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INTRODUCTION

Tyro3 (Sky), Axl and Mer are members of the TAM family of receptor tyrosine kinases sharing vitamin K-dependent ligands Gas6 and Protein S. TAM family receptors can be activated in both ligand-dependent and ligand-independent manner, resulting in receptor dimerization and autophosphorylation.1–4 TAM family receptors are overexpressed in cancers and are markers for poor prognosis and correlate with multidrug resistance.5–7 They also contribute to tumourigenesis by regulating migration and invasion, angiogenesis, cell survival and tumour growth.5,8,9 TAM family receptors,10 insulin-like growth factor I receptor,16 integrins17 and ErbB2/3,18 TAM family receptors are significantly overexpressed in schwannoma tissues.11 The relevance of Axl in merlin-deficient tumours is underlined by findings showing that Axl is negatively regulated by merlin in schwannoma and involved in increased proliferation of merlin-deficient meningioma and mesothelioma. Here, we demonstrated strong overexpression and activation of Axl receptor as well as its ligand Gas6 in human schwannoma primary cells compared to normal Schwann cells. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Stimulation of the Gas6/Axl signalling pathway recruits Src, focal adhesion kinase (FAK) and NFkB. We showed that NFkB mediates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK, leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. We conclude that Axl/FAK/Src/NFkB pathway is relevant in merlin-deficient tumours and is a potential therapeutic target for schwannoma and other merlin-deficient tumours.

Axl/Gas6/NFkB signalling in schwannoma pathological proliferation, adhesion and survival

S Ammoun1, L Provenzano1, L Zhou1, M Barczyk1, K Evans1, DA Hilton2, S Hafizi3 and CO Hanemann1

TAM family receptor tyrosine kinases comprising Tyro3 (Sky), Axl, and Mer are overexpressed in some cancers, correlate with multidrug resistance and contribute to tumourigenesis by regulating invasion, angiogenesis, cell survival and tumour growth. Mutations in the gene coding for a tumour suppressor merlin cause development of multiple tumours of the nervous system such as schwannomas, meningiomas and ependymomas occurring spontaneously or as part of a hereditary disease neurofibromatosis type 2. The benign character of merlin-deficient tumours makes them less responsive to chemotherapy. We previously showed that, amongst other growth factor receptors, TAM family receptors (Tyro3, Axl and Mer) are significantly overexpressed in schwannoma tissues. As Axl is negatively regulated by merlin and positively regulated by E3 ubiquitin ligase CRL4DCAF1, previously shown to be a key regulator in schwannoma growth we hypothesized that Axl is a good target to study in merlin-deficient tumours. Moreover, Axl positively regulates the oncogene Yes-associated protein, which is known to be under merlin regulation in schwannoma and is involved in increased proliferation of merlin-deficient meningioma and mesothelioma. Here, we demonstrated strong overexpression and activation of Axl receptor as well as its ligand Gas6 in human schwannoma primary cells compared to normal Schwann cells. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Stimulation of the Gas6/Axl signalling pathway recruits Src, focal adhesion kinase (FAK) and NFkB. We showed that NFkB mediates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK, leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. We conclude that Axl/FAK/Src/NFkB pathway is relevant in merlin-deficient tumours and is a potential therapeutic target for schwannoma and other merlin-deficient tumours.

INTRODUCTION

Tyro3 (Sky), Axl and Mer are members of the TAM family of receptor tyrosine kinases sharing vitamin K-dependent ligands Gas6 and Protein S. TAM family receptors can be activated in both ligand-dependent and ligand-independent manner, resulting in receptor dimerization and autophosphorylation.1–4 TAM family receptors are overexpressed in cancers and are markers for poor prognosis and correlate with multidrug resistance.5–7 They also contribute to tumourigenesis by regulating migration and invasion, angiogenesis, cell survival and tumour growth.5,8,9

