4 Association Analysis	215
2.12.5 Survival Analysis	215
2.12.6 Nottingham Prognostic Index (NPI)	216
CHAPTER 3. HIF-1α, GLUT-1 and p53 Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose	217
3.0 HIF-1α, GLUT-1 and p53 Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose	218
3.1 Northern Analysis of HIF-1α, VEGF, p53 and β-Actin mRNA Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia	221
3.2 HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia	228
3.3 Basal levels of HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell Lines Cultured Under Normoxia	236
3.4 HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell Lines Exposed To Short-Term High Glucose (3 day culture)	239

- 3.5 HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell243Lines Conditioned To Long-Term High Glucose and Exposed To Hypoxia243
- 3.6 HIF-1α mRNA Expression In Breast Carcinoma Cell Lines Exposed To25324 Hours Hypoxia and Subsequently Normoxia253
- 3.7 HIF-1α mRNA Expression In Breast Carcinoma Cell Lines Exposed To257Hypoxia and Hypoglycaemia257

3.8	Osmolarity and High Glucose Controls	260
3.9	Measurement of Lactic Acid	262
3.10	HIF-1 DNA Binding Activity In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose	266
3.11	HIF-1 Competition Assays	269
3.12	Comparison of Methodologies for Harvesting Nuclear Protein Extracts	272
3.13	HIF-1a Protein Expression In Breast Carcinoma Cell Lines Exposed to Hypoxia	274
3.14	GLUT-1 Protein Expression in Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose	278
3.15	Conclusion	282

CHAPTER 4. Expression of HIF-1 $\alpha$ and GLUT-1 in PBMCs Isolated From Patients with T1DM and Exposed to High Glucose		
4.0	Expression of HIF-1 $\alpha$ and GLUT-1 in PBMCs Isolated From Patients With T1DM and Exposed to High Glucose	286
4.1	Clinical Characteristics	287
4.2	HIF-1 $\alpha$ and GLUT-1 mRNA Expression	290
4.3	Basal Levels of mRNA Expression	304
4.4	Osmolarity and High Glucose Controls	307
4.5	Measurement of Lactic Acid	309
4.6	HIF-1 DNA Binding Activity	311
4.7	HIF-1a and GLUT-1 Protein Expression	315
4.8	Conclusion	319

CHAPTER 5. Expression of HIF-1α and GLUT-1 in Tumours of Patients with Breast Cancer	321
5.0 Expression of HIF-1α and GLUT-1 in Tumours of Patients with Breast Cancer	322
5.1 Clinical Characteristics	322
5.2 HIF-1a and GLUT-1 mRNA Expression	325
5.3 HIF-1a Protein Expression	331
5.4 Survival	334
5.5 Conclusion	343
CHAPTER 6. Expression of HIF-1 $\alpha$ , VEGF and p53 in Glial Cell Tumours	344
6.0 Expression of HIF-1 $\alpha$ , VEGF and p53 in Glial Cell Tumours	345
6.1 Clinical Characteristics	346
6.2 HIF-1α and VEGF mRNA Expression	348
6.3 HIF-1a Protein Expression	358
6.4 VEGF Protein Expression	361
6.5 p53 Protein Expression and Correlation with HIF-1 $\alpha$	363
6.6 Survival	365
6.7 Conclusion	380
CHAPTER 7. Polymorphisms in the p53 Family of Tumour Suppressor Genes and Susceptibility to Breast Cancer	381
7.0 Polymorphisms in the p53 Family of Tumour Suppressor Genes and Susceptibility to Breast Cancer	382
7.1 Clinical Characteristics	382
7.2 Genotyping of The p73 Dinucleotide Polymorphism	385
7.3 Genotyping of The p53 Codon 72 Polymorphism	393
7.4 p73 Genotype and p53 Isoform	400

#### LIST OF TABLES

۰.

## Page

Table 1.1.	The p53 tumour suppressor gene family	11
Table 1.2.	Endogenous positive and negative regulators of angiogenesis	28
Table 1.3.	Functional categorisation of target genes transactivated by HIF-1	52
Table 1.4.	Established and probable risk factors for breast cancer	54
Table 1.5.	Tumour Node Metastases (TNM) Classification of Breast Tumours	60
Table 1.6.	Correlation of the International Union Against Cancer (UICC) (1997) and TNM classifications of breast tumours with survival rates.	61
Table 1.7.	Recommendations of the World Health Organisation (WHO) for the grading of gliomas (Kliehues et al, 2001)	71
Table 1.8.	Pathogenic pathways of glucose toxicity	82
Table 1.9.	Characteristics of the extended facilitated glucose transporter family	<del>9</del> 9
Table 2.1.	Clinical characteristics of patients with breast cancer	116
Table 2.2.	Clinical characteristics of patients with T1DM and normal controls	117
Table 2.3.	Frequencies of the clinicopathological characteristics of patients with breast cancer and their tumours	120
Table 2.4.	Clinical characteristics of normal controls and patients with glioblastoma accessed in this study	121
Table 2.5.	Culture conditions of established cell lines	127
Table 2.6.	Characteristics of human breast carcinoma cell lines	130
Table 2.7.	Custom Oligonucleotide Amplimer sequences and PCR reaction conditions used in assays for DNA and RNA studies.	142
Table 2.8.	Characteristics of expression plasmids	150
Table 2.9.	Amplimer sequences and reaction conditions for HIF-1 $\alpha$ probe synthesis used in Digoxigenin (DIG) labelling.	169
Table 2.10.	T7 amplimer sequences and reaction conditions for RPA probe synthesis	186
Table 2.11.	Preparation of lactate standards for a lactate calibration curve from a 0.04 mg/ml stock standard by serial dilution.	1 <del>9</del> 4

Table 2.12.	Preparation of diluted BSA standards for protein curve from a 2 mg/ml stock standard by serial dilution.	196
Table 2.13.	Recipes for the preparation of SDS-polyacrylamide gels used in Western blotting	206
Table 2.14.	Antibodies, working dilutions and SDS-polyacrylamide gel percentages used in Western blotting assays.	209
Table 3.1.	Levels of VEGF, $p53$ and $\beta$ -actin mRNA in the T47D breast carcinoma cell line exposed to 60 and 120 mM cobalt chloride for 17.5 hours analysed by Northern blotting.	223
Table 3.2.	Levels of <i>HIF-1</i> $\alpha$ and <i>VEGF</i> mRNA in the MCF7 breast carcinoma cell line exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for up to 6 hours analysed by Northern blotting	225
Table 3.3.	Levels of <i>HIF-1</i> $\alpha$ mRNA expression in PBMCs of a normal healthy individual exposed to hypoxia ( $\leq 1\% O_2$ ) for up to 8 hours analysed by Northern blotting.	227
Table 3.4.	The fold change in <i>HIF-1 <math>\alpha</math></i> , <i>GLUT-1</i> and <i>p53</i> mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\% O_2$ ) for up to 48 hours.	232
Table 3.5.	Mean basal expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA at normoxia in all breast carcinoma cell lines	237
Table 3.6.	The fold change in HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression from normal culture conditions in breast carcinoma cell lines cultured for 3 days in moderate and hyperglycaemia.	240
Table 3.7.	The fold change in <i>HIF-1</i> $\alpha$ , <i>GLUT-1</i> and <i>p53</i> mRNA expression from normoxia in breast carcinoma cell lines exposed to long-term high glucose and exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for up to 48 hours	245
Table 3.8.	The fold change in <i>HIF-1</i> $\alpha$ mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for 24 hours and subsequently to normoxia for up to 24 hours	254
Table 3.9.	Levels of HIF-1 $\alpha$ mRNA in breast carcinoma cell lines exposed to both hypoglycaemia and hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for 24 hours	258
Table 3.10	Levels of HIF-1 $\alpha$ mRNA in breast carcinoma cell lines exposed for 7 days to hypoglycaemia or 20 mM L-glucose	259
Table 3.11	. Fold change in $HIF$ -1 $\alpha$ and $GLUT$ -1 mRNA expression in breast carcinoma cell lines exposed to 20 mM D-glucose supplemented with either 20 mM L-glucose or 20 mM mannitol	261

 Table 3.12.	Measured lactate concentrations (mmol/L) in breast carcinoma cell line culture media exposed to hypoxia.	263
Table 3.13.	Measured lactate concentrations (mmol/L) in breast carcinoma cell line culture media exposed to high glucose and osmolarity controls	264
Table 3.14.	Mean fold change in expression of HIF-1 DNA binding activity in nuclear extracts isolated from breast carcinoma cell lines exposed to hypoxia and long-term high glucose (20 mM D-glucose>30 days culture)	267
Table 3.15.	Expression of GLUT-1 protein in breast carcinoma cell lines exposed to hypoxia and long-term high glucose (20 mM D-glucose >30 days culture)	280
Table 4.1.	Clinical characteristics of normal controls and patients with T1DM studied in the HIF-1 $\alpha$ RPA.	288
Table 4.2.	Clinical characteristics of normal controls and all patients with T1DM studied in the GLUT-1 RPA.	289
Table 4.3.	<i>HIF-1</i> $\alpha$ mRNA expression in the PBMCs isolated from patients with T1DM exposed to normal glucose and high glucose for 3, 4 and 5 days	292
Table 4.4.	Overall mean fold change in <i>HIF-1</i> $\alpha$ and <i>GLUT-1</i> mRNA expression from normal glucose in PBMCs, isolated from normal controls and patients with T1DM, exposed to moderate and high glucose for 5 days.	297
Table 4.5.	Statistical analysis of the overall mean levels of $HIF$ -1 $\alpha$ and $GLUT$ -1 mRNA expression in PBMCs isolated from normal controls and patients with T1DM, exposed to moderate and high glucose.	298
Table 4.6.	Fold change in the levels of HIF-1 $\alpha$ and GLUT-1 mRNA in the PBMCs of patients with complications exposed to high glucose and supplemented with either 20 mM L-glucose or 20 mM mannitol	308
Table 4.7.	Measured lactate concentrations (mmol/L) in the culture media of PBMCs isolated from a normal control and from patients with T1DM exposed to high glucose.	310
Table 4.8.	Mean levels of HIF-1 DNA binding activity in nuclear extracts from the PBMCs of normal controls and patients with T1DM exposed to high glucose.	312
Table 4.9.	Expression of GLUT-1 protein in extracts isolated from the PBMCs of normal controls and patients with T1DM exposed to high glucose for 5 days.	317
Table 5.1.	Clinical characteristics of patients with breast tumours	323

Table 5.2.	Frequencies of the clinicopathological characteristics of patients with breast tumours	324
Table 5.3.	<i>HIF-1</i> $\alpha$ and <i>GLUT-1</i> mRNA expression and HIF-1 $\alpha$ protein expression in tumours from patients with breast cancer	328
Table 5.4.	Association between disease variables and HIF-1 $\alpha$ and GLUT-1 mRNA expression and HIF-1 $\alpha$ protein expression in the tumours of 22 patients with breast cancer	330
Table 5.5.	Univariate and multivariate analysis of cumulative overall survival of 22 patients with breast cancer	333
Table 5.6.	Nottingham Prognostic Index (NPI) of the 22 patients with breast cancer	342
Table 6.1.	Clinical characteristics of normal controls and patients with glial cell tumours	347
Table 6.2.	RPA analysis of HIF-1 $\alpha$ mRNA expression in 8 glioblastoma tissue samples	350
Table 6.3.	Statistical analysis of the mean ratios of $HIF$ -1 $\alpha$ to $VEGF$ mRNA expression between glioblastoma tumour types and coefficients of correlation	357
Table 6.4.	Immunohistochemical analysis of HIF-1 $\alpha$ , VEGF and p53 expression in glial cell tumours	359
Table 6.5.	Univariate and multivariate analysis of cumulative overall survival of 27 patients with glioblastoma	367
Table 6.6.	Univariate and multivariate analysis of cumulative overall survival of all 36 patients with glial cell tumours	368
Table 6.7.	Multivariant analysis of cumulative overall survival of all 36 patients with glial cell tumours based on HIF-1 $\alpha$ , VEGF and p53 immunohistochemical status	369
Table 7.1.	Clinical characteristics of patients with breast cancer	384
Table 7.2.	Frequency of p73 genotype in patients with breast cancer and control populations	387
Table 7.3.	Statistical analysis of patient and control populations for each p73 genotype	388
Table 7.4.	Frequency of disease variables and association with p73 genotype in patients with breast cancer.	389

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Table 7.5.	Frequency of p73 genotype with respect to age of diagnosis in patients with breast cancer.	390
Table 7.6.	Frequency of the p73 genotype in the Celtic population	391
Table 7.7.	Allele frequencies of the p73 genotype in patients with breast cancer and control populations	392
Table 7.8.	Frequency of the p53 isoform in patients with breast cancer and control populations.	394
Table 7.9.	Statistical analysis of patient and control populations for each p53 isoform	395
Table 7.10.	Frequency of disease variables and association with p53 isoforms in patients with breast cancer.	396
Table 7.11.	Frequency of p53 isoform with respect to age at diagnosis in patients with breast cancer	397
Table 7.12.	Frequency of the p53 isoforms in the Celtic population	398
Table 7.13.	Allele frequencies of the p53 isoforms in patients with breast cancer and control populations	399
Table 7.14.	Frequency of p73 genotype versus p53 Pro/Arg genotype in patients with breast cancer and control populations	401
Table 7.15.	Observed and expected frequencies of p73 and p53 allele combinations in observed patient and control populations.	402
Table 7.16.	Odds ratios (relative risk) for patients with breast cancer and control populations for each p73 and p53 genotype	403
Table 7.17.	Univariate analysis of cumulative overall survival of 97 patients with breast cancer	406
Table 7.18.	Multivariate analysis of cumulative overall survival of 97 patients with breast cancer.	407
Table 7.19	Correlation between survival variables and clinical characteristics in 97 patients with breast cancer	408
Table 7.20.	Nottingham Prognostic Index (NPI) for 97 patients with breast cancer	417

•

#### LIST OF FIGURES

Page

Figure 1.1.	Schematic representation of the cellular components and specific steps in the process of tumour angiogenesis regulated by proto-oncogenes and oncogenes	6
Figure 1.2.	Genomic imprinting in tumourigenesis	8
Figure 1.3.	Structural homology and structure of the p53, p73 $\alpha$ and p63 $\alpha$ proteins	13
Figure 1.4.	p73 allelic polymorphism	16
Figure 1.5.	Nucleotide substitution resulting in p53 Pro/Arg allelic expression	20
Figure 1.6.	Schematic representation of the initial stages of angiogenesis	27
Figure 1.7.	Structure of the VEGF gene and its isoforms	31
Figure 1.8.	A 3-D molecular model of the VEGF receptor binding domain	33
Figure 1.9.	Structure of hypoxia-inducible factor-1 $\alpha$ (HIF-1 $\alpha$ ) and HIF-1 $\beta$	41
Figure 1.10.	Proposed 3D molecular models of HIF-1	44
Figure 1.11.	Activation of HIF-1 by hypoxia and subsequent degradation in normoxia via ubiquitination	47
Figure 1.12.	Histologic classification of breast tumours	58
Figure 1.13.	A) Low-grade carcinoma of the breast. B) Moderate grade carcinoma of the breast. C) High-grade carcinoma of the breast.	64
Figure 1.14.	A) A low-grade astrocytoma showing small uniform astrocytic nuclei which are forming microcystic spaces with no mitotic activity. B) A highly cellular astrocytic tumour showing mitotic activity and nuclear pleomorphism	72
Figure 1.15.	A) Glioblastoma showing marked nuclear pleomorphic large atypical astrocytic cells. B) Glioblastoma showing 2 features: vascular proliferation to the left of the photo and necrosis to the right of the photo.	73
Figure 1.16.	Regulation of the polyol pathway in response to hyperglycaemia	83
Figure 1.17.	The four hypothetical pathways by which hyperglycaemia is thought to induce mitochondrial superoxide overproduction.	88
Figure 1.18.	Proposed ribbon model of the human facilitative glucose transporter GLUT-1	101

Figure 1.19.	Hypoxia induced GLUT-1 mediated glucose transport	104
Figure 1.20	Involvement of HIF-1 in disease	111
Figure 2.1.	Schematic representation of Nested Polymerase Chain Reaction	144
Figure 2.2.	Map of the pcDNA3 plasmid	151
Figure 2.3.	Map of the pCH110 eukaryotic assay vector	162
Figure 2.4.	Schematic Representation of the Ribonuclease Protection Assay	184
Figure 3.1.	Northern blot analysis of VEGF, $p53$ and $\beta$ -actin mRNA expression in T47D breast carcinoma cells.	222
Figure 3.2.	Northern blot analysis of HIF-1 $\alpha$ mRNA expression in MCF7 breast carcinoma cells.	224
Figure 3.3.	Northern analysis of HIF-1 $\alpha$ mRNA expression in PBMCs of a normal healthy individual	226
Figure 3.4.	RPA probes used in the analysis of samples	230
Figure 3.5.	<i>HIF-1</i> $\alpha$ , <i>GLUT-1</i> and <i>p53</i> mRNA expression in MCF7, SKBR3, T47D and ZR75 breast carcinoma cells that had been exposed to normoxia and to 2 to 48 hours hypoxia, analysed by RPA.	231
Figure 3.6.	The fold change in <i>HIF-1</i> $\alpha$ mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%O_2$ ) for up to 48 hours	233
Figure 3.7.	The fold change in <i>GLUT-1</i> mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%O_2$ ) for up to 48 hours.	234
Figure 3.8.	The fold change in $p53$ mRNA expression in breast carcinoma epithelial cell lines exposed to hypoxia (<1%O <sub>2</sub> ) for up to 48 hours	235
Figure 3.9.	Mean basal expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA cultured for 5 days at normoxia in all breast carcinoma cell lines.	238
Figure 3.10	Expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in MCF7, T47D and ZR75 breast carcinoma cell lines exposed to hyperglycaemia.	241
Figure 3.11	The fold change in HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression from normal culture conditions in breast carcinoma cell lines cultured for 3 days in moderate or hyperglycaemia	242
Figure 3.12	<i>HIF-1</i> $\alpha$ , <i>GLUT-1</i> and <i>p53</i> mRNA expression in MCF7 Hyper, SKBR3 Hyper and T47D Hyper breast carcinoma cells exposed to normoxia and to 2 to 48 hours hypoxia, analysed by RPA.	246

Figure 3.13.	The fold change in <i>HIF-1</i> $\alpha$ mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for up to 48 hours	247
Figure 3.14	The fold change in <i>HIF-1</i> $\alpha$ mRNA expression from normoxia in the MCF7, SKBR3 and T47D breast carcinoma cell line and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for up to 48 hours	248
Figure 3.15	The fold change in <i>GLUT-1</i> mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\% O_2$ ) for up to 48 hours	249
Figure 3.16	The fold change in <i>GLUT-1</i> mRNA expression from normoxia in MCF7, SKBR3, T47D and ZR75 breast carcinoma cell lines and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for up to 48 hours.	250
Figure 3.17	The fold change in $p53$ mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose and exposed to hypoxia (<1% O <sub>2</sub> ) for up to 48 hours	<b>25</b> 1
Figure 3.18	The fold change in $p53$ mRNA expression from normoxia in MCF7, SKBR3 and T47D breast carcinoma cell line and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia for up to 48 hours	252
Figure 3.19	The fold change in <i>HIF-1</i> $\alpha$ mRNA expression in breast carcinoma cell lines and breast carcinoma cell lines conditioned to long-term high glucose, exposed to hypoxia ( $\leq 1\%$ O <sub>2</sub> ) for 24 hours and subsequently to normoxia for up to 24 hours.	255
Figure 3.20	b. The fold change in <i>HIF-1</i> $\alpha$ mRNA expression in MCF7, SKBR3, T47D and ZR75 breast carcinoma cell lines and in the same cell lines conditioned to long-term high glcuose, exposed to hypoxia (<1% O <sub>2</sub> ) for 24 hours and subsequently to normoxia for up to 24 hours.	256
Figure 3.21	. Measured lactate concentrations (mmol/L) in breast carcinoma cell line culture media exposed to high glucose and osmolarity controls	265
Figure 3.22	2. The fold change in HIF-1 DNA-binding activity in breast carcinoma cell lines and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\% O_2$ ) for up to 48 hours	268
Figure 3.23	3. The effect of hypoxia and high glucose on HIF-1 DNA binding activity	270
Figure 3.24	I. Supershifting of HIF-1 DNA complexes	271

Figure 3.25.	Comparison of methodologies for harvesting nuclear protein extracts	273
Figure 3.26.	Separation of protein by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel.	276
Figure 3.27.	Western blot of breast carcinoma cell lines exposed to hypoxia demonstrating the detection of bovine serum albumin (BSA) by the HIF-1 $\alpha$ antibody in the nuclear extracts.	277
Figure 3.28.	Western blot analysis of GLUT-1 protein expression in breast carcinoma cell lines exposed to hypoxia and long-term high glucose	279
Figure 3.29.	GLUT-1 protein expression in extracts isolated from all breast carcinoma cell lines exposed to hypoxia for up to 48 hours	281
Figure 4.1.	<i>HIF-1</i> $\alpha$ mRNA expression in the PBMCs isolated from a normal healthy control and patients with T1DM exposed to normal glucose and high glucose, analysed by RT-PCR	291
Figure 4.2.	Illustration of $HIF$ -1 $\alpha$ and B) $GLUT$ -1 mRNA expression in PBMCs isolated from normal healthy controls and of patients with T1DM that had been exposed to normal glucose, moderate and high glucose, analysed by RPA	294
Figure 4.3.	The mean fold increase of $HIF$ -1 $\alpha$ mRNA in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate and high glucose.	299
Figure 4.4.	The mean fold change in <i>GLUT-1</i> mRNA in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate and high glucose	300
Figure 4.5.	<i>HIF-1</i> $\alpha$ mRNA expression in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate and high glucose	302
Figure 4.6.	GLUT-1 mRNA expression in PBMCs of normal controls and patients with T1DM exposed to normal glucose, moderate and high glucose	303
Figure 4.7.	Basal HIF-1 $\alpha$ mRNA expression in the PBMCs isolated from healthy normal controls and patients with T1DM exposed to normal glucose, moderate or high glucose for 5 days.	305
Figure 4.8.	Basal GLUT-1 mRNA expression in PBMCs isolated from normal controls and patients with T1DM exposed to normal glucose and high glucose for 5 days.	306
Figure 4.9.	EMSA of nuclear extracts prepared from the PBMCs of normal controls and patients with T1DM that had been exposed to normal glucose and high glucose	313

Figure 4.10	. Graphical expression of HIF-1 DNA binding activity in nuclear extracts from the PBMCs isolated from normal controls and patients with T1DM exposed to high glucose for 5 days.	314
Figure 4.11	. Western blot analysis of GLUT-1 protein expression in the PBMCs isolated from normal controls and from patients with T1DM exposed to high glucose	316
Figure 4.12	. GLUT-1 protein expression in extracts isolated from the PBMCs of normal controls and from patients with T1DM exposed to normal glucose and high glucose for 5 days.	318
Figure 5.1.	<i>HIF-1</i> $\alpha$ and $\beta$ -actin mRNA expression in tumours from patients with breast cancer.	326
Figure 5.2.	The fold change in HIF-1 $\alpha$ and GLUT-1 mRNA expression in tumours from patients with breast cancer as determined from RPA analysis	327
Figure 5.3.	Immunohistochemical analysis of HIF-1 $\alpha$ protein expression in tumours from patients with breast cancer.	332
Figure 5.4.	Cumulative overall survival of patients with breast cancer (n=22) differentiated by tumour stage and analysed by Kaplan-Meier	335
Figure 5.5.	Cumulative overall survival of patients with breast cancer (n=22) differentiated by node stage and analysed by Kaplan-Meier.	336
Figure 5.6.	Cumulative overall survival of patients with breast cancer $(n=22)$ differentiated by grade stage and analysed by Kaplan-Meier	337
Figure 5.7.	Cumulative overall survival of patients with breast cancer (n=22) with negative mRNA expression of $HIF$ -1 $\alpha$ , compared with patients with positive mRNA expression of $HIF$ -1 $\alpha$ , determined by RPA and analysed by Kaplan-Meier	338
Figure 5.8.	Cumulative overall survival of patients with breast cancer (n=22) with negative mRNA expression of $GLUT-1$ , compared with patients with positive mRNA expression of $GLUT-1$ , determined by RPA and analysed by Kaplan-Meier	339
Figure 5.9.	Cumulative overall survival of patients with breast cancer (n=22) with negative HIF-1 $\alpha$ nuclear protein expression, compared with patients with either strong HIF-1 $\alpha$ cytoplasmic protein expression or positive HIF-1 $\alpha$ nuclear protein expression, determined by immunohistochemistry and analysed by Kaplan-Meier	340
Figure 6.1.	HIF-1 $\alpha$ , VEGF and $\beta$ -actin mRNA expression in glial cell tumours analysed by RT-PCR and RPA	349

Figure 6.2.	<i>HIF-1</i> $\alpha$ splice variant mRNA expression in glial cell tumours analysed by RT-PCR	351
Figure 6.3	(A). HIF-1 $\alpha$ mRNA expression in individual tumour samples from patients with glioblastoma, lower grade tumours and normal brain analysed by RT-PCR	352
Figure 6.3	(B). Mean HIF-1 α mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain analysed by RT-PCR	353
Figure 6.4.	Mean <i>VEGF</i> mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain analysed by RT-PCR	355
Figure 6.5.	Mean HIF-1 $\alpha$ and VEGF mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain analysed by RT-PCR	356
Figure 6.6.	Immunohistochemical analysis of HIF-1 $\alpha$ expression in glioblastoma	360
Figure 6.7.	Immunohistochemical analysis of VEGF expression in glioblastoma	362
Figure 6.8.	Immunohistochemical analysis of p53 expression in glioblastoma	364
Figure 6.9	Cumulative overall survival of patients with glioblastoma (n=27) differentiated by sex and analysed by Kaplan-Meier	371
Figure 6.1	0. Cumulative overall survival of patients with glioblastoma (n=27) differentiated by HIF-1α protein expression and analysed by Kaplan-Meier	372
Figure 6.1	<ol> <li>Cumulative overall survival of patients with glioblastoma (n=27) differentiated by VEGF protein expression and analysed by Kaplan-Meier</li> </ol>	373
Figure 6.1	<ol> <li>Cumulative overall survival of patients with glioblastoma (n=27) differentiated by p53 protein expression and analysed by Kaplan-Meier</li> </ol>	374
Figure 6.1	3. Cumulative overall survival of all patients with glial cell tumours analysed by Kaplan-Meier	375
Figure 6.1	<ol> <li>Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by sex and analysed by Kaplan-Meier.</li> </ol>	376
Figure 6.1	<ol> <li>Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by HIF-1α protein expression and analysed by Kaplan-Meier</li> </ol>	377

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ľ

Figure 6.16.	Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by VEGF protein expression and analysed by Kaplan-Meier	378
Figure 6.17.	Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by p53 protein expression and analysed by Kaplan-Meier	379
Figure 7.1.	Analysis of p73 allelic expression and p53 Pro/Arg polymorphisms in the lymphocytes of patients with breast cancer.	383
Figure 7.2.	Automated sequencing of p73 amplified and genotyped DNA from the lymphocytes of two patients with breast cancer.	386
Figure 7.3.	Cumulative overall survival of patients with breast cancer (n=97) with the p73 GC/AT genotype compared with patients with the p73 GC/GC, analysed by Kaplan-Meier	409
Figure 7.4.	Cumulative overall survival of patients with breast cancer (n=97) with p53 Pro, p53 Arg and p53 Pro/Arg genotypes, analysed by Kaplan-Meier	410
Figure 7.5.	Cumulative overall survival of patients with breast cancer (n=97) differentiated by T stage and analysed by Kaplan-Meier	411
Figure 7.6.	Cumulative overall survival of patients with breast cancer (n=97) differentiated by N stage and analysed by Kaplan-Meier	412
Figure 7.7.	Cumulative overall survival of patients with breast cancer (n=97) differentiated by G stage and analysed by Kaplan-Meier	413
Figure 7.8.	Cumulative overall survival of patients with breast cancer (n=97) who have had treatment with chemotherapy, compared with patients who have not had treatment with chemotherapy, analysed by Kaplan-Meier.	414
Figure 7.9.	Cumulative overall survival of patients with breast cancer (n=97) who have had treatment with radiotherapy, compared with patients who have not had treatment with radiotherapy, analysed by Kaplan-Meier.	415
Figure 7.10	Cumulative overall survival of patients with breast cancer (n=97) who smoke compared with patients who do not smoke, analysed by Kaplan-Meier	416
Figure 9.1.	Illustration of p53 Pro and p53 Arg isolated plasmid DNA and subsequent double digestions using HindIII and EcoRI restriction enzymes.	455

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#### ABBREVIATIONS

$\alpha^{32}P dCTP$	alpha phosphate-32 deoxycytidine-5'-triphosphate
$\alpha^{32}P dUTP$	alpha phosphate-32 deoxyuridine-5'-triphosphate
$\gamma^{32}$ P dATP	gamma phosphate-32 deoxyadenosine-5'-triphosphate
λ	wavelength
χ²	chi-squared
AEBSF	4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride
Amp <sup>8</sup>	ampicillin sensitive
ATCC	American Type Culture Collection
АТР	adenosine-5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
CO <sub>2</sub>	carbon dioxide
CoCl <sub>2</sub>	cobalt chloride
CSA	catalysed signal amplification
CsCl	cesium chloride
СТР	cytidine-5'-triphosphate
DAB	3,3'-diaminobenzidine
DC	diabetic control
DEPC	diethyl pyrocarbonate
DIG	digoxygenin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DN	diabetic nephropathy
DNA	deoxyribonucleic acid

• DIT · . · · · · · · ·	dithiothreitol
dNTPs	2' deoxyribonucleotide 5'-triphosphates
ECACC	European Collection of Animal Cell Cultures
EDTA -	ethylene diamine tetra-acetic acid
EMSA	electrophoretic mobility shift assay
FBS	foetal bovine serum.
g	gravitational force
GEUT-1	Glucose Transporter 1
GTP	guanosine-5'-triphosphate
HBSS	Hank's Balanced Salt Solution
HIF-1a	Hypoxia-Inducible Factor 1 Alpha
HIF-1β	Hypoxia-Inducible Factor 1 Beta
HSP	heat shock protein
IMS	Industrial Methylated Spirits
IPTG	isopropyl β-D-thiogalactopyranoside
Kan <sup>R</sup>	kanamycin resistant
Kb	kilo base
KCI	potassium chloride
kDa	kilo dalton
: <b>L:B</b> :	Luria-Bertaini
ĽD	lactate dehydrogenase
MgCl <sub>2</sub>	magnesium chloride
mRNA	messsanger ribonucleic acid
MOPS	3-[N-morpholino]propane-sulphonic acid
MW	molecular weight
'n	number of subjects

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N <sub>2</sub>	nitrogen
NaCi	sodium chloride
NAD+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NC	normal control
N/D	not done
NFDM	non-fat dairy milk
NIDDM	non-insulin dependent diabetes mellitus
NPI	Nottingham Prognostic Index
ns	not significant
O <sub>2</sub>	oxygen
OD	optical density
p	p value
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
РНА	phytohaemaglutinin
PMSF	phenylmethylsulphonylfluoride
PNK	polynucleotide kinase
PVP	polyvinylpyrolidone
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RPA	ribonuclease protection assay

rpm	rotations per minute
RPMI	Roswell Park Memorial Institute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecył sulphate
SOB	Hanahan's broth
SSC	saline-sodium citrate
SSPE	saline-sodium phosphate-EDTA
T1DM	Type 1 Diabetes Mellitus
TBA	2-thiobarbituric acid
TBE	tris-borate-EDTA
TCA	trichloroacetic acid
TE	tris-EDTA
Tet <sup>s</sup>	tetracycline sensitive
TTP	thymidine-5'-triphosphate
UTP	uridine-5'-triphosphate
UV	ultraviolet
VEGF	vascular endothelial growth factor
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-indolyl β-D-galactopyranoside

#### UNITS OF MEASUREMENT

Ci	curi
Ci/mmol	curi per millimole
cm	centimetre
cpm	counts per minute
g	grams
ng	nanogram
nm	nanometre
μCi/μl	micro-Curi per micro-litre
μg	micro-gram
μΙ	micro-litre
μg/μl	micro-gram per micro-litre
μg/ml	micro-gram per milli-litre
μM	micro-molar
М	molar
mg/dL	milligram per deci-litre
ml	milli-litre
mM	milli-molar
mmol/L	milli-mole per litre
pg	pico-gram
psi	pounds per square inch
U	units
U/ml	units per milli-litre
v/v	volume per volume
w/v	weight per volume

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## **AUTHOR'S DECLARATION**

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# **CONFERENCES**

Scientific seminars and conferences were attended, at which the following work was presented:

- British Association For Cancer Research 1998 Winter Meeting (London, UK).
   Both a poster presentation and oral communication were presented.
- <u>Cancer Research Campaign 1999 Beatson International Cancer Conference</u> (Glasgow, UK)
   A poster presentation was presented.
- British Diabetic Association Annual Professional Conference 2000 (Brighton,UK). An oral communication was presented.
- 4) <u>The Renal Association 50<sup>th</sup> Anniversary Meeting 2000</u> (Cambridge, UK).
   A poster presentation was presented.

### **PUBLICATIONS**

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Chapter 1.

# Introduction

# **1.0 Introduction**

There are many ancient, medieval and modern day researchers who have offered theories about the group of diseases known as cancer. The oldest recorded description of cancer dates back to 1600 B.C. Egypt, where tumours of the breast were written in papyrus. Hippocrates (460-370 B.C.) originated the word cancer having used the word 'carcinoma' to describe different types of tumours. He believed that an excess of black bile produced by the stomach and spleen caused cancer (Coagner, 1991). Modern research has concluded that cancer is a combination of uncontrolled cellular proliferation and/or lack of apoptosis. Rigorous scientific research during the past twenty to thirty years has resulted in an enormous accumulation of knowledge underlying the molecular and cellular basis of cancer. A major area of investigation has been the analysis of genes that determine tumour cell proliferation and viability.

### 1.01 Knudson Hypothesis

One of the most significant theories in modern biology, Knudson's 'two-hit' hypothesis (Knudson, 1971) was proposed from the evidence in familial retinoblastoma cases to elucidate the mechanism by which common cancers, such as breast cancer, become more prevalent with age. The hypothesis explains the relationship between the hereditary and nonhereditary, or sporadic, forms of cancer. The simplified model suggests that two separate genetic events are required for the development of a cancer. The first event is inherited in the familial form and the time required for the second hit to occur is shorter than two hits to occur in a normal person. This hypothesis explains why the familial form of retinoblastoma occurs at an earlier age and is usually bilateral. Thus, inherited cancer occurs earlier whereas sporadic cancer has later onset.

#### 1.02 Multistage Model

It is largely acknowledged that cancer is a genetic disease that arises from acquired mutagenetic changes. The resulting damage to a cells' genetic material is both a multi-hit (genetic and factorial) and multi-step (transformation, promotion, overt) process which occurs over a prolonged period of time affecting either or both proto-oncogenes and tumour suppressor genes (Cohen and Ellwein, 1991).

Initial studies into the development of cancer, or carcinogenesis, believed it to be a two-stage process (Berenblum and Shubik, 1949). Later investigations proposed that carcinogenesis was a six to seven stage process (Armitage and Doll, 1954). It is now understood that multistage carcinogenesis is analogous to a 'two-hit' model of tumour development and is often characterised by four sequential stages: initiation, promotion (which leads to the appearance of benign tumours), progression and malignant conversion (Harris *et al*, 1992). Genetic changes which cause initiation or promotion could involve mutations that activate a cellular oncogene, inactivate a tumour suppressor gene, or inactivate an anti-metastasis gene. The overall process can occupy a major fraction of the life span of the individual. Evidence also suggests that failure to activate apoptosis after DNA injury may be an additional route to carcinogenesis (section 1.2.3) (Griffiths *et al*, 1997).

Tumour formation usually arises as a consequence of alterations in the control of cell proliferation and disorders in the interactions between cells and their surroundings that result in invasion and metastasis. Although a particular genetic alteration may be necessary for tumourigenesis, in most cases a single event, such as oncogene inactivation, is not sufficient. Early research demonstrated that tumour progression occurs in a stepwise fashion with each step determined by the activation, mutation or loss of specific genes (Foulds, 1957). The control of cell proliferation is highly complex as a cell driven through its proliferation cycle by a series of co-ordinated stepwise genetic events which determine

entry into S-phase and mitosis. Proto-oncogenes and tumour suppressor genes may modify these steps. The rate of cell proliferation is probably a balance of the growth promoting effects of the proto-oncogenes and the constraints imposed by the action of tumour suppressor genes (Cooke & Stanton 1994, Marshall 1991). Thus, the cumulative effects of genetic abnormalities are required to convert a normal cell into a cancer cell.

Although the majority of tumours are angiogenic and able to induce the formation of new vasculature, the normal cells from which these tumours arise are commonly antiangiogenic. They frequently secrete low levels of inducers that are masked by high levels of inhibitors of angiogenesis (Kerbel, 2000). As these normal cells progress to malignancy they become potently angiogenic (Rastinejad & Bouck, 1997). Cells in a developing tumour acquire their hyper-angiogenic phenotype as a result of the sequential activation of oncogenes and inactivation of tumour suppressor genes. These mutations have been shown to increase the proliferation and/or viability of cultured tumour cells (Hanahan and Weinberg, 2000; Semenza, 2000). Both oncogenes and tumour suppressor genes also control the production of a variety of secreted molecules that regulate angiogenesis.

# **1.1 Proto-Oncogenes and Oncogenes**

Proto-oncogenes encode elements of intracellular signal transduction pathways leading from growth stimuli outside a cell to DNA within the cell nucleus. They are expressed in normal cells and may code for growth-factor receptors, intracellular signal-carrying molecules and transcription factors. The expression of proto-oncogenes is tightly controlled by a number of regulatory mechanisms and their expression is usually short lived so that the gene product only exists at the required level when a growth response is required. This may be achieved by transcriptional up-regulation, enhancement of mRNA stability, post-translational modification or a combination of these events (Cooke & Stanton, 1994).

- 4 -

Cancer cells commonly express oncogenes that can arise in cells via two mechanisms: infection of cells by tumour viruses and conversion of cellular protooncogenes to oncogenes. Oncogenes are generally derived from the genetic material within each normal proto-oncogene, thus oncogenes are proto-oncogene conversions generally having resulted from proto-oncogene mutation, amplification, chromosomal translocation or retroviral activation (Varmes, 1984). Oncogenes contribute to tumourigenesis by activation, overexpression or amplification resulting in disordered proliferation or differentiation. They exert a positive driving force for cell growth by their inability to cease in response to the absence of stimulation.

A number of oncogenes and proto-oncogenes play a key role in the multi-step induction of tumour angiogenesis. Tumour angiogenesis requires that endothelial cells receive angiogenic signals from their environment. The expression of oncogenes within cells is a critical event in the early stages of tumour formation and it is believed that tumours originate from the oncogenic transformation of a single cell. Oncogenes enable tumours to provide these signals by enhancing the secretion of angiogenic factors, by limiting the production of inhibitors and by stimulating the degradation of the extracellular matrix. These stimuli activate proto-oncogenes within endothelial cells as they respond to new blood vessels.

The involvement of oncogenes and cellular proto-oncogenes in mediating angiogenesis and tumour growth is depicted in Figure 1.1. Angiogenic factors belonging to the growth factor class of oncogenes can simultaneously support tumour cell growth and sustain angiogenesis (reviewed in section 1.3) (Rastinejad & Bouck, 1997).

- 5 -

Chapter 1. Introduction



Figure 1.1. Schematic representation of the cellular components and specific steps in the process of tumour angiogenesis regulated by proto-oncogenes and oncogenes. The cellular position of some cell cycle regulators is demonstrated. Nuclear onco-proteins, transcriptional modifiers, are expressed in most cells however, when a mutation occurs their expression becomes abnormal triggering a series of processes resulting in tumour formation.

# **1.2 Tumour Suppressor Genes**

Tumour suppressor genes (anti-oncogenes) can be defined as genes in which their deletion, reduced expression or inactivation contributes to carcinogenesis. Tumour suppressor genes are foremost among the protective mechanisms that have developed to reduce the chances of developing cancer. They prevent tissue overgrowth, nullify cells with damaged genomes and metastases. Their products act downstream from growth-signal pathways and as long as they are fully active, these genes serve as guardians against tumour progression. It is only when one or more of them become defunct that uncontrolled cell multiplication can occur leading to cancer. The loss of a tumour-suppressor gene is generally characterised by a mutation in one copy of the gene and loss of the homologous copy (Levine, 1995).

Turnour suppressor genes can exert an influence on angiogenesis by stimulating the cells in which they are expressed to secrete high levels of inhibitors of angiogenesis. A variety of cells expressing turnour suppressor genes switch their phenotypes from antiangiogenic to angiogenic when a turnour suppressor gene is inactivated. This switch may be a result of decreased synthesis and secretion of an inhibitor of angiogenesis, such as thrombospondin-1 (Dameron *et al*, 1994).

# **1.2.1 Genomic Imprinting**

Genomic imprinting, a recently discovered mechanism, involves tumour suppressor gene inactivation through mutation or parent-specific inactivation of a non-mutated gene by imprinting. The activity of genes may be altered epigenetically in a manner dependent on the parent of origin of each of the two alleles (Figure 1.2). The effects of genomic imprinting can be seen by differences in the expression of an inherited disease depending on the sex of the transmitting parent. Evidence also exists that genomic imprinting may play a role in the pathogenesis of some embryonal tumours (Ferguson-Smith *et al*, 1990).

- 7 -

Chapter 1. Introduction



Figure 1.2. Genomic imprinting in tumourigenesis.

The model suggests that preferential retention of paternal alleles occurs in the tumour tissue. (Adapted from Ferguson-Smith et al, 1990).

# 1.2.2 p53

Perhaps the best known of all tumour suppressor genes is p53. Alterations of the p53 gene complex are one of the most frequently encountered genetic events in human malignancy where half of all tumours have an altered form of the gene (Hollstein *et al*, 1994). Genetic alterations in the p53 tumour suppressor gene occur in the majority of human cancers, are complex and are a significant factor in the development of malignant progression, including carcinoma of the breast, where half of all tumours have an altered form of the gene (Hollstein *et al*, 1991, Nigro *et al*, 1989). The wild-type p53 tumour suppressor gene is fully active when the p53 protein is expressed at normal ambient levels in the cell and does not depend on overexpression of the tumour suppressor protein.

p53 functions biochemically as a transcription factor and transcription activation is lost in mutant proteins. The p53 gene is located on chromosome 17q13.1 (Benchimole *et al*, 1985; Umesh *et al*, 1988) and corresponds to approximately 20 kb of genomic DNA consisting of 11 exons, of which the first exon is non-coding, followed by a large 10 kb intron (Lamb and Crawford, 1986). Gene transcription is controlled by two regulatory sites; a 400 bp element 5' upstream of exon 1 (P<sub>1</sub>) and a second promoter element P<sub>2</sub> located in intron 1 approximately 1 kb downstream of P<sub>1</sub> (Harlow *et al*, 1985, Reisman *et al*, 1988). The entire p53 transcript is 2.8 kb long and produces a 53 kDa phosphoprotein containing 393 amino acids.

p53 is thought to monitor the integrity of the cellular genome by acting as a cell regulator as it is often described as a G1 Check Point. p53 is activated in response to DNA damage and elicits apoptosis, cell-cycle arrest or senescence thereby maintaining healthy cells and preventing formation of tumours. Depending on the extent of DNA damage, p53will respond by inducing either G1 arrest, which allows for DNA repair to take place before S phase entry (survival), or apoptosis. Cells that cannot be repaired will enter the apoptotic pathway. Cells deficient in p53 or expressing mutant p53 have a block in the G1

-9-

phase of the cell (Levine *et al*, 1991), thus a mutated form of p53 looses its regulating and arresting activity.

The p53 phosphoprotein is a nuclear protein that is expressed in very low levels in normal cells and tissues and it has a short half-life. The C-terminal region of the protein contains signals for nuclear localisation (Dang and Lee 1989, Addison *et al*, 1990, Shaulsky *et al*, 1990) whereas mutant proteins with deletions in the C-terminal region show cytoplasmic localisation (Sturzbecher *et al*, 1988). Thus the protein displays nuclear and/or cytoplasmic localisation.

The p53 protein has the ability to regulate transcription by binding to DNA. There are 5 highly conserved regions in the p53 protein (I-V) corresponding to codons 12 to 290 (Nigro *et al*, 1989; Levine *et al*, 1991, 1994; Vogelstein and Kinzler, 1992), which function as a specific DNA-binding domain. Missense mutations occur predominately between codons 120 and 290, which contains 86 % of p53 mutations, whereas non-sense mutations tend to occur outside this region (Levine *et al*, 1993). Missense mutations of abnormal p53 will result in a defective protein product that cannot bind to a target gene with a p53 responsive element. Hence, these conserved regions serve as important functional domains.

### 1.2.2.1 The p53 Gene Family

Recently it has been suggested that p53 may not control cancer alone but may be part of a larger family (Oren, 1998). The discovery of new p53 family members has raised the possibility that p53 is assisted in preventing tumour formation and has dispelled the opinion that p53 was unique in form and function. In addition to the newly discovered p53 homologue p73, p53CP (p53 competing protein), p40, NBP (non-p53 p53RE-binding protein), p51 and p63 have been proposed as new additional members of the family (Bain and Sun, 1997; Trink *et al*, 1998; Zeng *et al*, 1998; Osada *et al*, 1998; Yang *et al*, 1998)

# Chapter 1. Introduction

p53 Family Member	Isoforms	References	
р63	α	Yang et al, 1998;	
	β	Schmale and Bamberger, 1997	
	γ	Yang et al, 1998; Schmale & Bamberger, 1997	
	ΔNp63	Yang et al, 1998	
	p40	Trink et al, 1998	
	p51	Osada et al, 1998	
	p53CP	Bain and Sun, 1997; Tan et al, 2001	
	NBP	Zeng et al, 1998; Zeng et al, 2001	
	p73L	Senoo et al, 1998	
p73	α	Kaghad et al, 1997; Ichimiya et al, 1999	
	β	Kaghad et al, 1997; Zaika et al, 1999	
	γ	De Laurenzi et al, 1998	
	δ	De Laurenzi et al, 1998	
	3	De Laurenzi et al, 1999	
	ζ	De Laurenzi et al, 1999	
	η	Ishimoto et al, 2002	
	ΔNp73	Ishimoto et al, 2002	

Table 1.1. The p53 tumour suppressor gene family.

p53 CP = p53 competing protein

NBP = non-p53 p53 responsive DNA element (RE) binding protein

(Table 1.1). Both p73 and p63 have an additional C-terminal extension found in p53 that undergoes alternative splicing resulting in multiple isoforms with different biological activities. While it is well established that p53 is a tumour suppressor gene, experiments with knock-out mice have determined that p63 plays a major role in ectodermal differentiation (Yang *et al*, 1999) and that p73 is involved in neurogenesis (Yang *et al*, 2000), (reviewed in Yang *et al*, 2002).

# 1.2.2.2 p73

p73 was the first p53 homologue described and is mapped to chromosome 1p36.33 (Kaghad *et al*, 1997), a region frequently deleted in several cancers, including breast (Caron *et al*, 1993, Nagai *et al*, 1995, Schwab *et al*, 1996, Thompson *et al*, 1997, White *et al*, 1995). p73 is both structurally and functionally similar to p53 where over-expression can activate transcription of p21<sup>Waf1/Cip1</sup>(wild-type p53 activated fragment/cdk-interacting protein 1), a cyclin-dependent kinase inhibitor gene, and induce apoptosis in a p53 independent manner (Kaghad *et al*, 1997, Jost *et al*, 1997), as well as activating the transcription of p53-responsive genes involved in cell-cycle control (Jost *et al*, 1997).

The structural homology between p53 and p73 is most extensive within the conserved activation (29%), sequence specific DNA binding (63%) and oligomerisation domains (42%), (Figure 1.3). p73 encodes 8 distinct polypeptides ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\varepsilon$ ,  $\zeta$ ,  $\eta$ , and  $\Delta$ N), whose profiles vary between tumours and normal tissues and have different biological activities (Ichimiya *et al*, 1999; Zaika *et al*, 1999; DeLaurenzi *et al*, 1998; DeLaurenzi *et al*, 1999; Ishimoto *et al*, 2002). These different p73 variants exist in cells, resulting in a family of proteins that further complicates the understanding of p73 signalling in cancer cells. The p73 $\alpha$  and p73 $\beta$  isoforms were the first originally described. p73 $\alpha$  encodes 14 exons and consists of 636 amino acids whereas p73 $\beta$  consists of 499 amino acids, derived from an alternative splicing of exon 13 and lacks a



Figure 1.3. Structural homology and structure of the p53, p73a and p63a proteins.

The percent identity at the amino acid level relative to p53 is indicated for each domain. The highest homology between the three proteins is within the central sequence-specific DNA binding domain with significant similarity in the N-terminal transactivation and C-terminal oligomerisation domains. Only p73 and p63 contain SAM-like domains. AA= amino acids, SAM = sterile alpha motif

C-terminal tail (Kaghad *et al*, 1997). p73 is monoallelically expressed in neuroblastoma cell lines (Kaghad *et al*, 1997) and it is found to have marked peculiarity as variations in its expression have been found from tissue to tissue and from person to person (Nomoto *et al*, 1998; Tsao *et al*, 1999). The human p73 promoter has recently been characterised (Ding *et al*, 1999) and is found to contain a TATA-like box which has low homology to the p53 promoter (Levrero *et al*, 2000).

The role of the p73 gene was originally thought to be that of a classical tumour suppressor gene (Kaghad et al, 1997). This however has not been supported by recent studies in malignant tumours where it has been frequently found to be expressed and not mutated (Nomoto et al, 1998, Sunahara et al, 1998, Takahashi et al, 1998). Consistent loss of heterozygosity in tumours can be used as an indication of the presence of a tumour suppressor gene. Loss of heterozygosity studies have also been used to indicate the location of inherited genes predisposing to familial forms of tumours (Ponder, 1988). p73 is expressed at low levels in all normal tissues and it is not expressed in neuroblastomas that contain deletions in chromosome 1p. As the maternal chromosome is preferentially lost in some neuroblastomas, this suggests that at least one tumour suppressor on 1p is an imprinted gene. Experiments with normal peripheral blood specimens showed that expression of p73 was exclusively from the maternally derived chromosome (Kaghad et al, 1997). Additionally, monoallelic expression of p73 was found to occur in a number of cancer cell lines and in normal tissue (Kaghad et al, 1997) and activation of the silent allele was found in renal cell carcinoma and lung cancer that is similar to loss of imprinting (Mai et al, 1998; Mai et al, 1998b). However, biallelic expression of p73 was observed in some normal tissue and lymphocytes as well as in bladder cancer and normal bladder suggesting that p73 expression could be tissue independent and that it is not monoallelically expressed in all human tissues (Kaghad et al, 1997; Yokomizo et al, 1999). A recent study has shown that the p73 gene is not an imprinted gene in ovarian cancer but could be involved in advanced ovarian cancer through overexpression (Chen et al, 2000).

Within the 5' untranslated region of the p73 gene there is a double nucleotide substitution at position 4 and 14 of exon 2 (Kaghad *et al*, 1997, DeLaurenzi *et al*, 1998), (Figure 1.4). The frequency of these polymorphisms has been studied in a number of patients with different tumours including bladder, colorectal, oesphageal, liver, lung, neural, prostrate and renal cell carcinoma, and most recently breast cancer (Soulitzis *et al*, 2002; Yokomizo *et al*, 1999, Sunahara *et al*, 1998, Nimura *et al*, 1998, Mihara *et al*, 1999, Nomoto *et al*, 1998, Kovalev *et al*, 1998, Takahashi *et al*, 1998, Mai *et al*, 1998, Ahomadegbe *et al*, 2000). Although p73 has frequent heterozygous deletions, mutations in the remaining allele are extremely rare. To date no frameshift mutations in p73 have been found in human primary tumours. In breast cancer, only one somatic missense mutation has been discovered (Han *et al*, 1999). Overexpression of wild type p73 mRNA has also been observed in carcinoma of the breast (Zaika *et al*, 1999), however the exact mechanism of this modification is not understood. The studies would suggest the p73 may augment rather than inhibit tumour development suggesting a contradictory role for p73 in malignancy (reviewed in Stiewe and Putzer, 2002).

# 1.2.2.3 p63

Various reported homologs of p53 are now known to be isoforms of the same gene, p63 (Hibi *et al*, 2000). p53CP shows similar binding specificity and also binds both double and single-stranded DNA oligonucleotides. p53CP may have p53-like functions by binding and transactivating p53 downstream target genes. A recent study has proposed that p53CP is p63, the official third member of the p53 gene family (Tan *et al*, 2001). Both p51 and p40 map to human chromosome 3q, a region where deletions are commonly associated with bladder and other types of cancer. *p40* has recently been reported to be

- 15 -





Figure 1.4. p73 allelic polymorphism.

The 5' untranslated region of the gene consists of a double nucleotide substitution at position 4 and 14 (Kaghad et al, 1997, DeLaurenzi et al, 1998).

oncogenic (Hibi *et al*, 2000). p51, is found to be highly expressed in skeletal muscle and placenta, and appears to bear more similarity to p73 than to p53. It has been concluded that p51 and p63 are actually the same molecule and it is sometimes referred to as p51/p63 (Osada *et al*, 1998). Another study has also concluded that NBP is not p73 but is actually the p53 homologue p63 (Zeng *et al*, 1998; Zeng *et al*, 2001). Thus, NBP and p53CP appear to be the same family encoded by the *p53* analogue *p63*.

Unlike p53, but like p73, p63 encodes 3 polypeptides ( $\alpha$ ,  $\beta$ ,  $\gamma$ ) that are translated from RNA molecules that share a common 5' end and differ at their 3' end due to alternative splicing (Yang *et al*, 1998; Schmale and Bamberger, 1997). *p53* encodes one major transcript however both *p63* and *p73* each contain two separate promoters that direct expression of two different classes of proteins; TAp63/p73 which contains an acidic N terminus with homology to the transactivation domain of p53, and  $\Delta$ Np63/73 which contain N-terminally truncated products that lack the TA domain (Yang *et al* 1998; Yang *et al*, 2000). The transactivation competent (TA) variants of p63 and p73 can activate many p53 target genes and can in some circumstances double for p53 in its anti-proliferative action (Jost *et al*, 1997; Yang *et al*, 1998). In contrast, variants lacking the transactivation domain ( $\Delta$ N) actually antagonise TA family members, including p53 (Pozniak *et al*, 2000; Liefer *et al*, 2000). Thus, different variants exert opposing effects on cell fate and cell behaviour (reviewed in Michael and Oren, 2002; Little and Jochemsen, 2002).

Both p73 and p63 contain a SAM (sterile alpha motif)-like domain at the Cterminus that is involved in protein-protein interactions (Chi *et al*, 1999). The SAM domain is a compact globular domain composed of five helices (Levrero *et al*, 1999). When both p63 and p73 are overexpressed they carry out many of the same function as p53, such as transactivating some of the same target genes and inducing apoptosis or cell cycle arrest (Jost *et al*, 1997; Yang *et al*, 1998). Isoforms encoded by splice variants or generated by transcription at alternative initiation sites are known to antagonise the effects

- 17 -

(Lohrum and Vousden, 2000; Yang *et al*, 2000). It has also been demonstrated that mice lacking p53 give rise to spontaneous tumours, whereas those lacking p73 or p63 are not tumour prone (Yang *et al*, 2000). In contrast to p53, no known viral oncoproteins bind to or inactivates p73 or p63 (Marin *et al*, 1998; Dobblestein *et al*, 1998; Steegenga *et al*, 1999). However, p73 deficient mice exhibit inflammation of many organs suggesting a role for p73 in the inflammation response (Yang *et al*, 2000).

Although these proteins belong to the same gene family, there are substantial differences in their normal physiological functions. Biologically, p73 appears to be more involved in neurogenesis and p63 in embryogenesis rather then in cancer (Yang *et al*, 1999; Yang *et al*, 2000; Mills *et al*, 1999). A mutational study showed very low frequency in cancers (Han *et al*, 1999; reviewed in Kaelin, 1999). However, it is now established that both of these p53 homologues have distinct and separate roles and may regulate a multitude of cellular and homeostatic processes (reviewed in Yang *et al*, 2002).

# 1.2.2.4 p53 and Human Papilloma Virus (HPV)

Since p53 was originally discovered as a protein that coprecipitates with the large T antigen of Simian Virus-40 (SV-40) (Lane and Crawford, 1979; Linzer and Levine, 1979), other viral oncoproteins have been reported to bind to and inactivate p53, including E6 of human papilloma virus (HPV) (Sarnow *et al*, 1984). Studies have shown that p53 may be inactivated by the E6 protein of cervical associated HPV, which belongs to the papovavirus family of oncogenic DNA viruses (Lechner *et al*, 1992). Once infected into a host cell, these viruses produce proteins that interact with host-derived proteins to bring about transformation. HPVs, especially HPV 16 and 18, are associated with high-grade cervical intraepithelial neoplasia (CIN) and invasive squamous carcinomas. The integration of these viruses in the genome results in increased expression of E6 and E7 proteins which have the ability to transform cells into a neoplastic state. It is known that the E6 protein binds to p53

(Werness *et al*, 1990) using the E6-associated protein (E6-AP) after which a rapid degradation of the p53 protein occurs which is mediated by ubiquitin-dependent proteolysis (Huibregtse *et al*, 1993). The introduction of a single HPV16-E6 gene causes the immortalisation of cells and dramatically reduces p53 protein levels (Band *et al.*, 1991). Tumour development is thought to arise through p53 mutations or by neutralisation of wild-type p53 by interaction with HPV proteins. Hence, formation of a p53-E6 complex sequesters the p53 protein thereby preventing it from performing its normal suppressor function. There are currently no known viral proteins that binds to or inactivates p73 or p63 (Marin *et al*, 1998; Dobblestein and Roth, 1998).

### 1.2.2.5 p53 and p73

The gene coding for p53 contains a single nucleotide polymorphism that results in either a proline or arginine at codon 72 (Figure 1.5). It has recently been shown that the arginine form of p53 is more susceptible to degradation by the human papillomavirus (HPV) E6 protein (Matlashewski *et al*, 1987), whilst individuals homozygous for p53 Arg are 7 times more likely to develop HPV-associated cancer (Storey *et al*, 1998). However, this has not been confirmed by studies in other populations (Hayes *et al*, 1998, Rosenthal *et al*, 1998, Helland *et al*, 1998, Josefsson *et al*, 1998, Hildestein *et al*, 1998). p73 has also recently been reported to be resistant to E6-ubiquitin mediated proteolysis with both the p73 $\beta$  variant and mutant p73 being potent inhibitors of the growth of HPV E6 expressing human cancer cells (Prabhu *et al*, 1998). It is important to determine whether members are involved in the same pathway, or whether they act as back-up genes for one another.



Figure 1.5. Nucleotide substitution resulting in p53 Pro/Arg allelic expression. (AA= amino acids)

### 1.2.2.6 p53 and MDM-2

The MDM-2 (mouse double minute 2) oncoprotein (HDM-2 in humans) is a wellknown regulator of p53 and p73. MDM-2 binds directly to the N-terminus of p53, where it is then neutralised and degraded through ubiquitin-dependent proteolysis (Kubbutat *et al*, 1997; Haupt *et al*, 1997). p53 induces the expression of MDM-2 that will then control p53 activity and stability resulting in an autoregulatory feedback loop (Strano *et al*, 2001; Michael and Oren, 2002). HDM-2 can bind to p73 where it promotes its stability and reduces its transcriptional activity but it does not degrade it (Zeng *et al*, 1999; Ongkeko *et al*, 1999).

Additional studies have suggested that the p14  $^{ARF}$  (alternative reading frame) protein is critical for p53 to respond to certain oncogenic stimuli, perhaps by modulating the interaction of HDM-2 with p53 (Zhang *et al*, 1998; Kamijo *et al*, 1998). It is thought that the destruction of p53 is mediated by binding to complexes containing HDM-2 and p300 (Grossman *et al*, 1998). The p300/CBP complex also binds to the N-terminus of p73 promoting its transcriptional activity (Steegenga *et al*, 1999). It is known that p14<sup>ARF</sup> can physically interact with both HDM-2 and p53 thereby antagonising the effects of HDM-2 on p53 (Zhang *et al*, 1998; Kamijo *et al*, 1998). However, p73 is not ubiquitinated by HDM-2 (Zeng *et al*, 1999) and HDM-2 is known to enhance p63-dependant transcription (Zeng *et al*, 2001).

# 1.2.3 Apoptosis

Two types of cell death have been characterised: necrosis and apoptosis. Necrosis is a pathological form of cell death that is not implicated in normal cell kinetics. It is associated with exogenous cell damage due to severe departures from ideal physiological conditions such as hypoxia and ischemia (Arends & Wyllie, 1991). Apoptosis is however involved in tissue kinetics and is typically triggered by a variety of stimuli including growth factor withdrawal (Williams *et al*, 1990), ionising radiation (Gobe *et al*, 1988), heat and cytotoxic drugs (Lowe *et al*, 1993). Apoptotic cell death is characterised by distinctive morphological changes which include membrane blebbing, cytoplasmic and nuclear condensation, chromatin fragmentation and formation of apoptotic bodies (Wyllie, 1980). It is an active process in which apoptotic cells are recognised by phagocytes and removed before they disintegrate. Apoptosis plays important roles in a variety of developmental events and in the control of tissue homeostasis (Oppenheim, 1991; Raff, 1992) and in disease processes, including cancer (Thompson, 1995).

Both proto-oncogenes and oncogenes have been shown to be involved in the control of apoptosis, or programmed cell death. Genes such as c-Myc (Koskinen and Alitalo, 1993) and p53 (van Slooten *et al*, 1998) play a role in the induction of apoptosis, whereas Bcl-2 acts specifically to block apoptosis (van Slooten *et al*, 1998). It is known that Bcl-2 family proteins are key regulators of the apoptotic pathway and play an important role in tumourigenesis (reviewed in Schmitt and Lowe, 1999; Zörnig *et al*, 2001).

There are three main theories as to how cell might decide between apoptosis and growth arrest in response to p53: the dose-response model proposes that low levels of p53 induce growth arrest and that high levels trigger apoptosis, the cell-background model proposes that a response to p53 is determined by the cell milieu, and that the promoters of different p53-responsive genes are recognised by differently modified forms of p53 (Lane, 2001). However, it is also known that a p53-independent pathway of apoptosis also exists (Strasser *et al*, 1994; Naik *et al*, 1996). Many cell types are capable of executing apoptosis during embryogenesis through a route independent of p53 (Donehower *et al*, 1992). DNA repair systems are intricately involved in apoptotic signalling and the loss of mismatch repair or base excision and double-strand-break repair systems weakens that signalling (Hickman, 2002).

- 22 -

Normal tissue homeostasis is maintained through the regulation of cell proliferation and apoptosis. Thus, inhibition of cell death by suppression of genes that induce cell death or activation of genes that are responsible for cell survival, contribute to the development of tumours. Angiogenesis also plays a critical role in the inhibition of apoptosis by increasing the levels of growth factor thereby contributing to tumour progression (Naik *et al*, 1996). The formation of tumours is a complex process that also involves the accumulation of lesions in genes that normally regulate the pathways of cell proliferation, differentiation and death required for tissue development.

Some cells engage apoptosis more readily than others at the same levels of damage or when survival signals are withdrawn suggesting that certain types of cells can survive carcinogenic damage better than others. Some tissues have a higher incidence of cancer then others. The high prevalence of epithelial tumours would indicate that epithelial cells have a higher survival threshold than other cell types (Hickman, 2002).

# **1.3 Tumour Angiogenesis**

Angiogenesis, the process by which new blood vessels are formed, is an important event in physiological or pathological conditions as it is fundamental to healing, reproduction, and embryonic development. Accumulating evidence indicates that progressive tumour growth is dependent on angiogenesis (Harris, 1997). Tumours induce blood vessels from the host tissue to sprout capillary tips that migrate towards and ultimately penetrate the tumour, providing it with a circulating blood supply and, therefore, an almost limitless source of nutrients. Thus, cancer is driven by persistent unregulated angiogenesis.

It has been thirty years since it was first demonstrated that tumour growth is dependent upon adequate vascularisation (Folkman *et al*, 1971, Folkman 1972). Subsequently, two distinct phases of tumour growth were identified: the avascular and vascular phases (Folkman, 1975). The avascular phase of tumour growth consists of a selflimiting growth phase due to constraints on the diffusion of nutrients and catabolic products imposed by the tumour surface area in relation to its volume. In contrast, the vascular phase of tumour growth comprises a phase of rapid exponential expansion.

Within humans, most tumours persist *in situ* for a long period of time in an avascular, quiescent state, from months to years. In this phase the tumour may contain only a few million cells as the cancerous tumour remains limited and will not grow beyond 2 mm in size unless vascularisation has occurred (Weidner *et al*, 1991, Folkman, 1995). The process of neovascularisation of the tumour is a critical step in the progression of the disease. Without neovascularisation, even cells that are perfectly capable of growing without constraint cannot form tumours of clinically relevant size as the limited diffusion of  $O_2$  from nearby host vessels limits their growth. Additionally, cell division is balanced by cell death. When a subgroup of cells within the tumour switches to an angiogenic phenotype by changing the local equilibrium between positive and negative regulators of

angiogenesis, tumours will start to grow rapidly and become clinically detectable. The different properties of endothelial cells can also influence their response to angiogenic and antiangiogenic molecules as the microvascular and capillary endothelial cells in distinct tissues display different biological properties depending on microenvironmental or genetic backgrounds.

Angiogenesis is the only mechanism by which new vessels develop in adults. In an adult, the proliferation rate of endothelial cells is very low compared to many other cell types in the body. More than  $10^{13}$  endothelial cells line the inside of blood vessels and cover an area of approximately 1000 square meters in a 70 Kg adult. The turnover time of these cells can exceed 1000 days. Tumour endothelial cells divide much more rapidly than normal endothelial cells, up to 50 times as fast as in breast cancer (Harris, 1997). Excessive angiogenesis also contributes to the pathology of a number of diseases including; cancer (Weidner *et al*, 1991), atherosclerosis (Shweiki *et al*, 1992), arthritis (Peacock *et al*, 1992), psoriasis (Nickoloff *et al*, 1994), diabetic retinopathy (Adamis *et al*, 1994), endometriosis (Shifren *et al*, 1996). In the disease process, angiogenesis can be the key contributor to the pathologic process or a beneficial compensatory response.

#### **1.3.1 Angiogenic Process**

The angiogenic process is complex involving not only endothelial cell proliferation but also the digestion of the extracellular matrix surrounding the capillaries by collagenases and proteases. Consequently, endothelial cells migrate and new capillaries differentiate. New capillaries arise mainly from the small venules in response to the angiogenic stimulus imparted by the tumour. The multi-step process requires the interaction of numerous factors able to stimulate the growth and development of new blood vessels. This may occur either directly, by activating the endothelial cells or promoting

- 25 -

enzyme synthesis for the release of angiogenic factors, or indirectly, by stimulating the stromal cells to produce enzymes (collagenases) that cause the degradation of the extracellular matrix and induce angiogenesis, thus facilitating the release of endothelial cells (Hannahan and Folkman, 1996). Local dissolution of the basement membrane, due to proteases, allows endothelial cells to migrate towards the source of the angiogenic factor and align themselves end to end to form a sprout, which subsequently develops a lumen (Ausprunk and Folkman, 1977). A confluence of sprouts permits blood flow resulting in the formation of a new basement membrane around the immature vessel (Jain *et al*, 1997). The angiogenic process is demonstrated in Figure 1.6.

#### 1.3.2 Regulators of Angiogenesis

Multiple angiogenic-mitogenic molecules, released by both tumour cells and host cells, mediate the induction of angiogenesis. The increase in vasculature allows for the rapid growth of a tumour and increases the probability of tumour cells entering the circulation and, hence, subsequent dissemination (Ellis and Fidler, 1995). To assure efficient exchange of oxygen and nutrients, every cell of the body needs to be sufficiently close to a blood capillary. Neovascularisation is critical for sustained tumour growth as it allows oxygenation and nutrient perfusion of the tumour as well as removal of waste products (Skobe *et al*, 1997). The vascular and lymphatic systems are the major vehicles of transport of cancer cells released from the primary tumour. However, the extent of neovascularisation varies in different regions of the same tumour.

Several positive and negative regulators of angiogenesis have been identified which are produced by tumour cells as well as by inflammatory cells (**Table 1.2**). These cells are stored in the extracellular matrix in a bioactive form. The endothelial cell surface also contains receptors for angiogenesis factors. Most of these factors act as paracrine factors produced by local cells or by recruited monocytes.

- 26 -

Chapter 1. Introduction



Figure 1.6. Schematic representation of the initial stages of angiogenesis.

A capillary sprout emerging from a functional microvessel lined with endothelial cells in the interstitium is shown. During the process the basement membrane is degraded by proteases, endothelial cells migrate towards the stimulus, endothelial cells trailing behind the leading cell(s) proliferate, the lumen forms (canalisation) in the endothelial sprout, branches and loops form by confluence of sprouts to permit blood flow, the vessel is invested with pericytes and a basement membrane forms around the immature vessel (Jain *et al*, 1997).

$\mathbf{A} = \text{Activation}$	$\mathbf{E} = $ Permeability	
$\mathbf{B} =$ Proteolysis of extracellular matrix	$\mathbf{F} = \mathbf{M}$ igration and chemotaxis	
$\mathbf{C} = \mathbf{D}$ isruption of cellular adhesion	$\mathbf{G} = $ Inhibition and growth	
$\mathbf{D} = $ Proliferation of endothelial cells	$\mathbf{H} = $ Angiogenic factors/stimulus	

(Adapted from Sage, 1996).

Positive Regulators	Function	Reference
Angiogenin	Stabilise vessels and inhibit permeability	Fett et al, 1985
Fibroblast growth factors (Basic and Acidic)	Stimulates angio/arteriogenesis	Shing <i>et al</i> , 1984; Maciag <i>et al</i> , 1984
Granulocyte colony-stimulating factor	Endothelial mitogen	Bussolino et al, 1991
Hepatocyte growth factor	Stimulates angio/arteriogenesis Endothelial mitogen	Rosen et al, 1993; Bussolino et al, 1993
Interleukin-8	Endothelial mitogen and motogen	Koch et al, 1992
Placental growth factor	Weak endothelial mitogen	Maglione et al, 1991
Platelet-derived endothelial cell growth factor	Recruits smooth muscle cells, causes DNA synthesis	Ishikawa <i>et al</i> , 1989
Proliferin	Endothelial motogen	Jackson <i>et al</i> , 1994
Transforming growth factors $\alpha$ and $\beta$	Angiogenic in vivo	Schreiber <i>et al</i> , 1986; Roberts <i>et al</i> , 1986
Tumour Necrosis Factor $\alpha$	Stimulates formation of tubular structure of endothelial cells <i>in vitro</i>	Frâter-Schröder et al, 1987; Leibovich et al, 1987
Vascular endothelial growth factor	Stimulates angio/vasculogenesis, permeability, leukocyte adhesion	Senger et al, 1983; Ferrara et al, 1989
Negative Regulators	Function	Reference
Angiostatin (38 kDa fragment of plasminogen)	Inhibits proliferation, chemotaxis	O'Reilly et al, 1995
bFGF soluble receptor	Mitogen and chemotactic to endothelial cells	Schweigerer et al, 1987
Interferon alpha	Inhibition of endothelial cell motility and growth	Sidky <i>et al</i> , 1987
Metallo-proteinase inhibitors	Suppresses pathological angiogenesis	Moses et al, 1990
Placental proliferin-related protein	Inhibition of endothelial cell motility	Jackson et al, 1994
Platelet factor 4	Inhibits proliferation and VEGF-A activity, chemotaxis	Sharpe <i>et al</i> , 1990; Taylor and Folkman, 1982
Prolactin (16 kDa fragment)	Inhibition of endothelial cell motility and growth	Clapp <i>et al</i> , 1993
Thrombospondin-1	Inhibits proliferation, chemotaxis	Rastinejad et al, 1989; Iruela-Arispe et al, 1991
Transforming growth factor β	Inhibits proliferation of endothelial cells in vitro	Roberts et al, 1986

# Table 1.2. Endogenous positive and negative regulators of angiogenesis.

### **1.3.3 Promoters of Angiogenesis**

Some of the positive regulators of angiogenesis include; acidic and basic fibroblast growth factor (aFGF and bFGF), transforming growth factors  $\alpha$  and  $\beta$  (TGF- $\alpha/\beta$ ), tumour necrosis factor (TNF- $\alpha$ ), angiogenin, and vascular endothelial growth factor (VEGF). The FGFs were among the first angiogenic factors to be well characterised. FGF is not a specific endothelial cell growth factor and has many cell targets including smooth muscle cells and neurons (Klagsburn and Moses, 1999). These inducers of angiogenesis can be exported from tumour cells, mobilised from extracellular matrix or released by macrophages attracted to the tumour (Folkman, 1995). bFGF is distributed widely in normal and neoplastic tissues and its expression has been reported in a variety of human tumours, including brain (Folkman, 1995).

#### **1.3.3.1 Vascular Endothelial Growth Factor**

Vascular endothelial growth factor (VEGF) is the most potent and specific growth factor for endothelial cells. It plays a major role in regulating normal embryonic vasculogenesis and angiogenesis as well as tumour angiogenesis (reviewed in Dvorak *et al*, 1999; Ferrara, 1999). It was originally isolated as the vascular permeability factor (Senger *et al*, 1983) and subsequently as a growth factor which induced proliferation of endothelial cells a few years later (Ferrara and Henzel, 1989). VEGF is a 46 kD dimeric glycoprotein and is a specific endothelial cell mitogen that has a high affinity for heparin binding (Neufield *et al*, 1999). The human gene has been assigned to chromosome 6p21.3 (Vincenti *et al*, 1996) and is organised in eight exons, separated by 7 introns, and by its coding regions which spans approximately 14 kb (Tischer *et al*, 1991) (Figure 1.7).

There are currently four family members which are dimeric glycoproteins; VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF) (Senger *et al*, 1983; Olofsson *et al*, 1996; Joukov *et al*, 1996; Achen *et al*, 1998; Maglione *et al*, 1991). VEGF-

A is found in at least six splice variant isoforms that have been designated VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>183</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub>, which refers to the number of amino acid residues in the mature protein (Leung *et al*, 1989; Poltorak *et al*, 1997; Keck *et al*, 1989; Houck *et al*, 1991; Jingjing *et al*, 1999; Tischer *et al*, 1991). The isoforms arise from alternative splicing of mRNA transcripts and each exhibit different heparin-binding affinity (Houck *et al*, 1992). The independent roles of each isoform are poorly understood.

All five peptides are biologically active but differ in their physiochemical properties. The N-terminal region of VEGF contains eight cysteine residues that enable the isoforms to dimerise. All of the known exons of the VEGF gene are expressed in VEGF<sub>206</sub>, whereas the other isoforms are characterised by partial or complete deletion of exon 6 (Jingjing et al, 1999) (Figure 1.7). The major subtype VEGF<sub>121</sub>, consists of exons 1-5 encoding 141 amino acids of the N-terminal region and six C-terminal amino acids encoded by exon 8 (Tischer *et al*, 1991). Complete deletion of exon 6 is found in VEGF<sub>121</sub> and VEGF<sub>165</sub>, whereas only a part of exon 6 is missing in VEGF<sub>145</sub>, VEGF<sub>183</sub>, and VEGF<sub>189</sub>. Exon 6 encodes a stretch of highly basic amino acids and the exon 7 encoded region of VEGF displays a mild heparin-binding ability (Ferrara et al, 1991). VEGF<sub>121</sub> is a soluble form of VEGF and possess no heparin binding ability whereas VEGF<sub>189</sub> and VEGF 165 do bind heparin but VEGF<sub>189</sub> binds more strongly than does VEGF<sub>165</sub> (Houck et al, 1992). VEGF<sub>165</sub> is the most abundant of the five isoforms in vivo and is commonly used in studies investigating the biological effects of VEGF (Ferrara, 1993). The different isoforms have distinct biological activity where the differences in their ability to regulate vascular permeability or their mitogenic activity may involve splicing out these important domains (Keyt et al, 1996).

Chapter 1. Introduction



Figure 1.7. Structure of the VEGF gene and its isoforms.

Structural characterisation of the human VEGF gene shows that it comprises eight exons. VEGF isoforms arise from alternative splicing, where VEGF<sub>121</sub> lacks exons 6 (composed of exons 6A, 6B and 6C) and 7, VEGF<sub>165</sub> lacks exon 6, whilst only part of exon 6 is missing is VEGF<sub>124</sub>, VEGF<sub>183</sub> and VEGF<sub>189</sub>. All known exons are expressed in VEGF<sub>206</sub>. (Adapted from Tischer *et al*, 1991; Jingjing *et al*, 1999).

### 1.3.3.1.1 VEGF Receptor Family

VEGF is specific for vascular endothelium as it binds to membrane receptors that belong to the tyrosine kinase family of growth factor receptors, which are found only on vascular endothelial cells and may be up-regulated in many tumours (Guillemin & Krasnow, 1997). VEGF binds the receptor tyrosine kinases and stimulates the local proliferation of blood capillaries to increase oxygen delivery. A 3-D molecular model of the VEGF receptor binding domain is shown in **Figure 1.8** (Müller *et al*, 1997).

The VEGF receptor family includes flt-1 (fms-like-tyrosine kinase) (Shibuya et al, 1990; Finnerty et al, 1993), flt-4 (Pajusola et al, 1992; Finnerty et al, 1993; Pajusola et al, 1994), flk-1 (also known as KDR) (Matthews et al, 1991, Sarzani et al, 1992) and neuropilin receptors 1 and 2 (Neufeld et al, 1999). The receptors are transmembrane proteins that contain an extracellular, a transmembrane and an intracellular tyrosine kinase domain. The flt-1 gene encodes the VEGF receptor 1 (VEGFR-1), flk-1 genes encode the VEGFR-2 and the flt-4 gene encodes VEGFR-3, all of which can be upregulated by exposure to VEGF. The VEGFR-1 is generated by mRNA splicing and both VEGFR-1 and VEGFR-2 bind VEGF-A. The flt-1 and flk-1 genes were the first discovered and therefore the best characterised. The flt-1 gene contains a HIF-1 $\alpha$  binding site and responds to hypoxic induction. Although the flk-1 gene also responds to hypoxic induction with increased expression, it does not contain a HIF-1a binding site and this response is believed to occur via a posttranscriptional mechanism (Giordano, 1999). The flt-4 receptor preferentially binds to VEGF-C and -D. As both VEGF and its receptors are required for angiogenesis, they are considered to be one of the most crucial regulatory pathways in angiogenesis.



Figure 1.8. A 3-D molecular model of the VEGF receptor binding domain (Müller *et al*, 1997)

#### **1.3.3.1.2 VEGF Induction and Expression**

VEGF is known to be a major factor in the neovascularisation of tumours as it is a potent angiogenic factor. VEGF functions as a survival factor for newly formed blood vessels during developmental neovascularisation but it is not required for the maintenance of mature vessels (Benjamin and Kishat, 1997). VEGF is essential for angiogenesis and is produced by many normal and tumour cells, including glioma and carcinomas (Thomas, 1996). VEGF gene expression is induced by environmental stresses such as hypoxia (Shweiki *et al*, 1992), hypoglycaemia (Schweiki *et al*, 1995), in the presence of growth factors (Horiuchi *et al*, 1997), oncogenes (Mukhopadhyay *et al*, 1995) and tumour suppressor mutations (Fontanini *et al*, 1997).

The major control point for the hypoxic induction of the VEGF gene is the regulation of the steady state levels of mRNA (Ikeda *et al*, 1995; Levy *et al*, 1996). Hypoxia is a potent inducer of VEGF mRNA expression *in vitro* and VEGF up-regulation is achieved by increased RNA stability (Levy *et al*, 1996). Hypoxia up-regulates VEGF expression by activating a hypoxia-inducible factor–1 (HIF-1) binding sequence in the 5'-flanking region of the VEGF promoter, which results in increased VEGF mRNA transcription and stability (Liu *et al*, 1995). This region functions as a cis-element regulating the hypoxic induction of VEGF. It has been suggested that an adjacent sequence located immediately downstream within the hypoxia response elements is also essential for the hypoxic induction of the promoter (Lui *et al*, 1995). A discrepancy between the transcriptional rate and the steady-state mRNA level may be induced by hypoxia (Levy *et al*, 1996). It has been demonstrated that hypoxic induction of VEGF in C6 glioma cells is due to both transcriptional activation and increased stability of mRNA mediated by the presence of hypoxia responsive elements in the VEGF gene (Ikeda *et al*, 1995).

VEGF is the most widely studied angiogenic factor. However, little is known about the molecular mechanism of hypoxic induction and activation of expression of VEGF in
tumours. VEGF is thought to act as a paracrine angiogenic factor, that is, it is produced and secreted by the tissue to which new capillaries grow (Klagsburn and Moses, 1999). Thus, its expression results in the delivery of oxygen and nutrients required for tumour growth. To date there is no evidence for translational or post-translational control of VEGF expression. p53 protein depresses VEGF production and supports the secretion of thrombospondin-1, a multifunctional angiogenesis inhibitor that modulates endothelial cell adhesiveness (Bussolino *et al*, 1997). Thus, p53 mutations induce angiogenesis.

