New pathways and mechanisms regulating and responding to Delta-like ligand 4–Notch signalling in tumour angiogenesis

Chern Ein Oon and Adrian L. Harris
Molecular Oncology Laboratories, Department of Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9D5, U.K.

Abstract
Notch signalling is a key pathway controlling angiogenesis in normal tissues and tumours. This has become a major focus of development of anticancer therapy, but to develop this appropriately, we need further understanding of the mechanisms of regulation of Dll4 (Delta-like ligand 4), a key endothelial Notch ligand. Dll4 and VEGF (vascular endothelial growth factor) cross-talk, with VEGF up-regulation of Dll4 and Dll4 down-regulating VEGFR (VEGF receptor) signalling. Both are essential for normal angiogenesis, and blockade of one may produce compensatory changes in the other. The present review considers recent developments in the regulation of Dll4 expression and functions, its role as a mechanism of resistance to anti-angiogenic therapy, and methods needed to develop effective therapy against this target.

Introduction
Notch signalling is an evolutionarily conserved pathway, which is mediated by cell–cell contact (Figure 1). It is involved in a variety of developmental processes and has an essential role in vascular development and angiogenesis. Dll4 (Delta-like ligand 4) is a Notch ligand, which is up-regulated during angiogenesis. It is expressed in endothelial cells and regulates the differentiation between tip cells and stalk cells of neovascularature. There has been extensive review of the roles of the Notch ligands and receptors [1–4], so the present article will focus on recent developments in the regulation and function of the Notch ligand Dll4, which is the ligand most specific to endothelium. Particular consideration is also given to optimizing therapeutic possibilities of this molecule for anticancer therapy.

Regulation of Dll4 expression
Although Dll4 is commonly up-regulated preferentially in tumour vasculature compared with normal vasculature [5–8], the mechanisms are still poorly understood. Previously, Dll4 itself, FGF (fibroblast growth factor), VEGF (vascular endothelial growth factor) and hypoxia [9] were shown to be important [5,10], but more recently, another layer of interactions has been found. These include a pathway via angiopoietin-1/Tie2, which activates PI3K (phosphoinositide 3-kinase)/Akt signalling and hence β-catenin transcriptional activation. β-Catenin complexed with NICD (Notch intracellular domain) and RBP-Jκ (recombination signal-binding protein 1 for Jκ), binding to intron 3 of Dll4 and enhancing expression. Although HIF (hypoxia-inducible factor)-1α is known to regulate Dll4 in HUVECs (human umbilical vein endothelial cells), in murine pulmonary artery endothelial cells with HIF-2 deletion, Dll4 was also suppressed, implying that Dll4 is regulated by different pathways in different tissues [11,12]. Another extracellular pathway involves laminin and the laminin-binding integrins α2 and α6, which up-regulate Dll4 through integrin signalling [13].

However, the β-catenin pathway is activated much earlier in embryo development. Endothelial-specific stabilization of Wnt/β-catenin signalling alters early vascular development in the embryo in a manner similar to Notch activation [14]. β-Catenin up-regulates Dll4 transcription and strongly increases Notch signalling in the endothelium, resulting in a lack of vascular remodelling, altered elongation of the intersomitic vessels, defects in branching and loss of venous identity. The functional consequences of β-catenin signalling depend on the stage of vascular development and are lost when a gain-of-function mutation is induced at a late stage of development or postnatally. These findings establish a link between Wnt and Notch signalling in vascular development.

Additional mechanisms regulate the interactions of Notch and Wnt signalling. Nrarp (Notch-regulated ankyrin repeat protein) is induced by Dll4, limits Notch signalling and enhances Wnt signalling in stalk cells. In vivo loss of Nrarp causes vessel regression, suggesting that the signalling between Wnt and Notch determines whether new vessel connections form [15,16].
CCMs (cerebral cancerous malformations) are caused by mutations in one of the CCM genes. CCM1 stabilizes endothelial functions and inhibits endothelial proliferation and sprouting angiogenesis. It strongly induces Dll4–Notch signalling, and blockade of Notch signalling reduces the effects of CCM1 [17]. Some of the effects of CCM1 are mediated by the integrin cytoplasmic domain-associated protein-1, which recruits CCM1 to the endothelial cell membrane and activates CCM1. The effects are mediated via Dll4–Notch signalling [18].

An interesting link of activation of endothelial Notch signalling to metabolism is the demonstration that the NICD is stabilized by acetylation, and deacetylation is mediated by the NAD(+) -dependent deacetylase SIRT1 (sirtuin 1). SIRT1 binds to the NICD and destabilizes it. Endothelial cells with low SIRT1 levels are sensitized to Dll4–Notch signalling [19]. This provides a new metabolic link to Notch signalling.