Mutations in the gene coding for a tumour suppressor, merlin, cause the development of multiple tumours of the nervous system such as schwannomas, meningiomas and ependymomas occurring either spontaneously or as a hereditary disease, neurofibromatosis type 2. The benign character of merlin-deficient tumours makes them relatively unresponsive to conventional chemotherapies, leaving invasive surgery or radiosurgery as the main treatment options. However, these treatments carry significant risks, particularly when the tumours are close to important neurological structures or if the tumours are multiple as in neurofibromatosis type 2. The benign character of merlin-deficient tumours makes them less responsive to chemotherapy. We previously showed that, amongst other growth factor receptors, TAM family receptors (Tyro3, Axl and Mer) are significantly overexpressed in schwannoma tissues. As Axl is negatively regulated by merlin and positively regulated by E3 ubiquitin ligase CRL4DCAF1, previously shown to be a key regulator in schwannoma growth we hypothesized that Axl is a good target to study in merlin-deficient tumours. Moreover, Axl positively regulates the oncogene Yes-associated protein, which is known to be under merlin regulation in schwannoma and is involved in increased proliferation of merlin-deficient meningioma and mesothelioma. Here, we demonstrated strong overexpression and activation of Axl receptor as well as its ligand Gas6 in human schwannoma primary cells compared to normal Schwann cells. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Stimulation of the Gas6/Axl signalling pathway recruits Src, focal adhesion kinase (FAK) and NFkB. We showed that NFkB mediates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK, leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. We conclude that Axl/FAK/Src/NFkB pathway is relevant in merlin-deficient tumours and is a potential therapeutic target for schwannoma and other merlin-deficient tumours.

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Using our schwannoma in vitro model, we demonstrate strong overexpression of all three members of the TAM receptor family, and their ligand Gas6 in human schwannoma. We show that Gas6 is mitogenic and increases schwannoma cell-matrix adhesion and survival acting via Axl in schwannoma cells. Furthermore, Gas6 signalling via specifically Axl involves focal adhesion kinase (FAK) and Src, but not the ERK1/2, JNK1/2 and AKT signalling pathways. We also demonstrate the role of NFkB, which regulates Gas6/Axl-mediated overexpression of survivin, cyclin D1 and FAK leading to enhanced survival, cell-matrix adhesion and proliferation of schwannoma. NFkB expression was found to be merlin-dependent and its activity depended on Axl.

RESULTS

Axl, Tyro3 and Mer receptors are overexpressed in schwannoma TAM family receptors, Axl, Tyro3 and Mer are expressed at higher levels in human primary schwannoma compared with normal Schwann cells (Figures 1a, c, and e). The expression of Axl and Tyro3 but not Mer was decreased by the reintroduction of merlin (Figures 1b, d and f). Thus Axl and Tyro3 but not Mer are under direct merlin control.

Gas6 is expressed and released from both schwannoma and Schwann cells

The TAM receptor ligand, Gas6, was also expressed at higher levels in schwannoma than in Schwann cells (Figure 1g). Furthermore, Gas6 was also released from both cell types, with slightly higher levels (not significant) being observed from schwannoma cells as compared with Schwann cells (Figure 1h). Moreover, Gas6 expression was decreased upon reintroduction of merlin, suggesting that this TAM ligand is regulated by merlin (Figure 1i).

Gas6 increases schwannoma proliferation, cell-matrix adhesion and survival and activates Axl receptor

Proliferation. Gas6-containing medium (40 ng/ml, 24 h) increased proliferation of schwannoma cells by ~80% with the same efficiency as 10% FCS, while mock had no effect (Figure 2a). The specificity of Gas6-mediated proliferation was confirmed using recombinant human Axl and Tyro3 ectodomain-Fc chimeras to capture Gas6 from media (Figure 2b), and the chimeras’ specificities were confirmed by using Insulin-like growth factor-1 (IGF-I), to which they did not bind (Figure 2c). Axl shRNA completely inhibited the Gas6-mediated increase in proliferation of schwannoma cells (Figure 2d). Moreover, Gas6 increased the phosphorylation/activity of Axl (Figure 2e). Thus, Gas6-mediated proliferation of schwannoma cells is mediated via the Axl receptor.

