Delta-like 4/Notch Signaling and Its Therapeutic Implications
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Abstract
Intense research efforts have been focused toward the identification of regulators of angiogenesis and the development of antiangiogenesis-based cancer therapies. The approval of anti–vascular endothelial growth factor (VEGF) monoclonal antibody (bevacizumab) for use in colorectal and lung cancer provides clinical validation for targeting angiogenesis for the treatment of cancer. Delta-like 4 (Dll4)–mediated Notch signaling represents another key pathway essential for vascular development. Recent studies yield substantial insights into the role of Dll4 in angiogenesis. Dll4 is downstream of VEGF signaling and its activation triggers a negative feedback that restrains the effects of VEGF. Attenuation of Dll4/Notch signaling results in chaotic vascular network with excessive branching and sprouting. In preclinical studies, blocking of Dll4/Notch signaling is associated with a paradoxical increase in tumor vessel density, yet causes marked growth inhibition due to functionally defective vasculature. Dll4 blockade holds promise as an additional strategy for angiogenesis-based cancer therapy, especially when resistance to and/or escape from existing therapies evolve.

Background
The Notch pathway regulates numerous cell fate/lineage decisions in multicellular organisms during embryogenesis, postnatal development, and in the adult. Notch signaling is very dependent on context and influences cell growth, differentiation, and death in a variety of tissue types (1, 2). In mammals, the Notch system consists of four single pass transmembrane receptors (Notch1-Notch4) and at least five membrane-anchored ligands [Jagged1, Jagged2, Delta-like (Dll)-1, Dll3, and Dll4]. Notch receptors are constrained in a dormant state before ligand-induced activation that initiates a series of successive proteolytic cleavages (3). The final intramembrane cleavage is catalyzed by γ-secretase, a multisubunit protein complex, and leads to the release of Notch intracellular domain. This protein fragment then translocates into the nucleus and functions as a cofactor to regulate transcription of Notch target genes (Fig. 1A).

Several components of the Notch pathway are prominently expressed in the vasculature. Of note, Dll4 is an endothelium-specific Notch ligand (4–6). In the developing embryo, expression of Dll4 is initially restricted to large arteries, whereas in adult mice its expression is limited to small arteries and microvessels (7). Haploinsufficiency of Dll4 in mice results in embryonic lethality at approximately E10.5 due to defective vascular development (7–9). Remarkably, Dll4 and vascular endothelial growth factor (VEGF) are the only known genes where loss of a single allele results in embryonic lethality due to failure to form a functional vasculature. Dll4-deficient mouse embryos exhibit several vascular defects including impaired arteriogenesis as evidenced by loss of expression of arterial markers and lack of well-defined major arteries, disrupted vascular hierarchy, and enhanced vascular density with reduced vessel caliber (7–9).

Several new studies have shed further insights into the function of Dll4/Notch signaling in angiogenesis and the underlying mechanism of vascular defects resulting from attenuated Dll4/Notch activity (10–17). The experimental systems used in these studies include in vitro three-dimensional culture of primary endothelial cells, developing zebrafish, neonatal mouse retina, mouse embryogenesis, and murine tumor models. Excessive angiogenic sprouting and branching was common to all of these models on inhibition of Notch signaling. Dll4/Notch signaling is apparently required to restrain the exploratory behavior of endothelial cells in response to angiogenic signals. Blockade of Dll4/Notch signaling results in more endothelial cells adopting a tip-cell fate and encourages endothelial cells to extend their filopodia and migrate along the VEGF gradient.

Interaction of VEGF and Dll4/Notch pathways
VEGF is a major growth factor that orchestrates the formation of the primary vascular network and drives secondary angiogenesis during vascular development (18, 19). It plays an essential role in the specification, morphogenesis, and homeostasis of vessels by influencing several fundamental aspects of endothelial cell biology, including proliferation, migration, and survival. It is unclear how VEGF signaling is integrated into more complex processes during vascular development, such as arteriovenous differentiation, vessel caliber determination, and the establishment of hierarchical vascular organization. Those events apparently demand additional highly coordinated signaling pathways.