#### 1.3.3.1.3 VEGF and Angiogenesis

It has long been recognised that increased expression of VEGF is essential for the establishment of angiogenesis in most solid tumours. A model for this induction has recently been put forth suggesting that increased levels of VEGF result from the synergistic effects of tumour hypoxia and tumour-specific genetic alterations (mutations) involving oncogenes and tumour suppressor genes. The increased expression of VEGF results in the formation of dysfunctional vasculature that cannot adequately perfuse the complete tumour. Tumour progression is then dependent upon the cellular adaptation of hypoxia that is independent of angiogenesis. Consequently, most solid tumours share the similar characteristic that poor clinical outcome is significantly correlated with vascular density and tumour hypoxia (Semenza, 2000).

It is known that VEGF plays an important role in embryogenesis and it functions as a major angiogenic factor in both normal and in pathological conditions. It has been proposed that VEGF participates in the development of diabetic retinopathy. In simple diabetic retinopathy, VEGF may also be responsible for retinal microvascular hyperpermeability, an early functional disorder which occurs in this phase of diabetic retinopathy (Murata *et al*, 1996). It has also been suggested that the overexpression of VEGF plays an important role in the development of blood-retinal barrier (BRB)

- 35 -

breakdown in simple diabetic retinopathy (Matthews et al, 1997).

Recently, a study has shown that the potential tumour suppressor gene p73 can function as a transcriptional repressor of the VEGF gene promoter suggesting a role for p73 in angiogenesis by repressing VEGF expression (Salimath *et al*, 2000). p73 behaves similarly to p53 in its inhibitory effect on VEGF expression. Tumour suppressor genes such as p53 and p73 play a role in tumour cell proliferation and are also able to modulate cell survival functions by inhibiting VEGF expression.

#### **1.3.4 Inhibitors of Angiogenesis**

Several angiogenesis inhibitors have recently been described (Table 1.2). Some of these inhibitors are natural molecules that act directly on endothelial cells to block their migration, proliferation and/or their ability to form capillary-like tubes, and include proteins such as angiostatin, endostatin, platelet factor-4, and thrombospondin-1 (reviewed in Hanahan and Folkman, 1996).

Thrombospondin-1 (TSP-1) was one of the first natural angiogenesis inhibitors to be described, where its expression is inversely correlated with angiogenic activity (Rastinejad *et al*, 1989). It is a secreted heparin binding glycoprotein found in the circulation and is associated with the extracellular matrix. It is also constitutively secreted by certain types of normal cells (Folkman, 1995). It is known that TSP-1 is regulated by p53, where loss of p53 results in suppression of TSP-1 and a concomitant increase in angiogenic activity (Dameron *et al*, 1994).

Angiostatin is an angiogenesis inhibitor that forms as a proteolytic fragment of plasminogen by the action of elastase or matrix metalloproteinase-2 (O'Reilly *et al*, 1994; Giordano, 1999). Endostatin is a proteolytic fragment of the carboxyl terminus of collagen XVIII and is a potent inhibitor of the growth of primary and metastatic tumours (O'Reilly *et al*, 1997) and induces apoptosis in endothelial cells (Dhanabal *et al*, 1999).

Additionally, VEGF can be neutralised by specific anti-VEGF antibodies and sequestered by soluble receptors (Presta *et al*, 1997; Goldman *et al*, 1998). Moreover, specific inhibitors of VEGF receptor kinases can inhibit VEGF-mediated signal transduction (Fong *et al*, 2002).

# 1.4 Tumour Hypoxia

Angiogenesis can be initiated by a variety of physical signals, including tissue hypoxia. As tumours grow and divide rapidly they exhaust local supplies and central regions of tumour become hypoxic. Resistant tumour cells tend to develop in oxygen deprived areas of the tumour (Harris, 1997). The blood vessels supplying tumours are relatively poorly organised and disordered compared with those of normal tissues, leading to inefficient delivery of oxygen and other nutrients to many of the cells in tumours. As oxygen diffuses from blood capillaries through the mass surrounding cells it is depleted by normal cell metabolism.

The degree of hypoxia experienced by a cell *in vivo* is determined by its proximity to the arterial blood supply and rates of oxygen consumption by the cell and its neighbours. The human body maintains sufficient oxygen to meet the metabolic demands of each cell thus, hypoxia is a cellular phenomenon characterised by oxygen levels that are inadequate to meet metabolic demands (Jiang *et al*, 1996). Cells, which are hypoxic because of their distance from blood vessels, are likely to also be deprived of their nutrients and exposed to acidic metabolites such as lactic acid. Transient changes in the perfusion of individual blood vessels may further reduce oxygen transport and change the oxygenation status of some cells near to blood vessels over relatively short time periods (Webster, 1998).

Hypoxia occurs in many common pathological conditions and moderate levels of hypoxia are also known to exist under normal conditions in some normal tissues, for example, skin, oesophagus and liver (Webster, 1998). When oxygen becomes rate limiting

## Chapter 1. Introduction

the cell responds by undergoing the 'Pasteur effect'; as ATP generation by oxidative phosphorylation begins to fall off due to lack of oxygen, the cell shuts down nonessential cell functions and the resulting energetic deficit is made up by activation of anaerobic ATP supply pathways (Hochachka *et al*, 1996). Hence, the cell abandons oxidative phosphorylation and relies solely on glycolysis for energy production. As a consequence, the expression of glucose transporters and glycolytic enzymes is increased and transcriptionally upregulated to compensate for the decrease in the oxidative capacity to generate ATP. Expression of genes encoding components of the electron transport chain is repressed, whereas transcription of genes encoding enzymes of the glycolytic pathway is activated (Semenza *et al*, 1994). Factors that promote angiogenesis are then released. Hypoxia is also able to stimulate p53 levels and activate the p53 protein which may trigger apoptosis (Graeber *et al*, 1996). Under conditions of severe hypoxic stress, cell proliferation is inhibited by the tumour suppressor p53 and cell death follows through the transactivation of genes such as  $p21^{Wa/I/Ctp1}$  and the pro-apoptotic gene *bax*. Tumours that do not adapt to severe oxygen and nutrient deprivation undergo apoptosis or necrosis.

Many biological processes are concerned with adaptation to the availability of oxygen and oxygen tension is an important regulator of gene expression. Hypoxia influences the proliferation tumour cells (Carmeliet *et al*, 1998), the rate of apoptotic death (Graeber *et al*, 1996) and metastasis (Brizel *et al*, 1996). Multiple hypoxic responses including adaptation to anaerobic metabolism (glycolysis), erythropoiesis, angiogenesis, vasodilation and genetic alterations are all under the control of a single transcription factor, hypoxia inducible factor-1 (HIF-1). As tissue hypoxia is a common and prognostically important feature of rapidly growing tumours (Höckel *et al*, 1996), HIF-1 plays a potential role in cancer as well as a number of other diseases.

#### 1.4.1 Hypoxia Inducible Factor-1 (HIF-1)

The discovery that HIF-1 is a key regulator of  $O_2$  homeostasis initially resulted from analysis of the molecular mechanisms by which erythropoietin gene transcription was activated in response to hypoxia (Semenza *et al*, 1991; Semenza, 1999). The identification of a critical DNA control-sequence termed the EPO 3' enhancer, and its corresponding transcription factor (Madan and Curtin, 1993; Beck *et al*, 1993), led to the characterisation of a short sequence that functions as a hypoxia response element (HRE). Transfection studies of this enhancer sequence found that is was involved in the regulation of genes encoding different gene products, such as glycolytic enzymes (Maxwell *et al*, 1993). Further analysis of the erythropoietin 3' enhancer defined HIF-1 as a DNA binding complex that is critical for the function of this sequence (Wang *et al*, 1995).

HIF-1 is a transcription factor that is a central component of the hypoxic response pathway as it regulates the transcriptional response of a number of genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), glucose transporter 1 (GLUT-1) and glycolytic enzymes under conditions of hypoxia (Semenza and Wang, 1992; Levy *et al*, 1995; Melillo *et al*, 1995; Firth *et al*, 1994; Firth *et al*, 1995; Semenza *et al*, 1994; Guillemin and Krasnow 1997). More recently a novel HIF-1 responsive gene, RTP801 has been identified and is known to be involved in apoptosis (Shoshani *et al*, 2002).

# 1.4.1.1 Structure of HIF-1

HIF-1 is a heterodimer consisting of an alpha ( $\alpha$ ) and beta ( $\beta$ ) subunit with molecular weights of 120 kDa and 91-94 kDa respectively. Both subunits are members of the basic helix-loop-helix (bHLH)-*per-arnt-sim* (PAS) family of transcription factors (Wang *et al*, 1995, Guillemin and Krasnow, 1997) and contain bHLH domains responsible for heterodimerisation through the two helices and DNA binding through their basic domain (Figure 1.9). Both heterodimers also contain a PAS motif that is found in a number of transcription factors including the Drosophila proteins Period, Single-minded, Trachealess and mammalian proteins such as Aryl Hydrocarbon Receptor (AHR). PAS domains are also required for dimerisation and target gene specifity (Jiang *et al*, 1996; Zelzer *et al*, 1997). The C-terminal half of HIF-1 $\alpha$  contains domains required for hypoxia-induced nuclear translocalisation, protein stabilisation and transactivation (Huang *et al*, 1998; Jiang *et al*, 1996; Kallio *et al*, 1998). The HIF-1 $\alpha$  subunit is composed of 826 amino acids whereas the HIF-1 $\beta$  subunit is composed of 789 amino acids (Wang *et al*, 1995).

HIF-1 $\alpha$  is encoded by the human HIF1A gene (Iyer *et al*, 1998). Recent sequencing of the HIF1A gene promoter found that it belongs to the TATA-less promoter gene family (Iyer *et al*, 1998). The HIF1A promoter is composed of a GC rich sequence containing several Sp1 putative binding sites, which are located downstream from the transcription initiation site. The promoter also contains several putative HREs which are HIF-1 *cis* acting elements (Iyer *et al*, 1998). Constitutive transcription of the TATA-less promoters is dependent on Sp1, NF-1 binding sites or Initiator sequences located upstream or downstream from the +1 transcription start site (Minet *et al*, 1999).



Figure 1.9. Structure of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-1 $\beta$ .

Under hypoxia, HIF-1 $\alpha$  accumulates, translocates to the nucleus, dimerises with HIF-1 $\beta$ , and binds to the target DNA sequence within the hypoxiaresponsive element found in the promoter region of different oxygen-regulated genes. The N-terminal half of the molecule containing the bHLH-PAS domain is required for dimerisation and DNA binding (Jiang *et al*, 1996). The C-terminal contains domains required for hypoxia-induced nuclear localisation, protein stabilization and transactivation (Huang *et al*, 1998; Jiang *et al*, 1996; Kallio *et al*, 1998).

#### 1.4.1.2 HIF-1 Transcriptional Activity

It has been demonstrated that elements in the C-terminal transactivating domain (775-826 AA) of HIF-1 $\alpha$  are responsive to low oxygen concentrations (Huang *et al*, 1998; Pugh *et al*, 1997). Under hypoxia, a rapid accumulation of HIF-1 $\alpha$  occurs in the nucleus where it then dimerises with HIF-1 $\beta$  (Wang *et al*, 1995). Both dimerisation and DNA binding are essential for the function of HIF-1 (Jiang *et al*, 1996); the transactivation domains in the carboxy-terminal portion are then able to participate in gene transcription. The amount of HIF-1 $\alpha$  protein in the nucleus is rate limiting and determines the functional activity of the HIF-1 complex (Semenza, 1999). A molecular model for the dimerised subunits through the bHLH domain was recently determined and is shown in Figure 1.10A (Michel *et al*, 2000).

The HIF- $\beta$  factor is also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 $\beta$  can form heterodimers with other proteins, such as the aryl hydrocarbon receptor, and it is abundantly present (Reyes *et al*, 1992). Although both heterodimers contain C-terminal transactivation domains, the HIF-1 $\beta$  transactivation domain is not implicated in the hypoxic response (Wood *et al*, 1996). A conflict regarding the transcriptional regulation of HIF-1 $\alpha$  exists in the literature. The expression of the HIF-1 $\alpha$  gene has been demonstrated to be rapidly up-regulated in response to low cellular oxygen tension (Huang *et al*, 1996; Bergeron *et al*, 1999), however other studies have reported that at the mRNA level HIF-1 $\alpha$  and HIF-1 $\beta$  are both constitutively expressed and do not seem to be significantly modified by hypoxia (Morel and Barouki, 1999). Conversely, while the HIF-1 $\beta$  protein is found in normoxic cells, nearly no HIF-1 $\alpha$  protein can be detected under these conditions.

Transcriptional activation is mediated by the binding of HIF-1 to the 5' flanking sequence of the VEGF gene, a *cis*-acting hypoxia response element (HRE) located 1 kb 5'

to the transcriptional start site (Forsythe *et al*, 1996; Carmeliet *et al*, 1998; Ryan *et al*, 1998), or to the target sequence within the HRE found in the promoter region of a number of different oxygen-related genes. The HRE is a specific 8 bp motif (5' (G/C/T)-ACGTGC-(G/T) 3') target sequence and was first identified in the erythropoietin gene promoter (Semenza and Wang, 1992), a glycoprotein hormone that regulates mammalian erythrocyte production and oxygen delivery to tissues. A 3D molecular model for the binding of HIF-1 to its DNA consensus sequence HRE has been proposed and is shown in **Figure 1.10B** (Michel *et al*, 2000).

#### 1.4.1.3 Mediators of HIF-1 Transcriptional Activity

In addition to hypoxia, the HIF-1 complex is inducible by particular transition elements such as cobalt ions and iron chelating agents such as desferrioxamine (DFO) but not by inhibitors of mitochondrial respiration such as cyanide or azide (Pugh *et al*, 1997). This suggests that at least one key component in the pathway is a heme protein (Kallio *et al*, 1997). The underlying mechanisms of action are not known however, it has been suggested that they increase (Chandel *et al*, 1998) or decrease (Bunn and Poyton, 1996) the level or production of reactive oxygen species, which may serve as signaling molecules or mediators of hypoxia-induced gene transcription (Semenza, 1999). It has recently been suggested that the oxygen signal is converted to a redox signal that then may trigger a kinase cascade and/or regulate HIF-1 directly (Chandel *et al*, 2000; Haddad *et al*, 2000; Chandel and Schumacker 2000).

It is known that HIF-1 $\alpha$  binds directly to and stabilises p53 (An *et al*, 1998) and HIF-1 $\alpha$  mediated transcriptional activity is also inhibited by p53 through interaction with p300 (Blagosklonny *et al*, 1998). The transactivational activator/histone acetyltransferase proteins CBP (CREB binding protein) and p300 have been shown to be directly associated with HIF-1 $\alpha$  and increase transactivational activity through their cysteine-histidine rich



Figure 1.10. Proposed 3D molecular models of HIF-1. A) 3D molecular model of the HIF-1 $\alpha$ /HIF-1 $\beta$  (ARNT) heterodimer through the bHLH domain. B) 3D molecular model for the binding of HIF-1 to its DNA consensus sequence HRE (Michel *et al*, 2000). domain CH1 (Ebert *et al*, 1998; Kallio *et al*, 1998). The interaction of the transactivational domains in HIF- 1 $\alpha$  with coactivators such as CBP, p300, SRC-1 and TIF2, is regulated by the cellular oxygen concentration and redox state (Kallio *et al*, 1998; Ema *et al*, 1999; Carrero *et al*, 2000).

An additional study suggests that p53 accumulation during hypoxia requires an increase in a mitochondrial dependent ROS signal in human breast carcinoma MCF-7 cells (Chandel *et al*, 2000). Thus, it is thought that mitochondria regulate p53 protein levels during hypoxia through a redox-dependent mechanism involving ROS that create an oxidising signal in the cytosol. The cell presumably senses its oxygen concentration through reactive oxygen species so that the stabilisation of the HIF-1 $\alpha$  protein is redox induced (Salceda and Caro, 1997).

A recent report has found that advanced glycation end products (AGEs) induce HIF-1 $\alpha$  accumulation through an ERK dependent pathway (Treins *et al*, 2001). AGEs and their intermediates have been implicated in the pathophysiological dysfunction associated with the vascular complications of diabetes mellitus (Brownlee, 1995; 2001). ERK activates HIF-1 transcriptional activity by promoting the phosphorylation of HIF-1 $\alpha$ subunit (Richard *et al*, 1999; Minet *et al*, 2000). The PI3K dependent pathway is also involved in HIF-1 activation (Jiang *et al*, 2000; Zundel *et al*, 2000; Zhong *et al*, 2000). Overexpression of an activated form of phosphatidylinositol 3-kinase (PI3K) or PKB, or expression of dominant-negative phosphatase and tensin homologue stimulates HIF-1 $\alpha$ expression in response to hypoxia and induces angiogenesis. PI3K catalyses the conversion of phosphatidylinositol 4-phosphate and phosphatidylinositol 3,4,5-triphosphate to phosphatidylinositol 3,4 phosphate and phosphatidylinositol 3,4,5-triphosphate. All of these products are allosteric activators of phosphatidylinositol-dependent kinase 1 (Sandau *et al*, 2000). Additionally, epidermal growth factor regulates HIF-1 $\alpha$  expression through the signalling cascade PI3K/PKB/FRAP (FKBP-rapamycin-associated protein) (Zhong et al, 2000).

# 1.4.1.4 HIF-1a Degradation

The HIF-1 $\alpha$  half-life has been demonstrated to be less than 5 minutes in normal oxygen concentrations (Huang *et al*, 1998; Wang *et al*, 1995), making it one of the most quickly degraded proteins found in mammals. The formation of HIF-1 is dependent on the stability of the HIF-1 $\alpha$  subunit, which is protected from ubiquitination and proteasomal degradation in hypoxic conditions (Huang *et al*, 1996; Huang *et al*, 1998). In oxygenated cells HIF-1 $\alpha$  is rapidly ubiquitinated and degraded by the ubiquitin-proteasome system, thus keeping its protein level extremely low (Salceda and Caro, 1997; Huang *et al*, 1998; Kallio *et al*, 1999). HIF-1 $\alpha$  is stabilised by blockage of the ubiquitin-proteasome pathway with specific proteasome inhibitors thereby stabilising the HIF-1 complex under normoxic conditions (Figure 1.11). Ubiquitin-mediated proteolysis functions by covalently linking the protein for destruction to a small, highly conserved ubiquitin protein. This covalent attachment signals their destruction by the 26S proteasome, a large complex with multiple proteolytic activities (DeMartino and Slaughter, 1999).

An area in HIF-1 $\alpha$ , located in the central region of the protein (401-603 AA), has been found to be responsible for oxygen dependent degradation and had been labelled as the oxygen dependent degradation domain (ODD) (Huang *et al*, 1998). The whole ODD domain may be required for the ubiquitin/proteasome-dependent degradation of HIF-1 $\alpha$  as it remains stable even under normoxic conditions when part of the ODD is deleted (Huang *et al*, 1998). The region also contains two PEST motifs (proline, glutamic acid, serine, threonine) which have been demonstrated to be a trademark of rapid intracellular degradation (Rogers *et al*, 1986). An additional highly conserved sequence has been found

Chapter 1. Introduction



Figure 1.11. Activation of HIF-1 by hypoxia and subsequent degradation in normoxia via ubiquitination.

During hypoxia, ubiquitination is prevented and HIF-1 $\alpha$  is stabilised. It then forms a stable heterodimer with HIF-1 $\beta$  (ARNT) and the HIF-1 $\alpha\beta$  heterodimer translocates to the nucleus where it binds to hypoxia-response elements (HRE) in the 5' intronic and 3' sequences of genes that are regulated by hypoxia, promoting gene transcription. CBP and p300 are transcriptional coactivators, +1 indicates the transcriptional start site. Under normoxic conditions, an unknown oxygen sensor targets HIF-1 $\alpha$  for proteolytic degradation. HIF-1 $\alpha$  is rapidly ubiquitinated (Ub) by a characterised complex containing pVHL, Cul2 (cullin 2), E2 (unknown ubiquitin-conjugating enzyme), Rbx1 (a RING-H2 finger protein), and then destroyed by the proteasome.

between residues 557-571 of the HIF-1 $\alpha$  protein that was shown to be involved in hypoxic regulation (Srinivas *et al*, 1999). These areas found within the HIF-1 $\alpha$  protein help to understand why the protein degrades so rapidly however they have yet to explain the mechanism or mechanisms involved in its degradation. It has been suggested that phosphorylation or association with interacting proteins may play a role (Pugh *et al*, 1999; Srinivas *et al*, 1999), however further studies are required to confirm or eliminate these theories.

The product of the Von Hippel Lindau (pVHL) tumour suppressor gene has also been suggested to be involved in HIF-1 $\alpha$  degradation. Inactivation of the VHL gene is associated with several types of cancer, most notably central nervous system haemangioblastomas and renal carcinoma. pVHL is also associated with ubiquitin-protein ligase activity (Lisztwan *et al*, 1999). The association of HIF-1 $\alpha$  with pVHL is dependent on the HIF-1 $\alpha$  ODD domain (Maxwell *et al*, 1999), within which is a highly conserved region that contains a binding site for pVHL. pVHL, along with elongin B and elongin C, will bind directly to HIF-1 $\alpha$  subunits in the presence of oxygen and targets them directly for polyubiquitination and destruction (Cockman *et al*, 2000; Kamura *et al*, 2000). The HIF-1 complex has been demonstrated to be active, in addition to HIF-1 $\alpha$  being constitutively expressed, in cells defective in VHL (Maxwell *et al*, 1999). In cells that constitutively lack the VHL protein (pVHL), HIF-1 $\alpha$  subunits are stable and HIF-1 is active, overproducing mRNAs encoded by HIF target genes. The cells have a high expression of HIF-1 induced genes and glucose transporter 1 (GLUT-1) under both normoxia and hypoxia.

A recent study has found that HIF-1 $\alpha$  targeted for ubiquitination destruction is regulated through hydroxylation of a proline residue (HIF-1 $\alpha$  P564) by an enzyme termed HIF- $\alpha$  prolyl-hydroxylase (HIF-PH), which functions as an oxygen sensor as it requires

- 48 -

both molecular oxygen and iron as a cofactor (Jaakkola *et al*, 2001; Ivan *et al*, 2001; Masson *et al*, 2001). HIF-PH is thought to regulate the HIF transcriptional cascade as it mediates the binding of the HIF-1 $\alpha$  subunit to pVHL and the subsequent assembly of a complex that activates ubiquitination. However, under hypoxic conditions, the proline hydroxylation is inhibited and HIF-1 $\alpha$  is stabilised. This discovery is a further step towards deciphering how HIF controls gene expression in response to changes in oxygen tension.

In addition to stabilising HIF-1 $\alpha$ , hypoxia also induces the nuclear translocation of the protein, providing a potential means by which it escapes from proteasomal degradation (Kallio *et al*, 1999). HIF-1 $\alpha$  nuclear localisation has been proposed as a regulatory step involved in its stabilisation (Tanimoto *et al*, 2000). A recent report contradicts this finding by suggesting that nuclear translocation is not necessary for HIF-1 $\alpha$  stabilisation and that both nuclear and cytoplasmic proteasomes are fully competent for HIF-1 $\alpha$  degradation in an O<sub>2</sub> dependent manner (Berra *et al*, 2001). This also contrasts with the mechanism that controls p53 degradation via MDM2 (Freedman and Levine, 1998) in that HIF-1 $\alpha$  protein is degraded in both subcellular locations providing a possible explanation for the extremely short half-life of HIF-1 $\alpha$  protein.

# 1.4.1.5 HIF Splice Variants and Related Proteins

Several alternatively spliced variants of human HIF-1 $\alpha$  mRNA have been recently identified (Gothié *et al*, 2000; Yang-Sook *et al*, 2002). Alternative splicing between exons 1 and 2 produces an additional 3 bp at their junctions without a frame shift, and alternative splicing between 13 and 14 results in the loss of exon 14, producing a frame shift and an immediate termination codon (Gothié *et al*, 2000). The latter variant displays oxygentension-dependent regulation, as it retains the whole of the ODD domain, and transactivation by the VEGF promoter. A more recent alternative variant lacks exons 11

and 12, resulting in a frame shift and a shorter form of HIF-1 $\alpha$  which lacks part of the ODD domain, both TADs and the C-terminal nuclear localisation signals (Yang-Sook *et al*, 2002).

Several proteins that are structurally related to HIF-1 $\alpha$  and HIF-1 $\beta$  have also been identified. These include HIF-2 $\alpha$  (Ema *et al*, 1997; Flamme *et al*, 1997, Tian *et al*, 1997) and HIF-3 $\alpha$  (Gu *et al*, 1998), as well as ARNT2 (Hirose *et al*, 1996) and ARNT3, which is also known as MOP 3 (Ikeda and Nomura, 1997; Takahata *et al*, 1999). HIF-2 $\alpha$  was initially described as endothelial and foetal specific and named endothelial PAS protein-1 (EPAS-1)/HIF-related factor (HRF)/HIF-like factor (HLF) and MOP 2 (Tian *et al*, 1998, Flamme *et al*, 1997; Ema *et al*, 1997). HIF-2 $\alpha$  exhibits similar characteristics to HIF-1 $\alpha$  and its mRNA is abundantly expressed in a variety of organs in a normoxic state in patterns similar to those of VEGF mRNA, whereas HIF-1 $\alpha$  mRNA is ubiquitous at much lower levels (Wenger *et al*, 1997). Their biological functions have yet to be determined apart from the finding that mice lacking HIF-2 $\alpha$  die at midgestation due to catecholamine deficiency and heart failure (Tian *et al*, 1998).

Recently, a novel ARNT-interacting cytosolic protein (AINT) has been described in mice that is reported to interfere with the formation of HIF-1 by sequestering ARNT in the cytoplasm (Sadek *et al*, 2000). The finding of these new family members suggests that they may play a role in mediating the response to hypoxia and demonstrates the complexity involved in determining the precise function and involvement of HIF-1 in human disease.

# 1.5 HIF-1 and Human Disease

There have been several recent publications that have demonstrated the involvement of HIF-1 in human disease (Zagzag *et al*, 2000; Treins *et al*, 2001). HIF-1 is thought to play an important role in the pathogenesis of a number of conditions including myocardial ischemia, cerebral ischemia, lung disease and tumour vascularisation (Martin *et al*, 1998; Yu *et al*, 1999; Forsythe *et al*, 1996). Over 40 different target genes have been shown to be activated by HIF-1 including VEGF, erythropoietin, insulin-like growth factor 2, glycolytic enzymes and glucose transporter 1 (Semenza, 2002; Semenza *et al*, 1996, Forsythe *et al*, 1994, Ebert *et al*, 1995). The overexpression of HIF-1 $\alpha$  may promote key aspects of tumour progression through the activation of these target genes including metabolic adaptation to hypoxia, the promotion of angiogenesis, cell survival and proliferation (**Table 1.3**). Activation of HIF-1 is known to occur as a primary genetic event and as a result of local hypoxia in the tumour microenvironment.

# 1.5.1 Cancer

HIF-1 $\alpha$  is overexpressed in the majority of common human cancers including breast and brain (Zhong *et al*, 1999), and significant correlations have been found between HIF-1 $\alpha$  protein expression, tumour grade and tumour vascularisation (Zagzag *et al*, 2000). Thus, HIF-1 plays an important role in promoting tumour progression in these and other cancers (Semenza, 2000).

Gene Product	Reference	
Cell Proliferation and Viability (Increased O <sub>2</sub> delivery)		
p21	Carmeliet et al. 1998	
Insulin-like growth factor 2 (IGF-2)	Feldser et al, 1999	
IGF binding protein 1	Tazuke et al, 1998	
IGF binding protein 3	Feldser et al, 1998	
Transforming Growth Factor $\beta_3$	Caniggia et al, 2000	
Energy Met	tabolism	
Adenylate kinase 3	Wood <i>et al</i> , 1998	
Aldolase A	Iyer et al, 1998	
Aldolase C	Iyer et al, 1998	
Enclase 1	Iyer <i>et al</i> , 1998	
Glucose transporter 1	Iyer <i>et al</i> , 1998	
Glucose transporter 3	Iyer <i>et al</i> , 1998	
Glyceraldehyde phosphate dehydrogenase	Iyer <i>et al</i> , 1998	
Hexokinase 1	Iyer <i>et al</i> , 1998	
Hexokinase 2	Iyer <i>et al</i> , 1998	
Lactate dehydrogenase A	Iyer <i>et al</i> , 1998	
Phosphofructokinase L	Iyer et al, 1998	
Phosphoglycerate kinase 1	Carmeliet et al, 1998	
Pyruvate kinase M	Iyer et al, 1998	
Erythrop (Increased O	ooiesis delivery)	
Ceruloplasmin	Mukhopadhyay et al. 2000	
Erythropoietin	Jiang et al 1996	
Transferrin	Rolfs et al. 1997	
Transferrin Receptor	Tacchini et al, 1999	
Vasodilation and	Vascularisation	
(Increased of		
neme oxygenase-1 Vesetaler en detheliel ereveth forter (UPCOD)	wood <i>et al</i> , 1998	
vascular endotnellal growth factor (VEGF)	Carmellet et al, 1998	
Vasomotor I (Increased O <sub>2</sub>	Regulation delivery)	
Endothelin-1	Hu et al. 1998	
Nitric oxide synthase 2	Palmer et al. 1998	
	··· ··· ··· ··· · · · · · · · · · · ·	

Table 1.3. Functional categorisation of target genes transactivated by HIF-1.

Each of the genes contains a HRE sequence of >100 bp that includes one or more HIF-1 binding sites containing the core sequence 5'-TACGTG-3'. HIF-1 mediates increased oxygen delivery as well as adaptation to decreased oxygen availability. (Adapted from Semenza, 2001).

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#### 1.5.1.1 Breast Cancer

Breast cancer is one of the first cancers to be described with the earliest description being recorded in 1600 BC in ancient Egypt. Despite recent advances in molecular science that have led to a better understanding of the molecular events important in the pathogenesis of breast cancer, it remains one of the most common malignancies in women, comprising 18% of all female cancers, with nearly 600 000 new cases diagnosed in the world each year (McPherson *et al*, 2000). Breast cancer is the second leading cause of cancer death for all women and the leading cause of cancer death in all women between the ages of 35 and 54. It affects approximately 1 in 10 women and is responsible for 23.7% of all cancer deaths (Breast Cancer Statistics, 2000). The United Kingdom has the highest age standardised incidence and mortality in the world with more than 15 000 deaths each year (McPherson *et al*, 2000). It is currently believed that breast cancer presents as a spectrum of disease from local disease to systemic disease when first detected, requiring both local and systemic therapy (Coagner, 1991).

# 1.5.1.1.1 Risk Factors

There are several established and probable risk factors associated with the development of breast cancer (Table 1.4). Age is a major aspect related with the development of breast cancer, as it is known that the incidence of breast cancer increases with age, doubling every ten years until the menopause, where the rate then slows dramatically (Breslow and Kessler, 1995). Additionally, the age of menarche and menopause is important as women who begin menstruating early in life or who reach the menopause after the age of 55 have been shown to be twice as likely to develop breast cancer as women who reach the menopause prior to the age of 45. Women aged over 70 years account for 40% of all breast cancers (McPherson *et al*, 2000).

Factor	Relative Risk	High Risk Group
Age	>10	Elderly
Age at Menarche	3	Menarche before age 11
Age at Menopause	2	Menopause after age 54
Age at First Full Pregnancy	3	First child in early 40's
Geographical Location	5	Developed country
Socio-economic Group	2	Groups I and II
Family History	≥2	Breast cancer in first degree relative when young
Previous Benign Disease	4 - 5	Atypical hyperplasia
Cancer in other Breast	> 4	
Diet	1.5	High intake of saturated fat
Body Weight Premenopausal	0.7	Body mass index >35
Body Weight Postmenopausal	2	Body mass index > 35
Alcohol Consumption	1.3	Excessive intake
Exposure to Ionising Radiation	3	Abnormal exposure in young females after age 10
Oral Contraceptives	2	Use for $\geq$ 4 years when young
Hormone Replacement Therapy	1.24	Use for $\geq 10$ years
Diethylstilbestrol	2	Use during pregnancy

Table 1.4. Established and probable risk factors for breast cancer.

(Adapted from McPherson et al, 2000)

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Other factors such as age of first pregnancy (Kerlikowske *et al*, 1997), geographical location and genetic disposition also play a significant role in the lifetime risk of developing breast cancer. The difference in the incidence of breast cancer between Western and Far Eastern countries is approximately five fold and up to 10 % of patients with breast cancer in Western countries have a genetic abnormality that predisposes them to develop the disease (McPherson *et al*, 2000).

#### 1.5.1.1.2 Genetic Factors

The susceptibility to nearly any disease has some element of genetic predisposition. To date, two genes have been associated with susceptibility to breast cancer, BRCA 1 (Miki et al, 1994), located on the long arm of chromosome 17, and BRCA 2 (Wooster et al, 1994) mapped to the long arm of chromosome 13, with the possibility that more may exist. A recent study reported evidence for a novel breast cancer susceptibility locus on chromosome 13q21 (Kainu et al, 2000). This candidate BRCA3 locus was further studied in genetic linkage analysis of breast cancer families of European origin with the finding that its contribution to familial breast cancer was very small (Thompson et al, 2002). Germline mutations in either the BRCA1 or the BRCA2 genes are responsible for the majority of hereditary breast cancers, each of then accounting roughly for half of the cases (Feunteun and Lenoir, 1996). Approximately one third of familial cases are believed to be due to mutations in the BRCA 1 gene and studies have shown that tumours with BRCA1 or BRCA2 mutations have a higher frequency of specific somatic genetic alterations (Tirkkonen et al, 1997). It is also known that mutations in the p53 tumour suppressor gene on the short arm of chromosome 17 is involved in the development of the disease, providing strong evidence for the involvement of a tumour suppressor gene in breast cancer (Malkin et al, 1990; Kleihues et al, 1997).

Allele loss studies (loss of heterozygosity) indicate chromosomal regions that may contain tumour suppressor genes that exhibit an important two-hit mechanism involving both loss of heterozygosity and mutation that is commonly involved in breast cancer. In addition, studies of loss of heterozygosity have suggested that chromosome 1 may house genes involved in breast cancer (Devilee *et al*, 1991). Chromosome arms including 1p, 1q, 3p, 6p, 7q, 11p, 13q, 17p, 17q, 18q show moderate (20-40%) to high (>50%) frequency of loss of heterozygosity in invasive ductal breast cancer (Gendler *et al*, 1990, Bièche *et al*, 1994). The growing family of p53 tumour suppressor genes, including p73, have been mapped to these chromosomes. Many breast tumours show changes in ploidy indicative of severe nuclear disarrangement.

There is genetic evidence that the angiogenic pathway plays a major role in carcinogenesis of the breast and may involve different mechanisms at later stages of tumour progression (Hellelginger *et al*, 1999). A small hypoxic tumour, which is genetically unstable, will be exposed to a hostile environment and mutations (notably p53) producing a phenotype able to resist cell death (apoptosis) will have a survival advantage. Mutations enable cells to survive under hypoxic conditions and also switch on the angiogenic phenotype by enhancing VEGF production. A strong correlation has been found between the expression of VEGF and increased metastasis and prognosis (Saaristo *et al*, 2000).

#### 1.5.1.1.3 Classification

As tumours show characteristic patterns of growth and cellular morphology, definite types of breast cancer can be identified and classified (Figure 1.12). Breast cancers are derived from the epithelial cells that line the terminal duct lobular unit. Tumours that contain specific features are described as invasive carcinomas of special type, whereas all others are described as of no special type (Sainsbury *et al*, 2000). As long

as the tumour remains within the confines of the ductal basement membrane, it constitutes a noninfiltrating intraductal carcinoma. It begins as an atypical proliferation of ductal epithelium that eventually completely fills the ducts with neoplastic cells (Cotran *et al*, 1999). An invasive breast cancer is one in which there is dissemination of cancer cells outside the basement membrane of the ducts and lobules into the surrounding adjacent normal tissue. Invasive breast cancers are most commonly classified as either ductal or lobular types (Sainsbury *et al*, 2000), however there are a wide range of other histological types (World Health Organisation, 1981). Cancer cells that remain within the basement membranes of the elements of the terminal duct lobular unit and the draining duct are classified as *in situ* or non-invasive. Ductal carcinoma *in situ* is the most common form of non-invasive carcinoma (Page *et al*, 1995). Lobular carcinoma *in situ* is manifested by proliferation of cells, in one or more of the terminal ducts, which are large, loosely cohesive and have rare mitoses (Cotran *et al*, 1999).

Tumours of no special type are graded by the degree of differentiation and are assessed semi-quantitatively by three characteristics: the degrees of glandular formation, nuclear pleomorphism and mitotic frequency. These values are combined and the tumour is then assigned one of three histological grades: Grade I (score 3-5), Grade II (scores 6-7) and Grade III (scores 8-9), which correlate directly with survival. This histological grading system, known as the Scraff, Bloom and Richardson grade after the creators of the system, has become an important predictor of both disease free and overall survival (Bloom and Richardson, 1957; Sainsbury *et al*, 2000).

Chapter 1. Introduction



Figure 1.12. Histologic classification of breast tumours.

(Adapted from the World Health Organisation, 1981).

#### 1.5.1.1.4 Staging

Prognosis and survival in breast cancer is directly related to the stage of disease at presentation. When an invasive breast cancer is diagnosed the extent of the disease is then assessed and the tumour staged. There are currently two staging classifications in use, both of which are inappropriate for breast cancer due to its complexity. The first system, the tumour node metastases system (TMN), is dependent upon the inaccurate methods of clinical assessment and measurement of lymph node status (**Table 1.5**) (Sobin and Wittekind, 1997; American Joint Committee on Cancer, 1997). The second system, the International Union against Cancer (UICC) system incorporates the TMN classification system (**Table 1.6**) (International Union Against Cancer, 1997). A separate pathological classification, where tumour size and node status are assessed by a pathologist, has been added to the TMN system in an attempt to improve the accuracy of classification (Fleming, 1997).

Both systems of breast tumour classification consider the size of a tumour (T), lymph node involvement (N) and metastatic disease (M) (Rosen and Oberman, 1993). As the pathological size of a tumour correlates directly with survival, patients with smaller tumours have a better survival rate than those with large tumours. Stage I cancer defines a tumour of a small size that has not progressed outside of its original site whereas a stage IV growth is large and has spread widely. The number of axillary nodes involved is also directly correlated with survival. The presence of cancer cells in lymphatic vessels indicates a more aggressive disease. Patients whose cancer has spread beyond the axillary or internal mammary nodes ( $M_1$  or stage IV disease) are known to have a much worse survival rate than patients whose disease is localised (Miller *et al*, 1995).

A low-grade tumour histologically resembles the tissue of origin, whereas a highgrade tumour undergoes several changes that it marginally resembles the tissue of origin. Both the stage and grade of a tumour are important as they measure different parameters of

- 59 -

Classification	Description
Primary Tumour	
T <sub>is</sub>	Cancer in situ
Tı	$\leq 2$ cm, (T <sub>1a</sub> $\leq 0.5$ cm, $> 0.5$ cm $- 1$ cm, T <sub>1c</sub> $> 1$ cm $- 2$ cm)
T <sub>2</sub>	> 2  cm - 5  cm
T <sub>3</sub>	> 5 cm
T <sub>4a</sub>	Involvement of chest wall
T <sub>4b</sub>	Involvement of skin (includes ulceration, direct infiltration, peau d'orange and satellite nodules
T <sub>4c</sub>	$T_{4a}$ and $T_{4b}$ together
T <sub>4d</sub>	Inflammatory cancer
Lymph Nodes	
N <sub>0</sub>	No regional node metastases
N <sub>1</sub>	Palpable mobile involved ipsilateral axillary nodes
N <sub>2</sub>	Fixed involved ipsilateral axillary nodes
$N_3$	Ipsilateral internal mammary node involvement (rarely clinically detectable)
Metastases	
M <sub>0</sub>	No evidence of metastasis
M1	Distant metastasis (includes ipsilateral supraclavicular nodes)

Table 1.5. Tumour Node Metastases (TNM) Classification of Breast Tumours

(Adapted from Sainsbury et al, 2000)

UICC Stage	TNM Grouping	Clinical Description	5 Year Survival
I	T <sub>1</sub> , N <sub>0</sub> , M <sub>0</sub>	Tumours ≤2 cm, no nodal involvement, no metastases	87 %
П	T <sub>1</sub> , N <sub>1</sub> , M <sub>0</sub> T <sub>2</sub> , N <sub>0-1</sub> , M <sub>0</sub>	Tumours ≤5 cm, with involved but movable axillary nodes, no metastases, or tumour >5 cm, no nodal involvement, no metastases	75 %
Ш	Any T, N <sub>2-3</sub> , M <sub>0</sub> T <sub>3</sub> , any N, M <sub>0</sub> ; T <sub>4</sub> , any N, M <sub>0</sub>	Breast cancers of any size with possible skin involvement, pectoral and chest wall fixation, nodal involvement, no metastases	46 %
IV	Any T, any N, M <sub>1</sub>	Breast cancers with/without nodal involvement, pectoral or chest wall fixation, skin ulceration, disseminated metastases	13 %

Table 1.6. Correlation of the International Union Against Cancer (UICC) (19	97) and
TNM classifications of breast tumours with survival rates.	

For TNM Grouping definitions refer to Table 1.5.

(Adapted from Sainsbury et al, 2000)

the cancer and are used to predict the direction of the cancer (Sainsbury *et al*, 2000). Figure 1.13 illustrates histologically low, moderate and high-grade tumours of the breast.

# 1.5.1.1.5 Angiogenic Factors

Breast cancer angiogenesis has been studied extensively and now serves as a paradigm for understanding the biology of angiogenesis and its effects on tumour outcome and patient prognosis (Leek, 2001). Studies have shown that primary breast cancers express multiple angiogenic factors belonging to different growth factor families (Relf *et al*, 1997; De Jong *et al*, 1998). Other studies have shown that quantitation of angiogenesis can be used as an independent prognostic factor (Obermair *et al*, 1997; Gasparini *et al*, 1999; 2001).

Among the angiogenic factors tested at the tumoural level in breast cancer, VEGF is the only factor that provides the most relevant prognostic information (Veikkola *et al*, 2000). Several studies have determined VEGF expression to be of prognostic significance in operable breast cancer (Gasparini *et al*, 1999; Linderholm *et al*, 2000; reviewed in Gasparini, 2001). Additionally, soluble vascular endothelial growth factor receptor-1 (sVEGFR1), a naturally occurring soluble form of VEGFR1 and inhibitor of VEGF, is frequently co-expressed with VEGF in primary breast cancer tissues and it is thought that the intratumoral balance between sVEGFR1 and VEGF levels might be crucial for the progression of breast cancer (Toi *et al*, 2002). Other studies have shown that the expression of VEGF correlates with mutant p53 and poor prognosis in human breast cancer (Linderholm *et al*, 2001).



Figure 1.13. A) Low-grade carcinoma of the breast. The photo shows well differentiated carcinoma cells forming tubular structures infiltrating breast tissue (magnification X500). B) Moderate grade carcinoma of the breast. Groups of moderately differentiated carcinoma cells infiltrating fibroadipose breast tissue (magnification X500). C) High-grade carcinoma of the breast. Poorly differentiated adenocarcinoma composed of pleomorphic tumour cells with prominent nucleoli and numerous mitotic figures (magnification X500) (haematoxylin counterstain). (Photographs courtesy of Dr. David Hilton, Derriford Hospital, Plymouth, UK)

#### 1.5.1.1.6 Tumour Hypoxia

It has been shown that intratumoral hypoxia is associated not only with genetic instability but also with resistance to chemotherapy and radiation allowing for a possible recurrence of the tumour (Yuan and Glazer, 1998). It is known that independent of treatment, solid tumours have a poor prognosis. Thus, hypoxic conditions select for chemotherapy-resistant cells, which might subsequently clonally metastasise.

As radiotherapy relies upon the formation of reactive oxygen species to promote radiation-induced cell death, decreased oxygen concentrations in the tumour would decrease their formation. It has also been suggested that hypoxic tumour cells express survival factors that mediate protection against any apoptosis-inducing stimulus (Semenza, 2000). Cultured glioma cells exposed to hypoxia have demonstrated increased survival after exposure to chemotherapeutic agents (Liang, 1996). The expression of insulin-like growth factor 2 (IGF-2) under hypoxic conditions is considered to be a survival factor which is regulated by HIF-1 (Feldser *et al*, 1999; Semenza, 2000).

A recent study has suggested that a hypoxic tumour microenvironment may downregulate both oestrogen receptor expression and function in hormone responsive breast cancer cells and reduces their responsiveness to hormonal agents (Kurebayashi *et al*, 2001). This would indicate that hypoxia may play a key role in the development of acquired resistance to hormonal agents.

# 1.5.1.1.7 Treatment of Breast Cancer

The aim of current treatments for cancer is to maximise the effect on the cancer and to minimise any adverse side effects on normal tissues. It is known that the earlier that a cancer is detected, the fewer changes it will have undergone, resulting in a better response to treatment.

# 1.5.1.1.7.1 Surgery

Established cancers indicate increased cellular changes and increased aggressiveness. For any cancer that has already established, surgical removal remains the best approach but has limitations as cancers can metastasise to inaccessible sites, resulting in a relapse at local or distant sites, and undetectable micrometastases may also be present at the time of diagnosis (Saphner *et al*, 1996). However, surgery is now less aggressive then 10 years ago. Treatment of primary, operable breast cancer involves the use of surgery with or without adjuvant systemic therapy. Conservative breast surgery combined with radiotherapy is the standard procedure for all unifocal cancers less than 3-4 cm in size (Early Breast Cancer Treatment Collaborative Group, 1995).

#### 1.5.1.1.7.2 Radiotherapy

Radiotherapy is used for about half of all patients who develop cancer in the United Kingdom each year (Greenlee *et al*, 2001) and relies on the generation of reactive oxygen species from molecular oxygen to damage DNA and inevitability cause cell death via apoptosis (Yamada *et al*, 1988; Allan, 1992). Reactive oxygen species, or free radicals, are extremely unstable and interact with neighbouring molecules to produce chemically stable lesions (Ward, 1990). As all tumours contain regions of hypoxia related to poor blood supply, cells found in these regions are usually radioresistant (Weichselbaum *et al*, 1993). Thus, poor blood perfusion of the tumour or poorly developed microvasculature in the

tumour, results in reduced oxygenation, leading to decreased effectiveness of radiotherapy. Radiotherapy is therefore confined to the treatment of localised disease in tumours that are radiosensitive, or as an adjunct to surgery or chemotherapy.

## 1.5.1.1.7.3 Chemotherapy

Chemotherapy refers to treatment based on the delivery of drugs that affect cell processes such as DNA synthesis (mitosis) and cell proliferation. Chemotherapy is very effective against rapidly dividing cells and its efficacy is dependent upon dosage and duration. Primary or neoadjuvant chemotherapy is used as a first line of treatment for local disease, whereas adjuvant chemotherapy is used in conjunction with another treatment, such as surgery. Adjuvant chemotherapy is also used to treat advanced disease in an attempt to improve the quality of life when surgery is not an alternative. A tumour larger than 5 cm or a smaller tumour with signs of locally advanced disease ( $T_3/T_4$ ,  $N_2$ ,  $N_3$ ) are currently treated with neo-adjuvant chemotherapy (Early Breast Cancer Treatment Collaborative Group, 1995).

Treatment that results in DNA damage will activate the apoptotic pathway. Taxanes are plant alkaloids that promote tubulin assembly and induce apoptosis of tumour cells by a p53-independent  $G_2/M$  cell cycle checkpoint (Lanni *et al*, 1997). The development of new cytotoxic agents and radiation therapy techniques, administered as adjuvant therapy after surgery, has led to a reduction in the risk of relapse of only 20-40 %, depending on the chemotherapeutic agents used (Bange *et al*, 2001; Hortobagyi, 2000). Moreover, it has been demonstrated that simultaneous combination of two or more agents provide more effective treatment (Fossati *et al*, 1998).

#### 1.5.1.1.7.4 Anti-Oestrogen Receptors

As steroid hormones are required for the growth of breast cancer, hormone treatment is often given as it generates fewer side effects. Treatment is based on the principle of depriving the cancer cells of mitogenic hormone by either preventing steroid synthesis or by blocking their effects at the target level via the receptor machinery. As relatively few cell types contain hormone receptors, drugs aimed at disrupting them remain highly specific. Response to treatment is dependent upon cellular localisation. Cells situated at a distance from a blood vessel will not receive a sufficient dose of drugs to destroy it. Areas of a tumour that are well vascularised tend to respond better to treatment then those areas that are not.

Tamoxifen, an antagonist of the oestrogen receptor in breast tissues, reduces the incidence of cancer relapse in hormone receptor positive tumours and can delay or prevent the development of breast cancer in women at high risk (Peto and Mack, 2000). A large 5 year study of women with an increased risk of breast cancer treated daily with Tamoxifen has shown a statistically significant 49 % reduction in the incidence of breast cancer (Fisher *et al*, 1998). However, long term usage of the drug has produced a number of side effects which are dependent upon age and race (Gail *et al*, 1999). New oestrogen receptor drugs, such as raloxifen, which produce fewer side-effects are now being studied (Rosenbaum and Osborne, 2000).

# 1.5.1.1.7.5 Molecular Targets

The development of cDNA array analysis has allowed for the pathological characterisation of tumours using gene expression profiling, through which gene clusters can be identified and localised. A recent study found different gene profile phenotypes among 65 tumour samples using cDNA array analysis (Perou *et al*, 2000). Several new drugs have recently emerged including; trastuzumab (*Herceptin*) (Baselga, 2001; Slamon

et al, 2001), the monoclonal antibody against human epidermal growth factor receptor 2 ((HER-2), also known as ERBB2), and the cyclooxygenase inhibitor celecoxib (Harris et al, 2000). ERBB2 is known to be an essential breast cancer oncogene as mitogenic signalling has been associated with increased activation of cyclin D1, an essential factor of G1/S phase transition of the cell cycle (Lee et al, 2000). A recent study has shown that *Herceptin* induces normalisation and regression of the vasculature in an experimental human breast tumour that overexpresses ERBB2 in mice by modulating the effects of different pro- and anti-angiogenic factors (Izumi et al, 2002).

Inhibition or the prevention of angiogenesis has been a main focus of cancer treatment. There are more than 20 anti-angiogenetic drugs which prevent endothelial cell proliferation, block activators of angiogenesis, such as VEGF, or inhibit extracellular matrix breakdown by proteases, undergoing evaluation in phase I, II or III clinical trials (Bange *et al*, 2001). In the absence of new blood vessel formation, oxygen and essential nutrients become limited, cell functions are not maintained and necrotic death follows. The continued identification and characterisation of signalling mechanisms the govern cell growth, differentiation, motility and apoptosis will continue to result in the development of new molecular therapeutic approaches in the treatment of breast cancer.

### 1.5.1.2 Glioblastoma

Primary malignant brain tumours are amongst those human neoplasms that exhibit the greatest angiogenic activity thus they are very suitable for angiogenesis studies. Glioblastoma multiforme (GBM) is the most common malignant brain tumour in humans and is characterised histologically by necrosis and microvascular proliferation (Shweiki *et al*, 1992). GBM is highly invasive and has a median survival of less than one year following diagnosis, irrespective of treatment (Burger and Scheithauer, 1993).

To metastasise to the brain, malignant tumour cells must attach to microvessel endothelial cells, respond to brain-derived invasive factors, invade the blood-brain barrier and respond to survival and growth factors (Mentor *et al*, 1995). Malignant glioma cells may be able to induce neovascularisation, however vascular supply may not be sufficient for tumour areas with high cell proliferation and the resultant oxygen and nutritional deprivation induces extensive necrosis (Russel and Rubinstein, 1989).

## 1.5.1.2.1 Genetic Factors

Alterations (loss or mutation) of the p53 gene are one of the most common abnormalities in gliomas and may be an early event in malignant transformation of glioma cells. Germline mutations of the p53 gene are associated with increased susceptibility to glioma. Also, chromosome 17p deletions and p53 gene mutations are found frequently in sporadic gliomas of all malignancy stages (Van Meyel *et al*, 1994). It is known that under hypoxic conditions, HIF-1 $\alpha$  accumulates and binds to p53 thereby inhibiting its degradation (An *et al*, 1998). The role of p53 in hypoxic or ischaemic brain tissue from patients with GBM is not known nor is the relation of severity and duration of hypoxia to the expression of p53.

Recent studies suggest that PTEN, a tumour suppressor originally isolated from a homozygous deletion on chromosome 10q23 in GBM (Li et al, 1998, Steck et al, 1997),

attenuates hypoxia-mediated HIF-1 $\alpha$  stabilisation (Zundel *et al*, 2000). PTEN downregulates HIF-1 $\alpha$  expression and HIF-1 $\alpha$  mediated transcription in human prostate and glioma cells (Zhong *et al*, 2000; Zundel *et al*, 2000). Thus, PTEN loss-of-function increases HIF-1 $\alpha$  expression.

It has been shown that there is a significant correlation between tumour grade and HIF-1 $\alpha$  expression in GBM, which is unlikely to be due to more extensive necrosis within higher-grade tumours (Zagzag *et al*, 2000). It is probable that several types of somatic mutations common to gliomas contribute to increased HIF-1 $\alpha$  expression resulting in increased HIF-1 mediated transactivation of downstream target genes. An investigation of the expression of HIF-1 $\alpha$ , as well as VEGF and p53 in glioblastoma multiforme may help elucidate their roles in this disease.

## 1.5.1.2.2 Grading

Astrocytic tumours are classified according to the recommendations of the World Health Organisation (WHO) (Kleihues *et al*, 2001), where they are divided into four grades: pilocytic astrocytoma (Grade I), astrocytoma (Grade II), anaplastic astrocytoma (Grade III), and glioblastoma (Grade IV), (Table 1.7). The histological characteristic of nuclear atypia is usually required for tumours of grades II to IV, which includes coarse nuclear chromatin, nuclear pleomorphism, multinucleation and pseudoinclusions. Also, for the higher grades of tumour the additional criteria of mitotic activity (Grades III and IV), cellularity, vascular proliferation (Grade IV) and necrosis (Grade IV) are also required. Grading in usually based on areas showing the highest degree of anaplasia, on the assumption that the tumour cell population eventually determines the course of the disease (Kleihues and Cavenee, 1997). Figures 1.14 and 1.15 illustrate a low-grade astrocytoma, an anaplastic astrocytoma and a high-grade glioblastoma.
Table 1.7. Recommendations of the World Health Organisation (WHO) for the grading of gliomas (Kliehues et al, 2001).

WHO Grade	WHO Designation	Histological Criteria
Ι	Pilocytic astrocytoma	
П	Astrocytoma (low-grade diffuse)	Nuclear atypia
Ш	Anaplastic astrocytoma	Nuclear atypia, mitotic activity
IV	Glioblastoma	Nuclear atypia, mitoses, endothelial proliferation and/or necrosis

(Adapted from Kleihues and Cavenee, 1997)



**Figure 1.14**. **A)** A low-grade astrocytoma showing small uniform astrocytic nuclei which are forming microcystic spaces with no mitotic activity. **B)** A highly cellular astrocytic tumour showing mitotic activity and nuclear pleomorphism (haematoxylin counterstain). (Photographs courtesy of Dr. David Hilton, Derriford Hospital, Plymouth, UK)



Figure 1.15. A) Glioblastoma showing marked nuclear pleomorphic large atypical astrocytic cells. B) Glioblastoma showing 2 features: vascular proliferation to the left of the photo and necrosis to the right of the photo (haematoxylin counterstain). (Photographs courtesy of Dr. David Hilton, Derriford Hospital, Plymouth, UK).

# 1.5.1.2.3 Glioma-Induced Angiogenesis

One event that accompanies glioma progression is the up-regulation of angiogenesis. Low-grade gliomas are moderately vascularised tumours whereas highgrade gliomas show predominately microvascular proliferation and areas of high vascular density (Plate *et al*, 1992). Hypoxia seems to play a critical role in the induction of VEGF expression during glioma progression as VEGF is highly expressed in high-grade gliomas and has been shown to be hypoxia inducible in glioma cells in vitro (Stratmann *et al*, 1997).

Transcription of VEGF is greatly enhanced in GBM and it is an important regulator of vascular functions in glioma-induced angiogenesis. A characteristic feature of GBM is the presence of necrotic regions surrounded by palisading cells, viable cells which are furthest removed from a blood vessel, that express high levels of VEGF mRNA (Plate *et al*, 1992). The regulation of expression of VEGF in GBM is poorly understood but is probably modulated by binding of HIF-1 $\alpha$  to the HRE site in the flanking region of the gene (Dachs *et al*, 1997). There are few published data on the expression of HIF-1 $\alpha$  in GBM. Recent immunohistochemical analysis of GBMs found that HIF-1 $\alpha$  is expressed in the palisading cells and localised in areas adjacent to necrotic zones and within infiltrating tumour cells (Zagzag *et al*, 2000), suggesting a role for HIF-1 $\alpha$  in GBM. These results, together with previous findings imply that hypoxia induces HIF-1 $\alpha$  and VEGF expression, as well as tumour vascularisation.

# 1.5.1.2.4 Survival

The survival of a patient with glioblastoma is dependent upon a variety of clinical parameters that include patient age, tumour location and treatment. Treatments available include surgical resection, radiotherapy and chemotherapy. The average range of survival for patients with low-grade diffuse astrocytomas is more than 5 years, for patients with anaplastic astrocytomas is from 2 to 5 years, and for patients with glioblastoma it is less than one year (Kleihues and Cavenee, 1997).

### 1.5.2. Diabetes Mellitus

Diabetes mellitus (DM) is a disorder characterised by abnormally high levels of glucose in the blood due to either a deficiency of insulin secretion or to resistance of the body's cells to the action of insulin, or to a combination of the above (Kuzuya, 2000). Two differentiated forms of the disease were first documented in 600 B.C. by two Indian physicians, Chakrata and Susrata, however it wasn't until the mid 1930's that two clinical types of diabetes mellitus were proposed to exist, an insulin sensitive and insulin insensitive form, the former being due to insulin deficiency (Himsworth, 1936). The development of a bioassay for insulin in 1951 confirmed these clinical observations (Bornstein and Lawrence, 1951) and continued research in the later half of the 20<sup>th</sup> century that has led to the recognition that DM is a syndrome that is comprised of a genetically heterogeneous collection of disorders characterised by high blood glucose levels (American Diabetes Association Expert Committee, 2001).

In 1979 an international workgroup sponsored by the National Diabetes Data Group (NDDG) of the National Institutes of Health developed a classification for DM based on scientific research conducted during the previous decades (National Diabetes Data Group, 1979). DM was officially recognised as a syndrome that included a collection of disorders having hyperglycaemia and glucose intolerance as their feature characteristic. The World Health Organisation (WHO) Expert Committee on Diabetes approved and supported the recommendations of the NDDG in 1980 and both groups went on the further distinguish two major forms of DM in the Western hemisphere which were termed insulindependent diabetes (T1DM) and non-insulin-dependent diabetes (T2DM) (World Health Organisation, 1980; 1999). An expert committee of the American Diabetes Association proposed further changes to the NDDG/WHO classification having considered the research findings of the last 25 years (American Diabetes Association Expert Committee, 1997; 2001). These proposals include changes to diagnostic criteria as well as the elimination of the terms *insulin-dependent DM* and *non-insulin-dependent DM* and their acronyms, IDDM and NIDDM, however the terms T1DM and T2DM were proposed to be retained. Additionally, it was proposed that forms of DM involving pancreatic  $\beta$ -cell destruction be included under T1DM (American Diabetes Association Expert Committee, 1997; 2001).

Different genetic, metabolic, environmental and lifestyle factors can result in similar diabetic phenotypes (hyperglycaemia and microvascular complications) implying. strong heterogeneity within the syndrome of DM (Kuzuya, 2000). However, chronic hyperglycaemia has been identified as the primary risk factor in the development of diabetic complications (Nathan, 1996; The Diabetes Control and Complications Trial Research Group, 1993; The United Kingdom Prospective Diabetes Study Group, 1998). Despite decades of intensive research, the exact causes of T1DM and T2DM remain unknown although both can be accompanied by ketoacidosis, blindness, kidney failure, premature cardiac disease, stroke, amputations and other diabetic complications (section 1.5.2.3).

# 1.5.2.1 Type 1 Diabetes Mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) comprises approximately 5 to 10 % of cases in the DM syndrome, of which 15 to 30 % of all cases are diagnosed after 30 years of age (Laakso and Pyorala, 1985; Kuzuya, 2000). Incidence rates are increasing in a number of countries around the world (Rewers and Norris, 1996) where currently about 0.5% of the population in developed countries is affected (Mathis *et al*, 2001).

T1DM results from the autoimmune-mediated destruction of pancreatic  $\beta$ -cells islets that leads to nearly total loss of insulin secretion and absolute insulin deficiency (WHO, 1999). Early systematic and quantitative studies of the pancreas resulted in the description of a specific loss of insulin-producing  $\beta$ -cells in association with the clinical onset of T1DM (Gepts, 1965). T1DM has since been differentiated into two subclasses, an

autoimmune class and an idiopathic class. The autoimmune form of T1DM is a chronic disease characterised by cellular-mediated (T lymphocytes) autoimmune destruction of the insulin producing  $\beta$ -cells in the pancreatic islets of Langerhans (Atkinson and Eisenbarth, 2001). Autoimmunity results from the failure to regulate or eliminate autoreactive T cells. When most of the  $\beta$ -cells have been killed off, there is no longer sufficient insulin production to regulate blood glucose levels, resulting in hyperglycaemia (Mathis *et al*, 2001). This destructive process may occur over a prolonged period of time prior to the development of clinical symptoms from hyperglycaemia. During this time antibodies to pancreatic antigens and abnormalities in pancreatic  $\beta$ -cell may be detected (Hansen *et al*, 1997). The presence of anti-islet autoantibodies is a distinguishing feature of T1DM, which has since become a classic pre-diabetes marker used to follow relatives of T1DM probands (Bottazzo *et al*, 1974).

### 1.5.2.2. Pathogenesis of T1DM

The development of T1DM has been divided into a series of stages in which an individual may progress through some or all of these stages in the advancement of the disease. Stage 1 involves genetic predisposition, stage 2 is environmental triggering of autoimmunity, stage 3 is the development of a series of autoantibodies, stage 4 is loss of  $\beta$ -cell function through metabolic defects, stage 5 is overt DM and stage 6 is total or near total  $\beta$ -cell destruction (Eisenbarth, 1986). The autoimmune response to islet  $\beta$ -cells is believed to occur in persons who possess certain susceptibility alleles and also lack other protective alleles of the HLA or major histocompatibility complex (MHC) genes, which regulate immune responses. It is thought that a genetic susceptibility to altered immune regulation predisposes certain individuals to the development of autoimmunity of islet beta cells (Atkinson and MacLaren, 1994). The autoimmunity may develop spontaneously or may be triggered by environmental agents such as a virus, chemical or unknown toxin,

resulting in acute insulitis (Myers *et al*, 2001). Non-MHC genes may also contribute to the autoimmune response, however it is clear that genetics alone are not sufficient to explain the development of T1DM.

## 1.5.2.2.1. Insulin Metabolism

The human insulin gene was one of the first human genes cloned. It is encoded on the short arm of chromosome 11p15.5 and is expressed in the beta cells of the pancreatic islets, where mature insulin mRNA is transcribed (Owerbach *et al*, 1980). The most important stimulant of insulin release is glucose, which also initiates insulin synthesis. Pancreatic  $\beta$ -cells release insulin into the circulation in response to rising blood glucose levels, where it binds to specific receptors present on cell surfaces. This binding signals uptake of nutrients, storage of energy and growth (Gustafson *et al*, 1999; Virkamaki *et al*, 1999).

Insulin is a major anabolic hormone (Kahn, 1988) and is necessary for; transmembrane transport of glucose and amino acids, glycogen formation in the liver and skeletal muscles, glucose conversion to triglycerides, nucleic acid synthesis and protein synthesis. Its prime metabolic function is to increase the rate of glucose transport into striated muscle cells, myocardial cells, fibroblasts and fat cells in the body. Insulin interacts with target cells by first binding to the insulin receptor, which is composed of two  $\alpha$  and two  $\beta$  glycoprotein subunits (Patti and Kahn, 1998). Insulin binds to the extracellular portion of the  $\alpha$ -subunit and this in turn activates the tyrosine-specific protein kinase activity of the cytoplasmic portion of the  $\beta$ -subunit (Patti and Kahn, 1998). The  $\beta$ -subunit tyrosine kinase then catalyses phosphorylation of the  $\beta$ -subunit itself as well as other intracellular proteins. This is followed by a number of cellular responses, including activation or inhibition of insulin-sensitive enzymes in mitochondria, protein synthesis and DNA synthesis.

#### **1.5.2.3 Diabetic Complications**

The pathogenesis of chronic diabetic complications is thought to be complex and multifactorial, although all forms of diabetes are characterised by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology (Brownlee, 2001). Considerable amounts of epidemiologic and animal data support the link between chronic hyperglycaemia and long-term microvascular complications of T1DM, including retinopathy, neuropathy and nephropathy (Raskin *et al*, 1983; Krolewski *et al*, 1988; The Diabetes Control and Complications Trial Research Group, 1993) as well as in T2DM (The United Kingdom Prospective Diabetes Study Group, 1998). In addition to microvascular complications, hyperglycaemia also contributes to macrovascular disease, affecting arteries that supply the heart, brain and lower extremities, as well as impaired cellular immunity in T1DM (Rich, 1997).

It is known that intracellular hyperglycaemia causes abnormalities in blood flow and increased vascular permeability (Brownlee, 2001). Consequently, there is decreased activity of vasodilators such as nitric oxide (section 1.5.2.3.7), increased activity of vasoconstrictors such as angiotension II and increases in permeability factors such as VEGF (sections 1.3.3.1 and 1.5.2.3.9) (Brownlee, 2001). The combined effect of these changes can lead to ischaemia and hypoxia-induced neovascularisation in the retina, proteinuria, mesangial matrix expansion and glomerulosclerosos in the kidney, and multifocal axonal degeneration in peripheral nerves.

Diabetic complications are believed to result from hyperglycaemia through four hypotheses involving signal transduction pathways (**Table 1.8**): increased flux through the polyol pathway (section 1.5.2.3.1); increased advanced glycation end-product (AGE) formation (section 1.5.2.3.2); activation of protein kinase C (PKC) isoforms (section 1.5.2.3.3); and increased hexosamine pathway flux (section 1.5.2.3.4). The common element linking these four hypothesis is the hyperglycaemia-induced overproduction of superoxide by the mitochondrial electron-transport chain (Du et al, 2000; Nishikawa et al, 2000) (section 1.5.2.3.5).

## 1.5.2.3.1 The Polyol Pathway

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Activation of the polyol pathway by hyperglycaemia occurs in numerous cells and tissues expressing aldose reductase and sorbitol dehydrogenase (Dvornik, 1987). Under conditions of hyperglycaemia, excess glucose is metabolised to sorbitol and fructose by nicotinamide adenine dinucleotide (NAD) linked oxidoreductases that further influence cellular metabolism, redox potential and signal transduction. Excessive polyol pathway activity, induced through a high cellular glucose flux, results in a change in the homeostasis of oxidised and reduced nicotinamide adenine dinucleotides (NADP) redox couples. Increased oxidation of NADPH to NADP<sup>+</sup> occurs that is coupled to the reduction of glucose to sorbitol by aldose reductase. An increased reduction of NAD<sup>+</sup> to NADH then follows which is coupled to the oxidation of sorbitol to fructose by sorbitol dehydrogenase (reviewed in Oates and Mylari, 1999) (Figure 1.16).

Glucose and its metabolites mediate their adverse affects by altering various signal transduction pathways, which are used by vascular and mesangial cells to maintain cellular integrity. These effects can vary from cell to cell in complex heterogeneous tissues and can result in the development of diabetic complications in target tissues. Conversely, it has been shown that cultured human endothelial cells chronically exposed to high glucose concentrations exhibit important abnormalities in cell function that could not be attributed to alteration of the polyol pathway (Lorenzi, 1992).

Table 1.8. Pathogenic pathways of glucose toxicity.

Non-enzymatic Pathway	Reference	
Increased formation of AGEs	Brownlee et al, 1998	
Oxidative stress via AGE/RAGE interaction	Yan <i>et al</i> , 1994	
Metabolic Pathways	Reference	
Polyol Pathway	Schleicher and Nerlich, 1996	
Activation of PKC	Ayo et al, 1991	
Hexosamine Pathway	Kolm-Litty et al, 1998	
Increase in oxidative stress	Du <i>et al</i> , 1998	

(Adapted from Lehmann and Schleicher, 2000)

AGE = advanced glycation end product RAGE = receptor for AGE PKC = protein kinase C



Figure 1.16. Regulation of the polyol pathway in response to hyperglycaemia

During hyperglycaemia glucose enters the cell via glucose transporters. A high glucose flux through aldose reductase and sorbitol dehydrogenase oxidises the NADPH/NADP<sup>+</sup> co-factor, (which is shared by aldose reductase, glutathione reductase and nitric oxide synthase) and reduces the NADH/NAD<sup>+</sup> redox couples thereby increasing intracellular stress. Oxidation of the NADPH/NADP<sup>+</sup> co-factor results in depletion of reduced glutathione and increased production of superoxide radicals. Hyperglycaemia induced variations in nicotinamide adenine dinucleotide (NAD) homeostasis is thought to increase susceptibility to oxidative tissue damage. (Adapted from Stevens *et al*, 2000).

Hyperglycaemia contributes to the development of diabetic complications by directly affecting glycolysis and polyol pathway activity. Several mechanisms have been proposed explaining the detrimental effects of increased flux through the polyol pathway including; sorbitol-induced osmotic stress, decreased (Na<sup>+</sup> + K<sup>+</sup>) ATPase activity, increase in cytosolic NADH/NAD<sup>+</sup> and a decrease in cytosolic NADPH (Brownlee, 2001). These proposals have been further investigated clarifying the effects of these potential mechanisms.

Accumulation of intracellular sorbitol, in excess of that required to buffer extracellular tonicity, produces intracellular osmotic stress and is believed to result in the depletion of myo-inositol, a precursor for phosphoinositide synthesis, and other intracellular osmolytes and taurine, a  $\beta$ -amino acid (Greene et al, 1992; Stevens et al, 1993; Burger-Kentischer et al, 1999). It was believed that sorbitol does not easily diffuse across cell membranes resulting in osmotic damage to microvascular cells. However, measurement of sorbitol concentrations in diabetic vessels and nerves show that they are too low to cause osmotic damage (Brownlee, 2001). Additionally, decreased (Na<sup>+</sup> + K<sup>+</sup>) ATPase activity has recently been shown to result from activation of PKC, increasing cytosolic phospholipase activity which increases the production of two  $(Na^+ + K^+)$  ATPase inhibitors (Xia et al, 1995). Moreover, although hyperglycaemia increases the cytosolic NADH/NAD<sup>+</sup> ratio in endothelial cells, through oxidation of sorbitol by NAD<sup>+</sup>, and inhibits the activity of glyceraldehydes-3-phosphate (GADPH), a marked decrease in the concentration of NAD<sup>+</sup> occurs due to its consumption by activated poly (ADP-ribose) polymerase (PARP), as opposed to a reduction in NAD<sup>+</sup> to NADH (Garcia-Soriano et al, 2001).

Finally, the proposal that decreases in cytosolic NADPH, due to the consumption of NADPH by the reduction of glucose to sorbitol, is a likely mechanism by which increased flux through the polyol pathway causes detrimental effects has been supported

- 84 -

by recent studies using transgenic and knockout mice (Lee and Chung, 1999; Brownlee, 2001). NADPH is required for regenerating reduced glutathione (GSH), thus a decrease in concentration would induce intracellular oxidative stress due to aldose reductase competing with glutathione reductase for NADPH (DeMattia *et al*, 1994). Decreased levels of GSH were found in transgenic mice overexpressing aldose reductase (Lee and Chung, 1999) and studies with knockout mice deficient in aldose reductase showed that diabetes did not decrease the GSH content nor reduce motor nerve conduction (Brownlee, 2001). Both aldose reductase mediated NADPH oxidation and sorbitol dehydrogenase mediated NAD reduction were found to increase free cytosolic NADP+/NADPH and decrease free cytosolic NAD+/NADH ratios (Lee and Chung, 1999).

## 1.5.2.3.2 Advanced Glycation End-Products (AGEs)

Glucose may react with proteins without enzymatic action by the chemical reactivity of its carbonyl group. This nonenzymatic glycation of proteins and DNA occurs during hyperglycaemia and results in the production of advanced glycosylation end-products (AGEs), potentially altering enzymatic activity and DNA integrity (Brownlee, 1995; 2001). AGEs attach to collagen in blood vessel walls and irreversibly cross-link to plasma proteins, which become resistant to enzymatic degradation. They form both intracellularly and extracellularly as a function of glucose concentration (Brownlee, 1995).

High concentrations of AGEs have been found in diabetic retina vessels (Stitt *et al*, 1997) and renal glomeruli (Horie *et al*, 1997). The receptor for AGE (RAGE) (Vlassara *et al*, 1986) is known to be expressed on vascular cells including macrophages, vascular endothelial and smooth muscle cells and mesangial cells (Bierhaus *et al*, 1998). Stimulation of RAGE by AGE-modified proteins results in increased oxidative stress to cells (Yan *et al*, 1994).

AGEs were originally thought to arise from non-enzymatic reactions between extracellular proteins and glucose; from autooxidation of glucose, cleavage of Amadori product 1-amino-1-deoxyketose and fragmentation of glycolysis-derived triose phosphates (Shinohara *et al*, 1998). However, it is now thought that intracellular hyperglycaemia is the primary initiating event in the formation of both intracellular and extracellular AGEs (Degenhardt *et al*, 1998).

There are three general mechanisms by which AGE formation may cause pathologic tissue changes: rapid intracellular AGE formation by glucose or fructose can alter intracellular protein function in tissues; modification of the extracellular matrix by AGE precursors can interfere with normal matrix function; and modification of plasma proteins by AGE precursors can induce receptor-mediated cytokine and hormone production (Matsumura *et al*, 2000).

## 1.5.2.3.3 Activation of Protein Kinase C (PKC)

Intracellular hyperglycaemia has been shown to increase PKC activity in cultured vascular cells (Xia *et al*, 1994) and in the retina and the renal glomeruli of diabetic animals (Koya and King, 1998). It is believed that this is achieved in part through an increase in the *de novo* synthesis of the PKC regulator, diacylglycerol (DAG) from the glycolytic intermediate dihydroacetone phosphate (DHAP), where it is then reduced to glycerol-3-phosphate and subsequently acylated (Koya and King, 1998). Hyperglycaemia may also activate PKC indirectly through ligation of AGE receptors (Portilla *et al*, 2000) and increased polyol pathway activity (Keogh *et al*, 1997).

#### 1.5.2.3.4 The Hexosamine Pathway

It is thought that hyperglycaemia induced activation of the hexosamine pathway may result in changes in both gene expression and protein function which together may

- 86 -

contribute to the pathogenesis of diabetic complications (Brownlee, 2001). The hexosamine pathway plays an important role in hyperglycaemia induced and fat-induced insulin resistance (Marshall *et al*, 1991; Hawkins *et al*, 1997). Hyperglycaemia increases hexosamine pathway flux by increasing fructose-6-phosphate levels and providing more glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of the pathway, due to the inhibition of GAPDH by reactive oxygen species (Du *et al*, 2000). Fructose-6-phosphate is diverted from glycolysis to provide substrates for reactions that require UDP-N-acetylglucosamine (Brownlee, 2001). Inhibition of GFAT in the conversion of glucose to glutamine, blocks hyperglycaemia induced transcription of TGF- $\alpha$  and TGF- $\beta$ 1 (Kolm-Litty, 1998). Other transcription factors, such as SP-1 and plasminogen activator inhibitor-1 (PAI-1), have also been found to be regulated by increased flux through the hexosamine pathway (Chen *et al*, 1998; Goldberg *et al*, 2000). Sp-1 is known to be modified by N-acetylglucosamine (GlcNAc) (Chen *et al*, 1998), which may in turn activate other glucose-responsive genes.

## 1.5.2.3.5 Mitochondrial Electron Transport Chain

Hyperglycaemia has been shown to induce the overproduction of superoxides through the mitochondrial electron transport chain (Du *et al*, 2000; Nishikawa *et al*, 2000), which is believed to be the common element linking the four-mechanisms of hyperglycaemia-induced damage (Brownlee, 2001). This overproduction of superoxides increases the flux through both the polyol and hexosamine pathways, AGE formation and PKC activity (Figure 1.17). Mitochondrial superoxides have been shown to induce a 66% decrease in GAPDH activity (Du *et al*, 2000). This inhibition of GADPH activity may activate PARP and deplete NAD<sup>+</sup> (Garcia-Soriano *et al*, 2001), increase triose phosphates levels (Shinohhara *et al*, 1998), increase dihydroxyacetone phosphate levels (Du *et al*, 2000), and increase fructose-6-phosphate levels (Du *et al*, 2000).



Figure 1.17. The four hypothetical pathways by which hyperglycaemia is thought to induce mitochondrial superoxide overproduction. The glycolytic enzyme GADPH is partially inhibited by excess superoxide, which then diverts upstream metabolites from glycolysis into pathways of glucose overutilisation., resulting in increased flux through the various pathways. (Adapted from Brownlee, 2001). GFAT = glutamine:fructose-6-phosphate amidotransferase, UDP-GlcNAc = UDP-*N*-acetylglucosamine, DHAP = dihydroxyacetone phosphate, DAG = diacylglycerol, PKC = protein kinase C, AGE = advanced glycation end-product. Reductive stress can also trigger production of reactive oxygen species through overload of mitochondrial matrix and coenzyme shuttles with NADH and by reaction of NADH with NADH oxidase (Oates and Mylari, 1999). It is also believed that mutations in mitochondrial DNA may be induced by the overproduction of superoxides resulting in the production of defective subunits of the electron-transport complexes. This would eventually result in further increases in superoxide production at physiological concentrations of glucose, eventually activating all four pathways in the absence of hyperglycaemia (Brownlee, 2001).

#### 1.5.2.3.6 Oxidative Stress

Free radicals are highly reactive molecules as they have unpaired electrons in their outer orbits and they become damaging to cellular structures and enzymes when they are produced excessively or when there are inadequate antioxidant defence mechanisms (Halliwell and Gutteridge, 1999). Oxygen free radicals include the hydroxyl radical (OH), superoxide anion ( $O_2$ ), hydrogen peroxide and singlet oxygen and their generation can oxidise membrane lipids or proteins and inactive enzymes which can then impair cellular transport and function eventually leading to cell death (Halliwell, 2001). Oxidative stress thus results from an imbalance between the generation of reactive oxygen species and the ability of the organism to dispose of them. A forced regulation of osmolytes occurs where intracellular concentrations are either increased or decreased through the induction or suppression of enzymes.

It is believed that reactive oxygen species generated as a result of hyperglycaemia play a role in the development of diabetic complications such as nephropathy, retinopathy and neuropathy. The oxidative stress hypothesis suggests that fiber and microvessel damage is promoted by defective antioxidant resistance in diabetes (Nagamatsu *et al*, 1995; Low *et al*, 1997). Many studies have shown that diabetes and hyperglycaemia increase oxidative stress (Guigliano *et al*, 1996). Hyperglycaemia can increase the formation of free radicals and lipid peroxides through several mechanisms including direct auto-oxidation of glucose (glycoxidation), where glucose initiates free radical production and alters the ratio of reduced NADH to NAD<sup>+</sup>, induction and activation of various lipoxygenase enzymes, activation of glycation pathways, interaction of nitric oxide with superoxide anions, and reduction of the activity of the antioxidant defence mechanisms (Ido *et al*, 1997; Giuglioano *et al*, 1996; Baynes and Thorpe, 1999).

### 1.5.2.3.7 Nitric Oxide (NO)

Nitric oxide (NO) is produced from the oxidation of L-arginine by nitric oxide synthase (NOS) (Moncada *et al*, 1991). It serves as an intracellular or intercellular mediator and is released from the endothelium and a variety of other tissues. It is a free radical that can act in a paracrine or autocrine manner to produce both beneficial and detrimental cellular responses. It has been demonstrated in the physiology of every organ system featuring prominently in the control of blood vascular, immune and nervous activities and the regulation of gene expression (Ignarro *et al*, 1996; 1999).

NO was originally identified as a potent endothelial-derived relaxing factor for vascular smooth muscle. The human body contains three known forms of NOS defined in terms of their original cellular source; endothelial NOS (ecNOS or type III NOS), macrophage/cytokine inducible NOS (iNOS or type II NOS) and neuronal NOS (ncNOS or type I NOS) (Moncada *et al*, 1991). Both the ecNOS and ncNOS forms are constitutive, calcium/calmodium-dependent enzymes that synthesise small basal quantities of NO (Knowles and Moncada, 1994). The activity and expression of the iNOS form is low or absent in resting cells but can be rapidly induced by the action of certain cytokines and lipopolysaccharide and is independent of intracellular calcium concentrations. iNOS can be expressed in many cells including pancreatic β-cells, macrophages, fibroblasts, vascular

endothelial cells, masangial cells and cardiac myocytes (Knowles and Moncada, 1994; Xie and Nathan, 1994).

## 1.5.2.3.8 Mitogen-Activated Protein Kinases (MAPKs)

The four major hypothesis believed to contribute towards the development of diabetic complications also share the capacity to activate mitogen-activated protein kinases (MAPKs) which in turn can trigger cellular events necessary for the development of diabetic retinopathy, nephropathy and neuropathy (Tomlinson, 1999). Three main groups of MAPKs are known; the extracellular signal regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases, all of which have subtypes. JNKs respond to several forms of cellular stress (Ip and Davis, 1998) and ERKs are primarily regarded as growth factor signalling kinases (Davis, 1995). p38 is an osmotic response element that can be activated by glucose or diabetes (Igarashi *et al*, 1999). Cellular oxidative stress is known to activate MAPKs (Wang *et al*, 1998) and it has been shown that hydrogen peroxide can activate all three MAPKs (Guyton *et al*, 1996; Clerk *et al*, 1998). Ligand binding to RAGE (Lander *et al*, 1997) and PKC (Clerk *et al*, 1998) can also activate MAPKs.

