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http://hdl.handle.net/10026.1/10300

10.2741/3438
Front Biosci (Landmark Ed)

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Crosstalk of VEGF and Notch pathways in tumour angiogenesis: therapeutic implications

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1. ABSTRACT

Angiogenesis is regulated by a number of angiogenic factors through many signalling pathways. The VEGF pathway and Notch signalling are perhaps two of the most important mechanisms in regulation of embryonic vascular development and tumour angiogenesis. Blockade of the VEGF pathway effectively inhibits tumour angiogenesis and growth in preclinical models. The successes in phase III trials have added anti-VEGF agents to standard cancer therapy in several major cancers. A recent flurry of findings indicate that DLL4/Notch signalling decreases angiogenesis by suppressing endothelial tip cell formation; importantly, blockade of DLL4/Notch signalling strikingly increases non-productive angiogenesis but significantly reduces the growth of VEGF-sensitive and VEGF-resistant tumours. The VEGF pathway interplays at several levels with DLL4/Notch signalling in vasculature. VEGF induces DLL4/Notch signalling while DLL4/Notch signalling modulates the VEGF pathway. DLL4 and VEGF emerge to be the yin and yang of angiogenesis. Combination therapy by blocking DLL4/Notch and VEGF pathways synergistically inhibits tumour growth in preclinical models. Thus, targeting the DLL4/Notch pathway, though still at an early stage, may lead to exciting new therapies for clinical application.

2. INTRODUCTION

The maintenance, growth and metastasis of solid tumours requires angiogenesis, a complex process involving matrix breakdown, endothelial sprouting, proliferation, migration, differentiation, and recruitment of pericytes/smooth muscle cells (1). Tumour angiogenesis is triggered by a variety of pro-angiogenic molecules, largely produced by tumour and stromal cells and regulated by many angiogenic pathways. Of these, the most prominent and best characterised is perhaps the vascular endothelial growth factor (VEGF) pathway (2). Blockade of the VEGF pathway has been shown to reduce tumour vascular density and inhibit xenograft tumour growth in various preclinical mouse models (3, 4). The phase III clinical trials have shown that blockade of the VEGF pathway inhibits tumour progression and prolongs patient survival in several major cancers (5-8). However, a number of tumours did not respond or responded in an early stage but became resistant in a late stage to VEGF inhibition in preclinical mouse models (9-12). In addition, anti-VEGF therapy alone appears to be ineffective in some clinical trials (13). It is, therefore, suggested that other factors or pathways are also important for tumour angiogenesis and growth, and that additional anti-angiogenic therapies are required for tumours that are resistant to VEGF inhibition.
Crosstalk of VEGF and Notch signalling pathways

Figure 1. The VEGF pathway. There are five ligands (VEGF/VEGF-A, PLGF, VEGF-B, VEGF-C and VEGF-D) and five receptors (VEGFR1, VEGFR2, VEGFR3, NRP1, and NRP2). VEGFR1 and VEGFR2 are expressed in the cell surface of most blood ECs while VEGFR3 is largely restricted to lymphatic ECs. VEGF-A binds VEGFR1, VEGFR2, NRP1 and NRP2; VEGF-B interacts with VEGFR1 and NRP1; VEGF-C binds VEGFR2, VEGFR3 and NRP2; VEGF-D interacts with VEGFR2 and VEGFR3; and PLGF interacts with VEGFR1, RPN1 and NRP2. Various strategies have been used to block the VEGF pathway: to target VEGF-A with monoclonal antibodies such as bevacizumab (A) and VEGF-trap (B), to inhibit VEGFR2 with specific antibodies (C) and a variety of small-molecule VEGF RTK inhibitors that inhibit ligand-dependent autophosphorylation of VEGFR2 (D), to disrupt VEGFR1 with anti-VEGFR1 antibodies (E), and to block the interaction between VEGF-A and VEGFR2 with soluble VEGFR1 protein (F). Additional approaches to disrupt VEGF signalling include antisense and siRNA targeting VEGF-A or its receptors and anti-PLGF antibodies targeting PLGF.

Many processes involved in tumour angiogenesis are mirrored during embryonic vascular development (14). In past decade, the Notch pathway has been shown to play a key role in vascular development (15, 16). Recently, several studies have shown that DLL4/Notch signalling plays an important role in tumour growth by increasing angiogenesis but improving vessel structure and function. Interruption of DLL4/Notch signalling increased non-productive angiogenesis but dramatically inhibited the growth of either VEGF-sensitive or VEGF-resistant tumours (17-20). The emerging evidence has shown that Notch signalling is highly interacted with the VEGF pathway: VEGF induces DLL4/Notch signalling at several levels while Notch signalling modulates the VEGF pathway, leading to an appropriate formation of functional vasculature in embryonic development and in tumour angiogenesis.

3. THE VEGF PATHWAY

3.1. VEGF ligands and receptors

There are five ligands (VEGF/VEGF-A, PLGF, VEGF-B, VEGF-C, and VEGF-D) and five receptors (VEGFR1, VEGFR2, VEGFR3, NRP1 and NRP2) in the VEGF pathway (Figure 1), of which VEGF and VEGFR2 appear to be the primary players in endothelial cells (ECs). Alternative exon splicing of the VEGF gene yields five isoforms ranging from 121 to 206 amino acids after signal sequence cleavage (VEGF121, VEGF1145, VEGF165, VEGF189 and VEGF206). VEGF121 is a freely diffusible protein. VEGF189 and VEGF206 are almost completely sequestered in the extracellular matrix. VEGF165, exists as the predominant and most physiologically relevant isoform, and has intermediary properties as it is secreted, but a
significant fraction remains bound to the cell surface and the matrix (21). VEGF binds to two related receptor tyrosine kinases (RTKs), VEGFR1 (Flt-1) and VEGFR2 (Flk-1, KDR), of which VEGFR2 is the major mediator of VEGF effects. The third receptor, VEGFR3 (Flt-4), binds VEGF-C and VEGF-D rather than VEGF. Neuropilins (NRP1 and NRP2) bind class 3 semaphorins and mediate repulsive signals during neuronal guidance (22). NRP1 also binds to VEGF, PLGF and VEGF-B while NRP2 interacts with VEGF, PLGF and VEGF-C. NRP1 and NRP2 act as co-receptors, enhancing VEGF-VEGFR2 interactions and promoting VEGF stimulated signalling (23, 24). VEGFR1 and VEGFR2 are predominantly expressed on the surface of vascular ECs whereas VEGFR3 is present on all ECs in developing blood vessels but in adult becomes largely restricted to lymphatic ECs and certain fenestrated blood ECs (25). Activation of VEGF R2 results in autophosphorylation and downstream signalling through pathways such as PI3K/Akt and thus mediates the mitogenic, angiogenic, anti-apoptotic and permeability-enhancing effects of VEGF. Activation of VEGFR1 may have a decoy effect on ECs, suppressing the availability of VEGF to VEGFR2 (21). There is growing evidence that VEGFR1 may also have important roles in haematopoiesis and in the recruitment of angiocompetent bone marrow progenitors that may home in on the tumour vasculature and promote angiogenesis (26, 27).