Tel is an Ets repressor that partners another repressor CtBP (C-terminal-binding protein). In endothelial cells, the Tel–CtBP complex is transiently dissociated by VEGF cell-autonomously and results in Dll4 expression, and thus links VEGFR (VEGF receptor) signalling to Dll4–Notch signalling [20]. The effect depends on pulsed VEGF and is lost with continuous exposure. The complex also controls branching by regulating expression of other factors that inhibit angiogenesis such as sprouty family members and VE-cadherin (vascular endothelial cadherin), which are induced by VEGF when given in a pulsatile manner. Thus this complex acts as a sensor to balance transient effects between the two pathways. Interestingly, NAD(H) optimizes the interaction of the two repressors (see previous paragraph) by binding. Previous studies showed that a lack of Foxc (forkhead box c) 1 and Foxc2 results in a failure of arterial cell specification during development and that Foxc1 and Foxc2 directly induce Dll4 transcription. Further investigation showed that Foxc2 interacted with a Notch transcriptional activation complex containing Su(H) and NICD to induce Hey2 promoter activity [21]. The Dll4 and Hey2 promoters were induced by VEGF in combination with either Foxc1 or Foxc2 more than by any one alone. The relative importance of this pathway compared with the others is not known.
Mechanisms of Dll4 signalling via the circulation

Exosomes
It is clear that Notch signalling requires cell–cell interaction, but recently there has been much interest in signalling mediated by secreted cell membranes such as exosomes, small particles containing cell-surface proteins on their external shell. They are produced from internalized cell-surface proteins via caveolae, then endosomes and multivesicular bodies. It has been speculated that Dll4 is incorporated into exosomes. The reason for this stems from a number of studies that have identified endocytosis of Dlls as a key step during Notch signalling in Drosophila. Since internalization and modification of Dlls is required for their function, we investigated whether Dll4 could be re-expressed on the surface of these cell-free microvesicles and mediate Notch signalling at a distance. We demonstrated that, upon induction of Dll4 in HUVECs, the protein is indeed incorporated into exosomes. This also occurs in the U87 human tumour cell line transfected with Dll4. The exosomes had a proteomic profile, which matched published results [22].

These exosomes can transfer the Dll4 protein to other endothelial cells and incorporate it into their cell membrane, resulting in an inhibition of Notch signalling and a loss of Notch receptor. Transfer of Dll4 was also shown in vivo from tumour cells to host endothelium. Addition of Dll4–exosomes confers a tip cell phenotype on the endothelial cell resulting in a high Dll4/Notch receptor ratio, low Notch signalling and filopodia formation. This was further evident from increased branching in a tube-formation assay and in vivo. This reversal in phenotype appears to enhance vessel formation and is a new form of signalling for Notch ligands which expands their signalling potential beyond cell–cell contact. The exosomes therefore appear to be able to confer a tip cell phenotype on the receiving cells and may represent a mechanism by which stalk cell differentiation is restricted to allow tip cell formation to reoccur to produce an even network of vessels.

Circulating BMVPCs (bone marrow vascular progenitor cells) expressing Dll4
BMVPCs may be recruited by tumour VEGF or SDF-1 (stromal-cell-derived factor 1) and contribute to tumour vasculature. Real et al. [23] showed by transplantation experiments involving BMVPCs with low Dll4 levels that such cells inhibited tumour growth and angiogenesis [23]. In vitro BMVPCs activated Notch signalling in mature endothelium. This provides a further mechanism for Notch activation and may provide an assay to select for anti-Dll4 therapy.

Tip cell competition
In contrast with previous views on tip cell migration, time-lapse video microscopy of mosaic sprouts shows a dynamic competition to become a tip cell [26]. This is mediated via Notch signalling, which down-regulates VEGFR2, but up-regulates VEGFR1, and the receptor profile will determine migration response to VEGF [27]. This demonstrates just how dynamic the process is and the mechanism regulating how the cells move and compete will be of major interest [28].

Role of macrophages
Macrophages have an intimate interaction with endothelial cells during angiogenesis, and, in mice with myeloid-specific loss of Notch1, there was a defect in vascular sprouting anastomosis. These macrophages usually localize between Dll4-positive tip cells and branch points and have activated Notch signalling, implying a role for Dll4 tip cell–macrophage communication in vascular anastomosis [29].