# 1.5.2.3.9 Vascular Endothelial Growth Factor (VEGF)

VEGF (described and characterised in section 1.3.3.1) induces vascular endothelial cell proliferation, migration and vasopermeability in many cells and tissues (Aiello and Wong, 2000). *In vivo*, VEGF has been identified as a primary initiator of proliferative diabetic retinopathy and as a potential mediator of non-proliferative retinopathy (Aiello *et al*, 1994). It has been known that intraocular VEGF levels are increased in diabetic patients and that a correlation exists between the levels of glycated proteins and the development of retinopathy (Singh *et al*, 2001). VEGF has also been implicated in the development of

neuropathy and nephropathy in patients with diabetes (Aiello and Wong, 2000).

AGEs stimulate VEGF expression in epithelial cells and in vascular smooth muscle cells (Lu *et al*, 1998; Yamagishi *et al*, 1997). A recent study has found that AGEs stimulate VEGF expression in mice retina through an increase in HIF-1 $\alpha$  accumulation and activation of HIF-1 through an ERK dependent pathway (Treins *et al*, 2001).

It has also been reported that PKC is involved in the generation of VEGF (Doanes *et al*, 1999). PKC activation enhances angiogenesis by participating in the intracellular signalling of VEGF in endothelial cells (Takahashi *et al*, 1999; Wu *et al*, 2000). A study has shown that elevated glucose concentrations increase the expression of VEGF in human aortic smooth muscle cells which was inhibited by PKC inhibitors (Williams *et al*, 1997). Additionally, it is known that VEGF can activate several PKC isoforms (Xia *et al*, 1997; Aiello *et al*, 1997). Thus, it is possible that the production of VEGF is stimulated synergistically by activated PKC and hypoxia derived from oxidative stress and increased flux through the polyol pathway (Tomlinson, 1999).

### 1.5.2.3.10 Retinopathy

Patients with T1DM are at higher risk for development of severe retinal complications and visual loss. Damage is caused by both microvascular leakage due to breakdown of the inner blood-retinal barrier and microvascular occlusion (Watkins, 1998).

Diabetic retinopathy involves 5 basic pathological processes that occur at the level of the retinal capillary: formation of microaneurysm, excessive vascular permeability, vascular occlusion, proliferation of new blood vessels and fibrous tissue on the retina, and contraction of the fibrovascular proliferation and the vitreous. The classification of diabetic retinopathy is based on the severity of intraretinal microvascular changes and the presence or absence of retinal neovascularisation. Nonproliferative diabetic retinopathy occurs when only intraretinal microvascular changes occur whereas proliferative diabetic retinopathy

### Chapter 1. Introduction

involves the formation of new vessels or fibrous tissue on the retina (Chew, 2000). Proliferative retinopathy occurs in response to severe ischemia and hypoxia of the retina.

Hyperglycaemia is known to be important in the development of tissue in the lens and optic nerve (Greene *et al*, 1988; Kinoshita, 1986). Tissue injury can result from excessive intracellular production of sorbitol from glucose caused by an increase flux through the polyol pathway (Brownlee, 2001). Transport of glucose into the retina across the blood-retinal barrier occurs via the GLUT-1 transporter (Pessin and Bell, 1992). There have been few reports on the *in vivo* expression of GLUT-1 in retina and its microvessels (Kumagai *et al*, 1996, Badr *et al*, 2000). However, a recent study has shown that diabetes results in the downregulation of GLUT-1 expression in retinal microvessels, but not in the retinal pigment epithelium of rats, suggesting that glucose entering the retina in diabetes is likely to be greater across the retinal pigment epithelium than across the retinal vasculature (Badr *et al*, 2000).

Diabetic retinopathy is also mediated by the hypoxic regulation of angiogenic growth factors including VEGF (Singh *et al*, 2001). Retinal epithelial cells produce VEGF and the synthesis and release of VEGF is increased when the cells are grown under reduced oxygen tension (Aiello *et al*, 1995). Thus, VEGF overexpression can have a detrimental effect in the retina. It is also known that retinal endothelial cells possess many VEGF receptors (Thieme *et al*, 1995). The correlation between elevated VEGF levels and the development of a disordered vascular network has been extensively studied in both animal models (Murohara *et al*, 1998; Rivard *et al*, 1999; Rivard *et al*, 1999b) and human subjects (Losordo *et al*, 1998; Isner *et al*, 1999; Kalka *et al*, 2000; Lathi *et al*, 2001; Bashir *et al*, 2002). It is believed that multiple signals converge and augment the angiogenic signal through a VEGF signalling cascade. It is clear that the interaction of several signals result in neovascularisation. A recent study has reported that the stimulation of VEGF by AGEs, through the activation of HIF-1, could play an important role in the development of diabetic retinopathy (Treins *et al*, 2001). It is known that HIF-1 $\alpha$  expression is induced during normal retinal development however, it is downregulated by hyperoxia, and upregulated upon returning to normoxic conditions (Ozaki *et al*, 1999). Retinal hypoxia in the diabetic state due to reduction of blood flow stimulates the expression of VEGF and results in retinal neovascularisation and increased retinal vascular permeability (Veves and King, 2001).

## 1.5.2.3.11 Nephropathy

Thirty to forty percent of patients with T1DM develop nephropathy within 10 years of diagnosis (Ibrahim and Vora, 1999). Increased excretion of albumin and other proteins in the urine is an early and progressive marker or renal dysfunction in diabetes (Mogensen *et al*, 1995). Diabetic nephropathy is clinically defined as the presence of persistent proteinuria (total urinary protein excretion > 0.5g/24 hours) in sterile urine of diabetic patients with concomitant retinopathy, but without other renal disease or other heart failure (American Diabetes Association, 1998). Overt diabetic nephropathy is characterised by a progressive decline in renal function, resulting in end-stage renal disease.

Diabetic nephropathy occurs in several distinct and interconnected phases; an early phase of physiologic abnormalities of renal function, a microalbuminuria phase and a clinical phase with persistent clinical proteinuria progressing to end-stage renal failure (Mogensen, 1995; 1997). Microalbuminuria is defined as an increase in the albumin excretion rate to a range of 20 to 200  $\mu$ g/minute. Albumin excretion rates in healthy individuals range from 1.5 to 20  $\mu$ g/minute (median 6.5  $\mu$ g/minute). As the disease progresses albuminuria increases until end stage nephropathy, when it may decrease (Ibrahim and Vora, 1999). Large studies have shown that high albumin excretion rates are

the most important risk factor for the later development of incipient and overt nephropathy in T1DM (Microalbuminuria Collaborative Study Group, 1993; Mathiesen *et al*, 1995).

The development of diabetic nephropathy is characterised by progressive thickening of the glomerular and tubular basement membranes, expansion of the mesangial matrix, and increased synthesis of collagens due to increased proliferation of mesangial cells, accumulation of the extracellular matrix and obliteration of glomerular capillaries (Lehmann and Scheicher, 2000). These characteristics can influence the glomerular filtration capacity and hemodynamics as well as being responsible for the increased secretion of albumin and decreased filtration that occurs in patients with diabetic nephropathy (Khasigov *et al*, 2000).

It is known that chronic hyperglycaemia can lead to sorbitol accumulation in a variety of tissues, including renal tubuli and glomeruli, which then leads to tissue damage through a disruption of cellular osmoregulation in addition to depletion of myoinositol (Greene, 1988). However, it is believed that renal damage in the diabetic kidney is unlikely to occur through a mechanism involving the polyol pathway (Schleicher and Nerlich, 1996). It is known that glucose transporter-1 (GLUT-1) is the main transporter of glucose in the glomeruli (Heilig *et al*, 1995; Heilig *et al*, 1997) (section 1.5.2.4.1). Experiments involving the administration of an aldose reductase inhibitor to streptozotocin-induced diabetic rats have shown that histologic lesion of glomerular disease remained unaffected (Rasch and Osterby, 1990).

The role of VEGF in the development of diabetic nephropathy has recently been explored. VEGF expression is found to be upregulated in glomerular endothelial, tubular and mesangial cells by hypoxia, hyperglycaemia induced PKC activation or transforming growth factor- $\beta$  (TFG- $\beta$ ) mediated pathways (Del Prete *et al*, 1998; Cooper *et al*, 1999). Increased expression of VEGFR-2 receptors have also been observed in diabetic rat models (Cooper *et al*, 1999). Studies have also demonstrated that VEGF isoforms mediate repair in glomerular cells (Ostendorf *et al*, 1999) suggesting that increased VEGF expression may be a repair response and/or a cause of pathological changes in diabetic nephropathy.

### 1.5.2.3.12 Neuropathy

Diabetic neuropathy is associated with risk factors for macrovascular disease and with other microvascular complications such as poor metabolic control, dyslipidaemia, body mass index, smoking, microalbuminuria and retinopathy (Cameron *et al*, 2001). The most common form of diabetic neuropathy, a heterogeneous condition, is distal symmetric polyneuropathy which damages distal peripheral nerves together with the autonomic nervous system. Neuropathies may be sensory, motor or mixed and may involve primarily small or large nerve fibers (Brown and Asbury, 1984; Tomlinson *et al*, 1997). Diffuse peripheral neuropathy also causes diffuse damage to the autonomic nerves, both parasympathetic and sympathetic. Studies in human and animal models have shown reduced nerve perfusion and endoneurial hypoxia.

Investigations on biopsy material from patients with mild to severe neuropathy show graded structural changes in nerve microvasculature including basement membrane thickening, pericyte degeneration and endothelial cell hyperplasia (Cameron *et al*, 2001). Increased polyol pathway activity by hyperglycaemia results in the accumulation of sorbitol and fructose in nerves, which in turn cause damage by an unknown mechanism. Decreased myoinositol uptake, inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase and changes in the specific activity of PKC then occur, resulting in Na<sup>+</sup> retention, edema, myelin swelling, axoglial disjunction and nerve degeneration (Vinik *et al*, 2000). This complex interaction of metabolic factors accounts for endothelial dysfunction, reduced nerve perfusion and function. Thus, vascular dysfunction, driven by metabolic change, is a main factor in the development of diabetic neuropathy.

### 1.5.2.4 Glucose Transporter Gene Family

Glucose transport has been highly investigated in diabetes research and has led to the discovery of a family of glucose transporters. The glucose transport (GLUT) family of integral membrane glycoproteins mediates the facilitative transport of glucose across mammalian cell plasma membranes. There were originally 7 members which have been designated GLUT-1 through 5 in order of their identification by cDNA cloning and share 39 % to 65 % identity with each other and 50-76 % similarity between amino acid sequences (Mueckler, 1994; Baldwin, 1993, Pessin and Bell, 1992). Two additional members were included in the family however *GLUT-6* was found to encode a pseudogene (Kayano *et al*, 1990) and the existence of GLUT-7 is disputed (Burchell, 1998).

Recently, homology searches of the EST databases have identified several glucoselike sequence fragments from which full-length cDNAs were isolated and characterised using PCR and RACE technologies (Doege et al, 2000a; 2000b; 2001a; Ibberson et al, 2000; Phay et al, 2000; McVie-Wylie et al, 2001). These searches have resulted in the identification of a total of 7 new members of the GLUT family that exhibit significant similarity (>28 % identical amino acids) with GLUT-1 were identified; GLUT-6, 8, 9, 10, 11, 12 and HMIT1 (reviewed in Joost and Thorens, 2001) (Table 1.9). One of the original GLUT proteins that was designated GLUT-9 (Doege et al, 2000a) was recently renamed GLUT-6 (Joost et al, 2001). Additional homology searches have identified 4 presumed pseudogenes that exhibit significant similarity with the GLUT family. The extended GLUT family has been subsequently divided into three sub-classes; Class I comprises the totally characterised glucose transporters GLUT-1 to 4, Class II comprises the fructose-specific transporter GLUT-5 and related proteins GLUT-7, 9, and 11, and Class III comprises five isotypes GLUT-6, 8, 10, 12 and HMIT1 (Doege et al, 2001; Joost and Thorens, 2001). The GLUT proteins are expressed in a cell specific manner in different tissues and display unique regulatory functions, which indicates that each transporter plays a precise role in glucose metabolism.

Glucose transporters are widely distributed and are found on the surface of all mammalian cells in either inward or outward facing conformations. All family members have a similar membrane topology that includes 12 membrane-spanning  $\alpha$ -helical segments consisting of a large exofacial (outward facing) loop between membrane spanning segment 1 (M1) and M2 and a large hydrophilic segment connecting M6 and M7 (Bell *et al*, 1990; Mueckler, 1994). This region of the molecule is believed to form the hexose pore (Leinhard *et al*, 1992). The amino and carboxy termini are exposed to the cytoplasmic face. A sugar moiety of variable length is attached to the exofacial domain that is involved in determining the cellular location of the proteins (Kumagai *et al*, 1994). A single cell can express more than one transporter isoform and most tissues express several. The glucose transporter genes are not located at one specific site; rather they are dispersed on different chromosomes or on different regions of a specific chromosome.

### 1.5.2.4.1 Glucose Transporter 1 (GLUT-1)

The human GLUT-1 gene was the first to be characterised, it is located on chromosome 1p35-p31.3 and found to consist of 10 exons of 2544 nucleotides in length (Mueckler *et al*, 1985; Fukumoto *et al*, 1988). The introns of the GLUT-1 gene interrupt the protein-coding region, as in all the family members. GLUT-1 is the most widely distributed isoform and is expressed in foetal and adult tissues (Birnbaum *et al*, 1986; Mueckler *et al*, 1985). It is abundant in human erythrocytes, placenta and endothelia and mediates basal glucose uptake and transport across blood-brain barrier and other barrier tissues. GLUT-1 is associated with cells undergoing proliferation and is induced in growth-factor stimulated cells. In addition, the level of expression of GLUT-1 correlates with the glycolytic activity of the cells in which it is expressed. It is known that GLUT-1 and GLUT-4 continuously recycle in living cells even in the absence of insulin (Czech and Buxton, 1993; Davies *et al*, 1990). A three-dimensional model of the human facilitative

Isoform	Size (Amino Acids)	Function	Tissue Location (Sites of Expression)	Reference
GLUT-1	492	Basal transport	Erythrocytes, brain, placenta, kidney, colon	Mueckler et al, 1985
GLUT-2	524	Glucose sensor, low- affinity transporter	Liver, pancreatic β-cells, kidney, small intestine	Fukumoto <i>et al</i> , 1988
GLUT-3	496	Basal transport, high- affinity transporter	Brain, placenta, testes, kidney, neurons	Kayano <i>et al</i> , 1988
GLUT-4	509	Insulin-regulated glucose transport	Fat, muscle, heart, skeletal muscle	Fukumoto et al, 1989
GLUT-5	501	High-affinity fructose transporter	Small intestine, testes, skeletal muscle, fat	Kayano et al, 1990
GLUT-6 (formerly GLUT-9)	507	Low-affinity transporter	Brain, spleen, leukocytes	Doege <i>et al</i> , 2000a; Joost <i>et al</i> , 2001b
GLUT-7	528	Mediated glucose release from the ER	Liver	Burchell, 1998
GLUT-8 (formerly GLUTX 1)	477	High-affinity glucose transporter	Testes, brain, blastocyst, heart, skeletal muscle	Doege et al, 2000b; Carayannopoulos et al, 2000; Ibberson et al, 2000
GLUT-9	540	No data published	Liver, kidney, lung	Phay <i>et al</i> , 2000
GLUT-10	541	Glucose transporter	Liver, pancreas, heart, kidney, skeletal muscle	McVie-Wylie et al, 2001; Dawson et al, 2001
GLUT-11 (formerly GLUT10)	496	Fructose transporter	Heart, skeletal muscle	Doege et al, 2001a; 2001b Sasaki et al, 2001
GLUT-12 (formerty GLUT-8)	617	Possible glucose transporter	Heart, prostate, skeletal muscle, small intestine, fat	Rogers <i>et al</i> , 1998; Rogers <i>et al</i> , 2002
HMIT	629	High-affinity myo- inositol transporter	Brain	Uldry et al, 2001

Note: GLUT-9 was re-named GLUT-6 which was previously used for a pseudogene (Kayano *et al*, 1990). (Adapted from Joost and Thorens, 2001).

glucose transporter GLUT-1 has been recently proposed and is shown in Figure 1.18 (Zuniga et al, 2001).

GLUT-1 is often the predominant isoform expressed in cultured cells. The level of glucose transporter activity, GLUT-1 protein and mRNA in cultured cells can be altered by a number of different stimuli including stress. However, the effect of additional stimuli on glucose transporter expression is often specific for a given cell line or cell type.

### 1.5.2.4.1.2 Glucose Metabolism

Glucose is the main source of energy for most cells and transport across plasma membranes is rate-limiting for glucose metabolism in the majority of mammalian cells (Klip *et al*, 1994; Baldwin 1993). After glucose is transported into cells or tissue, it is either converted into glycogen or it undergo glycolysis where it is released as lactate or oxidised in the Krebs cycle. The enzyme glycogen synthase regulates the incorporation of glucose into glycogen and the pyruvate dehydrogenase multienzyme complex regulates the entry of glucose carbon into the Krebs cycle for oxidation. Glucose is eventually completely oxidised to  $CO_2$  and  $H_2O$  in the mitochondria. Increased glycolysis is associated with upregulation of glycolytic enzymes and abnormal expression of glucose transporters (Hennipman *et al*, 1987). In the majority of cells and tissues intracellular glucose is low and glucose transport is rate-limiting for glucose utilisation. Thus, glucose homeostasis requires a controlled regulation of glucose flux into and out of cells or organs.



Figure 1.18. Proposed ribbon model of the human facilitative glucose transporter GLUT-1. Helices 1-5, 8 and 10-12 (coloured) are arranged in a 9-member barrel-like manner. Loops are white. (Zuniga *et al*, 2001).

# 1.5.2.4.1.3 Glucose Transport

Glucose transport in most tissues is a passive process as it occurs down a concentration gradient by facilitative diffusion. As the plasma membrane is impermeable to large polar molecules, the cellular uptake of glucose occurs using membrane-associated carrier proteins that bind glucose and carry it across the lipid bilayer (Bell *et al*, 1990). The glucose molecules bind to the outward facing form of the transporter, which then undergoes a conformational change and transports the sugar into the cell. Glucose transporters can be regulated acutely, chronically or both. Acute stimulation of glucose transporters occurs through either translocation of the transporter to the plasma membrane or activation of glucose transporters that pre-exist at the cell surface (Behrooz and Ismail-Beigi, 1999). Insulin increases the rate of glucose transport across the cell membrane in both muscle and adipose tissue by increasing the number of glucose transporters in the plasma membrane, which in turn increases the net rate of translocation of the transporter from an intracellular store to the cell membrane (Kahn, 1996). Under high glucose transport activity the rate of glucose transport is not susceptible to insulin and glucose transport occurs with near equilibrium in both directions across the cell membrane.

### 1.5.2.4.1.4 Glucose Transport and Cancer

It has been long known that there is increased glycolysis in cancer cells compared to normal cells (Warburg, 1956). Tumour cells exhibit high glycolytic activity and accumulate high levels of lactate compared to normal cells, which can be attributed to an increased expression of glucose transporters. The expression of glucose transporters has been studied in a wide variety of human tumour types. Increased GLUT-1 expression has been observed under hypoxic conditions in cancer cells (Clavo *et al*, 1995) and in cells transformed by oncogenes (Flier *et al*, 1987, Godwin and Lieberman, 1991). Glucose uptake has been shown to be up-regulated in breast cancer cell lines where increased GLUT-1, GLUT-2, GLUT-4 and GLUT-5 expression was demonstrated (Zamora *et al*, 1996). In breast cancers with higher tumour grade and proliferative activity, increased expression of GLUT-1 was found (Wang *et al*, 1996).

### 1.5.2.4.1.5 Glucose Transport and Hypoxia

Several stimuli, such as hypoxia, inhibition of oxidative phosphorylation and exposure to alkaline pH, have been shown to increase the rate of glucose transport in a variety of experimental models (Wheeler, 1998; Shetty *et al*, 1992; Hakimian and Ismail-Beigi, 1991). It is thought that hypoxia mediates the enhancement of GLUT-1 mediated glucose transport by at least two independent cell-signalling pathways, which involve multiple mechanisms. One pathway involves the inhibition of oxidative phosphorlyation after hypoxia, which leads to acute stimulation of glucose transport and a delayed induction of GLUT-1 expression. A second pathway involves the induction of an oxygen sensor mechanism that also stimulates GLUT-1 gene transcription (Behrooz and Ismail-Beigi, 1999) (Figure 1.19).

During aerobic metabolism, molecular oxygen becomes an electron acceptor, which results in the production of ATP or cellular energy, and oxidative phosphorylation occurs. Inhibition of this process through production of hypoxic conditions or exposure to pharmacological inhibitors results in an increase in the cellular demand for glucose metabolism through the glycolytic pathway to generate lactate (Behrooz and Ismail-Beigi, 1999). Thus, decreased mitochondrial respiration and ATP synthesis activates the stimulation of glucose transport. It has been demonstrated that decreased levels of intracellular ATP increase cytosolic calcium concentrations and enhance GLUT-1 function and expression. However, not all cells exposed to hypoxia exhibit decreases in intracellular ATP levels suggesting that localised changes in intracellular ATP levels may modulate glucose transport (Mitani *et al*, 1996).

Chapter 1. Introduction



Figure 1.19. Hypoxia induced GLUT-1 mediated glucose transport.

At least two independent pathways are involved in hypoxia enhanced GLUT-1 mediated glucose transport, an oxygen-sensing and signalling pathway involving hypoxia inducible factor 1 (HIF-1), and inhibition of oxidative phosphorylation which results in increased GLUT-1 mRNA stability and activation and translocation of pre-existing GLUT-1 molecules. (Adapted from Behrooz and Ismail-Beigi, 1999).

As many cellular processes are oxygen dependent, several oxygen consuming reactions could serve as second messangers that trigger a hypoxic response. It is believed that these reactions work independently or in conjunction to modulate specific changes in gene expression and gene product function. Oxygen sensing involves the oxygen molecule binding reversibly to intracellular receptors under hypoxic conditions, which then transmit a hypoxic signal. It is believed that heme-containing molecules act as oxygen sensors which are able to bind to oxygen as well as certain divalent cations such as  $Co^{2+}$ , Ni<sup>2+</sup>, and  $Mn^{2+}$  (Goldberg *et al*, 1988). These metal ions can substitute for iron in the sensors' heme moiety and maintain the molecule in an active state. This oxygen-sensing system and signalling pathway is ubiquitous and in addition to controlling GLUT-1 gene expression through transcription, it also regulates a number of hypoxia-inducible genes in a variety of oxidative phosphorylation or decreases in intracellular ATP content (Behrooz and Ismail-Beigi, 1997).

## 1.5.2.4.1.6 Neurons

Neurons are dependent upon glucose and oxygen for energy. The majority of adenosine triphosphate (ATP) generated in neurons for normal functions is dependent upon the aerobic oxidation of glucose and the integrity of the oxidative phosphorylation pathway. The facilitative uptake of glucose into mammalian brain cells occurs via GLUT-1 and GLUT 3. GLUT-1 is most abundant in brain microvessels (Maher *et al*, 1994) and its mRNA is distributed in a diffuse pattern throughout the brain (Bondy *et al*, 1992). A recent study found that hypoxia stimulates a reversible increase in GLUT-1 steady-state mRNA levels in primary cultures of rat neurons and glia, due to enhanced RNA stability and not increased transcription, and is temporally associated with increased GLUT-1 protein and transporter activity (Bruckner *et al*, 1999). Thus, hypoxia and glucose deprivation synergise to increase GLUT gene expression.

# **1.6 HIF-1 Interactions**

HIF-1 $\alpha$  activates transcription of a large number of different glucose transporters and glycolytic enzymes, and a number of stress response and oxygen regulated genes including p53, VEGF and iNOS, all of which are implicated in vasodilation, neovascularisation and tumour metastasis (Iyer *et al*, 1998; Ryan *et al*, 1998; Carmeliet *et al*, 1998; Zhong *et al*, 1999). In response to hypoxia, or stress, oncoproteins provide hypoxic tumour cells with a growth advantage, angiogenic factors attract new vasculature to increase oxygenation and glucose transporters and glycolytic enzymes allow to switch to energy saving glycolysis (Dachs and Tizer, 2000).

#### 1.6.1 Tumour Suppressor: p53

There have been many recent advances in understanding the functional role of p53 in tumourigenesis, however the mechanisms underlying the stabilisation of p53 in hypoxia have yet to be determined. It is known that increased HIF-1 expression is associated with multiple genetic alterations that promote tumour angiogenesis, such as loss-of-functions mutations in tumour suppressor genes and by oncogene activation. As HIF-1 is a key transcriptional activator of genes encoding glucose transporters and glycolytic enzymes (Iyer *et al*, 1998), genetic alterations may also contribute to the metabolic adaptation and increased survival of tumour cells in hypoxic environments (Ravi *et al*, 2000).

A recent study proposed that the HIF-1 $\alpha$  subunit binds to p53 and stabilises p53 by protecting it from proteasomal degradation (An *et al*, 1998), however another study has proposed that mechanisms other than HIF-1 $\alpha$  activation contribute to oxygen-regulated p53 induction (Wenger *et al*, 1998). As HIF-1 $\alpha$  and p53 both bind to distinct regions of the transcriptional coactivator p300 (Blagosklonny *et al*, 1998), which is required for full activity of both transactivators, it has been proposed that the interaction of HIF-1 $\alpha$  and p53 may be indirect and insufficient for hypoxic stabilisation of p53. High levels of p53 inhibit
HIF-inducible transcription via p300 (Blagosklonny *et al*, 1998), however up-regulation of HIF-1 $\alpha$  is not sufficient for p53 induction (Wenger *et al*, 1998). It is possible that both p53 and HIF-1 $\alpha$  can bind simultaneously to p300 as the HIF binding site has been localised to amino acids 346 to 400 (Arany *et al*, 1996) and that for p53 to a p300 fragment containing amino acids 1514 to 1922 (Gu *et al*, 1997).

Another recent study has shown that loss of p53 function increases HIF-1 activity resulting in the overexpression of VEGF that is commonly observed in a wide variety of cancers (Ravi *et al*, 2000). It was found that p53 interacts with HIF-1 $\alpha$  *in vivo* and inhibits the hypoxia-induced expression of HIF-1 $\alpha$  by assisting its ubiquitination, through the binding of the ubiquitin-protein ligase MDM2 to p53, and consequential degradation. Thus, p53 decreases the stability of HIF-1 $\alpha$  in an MDM2-dependent manner, indicating that HIF-1 $\alpha$  is the preferential target of MDM2 (Ravi *et al*, 2000). This mechanism of tumour suppressor action is similar to that proposed for the von Hippel-Lindau (VHL) tumour suppressor which affects HIF-1 $\alpha$  degradation through ubiquitination. Thus p53 mediated inhibition of VEGF expression, in conjunction with its ability to up-regulate inhibitors of angiogenesis, such as thrombospondin-1 (Dameron *et al*, 1994), indicate that p53 regulates angiogenesis using dual functions.

# 1.6.2 Angiogenic Factor: VEGF

HIF-1 is the best-characterised regulator of VEGF gene transcription. It plays a central role in the hypoxic induction of the VEGF gene by binding to its target DNA sequence. A hypoxic response element is located approximately 1 kb 5' to the transcription start site of the human VEGF gene (Forsythe *et al*, 1996; Lui *et al*, 1995). Studies have shown a strong correlation between the level of hypoxia-induced HIF-1 DNA-binding activity and VEGF mRNA expression (Forsythe *et al*, 1996; Wood *et al*, 1996).

VEGF expression is induced in both transformed and nontransformed cells exposed to various cytokines and growth factors (Jackson *et al*, 1997; Ryuto *et al*, 1996) all of which have also been shown to induce HIF-1 $\alpha$  protein expression and/or HIF-1 DNA binding activity (Zhong *et al*, 2000). Further immunohistochemical studies have also shown that VEGF and HIF are both highly expressed in pseudopalisading cells surrounding areas of necrosis in GBM (Plate *et al*, 1992; Zagzag *et al*, 2000) suggesting that HIF-1 mediates hypoxia-induced VEGF expression in GBM. Moreover, a recent study has shown that AGEs stimulate VEGF mRNA expression in mice retina through and increase in HIF-1 $\alpha$  accumulation and activation of HIF-1 (Treins *et al*, 2001). Thus, AGE induced VEGF expression, through the activation of HIF-1, could play an important role in the development of diabetic complications.

# 1.6.3 Metabolite: GLUT-1

The key regulator of the oxygen sensing and signalling pathway is HIF-1 (Semenza, 2002). There are no less than two independent pathways involved in hypoxia enhanced GLUT-1 mediated glucose transport, an oxygen-sensing and signalling pathway involving hypoxia inducible factor 1 (HIF-1), and inhibition of oxidative phosphorylation which results in increased GLUT-1 mRNA stability and activation and translocation of pre-existing GLUT-1 molecules (Behrooz and Ismail-Beigi, 1999). As in other oxygen-regulated genes, both the mouse and rat GLUT-1 gene contains a *cis*-acting element located in the 5' flanking region of the gene that is necessary for transcriptional response to hypoxia (Ebert *et al*, 1995; Behrooz and Ismail-Beigi, 1997). Exposure to hypoxia for prolonged periods of time results in the induction of mRNA and GLUT-1 protein (Bashan *et al*, 1992), however the half-life of GLUT-1 mRNA is decreased (Stein *et al*, 1995). The response is mediated by transcriptional and post-transcriptional regulatory mechanisms that lead to an overall increase in cell and plasma membrane GLUT content. Enhanced

transcription occurs to increase the availability of glucose so that the cell can survive metabolic alterations. Increases in glucose uptake and glycolysis are thought to help the cell preserve high-energy phosphates and sustain the cells from lethal injury during hypoxia.

#### 1.6.4 Vasoactive Agent: Nitric Oxide

HIF-1 is essential for iNOS induction under hypoxia conditions (Jung *et al*, 2000), and NO is known to modulate the expression of HIF-1 in hypoxia (Liu *et al*, 1998; Huang *et al*, 1999; Kimura *et al*, 2000). This interaction has not been thoroughly defined. It is thought that the thiol groups in HIF-1 or the proteins that are involved in the regulation of HIF-1 are potential targets for nitric oxide (Palmer *et al*, 2000). Additionally, it has recently been demonstrated that NO generated by iNOS expression inhibits HIF-1 activity in hypoxic C6 cells suggesting a negative feedback loop in the HIF-1/iNOS cascade (Yin *et al*, 2000). The molecular mechanism of this NO-mediated inhibition of HIF-1 binding is not known, however several theories have been suggested. One theory suggests that NO may bind to ferric heme oxygen and superoxide which may affect the signal pathway for HIF activation (Stamler, 1994). Enhanced oxidative stress enhances the cellular redox state and thereby determines HIF-1 $\alpha$  binding activity (Haddad and Land, 2000).

It is also known that VEGF expression is regulated by NO, where NO has been shown to stimulate VEGF expression by stabilising HIF-1 expression (Klagsbrun and D'Amore, 1996). Additionally, it has been shown that NO down-regulates the expression of VEGF (Liu *et al*, 1998; Huang *et al*, 1999). The exact role that NO plays in this regulation remains unclear. Studies have shown that NO induces VEGF expression in glioblastoma and hepatoma cells (Chin *et al*, 1997; Kimura *et al*, 2000). A recent study in breast carcinoma cell found that VEGF contributes to tumour growth through inhibition of apoptosis and increased NOS activity (Harris *et al*, 2002). It has recently been demonstrated that the additional HIF-1 binding site located downstream within the HRE, now deemed the HIF-1 ancillary sequence (HAS), is a novel *cis*-element for VEGF gene induction by NO and hypoxia (Kimura *et al*, 2001).

Figure 1.20 summarises the response mechanisms of HIF-1 to hypoxia and cellular stresses.

Chapter 1. Introduction



Figure 1.20. Involvement of HIF-1 in disease.

The diagram summarises HIF-1 response mechanisms when induced by hypoxia and/or cellular stresses.

# 1.7 Aims of the Project

The aims of this study were to determine the role of HIF-1 in disease including carcinoma of the breast, brain tumours and diabetes mellitus through:

• An investigation of the effect of the cellular stresses of hypoxia and hyperglycaemia on HIF-1 a, p53, VEGF, and GLUT-1 gene and protein expression and regulation in established breast carcinoma cell lines and in isolated PBMCs from patients with T1DM and normal healthy controls.

The primary cellular response to either excess or depletion of local glucose or oxygen concentrations is a shift in metabolic pathways usually via glycolysis. Exactly how conditions of stress, such as hypoxia, hypoglycaemia or hyperglycaemia act at the molecular level to initiate disease progression is not clearly understood. Changes in gene pattern expression prompted by cellular stress and specific transcription factor involvement have only recently begun to be investigated.

Since the discovery of HIF-1 (Wang and Semenza, 1992), the characteristics of HIF-1 and other stress regulated genes have been explored in studies using cultured cell lines. Gene expression in response to hypoxia is complex, differs between models and may be significantly different in *in-vitro* cell culture compared to the *in-vivo* environment (Sander *et al*, 1997). This project used established breast carcinoma cell lines as a model system for breast tumour tissue as well as cultured PBMCs in exploring the characteristics of HIF-1.

• An investigation of the levels of HIF-1 $\alpha$ , VEGF, p53 and GLUT-1 expression and localisation in breast and brain tumour tissue and determining whether levels of some of these genes are associated with disease progression and survival.

The ability of cancer cells to respond to and survive under hypoxia through the induction of oxygen related genes relates to their capacity to sense hypoxia and to activate the HIF pathway. It is possible that constitutive up-regulation or increased inducibility of HIF-1 $\alpha$  may be associated with increased induction of oxygen related genes, tumour growth and disease progression.

• An investigation of the p53 and putative p73 gene tumour suppressor involvement in breast carcinoma by investigating its genomic and expression status in isolated PBMCs of patients with breast cancer and normal healthy control populations, as well as determine whether p53 and p73 gene status is related to susceptibility, aggressiveness of the disease and patient survival.

The candidate tumour suppressor gene p73, which is implicated in the pathogenesis of several different types of cancer, may also be involved in the development of carcinoma of the breast. To elucidate the role of p73 in the development of breast cancer and the allelic-specific expression as a potential imprinted tumour suppressor, the allelic expression of the p73 gene was investigated in the PBMCs of patients with breast cancer.

p53 has been implicated in the regulation of angiogenic balance through control of the expression of VEGF. p73 has been found to down-regulate endogenous expression of VEGF gene expression at mRNA and protein levels (Salimath *et al*, 2000). Chapter 2. Materials and Methods

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# Chapter 2.

# **Materials and Methods**

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# 2.1 Subjects

# 2.1.1 Patients with Breast Cancer

Ninety-eight patients with breast cancer attending the Breast Cancer Clinic (Dr. S. Kelly) at Derriford Hospital, Plymouth, U.K., were used in DNA genotyping studies. Local ethical committee approval had been obtained. At presentation the ages ranged from 27 to 80 years (mean  $\pm$  SD = 52  $\pm$  12.3 years). The clinical characteristics of the patients are presented in Table 2.1.

# 2.1.2 Patients with Type-1 Diabetes Mellitus (T1DM)

Thirty-eight British Caucasoid patients with T1DM, as defined by the National Diabetes Group 1979, attending the Diabetic Clinic (Dr. B.A. Millward) at Derriford Hospital, Plymouth, UK, were used in RNA studies. Local ethical committee approval had been obtained. At presentation the ages ranged from 17 to 70 years (mean  $\pm$  SD = 42.4  $\pm$  12.8 years). The patients consisted of individuals without complications (diabetic controls (DC) section 2.1.3.2) and with complications, patients who have had T1DM for more than 10 years with nephropathy, neuropathy and/or retinopathy. The clinical characteristics of all patients are presented in Table 2.2.

Variable	Frequency (n)			
Patients	97 (100)			
Age (median 52 year	s)			
<52	49.5 (48)			
≥52	50.5 (49)			
T stage				
1	51.5 (50)			
2	35.1 (34)			
3	11.3 (11)			
4	2.1 (2)			
N stage				
0	53.6 (52)			
1	26.8 (26)			
2	10.3 (10)			
3	9.3 (9)			
Grade				
1	9.3 (9)			
2	30.9 (30)			
3	59.8 (58)			
Smoking habits				
Non-smoker	82.5 (80)			
Smoker	17.5 (17)			
Chemotherapy				
Yes	63.9 (62)			
No	36.1 (35)			
Radiotherapy				
Yes	84.5 (82)			
No	15.5 (15)			

Table 2.1. Clinical characteristics of patients with breast cancer (section 2.1.1).

Mean age at diagnosis was  $52.0 \pm 12.3$  years (27 – 80 years), mean survival time was  $5.5 \pm 3.9$  years (0.70 - 24.2 years). ( $\pm$  standard deviation). n = actual number of patients (in parenthesis).

Characteristics	Normal	Patients with T1DM				
	Controls	Without Complications	With Complications	All Patients		
Number of subjects	6	9	29	38		
Sex (m:f)	4:2	2:7	12:17	14:24		
Age (years)	35 ± 6.4	44.9 ± 15.0	41.6 ± 12.3	42.4 ± 12.8		
(Range)	(27-44)	(27-70)	(17-69)	(17-70)		
Age at onset (years) (Range)	-	19.0 ± 14.0 (2-42)	14.2 ± 9.7 (1-37)	15.4 ± 10.9 (1-42)		
Duration (years) (Range)	-	28.4 ± 11.7 (13-51)	27.4 ± 9.0 (8-43)	27.7 ± 9.6 (8-51)		
HbA1c (Range)	-	7.92 ± 0.7 (7.2–9.2)	8.73 ± 1.5 (6.1–12.4)	8.54 ± 1.4 (6.1-12.4)		
Insulin Dose (Units) (Range)	-	49.8 ± 29.9 (20-118)	40.7 ± 18.6 (10-94)	42.8 ± 21.7 (10-118)		

Table 2.2. Clinical characteristics of patients with T1DM and normal controls (section 2.1.2)

All patients had T1DM as defined by the National Diabetes Data Group, 1979. The data are expressed as mean values (± standard deviation).

#### 2.1.3 Controls Subjects

#### 2.1.3.1 Normal Controls (NC)

a) Controls used for DNA genotyping consisted of 142 sequential cord blood samples, obtained after normal obstetric delivery at the Maternity Unit, Derriford Hospital, Plymouth. No individual or family history of T1DM or any other autoimmune disease was associated with the subjects. The characteristics of the normal controls are listed in **Table 2.2**.

b) Eighty-five individuals of Cornish Celtic ancestry (at least three generations of both maternal and paternal relatives have resided in Cornwall) were used as an additional control population in the DNA genotyping studies.

c) Controls used in RNA studies consisted of 10 Caucasoid healthy individuals with no personal history of diabetes or any other autoimmune disease.

#### 2.1.3.2 Diabetic Controls (DC)

Patients with T1DM, as defined by the National Data Group 1979, who have no history of, nor documented long term diabetic complications after 20 or more years of the disease were used as normal controls in the RNA studies. These patients have had T1DM for at least 20 years but remain free of retinopathy (fewer than 5 dots or blots per fundus), proteinuria (negative on urine Albustix on at least three consecutive occasions over then previous 12 months) and no overt neuropathy. The clinical characteristics of the patients are listed in Table 2.2 (patients with T1DM without complications).

# 2.2 Tumour Samples

# 2.2.1 Breast Cancer

Approximately a 1 cm<sup>2</sup> sample of fresh breast tumour from 22 individuals with breast cancer was collected immediately following operative removal, snap-frozen and stored in liquid nitrogen prior to RNA extraction. Tumours were classified according to WHO guidelines (Kleihues *et al*, 1993). No normal tissue was obtained for study. At presentation the ages ranged from 42 to 91 years (mean  $\pm$  SD = 66.7  $\pm$  15.8 years). Local ethical committee approval had been obtained. The clinical characteristics of the patients are presented in Table 2.3.

# 2.2.2 Glioblastoma Multiforme

Forty-one samples of formalin fixed and paraffin embedded sections of brain tumours were studied. Tissue from 34 of these tumour samples was snap-frozen and stored in liquid nitrogen prior to RNA extraction. Tumours were classified according to current World Health Organisation (WHO) guidelines (Kleihues *et al*, 1993): 27 glioblastomas, 4 anaplastic astrocytomas, 5 diffuse astrocytomas (only 3 with frozen tissue), 5 pilocytic astrocytomas (none with frozen tissue). Two frozen samples of normal brain tissue obtained at autopsy were also used. Local ethical committee approval had been obtained. At presentation the ages ranged from 25 to 80 years (mean  $\pm$  SD = 55  $\pm$  15.3 years). The clinical characteristics of the patients are presented in Table 2.4.

 Table 2.3. Frequencies of the clinicopathological characteristics of patients with breast cancer and their tumours (section 2.2.1).

Variable	Frequency (n)		
Patients	100 (22)		
Age (median 66 years)			
<66	45.5 (10)		
≥66	54.5 (12)		
T stage			
1	68.2 (15)		
2	31.8 (7)		
3	0 (0)		
4	0 (0)		
N stage			
0	45.5 (10)		
1	36.4 (8)		
2	9.1 (2)		
3	9.1 (2)		
Grade			
1	4.5 (1)		
2	36.4 (8)		
3	59.1 (13)		

The mean age at diagnosis was  $66.7 \pm 15.8$  years (42-91 years), mean survival time was 3.4  $\pm$  1.4 years (1.2-8.0 years) ( $\pm$  standard deviation). n = actual number of patients (in parenthesis).

Table 2.4.	Clinical characteristics of normal controls and patients with glial cell tumours
	(section 2.2.2).

Characteristics	Normai Controls	Diffuse Astrocytomas	Anaplastic Astrocytomas	Glioblastomas	All Patients	
Number of subjects	2	3	4	27	34	
Sex (m:f)	2:0	0:3	1:3	18:9	19:15	
Age at Diagnosis (years) (Range)	74.5 ± 19.1 (61-88)	30.7 ± 5.13 (25-35)	43.5 ± 11.3 (32-59)	59.4 ± 13.1 (35-80)	55.0 ± 15.3 (25-80)	
Survival (years) (Range)	•	4.46 ± 0.23 (4.01-4.73)	2.36 ± 0.71 (1.08-4.05)	0.58 ± 0.51 (0.01-2.11)	1.13 ± 1.4 (0.01-4.73)	
Survival (weeks) (Range)	-	232.0 ± 11.72 (208.74-245.97)	122.9 ±·37.0 (56.14–210.67)	30.1 ± 26.3 (0.74-109.47)	58.8 ± 70.5 (0.74-245.97)	

The data are expressed as mean values ( $\pm$  standard deviation).

# 2.3 Materials

# 2.3.1 Water

All general purpose, specialised and stock solutions were prepared using either sterile bottled water (Baxter Healthcare, Thetford, UK) or double distilled tap water obtained from an Elix S Millipore water filtration system (Millipore Ltd, Watford, UK). All PCR based applications were prepared solely with sterile bottled water.

# 2.3.2 Reagents

Reagents used in all experiments were of molecular biology grade, analytical grade or equivalent. All general-purpose reagents were acquired from four main suppliers:

a) BDH Laboratory Supplies, Merck Limited (Lutterworth, UK)

Acetic acid, ethylene diamine tetra-acetic acid, hydrochloric acid, magnesium chloride, maleic acid, orthoboric acid, sodium chloride, sodium citrate, sodium hydroxide, Tween-20 (poly-oxethylenesorbitanmonolaurate)

b) Sigma Chemicals (Poole, UK).

Ammonium persulfate, Aprotonin, Brilliant Blue, Ethidium bromide (2,7-diamino-10ethyl-9-phenly-pheanthridinium), Dimethyl sulphoxide (DMSO), Dithiothreitol (DTT), Formamide, Glycine, Glycerol, HEPES, MOPS, N-Laurosarcosine, Phytohaemaglutinin (PHA, Lectin), Leupeptin, Pepstatin, Phenylmethylsulphonylfluoride (PMFS), Potassium Chloride, Sodium Dodecyl Sulphate, Sodium Floride, Sodium Orthovanadate, Sodium Sulfide, Sucrose, Temed, Tris (hydroxymethyl) aminomethane, Triton X-100, Trypan blue, Xylene cyanol c) Fischer Scientific (Loughborough, UK).

Orange G, Cryovials.

d) Rathburn Limited (Walkerburn, UK).

Chloroform, Ethanol, Industrial Methylated Spirit and Methanol.

## 2.3.2.1 Specialised Reagents and Materials

• Agarose, Expand<sup>TM</sup> high fidelity PCR system, 100 bp and 123 molecular weight markers, Random primer labelling kit, Restriction enzymes and buffers were obtained from Roche Diagnostics (Lewes, UK).

• Deoxynucleoside 5'-triphosphates (dNTPs), Ficoll Type 400, radioactive isotopes ( $\alpha^{32}$ P dUTP,  $\gamma^{32}$ P dATP), Rainbow Protein marker, Rapid Hybridisation buffer, T4 Polynucleotide kinase (T4 PNK), were supplied by Amersham Pharmacia Biotech (Little Chalfont, UK).

• Super Taq polymerase and 10X Super Taq buffer were obtained from HT Biotechnology (Cambridge, UK).

• Custom oligonucleotide amplimers were produced by MWG Biotechnology (Germany) and Invitrogen Life Technologies (Paisley, UK). Superscript<sup>TM</sup> preamplification system for first strand cDNA system was also obtained from Invitrogen Life Technologies (Paisley, UK).

• Nucleon<sup>®</sup> DNA extraction kit was purchased from Scotlab (Coatbridge, UK).

• RNA Stat-60<sup>TM</sup> was supplied by Biogenesis (Poole, UK). MAXIScript<sup>TM</sup> and RPA II kits were obtained from AMS Biotechnology Limited (Oxfordshire, UK).

• All tissue culture reagents were attained from Invitrogem Life Technologies (Paisley, UK) and included; Lymphoprep<sup>TM</sup>, Fetal calf serum (FCS), Dulbecco's Modified Eagles Medium (DMEM), 20% glucose solution, Hank's balanced salt solution (HBSS), L-glutamine, non-essential amino acids (NEAA), Penicillin/streptomycin, Phosphate buffered saline (PBS), RPMI 1640, Trypsin-EDTA, Neomycin (G418).

• All tissue culture plastics were supplied by Fahrenheit (Milton Keyes, UK).

• E. Coli strain MC1061/P3 (carrying the P3 plasmid) was obtained from Invitrogen Life Technologies (Paisley, UK). The pCH110 Eukaryotic Assay Vector and JM109 Competent cells were purchased from Promega (Southampton, UK).

• Ampicillin, Dialysis tubing, 25% Gluteraldehyde, IPTG, Kanamycin, Potassium Ferricyanide, Potassium Ferrocyanide, Sodium Sulfide, Sulfuric Acid, Tetracycline, X-gal were supplied by Sigma Chemicals Ltd. (Poole, UK).

• Tryptone, Yeast Extract and Agar were obtained from Difco, USA.

• 2% Bisacrylamide, 30% and 40% (19:1) Acrylamide were attained from Bio-Rad Laboratories (Hemel Hempstead, UK).

• Antibodies were obtained from several suppliers; Anti-mouse IgG peroxidase conjugate and Horseradish Peroxidase Rabbit Anti-mouse IgG from Sigma (Poole, UK), HIF-1 $\alpha$ from AbCam (Cambridge, UK), Glucose transporter 1 (GLUT-1) from Biogenesis Ltd. (Poole, UK), p53 from Novacastra (Newcastle upon Tyne, UK), Vascular endothelial growth Factor (VEGF) from CN Biosciences (Nottingham, UK). Catalysed signal amplification system from DAKO (Cambridgeshire, UK).

• Coomassie Plus Protein Assay Reagent Kit and SuperSignal<sup>R</sup> Chemiluminescent Substrate were provided by Perbio Science UK Ltd. (Chester, UK).

# 2.4 Autoclaving

All glassware, solutions, instruments and plastics used in experiments involving DNA, RNA and protein analysis or tissue culture were autoclaved at a temperature of 121 °C under a pressure of 15 psi for a duration of 30 minutes in a top loading autoclave (PriorClave Ltd, Woolwich, UK).

# 2.5 Cell Culture

All cell culture work and cell maintenance was performed in a Microflow class II biological safety cabinet (Microflow Ltd., Hampshire, UK) using sterile techniques. The cabinet was swabbed with alcohol both before and after work was performed in it. All plastics were obtained from Fahrenheit (Milton Keynes, UK) and consisted of: 1 ml, 5 ml, and 10 ml individually wrapped sterile pipettes, sterile vented 25 cm<sup>2</sup> and 75 cm<sup>2</sup> flasks, sterile individually wrapped cell scrappers, sterile 15 ml and 50 ml tubes. Cell cultures were maintained at a constant temperature of 37 °C in an atmosphere of 5 % CO<sub>2</sub> (BOC, Bristol, UK) in dry Leec incubator (Jencons-PLS, Leighton Buzzard, UK). Cells cultures used in hypoxia experiments were incubated at 37 °C in an atmosphere of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, 95% N<sub>2</sub> (BOC, Bristol, UK) in a dry CO<sub>2</sub>/O<sub>2</sub> incubator (NuAire<sup>TM</sup>, Minnesota, USA). Cell cultures were viewed on an inverted Leica DMIL microscope (Jencons-PLS, Leighton Buzzard, UK).

#### 2.5.1 Established Cell Lines

Established breast carcinoma cell lines were generously provided by Dr. Corine L'Hôte from the Ovarian Cancer Group at the Imperial Cancer Research Fund, Oxford, UK, having originated from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK) or the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Saos-2 cells were kindly made available by Dr. Alan Storey from the Imperial Cancer Research Fund Skin Tumour Laboratory, London, UK. Cells were monitored daily, passaged at a dilution of 1/10 weekly, or more frequently when required, and stocks of each cell line were stored in liquid nitrogen. A summary of the culture conditions for the established cell lines is listed in **Table 2.5**.

Cell Line	Origin	Media	Passage
MCF7	ECACC (# 86012803) Pleural effusion from breast tissue of a female Caucasian (69 years)	DMEM 10% FCS, 1 mM glutamine, 100 U/ml pen-strep	At confluence 1:6 weekly
Saos-2	ECACC (#89050205) Primary osteogenic sarcoma from bone tissue of a female Caucasian (11 years)	DMEM 10% FCS, 100 U/ml pen-strep	Prior to confluence 1:4 weekly
SKBR3	ATCC (# HTB-30) Pleural effusion adenocarcinoma of the breast from a female Caucasian (43 years)	RPMI 1640 10% FCS, 50 U/ml pen-strep	At confluence 1:4 weekly
T47D	ECACC (# 85102201) Pleural effusion infiltrating ductal carcinoma of the breast from a female Caucasian (54 years)	DMEM 10% FCS, 100 U/ml pen-strep	At confluence 1:10 weekly
ZR75	ECACC (# 87012601) Ascitic effusion infiltrating ductal carcinoma of the breast from a female Caucasian (63 years)	RPMI 1640 10% FCS, 50 U/mi pen-strep	At confluence 1:10 weekly

# Table 2.5. Culture conditions of established cell lines.

ATCC = American Type Culture Collection

ECACC = European Collection of Animal Cell Cultures

Pen-strep = penicillin-streptomycin

#### T47D (ECACC # 85102201)

Adherent human breast epithelial cells established from the pleural effusion of an infiltrating ductal carcinoma of the breast of a 54 year-old postmenopausal female. The cells carry receptors for a variety of steroids and tend to form monolayers. Cells were grown in Dublecco's Modified Eagles Medium (DMEM) supplemented with 10 % FCS and 100 units (U)/ml of penicillin-streptomycin (10000U/ml, Life Technologies Ltd., Paisley, UK). Cells were passaged at confluence 1:10 weekly.

#### MCF7 (ECACC # 86012803)

Adherent cells were derived from a pleural effusion obtained from a 69 year-old female Caucasian. The cells express the wildtype and variant oestrogen receptors, as well as the progesterone receptor. Cells exhibit some features of differentiated mammary epithelium including oestradiol synthesis and formation of domes and may carry a B or C type retrovirus. Cells were cultured in DMEM medium supplemented with 10 % FCS, 1 mM glutamine (Invitrogen Life Technologies, Paisley, UK) and 100 U/ml of penicillinstreptomycin. Cells were passaged at confluence 1:6 weekly.

# Saos-2 (ECACC # 89050205)

Adherent human epithelial cells derived from a primary osteogenic sarcoma from the bone tissue of an 11 year-old female Caucasian. These cells are p53 null as p53 expression over a longer period of time than a transient transfection leads to apoptosis. Cells were cultured in DMEM supplemented with 10 % FCS and 100 U/ml of penicillin-streptomycin. Cells were passaged prior to confluence 1:4 weekly.

#### SKBR3 (ATCC # HTB-30)

Adherent human breast epithelial cells derived from a malignant pleural effusion adenocarcinoma of the breast of a 43 year-old female Caucasian. Cells were cultured in RPMI 1640 media supplemented with 10 % FCS and 50 U/ml of penicillin-streptomycin. Cells were passaged at confluence 1:4 weekly.

# ZR75 (ECACC # 87012601)

These colony forming adherent human breast epithelial cells were originally derived from a malignant ascitic effusion in a 63 year-old female Caucasian with infiltrating ductal carcinoma. The cells have receptors for both wild-type and variant oestrogen receptors, progesterone receptor and other steroid hormones. Cells were cultured in RPMI 1640 media supplemented with 10 % FCS and 50 U/ml of penicillin-streptomycin. Cells were passaged at confluence 1:10 weekly. The cells grow very slowly, do not form a confluent monolayer and attach only lightly to the substrate.

# 2.5.2 Characteristics of Human Breast Carcinoma Cell Lines

The characteristics of the human breast carcinoma cell lines used in this study are listed in **Table 2.6**. These cell lines were chosen to reflect the heterogeneity of the human breast cancer progression pathway. The cell lines represent different stages and phenotypes of breast carcinoma.

Cell Line	ER Status	Tumorigenic Potential	p53 Status	Other	Reference
MCF7	+	+	Wild-type	Growth inhibited by TNFα Secrets IGFBP's	Sugarman <i>et al</i> , 1985; Pratt and Pollack, 1993
SKBR3	-	++	Mutant	HER2/c-erbB2+	Fogh <i>et al</i> , 1977; Hynes <i>et al</i> , 1989
T47D	+	+ (with oestrogen)	Single allele mutant	54	Mauvais-Jarvis <i>et al</i> , 1986
ZR75	+	++	Wild-type	Interferon+	Landers et al, 1997; Couillard et al, 1998

<b>Table 2.6</b> . Characteristics of human breast carcinoma cell lin
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(+) = positive

(-) = negative/ not known

ER = oestrogen receptor

 $TNF\alpha$  = tumour necrosis factor alpha

IGFBP = insulin-like growth factor binding protein

#### 2.5.3 Peripheral Blood Mononuclear Blood Cells (PBMCs)

Isolated PBMCs (section 2.6.9) were cultured for 5 days in 20 ml of RPMI 1604 media supplemented with 10 % FCS, 50 U/ml of penicillin-streptomycin and 5  $\mu$ g (0.25  $\mu$ g/ml) of PHA in a 75 cm<sup>2</sup> flask, under both normal and experimental conditions (section 2.5.8) prior to RNA extraction (section 2.10.2.2).

#### 2.5.4 Cell Passage

Adherent cell lines grown in 75 cm<sup>2</sup> flasks (Fahrenheit, Milton Keynes, UK) where passaged when each cell monolayer was confluent. The cell monolayer was first washed twice in 10 ml of Hank's Balanced Salt Solution (HBSS) medium (Invitrogen Life Technologies, Paisley, UK) then 2 ml of 1X Trypsin/EDTA solution (Invitrogen Life Technologies, Paisley, UK) was added to each flask and incubated at room temperature for 10-40 minutes until total cell detachment was achieved. An equal volume (2 ml) of Foetal Calf Serum (FCS) was then added to the flask to stop the trypsinisation reaction; the cell suspension was transferred to a sterile 15 ml tube (Fahrenheit, Milton Keynes, UK) and centrifuged at 1400 rpm for 4 minutes in a MSE Mistral centrifuge (MSE Scientific Instruments, Leicester, UK). The supernatant was then carefully decanted and the cell pellet was resuspended and washed in 5 ml of HBSS medium with gentle pipetting or vortexing. The tube was again centrifuged at 1400 rpm for 4 minutes, the supernatant decanted and the pellet washed a second time in 5 ml of HBSS. Following a final centrifugation at 1400 rpm for 4 minutes, the supernatant was decanted and the cells were resuspended in 5-10 ml of appropriate culture medium. The cells were then counted using a Neubauer haemocytometer (Fischer Scientific, Loughborough, UK). For cell passage, the cell densities were adjusted to  $10^{5}$ - $10^{6}$  cells/ml and 1 ml of the cell suspension was then transferred to a sterile 75 cm<sup>2</sup> flask containing 19 ml of appropriate culture medium. For cell experiments, the remaining cell suspensions were split as required into separate 75 cm<sup>2</sup> flasks.

#### 2.5.5 Cell Counting

Culture and experimental cell conditions were standardised by determining cell densities. The concentration of cell suspensions was determined by individual cell counting using a Neubauer haemocytometer under an inverted Leica DMIL microscope (Jencons-PLS, Leighton Buzzard, UK). The cell number within a defined area of known depth within the chamber was counted and the cell concentration derived from the count. Cell suspensions were mixed with gentle pipetting, then a 50  $\mu$ l aliquot was transferred to the edge of the haemocytometer where the suspension was drawn into the chamber by capillary action. Cells lying within a 1 mm<sup>2</sup> bounded area were counted in each of the separate chambers and averaged. The total cell number was determined using the following formula;

$$c = (n \ge 10^4)$$

where c is the cell concentration (cells/ml), and n is the number of cell counted. The  $10^4$  multiplication factor is derived from the volume counted and is based on the assumption that the depth of the Neubauer chamber is 0.1 mm and that only the central 0.1 mm<sup>2</sup> area is used, hence 0.1 mm<sup>3</sup> or  $10^{-4}$  ml.

# 2.5.6 Cryopreservation

Adherent cell lines were grown to near confluence, detached, washed and counted as above. Cell densities were adjusted to  $5 \times 10^6$  cells/ml in the appropriate culture medium. Cells were prepared for freezing by slowly mixing 1 ml of cell suspension with 0.6 ml of FCS (Life Technologies, Paisley, UK) and 0.2 ml of DMSO (Sigma Chemicals Ltd., Poole, UK) in a sterile 15 ml tube. A 1 ml volume of the cell mixture (10% DMSO, 30% FCS, 50% cell suspension) was then aliquoted into 2 separate 2 ml cryovials (Nalgene, Fischer Scientific, Loughborough, UK) and tightly closed. The cells were allowed to freeze slowly by first storing the cryovials at -20 °C for 6 hours, then transferring to -80 °C for 12-16 hours prior to final transfer to liquid nitrogen for indefinite storage.

# 2.5.7 Cell Resuscitation

A frozen ampoule containing cells was carefully removed from storage in a liquid nitrogen canister (Jencons-PLS, Leighton Buzzard, UK) and left to thaw at room temperature for approximately 1 minute. The ampoule was then transferred to a 37 °C waterbath (Grant, Cambridge, UK) for 1-2 minutes until fully thawed and then wiped with a tissue soaked in 70% alcohol prior to opening. Once opened, the contents of the vial were transferred to a 25 cm<sup>2</sup> vented flask, which contained 9 ml of pre-warmed media. An additional 1 ml of media was added to the ampoule to wash and collect any remaining cells. This was then added to the flask, which was then closed and placed in a 37 °C, 5% CO<sub>2</sub> Leec incubator (Jencons-PLS, Leighton Buzzard, UK) overnight. The next day the media was carefully removed by aspiration, replaced with the appropriate growth media and the flask returned to the incubator.

#### 2.5.8 Experimental Culture Conditions

#### 2.5.8.1 High Glucose

Established breast carcinoma cell lines and PBMCs were cultured at normal glucose levels (no supplement), moderate glucose levels with a supplement of 10 mM glucose, or at high glucose levels with a supplement of 20 mM glucose. The basal level of glucose was determined for each media used, RPMI 1640 medium contained 2000 mg/litre of D-glucose, equivalent to 11 mM, DMEM medium contained 4500 mg/litre of D-glucose, equivalent to 25 mM. A supplement of 10 mM D-glucose (moderate glucose) corresponded to an addition of 184  $\mu$ l of a 20 % solution of D-glucose for cells cultured in RPMI 1640 medium and 35 mM total D-glucose for cells cultured in DMEM medium. A supplement of 20 mM D-glucose (high glucose) corresponded to an addition of 360  $\mu$ l of a 20 % solution of D-glucose for cells cultured in RPMI 1640 medium and 35 mM total D-glucose for cells cultured in DMEM medium.

equating to 31 mM total D-glucose for cells cultured in RPMI 1640 medium and 45 mM total D-glucose for cells cultured in DMEM medium.

# 2.5.8.2 Osmolarity

# 2.5.8.2.1 Mannitol

Mannitol is a non-metabolisable analogue of D-glucose. PBMCs and established breast carcinoma cell lines were cultured in mannitol to control for osmolarity. Mannitol was added to the cultures in an amount equal to the amount of excess D-glucose over the basal glucose level in the media. For cells cultured in high glucose conditions (a supplement of 20 mM D-glucose), a parallel culture was established where 20 mM mannitol was used as a supplement. This corresponded to the addition of 7.3  $\mu$ l of a 500 mg/ml mannitol stock solution to a 75 cm<sup>2</sup> flask containing 20 ml of culture medium.

# 2.5.8.2.2 L-Glucose

L-glucose is a non-metabolisable analogue of D-glucose as it does not contain a reactive aldehydic group at the C1 carbon, thus it can be used as a control for glycation reactions that are non-enzymatic that D-glucose can undergo. PBMCs and established breast carcinoma cell lines were cultured in an equivalent concentration of L-glucose to control for high glucose. For cells cultured in high glucose conditions (a supplement of 20 mM Dglucose), a parallel culture was established where 20 mM L-glucose was used as a supplement. This corresponded to the addition of 7.2  $\mu$ l of a 500 mg/ml L-glucose stock solution to a 75 cm<sup>2</sup> flask containing 20 ml of culture medium.

# 2.5.8.3 Hypoxic Exposure

# 2.5.8.3.1 Cobalt Chloride

Cobalt chloride (CoCl<sub>2</sub>) is an agent known to mimic hypoxia through chemical induction. Cells were cultured in 60-500  $\mu$ M CoCl<sub>2</sub> (Sigma Chemicals Ltd., Poole, UK) for 6, 24 and 48 hours prior to harvesting.

# 2.5.8.3.2 CO<sub>2</sub>/O<sub>2</sub> Incubator

A hypoxic environment was created in a 37 °C CO<sub>2</sub>/O<sub>2</sub> incubator (NuAire<sup>TM</sup>, Minnesota, USA), which was able to produce an internal hypoxic atmosphere of 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 95% N<sub>2</sub>. The incubator required approximately 16 hours to reach the set internal atmospheric conditions. Both O<sub>2</sub> and CO<sub>2</sub> percentages inside the incubator chamber were measured using a Fyrite<sup>TM</sup> gas analyser (Jencons-PLS, Buzzard Leighton, UK), (section 2.5.8.4.3).

Established cell lines were passaged and transferred to new 75 cm<sup>2</sup> flasks 2 days prior to exposing to the hypoxic environment and the media was replenished with 20 ml the appropriate media. For hypoxic conditions, cells were exposed to 1% O<sub>2</sub> (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37 °C CO<sub>2</sub>/O<sub>2</sub> incubator (NuAire<sup>TM</sup>, Minnesota, USA) for 2,4,6,8,24 and 48 hours. As a control, an aliquot of cells of the respective cell line exposed to hypoxic conditions was exposed to normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>) for the same period of time. At each established time point, 2 ml of supernatant was collected from the flask and stored in cryovials at -70 °C.

#### 2.5.8.3.3 Fyrite® Gas Analyser

The Fyrite® Gas analyser allows for the determination of the percentage of carbon dioxide or oxygen in an atmospheric sample. The technique employs the 'Orsat' method of volumetric analysis involving chemical absorption of a sample gas and it allows accurate readings to within +/- 0.5 % compared to actual value. The sampling assembly, consisting of a centre tube connected to two reservoirs, contains an absorbing fluid that will absorb either O<sub>2</sub> or CO<sub>2</sub>. The reagent used to absorb O<sub>2</sub> is chromous chloride, and CO<sub>2</sub> is potassium hydroxide. Each sampling assembly containing the appropriate absorbing fluid was first vented of air by depressing the plunger valve at the top of the assembly. The assembly was inverted and vented, then held vertical whilst the volume scale was set at the top level of the fluid column to zero. The air sample inside the incubator was pumped into the sampling assembly by attaching one end of a sampling rubber connecter tube to an airsampler valve on the exterior surface of the incubator and placing the other end over the plunger valve. The plunger valve was depressed and the sample pumped into the sampling assembly by squeezing and releasing an aspirator bulb 18 times. The sample gas was absorbed into the fluid by inverting the assembly then allowing all fluid drops to drain to the bottom of the assembly prior to reading the volume scale. The % reading on the volume scale corresponded to the actual % gas inside the incubator.

# 2.6 Deoxyribonucleic Acid (DNA) Methodologies

# 2.6.1 Extraction of Total DNA

High molecular weight DNA was extracted from whole blood using a Nucleon<sup>®</sup> BACC (blood and cell culture) genomic DNA extraction kit (Scotlab, Coatbridge, UK). Up to 25 ml of peripheral venous blood was collected in 5% ethylenediaminetetracetic acid (EDTA) vacutainer tubes (Becton Dickinson, Oxford, UK). The blood was transferred to 50 ml sterile Falcon tubes (Fahrenheit, Milton Keynes, UK) and a 4 X volume of Nucleon<sup>®</sup> A (10mM Tris-HCL, 320 mM sucrose, 5mM MgCl<sub>2</sub>, 1% Triton X-100, pH 8.0) was added and the solution was gently shaken for 4 minutes at room temperature. The samples were then centrifuged in a MSE Mistral 1000 centrifuge (MSE Scientific Instruments, Leicester, UK) at 1300 X g for 4 minutes, the supernatant containing lysed red cells was discarded and 2 ml of Nucleon® B (400 mM Tris-HCL, pH 8.0, 60 mM EDTA, 150 mM NaCl, 1% SDS) was added to the tubes. The pellets were resuspended by vortexing and the solutions were incubated in a 37 °C waterbath (Grant, Cambridge, UK) for 10-15 minutes to facilitate nuclear membrane disruption and protein denaturation. The solutions were then transferred to 15 ml sterile polypropylene tubes where 500µl of 5M sodium perchlorate was added and the tubes were inverted 10-15 times to emulsify the phases. The samples were then centrifuged at 1300 X g for 3 minutes and 200 µl of Nucleon silica resin was added to each tube without disrupting the phases and then centrifuged at 1,300 X g for 3 minutes. The upper aqueous phase containing the DNA was carefully removed from each tube and transferred to clean 15 ml sterile tubes ensuring that no resin had been carried over. The tubes were then centrifuged for 1 minute at 1300 X g and the supernatants were again transferred to clean 15 ml sterile tubes where the DNA was precipitated out by the addition of a 2X volume of ice-cold 100 % (v/v) ethanol (Rathburn Ltd., Walkerburn, UK). The tubes were inverted several times and the DNA was hooked out using sterilised glass pipettes with sealed tips. The DNA was then washed in 70% (v/v) ethanol, placed in 1.5 ml sterile micro-centrifuge tubes and resuspended in 500  $\mu$ l of sterile water. DNA samples were kept at 4 °C for 24 hours to ensure complete dissolution, after which DNA samples were stored at -20 °C.

#### 2.6.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is used to enzymatically amplify *in vitro* specific DNA sequences using oligonucleotide primers that flank the region of interest in the target DNA. PCR was first described by Kleppe and colleagues in 1971 (Kleppe *et al*, 1971), but was devised and named by Mullis and colleagues at Cetus Corporation in California, USA, in 1986. (Mullis *et al*, 1986; Mullis & Faloona 1987). The technique is based on repeated cycles of high temperature template denaturation, oligonucleotide primer (amplimer) annealing and polymerase mediated primer extension. The extension product of one cycle of the PCR reaction serves as template in the next cycle, causing an approximate doubling of the amount of the particular nucleic acid with each cycle and an exponential accumulation of it overall. Assuming 100% efficiency and a doubling of the desired target in each cycle, 30 cycles could in theory produce  $1 \times 10^9$  copies from one target copy. The simplicity of the reaction, as well as its speed and sensitivity, makes it ideally suited for a wide variety of applications, including the diagnosis and characterisation of genetic diseases, as well as cancer.

# 2.6.3 Optimisation of PCR

PCR reaction conditions were optimised for each set of amplimers. Optimising reaction conditions included varying essential components in each assay. Key parameters for PCR optimisation include:

- a) Cycle conditions
- b) Magnesium concentration
- c) Deoxynucleotide (dNTP) concentration
- d) Amplimer concentration
- e) Enzyme concentration

Optimisation techniques included using an initial Hot Start, where all reaction components were heated to above 80 °C prior to the addition of the last component, which preceded programme cycling in an attempt to avoid amplification of non-target sequences in the DNA background (mispriming) and primer dimerisation. Optimisation of cycling conditions involved varying the temperatures in the amplification reaction, denaturation (92-96 °C), amplimer annealing (50-75 °C) and DNA extension (68-72 °C). Initial annealing temperatures were determined using either Touchdown PCR, where cycling is started with a relatively high annealing temperature, or by using a temperature 2-5 °C below the true melting temperature ( $T_m$ ) of the amplimers. Balanced dNTP concentrations of between 0.2 and 1.0  $\mu$ M of each amplimer were optimal. Magnesium concentrations (1.0 to 3.0 mM) and Taq DNA polymerase (0.5 to 5.0 units per 50  $\mu$ l reaction) were determined empirically for each reaction. In some assays, an additional final incubation of either 68 °C or 72 °C for 10 minutes immediately after cycling was added to optimise the yield of PCR products.

# 2.6.4 Custom Oligonucleotides (Amplimers)

For either DNA or RNA studies, gene sequences of interest were obtained from a public databank (Genebank, <u>www.ncbi.nlm.nih.gov</u>). Sense (5' to 3') amplimers were designed to be complimentary to the DNA template sequence of interest and anti-sense (3' to 5') amplimers were designed reverse complimented. Amplimers ranged in length from 18–35 bases and contained a GC content of 50% or greater. Custom oligonucleotide amplimers

were produced by MWG Biotechnology (Germany) or Invitrogen Life Technologies (Paisley, UK). All amplimers were supplied lyophilised and were resuspended in 1 ml of sterile water (Baxter Healthcare, Thetford, UK) prior to dilution to a working concentration of 1 pmol/ml. Table 2.7 lists custom oligonucleotides and their respective PCR reaction conditions used for all DNA or RNA studies.

# 2.6.5 PCR Reactions

All standard PCR reactions were performed in 0.2 ml thin walled micro-centrifuge tubes (Advanced Biotechnology, Epsom, UK) in a final reaction volume of 50  $\mu$ l. Generally, 100-500 ng of genomic DNA or cDNA, 7.5 pmol of each amplimer pair, 1.0-2.0 mM of MgCl<sub>2</sub>, 0.125 mM of each dNTP, 1 X Super Taq Buffer (50 mM Tris-HCl, pH 9.0, 1.5 mM MgCl<sub>2</sub>, 250 mM KCl, 1% Triton X-100, 0.1% (w/v) gelatine), and 0.8 units of SuperTaq DNA polymerase were used in each reaction mixture. All PCR reactions were performed on a 48/48 well DNA Engine Thermocycler (Genetic Research Instrumentation, USA) and stored at 4 °C pending analysis by gel electrophoresis.

### 2.6.6 Nested PCR

Nested PCR involves using two sets of amplimers. The first amplimer set is targeted within the amplification product of the second set; hence two rounds of amplification are required to amplify the specific target DNA. The amplicon of the second reaction is shorter than that of the first (**Figure 2.1**). The procedure is designed to increase the sensitivity of PCR by directly reamplifying the product from a primary PCR reaction with a second PCR. The advantage of nested PCR is increased sensitivity and specificity of the reaction, since the internal primers anneal only if the amplicon has the corresponding expected sequence. Nested PCR was used to identify p73 alleles in the high molecular weight DNA extracted from PBMC's of patients with breast cancer. In the first round of PCR, intronic amplimers were employed in a 50 $\mu$ l reaction volume. In the second round of amplification, a 1  $\mu$ l aliquot of the first round product was transferred to a new 0.2 ml thin-walled tube and amplified using a second set of internal amplimers (see **Table 2.7** for amplimers and cycle conditions). The amplified DNA products were visualised by running 10  $\mu$ l of each sample mixed with 2  $\mu$ l of loading buffer on a 1% agarose gel containing 0.01% ethidium bromide. The PCR products were subsequently digested with the restriction endonuclease Sty 1 (Roche Diagnostics, Lewes, UK).

# 2.6.6.1 Restriction Enzyme Digestion of PCR Products

Identification of p73 alleles from patients with breast cancer required digesting the amplified PCR products with Sty 1 restriction endonuclease (Roche Diagnostics, Lewes, UK). Amplimers and PCR cycle conditions used in the assay are listed in **Table 2.7**. The DNA was digested by mixing 5-10  $\mu$ g of amplified product with 10 units of Sty 1 restriction endonuclease, 1/10<sup>th</sup> volume of endonuclease buffer (Roche Diagnostics, Lewes, UK), 0.1  $\mu$ l of acetylated bovine serum albumin (BSA) (10  $\mu$ g/ $\mu$ l) and distilled water to a final reaction volume of 20  $\mu$ l in a 0.5 ml micro-centrifuge tube. The reaction was incubated for 1 hour at 37 °C in a waterbath (Grant, Cambridge, UK). Digested products were visualised by running 10  $\mu$ l of each sample mixed with 2  $\mu$ l of loading buffer on a 1.5% agarose gel containing 0.01% ethidium bromide for 1 hour at 200 volts.

Amplimer Sequences	Length (mer)	Gene	Cycles	Cycle Conditions	Product (base pairs)	Genebank Accession #	Reference
5' TGACGGGGTCACCCACACTGTGCCCATCTA 3' (sense) 5' CTAGAAGCATTTGCGGTGGACGATGGAGGG 3' (antisense)	30 30	β-actin	35	Initial 60 °C for 5 minutes, 72 °C for 1.5 minutes 94 °C for 45 seconds 60 °C for 45 seconds Additional 72 °C for 10 minutes	661 bp	M10277	Nakajima-Iijima <i>et al</i> , 1985
5' CCACTTCCACATAATGTGAGTTCGG 3' (sense) 5' GGTTCACAAATCAGCACCAAGCAGG 3' (antisense)	25 25	HIF-1a	30	94 °C for 1 minute 55 °C for 2 minutes 72 °C for 3 minutes	520 bp	U22431	Wang <i>et al</i> , 1995
5' GTCGGACAGCCTCACCAAACAGAGC 3' (sense) 5' GTTAACTTGATCCAAAGCTCTGAG 3' (antisense)	25 24	HIF-1α splice variant	35	95 °C for 30 seconds * 55 °C for 1 minute 72 °C for 2.5 minutes Additional 72 °C for 10 minutes	487 bp (with exon 14) 350 bp (without exon 14)	U22431	Gothié <i>et al</i> , 2000
5' ATGCCTCCACAGAGGCTATGCC 3' (sense) 5' GCACTAAGGAACGTCTGTAGGC 3' (antisense)	22 22	VEGF	30	95 °C for 1 minute 61 °C for 1.5 minutes 72 °C for 1.5 minutes	441 bp	M63971	Tischer <i>et al</i> , 1991
5' GGCTATGGCCACCCGTTCTGCTGGC 3' (sense) 5' GCCGTTGCAGTGGTTGCAACCC 3' (antisense)	25 22	GLUT 1	30	95 °C for 1 minute 54 °C for 1.5 minutes 72 °C for 1.5 minutes	525 bp	K03195	Mueckler <i>et al</i> , 1985

Table 2.7. Custom Oligonucleotide Amplimer sequences and PCR reaction conditions used in assays for DNA and RNA studies.

Note: All PCR cycle conditions included one Hot Start cycle of 94°C for 4 minutes and all reactions had a total reaction volume of 50  $\mu$ l using 1.5 mM MgCl<sub>2</sub>. (HMW) = High Molecular Weight DNA, \* = Expand High Fidelity PCR System (Roche Diagnostics, Lewes, UK) was used in this assay.
Amplimer Sequences	Length (mer)	Gene	Cycles	Cycle Conditions	Product (base pairs)	Genebank Accession #	Reference
1 <sup>st</sup> Round	18			Nested PCR	GC allele		
5' AAAATAGAAGCGTCAGTC 3' (antisense)	18	73	30	58 °C for 1 minute 68 °C for 2.5 minutes	482 & 284 bp (undigested)	Y11416	Kaghad <i>et al</i> , 1997
2 <sup>nd</sup> Round		F -		Additional 68 °C for 10 minutes	AT allele 376 & 106 bp 234 & 50 bp (digested)		
5' CTGTCCCCAAGCTGATGA 3' (antisense)	18		30	Followed by Sty 1 restriction enzyme digestion			
5' GCCAGAGGCTGCTCCCCC 3' (sense)	18	p53 Pro		95 °C for 30 seconds			Storev et al.
5' CGTGCAAGTCACAGACTT 3' (antisense)	18		30	68 °C for 2.5 minutes	177 bp	M22887	1998
				Additional 68 °C for 10 minutes			
5' TCCCCCTTGCCGTCCCAA 3' (sense)	18	-52 4-5	20	95 °C for 30 seconds			Starran I.
5' CTGGTGCAGGGGCCACGC 3' (antisense)	18	pos Arg	3 Arg 30	61 °C for 1 minute 68 °C for 2.5 minutes	141 bp	M22887	1998
				Additional 68 °C for 10 minutes			
5' TTCCTCTTCCTGCAGTACTCC 3' (sense)	21			95 °C for 1 minute		M22887	Buchman et al,
5' AGTTGCAAACCAGACCTCAGGC 3' (antisense)	22	p53	30	56 °C for 1.5 minutes 72 °C for 1.5 minutes	144 Dp		1988

Table 2.7. (continued) Custom Oligonucleotide Amplimer sequences and PCR reaction conditions used in assays for DNA and RNA studies.

Note: All PCR cycle conditions included a Hot Start of 94°C for 4 minutes and all reactions had a total reaction volume of 50  $\mu$ l using 1.5 mM MgCl<sub>2</sub>. (HMW) = High Molecular Weight DNA



Figure 2.1. Schematic representation of Nested Polymerase Chain Reaction .

# 2.6.7 Agarose Gel Electrophoresis

Agarose gels (1.5%) were prepared by dissolving 1.5 grams of multi-purpose agarose in 100 ml of 1X TBE (89µM Tris base, 89µM boric acid, 200µM EDTA, pH 8.0) in a 500 ml Duran glass bottle by boiling. The agarose solution was then cooled to 60-65 °C prior to adding 10 µl of a 10 mg/ml solution of ethidium bromide (0.01% v/v). The solution was gently mixed and then poured into a 14 X 11 cm perspex gel mould tray (Life Technologies, Paisley, UK) that was sealed at both ends with masking tape. Two plastic 14 well tooth-combs were positioned 1 mm above the tray and the gel was then allowed to set at room temperature for 30 minutes. Once set, the combs and masking tape were carefully removed and the gel and tray were placed into a horizontal gel electrophoresis tank (Life Technologies, Paisley, UK) containing 0.5 X TBE buffer sufficient to cover the gel. Samples were prepared for loading by mixing 10  $\mu$ l of DNA product with 2  $\mu$ l of either 6 X gel loading buffer (0.25% w/v bromophenol blue, 0.25 % w/v xylene cyanol, 15% v/v Ficoll Type 400) or Orange G loading buffer (0.25% w/v xylene cyanol, 10% v/v glycerol in 10 X TBE) and loaded into the gel. The gel was run at 200 volts for 30 minutes to 1 hour or until the dye fronts migrated an appropriate distance through the gel. DNA molecular weight markers of either 100 base pairs (bp) or 123 bp were run concurrently to facilitate fragment sizing. The gels were visualised via irradiation with UV light on a transilluminator (UVP International, Cambridge, UK) at  $\lambda 302$  nm and photographed using a gel imager software package (Vision Works 3.0, UVP International, Cambridge, UK) and video linked to a Sony graphic printer using type IV UPP-11-HA thermo film (Sony Corporation, Tokyo, Japan).

#### 2.6.8 Purification of PCR Fragments

Following agarose gel electrophoresis, PCR fragments were recovered and purified using one of the below methods.

## 2.6.8.1 Glass Wool

PCR fragments were purified after gel electrophoresis by spinning through glass wool. DNA bands were excised from 1% agarose gels (1 gram of agarose in 100 ml of 0.5 X TBE) containing 0.01% v/v ethidium bromide, using a clean, sharp scalpel blade while visualised with UV light on a transilluminator. Excised bands were placed into a 0.5 ml micro-centrifuge tube containing a loosely packed piece of glass wool and of which the bottom had been pierced with a needle. The 0.5 ml tube was then placed in a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 10 minutes. The DNA in the collected eluant was then precipitated out by the addition of a  $1/10^{th}$  volume of 3M sodium acetate, pH 5.2 and 2 –3 volumes of 100% ethanol. The solution was mixed and placed at -20 °C for at least one hour. The DNA was then collected by centrifugation at 13,000 rpm for 15 minutes, the supernatant decanted and the pellet washed with 3 volumes of 70% ethanol. The DNA was then centrifuged at 7,500 for 5 minutes, the supernatant removed and the sample air-dried for 5 –10 minutes and subsequently resuspended in 25-50 µl of water.