Adhesion. Similarly to the increased proliferation, Gas6 (40 ng/ml) potentiated schwannoma cell-matrix adhesion by 40% (Figure 2f), while medium had no effect (Figure 2f). Axl shRNA completely inhibited the Gas6-mediated increased cell-matrix adhesion (Figure 2g). Thus, Gas6-mediated adhesion occurs via the Axl receptor. Controls for the specificity of Axl shRNA are shown in Figure 2h, where only Axl downregulation but not Tyro3 or Mer were observed (Figure 2h).

Survival. Schwannoma cells cultured in DMEM without any supplements, for 5 days displayed only 6% of cell death, which was rescued by Gas6 40 ng/ml to ~2% (n = 5) (Figure 2i). The data suggest that schwannoma cells develop pro-survival mechanisms during starvation, which confirms our previous findings, and that Gas6 seem to have a role in the increase of cell viability.

Tissue expression. As Gas6-mediated proliferation and cell-matrix adhesion are fully dependent on Axl receptor, we went on to explore Axl-mediated signalling in schwannoma. Firstly, by immunohistochemistry, we were able to show strong staining for both Axl (Figure 2j) and phospho-Axl (Figure 2k), in human schwannoma tissues compared with normal nerves, with both cytoplasmic and widespread nuclear immunoreactivity in the tumour cells. Next, we dissected potential downstream signalling pathways from Axl in human schwannoma primary cells.

Signalling pathways activated by Gas6 via Axl in schwannoma

Gas6 leads to the activation of ERK1/2, JNK, AKT, FAK and Src in schwannoma cells. Stimulation of schwannoma cells with Gas6 (40 ng/ml) for 5 min lead to strong activation/phosphorylation of ERK1/2, JNK and AKT (Figures 3a, b and c), which have previously been shown to contribute to increased proliferation, cell-matrix adhesion and survival in schwannoma. Moreover, it has been previously shown to be overexpressed and strongly activated in schwannoma, contributing to increased proliferation and cell-matrix adhesion, is also activated/phosphorylated by Gas6 at autophosphorylation site Y937 and Src phosphorylation site Y925 (Figures 3d and e). Levels of active/phosphorylated Src P-Y416, previously demonstrated to have an important role in schwannoma development by increasing proliferation, were also increased by Gas6 (Figure 3f).

Dissection of Gas6-mediated pathways towards activation of ERK1/2, JNK and AKT in schwannoma cells. Using various inhibitors SU6656 (Src), FTI-277 (Ras), Wortmannin (PI3K), GF109203x (PKC), previously validated in our cells, we found that Gas6 stimulated ERK and JNK, recruiting Src and PI3K but not involving Ras and PKC (Figures 3g and h). Gas6-mediated AKT activation proceeded via Src and PI3K but not via Ras and PKC (Figure 3i). Thus the ERK, JNK and AKT pathways share similar mediators such as Src and PI3K.

Axl triggers Gas6-mediated activation of Src and FAK but not ERK, JNK or AKT. As Gas6 is an agonist for all TAM family receptors and Gas6-induced proliferation, and cell-matrix adhesion can be blocked by Axl knockdown, we used Axl shRNA to monitor which signalling pathways are triggered by this particular receptor. We demonstrate that Axl shRNA decreased Gas6-mediated Src and FAK (Figures 4d–f) but not ERK1/2, JNK or AKT activity (Figures 4a–c). These data therefore suggests that Gas6-mediated activation of ERK1/2, JNK and AKT (as shown in Figures 3a–c) probably proceeds via other TAM family receptors overexpressed in schwannoma such as Tyro3 and Mer.