Dll4 as a target for cancer therapy and biomarker development
Considering that VEGF induces Dll4 and Dll4 induces vascular quiescence and differentiation, and down-regulates VEGFR2 [30], it is clear that the balance of these two pathways will be critical in the outcome of therapeutically modifying their signalling. There are several VEGF kinase inhibitors and one anti-VEGF antibody approved for a wide range of cancers, and all show slowing of tumour growth, but not prolongation of overall survival. Thus it is of major interest
to assess mechanisms of acquired or first-line resistance and to use combination therapy to build on these single agent effects.

**Dll4 inhibitory antibodies and γ-secretase inhibitors**

Several groups have now clearly shown the anti-tumour effects of Dll4 blockade alone [31]. γ-Secretase inhibitors also affect tumour growth through multiple mechanisms, as its targets include many substrates besides Notch cleavage [32]. There has been dose-limiting gastrointestinal toxicity with those inhibitors, because of the key role of Notch signalling in differentiation of the gut. However, antibodies targeting individual Notch receptors do not have this effect, and combined blockade of at least two receptors is necessary [33,34]. Similarly, Dll4-blocking antibodies do not have gut toxicity, but have caused vascular abnormalities.

Yan et al. [34a] reported that an anti-Dll4 antibody did not cause gastrointestinal toxicity, but produced marked histopathological changes in the liver of mice, rats and monkeys. These included centrilobular heptocyte atrophy, sinusoidal dilation and bile duct proliferation. They also found that chronic Dll4 blockade was associated with the development of vascular neoplasms in rats in a dose-related manner [34]. To be considered malignant, evidence is required to show that the tumours do not show involution on the discontinuation of Dll4 blockade and show aggressive local invasion and/or distant metastasis, which was not the case here [35]. These observations were not reported in other species such as mice and monkeys, and a histopathological study of the natural history of these tumours, with continued therapy and on cessation of therapy, is critical to inform their behaviour. However, these findings will need to be taken into account in monitoring trials.

Because the multiple downstream genes regulated by Dll4–Notch are also regulated by other pathways, it is of major interest to investigate whether combination therapy against both the upstream pathway and downstream output can increase effectiveness of anti-angiogenic therapy. Neutralizing antibodies against Dll4 and soluble antagonist EphrinB2 were used in vivo to show that VEGF and Dll4 induced EphrinB2, and blockade of Dll4 reduced the induction of EphrinB2 by VEGF. Both anti-Dll4 and soluble EphrinB2 blocked in vivo tumour growth and produced non-productive angiogenesis. These results showed that EphrinB2 played a crucial role in non-productive angiogenesis induced by Dll4 blockade [36]. There are, however, many other angiogenic genes regulated by Dll4 [37].

Continuing this line of investigation, it has been shown in a RIP1-Tag2 tumour model, Dll4 deletions or an sDll4 (soluble Dll4) antagonist or EphB4 soluble antagonist (to reduce EphrinB2–EphB4 interactions), all reduced tumour growth [38]. The former increased tumour vessel density and reduced perfusion, whereas the latter reduced vessel density and perfusion. The combination was more effective than either alone. Dll4 antagonism alone causes hepatic vascular alterations, which were prevented by concomitant EphB4, thus potentially increasing safety.

Similar results in principle were reported by Li et al. [39], in that soluble EphB4 or anti-EphrinB2 antibodies could reduce tumour growth and vessel density in the presence of increased Dll4 signalling from brain tumour cells. However, there was little effect when only basal Dll4 signalling was involved. This emphasizes the heterogeneity of models and shows the context-dependence of these angiogenic pathways. This highlights the need for biomarkers to guide single-agent and combination anti-angiogenic therapy.

**Immune therapy**

An unusual method of anti-angiogenic therapy, vaccination against Dll4, showed therapeutic activity in a mouse model of breast cancer [40]. A DNA vaccine was used and did not produce toxicity or a delay in wound healing. Vaccines would be difficult to develop in metastatic disease because of immunosuppression and growth rate of tumours, but could be a novel approach in adjuvant therapy.