# 2.6.8.2 Quick Precip™ (Advanced Biotech Corp., USA)

Following purification by spinning through glass wool, the DNA was precipitated by adding 2  $\mu$ l of Quick Precip<sup>TM</sup> (Advanced Biotech Corp., USA), 1/10<sup>th</sup> volume of 5 M sodium chloride and 2-3 volumes of ice-cold 100 % ethanol to the tube, which was then mixed by vortexing. The solution was then centrifuged at 13,000 rpm for 3 minutes, the supernatant decanted and the pellet washed with 1 volume of 70 % ethanol. The solution was again centrifuged at 13,000 rpm for 30 seconds, the supernatant decanted, the sample

air-dried for 5 minutes and the DNA pellet was then resuspended in 50  $\mu$ l of sterile water. The DNA was then stored at – 20 °C.

# 2.6.8.3 Wizard™ PCR Preps DNA Purification System (Promega, Southampton, UK)

PCR fragments were separated by electrophoresis through a 1% low-melting point agarose gel run in 1 X Tris-Acetate (TAE) buffer as described above (section 2.6.7). DNA bands were excised from the gel using a clean, sharp scalpel blade while visualised with UV light on a transilluminator. Excised bands were placed into a 1.5 ml sterile micro-centrifuge tube and incubated in a waterbath at 70 °C until the agarose gel had completely melted. Next, 1 ml of resin was added to the tube, the sample mixed gently by pipetting up and down, then it was transferred a 2 ml disposable syringe barrel attached to the column extension of a Wizard<sup>™</sup> minicolumn. The syringe plunger was inserted into the barrel and the resin/DNA solution was gently pushed into the minicolumn. The column was washed in the same manner by pushing 2 ml of 80 % isopropanol through the column. The syringe was removed and the minicolumn was transferred to a sterile 1.5 ml micro-centrifuge tube and centrifuged at 13000 rpm for 2 minutes to dry the resin. The minicolumn was again transferred to a new 1.5 ml micro-centrifuge tube where 50 µl of sterile water was applied to the column, incubated at room temperature for 1 minute prior to centrifugation at 13000 rpm for 30 seconds. The minicolumn was removed and the eluted DNA was stored at -20°C.

2.6.9 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) From Whole Blood Peripheral blood mononuclear cells (PBMCs) were isolated from 20-50 ml of peripheral collected from both patients and healthy controls into 5% venous blood ethylenediaminetetracetic acid (EDTA) vacutainer tubes (Becton Dickinson, Oxford, UK). using Lymphoprep<sup>™</sup> (Invitrogen Life Technologies, Paisley, UK). The blood was transferred to 50 ml sterile Falcon tubes (Fahrenheit, Milton Keynes, UK) where it was diluted with an equal volume of PBS (Invitrogen Life Technologies, Paisley, UK) and mixed by inverting. Next, 7 ml of the diluted blood was carefully laid on top of an equal volume (7 ml) of Lympoprep<sup>™</sup> in sterile 15 ml tubes which were then centrifuges at 2000 rpm at room temperature for 30 minutes in a MSE Mistral centrifuge (MSE Scientific Instruments, Leicester, UK) resulting in 4 layers separated out by density gradient. The white mononuclear cells layer was collected using a Pasteur pipette (Richardsons of Leicester, Leicester, UK), transferred to a clean 15 ml tube and washed by adding a 4 times volume of PBS and gently inverting. The solution was then centrifuged at 1400 rpm at room temperature for 15 minutes, the supernatant discarded and the cells washed with another 4 times volume of PBS. After a final centrifugation at 1200 rpm at room temperature for 10 minutes, the supernatant was again discarded and the resulting cell pellet resuspended in 1 ml of RPMI 1640 medium supplemented with 10 % FCS, 50 U/ml of penicillin-streptomycin and 5 µg (0.25 µg/ml) of phytohaemaglutinin (PHA) (Sigma Chemicals Ltd, Poole, UK) with gentle pipetting and counted using a haemocytometer. The cell density was adjusted to  $1 \times 10^6$  cells/ml and the cells suspension was then divided into the appropriate number of flasks required for culture experiments.

# 2.7 Plasmid DNA Methodologies

Expression vectors express cloned DNA sequences if they are fused to appropriate transcription and translation start signals. Both vector and target DNA can be cleaved at appropriate sites, covalently joined together and used to amplify the amount of material or express a particular sequence by replicating autonomously in an appropriate host (*E.coli*).

Four individual expression plasmids were used in this work; pcDNA3, pCH110, pUC18 and pGEM<sup>®</sup>Z. A summary of their characteristics is found in **Table 2.8**.

# 2.7.1 pcDNA3 Plasmid (Invitrogen Life Technologies, Paisley, UK)

pcDNA3 is a P3 low-copy 60 Kb episomal plasmid which encodes a kanamycin resistance gene as well as amber mutants of the tetracycline and ampicillin resistance genes. *E.coli* harbouring the plasmid P3 permit selection and maintenance of plasmids that encode the tRNA suppressor F gene (*sup*F). When E.coli carrying the P3 plasmids are transformed with *sup*F plasmids, such as pcDNA3, they are rendered resistant to both tetracycline and ampicillin by suppression of the amber mutants. As spontaneous reversion of the amber point mutations on the P3 episome is high, colonies were selected for both tetracycline and ampicillin resistance.

Two pcDNA3 plasmids (5.4 Kb, Invitrogen Life Technologies, Paisley, UK) containing p53 DNA encoding the arginine (Arg) and proline (Pro) alleles (approximately 1.8 Kb each) cloned at the Bam HI site, were made available by Dr. Alan Storey from the Imperial Cancer Research Fund Skin Tumour Laboratory, London, UK (Storey *et al*, 1998). Total plasmid sizes were approximately 7.2 Kb each. A map of the pcDNA3 plasmid is shown in **Figure 2.2**.

Plasmid	Supplier	Size	Resistance	E.Coli Host
pcDNA3	Invitrogen Life Technologies, Paisley, UK	5400 bp	ampicillin, kanamycin, tetracycline	MC1061/P3
pCH110	Amersham Pharmacia Biotech, Little Chalfont, UK	7128 bp	ampicillin	JM109
pUC18	Amersham Pharmacia Biotech, Little Chalfont, UK	2686 bp	ampicillin	JM109
pGEM <sup>®</sup> Z	Promega, Southampton, UK	3197 bp	ampicillin	JM109

Table 2.8. Characteristic	s of expressi	on plasmids.
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bp = base pairs

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Figure 2.2. Map of the pcDNA3 plasmid.

The pcDNA3 plasmid is a low copy number plasmid that carries drug resistance markers for kanamycin, tetracycline and ampicillin. The kanamycin gene is fully active and is used to select for cells carrying the pcDNA3 plasmid. (Invitrogen Life Technologies, Paisley, UK).

## 2.7.2 Preparation of Competent Cells

The plasmid DNA was transformed into competent cells of the appropriate host strain, JM109 cells for pUC18 plasmid with insert, pGEM<sup>®</sup>Z plasmid with insert and pCH110 eucaryotic control plasmid, and MC1061/P3 cells for pcDNA3 plasmids.

### 2.7.2.1 JM109

The bacterial host strain was streaked onto Luria-Bertani (LB) agar plates (LB medium with 1.5 % w/v agar) with 100 µg/ml ampicillin and without ampicillin using a flame sterilised loop and left to grow overnight in a 37 °C incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). A 25 ml aliquot of LB medium (1% Tryptone, 0.5 % Yeast Extract, 1% NaCl) was then inoculated with a single colony and incubated overnight in a waterbath (Grant, Cambridge, UK) at 37 °C with vigorous shaking at 200 rpm. Two sterile 500 ml flasks of LB medium were then inoculated with 5 ml of cells from the overnight cultures and shaken at 200 rpm at 37 °C in a waterbath until the OD<sub>600</sub> reached 0.45-0.55. Each solution was then chilled on ice for two hours and subsequently collected by centrifugation at 2500 X g for 15 minutes at 4 °C. The pellet was resuspended in 10-20 ml of ice-cold trituration buffer and diluted to 500 ml with the same solution, then incubated on ice for 45 minutes and centrifuged at 1800 X g for 10 minutes. The supernatant was decanted and 50 ml of ice-cold trituration buffer was added to the cells that were gently resuspended by pipetting up and down. Next, 80% glycerol was added drop-wise with gentle swirling to a final concentration of 15% v/v. The competent cells were then aliquoted in 1 ml volumes into sterile 1.5 ml micro-centrifuge tubes, snap frozen in liquid nitrogen and stored at -70 °C.

## 2.7.2.1 MC1061/P3

Competent cells were prepared for MC1061/P3 cells containing P3 for transformation with a SupF plasmid from a non-revertant colony sensitive to ampicillin (Amp<sup>s</sup>) and tetracycline (Tet<sup>R</sup>), triple antibody selection. Using a flame-sterilised loop, MC1061/P3 cells were streaked onto LB plates containing 50 µg of kanamycin for selection of E. coli containing P3 and left to grow overnight in a 37 °C incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). Approximately 20-30 single isolated kanamycin resistant (Kan<sup>K</sup>) colonies were then patched onto separate LB plates containing 100 µg/ml of ampicillin only, 15 µg/ml of tetracycline only and 50 µg/ml of kanamycin only and left to grow overnight in a 37 °C incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). Single isolated colonies exhibiting sensitivity to both tetracycline and ampicillin (Kan<sup>R</sup> Tet<sup>s</sup> Amp<sup>s</sup>) were selected to prepare competent cells. A 25 ml aliquot of LB medium was then inoculated with a single colony and incubated overnight in a 37 °C waterbath (Grant, Cambridge, UK) with vigorous shaking at 200 rpm. Next, 1 ml of the overnight culture was added to two 500 ml sterile flasks containing 100 ml of LB medium. The cultures were shaken at 200 rpm at 37 °C until the OD<sub>600</sub> reached 0.45-0.55. Each solution was then aliquoted into pre-chilled sterile 50 ml tubes, left on ice 10-15 minutes, and centrifuged at 4000 rpm at 4 °C for 15 minutes. The supernatant was decanted and the resulting cell pellets resuspended in 33 ml of FSB transformation (10 mM potassium acetate, pH 7.5, 45 mM MnCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 100 mM KCl, 3 mM Hexaaminecobalt (III) chloride, 10 % glycerol) buffer by gentle vortexing, then incubated on ice a further 10-15 minutes. The supernatant was again decanted and the cell pellets were resuspended in 8 ml of FSB transformation buffer and resuspended with gentle vortexing. A 280 µl aliquot of DMSO (v/v 3.5 %) was then added to the suspension, mixed immediately by swirling and incubated on ice for 5 minutes. A second 280 µl aliquot of DMSO was added (final concentration 7 %), the tube mixed by swirling and then incubated on ice for 10-20 minutes. The competent cells were then aliquoted in 210  $\mu$ l volumes into sterile 1.5 ml micro-centrifuge tubes, snap frozen in liquid nitrogen and stored at -70 °C.

## 2.7.3 Transformation of Competent Cells

A pUC18 plasmid with insert and a  $pGEM^{\oplus}Z$  plasmid with insert were used as positive controls in the transformation assays. JM109 competent cells were also used for amplification of PCR products.

# 2.7.3.1 JM109

A 600  $\mu$ l aliquot of competent cells was thawed on ice and 18 ng of plasmid DNA was added to 3 separate aliquots of 200  $\mu$ l and incubated on ice for 30 minutes. After incubation, cells were heat shocked in a waterbath at 42 °C for 5 minutes and immediately cooled on ice for one minute. Cells were then allowed to recover by adding 2 ml of LB medium to each tube and incubating in a waterbath (Grant, Cambridge, UK) at 37 °C with shaking at 200 rpm for 1 hour. Cells were then plated directly in 50-200  $\mu$ l aliquots onto LB plates containing 50  $\mu$ g/ml of ampicillin with a top gel containing 40  $\mu$ l of 2% 5bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-gal) (Sigma Chemicals Ltd., Poole, UK) and 40  $\mu$ l of 100 mM Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG). The plates were left to incubate at room temperature for 30 minutes, then inverted and incubated in a 37 °C incubator (Leec, Jencons-PLS, Leighton Buzzard, UK) for 12-16 hours. Blue/white screening of colonies for recombinants was used, where white colonies were selected.

### 2.7.3.2 MC1061/P3

Four 210  $\mu$ l aliquots of competent cells were thawed at room temperature until barely liquid and then placed on ice for 10 minutes prior to the addition of DNA. A 0.5  $\mu$ l (750 ng) and 1 $\mu$ l (1500 ng) aliquot of each plasmid DNA was added to the thawed tubes, mixed by swirling and then incubated on ice for 30 minutes. Cells were heat shocked in a waterbath at 42 °C for exactly 90 seconds and immediately cooled on ice for 1-2 minutes. Cells were allowed to recover by adding 400  $\mu$ l of SOC medium (2 % Tryptone, 0.5 % Yeast Extract, 0.05 % NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 20 mM glucose) to each tube and incubating in a waterbath (Grant, Cambridge, UK) at 37 °C with shaking at 200 rpm for 1 hour. Transformed cells were then plated directly in 50–200  $\mu$ l aliquots onto LB selective agar plates containing 100  $\mu$ g/ml of ampicillin and 15  $\mu$ g/ml of tetracycline and incubated in a 37 °C incubator (Leec, Jencons-PLS, Leighton Buzzard, UK) for 12-16 hours.

# 2.7.4 Isolation of Plasmid DNA

To each of four sterile 50 ml tubes, 10 ml of autoclaved LB medium or SOB medium (2 % Tryptone, 0.5 % Yeast Extract, 0.05 % NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>) and 10  $\mu$ l of ampicillin were added. Each medium was then inoculated with a single transformed competent bacterial colony and grown at 37 °C, with shaking (250 cycles per minute), to late log phase. Four sterile 500 ml flasks of pre-warmed LB medium containing 500  $\mu$ l of ampicillin each were then inoculated with 1 ml of the late log phase cultures and incubated overnight in a waterbath (Grant, Cambridge, UK) at 37 °C with vigorous shaking at 250 cycles per minute. The cultures were then transferred to 250 ml Oak Ridge centrifuge tubes (Nalgene, USA) and centrifuged at 6000 rpm for 20 minutes at 4 °C. The supernatant was then discarded and the plasmid DNA was extracted from the resulting pellet via either small or large-scale methods.

#### 2.7.5 Extraction of Plasmid DNA

Plasmid DNA was extracted from bacterial cell cultures following the method of Birnboim and Doly, 1979, using either small or large-scale methods.

#### 2.7.5.1 Small-Scale Preparation

Bacterial cells were harvested by placing 1.5 ml aliquots into sterile 1.5 ml microcentrifuge tubes and centrifuging at 13000 rpm for 1 minute at room temperature. The supernatant was decanted and the pellet was resuspended in 100 µl of Solution I (50mM glucose, 25 mM Tris-HCl, 10 mM EDTA) by gentle vortexing. Next, 200 µl of Solution II (1% SDS, 0.2N NaOH) was added, mixed by gently inverting the tube and then incubated on ice for 5 minutes. Following the incubation, 150 µl of Solution III (3M sodium acetate, pH 4.8) was added, mixed by gently inverting the tube, incubated on ice for 5 minutes, and centrifuged at 13000 rpm for 5 minutes at room temperature. A 400 µl aliquot of the supernatant was carefully transferred to a new sterile 1.5 ml micro-centrifuge tube, 1 ml of ice-cold 100 % ethanol added, mixed by vortexing and then incubated on ice for 10 minutes. The tube was then centrifuged at 13000 rpm for 3 minutes at room temperature to collect the precipitated plasmid DNA. The supernatant was then discarded and the DNA pellet was resuspended in 100 µl of Solution IV (50 mM Tris-HCl, pH 8.0, 100 mM sodium acetate) by gentle vortexing. The DNA was then reprecipitated by adding 250 µl of ice-cold 100 % ethanol, mixing by vortexing and incubating on ice for 10 minutes. The DNA was again collected by centrifugation at 13000 rpm for 3 minutes at room temperature, the supernatant discarded and the pellet washed with 1 volume of 70 % ethanol. After a final centrifugation at 13000 rpm for 1 minute, the supernatant was discarded and the pellet was resuspended in 15  $\mu$ l of TE buffer, pH 8.0.

### 2.7.5.2 Large-Scale Preparation

Bacterial cells were harvested by aliquoting into sterile 50 ml tubes and centrifuging at 6000 rpm in a MSE Europa 24M centrifuge (MSE Scientific Instruments, Leicester, UK) at 4 °C for 20 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml of Solution I (50mM glucose, 25 mM Tris-HCl, 10 mM EDTA). Next, 20 ml of Solution II (1% SDS, 0.2N NaOH) was added, mixed and kept on ice for 5 minutes to precipitate out chromosomal DNA, high molecular weight RNA and protein/membrane complexes. Following incubation on ice, 15 ml of Solution III (3M sodium acetate, pH 4.8) was added, mixed and again incubated on ice for 10 minutes, followed by centrifugation at 7,000 rpm at 4 °C for 20 minutes. The supernatant was carefully decanted and allowed to warm to room temperature after which a 0.6 volume of isopropanol was added. The solution was mixed and then left at room temperature for 15-30 minutes. The solution was then centrifuged at 9000 rpm at 15 °C for 15 minutes, the supernatant decanted and the pellet washed with 1 ml of 70 % ethanol. The ethanol was decanted and the solution was allowed to air dry for 10 minutes before it was dissolved in 2.5 ml of TE buffer, pH 8.0.

## 2.7.6 Purification of Plasmid DNA

Plasmid DNA was purified by equilibrium centrifugation in cesium chloride (CsCl) ethidium bromide continuous gradients (Sambrook *et al*, 1989). The DNA present in the centrifuged CsCl solution will move to a position of equilibrium in the gradient equivalent to its buoyant density. In CsCl solutions of appropriate density, the DNA will form bands near the centre of the tube, RNA will pellet at the bottom and proteins float near the top.

The exact volume of the DNA solution was determined and one gram of CsCl was added for every ml of solution. For a 2.5 ml sample volume, 2.5 g of CsCl was added to the DNA solution followed by 0.25 ml of ethidium bromide solution (10 mg/ml). The solution was mixed thoroughly then centrifuged at 13,000 rpm for 3 minutes. The supernatant was transferred to a 13 x 23 mm Quick-Seal centrifuge tube (Beckman, USA) which was then sealed by heating and centrifuged at 80,000 rpm in a fixed angle rotor ultracentrifuge at 20 °C for 24 hours (Beckman TL-100, USA). The superhelical plasmid DNA formed from the CsCl gradient was collected from the tube observed under UV light by inserting a needle with a syringe just below the band of plasmid DNA. A second needle was inserted into the top if the tube to form a vent. The collected DNA was then transferred to a sterile 15 ml tube where the ethidium bromide was removed by adding an equal volume of water saturated isopropanol and mixed. When the two phases settled and separated, the upper phase containing the ethidium bromide was removed and discarded and an equal volume of water-saturated isopropanol was

Again added and mixed. The extraction procedure was repeated until both the aqueous and organic phases were clear in colour. The DNA was then transferred to a sterile 1.5 ml micro-centrifuge tube and then precipitated by adding a  $1/10^{\text{th}}$  volume of 3M sodium acetate and 3 volumes of ice-cold 100 % ethanol. The sample was stored at -20 °C for at least 1 hour then centrifuged at 13,000 rpm for 10 minutes. The supernatant was decanted and the pellet washed with 70% ethanol. The DNA pellet was air-dried for 5-10 minutes and resuspended in 100 µl of distilled water. The quality of the DNA was determined spectrophotometrically and its quality was assessed by electrophoresis through a 1 % agarose gel in 0.5 X TBE buffer. The DNA was stored at -20 °C until required.

# 2.7.7 Restriction Endonuclease Mapping of Inserts

The concentrations of the purified plasmid recombinants containing DNA inserts were determined spectrophotometrically at  $OD_{260}$ , where up to 170 µg of purified plasmid was obtained. Approximately 1 µg of each purified plasmid was digested using restriction enzymes *BamH* I, *EcoR* I and *Hind* III (1 unit/µg DNA, Roche Diagnositics, Lewes, UK).

A 5  $\mu$ l aliquot of purified plasmid was added to a sterile 1.5 ml micro-centrifuge tube containing 1 unit of the appropriate restriction enzyme, 1.5  $\mu$ l of 10 X restriction buffer and sufficient water to a 15  $\mu$ l total reaction volume. The samples were then incubated in a waterbath at 37 °C for 1.5 hours. Following digestion, 10  $\mu$ l of the sample was run on a 2 % horizontal agarose gel inc 0.5 X TBE buffer along with 5  $\mu$ l of the undigested sample and a 23 Kb molecular weight marker (Roche Diagnostics, Lewes, UK). The gel was visualised via irradiation with UV light on a transilluminator (UVP International, Cambridge, UK) at  $\lambda$ 302 nm and photographed using a gel imager software package (Vision Works 3.0, UVP International, Cambridge, UK) and video linked to a Sony graphic printer using type IV UPP-11-HA thermo film (Sony Corporation, Tokyo, Japan).

## 2.7.8 Transfection of Saos-2 Cells

Saos-2 cells were transfected with purified recombinant plasmid containing DNA inserts using highly efficient calcium phosphate precipitation where the transfected DNA enters the cytoplasm of the cell by endocytosis and is then transferred to the nucleus.

# 2.7.8.1 pcDNA3 (containing p53 Pro and p53 Arg DNA Inserts)

Approximately 24 hours prior to transfection, exponentially growing Saos-2 cells were harvested by trypsinisation and replated at a density of 2 X  $10^5$  cells/cm<sup>2</sup> in 60 mm tissue culture dishes in 4 ml of appropriate medium and incubated for 24 hours at 37 °C in an atmosphere of 5 % CO<sub>2</sub> in a dry CO<sub>2</sub>/O<sub>2</sub> incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). Next, 10-40 µg of each DNA sample to be transfected was added to a sterile 1.5 ml micro-centrifuge tube, sufficient water added to 180 µl, the sample mixed by pipetting up and down and then 20 µl of 2.5 M CaCl<sub>2</sub> was added and again mixed. A 200 µl aliquot of 2 X HBS (2 X HEPES buffered saline, 280 mM NaCl, 50 mM HEPES, 1.5 mM sodium phosphate, pH 7.15) was added to a separate sterile 15 ml conical tube and using a mechanical pipettor attached to a sterile 1 ml pipette, the solution was bubbled. The diluted calcium phosphate-DNA suspension was then added dropwise with a Pasteur pipette into the bubbling solution and allowed to precipitate for 20-30 minutes at room temperature. The medium in the 60 mm tissue culture dishes was aspirated off and 3.6 ml of appropriate fresh medium added. The DNA precipitate was then added dropwise with a Pasteur pipette into the medium above the cell monolayer, the plate was gently swirled and incubated 6-14 hours at 37 °C in an atmosphere of 5 % CO2 in a dry CO2/O2 incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). The DNA precipitate was aspirated off and the cells were then washed with 4 ml of 1 X versene (0.2 g/litre in PBS; 8g of NaCl, 0.2 g of KCl, 1.2 g of sodium phosphate, 0.2 g of potassium phosphate, 0.2 g of EDTA, 1.5 ml of phenol red) followed by 4 ml of PBS which was aspirated off. The cells were glycerol shocked by incubation in 0.5 ml of 20 % glycerol in PBS for 1 minute then 3 ml of complete medium was added to the dish and mixed by gentle swirling. The solution was aspirated off and the cells were washed in 3 ml of complete medium. The medium was aspirated off, 4 ml of complete medium was added to the dish and the cells were incubated at 37 °C in an atmosphere of 5 % CO<sub>2</sub> in a dry CO<sub>2</sub>/O<sub>2</sub> incubator (Leec, Jencons-PLS, Leighton Buzzard, UK) for 48 hours.

2.7.8.2 pCH110 Eukaryotic Assay Vector (Amersham Pharmacia Biotech, Little Chalfont, UK) The pCH110 plasmid contains a functional lacZ gene that is expressed from the SV40 early promoter in eukaryotes (Hall *et al*, 1983).  $\beta$ -galactosidase, the product of the lacZ gene, can be easily assayed as it is transiently expressed thereby providing an internal marker for monitoring expression. Cells transfected with the vector can be fixed and stained using a colourmetric assay to determine transfection efficiency. A map of the pCH110 eukaryotic assay vector is shown in Figure 2.3. The pCH110 plasmid was transformed into competent JM109, amplified and isolated as described above (sections 2.7.3.1 and 2.7.4). Soas-2 cells were trypsinised, plated at 2-3 X  $10^5$  cells in a sterile 60 mm tissue culture dish and transfected with 10 µg of pCH110 vector as described above. After 48 hours incubation at 37 °C in an atmosphere of 5 % CO<sub>2</sub> in a dry CO<sub>2</sub>/O<sub>2</sub> incubator, the cells were washed twice with a 4 ml volume of PBS which was then removed by aspiration. The cells were then fixed in 4 ml of 0.1 % gluteraldehyde (Sigma Chemicals Ltd., Poole, UK) in PBS for 10 minutes and washed twice with 4 ml of PBS and removed with aspiration. The washed cells were then stained in 4 ml of freshly made  $\beta$ -galactosidase stain (3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 0.08 % X-gal, 2 mM MgCl<sub>2</sub>) for 24 hours. Cells were verified for blue staining indicating the presence of the transfected vector.



Figure 2.3. Map of the pCH110 eukaryotic assay vector.

The pCH110 plasmid contains a functional lacZ gene which is expressed from the SV40 early promoter in eukaryotics (Hall *et al*, 1983).  $\beta$ -galactosidase, the product of the lacZ gene, can be easily assayed as it is transiently expressed thereby providing an internal marker for monitoring expression. (Amersham-Pharmacia Biotech, Little Chalfont, UK)

#### 2.7.9 Selection of Transfected Saos-2 Cells

Geneticin (G418) was used to select for transfected cells. G418 blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside that is similar in structure to neomycin, gentamycin and kanamycin. Selection conditions were determined by adding varying concentrations of Geneticin (G418) (50 mg/ml, Invitrogen Life Technologies, Paisley, UK), ranging from 100-800  $\mu$ g/ml, to tissue culture dishes containing Soas-2 cells and cultured for up to 10 days to determine the optimal potency. The dishes were examined for viable cells and the optimal concentration of antibiotic determined. Approximately 48 hours after transfection, cells were trypsinised, split 1:10 into an appropriate number of sterile 60 mm tissue culture dishes and incubated 12-16 hours at 37 °C in an atmosphere of 5 % CO<sub>2</sub> in a dry CO<sub>2</sub>/O<sub>2</sub> incubator (Leec, Jencons-PLS, Leighton Buzzard, UK). Selection for stable transformants was performed by adding 500  $\mu$ g/ml of Geneticin (G418) to the medium. The medium was changed every 2-4 days for 2-3 weeks to remove the debris of dead cells and replated in new tissue culture dishes.

### 2.7.10 Storage of Plasmid Cultures

### 2.7.10.1 Short Term Storage of Bacteria

Colonies containing recombinant plasmid were picked from the original agar plates and streaked onto fresh LB agar plates containing antibiotics and incubated at 37 °C for 12-16 hours. After incubation, well-isolated single colonies were picked with a sterile inoculating loop and streaked onto fresh LB agar plates containing the appropriate antibiotics and again incubated at 37 °C for 12-16 hours. The plates were then sealed with Nescofilm<sup>TM</sup> and stored at 4 °C for 2-3 months.

# 2.7.10.2 Long Term Storage of Bacteria

Colonies containing recombinant plasmid were picked from the original agar plates and streaked onto fresh LB agar plates containing antibiotics and incubated at 37 °C for 12-16 hours. After incubation, well-isolated single colonies were picked with a sterile inoculating loop and grown in 10 ml of LB culture medium in sterile 50 ml tubes at 37 °C with shaking (250 cycles per minute) in a waterbath (Grant, Cambridge, UK) for 12 –16 hours. The cultures were prepared for storage by mixing each thoroughly with 15 % glycerol (0.15 ml of glycerol to 0.85 ml bacterial culture). Aliquots of 1 ml were then transferred into 1.8 ml sterile cryogenetic vials (Corning, New York, USA), snap frozen in liquid nitrogen and stored at -70 °C indefinitely.

# 2.7.11 Recovery of Bacteria From Long Term Storage

A cryovial of frozen bacterial culture was removed from storage at -70 °C. While still frozen, a sample of culture was scraped from the surface using a sterile inoculating loop and streaked onto the surface of a fresh LB agar plate containing antibiotics. The remaining frozen culture was returned to storage at -70 °C and the LB agar plate was incubated at 37 °C in a Leec incubator (Jencons-PLS, Buzzard Leighton, UK) for 12-16 hours.

# 2.8 Labelling of DNA

### 2.8.1 Radiolabelling

#### 2.8.1.1 Random Primed Labelling of DNA

DNA probes used in hybridisation reactions were radiolabelled with  $\alpha^{32}P$  dCTP using a Random Primed DNA Labelling Kit (Roche Diagnostics, Lewes, UK). The method is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complimentary strand is synthesised from the 3'OH terminal of the random hexanucleotide primer using Klenow enzyme, labelling grade. Approximately 25 ng of DNA to be labelled was added to a sterile 0.2 ml micro-centrifuge tube and denatured by heating in a waterbath at 100 °C for 10 minutes followed by immediately cooling on ice for at least 1 minute. To the same tube, 1 µl each of dATP, dGTP, and dTTP (0.5 mM) were added, 2 µl of reaction mixture (hexanucleotide mixture in 10 X reaction buffer), 5 µl of  $\alpha^{32}P$  dCTP (3000 Ci/mmol), sufficient water to make up to 19 µl reaction volume and 1 µl of Klenow enzyme (2 units/µl). The solution was mixed by pipetting up and down and then incubated in a waterbath at 37 °C for 30 minutes. The reaction was stopped by the addition of 2 µl of 0.2 M EDTA, pH 8.0.

## 2.8.1.2 5' End Labelling of DNA

DNA molecular weight markers were radiolabelled using a 5'-End labelling system, Ready-To-Go<sup>TM</sup> T4 Polynucleotide Kinase (PNK) (Amersham Pharmacia Biotech, Little Chalfont, UK). A tube containing 8-10 units of FPLC*pure*<sup>TM</sup> T4 polynucleotide Kinase (50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, pH 8.0, 0.2  $\mu$ M ATP and stabilisers) was reconstituted in 25  $\mu$ l of sterile water, incubated at room temperature for 5-10 minutes, then mixed by gentle pipetting up and down. Next, 5-10 pmol of DNA was added to a sterile 0.2 ml micro-centrifuge tube and denatured by heating at 65 °C in a waterbath for 5 minutes, then cooled for 5 minutes at room temperature. The heat denatured DNA was then added to the tube along with sufficient water to make a final reaction volume of 49 µl and 1 µl of  $\gamma^{32}$ P dATP (3000 Ci/mmol, 10 µCi/µl). The solution was gently mixed by pipetting up and down, centrifuged at 13000 rpm for 30 seconds and incubated at 37 °C in a waterbath for 30 minutes. The reaction was then stopped by the addition of 5 µl of 250 mM EDTA. The sample was precipitated to remove unincorporated nucleotides by adding 2 µl of Quick Precip<sup>TM</sup> (Advanced Biotech Corp., USA), 1/10<sup>th</sup> volume of 5 M sodium chloride and 2-3 volumes of ice-cold 100 % ethanol to the tube, which was then mixed by vortexing. The solution was then centrifuged at 13000 rpm for 3 minutes, the supernatant decanted and the pellet washed with 1 volume of 70 % ethanol. The solution was again centrifuged at 13000 rpm for 30 seconds, the supernatant decanted, the sample air-dried for 5 minutes and the labelled DNA was then resuspended in 50 µl of sterile water. The labelled DNA was then stored at -20 °C for up to 1 week.

#### 2.8.1.2.1 Removal of Unincorporated Radiolabelled Nucleotides

Labelled DNA was separated from unincorporated deoxyribonucleoside triphosphates by column chromatography through a Sephadex G-50 column (Sambrook *et al*, 1989). A slurry of Sephadex G-50 (Sigma Chemicals Ltd., Poole, UK) was prepared by adding 30 grams of Sephadex G-50 to 250 ml of TE buffer, pH 7.6, in a 500 ml Duran bottle an incubating at room temperature for 12 - 16 hours. The solution was then heated in a waterbath at 65 °C for 2 hours and then left to cool to room temperature for 3 hours. The supernatant was then decanted and an equal volume of TE buffer, pH 7.6, was added to equilibrate the resin. A Sephadex G-50 chromatography column was prepared by pushing a small amount of sterile glass wool to the bottom of a disposable glass Pasteur pipette using a sterile bio-loop. The equilibrated Sephadex G-50 resin was mixed well and gently added

to the plugged pipette using a sterile plastic Pasteur pipette, avoiding the formation of air bubbles, until about 1 cm below the top of the column. The column was clamped to a holding stand and continually irrigated with TE buffer, pH 7.6, to avoid it drying out or cracking. Immediately following column packing and irrigation the labelled DNA sample, mixed with 180  $\mu$ l of TE buffer, pH 7.6, was added to the top of the column. As soon as the labelled DNA sample has entered the resin another 180  $\mu$ l of TE Buffer, pH 7.6, was added to the column to wash the DNA sample through. Elution fractions were collected into separate sterile 0.2 ml micro-centrifuge tubes. The sample was washed through the column using an additional 11 aliquots of 180  $\mu$ l of TE buffer, pH 7.6, with eluted fractions collected for each.

# 2.8.1.2.2 Labelling Efficiency

The degree of labelling was determined by comparison of incorporated with total input radioactivity in the collected reaction aliquots. The specific activity was determined using liquid scintillation counting of each collected sample in a scintillation counter (Beckman Instruments, USA). Unincorporated nucleotides will elute more slowly through the resin than labelled DNA therefore an elution profile consists of a leading peak of radioactivity consisting of nucleotides incorporated into DNA followed by a trailing peak of unincorporated radioactivity deoxyribonucleoside consisting of triphosphates. Incorporation was routinely > 70 %, with specific activities of  $10^9$  cpm/ µg of DNA. Collected fractions containing the incorporated nucleotides from the leading peak were pooled and stored in a labelled sterile 1.5 ml micro-centrifuge tube at - 20 °C for up to 1 week.

# 2.8.2 Digoxigenin (DIG) Labelling

labelling reaction using Digoxigenin-11-2'-deoxy-uridine-5'-A random primed triphosphate (DIG-11-dUTP) (Roche Diagnostics, Lewes, UK) was used to label two separate amplified HIF-1a DNA probes (Table 2.9). Approximately 3.5 µg of DNA was first denatured by heating in a waterbath (Grant, Cambridge, UK) at 95 °C for 10 minutes then chilled on ice for at least 1 minute. Next, the freshly denatured DNA was added to a sterile 0.2 ml micro-centrifuge tube along with 2µl of 10X hexanucleotide mixture, 2µl of 10 X DIG DNA labelling mixture, 1 µl of labelling grade Klenow enzyme and sufficient sterile water to a final reaction volume of 19 µl. The tube was mixed, briefly centrifuged and incubated in a 37 °C waterbath for 1 hour, after which 2 µl of 0.2 M EDTA, pH 8.0 was added to stop the reaction. The DIG-labelled DNA was then precipitated with 2.5 µl of 3M sodium acetate, pH 5.2 and 75 µl of pre-chilled 100 % (v/v) ethanol for 2 hours at -20 °C. The tube was then centrifuged at room temperature at 13000 rpm for 10 minutes, the supernatant decanted and the pellet washed with 50  $\mu$ l of 70 % (v/v) ethanol. The tube was again centrifuged at 13000 rpm for 5 minutes, the supernatant decanted and the pellet airdried for 10 minutes prior to being resuspended in 50µl of TE buffer. The DIG-labelled DNA was then stored at -70 °C.

 Table 2.9. Amplimer sequences and reaction conditions for HIF-1α probe synthesis used in Digoxigenin (DIG) labelling.

Amplimer Sequence	Length (mer)	Gene	Cycle Conditions	Cycles	Product (base pairs)
5' GCCACTTCGAAGTAGTGCTGACC 3' (sense) 5' GCTTCCATCGGAAGGACTAGG 3' (antisense)	23 21	HIF-la	94 °C for 1 minute 55 °C for 1 minute 68 °C for 1.5 minutes Additional 68 °C for 10 minutes	30	135 bp
5' CACCTATGACCTGCTTGGTGC 3' (sense) 5' GGCAAGCATCCTGTACTGTCC 3' (antisense)	· 21 21	HIF-1α	94 °C for 1 minute 55 °C for 1 minute 68 °C for 1.5 minutes Additional 68 °C for 10 minutes	30	283 bp

# 2.9 Sequencing of PCR products

PCR products were sequenced using two separate methods based on the chain-termination DNA sequencing methods (Sanger *et al*, 1977).

# 2.9.1 Sequenase PCR Product Sequencing Kit (Amersham Pharmacia Biotech, Little Chalfont, UK)

This method requires no purification of the PCR product and all gel purifications, sedimentations and filtrations are eliminated by the use of two enzymes which effectively remove excess dNTPs and primers from the DNA produced in the PCR amplification.

### 2.9.1.1 Enzymatic Pre-Treatment of PCR product

The PCR product was treated with a combination of Exonuclease I and Shrimp Alkaline Phosphatase. Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with the labelling step of the sequencing process. A 5  $\mu$ l aliquot of PCR amplified product was added to a sterile 0.5 ml micro-centrifuge tube containing 1  $\mu$ l of Exonuclease (10.0 units/ $\mu$ l) and 1  $\mu$ l of Shrimp Alkaline Phosphatase (2.0 units/ $\mu$ l) and mixed by pipetting up and down. The solution was then incubated in a waterbath at 37 °C for 15 minutes, and the enzymes inactivated by heating to 80 °C in a waterbath for 15 minutes.

# 2.9.1.2 Annealing

Next, a 3.5  $\mu$ l aliquot of the treated PCR product was transferred to a new sterile 0.5 ml micro-centrifuge tube and 6.5 $\mu$ l of primer (1 pmol/ $\mu$ l) was added to the tube to make a 10  $\mu$ l total reaction volume. The DNA was then denatured by heating for 2-3 minutes at 100 °C in a thermal cycler (Techne Ltd., Cambridge, UK) and quickly cooled by placing the

vial directly in ice for 5 minutes. The solution was centrifuged at 13000 rpm for 30 seconds then 2  $\mu$ l of 5 X reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mm MgCl<sub>2</sub>, 250 mM NaCl), 1  $\mu$ l of 0.1M DTT, 2  $\mu$ l of 1:5 diluted labelling mixture (7.5  $\mu$ M dCTP, 7.5  $\mu$ M dGTP, 7.5  $\mu$ M dTTP), 0.5  $\mu$ l of  $\alpha^{32}$ P dATP (10 $\mu$ Ci/ $\mu$ l) and 2  $\mu$ l of Sequenase version 2.0 T7 DNA Polymerase (1.6 units/ $\mu$ l) with 2 units/ $\mu$ l of inorganic pyrophosphatase in 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.1 mM EDTA, 50 % glycerol) were added to the tube to make a total reaction volume of 17.5  $\mu$ l. The solution was mixed by pipetting up and down and incubated at room temperature for 5 minutes.

# **2.9.1.3 Termination Reaction**

Sterile 0.5 ml micro-centrifuge tubes were labelled G, A, C, T and 2.5  $\mu$ l of appropriate termination mixture was added to each and pre-warmed in a waterbath at 37 °C for 1 minute (ddG termination mixture: 80 $\mu$ M dGTP, 80 $\mu$ M dATP, 80 $\mu$ M dCTP, 80 $\mu$ M dTTP, 8  $\mu$ M dGTP, 50 mM NaCl; ddA termination mixture; 80 $\mu$ M dGTP, 80 $\mu$ M dATP, 80 $\mu$ M dTTP, 8 $\mu$ M dCTP, 50 mM NaCl; ddC termination mixture: 80 $\mu$ M dGTP, 80 $\mu$ M dATP, 80 $\mu$ M dATP, 80 $\mu$ M dCTP, 80 $\mu$ M dTTP, 8 $\mu$ M dCTP, 50 mM NaCl; ddC termination mixture: 80 $\mu$ M dGTP, 80 $\mu$ M dATP, 80 $\mu$ M dCTP, 80 $\mu$ M dTTP, 8 $\mu$ M dCTP, 50 mM NaCl). A 3.5  $\mu$ l aliquot of the labelling reaction was added to each termination tube, the solution mixed by pipetting up and down and then incubated in a waterbath at 37 °C for 5-10 minutes. The reactions were stopped by the addition of 4  $\mu$ l of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanol FF) and then heated in a waterbath at 75 °C for 2 minutes immediately prior to loading into the sequencing gel.

#### 2.9.1.4 Denaturing Gel electrophoresis For Manual Sequencing

A Bio-Rad Sequence Gen II<sup>™</sup> electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, UK) was used which consisted of two 21 X 40 cm glass plates with 0.25 cm spacers, 2 clamps and a 24 well vinyl sharktooth comb. The glass plates were washed and then dried with 70% industrial methylated spirit (IMS). The inner surface of the back plate was then covered in a thin layer of Repelcote (2% solution of dimethydichlorosilane in octamethylcyclotetra-siloxane, Merck Ltd., Lutterworth, UK) to prevent the gel from tearing as the plates are pried apart after electrophoresis. A small amount of petroleum jelly was applied to the edges of the back plate and the two spacers were placed at the sides of the plate. The front plate was laid on top of the back plate and spacers and clamped together. The sandwiched plates were then placed in a casting tray at a 45° angle. A 6 % denaturing polyacrylamide gel was prepared by mixing 7.5 ml of 20 X glycerol tolerant gel buffer (prepared by mixing 216 grams of Tris-base with 72 grams of taurine and 4 g of EDTA in sterile water to a final volume of 1 litre), with 7.5 ml of water, 99 ml of Ultra Pure<sup>™</sup> Sequagel concentrate solution, and 36 ml of Ultra Pure<sup>™</sup> Sequagel diluent (Sigma Chemicals Ltd, Poole, UK). Half of the gel mix was transferred to a 100 ml conical flask and 0.8 ml of ammonium persulfate and 40 µl of TEMED was added to the flask, mixed by gentle swirling, poured immediately into the casting tray and allowed to polymerise for 30 minutes to seal the bottom of the plates. Following polymerisation, 0.8 ml of ammonium persulfate and 40 µl of TEMED was added to the remaining gel mix, the solution was drawn into a syringe and carefully injected into the gel plate sandwich until it reached the edge of the plates. Air bubbles were removed by gently tapping the glass. The flat edge of the comb was then inserted halfway into the space between the plates and the gel was allowed to polymerise for 1 hour at room temperature. When the gel was set the casting tray was removed and the gel sandwich was placed vertically into the lower buffer tank that contained 1 X glycerol tolerant buffer. The upper buffer chamber was also filled with 1 X glycerol tolerant buffer, the system was connected to a constant power supply (PC3000 Powerpac, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) and the gel was pre-run at 1600 volts for approximately 30 minutes until the temperature of the gel reached 50-55 °C. The gel system was disconnected, the vinyl comb was removed and carefully reinserted with the sharkteeth downwards to create wells. The sequencing samples were then loaded into the wells in the order of G, A, T and C using a 10 µl Drummond sequencing pipette (Drummond Laboratories, USA) and run at 2000 volts for 2-4 hours. After electrophoresis, the gel apparatus was disassembled and the gel plates gently pried apart leaving the gel attached to one of the gel plates which was then soaked in a 5 % acetic acid, 15 % methanol solution for 5 - 15 minutes to remove the urea and detach the gel form the plate. A piece of 3 MM filter paper (Amersham Pharmacia Biotech, Little Chalfont, UK) was laid on top of the gel and the gel and filter paper were covered in Saran Wrap<sup>™</sup> and dried at 75 °C for 2 hours on a Sue 300 gel dryer (Heto Laboratory equipment, Surrey, UK). The dried gel was then placed in an Cronex autoradiography cassette (Kodak, UK) containing lighting and intensifying screens. In a dark room, a piece of Kodak X-OMAT<sup>™</sup> LS X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the gel and the cassette was firmly closed. The cassette was placed at -80 °C where the film was exposed for at least 24 hours. The film was then developed in Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stop-bath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried.

2.9.2 Automated (ABI Prism 377 Automated Sequencer, Perkin Elmer, Chesire, UK) Automated sequencing on purified PCR fragments was performed on 4.25 % gels with urea on an ABI 377 Prism automated sequencer (Perkin Elmer, Chesire, UK) using BigDye terminator cycle sequencing kits (Perkin Elmer, Chesire, UK) at the Molecular Genetics Laboratory, Exeter, UK. Sequencing results were analysed using Factura software and Sequence Navigator, which aligns sequences using Clustal software (Perkin Elmer, Chesire, UK).

# 2.10 Ribonucleic Acid (RNA) Methodology

### 2.10.1 Ribonuclease Free Environment

The successful isolation of intact RNA by any procedure requires that four important steps be performed (Sambrook *et al*, 1989):

- 1) effective disruption of cells or tissues
- 2) denaturation of nucleoprotein complexes
- 3) inactivation of endogenous ribonuclease (RNase) activity, and
- 4) purification of RNA away from contaminating DNA and proteins.

The most important of these steps is the immediate inactivation of endogenous RNase activity, which is released from membrane bound organelles upon cell disruption. RNases are active and stable enzymes that are difficult to inactivate. Thus, all glassware, plastics and instruments used in RNA work was soaked overnight at room temperature in a solution of 0.1% diethylpyrocarbonate (DEPC) to remove RNase and then autoclaved at 121°C, 15 psi for 30 minutes to remove residual traces of DEPC which may cause carboxymethylation of RNA. Tubes used in the preparation of first strand cDNA synthesis were dipped in a silicone solution (Repelcote, BDH Laboratory Supplies, Merck Ltd., Lutterworth, UK) after DEPC treatment and prior to autoclaving. All solutions were made

using 0.1% DEPC treated water and subsequently autoclaved. Latex gloves were worn throughout all procedures and were changed frequently. When required, all reagents and solutions were kept on ice to avoid degradation of RNA by endogenous or residual RNases.

## 2.10.2 Isolation of RNA

Two separate methods were used in isolating RNA from peripheral blood mononuclear cells (PBMCs), established cell lines, and breast and brain tumour tissue.

# 2.10.2.1 RNeasy<sup>TM</sup> Total RNA Purification Kit (QIAGEN Ltd., Crawley, UK)

RNA from near confluent adherent cell lines was obtained by direct lysis with the addition of 600  $\mu$ l of Lysis Buffer RTL (containing guanidinium isothiocyanate (GTC) and  $\beta$ mercaptoethanol ( $\beta$ -ME)) to 75 cm<sup>2</sup> tissue cultures flasks after complete aspiration of the cell culture medium. The resulting lysate was transferred to a RNase free 1.5 ml microcentrifuge tube and homogenised by rapid shearing through a needle and syringe for 30 seconds. The lysate was then centrifuged at 13,000 rpm for 3 minutes in a Biofuge 13 microcentrifuge (Heraeus Sepatech, Germany). A 600  $\mu$ l volume of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The mixture was then applied to an RNeasy spin column that was held in a 1.5 ml micro-centrifuge tube and centrifuged at 10,000 rpm for 15 seconds. The flow-through was then discarded and the column was washed by the addition of 700  $\mu$ l of Wash Buffer RW1 by an additional centrifugation at 10,000 rpm for 15 seconds. The flow-through was again discarded and the column was washed twice with 500 $\mu$ l of Wash Buffer RPE (containing 80% ethanol) and centrifuged as above. After discarding the flow-through, the column membrane was then dried by centrifugation at 13,000 rpm for 2 minutes, ensuring the evaporation of any residual ethanol. The RNA was then eluted into a new 1.5 ml collection tube by the addition of 50  $\mu$ l of DEPC treated water and centrifugation at 10,000 rpm for 1 minute.

# 2.10.2.2 RNA STAT-60<sup>TM</sup> Total RNA Isolation Reagent (Biogenesis, Poole, UK)

RNA STAT- 60<sup>TM</sup>, a mono-phase solution containing guanidinium thiocyanate and phenol, is a single-step method for the isolation of total RNA. As above, RNA from near confluent adherent cell lines was obtained by direct lysis with the addition of 1 ml of RNA STAT- $60^{\text{TM}}$  per 5-10 X  $10^6$  cells, to 75 cm<sup>2</sup> tissue cultures flasks after complete aspiration of the cell culture medium. The cell lysate was collected to a corner of the flask using a cell scrapper where the homogenate was then sheared through was a 1 ml pipette several times prior to being transferred to an RNase free 1.5 ml micro-centrifuge tube. Whole tissue samples (up to 100 mg) were first homogenised in a borosilicate glass 7 ml Dounce tissue grinder (Wheaton Science Products, Millville, N.J., USA) using 10 strokes and 1 ml of RNA STAT-60<sup>TM</sup> per 50-100 mg of tissue. The homogenised lysate was then transferred to a 1.5 micro-centrifuge tube. The homogenates were stored for 5 minutes at room temperature to allow for complete dissociation of nucleoprotein complex. Next, 200µl of chloroform per 1 ml of RNA STAT-60<sup>™</sup> was added, the sample was shaken vigorously for 15 seconds and left at room temperature for 2-3 minutes. Following centrifugation at 13,000 rpm for 15 minutes at 4°C, the upper aqueous layer was transferred to a new 1.5 ml micro-centrifuge tube and precipitated with 0.5 ml of isopropanol, per 1 ml of RNA STAT- $60^{\text{TM}}$  used in homogenisation. The sample was mixed and stored at room temperature for 5-10 minutes then centrifuged at 13,000 rpm for 10 minutes at 4°C. The pelleted RNA was then washed by vortexing with 1 ml of 75% ethanol per 1 ml of RNA STAT-60<sup>TM</sup> used in the initial homogenisation. The sample was then centrifuged at 7,500 rpm for 5 minutes at  $4^{\circ}$ C, the supernatant removed and the sample air-dried for 5 –10 minutes and subsequently resuspended in 50 µl of DEPC treated water.

#### 2.10.3 Quantitation of DNA and RNA

The concentration and purity of the isolated DNA or total RNA was determined spectrophotometrically using a scanning wavelength ( $\lambda$ 240 nm to  $\lambda$ 280 nm) Cecil 5000 spectrophotometer (Cecil Instruments, Cambridge, UK) blanked with water. In determining the nucleic acid concentrations it is implicit that an absorbance reading of 1.0 at 260 nm is equivalent to a 40 µg/ml RNA solution, a 40 µg/ml single stranded DNA solution or a 50 µg/ml double stranded DNA solution (Sambrook *et al*, 1989). Concentrations of either DNA or RNA samples were determined by multiplying the absorbance reading at  $\lambda$ 260 nm by the dilution factor and the relevant nucleic acid concentration. The purity of samples was determined by calculating the absorbance ratio of the optical density (OD)<sub>240</sub> to OD<sub>280</sub>. Samples with a ratio greater than 1.80 were considered pure and free from protein contamination.

### 2.10.4 Reverse Transcription of Total RNA

First strand cDNA was prepared using a SUPERSCRIPT<sup>M</sup> Preamplification System (Invitrogen Life Technologies, Paisley, UK). Four  $\mu$ g of total RNA was mixed with 1  $\mu$ l of Oligo dT<sub>12-18</sub> (0.5  $\mu$ g/ $\mu$ l), which hybridises to the 3' poly (A) tails, and DEPC-treated water to a final reaction volume of 12  $\mu$ l. in a sterile 0.5 ml siliconised micro-centrifuge tube. The sample was then incubated at 70 °C in a waterbath (Grant, Cambridge, UK) for 10 minutes and cooled on ice for at least 1 minute. Next, 2  $\mu$ l of 10X PCR buffer (Invitrogen Life Technologies, Paisley, UK), 2 $\mu$ l of 25 mM MgCl<sub>2</sub>, 1  $\mu$ l of 10 mM dNTP mix and 2  $\mu$ l of 0.1 M dithiothreitol (DTT) were added to the sample, gently mixed, briefly centrifuged, then incubated at 42 °C for 5 minutes. After incubation, 1  $\mu$ l (200 units) of SUPERSCRIPT II RNase H<sup>-</sup> reverse transcriptase (RTase) was added to catalyse the cDNA synthesis, the sample was mixed and again incubated at 42 °C for 50 minutes. The reaction was then

terminated by incubating the sample at 70 °C for 15 minutes, followed by chilling on ice for at least 1 minute. The sample was collected by brief centrifugation, then 1µl of RNase H was added and the sample incubated at 37 °C for 20 minutes. The resulting cDNA was then either used immediately in subsequent PCR amplification reactions (reverse transcription polymerase chain reaction (RT-PCR)) or stored at -20 °C.

### 2.10.5 Northern Blotting

### 2.10.5.1 Separation of Total RNA by Agarose Formaldehyde Gel Electrophoresis

Total RNA was separated by electrophoresis in a 1.5 % horizontal slab agarose formaldehyde gel prepared by dissolving 1.5 grams of multi-purpose agarose in 10 ml of 10 X MOPS (0.2 M 3-[N-Morpholino]propane-sulphonic acid, 0.5M sodium acetate, pH 7.0, 0.01 M EDTA) and 73 ml of water in a 500 ml Duran glass bottle by boiling. The agarose solution was then cooled to 50-55 °C prior to adding 10 µl of a 10 mg/ml solution of ethidium bromide (0.01% v/v) and 17 ml of formaldehyde (37 % v/v, Sigma Chemicals Ltd., Poole, UK). The solution was gently mixed and then poured into a 11 X 14 cm perspex gel mould tray (Life Technologies, Paisley, UK) that was sealed at both ends with masking tape in a fume hood. A plastic 14 well tooth-comb was positioned 1 mm above the tray and the gel was then allowed to set at room temperature for 30 minutes. Once set, the comb and masking tape were carefully removed and the gel and tray were placed into a horizontal gel electrophoresis tank (Life Technologies, Paisley, UK) containing 1 X MOPS buffer sufficient to cover the gel. Up to 100 µg of total RNA in a 6 µl volume was added to a sterile 1.5 ml micro-centrifuge tube containing 12.5 µl of formamide, 2.5 µl of 10 X MOPS buffer and 4  $\mu$ l of 37 % (w/v) formaldehyde. The solution was mixed by pipetting up and down, incubated in a waterbath at 65 °C for 5 minutes and then chilled on ice for at least 1 minute prior to adding 2.5 µl of loading buffer (50 % (v/v) glycerol containing 0.1
mg/ml bromophenol blue). The samples were loaded onto the gel and a RNA molecular weight marker II (5  $\mu$ l of a 1 mg/ml stock solution, 1.6-7.4 Kb, Roche Diagnostics, Lewes, UK), prepared as described above, was run concurrently. The gel was run at 100 volts for 3-4 hours or until the dye front had migrated approximately 8 cm through the gel. Following electrophoresis, the gel was visualised via irradiation with UV light on a transilluminator (UVP International, Cambridge, UK) at  $\lambda$ 302 nm, a ruler was placed alongside the gel and photographed using a gel imager software package (Vision Works 3.0, UVP International, Cambridge, UK) and video linked to a Sony graphic printer using type IV UPP-11-HA thermo film (Sony Corporation, Tokyo, Japan).

## 2.10.5.2 Transfer

Immediately following electrophoresis, the molecular weight marker and any unused areas of the gel were trimmed away with a scalpel blade and the bottom left hand corner of the gel was cut to serve as an orientation point. Two sheets of 3MM Whatman chromatography paper were placed over a 30 cm X 20 cm plastic support so that the ends of the paper fell into a large plastic container. The container was filled with 20 X SSC blotting buffer until the level of the liquid almost reached the top of the support. Capillary action wet the 3MM paper and when it was thoroughly wet any air bubbles were removed by smoothing with a glass rod. The gel was inverted and placed on the support. A sheet of Hybond<sup>™</sup>-N+ positively charged nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) was cut to the size of the gel and placed on top of it avoiding trapping any air bubbles beneath it. Two sheets of 3MM paper cut to the size of the gel were soaked in blotting 20 X SSC were placed on top of the nylon membrane with any air bubbles smoothed out. A stack of absorbent paper towels was placed on top of the 3 MM paper approximately 5 cm high, followed by a glass plate and a 500 g weight. The RNA was allowed to transfer 6-18 hours. After blotting, the towels and Whatman paper were removed from the gel, the membrane was marked with a pencil, removed from the gel and washed in 3 X SSC for 10 minutes. The membrane was either UV fixed (254nm, 2 minutes) or oven baked between 2 sheets of 3MM Whatman paper at 80 °C for 2 hours and stored at room temperature until probed.

#### 2.10.5.3 Radiolabelled Probes

Detailed description and synthesis of probes for HIF-1 $\alpha$ , VEGF, p53 and  $\beta$ -actin are described in Table 2.10. Probes used in Northern hybridisations were radiolabelled and separated as described in section 2.8.1.1.

## 2.10.5.4 Hybridisation

Hybridisation of radiolabelled probes to immobilised RNA was performed in sealed revolving cylindrical siliconised borosilicate glass tubes 20 cm in length X 3 cm in diameter or 20 cm in length X 8 cm in diameter, with screw caps in a Techne HB-ID hybridisation oven (Techne Ltd., Cambridge, UK), using two separate hybridisation buffers.

## 2.10.5.4.1 Hybridisation Buffer (Sambrook et al, 1989)

The membrane carrying immobilised probe was first blocked with non-homologous DNA to mask non-specific binding sites. Sonicated salmon sperm DNA (10 mg/ml) was denatured by heating at 100 °C in a waterbath for 5 minutes, cooled on ice for at least 1 minute and then added to pre-hybridisation solution (5 X SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7), 5 X Denhardt's solution (0.1 % (w/v) BSA, 0.1 % (w/v) Ficoll<sup>TM</sup>, 0.1 % (w/v) polyvinylpyrrolidone (PVP)), 0.5 % (w/v) SDS). The membrane was then pre-hybridised at 65 °C with rotation for 1 hour in 10 ml of pre-hybridisation buffer. The labelled probe, with specific activity > 5 X 10<sup>6</sup> cpm (section

**2.8.1.1.2**) was denatured by heating at 100 °C in a waterbath for 5 minutes, cooled on ice for 10 minutes, added to the tube containing the membrane and pre-hybridisation solution and incubated with rotation at 65 °C for 12 –16 hours. Following hybridisation, the pre-hybridisation solution was discarded and the membrane was washed twice by incubating the membrane in 50 ml of 2 X SSPE, 0.1 % (w/v) SDS with rotation for 10 minutes each at room temperature. The membrane was then washed in 50 ml of pre-warmed 1 X SSPE, 0.1 % (w/v) SDS with rotation at 65 °C for 15 minutes, followed by a wash in 50 ml of pre-warmed 0.1 X SSPE, 0.1 % (w/v) SDS with rotation at 65 °C for 10 minutes.

## 2.10.5.4.2 Rapid-Hyb Buffer (Amersham Pharmacia Biotech, Little Chalfont, UK)

Hybridisation with commercial Rapid-Hyb buffer eliminates the necessity of adding nonhomologous DNA to mask non-specific binding sites on the membrane, as it contains chemical blocking agents. A 10 ml aliquot of the buffer was added to a hybridisation tube and pre-warmed to 65 °C in a hybridisation oven. The membrane containing immobilised RNA was then immersed completely in the pre-warmed buffer and incubated with rotation at 65 °C for 15 minutes. The labelled probe, with specific activity > 5 X 10<sup>6</sup> cpm was denatured by heating at 100 °C in a waterbath for 5 minutes, cooled on ice for 10 minutes, added to the tube containing the membrane and pre-hybridisation solution and incubated with rotation at 65 °C for 2-4 hours. Following hybridisation, the pre-hybridisation solution was discarded and the membrane was washed by incubating the membrane in 50 ml of 2 X SSC, 0.1 % (w/v) SDS with rotation for 20 minutes at room temperature. The membrane was then washed twice in 50 ml of pre-warmed 1 X SSC, 0.1 % (w/v) SDS with rotation at 42 °C for 15 minutes each, followed by 2 washes in 50 ml of 0.1 X SSC, 0.1 % (w/v) SDS with rotation at 42 °C for 10 minutes each.

### 2.10.5.5 Autoradiography

Following washings, the membrane was then drained and placed on a piece of SaranWrap<sup>TM</sup> where it was enclosed in the wrap and air bubbles removed. The wrapped membrane was then placed in a Cronex autoradiography cassette containing lighting and intensifying screens. In a dark room, a piece of Kodak XLS5 X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the wrapped membrane and the cassette was firmly closed. The cassette was placed at -80 °C where the film was exposed for 3-7 days. The film was then developed in Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stop-bath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried. Membranes were stored wet at 4 °C wrapped in SaranWrap<sup>TM</sup>.

## 2.10.5.6 Membrane Stripping

Wet membranes were stripped of bound probes by boiling a solution of 0.5 % (w/v) SDS, pouring it on the membrane and allowing the solution to cool to room temperature. Membranes were then rehybridised as described above (section 2.10.6.4) or stored wet at 4 °C wrapped in SaranWrap<sup>™</sup>.

# 2.10.6 Ribonuclease Protection Assay (RPA)

The ribonuclease protection assay (RPA) is a sensitive technique used for the detection and quantitation of target RNA sequences and related RNAs. A radiolabelled RNA probe is allowed to hybridise to target RNA in solution after which remaining single-strand probe is removed from the reaction by incubation with ribonuclease (RNase). Reaction products are resolved by polyacrylamide gel electrophoresis to quantitate the amount of protected probe (**Figure 2.4**). The technique does not require the transfer of RNA to a solid support and multiple mRNA species can be probed in a single reaction. RPAs were performed using a RPA II<sup>™</sup> kit (AMS Biotechnology Ltd., Oxfordshire, UK).

## 2.10.6.1 RPA Probes

RPA probes were synthesised by RT-PCR (section 2.10.4) from total RNA isolated from PBMCs or established breast carcinoma cell lines using a MAXIscript<sup>™</sup> In Vitro transcription Kit (AMS Biotechnology Ltd., Oxfordshire, UK). A 23 base T7 phage promoter sequence was appended to either the sense or antisense PCR amplimers. Amplification of the target DNA then yields a PCR product that contains the T7 promoter either upstream or downstream of the sequence of interest (Table 2.10).

• The probe for HIF-1 $\alpha$  mRNA was synthesised from 5 µg of total RNA from the breast carcinoma cell line T47D exposed to hypoxia (< 1 % O<sub>2</sub>) for 24 hours. The length of the HIF-1 $\alpha$  probe was 543 bp, corresponding to positions 161-681 bp, and produced a protected fragment size of 520 bp.





• The probe for VEGF mRNA was synthesised from 5  $\mu$ g of total RNA from glioblastoma tissue. The length of the VEGF probe was 464 bp, corresponding to positions 779-1220 bp, and produced a protected fragment size of 441 bp.

• The probe for p53 mRNA was synthesised from 5  $\mu$ g of total RNA from PBMCs of a normal healthy individual. The length of the p53 probe was 463 bp, corresponding to positions 244-684 bp, and produced a protected fragment size of 440 bp.

• The probe for GLUT-1 mRNA was synthesised from 5  $\mu$ g of total RNA from PBMCs of a normal healthy individual. The length of the GLUT-1 probe was 548 bp, corresponding to positions 2106-2631 bp, and produced a protected fragment size of 525 bp.

A  $\beta$ -actin antisense probe was synthesised from a pTRI-Actin-Mouse DNA template, linearised pTRIPLEscript DNA, supplied with the kit. The  $\beta$ -actin gene fragment is a 250 bp *KpnI-Xba*I fragment of the mouse  $\beta$ -actin gene subcloned from pAL41 (Alonso *et al*, 1986) that produces an expected transcript size of 304 bases. Due to the high abundance of the  $\beta$ -actin transcript, the probe was synthesized at a lower specific activity.

T7 Amplimer Sequence	Length (mer)	Gene	Protected fragment (base pairs)	Cycles	Cycle Conditions
5' TAATACGACTCACTATAGGGAGGCCCTGCTTGGTGCTGATTTGTGAACC 3' (T7)	49		542 bp	20	94 °C for 1 minute
5' GGTTCACAAATCAGCACCAAGCAGG 3' (antisense)	25	HIF-IA	position 161-681	30	55 °C for 2 minutes 72 °C for 3 minutes
5' ATGCCTCCACAGAGGCTATGCC 3' (sense)	22				
5' GCACTAAGGAACGTCTGTAGGC 3' (antisense)	22	VEGF	464 bp (441 protected) position 779-1220	30	95 °C for 1 minute 61 °C for 1.5 minutes 72 °C for 1.5 minutes
a) 5' TAATACGACTCACTATAGGGAGGGCACTAAGGAACGTCTGTAGGC 3' (T7)	45				
b) 5' TAATACGACTCACTATAGGGAGGCGGTATGGTGTCCTAAGGC 3' (T7)					
5' GCTCCACTGAACAAGTTGGCC 3' (sense)	21		463 bp		95 °C for 1 minute
5' CCTCTACCTAACCAGCTGCC 3' (antisense)		p53	(440 bp protected) position 244-684	30	60°C for 1.5 minutes 72 °C for 1.5 minutes
			-		72 C Ioi 1.5 minutos
					1 <sup>st</sup> round (amplimers Table X)
5' TAATACGACTCACTATAGGGAGGGCCGTTGCAGTGGTTGCAACCC 3' (T7)	45				95 °C for 1 minute 56 °C for 1.5 minutes
		GLUT-1	548 bp (525 bp protected)	30	72 °C for 1.5 minutes
5' GCCGTTGCAGTGGTTGCAACCC 3' (antisense)			position 2106-2631		2 <sup>m</sup> round (T7 amplimers)
					95 °C for 1 minute
					72 °C for 1.5 minutes

 Table 2.10.
 T7 amplimer sequences and reaction conditions for RPA probe synthesis.

Note: All PCR cycle conditions included a Hot Start of 94°C for 4 minutes and all reactions had a total reaction volume of 50  $\mu$ l using 1.5 mM MgCl<sub>2</sub>. First round PCR reactions amplimers and cycle conditions are listed in **Table 2.7**. T7 amplimers were used with first round sense or antisense amplimers and 1  $\mu$ l of the first round product.

#### 2.10.6.2 Transcription

Amplified PCR product was added directly to the transcription reaction without purification. Either 5 µl of a 50 µl PCR reaction volume, or a 5 µl resuspended volume from 10 µl of a 50 µl ethanol precipitated PCR reaction volume (corresponding to approximately 1 µg of DNA), was combined with 2 µl of 10 X Transcription buffer (containing DTT and spermidine), 1 µl each of 10 mM ATP, CTP and GTP solutions, 1µl of a 1:100 diluted solution of 10 mM UTP, 2.5  $\mu$ l of  $\alpha^{32}$ P UTP (30 TBq/mmol, 800 Ci/mmol, 10 mCi/ml, Amersham Pharmacia Biotech, Little Chalfont, UK), 2µl of T7 RNA polymerase (5 U/ $\mu$ l) and sufficient water to produce a final reaction volume of 20  $\mu$ l in a sterile 1.5 ml micro-centrifuge tube. The solution was mixed by pipetting up and down, centrifuged at 13000 rpm for 30 seconds and incubated at 37 °C in a waterbath for 1 hour. Next, 1  $\mu$ l of RNase free DNase I (2 U/ $\mu$ l) was added to the solution, mixed by flicking and incubated at 37 °C for a further 15 minutes after which 1 µl of 0.5 M EDTA was added to the tube to terminate the reaction. An equal volume (20 µl) of Gel Loading Buffer was added to the labelled probe, the tube was vortexed briefly, heated at 92 °C for 5 minutes in a waterbath (Grant, Cambridge, UK) then loaded onto a 5 % polyacrylamide 8 M urea gel, purified and eluted (section 2.10.5.5).

The  $\beta$ -actin probe was synthesised as in the above 20 µl transcription reaction with the exception of having utilising 1 µl of 1 mM UTP (50 µM) and 1.6 µl of  $\alpha^{32}$ P UTP (1µM). A Century<sup>TM</sup> RNA molecular weight marker (100-500 MW, AMS Biotechnology Ltd., Oxfordshire, UK) was labelled as described above in a 20 µl transcription reaction volume with the exception of having used 1 µl of 10 mM UTP.

#### 2.10.6.3 Hybridisation

For each sample, 10  $\mu$ g of total RNA was mixed with approximately 500 pg of radiolabelled probe (1-4  $\mu$ l) in a sterile 1.5 ml micro-centrifuge tube. A 1/10<sup>th</sup> volume of 5M ammonium acetate and 2.5 volumes of 100 % ice-cold ethanol were added to each tube, briefly vortexed, centrifuged at 13000 rpm for 30 seconds and incubated at -20 °C for a minimum of 15 minutes. Precipitated RNA and probe were then centrifuged at 13000 rpm for 15 minutes at 4 °C, the supernatant drawn off using a pipette and the pellet resuspended in 20  $\mu$ l of hybridisation buffer (80 % formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, 1 mM EDTA) by vortexing. The tubes were then centrifuged at 13000 rpm for 30 seconds prior to incubating in a waterbath at 42 °C for 12–16 hours.

### 2.10.6.3 RNase Digestion

Following hybridisation, 200  $\mu$ l of RNase A/ RNase T1 mix was added to each sample, mixed by vortexing and centrifuged at 13000 rpm for 30 seconds. The samples were then incubated at 37 °C for 30 minutes in a waterbath to digest any unprotected single stranded RNA. Next, 300 $\mu$ l of RNase inactivation/precipitation solution was added to each sample, mixed by vortexing, centrifuged at 13000 rpm for 30 seconds and incubated at -20 °C for at least 15 minutes to precipitate out protected fragments. Samples were then centrifuged at 13000 rpm for 15 minutes at 4 °C, the supernatant decanted and resuspended in 8  $\mu$ l of Gel Loading Buffer II with vigorous vortexing for 1 minute. The samples were then heated at 94 °C for 4 minutes in a waterbath (Grant, Cambridge, UK), vortexed briefly, centrifuged at 13000 rpm for 30 seconds, then separated on a 5 % polyacrylamide 8 M urea gel.

#### 2.10.6.4 Denaturing Polyacrylamide Gel Electrophoresis

Samples mixed with gel loading buffer were loaded onto a vertical 0.75 mm 5 % polyacrylamide 8 M urea gel run in 1 X Tris-Boric Acid (TBE) in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hemel Hempstead, UK), which consisted of a electrophoresis unit with clamps which holds a gel sandwich unit, glass plates, casting stand and buffer tank. Two glass sandwich plates, both 9 cm X 10 cm with one containing 0.75 mm spacers, were washed and dried with 70% industrial methylated spirit (IMS). The glass plates were clamped together and secured in a gel-casting tray. A 5 % acrylamide/8 M urea gel was prepared by mixing 4.8 g of urea, 1 ml of 10 X TBE, 1.25 ml of 40 % acrylamide with 7.75 ml of sterile water in a 100 ml conical flask. The solution was mixed with gentle heating on a Jenway 1000 stirrer and hotplate (Jencons-PLS, Buzzard Leighton, UK) for at least 1 hour prior to adding 80 µl of 10 % ammonium persulfate and 10 µl of TEMED. The gel solution was then immediately poured into a gel sandwich unit using a 10 ml syringe, a 10 well plastic comb was inserted into the top of the gel carefully avoiding any air bubbles and the gel was left to polymerise at room temperature for at least 30 minutes. The set gel was then removed from the casting stand and clamped to the electrophoresis cell creating a buffer chamber which was filled with 1 X TBE. The comb was gently removed from the gel, the wells rinsed with 1 X TBE, the buffer tanked was filled to approximately 3 cm above the electrophoresis cell and gel assembly with 1 X TBE. Samples mixed with gel loading buffer and heated were then loaded onto the gel and electrophoresis was performed at 100-200 volts for 40 minutes to 1 hour until the dye front reached the bottom of the gel. The gel apparatus was then disassembled and the gel plates gently pried apart leaving the gel attached to one of the gel plates which was then laid onto a piece of 3MM filter paper (Amersham Pharmacia Biotech, Little Chalfont, UK). The gel and filter paper where covered in Saran Wrap<sup>™</sup> and placed in a Cronex autoradiography cassette containing lighting and intensifying screens. In a dark room, a piece of Kodak X-

OMAT<sup>TM</sup> LS X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the gel and the cassette was firmly closed. The cassette was placed at --80 °C where the film was exposed for at least 24 hours. The film was then developed in Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stop-bath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried.

## 2.10.6.5 Gel Purification of Probe

Following electrophoresis, the gel apparatus was disassembled and the gel plates gently pried apart leaving the gel attached to one of the gel plates that was then wrapped in SaranWrap<sup>™</sup> and placed in a Cronex autoradiography cassette containing lighting and intensifying screens. In a dark room, a piece of Kodak X-OMAT<sup>™</sup> LS X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the gel, secured with masking tape and an outline of the gel was traced onto the film with a felt permanent marker pen. The cassette was firmly closed and the film exposed for 10-30 seconds. The film was then developed in Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stop-bath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried. The film was used to precisely locate the area of the gel containing the full-length transcript, the slowest migrating and most intense band, by aligning it with the gel and tracing the area on the gel. The transcript was then excised with a scalpel, placed into a sterile 1.5 ml micro-centrifuge tube containing 350 µl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1 % SDS) and incubated in a waterbath at 37 °C for 12-16 hours allowing approximately 95 % recovery of the labelled probe from the gel. The eluted probe was then labelled and stored at -70 °C.

## 2.10.6.6 Quantitation of mRNA Gene Expression

Signal band intensities were quantitated by densiometric scanning of the autoradiographs using a Fluor-S<sup>TM</sup> imager and Multi-Analyst<sup>®</sup> image analysis software (Bio-Rad Laboratories, Hemel Hempstead, UK). Background intensity was subtracted from band intensity values. Results are expressed as fold increase from control levels or as the ratio of intensity of the expression of the gene of interest compared to the housekeeping gene  $\beta$ -actin. Statistical significance was assessed on the data groups using analysis of variance (ANOVA) followed by F-test and the Student's *t*-test for comparison of means with values of p<0.5 considered as significant.

# 2.11 Protein Methodologies

## 2.11.1 Lactate Assay

Lactate acid is a by-product of carbohydrate metabolism, where it is oxidised to pyruvate to produce energy. Measurement of lactate acid in cell culture supernatants can be used to indicate changes in cell glycolysis. Lactate acid production in cultures cells was measured using a Sigma Diagnostics Lactate kit (Sigma Chemicals, Poole, UK). The assay is based on an enzymatic reaction where the addition of excess nicotinamide adenine dinucleotide (NAD) and lactate dehydrogenase (LD) to the sample of interest produces a catalytic reaction where lactate is then measured spectrophotometrically at 340 nm in terms of the increased generation of reduced nicotinamide adenine dinucleotide (NADH). Formed pyruvate and hydrazine become trapped which them force the reaction to completion. An increased absorbance reading at 340 nm due to the increased formation of NADH becomes the measurement of any lactate originally present.

## 2.11.1.1 Deproteinisation

Cell culture supernatants were first deproteinised by mixing 0.2 ml of supernatant sample with 0.4 ml of 10 % Trichloroacetic acid (TCA) in a sterile 1.5 ml micro-centrifuge tube by vortexing for 30 seconds. The samples were incubated on ice for 5 minutes, centrifuged at 1500 X g at 4 °C for 10 minutes. The supernatant was then transferred to a sterile 1.5 ml micro-centrifuge tube ready for use.

#### 2.11.1.2 Measurement of Lactic Acid

The appropriate number of vials containing purified NAD (1 ml of NAD/LD solution required per sample tested) were each resuspended in 2 ml of glycine buffer, 4 ml of sterile water and 0.1 ml of LD by inverting several times. Following this, 1 ml of the NAD/LD solution was added to 50  $\mu$ l of each sample in sterile 1.5 ml micro-centrifuge tubes, mixed by vortexing and incubated at 37 °C in a waterbath (Grant, Cambridge, UK) for 15 minutes. A blank for the spectrophotometer was prepared by mixing with 1 ml of NAD/LD solution and incubating at 37 °C in a waterbath for 15 minutes. Absorbance readings at 340 nm were then measured for each sample against a blank of 50  $\mu$ l of 10 % TCA mixed with 1 ml of the NAD/LD solution incubated at 37 °C for 15 minutes.

#### 2.11.1.3 Quantitation of Lactate

A lactate calibration curve was prepared to access the linearity of the spectrophotometer and cuvettes used in the assay. The Lactate Standard Solution (40 mg/dL) was diluted 1:10 by adding 1 ml of Lactate Standard to a 15 ml sterile tube containing 9 ml of sterile water. An appropriate number of NAD vials were reconstituted as described above with the exception of the addition of 0.23 ml of water to each vial, as opposed to 4 ml. Lactate standards ranging from 0-13.32 mmol/L were prepared by serial dilution from the diluted Lactate Standard (0.04 mg/ml) in 10 ml borosciliate glass test tubes (Fischer Scientific, Loughborough, UK), (**Table 2.11**). The tubes were mixed well and incubated at 37 °C in a waterbath (Grant, Cambridge, UK) for 15 minutes. Next, 1 ml of each sample was transferred to a disposable cuvette and the absorbance read at 340 nm using standard 1 as a reference blank. The absorbance values versus corresponding lactate concentrations were then plotted. Quantities of lactate in each sample, in millimoles per litre (mmol/L), were determined using the following equation:

 $(A_{340} \times RV) / (6.22 \times SV \times 1)$ 

Table 2.11.	Preparation of lactate standards for a lactate calibration curve from a 0.04
	mg/ml stock standard (Sigma Chemicals Ltd., Poole, UK) by serial dilution.

Standard	Volume of water (ml)	Volume of lactate diluted standard (0.04 mg/ml) (ml)	Volume of prepared NAD solution (ml)	Final lactate concentration (mmol/L)
1	2.0	0	1.0	0
2	1.9	0.1	1.0	1.33
3	1.7	0.3	1.0	4.00
4	1.5	0.5	1.0	6.66
5	1.2	0.8	1.0	10.66
6	1.0	1.0	1.0	13.32

where  $A_{340}$  is the final absorbance at 340 nm, RV is the reaction volume in millilitres, 6.22 is the millimolar absorptivity of NADH at 340 nm, SV is the sample volume in the cuvette and 1 is the lightpath in centimetres. To determine lactate quantities in milligrams per decilitre (mg/dL), the above equation was multiplied by 90, the molar weight of lactic acid, and divided by 10, the conversion of results in terms of 1 litre to 100 ml.

## 2.11.2 Quantitation of Protein

Protein concentrations were determined using the Coomassie<sup>®</sup> Plus Protein Assay reagent Kit (Perbio Science Ltd., Chester, UK), a dye-binding colourimetric method based on the Bradford method of total protein quantitation (Bradford, 1976). When the Coomassie<sup>®</sup> binds protein in an acidic medium, an immediate absorbance shift occurs from 465 nm to 595 nm with a simultaneous colour change of the reagent from red/brown to blue. The absorbance reading of each sample at 595 nm can then be compared against a BSA protein standard curve to determine the total protein concentration.

Protein standards ranging from 0.025-2.0 mg/ml were prepared by serial dilution from a 2 mg/ml bovine serum albumin (BSA) stock standard with sterile water in 10 ml borosciliate glass test tubes (Fischer Scientific, Loughborough, UK), (**Table 2.12**). A 50 µl aliquot of each standard and 5-50 µl of each unknown sample were pipetted into appropriately labelled 10 ml borosciliate glass test tubes. Sample volumes of less than 50 µl were completed with sufficient sterile water. A blanking tube was prepared using 50 µl of sterile water. Next, 1.5 ml of Coomassie<sup>®</sup>Plus dye reagent was added to each tube and mixed by vortexing. A 1 ml aliquot of the mixed sample and dye solution was added to a disposable polystyrene cuvette (Sigma Chemicals Ltd., Poole, UK), which was then placed into a fixed wavelength WPA UV1101 Biotech Photometer (Jencons-PLS, Buzzard Leighton,

 Table 2.12. Preparation of diluted BSA standards for protein curve from a 2 mg/ml stock standard (Perbio Science Ltd., Chester, UK) by serial dilution.

Volume of BSA (µl)	Volume of water (µl)	Final BSA concentration (mg/ml)	Sample ID
300 µl of stock	0 µl	2.0 mg/ml	~
375 μl of stock	125 μl	1.5 mg/ml	A
325 µl of stock	325 μl	1.0 mg/ml	В
175 µl of sample A	175 μl	0.75 mg/ml	С
325 µl of sample B	325 μl	0.50 mg/ml	D
325 μl of sample D	325 μl	0.25 mg/ml	E
325 µl of sample E	325 μl	0.125 mg/ml	F
100 µl of sample F	400 µl	0.025 mg/ml	G

UK) previously blanked with water, and the absorbance at 595 nm was measured for each tube. A standard curve was then prepared by plotting the absorbance reading at 595 nm for each BSA standard versus its concentration. Using the standard curve, the protein concentration was then determined for each unknown sample considering any dilution factor.