3.2. Role in embryonic vascular development

Genetic alternations have yielded insights into fundamental functions of the VEGF pathway in embryonic vascular development. Mice deficient for various components of the VEGF pathway die in utero of severe vascular abnormalities. Haploinsufficiency of VEGF results in embryonic lethality between embryonic day E11 and E12 (28, 29). VEGF deficiency impaired most steps of early vascular development, including differentiation of blood islands, sprouting from pre-existing vessels, the formation of large vessels, the establishment of interconnections and the spatial organisation of intra- and extra-embryonic vessels. Embryos homozygous for VEGFR2 mutation die between E8.5 and E9.5 due to lack of development of the blood islands, embryonic vasculature and haematopoietic cells. Organised blood vessels could not be observed in the embryo or yolk sack at any stage, suggestive of an essential role of VEGFR2 in early developmental vasculogenesis and angiogenesis (30). In contrast, embryos homozygous for VEGFR1 mutation not only formed ECs in both embryonic and extra-embryonic regions but also increased endothelial progenitors, resulting in abnormal vascular disorganisation and thus died at mid-somite stages (between E8.5 and E9.5) (31, 32), indicating that VEGFR1 is a negative regulator of the VEGF pathway during early development. Mice lacking only the kinase domain of VEGFR1 appeared rather normal except for slightly impaired angiogenesis during pathological conditions, consistent with the notion that the primary role of VEGFR1 may be that of a decoy receptor (33, 34). Targeted inactivation of VEGFR3 resulted in defective blood vessel development in early mouse embryos. Vasculogenesis and angiogenesis occurred but large vessels became abnormally organised with defective lumens, leading to embryonic lethality at E9.5 prior to the initiation of lymphangiogenesis (35). However, VEGFR3, like VEGF-C, is also essential for development of lymphatic vessels (36, 37). NRP1 gene targeted mice die at E13 from vascular defects such as insufficient development of yolk sac vascular networks, deficient neural vascularisation and transposition of large vessels (38). Although NRP2-deficient mice have normal vasculature, double NRP1/NRP2 knockout mice die in utero at E8.5, with an abnormal vascular phenotype resembling those of the VEGF and VEGFR2 knockouts (39).

Developing zebrafish embryos are almost transparent, making them ideal for high-resolution imaging studies of segmental and intersegmental vessel development. Knockout of VEGF using morpholino oligonucleotides results in severe defects of the dorsal aorta and intersegmental arteries and reduces artery-specific gene expression, whereas veins are largely unaffected (40). Formation of arteries and veins in the zebrafish embryo is actually governed in part by different combinations of VEGFR2a, VEGFR2b and VEGFR3 (41).

3.3. Role in tumour angiogenesis

VEGF is overexpressed by the vast majority of solid human tumours and in a variety of haematological malignancies. Increased VEGF expression has been shown to be associated with malignant progression in many tumours and patient survival in various cancers (21). In fact, many tumour cell lines secrete a large amount of VEGF in vitro (3, 42). Although tumour cells represent the major source of VEGF, tumour-associated stromal cells including ECs and macrophages are also important sites of VEGF production. VEGF expression is upregulated by numerous growth factors including EGF, TGF-alpha, TGF-beta, IGF-1, HGF and bFGF in a local tumour environment, by hypoxia through HIF-1alpha, a characteristic feature of solid tumours, by inflammatory cytokines such as IL-lalpha and IL-6, and by activation of oncogenes such as Ras, Src HER2/neu and Bcr/Abl or inactivation of tumour suppressor genes such as p53 and PTEN, an intrinsic characteristic of many tumours (21, 43). Elevated VEGF induces endothelial proliferation, migration, survival and vessel formation in tumours (Figure 1). It is well known that expressions of VEGFR1, VEGFR2 and VEGFR3 are upregulated in tumour ECs and tumour hypoxia increases expressions of VEGFR1 and VEGFR2.

Due to its critical role in tumour angiogenesis and growth, the VEGF pathway has become an important target for anticancer therapy. Early works revealed that anti-VEGF monoclonal antibodies exert a potent inhibitory effect on the growth of several tumour cell lines in nude mice, whereas the antibody has no effect on tumour cells in vitro (44). Subsequent studies have shown that many other tumour cell lines, regardless of tumour origins, are also inhibited by anti-VEGF antibodies in various preclinical mouse models (3). Indeed, inhibition of tumour angiogenesis and tumour progression in numerous xenograft tumour models have been further demonstrated using different strategies (Figure 1) for targeting either ligands (VEGF, PLGF) or receptors (VEGFR1, VEGFR2...
and VEGFR3) with specific antibodies, soluble VEGF receptors, VEGF-traps, aptamers, and small-molecule VEGFR tyrosine kinase inhibitors (3, 4, 42, 45-48).

Importantly, in randomised phase III clinical trials, two approaches of blockade of the VEGF pathway have yielded survival benefit in patients with several different metastatic cancers. Addition of bevacizumab, a humanised specific anti-human VEGF monoclonal antibody, to a standard chemotherapy in 4 different phase III trials improved overall survival in colorectal (20.3 months versus 15.6 months in total 813 cases, hazard ratio=0.66, P<0.001) and lung (12.3 months versus 10.3 months in total 878 cases, hazard ratio=0.79, P=0.003) cancer patients (6, 8) or progression-free survival in breast (11.8 months versus 5.9 months in total 722 cases, hazard ratio=0.60, P<0.001) and renal-cell (10.2 months versus 5.4 months in total 649 cases, hazard ratio=0.63, P=0.0001) cancer patients (5, 7). The second approach is to target both ECs and tumour cells with small molecule inhibitors that block VEGF receptor and other tyrosine kinases with or without chemotherapy. Sunitinib (SU11248), a multi-targeted inhibitor of VEGF/VEGFR2, PDGFR-beta, Flt-3 and c-Kit, significantly improved progression-free survival of patients with metastatic renal-cell carcinoma when compared with interferon-alpha control (11 months versus 5 months in total 750 cases, hazard ratio for progression of 0.42, P<0.001 (49)). Sorafenib (BAY 43-9006), targeting VEGFR2, VEGFR3, PDGFR-alpha, PDGFR-beta, Raf, Flt-3 and c-Kit, when compared with placebo control prolonged progression-free survival (5.5 months versus 2.8 months in total 903 cases, hazard ratio for progression of 0.44, P=0.01) in patients with advanced clear-cell renal-cell carcinoma in whom previous therapy has failed (50).