NFkB is upregulated in schwannoma

We observed that Axl receptor activation leads to downstream activation of Src and FAK, but not of ERK1/2, JNK or AKT upon stimulation with Gas6. However, Axl activation by Gas6 does increase schwannoma proliferation and cell-matrix adhesion. We therefore hypothesized that an additional pathway must be involved in Gas6/Axl-mediated signalling towards increased proliferation and cell-matrix adhesion. The transcription factor NFkB regulates expression of FAK, which localizes to the nucleus in schwannoma cells leading to increased proliferation and cell-matrix adhesion. NFkB is known to be an anti-apoptotic factor in Schwann cells. We therefore investigated the expression and signalling of NFkB in human schwannoma downstream of Axl.

We demonstrate that NFkB (p65 subunit) is strongly overexpressed in schwannoma cells (Figure 5a) owing to merlin deficiency as merlin reintroduction into primary human schwannoma cells decreased NFkB (p65) expression (Figure 4b). Immunohistochemistry showed nuclear localization of NFkB in both Schwann and schwannoma cells (Figure 5c) as expected. The levels of phosphorylated NFkB (P-Ser529), which increases NFkB transcriptional activity was also elevated in schwannoma cells (Figure 5d).
NFκB (p65 subunit) activity was potentiated by Gas6 (40 ng/ml) from 5 min up to 5-h stimulation, as shown by western blotting (Figure 5e) and by luciferase reporter gene assay (Figure 5f). By 24 h after stimulation with Gas6 (40 ng/ml), NFκB activity had decreased (Figures 5f and g). Axl shRNA decreased the levels of phosphorylated/active (Gas6 40 ng/ml, 5 min) NFκB (p65 subunit) (Figure 5h).
The expression of survivin, cyclin D1 and FAK are potentiated by Gas6 in a NFκB-mediated manner in schwannoma cells. As both Gas6 and NFκB are known to regulate the survival and proliferation of Schwann cells\(^30\) and are linked to cyclin D1 and FAK expression,\(^{29,31}\) we investigated the role of the Gas6/Axl/NFκB signalling axis in the expression of survivin, cyclin D1, and FAK.

**Survivin.** Survivin was overexpressed in primary human schwannoma cells (Figure 6a). Gas6 (40 ng/ml, 24 h) potentiated the expression of survivin (Figure 6b) in a merlin-dependent manner (Supplementary Figure 2A).\(^{11}\) NFκB was found to be involved in this process as both the NFκB inhibitor SNS0 and NFκB shRNA (Supplementary Figure 1) significantly inhibited the Gas6-mediated expression of survivin (Figures 6c and d). These data suggest that the Gas6/Axl/NFκB signalling axis may have a role in the pathological survival of schwannoma cells.

**Cyclin D1.** Gas6 (40 ng/ml, 24 h) potentiated the expression of cyclin D1 (Figure 6e), which is overexpressed in schwannoma cells\(^{32}\) in a merlin-dependent manner (Supplementary Figure 2B). NFκB acted as a mediator in the potentiation of cyclin D1 expression (Figures 6f and g). These data therefore suggest that the Gas6/Axl/NFκB signalling axis may have a role in the pathological proliferation of schwannoma cells.

**FAK.** The expression of FAK was increased by Gas6 (40 ng/ml, 24 h) (Figure 6h). Reintroduction of merlin decreased FAK expression (Supplementary Figure 2C). The inhibition of NFκB by SN50 and NFκB shRNA (Supplementary Figure 1) decreased the Gas6-mediated increase of FAK expression (Figures 6i and j). These data therefore suggest that the Gas6/Axl/NFκB signalling axis may have a role in the pathological proliferation of schwannoma cells.
Gas6/Axl-mediated proliferation of schwannoma cells depends on NFκB

We observed that NFκB is downstream of Axl regulating the expression of cell cycle protein cyclin D1 and also FAK in human schwannoma. Previously we have shown that FAK localizes to the nucleus and, together with Src, is responsible for integrin β1/IGFBP-1-mediated proliferation independently of ERK1/2 and JNK.12 We therefore investigated here the role of NFκB in Gas6/Axl-mediated proliferation.