# 2.11.3 Electrophoretic Mobility Shift Assay (EMSA)

The Electrophoretic Mobility Shift Assay (EMSA) is used to detect the interaction of DNA binding proteins with their cognate DNA recognition sequences, in both a qualitative and quantitative manner. Purified proteins or crude cell extracts are incubated with a <sup>32</sup>P radiolabelled DNA probe containing a puntative protein-binding site. The reaction complexes are then separated from the free probe through a nondenaturing polyacrylamide gel. The DNA-protein complexes migrate more slowly than the unbound probe. The specificity of the DNA binding protein for the puntative binding site can be determined with competition experiments using oligonucleotides containing a binding site for the protein of interest (specific competitor) or another unrelated sequence (non-specific competitor). Specific interactions can then be identified by the differences in the nature and intensity of the complex formed in the presence of either competitor.

## 2.11.3.1 Preparation of Nuclear Extract

Two separate methods were used in the preparation of crude nuclear extract from peripheral blood mononuclear cells (PBMCs) (section 2.5.3), and established breast carcinoma cell lines (section 2.5.1).

#### 2.11.3.1.1 Dignam, Lebovitz and Roeder, 1983

Nuclear extracts were prepared with Dignam Buffers A and C containing 0.5 mM AEBSF, 0.184 mg/ml sodium orthovanadate, 0.42 mg/ml sodium fluoride and 2.2 µg/ml aprotonin. After exposure to 1% O<sub>2</sub> or 21% O<sub>2</sub> for predetermined time intervals, adherent cells were washed twice by adding 5 ml of PBS directly to the 75 cm<sup>2</sup> flask and aspirating off. A 0.5 ml aliquot of Dignam Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.2 % NP-40) was added to the flask and the cell lysate was collected to a corner of the flask using a sterile cell scrapper. Cells grown under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>) were also processed in the same manner as a control. PBMC suspensions were collected into 50 ml sterile tubes, centrifuged at 1400 rpm for 4 minutes and the supernatant decanted. A 0.5 ml aliquot of Dignam Buffer A was added to the 50 ml tube and resuspended by pipetting up and down. Cells were collected with a 1 ml pipette, placed into a sterile 1.5 ml micro-centrifuge tube, incubated on ice for 20 minutes and then centrifuged at room temperature at 13000 rpm for 15 minutes. The supernatant was transferred to a sterile 1.5 ml micro-centrifuge tube, labelled and stored at -70 °C as the cytoplasmic protein. The remaining pellet was then resuspended in 50 µl of Dignam Buffer C (20mM HEPES, pH 7.9, 25 % glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.2 mM EDTA) with pipetting and incubated on ice for 10 minutes with occasional mixing. The solution was then centrifuged at room temperature at 13000 rpm for 1 minute. The supernatant was then transferred to a sterile 1.5 ml micro-centrifuge tube, labelled and stored at -70 °C as the crude nuclear extract.

#### 2.11.3.1.2 Semenza and Wang, 1992

Nuclear extracts were prepared with Buffers A and C containing 0.5 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 2  $\mu$ g/ml of leupeptin, 2 $\mu$ g/ml of aprotinin, 2  $\mu$ g/ml of pepstatin and 1 mM sodium vanadate. After exposure to 1% O<sub>2</sub> or

21% O<sub>2</sub> for predetermined time intervals, adherent cells were washed twice by adding 5 ml of PBS directly to the 75 cm<sup>2</sup> flask and aspirating off. Cells grown under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>) were also processed in the same manner as a control. An additional 5 ml of PBS was then added to the flask and cells were scraped off using a sterile cell scrapper. Cells were collected with a 1 ml pipette, placed into a sterile 15 ml tube and centrifuged in a Mistral 1000 centrifuge (MSE Scientific Instruments, Leicester, UK) at 1500 rpm at room temperature for 5 minutes. The supernatant was decanted and the pellet was washed once with 4 packed cell volumes (PCV) of Buffer A (10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl), centrifuged at 1500 rpm for 2 minutes, the supernatant decanted and the pellet resuspended in 4 PCVs of Buffer A by vortexing and then incubated on ice for 10 minutes. Cells grown under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>) were also processed in the same manner as a control. PBMC suspensions were collected into 50 ml sterile tubes, centrifuged at 1400 rpm for 4 minutes and the supernatant decanted. The pellet was washed once with 4 PCV of Buffer A, centrifuged at 1500 rpm for 2 minutes, the supernatant decanted and the pellet resuspended in 4 PCVs of Buffer A by vortexing and then incubated on ice for 10 minutes. The cell suspension was then homogenised with a type B pestle of Dounce homogeniser (Wheaton Science Products, Millville, N.J., USA) using 10 strokes. The homogenate was then transferred to a 1.5 ml sterile micro-centrifuge tube and centrifuged at 3500 rpm at 4 °C for 5 minutes. The supernatant was then decanted and the pellet was resuspended in 2 PCVs of Buffer C (20 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 420 mM KCl, 20 % glycerol), and mixed on a Luckham R100/TW Rotatest shaker on ice for 30 minutes. The nuclear debris was then pelleted by centrifugation at 13500 rpm at 4 °C for 30 minutes. The supernatant was transferred to dialysis tubing (Sigma Chemicals Ltd, Poole, UK) and dialysed against 500 ml of Buffer D (20 mM Tris-HCl, pH 7.8, 100 mM KCL, 0.2 mM EDTA, 20 % glycerol) at 4 °C for 4 hours, changing the dialysis buffer once at mid dialysis. The

dialysate was then collected and transferred to a sterile 1.5 ml micro-centrifuge tube and centrifuged at 13500 rpm at 4 °C for 10 minutes. The supernatant was then transferred to another sterile 1.5 ml micro-centrifuge tube and snap-frozen in liquid nitrogen. The nuclear extracts were then stored at -70 °C.

## 2.11.3.1.2.1 Preparation of Dialysis Tubing

Glycerin was removed from the dialysis tubing by washing it in running water for 3-4 hours. Sulphur compounds were removed by treating the tubing with 0.3 % (w/v) sodium sulfide at 80 °C for 1 minute. The tubing was then washed with hot water (60 °C) for 2 minutes then transferred to a solution of 0.2% (v/v) sulphuric acid. The tubing was then rinsed with hot water to remove the acid and stored in a 500 ml Duran bottle in distilled water.

### 2.11.3.2 Oligonucleotide Probe Labelling

The EMSA was performed using a 5' end labelled HIF-1 $\alpha$  coding sense strand oligonucleotide sequence, 5' AGCTTGCCCTACGTGCTGTCTCAG 3' (HIF-1 binding site underlined), which corresponded to nucleotides 1-18 of the erythropoiesis (EPO) enhancer (highlighted). A 2  $\mu$ l aliquot of HIF-1 $\alpha$  sense oligonucleotide (1.75 pmol/ $\mu$ l) was added to a 0.2 ml sterile micro-centrifuge tube containing 1 µl of T4 polynucleotide kinase 10X buffer (Promega, Southampton,UK), 1  $\mu$ l of  $\gamma^{32}$ P ATP (3000 Ci/mmol at 10 mCi/ml), 5µl of sterile water and 1 µl of T4 polynucleotide kinase. The labelling reaction was incubated at 37 °C for 10 minutes in a waterbath (Grant, Cambridge, UK), the reaction stopped with the addition of 1  $\mu$ l of 0.5 M EDTA and then diluted with 89  $\mu$ l of TE buffer, pH 8. The radiolabelled oligonucleotide HIF-1 probe was then annealed to a 6 fold molar excess of cold unlabelled HIF-1 antisense strand oligonucleotide 5' ACGGGATGCACGACAGAGTCTTAA 3' and used as a DNA consensus probe in subsequent binding reactions. Probe annealing was performed by mixing a 50  $\mu$ l aliquot of the HIF-1 radiolabelled sense oligonucleotide in a 0.2 ml sterile thin-walled microcentrifuge tube with 10  $\mu$ l of unlabelled HIF-1 antisense oligonucleotide (1.75 pmol/ $\mu$ l) and incubating the sample at 70 °C for 5 minutes in a Cyclogene thermocycler (Techne Ltd., Cambridge, UK). The solution was then left at room temperature to cool overnight. The consensus probe was then stored at -80 °C.

### 2.11.3.3 Binding Reaction

A 15  $\mu$ g sample of nuclear protein extract was added to a 0.2 ml sterile micro-centrifuge tube containing 2  $\mu$ l of binding buffer. Sufficient sterile water was added to make a final reaction volume of 10-20  $\mu$ l and the solution was then pre-incubated at room temperature for 10 minutes. Next, 1  $\mu$ l of labelled consensus probe was added to the reaction tube and the incubation continued for an additional 20 minutes. The samples were then mixed with 5 $\mu$ l of 10X gel loading buffer (250 mM Tris-HCl, pH 7.5, 0.2 % bromophenol blue, 40 % glycerol).

### 2.11.3.4 Non-Denaturing Gel Electrophoresis

Samples mixed with gel loading buffer were loaded onto a vertical 0.75 mm 4 % nondenaturing polyacrylamide gel run in 0.5 X Tris-Boric Acid (TBE) in a Protean II electrophoresis cell (Bio-Rad laboratories, Hemel Hempstead, UK), which consisted of a electrophoresis unit with clamps which holds a gel sandwich unit, glass plates, casting stand and buffer tank. Two glass sandwich plates, 20 cm X 20 cm and 16 X 20 cm, were washed and dried with 70% industrial methylated spirit (IMS). The plates were separated by 0.75 mm spacers, clamped together and secured in a gel-casting tray. A 4 % nondenaturing polyacrylamide gel was prepared by mixing 1 ml of 10 X TBE, 0.5 ml of 2 % bisacrylamide, 2 ml of 40 % acrylamide, 0.625 ml of 80 % glycerol with 15.9 ml of sterile water in a 100 ml conical flask. Next, 150 µl of ammonium persulfate and 10 µl of TEMED were added to the flask to polymerase the gel, the solution was mixed and then poured into the gel plate assembly along an edge of one of the spacers using a sterile 10 ml plastic pipette. A plastic 0.75 mm 14 well comb was inserted into the top of the gel, avoiding the formation of air bubbles, and the gel was left to polymerise for 1 hour at room temperature. The set gel was then removed from the casting tray and clamped to the electrophoresis cell creating a buffer chamber which was filled with 0.5 X TBE. The comb was gently removed from the gel, the wells rinsed with 0.5 X TBE, the buffer tanked was filled to approximately 3 cm above the electrophoresis cell and gel assembly with 0.5 X TBE and the unit was pre-run at 100 volts for 30 minutes. Samples mixed with gel loading buffer were then loaded onto the gel and electrophoresis was performed at 150 volts for 3-4 hours until the dye front reached the bottom of the gel. The gel apparatus was then disassembled and the gel plates gently pried apart leaving the gel attached to one of the gel plates which was then laid onto a piece of 3MM filer paper (Amersham Pharmacia Biotech, Little Chalfont, UK). The gel and filter paper where covered in Saran Wrap<sup>™</sup> and placed in a Cronex autoradiography cassette containing lighting and intensifying screens. In a dark room, a piece of Kodak XLS5 X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the gel and the cassette was firmly closed. The cassette was placed at -80 °C where the film was exposed for at least 24 hours. The film was then developed in Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stop-bath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried.

### 2.11.3.5 Competition and Supershift Assays

Competition assays were performed by pre-incubating a 10 fold excess of unlabelled HIF-1 antisense oligonucleotide with nuclear extract and binding buffer for 30 minutes at room temperature prior to the addition of the HIF-1 labelled consensus probe. Supershift assays were performed by pre-incubating 1-3  $\mu$ l of HIF-1 $\alpha$  monoclonal IgG 2b antibody (clone H1 $\alpha$ 67, 2 separate lots from Balb/c mouse ascites, one lot from cell max culture supernatant, Abcam, Cambridge, UK) with the nuclear extract and binding buffer mixture at either room temperature or on ice for 30 minutes prior to the addition of the HIF-1 labelled consensus probe.

#### 2.11.4 Western Blotting

#### **2.11.4.1 Preparation of Protein**

Protein samples were prepared as either whole cell lysates or as isolated cytoplasmic protein from peripheral blood mononuclear cells (PBMCs) (section 2.5.3), and established breast carcinoma cell lines (section 2.5.1).

### 2.11.4.1.1 Cytoplasmic Protein

Cytoplasmic protein was isolated from adherent cells and PBMCs exposed to  $1\% O_2$  or  $21\% O_2$  for predetermined time intervals as described above in the preparation of nuclear extracts (section... method of Dignam, Lebovitz and Roeder, 1983). Cells grown under normoxic conditions ( $21\% O_2$ ,  $5\% CO_2$ ,  $74\% N_2$ ) were also processed in the same manner. Sample protein concentrations were determined (section 2.11.2) and then stored in labelled sterile 1.5 ml micro-centrifuge tubes at -70 °C.

#### 2.11.4.1.2 Whole Cell Lysate

After exposure to 1%  $O_2$  or 21%  $O_2$  for predetermined time intervals, adherent cells were washed twice by adding 5 ml of PBS directly to the 75 cm<sup>2</sup> flask and aspirating off. An additional 1.5 ml of PBS was then added to the flask and cells were scraped off using a sterile cell scrapper. Cells were collected with a 1 ml pipette, placed into a sterile 1.5 ml micro-centrifuge tube and centrifuged at 7500 rpm at room temperature for 1 minute. The supernatant was decanted and the pellet was resuspended in 500 µl of Laemmli sample buffer. Cells grown under normoxic conditions (21%  $O_2$ , 5%  $CO_2$ , 74%  $N_2$ ) were also processed in the same manner. PBMC suspensions were collected into 50 ml sterile tubes, centrifuged at 1400 rpm for 4 minutes, the supernatant decanted and the pellet was resuspended in 500 µl of Laemmli sample buffer. The solution was then transferred to a sterile 1.5 ml micro-centrifuge tube. The resuspended cell suspensions were then sonicated for at least 1 minute until the lysate was homogenous. Sample protein concentrations were determined (section 2.11.2), the samples were labelled and stored at -70 °C.

## 2.11.4.2 SDS-Polyacrylamide Gel Electrophoresis

One-dimensional gel electrophoresis under denaturing conditions separates proteins based on molecular size as they move through a polyacrylamide gel matrix towards an anode. Sample proteins are solubilised by boiling in the presence of SDS and  $\beta$ -mercaptoethanol, which reduces disulfide bonds.

A Protean II electrophoresis cell (Bio-Rad laboratories, Hemel Hempstead, UK) and glass plate sandwich was assembled as for non-denaturing gel electrophoresis (section 2.11.3.4). SDS-denatured protein samples were electrophorised on a 6 % or 12 % SDSpolyacrylamide resolving gel topped with a 4 % stacking gel (see Table 2.13 for SDSpolyacrylamide gel recipes). The desired percentage of acrylamide in the resolving gel is

Table 2.13.	Recipes for the preparation of SDS-polyacrylamide gels used in Western
	blotting.

	Stacking Gel	Resolving Gel		
Reagents	4 %	6%	12 %	
0.5 M Tris-HCl. pH 6.8	6.3 ml	-	-	
1.5 M Tris-HCl, pH 8.8	-	10 mi	10 ml	
30% Arcylamide/ 0.8 % Bisacrylamide	3.3 ml	8 ml	16 ml	
10 % SDS	0.25 ml 0.4 m		0.4 ml	
Water	15.9 ml	21.2 ml	13.2 ml	
10 % (w/v) Ammonium Persulfate	0.125 ml	0.4 ml	0.4 ml	
TEMED	0.025 ml	0.032 ml	0.016 ml	
Total Volume	25 ml	40 ml	40 ml	

### 2.11.4.3 Transfer

Immediately following electrophoresis, the gel apparatus was disassembled and the gel plates gently pried apart leaving the gel attached to one of the gel plates. The stacking gel was removed from the resolving gel and any unused areas of the gel were cut away using a scalpel blade. One corner of the gel was removed to serve as an orientation point and the gel was then quickly rinsed in transfer buffer to achieve equilibration. The electroblotting cassette was assembled by first placing a fibre pad pre-soaked in transfer buffer (20 mM Tris, pH 8.0, 150 mM glycine, 20% methanol) on the panel of the gel holder, then 3 pieces of 3M filter paper cut to the dimensions of the gel and saturated in transfer buffer, followed by the gel which was carefully placed on top. A sheet of Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK), cut to the dimensions of the gel and soaked in transfer buffer for 1 hour prior to transfer, was carefully placed on top of the gel avoiding trapping any air bubbles beneath it. A further 3 pieces of sized and saturated 3M filter paper were placed on top of the membrane followed by a final presoaked fibre pad. The cassette was closed by securing the latch of gel holder and then placed between the electrodes in the Trans-Blot Cell (Bio-Rad Laboratories, Hemel Hempstead, UK) with the membrane facing the anode side. A cooling coil was inserted into the Trans-Blot Cell, the tank filled with transfer buffer, covered and the protein allowed to transfer at room temperature for 12-16 hours at 3 volts.

#### 2.11.4.5 Immunodetection

Following transfer, the Trans-Blot cell was disassembled and the membrane removed from the blotting cassette. The orientation of the gel was marked on the membrane, which was then quickly rinsed in transfer buffer prior to being placed in a solution of 5 % (w/v) nonfat dry milk in TBS-Tween (NFDM), to block non-specific sites on the membrane, and incubated at room temperature for 1.5 hours with gentle shaking on a Luckham R100/TW Rotatest shaker (Jencons-PLS, Buzzard Leighton, UK). After blocking, the membrane was incubated in primary antibody (see Table 2.14 for antibodies and working dilutions) diluted in NFDM for 1.5 hours at room temperature with gentle shaking or at 4 °C for 12-16 hours. The membrane was then washed 3 times (1 X 15 minutes, 2 X 10 minutes) in TBS-Tween (20 mM Tris-HCl, pH 7.6, 100mM NaCl, 0.1 % Tween-20) at room temperature for 35 minutes with gentle shaking. Following this, the membrane was then incubated in secondary antibody diluted in NFDM at room temperature for 1 hour with gentle shaking and subsequently washed 3 times (1 X 15 minutes, 2 X 10 minutes) in TBS-Tween (20 mM Tris-Hcl, pH 7.6, 100mM NaCl, 0.1 % Tween-20) at room temperature for 35 minutes with gentle shaking. The membrane was drained, placed on SaranWrap<sup>™</sup>, covered with SuperSignal<sup>®</sup> Chemiluminescent Substrate (SuperSignal<sup>®</sup> Chemiluminescent Substrate for Western Blotting, Perbio Science Ltd., Chester, UK) and incubated at room temperature for at least 15 minutes. The membrane was then drained and placed on a new piece of SaranWrap<sup>™</sup> where it was enclosed in the wrap and air bubbles removed. The wrapped membrane was then placed in a Cronex autoradiography cassette containing lighting and intensifying screens. In a dark room, a piece of Kodak XLS5 X-ray film (Scientific Imaging Systems Ltd., Cambridge, UK) was placed on top of the wrapped membrane and the cassette was firmly closed. The film was exposed repeatedly for an appropriate length of time, ranging form 1 to 30 minutes. The film was then developed in

Primary Antibody	Host	Supplier	Working Dilution	Secondary Antibody	Supplier	Working Dilution	Resolving Gel
Anti-HIF-1α Monoclonal IgG 2b	Cell Max System, culture supernatant	Abcam, (Cambridge, UK) Clone H1α67	1:500	Anti-mouse IgG, peroxidase conjugate	Sigma Chemicals Ltd. (Poole, UK)	1:2000	6 %
Anti-HIF-1α Monoclonal IgG 2b	Balb/c mouse	Abcam (Cambridge, UK) Clone H1α67	1:500	Anti-mouse IgG, peroxidase conjugate	Sigma Chemicals Ltd. (Poole, UK)	1:2000	6%
GLUT-1 polyclonal	Rabbit	Biogenesis (Poole, UK)	1:100	Anti-rabbit IgG, peroxidase conjugate	Sigma Chemicals Ltd. (Poole, UK)	1:2000	12 %

Table 2.14. Antibodies, working dilutions and SDS-polyacrylamide gel percentages used in Western blotting assays.

Note: All antibodies were prepared by dilution in TBS-Tween containing 5 % non-fat dry milk.

HIF-1 $\alpha$  = Hypoxia Inducible Factor-1 $\alpha$ 

GLUT-1 = Glucose Transporter 1

Kodak X-ray developer (Anachem, Luton, UK) for 3 minutes, placed in an indicator stopbath solution (3 % sulphuric acid) for 30 seconds, and fixed in a liquid fixer solution for at least 1 minute (Anachem, Luton, UK). The film was then rinsed for 1 minute under tap water and air-dried. Membranes were stored wet at 4 °C wrapped in SaranWrap<sup>™</sup>.

# 2.11.4.6 Membrane Stripping

Membranes can be stripped of bound antibodies and reprobed several times. Membranes were placed in stripping buffer (100 mM  $\beta$ -mercaptoethanol, 2 % SDS, 62.5 mM Tris, pH 6.7) and incubated at 56 °C for 30 minutes. The membrane was then washed at room temperature for 15 minutes in several changes of TBS-Tween.

## 2.11.4.7 Gel Staining

SDS-polyacrylamide gels were stained for 1 hour in Coomassie Blue staining solution (10 % acetic acid, 20 % methanol, 0.05 % (v/v) Coomassie brilliant blue R-250) with gentle shaking on a Luckham R100/TW Rotatest shaker (Jencons-PLS, Buzzard Leighton, UK) and destained in several changes of water to verify efficiency of protein transfer.

### 2.11.5 Immunocytochemistry

Immunocytochemistry was performed with the aid of the Histopathology Department at Derriford Hospital, Plymouth.

## 2.11.5.1 Hypoxia Inducible Factor-1α (HIF-1α)

Immunocytochemical detection of HIF-1a was performed on Glioblastoma and Breast tumour tissue using a DAKO<sup>®</sup> catalysed signal amplification (CSA) system (DAKO, Cambridgeshire, UK), which results in a greater amplification of signal allowing for the detection of small quantities of target antigen. First, 5µm sections of paraffin-embedded tissue were collected onto 3-aminopropyltriethoxysilane coated glass slides, placed in holding trays, dewaxed in 3 X 3 minute washes in xylene and subsequently rehydrated with 3 X 3 minute washes in graded alcohol, in Hellendahl jars. The slides were then rinsed in de-ionised water for 3 minutes. Antigen retrieval was performed by pre-treating the slides in a microwave oven for 25 minutes on full power in 10 mM citrate buffer, pH 6.0, followed by rinsing in de-ionised water. The slides were then blocked in a 3 % solution of hydrogen peroxide at room temperature for 5 minutes to quench endogenous peroxidase activity. The solution was then tapped off the slides and the tissue sections were covered with a few drops of Avidin D blocking solution and incubated at room temperature for 15 minutes. The slides were then rinsed in TBST (0.05 M Tris-HCl, pH 7.6, 0.3 M NaCl, 0.1 % Tween 20) and incubated at room temperature in biotin blocking solution for 15 minutes. The solution was tapped off the slides and HIF-1a monoclonal antibody (clone OZ12, Neomarkers, Fremont, USA), diluted 1:1000 in TBST, was applied and incubated 12-16 hours at room temperature. The sections were then washed 3 X 5 minutes in TBST and a link antibody (biotinylated rabbit anti-mouse immunoglobulins) was applied and the slides incubated at room temperature for 15 minutes. The slides were washed 3 X 5 minutes in TBST and incubated at room temperature in Streptavidin-Biotin complex for 15 minutes. The slides were washed 3 X 5 minutes TBST and then incubated at room temperature for 15 minutes in Amplification reagent (Biotinyl tyramide and hydrogen peroxide in PBS containing carrier protein). The slides were washed 3 X 5 minutes in TBST and then incubated in Streptavidin-peroxidase at room temperature for 15 minutes. The slides were again washed 3 X 5 minutes in TBST and then incubated in 3,3'diaminobenzidine (DAB) substrate-chromogen solution for 5 minutes. The slides were rinsed for 2 minutes in tap water, incubated in DAB enhancer solution (0.02 M copper sulphate, 0.12 M NaCl) for 2 minutes and subsequently rinsed again in tap water for 2 minutes. The slides were then conterstained in 10 % haematoxylin for 20 seconds, rinsed thoroughly in tap water, dipped in acid alcohol (10 % HCl, 70 % Industrial Methylated Spirits (IMS)) for 3 seconds and washed in Scott's Tap Water substitute (0.16 M magnesium sulphate, 0.017 sodium hydrogen carbohydrate). Slides were then dehydrated by washing 3 X 1 minute in alcohol followed by 3 X 1 minute in xylene. The slides were mounted with cover slips in a Tissue-Tek coverslipping machine. A negative control consisted of a slide containing sample tissue incubated in TBST followed through the same procedure. A positive control consisted of an additional slide of sample tissue known to stain positive in optimisation assays.

## 2.11.5.2 Vascular Endothelial Growth Factor (VEGF)

Immunocytochemistry was performed as for HIF-1 $\alpha$  without the use of the catalysed signal amplification (CSA) system, using an antibody to Vascular Endothelial growth Factor (AB-5, CN Biosciences, Nottingham, UK). Initial slide dewaxing, rehydrating blocking were performed as above. Antigen retrieval was performed by pre-treating the slides in a microwave oven for 30 minutes on full power in 1 mM EDTA, pH 8.0, followed by rinsing in de-ionised water. Slides were incubated with primary antibody diluted 1:350 in TBST at room temperature for 12-16 hours. Washes consisted of 2 X 5 minutes in

TBST. Both secondary and tertiary antibodies were applied at a dilution of 1:50 and incubated at room temperature for 30 minutes. Slides were incubated in DAB substratechromogen solution, enhanced, counterstained, dehydrated and mounted as described above. A negative control consisted of a slide containing sample tissue incubated in TBST followed through the same procedure. A positive control consisted of an additional slide of sample tissue known to stain positive in optimisation assays.

# 2.11.5.3 p53

Immunocytochemistry was performed as for HIF-1 $\alpha$  without the use the catalysed signal amplification (CSA) system, using primary antibodies to p53 (polyclonal CM1, Novacastra, Newcastle Upon Tyne, UK). Slides were blocked in normal goat serum prior to incubation with primary antibody diluted 1:64000 in TBST at room temperature for 12-16 hours. All further procedures were performed as described above.

# 2.12 Statistical Analysis

## 2.12.1 Allele and Genotype Frequencies

Allele frequencies were determined as the percentage of the total number of copies of individual alleles present in the populations studied. Genotype frequencies were determined as the percentage of the total number of each particular genotype found in the populations studied. Comparison of allele and genotype frequencies between patients and controls were determined using the  $\chi^2$  test with contingency tables. The  $\chi^2$  value was calculated using the following equation:

 $[Observed (n) - Expected (n)]^2 / Expected (n)$ 

where (n) represents the number of observations. Some  $\chi^2$  values were determined using a Statcalc program found in the statistical program Epi Info 6 (Epiglue 1.0; World Health Organisation, Geneva, Switzerland). The p values were then corrected (pc) for the number of variables using the Bonferroni inequality method and were considered significant when pc<0.05 (Bonferroni, 1936; Thompson, 1941).

# 2.12.2 Hardy-Weinberg Equilibrium

The Hardy-Weinberg Equilibrium test is used to determine whether a bi-allelic polymorphism follows a normal distribution within a population and is represented by the following equation:

$$p^2 + 2(pq) + q^2 = 1$$

where p and q represent the frequencies (f) of each allele within the population. The Hardy-Weinberg principle (Hardy, 1908) states the allele frequencies remain constant from generation to generation thus Hardy-Weinberg equilibrium occurs if the frequency is not significantly different from the expected frequency using a p value <0.05.

#### 2.12.3 Odds Ratio

The Odds Ratio is used to determine the odds of developing a disease condition based on the number of individuals with a particular genotype within a population versus a control population. It is represented by the following equation:

#### (pqA X cB/(pqB X cA))

where pqA represents the number of individuals with the genotype of interest in the sample population, pqB represents the number of individuals in the same population without the genotype of interest, cA represents the number of individuals in a control population with the genotype of interest, and cB represents the number of individuals in the same control population without the genotype of interest.

2

#### 2.12.4 Association Analysis

Statistically significant associations between independent samples were determined using the statistical package SPSS (version 9.0.0; SPSS Inc., Chicago, USA) using nonparametric Mann-Whitney U, Kruskal-Wallis H or Spearman's coefficient of correlation tests. Analysis of variance (ANOVA) was also performed followed by the F-test and the Student's *t*-test for comparison of means with values of p<0.05 considered as significant.

#### 2.12.5 Survival Analysis

Statistical analyses were performed using the statistical program SPSS (version 9.0.0; SPSS Inc., Chicago, USA). Univariate analysis of overall survival was performed as outlined by Kaplan and Meier (Kaplan & Meier, 1985), where the log-rank test was used to determine significant differences between these. The cox proportional hazards model was used for multivariate analysis, although statistical power was limited due to relatively low number of cases in each study. Multivariate analysis was performed using a backward elimination procedure to remove variables with  $p \ge 0.10$ . The qualifying criteria for
inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis. Hazard ratios and p values are noted for each variable in multivariate analysis. For all tests, a p value of less than or equal to 0.05 was considered as significant. All p values given are results of two-sided tests.

#### 2.12.6 Nottingham Prognostic Index (NPI)

The NPI is a prognostic index based on multiple factors and is used to predict overall survival in patents with breast cancer (Haybittle *et al*, 1982). The factors include tumour size, histological grade and lymph node stage, all of which are used to create an index predicting survival:

 $NPI = size (cm) \times 0.2 + Grade (1-3) + Node stage (1-3)$ 

where grades 1 to 3 define well, moderate or poor differentiation, and node stages 1 to 3 define no nodal involvement, involvement of up to 3 nodes, or involvement of 4 or more nodes. The higher the value for NPI the worse the prognosis.

### Chapter 3.

## HIF-1α, GLUT-1 and p53 Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose

#### 3.0 HIF-1α, GLUT-1 and p53 Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose

Hyperglycaemia is known to cause oxidative and reductive stress and it has been proposed that this will ultimately lead to a state of redox imbalance within the cell. It has been suggested that hyperglycaemia-induced redox imbalance mimics the effects of hypoxia (Williamson *et al*, 1993). GLUT-1 is responsible for basal glucose transport and was the first in the family of glucose transporters to be cloned (Meuckler *et al*, 1985). Under hypoxic conditions a cell must undergo metabolic adaptations to survive which includes an increase in glucose uptake facilitated by upregulation of *GLUT-1* mRNA expression. Hypoxia is a potent stimulus for *GLUT-1* mRNA induction in a variety of tissue types including various tumour cell lines (Ebert *et al*, 1995) and in a variety of human cancers (Younes *et al*, 1996).

Presently little is known about the hyperglycaemia-induced expression of HIF-1 $\alpha$ in breast carcinoma cell lines. The expression profiles of both the *HIF-1* $\alpha$  and *GLUT-1* genes under hypoxic and hyperglycaemic conditions may offer a possible explanation as to how cancer cells behave under conditions of extreme stress. Further information about HIF-1 DNA binding activity and HIF-1 $\alpha$  and GLUT-1 protein expression levels will also offer additional insight. Thus, one of the aims of this study was to quantitate the expression of *HIF-1* $\alpha$  and *GLUT-1* mRNA in breast carcinoma cell lines exposed to hypoxia and high glucose.

It is known that HIF-1 $\alpha$  expression is induced by exposure to hypoxia or treatment with cobalt chloride (Wang *et al*, 1995), a hypoxia-mimicking agent. HIF-1 $\alpha$ , VEGF, p53and  $\beta$ -actin expression were first studied using Northern blotting, in breast carcinoma cells treated with varying concentrations of CoCl<sub>2</sub> (60 mM to 500 mM) for up to 24 hours, to determine whether HIF-1 $\alpha$  expression could be induced in these cells. CoCl<sub>2</sub> has been used previously to induce HIF-1 in Hep3B cells (Semenza *et al*, 1994; Wang and Semenza, 1993). Probes for each gene were created by PCR amplification of T47D cell genomic DNA, were radiolabelled and then hybridised to blots containing 50 to 125  $\mu$ g of mRNA.

Subsequently, the expression of HIF-1 $\alpha$ , p53 and GLUT-1 mRNA was quantitated in cell lines exposed to hypoxia, high glucose, both hypoxia and high glucose, and subsequently normoxia, using the RPA. A probe for HIF-1 $\alpha$  was amplified via RT-PCR from cDNA synthesised from total RNA isolated from the T47D breast carcinoma cell line exposed to hypoxia (<1% O<sub>2</sub>) for 24 hours. Probes for p53 and GLUT-1 were amplified as above from genomic DNA but without exposure to hypoxia.

Four individual breast carcinoma epithelial cell lines (T47D, MCF7, SKBR3, ZR75) were cultured in their recommended tissue culture media at normal glucose levels (no supplement), moderate glucose (supplemented with 10 mM D-glucose) or high glucose (supplemented with 20 mM glucose). For hypoxic conditions, cells were exposed to  $1\% O_2$  (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37° C CO<sub>2</sub>/O<sub>2</sub> incubator for 2, 4, 6, 8, 24 and 48 hours. An aliquot of cells was also exposed to normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub>) for the same period of time. The cell lines were also conditioned to long-term high glucose by culturing for more than 30 days in media supplemented with 20 mM D-glucose. For experiments where hypoxic cells were returned to normoxic conditions, cell were firstly exposed to 1% O<sub>2</sub> (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37° C CO<sub>2</sub>/O<sub>2</sub> incubator for 24 hours and subsequently exposed to 1% O<sub>2</sub> (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37° C CO<sub>2</sub>/O<sub>2</sub> incubator for 24 hours and subsequently exposed to 1% O<sub>2</sub> (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37° C CO<sub>2</sub>/O<sub>2</sub> incubator for 24 hours and subsequently exposed to 1% O<sub>2</sub> (5% CO<sub>2</sub>, 95% N<sub>2</sub>) in a 37° C CO<sub>2</sub>/O<sub>2</sub> incubator for 24 hours and subsequently exposed to normoxia (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 95% N<sub>2</sub>) for 1, 2, 4, 6, 8 and 24 hours.

A further aim of this study was to investigate the HIF-1 DNA binding activity and HIF-1 and GLUT-1 protein expression in cellular and nuclear extracts isolated from breast carcinoma cells exposed to hypoxia and long-term high glucose (20 mM D-glucose >30 days culture). Several cellular steps are required to activate HIF-1: the accumulation of HIF-1 $\alpha$ , the nuclear translocation of HIF-1 $\alpha$  and HIF-1 $\beta$ , the formation of the HIF-1 complex and the binding to DNA (Chilov *et al*, 1999). HIF-1 forms DNA-binding complexes containing the p300/camp-response-element-binding protein when bound to its target HIF-1 binding site under hypoxic conditions (Arany *et al*, 1996). It is known that the cellular redox state determines HIF-1 $\alpha$  binding activity (Haddad and Land, 2000; Haung *et al*, 1996).

The DNA-binding activity of HIF-1 was analysed by electrophoretic mobility shift assays (EMSA), using a double stranded oligonucleotide probe containing the HIF-1 binding site from the *EPO* enhancer (Semenza & Wang, 1992) that binds both HIF-1 and constitutively expressed factors. Protein expression levels were determined by Western blot analysis using a monoclonal antibody to HIF-1 $\alpha$  and a polyclonal antibody to GLUT-

1.

#### 3.1 Northern Analysis of HIF-1α, VEGF, p53 and β-Actin mRNA Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia

Figure 3.1 illustrates Northern blot analysis of T47D breast carcinoma cells exposed to 60 and 120  $\mu$ M cobalt chloride for 17.5 hours. Blots were hybridised with VEGF, p53 and  $\beta$ -actin DNA probes and RNA expression was normalised with 28S:18S RNA expression. Upregulation of both *VEGF* and *p53* mRNA is seen at exposure to both concentrations of cobalt chloride (Table 3.1).

Breast carcinoma cells and peripheral blood mononuclear cells (PBMC) from a normal healthy individual were also exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 8 hours. Northern blot analysis of 100 µg of mRNA from MCF7 breast carcinoma cells exposed to hypoxia for up to 6 hours of is shown in Figure 3.2 where no up-regulation of *HIF-1* $\alpha$ mRNA expression can be seen, only consistent expression, however up-regulation of *VEGF* mRNA is observed after 4 hours exposure to hypoxia (Table 3.2). Figure 3.3 illustrates Northern blotting of PBMCs for *HIF-1* $\alpha$  mRNA expression where upregulation can be seen at 2 hour exposure to hypoxia (Table 3.3).

As Northern blotting was found to require large quantities of mRNA for genetic expression and proved difficult to optimise, the technique was abandoned in favour of the relatively new and more sensitive RPA.



Control 60 µM 120µM CoCl<sub>2</sub>

Figure 3.1. Northern blot analysis of *VEGF*, p53 and  $\beta$ -actin mRNA expression in T47D breast carcinoma cells. Cells were exposed to 60 and 120 mM cobalt chloride (CoCl<sub>2</sub>) for 17.5 hours, RNA was isolated and up to 70 to 125 µg were analysed by Northern blotting using a A) VEGF, B) p53 and C)  $\beta$ -actin DNA probe, following blot stripping. In this example 70 µg of mRNA was blotted and probed. D) Both 28S and 18S mRNA bands are shown. Levels of expression are given in Table 3.1.

**Table 3.1**. Levels of *VEGF*, *p53* and  $\beta$ -actin mRNA in the T47D breast carcinoma cell line exposed to 60 and 120 mM cobalt chloride for 17.5 hours analysed by Northern blotting.

Cobalt Chloride	Fo	Ratio of 28S:18S		
(CoCl <sub>2</sub> )	VEGF	p53	β-actin	RNA
60 mM	1.51	1.67	1.07	1.87
120 mM	5.02	1.92	0.90	1.40

Results are expressed as the fold change (increase or decrease) in mRNA against the level obtained under normoxic conditions (21%  $O_2$ , 5%  $CO_2$ , 74%  $N_2$  at 37° C), after normalisation for 28S:18S RNA expression. Average ratio of 28S:18S RNA is 1.84 (control ratio = 2.26).



Figure 3.2. Northern blot analysis of  $HIF-1\alpha$  mRNA expression in MCF7 breast carcinoma cells. Cells were exposed to hypoxia ( $\leq 1 \% O_2$ ) for up to 6 hours, RNA was isolated and 100 µg was analysed by Northern blotting using a A) HIF-1 $\alpha$  and B) VEGF probe. C) Both 28S and 18S mRNA bands are shown. Results show no up-regulation of  $HIF-1\alpha$  mRNA however up-regulation of VEGF mRNA is seen after 4 hours of hypoxic exposure. Levels of expression are given in Table 3.2.

**Table 3.2**. Levels of *HIF-1* $\alpha$  and *VEGF* mRNA in the MCF7 breast carcinoma cell line exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 6 hours analysed by Northern blotting.

Нурохіс Exposure	Fold mRNA	Ratio of 28S:18S	
(≤1% O₂)	HIF-1a	VEGF	RNA
1 hour	0.72	0.78	1.54
2 hours	0.60	0.89	1.69
3 hours	0.60	1.01	1.78
4 hours	0.49	1.24	1.66
5 hours	0.29	1.16	1.76
6 hours	0.48	1.21	1.63

Results are expressed as the fold change (increase or decrease) in mRNA against the level obtained under normoxic conditions (21%  $O_2$ , 5%  $CO_2$ , 74%  $N_2$  at 37° C), after normalisation for 28S:18S RNA expression. Average ratio of 28S:18S RNA is 1.64 (control ratio = 1.42).



Control 2 4 8 hours hypoxia

Figure 3.3. Northern analysis of *HIF-1* $\alpha$  mRNA expression in PBMCs of a normal healthy individual. A) Cells were exposed to hypoxia ( $\leq 1 \% O_2$ ) for up to 8 hours, RNA was isolated and 50 µg was analysed by Northern blotting using a HIF-1 $\alpha$  probe. B) Both 28S and 18S mRNA bands are shown. Results show up-regulation of *HIF-1* $\alpha$  mRNA at 2 hours exposure to hypoxia. Levels of expression are given in Table 3.3.

Table 3.3.	Levels of HIF-1 $\alpha$ mRNA expression in PBMCs of a normal healthy individual
	exposed to hypoxia (≤ 1% O <sub>2</sub> ) for up to 8 hours analysed by Northern blotting.

Hypoxic Exposure (≤1% O <sub>2</sub> )	Fold HIF-1α mRNA expression	Ratio of 28S:18S RNA
2 hours	2.94	1.74
4 hours	1.19	1.78
8 hours	1.38	1.78

Results are expressed as the fold change (increase or decrease) in mRNA against the level obtained under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37° C), after normalisation for 28S:18S RNA expression. Average ratio of 28S:18S RNA is 1.82 (control ratio = 1.97). Up-regulation of *HIF-1* $\alpha$  mRNA is seen after 2 hours exposure to hypoxia.

#### 3.2 HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia

The effect of hypoxia on the induction of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in breast carcinoma established cell lines was analysed by RPA. Probes to all 3 genes were prepared as described above and in **chapter 2**, section 2.10.6.1. The intensity and size of the HIF-1 $\alpha$ , p53, VEGF and the GLUT-1 probes used in the RPAs is shown in figure 3.4. Despite the synthesis of at least 2 separate and intensely strong VEGF probes, limited success was obtained when used in RPA.

The fold changes in mRNA expression from normoxia for each gene in each of the breast carcinoma cell lines exposed to hypoxia for up to 48 hours are listed in Table 3.4 (Figure 3.5). A change of less than 2 fold expression from normoxic levels (increase or decrease) was considered as non-significant due to background noise or other inconsistencies.

In 3 of the 4 cell lines (SKBR3, T47D, ZR75) that were exposed to hypoxia (<1 %  $O_2$ ) for up to 48 hours, little or no change in *HIF-1*  $\alpha$  mRNA expression was observed from normoxic levels (**Table 3.4; Figure 3.6**). However, the SKBR3 cell line displayed a 2.7 ± 0.7 fold increase in *HIF-1*  $\alpha$  mRNA expression from normoxic levels. Considering the standard error for this time point, the increase in mRNA expression was probably negligible. Thus, prolonged exposure to hypoxia failed to increase *HIF-1*  $\alpha$  mRNA expression in these breast carcinoma cell lines.

As with *HIF-1* $\alpha$  mRNA expression, 3 of the 4 cell lines (MCF7, SKBR3, ZR75) displayed minor but non-significant variations in *GLUT-1* mRNA expression from normoxic levels (**Table 3.4**) These cell lines showed decreased expression for *GLUT-1* mRNA after 2 and 4 hours of hypoxic exposure (mean 0.45 and 0.61 fold expression, respectively, equivalent to 2.2 and 1.6 fold decrease from normoxic levels), (**Figure 3.7**). However, the T47D breast cell line showed an increase in *GLUT-1* mRNA expression with

prolonged exposure to hypoxia, up to an 8.8 fold increase at 24 hours exposure. With the exception of the T47D cell line, prolonged hypoxic exposure seemed to decrease *GLUT-1* mRNA expression in the majority of breast carcinoma cell lines.

Decreased expression of p53 mRNA from normoxic levels was observed in the majority of the breast carcinoma cell lines (Table 3.4; Figure 3.8). As with *GLUT-1*, hypoxia decreased p53 mRNA expression under hypoxia, however this was non-significant.





520 bp 520 bp 553 bp 441 bp 525 bp 440 bp Protected fragment

Figure 3.4. RPA probes used in the analysis of samples. Lane 1 and 2 contain the HIF-1 $\alpha$  probe, lane 3 and 4 contain 2 separate VEGF probes, lane 5 contains the GLUT-1 probe and lane 6 contains the p53 probe (M = 100 bp ladder). PCR assays consisted of 2 separate rounds of PCR using 2 amplimer sets for each probe; conditions were optimised by varying magnesium concentrations and annealing temperatures.

Chapter 3. Results





Hypoxic Exposure (≤1% O <sub>2</sub> )	MCF7			SKBR3			T47D			ZR75		
	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53
2 hours	0.8 ± 0.1	0.3	0.9	2.2 ± 1.0	0.3	1.0	1.6 ± 0.8	0.5	0.7	1.4	0.6	0.6
4 hours	0.8± 0.03	0.8	1.0	$2.7\pm0.7$	0.2	0.6	1.3 ± 0.4	0.9	0.8	1.0	0.5	0.2
6 hours	0.8±0.6	1.3	0.1	1.1 ± 0.3	0.2	0.9	1.4 ± 0.4	2.2	0.2	0.3	0.3	0.3
8 hours	0.6 ± 0.1	1.0	0.8	1.4 ± 0.1	0.4	1.2	1.0 ± 0.2	5.9	0.4	0.6	0.3	0.2
24 hours	$0.4\pm0.2$	1.3	0.0	0.8 ± 0.3	1.1	1.0	$0.8\pm0.3$	8.8	0.6	1.0	0.9	0.2
48 hours	1.0	0.9	0.6	0.4	0.2	1.0	1.2	0.6	0.7	1.8	0.8	0.9

Table 3.4. The fold change in HIF-1α, GLUT-1 and p53 mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia (≤1% O<sub>2</sub>) for up to 48 hours.

Results are expressed as mean values  $\pm$  standard error from at least 2 separate experiments of the fold change (increase or decrease) in mRNA against the level obtained under normoxic (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37° C) conditions. Where no standard error is shown, only one experiment was performed. Results are displayed graphically in **Figures 3.6, 3.7 and 3.8**.

Chapter 3. Results



Figure 3.6. The fold change in *HIF-1* $\alpha$  mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%O_2$ ) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant. The SKBR3 cell line expressed up to a 2.7 fold increase in *HIF-1* $\alpha$  mRNA expression after 4 hours of hypoxic exposure which steadily decreased with prolonged exposure to hypoxia (**Table 3.4**).





Figure 3.7. The fold change in *GLUT-1* mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%O_2$ ) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant. The T47D cell line expressed up to an 8.8 fold increase in *GLUT-1* mRNA expression after 24 hours of hypoxic exposure which dropped off at 48 hours of hypoxic exposure (Table 3.4).

Chapter 3. Results



Figure 3.8. The fold change in p53 mRNA expression in breast carcinoma epithelial cell lines exposed to hypoxia (<1%O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant. Overall, the cell lines displayed decreased expression of p53 mRNA under prolonged hypoxia (Table 3.4).

## 3.3 Basal levels of *HIF-1a*, *GLUT-1* and *p53* mRNA Expression In Breast Carcinoma Cell Lines Cultured Under Normoxia

The mean expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA, analysed by RPA, for each cell line cultured for 5 days at normoxic culture conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37° C) was used to determine the corresponding basal levels and ratios of mRNA expression (**Table 3.5**; **Figure 3.9**). Under normoxic conditions, basal expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA were detected at varying levels in all of the cell lines studied. The abundance of each mRNA varied between cell lines over a range of approximately 9 fold. For HIF-1 $\alpha$  mRNA expression, the cell lines conditioned to long-term high glucose displayed increased basal levels to those not conditioned to high glucose, ranging between 1 and 2.3 fold difference. The basal level of HIF-1 $\alpha$  mRNA varied approximately 3 fold from the lowest expressing cell line SKBR3, to the highest expressing cell line ZR75.

For GLUT-1, the basal level of mRNA varied approximately 5.7 fold from the lowest expressing cell line T47D, to the highest expressing cell line ZR75. Basal levels p53 mRNA expression varied approximately 3.5 fold from the lowest expressing cell line ZR75, to the highest expressing cell line T47D. In the MCF7 and SKBR3 cell lines, basal expression of GLUT-1 and p53 mRNA was approximately 3 to 4 fold higher than that of HIF-1 $\alpha$ . Interestingly, basal expression levels of p53 mRNA was 4 to 5 fold higher than both HIF-1 $\alpha$  and GLUT-1 mRNA in the T47D cell line.

Table 3.5.	Mean basal expression of HIF-1 a, GLUT-1 and p53 mRNA at normoxia (21%
	O2, 5% CO2, 74% N2 at 37° C) in all breast carcinoma cell lines.

Cell Line	Mean I	Ratio		
	HIF-1a	HIF-1:GLUT-1: p53		
MCF7	n= 10 1.03 ± 0.30 (0.19 - 3.49)	n= 2 3.46 ± 1.55 (1.91 - 5.01)	$n=2 3.77 \pm 0.9 (2.87 - 4.67)$	1 : 3.35 : 3.52
MCF7 Hyper	n=6 1.04 ± 0.28 (0.34 - 2.29)	n=2 2.85 ± 0.96 (1.89 - 3.80)	n=1 3.56	1 : 2.74 : 3.42
Ratio	1:1	1: 0.82	1: 0.94	
SKBR3	n= 9 0.86 ± 0.29 (0.09 - 2.87)	n= 2 3.36 ± 0.98 (2.38 - 4.34)	n= 1 2.65	1 : 3.95 : 3.08
SKBR3 Hyper	n=5 1.94 ± 0.56 (0.89 - 3.56)	n=2 3.35 ± 1.62 (1.73 - 4.92)	n=1 3.26	1 : 1.73 : 1.68
Ratio	1: 2.25	1:1	1: 1.23	
T47D	n=9 1.06 ± 0.35 (0.14 - 3.14)	n=3 0.64 ± 0.17 (0.31 - 0.87)	n=2 5.57 ± 1.16 (4.41 - 6.73)	1 : 0.60 : 5.25
T47D Hyper	n=5 1.37 ± 0.61 (0.43 - 3.75)	n=2 1.27 ± 0.22 (1.05 - 1.49)	n=1 5.15	1 : 0.93 : 3.76
Ratio	1: 1.29	1: 1.98	1: 0.92	
ZR75	n=7 2.34 ± 0.99 (0.12 - 5.99)	$n=33.62 \pm 0.94(1.76 - 4.82)$	n=2 1.61 ± 1.41 (0.20 - 3.02)	1 : 1.55 : 0.69
ZR75 Hyper	n=2 2.77 ± 2.31 (0.46 - 5.08)	n=2 4.12 ± 1.44 (2.68 - 5.56)	N/D	1 : 1.49
Ratio	1: 1.15	1: 1.14	-	

The mean expression for each cell line is given in arbitrary units ( $\pm$  standard deviation) together with the range (in parentheses) and the number (n) of times the experiment was repeated, where applicable. The mean expression is relative to an equivalent area analysed for each sample (5.4 mm<sup>2</sup>). N/D = not done. Results are expressed graphically in Figure 3.9.

Chapter 3. Results



Figure 3.9. Mean basal expression of  $HIF-1\alpha$ , GLUT-1 and p53 mRNA cultured for 5 days at normoxia (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37 °C) in all breast carcinoma cell lines. Results are expressed in arbitrary units ± standard deviation. The basal ratio of  $HIF-1\alpha$ : GLUT-1: p53 mRNA expression for each cell line is listed in Table 3.5.

# 3.4 *HIF-1α*, *GLUT-1* and *p53* mRNA Expression In Breast Carcinoma Cell Lines Exposed To Short-Term High Glucose (3 day culture)

The effect of short-term high glucose (3 day culture) on the induction of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in breast carcinoma established cell lines was analysed by RPA (Figure 3.10). The fold changes in mRNA expression from normal culture conditions for each gene in each of the breast carcinoma cell lines exposed to short-term high glucose (3 day cultures supplemented with 20 mM D-glucose) are listed in Table 3.6. A change of less than 2 fold expression from normal culture conditions (increase or decrease) was considered as non-significant due to background noise or other inconsistencies.

When the breast carcinoma cell lines were exposed to high glucose (supplemented with 20 mM D-glucose) for 3 days, a 2.1 fold (MCF7) and a 12.9 fold (ZR75) increase in the expression of *HIF-1* $\alpha$  mRNA and a 1.9 fold decrease (T47D) in *HIF-1* $\alpha$  mRNA expression was observed compared to those cells not subjected to high glucose (**Table 3.6**; **Figure 3.11**). Similar results were observed for *GLUT-1* and *p53* mRNA expression in the same cell lines. A 1.1 fold (MCF7) and a 2.9 fold (ZR75) increase in the expression of *GLUT-1* mRNA and a 1.6 fold decrease (T47D) in *GLUT-1* mRNA expression was observed to cells not subjected to high glucose (**Table 3.6**; **Figure 3.11**). Similarly, a 1.3 fold (MCF7) and a 5.0 fold (ZR75) increase in the expression of *p53* mRNA and a 1.5 fold decrease (T47D) in *p53* mRNA expression was observed compared to cells not subjected to high glucose (**Table 3.6**; **Figure 3.11**).

The ZR75 cell line exhibited the greatest increase in mRNA expression for all three genes. In contrast to the effects of hypoxia seen on the T47D cell line (section 3.2; Figure 3.6), short-term exposure to high glucose decreased the expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression in this cell line.

Table 3.6. The fold change in *HIF-1α*, *GLUT-1* and *p53* mRNA expression from normal culture conditions in breast carcinoma cell lines cultured for 3 days in moderate and high glucose.

Glucose Concentration		Fold change HF-1α mR1	in NA	F G	old change i LUT-1 mRN	in IA	Fold change in <i>p53</i> mRNA			
	MCF7	T47D	ZR75	MCF7	T47D	ZR75	MCF7	T47D	ZR75	
Moderate (10 mM D-glucose)	2.1 ± 1.4	$0.5 \pm 0.1$	5.0 ± 0.2	0.9	1.2	2.4	1.2	1.0	3.4	
High (20 mM D-glucose)	2.1 ± 1.5	0.7±0.5	12.9 ± 4.9	1.1	0.6	2.9	1.3	0.7	5.0	

Results are expressed as mean values  $\pm$  standard error from at least 2 separate experiments of the fold change (increase or decrease) in mRNA exposed to high glucose against the level obtained under normal culture conditions. Where no standard error is shown, only one experiment was performed. Results are displayed graphically in **Figure 3.11**.



Figure 3.10. Expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in MCF7, T47D and ZR75 breast carcinoma cell lines exposed to high glucose. Cell lines were exposed to normal glucose, moderate and high glucose in cultures for 3 days. MCF7 and ZR75 cell lines displayed increases in mRNA expression for all 3 genes and T47D cells displayed a decrease in expression for all 3 genes (Table 3.6). Results are expressed as the value of the fold change in mRNA exposed to high glucose compared to the level obtained under normal glucose and are expressed graphically in Figure 3.11. N= normal, M= moderate, H= high.





Figure 3.11. The fold change in HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression from normal culture conditions in breast carcinoma cell lines cultured for 3 days in moderate or high glucose (Table 3.6; Figure 3.10).

### 3.5 HIF-1α, GLUT-1 and p53 mRNA Expression In Breast Carcinoma Cell Lines Conditioned To Long-Term High Glucose and Exposed To Hypoxia

The breast carcinoma cell lines were conditioned to long-term high glucose (20 mM D-glucose >30 days culture), exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours and analysed by RPA. The fold changes in mRNA expression from normoxia for each gene in each of the breast carcinoma cell lines exposed to hypoxia are listed in **Table 3.7 (Figure 3.12)**. A change of less than 2 fold expression from normoxic levels (increase or decrease) was considered as non-significant due to background noise or other inconsistencies.

Minor non-significant responses were observed in the cell lines in HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression. All lines showed an initial decrease in the expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA (Table 3.7). Both the MCF7 Hyper and SKBR3 Hyper cell lines displayed decreased HIF-1 $\alpha$  mRNA expression throughout the 48 hours of hypoxic exposure, whereas the T47D Hyper cell lines showed increased expression from 4 to 24 hours of hypoxic exposure, dropping after 48 hours of hypoxic exposure (Figure 3.13). With the exception of the T47D Hyper cell line, the remaining cell lines showed decreased expression of GLUT-1 mRNA for up to 8 hours of hypoxic exposure (Table 3.7; Figures 3.15). p53 mRNA expression increased in the MCF7 Hyper cell line from 6 hours of hypoxic exposure (1.1 fold) to 24 hours (1.3 fold), where it then decreased after 48 hours of exposure (2.6 fold) (Figure 3.17). The remaining cell lines all expressed decreased levels of p53 mRNA expression at all time points.

Direct comparison of the normal cell lines and those conditioned to long-term high glucose and exposed to hypoxia for *HIF-1* $\alpha$ , *GLUT-1* and *p53* mRNA expression are displayed graphically in Figures 3.14, 3.16 and 3.18. With the exception of the T47D cell lines, the extreme combined stresses of hypoxia and high glucose decrease the expression of *HIF-1* $\alpha$  and *GLUT-1* mRNA. *p53* was the only decreased mRNA expression seen in

the T47D cell lines. However, the observed decreases remain below 2 fold from control levels and are considered as non-significant.

Hypoxic Exposure	MCF7 Hyper			SKBR3 Hyper			T47D Hyper			ZR75 Hyper		
(<1% O <sub>2</sub> )	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53	HIF-1a	GLUT-1	p53
2 hours	$0.8 \pm 0.3$	0.7	1.0	0.8±0.2	0.5	0.9	$0.8 \pm 0.3$	2.7	0.5	N/D	0.6	N/D
4 hours	0.4 ± 0.1	0.3	0.6	0.4 ± 0.1	0.4	0.9	$1.2 \pm 0.5$	0.1	0.6	N/D	0.2	N/D
6 hours	0.9 ± 0.2	1.0	1.1	0.4 ± 0.1	0.5	0.5	1.4 ± 0.6	0.2	0.7	N/D	0.1	N/D
8 hours	0.5 ± 0.1	0.8	1.1	0.7 ± 0.2	0.6	1.0	$1.0 \pm 0.1$	0.3	0.8	N/D	0.2	N/D
24 hours	0.5 ± 0.1	1.9	1.3	0.2 ± 0.1	0.5	0.7	1.2 ± 0.6	2.9	0.6	N/D	0.7	N/D
48 hours	0.5 ± 0.04	0.8	0.4	0.5 ± 0.4	0.6	0.8	0.8	0.3	0.7	N/D	0.7	N/D

**Table 3.7**. The fold change in *HIF-1* $\alpha$ , *GLUT-1* and *p53* mRNA expression from normoxia in breast carcinoma cell lines exposed to long-term high glucose and exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours.

Results are expressed as mean values  $\pm$  standard error from at least 2 separate experiments of the fold change (increase or decrease) in mRNA against the level obtained under normoxic (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37° C) conditions. Where no standard error is shown, only one experiment was performed. Results are displayed graphically in Figures 3.13, 3.15 and 3.17. N/D = not done

Chapter 3. Results



Figure 3.12. *HIF-1* $\alpha$ , *GLUT-1* and *p53* mRNA expression in MCF7 Hyper, SKBR3 Hyper and T47D Hyper breast carcinoma cells exposed to normoxia (N) and to 2 to 48 hours hypoxia, analysed by RPA. Results show a varied response through both an increase and decrease in mRNA expression (**Table 3.7**). A 100 bp marker was run to ensure correct protected fragment size.

Chapter 3. Results



Figure 3.13. The fold change in *HIF-1* $\alpha$  mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose (20 mM D-glucose >30 days) and exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).





Figure 3.14. The fold change in *HIF-1* $\alpha$  mRNA expression from normoxia in the MCF7, SKBR3 and T47D breast carcinoma cell line and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).

Chapter 3. Results



Figure 3.15. The fold change in *GLUT-1* mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose (20 mM D-glucose >30 days) and exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).

Chapter 3. Results



Figure 3.16. The fold change in *GLUT-1* mRNA expression from normoxia in MCF7, SKBR3, T47D and ZR75 breast carcinoma cell lines and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).

Chapter 3. Results



Figure 3.17. The fold change in p53 mRNA expression from normoxia in breast carcinoma cell lines conditioned to long-term high glucose (20 mM D-glucose >30 days) and exposed to hypoxia (<1% O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).




Figure 3.18. The fold change in p53 mRNA expression from normoxia in MCF7, SKBR3 and T47D breast carcinoma cell line and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia (<1% O<sub>2</sub>) for up to 48 hours. Polynomial trend lines, with respective R<sup>2</sup> values, are indicated for each cell line. A change of less than 2 fold expression from normoxic levels was considered non-significant (Table 3.7).

#### 3.6 HIF-1α mRNA Expression In Breast Carcinoma Cell Lines Exposed To 24 Hours Hypoxia and Subsequently Normoxia

The effect of hypoxia, and subsequent exposure to normoxia, on the induction of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in breast carcinoma established cell lines was analysed by RPA (Figures 3.19 and 3.20). The fold changes in mRNA expression from normoxia for each gene in each of the breast carcinoma cell lines exposed to hypoxia for up to 48 hours are listed in Table 3.8. A change of less than 2 fold expression from normoxic levels (increase or decrease) was considered as non-significant due to background noise or other inconsistencies.

When all of the cell lines, both the normal and those conditioned to long-term high glucose, were exposed to 24 hours hypoxia and subsequently to normoxia (21% O<sub>2</sub>, 5% CO<sub>2</sub>, 74% N<sub>2</sub> at 37° C) for up to 24 hours, varying responses were observed in *HIF-1* $\alpha$  mRNA expression (**Table 3.8**; **Figures 3.19 and 3.20**). Both the MCF7 and MCF7 Hyper cell lines, as well as the SKBR3 Hyper cell line displayed increased *HIF-1* $\alpha$  mRNA expression after one hour recovery in normoxia. In the majority of the cell lines, prolonged exposure to hypoxia and recovery in normoxia resulted in *HIF-1* $\alpha$  mRNA levels remaining similar to or just below that of normal control levels.

**Table 3.8**. The fold change in *HIF-1*  $\alpha$  mRNA expression from normoxia in breast carcinoma cell lines exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for 24 hours and subsequently to normoxia for up to 24 hours.

Normoxic Exposure	MCF7	SKBR3	T47D	ZR75	MCF7 Hyper	SKBR3 Hyper	T47D Hyper	ZR75 Hyper
1 hours	7.94	0.81	1.26	0.10	3.31	2.38	0.97	0.16
2 hours	1.72	0.82	3.15	0.26	0.55	0.41	0.48	0.35
4 hours	1.23	0.79	1.17	0.19	0.51	0.53	0.15	0.51
6 hours	4.65	0.63	2.49	0.15	2.22	0.51	0.44	0.09
8 hours	0.66	0.81	0.83	0.28	1.29	0.27	0.36	0.43
24 hours	3.09	0.99	4.89	0.44	3.1	0.31	1.58	0.77

Results are expressed as the value of the fold change in mRNA against the level obtained under normoxic conditions.

Results are expressed graphically in Figures 3.19 and 3.20.





Figure 3.19. The fold change in *HIF-1* $\alpha$  mRNA expression in breast carcinoma cell lines and breast carcinoma cell lines conditioned to long-term high glucose, exposed to hypoxia ( $\leq$ 1% O<sub>2</sub>) for 24 hours and subsequently to normoxia for up to 24 hours. Results are expressed as the value of the fold change in mRNA against the level obtained under normoxic conditions (**Table 3.8**).

Chapter 3. Results



Figure 3.20. The fold change in *HIF-1* $\alpha$  mRNA expression in MCF7, SKBR3, T47D and ZR75 breast carcinoma cell lines and in the same cell lines conditioned to long-term high glucose, exposed to hypoxia (<1% O<sub>2</sub>) for 24 hours and subsequently to normoxia for up to 24 hours. Results are expressed as the value of the fold change in mRNA against the level obtained under normoxic conditions (Table 3.8).

#### 3.7 *HIF-1α* mRNA Expression In Breast Carcinoma Cell Lines Exposed To Hypoxia and Hypoglycaemia

The combined stresses of hypoglycaemia and hypoxia on the expression of HIF-1 $\alpha$  mRNA was also analysed in the cell lines by RPA. As seen with the combined stress of hypoxia and high glucose, all cell lines with the exception of T47D, expressed decreased levels of HIF-1 $\alpha$  mRNA when exposed for 24 hours to hypoglycaemia and hypoxia (Table 3.9).

The T47D and T47D Hyper cell lines were subsequently exposed to 7 days culture in hypoglycaemia where again an increase in *HIF-1* $\alpha$  mRNA was observed (**Table 3.10**). Exposure of these two cell lines, as well as the MCF7 and MCF7 Hyper cell lines, to 7 days culture in media supplemented with 20 mM L-glucose resulted in an increased expression of *HIF-1* $\alpha$  mRNA (**Table 3.10**). This preliminary result would indicate that increased expression may be due to differences in osmolarity, however the experiments should be repeated several times to confirm this.

Table 3.9. Levels of HIF-1 $\alpha$ mRNA in breast carcinoma cell lines exposed to both
hypoglycaemia and hypoxia (≤1% O <sub>2</sub> ) for 24 hours.

Culture Condition	Fold change in <i>HIF-1α</i> mRNA							
	MCF7	SKBR3	T47D	ZR75				
24 hour exposure to hypoglycaemia + hypoxia (≤1% O₂)	0.58	0.53	1.14	0.71				

Results are expressed as the values of the fold change in mRNA against the level obtained under normoxic conditions.

**Table 3.10.** Levels of HIF-1 $\alpha$  mRNA in breast carcinoma cell lines exposed for 7 daysto hypoglycaemia or 20 mM L-glucose.

Culture Condition	Fold change in <i>HIF-1α</i> mRNA						
	MCF7	MCF7 Hyper	T47D	T47D Hyper			
Hypoglycaemia	N/D	N/D	1.41	6.64			
20 mM L-glucose	4.77	2.85	5.61	6.61			

Results are expressed as the value of the fold change in mRNA against the level obtained at normal culture conditions.

N/D = not done.

#### 3.8 Osmolarity and High Glucose Controls

To control for osmolarity and high glucose, and to determine whether the increased or decreased expression of HIF-1 $\alpha$  and GLUT-1 was specific for D-glucose each of the breast carcinoma cell lines were stimulated with either L-glucose, which competes with Dglucose for the transporter, or mannitol, an osmolyte and analysed by RPA. In the first experiment, 20 mM mannitol was used as a supplement with the 20 mM D-glucose. A second, parallel culture consisted of 20 mM L-glucose being used as a supplement with the 20 mM D-glucose. Mannitol is known to activate p38 mitogen activated protein kinase (Igarashi *et al*, 1999).

When the tissue culture media for the cell lines was supplemented with either 20 mM L-glucose or 20 mM mannitol, up to a 100 fold decrease was seen in the expression of HIF-1 $\alpha$  mRNA (Table 3.11). Additionally, in contrast to initial results where cultures supplemented with 20 mM D-glucose alone for 3 days displayed an increased expression of HIF-1 $\alpha$ , a decrease in HIF-1 $\alpha$  mRNA was observed.

GLUT-1 mRNA expression was also quantitated in the same cultures where GLUT- I mRNA was induced by D-glucose, L-glucose and mannitol in the MCF7 and ZR75 cell lines and by mannitol and long-term exposure to D-glucose in the T47D cell line (**Table 3.11**). These results would indicate that the expression of HIF-1 $\alpha$  and GLUT-1 mRNA in the breast carcinoma cell lines is not specific for D-glucose alone but possibly due to differences in osmolarity. Although cell lines supplemented with either 20 mM L-glucose or 20 mM mannitol show an increase in GLUT-1 mRNA, this increase is not as great as that found in cell lines cultured in long-term high glucose (with the exception of the ZR75 cell line). As only one experiment was performed for each cell line under each culture condition, several repeated experiments are required to confirm these observations.

# **Table 3.11.** Fold change in *HIF-1* $\alpha$ and *GLUT-1* mRNA expression in breast carcinoma cell lines exposed to 20 mM D-glucose supplemented with either 20 mM L-glucose or 20 mM mannitol.

Culture Conditions	Fold	Fold change in <i>HIF-1</i> $\alpha$ mRNA				Fold change in <i>GLUT-1</i> mRNA		
	MCF7	SKBR3	T47D	ZR75	MCF7	T47D	<b>ZR75</b>	
High Glucose (20 mM D-glucose)	0.36	2.51	0.48	0.02	1.12	0.43	1.16	
L-glucose (20 mM D-glucose + 20 mM L-glucose)	0.59	1.31	0.92	0.32	1.20	0.97	2.34	
Mannitol (20 mM D-glucose + 20 mM mannitol)	0.84	1.96	0.43	0.01	1.67	1.02	1.14	
Long-term High glucose (20 mM D-glucose <30 days exposure)	0.71	2.63	1.21	0.12	1.69	1.22	1.8	

Results are expressed as the fold change in mRNA against the level obtained at normal

culture conditions.

#### 3.9 Measurement of Lactic Acid

Measurement of lactic acid was determined for each cell line exposed to hypoxia, high glucose as well as the osmolarity controls (**Tables 3.12 and 3.13**). Basal levels of lactic acid were found to be relatively high in the MCF7 and ZR75 cell lines, dropping approximately 3 fold in both cell lines after 2 hours exposure to hypoxia. Increased production was then observed with increased exposure time to hypoxia, surpassing normoxia levels after 24 to 48 hours of exposure (**Table 3.12**). In cell lines exposed to high glucose, L-glucose and mannitol, lactate levels remained similar in all cell lines in all conditions with decreased production of lactate when cultured in long-term high glucose. The T47D cell line displayed the highest basal level of lactic acid, two fold greater then the lowest basal level observed in the ZR75 cell line (**Figures 3.21**).

 Table 3.12.
 Measured lactate concentrations (mmol/L) in breast carcinoma cell line culture media exposed to hypoxia.

Hypoxic Exposure	M	CF7	ZR75		
(>1% O <sub>2</sub> )	OD (340 nm)	Lactate Conc. (mmol/L)	OD (340 nm)	Lactate Conc. (mmol/L)	
Normoxia (21% O <sub>2</sub> )	0.85	8.66	0.81	8.30	
2 hours	0.26	2.60	0.30	3.08	
4 hours	0.30	3.04	0.35	3.52	
6 hours	0.31	3.12	0.43	4.38	
8 hours	0.37	3.81	0.43	4.41	
24 hours	0.69	6.99	0.83	8.45	
48 hours	1.13	11.50	1.12	11.45	

Lactate concentrations were calculations as described in Chapter 2, section 2.11.1.3.

 Table 3.13.
 Measured lactate concentrations (mmol/L) in breast carcinoma cell'line culture media exposed to high glucose and osmolarity controls.

	Μ	MCF7		SKBR3		T47D		ZR75	
Culture Conditions	OD (340nm)	Lactate Conc. (mmol/L)							
Normal media (basal glucose)	0.93	9.51	1.07	10.88	1. <b>74</b>	17.71	0.84	8.55	
20 mM D-glucose	0.93	9.51	1.07	10.88	2.06	21.01	0.75	7.67	
20 mM D-glucose + 20 mM L-glucose	0.75	7.63	1.02	10.44	1.94	19.75	0.62	6.28	
20 mM D-glucose + 20 mM Mannitol	0.98	10.04	0.87	8.85	2.01	20.55	0.64	6.54	
Long-term high glucose (20 mM D-glucose >30 days)	0.80	8.18	0.61	6.25	1.67	17.03	0.48	4.93	

Lactate concentrations were determined as described in Chapter 2, section 2.11.1.3. Results are expressed graphically in Figure 3.21.

Chapter 3. Results



Figure 3.21. Measured lactate concentrations (mmol/L) in breast carcinoma cell line culture media exposed to high glucose and osmolarity controls. The graph displays the results of all culture conditions per cell line and shows similar decreased production of lactate in all cell lines during culture in long-term high glucose (Table 3.13).

#### 3.10 HIF-1 DNA Binding Activity In Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose

HIF-1 DNA-binding activity was induced in nuclear extracts made from all breast carcinoma epithelial cells exposed to hypoxia for up to 48 hours (Figure 3.22) as well as in extracts exposed to long-term high glucose and hypoxia (Table 3.14; Figure 3.22). DNAbinding of HIF-1 functional complexes was also observed under normoxic conditions in the cell lines.

The relative amounts or intensity of the bands seen varied in all extracts. Of the cell lines exposed to hypoxia, the MCF7 cell line displayed a decrease in binding activity. The SKBR3 cell line displayed an initial increase in DNA-binding activity but then decreased below normoxic control levels. This was also observed in the SKBR3 Hyper cell line. Extracts from the T47D and ZR75 cell lines displayed increases in binding activity when exposed to prolonged hypoxia. Extracts from the ZR75 cell line exposed to hypoxia displayed the highest binding activity, where up to a 3 fold increase in activity was seen over the level observed in the other cell lines. DNA-binding activity in the cell lines conditioned to long-term high glucose and exposed to hypoxia was similar to those exposed to hypoxia alone (**Table 3.14**).

Hypoxic Exposure (<1% O <sub>2</sub> )	MCF7	SKBR3	T47D	ZR75	MCF7 Hyper	SKBR3 Hyper	T47D Hyper	ZR75 Hyper
2 hours	0.6±0.08	1.1	$1.1 \pm 0.02$	4.5 ± 3.6	0.6 ± 0.13	1.3	$1.5 \pm 0.45$	1.3 ± 0.02
4 hours	N/D	1.3	1.4 ± 0.09	N/D	$0.4 \pm 0.05$	1.5	$1.4 \pm 0.05$	N/D
6 hours	$0.6 \pm 0.12$	1.1	1.1 ± 0.01	4.9 ± 3.5	1.0 ± 0.17	0.5	$1.4 \pm 0.41$	$1.2 \pm 0.01$
8 hours	0.6 ± 0.01	0.4	1.8 ± 0.05	4.5 ± 1.9	1.1 ± 0.06	0.1	1.5 ± 0.05	1.3 ± 0.08
24 hours	$0.6 \pm 0.02$	N/D	1.7 ± 0.04	4.4 ± 3.1	1.1 ± 0.05	N/D	N/D	1.3 ± 0.02
48 hours	0.5 ± 0.01	N/D	1.0 ± 0.12	N/D	1.0 ± 0.04	N/D	1.2 ± 0.35	N/D
Hypoxia + excess unlabelled probe	0.1 ± 0.01	0.1	0.7±0.41	0.5 ± 0.08	0.2 ± 0.08	0.1	0.3 ± 0.18	$0.7 \pm 0.01$

Table 3.14. Mean fold change in expression of HIF-1 DNA binding activity in nuclear extracts isolated from breast carcinoma cell lines exposed to hypoxia and long-term high glucose (20 mM D-glucose>30 days culture).

Results are expressed as the mean value of the fold change in HIF-1 DNA-binding activity in nuclear extracts isolated from cultures exposed to hypoxia compared to the level obtained under normoxic culture conditions. Results represent the mean of 2 separate experiments and are expressed graphically in figure 3.22. Only one experiment was performed on the SKBR3 and SKBR3 Hyper cell lines. N/D = Not Done

Chapter 3. Results



Figure 3.22. The fold change in HIF-1 DNA-binding activity in breast carcinoma cell lines and in the same cell lines conditioned to long-term high glucose and exposed to hypoxia  $(\leq 1\% O_2)$  for up to 48 hours. The cell lines displayed varied responses to hypoxia. The addition of excess unlabelled probe in competition assays displaced HIF-1 binding complexes (Figure 3.23).

#### **3.11 HIF-1 Competition Assays**

In competition assays, HIF-1 bands induced by hypoxia were displaced by excessive unlabelled probe (up to 6 fold excess) in the extracts of all cell lines tested, suggesting that these bands are specific for the HIF-1 binding site (Figure 3.23). The same result was obtained with hypoxia and high glucose-treated extracts where again bands induced by these treatments were displaced with excess unlabelled probe (Figure 3.23).

Several attempts were made to supershift the HIF-1 DNA complexes with the addition of a monoclonal HIF-1 antibody without success (Figure 3.24). Two separate monoclonal HIF-1 antibodies were used with 2 separate assay conditions: one involved an incubation of the reaction mixture on ice for 1 hour and the other an incubation of the reaction mixture for 1 hour, after the addition of the antibody. Although these antibodies specific for HIF-1 $\alpha$  could not supershift the complexes, the hypoxia/high glucose induced DNA-binding activity was due to HIF-1 as the binding complex was knocked out in the competition assays.



### N 2 6 8 24 48 Excess N 2 6 8 24 48 Excess Neg Probe Probe Probe control MCF7 MCF7 Hyper

Figure 3.23. The effect of hypoxia and high glucose on HIF-1 DNA binding activity. Nuclear protein was extracted from the nuclei of MCF7 cells and MCF7 Hyper cells (conditioned to long-term high glucose) subjected to normoxia (N), or to 2, 6, 8, 24, and 48 hours hypoxia and analysed by EMSA. 15  $\mu$ g of nuclear protein was analysed. HIF-1 binding complex (HIF-1) bands are indicated. The addition of excess unlabelled probe in competition assays displaced HIF-1 binding complexes.



N 2 8 8+Ab1 8+E Neg control



Figure 3.24. Supershifting of HIF-1 DNA complexes. Two separate monoclonal antibodies to HIF-1 (Ab1 and Ab2) were added to the EMSA reactions containing nuclear protein from ZR75 cells subjected to normoxia (N), or to 2 and 8 hours hypoxia in an attempt to supershift the HIF-1 DNA complexes without success. The complex was displaced with excess unlabelled probe (E). 15  $\mu$ g of nuclear protein was analysed. HIF-1 binding complex (HIF-1) bands are indicated.

#### 3.12 Comparison of Methodologies for Harvesting Nuclear Protein Extracts

Several different methodologies have been published describing appropriate techniques for harvesting nuclear protein extracts for investigation of HIF-1 DNA-binding activity by EMSA. As HIF-1 has a very short half-life, it is important to ascertain that the correct methodology is used. Two of the most tried and popular methodologies have been used in this study. As both techniques are very different in their methodology and processing time, nuclear extracts prepared from each technique were compared for any notable differences in the detection of HIF-1 DNA-binding activity.

Figure 3.25 displays a graphical comparison of the 2 techniques in 4 cell lines. Both methods produced extracts from the cell lines which display HIF-1 DNA binding activity with minor differences in the levels. The most notable difference is in the ZR75 cell line exposed to hypoxia which displays a 3 fold increase in the level of activity of one technique versus the other. Although the MCF7 cell line exposed to hypoxia shows a decreased level of binding activity after exposure to hypoxia in one extraction methodology, the second extraction methodology shows an equal level of binding activity with the normoxia control. Thus, no increase in binding activity occurred. As the second methodology was accessed only once due to time constraints, this is not an accurate comparison but nevertheless shows that both methodologies are adequate for harvesting nuclear protein extracts for the investigation of HIF-1 binding activity.

Chapter 3. Results



HIF-1 Binding Activity

**Figure 3.25**. Comparison of methodologies for harvesting nuclear protein extracts. Two different methodologies for the harvesting of nuclear protein extracts for investigation of HIF-1 DNA-binding activity were accessed with slight differences observed in the level of binding activity.

#### 3.13 HIF-1a Protein Expression In Breast Carcinoma Cell Lines Exposed to Hypoxia

Several different protocols were followed in attempts to obtain a signal from the HIF-1 $\alpha$  antibody used in the Western blotting experiments analysing breast carcinoma cell lines exposed to hypoxia.

Following electrophoresis and transfer to nitrocellulose paper, gels were stained with Coomassie blue to access the success of the transfer. Good separation of the protein was obtained however on a few occasions the protein transfer was incomplete in the upper quadrant of the gel (Figure 3.26). Despite this, protein expression could be obtained using the GLUT-1 antibody but not with the HIF-1 $\alpha$  antibody.

Although the suppliers' recommended protocol for use with the antibody was followed and adapted where necessary, in addition to trying various protocols from published papers, a signal could not be detected. Additional attempts to solve the detection problem included accessing whole cell protein extracts, cytoplasmic protein and nuclear protein extracts from the breast carcinoma cell lines that had been exposed to hypoxia. The amount of protein separated and blotted was also increased (up to 90  $\mu$ g), in attempts to detect the correct size fragment (120 kDa for HIF-1 $\alpha$ ). A fragment approximately 66 kDa in size was detected on several occasions which was confirmed to be bovine serum albumin (BSA) by subsequent spiking experiments (Figure 3.27).

Contact with the supplier revealed that there had been several complaints from customers about the particular batch of antibody that had been released. The company confirmed through in house testing that specificity problems were found with the batch of antibody used in the Western blotting experiments. A second different HIF-1 $\alpha$  antibody was received from the company and tested but a signal still could not be obtained. An eventual follow-up response from the technical assistance department at the company

found that the antibody could detect HIF-1a protein from MCF7 breast carcinoma cell lines exposed to hypoxia when 120 ug of protein was used.



**Figure 3.26**. Separation of protein by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel. In this example, 40  $\mu$ g of total protein from MCF7, ZR75 and T47D breast carcinoma cell lines that had been exposed to hypoxia for 4 hours was separated and transferred onto a nitrocellulose membrane. The corresponding immunoblot demonstrates increased expression of GLUT-1 protein. One of the difficulties of the procedure was attaining complete transfer of the protein to the nitrocellulose membrane. The gel pictured in (**A**) had been transferred overnight at 3 mA, stained with Coomassie blue and lightly destained. The corresponding blot was then probed with a monoclonal anti-HIF-1 or a polyclonal anti-GLUT-1 antibody and detected by ECL chemiluminescence (**B**).





**Figure 3.27.** Western blot of breast carcinoma cell lines exposed to hypoxia demonstrating the detection of bovine serum albumin (BSA) by the HIF-1 $\alpha$  antibody in the nuclear extracts. Cells were incubated in complete media under hypoxic conditions for 6 hours prior to nuclear extract preparation. Lane 1 and 2 contain 70 µg and 50 µg, respectively, of nuclear protein from T47D cells exposed to hypoxia for 4 hours, lane 3 contains 50 µg of nuclear protein from ZR75 cells exposed to hypoxia for 6 hours. Lanes 4 and 5 are the same as lanes 2 and 3 but spiked with 50 µg of BSA, lane 6 contains 50 µg of BSA alone. The HIF-1 $\alpha$  antibody detected BSA in the cell lines but not HIF-1 $\alpha$ . M = marker, Phos b = Phosphorylase b

#### 3.14 GLUT-1 Protein Expression in Breast Carcinoma Cell Lines Exposed To Hypoxia and High Glucose

GLUT-1 protein expression was also assessed in breast carcinoma cell lines exposed to hypoxia and long-term high glucose. A 55 kDa band was detected in all cell lines studied suggesting a moderate basal level in all cells at normoxia (Figure 3.28). GLUT-1 protein levels varied in response to hypoxia and long-term high glucose in the majority of cell lines.