4. THE NOTCH PATHWAY

4.1. Notch ligands and receptors

The Notch pathway is an evolutionarily conserved intercellular signalling pathway affecting many biological processes including cell-fate determination, cellular differentiation, proliferation, survival and apoptosis (51-53). Five Notch ligands [Jagged1, Jagged2, delta-like 1 (DLL1), DLL3 and DLL4] and four Notch receptors (Notch1-Notch4) have been described in mammals. Both ligand and receptor are transmembrane proteins with large extracellular domains that consist of epidermal growth factor (EGF)-like repeats. Activation of Notch signalling is initiated by ligand binding of Notch receptor between bordering cells, resulting in two proteolytic cleavages of the Notch receptor (Figure 2). The first cleavage is mediated by ADAM-family metalloproteases (ADAM10 or TACE (TNF-alpha-converting enzyme; also known as ADAM17)) while the second is catalysed by gamma-secretase, a protein complex that is composed of presenilin, nicastrin, PEN2 and APH1 (54). The last cleavage releases the Notch intracellular domain (NICD) from the cell membrane, which subsequently translocates to the nucleus. NICD then interacts with the DNA-binding protein CBF1/RBP-Jkappa and cooperates with Mastermind to displace corepressor proteins from RBP-Jkappa, thus activating the transcription of Notch target genes. Members of the Hes (hairy/enhancer of split) and Hey families (such as Hes1, Hes5, Hes7, Hey1, Hey2 and HeyL) of basic helix-loop-helix (bHLH) transcription factors and EphrinB2 are perhaps the best characterised downstream targets of the Notch pathway (55-58).

4.2. Role in vascular development and homeostasis

The Notch pathway is involved in multiple aspects of vascular development. Major components of the Notch pathway expressed in vasculature comprise four ligands (DLL1, DLL4, Jagged1 and Jagged2), three receptors (Notch1, Notch3 and Notch4), and three downstream targets (Hey1, Hey2 and HeyL) (59-61). Gene alteration strategies in mice and zebrafish have provided clear evidence for an absolute requirement of Notch signalling for vascular development and homeostasis.

Targeted deletion of Notch1 leads to embryonic lethality at approximately E10.5 (62-64). Although the initial formation of a primitive vascular network proceeds normally, some homozygous embryos display severe defects in yolk sac vascular remodelling, massive embryonic haemorrhages, enlarged pericardial sacs, absence or loss of large embryonic blood vessels, and impaired placental development (63, 65). Mice deficient for Notch4 are viable and exhibit no obvious mutant phenotype. However, Notch1/Notch4 double mutants display even more severe defects than the embryos deficient in Notch1 alone in angiogenic vascular remodelling that affected the embryo, yolk sac and placenta, suggesting a partial functional redundancy of Notch1 and Notch4 (65). Interestingly, overexpression of activated Notch4 in ECs results in embryonic lethality around E10. The mutants display substantial defects in the embryonic and extraembryonic vasculature, resembling those in Notch1/Notch4 double knockouts (66), suggesting that an optimal window of Notch signalling is critical for proper vascular development.

Both DLL1 and Jagged1 homozygous mutant embryos die from vascular defects and haemorrhaging at approximately E10.5 (67, 68). Mice lacking Jagged1 develop some of the vascular abnormalities seen in the Notch1-null mutants. DLL4, the latest identified Notch ligand, is initially restricted to ECs of large arteries in the embryo while in adult it is specifically expressed in smaller arteries and microvessels, with a striking break in expression just as capillaries merge into venules (65, 69-71). Mice heterozygous for DLL4 display vascular defects similar in patterns to those seen in Notch1/Notch4 double knockouts. Interestingly, haploinsufficiency of DLL4 also results in embryonic lethality from severe vascular defects at E10.5 (69, 72, 73). Haploinsufficiency for angiogenic factors is uncommon and highlights the importance of DLL4 over other components of the Notch pathway. Such embryonic lethality caused by gene deletion of single-copy has been only described in VEGF knockout mice (28, 29).

Hey1-knockout mice have no apparent phenotypical defect whereas Hey2-deficient mice exhibit a quite strong, albeit variable, phenotype of cardiac impairment with high postnatal lethality. The combined
Figure 2. The Notch pathway. Notch is synthesised as a precursor protein that is processed by a furin-like convertase (S1 cleavage) in the Golgi before being transported to the cell surface, where it resides as a heterodimer. Interaction of Notch receptors with Notch ligands, such as Delta-like or Jagged, between two bordering cells leads to a cascade of proteolytic cleavages. The first cleavage (S2 cleavage) is mediated by ADAM-family metalloproteases such as ADAM10 or TNF-alpha-converting enzyme (TACE, also known as ADAM17), generating a substrate for S3 cleavage by the gamma-secretase complex. This cleavage releases the Notch intracellular domain (NICD) from the cell membrane. NICD then translocates to the nucleus, where it interacts with the DNA-binding protein RBP-Jkappa (also known as CBF1) and cooperates with Mastermind to displace corepressor proteins, thus activating the transcription of Notch target genes. The basic helix-loop-helix proteins hairy/enhancer of split (such as Hes1, 5 and 7) and Hes-related proteins (Hey1, 2 and L) and EphrinB2 are the best characterised downstream targets. Blockade of Notch signalling has been achieved by using different strategies, including (A) anti-DLL4 monoclonal antibodies, (B) gamma-secretase inhibitors such as DBZ and DAPT, (C) soluble DLL4-Fc, (D) anti-Notch1 neutralising antibodies, and (E) Notch1-trap.

loss of Hey1 and Hey2, however, results in embryonic lethality after E9.5 with a global lack of vascular remodelling and massive haemorrhage, reminiscent of the phenotypical changes in Notch1-null mice (74). Mice nullizygous for RBP-Jkappa display similar vascular defects to those observed in Notch1/Notch4 double mutant embryos (73). A similar phenotype is also seen in presenilin1/presenilin2 double mutant embryos (75).

