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Review

Notch: A key regulator of tumor angiogenesis and metastasis

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Summary. The Notch signaling pathway is critical for many developmental processes including physiologic angiogenesis. Notch is also implicated in having a key role in tumor angiogenesis. Preclinical and clinical experience with anti-angiogenic strategies indicates that they may be limited by tumor resistance and recurrence, which has led to the search for alternative angiogenic treatment strategies. Significant progress has been made in shedding light on the complex mechanisms by which Notch signaling can influence tumor growth by disrupting vasculature in an array of tumor models (Ridgway et al., 2006). These results have led to the consideration of Notch as an attractive target to block tumor angiogenesis and inhibit growth.

However, studies of inhibition of Notch signaling in different tumor models have uncovered similarly variable results, and some unexpected adverse effects. The ability of Notch to function in a context-dependent manner as a determinant of cell fate, a tumor suppressor, and an oncogene may partially explain the complexity in interpreted the role of Notch signaling inhibitors in preclinical tumor studies. In addition, Notch may also play an important role in metastasis via its direct effects on the vasculature and by modulation of epithelial-mesenchymal transition in tumor cells. Here we present a current understanding of Notch signaling in tumor angiogenesis, and discuss recent work on the role of Notch in tumor metastatic progression.

Key words: Angiogenesis, Notch, Cancer, Migration, Metastasis

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Introduction

The Notch family of proteins is part of an evolutionarily conserved pathway that is involved in many key developmental processes. Notch cell surface receptors are expressed by various cell types and are involved in cell fate, differentiation, and proliferation. Notch is particularly integral to proper vascular formation during embryonic development, and in vascular maintenance and remodeling during postnatal angiogenesis (Takeshita et al., 2007). In mammals, the Notch family of proteins consists of four Notch receptors (Notch1-4) and five ligands (Jagged 1-2, Delta-like1, 3, 4), which are expressed on the cell's surface. Depending on organ and tissue type, Notch signaling can inhibit as well as induce differentiation, proliferation, and cell survival. The effect of Notch on cell-fate decisions is based on the expression of certain genes in cell-type specific manner (Rehman and Wang, 2006). Of the Notch receptors, only Notch1, 2, and 3 are expressed on vascular endothelial cells and are critical to the proper formation of a functional vasculature during development (Iso et al., 2003). The Notch pathway is also implicated as a key participant in tumor angiogenesis (Dufraine et al., 2008). Further, Notch signaling interacts with other angiogenic pathways (Alva and Iruela-Arispe, 2004; Thurston and Kitajewski, 2008). These findings have led to an interest in better elucidating the role of Notch in tumor vessel assembly, with the ultimate aim of improving therapeutics.

The Notch receptor is a single-pass transmembrane protein consisting of a single peptide, an extracellular component that is responsible for ligand interaction, a transmembrane domain responsible for receptor activation, and an intracellular signaling domain. The expression of Notch proteins on the cell surface coincides with their role in regulating cell fate decisions by direct cell-to-cell interactions. The Notch ligands

interact with receptors on adjacent cells by a process termed lateral inhibition. The Notch ligand expressed on one cell binds to the Notch receptor of an adjacent cell, leading to activation of the Notch pathway in that cell and suppression of activity in the adjacent cells. Upon activation of the Notch receptor, the intracellular portion is released and translocates into the nucleus where it interacts with recombining binding protein suppressor of hairless (RBP-J). This results in the release of transcriptional co-repressor proteins, which lead to the activation of numerous basic helix-loop-helix transcriptional repressors including Hairy/Enhancer of Split (Hes1, -5, -7) and HES-related with YRPW (Hey1, -2, -L).

It is clear that proper Notch signaling is critical in vascular development, differentiation, and proliferation. The Notch ligands and receptors are all expressed in arteries but not in veins, and they are critical in arterial differentiation during early embryogenesis (Villa et al., 2001). Notch1 is critical in early embryonic vascular differentiation and remodeling. Homozygous deletion of Notch1 in mice is embryonic lethal with widespread cell death by gestational day 11.5 (Swiatek et al., 1994). Deletion of Notch4 does not have the same effect on vascular development; however, double knockouts for Notch1 and Notch 4 have a more severe vascular phenotype than Notch1 knockout alone (Krebs et al., 2000). These results suggest that Notch1 and Notch4 have partially redundant roles during embryonic development. Haploinsufficiency for Dll4 leads to embryonic lethality at around 10.5 days of gestation, with defects in vascular development and remodeling (Duarte et al., 2004; Gale et al., 2004). In Notch mutant mouse embryos, formation of the primary vascular plexus is unaffected. Instead, there is a failure to reorganize these rudimentary vessels into large vessels and branching capillaries, suggesting that Notch may not be required for vasculogenesis but is critical for vessel assembly during physiologic angiogenesis (Xue et al., 1999; Krebs et al., 2000).

Notch and physiologic angiogenesis

Angiogenesis is the process by which new blood vessels are generated from an existing vascular network. It is a complex process involving the normally quiescent vasculature responding to various angiogenic stimuli, with the primary driver of angiogenesis being hypoxia. In reaction to a pro-angiogenic stimulus, the existing vasculature undergoes degradation of the extracellular matrix, budding, proliferation, migration, tube formation, maturation and the primary driver of angiogenesis is hypoxia. The sprouting of the existing vasculature occurs toward a gradient of angiogenic stimulation. The best-studied proangiogenic stimulator is vascular endothelial growth factor (VEGF), which is secreted by cells under conditions of hypoxia. During angiogenesis, endothelial cells must adopt various roles in order to ensure a stable vasculature. At the apex of the sprout, endothelial cells differentiate into a tip cell. These tip cells are a nonproliferative, highly motile, tubeless cell type that is restricted to the tip of the sprout. The tip cells migrate and extend numerous filopodia to sense their microenvironment and lead the direction of the new sprout. Adjacent to these tip cells are the stalk cells which consist of a different type of endothelial cell and form the trunk of the new blood vessel. These cells are highly proliferative, motile, and vacuolated. Further from the tip are another series of endothelial cells that form tube cells. These tube cells are the stable, lumen containing, nonproliferating, immobile cells that will make up the final blood carrying vasculature. The formation of a new vascular sprout is highly regulated by Notch signaling.