In the MCF7 cell line exposure to hypoxia increased GLUT-1 protein levels, however long-term exposure to high glucose (MCF7 Hyper cell line) and exposure to hypoxia was found to decrease the response (**Table 3.15; Figure 3.29**). No increase in the expression of GLUT-1 protein from normoxic levels was seen in the SKBR3 and ZR75 cell lines after exposure to hypoxia. The T47D cell line showed an initial decrease in GLUT-1 protein after exposure to hypoxia which then increased to 1.5 fold above that seen at normoxia. All of the cell lines conditioned to long-term high glucose and exposed to hypoxia, with the exception of the T47D hyper cell line, showed decreased expression of GLUT-1 protein from that seen at normoxia.

The level of GLUT-1 protein detected by Western blotting is consistent with results obtained at the mRNA level with the SKBR3, SKBR3 Hyper, ZR75 and ZR75 Hyper cell lines all displaying decreased GLUT-1 expression at both the mRNA and protein levels. GLUT-1 mRNA and protein levels are consistent in the MCF7 cell line at 24 hours exposure to hypoxia and in the MCF7 Hyper cell line at 4 hours exposure to hypoxia. Consistent expression in GLUT-1 at the mRNA and protein levels in seen in the T47D cell line at 4 hours of exposure to hypoxia. As only a few time points were sampled it is difficult to assess the behaviours of all the cell lines throughout the entire time period.



Figure 3.28. Western blot analysis of GLUT-1 protein expression in breast carcinoma cell lines exposed to hypoxia and long-term high glucose. After exposure to normoxia and hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours, cellular proteins (60 µg/lane) were separated by 12 % SDS-Page, blotted on nitrocellulose and probed with a polyclonal anti-GLUT antibody. A 55 kDa band was observed and quantified (**Table 3.15**). N= Normoxia

Cell Line	Hypoxic Exposure (≤1% O <sub>2</sub> )							
	2 hours 4 hours		24 hours	48 hours				
MCF7	2.29	N/D	2.77	N/D				
MCF7 Hyper	N/D	0.73	0.32	N/D				
SKBR3	N/D	0.80	N/D	N/D				
SKBR3 Hyper	N/D	0.63	N/D	N/D				
T47D	N/D	0.45	N/D	1.54				
T47D Hyper	N/D	1.51	N/D	N/D				
ZR75	0.99	0.65	0.64	N/D				
ZR75 Hyper	N/D	0.82	0.94	N/D				

<b>Table 3.15</b> .	Expression of GLUT-1 protein in breast carcinoma cell lines exposed to
	hypoxia and long-term high glucose (20 mM D-glucose >30 days culture).

Results are expressed as the fold change in GLUT-1 protein expression in extracts isolated from cultures exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for up to 48 hours, compared to the level obtained under normoxia. Results are expressed graphically in figure 3.29. N/D = Not Done.

Chapter 3. Results



Figure 3.29. GLUT-1 protein expression in extracts isolated from all breast carcinoma cell lines exposed to hypoxia for up to 48 hours. The expression of GLUT-1 protein in the cell lines varied. The MCF7, T47D and T47D Hyper cell lines displayed increased GLUT-1 protein expression after exposure to hypoxia whereas the remaining cell lines displayed decreased GLUT-1 protein expression after exposure to hypoxia. Results are expressed as the fold change in GLUT-1 protein expression in cell lines exposed to hypoxia compared to the level obtained under normoxia (Table 3.15).

#### 3.15 Conclusion

This is a preliminary report of HIF-1 $\alpha$  and p53 mRNA expression in high glucose and of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression in the combined stresses of hypoxia and high glucose. Each cell line studied produced minor genetic expression responses to the stresses of hypoxia, high glucose or the combined stresses of both, displaying slight increased expression of both the hypoxia-regulated genes HIF-1 $\alpha$  and GLUT-1. It was found that short-term exposure (3 days) to high glucose increased the expression of HIF- $1\alpha$ , GLUT-1 and p53 mRNA in 2 of 3 breast cancer cell lines studied. However, the combined stresses of hypoxia and long-term exposure to high glucose decreased the expression of HIF-1 $\alpha$ , GLUT-1 and p53 mRNA in the majority of breast cancer cell lines studied.

Under normoxic conditions,  $HIF-1\alpha$ , GLUT-1 and p53 mRNA was detected at varying concentrations in all of the breast carcinoma cell lines studied. Basal levels of expression of each gene varied by up to 9 fold in all cell lines, with  $HIF-1\alpha$  mRNA expression increasing up to 2.3 fold in the cell lines conditioned to long-term high glucose. Basal levels of  $HIF-1\alpha$  mRNA expression have been shown to vary according to differences in culture conditions (Semenza, 1998).

Exposure of the breast carcinoma cell lines to hypoxia demonstrated a small but non-significant increase in the expression of *HIF-1* $\alpha$  mRNA, *GLUT-1* and *p53* mRNA expression in the majority of cell lines. This slight increase may not be a consequence of low oxygen tension but may instead represent a general adaptation response to of the cells during cultivation. This hypoxic exposure resulted in an initial decrease in the level of lactate from normoxic levels that steadily increased over normoxic levels after 24 and 48 hours of exposure. The initial decrease in lactate levels would indicate recovery from the hypoxic stress followed by an increase in the rate of glycolysis. A decrease in the expression of *HIF-1* $\alpha$  and *GLUT-1* mRNA was observed in the cell lines exposed to the combined stresses of high glucose and hypoxia. Experiments controlling for osmolarity determined that the limited responses were not specific for D-glucose and were probably due to differences in osmolarity as no significant increase in mRNA expression was observed in the cell lines exposed to L-glucose and mannitol. However, as only one experiment was performed for each cell line under each culture condition, several repeats are required to confirm these observations. As oxygen deprivation and excess glucose represent an additional disturbant of glucose transporter expression in human breast carcinoma cells, it is possible that a redox effect may promote any observed induction of *HIF-1* $\alpha$  and *GLUT-1* mRNA in these cell lines and not hypoxia or D-glucose alone.

HIF-1 DNA binding activity was observed in breast carcinoma cells exposed to normoxia, hypoxia and long-term high glucose. HIF-1 binding to its cognate DNA sequence increased after only 2 hours exposure to hypoxia in the majority of breast carcinoma cell lines investigated. The observed HIF-1 binding was specific as it could be effectively inhibited with excess unlabelled probe in competition experiments.

Methodological difficulties with Western blotting prevented the assessment of HIF-1 $\alpha$  protein expression in the breast carcinoma cell lines exposed to hypoxia and long-term high glucose. However, Western blot analysis of GLUT-1 protein expression showed no increase in the majority of breast carcinoma cell lines exposed to hypoxia for up to 24 hours, which was consistent with observed mRNA levels. Cell lines conditioned to longterm high glucose and exposed to hypoxia displayed a decrease in GLUT-1 protein expression. The MCF7, T47D and the T47D Hyper cell lines were the only cell lines which expressed an increase in GLUT-1 protein expression from normoxic levels after exposure to hypoxia for up to 24 hours. These experimental results confirm that hypoxic regulation of HIF-1 $\alpha$ , GLUT-1 and p53 occurs post-transcriptionally and the variation in response demonstrates the difficulties in working with cell lines.

## Chapter 4.

## Expression of HIF-1a and GLUT-1 In PBMCs Isolated From Patients with T1DM and Exposed to High Glucose

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#### 4.0 Expression of HIF-1α and GLUT-1 in PBMCs Isolated From Patients with T1DM and Exposed to High Glucose

Many of the genes that are induced by hypoxia through HIF-1 $\alpha$ , such as VEGF and GLUT-1, are up-regulated in response to glucose in both experimental and clinical diabetes. Hence the stimulation of glucose transport in the adaptive response of cells and tissues to hypoxia is of particular importance (Zhang, 1999). Moreover, it is known that the cellular redox state determines HIF-1 $\alpha$  binding activity (Haddad and Land 2000; Huang *et al*, 1996) and that hyperglycaemia-induced redox imbalance mimics the effects of hypoxia (Williamson *et al*, 1993).

Presently little is known about the hyperglycaemia-induced expression of HIF-1 $\alpha$  in the PMBCs of patients with or without diabetic complications. The expression profiles of both the *HIF-1\alpha* and *GLUT-1* genes under hyperglycaemic conditions may offer an explanation as to why certain individuals with diabetes develop complications whilst others do not. Further information about HIF-1 DNA binding activity and HIF-1 $\alpha$  and GLUT-1 protein expression levels will also offer additional insight.

One of the aims of this work was to study the expression of HIF-1 $\alpha$  by RT-PCR and quantitate the expression of HIF-1 $\alpha$  and GLUT-1 mRNA in the PBMCs of patients with T1DM exposed to high glucose using the RPA. A probe for HIF-1 $\alpha$  was amplified via RT-PCR from cDNA synthesised from total RNA, which was isolated from the T47D breast carcinoma cell line exposed to hypoxia ( $\leq 1\%$  O<sub>2</sub>) for 24 hours. For both HIF-1 $\alpha$  and GLUT-1 mRNA expression analysis, PBMCs from patients with T1DM and normal healthy controls were exposed to normal glucose (11 mM D-glucose basal level within the media), moderate glucose (supplemented with 10 mM D-glucose) or high glucose (supplemented with 20 mM D-glucose) in PHA stimulated cultures for 5 days.

Another aim of this study was to investigate the HIF-1 DNA binding activity and HIF-1 and GLUT-1 protein expression in cellular and nuclear extracts isolated from

PBMCs of normal controls and patients with T1DM exposed to high glucose. The DNAbinding activity of HIF-1 was analysed by electrophoretic mobility shift assays (EMSA), using a double stranded oligonucleotide probe containing the HIF-1 binding site from the *EPO* enhancer (Semenza & Wang, 1992) that binds both HIF-1 and constitutively expressed factors. Protein expression levels were determined by Western blot analysis using a monoclonal antibody to HIF-1 $\alpha$  and a polyclonal antibody to GLUT-1.

#### 4.1 Clinical Characteristics

The clinical characteristics of all subjects studied for HIF-1 $\alpha$  mRNA expression are listed in Table 4.1 and those studied for GLUT-1 mRNA expression are listed in Table 4.2. Of the 38 patients analysed in the HIF-1 $\alpha$  study, 25 patients had diabetic nephropathy (DN), 27 had diabetic retinopathy and 11 had remained free of microvascular disease (absence of background retinopathy, microalbuminuria and overt neuropathy) after 20 years duration of diabetes (without complications). Of the 39 patients analysed in the GLUT-1 study, 26 patients had diabetic nephropathy, 27 had diabetic retinopathy and 12 had remained free of microvascular disease after 20 years duration of diabetes (without complications). No statistically significant differences were found between the HbA1c and Insulin values between the patient groups in either study group.
Characteristics	Normal	Patients with T1DM			
	Controls	Without Complications	With Complications	All Patients	
Number of subjects	6	11	27	38	
Sex (m:f)	4:2	3:8	11:16	14:24	
Age (years)	35 ± 6.4	<b>42.2</b> ± 15.1	42.5 ± 12.1	42.4 ± 12.8	
(Range)	(27-44)	(27-70)	(17-69)	(17-70)	
Age at onset (years) (Range)	-	17.8 ± 12.9 (2-42)	14.4 ± 10.1 (1-38)	15.4 ± 10.9 (1-42)	
Duration (years) (Range)	-	26.5 ± 11.7 (11-51)	28.2 ± 8.7 (8-43)	27.7±9.6 (8-51)	
HbA1c (Range)	-	8.1 ± 0.8 (7.2–9.8)	8.7 ± 1.58 (6.1–12.4)	8.5 ± 1.4 (6.1–12.4)	
Insulin (Units/kg) (Range)	-	45.1 ± 28.9 (16-118)	41.9 ± 18.5 (10-94)	42.8 ± 21.7 (10-118)	

 
 Table 4.1. Clinical characteristics of normal controls and patients with T1DM studied in the HIF-1α RPA.

Thirty-eight patients with T1DM were considered in this study. Twenty-five of these had diabetic nephropathy, 27 had diabetic retinopathy and 11 had remained free of microvascular disease (absence of background retinopathy, microalbuminuria and overt neuropathy) after 20 years duration of diabetes (without complications). Six normal healthy controls were also studied. The data are expressed as mean values ( $\pm$  standard deviation). Neither the HbA1c values (T-test = 1.61, p = 0.12) nor the Insulin values (T-test = -0.40, p = 0.69) were significantly different between the patient groups.

<b>Table 4.2</b> .	Clinical characteristics of normal controls and all patients with T1DM studied
	in the GLUT-1 RPA.

Characteristics	Normal	Patients with T1DM				
	Controls	Without Complications	With Complications	All Patients		
Number of subjects	7	12	27	39		
Sex (m:f)	4:3	2:10	10:17	12:27		
Age (years)	37.9 ± 7.0	36.3 ± 12.6	37.3 ± 10.6	37.2 ± 11.1		
(Range)	(31-49)	(22-70)	(13-56)	(13-70)		
Age at onset (years)	-	13.0 ± 8.6	13.0 ± 8.6	13.0 ± 8.5		
(Range)		(2-30)	(1-28)	(1-30)		
Duration (years)	-	25.7 ± 11.7	25.3 ± 8.0	25.4 ± 9.1		
(Range)		(11-51)	(8-38)	(8-51)		
HbA1c	-	8.3 ± 1.1	9.1 ± 1.5	8.8 ± 1.4		
(Range)		(6.8-10.1)	(6.4-12.4)	(6.4-12.4)		
Insulin (Units/kg)	-	$53.6 \pm 24.7$	$47.4 \pm 26.0$	$49.3 \pm 25.4$		
(Ivange)		(20-100)	(10-128)	(10-128)		

Thirty-nine patients with T1DM were considered in this study. Twenty-six of these had diabetic nephropathy, 27 had diabetic retinopathy and 12 had remained free of microvascular disease (absence of background retinopathy, microalbuminuria and overt neuropathy) after 20 years duration of diabetes (without complications). Seven normal healthy controls were also studied. The data are expressed as mean values ( $\pm$  standard deviation). Neither the HbA1c values (T-test = 1.89, p = 0.07) nor the Insulin values (T-test = -0.70, p = 0.49) were significantly different between the patient groups.

# 4.2 HIF-1α and GLUT-1 mRNA Expression

The effect of high glucose on inducing the expression of HIF-1 $\alpha$  and mRNA in the PBMCs of patients with T1DM and normal controls was determined by RT-PCR and RPA. RT-PCR was performed using amplimers for an alternative splice variant to HIF-1 $\alpha$  (Gothié *et al*, 2000). In figure 4.1, increased and decreased expression of HIF-1 $\alpha$  can be seen in the PBMCs isolated from patients with T1DM, cultured in normal glucose or high glucose, by the intensity of the amplified DNA which were confirmed by RPA analysis (Figure 4.2).

The optimal experimental culture time for the induction of HIF-1 $\alpha$  mRNA expression was determined by comparing the expression of *HIF-1\alpha* mRNA in the PBMCs of two patients with T1DM cultured in high glucose for 3, 4 or 5 days (**Table 4.3**). As minor variations were observed in the expression of *HIF-1\alpha* mRNA between the culture time periods, 5 days was chosen to obtain maximal quantities of mRNA. The same culture times were used for experiments investigating *GLUT-1* mRNA expression. It was not possible to analyse the same patient population that was studied for *HIF-1\alpha* mRNA expression due to limited amounts of mRNA. Further, PBMCs from 13 patients were only exposed to normal and high glucose and not to moderate glucose due to low yields of PBMCs.





N H H H H Condition N N N H H H N N N ↑ DC ↓ DN 1 DC 1 DC Expression ≅ DN ≅ DN ≅ NC

Figure 4.1. *HIF-1* $\alpha$  mRNA expression in the PBMCs isolated from a normal healthy control and patients with T1DM exposed to normal glucose (N), high glucose (H), analysed by RT-PCR. Amplification products were 487 bp for HIF-1 $\alpha$  amplified product containing exon 14 and 350 bp for HIF-1 $\alpha$  amplified product without exon 14 (M = 100 base pair ladder). Increased and decreased expression of HIF-1 $\alpha$  can be seen by the intensity of the amplified DNA which was confirmed by RPA analysis. Arrows ( $\uparrow\downarrow$ ) indicate increased or decreased expression or ( $\cong$ ) near equivalent expression from normal glucose cultures.

NC = Normal Control, DC = Diabetic Control, DN = Diabetic Nephropath

Table 4.3. *HIF-1* $\alpha$  mRNA expression in the PBMCs isolated from patients with T1DM exposed to normal glucose and high glucose for 3, 4 and 5 days.

High Glucose (20 mM D-glucose)	Fold change in HIF-1α mRNA
3 days	1.39 ± 0.05
4 days	1.29 ± 0.06
5 days	1.24 ± 0.10

Results are expressed as the mean fold increase in mRNA expression above the level obtained under normal conditions (± standard deviation).

An illustration of the expression of *HIF-1* $\alpha$  and *GLUT-1* mRNA in PBMCs isolated from patients with T1DM, as well as normal healthy controls, exposed to normal glucose, moderate glucose and high glucose and analysed by RPA is shown in figure 4.2 A and 4.2B. To control for mRNA quality and loading variations, the level of  $\beta$ -actin expression was determined in the mRNA from the PBMCs of patients with diabetic nephropathy stimulated with either normal glucose, moderate or high glucose by RPA. Expression levels of mRNA were measured as fold increase or decrease in expression (Figure 4.2C). Similar levels of expression between the samples ensure that variations in gene expression are true and not due to loading errors (Hodgkinson *et al*, 2001). Additionally, an 18S rRNA internal control was run on 5 diabetic PBMCs exposed to normal glucose and high glucose, showing that expression was consistent and equal with a mean fold difference of 1.00 ± 0.08 from normal glucose.

Chapter 4. Results



Figure 4.2. A) Illustration of *HIF-1* $\alpha$  and **B**) *GLUT-1* mRNA expression in PBMCs isolated from normal healthy controls and of patients with T1DM that had been exposed to normal glucose (N), moderate (M) and high glucose (H), analysed by RPA. C) Illustration of  $\beta$ -actin mRNA expression in PBMCs isolated from a patient with complications exposed to normal glucose, moderate and high glucose. Similar levels of expression between the samples ensured mRNA quality, equal loading and true gene expression (published in Hodgkinson *et al*, 2001). A 100 bp MW marker was run to ensure correct protected fragment size.

The overall mean levels of HIF-1 $\alpha$  and GLUT-1 mRNA expression in the PBMCs isolated from normal controls, all patients with T1DM as well as their subgroups (patients with and without complications) and exposed to normal glucose, moderate and high glucose are listed in **Table 4.4** and displayed graphically in figures 4.3 and 4.4. The results are expressed as the fold change in mRNA from the level obtained under normal culture conditions. Statistical analyses of the overall mean levels of expression are listed in **Table 4.5**.

In the patients with complications, HIF- $1\alpha$  mRNA expression did not change from normal glucose levels when exposed to moderate glucose,  $1.1 \pm 0.12$  ( $\pm$  standard error), whilst in high glucose it increased slightly by  $1.3 \pm 0.33$  fold. HIF- $1\alpha$  mRNA expression in the normal controls did not change with increasing concentration of D-glucose,  $1.1 \pm$ 0.08 in moderate glucose to  $1.2 \pm 0.20$  in high glucose. Patients without complications demonstrated a similar fold increase in HIF- $1\alpha$  mRNA expression in both moderate glucose,  $2.0 \pm 0.53$ , and high glucose  $2.2 \pm 0.56$ .

*GLUT-1* mRNA expression remained constant in the patient group with complications with increasing concentration of D-glucose,  $1.5 \pm 0.41$  in moderate glucose and  $1.4 \pm 0.20$  in high glucose. Patients without complications demonstrated a decrease in *GLUT-1* mRNA expression from  $2.22 \pm 0.62$  in moderate conditions to  $1.82 \pm 0.39$  in high glucose, however this change was not significant (**Table 4.4 and Figure 4.4**). Moreover, *GLUT-1* mRNA expression was 1.5 fold higher in patients without complications (p= 0.14) then that of patients with complications at moderate glucose and 1.3 fold higher (p= 0.07) at high glucose. Although the expression of *GLUT-1* mRNA was notably lower in the normal controls at high glucose then in the patients with complications, this decrease was not significant (p = 0.80). However, a significant difference in *GLUT-1* mRNA expression was found between normal controls and patients without complications (p=0.03). (**Table** 

4.5).

When all patients with T1DM were analysed together, there was no significant difference in HIF-1 $\alpha$  or GLUT-1 mRNA expression at both concentrations of D-glucose. The increases observed in HIF-1 $\alpha$  mRNA expression in all patients studied at both moderate glucose and high glucose were not significantly different from normal controls. Although GLUT-1 mRNA expression decreased with D-glucose concentration in all patients analysed, the expression was still greater than that found in the normal controls exposed to high glucose.

Chapter 4. Results

<b>Table 4.4</b> .	Overall mean fold change in HIF-1 $\alpha$ and GLUT-1 mRNA expression from normal glucose in PBMCs, isolated from normal controls
	and patients with T1DM, exposed to moderate and high glucose for 5 days.

Glucose	Normal	Controls	Patients v	vithout comp	lications	Patients	with compli	cations	All pat	tients with T	IDM
Concentration	HIF-1a (n=6)	GLUT-1 (n=7)	HIF-1α (n=9)	GLUT-1	p value	HIF-1α (n=29)	GLUT-1	p value	HIF-1α (n=38)	GLUT-1	p value
Moderate (10 mM D-glucose) (Range)	<b>1.1</b> ± 0.08 (0.9-1.4)	N/D	<b>2.2</b> ± 0.56 (0.3-4.6)	(n=6) 2.2 ± 0.62 (0.9-4.5)	p= 0.65	<b>1.1</b> ± 0.12 (0.4-3.5)	(n=20) <b>1.5</b> ± 0.41 (0.2-8.8)	p = 0.59	<b>1.3</b> ± 0.18 (0.3-4.6)	(n=26) 1.7 ± 0.34 (0.2-8.8)	p = 0.44
High Glucose (20 mM D-glucose) (Range)	<b>1.2</b> ± 0.20 (0.7-2.1)	<b>0.9</b> ± 0.10 (0.6-1.2)	<b>2.0</b> ± 0.53 (0.3-4.2)	(n=10) <b>1.8</b> ± 0.39 (0.5-4.0)	p= 0.71	<b>1.3</b> ± 0.33 (0.2-10.2)	(n=29) 1.4 ± 0.20 (0.3-6.1)	p = 0.70	<b>1.4</b> ± 0.28 (0.2-10.2)	(n=39) 1.4 ± 0.19 (0.3-6.1)	p = 0.42
p value	p = 0.75	-	p = 0.77	p = 0.90	-	p= 0.65	p= 0.81	-	p= 0.64	p= 0.55	-

Results are expressed as mean values of the fold increase or decrease in mRNA against the level obtained at normal glucose ( $\pm$  standard error). Statistical analyses were performed using the Mann-Whitney U-test. No statistically significant differences were found within any of the patient groups. Overall mean *HIF-1*  $\alpha$  and *GLUT-1* mRNA levels are expressed graphically in Figures 4.3 and 4.4, respectively. N/D= not done, n= number of subjects.

Table 4.5. Statistical analysis of the overall mean levels of HIF-1 $\alpha$ and GLUT-1	I mRNA expression in PBMCs isolated from normal controls and
patients with T1DM, exposed to moderate and high glucose.	

Statistical Comparison	HI	F-1a	GLUT-1		
	Moderate	High Glucose	Moderate	High Glucose	
Patients with complications vs. Patients without complications	p = 0.20	p= 0.47	p= 0.14	p= 0.07	
Patients with complications vs. Normal controls	p = 0.62	<b>p=</b> 0.62	-	p= 0.80	
Patients without complications vs. Normal controls	p= 0.51	p= 0.80	-	p= 0.03 *	
All patients with T1DM vs. Normal controls	p=0.91	p <del>=</del> 0.80	-	p= 0.41	

Statistical analyses were performed using the Mann-Whitney U test. A p value of <0.05 was considered significant. HIF-1 $\alpha$  and GLUT-1 mRNA expression levels are listed in Table 4.4 and expressed graphically in Figures 4.4 and 4.5, respectively. \*= statistically significant

Chapter 4. Results



Figure 4.3. The mean fold increase of *HIF-1* $\alpha$  mRNA in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate and high glucose. No statistically significant difference was found between culture conditions and patient subgroups (Table 4.4).

Chapter 4. Results



Figure 4.4. The mean fold change in *GLUT-1* mRNA in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate and high glucose. *GLUT-1* mRNA expression levels and patient numbers are listed in Table 4.4. A statistically significant difference was found between the patient group without complications and normal controls (\* p=0.03, Mann-Whitney U test) (Table 4.5).

Analysis of the ratios of HIF-1 $\alpha$  and GLUT-1 mRNA expression in each of the patients groups studied showed 2 distinct trends compared to normal controls (Figures 4.5 and 4.6). Overall, patients with T1DM displayed an increase in HIF-1 $\alpha$  mRNA expression in both moderate and high glucose, similar but higher expression as that seen in the normal controls, yet not significant (Figure 4.5). The overall pattern of GLUT-1 mRNA expression in the patient population was an increase at moderate glucose followed by a decrease in expression at high glucose. The normal controls showed a slight decrease in expression at high glucose (Figure 4.6).

Chapter 4. Results



Figure 4.5. *HIF-1* $\alpha$  mRNA expression in PBMCs isolated from normal controls and patients with T1DM, exposed to normal glucose, moderate, and high glucose. The pattern of *HIF-1* $\alpha$  mRNA expression is charted, expressed as fold change, illustrating both distinct and interesting trends between the control and patient groups, and between the two patient groups with increasing only and decreasing only trends. Results are expressed as the mean fold increase or decrease in mRNA expression from the level obtained under normal conditions. There is no significant difference between normal controls and all patients with T1DM studied. n = number of subjects.

Chapter 4. Results



**Figure 4.6**. *GLUT-1* mRNA expression in PBMCs of normal controls and patients with T1DM exposed to normal glucose, moderate, and high glucose. The pattern of *GLUT-1* mRNA expression is charted, expressed as fold change, illustrating both distinct and interesting trends between the control and patient groups, and between the two patient groups with increasing only and decreasing only trends. Results are expressed as the mean fold increase or decrease in mRNA expression from the level obtained under normal conditions.

n = number of subjects

# 4.3 Basal Levels of mRNA Expression

The basal level of *HIF-1* $\alpha$  mRNA was found to be lower in the patients compared to the normal controls in all culture conditions after 5 days (Figure 4.7). The former had approximately 1.4 fold lower *HIF-1* $\alpha$  mRNA expression in normal, moderate or high glucose conditions when compared with normal controls. Although no statistically significant differences were observed between the control and patient groups, a near significant difference was observed between the control and patient groups exposed to moderate glucose levels (p=0.05; Mann-Whitney U test).

The basal level of *GLUT-1* mRNA expression was significantly lower in the patients with T1DM compared to the normal controls (Figure 4.8). Normal controls had a 1.81 fold higher *GLUT-1* mRNA expression in normal glucose (p=0.020; Mann-Whitney U test) and a 1.63 fold higher expression in high glucose (p=0.04; Mann-Whitney U test).

### Chapter 4. Results



Figure 4.7. Basal *HIF-1* $\alpha$  mRNA expression in the PBMCs isolated from healthy normal controls and patients with T1DM exposed to normal glucose, moderate and high glucose for 5 days. *HIF-1* $\alpha$  mRNA expression in PBMCs of normal controls was found to be 1.4 fold higher then those of patients with diabetes which is independent of glucose concentration. No statistically significant differences were found between the control and patient groups at all D-glucose concentrations (Mann-Whitney U-test).

Normal glucose: p = 0.18, Moderate glucose: p = 0.05, High glucose: p = 0.11



Figure 4.8. Basal *GLUT-1* mRNA expression in PBMCs isolated from normal controls and patients with T1DM exposed to normal glucose and high glucose for 5 days. *GLUT-1* mRNA expression in PBMCs of normal controls was found to be significantly higher than those of patients with diabetes at normal glucose and high glucose, 1.81 and 1.63 fold, respectively (Mann-Whitney U test). \* = statistically significant

Normal glucose (normal controls vs patients with T1DM): p = 0.02\*High glucose (normal controls vs patients with T1DM): p = 0.04\*

# 4.4 Osmolarity and High Glucose Controls

To control for osmolarity and high glucose, and to determine whether the increased or decreased expression of *HIF-1* $\alpha$  and *GLUT-1* mRNA was specific for D-glucose the PBMCs from 2 patients with nephropathy were stimulated with either L-glucose, which competes with D-glucose for the transporter, or mannitol, an osmolyte. In the first, 20 mM mannitol was used as a supplement with the 20 mM D-glucose. A second, parallel culture consisted of 20 mM L-glucose being used as a supplement with the 20 mM D-glucose.

When the tissue culture media containing the PBMCs isolated from patients with T1DM was supplemented with either 20 mM L-glucose or 20 mM mannitol, a 1.3 fold increase was seen in the expression of *HIF-1* $\alpha$  mRNA (Table 4.6). In cultures supplemented with 20 mM D-glucose alone, one patient displayed a 1.4 fold increase and the other a 1.2 fold decrease in *HIF-1* $\alpha$  mRNA expression. The mean of the two values for each experimental culture demonstrated that the supplements had no effect on *HIF-1* $\alpha$  mRNA expression.

GLUT-1 mRNA expression was also quantitated in the same cultures where GLUT-1 mRNA was induced by D-glucose, L-glucose and mannitol in one patient and by Dglucose and mannitol in the other (Table 4.6). As with HIF-1 $\alpha$  mRNA expression, the supplements had no effect on GLUT-1 mRNA expression.

# **Table 4.6**. Fold change in the levels of HIF-1 $\alpha$ and GLUT-1 mRNA in the PBMCs of patients with complications exposed to high glucose and supplemented with either 20 mM L-glucose or 20 mM mannitol.

Culture	Fold change in <i>HIF-1 a</i> mRNA			Fold change in <i>GLUT-1</i> mRNA		
conditions	DN 1	DN 2	Mean (± SE)	DN 1	DN 2	Mean (± SE)
High Glucose (20 mM D-glucose)	1.36	0.81	1.1±0.28	1.74	1.15	1.5 ± 0.30
L-glucose (20 mM D-glucose + 20 mM L-glucose)	1.32	0.98	1.2 ± 0.17	0.7	1.14	0.9 ± 0.22
Mannitol (20 mM D-glucose + 20 mM mannitol)	1.19	1.22	1.2 ± 0.02	1.03	1.28	1.2 ± 0.13

Results are expressed as the fold change in mRNA against the level obtained at normal glycaemia. SE = standard error

# 4.5 Measurement of Lactic Acid

The concentration of lactate was determined in the supernatant of cultures of PBMCs isolated from 4 patients without diabetic complications, 2 patients with diabetic complications and from 2 normal controls exposed to normal glucose and high glucose (**Tables 4.7**). Basal levels of lactate were near equivalent in both normal glucose and high glucose cultures in the normal control and in the patients with diabetic complications. In the patients without complications, 3 of the 4 patients displayed increased levels of lactate (up to 1.3 fold) in high glucose. Of the 6 cultures of PBMCs isolated from patients with T1DM, 4 displayed higher levels of lactate than the normal control. The mean concentration of lactate in the 6 cultures of PBMCs isolated from patients with T1DM was 1.3 fold higher than the normal control after exposure to both normal glucose and high glucose. None of these increases were statistically significant.

 Table 4.7. Measured lactate concentrations (mmol/L) in the culture media of PBMCs isolated from a normal control and from patients with T1DM exposed to high glucose.

PBMCs	Measurement	Culture Conditions		
Cultured		Normal glucose	High glucose	
	OD (340 nm)	1.34	1.45	
DC	Lactate Conc. (mmol/L)	13.67	14.82	
	OD (340 nm)	1.33	1.27	
DC	Lactate Conc. (mmol/L)	13.57	12.95	
	OD (340 nm)	1.38	1.44	
DC	Lactate Conc. (mmol/L)	14.10	14.66	
DC	OD (340 nm)	0.78	1.00	
	Lactate Conc. (mmol/L)	7.93	10.17	
DN	OD (340 nm)	0.99	0.97	
	Lactate Conc. (mmol/L)	10.10	9.86	
	OD (340 nm)	1.42	1.44	
DN	Lactate Conc. (mmol/L)	14.48	14.66	
Mean (±SE)	Lactate Conc. (mmol/L)	12.0 ± 0.96	12.5 ± 0.87	
	OD (340 nm)	1.01	1.01	
Normal	Lactate Conc. (mmol/L)	10.35	10.35	

Lactate concentrations were determined as described in chapter 2 (section 2.11.1.3).

DC = Diabetic Control, DN = Diabetic Nephropath. SE = standard error.

## 4.6 HIF-1 DNA Binding Activity

HIF-1 DNA-binding activity was observed in nuclear extracts prepared from the PBMCs of normal controls and patients with T1DM exposed to high glucose (Figure 4.9). Decreased HIF-1 binding activity, from the level observed at normal culture conditions, was seen in both the normal control and in the patient extracts with near equivalent levels seen in both groups (Table 4.8; Figure 4.10).

In competition assays, HIF-1 bands induced by exposure to high glucose were displaced by the addition of excess unlabelled probe (up to 6 fold excess) in the extracts from cultured PBMCs of normal controls and patients with T1DM under both normal glucose and high glucose, suggesting that these bands are specific for the HIF-1 binding site (Figure 4.9). Attempts to supershift the HIF-1 DNA complexes with the addition of a monoclonal antibody to HIF-1 proved unsuccessful, however the observed high glucose induced DNA-binding activity was due to HIF-1 as the binding complex was knocked out in the competition assays.

<b>Table 4.8</b> .	Mean levels of HIF-1 DNA binding activity in nuclear extracts from the
	PBMCs of normal controls and patients with T1DM exposed to high
	glucose.

Experimental Condition	Normal Controls (n=2)	Patients with T1DM (n=3)
High glucose (20 mM D-glucose)	$0.87\pm0.07$	$0.89 \pm 0.06$
High glucose + excess unlabelled probe	$0.58 \pm 0.02$	$0.21 \pm 0.04$
P value	T=4.35, p=0.049	T = 9.26, p = 0.03

Results are expressed as the mean value ( $\pm$  standard error) of the fold change in HIF-1 DNA-binding activity in nuclear extracts isolated from cultures exposed to high glucose compared to the level obtained under normal culture conditions. Results are expressed graphically in **figure 4.10**.



DN	DN	DN	NC	NC

Figure 4.9. EMSA of nuclear extracts prepared from the PBMCs of normal controls and patients with T1DM that had been exposed to normal glucose and high glucose. 15  $\mu$ g of nuclear protein was analysed. HIF-1 binding complex (HIF-1) bands are indicated. The addition of excess unlabelled probe in competition assays displayed HIF-1 binding complexes. Results are displayed graphically in figure 4.10. N= Normal glucose, H = High glucose, H+E = High glucose + Excess unlabelled probe, DN= Diabetic Nephropath, NC = Normal Control.

#### Chapter 4. Results



**HIF-1 Binding Activity** 

**Figure 4.10**. Graphical expression of HIF-1 DNA binding activity in nuclear extracts from the PBMCs isolated from normal controls and patients with T1DM exposed to high glucose for 5 days. HIF-1 DNA-binding activity decreased after exposure to high glucose in both cultures with near equivalent activity. The addition of excess unlabelled probe displaced the binding activity. Results are expressed as the value of the fold change in HIF-1 DNA-binding activity in nuclear extracts isolated from cultures exposed to high glucose compared to the level obtained under normal glucose.

# 4.7 HIF-1α and GLUT-1 Protein Expression

Methodological difficulties with Western blotting prevented the assessment of HIF-1 $\alpha$  protein expression in the PBMCs of patients with T1DM and normal controls (see **chapter 3, section 3.13** for initial experimental results).

However, the effect of high glucose on inducing the expression of GLUT-1 protein in the PBMCs of patients with T1DM and normal controls was determined by Western blotting (**Table 4.9**; **Figures 4.11 and 4.12**). Exposure to high glucose was found to increase GLUT-1 protein expression by up to 1.4 fold in protein extracts isolated from the PBMCs of 2 normal controls, and decrease GLUT-1 protein expression by half in protein extracts isolated from the PBMCs of a patient without complications and of a patient with complications.

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Figure 4.11. Western blot analysis of GLUT-1 protein expression in the PBMCs isolated from normal controls and from patients with T1DM exposed to high glucose. PBMCs were exposed for 5 days to high glucose, cellular proteins (30  $\mu$ g/lane) were separated by 12 % SDS-Page, blotted on nitrocellulose paper and probed with a polyclonal anti-GLUT-1 antibody. A 55 kDa band was observed and quantified (Table 4.9). N = Normal glucose, H= High glucose, DC = Diabetic Control, DN = Diabetic Nephropath, NC = Normal Control.

 Table 4.9. Expression of GLUT-1 protein in extracts isolated from the PBMCs of normal controls and patients with T1DM exposed to high glucose for 5 days.

High glucose (20 mM D-glucose)	GLUT-1 fold protein expression	Mean (± SE)	
Normal control 1	1.43	1.28 ± 0.15	
Normal control 2	1.13		
Diabetic Control	0.51	0.49 ± 0.03	
Diabetic Nephropath	0.46		

Results are expressed as the fold change in GLUT-1 protein expression in extracts isolated from cultures exposed to high glucose compared to the level obtained under normal glucose ( $\pm$  standard error). Results are expressed graphically in figure 4.12.

Normal controls vs. patients with T1DM : T-test = 5.23, p = 0.03

Chapter 4. Results



**Figure 4.12**. GLUT-1 protein expression in extracts isolated from the PBMCs of normal controls and from patients with T1DM exposed to normal glucose and high glucose for 5 days. The expression of GLUT-1 protein in the PBMCs of patients with T1DM exposed to high glucose decreased to half the level expressed under normal glucose whilst the GLUT-1 protein expression in normal controls increased up to 1.4 fold when exposed to high glucose. Results are expressed as the mean fold change in GLUT-1 protein expression in PBMCs exposed to high glucose compared to the level obtained under normal glucose.

#### 4.8 Conclusion

In this study, the expression of  $HIF-1\alpha$  and GLUT-1 mRNA in the PBMCs of patients with T1DM increased upon exposure to D-glucose. Although results showed that the patients without complications displayed a greater fold increase in mRNA expression for both genes, above that of normal controls and of patients with complications, this increase was only significant in GLUT-1 mRNA expression. The experiments controlling for osmolarity determined that this response was not specific for D-glucose and was probably due to differences in osmolarity as exposure of PBMCs to L-glucose and mannitol had no effect on mRNA expression. It is possible that the PBMCs from patients with T1DM may be preconditioned to hyperglycaemia and that the expression and function of glucose transporters differs between patients with and without complications.

A slight but non-significant increase in basal lactate levels was observed in cultures of PBMCs from patients with T1DM than in cultures of PBMCs from normal healthy controls, indicating that increased glycolysis occurs in patients with T1DM. Exposure to hyperglycaemia does not seem to further up-regulate glycolysis in these patients nor in normal controls. A possible explanation for this may be that the glucose receptors are saturated due to the high concentration of D-glucose in the cultures, thereby producing a slower observed response.

Although HIF-1 binding activity was observed in the PBMCs from normal healthy controls and from patients with T1DM, the binding activity decreased after exposure to high glucose. The observed HIF-1 binding was specific as it could be inhibited effectively with excess unlabelled probe in competition experiments.

Methodological difficulties with Western blotting prevented the assessment of HIF-1 $\alpha$  protein expression, however Western blot analysis of protein extracts from the PBMCs of normal controls exposed to high glucose displayed an increase in GLUT-1 protein expression whilst those from patients with T1DM exposed to high glucose showed a dramatic decrease in GLUT-1 protein expression. This would imply that the induction of *GLUT-1* mRNA is not mediated at the transcriptional level in patients with T1DM. As these initial results were obtained from analysis of only 2 patients with T1DM, one with and one without complications, further experiments are required on a larger patient population to elucidate the response seen.

This novel preliminary report suggests that HIF-1, in conjunction with GLUT-1, may be an important regulator of the cellular response to hyperglycaemia as abnormal expression of HIF-1 $\alpha$  and its target genes, through activation by hyperglycaemia, may contribute to diabetic complications. Further studies of subsequent gene and protein regulation by HIF-1 $\alpha$  are required as the molecular basis of the cellular response to hypoxia and hyperglycaemia has yet to be determined.

# Chapter 5.

# Expression of HIF-1 $\alpha$ and GLUT-1 In Tumours of Patients with Breast Cancer

# 5.0 Expression of HIF-1α and GLUT-1 in Tumours of Patients with Breast Cancer

 $HIF-1\alpha$  and GLUT-1 mRNA expression was assessed in the total RNA extracted from 22 tumour samples from patients with breast cancer by RT-PCR and RPA. HIF-1 $\alpha$ amplifications were performed using amplimers for a recently discovered alternative splice variant to HIF-1 $\alpha$  (Gothié *et al*, 2000). HIF-1 protein expression was assessed by immunocytochemistry, using a monoclonal antibody to the  $\alpha$  subunit (HIF-1 $\alpha$ ).

# **5.1 Clinical Characteristics**

The clinical characteristics of the 22 patients from which these tumours were derived are listed and summarised in **Tables 5.1 and 5.2**. The mean age of diagnosis was  $66.7 \pm 15.8$  years (42-91 years). The majority of patients had advanced T stage (31.8% T<sub>2</sub>. 3), grade (95.5% G<sub>2-3</sub>) or positive node stage (56.4% N<sub>1-3</sub>).

Characteristics	Patients with Breast Tumours
Number of subjects	22
Age at Diagnosis (years) (Range)	66.7 ± 15.8 (42-91)
Survival (years) (Range)	3.44 ± 1.4 (1.2-8.0)
Survival (weeks) (Range)	179.1 ± 72.9 (59.7-417.2)

Table 5.1. Clinical characteristics of patients with breast tumours.

The data are expressed as mean values ( $\pm$  standard deviation).
Table 5.2.	Frequencies	of the	clinicopa	thological	characteristics	of pa	tients	with	breast
	tumours								

Variable	Frequency (n)
Patients	100 (22)
Age (median)	
<66	45.5 (10)
≥66	54.5 (12)
T stage	
1	68.2 (15)
2	31.8 (7)
3	0 (0)
4	0 (0)
N stage	
0	45.5 (10)
1	36.4 (8)
2	9.1 (2)
3	9.1 (2)
Grade	
1	4.5 (1)
2	36.4 (8)
3	59.1 (13)

n = actual number of patients (in parenthesis).

T = tumour stage, N = node stage

### 5.2 HIF-1a and GLUT-1 mRNA Expression

The expression of *HIF-1* $\alpha$  mRNA was analysed by RT-PCR using amplimers for a recently discovered alternative splice variant to HIF-1 $\alpha$  (Gothié *et al*, 2000) (Figure 5.1). Only 1 µg of total RNA was available for cDNA synthesise, due to a low yield of total RNA from limited tissue samples, thus the assay was not performed under optimal conditions. When *HIF-1\alpha* mRNA expression was detected, the intensity of the amplified band ranged from very faint to strong resulting in 13 of 22 tumours being positive for *HIF-1\alpha* mRNA expression.

*HIF-1* $\alpha$  and *GLUT-1* mRNA expression was also analysed by RPA (**Table 5.3**, **Figure 5.2**). RT-PCR results were not entirely confirmed by this additional analysis, not due to the technique but rather due to a lack of cDNA available for analysis in the RT-PCR assay. As RPA is an extremely sensitive and quantitative technique, this method of analysis for mRNA expression is more likely to be accurate. Results are expressed as the fold increase in expression from the lowest expression level (**Table 5.3**). As no normal control tissue was available for analysis a visual cut off point of 0.40 for band intensity was taken as positive expression, thus values in bold indicate increased expression of the respective gene and are at least 2.5 fold higher then the lowest expression level. Both genes were moderately overexpressed in the breast tumours but the incidence and amplitude of overexpression varied. Of the 22 tumours analysed by RPA, 13 were considered positive for *HIF-1* $\alpha$  mRNA expression and 15 were considered positive for *GLUT-1* mRNA expression. Although the majority of tumours showed overexpression of both or neither of the two genes, 11 of the 22 tumours expressed significantly higher levels of *HIF-1* $\alpha$  of *GLUT-1* mRNA only.





Figure 5.1. *HIF-1* $\alpha$  and  $\beta$ -actin mRNA expression in tumours from patients with breast cancer. Amplification products were 487 bp for HIF-1 $\alpha$  amplified product containing exon 14, 350 bp for HIF-1 $\alpha$  amplified product without exon 14 and 661 bp for  $\beta$ -actin. (M = 100 base pair ladder). Lanes 3, 4, 7, 8 and 9 were scored positive for HIF-1 $\alpha$  (weak and strong expression).  $\beta$ -actin expression demonstrates the quality of cDNA and equal sample loading. The band products were quantified and are listed in Table 5.3.

Chapter 5. Results



HIF-1 and GLUT 1

Figure 5.2. The fold change in *HIF-1* $\alpha$  and *GLUT-1* mRNA expression in tumours from patients with breast cancer as determined from RPA analysis. Fold change represents the fold increase in expression from the lowest expression level in all patients, as no normal control tissue was available for analysis. Of the 22 tumours analysed, 13 were considered positive for *HIF-1* $\alpha$  mRNA expression and 15 were considered positive for *GLUT-1* mRNA expression using RPA analysis. Expression levels are listed in **Table 5.3**.

Chapter 5. Results

Table 5.3. HIF-1 $\alpha$ and GLUT-1 mRNA expression and HIF-1 $\alpha$ protein expression	sion in
tumours from patients with breast cancer.	

Patient #	Tumour Stage	HIF mRNA (RT-PCR)	HIF mRNA (RPA)	Fold Change	HIF Protein	GLUT-1 mRNA (RPA)	Fold Change
1	T1N0G1	0.44	0.11	1.00	++	1.72	10.75
2	T2N0G3	1.58	0.12	1.09		1.84	11.50
3	T2N1G3	0.01	0.6	5.45		0.88	5.50
4	T2N2G3	0.16	0.64	5.82		0.34	2.13
5	T1N0G3	0.15	0.94	8.55	- (c)	0.39	2.44
6	T1N1G3	0.71	2.05	18.64		0.4	2.50
7	T2N1G3	0.85	1.87	17.00		1.64	10.25
8	T1N1G2	0.56	0.41	3.73		0.83	5.19
9	T1N1G2	0.55	0.33	3.00	- (c)	1	6.25
10	T1N0G2	0.42	0.23	2.09	- (c)	0.16	1.00
11	T2N3G3	0.41	2.22	20.18	- (c)	4.41	27.56
12	T1N0G2	0.38	0.38	3.45		0.27	1.69
13	T2N1G2	0.36	0.39	3.55		0.54	3.38
14	T2N3G3	1.01	6.36	57.82	- (c)	9.9	61.88
15	T1N0G3	0	0.61	5.55	- (c)	0.5	3.13
16	T1N1G3	0.06	0.42	3.82	- (c)	2.25	14.06
17	T2N0G3	1.27	1.37	12.45	++	6.69	41.81
18	T1N0G2	1.2	0.94	8.55	+	6.9	43.13
19	T1N0G3	0.36	0.17	1.55	- (c)	0.33	2.06
20	T1N1G2	0.36	-	-	- (c)		-
21	T1N2G2	0.63	0.27	2.45	+	0.28	1.75
22	T1N0G3	1.28	0.94	8.55	+	4.23	26.44

RT-PCR and RPA expression levels are given as raw values minus background. Fold change represents the fold increase in expression from the lowest expression level (i.e. 0.11 for HIF-1 $\alpha$  mRNA, patient 1). For RT-PCR, values in bold indicate a visual band. As no normal control tissue was available for analysis a visual cut off point of 0.40 for RPA band intensity was taken as positive expression, thus values in bold indicate increased expression of the respective gene. Spearman's correlation between HIF-1 $\alpha$  and GLUT-1 mRNA expression was significant for analyses by RT-PCR (p=0.02) and by RPA (p=0.03).

For immunohistochemical analysis:

(-) = negative nuclear staining/expression , (- (c)) = negative nuclear staining but strong cytoplasmic staining, (+) = local/weak nuclear staining, (++) = strong nuclear staining

*HIF-1* $\alpha$  and *GLUT-1* mRNA expression was analysed statistically 2 ways: as numerical values which included all expression values (one group), and as categorical values which were divided into 2 groups, tumours which overexpressed or did not overexpress either mRNA. Analysis of all expression values in the tumours showed that there was a significant association between *HIF-1* $\alpha$  and *GLUT-1* mRNA expression (Spearman's non-parametric correlation p=0.03; Table 5.4). This association was lost when the mRNA expression was categorised (p=0.09). Additionally, a significant association was also found between *HIF-1* $\alpha$  mRNA expression and age (p=0.04), T stage (p=0.02), N stage (p=0.03) and grade (p=0.02). No associations were found with *GLUT-1* mRNA expression in the tumours using this analysis. When expression levels were categorised, the association between *HIF-1* $\alpha$  mRNA expression and T stage was lost (p=0.01), however an association was found between *GLUT-1* mRNA expression and age (p=0.03) and N stage (p=0.03) (**Table 5.4**). No significant association was found between *HIF-1* $\alpha$  or *GLUT-1* mRNA expression and HIF-1 $\alpha$  protein expression. Table 5.4. Association between disease variables and  $HIF-1\alpha$  and GLUT-1 mRNA expression and HIF-1 $\alpha$  protein expression in the tumours of 22 patients with breast cancer.

Analysed as	Test variable with corresponding p value (p)					
numerical variables (All expression levels)	<i>HIF-1α</i> mRNA expression	GLUT-1 mRNA expression	HIF-1a protein expression			
$HIF-1\alpha$ mRNA		0.47 <sup>a</sup> , p=0.03*	$-0.01^{a}$ , p = ns			
GLUT-1 mRNA	0.47°, p=0.03*	-	$0.34^{a}$ , p= ns			
HIF-1a protein	$-0.01^{a}, p = ns$	$0.34^{a}, p = ns$	-			
Survival (years)	$-0.23^{a}$ , p = ns	$-0.01^{a}, p = ns$	$-0.04^{a}$ , p = ns			
Age	-0.45°, p=0.04*	$-0.34^{a}, p = ns$	$0.03^{a}, p = ns$			
T stage	5.36°, p=0.02*	$2.01^{\circ}, p = ns$	$-0.27^{\circ}$ , p = ns			
N stage	6.75°, p=0.03*	$2.30^{\circ}, p = ns$	$-0.27^{\circ}$ , p = ns			
Grade	3.50°, p=0.02*	$0.21^{\circ}, p = ns$	-0.19°, $p = ns$			
Analysed as categorical variables (Overexpressed vs. not overexpressed)	HIF-1α mRNA expression	GLUT-1 mRNA expression	HIF-1α protein expression			
HIF-1 a mRNA	-	$2.91^{\rm d}, p = \rm ns$	$0.002^{d}$ , p = ns			
GLUT-1 mRNA	$2.91^{d}, p = ns$		$0.08^{\rm d}, p = ns$			
HIF-1a protein	$0.002^{d}, p = ns$	$0.08^{d}, p = ns$	-			
Survival (years)	$47.5^{\rm b}, p = {\rm ns}$	$29.0^{\rm b},  {\rm p} = {\rm ns}$	$0.20^{\rm b},  {\rm p} = {\rm ns}$			
Age	$23.5^{b}$ , p = 0.04*	$17.5^{\rm b}, p = 0.03*$	$0.001^{\rm b}$ , p = ns			
T stage	$2.52^{d}, p = ns$	$1.05^{\rm d},  {\rm p} = {\rm ns}$	$1.92^{\rm d}, p = {\rm ns}$			
N stage	$2.22^{d}, p = ns$	$9.24^{\rm d}, p = 0.03*$	$0.27^{\rm d},  {\rm p} = {\rm ns}$			
Grade	$7.77^{\rm d}, p = 0.02*$	$1.12^{d}, p = ns$	$0.60^{\rm d},  {\rm p} = {\rm ns}$			

Non-parametric analysis was performed using <sup>a</sup>Spearman's coefficient of correlation, <sup>b</sup>Mann-Whitney U, <sup>c</sup>Kruskal-Wallis H and <sup>d</sup>Pearson Chi-square tests. ns = not significant \* = statistically significant

## 5.3 HIF-1α Protein Expression

Immunohistochemical analysis of HIF-1 $\alpha$  protein expression was performed using antibodies to HIF-1 $\alpha$  that had just become commercially available, which had not yet been validated for immunohistochemical analysis. An immense amount of time was spent optimising and validating this procedure of which the results of at least four separate and consistent analyses are described below. All slides were assessed by a neuropathologist (Dr. David Hilton).

Tumour cell immunoreactivity was scored according to the nuclear staining. Both the extent of staining (relative number of HIF-1 $\alpha$  positive cells) and the intensity of the reaction were taken into account: (-) = negative nuclear staining/expression, -(c) = negative nuclear staining but strong cytoplasmic staining, (+) = local/weak nuclear staining, (++) = strong nuclear staining. For purposes of statistical analysis, HIF-1 $\alpha$  observed immunoreactivity was classified as 3 grades of staining: 0 = negative nuclear staining, 1 = strong cytoplasmic staining, and 2 = positive nuclear staining. Immunohistochemical results for HIF-1 $\alpha$  protein expression in the tumours from patients with breast cancer are shown in **Table 5.3**.

Of the 22 samples analysed, strong HIF-1 $\alpha$  immunoreactivity was seen in 2/22 tumours (Figure 5.3), 3/22 tumours displayed weak HIF-1 $\alpha$  immunoreactivity and 17 were negative for expression. Of the 16 scored negative for HIF-1 $\alpha$  expression, 9 specimens (40.9 %) displayed strong cytoplasmic staining. Cytoplasmic staining consisted primarily of a granular staining pattern distributed throughout the cell cytosol. Of the 5 samples which displayed HIF-1 $\alpha$  immunoreactivity, 3 also overexpressed *HIF-1\alpha* and *GLUT-1* mRNA. No significant association was found between HIF-1 $\alpha$  protein expression and *HIF-1\alpha* or *GLUT-1* mRNA expression or with clinicopathological characteristics (Table 5.4).



Figure 5.3. Immunohistochemical analysis of HIF-1 $\alpha$  protein expression in tumours from patients with breast cancer. Strong nuclear immunoreactivity is seen infiltrating the tumour margin (X150) (haematoxylin counterstain).

Table 5.5.	Univariate and multivariate analysis of cumulative overal	survival	of 22
	patients with breast cancer.		

Univariate Analysis	Hazard Ratio	95 % CI	p value
HIF-1 $\alpha$ (negative vs. positive mRNA)	0.02	1.52x10 <sup>-6</sup> -304.9	ns
GLUT-1 (negative vs. positive mRNA)	1.27	0.12-13.99	ns
HIF-1α (negative vs. positive protein)	3.48	0.32-38.7	ns
Age (continuous)	0.97	0.90-1.05	ns
T stage (T <sub>1-2</sub> )	0.04	2.35x10 <sup>-8</sup> -549.7	ns
N stage (N <sub>0-3</sub> ) N <sub>0</sub> vs. N <sub>1</sub> N <sub>0</sub> vs. N <sub>2</sub> N <sub>0</sub> vs. N <sub>3</sub>	61532 310102 390704	8.54x10 <sup>-185</sup> -4.43x10 <sup>193</sup> 4.30x10 <sup>-184</sup> -2.23x10 <sup>194</sup> 5.42x10 <sup>-184</sup> -2.82x10 <sup>194</sup>	ns ns ns ns
<b>Grade (G<sub>1-3</sub>)</b> G <sub>1</sub> vs. G <sub>2</sub> G <sub>1</sub> vs. G <sub>3</sub>	0.02 0.02	1.24x10 <sup>-14</sup> -3.13x10 <sup>10</sup> 9.03x10 <sup>-7</sup> -429.6	ns ns ns
Multivariate Analysis	Hazard Ratio	95 % CI	p value
<i>HIF-1</i> $\alpha$ (negative vs. positive mRNA)	0.03	1.11x10 <sup>-254</sup> -1.07x10 <sup>251</sup>	ns
GLUT-1 (negative vs. positive mRNA)	608	1.03x10 <sup>-168</sup> -3.58x10 <sup>178</sup>	ns
HIF-1α (negative vs. positive protein)	1.40	3.80x10 <sup>-73</sup> -5.17x10 <sup>72</sup>	ns
Age (continuous)	0.979	0.88-1.08	ns
T stage (T <sub>1-2</sub> )	6.89x10 <sup>-4</sup>	1.43x10 <sup>-35</sup> -3.32x10 <sup>28</sup>	ns
N stage (N <sub>0-3</sub> )			ns
$N_0$ vs. $N_1$	1.98x10 <sup>-4</sup>	5.83x10 <sup>-102</sup> -6.72x10 <sup>93</sup>	ns
N <sub>0</sub> vs. N <sub>2</sub>	0.74	0.04-12.59	ns
$N_0$ VS. $N_3$	0.01	1.29x10 <sup>-173</sup> -4.72x10 <sup>168</sup>	ns
Grade (G <sub>1-3</sub> )			ns
$G_1$ vs. $G_2$	2314	0-0	ns
$G_1$ vs. $G_3$	0.02	8.86x10 <sup>-252</sup> -6.32x10 <sup>247</sup>	ns

Hazard ratios (HR), p values and 95 % confidence intervals (CI) are indicated. Multivariate analysis was performed using a backward elimination procedure to remove variables with  $p \ge 0.10$ . The qualifying criteria for inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis. ns = not significant

### 5.4 Survival

Survival of the 22 patients with breast cancer from whom tumours were accessed was followed up, with overall survival being measured from the time of diagnosis to the date of death or until October 2001. Two groups of patients were formed with regard to *HIF-1* $\alpha$  and *GLUT-1* mRNA expression: positive or negative expression for *HIF-1* $\alpha$  and *GLUT-1* mRNA. Univariate analysis of overall survival was performed by Kaplan-Meier analysis (Kaplan and Meier, 1985). Although statistical power was limited because of the relatively low number of cases, the Cox proportional hazards model was used for multivariate analysis. For all tests, a p value of less than or equal to 0.05 was considered as significant. All p values are results of two-sided tests.

All but 3 patients remained alive at the time of follow up and all patients had an overall survival time averaging  $3.44 \pm 1.4$  years  $(179.1 \pm 72.9 \text{ weeks})$  (Table 5.1). Univariate and multivariate analysis of the patient group showed that none of the disease variables were significantly associated with survival in the patient group (Table 5.5). This was expected due to the small test group and the restricted variety in tumour grading.

Kaplan-Meier analysis revealed a significant influence of T stage (log-rank test, p=0.006; Figure 5.4) and N stage (log-rank test, p=0.009; Figure 5.5) on cumulative overall survival, where patients with a T stage of T1 surviving longer than patients with a T stage of T<sub>2</sub> and patients with a N stage of N<sub>0</sub> or N<sub>1</sub> surviving longer than patients with a N stage of N<sub>2</sub> or N<sub>3</sub>. Tumour grade did not show an influence due to the small number of patients with a low G<sub>1</sub> grade (n=1) (log-rank test, p=0.32; Figure 5.6). No significant influence from either *HIF-1* $\alpha$  or *GLUT-1* positive or negative mRNA expression was found on the cumulative overall survival in the patient group (log-rank test, p=0.16 and 0.85 respectively; Figures 5.7 and 5.8), or from HIF-1 $\alpha$  protein expression (log-rank test, p=0.47, Figure 5.9).

Chapter 5. Results



Figure 5.4. Cumulative overall survival of patients with breast cancer (n=22) differentiated by tumour stage (T stage) and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank test 7.57, p = 0.006\*.

\* = statistically significant

Chapter 5. Results



Figure 5.5. Cumulative overall survival of patients with breast cancer (n=22) differentiated by node stage (N stage) and analysed by Kaplan-Meier. N stages were grouped in order to produce the survival plot as the majority of patients remained alive at the time of analysis. Overall survival is defined from the day of diagnosis until day of death. Log rank test 6.87, p = 0.009\*.

\* = statistically significant

Chapter 5. Results



Figure 5.6. Cumulative overall survival of patients with breast cancer (n=22) differentiated by grade and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank test 2.26, p = 0.32.



Figure 5.7. Cumulative overall survival of patients with breast cancer (n=22) with negative mRNA expression of *HIF-1* $\alpha$ , compared with patients with positive mRNA expression of *HIF-1* $\alpha$ , determined by RPA and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank test 2.01, p = 0.16.

Chapter 5. Results



Figure 5.8. Cumulative overall survival of patients with breast cancer (n=22) with negative mRNA expression of *GLUT-1*, compared with patients with positive mRNA expression of *GLUT-1*, determined by RPA and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank test 0.04, p = 0.85.

Chapter 5. Results



Figure 5.9. Cumulative overall survival of patients with breast cancer (n=22) with negative HIF-1 $\alpha$  nuclear protein expression, compared with patients with either strong HIF-1 $\alpha$  cytoplasmic protein expression or positive HIF-1 $\alpha$  nuclear protein expression, determined by immunohistochemistry and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank test 1.5, p = 0.47.

The Nottingham Prognostic Index (NPI) was also assessed in the patient group (see **Chapter 2, section 2.12.6**). Calculated NPI's in the 22 patients with breast cancer in this study showed that the majority of subjects were in the range of the moderate prognostic group (63.6 %) (**Table 5.6**). Interestingly, all 3 of the patients who succumbed to the disease were determined to be in the poor prognostic group and had an average survival time of 2.14  $\pm$  0.90 years. A significant correlation was found between survival and NPI (Spearman's coefficient of correlation -0.466, p=0.03), with a hazard ratio of 7.96, p = 0.03 (1.24-50.92; 95% CI), log rank 18.36, p=0.0001, and between *HIF-1*  $\alpha$  mRNA expression and NPI (Spearman's coefficient of correlation 0.477, p=0.03).

1

NPI	Subjects	Subjects Surviv	
	n (%)	n (%)	Years (± SD)
Good Prognostic Group (GPG) (<3.4)	4 (18.2)	4 (100)	4.05 ± 1.91
Moderate Prognostic Group (MPG) (3.41-5.4)	14 (63.6)	14 (100)	3.58 ± 1.30
Poor Prognostic Group (PPG) (>5.41)	4 (18.2)	1 (25)	3.11

Table 5.6. Nottingham Prognostic Index (NPI) of the 22 patients with breast cancer.

Of the 3 patients with breast cancer assessed in this study that are deceased, all had an NPI greater than 5.41 and had an average survival time of  $2.14 \pm 0.91$  years. Survival time is measured from the time of diagnosis to the date of death or until October 2001 and is presented in years  $\pm$  standard deviation. A correlation was observed between survival and NPI (Spearman's coefficient of correlation 0.466, p=0.03\*; Kruskal-Wallis H test 5.89, p=0.05\*).

n = number of subjects.

\* = statistically significant

### 5.5 Conclusion

This study investigated the expression of HIF-1 $\alpha$  in breast tumours as well as its predictive potential and found a significant association between HIF-1 $\alpha$  and GLUT-1 mRNA expression in breast tumours (p=0.03). RPA analysis showed that 13 of 22 tumours were positive for HIF-1 $\alpha$  mRNA expression which correlated significantly with patient and disease characteristics of age and grade, but not with T stage or N stage. Expression of GLUT-1 mRNA was found in 15 of 22 tumours and correlated significantly with patient age and N stage, but not with T stage or grade. Only 5 of 22 tumours displayed HIF-1a immunoreactivity with 9 of 22 tumours displaying cytoplasmic staining. No association was found between HIF-1 $\alpha$  protein expression and HIF-1 $\alpha$  or GLUT-1 mRNA expression or with clinicopathological characteristics. Univariate and multivariate analysis did not find any association with disease variables and patient survival. However, Kaplan-Meier analysis revealed a significant influence of T stage (log-rank test, p=0.006) and N stage (log-rank test, p=0.009) on cumulative overall survival. No significant influence from either HIF-1 $\alpha$  or GLUT-1 positive or negative mRNA expression or from HIF-1 $\alpha$  protein expression was found on the cumulative overall survival in the patient group. However, a significant association was found between survival time and NPI and between HIF-1 $\alpha$ mRNA expression and NPI in the patient group.

This is a preliminary report of  $HIF-1\alpha$  and GLUT-1 mRNA expression in breast tumours. The results may suggest that overexpression of both  $HIF-1\alpha$  and GLUT-1 mRNA increase with disease progression. The significance of these results may reflect the weak statistical power of the small patient group analysed (n=22).

# Chapter 6.

# Expression of HIF-1α, VEGF and p53 In Glial Cell Tumours

### 6.0 Expression of HIF-1 $\alpha$ , VEGF and p53 in Glial Cell Tumours

Recent work has suggested a role for HIF-1 $\alpha$  in glioblastomas, although little is known about the mechanisms controlling its expression (Zagzag et al, 2000; Zhong et al, 1999). The expression of HIF-1, VEGF and p53 protein and mRNA was accessed in a range of astrocytic tumours, including glioblastomas. HIF-1 protein expression in multiforme specimens glioblastoma tissue assessed in forty-one by was immunocytochemistry, using a monoclonal antibody to the  $\alpha$  subunit (HIF-1 $\alpha$ ). VEGF and p53 protein expression was assessed in the same specimens using separate polyclonal antibodies to VEGF and p53. HIF-1 $\alpha$  and VEGF mRNA expression was assessed in the total RNA extracted from 34 tumour samples and 2 normal brain samples by reverse transcriptase PCR (RT-PCR) and the ribonuclease protection assay (RPA), including an 18S rRNA control.

 $HIF-1\alpha$  and VEGF mRNA expression were originally assessed by Northern blotting however, due to the limited specimen size and the high quantity of mRNA required to attain a signal, the above mentioned techniques were used. DIG-II labelled DNA probes to HIF-1 $\alpha$  were used in the initial assessment of mRNA expression in the tissue sections. Two separate probes, one of 135 bp and another of 283 bp, were prepared via RT-PCR. As the results were unsatisfactory, in that a signal could not be detected, the approach was changed to immunohistochemistry when the first commercially available antibody to HIF-1 $\alpha$  became available.

The aim of this study was to investigate the levels and expression of HIF-1 $\alpha$ , VEGF and p53 with respect to different grades of tumour. Results are expressed as the ratio of intensity of the expression of the gene of interest compared to the housekeeping gene  $\beta$ -actin. Statistical significance was assessed on the data groups using analysis of variance (ANOVA) followed by F-test and the student's t-test for comparison of means with values of p<0.05 considered as significant.

## **6.1 Clinical Characteristics**

The clinical characteristics of the patients from which these tumours were derived are listed in **Table 6.1**. There were 19 male patients and 15 female patients included in the study of which the overall mean age of diagnosis was  $55 \pm 15.3$  years (25-80 years).

Characteristics	Normal Controls	Diffuse Astrocytomas	Anaplastic Astrocytomas	Glioblastomas	All Patients
Number of subjects	2	3	4	27	34
Sex (m:f)	2:0	0:3	1:3	18:9	19:15
Age at Diagnosis (years) (Range)	74.5 ± 19.1 (61-88)	30.7 ± 5.13 (25-35)	43.5 ± 11.3 (32-59)	59.4 ± 13.1 (35-80)	55.0 ± 15.3 (25-80)
Survival (years) (Range)	-	4.46 ± 0.23 (4.01-4.73)	2.36 ± 0.71 (1.08-4.05)	0.58 ± 0.51 (0.01-2.11)	1.13 ± 1.4 (0.01-4.73)
Survival (weeks) (Range)	-	232.0 ± 11.72 (208.74-245.97)	122.9 ± 37.0 (56.14–210.67)	30.1 ± 26.3 (0.74-109.47)	58.8 ± 70.5 (0.74-245.97)

Table 6.1. Clinical characteristics of normal controls and patients with glial cell tumours.

Forty-one samples of formalin fixed and paraffin embedded sections of tumours samples were studied. Tissue from 34 of these tumour samples was snap-frozen in liquid nitrogen prior to RNA extraction. Tumours were classified according to current World Health Organisation guidelines (Kleihues *et al*, 2000). Of the 41 samples, 27 were glioblastomas, 4 were anaplastic astrocytomas, 5 were diffuse astrocytomas (only 3 with frozen tissue), and 5 pilocytic astrocytomas (none with frozen tissue). Two frozen samples of normal brain tissue obtained at autopsy were also used as controls. The data is expressed as mean values ( $\pm$  standard deviation).

### 6.2 HIF-1α and VEGF mRNA Expression

The expression of *HIF-1* $\alpha$  and *VEGF* mRNA was analysed by RT-PCR using specific amplimers for each (Figure 6.1), as well as by RPA. There were marked differences in the level of expression of *HIF-1* $\alpha$  mRNA in the tumour samples from the different study groups (Figures 6.3A and 6.3B). There was a highly significant increase in the mean ratios of HIF-1 $\alpha$  to  $\beta$ -actin expression in the glioblastomas when compared to the low grade diffuse astrocytomas (mean value 0.45 vs 0.25 respectively, p=0.005) as well as the anaplastic astrocytomas (mean value 0.45 vs 0.3 respectively, p=0.04). The differences in the HIF-1 $\alpha/\beta$ -actin ratio in the low-grade diffuse astrocytomas, the anaplastic astrocytomas as well as the glioblastomas were significantly different from the normal brain (mean values 0.25, 0.3, 0.45 and 0.1 respectively, p<0.05).

HIF-1 $\alpha$  amplifications were verified by performing an additional RT-PCR assay using amplimers for an alternative splice variant to HIF-1 $\alpha$  (Gothié *et al*, 2000) (Figure 6.2).

Results obtained from RT-PCR were confirmed by performing RPA on 8 glioblastoma samples where there was significant tissue for the assay (10µg of total RNA was required per sample for the assay). A glioblastoma sample negative for HIF-1 $\alpha$  expression was verified as having no HIF-1 $\alpha$  expression by RPA and was used as a control. The remaining 7 samples analysed confirmed upregulation of HIF-1 $\alpha$  mRNA expression varying from 8.5 to 23 fold (mean 16.3 fold), **Table 6.2**. Expression of 18S rRNA on the 8 samples showed that expression was consistent and equal. RPA analysis for *VEGF* mRNA expression was not consistent. Synthesis of a RPA probe for *VEGF* mRNA expression proved difficult and the assay could not be optimised due to an insufficient amount of sample material.



**Figure 6.1**. *HIF-1* $\alpha$ , *VEGF* and  $\beta$ -actin mRNA expression in glial cell tumours analysed by RT-PCR and RPA. (A) Amplification products were 520 bp for HIF-1 $\alpha$  and 441 bp for VEGF and 661 bp for  $\beta$ -actin. (M = 100 base pair ladder). Lanes 1,2,3,5,8,9,10 and 11 were scored positive for *HIF-1* $\alpha$ . All of the lanes with the exception of lanes 7 and 11 were scored positive for *VEGF*. *VEGF* expression varied from 1 to 100 fold (mean 25 fold).  $\beta$ actin expression demonstrates the quality of cDNA and equal sample loading. (B) HIF-1 $\alpha$ RT-PCR results were confirmed by performing RPA on 8 glioblastoma samples which confirmed up-regulation of *HIF-1\alpha* mRNA varying from 8.5 to 23 fold (mean 16.3 fold) against a negative control sample (C), (lane 1) (**Table 6.2**). AA = analplastic astrocytoma, LGA = low-grade astrocytoma, GBM = glioblastoma

Sample	Mean (OD)	Volume (OD x mm <sup>2</sup> )	Standard Deviation	Adjusted Volume (OD x mm <sup>2</sup> )	Fold Increase In Expression
1	0.04	8.52	0.02	0.26	-
2	0.60	12.14	0.15	3.89	14.96
3	0.92	14.23	0.14	5.89	22.65
4	0.41	10.93	0.12	2.68	10.31
5	0.34	10.48	0.13	2.23	8.58
6	0.83	13.62	0.12	5.37	20.65
7	0.71	12.89	0.14	4.63	17.81
8	0.77	13.27	0.19	5.02	19.31

**Table 6.2.** RPA analysis of HIF-1 $\alpha$  mRNA expression in 8 glioblastoma tissue samples

Signal band intensities were quantitated by densitometric scanning of the autoradiographs using a Fluor-S<sup>TM</sup> imager and Multi-Analyst<sup>®</sup> image analysis software (Bio-Rad Laboratories, Hemel Hempstead, UK). Background intensity was subtracted from band intensity values. Sufficient tissue was only available from the above samples for analysis in this assay (10µg of total RNA required per sample). Results are expressed as fold increase from the negative control level (sample 1) which was confirmed as having no HIF-1 $\alpha$  mRNA expression. A constant band area size of 6.5 mm<sup>2</sup> was used in the expression analysis of each sample. Results confirm up-regulation of HIF-1 $\alpha$  mRNA varying from 8.5 to 23 fold (mean 16.3 fold). A control probe of 18S rRNA was hybridised to samples to control for the quality of mRNA and ensure equal sample loading (mean adjusted volume 17.42 ± 0.05 SE).



GBM N N AA AA LGA GBM GBM GBM GBM

Figure 6.2. *HIF-1* $\alpha$  splice variant mRNA expression in glial cell tumours analysed by RT-PCR. Amplification products were 487 bp for the HIF-1 $\alpha$  amplified product containing exon 14, 350 bp for the HIF-1 $\alpha$  amplified product without exon 14. (M = 100 base pair ladder). Results coincide with and verify the original RT-PCR assay. In this example, showing a selection of samples from each of the tumour categories as well as normal controls, lanes 1, 4, 5, 8, 9 and 10 were scored positive for *HIF-1\alpha*, and lanes 2 and 3 (normal controls), 6 and 7 were scored negative for *HIF-1\alpha*.

N = normal control AA = anaplastic astrocytoma LGA = low-grade astrocytoma GBM = glioblastoma multiforme



Figure 6.3 (A). *HIF-1* $\alpha$  mRNA expression in individual tumour samples from patients with glioblastoma, lower grade tumours and normal brain, analysed by RT-PCR. Results are represented as the percentage change in abundance of *HIF-1* $\alpha$  with respect to  $\beta$ -actin and show the range of distribution between samples. n = number of subjects



Figure 6.3 (B). Mean *HIF-1* $\alpha$  mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain, analysed by RT-PCR. A highly significant increase in the mean ratios of *HIF-1* $\alpha$  to  $\beta$ -actin expression was observed in the glioblastomas (GBM) when compared to the low-grade diffuse astrocytomas (LGA) as well as the anaplastic astrocytomas (AA) (p<0.01). Additionally a significant difference was also observed between the normal brain and the two lesser glioblastoma classes (p<0.05 by students t-test). Mean of at least 4 independent experiments.  $\pm$  standard errors (SE) are shown. n = number of subjects

GBM vs. LGA (mean value 0.45 vs. 0.25 respectively, p = 0.005)\* GBM vs. AA (mean value 0.45 vs. 0.3 respectively, p = 0.04)\* GBM vs. normal brain (mean value 0.45 vs. 0.1 respectively, p = 0.00003)\* Normal brain vs. LGA (mean value 0.1 vs. 025 respectively, p = 0.03)\* Normal brain vs. AA (mean value 0.1 vs. 0.3 respectively, p = 0.01)\* Between group and within group variance of all 4 means (F-test = 1.46, p = ns)

\* = statistically significant, ns = not significant

In contrast to the results for HIF-1 $\alpha$ , there were no significant differences in the level of expression of VEGF with respect to grade or type of tumour or with normal brain tissue. The mean ratio of VEGF to  $\beta$ -actin levels was 0.18 for normal brain, 0.18 for low grade diffuse astrocytomas, 0.24 for anaplastic astrocytomas and 0.19 for glioblastoma (Figure 6.4). A significant difference was found in the medians of HIF-1 $\alpha$  and VEGF mRNA expression in the glioblastoma tumours samples (p=0.0003) but not in the other tumours types (Table 6.3).

No correlations were found between the expression of HIF-1 $\alpha$  and VEGF mRNA in the glioblastoma tumour samples (correlation coefficient = 0.18, p=0.36) (Figure 6.5), nor in the other tumour types (p=0.30 for low grade diffuse astrocytomas, p=0.40 for anaplastic astrocytomas). The mean ratio of HIF-1 $\alpha$  to VEGF levels was 0.26 for normal brain, 3.81 for low-grade diffuse astrocytomas, 4.66 for anaplastic astrocytomas and 8.01 for glioblastomas. Statistically significant differences were found in the HIF-1 $\alpha$ /VEGF ratio in the anaplastic astrocytomas and the glioblastomas from the normal brain (p=0.03 and p=0.000002, respectively), but not in the low-grade diffuse astrocytomas ( p=0.17).



Figure 6.4. Mean VEGF mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain, analysed by RT-PCR. Results are represented as change in abundance of VEGF with respect to  $\beta$ -actin. The mean ratio of VEGF to  $\beta$ -actin levels was 0.18 for normal brain, 0.18 for low-grade diffuse astrocytomas, 0.24 for anaplastic astrocytomas and 0.19 for glioblastoma. Mean of at least 4 independent experiments.  $\pm$  SE is shown. n= number of subjects. ns = not significant

GBM vs. LGA (mean value 0.19 vs. 0.18 respectively, p = ns) GBM vs. AA (mean value 0.19 vs. 0.24 respectively, p = ns) GBM vs. normal brain (mean value 0.19 vs. 0.18 respectively, p = ns) Normal brain vs. LGA (mean value 0.18 vs. 0.18 respectively, p = ns) Normal brain vs. AA (mean value 0.18 vs. 0.24 respectively, p = ns) Between group and within group variance of all 4 means (F-test = 0.71, p = ns)





Figure 6.5. Mean HIF-1 $\alpha$  and VEGF mRNA expression in tumour samples from patients with glioblastoma, lower grade tumours and normal brain, analysed by RT-PCR. Results are expressed as the percentage change in abundance of HIF-1 $\alpha$  with respect to VEGF. A significant increase in the mean ratios of HIF-1 $\alpha$  to VEGF was observed in the normal brain when compared to the anaplastic astrocytomas (p<0.05 by students t-test). A marked trend and correlation between the expression of HIF-1 $\alpha$  and VEGF mRNA in the GBM tumour samples was observed. Mean of at least 4 independent experiments.  $\pm$  SE is shown.

n = number of subjects. \* = statistically significant, ns = not significant

GBM vs. LGA (mean value 8.01 vs. 3.81 respectively, p = ns) GBM vs. AA (mean value 8.01 vs. 4.66 respectively, p = ns) GBM vs. normal brain (mean value 8.01 vs. 0.26 respectively, p = 0.000002)\* Normal brain vs. LGA (mean value 0.26 vs. 3.81 respectively, p = ns) Normal brain vs. AA (mean value 0.26 vs. 4.66 respectively, p = 0.03)\* Between group and within group variance of all 4 means (F-test = 1.56, p = ns)

Table 6.3.	Statistical analysis of the mean ratios of HIF-1 $\alpha$ to VEGF mRNA expression
	between glioblastoma tumour types and coefficients of correlation.

Tumour Type	HIF-1α mRNA expression (mean value)	VEGF mRNA expression (mean value)	p value	Correlation coefficient	p value
GBM	0.45	0.19	0.0003*	0.182	0.36
LGA	0.30	0.27	0.08	0.500	0.67
AA	0.25	0.48	0.27	-0.800	0.20
Normal brain	0.10	0.18	0.10	-1.0	1.00

Mann-Whitney U non-parametric test was employed for comparison of medians and Spearman's coefficient of correlation was employed for correlation analysis.

\* = statistically significant

GBM = Glioblastoma multiforme LGA = Low-grade astrocytoma AA = Anaplastic astrocytoma

### 6.3 HIF-1a Protein Expression

Immunohistochemical analysis of HIF-1 $\alpha$  protein expression was performed using antibodies to HIF-1 $\alpha$  that had just become commercially available, which had not yet been validated for immunohistochemical analysis. An immense amount of time was spent optimising this procedure of which the results of at least four separate and consistent analyses are described below. All slides were assessed by a neuropathologist (Dr. David Hilton, Derriford Hospital, Plymouth, UK).

Tumour cell immunoreactivity was scored according to the nuclear staining. Both the extent of staining (relative number of HIF-1 $\alpha$  positive cells) and the intensity of the reaction were taken into account, classified as 4 grades of staining: 0 = not detected, 1 =weak staining, 2 = moderate staining, and 3 = strong staining. Staining scores for HIF-1 $\alpha$ , VEGF and p53 expression are shown in **Table 6.4**.

In most of the samples scattered weakly immunoreactive tumour cell nuclei were seen. However, widespread HIF-1 $\alpha$  immunoreactivity was seen in tumour cell nuclei in 21/27 glioblastomas, 3/4 anaplastic astrocytomas, 2/10 low grade astrocytomas and in none of the 2 samples of normal brain. In glioblastomas HIF-1 $\alpha$  immunoreactivity was particularly strong in tumour cells around areas of necrosis (Figure 6.6A), which was predominantly present in palisading tumour cell nuclei (Figure 6.6B). In some cases, HIF-1 $\alpha$  immunoreactivity was seen in the larger more atypical nuclei (Figure 6.6C). Where tumour cells were seen infiltrating brain, immunoreactivity was much more prominent in neoplastic than reactive cells.