Understanding of the function of the Notch pathway in postnatal vascular homeostasis is directly clinical significant. In human, mutations in Jagged1 or Notch3 cause the autosomal dominant disorders Alagille syndrome and CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), respectively, and both display abnormal vascular phenotypes (76-78). In mice, Notch3 is required to generate functional arteries by regulating arterial differentiation and maturation of vascular smooth muscle cells. In adult Notch3-null mice, distal arteries display structural defects and arterial myogenic responses are defective (79). Notch3-transgenic mice, in which human Notch3 carrying the R90C mutation, a CADASIL archetypal mutation, is specifically expressed in vascular smooth muscles cells, could recapitulate the characteristic vascular lesions observed in CADASIL (80). Mice heterozygous for a null allele of Jagged1 and a hypomorphic allele of Notch2 phenocopy many of the
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defects of Alagille patients (81, 82). Targeted expression of constitutively active Notch4 (int3) (or active Notch1) in adult ECs has shown to cause reversible arteriovenous defects and mouse lethality within weeks of its expression. The int3-mediated vascular defects are accompanied by arteriolarisation, including ectopic venous expression of EphrinB2, increased smooth muscle cells and upregulation of endogenous Notch signalling (83). DLL1 has demonstrated to be an essential Notch ligand in postnatal arterial ECs, which regulates Notch signalling-dependent EphrinB2 expression and postnatal arterigenesis in response to ischemia (60).

Blood vessels in mouse retina develop only after birth, initiating from the avascular region of the optic nerve head and growing radially toward the periphery in a highly reproducible spatial and temporal pattern; during these stages, the retinal vasculature is accessible both for observation and for experimental manipulation with drugs or other agents. Recently, several independent studies in the mouse retinal model show that genetic inactivation of one allele of DLL4, inhibition of DLL4/Notch signalling using gamma-secretase inhibitors, soluble DLL4 and specific anti-DLL4 antibodies, or EC-specific inactivation of Notch1, all increase numbers of filopodia-extending endothelial tip cells, promote endothelial proliferation, and thus enhance angiogenic sprouting and branching, resulting in a much denser and more highly interconnected superficial capillary plexus (19, 84-86). DLL4 is prevalently expressed in tip cells and stalk cells that sit in close to the sprouting margin (84, 85, 87, 88) while Notch1 is frequently absent in tip cells but is prominently expressed in stalk cells that are in close to the tip cells (88).

In zebrafish DLL4, as in the mouse, is expressed in the dorsal aorta and intersegmental arterial ECs but not the posterior cardinal venous ECs. Knockout of DLL4, Notch1b, or Rbpsuh using specific morpholino oligonucleotides and inhibition of Notch signalling by the gamma-secretase inhibitor DAPT, all result in increased numbers of tip cells, excessive sprouting, aberrant branching of the intersegmental vessels, and severely reduced blood flow in the embryonic zebrafish; in contrast, activation of Notch signalling by overexpression of active NICD has opposite consequences (89, 90). The tip cells extend protrusions that sense the local environment and guide growth of these sprouts along the gradients of VEGF protein. The results from both the mouse retina and the zebrafish embryo indicate that DLL4 acts as a negative feedback regulator of VEGF-mediated vascular sprouting, ensuring the timely formation of well-differentiated and maturated vascular networks.

4.3. Role in tumour angiogenesis

Role of the Notch pathway in angiogenesis has been evaluated by manipulating the expression of different components in ECs, displaying either increased or decreased angiogenic processes such as endothelial proliferation, migration and tube formation in vitro, depending on the component, cell type and contexts (16, 91, 92). Information about Notch signalling in tumour angiogenesis in vivo was very limited until recently. It was reported that Jagged1 expressed in head and neck squamous cell carcinoma (HNSCC) cells activates Notch signalling in human dermal microvascular ECs and thus promotes tumour angiogenesis and tumour growth in a SCID mouse model. In human HNSCC clinical samples, Jagged1 expression is not only positively correlated with blood vessel density but also associated with HNSCC development (93).

DLL4 appears to play much more important roles in tumour angiogenesis than any other Notch component. We and others have previously shown that DLL4 is strongly upregulated in tumour vasculature in mouse models (18, 20, 69, 70, 94) and in human breast, kidney and bladder cancers (70, 95, 96). Knockdown of basal DLL4 levels in ECs inhibited multiple endothelial functions (96), whereas overexpression of DLL4 reduced endothelial proliferation, migration, tube-like formation and sprouting (97, 98), suggesting that an optimal level of DLL4 expression is essential for EC functions. These characteristics, together with the single-copy lethality, have attracted us and at least other three groups to further address the precise function of DLL4 in tumour angiogenesis and growth.

Regeneron (18) developed a mouse model in which rat C6 glioma tumour cells were transduced to express either mouse full-length DLL4 (mDLL4) or a soluble dimerised DLL4 in which the extracellular region conjugated with human IgG1 Fc constant region (soluble mDLL4-hFc). When implanted into mice, soluble mDLL4-hFc inhibited but mDLL4 stimulated Notch signalling in mouse stromal cells within C6 tumour. There were no clear effects of both on growth characteristics of the tumour cell line in vitro. Surprisingly, soluble mDLL4-hFc dramatically increased vascular density, angiogenic sprouting and branching in the tumour but significantly inhibited tumour growth; whereas mDLL4 significantly reduced vessel density and sprouting in the tumour but did not affect tumour growth. This increased vasculature was non-productive, as revealed by poor perfusion and increased tumour hypoxia. Systemic delivery of soluble mDLL4-hFc by intravenous injection of adenoviruses at the time of implanting C6 or mouse mammary tumours also inhibited tumour growth but increased tumour vessel density.

Genentech (19) generated a specific DLL4-neutralizing humanised phage antibody, YW152F. Systemic treatment of several tumours with YW152F in mouse models including human colon cancer (HM7 and Colo205), lung carcinoma (Calu6 and MV-522) and melanoma (MDA-MB-435) cells, and mouse leukaemia (WEHI-3) and lymphoma cells (EL4), starting from tumour size of ≥250mm3 or after tumours established, dramatically increased tumour vascular density but decreased tumour growth because of poor perfusion of tumour vasculature.