Both the NFκB inhibitor SN50 and shRNA for NFκB (p65 subunit) (Supplementary Figure 1) significantly decreased Gas6-mediated proliferation of schwannoma cells to basal levels (Figures 7a left panel and b) in parallel to preventing Gas6-induced increases in cyclin D1 (Figures 6f and g) and FAK (Figures 6i and j) expression. Schwannoma cell viability upon SN50 treatment for 24 h was not affected (Figure 7a right panel).

The role of NFκB in Gas6/Axl-mediated cell proliferation, cell-matrix adhesion and survival in schwannoma cells NFκB is specifically involved in Gas6/Axl-mediated proliferation in schwannoma, which converges with integrin β1 signalling. To test whether NFκB is specific for Gas6-mediated proliferation, we also investigated its role in PDGF-DD-, IGF-I- and β1-hereregulin-mediated proliferation, all known to be relevant for schwannoma pathology.10,11,26 We observed that NFκB is specific or selective for Gas6 signalling towards schwannoma proliferation, as SN50 had no effect on either PDGF-DD or IGF-I and β1-hereregulin-mediated proliferation (Figure 7c). Signalling triggered by IGFBP-1, acting via integrin β1, also utilized NFκB for proliferation (Figure 7c).

Moreover, both IGFBP-1 (100 ng/ml) and Gas6 (40 ng/ml) increased the expression of NFκB after 24 h of stimulation (Figure 7d). These data suggest a degree of convergence between Gas6/Axl and integrin β1/IGFBP-1 pathways towards increased proliferation of schwannoma cells via NFκB.

NFκB mediates Gas6/Axl-potentiated cell-matrix adhesion and survival of schwannoma cells. We found that similarly to proliferation, Gas6/Axl-mediated cell-matrix adhesion and survival also occurred via recruiting NFκB, as observed because of the blockade of Gas6-mediated increase in cell-matrix adhesion by the NFκB inhibitor SN50 and NFκB (p65 subunit) shRNA (Figures 7e and f; Supplementary Figure 1) and decrease of survival by the...
NFκB inhibitor SN50 (Figure 7g). To study the survival of schwannoma cells is difficult as these cells survive, when cultured under growth factor withdrawal conditions, for weeks displaying only very minor decrease in cell viability in contrast to Schwann cells as shown in Figures 2i and 7g and previously published in Utermark et al.22

**DISCUSSION**

In this manuscript, we have investigated the role of Axl receptor signalling in the development of merlin-deficient schwannoma. We formulated our hypothesis that Axl may contribute to schwannoma development on the basis of previous findings: (1) positive regulation of Axl by E3 ubiquitin ligase CRL4DCAF1, which we and others have shown to contribute to schwannoma tumour growth;16 (2) Axl interaction with oncogene Yes-associated protein, a member of Hippo pathway, which is under merlin regulation in schwannoma and is involved in increased proliferation of merlin-negative meningioma and mesothelioma;20 (3) negative regulation of Axl by merlin; (4) the previously reported mitogenic effect of Gas6 on human Schwann cells acting via Axl.2