Tumour Type	HIF-1 Score	VEGF Score	p53 Score	p53 (%)	Microvascular Proliferation	Necrosis
PA	1	1	N/D	N/D	0	0
PA	1	1	N/D	N/D	0	0
PA	1	1	N/D	N/D	0	0
PA	1	1	N/D	N/D	0	0
PA	1	1	N/D	N/D	0	0
LGA	1	1	N/D	N/D	0	0
LGA	1	1	N/D	N/D	0	0
LGA	2	2	N/D	N/D	0	0
LGA	2	1	N/D	N/D	0	0
LGA	1	1	2	30	0	0
AA	0	1	3	80	0	0
AA	2	1	3	60	0	0
AA	2	2	1	15	0	0
AA	2	1	0	5	0	0
GBM	2	2	3	70	1	1
GBM	1	3	N/D	N/D	î	i
GBM	1	N/D	3	60	i	i
GBM	1	3	2	30	1	1
GBM	1	2	1	10	i	1
GBM	1	3	1	15	1	i
GBM	1	2	2	50	1	1
GBM	3	2	2	20	1	1
GBM	N/D	2	1	10	1	1
GBM	3	N/D	3	80	1	1
GBM	2	2	3	80	1	1
GBM	2	2	1	10	1	i
GBM	1	2	3	80	1	1
GBM	N/D	3	1	20	1	1
GBM	1	2	3	80	1	1
GBM	1	3	3	80	1	1
GBM	2	1	2	30	1	1
GBM	2	3	2	50	1	1
GBM	2	3	2	50	1	1
GBM	3	2	3	80	1	1
GBM	3	3	1	20	1	1
GBM	2	1	1	20	1	1
GBM	3	0	2	30	1	1
GBM	1	3	1	20	1	1
GBM	2	2	3	80	1	1
GBM	1	3	1	10	1	1
GBM	1	1	0	5	1	1

Table 6.4. Immunohistochemica	l analysis of HIF-1α,	VEGF	and p53	expression in
glial cell tumours.				

PA = pilocytic astrocytoma LGA = low-grade diffuse astrocytoma

AA = anaplastic astrocytoma GBM = glioblastoma N/D = not done

HIF-1 $\alpha$  and VEGF tumour cell immunoreactivity was scored according to the nuclear staining: 0 =not detected, 1 = weak staining, 2 = moderate staining, and 3 = strong staining.

p53 tumour cell immunoreactivity was scored for nuclear expression on a four-point scale: 0 = < 10% of cell nuclei with positive staining, 1 = 10-20% of cell nuclei stained, 2 = 20-50% of cell nuclei with positive staining, and 3 = > 50-100% of cell nuclei with positive staining.


Figure 6.6. Immunohistochemical analysis of HIF-1 $\alpha$  expression in glioblastoma. A) Low power view (X50) of HIF-1 $\alpha$  immunoreactivity in a glioblastoma showing widespread nuclear immunoreactivity, which at high magnification (B), is particularly prominent in tumour cells surrounding foci of necrosis (X150) (haematoxylin counterstain). C) Strong HIF-1 $\alpha$  immunoreactivity is observed in glioblastoma cells, particularly those with large atypical nuclei (X200) (haematoxylin counterstain).

# 6.4 VEGF Protein Expression

Tumour cell immunoreactivity for VEGF expression was scored as described for HIF-1 $\alpha$  above. VEGF expression was consistently found in all but one specimen evaluated (**Table 6.4**; Figure 6.7A and 6.7B). Approximately 59% of all specimens and 84% of glioblastomas displayed greater than 20% of tumour cell nuclei positive. No correlation was found between VEGF, HIF-1 $\alpha$  or p53 immunoreactivity (Pearson's Chi-squared test, p=0.90 for HIF-1 $\alpha$  vs. p53, p=0.90 for VEGF vs. p53, and p= 0.69 for HIF-1 $\alpha$  and VEGF).



**Figure 6.7**. Immunohistochemical analysis of VEGF expression in glioblastomas. (**A**) Low power view (X50) of VEGF immunoreactivity showing strong cytoplasmic staining (brown) in tumour cells adjacent to necrosis (left of photo). (**B**) High-power view showing brown granular cytoplasmic staining (X150) (haematoxylin counterstain).

# 6.5 p53 Protein Expression and Correlation with HIF-1a

Tumour cell immunoreactivity for p53 expression was scored for nuclear expression on a four-point scale from 0 to 3. A score of 0 indicated < 10% of cell nuclei with positive staining, 1 indicated 10-20% of cell nuclei stained, 2 indicated 20-50% of cell nuclei with positive staining, and 3 indicated > 50-100 % of cell nuclei with positive staining.

Of all tumours studied, 0–80% of tumour cell nuclei showed p53 immunoreactivity, with 63% of all glioblastomas having greater than 20% of tumour cell nuclei positive (Figure 6.8A and 6.8B). A correlation between p53 immunoreactivity and either HIF-1 $\alpha$  or VEGF mRNA expression could not be demonstrated.

Chapter 6. Results



**Figure 6.8**. Immunohistochemical analysis of p53 expression in glioblastomas. **A**) A lowgrade astrocytoma shows strong nuclear p53 immunoreactivity in a proportion of tumour cells (X300). **B**) A glioblastoma shows strong nuclear p53 immunoreactivity in the majority of tumour cells (X300) (haematoxylin counterstain).

#### 6.6 Survival

Survival of the 34 patients with glioblastoma was followed up, with overall survival being measured from the time of diagnosis to the date of death. The patients were analysed as a whole group (n=36) to include all tumour types and also as patients who had glioblastoma tumours (n=27). Univariate analysis of overall survival was performed by Kaplan-Meier analysis (Kaplan and Meier, 1985). Although statistical power was limited because of the relatively low number of cases, the Cox proportional hazards model was used for both univariate and multivariate analysis. HIF-1 $\alpha$ , VEGF and p53 expression, sex and age at surgery (continuous) were entered into Cox regression. For all tests, a p value of less than or equal to 0.05 was considered as significant. All p values are results of two-sided tests.

Patients diagnosed with a diffuse astrocytoma all remained alive at the time of follow up and had the greatest survival time averaging  $4.46 \pm 0.23$  years. These patients also had the youngest age of diagnosis at  $30.7 \pm 5.13$  years. Patients diagnosed with an anaplastic astrocytoma averaged the second longest survival time of  $2.36 \pm 0.71$  years, presenting with an average age of  $43.5 \pm 11.3$  years at the time of diagnosis. As expected, patients diagnosed with glioblastoma, the most severe tumour type, had the shortest survival time of  $0.58 \pm 0.51$  years ( $30.1 \pm 26.3$  weeks) and presented with the greatest age at diagnosis,  $59.4 \pm 13.1$  years (**Table 6.1**). The median age for all patients was 55 years and the median survival following initial surgery was  $1.13 \pm 1.36$  years ( $58.8 \pm 70.5$  weeks).

Univariate analysis on the glioblastoma patient group showed that sex, HIF-1 $\alpha$ , VEGF or p53 expression were not associated with survival, however age was associated (p=0.01; Table 6.5). In multivariate analysis, age remained an independent prognostic factor on overall survival (p=0.006; Table 6.5). Univariate analysis on all patients to

include all tumour types showed a weak association of VEGF protein expression with overall survival (p=0.06; Table 6.6), which was significant with weak versus strong protein expression. Age and sex were also significantly associated with survival in the total patient group (p=0.0001 and p=0.009, respectively).

Multivariate analysis on all patients showed that VEGF protein expression was eliminated from the model, however HIF-1 $\alpha$  protein expression became a prognostic factor with overall significance of p=0.008, and low and moderate versus strong protein expression having p=0.002 and p=0.0006, respectively. Age and sex remained in the model and were significant prognostic factors with p=0.0001 and p=0.002, respectively (Table 6.6). A correlation was found between age of diagnosis and survival in both the glioblastoma patient group and in all patients analysed (Spearman's coefficient of correlation p=0.02 and p=0.0001, respectively).

When multivariate analysis was limited to protein expression, a statistically significant interaction was found between HIF-1 $\alpha$  and VEGF protein expression in all patients with brain tumours and overall survival (p=0.03 and p=0.01, respectively; **Table 6.7**) indicating that both HIF-1 $\alpha$  and VEGF protein expression are significant predictors of outcome as protein expression is associated with shorter survival (VEGF log-rank test 8.45, p=0.04; **Figure 6.16**). When p53 protein expression was included in the analysis, the overall significance in HIF-1 $\alpha$  and VEGF protein expression and overall survival was lost, however the significance with low to strong expression remained (p=0.01 for low and p=0.03 for high HIF-1 $\alpha$  expression; p=0.03 for low and p=0.04 for high VEGF expression) (**Table 6.7**).

Univariate Analysis	Hazard Ratio	95 % CI	p value
HIF-1a Expression			ns
1 vs 3	1.14	0.45-2.91	ns
2 vs. 3	1.73	0.57-5.25	ns
<b>VEGF</b> Expression			ns
0 vs. 3	0.98	012-7.86	ns
1 vs. 3	0.72	0.19-2.65	ns
2 vs. 3	0.78	0.32-1.89	ns
p53 Expression			ns
0 vs. 3	0.39	0.05-3.12	ns
1 vs. 3	0.77	0.30-1.96	ns
2 vs. 3	0.71	0.25-2.02	ns
Age (continuous)	1.05	1.01-1.10	0.01*
Sex (male vs. female)	1.19	0.52-2.76	ns
Multivariate Analysis	Hazard Ratio	95 % CI	p value
Age (continuous)	1.06	1.02-1.11	0.006*

 Table 6.5. Univariate and multivariate analysis of cumulative overall survival of 27 patients with glioblastoma.

Hazard ratios (HR), p values and 95 % confidence intervals (CI) are indicated. The HR for age as a continuous variable indicates the increase in hazard per year on a per year basis.

Multivariate analysis was performed using a backward elimination procedure to remove variables with  $p \ge 0.10$ . The qualifying criteria for inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis.

\* = statistically significant, ns = not significant

Univariate Analysis	Hazard Ratio	95 % CI	p value
HIF-1a Expression			ns
0 vs. 3	0.32	0.04-2.87	ns
1 vs. 3	0.37	0.12-1.12	0.08
2 vs. 3	0.41	0.14-1.26	ns
<b>VEGF</b> Expression			0.06
0 vs. 3	0.97	0.12-7.70	ns
1 vs. 3	0.21	0.07-0.64	0.007*
2 vs. 3	0.59	0.25-1.40	ns
p53 Expression			ns
0 vs. 3	0.47	0.10-2.16	ns
1 vs. 3	0.84	0.35-2.04	ns
2 vs. 3	1.26	0.47-3.36	ns
Age (continuous)	1.07	1.03-1.10	0.0001*
Sex (male vs. female)	2.82	1.29-6.15	0.009*
Multivariate Analysis	Hazard Ratio	95 % CI	p value
HIF-1a Expression			0.008*
0 vs. 3	0.39	0.04-4.16	ns
1 vs 3	0.08	0.01-0.40	0.002*
2 vs. 3	0.05	0.01-0.27	0.0006*
Age (continuous)	1.09	1.05-1.14	0.0001*
Sex (male vs. female)	6.21	1.91-20.16	0.002*

 Table 6.6. Univariate and multivariate analysis of cumulative overall survival of all 36 patients with glial cell tumours.

Hazard ratios (HR), p values and 95 % confidence intervals (CI) are indicated. The HR for age as a continuous variable indicates the increase in hazard per year on a per year basis.

Multivariate analysis was performed using a backward elimination procedure to remove variables with  $p \ge 0.10$ . The qualifying criteria for inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis.

\* = statistically significant, ns = not significant

All Patients (n=36)	Hazard Ratio	95 % CI	p value
HIF-1a Expression			0.03*
0 vs. 3	0.31	0.02-4.69	ns
1 vs. 3	0.09	0.02-0.46	0.004*
2 vs. 3	0.18	0.04-0.83	0.03*
VEGF Expression			0.01*
0 vs. 3	0.11	0.01-1.38	0.08
1 vs. 3	0.11	0.03-0.47	0.003*
2 vs. 3	0.30	0.11-0.85	0.02*
HIF-1a Expression			0.08
0 vs. 3	0.22	0.02-3.34	ns
1 vs. 3	0.12	0.02-0.60	0.01*
2 vs. 3	0.17	0.04-0.81	0.03*
VEGF Expression			0.09
0 vs. 3	0.12	0.01-1.54	ns
1 vs. 3	0.25	0.15-0.92	0.04*
2 vs. 3	0.34	0.12-0.98	0.04*
p53 Expression		( 	ns
0 vs. 3	0.94	0.13-6.83	ns
1 vs. 3	0.68	0.19-3.50	ns
2 vs. 3	0.92	0.26-3.33	ns
	1		

Table 6.7. Multivariate analysis of cumulative overall survival of all 36 patients with glialcell tumours based on HIF-1α, VEGF and p53 immunohistochemical status.

Tumours were scored for HIF-1 $\alpha$ , VEGF and p53 expression by immunohistochemistry (**Table 6.4**). A Cox multivariate analysis was performed to test for associations of immunohistochemical status with survival. Hazard ratios (HR), 95% confidence intervals (CI) and p values are indicated.

\* = statistically significant, ns = not significant

Kaplan-Meier analysis revealed no significant influence of sex (log-rank test, p=0.67; Figure 6.9), HIF-1 $\alpha$  (p=0.61; Figure 6.10), VEGF (p=0.94; Figure 6.11) or p53 (p=0.77; Figure 6.12) protein expression on cumulative overall survival in the glioblastoma patients. However tumour type had a significant influence on overall survival where analysis by Cox regression resulted in a hazard ratio of 9.16, p = 0.001 (2.35-35.8; 95% CI) (Figure 6.13). Kaplan-Meier analysis of all patients to include all tumour types did reveal a significant association of sex (p=0.007; Figure 6.14) and VEGF protein expression (p=0.04; Figure 6.16) on cumulative overall survival but not with HIF-1 $\alpha$  (p=0.30; Figure 6.15) or p53 protein expression (p=0.63; Figure 6.17).



Figure 6.9. Cumulative overall survival of patients with glioblastoma (n=27) differentiated by sex and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 0.18, p = ns.

Chapter 6. Results



Figure 6.10. Cumulative overall survival of patients with glioblastoma (n=27) differentiated by HIF-1 $\alpha$  protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 1.00, p = ns.





Figure 6.11. Cumulative overall survival of patients with glioblastoma (n=27) differentiated by VEGF protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 0.43, p = ns.



Figure 6.12. Cumulative overall survival of patients with glioblastoma (n=27) differentiated by p53 protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 1.12, p = ns.

Chapter 6. Results



Figure 6.13. Cumulative overall survival of all patients with glial cell tumours analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank 17.9, p=0.0001\*. Cox regression, hazard ratio=9.16, p = 0.001\* (2.35-35.8; 95% CI). \* = statistically significant

LGA = Low-grade astrocytoma AA = Anaplastic astrocytoma

GBM = Glioblastoma multiforme

Chapter 6. Results



Figure 6.14. Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by sex and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 7.41, p = 0.007\*.

\* = statistically significant

Chapter 6. Results



Figure 6.15. Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by HIF-1 $\alpha$  protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 3.67, p = ns.

Chapter 6. Results



Figure 6.16. Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by VEGF protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 8.45, p = 0.04\*.

\* = statistically significant

Chapter 6. Results



Figure 6.17. Cumulative overall survival of all patients with glial cell tumours (n=36) differentiated by p53 protein expression and analysed by Kaplan-Meier. Overall survival is defined from the day of surgery until day of death. Log rank test 1.72, p = ns.

## 6.7 Conclusion

Strong nuclear expression of HIF-1 $\alpha$  protein was seen in the majority of glioblastomas and anaplastic astrocytomas, particularly surrounding areas of necrosis in glioblastomas. In the majority of these tumours up-regulation of *HIF-1* $\alpha$  mRNA was also demonstrated, with a significant increase in glioblastoma multiforme compared to low grade forms of brain tumour as well as in all the tumour types versus normal brain. Additionally, a significant difference between the levels of *HIF-1* $\alpha$  and *VEGF* mRNA expression in anaplastic astrocytomas and glioblastoma multiforme was observed compared to normal brain. However, no correlation was found between the presence of HIF-1 $\alpha$  or VEGF protein and immunohistochemical expression of p53 protein. Moreover, no significant associations were found between HIF-1 $\alpha$ , VEGF or p53 protein expression with patient characteristics.

Both univariate and multivariate analysis showed that age had a significant influence on overall survival in addition to both HIF-1 $\alpha$  and VEGF levels of protein expression. Kaplan-Meier analysis of all 36 patients revealed a significant association of sex and VEGF protein expression on cumulative overall survival in all patients but not with HIF-1 $\alpha$  or p53 protein expression.

These findings are in keeping with an important role for HIF-1 $\alpha$  in the vascularisation of glioblastomas and suggest that up-regulation is at least partly at a transcriptional level. Moreover, that HIF-1 $\alpha$  and VEGF protein expression may be important prognostic factors in glioblastoma.

# Chapter 7.

Polymorphisms in the p53 Family of Tumour Suppressor Genes and Susceptibility to Breast Cancer

 $\mathcal{A}$ 

# 7.0 Polymorphisms in the p53 Family of Tumour Suppressor Genes and Susceptibility to Breast Cancer

p73 is a new member of the growing family of p53 tumour suppressor genes, all of which share high homology (Kaghad *et al*, 1997). Recent studies have suggested that the genes coding for p53 and p73 contain polymorphisms that may increase susceptibility to certain cancers (Storey *et al*, 1998). One of these studies has shown that individuals homozygous for p53 Arg are 7 times more likely to develop HPV-associated cancer (Storey *et al*, 1998). To elucidate the roles of both p53 and p73 in the susceptibility to carcinoma of the breast, polymorphisms of both p53 and p73 genes in 97 women with breast cancer and two normal healthy control populations (one of pure Cornish Celtic origin) were investigated. Polymorphisms of the p53 and p73 genes were determined by nested PCR followed by RFLP (Figure 7.1).

#### 7.1 Clinical Characteristics

The clinical characteristics of the 97 patients with breast cancer are listed and summarised in **Tables 7.1 and 7.4**. The mean age of diagnosis was  $52 \pm 12.3$  years (27-80 years). Interestingly, the cancer was diagnosed in near equivalent percentage of either the right (49.5 %) or left (47.4 %) breast, with 3.1 % diagnosed with bilateral disease. The majority of patients had advanced T stage (48.5 % T<sub>2-4</sub>), grade (90.7 % G<sub>2-3</sub>) or positive node stage (46.4 % N<sub>1-3</sub>), and were non-smokers (82.5 %). For treatment, 63.9 % had received chemotherapy and 84.5 % had received radiotherapy.

The mean age of the Celtic control population was  $55 \pm 15.8$  years (7-89 years).

Chapter 7. Results



**Figure 7.1**. Analysis of p73 allelic expression and p53 Pro/Arg polymorphisms in the lymphocytes of patients with breast cancer. **A)** p73 allelic expression was analysed in 97 patients with breast cancer using a C/T polymorphism found in exon 2. p73 products of 482 bp were amplified using nested PCR (PCR-RL). Biallelic expression was identified after RFLP using Sty1 restriction enzyme digestion. Restriction sizes for the GC allele are 482 bp and 284 bp, and for the AT allele 376 bp plus 106 bp and 234 bp plus 50 bp. Incomplete digestion is observed in some of the lanes. **B)** Sequence specific amplimers were used to amplify and detect p53 Pro/Arg polymorphisms in the lymphocytes of patients with breast cancer, producing a fragment size of 177 bp for the p53 Pro/Arg allele and 141 bp for the p53 Arg allele. The presence of both fragments indicates the p53 Pro/Arg allele genotype. All products were analysed on a 1.5 % agarose gel and visualised with ethidium bromide. (M = 100 bp ladder).

Characteristics	Patients with Brea Cancer		
Number of subjects	97		
Breast Affected			
Right	48		
Left	46		
Bilateral	3		
Age at Diagnosis			
(years)	$52.0 \pm 12.3$		
(Range)	(27 - 80)		
Survival (years)	5.42 ± 3.91		
(Range)	(0.70 - 24.2)		

Table 7.1. Clinical characteristics of patients with breast cancer.

Results are presented as mean values ( $\pm$  standard deviation).

#### 7.2 Genotyping of The p73 Dinucleotide Polymorphism

p73 genotypes were verified by automated sequencing of two of the obtained PCR products, a p73 GC/AT heterozygous genotype and a p73 GC/GC homozygous genotype (Figure 7.2). Although sequencing confirmed the p73 genotypes, some incomplete digestion of the PCR products was observed and further controls should have been used.

Out of the 98 patients with breast cancer, 36 (37.1%) had the p73 GC/AT heterozygous genotype compared to 35.2% (50 of 142) of the 142 normal controls (**Table 7.2**). As the p73 AT/AT homozygous genotype was not found in any of the patients with breast cancer nor the normal controls and was found in only 4 individuals in the Cornish control population, it would seem that the homozygous AT/AT genotype is very rare (1.25%). A statistically significant difference was found between the p73 AT/AT genotype and the p73 GC/GC and p73 GC/AT genotypes in the 2 control populations (p=0.02, pc=0.04), between the patients with breast cancer and the Celtic population (p=0.03, pc=0.05) and between all 3 populations (p=0.006, pc=0.012) (**Table 7.3**). The over-representation of the p73 GC/GC homozygous genotype does not appear to be linked to the populations.

The frequency of the p73 genotypes was categorised with disease variables in the patients with breast cancer and a significant association was found between age at diagnosis and p73 genotype ( $\chi^2$ = 4.75, p=0.03; **Table 7.4**). Nearly three times as many patients diagnosed after 52 years of age (median age) had the p73 GC/GC genotype (73.5%, 36 of 49) rather than the GC/AT polymorphism (26.5%, 13 of 49), (**Table 7.5**). Patients diagnosed before 52 years of age had a similar frequency of p73 genotypes. This same trend was not observed in the Cornish Celtic population (**Table 7.6**). Allele frequencies for the p73 genotypes for each population are given in **Table 7.7**.

Chapter 7. Results

	10	20	30	40
1 32.825 F	GY-	CCCAC-G	AG-CTGTCAC	AGGAGGACMG
22 33 B25 R	ACIONS-TCC-	GCCCCCACCG	AGGCIGICAC	AGGAGGACAG
3 34 B25 F	TGC-	CCCACCG	AG-CIGICAC	AGGAGGACAG
4535.B26 R	MCAKSCIGCC	GCCCCCACCG	AGGCTGTCAC	AGGAGGACAG
5				*-
6	*=*==*=*=*			
	50	60 1	70	80
1 32·B25 F	AGCACGAGTT	CCCAGGGTGC	TCARGTGTCA	TICCTICCIT
25 33 B25 R	AGCACGAGTT	CCCAGGGTGC	TCAGGTGTCA	TTCCTTCCTT
3 34 · B26 F	AGCACGAGTT	CCCAGGGTGC	TCAGGTGTCA	TICCTICCTT
45 35 B26 R	AGCACGAGTT	CCCAGGGTGC	TCAGGTGTCA	TTCCTTCCTT
5	*********		*	
6				
	94	iqo	J ne	120
1 32+B25 F	CCTGCAGAGC	EAGCTGCCCT	YGGAGGCCGG	CGTGGGGAAG
2 33 • B25 R	CCTGCAGAGC	NAGCTGCCCT	TGGAGGCCGG	CGTGGGGAAG
3 34 · B26 F	CCTGCAGAGC	GAGCTGCCCT	CGGAGGCCGG	CGTGGGGAAG
45 35 B26 R	CCTGCAGAGC	GAGCTGCCCT	CGGAGGCCGG	CGTGGGGAAG
5		*	*	
6		********	*	
	130	140	15	0 160
1 32·B25 F	ATGGCCCAGT	CCACCGCCAC	CTCCCCTGAT	GGGGGCACCA
25 33 B25 R	ATGGCCCAGT	CCACCGCCAC	CTCCCCTGAT	GGGGGGCACCA
3 34.B26 F	ATGGCCCAGT	CCACCGCCAC	CTCCCCTGAT	GGGGGGCACCA
45 35 B26 R	ATGGCCCAGT	CCACCGCCAC	CTCCCCTGAT	GGGGGGCACCA
5				
6				

**Figure 7.2.** Automated sequencing of p73 amplified and genotyped DNA from the lymphocytes of two patients with breast cancer. The sequences are of two individual p73 nested PCR products (forward sequence and reverse complimented sequence), one genotyped as a p73 GC/AT heterozygote (top sequence labelled B25), the other genotyped as a p73 GC/GC homozygote (third sequence labelled B26). The arrow points to the polymorphic substitution R (A) to Y (T) at nucleotide position 91 and 101 in exon 2 (red font). Only part of the sequence is shown.

Population	р <b>73 GC/A</b> Т	p73 GC/GC	p73 AT/AT
Breast Cancer Patients (n=97)	37.1 (36)	62.9 (61)	0.0 (0)
Celtic population (n=85)	35.3 (30)	60 (51)	4.7 (4)
Cord blood (n=142)	35.2 (50)	64.8 (92)	0.0 (0)

 Table 7.2. Frequency of p73 genotype in patients with breast cancer and control populations.

n= number of samples

Figures in parentheses represent actual numbers.

Populations	$\chi^2$ (p value)				
_	р <b>73 GC/A</b> Т	p73 GC/GC	р73 АТ/АТ		
Breast vs Cord vs Celtic	0.10 (0.95)	0.52 (0.77)	11.4 (0.003)* (pc=0.006)*		
Breast vs Cord	0.09 (0.76)	0.09 (0.76)	0 (0.00)		
Breast vs Celtic	0.06 (0.80)	0.16 (0.69)	4.67 (0.03)* (pc=0.05)*		
Celtic vs Cord	0 (0.99)	0.52 (0.47)	6.80 (0.02)* (pc=0.04)*		

Table 7.3. Statistical analysis of patient and control populations for each p73 genotype.

pc = corrected p value

**\*** = statistically significant

Table 7.4	. Frequency of disease	e variables a	nd association	with p73	genotype in	patients
	with breast cancer.					

Variable	Number of	p73 Ge	notype	Association
	patients	GC/AT	GC/GC	(test value)
Patients	97 (100)	36 (37.1)	61 (62.9)	$\chi^2 = 2.67^{\text{a}}, p = \text{ns}$
Age (median)			·	
<52	48 (49.5)	23 (47.9)	25 (52.1)	$\chi^2 = 4.75^{b}$
≥52	49 (50.5)	13 (26.5)	36 (73.5)	p=0.03 <b>*</b>
T stage	<u> </u>		·	
1	50 (51.5)	19 (38.0)	31 (62.0)	
2	34 (35.1)	12 (35.3)	22 (64.7)	$\chi^2 = 1.57^{b}$
3	11 (11.3)	5 (45.5)	6 (54.5)	p= ns
4	2 (2.1)	0 (0)	2 (100)	
N stage			<u> </u>	
0	52 (53.6)	22 (42.3)	30 (57.7)	
1	26 (26.8)	8 (30.8)	18 (69.2)	$\chi^2 = 1.44^{b}$
2	10 (10.3)	5 (50.0)	5 (50.0)	p= ns
3	9 (9.3)	1 (11.1)	8 (88.9)	
Grade				
1	9 (9.3)	3 (33.3)	6 (66.7)	$\chi^2 = 0.72^{b}$
2	30 (30.9)	13 (43.3)	17 (56.7)	p= ns
3	58 (59.8)	20 (34.5)	38 (65.5)	
Smoking habits				
Smoker	80 (82.5)	3 (17.6)	14 (82.4)	$\chi^2 = 3.35^a$
Non-smoker	17 (17.5)	33 (41.3)	47 (58.8)	p= ns
Chemotherapy				_
Yes	62 (63.9)	23 (37.1)	39 (62.9)	$\chi^2 = 0.00^a$
No	35 (36.1)	13 (37.1)	22 (62.9)	p= ns
Radiotherapy				
Yes	82 (84.5)	30 (36.6)	52 (63.4)	$\chi^2 = 0.06^{a}$
No	15 (15.5)	6 (40.0)	9 (60.0)	p≔ ns

Figures in parentheses represent percentages. Non-parametric analysis was performed using <sup>a</sup>Pearson Chi-square and <sup>b</sup>Kruskal-Wallis H tests. \* = statistically significant T stage = tumour stage, N stage = node stage, p = p value, ns = not significant

Age (n=97)	р <b>73 GC/A</b> Т	p73 GC/GC
20-30	0.0 (0)	2.1 (2)
31-40	8.2 (8)	8.2 (8)
41-51	15.5 (15)	15.5 (15)
52-60	6.2 (6)	21.6 (21)
61-70	4.1 (4)	9.3 (9)
71-80	3.1 (3)	6.2 (6)

 Table 7.5. Frequency of p73 genotype with respect to age of diagnosis in patients with breast cancer.

p73 GC/AT vs GC/GC genotype in patients diagnosed over 52 years of age (median, n=49) results in a  $\chi^2 = 4.75$ , p=0.03\* (corrected  $\chi^2 = 3.88$ , p=0.05)\*

n= number of subjects

Figures in parentheses represent actual numbers.

\* = statistically significant

Age (n=77)	р <b>73 GC/A</b> Т	p73 GC/GC	p73 AT/AT
<20	1.3 (1)	0.0 (0)	0.0 (0)
20-30	0.0 (0)	3.9 (3)	0.0 (0)
31-40	7.8 (6)	7.8 (6)	1.3 (1)
41-51	2.6 (2)	9.1 (7)	0.0 (0)
52-60	15.6 (12)	10.4 (8)	0.0 (0)
61-70	3.9 (3)	18.2 (14)	0.0 (0)
71-80	5.2 (4)	10.4 (8)	2.6 (2)

Table 7.6. Frequency of the p73 genotype in the Celtic population.

No significant differences were found between any of the groups.

p73 GC/AT vs GC/GC genotype in Celtic population over 52 years of age (n=51) results in a  $\chi^2 = 0.05$ , p=ns

n= number of subjects

ns= not significant

Figures in parentheses represent actual numbers.

## Chapter 7. Results

	p73 Genotype								
Population	GC Observed	C/AT Expected	GC Observed	C/GC Expected	AT Observed	/AT Expected	Allele Fi GC	requency AT	χ² (p<0.05)
Breast (n=97)	37.1 (36)	30.8 (29.9)	62.9 (61)	65.6 (63.6)	0.0 (0)	3.6 (3.5)	0.81	0.19	4.85
Celtic (n=85)	35.3 (30)	34.4 (29.2)	60.0 (51)	60.8 (51.7)	4.7 (4)	4.8 (4.1)	0.78	0.22	0.02
Cord controls (n=142)	35.2 (50)	29.5 (41.9)	64.8 (92)	67.3 (95.5)	0.0 (0)	3.2 (4.6)	0.82	0.18	6.30*

Table 7.7. Allele frequencies of the p73 genotype in patients with breast cancer and control populations.

Figures in parentheses represent actual numbers.

n = number of subjects, p = p value

\* = statistically significant ( $\chi^2$  distribution table, 2 degrees of freedom; Murdoch and Barnes, 1981)

### 7.3 Genotyping of The p53 Codon 72 Polymorphism

The frequency of p53 Pro or Arg polymorphism was determined in all populations (**Table 7.8**). Of the 97 patients with breast cancer studied, 42.3% (41 of 97) had the p53 Pro/Arg heterozygous isoform, 56.7% (55 of 97) had the p53 Arg/Arg homozygous isoform and 1% (1 of 97) had the p53 Pro/Pro homozygous isoform.

The Cornish Celtic population was also examined for the frequency of the p53 isoform. In this group 32.9% (28 of 85) had the p53 Pro/Arg isoform, 63.5% (54 of 85) had the p53 Arg/Arg and 3.53% (3 of 85) had the p53 Pro/Pro isoform. As with the p73 AT/AT genotype, the p53 Pro isoform is very rare having occurred in only 1 individual in the patients with breast cancer and in 3 individuals in the Celtic control population (1.25%). A statistically significant difference was found in the frequency of p53 isoforms between the control populations (p=0.03), which lost significance when corrected for variables (p=0.06, Table 7.9).

The frequency of the p53 isoforms was categorised with disease variables in the patients with breast cancer and no significant correlations were found between clinical features and genotype (Table 7.10). Comparison of the age of diagnosis versus the p53 isoform in patients with breast cancer did not result in a trend being observed for either the patients with breast cancer nor the Celtic population (Tables 7.11 and 7.12). Allele frequencies for the p53 Pro and p53 Arg isoforms for each population are given in Table 7.13.

Population	p53 Pro	p53 Arg	p53 Pro/Arg
Breast Cancer Patients (n=97)	1.0 (1)	56.7 (55)	42.3 (41)
Celtic population (n=85)	3.5 (3)	63.5 (54)	32.9 (28)
Cord blood (n=136)	0.0 (0)	64.7 (88)	35.3 (48)

 Table 7.8. Frequency of the p53 isoform in patients with breast cancer and control populations.

n= number of subjects

Figures in parentheses represent actual numbers.

Populations	$\chi^2$ (p value)		
	p53 Pro	p53 Arg	p53 Pro/Arg
Breast vs Cord vs Celtic	5.30 (0.07)	1.66 (0.44)	1.92 (0.38)
Breast vs Cord	1.41 (0.24)	1.53 (0.22)	1.17 (0.28)
Breast vs Celtic	1.32 (0.25)	0.88 (0.35)	1.67 (0.20)
Celtic vs Cord	4.87 (0.03)* (pc ≕0.06)	0.03 (0.86)	0.13 (0.72)

Table 7.9. Statistical analysis of patient and control populations for each p53 isoform.

Figures in parentheses represent the respective p value

pc = corrected p value

**\*** = statistically significant
Table 7.10. Frequency of disease	variables and association	with p53	isoforms in	patients
with breast cancer.				

Variable	Number of		p53 Isoform		Association
	patients	Pro	Arg	Pro/Arg	(test value)
Patients	97 (100)	1 (1.0)	55 (56.7)	41 (42.3)	$\chi^2 = 2.67^{a}$ , p= ns
Age (median)					
<52	48 (49.5)	1 (2.1)	28 (58.3)	19 (39.6)	$\chi^2 = 1.23^{a}$
≥52	49 (50.5)	0 (0)	27 (55.1)	22 (44.9)	p= ns
T stage					
1	50 (51.5)	0 (0)	28 (56.0)	22 (44.0)	
2	34 (35.1)	1 (2.9)	17 (50.0)	16 (47.1)	$\chi^2 = 2.54^{b}$
3	11 (11.3)	0 (0)	8 (72.7)	3 (27.3)	p= ns
4	2 (2.1)	0 (0)	2 (100)	0 (0)	
N stage					
0	52 (53.6)	0 (0)	26 (50.0)	26 (50.0)	
1	26 (26.8)	1(3.8)	17 (65.4)	8 (30.8)	$\chi^2 = 3.12^{b}$
2	10 (10.3)	0 (0)	6 (60.0)	4 (40.0)	p= ns
3	9 (9.3)	0 (0)	6 (66.7)	3 (33.3)	
Grade					
1	9 (9.3)	0 (0)	4 (44.4)	5 (55.6)	$\chi^2 = 0.92^{b}$
2	30 (30.9)	0 (0)	17 (56.7)	13 (43.3)	p= ns
3	58 (59.8)	1 (1.7)	34 (58.6)	23 (39.7)	
Smoking habits					
Smoker	80 (82.5)	0 (0)	10 (58.8)	7 (41.2)	$\chi^2 = 0.24^{a}$
Non-smoker	17 (17.5)	1 (1.2)	45 (56.3)	34 (42.5)	p= ns
Chemotherapy					
Yes	62 (63.9)	1 (1.6)	37 (59.7)	24 (38.7)	$\chi^2 = 1.35^{a}$
No	35 (36.1)	0 (0)	18 (51.4)	17 (48.6)	p= ns
Radiotherapy					
Yes	82 (84.5)	1 (1.2)	46 (56.1)	35 (42.7)	$\chi^2 = 0.24^{a}$
No	15 (15.5)	0 (0)	9 (60.0)	6 (40.0)	p= ns

Figures in parentheses represent percentages. Non-parametric analysis was performed using a <sup>a</sup>Pearson Chi-square and <sup>b</sup>Kruskal-Wallis H and tests.

T stage = tumour stage, N stage = node stage, p = p value, ns = not significant

Age (n=97)	p53 Pro/Arg	p53 Arg	p53 Pro
20-30	0.0 (0)	1.0 (1)	1.0 (1)
31-40	8.2 (8)	8.2 (8)	0.0 (0)
41-51	11.3 (11)	19.6 (19)	0.0 (0)
52-60	14.4 (14)	13.4 (13)	0.0 (0)
61-70	5.2 (5)	8.2 (8)	0.0 (0)
71-80	3.1 (3)	6.2 (6)	0.0 (0)

 Table 7.11. Frequency of p53 isoform with respect to age at diagnosis in patients with breast cancer.

p53 Pro/Arg vs p53 Arg vs p53 Pro isoforms in patients diagnosed over 52 years of age (n=49) results in a  $\chi^2 = 1.2$ , p = ns

n= number of subjects

ns = not significant

Figures in parentheses represent actual numbers.

Age (N=77)	p53 Pro/Arg	p53Arg	p53Pro
<20	0.0 (0)	0.0 (0)	1.3 (1)
20-30	3.9 (3)	0.0 (0)	0.0 (0)
31-40	1.3 (1)	14.3 (11)	1.3 (1)
41-51	2.6 (2)	9.1 (7)	0.0 (0)
52-60	14.3 (11)	10.4 <b>(8)</b>	1.3 (1)
61-70	6.5 (5)	5.6 (12)	0.0 (0)
71-80	6.5 (5)	11.7 (9)	0.0 (0)

 Table 7.12. Frequency of the p53 isoforms in the Celtic population.

p53 Pro/Arg vs p53 Arg isoforms in the Celtic population over 52 years of age (n=51) results in a  $\chi^2 = 2.0$ , p = ns

n= number of subjects

ns = not significant

Figures in parentheses represent actual numbers.

p53 Isoform									
Population	<b>Pro</b> Observed	/Arg Expected	A Observed	Expected	P Observed	ro Expected	Allele Fi Pro	requency Arg	χ² (p<0.05)
Breast (n=97)	42.3 (41)	34.3 (33.3)	56.7 (55)	60.8 (59)	1.0 (1)	4.9 (4.7)	0.22	0.78	4.96
Celtic (n=85)	32.9 (28)	32.0 (27.2)	63.5 (54)	64.0 (54.4)	3.5 (3)	4.0 (3.4)	0.20	0.80	0.07
Cord controls (n=136)	35.3 (48)	29.7 (40.4)	64.7 (88)	67.1 (91.4)	0.0 (0)	3.2 (4.4)	0.18	0.82	5.96

Table 7.13. Allele frequencies of the p53 isoforms in patients with breast cancer and control populations.

Figures in parentheses represent actual numbers.

n = number of subjects, p = p value

( $\chi^2$  distribution table, 2 degrees of freedom; Murdoch and Barnes, 1981)

## 7.4 p73 Genotype and p53 Isoform

A comparison of p73 genotypes with p53 isoforms in patients with breast cancer showed that 47.5% (29 of 61) of those with the p73 GC genotype also had the p53 Pro/Arg isoform whilst 50.8% (31 of 61) had the p53 Arg isoform (**Table 7.14**). In contrast, 33.3% (12 of 36) of those individuals with the p73 GC/AT genotype had the p53 Pro/Arg isoform and 66.6% (24 of 36) had the p53 Arg/Arg isoform. This trend is not significant but indicates that an association may exist between the p73 GC genotype and the p53 Pro/Arg isoform in patients with carcinoma of the breast. No correlation was found between the disease variables with either p73 genotype or p53 isoform in the patient group.

The opposite trend was found in both the cord blood and Celtic controls. In the cord bloods, 35.5% (22 of 62) of those individuals with the p73 GC/GC genotype had the p53 Pro/Arg isoform and 64.5% (40 of 62) had the p53 Arg/Arg isoform. In the Celtic population, 30.6% (15 of 49) of those with the p73 GC/GC genotype had the p53 Pro/Arg isoform whilst 67.3% (33 of 49) had the p53 Arg/Arg isoform. When the frequency of both the p73 genotypes and the p53 isoforms was compared between all three populations no significant correlation was found.

Analysis of the frequency of the p73 and p53 alleles showed that the p53 Pro/Pro isoform was more frequently found with the p73 GC allele than with the p73 AT allele. This occurs in all three populations (**Table 7.15**) and suggests that the p53 and p73 genes, despite being on separate chromosomes, appear to have a synergistic effect and certain combinations are inherited together more frequently then expected. The odds ratio and relative risk for the development of breast cancer is shown in **Table 7.16**.

Table 7.14.	Frequency of p73 genotype versus p53 Pro/Arg genotype in patients with
	breast cancer and control populations.

		p53 Isoform			
Population	p73 Genotype	Pro/Arg	Arg	Pro	
	GC/AT (36)	33.3 (12)	66.6 (24)	0.0 (0)	
Breast (n=97)	GC/GC (61)	47.5 (29)	50.8 (31)	1.2(1)	
	AT/AT (0)	0.0 (0)	0.0 (0)	0.0 (0)	
Celtic (n=84)	GC/AT (31)	41.9 (13)	51.6 (16)	6.5 (2)	
	GC/GC (49)	30.6 (15)	67.3 (33)	2.1 (1)	
	AT/AT (4)	0.0 (0)	100 (4)	0.0 (0)	
	GC/AT (31)	45.2 (14)	54.8 (17)	0.0 (0)	
Cord controls (n=93)	GC/GC (62)	35.5 (22)	64.5 (40)	0.0 (0)	
	AT/AT (0)	0.0 (0)	0.0 (0)	0.0 (0)	

No statistically significant differences were obtained between the breast population and cord controls or between the Celtic population and cord controls.

n= number of subjects

Figures in parentheses represent actual numbers.

Table 7.15. Observe	d and expected frequence	vies of p73 and p53	allele combinations in
observe	d patient and control po	pulations.	

Allele Combination	Observed	Expected	χ² (p<0.01)
p73GC-p53Pro	72	63.2	1.2
p73GC-p53Arg	331	339.6	0.2
p73AT-p53Pro	2	10.6	6.97*
p73AT-p53Arg	65	56.7	1.2

 $(\chi^2$  distribution table; 1 degree of freedom; Murdoch and Barnes, 1981)

Table 7.16. Odds ratios (relative risk)	) for patients with breast cancer and control
populations for each p73	and p53 genotype.

Populations	p73 Genotype			p53 Genotype		
	GC/AT	GC/GC	AT/AT	Pro/Arg	Arg	Pro
Breast vs Cord	1.11	0.90	0.00	0.00	0.70	1.38
Breast vs Celtic	1.11	1.10	0.00	0.28	0.73	1.53

## 7.5 Survival

Survival of the 97 patients with breast cancer was followed up, with survival time being measured from the time of diagnosis to the date of death or until October 2001.

Two groups of patients were formed with regard to p73 genotypes: GC/AT and GC/GC. Univariate analysis of overall survival was performed by Kaplan-Meier analysis (Kaplan and Meier, 1985) and Cox regression. The Cox proportional hazards model was used for multivariate analysis, where a backward elimination procedure was included to remove variables with  $p \ge 0.10$ . The qualifying criteria for inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis. For all tests, a p value of less than or equal to 0.05 was considered as significant. All p values are results of two-sided tests.

At the time of follow up, the mean survival of the 97 patients was  $5.42 \pm 3.91$  years (0.70-24.2 years) (Table 7.1). Univariate analysis of the patient group showed that T stage (p=0.02), grade (p=0.04) and treatment with chemotherapy (p=0.01) were significantly associated with survival (Table 7.17). Neither age, N stage, smoking habits, treatment with radiotherapy, p73 or p53 genotypes showed an association with survival (Figures 7.3 and 7.4). Multivariate analysis of the patient group showed that only treatment with chemotherapy became an independent prognostic factor (p=0.05) (Table 7.18; Figure 7.8). T stage, stages 1 versus 2, were also an independent prognostic factor in multivariate analysis (p=0.01). In contrast there was no influence of patient age, tumour N stage, grade, smoking habits nor treatment with radiotherapy on overall survival in multivariate analysis as they were eliminated from the model.

Although no correlation was found between p73 genotypes and p53 isoforms, a strong correlation was found between T stage and N stage (p=0.03) as well as between T stage and treatment with chemotherapy (p=0.04) (Table 7.18). Additionally, treatment with chemotherapy also correlated with age of diagnosis (p=0.001), N stage (p=0.001)

and grade (p=0.001). A correlation was also found between survival and N stage (p=0.03) and between N stage and grade (p=0.008).

Kaplan-Meier analysis revealed a significant influence of T stage (Log rank 12, p=0.007; Cox regression, hazard ratio =2.1, p=0.006; Figure 7.5) and grade (Log rank 7.64, p=0.02; hazard ratio =0.024, p=0.009; Figure 7.7) on cumulative overall survival. Treatment with chemotherapy also had a significant influence on overall survival (p=0.009; Figure 7.8) but not treatment with radiotherapy (p=0.37; Figure 7.9). No significant influence from either N stage (p=0.12; Figure 7.6) or smoking habits (p=0.14; Figure 7.10) was found on the cumulative overall survival in the patient group.

The Nottingham Prognostic Index (NPI) was also assessed in the patient group (see Chapter x, section x). Calculated NPIs in the 97 patients with breast cancer in this study showed that the majority of subjects were in the range of the moderate prognostic group (55.2 %) (Table 7.20). There is a significant correlation between survival time and NPI (Spearman's coefficient of correlation -0.29, p=0.005), with a hazard ratio of 2.86, p = 0.005 (1.38-5.91; 95% CI), log rank 10.48, p=0.005.

Variable	Hazard Ratio	95 % CI	p value
p73 Genotype			
(GC/AT vs GC/GC)	1.36	0.60-3.18	ns
p53 Genotype			ns
(Pro vs Pro/Arg)	2.02 x 10 <sup>-5</sup>	0.00	ns
(Arg vs Pro/Arg)	1.43	0.60-3.38	ns
Age (continuous)	0.99	0.96-1.03	ns
T stage (T1-4)			0.02*
$T_1$ vs. $T_2$	4.61	1.63-13.0	0.004*
$T_1$ vs. $T_3$	4.00	0.94-16.98	0.06
$T_1$ vs. $T_4$	10.43	1.16-93.41	0.04*
N stage (N <sub>0-3</sub> )			ns
N <sub>0</sub> vs. N <sub>1</sub>	2.72	0.86-8.55	ns
N <sub>0</sub> vs. N <sub>2</sub>	3.36	0.83-13.56	ns
N <sub>0</sub> vs. N <sub>3</sub>	2.09	0.45-9.69	ns
Grade (G <sub>1-3</sub> )			0.04*
$G_1$ vs. $G_2$	0.21	0.03-1.63	ns
G1 vs. G3	0.25	0.07-0.85	0.03*
Smoking (no vs. yes)	0.22	0.03-1.65	ns
Chemotherapy (no vs. yes)	4.66	1.38-15.75	0.01*
Radiotherapy (no vs yes)	1.80	0.42-7.74	ns

 Table 7.17. Univariate analysis of cumulative overall survival of 97 patients with breast cancer.

Hazard ratios (HR), p values and 95 % confidence intervals (CI) are indicated. The HR for age as a continuous variable indicates the increase in hazard per year on a per year basis. \* = statistically significant, ns = not significant

Variable	Hazard Ratio	95 % CI	p value
T stage (T <sub>1-4</sub> )			0.07
$T_1$ vs. $T_2$	4.11	1.36-12.46	0.01*
$T_1$ vs. $T_3$	3.81	0.83-17.42	ns
$T_1$ vs. $T_4$	8.28	0.88-78.10	0.07
Grade (G <sub>1-3</sub> )			0.09
G1 vs. G2	0.31	0.03-3.67	ns
G <sub>1</sub> vs. G <sub>3</sub>	1.30	0.14-11.84	ns
Smoking (no vs. yes)	5.89	0.77-44.85	ns
Chemotherapy (no vs. yes)	3.60	0.99-13.03	0.05*

 Table 7.18. Multivariate analysis of cumulative overall survival of 97 patients with breast cancer.

Hazard ratios (HR), p values and 95 % confidence intervals (CI) are indicated.

Multivariate analysis was performed using a backward elimination procedure to remove variables with  $p \ge 0.10$ . The qualifying criteria for inclusion in the multivariate analysis were p<0.1, or hazard ratios <0.5 or >2 in the univariate analysis.

\* = statistically significant, ns = not significant

<b>Table 7.19</b> .	Correlation between survival	variables and clinica	l characteristics in 97
	patients with breast cancer.		

Variables	Correlation (test value, p value)		
Age of diagnosis vs. Chemotherapy	430ª, p=0.0001*		
Chemotherapy vs. T stage	8.26°, p≕0.04 <b>*</b>		
Chemotherapy vs. N stage	17.10°, p=0.001*		
Chemotherapy vs. Grade	13.06°, p=0.001*		
T stage vs. N stage	18.41°, p=0.03*		
T stage vs. Grade	0.26°, p≔0.01 <b>*</b>		
Grade vs. N stage	17.51°, p=0.008*		
Survival vs. N stage	8.91°, p≕0.03*		

Non-parametric analysis was performed using <sup>a</sup>Mann-Whitney U, <sup>b</sup>Kruskal-Wallis H and <sup>o</sup>Pearson Chi-square tests.



Figure 7.3. Cumulative overall survival of patients with breast cancer (n=97) with the p73 GC/AT genotype compared with patients with the p73 GC/GC, analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 0.55, p=0.46, Cox regression, hazard ratio = 1.35, p=0.50 (0.57-3.21; 95% CI).



Figure 7.4. Cumulative overall survival of patients with breast cancer (n=97) with p53 Pro, p53 Arg and p53 Pro/Arg genotypes, analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 0.81, p=0.67. Cox regression, hazard ratio = 1.22, p=0.66 (0.53-2.96; 95% CI).



Figure 7.5. Cumulative overall survival of patients with breast cancer (n=97) differentiated by T stage and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 12, p=0.007\*. Cox regression, hazard ratio = 2.10, p=0.004\* (1.27-3.48; 95% CI).





Figure 7.6. Cumulative overall survival of patients with breast cancer (n=97) who differentiated by N stage and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 4.52, p=0.21. Cox regression, hazard ratio = 1.4, p= 0.12 (0.92-2.12; 95% CI).





Figure 7.7. Cumulative overall survival of patients with breast cancer (n=97) differentiated by grade and analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 7.64, p=0.02\*. Cox regression, hazard ratio = 2.80, p=0.02\*(1.14-6.85; 95% CI).



Figure 7.8. Cumulative overall survival of patients with breast cancer (n=97) who have had treatment with chemotherapy, compared with patients who have not had treatment with chemotherapy, analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 7.39, p=0.007\*. Cox regression, hazard ratio = 0.14, p=0.009\*(0.03-0.62; 95% CI).



Figure 7.9 Cumulative overall survival of patients with breast cancer (n=97) who have had treatment with radiotherapy, compared with patients who have not had treatment with radiotherapy, analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 0.64, p=0.42. Cox regression, hazard ratio = 0.51, p=0.37 (0.12-2.21; 95% CI).



Figure 7.10. Cumulative overall survival of patients with breast cancer (n=97) who smoke compared with patients who do not smoke, analysed by Kaplan-Meier. Overall survival is defined from the day of diagnosis until day of death. Log rank 3.28, p=0.07. Cox regression, hazard ratio = 0.22, p= 0.14 (0.29-1.65; 95% CI).

Table 7.20.	Nottingham Prognostic Index (NPI) for 97 patients with breast cancer
	accessed in this study.

NPI	Subjects	Survival	
	n (%)	n (%)	Years (± SD)
Good Prognostic Group (GPG) (<3.4)	17 (17.5)	14 (87.5)	7.10 ± 5.89
Moderate Prognostic Group (MPG) (3.41-5.4)	53 (54.6)	43 (81.1)	5.38 ± 3.47
Poor Prognostic Group (PPG) (>5.41)	27 (27.8)	18 (66.7)	4.84 ± 1.61

Survival time is measured from the time of diagnosis to the date of death or until October 2001 and is presented in years  $\pm$  standard deviation. There is a correlation between survival time and NPI (Spearman's coefficient of correlation -0.285, p=0.005\*; Kruskal Wallis H test 8.02, p=0.018\*). Cox regression, hazard ratio =2.86, p = 0.005\* (1.38-5.91; 95% CI). Log rank 10.48, p=0.005\*

n = number of subjects

## 7.6 Conclusion

This is a novel study of both p53 isoforms and p73 genotypes in individuals with breast cancer. Both the p73 AT/AT genotype and the p53 Pro isoform were found to be rare in all populations studied. A significant association was found with the p73 GC/GC genotype and patients with breast cancer diagnosed over 52 years of age (median). No association was found with the p53 codon 72 Pro/Arg polymorphism and breast cancer. The results suggest that polymorphisms of either p53 or p73 genes are not risk factors in the development of carcinoma of the breast. Both the p73 genotypes and p53 isoforms had no significant influence on overall survival in the patient group however, T stage, grade and treatment with chemotherapy were all found to have a significant influence on overall survival in the patient group. A significant correlation was also found between survival time and NPI in the patient group.

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# Chapter 8.

Discussion

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#### 8.0 Discussion

Cells respond to stress by regulating the expression of adaptive and/or pathological genes depending on the actual magnitude and duration of the stimulus or condition. This stress may be hypoxic, high glucose or oxidative, and include pH and temperature changes. The adaptive response to these stimuli is mediated in part by the transcription factor HIF-1 $\alpha$  by signalling through heterodimeric PAS family member interactions, resulting in the increased expression of glucose transporters, and glycolytic enzymes. Ultimately angiogenesis is enhanced and erythropoiesis increased (Guillemin and Krasnow, 1997). Under conditions of sustained metabolic stress, HIF-1 $\alpha$  stabilisation increases cellular p53 levels thereby up-regulating the transactivation of pathological genes and ultimately resulting in cell death (Halter man *et al*, 1999). However, p53 also decreases the stability of HIF-1 $\alpha$  by inhibiting the hypoxia-induced expression of HIF-1 $\alpha$  and assisting its ubiquitination and subsequent degradation (Ravi *et al*, 2000).

It is known that hypoxia is prevalent in solid tumours and it is the adaptation to hypoxic conditions that is the critical step in the survival and progression of tumours and disease. One major point of consideration is the definition and severity of hypoxia. Much debate has arisen over optimal hypoxia conditions as opposed to anoxia. HIF-1 $\alpha$  has been shown to be optimally induced at 0.5% O<sub>2</sub> (Wenger and Gassmann, 1997), however several studies have reported that oxygen concentrations found to induce the expression of p53 were indeed anoxic (0.02%) (Graeber *et al*, 1996; An *et al*, 1998), suggesting that anoxic mechanisms are involved in oxygen-dependent p53 regulation. A recent report found that the sensitivity of pathways to hypoxia varies in cell lines where both HIF-1 $\alpha$  and HIF-2 $\alpha$  were found to be detectable at a range of 0.1% to 3% O<sub>2</sub> suggesting that some tumours *in vivo* may be more able to respond to small changes in the microenvironment with less severe hypoxia (Blancher *et al*, 2000). All hypoxic experiments in this work were performed at  $\leq 1\%$  oxygen, as verified by a Fyrite<sup>®</sup> gas analyser.

# 8.1 HIF-1α, GLUT-1 and p53 Expression In Breast Carcinoma Cell Lines

Although cell lines derived from human tumours have been extensively used as experimental models of neoplastic disease, clear difficulties exist. Established cell lines differ from both normal and cancerous tissue, however they provide a convenient and unlimited source of human tissue. A recent study has shown that breast carcinoma cell lines are broadly representative of fresh tumours (Davidson *et al*, 2000). The majority of experimental work was performed on established cell lines. Cell lines were chosen to represent a spectrum of breast cancer types as well as reflect the heterogeneity of human breast cancer progression pathways.

Initial studies investigating the mRNA expression of HIF-1 $\alpha$ , VEGF, p53 and  $\beta$ actin in four individual breast carcinoma cell lines exposed to hypoxia were performed using Northern blot analysis and showed that upregulation occurred after exposure of cells to CoCl<sub>2</sub> and hypoxia. As it was found that large quantities of mRNA were required for genetic expression, the newer and more sensitive RPA was utilised for determining and quantitating mRNA expression.

Under normoxic conditions, HIF-1 $\alpha$ , GLUT-1 and p53 mRNA was detected at varying concentrations in all of the breast carcinoma cell lines studied. After prolonged exposure to hypoxia, the majority of cell lines studied exhibited little or no change in the expression of HIF-1 $\alpha$  mRNA. Initial studies found that the gene for HIF-1 $\alpha$  was transcriptionally induced under hypoxia (Wang *et al*, 1996) however, further studies have shown that HIF-1 $\alpha$  mRNA is expressed constitutively in a large number of human tissue and cell lines and that these steady-state expression levels are not further up-regulated by hypoxia (Huang *et al*, 1996; Kallio *et al*, 1997; Wenger *et al*, 1997). Conversely, studies have reported that the HIF-1 $\alpha$  mRNA level increases in hypoxia in the human Hep3B cell line, in bovine pulmonary artery endothelial cells and in rat brain and kidney (Ladoux and Frelin, 1997; Wiener *et al*, 1996; Palmer *et al*, 1998; Wenger *et al*, 1997). Thus, it may be that the presence of a transcriptional up-regulation mechanism for HIF-1 $\alpha$  is species and/or cell-type specific. Although little or no change in *HIF-1* $\alpha$  mRNA expression was observed in the majority of cell lines exposed to hypoxia, this may not be a consequence of low oxygen tension but may instead represent a general adaptation response of the cells during cultivation.

At the time of this study, conflicting reports remained as to the transcriptional induction of HIF-1 $\alpha$  which warranted further investigation considering that very little work had been performed with breast tissue or breast carcinoma cell lines. Little is known about the hyperglycaemia-induced expression of HIF-1 $\alpha$  in breast carcinoma cell lines and it is possible that the expression profiles of *HIF-1* $\alpha$  and *GLUT-1* genes under hypoxic and high glucose conditions may offer further insight as to how cancer cells behave under conditions of extreme stress.

The transcription of the GLUT-1 gene is enhanced in response to hypoxia, or cobalt chloride, to inhibition of oxidation phosphorylation by azide, to an ionophore-induced increase in the concentration of cytosolic calcium, and after transformation (Zhang *et al*, 1999; Wertheimer *et al*, 1991). The studies presented here on breast carcinoma cell lines determined that HIF-1 $\alpha$ , GLUT-1 and p53 mRNA expression increased in response to high glucose but decreased in response to the combined stresses of hypoxia and high glucose (D-glucose) from normoxic levels. Additionally, the combination stress of oxygen and glucose deprivation resulted in the decreased expression of HIF-1 $\alpha$  mRNA from normoxic levels in 3 of the 4 cell lines investigated. Controls for osmolarity determined that this response was not specific for either hypoxia or high glucose and was probably due to differences in osmolarity as increases in HIF-1 $\alpha$  and GLUT-1 mRNA expression were observed after exposure to either L-glucose or mannitol. This would imply that a redox effect promotes the induction of HIF-1 $\alpha$  and GLUT-1 mRNA in these cell lines and not

#### Chapter 8. Discussion

hypoxia or D-glucose alone. This finding is confirmed by a recent study which demonstrated that hyperosmolarity increases *GLUT-1* mRNA content in a non-transformed rat liver cell line, the response was mediated by both enhanced transcription and stabilisation of *GLUT-1* mRNA as was associated with increases in GLUT-1 content and glucose transport activity (Hwang and Ismail-Beigi, 2001). As this study also found that increased expression of aldose reductase is associated with significant increases in the cellular content of sorbitol, it would be interesting to study the effect of hypoxia, high glucose and hyperosmolarity on both aldose reductase mRNA expression and glucose transport via measurement of the cellular content of sorbitol in the breast carcinoma cell lines. Obtaining a description of mRNA profiles for genes encoding for enzymes for the entire metabolic pathway may provide a useful insight into the induction and regulation of these genes under conditions of extreme stress. Additionally, it is possible that other osmolytes such as dextrose, NaCl and urea may produce similar results and should therefore also be investigated.

It is possible that an increase or decrease in mRNA expression may be mediated by an increase or decrease in the stability of the mRNA. Therefore, both the stability and the transcriptional rate of *HIF-1* $\alpha$  and *GLUT-1* should be determined by nuclear runoff transcription assays to establish whether hypoxia and high glucose affect the transcript levels in both genes through enhanced or decreased mRNA. A wide disparity in *GLUT-1* half-lives has been observed in different cell types indicating cell/ tissue specific regulation of *GLUT-1* mRNA stability (Stein *et al*, 1995; Jain *et al*, 1997; Behrooz and Ismail-Beigi, 1997; Vinals *et al*, 1997). This factor may account for the varied responses observed in this study.

An additional factor may have been the high basal levels of D-glucose in the culture media. The DMEM culture media contained a basal D-glucose level of 25 mM, slightly above the moderate glucose conditions of 21 mM in the RPMI culture media. The

result of adding additional D-glucose to the DMEM culture media to create moderate and high glucose conditions may have instead slowed any observed effect on mRNA expression due to saturation of glucose receptors.

When DNA binding was investigated in the breast carcinoma cell lines, EMSA revealed a very efficient DNA-binding of functional HIF-1 complexes under normoxic conditions as well as induced binding activity under hypoxic and hyperglycaemic conditions. A recent study has also reported HIF-1 binding under normoxia in the MX-1 human breast cancer cell line (Marxsen et al, 2001). Competition experiments determined that the observed HIF-1 binding was specific as it could be effectively inhibited with excess unlabelled probe. The MCF7 breast cell line was the only cell line in which extracts displayed a decrease in binding activity from normoxic control levels. The largest induction in binding activity was observed in extracts from the ZR75 breast cell line exposed to hypoxia which exhibited a 3 fold greater binding activity than that observed in the other breast cell lines. These variations in binding activity may be attributed to different states of phosphorylation. DNA binding of HIF-1 is regulated by protein phosphorylation (Wang et al, 1995) and the status of phosphorylation can affect the mobility of the target protein in polyacrylamide gels. It is also possible that different coactivators may be involved in HIF-1 activation by hypoxia when compared with that activated by high glucose.

To better understand how extreme stress affected these cells, HIF-1 $\alpha$  and GLUT-1 protein expression was investigated. Unfortunately, attempts at determining HIF-1 $\alpha$ protein expression through Western blotting were unsuccessful. Spiking experiments determined that the only signal detected was that of BSA.

GLUT-1 protein expression was also investigated in the breast carcinoma cell lines exposed to hypoxia and long-term high glucose where no increase in protein expression was observed in the majority of cell lines exposed to hypoxia for up to 24 hours, results which are consistent with observed mRNA expression levels. However, the combined exposure to both stresses resulted in decreased protein expression from that observed at normoxic levels. A recent study involving Western analysis of MCF7 breast cell protein exposed to hypoxia for up to 4 hours did not show any increase in the levels of HIF-1 $\alpha$ , HIF-1 $\beta$ , p53, GLUT-1 or GLUT 3 (Burgman et al, 2001) suggesting that there may be a defect in the hypoxia-sensing signal transduction pathway in these cells. Conversely, this present study found increased expression of GLUT-1 protein in the MCF7 cell line after 2 and 24 hours exposure to hypoxia, indicating that longer exposure to hypoxia may be required to invoke a response in this particular cell line. However, GLUT-1 protein expression was not induced in the SKBR3 and ZR75 breast carcinoma cell lines after exposure to hypoxia. It has been reported that only GLUT-1 and GLUT 3 are expressed in MCF7 breast cells (Aloj et al, 1999) thus, it is possible that differences in GLUT species expressed in the various cell lines can account for the different expression patterns observed in each cell line under the various treatments. Moreover, consistent findings at the mRNA and protein levels in the majority of breast cell lines that exposure to hypoxia and long-term high glucose resulted in decreased GLUT-1 mRNA or protein expression, indicate that conditions of extreme stress overload and curtail the response pathway. Oxygen deprivation and excess glucose represent an additional disturbant of glucose transporter expression in human breast carcinoma cells.

It is possible that breast epithelial carcinoma cells have a decreased capacity to store glycogen and therefore do not have a reservoir of metabolisable substrates available under conditions of extreme stress. Exposure of breast carcinoma cell lines to hypoxia resulted in an initial decrease in the level of lactate from normoxic levels that steadily increased over normoxic levels after 24 and 48 hours of exposure. The initial decrease in lactate levels would indicate recovery from the hypoxic stress followed by an increase in the rate of glycolysis. Concurrent measurement of the ATP levels during hypoxic exposure would verify upregulation of glycolysis in these cells. It is known that part of the adaptation to hypoxia involves upregulation of genes that encode the enzymes required for anaerobic glycolysis, thus allowing cells to switch to this form of metabolism from oxidative phosphorylation. The slow recovery time observed by the decreased levels of lactate produced under prolonged exposure to hypoxia may indicate that the cells have not switched to anaerobic glycolysis. Therefore oxidative phosphorylation has not been inhibited and *GLUT-1* mRNA expression is not induced. Studies have shown that *GLUT-1* mRNA induction by hypoxia requires the inhibition of oxidative phosphorylation (Behrooz and Ismail-Beigi, 1997). It is however, difficult to understand the complex metabolic processes that occur at the cellular level.

It is interesting to note that the magnitude of change in GLUT-1 protein expression impeded by hypoxia or by the combined stresses of hypoxia and excess glucose, was slightly less than the observed decrease in *GLUT-1* mRNA expression levels. If these discrepancies occur *in vivo* than cell damage may be due in part to the inability of breast cancer cells to translate *GLUT-1* messages sufficiently during conditions of limited oxygen availability with over availability of glucose.

Each cell line studied produced varied genetic expression responses to the stresses of hypoxia, high glucose or the combined stresses of both, displaying limited increased expression of both the hypoxia-regulated genes HIF-1 $\alpha$  and GLUT-1. Although these experimental results confirm that hypoxic regulation of HIF-1 $\alpha$ , GLUT-1 and p53 occurs post-transcriptionally, this variation in response demonstrates the difficulties in working with cell lines. Although culture and experimental cell conditions were standardised by determining cell densities to ensure that experimental work was performed in an optimal exponential growth phase, cells may have exceeded their exponential phase through the duration of some experiments. Mild hypoxia may occur from cell-to-cell contact (Jones *et al*, 2001) and cell confluence can produce stresses, such as low glucose and acidosis, resulting in nutritional deprivation and possible hypoxia-triggered apoptosis (Schmaltz *et al*, 1998). The toxicity of hypoxia is therefore proportional to cell density and may contribute to the regulation of both HIF-1 $\alpha$  and GLUT-1.

In conclusion, experimental results produced from this study suggest that when breast carcinoma cells are subjected to extreme stress, such as the combined stresses of hypoxia and high glucose, the hypoxia response pathway is impeded. It is probable that hypoxia, high glucose, hypoglycaemia and hyperosmolarity do not share a common transcriptional stress response pathway. Various studies on the response of the *GLUT-1* gene to cellular stress have found that different regions of the *GLUT-1* promoter have been identified which mediate responses to stresses such as hypoxia, azide, transformation and hyperosmolarity (Behrooz and Ismail-Beigi, 1997; Ebert *et al*, 1995; Hwang and Ismail-Beigi, 2001). Moreover, a recent study has shown that the basal levels of *HIF-2a* are highly related to the inducible level of the HIF protein in breast carcinoma cell lines (Blancher *et al*, 2000). Thus, it would be of interest to elucidate the regulation and role of *HIF-2a* mRNA expression in these cell lines.

#### 8.2 HIF-1α and GLUT-1 Expression In PBMCs of Patients with T1DM

Excessive flux through the polyol/sorbitol pathway caused by hyperglycaemia is likely to lead to a number of metabolic and vascular defects that ultimately give rise to tissue hypoxia and ischaemia. Currently, little is known about the hyperglycaemia-induced expression of HIF-1 $\alpha$  and GLUT-1 in the PBMCs of patients with or without diabetic complications.

In this study, the basal levels of HIF-1 $\alpha$  and GLUT-1 mRNA expression in the PBMCs of normal healthy controls was found to be significantly higher then in those of patients with T1DM. It is known that basal levels of *HIF-1\alpha* mRNA expression varies according to differences in culture conditions (Semenza, 1998). As *HIF-1\alpha* and *GLUT-1* 

mRNA were present in high levels in the non-hypoxic PBMCs of normal controls, induction of expression of either gene under high glucose was not significant, however significant differences were found between the control and patient groups at both moderate and high glucose levels. The PBMCs from patients with T1DM may be preconditioned to high glucose and oxidative stress and therefore may behave in a different way to cells subjected to short term exposure to high glucose.

Two distinct trends were observed in the expression of both HIF-1 $\alpha$  and GLUT-1 mRNA in the PBMCs of patients with T1DM. Considering the response found in the breast carcinoma cell lines, where short-term exposure to high glucose increased HIF-1 $\alpha$  and GLUT-1 mRNA expression, it was unexpected to find a large group of patients with a marked decrease in HIF-1 $\alpha$  and GLUT-1 mRNA expression in high glucose. As seen in the breast carcinoma cell lines, controls for osmolarity determined that this response was not specific for D-glucose and was probably due to differences in osmolarity. Increases in HIF-1 $\alpha$  and GLUT-1 mRNA expression were observed after the PBMCs from two patients with diabetic complications were stimulated with mannitol and both an increase and decrease in both HIF-1 $\alpha$  and GLUT-1 mRNA was observed after stimulation with Lglucose.

Although the average expression of both HIF- $I\alpha$  and GLUT-I mRNA in the PBMCs of all patients investigated increased in high glucose, there may be a number of factors as to why an increase in HIF- $I\alpha$  and GLUT-I mRNA expression was not observed in all patients with T1DM. It is possible that the expression and function of glucose transporters differs between patients with and without complications. Patients without complications displayed a 1.5 and 2.0 fold increase in HIF- $I\alpha$  mRNA expression, and a 1.3 and 1.5 fold increase in GLUT-I mRNA expression in moderate glucose and high glucose, respectively, over patients with complications. Additionally, the increase or decrease in HIF- $I\alpha$  and GLUT-I mRNA expression may be mediated by transcription of

- 428 -

the genes as well as by stabilisation of the mRNAs. Moreover, the stimulation of HIF-1 $\alpha$ , and hence GLUT-1, mRNA by high glucose may be due to increased conversion of an inactive to active form of the protein due to changes in the redox state of the cells. It may be that patients with T1DM have more active protein in their cells.

Another possible factor may be that patients with T1DM may have an aldose reductase protective polymorphism. Studies have suggested that genetic variation in the aldose reductase gene may contribute to the genetic susceptibility to diabetic nephropathy (Shah *et al*, 1998; Moczulski *et al*, 2000). Polymorphisms in the aldose reductase gene may be markers of different levels of expression of the gene, of altered tissue expression or the ability to respond to stimuli. It would be interesting to obtain the mRNA profiles for genes coding for the aldose reductase genotypes, as well as sorbital dehydrogenase, to determine if the response to uptake and disposal of D-glucose is different between the groups of patients with and without complications and normal controls, as was found in a recent study (Hodgkinson *et al*, 2001).

Investigation of HIF-1 $\alpha$  DNA binding activity in the nuclear extracts from the PBMCs of patients with T1DM and normal controls revealed efficient DNA-binding of functional HIF-1 complexes under normoxic conditions. Binding activity was slightly decreased after exposure to high glucose in all extracts. Preliminary investigation of GLUT-1 protein expression in the PBMCs of normal controls exposed to high glucose displayed an increase in GLUT-1 protein expression whilst those from patients with T1DM, one patient with complications and one without, exposed to high glucose showed a dramatic decrease in GLUT-1 protein expression. This would imply that the induction of *GLUT-1* mRNA is not mediated at the transcriptional level in the PBMCs of patients with T1DM. As a similar finding was observed in breast carcinoma cell lines exposed to long-term high glucose and hypoxia, it is possible that the regulation of GLUT-1 expression and function may be mediated at multiple levels. Further protein studies are required to

elucidate the response seen and this hypothesis.

It is known that vascular changes induced by increased glucose or sorbitol levels are prevented by raising tissue pyruvate levels, which drives the oxidation of NADH to NAD<sup>+</sup> coupled to the reduction of pyruvate to lactate by lactate dehydrogenase (Brownlee, 2001). This same redox imbalance also develops in hypoxic and ischaemic tissues due to impaired oxidation of NADH to NAD<sup>+</sup> by the mitochondrial electron transport chain. Measurement of lactate levels in the culture medium of PBMCs of four patients without diabetic complications and of two patients with diabetic complications exposed to high glucose, showed only minor increases over normal culture levels. Exposure to high glucose does not seem to significantly up-regulate glycolysis in these patients nor in normal controls. Pyruvate has been shown to attenuate vascular dysfunction induced by hypoxia and ischemia, by acute high glucose in nondiabetic rats and by diabetes (Bunger *et al*, 1989; Cavallini *et al*, 1990).

This is a novel preliminary report of HIF-1 $\alpha$  and GLUT-1 mRNA expression in high glucose which suggests that HIF-1 $\alpha$  may be an important regulator of the cellular response to high glucose. It is possible that abnormal expression of HIF-1 $\alpha$  and its target genes, through activation by high glucose, may contribute to diabetic complications. As the molecular basis of the cellular response to hypoxia and high glucose has yet to be determined, further studies of subsequent gene and protein regulation by HIF-1 $\alpha$  are required.

#### **8.3 HIF-1α and GLUT-1 Expression In Breast Tumours**

Oxygen and glucose are important for tumour growth as they fuel tumour cell survival and proliferation. It has long been known that tumours display numerous metabolic changes compared with normal cells, such as increased glycolytic metabolism even under aerobic metabolism (Warburg, 1930). This increased glycolysis is managed by the increased uptake of glucose by GLUT-1, the first in the family of glucose transporters to be cloned (Mueckler *et al*, 1985). The mechanism by which hypoxia increases *GLUT-1* mRNA expression is fairly well understood, believed to be through anaerobic glycolysis and inhibition of oxidative phosphorylation (Behrooz and Ismail-Beigi, 1997).

At the time of this study there were no reports of HIF-1 $\alpha$  or GLUT-1 mRNA or HIF-1 $\alpha$  protein expression in breast tumours. Increases in GLUT-1 mRNA expression have however been described in tumours of the oesophagus, colon, pancreas (Yamamoto *et al*, 1990), lung (Ogawa *et al*, 1997) and brain (Boado *et al*, 1994; Tsukamoto *et al*, 1996). Normal control tissue for this study could not be obtained and limited tissue was available for analysis as the collaborating pathologist left at the beginning of the study and was not replaced for some time. This made the interpretation of HIF-1 $\alpha$  and GLUT-1 mRNA

In this preliminary study, *HIF-1* $\alpha$  and *GLUT-1* mRNA were overexpressed in 59% and 68% of the breast tumours assessed, respectively, however the amplitude of overexpression varied. A significant correlation was found between the expression of both genes (p=0.03) which was lost when the expression was compared categorically. Significant correlations were also found between *HIF-1* $\alpha$  mRNA expression with patient age at diagnosis and tumour Grade, and between *GLUT-1* mRNA expression with patient age at diagnosis and tumour N stage. Over-expression of HIF-1 $\alpha$  and GLUT-1 mRNA may promote an increase in tumour size by supporting glycolysis thereby enhancing tumour cell viability and providing energy for cell division and tumour growth.

Until recently, the significance of HIF-1 $\alpha$  expression in human tumours remained largely unexplored, as monoclonal antibodies available for immunohistochemistry have only recently been developed. Indeed, immunohistochemical analysis performed in this study used monoclonal antibodies to HIF-1 $\alpha$  which had just become commercially

- 431 -
available and had not yet been used widely for immunohistochemical analysis. Several studies have only just emerged accessing the expression of HIF-1 $\alpha$  in a variety of tumours types, including breast (Birner *et al*, 2001; Aebersold *et al*, 2001; Qin *et al*, 2001; Birner *et al*, 2001b). A recent report found that HIF-1 $\alpha$  protein was undetectable in normal breast tissue and benign breast lesions but was expressed in *in situ* and invasive breast cancers, with increased levels as dedifferentiation progressed (Bos *et al*, 2001). The report also found HIF-1 $\alpha$  positivity in normal tissue adjacent to tumour, thus emphasising the importance of obtaining control tissue from healthy control subjects.

In this study, immunohistochemical analysis revealed that only 23 % of breast tumours displayed nuclear HIF-1 $\alpha$  immunoreactivity. It is possible that there was less oxygen consumption and less metabolically driven hypoxia and therefore, less induction of HIF-1 $\alpha$  in the tumours. Although a high percentage of HIF-1 $\alpha$  immunoreactivity was found in the cytoplasm (41%), as a granular staining pattern distributed throughout the cytosol, the significance of this is not certain and is difficult to explain. While nuclear HIF is the active form, HIF-1 $\alpha$  is synthesised as well as degraded in the cytoplasm (Wang et al, 1995; Jiang et al, 1996). Cytoplasmic staining has also been reported in a number of recent studies (Zhong et al, 1999; Aebersold et al, 2001; Giatromanolaki et al, 2001) with possible explanations of diffusion of the HIF-1 $\alpha$  protein from within the nucleus, or sequestration of an inactive pool (Karth et al, 2000). As a sensitive biotin based method with amplification was used in this study, higher levels of background staining were observed. Given that biotin is in mitochondria, it is possible that the tumour cells analysed in this study may have contained large amounts of mitochondria which would also account for this observation, although staining was not seen with omission of the primary antibody. It has also been suggested that strong cytoplasmic HIF expression better reflects the HIF up-regulated pathway in paraffin-embedded material (Giatromanolaki et al, 2001).

### Chapter 8. Discussion

Although GLUT-1 protein expression was not investigated in this study, immunostaining for GLUT-1 has been described as a sensitive and specific means of detecting breast carcinoma in effusions (Burstein *et al*, 1998), although a more recent report has found that GLUT-1 expression is of limited value (Zimmerman *et al*, 2001). GLUT-1 has been investigated immunohistochemically in a variety of malignant and normal tissues, including tumours of the breast (Brown and Wahl, 1993; Younes *et al*, 1995), thyroid (Haber *et al*, 1997), head and neck (Mellanen *et al*, 1994), bladder (Chang *et al*, 2000), lung (Brown *et al*, 1999), renal cell carcinoma (Nagase *et al*, 1995) and more recently in gastric carcinoma (Kawamura *et al*, 2001), ovarian carcinoma (Cantuaria *et al*, 2001), and carcinoma of the cervix (Airley *et al*, 2001). In all cases, expression of GLUT-1 expression had been assessed, results suggest that overexpression is associated with tumour aggressiveness and poor survival (Kawamura *et al*, 2001; Cantuaria *et al*, 2001). An additional study has found that GLUT-1 expression correlates with tumour hypoxia (Airley *et al*, 2001).

In this study, breast tumours were observed in which only one of the two genes, *HIF-1* $\alpha$  or *GLUT-1* mRNA was overexpressed or where *GLUT-1* mRNA was overexpressed but HIF-1 $\alpha$  protein was not, suggesting that possible additional regulatory mechanisms exist. It is known that hypoxia regulates *GLUT-1* mRNA expression (Mueckler, 1994), mediated by HIF-1 which binds to a *cis*-acting binding sites located within the 5' flanking region of the *GLUT-1* gene (Ebert *et al*, 1995). Thus where the expression of *GLUT-1* mRNA is increased, an increase in HIF-1 $\alpha$  protein is also expected, however this did not always occur in this study. A recent report supports the findings in this study where although a progressive increase in *GLUT-1* mRNA expression with increasing abundance of HIF-1 $\alpha$  protein was observed in clear cell renal carcinoma, single tumours were observed in which *GLUT-1* was not overexpressed (Wiesener *et al*, 2001).

- 433 -

Analysis of p53 expression in the breast tumours would provide additional information as accumulation is a result of its stabilisation by HIF-1 $\alpha$  (An *et al*, 1998) and wild-type p53 promotes degradation of HIF-1 $\alpha$  protein (Ravi *et al*, 2000). Additionally, the breast tumours analysed in this study were classified as either T<sub>1</sub> (<2 cm) or T<sub>2</sub> (2-5 cm) tumour stage, the earlier stages. It is widely accepted that the development of hypoxia is caused by the rapid growth rate of the tumour that is not accompanied by an adequate neovascularisation (Dang *et al*, 1999). As the intra-cellular O<sub>2</sub> levels were not known, it is possible that these tumours were well vascularised and hence well oxygenated. Experiments have shown that an oxygen concentration of  $\leq 1.5\%$  up-regulates cellular glucose uptake independent of glucose deprivation (Clavo *et al*, 1995), thus any O<sub>2</sub> level above this would not up-regulate HIF-1 $\alpha$  protein or *GLUT-1* gene expression. However, previous studies have shown that tumour oxygenation is independent of tumour characteristics, such as tumour size and grading (Höckel *et al*, 1996; Brizel *et al*, 1996), thus tumour oxygenation is not a predictable factor and must be assessed in each tumour individually.

Overall cumulative survival was assessed in this small series of patients with breast tumours with no significant influence found from either *HIF-1* $\alpha$  or *GLUT-1* positive or negative mRNA expression. However, tumour T stage and N stage were found to be significantly associated with survival in this study group. Tumour grade was not expected to influence survival in this study group as only one G<sub>1</sub> grade tumour was available for analysis. Although statistical power was also limited due to the low number of cases analysed (n=22) with a relatively short follow-up, a significant correlation was found between survival time and the NPI. It is interesting to note that all 3 of the patients who succumbed to the disease were categorised in the poor prognosis group, implying that the NPI is a good prognostic indicator for patients with breast cancer.