We (17) generated five tumour cell lines including human glioblastoma (U87), prostate adenocarcinoma (PC3), breast carcinoma (MDA-MB-231) and fibrosarcoma (HT1080) cells, and mouse melanoma
(B16) cells which overexpress human full-length DLL4. DLL4 expressed in tumour cells significantly inhibited the growth of U87 and HT1080 but not of other three cell lines in vitro; however, after implanted into SCID mice, DLL4 promoted tumour growth for U87 and PC3 but not for other three tumour types. DLL4 expressed in tumour cells dramatically inhibited tumour angiogenesis, but surprisingly improved the structure and function of tumour vasculature by inducing larger vessels with large lumina (Figure 3) and increased vessel perfusion and tumour oxygenation. The promotion of tumour growth was, to some extent, due to a reduction of tumour hypoxia, apoptosis and necrosis. Effects of DLL4 on tumour vascular phenotype are consistent and reproducible in all five models. In sharply contrast, soluble DLL4-mFc secreted from co-implanted tumour cells or CHO cells reversed the phenotype of DLL4-overexpressing tumours, displaying decreased tumour growth but paradoxically increased vascular density with decreased vessel sizes in U87 and PC3 tumours. Strikingly, both upregulation and downregulation of DLL4 resulted in decreased pericyte coverage around tumour vessels, suggesting an important interaction of DLL4 with pericytes. We also found that DLL4 is upregulated in ECs and, to some extent, in tumour cells of human glioblastoma. Thus, the paracrine Notch signalling from tumour cells to ECs may be important for some specific tumours (16, 93).

Schenet et al. (20) overexpressed human full-length DLL4 or soluble DLL4 (sDLL4-Fc and sDLL4-His) in human colon carcinoma (HT29) and Kaposi sarcoma (KS-SLK) cells and implanted into athymic nu/nu mice. DLL4 was not found to affect the growth and vasculature of both HT29 and KS-SLK tumours; whereas, soluble DLL4 significantly inhibited tumour growth but increased vascular density in both tumours. The vessels in sDLL4-expressing tumour appeared thin and often lacking apparent lumen but showed more branching points. Soluble DLL4 resulted in more hypoxia, less perfusion and decreased coverage of alpha-SMA-positive pericytes around vessels in tumour. Treatment of the tumour by pre-mixing soluble DLL4 with either HT29 or KS-SLK cells just before the implantation also exhibited significantly reduced tumour growth over 2 weeks.

Taken together, all these studies (17-20) indicate that DLL4 in mouse tumour models functions as a negative regulator of tumour angiogenesis by reducing number of tumour vessels, but acts as a positive driver for tumour growth by improving the structure and function of tumour vasculature only in a few specified tumour types.

5. INTERACTIONS BETWEEN VEGF AND NOTCH PATHWAYS

5.1. Genetic interaction

Information on the interaction of the VEGF pathway and the Notch pathway initially came from genetic studies of vascular development in zebrafish embryos. Loss of Notch signalling in embryos such as mutation of the Hey2 homologue, gridlock, and blockade of Su(H) leads to molecular defects in arterial-venous differentiation, including loss of arterial specific markers such as EphrinB2a and ectopic expression of venous markers such as EphB4a and Flt4 within the dorsal aorta. Conversely, ectopic activation of Notch signalling results in repression of venous cell fate (99, 100). VEGF lies downstream of sonic hedgehog and acts in a common signalling cascade with Notch signalling to induce arterial differentiation (40). A reduction in VEGF activity results in a loss of arterial marker expression from the dorsal aorta and increases the ectopic arterial expression of vein markers. Exogenous expression of VEGF causes ectopic expression of arterial markers in the posterior cardinal vein in wild-type embryos but is incapable of eliciting this effect in mib mutant embryos that lack Notch activity. However, activation of the Notch pathway in response to exogenous Notch1 intracellular form is sufficient to induce arterial
differentiation in the absence of VEGF function. Thus, the results suggest that Notch signalling acts downstream of VEGF to induce arterial differentiation (40). Recently, it was reported that blood flow recovery and postnatal neovascularisation in response to ischemia in heterozygous Notch1 global or EC-specific knockout mice is impaired compared with wild-type mice. Expression of VEGF in response to ischemia, however, is comparable between wild-type and Notch1 mutant mice, suggesting that Notch1 functions downstream of VEGF signalling in postnatal neovascularisation (101). More recently, it was showed that Notch signalling directly upregulates VEGFR3 expression in ECs and Notch1 genetically interacts with VEGFR3 to regulate embryonic vascular development in mice (102).

5.2. VEGF induces Notch signalling

Activation of Notch signalling by the VEGF pathway has been demonstrated in studies of cultured mammalian cells in vitro. Addition of VEGF to cultured human ECs significantly increases DLL4 expression in human iliac and femoral arterial ECs (HIAECs and HFAECs) (103) and umbilical vein ECs (HUVECs) (19, 94, 96). Apart from DLL4, VEGF also induces expression of Notch1 (60, 103), DLL1 and EphrinB2 in human aortic ECs (HAECS) (60). Upregulation of Notch1 and EphrinB2, but not upregulation of DLL1, by VEGF appears to be gamma-secretase-dependent (60). In fact, VEGF has been shown to upregulate both presenilin and ADAM10 expression, increase presenilin proteolytic processing and gamma-secretase activity in HUVEC, and result in activation of Notch1 and Notch4, leading to increased expression of Hes1 and EphrinB2 and decreased expression of EphB4 (94, 101). The PI3K/Akt pathway seems to be critical for the induction of expression of DLL4 and Notch1 and for the activation of gamma-secretase and Notch1 by VEGF, because inhibitors and dominant-negative mutants of PI3K/Akt both completely inhibited the induction/activation while constitutively active forms of PI3K/Akt enhanced the induction/activation (101, 103). We and others have previously showed that DLL4, Hey1 and Hey2 are upregulated by hypoxia (70, 104) probably through HIF-1alpha in EC (96, 104). VEGF is one of main hypoxia regulated genes and, therefore, also acts as an important mediator in regulating DLL4/Notch signalling by the hypoxia pathway.