Genetic studies in zebrafish suggested that VEGF acts upstream of Notch during arterial-venous differentiation (20).
More recently, it has been described that VEGF induces the expression of DLL4 in cultured human endothelial cells (21–23). Whereas DLL4 acts downstream of VEGF, there is a negative feedback loop by which DLL4-mediated Notch signaling represses the response to VEGF. In cultured human umbilical vascular endothelial cells, blocking of DLL4/Notch signaling up-regulated VEGF receptor 2 whereas forced activation of Notch by DLL4 led to down-regulation of VEGF receptor 2 (14, 23, 24). Therefore, the increased angiogenesis resulting from DLL4/Notch blockade may reflect the sensitized response to VEGF signaling due to elevated VEGF receptor-2 expression. Similarly, it has been suggested that in zebrafish, Notch activation limits angiogenic cell behavior in developing segmental artery sprouts by repressing flt4, the zebrafish orthologue of VEGF receptor 3 (16).

**Therapeutic Targeting of the DLL4/Notch Pathway**

DLL4 is found to be expressed at sites of physiologic and pathologic angiogenesis (4). Mice carrying a lacZ-reporter under the control of DLL4 promoter (7, 15) showed a marked increase of DLL4 expression in the tumor vasculature. In human tumors, DLL4 expression is increased within the vasculature of clear-cell renal cell carcinoma (22) and in both superficial and invasive bladder carcinomas (25). A close correlation is seen between VEGF and DLL4 expression in cultured endothelial cells, preclinical tumor models, and human tumor samples (13, 21, 22, 25). In light of these observations, it is tempting to speculate the possible involvement of DLL4/Notch pathway in tumor angiogenesis.

Recently, several groups have investigated whether inhibition of the DLL4/Notch pathway might affect tumor angiogenesis and growth (refs. 13–15; Fig. 1A). Notch pathway blockade was achieved with either a DLL4-selective neutralizing antibody (14) or a soluble DLL4 fusion protein that presumably works by binding Notch receptors and preventing their activation by endogenous DLL4 (13, 15). These reagents showed robust antitumor activity in a variety of human and rodent tumor models. In our studies, anti-DLL4 mAb showed significant tumor growth inhibition in all models (>13) tested. However,
some models are more dependent on DLL4 with tumor growth inhibitions varying from 45% to >90%.

Histology of anti-DLL4–treated tumors revealed that the reduced tumor growth was associated with an apparent increase in tumor vascular density (Fig. 1B). However, labeling with an intravascular tracer found these blood vessels to be poorly perfused (13–15). Inefficient blood flow in the treated tumor vessels was also reflected by the increased hypoxia observed in tumors treated with soluble DLL4 (13). What contributes to the defective function of the vasculature following DLL4/Notch blockade? A plausible explanation is that excessive branching results in a highly chaotic vascular network that lacks the hierarchy essential for efficient directional blood flow. In a three-dimensional fibrin gel culture system, antagonizing DLL4/Notch not only resulted in increased branching and proliferation but also caused a morphologic change of endothelial sprouts, with the multilayered lumen-like structure being mostly absent (14). Impaired lumen formation and the presence of nonorganized EC clusters were also described in the aortas of DLL4+/− embryos (7). Conceivably, a similar change in tumor vessels may lead to the reduced vessel lumen size that is incompatible with passage of RBC. Detailed imaging studies may assist better understanding of the structural and functional alterations of tumor vessels in response to blocking DLL4/Notch signaling.

The existing data support the endothelial cell–autonomous role for DLL4/Notch signaling in restricting endothelial cell behavior (14, 16). The cross talk of this pathway that occurs between tumor compartment and endothelium has not been fully established, although one study has shown that forced expression of DLL4 in tumor cells affects the morphogenesis of tumor vasculature (13). The Notch pathway has been implicated in a variety of human cancers in connection with the genetic alterations and epigenetic events that lead to either constitutive Notch activation or sensitized response to ligand-induced activation (26–30). At present, however, there are no data to support a direct role of DLL4-mediated Notch signaling for tumor initiation or progression.