Our results demonstrate strong overexpression of TAM family receptors in human primary schwannoma cells compared with normal Schwann cells, supporting previous array data.11 Strong immunostaining of Axl and active/phospho-Axl was also observed in schwannoma biopsies, within both tumour nuclei and the cytoplasm, compared with normal nerve tissues. Overexpression of Axl and Tyro3 is merlin-dependent in human primary schwannoma cells as shown by western blotting and reporter gene assays, and which was both Gas6- and Axl-dependent, suggesting NFκB expression is also merlin-dependent. In addition, we found that transcriptional activity of NFκB was highly increased in schwannoma as shown by western blotting and reporter gene assays, and which was both Gas6- and Axl-dependent, suggesting NFκB as an important signalling molecule downstream of Gas6/Axl in schwannoma. The importance of NFκB in schwannoma is underlined by the discovery of its involvement in increased expression of pro-survival protein survivin,11 shown to be overexpressed in schwannoma due to Gas6 stimulation. In addition, NFκB was found to be recruited in the Gas6-mediated increase in expression of cyclin D1, previously shown in our pathobiology was ascertained by functional assays, which demonstrated strong mitogenic and pro-adhesive effects of Gas6 on primary human schwannoma cells. Gas6 stimulation increased both proliferation and cell-matrix adhesion and had a pro-survival effect on schwannoma cells acting via the Axl receptor, which was previously observed in human Schwann cells34 and other tumours.42 Even though Gas6, known to be an agonist for Axl, Tyro3 and Mer, activates ERK1/2, JNK, AKT, FAK and Src in schwannoma, Gas6/Axl receptor-dependent signalling employed only FAK and Src. We thus suggest the presence of signalling specificity among TAM family receptors in schwannoma. As Axl mediates both increased proliferation and cell-matrix adhesion, but does not stimulate activation of pro-proliferative ERK1/2 and JNK and cell-matrix adhesion-stimulating ERK1/2,10 we employed only FAK and Src. We thus suggest the presence of signalling specificity among TAM family receptors in schwannoma.
From the image, the text appears to discuss the role of NFkB in schwannoma, with a focus on the overexpression of NFkB (p65 subunit) in schwannoma cells compared to normal Schwann cells. The text mentions that the overexpression of NFkB is in a merlin-dependent manner and is mediated by Axl receptor (5-minute stimulation). Gas6 potentiates schwannoma basal expression of phosphorylated/active NFkB in a merlin-dependent manner.

The text also mentions the role of NFkB in transcriptional activity and the localization of NFkB to the nucleus and focal adhesions. It discusses the potential therapeutic targets for NFkB and Axl, including small-molecule inhibitors and Axl ectodomains that can neutralize Gas6.

The text concludes with a suggestion to investigate and test pathways for potential therapeutic targets in schwannoma, which could be easily detected in patients for diagnostic purposes. The authors recommend the use of Axl as a new candidate biomarker for spontaneous and unorthodox schwannomas.

**Figure 5.** The role of NFkB in schwannoma. NFkB (p65 subunit) is overexpressed in schwannoma cells (NF2+/−) compared to normal Schwann cells (NF2−/+). (a) In merlin-dependent manner (n = 6). (b) NFkB (p65 subunit) localizes to the nucleus in both Schwann (NF2−/+−) and schwannoma cells (NF2−−/−) (n = 3). (c) Immunocytochemistry. Phosphorylated/active NFkB (p65 subunit) is overexpressed in schwannoma cells (n = 3). Gas6 potentiates schwannoma basal expression of phosphorylated/active NFkB (n = 6). (e) p65 subunit, western blotting) and NFkB transcriptional activity (n = 3) (f, reporter gene assay) upon stimulation for 5 min, 1 and 5 h, respectively. The levels of transcriptionally active NFkB (f) and of phosphorylated/active NFkB (p65 subunit) (n = 3) (g) are decreased to the basal levels after 24 h of stimulation with Gas6. The increased phosphorylation/activity of NFkB (p65 subunit) (Gas6 40 ng/ml, 5 min) in schwannoma is Axl dependent (n = 3) (h). In (b), the cells were infected with either mock (white bar) or wild-type (wt) merlin (grey bar) containing adenovirus. In H the cells were infected with mock or Axl shRNA lentivirus. The data in B and H are normalized to mock (white bars). In An and D the data are normalized to normal Schwann cells (NF2−+/−), in E, F and G to basal (non-stimulated, white bars) levels. N represents number of samples from different patients (*P < 0.05, **P < 0.01). RhoGDI is used as a loading control as previously described in Hanemann et al.22