As described previously, VEGF expression is upregulated by numerous growth factors including bFGF, EGF, TGF-alpha, TGF-beta and HGF. bFGF was reported to increase expression of DLL4 in HFAECs and HUVECs alone (96, 103) and, together with VEGF, induce expression of DLL1, DLL4, Notch1 or Notch4 in HUVECs and HAECS synergistically (60, 96, 103). The synergistic activation of Notch signalling by induction of DLL1 seemed to be necessary and sufficient to regulate EphrinB2 and to induce EphrinB2 and EphB4-dependent branching morphogenesis in human arterial EC (60). Interestingly, HGF, EGF and TGF-alpha are able to induce the protein expression of Jagged1 in HNSCC cells by activation of the MAPK pathway but not by the PI3K/Akt pathway and thus activate Notch signalling from tumour cells to ECs (93)

Evidence on the induction of Notch signalling by VEGF signalling has obtained from in vivo studies as well. The first in vivo clue came from transgenic mouse studies, in which VEGF overexpression in cardiac muscle, probably through Notch signalling, increased the number of EphrinB2-positive capillaries but reduced the number of EphB4-positive venules in the mouse heart (105). Studies on mouse tumour and retinal models have yielded clear insights on regulation of DLL4 expression in vivo by the VEGF pathway. Blockade of VEGF signalling by administration of bevacizumab or VEGF-trap caused a rapid and profound decrease of endogenous DLL4 expression in tumour ECs in several mouse tumour models, demonstrating that DLL4 expression in tumour vasculature depends on VEGF (17, 18). During normal retinal vascular development, DLL4 expression is most pronounced at the growing front of the superficial vascular network where VEGF is expressed at the highest levels. Similarly, disruption of the VEGF pathway by intracocular administration of soluble VEGFR1 or VEGF-trap significantly decreased DLL4 expression at the leading front of the growing superficial vascular plexus (85, 86). Conversely, enhanced VEGF signalling by intravitreal injection of VEGF protein increased DLL4 expression in retinas within 24 hours (85). In human clinical samples, the expression level of DLL4 mRNA is 9-fold higher in clear-cell renal-cell carcinoma and 2-fold higher in superficial bladder cancer than those in corresponding normal tissues and is strongly associated with high levels of VEGF for both tumours (95, 96).

5.3. Notch signalling regulates VEGF signalling

Although VEGF induces Notch signalling, Notch signalling is also capable of regulating the VEGF pathway by altering expression of its ligands (VEGF and PLGF) and receptors (NRP1, NRP2, VEGFR1, VEGFR2 and VEGFR3). Early studies showed that Hey2 (CHF1/HRT2/HESR2, gridlock in zebrafish) interacts with arydrocarbon receptor nuclear translocator (ARNT) in a yeast two-hybrid screen and inhibits binding of ARNT/EPAS1 (HIF-2) to VEGF promotor, suggesting that Hey2 may downregulate expression of VEGF (106). Recently, we showed that activation of DLL4/Notch signalling significantly downregulates expression of PLGF and inhibits angiogenesis in vitro (97). Apart from PLGF, DLL4/Notch signalling also downregulates mRNA expression of NRP1 and NRP2 in ECs and consequently may alter angiogenic processes (97, 98). The possibility that expression of NRP1 and NRP2 may be regulated by Notch signalling was raised by a recent discovery that the larger arteries developed in Notch1 homozygous or Hey1/Hey2 double mutant mice did not express NRP1 (74). In addition, expression of VEGFR1 appears to be also regulated by Notch signalling. DLL4 heterozygous mice displayed decreased expression of VEGFR1 in retinal vessels, potentially increasing their responsiveness to VEGF (86). In contrast, activation of Notch signalling by DLL4 led to increased expression of VEGFR1 at both mRNA and protein levels in cultured HUVECs; however, the soluble splice variant of VEGFR1 (sVEGFR1) was also upregulated in response to DLL4 contributing to the impairment of VEGF signalling (97).
A stream of evidence has been accumulated on downregulation of VEGFR2 by Notch signalling. Overexpression of Hey1 (CHF2/HRT1/HESR1), N1ICD or N4ICD in ECs decreased the luciferase activity driven by VEGFR2 promoter, mRNA expression of VEGFR2 and proliferative responses to VEGF in vitro (107-109). Activation of Notch signalling in HUVECs by DLL4, either transduced in ECs or immobilised on culture plate, reduced expression of VEGFR2 mRNA and protein and inhibited VEGF-induced EC function in vitro (19, 97, 98). Reciprocally, inhibition of Notch signalling by the gamma-secretase inhibitor dibenzazepine (DBZ) or specific anti-DLL4 antibodies increased expression of VEGFR2 in vitro (19). In addition, increased expression of VEGFR2 in vivo was observed in the region of retinal hyperfused vascular plexus in DLL4 heterozygous mice (86). Interestingly, in the xenograft tumour model of U87 DLL4 expressed in tumour cells downregulated expression of mouse VEGFR2 only in large vessels within tumour (17).

More recently, VEGFR3 was demonstrated to be a direct downstream target of Notch signalling (102). Activation of Notch signalling by overexpressing Notch4/Int-3 (or N1ICD) significantly induced VEGFR3 expression in human primary ECs (HUVECs, HUAECc and HMVECs) in vitro. Cocultures containing either Jagged1- or DLL4-expressing HUVECs mixed with Notch4-expressing HUVECs enhanced the induction of VEGFR3, indicating that ligand-mediated Notch4 signalling also induces VEGFR3. In vitro, Notch in complex with RBP-Jkappa bound the VEGFR3 promoter and transactivated VEGFR3 specifically in ECs. Through induction of VEGFR3 expression but reduction of VEGFR2 expression, Notch signalling modulated the response of ECs to angiogenic factors by making them more responsive to VEGF-C but less responsive to VEGF-A, promoting EC survival and morphological changes. In transgenic embryos, activated Notch4 induced VEGFR3 expression within the intersomitic ECs but not within other EC types. In the adult, Notch4, Notch1 and VEGFR3 are actually coexpressed in the vasculature of mouse ovarian follicles, suggesting a role for Notch/VEGFR3 signalling in follicular angiogenesis. In human invasive micropapillary breast carcinomas, Notch1 and Notch4 are coexpressed in the extratumoural blood and lymphatic vasculature with VEGFR3. The cleaved and activated Notch1 was present in the majority of the lymphatic endothelial nuclei, indicating that Notch1 is not only expressed but also activated in tumour lymphatic vessels. Thus, Notch/VEGFR3 signalling may participate in tumour lymphangiogenesis and tumour metastasis in human breast cancer.