# 8.4 HIF-1a, VEGF and p53 Expression In Glioblastoma

Glioblastoma multiforme is one of the most frequent forms of brain tumour and accounts for approximately 12-15% of all intra-cranial neoplasms (Zulch, 1986). It is characterised by high proliferation rates, necrosis and prominent angiogenesis. HIF-1 $\alpha$  plays a critical role in the regulation of many genes which have important roles in neovascularisation and tumour metabolism, such as *VEGF* and *p53*. *VEGF* has been shown to be upregulated by hypoxia in glioma cells, where it is found in both low and high-grade gliomas and in brain metastasis from different primary tumours (Machein and Plate, 2000).

Until recently, no data existed on the expression of HIF-1 $\alpha$  in human brain tumours. At the time of this study there were no reports of *HIF-1\alpha* mRNA or protein expression in glioblastoma. Recent data have suggested a role for HIF-1 $\alpha$  expression in glioblastoma, although little is known about the mechanism controlling its expression (Zagzag *et al*, 2000; Zhong *et al*, 1999).

In this preliminary study, 77 % of high-grade astrocytic astrocytic tumours were found to over-express HIF-1 $\alpha$  protein and 84 % VEGF protein. As noted in previous studies (Zagzag *et al*, 2000; Zhong *et al*, 1999), HIF-1 $\alpha$  protein expression was particularly strong surrounding areas of necrosis in glioblastomas, which is in keeping with a direct role for HIF-1 in the regulation of VEGF (Plate *et al*, 1992), with prominent nuclear expression being consistent with HIF-1 $\alpha$  acting as a transcription factor. Hypoxia is known to stabilise HIF-1 $\alpha$  protein (Huang *et al*, 1996) and may explain expression adjacent to necrosis, but HIF-1 $\alpha$  was present more diffusely in high grade astrocytic tumours, and was stronger in infiltrating tumour cells then reactive glia suggesting that expression is aberrantly controlled.

There were clear differences in the level of expression of HIF-1 $\alpha$  between the glioblastoma samples compared to lower grade tumours and normal brain. All

- 435 -

glioblastoma samples displayed similar degrees of necrosis and vascular proliferation. In many of the tumours over-expressing HIF-1 $\alpha$ , increased expression of HIF-1 $\alpha$  mRNA was also found, suggesting that control is at least partly at the transcriptional level. However, in several tumours over-expressing HIF-1 $\alpha$  protein, no increase in HIF-1 $\alpha$  mRNA was detected. Although this finding could be due to sampling error, as relatively small samples were available for mRNA analysis, it is also possible that post-transcriptional mechanisms may play a role. Three possible mechanisms are reduced HIF-1  $\alpha$  degradation due to loss of p53, PTEN and/or VHL function. PTEN is a tumour suppressor gene originally isolated from a homozygous deletion on chromosome 10q23 in glioblastoma (Li et al, 1998; Steck et al, 1997). Recently it has been shown that wild-type PTEN mediates the degradation of HIF-1 $\alpha$  and therefore the frequent loss of PTEN in glioblastoma (Wang et al, 1997), which may facilitate aberrant expression of HIF-1a (Zundel et al, 2000). Wild-type p53 promotes degradation of HIF-1a protein (Ravi et al, 2000) and it might be expected that inactivation of p53 may lead to increased HIF-1a expression. As most solid tumours contain hypoxic regions, a mutated p53 gene may confer a survival advantage to tumour cells under very low oxygen conditions (Graeber et al, 1996).

Although all of the glioblastoma samples displayed some degree of immunohistochemical p53 expression in some tumour cells, over-expression of the protein only occurred in 64% of samples. Mutations in *p53* are usually missense, which prolong the half-life of the protein and render it immunodetectable (Simmons *et al*, 2001). No correlation was found between p53 over-expression with either HIF-1 $\alpha$  protein or mRNA expression, a finding supported by a recent report where p53 over-expression was found in only 2 of 51 specimens of oligodendrogliomas (Birner *et al*, 2001). However, this does not preclude an association between HIF-1 $\alpha$  and p53 in glioblastoma as p53 protein over-expression in gliomas is not always due to a gene mutation and the relationship between HIF-1 $\alpha$  and p53 is complex with wild-type p53 being stabilised by HIF-1 $\alpha$  (An *et al*,

1998). The results in this study concur with a previous study that found no evidence for increased expression of the *VEGF* gene in glioma cells that contain a mutated form of p53 (Plate *et al*, 1994). It is also known that levels of p53 in cultured neurons increases with the duration of hypoxia, thus changes in the duration of hypoxia can alter cellular levels of p53 (Banasiak *et al*, 1998). Western blot analysis of p53 protein expression and screening p53 gene abnormalities in these tumours may help in further clarifying this association.

Another possible mechanism is that loss of VHL function may lead to HIF-1 $\alpha$  accumulation. The VHL gene product (pVHL) is required for regulation of the HIF-1 transcriptional control system (Maxwell *et al*, 1999) and the VHL gene may be mutated in glial neoplasms (Kanno *et al*, 1997). VHL forms a complex with elongins B and C, Cul 2 and the Ring-H2 finger protein Rbx1 (VCBCR complex) by binding to the oxygen dependent destruction domain of HIF-1 $\alpha$  subunits through the  $\beta$ -domain of pVHL (Lonergan *et al*, 1998; Kamura *et al*, 2000; Ohh *et al*, 2000). pVHL then acts as the recognition component of the E3 ubiquitin ligase complex mediating HIF-1 $\alpha$  destruction (Cockman *et al*, 2000; Iwai *et al*, 1999; Tanimoto *et al*, 2000). Under hypoxia, VHL-mediated degradation of HIF-1 $\alpha$  does not occur leading to accumulation of HIF-1 $\alpha$ . Additionally, non-functional VHL also results in the accumulation of HIF-1 $\alpha$  in normoxic conditions (Maxwell *et al*, 1999).

The increased expression of both HIF-1 $\alpha$  and VEGF support recent *in-vitro* studies showing that transcription of the VEGF gene is regulated by HIF-1 in cells grown under hypoxic conditions (Forsythe *et al*, 1996). The amplimers did not differentiate between the known VEGF isoforms, however previous studies have shown that in glioblastoma the 121, 165 and 189 isoforms are all present with the 165 isoform being most abundant (Berkman *et al*, 1993). Although previous studies found over-expression of VEGF in glioblastoma, this feature was not demonstrated in this study. This may be due to technical difficulties occurred within the assay. It is clearly difficult to compare the data obtained in

- 437 -

this study with those obtained in other studies using disparate techniques. Further, in this study it was not possible to compare *VEGF* mRNA with the protein or, rate of transcription of this gene in the tumour.

However, there was a marked correlation between HIF-1 $\alpha$  and VEGF expression in the glioblastoma samples, which is probably a reflection of the small sample size used in this study. Factors such as the quality and the quantity of the tumour tissue collected may explain this lack of correlation as it was not possible to control for sample size, location, nor the cross-sectional heterogeneity of the tumour samples at the time of collection. Additionally, the expeditious expression of 1 to 2 hours for HIF-1 $\alpha$  mRNA (Wang *et al*, 1995) makes its labile compared to *VEGF*.

Glioblastomas are the most common type of primary brain tumour, and despite treatment has a median survival of only 9-12 months (Walker *et al*, 1980). In contrast to most lower grade astrocytic tumours, glioblastomas show prominent new blood vessel formation, which is an important independent indicator of poor prognosis (Daumas-Duport *et al*, 1988). To investigate whether HIF-1 $\alpha$ , VEGF and p53 protein expression could be used as a prognostic factor in the pathogenesis of glioblastoma, overall cumulative survival was assessed in all patients with brain tumours as well as in patients with glioblastomas. To date, the most consistent predictor of survival in malignant gliomas is patient age at diagnosis. Multiple studies have shown that younger patients with these tumours live longer following initial diagnosis, even after adjustments for histological grade, size of tumour, extent of resection and treatment following biopsy/resection (Salmon *et al*, 1994, Kim *et al*, 1991, Rasheed *et al*, 1994; Sneed *et al*, 1995). Results from this study support these previous findings where age at diagnosis was found to be an independent prognostic factor on overall survival.

When survival analysis was limited to protein expression, both HIF-1 $\alpha$  and VEGF protein expression were found to be significantly associated with overall survival in all

- 438 -

patients with brain tumours but not in patients with glioblastoma. A recent study found that the relative intensity of HIF-1 $\alpha$  protein expression is of independent prognostic significance in oligodendrogliomas, as well as extent of resection in univariate and histologic grading in multivariate analysis (Birner *et al*, 2001). Univariate analysis of all patients with brain tumours and of patients with glioblastoma in this study did not find that HIF-1 $\alpha$  protein expression is an independent prognostic factor in overall survival. As the number of patients with various tumour types analysed were relatively small, it would be interesting to see if these findings were consistent in a larger patient population.

The recent discovery of two new members of the HIF family, HIF-2 $\alpha$  (Ema *et al*, 1997; Flamme *et al*, 1997; Tian *et al*, 1997) and HIF-3 $\alpha$  (Gu *et al*, 1998), demonstrates the complexity involved in the regulation of angiogenesis. These new family members may also play a role in mediating the response of the cell to hypoxia. Recent studies have shown that HIF-2 $\alpha$  is an important co-factor in the regulation of VEGF in bladder cancer (Jones *et al*, 2001) as well as being an independent prognostic indicator in non-small cell lung cancer (Giatromanolaki *et al*, 2001). Further studies are now required using a larger sample population to include Western blot analysis of HIF-1 $\alpha$  protein, in order to determine the precise role of HIF-1 in the response to hypoxia and angiogenesis in these tumours. This study has demonstrated a marked increase in the level of expression of the transcription factor HIF-1 $\alpha$  in glioblastoma multiforme when compared to lower grade tumours or normal brain tissue, suggesting that it has an important role in this disease.

# 8.5 p53 and p73 Expression In Patients with Breast Cancer

p73, like p53, is ubiquitously expressed in peripheral blood cells and has been reported to be monoallelically expressed and possibly imprinted (Kaghad *et al*, 1997). Although polymorphisms of the p73 gene have been studied in a number of tumours including bladder, colorectal, oesophageal, liver, lung, neural, prostate, and renal cell carcinoma (Yokomiza *et al*, 1999; Sunahara *et al*, 1998; Nimura *et al*, 1998; Mihara *et al*, 1999; Nomoto *et al*, 1998; Kovalev *et al*, 1998; Takahashi *et al*, 1998; Mai *et al*, 1998), at the time of this study there were no reports of p73 expression in breast cancer. These reports have found several p73 polymorphisms and showed that p73 somatic mutations are very rare in solid tumours (Han *et al*, 1999). Recently however, p73 expression has been reported in several breast cell lines and tumours (Zaika *et al*, 1999; Shishikura *et al*, 1999; Ahomadegbe *et al*, 2000).

In this study, where p73 genotypes were ascertained in patients with breast cancer and normal control populations; similar genotype frequencies were observed in all three populations. The frequency of the p73 AT/AT homozygous genotype was found to be very rare in all 3 populations studied, with a statistically significant difference found between the expression of the p73 AT/AT genotype and the remaining genotypes in all 3 populations studied. The over-representation of the p73 GC/GC genotype was not linked to the population of patients with breast cancer as similar results were obtained in the 2 control populations. This finding was confirmed in a recent report where only 18% of the sample population were found to have the p73 GC/AT heterozygous genotype with the AT allele being preferentially silent in 10 of 12 breast tumours (Ahomadegbe et al, 2000). Another study in primary gliomas found the p73 AT/AT genotype to be rare, being expressed in only 1 of 27 tumours (4%) (Watanabe et al, 2002). Although the initial analysis on lymphocytes from five healthy individuals and a neuroblastoma cell line described only monoallelic expression of the p73 gene, subsequent analysis on normal lung (Nomoto et al, 1998), thyroid, lymphocytes, neuroblastoma (Kovalev et al, 1998), kidney (Mai et al, 1998) and melanoma (Tsao et al, 1999) demonstrated biallelic expression in the majority of the cases studied. Consistent with these data, results from this study also indicate biallelic expression, which were confirmed in another report assessing breast cancer tissue where p73 was found to be biallelically expressed in 57% of tumours (Zaika *et al*, 1999). It can be concluded that monoallelic expression of p73 is highly uncommon and varies from tissue to tissue and person to person.

As carcinoma of the breast affects predominately older women, the frequency of the p73 genotypes was compared with the age at diagnosis. A significant association was found with the frequency of the p73 GC/GC genotype and patients with breast cancer diagnosed over 52 years of age (median). This association was not observed in the age matched Celtic control population. These results suggest that there may be an association between the p73 GC/GC polymorphism and carcinoma of the breast.

A single point substitution at nucleotide position 216 leads to a change at residue 72 of the p53 polypeptide chain. A recent report suggests that the p53 polymorphism is associated with increased risk of developing HPV-associated cancers (Storey *et al*, 1998). However, further reports contradict this finding and have found that these p53 polymorphisms are not risk factors in the development of these cancers (Hayes *et al*, 1998; Rosenthal *et al*, 1998; Helland *et al*, 1998; Josefsson *et al*, 1998; Hildesheim *et al*, 1998). In this study two separate populations were screened, patients with breast cancer and a Cornish Celtic population, for p53 polymorphisms and no significant differences between the populations were found. The frequency of the p53 polymorphisms in the patients with breast cancer (57% p53 Arg/Arg, 42% p53 Pro/Arg, 1% p53 Pro/Pro) were similar to those found in another study where 109 patients with breast cancer were used as a control population for patients with cervical cancer (58% p53 Arg/Arg, 37% p53 Pro/Arg, 5% p53 Pro/Pro) (Helland *et al*, 1998).

Large differences exist in the frequency of the p53 Pro and p53 Arg alleles between different populations (Beckman *et al*, 1994; To-Figueras *et al*, 1996) thus, screening an additional control population of known Celtic origin has eliminated the possibility of variation in p53 isoform expression due to mixed ethnic origin. Consequently the results obtained support recent reports that the p53 polymorphism may not be a risk factor in the development of certain cancers. As with the p73 AT/AT genotype, the p53 Pro isoform was found to be rare in all 3 populations studied.

At the present time the function of p73 is not known, however it is thought to be similar to p53 as both inhibit cell growth, possibly through different induction pathways, induce apoptosis and strongly interact with each other. A recent report suggests a functional interaction between p53 and p73 in a breast carcinoma cell line leads to enhanced induction of apoptosis (Zhu et al, 2001). Comparison of the frequencies of the p73 genotypes versus p53 Pro/Arg isoforms in all three populations showed no statistically significant correlation between either the breast patient population or the Celtic population with the control cord bloods. However, a synergistic effect was observed between p73 and p53 in all three populations. Despite being located on separate chromosomes, certain p73and p53 alleles were found together more frequently than expected. It is possible that particular combinations of p73 and p53 alleles have an enhanced function for maintenance of genome integrity compared to other combinations of alleles. This may have an important role in embryonic development where genome integrity is of critical importance for the survival of the zygote. It has already been proposed that p73, an ancestral p53, may play a role in development through induction of differentiation of certain tissues (Kaghad et al, 1997), a significant instance where loss of p53 has no phenotypical consequence.

To investigate whether p73 genotyping and p53 isoforms could be used as prognostic factors in the pathogenesis of breast cancer, cumulative overall survival was assessed and correlated with both p73 genotypes and p53 isoforms and clinical characteristics in the 97 patients with breast cancer. Correlation studies between both genotypes and disease variables in breast carcinoma did not demonstrate any significant associations, however tumour T stage, G stage and patient treatment with chemotherapy were found to be significantly associated with survival in this study group. To date, only one study has been reported which suggests that allelic loss of heterozygosity (LOH) in the p73 region could be pathologically related to breast cancer and possibly to poor tumour prognosis (Dominguez *et al*, 2000). Previous studies have not found any correlations between breast tumours with LOH in the p73 region or with p73 allelic expression and certain pathological parameters associated with poor prognosis (Shishikura *et al*, 1999; Ahomadegbe *et al*, 2000). Allelic loss in several chromosomal regions in breast cancer has been used as a prognostic factor (Harada *et al*, 1994). Allelic loss in the p73 region could indicate gene inactivation if monoallelic expression and LOH occurred at the same allele.

There has been extensive research into identifying clinically useful prognostic factors in the pathogenesis of primary breast cancer. The Nottingham prognostic index (NPI) is considered a reliable standard for prognosis in breast cancer patients as it is based on the morphopathologic features of lymph node stage (N stage), tumour size (T stage) and histologic grading of malignancy (Grade) (Haybittle *et al*, 1982). The NPI has been validated in several studies and has proven to maintain over time a valuable discriminating power in differentiating patients in low, intermediate and high risk subsets (Galea *et al*, 1992; Balslev *et al*, 1994). The NPI was assessed in this study group with a significant correlation found between survival time and NPI. When both p73 genotypes and p53 isoforms were correlated with the NPI no association was found, thus p73 genotypes and p53 isoforms do not contribute prognostically to the NPI.

It is believed that wild-type p73 functions similarly to p53, as a guardian when overexpressed and as a cell cycle regulator as a tumour suppressor gene. It is possible that wild type p73, not mutant p73, is overexpressed in cancers and it has been proposed that p73 may also function as an oncogene in the upregulation of cell growth (Nomoto *et al*, 1998) and silences p53 function by binding to its functional binding site (Vikhanskaya *et al*, 2000). To date, overexpression of p73 has been found in tumours of the breast, lung, neuroblastoma, oesophagus, stomach, colon, bladder, ovary, ependymoma, hepatocellular

### Chapter 8. Discussion

carcinoma and myeloid leukaemia (Zaika *et al*, 1999; Nomoto *et al*, 1998; Kovalev *et al*, 1998; Mai *et al*, 1998; Cai *et al*, 2000; Kang *et al*, 2000; Sunahara *et al*, 1998; Imyanitov *et al*, 1999; Peters *et al*, 1999; Yokomizo *et al*, 1999; Loiseau *et al*, 1999; Herath *et al*, 2000). In contrast, analysis of normal human tissue determined that p73 is expressed at very low levels (Kovalev *et al*, 1998; Ikawa *et al*, 1999; Zaika *et al*, 1999). Overexpression of wild-type p73 mRNA is to date the most relevant alteration involving p73 in breast cancer. However, a recent contradictory report has found that LOH, allele silencing and decreased expression of the p73 gene may play a role in breast cancer (Ahomadegbe *et al*, 2000). This contradiction in results may be explained by the methods of quantitation used and the multiple origin of the normal tissue analysed, which may show a different pattern of p73 *in vivo*, where overexpression of cellular and viral oncogenes up-regulate endogenous p73 proteins and activate their transactivation function (Zaika *et al*, 2001).

As the p53 family of tumour suppressor genes grows (Osada *et al*, 1998; Trink *et al*, 1998; Yang *et al*, 1998), the necessity for genotype screening increases. This is the first report of both p73 genotypes and p53 isoforms in individuals. At the time of this study this was the first report of p73 in breast cancer, however a further study has recently been reported with findings that p73 somatic mutations were very rare in breast cancer (Han *et al*, 1999). It is becoming clearer that p73 is not a tumour suppressor gene in a classic Knudson two-hit manner as p73 is infrequently mutated in many human cancers, unlike p53. In extensive searches very few mutations have been found in a variety of primary cancers analysed (Ichiyama *et al*, 1999; Han *et al*, 1999).

Overall the results in this study indicate that no association exists between p73 genotypes and p53 isoforms in patients with breast cancer and are therefore, not risk factors in the development of carcinoma of the breast in the British Caucasoid population.

# **8.6 Conclusions**

The principle findings of this work are as follows;

+ *HIF-1* $\alpha$ , *GLUT-1* and *p53* mRNA expression increased in response to high glucose but decreased in response to the combined stresses of hypoxia and high glucose from normoxic levels in breast carcinoma cell lines. *HIF-1* $\alpha$  mRNA levels were observed in breast carcinoma cell lines under normoxic conditions which displayed both increased and decreased expression in response to exposure to hypoxia and/or high glucose as well as to controls for osmolarity. These combined results confirm that hypoxic regulation of HIF-1 $\alpha$ , p53 and possibly GLUT-1 occurs post-transcriptionally. *HIF-1\alpha*, in conjunction with *GLUT-1* and *p53* may be an important regulator of the cellular response to osmotic stress and high glucose.

Solutions Up-regulation of HIF-1 $\alpha$  and GLUT-1 mRNA was observed in the PBMCs from patients with T1DM exposed to high glucose. The GLUT-1 mRNA up-regulation observed in patients without complications was significantly different from that seen in normal controls, where up to a 2.1 fold increase in mRNA expression was seen over that expressed in patients with complications. This would indicate that the expression and function of glucose transporters may differ in these patients, potentially leading to fewer complications.

A significant correlation was found between the expression of HIF-1 $\alpha$  and GLUT-1 mRNA in patients with breast tumours. Additional significance was found between HIF- $I\alpha$  mRNA expression and age of diagnosis and grade, and between GLUT-1 mRNA expression and age of diagnosis and N stage, in the patient group. These results suggest that both HIF-1 $\alpha$  and *GLUT-1* mRNA expression increases with disease progression and may contribute to tumour aggressiveness.

Superegulation of HIF-1 $\alpha$  mRNA was observed in glioblastomas with significant differences found between normal tissue and low-grade diffuse (LGA), anaplastic astrocytomas (AA), and glioblastomas (GBM) and between GBM and LGA and AA, indicating that up-regulation of HIF-1 $\alpha$  is partly at the transcriptional level. No correlation was found between HIF-1 $\alpha$  and p53 protein expression in these tumours however, age at diagnosis and HIF-1 $\alpha$  protein expression, as well as HIF-1 $\alpha$  and VEGF protein expression, were significant prognostic factors in cumulative overall survival. Although a small number of samples was studied, a significant association was also found between sex and VEGF protein expression on cumulative overall survival.

Polymorphisms of either p53 or p73 genes are not risk factors in the development of carcinoma of the breast. However, subtle interactions may occur between these genes and these may be involved in the pathogenesis of the disease. A significant association was found with the frequency of the p73 GC/GC genotype and patients with breast cancer diagnosed over 52 years of age (median). Both p73 genotype and p53 isoforms have no significant influence on overall survival in patients with breast cancer.

It has been seven years since the purification of HIF-1 protein (Wang and Semenza, 1995) and cloning of HIF-1 $\alpha$  cDNA sequences (Wang *et al*, 1995). Throughout this time, rapid progress has been made in elucidating the role that this transcription factor plays in normal development and physiology as well as in cancer biology. The accelerated rate of discovery and expanding knowledge about HIF-1 $\alpha$  certainly limits the work that one researcher can perform in a restricted time and environment. However, the limited studies

presented here represent interesting, informative and novel observations into how HIF-1 $\alpha$ and other oxygen related genes behave in different disease and disease models in man. It is hoped that this work will offer new insights and pose new questions as to how these diseases may be linked through a common factor, HIF-1.

# 8.7 Future Work

## 8.7.1 HIF-1α

Most studies have focused on HIF-1 $\alpha$  as a regulatory component of HIF-1. Future studies should investigate the roles of HIF-2 $\alpha$  and HIF-3 $\alpha$  in disease. HIF-2 $\alpha$  is a structurally related alternative dimerisation partner of HIF-1 $\beta$  which can also transactivate reporter genes via HIF DNA recognition sites (Wenger and Gassmann, 1997). *In vitro* it is regulated in an oxygen-dependent fashion very similar to that for HIF-1 $\alpha$  (Wiesener *et al*, 1998) where studies have shown that VHL deficiency also stabilises HIF-2 $\alpha$  (Maxwell *et al*, 1999). Moreover, a recent study has shown that the basal levels of *HIF-2\alpha* are highly related to the inducible level of the HIF protein in breast carcinoma cell lines (Blancher *et al*, 2000) and HIF-2 $\alpha$  expression has been seen in solid tumours (Talks *et al*, 2000). Additionally, overexpression of *HIF-2\alpha* mRNA has been reported in hemangioblastomas (Flamme *et al*, 1998). It would therefore be interesting to address in future studies the extent to which HIF-2 and HIF-3 are induced in breast and brain tumours, and how it may contribute to and amplify the induction of HIF-responsive genes.

## 8.7.2 VEGF

VEGF is constitutively expressed by many tumour cells, transformed cell lines and by some normal cells, however VEGF expression is substantially up-regulated by hypoxia, cytokines, hormones, and certain oncogenes including activated forms of *Ras* and *Src* (Rak *et al*, 1995; Pal *et al*, 2001; Mukhopadhyay *et al*, 1995). Although it has been demonstrated that breast cancer cells express both VEGF and VEGF receptors (Yoshiji *et al*, 1996; Speirs and Atkin, 1999; Blancher *et al*, 2000; Price *et al*, 2001), little is known about the regulation of VEGF expression in breast cancer cells. A recent study demonstrated that *p53* plays an important role in regulating breast tumour angiogenesis through inhibition of VEGF transcriptional activation, under both normoxic and hypoxic conditions, by inhibiting the binding of Sp1 to the promoter region of the VEGF gene (Pal *et al*, 2001). Additionally, VEGF was recently shown to require extracellular matrix (ECM) binding components, such as fibronectin, heparin and heparin sulfate proteoglycans to induce a mitogenic response and cell migration in breast carcinoma cells (Miralem *et al*, 2001). Thus, VEGF obviously plays an important role in the pathogenesis of breast cancer and warrants further investigation.

### 8.7.3 GLUT-1

As an increase in glucose transport is observed in malignancies and increased uptake of glucose has potential value as a prognostic indicator (Minn *et al*, 1997) it would be interesting to determine glucose transporter activity (measurement of 2 DG accumulation) in the breast carcinoma cell lines exposed to hypoxia as well as in breast and brain tumours and correlate the results with survival. Experiments have shown that an oxygen concentration of  $\leq 1.5\%$  up-regulates cellular glucose uptake independent of glucose deprivation (Clavo *et al*, 1995).

#### 8.7.4 Other HIF-1 Induced Genes

Most tumour cells retain the ability to undergo apoptosis in response to hypoxic stress (Shimizu *et al*, 1996), resulting in cells that are more resistant to treatment and which may contribute to a reoccurrence of the tumour (Schmaltz *et al*, 1998). HIF-1 inducible genes are well documented however, genes other than anaerobic metabolism associated genes may also play a role in tumour growth and survival as well as in resistance to apoptosis. It is probable that these genes may function as anti-apoptotic factors when induced by hypoxia and glucose deprivation. Thus, it would be interesting to screen for genes induced by hypoxia and hypoglycaemia in breast carcinoma cell lines using gene expression arrays (DNA microarray system).

A recent report detected the up-regulation of BNIP3 (Bcl2/adenovirus EIB 19 kDinteracting protein 3) by hypoxia in the breast carcinoma cell line T47D using gene expression arrays (Sowter *et al*, 2001). BNIP3 is a pro-apoptotic mitochondrial protein that was isolated through its interaction with E1B 19K and Bcl-2 (Boyd *et al*, 1994). Other genes found to be regulated by HIF-1 include the transferring receptor (Tacchini *et al*, 1999) and ceruloplasmin (Mukhopodhyay *et al*, 2000) genes. Conversely, screening for genes induced by hypoxia and other extreme stresses, such as high glucose, in breast carcinoma cell lines would be equally worthwhile.

## 8.7.5 p73

It has been widely reported that p73 is highly expressed in cancer but not in normal cells or benign tumours with contradictory reports. This paradox requires further investigation, particularly as the conflicting reports have been reported in breast cancer. One of the effects of p73 up-regulation is to steer cells into apoptosis (Jost *et al*, 1997; Zhu *et al*, 1998) and to block cells in the  $G_0/G_1$  phase of the cell cycle (DeLaurenzi *et al*, 2000). Mutant p53 is known to physically interact with p73 to inhibit the induction of apoptosis by p73 (DiComo *et al*, 1999). It is possible that enhanced p73 expression could act as a -449-

feedback mechanism to compensate for the loss of p73 function. The exact function of p73 protein is not completely known and its expression in various cancers has not been extensively analysed (Peters *et al*, 1999; Tannapfel *et al*, 1999; Zwahlen *et al*, 2000). Little is known about the upstream signals that induce a p73 response, in contrast to p53 protein which is stabilised and activated in response to a variety of cellular stresses. p73 protein may have additional important functions in normal cells and cancer cells which have not been elucidated. Thus it is important to analyse and quantitate the expression of p73 in a large sample of breast tumours with their corresponding normal tissue, as well as determine protein expression to establish whether p73 protein plays an important in the pathogenesis of breast cancer. p53 phenotyping would also be required to assess wild-type or mutant status. Difficulties in detecting p73 protein expression have been reported in both breast tissue and breast carcinoma cell lines (Zaika *et al*, 1999; Zhu *et al*, 2001) using commercially available monoclonal antibodies, thus a more sensitive radioactive RT-PCR method (Marin *et al*, 1998; Sunahara *et al*, 1998) and better quality antibodies would be required.

## 8.7.6 Apoptosis

Angiogenesis and apoptosis have been shown to play a role in the development of solid tumours. As both HIF-1 $\alpha$  and the apoptotic index represent individual markers of these complex processes, it would be interesting to determine the apoptotic rate in both breast and brain tumour samples using an indirect TUNEL (terminal deoxynucleotidyl transferase-mediated nick end-labelling) assay and compare it with HIF-1 $\alpha$  expression to identify a possible survival advantage. Bcl-2 expression should also be determined as hypoxia inhibits Bcl-2, an anti-apoptotic factor, via HIF-1 (Carmeliet *et al*, 1998). A previous study found that HIF-1 $\alpha$  was strongly positively associated with the apoptotic rate increases

during breast carcinogenesis (Carmeliet et al, 1998) and is high in highly proliferative and poorly differentiated cancers (De Jong et al, 2000).

# Chapter 9.

# Appendix

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# 9.0 p53 Allelic Response To Hypoxia

It is known that increased HIF-1 $\alpha$  expression is associated with multiple genetic alterations, such as loss of function mutations in the p53 tumour suppressor gene. HIF-1 $\alpha$ binds directly to and stabilises p53 (An *et al*, 1998). Additionally, p53 interacts with HIF-1 $\alpha$  in vivo and inhibits expression of HIF-1 $\alpha$ , thereby inducing its degradation through ubiquitination. Thus, it is possible that p53 mutants may correlate with the ability of a cell to respond to hypoxia through differential binding to HIF-1 $\alpha$ . Moreover, it is also possible that HIF-1 $\alpha$  contains polymorphisms in its binding region and may preferentially recognise either of the two forms of p53.

### 9.1 Transfection of Saos-2 cells with p53 Pro/Arg plasmids

To test these hypotheses, p53 null Saos-2 cells were transfected with plasmids expressing either p53 Pro or p53 Arg to study their ability to induce HIF-1 $\alpha$  mRNA expression, and other oxygen regulated genes, under hypoxia (Dr. A. Storey, Imperial Cancer Research Fund, London, (Storey *et al*, 1998).

Saos-2 cells harbour a homozygous deletion of the p53 gene locus and do not produce a p53 protein (Diller *et al*, 1990). Plasmids containing p53Pro and p53 Arg cDNAs were cloned, isolated (**Figure 9.1**) and transfected by calcium phosphate precipitation into Saos-2 cells. Transfection efficiencies were monitored by co-transfecting a *lacZ*-expressing plasmid on parallel plates and staining for LacZ expression.

Although transfections of both plasmids were successful, the cells failed to proliferate. Limited growth resulted in an insufficient quantity of cells following selection to further proceed. Selection conditions were optimised. Transfections were performed successfully on 4 separate occasions, verified by staining for  $\beta$ -galactosidase, each time resulting in a lack of cell proliferation. To alleviate the possibility of failure of the methodology, consultations were sought with Dr. Storey in London who also could not

determine the problem. Time constraints for the research degree resulted in a decision to abandon this part of the project and continue with another area of research.





- Whole plasmid DNA

- Insert DNA

p53 Pro p53 Arg

**Figure 9.1**. Illustration of p53 Pro and p53 Arg isolated plasmid DNA and subsequent double digestions using HindIII and EcoRI restriction enzymes. Lane 2 contains p53 Pro plasmid DNA and lane 4 contains p53 Arg plasmid DNA, producing a DNA insert of approximately 2 Kb. (MW= 23 Kb ladder).

# Chapter 10.

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# Chapter 11.

# Publications

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# Expression of hypoxia-inducible factor 1a in tumours of patients with glioblastoma

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#### Expression of hypoxia-inducible factor 1a in tumours of patients with glioblastoma

Angiogenesis is a prominent feature of glioblastomas but the mechanisms involved in the control of this process are poorly understood. We have investigated the potential role of a recently described transcription factor. hypoxia-inducible factor 1 (HIF-1), which initiates the transcription of a number of hypoxiainducible genes. including those encoding vascular endothelial growth factor and its receptors. HIF-1 protein expression was assessed by immunocytochemistry, using a monoclonal antibody to the  $\alpha$  subunit (HIF-1 $\alpha$ ). HIF-1 mRNA expression was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) and the ribonuclease protection assay (RPA). Strong nuclear expression of HIF-1 $\alpha$  protein was seen in the majority of glioblastomas and anaplastic astrocytomas. particularly surrounding areas of necrosis in glioblastomas. In the majority of these tumours upregulation of HIF-1 $\alpha$ mRNA was also demonstrated, with a significant increase in glioblastomas compared to lower grade tumours. No correlation was found between the presence of HIF-1 $\alpha$  protein and immunohistochemical expression of p53 protein. These findings are in keeping with an important role of HIF-1 $\alpha$  in the vascularization of glioblastomas and suggest that upregulation is at least partly at a transcriptional level.

Keywords: hypoxia-inducible factor 1, p53, angiogenesis. glioblastoma

# Introduction

Glioblastomas are the most common type of primary brain tumour and, despite treatment, have a median survival of only 9–12 months [34]. In contrast to most lower grade astrocytic tumours, glioblastomas show prominent new blood vessel formation, which is an important independent indicator of poor prognosis [4]. Angiogenesis is also associated with more aggressive tumour growth in several other human malignancies [10]. Experimental evidence and early reports of human trials suggest that inhibition of angiogenesis in

Correspondence: Dr A. G. Demaine. Department of Molecular Medicine. ITTC Building, Tamar Science Park. 1 Davy Road, Plymouth PL6 8BX, UK, E-mail: ademaine@plymouth.ac.uk glioblastomas may reduce tumour growth. A better understanding of the underlying mechanisms is clearly important to the development of new antiangiogenic treatment strategies [30,32].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric nuclear transcription factor that plays a critical role in the cellular response to low oxygen tension [35,37]. It is composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits which are both members of the basic helix-loop-helix (bHLH) PAS family of transcription factors [37]. Activation of HIF-1 $\alpha$ in cells exposed to hypoxia is mediated by stabilization of the HIF-1 $\alpha$  protein and possibly increased expression of the gene [15]. HIF-1 $\alpha$  is thought to play an important role in the pathogenesis of a number of conditions, including myocardial infarction, cerebrovascular disease. hypoxic lung disease and tumour vascularization [9.22.38]. Under hypoxic conditions. HIF-1 $\alpha$  binds to the hypoxia responsive elements (HREs) that are located in either the 5' or the 3' flanking region of the gene. Approximately 30 genes have been shown to be activated by HIF-1. including vascular endothelial growth factor (VEGF). erythropoletin, insulin-like growth factor 2, glycolytic enzymes and glucose transporter 1 [5.7.9.27.28].

Recent work has suggested a role for HIF-1 $\alpha$  in glioblastomas, although little is known about the mechanisms controlling its expression [39,40]. We have assessed the expression of HIF-1 protein and mRNA, and p53 protein. in a range of astrocytic tumours, including glioblastomas. The aim of this study was to investigate the levels and expression of these factors with respect to different grades of tumour.

#### Materials and methods

#### **Tumour** material

Forty-one samples of formalin-fixed and paraffinembedded sections of tumour samples were studied. Tissue from 34 of these tumour samples was snap-frozen and stored in liquid nitrogen prior to RNA extraction. Tumours were classified according to current World Health Organization guidelines [19]. Twenty-seven glioblastomas, four anaplastic astrocytomas, five diffuse astrocytomas (only three with frozen tissue) and five pilocytic astrocytomas (none with frozen tissue) were identified. Two frozen samples of normal brain tissue obtained at *post-mortem* were also used for the study.

# RNA isolation and reverse transcriptasepolymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from the frozen tissue using RNA Stat-60<sup>TM</sup> (Biogenesis, Poole, UK) according to the manufacturer's instructions. The concentration of the RNA was determined by optical density. Five μg of this RNA was used to generate first-strand cDNA using the Superscript<sup>TM</sup> preamplification system (Life Technologies, Paisley, UK) according to the manufacturer's instructions. One-twentieth of the cDNA was amplified in a polymerase chain reaction using amplimers specific for HIF-1α. Amplimer sequences for HIF-1 $\alpha$  were designed from a known sequence (accession no. U22431 [35]) as follows: 5' CACTTCCACATAATGTGAGTTCGG (sense) and 5' GGTTCACAAATCAGCACCAAGCAGG (antisense) using an initial denaturation step of 94°C for 3 min followed by 30 cycles of 94°C for 1 min. 55°C for 2 min and 72°C for 3 min. HIF-1 $\alpha$  amplifications were verified by performing an additional assay using amplimers for a recently discovered alternative splice variant to HIF-1 $\alpha$  [11].

The efficiency of amplifications was determined using β-actin amplimers as an internal standard to control for the quality of the cDNA in each sample. The reaction was performed in duplicate, first in the same sample reaction tube and then in separate reaction tubes. All amplification products were analysed by gel electrophoresis on a single 1.5% agarose gel and fragments were visualized using ethidium bromide. The intensity of the B-actin and HIF-1 a amplified products was quantified using a gel documentation and analysis system (Bio-Rad, Hertfordshire, UK). Background Intensity was subtracted from band intensity values. This was repeated several times with consistent findings, and the ratios of HIF-1a to β-actin are presented as a percentage expression where ratio values represent the mean of four separate experiments.

#### Ribonuclease protection assay (RPA)

A probe for the quantification of HIF mRNA was synthesized as follows: 4 µg of total RNA from the renal cell carcinoma cell line ACHN that had been exposed to hypoxia for 24 h was used as a template for cDNA using a Superscript<sup>TM</sup> preamplification system (Life Technologies) according to the manufacturer's instructions. An aliquot of the cDNA was then used to amplify the HIF sequence with the following cycle conditions: denaturation 4 min at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 1.5 min and 72°C for 2 min. The length of the HIF probe was 280 bp (accession number U22431). The antisense RNA probes were synthesized using a T7 in vitro synthesis kit (MaxiScript<sup>TM</sup>, Ambion, Austin, UK) in the presence of [<sup>32</sup>P] uridine triphosphate (30 TBq/mmol) (Amersham, Little Chalfont, UK).

The RPA was performed using an RPA II<sup>TM</sup> kit (Ambion. Austin, USA) according to the manufacturer's instructions. Briefly. 10 μg of RNA was hybridized with

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#### 212 K. L. Søndergaard et al.

500 pg of radioactively labelled HIF-1 probe in 80% formamide. 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4) and 1 mM ethylenediamine-tetraacetic acid (EDTA) at  $42^{\circ}$ C overnight. Samples were then digested with RNase A/RNase T1 for 30 min at 37°C and then inactivated and the RNA precipitated.

The protected fragments were separated on an 8 M urea/5% polyacrylamide denaturing gel and subsequently exposed to Kodak X-OMAT<sup>TM</sup> LS film. A control probe of 18S rRNA was run on samples to control for the quality of mRNA and equal sample loading. Signals were quantified from the gel using direct radioactivity measurement with a Fluor-S<sup>TM</sup> imager and Multi-Analyst<sup>6</sup> image analysis software (Bio-Rad). Results are expressed as the percentage expression of the mRNA against negative control levels.

## p53 immunocytochemistry

Primary antibodies to p53 were used (polyclonal CM1. Novacastra. Newcastle upon Tyne. UK). Sections (5  $\mu$ m) of paraffin-embedded tissue were collected onto 3-aminopropyltriethoxysilane-coated glass slides. The dewaxed sections were given microwave pretreatment for 12 min in sodium citrate. pH 6.0, then blocked with 3% hydrogen peroxide followed by normal goat serum. prior to overnight incubation with the primary antibody at a dilution of 1:64000. Detection was with biotinylated goat antimouse/rabbit immunoglobulin and streptavidin-biotin horseradish peroxidase amplification (DAKO, Cambridgeshire, UK). Colour was developed with diaminobenzidine.

#### HIF-1a immunocytochemistry

Immunocytochemistry for HIF-1 $\alpha$  was performed as above using a monoclonal antibody to HIF-1 $\alpha$  (clone OZ12. Neomarkers. Fremont, USA) at 1:1000 dilution and detection with the catalysed signal amplification system (DAKO).

#### Statistical analysis

Results are expressed as the ratio of intensity of the expression of the gene of interest compared to the housekeeping gene  $\beta$ -actin. Statistical significance was assessed on the data groups using analysis of variance (ANOVA) followed by *F*-test and the students *t*-test for comparison of means. with values of *P*<0.05 considered as significant.

# Results

#### HIF-1a mRNA expression

The expression of HIF-1 $\alpha$  mRNA was analysed by RT-PCR using specific amplimers for each (Figure 1) and by RPA. There were marked differences in the level of expression of HIF-1 $\alpha$  mRNA in the tumour samples from the different study groups (Figure 2a). There was a highly significant increase in the mean ratios of HIF-1 $\alpha$  to  $\beta$ -actin expression in the glioblastomas when compared to the low-grade diffuse astrocytomas (mean value 0.45 vs. 0.3. respectively. P=0.005) as well as the anaplastic astrocytomas (mean value 0.45 vs. 0.25, respectively, P<0.05) (Figure 2b). There was a marked



Figure 1. HIF-1 $\alpha$  and  $\beta$ -actin mRNA expression in glioblastoma. Amplification products were 520 base pairs (bp) for HIF-1 $\alpha$  and 661 bp for  $\beta$ -actin (M=100 bp ladder). Lanes 1. 2, 3, 5, 8, 9, 10 and 11 were scored positive for HIF-1 $\alpha$ . HIF-1 $\alpha$  mRNA expression was confirmed with RPA where expression varied from 8.5- to 23-fold (mean 16.4-fold).  $\beta$ -Actin expression demonstrates the quality of cDNA and equal sample loading.

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**Figure 2.** (a) Hypoxia-inducible factor 1 (HIF-1 $\alpha$ ) mRNA expression in individual tumour samples from patients with glioblastoma, lower grade tumours and normal brain. Results are represented as the percentage change in abundance of HIF-1 $\alpha$  with respect to  $\beta$ -actin and show the range of distribution between samples. (b) A highly significant increase in the mean ratios of HIF-1 $\alpha$  to  $\beta$ -actin mRNA expression was observed in the glioblastomas when compared to the low-grade diffuse astrocytomas and to the anaplastic astrocytomas (P < 0.01). Additionally, a significant difference was also observed between the normal brain and the two lesser glioblastoma classes (P < 0.05 by students *t*-test). Means of at least four independent experiments.  $\pm$  standard errors (se) are shown.

increase in the mean ratio of HIF-1 $\alpha$  to  $\beta$ -actin expression in the glioblastomas when compared to the normal brain (0.43 vs. 0.1). The differences in the HIF-1 $\alpha/\beta$ -actin ratio in the low-grade diffuse astrocytomas as well as the anaplastic astrocytomas were significantly different from the normal brain (0.3, 0.25 and 0,1, respectively, P < 0.05).

Results obtained from RT-PCR were confirmed by performing RPA on eight glioblastoma samples where there was sufficient tissue for the assay. A glioblastoma sample negative for HIF-1 $\alpha$  mRNA expression was verified as having no HIF-1 $\alpha$  expression by RPA and was used as a control. The seven remaining samples analysed confirmed upregulation of HIF-1 $\alpha$  mRNA expression varying from 8.5- to 23-fold (mean 16.4-fold) (data not shown).

# HIF-1a protein expression

In most of the samples scattered weakly immunoreactive tumour cell nuclei were seen. However, widespread HIF-1 $\alpha$  immunoreactivity was seen in tumour cell nuclei in 21/27 glioblastomas, 3/4 anaplastic astrocytomas, 2/10 low-grade astrocytomas and in none of the two samples of normal brain. In glioblastomas HIF immunoreactivity was particularly strong in areas surrounding necrosis (Figure 3a), which was predominantly present in palisading tumour cell nuclei (Figure 3b). In some cases, HIF immunoreactivity was seen in the larger, more atypical, nuclei (Figure 4). Where tumour cells were seen infiltrating brain, immunoreactivity was much more prominent in neoplastic than in reactive cells.

# p53 protein expression and correlation with HIF-1α

Between 0% and 80% of tumour cell nuclei showed p53 immunoreactivity, with 65% of glioblastomas having greater than 20% of tumour cell nuclei positive (Figure 5). A correlation between p53 immunoreactivity and either HIF-1α or mRNA expression could not be demonstrated.

## Discussion

In this study, 77% of high-grade astrocytic tumours were found to overexpress HIF-1 $\alpha$  protein. As noted in previous reports [39,40]. HIF-1 $\alpha$  protein expression was particularly strong surrounding areas of necrosis in glioblastomas, which is in keeping with a direct role for HIF-1 in the regulation of VEGF [25]. Hypoxia is known to stabilize HIF-1 $\alpha$  protein [15] and might explain expression adjacent to necrosis, but HIF-1 $\alpha$  was present more diffusely in high-grade astrocytic tumours and was stronger in infiltrating tumour cells than reactive glia, suggesting that expression is aberrantly controlled.

In many of the tumours overexpressing HIF-1a, increased expression of HIF-1a mRNA was also found,

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Figure 3. Immunohistochemical analysis of HIF-1 $\alpha$  expression in glioblastoma. (a) Low-power view (original magnification  $\times$  50) of HIF-1 $\alpha$  immunoreactivity in a glioblastoma showing widespread nuclear immunoreactivity, which at higher magnification (b) is particularly prominent in tumour cells surrounding foci of necrosis ( $\times$  150).

suggesting that control is at least partly at the transcriptional level. However, in several tumours overexpressing HIF-1 $\alpha$  protein we could not detect an increase in HIF-1 $\alpha$  mRNA. Although this finding could be due to sampling error (relatively small samples were available for mRNA analysis), it is also possible that posttranscriptional mechanisms might play a role. Three possible mechanisms are reduced HIF-1 $\alpha$  degradation due to loss of p53, PTEN and/or VHL function. PTEN is a tumour suppressor gene originally isolated from a homozygous deletion on chromosome 10q23 in glioblastoma [20,29]. Recently it has been shown that wild-type PTEN mediates the degradation of HIF-1 $\alpha$ and therefore the frequent loss of PTEN in glioblastomas



Figure 4. Strong HIF-12 immunoreactivity in glioblastoma tumour cells, particularly those with large atypical nuclei (×200).



Figure 5. A glioblastoma shows strong nuclear p53 immunoreactivity in the majority of tumour cells (×300).

[36], which might facilitate aberrant expression of HIF-1 $\alpha$  [42].

Wild-type p53 promotes degradation of HIF-1 $\alpha$  protein [26] and it might be expected that inactivation of p53 would lead to increased HIF-1 $\alpha$  expression. We did not find a correlation between p53 overexpression with either HIF-1 $\alpha$  protein or mRNA expression. However, this does not preclude an association between HIF-1 $\alpha$  and p53 in glioblastomas, as p53 protein overexpression in gliomas is not always due to a gene mutation and the relationship between HIF-1 $\alpha$  and p53 is complex with wild-type p53 being stabilized by HIF-1 $\alpha$  [1, 12, 13]. It is also known that levels of p53 in cultured neurones increases with the duration of hypoxia, thus changes in

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the duration of hypoxia can alter cellular levels of p53 [2]. Western blot analysis of p53 protein expression and screening p53 gene abnormalities in these tumours might help to further clarify this association.

Another possible mechanism is that loss of VHL function might lead to HIF-1 a accumulation. The VHL gene product (pVHL) is required for regulation of the HIF-1 transcriptional control system [23] and the VHL gene might be mutated in glial neoplasms [18]. VHL forms a complex with elongins B and C. Cul 2 and the Ring-H2 finger protein Rbx1 (VCBCR complex) by binding to the oxygen-dependent destruction domain of HIF-1a subunits through the B-domain of pVHL [17.21.24]. pVHL then acts as the recognition component of the E3 ubiquitin ligase complex mediating HIF-1a destruction [3.16.31]. Under hypoxia, VHLmediated degradation of HIF-1a does not occur, leading to accumulation of HIF-1a. Additionally, nonfunctional VHL also results in the accumulation of HIF-1a in normoxic conditions [23].

The recent discovery of two new members of the HIF family, HIF-2 $\alpha$  [6,8,33] and HIF-3 $\alpha$  [14], demonstrates the complexity involved in the regulation of angiogenesis. These new family members might also play a role in mediating the response of the cell to hypoxia. Further studies are now required using a larger sample population to include Western blot analysis of HIF-1 $\alpha$  protein to determine the precise role of HIF-1 in the response to hypoxia and angiogenesis in these tumours.

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# Aldose reductase expression is induced by hyperglycemia in diabetic nephropathy

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#### Aldose reductase expression is induced by hyperglycemia in diabetic nephropathy.

Background. Despite good metabolic control, many patients with type 1 diabetes still develop nephropathy, implicating a role for genetic factors. Recent studies examining the regulatory region of the aldose reductase (ALR2) gene, the ratelimiting enzyme of the polyol pathway, support its role as a candidate gene for nephropathy. Here we report the quantitation of ALR2, together with sorbitol dehydrogenase mRNA in the peripheral blood mononuclear cells (PBMCs) of type 1 diabetic patients with (N = 29) and without nephropathy (N =11) following stimulation with high levels of D-glucose.

Methods. PBMCs from patients and normal controls were cultured for five days with phytohemagglutinin in either normoglycemia (11 mmol/L D-glucose) or supplemented with 10 mmol/L D-glucose (moderate hyperglyemia) or 20 mmol/L D-glucose (hyperglycemia). The RNA was extracted and analyzed by ribonuclease protection assay.

Results. ALR2 mRNA levels were significantly elevated with increasing *D*-glucose concentration (normal to hyperglycemic) in those patients with nephropathy (P < 0.0001). In marked contrast, in those without nephropathy and in the normal healthy controls, there was no change in mRNA expression. Furthermore, those patients with nephropathy and the Z-2/X susceptibility genotype had the greatest increase in ALR2 mRNA compared with those with low-risk genotypes (P < 0.007).

Conclusion. These results show that patients with nephropathy exhibit marked disturbances in the expression of the enzyme components of the polyol pathway. Ultimately this leads to tissue damage and ischemia.

There is increasing evidence to suggest that genetic factors are involved in the pathogenesis of diabetic nephropathy in patients with type 1 diabetes [1-3]. The incidence peaks between 15 and 20 years duration of diabetes and subsequently declines rapidly [4, 5]. This is

Key words: sorbitol dehydrogenase, gene expression, blood sugar, type 1 diabetes, ALR2 gene.

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supported by the familial clustering of nephropathy in those patients with type 1 or type 2 diabetes [6, 7].

Recent studies have suggested that genetic variation in the aldose reductase (ALR2) gene may contribute to the genetic susceptibility to diabetic nephropathy [8-12]. Aldose reductase is the first and rate-limiting enzyme of the polyol pathway and converts glucose to sorbitol in a nicotinamide adenine dinucleotide phosphate (NADPH)dependent reaction. Sorbitol is metabolized to fructose by sorbitol dehydrogenase (SORD) using NAD as a cofactor [13-16]. Excessive flux through the polyol pathway caused by hyperglycemia is likely to lead to a number of metabolic and vascular defects that ultimately may give rise to tissue hypoxia and ischemia. Patients with diabetic microvascular complications have been shown to have raised enzymatic activity of aldose reductase as well as an increased amount of the protein compared with those with no microvascular complication [17, 18]. Patients with type 1 diabetes and nephropathy also have been shown to have increased levels of ALR2 mRNA [19]. This increase in mRNA appears to be linked to a genetic variation in the promoter region of the ALR2 gene [11].

Two polymorphic regions in the promoter region of ALR2 have been identified and shown to be associated with susceptibility to nephropathy in Caucasoid patients who have type 1 diabetes. A CA dinucleotide repeat region designated 5'ALR2 is located at approximately 2.1 kb upstream of the initiation site in close proximity to the osmotic response elements [20]. A second polymorphism, the C(-106)T site, occurs in the basal promoter region [21]. The Z-2 5'ALR2 allele, where Z is the most common allele and consists of 24 CA repeats, has been shown to occur with increased frequency in patients with type 1 diabetes and nephropathy. Patients with either type 1 or type 2 diabetes and retinopathy from a number of ethnic groups, including Caucasoid, Japanese, and Chinese origin, have also been found to have an increased frequency of the Z-2 allele [8-12, 20, 21].

The level of mRNA expression of ALR2 has been shown to be increased in the peripheral blood mononu-

Table 1. Clinical characterization of patient subgroups and normal controls

	Diabetic nephropaths	Diabetic controls (uncomplicated)	Normal controls
No. of subjects	29	11	10
Age years (range)	43 ± 11.3 (24-69)	46.5 ± 13.4 (27-70)	$35.4 \pm 8.4 (24-49)$
Age at onset of diabetes years (range)	12.6 ± 9.0 (1-36)	$19.6 \pm 11.1 (4-42)$	
Duration of diabetes (range)	30.6 ± 7.9 (15-43)	$31 \pm 10.5$ (22–51)	
Hemoglobin A <sub>1c</sub> (range)	$9.2 \pm 1.6 (6.1 - 12.2)$	$8.5 \pm 1.5$ (7.2–11.2)	
Insulin dosage units	38.2 ± 10.4 (24-70)	$61.1 \pm 31.7$ (20-118)	
Sex male:female	12:17	4:7	7:3

Data are expressed as mean values ± standard deviation together with the range (in parentheses). All patients had type 1 diabetes mellitus as defined by the National Diabetes Data Group. The hemoglobin A<sub>10</sub> values were not significantly different between the patient groups.

clear cells (PBMCs) of Caucasoid patients with type 1 diabetes and nephropathy who have the Z-2 allele [11]. The Z-4 allele of Japanese patients with type 2 diabetes and retinopathy has been shown to confer increased gene expression in reporter assays using human retinal pigment epithelial cells exposed to hyperglycemia [22]. However, at the present time, nothing is known about hyperglycemia-induced expression of the ALR2 and SORD genes in the PBMCs of patients with or without nephropathy. The expression profile of ALR2 and SORD genes under hyperglycemic conditions may explain why certain individuals with diabetes develop complications while others do not.

Here we provide compelling evidence to show that the expression profile of the ALR2 and SORD genes differs substantially between those patients with nephropathy compared with those who remain free of microvascular complications after 20 years of diabetes.

# METHODS

# Study subjects

Forty patients were recruited from the Diabetic Out-Patient Clinic (Dr. Millward, Derriford Hospital, Plymouth, UK). Local ethical committee approval was obtained. The patients were classified according to their microvascular complications, as previously described [8–10].

Uncomplicated patients (N = 11). These patients had type 1 diabetes for at least 20 years but remained free of retinopathy (fewer than 5 dots or blots per fundus), proteinuria (negative on urine Albustix on at least three consecutive occasions over the previous 12 months), and overt neuropathy. Overt neuropathy was defined if there was any clinical evidence of peripheral or autonomic neuropathy.

Nephropaths (N = 29). These patients had type 1 diabetes for more than 10 years and had proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three successive total urinary protein excretion rates of greater than 0.5 g/24 h) in the absence of hematuria or infection on midstream urine samples. Diabetic nephropathy was always associated with retinopathy. Retinopathy was defined as more than five dots or blots per eye; hard or soft exudates, new vessels, or fluorescein angiographic evidence of maculopathy or previous laser treatment for preproliferative or proliferative retinopathy; and maculopathy or vitreous hemorrhage. Fundoscopy was performed by both a diabetologist and ophthalmologist (the clinical characteristics of the subjects are shown in Table 1).

Normal healthy controls (N = 10). Peripheral blood samples were obtained from 10 healthy volunteers with no family history of type 1 or type 2 diabetes.

# Isolation and culturing of peripheral blood mononuclear cells from whole blood

Twenty milliliters of peripheral blood were collected into 5% ethylenediaminetetraacetic acid (EDTA) Vacutainers (Becton Dickinson, Oxford, UK). The blood was diluted 1:1 in phosphate-buffered saline (PBS), and the PBMCs were harvested following standard density gradient centrifugation. The PBMCs were washed four times and resuspended at a concentration of  $0.5 \times 10^{\circ}/mL$  in RPMI 1640 (11 mmol/L D-glucose) supplemented with penicillin/streptomycin (GIBCO GRL, Paisley, Scotland, UK), 10% fetal calf serum (GIBCO) and L-glutamine (Sigma Chemicals, Poole, Dorset, UK) and separated into three flasks. The first of these was cultured for five days with phytohemagglutinin (11 mmol/L D-glucose concentration within the media normoglycemia), and the second was supplemented with 10 mmol/L p-glucose (moderate hyperglycemia) and the third with 20 mmol/L D-glucose (hyperglycemia) before culture. Additional experiments using cultures of PBMCs were established to control for osmosity and hyperglycemia. In the first, 20 mmol/L mannitol were used as a supplement with the 20 mmol/L D-glucose. A second, parallel culture consisted of 20 mmol/L L-glucose being used as the supplement with the 20 mmol/L D-glucose.

# **Extraction of RNA**

At the end of the incubation period, the cells were pelleted by centrifugation. The supernatant was removed, and the cells were resuspended and lyzed in RNA Stat  $60^{14}$  (Biogenesis, Poole, UK). The solution was then transferred to an RNase-free Eppendorf; 200  $\mu$ L of chloroform were added (Sigma Chemicals), vortexed, and centrifuged at 13,000 × g for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean Eppendorf, mixed with 500  $\mu$ L of isopropanol and centrifuged. The supernatant was discarded, and the pellet was washed in 75% ethanol and resuspended in 50  $\mu$ L diethyl pyrocarbonate (DEPC)-treated water by vortexing. The approximate amount of total RNA extracted was determined using a Cecil 5500 spectrophotometer scanning 240 to 280 nm (Cecil Instruments Ltd., Nottingham, UK).

# Preparation, amplification, and radiolabeling and purification of antisense RNA probes

High-molecular-weight DNA was prepared from 10 mL of peripheral blood using Nucleon extraction kits (Scotlab, Paisley, Scotland, UK). An aliquot of this DNA was amplified using the polymerase chain reaction (PCR). Exons of ALR2 and SORD were amplified using three separate primers for each gene. The first round amplification reaction was performed in 30 µL volumes containing the amplimers for ALR2: sense, 5'-CTG TAG CTC CCA CAA CGA TTA CCC-3'; antisense, 5'-CAA GCA GTC AAA ACT CAA CCG TTA G-3'; and for SORD: sense, 5'-CAA AGC CAT CCT GGG CTG CAT GTG GG-3'; antisense, 5'-CTT TCC TGG CGA CCT GAG GGC-3'; 10 mmol/L dNTPs (Pharmacia Biotech, UK), 10 × buffer solution, 10 mmol/L MgC12, 1 U Taq polymerase (HT Biotech, Mayfield, UK). The samples were subjected to an initial cycle of denaturation for two minutes at 96°C. This was followed by 30 cycles of amplification that consisted of denaturation for 30 seconds at 94°C, annealing for 1 minute at 50°C, and extension for 1 minute at 72°C in a PTC-200 Thermal Cycler (MJ Research, Essex, UK). A second round of amplification was then employed using the antisense amplimer with an RNA polymerase T7 adapter and stuffer nucleotides, ALR2: Antisense + T7 - 5'-TAA TAC GAC TCA CTA TAG GGA GG CAA GCA GTC AAA ACT CAA CCG TTA G-3'; SORD: Antisense + 7 – 5'-TAA TAC GAC TCA CTA TAG GGA GG TTT TCC TGG CGA CCT GAG GGC-3'; 10 mmol/L NTPs, 10 × buffer solution, 10 mmol/L MgCl<sub>2</sub>, 1 U Taq olymerase. The samples were then subjected to DNA mplification as described previously in this article. One hicrogram of the product was used to generate the radioctive RNA probes by incorporating a<sup>32</sup>P UTP (Amerham Pharmacia Biotech, Amersham, Bucks, UK) using he MAXIscript System (Ambion, Abingdon, UK).

An equal volume of gel loading buffer (Ambion) was dded to the reaction and heated to 85 to 95°C for 3 to minutes and then loaded onto a 5% acrylamide/8 mol/L rea gel and run at approximately 100 to 200 V for 40 inutes to 1 hour. The gel was wrapped in SaranWrap nd exposed to Kodak X-Omat film (Scientific Imaging Systems, Cambridge, UK) for 10 to 30 seconds. The probe was excised, immersed in 350 µL of elution buffer (Ambion), and then incubated overnight at 37°C to obtain 95% recovery.

# Hybridization of ribonuclease protection assay probe to RNA sample

The RNA sample and the ribonuclease protection assay (RPA) antisense probe were mixed together and precipitated with ethanol and ammonium acetate. The RNA mix was pelleted by centrifugation, dissolved in 20 µL of hybridization buffer (Ambion), and then centrifuged to collect at the bottom of the tube. The sample was then briefly incubated at 92°C, vortexed, centrifuged, and then incubated overnight at 42°C. For the Rnase digestion, 200 µL of diluted RNase mix (Ambion) was then added, and the mixture was incubated for 30 minutes at 37°C, after which 300 µL of RNase inactivation/precipitation solution (Ambion) was added, vortexed, and centrifuged. The reaction was then transferred to -20°C for at least 15 minutes. The protected fragments were separated and detected by centrifugation of the sample for 15 minutes at 4°C, and the pellet was resuspended in gel loading buffer. The sample was then heated for three to four minutes at 94°C and loaded onto a 5% acrylamide gel, and electrophorosed for approximately 40 minutes to 1 hour at 200 V, transferred to filter paper (Whatman, Maidstone, UK), and exposed to Kodak X-Omat film at -80°C overnight. The bands were analyzed using a phospho-imager (Biorad, Hertfordshire, UK). An internal control B-actin was set up to ascertain any variations in the amount of RNA used in the RPA. pTRI-actin, a DNA template, was provided in the RPA kit (Ambion) and used for the transcription of an antisense B-actin RNA probe.

#### Statistical analysis

Results are expressed as means  $\pm$  SE. Statistical significance was assessed by Student *t* tests after analysis of variance (ANOVA) or Mann-Whitney *U* test. A *P* value of <0.05 was considered to be significant.

## RESULTS

Forty patients with type 1 diabetes were recruited for the study. Twenty-nine of these had diabetic nephropathy (nephropaths), and 11 had remained free of microvascular disease (absence of background retinopathy, microalbuminuria, and overt neuropathy) after 20 years duration of diabetes (uncomplicated). Ten normal healthy controls were also studied. The clinical characteristics of the subjects are shown in Table 1. Figure 1A shows the expression of the housekeeping gene  $\beta$ -actin mRNA in a patient with diabetic nephropathy. The level of  $\beta$ -actin expression was similar between the samples of PBMCs



Fig. 1. (A) An example of mRNA from peripheral blood mononuclear cells (PBMCs) of a patient with diabetic nephropathy that had been exposed to normal, moderate, and severe hyperglycemia and hybridized to B-actin in a ribonuclease protection assay. The fold differences in B-actin mRNA between the normal, moderate, and severe samples were 1.2 and 1.0, respectively. (B) The same mRNA samples from the patient with nephropathy as depicted in (A) as well as those from an uncomplicated patient. In contrast to the B-actin results shown in (A), there was a 2.0- and 7.6-fold increase in the expression of aldose reductase (ALR2) mRNA in PBMCs exposed to moderate and severe hyperglycemia in the patient with diabetic nephropathy. There was no change in the ALR2 mRNA expression with hyperglycemia in the uncomplicated patient.

stimulated with different concentrations of D-glucose (fold increase in the samples supplemented with D-glucose was up to 1.2 compared with normal samples). This demonstrates that equal amounts of study RNA (10  $\mu$ g) were loaded and that variations in *ALR2* and *SORD* mRNA expression were real and not caused by loading errors. Figure 1B shows the expression of *ALR2* mRNA in PBMCs in the same patient with diabetic nephropathy as well as an uncomplicated subject stimulated with different concentrations of D-glucose. The fold increases of *ALR2* mRNA in the diabetic nephropath were 2.0 and 7.6 for the PBMCs exposed to moderate or hyperglycemia, respectively.

The mean levels of ALR2 as well as SORD mRNA in PBMC exposed to normal, moderate (supplemented with 10 mmol/L p-glucose), or severe hyperglycemia (supplemented with 20 mmol/L p-glucose) in the nephropaths, uncomplicated, and the normal controls are shown in Table 2 and Figures 2 and 3. The results are expressed as fold increase in mRNA above the level obtained under normal conditions. In the nephropaths, the mean fold increase of ALR2 mRNA in moderate hyperglycemia was  $1.8 \pm 0.2$ -fold, while in severe hyperglycemia there was a 3.8  $\pm$  0.8-fold increase (P < 0.0001). In marked contrast, in the uncomplicated group of patients as well as the normal controls, there was no elevation of ALR2 mRNA expression with increasing D-glucose concentration. Indeed, in both these groups, there was a slight decrease  $(0.9 \pm 0.02 \text{ and } 0.94 \pm 0.1 \text{ for moderate hyper-}$ glycemia, respectively, and 0.8  $\pm$  0.05 and 0.8  $\pm$  0.1 for severe hyperglycemia, respectively; Table 2). The differences in response of ALR2 between the nephropaths and

214

Table 2. Overall mean levels of ALR2 and SORD mRNA in mod	lerate and hyperglycemic conditions in the study groups
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Glucose concentration	Diabetic nephropaths $N = 29$		Diabetic controls $N = 11$		Normal controls $N = 10$	
	ALR2	SORD	ALR2	SORD	ALR2	SORD
Moderate	1.8±0.2*	1.4±0.1*	$0.9 \pm 0.02$	$0.9 \pm 0.05$	$0.94 \pm 0.1$	$0.94 \pm 0.07$
Hyperglycemic	$3.8 \pm 0.8^{\text{b}}$	2.2 ± 0.3°	$0.8\pm0.05$	$0.9\pm0.05$	$0.8 \pm 0.1$	$0.84 \pm 0.07$

The results are expressed as mean values ± standard error.

\*vs. uncomplicated. P = 0.00002, and vs. normal controls. P = 0.0004\*vs. uncomplicated. P = 0.0001, and vs. normal controls. P = 0.001

\*vs. uncomplicated, P = 0.01, and vs. normal controls, P = 0.001

<sup>4</sup>vs. uncomplicated, P = 0.006, and vs. normal controls, P = 0.005



Fig. 2. Mean fold increase of ALR2 mRNA in PBMCs from nephropaths ( $\blacklozenge$ ) and uncomplicated ( $\Box$ ) and normal ( $\triangle$ ) controls cultured under normal and hyperglycemic conditions. Glucose concentration vs. ALR2 mRNA, P < 0.00001. Moderate conditions: nephropaths vs. uncomplicated, P = 0.0002, nephropaths vs. normal controls, P = 0.004. Severe conditions: nephropaths vs. uncomplicated, P = 0.0001, nephropaths vs. normal controls, P = 0.004. Severe conditions: nephropaths vs. uncomplicated, P = 0.0001, nephropaths vs. normal controls, P = 0.004.

uncomplicated as well as the normal controls was highly significant (moderate conditions, P = 0.00002 nephropaths vs. uncomplicated nephropaths, and P = 0.004 vs. normal control; severe conditions, P = 0.0001 nephropaths vs. uncomplicated; Table 2). Figure 2 shows an example of the increase in ALR2 mRNA of PBMCs stimulated with D-glucose from a nephropath in contrast to the lack of a response of PBMCs from an uncomplicated patient who was treated in the same way. To determine whether the increased expression of ALR2 in the nephropaths was specific for D-glucose the PBMCs from two patients with nephropathy were stimulated with either L-glucose, which competes with D-glucose for the transporter, or mannitol, an osmolyte that at high concentrations can activate p38 mitogen-activated protein kinase [23]. When the tissue culture media for the PBMCs was also supplemented with either 20 mmol/L L-glucose or

20 mmol/L mannitol, there was a reduction in the expression of ALR2 (Fig. 4). In contrast, with D-glucose alone (supplemented with 20 mmol/L D-glucose), there was an expected increase in ALR2 mRNA (Fig. 4).

When the SORD mRNA was measured in the same samples again, the nephropaths had a significant increase in mRNA expression with increasing concentrations of D-glucose ( $P < 0.01, 1.4 \pm 0.1$  and  $2.2 \pm 0.3$  for moderate and severe hyperglycemia, respectively; Table 2 and Fig. 3). There was a slight decrease in SORD mRNA expression in the uncomplicated and normal controls with increasing concentration of D-glucose (Table 2 and Fig. 3).

In the nephropaths, there was a significant correlation between the increase in ALR2 mRNA expression under severe hyperglycemic conditions and the Z-2/X (where X is not Z+2) 5'ALR2 susceptibility genotype (Table 3). In those patients with this genotype, there was an 8.4 ±



Fig. 3. Mean fold increase of SORD mRNA in PBMCs from nephropaths ( $\blacklozenge$ ) and uncomplicated ( $\blacksquare$ ) and normal ( $\blacktriangle$ ) controls cultured under normal and hyperglycemic conditions. Glucose concentrations vs. SORD mRNA, P = 0.01. Moderate conditions: nephropaths vs. uncomplicated, P = 0.01; nephropaths vs. normal controls, P = 0.03. Severe conditions: nephropaths vs. uncomplicated, P = 0.006; nephropaths vs. normal controls, P = 0.03.



Fig. 4. ALR2 mRNA expression in PBMC of patients with diabetic nephropathy. ALR2 mRNA is induced by p-glucose, but not by mannitol or L-glucose. Symbols are: (**II**), nephropath 1; (**II**), nephropath 2.

3.5 increase of ALR2 mRNA in contrast to those with the Z+2/Y (where Y is not Z-2) genotype, who only had a 2.9  $\pm$  0.2 increase in ALR2 mRNA (P < 0.04). Surprisingly, those nephropaths with the Z-2/Z+2 genotype had the lowest increase in ALR2 mRNA (1.3  $\pm$ 0.3). Indeed, when the increase in the Z-2/X genotype group is compared with the Z+2/Y and the Z-2/Z+2 as a whole, this difference was significant (P < 0.007). Also, it is clear from the SORD mRNA results with respect

to the 5'ALR2 genotype that those nephropaths with the highest ALR2 expression and the Z-2/X genotype did not necessarily have the most SORD mRNA. In the uncomplicated group, there was no correlation with 5'ALR2 genotype, as they did not increase their expression of ALR2 with hyperglycemia (data not shown). Four of the 11 uncomplicated patients had the Z-2/X genotype. Six had the Z+2/Y genotype, and the last subject had the X/Y genotype.

Table J. Aldose reductase	and SORD mRNA levels with respect
to 5'ALR2 genotype in	patients with diabetic nephropathy

	ALR2	SORD	ARL2/SORD ratio
Z - 2/X (N = 10)	8.4 ± 3.5*	$2.14 \pm 0.8$	4.4±1.5
Z+2/Y (N=6)	$2.9 \pm 0.2$	$2.9 \pm 1.0$	$1.9 \pm 0.2$
Z - 2/Z + 2 (N = 4)	$1.3 \pm 0.3$	$1.65 \pm 0.1$	$0.6 \pm 0.07$
X/Y (N = 9)	$4.3 \pm 1.0$	$1.6 \pm 0.3$	3.8±1.2

The results are expressed as mean fold increases of the PBMC exposed to severe hyperglycemic compared with those cultured under normal conditions ± standard error.

X = any allele other than Z+2; Y = any allele other than Z-2. \*vs. ALR2 Z+2/Y, P = 0.04

\*vs. ALR2 Z+2/Y plus Z-2/Z+2, P = 0.007

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# DISCUSSION

This study shows, to our knowledge for the first time, that the expression of both the ALR2 and SORD genes is induced by p-glucose in PBMCs in those patients with nephropathy. This response was specific for n-glucose and was not due to differences in osmolarity, as demonstrated by the lack of response to L-glucose and mannitol. However, the major finding from this study is the quite striking and unexpected lack of a response of the ALR2 gene to D-glucose in the PBMCs of the uncomplicated patients (no microvascular complications after 20 years duration of type 1 diabetes) as well as the normal controls. The reasons for this disparity are unclear at the present time, but obviously have major implications for understanding and possibly predicting diabetic nephropathy and other microvascular complications of diabetes. It is possible that these differences can be accounted for by the expression and function of glucose transporters between those with and without nephropathy. We and others have recently shown that polymorphisms in GLUT1, the major facilitative glucose transporter in insulin-nonresponsible tissues, such as the retinal epithelial cells and glomeruli, is associated with susceptibility to diabetic nephropathy in patients with type 1 and type 2 diabetes [24, 25]. This association is related to the 5'ALR genotype. Therefore, the availability of glucose as a substrate may be an important factor, although this may not necessarily be related to glycemic control. Indeed, the nephropaths and uncomplicated group had similar HbA1e results. It is possible that the uncomplicated group and normal controls did respond to D-glucose, but this was before the five-day end point by which time the response may have been down-regulated. Natural cellular inhibitors of the polyol pathway have not been found, and it is assumed that the flux through the pathway is simply regulated by the concentration of the substrate and cofactors. It remains unclear why high concentrations of p-glucose should fail to stimulate the expression of the ALR2 gene in these groups of subjects. However, it has been recently shown that human retinal epithelial cells that have a high level of expression of ALR2 are downregulated by a post-transcription mechanism, the taurine

cotransporter, when the cells are exposed to high glucose [26]. This effect can be reversed by the addition of aldose reductase inhibitors. The increase in SORD mRNA found in the PBMCs of the patients with nephropathy is in contrast to the response seen in rat inner medullary collecting duct cells exposed to high osmolarity [27]. In this model using a reverse transcription-PCR strategy, a fourfold increase in the level of aldose reductase mRNA is found within 24 hours of exposure to high osmolarity, but no difference in the expression of SORD is seen. It is also possible that the response to D-glucose in the nephropaths is due to the underlying renal dysfunction in these patients. This response may also occur in patients with nondiabetic renal disease.

While nephropaths showed an increase in expression of the ALR2 gene with increasing concentration of D-glucose, the degree of the response varied. This correlated with the 5'ALR2 genotype with those possessing the Z-2/X type having the greatest response. An examination of both the ALR2 and SORD mRNA expression suggests that there may be at least two profiles of flux through the polyol pathway under hyperglycemic conditions. Those nephropaths with the Z-2/X genotype had the greatest increase in ALR2 mRNA, but only a modest increase in SORD mRNA. Consequently, it is possible that in these individuals there is an accumulation of sorbitol in the cell. In contrast, those nephropaths with the Z+2/Y as well as the Z-2/Z+2 genotype had a similar increase of both ALR2 and SORD mRNA. Sorbitol would not be expected to accumulate in these cells. Instead, the exaggerated flux through the entire polyol pathway would be important, and this would give rise to increased levels of fructose and perhaps depletion of the cofactors NADPH and NAD<sup>+</sup>. It is also apparent that the presence of the Z+2 allele is dominant over the Z-2 allele. This is somewhat surprising and may be related to the affinity of the transcription factors for the regulatory response elements, which is perhaps modified by the presence of additional CA dinucleotide repeats. It has already been shown that the Z-4 allele can modify the efficiency of transcription in luciferase gene reporter assays [22].

In conclusion, this is the first description of mRNA profiles for genes coding for enzymes of an entire metabolic pathway. We have found striking differences in the expression profile of ALR2 of patients with nephropathy compared with those without and the normal controls. The response to uptake and disposal of D-glucose may be quite different between these groups of patients and provides a novel insight into the role of the polyol pathway in the pathogenesis of diabetic nephropathy.

## ACKNOWLEDGMENTS

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The Origin of the Universe

A. MARS, B. JUPITER and C. PLUTO

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The origins of the universe has long been in doubt but recent experiments have indicated that a computing error may have been the cause...

Expression of the gene for hypoxia inducible factor-1 and the molecular basis for oxidative stress and hypoxia in diabetes

K.L. SONDERGAARD, A. HODGKINSON and AG DEMAINE Molecular Medicine Research Group, Postgraduate Medical School, University of Plymouth, UK.

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that is intricately involved in the cellular response to hypoxia. Hyperglycaemia is associated with a cascade of metabolic abnormalities including oxidative stress and hypoxia. Hypoxia stimulates the expression of HIF-1 mRNA which in turn binds and activates a number of genes including vascular endothelial growth factor and glucose transporter 1. The aim of this study was to quantitate HIF-1 mRNA levels in cell lines exposed either to normal, moderate (14mM glucose) or hyperglycaemia (28mM glucose) for a minimum period of 14 days. The cell lines were also exposed to hypoxia for up to 24 hours. Lymphocytes from 3 patients with type 1 diabetes were also exposed to hyperglycaemia for 5 days. RNA was prepared from the cells and a ribonuclease protection assay (RPA) was used to quantitate the expression of HIF-1 using radioactively labelled riboprobes together with a Phosphor-Imager system. Exposure of 2 of the cell lines (MCF7 and ZR75) to hyperglycaemia caused up to 16 fold increase in expression of HIF-1 (3 and 16 fold respectively) compared to baseline. Exposure of the cell lines to hypoxia for up to 24 hours gave a similar increase in HIF-1 mRNA expression. HIF-1 mRNA was increased in the RNA of lymphocytes from patients by up to 19 fold compared to baseline. In conclusion, this is the first report of HIF-1 mRNA expression in hyperglycaemia and has important implications for understanding the molecular basis of diabetic complications.

F. Author for correspondence

Name K SONDEGATARD Institution UN: VERSITY OF PLAMOUTH Address MOLECVIAR MEDICINE ITTC BULDING PLAMOUTH PL68BX Telephone 01752 764236 Fax 01752 764234 formail advingine e planut accel Date 17/11/99 Signature Because angiopoietin-1 (Ang-1) promotes endothelial cell survival in vitro, we wanted to test if angiopoietin-2 (Ang-2), a naturally occurring antagonist of Ang-1, could be linked to this process. Fluorescent-labeled glioma 261 cells were implanted in the brains of 28 mice. Seven animals were sacrificed each week, over four weeks. We investigated the expression of Ang-2 by in situ hybridization and compared it to the distribution of apoptotic cells identified by TUNEL method and transmission electron microscopy (TEM). Ang-2 was detected as early as one week after implantation, in vascular cells surrounded by tumor cells. At this time, no apoptosis was seen by TUNEL or TEM. TEM showed tumor cells adjacent to the vascular cells "lifting up" the normal astrocytic feet processes away from the endothelial cells and disrupting normal pericytic cuffing. By two weeks, the number of perivascular glioma cells had increased. No increase in the number of blood vessels was detected at this time. Vascular cells remained positive for Ang-2 and rare cells were TUNEL positive. TEM showed closely packed proliferating perivascular tumor cells. At three weeks, there was vascular involution with scant zones of tumor necrosis. Ang-2 was again detected in vascular cells but now numerous vascular cells were TUNEL positive and apoptotic by TEM. At week four, there were extensive areas of tumor necrosis with adjacent angiogenesis. Ang-2 was detected in cells of newly formed vessels at the edge of the tumors. Both at 3 and 4 weeks most of the TUNEL-positive tumor cells displayed morphological features consistent with necrotic cell death by TEM. Only rare tumor cells appeared truly apoptotic. In contrast, the TUNELpositive endothelial cells and pericytes were round and shrunken, with condensed nuclear chromatin by TEM suggesting apoptotic cell death. Thus, Ang-2 is detected prior to the onset of apoptosis in vascular cells and could be linked to vascular involution which leads to tumor necrosis and angiogenesis.

## C46-07 Hypoxia Inducible Factor-1a Expression in Glioblastomas

Hilton, D'; Sondergaard, K<sup>1</sup>; Penney, M'; Demaine, A<sup>2</sup> 'Demiford Hospital, UK; <sup>2</sup>Plymouth University, UK

Introduction: Angiogenesis is crucial to the development of most malignant tumours, and is a particularly prominent feature of glioblastomas. The mechanisms underlying microvascular proliferation in glioblastomas is not fully understood, although over-expression of vascular endothelial growth factor (VEGF) occurs. Hypoxia inducible factor-1a (HIF-1a) is a transcription factor

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that is widely expressed in tissues under hypoxic conditions and controls several hypoxia-inducible genes, including VEGF. Recent studies have demonstrated increased expression of HIF-1a in a number of tumours, although little is known about its expression in glioblastomas. We have investigated the expression of HIF-1a mRNA and protein in glioblastomas, lower grade tumours and normal brain.

Methods: Immunocytochemistry was used to demonstrate HIF-1a protein in paraffin-embedded tissue sections using a commercially available monoclonal antibody, after microwave antigen retrieval. In cases where frozen tissue was available, RT-PCR was carried out using primers to HIF-1a and b-actin mRNA. Scanning densitometry was used to semiquantitate reaction products.

Results: Strong nuclear HIF-1a immunoreactivity was seen in 10/10 glioblastomas, 4/6 anaplastic astrocytomas and 2/10 low grade astrocytomas, but was not seen in normal brain. In positive cases immunoreactivity was present in the majority of tumour cell nuclei, however, in glioblastomas immunoreactivity was particularly strong in tumour cells surrounding foci of necrosis. Analyses of the RT-PCR products revealed upregulation of HIF-1a mRNA, compared to b-actin mRNA, in some of the glioblastomas.

Conclusions: HIF-1a protein over-expression is a consistent finding in glioblastomas, and is particularly prominent in tumour cells surrounding areas of necrosis. In some of the glioblastomas upregulation of HIF-1a mRNA was also found. These findings are in keeping with a role for HIF-1a in VEGF expression and angiogenesis in glioblastomas.

Poster Session C46: Tumours

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