**MATERIALS AND METHODS**

**Cell culture conditions**

The primary human Schwann and schwannoma cells were obtained after patient’s consent, as described elsewhere14 and cultured in DMEM.
supplemented with 10% FCS, 0.5 mM forskolin, 10 nM \(\beta\)-heregulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 2.5 mg/ml insulin (complete medium, growth factor medium). In every experiment, minimum three samples from three different patients and donors were included. In every experiment, minimum three samples from three different patients and donors were included. For the activation of different signalling pathways, the cells were stimulated for 5 min as we previously showed that, in schwannoma, the maximum activation of signalling pathways is obtained upon short period of stimulation.10 Proliferation assays and the increase of protein expression were performed upon 24-h post-stimulation.

Inhibitors and chemicals
SUs656 and FTI-277 were from Calbiochem (La Jolla, CA, USA), GF109203x from Tocris Bioscience (Bristol, UK), SP600125 from Affiniti Research Products, Ltd. (Exeter, UK), Wortmannin from Tocris and SN50 from Calbiochem. Axl shRNA and NF\(\kappa\)B shRNA were from Open Biosystems (Huntsville, AL, USA). DAPI and propidium iodide were from Sigma (St Louis, MO, USA) and U0126 from Promega (Madison, WI, USA). Human Gas6 was a gift from Dr Sassan Hafisi (Portsmouth University, UK). Recombinant Axl Fc Chimera and Dtk (Sky) Fc Chimera were from R&D Systems (Minneapolis, MN, USA). Most of these drugs have been previously tested in our human primary schwannoma model.10,11,27,43 The concentrations of inhibitors have been previously confirmed by others.41,42,44–49

shRNA knockdown
GIPZ-shRNAmir lentiviral particles encoding a short-hairpin RNA (shRNA) with nonsilencing sequence (mock) or sequences targeting specific genes were used. Infections were performed, as previously described in Ammoun et al.12,16

Sequences of Axl shRNA lentivirus. TRCN0000000574: 5'-CCGGCCGAAAGAAGGAGACCCGTTATCTCGAGATAACGGGTCTCCTTCTTTCGTTTTT-3' TRCN0000001041: 5'-CCGGGCTGTGAAGACGATGAAGATTCTCGAGAATCTTCATCGTCTTCACAGCTTTTT-3'

Sequences of NF\(\kappa\)B shRNA lentivirus. V3LHS_633760: 5'-TGCTGTTGACAGTGAGCGACAAGCTGATGTGCACCGACAATAGTGAAGCCACAGATGTATTGTCGGTGCACATCAGCTTGCTGCCTACTGCCTCGGA-3'

Figure 6. The role of NF\(\kappa\)B in increased expression of Src, cyclin D1 and FAK in schwannoma. Pro-survival protein survivin is overexpressed in schwannoma (NF2\(-/-\)) (n = 4) (a). Survivin (n = 3) (b), cyclin D1 (n = 10) (e) and FAK (n = 6) (h) expression is potentioted by Gas6 upon 24-h stimulation. Gas6-mediated increase in the expression of survivin (c (n = 3), d (n = 3)), cyclin D1 (f (n = 6), g (n = 3)) and FAK (i (n = 6), j (n = 4)) are dependent on NF\(\kappa\)B. In (a) the data are normalized to normal Schwann cells (NF2\(+/-\)), in (c) and (f) to the basal (nonstimulated) cells, in (d), (g) and (j) to basal mock-infected cells. In (b, c, e, f, h and i) statistical comparisons were made between nonstimulated (basal, white bars) and stimulated (Gas6, grey bars). Both NF\(\kappa\)B inhibitor SN50 (c, f and i) and shRNA (d, g and j) were used to inhibit NF\(\kappa\)B-mediated signalling. N represents the number of samples from different patients (*P < 0.05, **P < 0.01). RhoGDI is used as a loading control as previously described in Hanemann et al.52

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Re-introduction of merlin
Mock (control adenovirus containing a GFP) and merlin wild-type (recombinant adenovirus AdNF2) were gift from J Testa. Cells were treated with virus for 24 h, followed by incubation in fresh growth factor medium for additional 24 h.