Notably, in zebrafish ectopic Notch activation repressed expression of Flt4, the zebrafish orthologue of VEGFR3, in all blood vessels (99), whereas in Rbpsi deficient embryos loss of Notch signalling induced expression of Flt4 in the dorsal aorta and segmental arteries (90). Thus, the effects of Notch signalling on VEGF signalling seems to depend on species, microenvironments and specific cell types.

5.4. Role in tumour angiogenesis

Clearly, proper coordination of the VEGF pathway with Notch signalling in tumour is critical for tumour angiogenesis and growth (17-19). DLL4 and VEGF emerge to be the yin and yang of tumour angiogenesis. At an early stage, tumour cells may secrete VEGF that acts as a driver to induce endothelial proliferation and migration toward tumour cells from the surrounding tissue, leading to growth of new vessels. The resulting vasculature is structurally and functionally abnormal and tumour is hypoxic. Tumour hypoxia not only increases expression of VEGF by tumour and stromal cells but also induces expression of DLL4, Hey1 and Hey2. Increased VEGF in tumour tissue further induces vascular sprouting and branching. However, VEGF also induces expression of DLL4 in a subset of tumour ECs, particular tip cells. DLL4/Notch signalling modulates the actions of VEGF on tumour ECs, particularly on stalk cells that are adjacent the sprouting tip cells by downregulating expression of VEGFR2 and upregulating VEGFR1 and sVEGFR1 and consequently decreases vascular sprouting and branching by suppressing the formation of tip cells. Therefore, DLL4 seems to act as a caretaker to make sure that the vascularisation induced by VEGF do not go out of control, promoting the timely and spatially formation of functional vasculature. Uregupulating expression of VEGFR3 by DLL4/Notch signalling might help maintain sufficient amounts of functional blood and lymphatic vessels in response to stimulation of VEGF-C and/or VEGF-D for tumour growth and metastasis. However, it should be noted that VEGF signalling regulates a number of downstream pathways and DLL4/Notch signalling is only one of these pathways. DLL4 is also regulated by other pathways including Notch signalling itself (17, 19, 83, 97, 102). In addition, many connections between the Notch pathway and other signalling pathways such as hypoxia, TGF-beta, Hedgehog and Wnt (52, 110) may also contribute to the complexity of tumour angiogenesis.

Recent findings, although not directly from the studies in ECs, showed that the hypoxia pathway integrates with Notch signalling at different levels: a) HIF-1alpha can interact with NICD under hypoxia to stabilise the NICD in nucleus and thus increase the Notch downstream response (111); b) factor-inhibiting HIF-1 (FIH-1), apart from the regulation of HIF activity, can also hydroxylate NICD at two critical residues (N1945 and N2012) and thus negatively regulate Notch signalling in vivo (112, 113); and c) hypoxia can directly upregulate expression of DLL1 and Hes1 and increase preexisting Notch signalling in various tumour cell lines (114). Conversely, Notch signalling can mediate hypoxia-induced epithelial-mesenchymal transition, increased motility and invasiveness either by upregulating Snail-1 transcription through NICD directly or by increasing HIF-1alpha recruitment to the lysyl oxidase (LOX) promoter and elevated the hypoxia-induced upregulation of LOX, which stabilises the Snail-1 protein (114). In addition, we and others have shown that DLL4/Notch and Jagged1/Notch signalling upregulates expression of Slug (Snail-2) and that block of Notch signalling inhibits tumour growth and metastasis in an in vivo tumour (97, 115).
Crosstalk of VEGF and Notch signalling pathways

6. THERAPEUTIC COMBINATION BY DISRUPTION OF VEGF AND DLL4/NOTCH PATHWAYS

As described previously, blockade of VEGF signalling inhibits tumour growth and angiogenesis by extensive pruning of the rapidly growing tumour vasculature in numerous preclinical models. To some extent, blockade of VEGF may also normalise the remaining vessels in tumour to help deliver nutrients and oxygen (116). In phase III clinical trials, blocking VEGF prolongs patient survival for several major cancers. However, there are a number of tumours that did not respond at all or responded in an early stage but became resistant in a late stage to VEGF inhibition in preclinical mouse models (9-12). In addition, anti-VEGF therapy alone appears to be ineffective in most, if not all, clinical trials (13). Thus, additional factors/pathways may directly drive tumour angiogenesis or switch on at certain stages to regulate tumour angiogenesis and growth in anti-VEGF-resistant tumours and combined approaches for interrupting the VEGF pathway and additional pathways may improve anti-angiogenic therapeutic efficacy.

DLL4/Notch signalling may represent such an additional pathway. Recently, we and others have shown that therapeutic treatment of tumours in various preclinical models by disruption of DLL4/Notch signalling (Figure 2) remarkably inhibit tumour growth in vivo (17-20). It has been thought that tumour growth is positively correlated with tumour vascular density and more angiogenesis always translates to more aggressive tumours. Thus, conventional angiogenesis-based treatments of tumours, for example, anti-VEGF therapy, have focused on blocking angiogenesis. However, blockade of DLL4/Notch signalling resulted in a marked increase in tumour angiogenesis but a dramatic decrease in vessel function, providing a striking example of an uncoupling of tumour growth from tumour vascular density (117). The fact that blockade of DLL4/Notch signalling and VEGF-inhibition have paradoxical effects on tumour vasculature but both consistently reduce tumour growth accentuates that in tumour, vessel function is much more important than vessel density for tumour growth. Fewer but larger vessels can be as efficient as a much greater number of smaller vessels within tumour (17). Blockade of DLL4/Notch signalling could treat a wide range of tumour types. Regeneron has reported the effects on all 10 tumour lines tested in mice and Genentech also observed tumour inhibition in 13 tumour lines in mouse models (118, 119).

Importantly, blockade of DLL4/Notch signalling is also effective in growth inhibition of VEGF-resistant tumours. Systemic treatment of mice bearing resistant HT1080-RM tumours or mouse mammary tumours with soluble DLL4-Fc or blocking DLL4 antibodies resulted in a prolonged suppression of tumour growth, whereas anti-VEGF treatment with either bevacizumab or VEGF-trap had almost no impact on tumour growth (18). WEHI3 tumours were highly resistance to anti-VEGF mAb therapy; however, treatment with anti-DLL4 antibodies (YW152F) significantly suppressed tumour growth (19). PC3 tumours, when implanted in mouse, were hardly responsive to bevacizumab, but soluble DLL4-mFc secreted from co-implanted tumour cells resulted in a significant decrease in tumour growth (17). Thus, targeting DLL4/Notch signalling may have become an alternative therapy for anti-VEGF-resistant cancers.