---

**Table 1 | The role of mammalian Dll4 and Jag1 Notch ligands in angiogenesis**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sites of expression</th>
<th>Phenotype/role</th>
<th>Effect of loss of expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dll4</td>
<td>ECs and SMCs</td>
<td>Positive driver for tumour growth; involved in tip and stalk cell differentiation; reduced EC migration, proliferation and sprouting; modulates SMC differentiation and maturation</td>
<td>Increased vessel sprouting and EC proliferation; inhibited tumour growth</td>
<td>[5,31,32,44–52]</td>
</tr>
<tr>
<td>Jag1</td>
<td>EC, SMC and tumour cells</td>
<td>Positive driver for tumour growth; involved in tip and stalk cell differentiation; pro-angiogenic regulation; modulates SMC differentiation and maturation</td>
<td>Reduced vessel sprouting and EC proliferation; reduced SMC coverage</td>
<td>[24,25,53–59]</td>
</tr>
</tbody>
</table>
**Radiation therapy**

Although VEGF antagonism has been extensively investigated in combination chemotherapy with which it is routinely used, there are minimal data on anti-Dll4 therapy. We hypothesized that tumours with high Dll4 in their vasculature would have well-perfused tumours and may be sensitive to radiotherapy. Blocking Dll4 may prevent regeneration of vessels damaged by radiation and therefore synergize with radiotherapy. However, blocking Dll4 before radiotherapy would increase tumour hypoxia and cause resistance. We therefore used anti-Dll4 antibodies immediately after a single dose of radiation, both given at a clinically relevant level [41].

There was a marked delay in tumour recurrence (up to 4-fold), with no increased local or systemic toxicity. The mechanism included partly an increase in necrosis caused by the antibody, but there was minimal necrosis with radiotherapy alone. One possibility is a direct effect of radiation on vasculature, not otherwise detectable until vessel regeneration requiring Dll4 is induced. The clinical application of this to locally recurrent tumours, which cause approximately 20% of all cancer mortality, should be investigated. The rational and cost-effective use of combinations of anti-Dll4 therapy with other anti-angiogenic agents, drugs and radiation needs further pre-clinical development, but the combination of anti-Dll4 antibodies and radiotherapy could be investigated now.

**Biomarkers for Dll4 signalling and patient selection**

To increase cost-effectiveness of anti-angiogenic therapy, clearly markers that predict response are desirable. An obvious one would be for expression of Dll4 in tumour vessels. Unfortunately, we still do not have a biomarker for response to anti-VEGF therapy, and this has held up the field. Furthermore, the vessels we usually have to study are from the primary cancers, yet it is the metastases we are trying to treat, and there are little data on primary compared with metastatic cancers. Therefore a marker that could be studied serially and that also reflected the status of vessels currently would be valuable. One possibility is the use of exosomes, which are readily detected in peripheral blood, and accurate quantification and definition of the proteins on their surface by immunoassay is possible. Since Notch receptors and ligands are released extracellularly on activation, these soluble forms may be helpful for analysis.

There is strong pre-clinical evidence that Dll4 activation in tumour endothelium or expression of Dll4 in tumour cells can mediate resistance to VEGF inhibition [39]. This applies to first-line resistance, i.e. tumours resistant to VEGF blockade *ab initio*, and those where VEGF blockade was effective and could be enhanced. However, detailed evaluation of tumours with acquired resistance to VEGF pre-clinically has not yet been described. We investigated, however, whether expression of Dll4 in tumour vessels before anti-VEGF therapy with Avastin (bevacizumab) was associated with resistance. We studied expression of Dll4 in the vessels of primary tumour samples from a randomized clinical trial of bevacizumab added to oral capecitabine in advanced breast cancer. Although the trial was previously reported as negative for an effect on progression-free survival, in those patients without Dll4 expression, there was a significant benefit [42]. This study was carried out on only approximately one-third of the original population, but showed a positive effect. This emphasizes how the use of predictive biomarkers would enhance the rapidity of carrying out trials, lower the costs, lower the risk for patients and make better use of resources.

Imaging is another major modality for assessing response to anti-angiogenic therapy. Because of the abnormal vasculature with reduced flow induced by Dll4 blockade, contrast ultrasound is likely to be useful and shows effects within a few days in animal models [41]. It is possible that DCE-MRI (dynamic contrast enhanced magnetic resonance imaging) may show a change in permeability, i.e. an increase, because of increased VEGF signalling associated with Dll4 inhibition.

**Conclusions**

Dll4–Notch signalling has reached an exciting point in cancer therapy, with Phase I trials ongoing, potentially low toxicity and synergy with existing drugs and modalities. Nevertheless, biomarkers to select patients initially, and dynamic assessment of response will be critical for effective development and avoidance of some of the issues raised by anti-VEGF therapy [43].

**Funding**

This work was supported by Cancer Research UK and European Union Sixth Framework Grant Angiotargeting and funding by the Ministry of Higher Education Malaysia.

**References**


©The Authors Journal compilation ©2011 Biochemical Society


Received 6 September 2011
doi:10.1042/BST20110721