**Clinical-Translational Perspectives**

VEGF is fundamentally important during vascular development in embryos and is also hijacked by many types of cancer to promote tumor angiogenesis. Agents that block the VEGF pathway have been shown to be effective at inhibiting tumor angiogenesis and growth in preclinical tumor models. Some of them have been clinically validated and now become an important part of standard cancer treatment (31, 32). Different tumors, however, exhibit varied sensitivity to VEGF blockade. Furthermore, some tumors that are initially sensitive to a block in VEGF signaling may eventually progress while still on treatment. This “resistance” is generally believed to result from the recruitment of additional angiogenic signals beyond VEGF. Therefore, it is of clinical importance to develop alternative antiangiogenesis-based therapeutics anchored on distinctive signaling pathways to synergize with VEGF blockade.

In addition to tumor studies using anti-DLL4 as a single agent, additive antitumor activity was observed in combination with anti-VEGF therapy in a majority of the tumor models. In vitro studies have shown that the increased angiogenic sprouting after DLL4 blockade is still VEGF dependent (14), supporting the hypothesis that anti-DLL4 may result in a tumor microvasculature that is particularly dependent on a VEGF-mediated survival signal. Removal of VEGF could result in endothelial cell apoptosis and vascular regression. In this context, newly formed vessels would be particularly vulnerable to VEGF blockade.

In anti-DLL4–treated neonatal mouse retinas, there was a defect in arteriogenesis and a complete absence of pericyte coverage of the retinal vessels (14). Soluble DLL4 was also able to reduce the recruitment of pericytes in a murine xenograft tumor model (15). Therefore, DLL4 blockade may also impair remodeling of the tumor vasculature in response to VEGF blockade thereby preventing the progression to more mature, stabilized vessels.

Because it is possible that DLL4 pathway may have a broad and diverse interaction with angiogenic pathways beyond VEGF signaling, DLL4/Notch blockade might be beneficial in tumors that either are intrinsically less dependent on VEGF or have progressed due to a shift to other angiogenic pathways. Indeed, preclinical studies have shown that blockade of DLL4 was effective in inhibiting the growth of tumors that are resistant to VEGF inhibition (13, 14).

It becomes increasingly evident that targeted cancer therapies will be most effective when used early in the course of the disease and in combination with other agents that act through complementary mechanisms. Because DLL4 blockade apparently reduced the perfusion of tumor vessels, the concern arises that anti-DLL4 might interfere with the delivery of chemotherapy agents. Preliminary studies show that DLL4 blockade does not interfere with the antitumor effects of common chemotherapeutics.1 The hyperproliferative state of endothelial cells in the tumor vessels resulting from DLL4/Notch blockade might selectively amplify the endothelial cell–targeting effects of chemotherapy and ultimately lead to improved anticancer activity.

γ-Secretase inhibitors provide another approach to affect DLL4/Notch signaling because this protease complex plays a pivotal role in the activation of Notch signaling (33, 34). This strategy, however, would indiscriminately block all Notch signaling, not just that through DLL4. In addition, global Notch blockade is confounded by a significant risk for toxicity in gastrointestinal tract due to an increase of goblet cells within the crypt compartment (35–38). Merck has initiated phase I clinical trials with a γ-secretase inhibitor, MK-0752, in advanced breast cancer and in patients with T-cell acute lymphoblastic leukemia that is driven by Notch activation. It would be interesting to test if this inhibition has an antiangiogenic effect, in addition to a direct effect, on tumor cells.

 Whereas most of current antiangiogenesis approaches act through a reduction or elimination of tumor blood vessels, DLL4 blockade results in the formation of a nonfunctional vasculature that is unable to support tumor growth. This paradoxical strategy for targeting tumors will be the subject of intense research for years ahead.

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1 M. Yan, unpublished results.
References


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