The potent anti-tumour activity observed with DLL4/Notch signalling blockade and its dependence on VEGF raised the exciting possibility that anti-DLL4/Notch and anti-VEGF combination therapy may improve anti-angiogenic efficacy. Indeed, Genentech has already shown that in MV522 xenograft tumours, treatment with anti-DLL4 or anti-VEGF antibodies alone was only modestly effective at reducing tumour growth, revealing some intrinsic resistance of this tumour to each therapy; strikingly, the combination therapy with both antibodies resulted in a robust additive inhibition of tumour growth (19). In HT1080 xenograft tumours, we have found that therapeutic administration of either bevacizumab or DBZ had little effects on tumour growth; however, the combination therapy yielded a synergistical suppression of tumour growth (Li et al., unpublished data). In addition, we have revealed that in U87 glioblastoma xenograft tumours, the combination therapy with bevacizumab and DBZ resulted in the synergistic effect on tumour progression (Li et al., unpublished data). Therefore, blockade of DLL4/Notch signalling may have provided a potent option for combination therapy with anti-VEGF agents for solid tumours.

It has not been clear yet how blockade of DLL4/Notch signalling is capable of suppressing the growth of both VEGF-sensitive and VEGF-resistant tumours and enhancing the anti-VEGF therapeutic efficacy. Apart from VEGF signalling, DLL4/Notch signalling might have broad and diverse interactions with other angiogenic pathways and thus DLL4/Notch blockade becomes effective in tumours that either are intrinsically VEGF-independent or become VEGF-resistant at certain stages when other angiogenic pathways switch on. Another potential explanation is that blockade of DLL4/Notch signalling upregulates VEGFR2 and consequently make tumour ECs more sensitive to anti-VEGF therapy. A third possibility is that blockade of DLL4/Notch signalling reduces the recruitment of pericytes and accordingly tumour vasculature becomes more vulnerable to VEGF-inhibition. Finally, blockade of DLL4/Notch signalling may also have some non-EC effects, that is, DLL4/Notch signalling between tumour and/or other stromal cells in the tumour microenvironment, such as macrophages and dendritic cells. Clearly, to understand detailed mechanisms will facilitate extrapolation of anti-DLL4/Notch treatments to the clinic.

7. CONCLUDING REMARKS

The formation of a hierarchical vascular network in embryonic development and tumour angiogenesis in postnatal growth require highly coordinated interactions of various signalling pathways. VEGF signalling and DLL4/Notch signalling are perhaps two most important
pathways during vascular development and tumour angiogenesis. Agents that block the VEGF pathway effectively inhibit tumour angiogenesis and growth in preclinical tumour models. Some of them have been validated in phase III clinical trials and already become an integral part of standard cancer therapy. Both in vitro and in vivo studies have demonstrated that the VEGF pathway interacts at several levels with DLL4/Notch signalling. VEGF induces DLL4/Notch signalling while DLL4/Notch signalling modulates the VEGF pathway. The recent flurry of findings have shown that DLL4/Notch signalling regulates angiogenesis by suppressing the formation of endothelial tip cells and blockade of DLL4/Notch signalling strikingly induces non-productive angiogenesis but remarkably reduces the growth of tumours that are either sensitive or resistant to anti-VEGF therapy. Combination therapies by blocking DLL4/Notch signalling and the VEGF pathway synergistically inhibit tumour growth in preclinical mouse models. Thus, targeting the DLL4/Notch pathway may lead to exciting new therapies for clinical investigation.

However, a host of issues still remains. The diverse functions of DLL4/Notch signalling in vascular development and tumour angiogenesis can be only partially explained by its interaction with the VEGF pathway. The role of other signalling pathways and relevant interaction partners will need to be addressed. Given that four Notch ligands (DLL1, DLL4, Jagged1 and Jagged2) exist in vasculature, we wonder whether all of these ligands are involved in tumour angiogenesis or not and how Notch signalling initiated by different ligands is coordinated in tumour angiogenesis. For example, Jagged1-triggered Notch signalling stimulates tumour angiogenesis and growth in HNSCC xenograft tumours while DLL4-initiated Notch signalling inhibits tumour angiogenesis in various preclinical tumour models. It will be interesting to investigate which Notch signalling affects tumour angiogenesis and growth if both are activated in the same tumour type. Due to the lethality of the global knockout mice, we have only known relatively little about DLL4/Notch function in postnatal vasculature and in tumour malignant progress. Establishment of preclinical tumour models by using EC-specific inducible DLL4-knockout and DLL4-knockin mice will help address this question. Apart from indirect vessel-spacing effects, DLL4/Notch signalling could have direct effects on tumour growth. For example, DLL4 binding to Notch receptors on tumour cells could help maintain tumour stem cell populations because Notch is crucial for maintaining the stem cell niche. It becomes increasingly evident that targeted cancer therapies are normally more effective when started treatment at an early course of tumours and combined with other agents that target a complementary pathway or other therapies such as anti-angiogenic therapy, chemotherapy and radiation therapy. It will be important to investigate whether disruption of DLL4/Notch signalling could make tumour cells more vulnerable to chemotherapy and radiation therapy by increasing the number of rapidly dividing ECs in the expanding vascular network.

Before anti-DLL4/Notch treatments extrapolate to the clinic, it will be essential to further examine the effects of DLL4/Notch blockers on normal vessels, particularly those in female ovarian follicles and uterine endometrium (70, 120), and on non-vascular cells, particularly those in brain neural tissues (70, 121, 122) and immune systems such as macrophages, lymphocytes and thymus (70, 123) that are known to express DLL4. In addition, it will be interesting to investigate whether or not DLL4/Notch inhibitors affect stem cell populations in normal tissues since Notch signalling is important in regulation of stem cell functions (111). Understanding the side-toxic effects of anti-DLL4/Notch treatment will help establish a therapeutic index and yield the highest benefits for clinical patients of cancers.

8. ACKNOWLEDGEMENTS

Work on Notch signalling in authors’ laboratory is financially supported by the Cancer Research UK and European Union 6th Framework Angiotargeting programme.

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**Key Words:** VEGF, VEGF receptor, Notch, Delta, Jagged, DLL4, Bevacizumab, Angiogenesis, Xenograft, Anti-Angiogenic Therapy, Review

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