Immunoblot analysis
Following electrophoresis and western blotting, detection of proteins was performed using anti-Axl, anti P-Axl (Y779), anti-Tyro3 and anti-Gas6 (R&D Systems), anti-Mer (Abcam, Cambridge, UK), anti-integrin β1 and anti-phospho NFkappaB p65 Ser529 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-active-MAPK (anti-pThr(183)-pTyr (185)-ERK1/2) (Promega), anti-phospho-AKT (Ser473) (Cell Signaling), anti-phospho-FAK (Tyr925) (Cell Signaling), anti-phospho-FAK (Tyr397) (Chemicon International, Temecula, CA), anti-phospho-JNK (Thr183/Tyr185) (Cell Signaling), and HRP-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA). ECL-plus (Amer sham, Buckingham- hamshire, UK) was used as a substrate for HRP. As short-term stimulations do not affect protein expression levels, we have used a generic loading controls Rho-GDI previously established for our system (Anti-RhoGDI antibody; Santa Cruz Biotechnology)\(^2\) and GAPH (Millipore, Watford, UK). Images were scanned and processed using Corel Paint Shop Pro Photo XI software (Corel, Ottawa, ON, Canada). Images represent the original data.

Immunohistochemistry
Immunohistochemistry was performed as previously described in Ammoun et al.\(^1\) Cells were stimulated with 40 ng/ml Gas6 (medium collected form Gas6 producing HEK293 cells) or mock (from conditioned medium collected from equivalent, non-Gas6-producing HEK293) or growth factor medium, as described in Ammoun et al.\(^1\) Inhibitors pre-incubation was performed, as described in Ammoun et al.\(^1\) Cell count and DAPI, PI staining, as previously described Ammoun et al.\(^1\) Tissue from five schwannomas and five controls (normal and reactive nerve) were used.

Cell proliferation and viability assays
Cells were cultured for 24 h in DMEM alone, DMEM containing 40 ng/ml Gas6 (from conditioned medium collected form Gas6 producing HEK293 cells) or mock (from conditioned medium collected from equivalent, non-Gas6-producing HEK293) or growth factor medium, as described in Ammoun et al.\(^1\) Inhibitors pre-incubation was performed, as described in Ammoun et al.\(^1\) Cell count and DAPI, PI staining, as previously described Ammoun et al.\(^1\)

Adhesion assay
Adhesion assay was performed, as previously described in Utermark et al.\(^1\)

Cells were stimulated with 40 ng/ml Gas6 (medium collected form Gas6...
producing cells) or mock (medium collected from cells unable produce Gas6) for 3 h. Inhibitors were added 40 min before stimulation.

Luciferase reporter assay

The cells were seeded onto 96-well pre-coated plates.14 The day after the cells were infected with ready-to-transduce lentiviral particles expressing either an inducible transcription factor-responsive reporter gene (firefly luciferase) or expressing firefly luciferase without any additional transcriptional response elements (negative control) or constitutively expressing firefly luciferase (positive control), as described by the manufacturer (SABiosciences Corporation, Qiagen company; MD, USA). On day 3, medium containing Cignal lentiviral particles was replaced with fresh GFDM (growth factor medium) culture medium. Day 4, medium was changed to serum-free DMEM alone or containing 40 ng/ml Gas-6. Detection was performed using Firefly Luciferase Assay System (Promega), as described by the manufacturer.

Data analysis

For statistical analysis student's two-tailed t-tests and ANOVA were used. Experiments were performed in at least triplicates using at least three independent batches of cells from different individuals. ns (not significant): P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001. In figures, mean ± s.e.m. is given.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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REFERENCES


Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)