Notch plays a critical role in the maintenance of vascular homeostasis by repressing endothelial cell proliferation. Notch1 signaling is essential in endothelial cell differentiation between tip or tube phenotypes (Dufraine et al., 2008). Endothelial cells begin the process of tube formation toward a gradient of VEGF stimulation. Endothelial tip cells begin to express high levels of Dll4 and low levels of Notch activity in response to VEGF. These Dll4 expressing tip cells activate Notch1 signaling in adjacent cells (Claxton and Fruttiger, 2004). In response to Notch signaling, stalk endothelial cells down regulate VEGF receptors in order to inhibit excess sprout formation. In retinal models of angiogenesis, disruption of Dll4 signaling increases vascular density with an excess of angiogenic sprouts. In vitro models demonstrate that Jagged1 antagonizes Dll4-mediated Notch signaling within the sprout, and promotes new vessel growth (Benedito et al., 2009). Based on these results, Dll4 and Jagged1 function in opposing roles to regulate the growth of the new vasculature. This allows for the integration of different pro- or antangiogenic stimuli into a single coordinated biological process of tip cell formation. The function of Notch in regulating angiogenesis has made it an alluring target for inhibiting tumor angiogenesis.

The effect of Notch on angiogenesis is the dependent on crosstalk between multiple angiogenic pathways. The angiopoietin-1/Tie2 pathway, an important angiogenic-signaling pathway, augments Notch signaling to regulate vascular quiescence through AKT-mediated activation of b-catenin (Zhang et al., 2011). The relationship between VEGF and Notch is still not fully elucidated, but appears to involve cross-regulation. Therefore, it is likely that these pathways cooperate to maintain a properly functioning vasculature. In murine retinal models, inhibition of VEGF results in decreased sprouting and decreased expression of Dll4 on retinal vessels (Suchting et al., 2007). Similarly, inhibition of VEGF in tumors resulted in a rapid decrease in Dll4 expression in tumor vessels (Noguera-Troise et al., 2006).

Notch and tumor angiogenesis

Aberrant activation of the Notch signaling pathway

is implicated in various neoplastic processes. Notch's role in pediatric malignancies is particularly important given the function of Notch in many developmental processes (Zweidler-McKay, 2008). Notch was first documented to have a role in human T-cell acute lymphoblastic leukemia (T-ALL) involving a translocation of intracellular domain to the T-cell receptor-B promoter region resulting in overexpression of NICD (Matthay et al., 1999). While Notch1 signaling is essential for normal development of T-cell progenitors, constitutive activation of Notch1 is associated with T-ALL. Approximately 50% of T-ALL carries activating Notch1 mutations. These findings stimulated further investigation of the role of aberrant Notch signaling in tumorigenesis.

However, it has since become apparent that altered Notch status is associated with both pro- and anti- tumor suppressive roles. The effects of Notch signaling on tumor behavior are dependent on cellular context and the interaction with various signal transduction pathways. While Notch1 can function as an oncogene as outlined above, Notch1 can also function as a tumor suppressor in murine skin models by inducing Waf1 and repressing Shh and Wnt signaling (Radtke and Raj, 2003). Mice with epidermal and corneal epithelial specific deletion of Notch1 demonstrate epidermal and corneal hyperplasia within 25 days and develop spontaneous basal-cell carcinoma-like tumors over time (Nicolas et al., 2003). Notch1 loss in epidermal keratinocytes promotes tumorigenesis by impairing skin-barrier integrity and creating a wound-like microenvironment within the skin. This tumor-promoting effect of Notch1 loss involves crosstalk between the disrupted epidermis and its stroma (Demehri et al., 2009).

Notch proteins may interact with alternative signaling pathways via direct communication between tumor cells and adjacent stroma. For example, mitogen-activating protein kinase (MAPK) can induce expression of Jagged1 and trigger Notch activation in neighboring endothelial cells, promoting angiogenesis in a head and neck squamous cell carcinoma model (Zeng et al., 2005). Notch1 may also have a role in suppressing formation of vascular tumors. Mice engineered to develop spontaneous loss of Notch1 form vascular tumors in the liver with decreased survival (Liu et al., 2011). Also, chronic treatment of mice with blocking Dll4 antibodies can lead to the induction of vascular neoplasms (Yan et al., 2010). This suggests a role for specific Notch receptors and ligands in suppressing neoplasia in some vascular beds, and raises concerns for long-term use of Notch inhibitors in clinical settings.

Aberrant Notch signaling has been linked to tumor formation and progression in other human tissues. High expression of Jagged1 and/or Notch1 in human breast cancer is linked to poor survival rates, and may be related to a crosstalk between Notch and the estrogen receptor (Reedijk et al., 2005; Rizzo et al., 2008). Jagged1 is highly expressed in metastatic prostate cancer compared to either localized prostate cancer or benign prostatic tissues (Santagata et al., 2004). In addition, Notch1 signaling was found to be constitutionally active in many types of renal cell carcinomas and that blocking Notch resulted in inhibition of tumor growth in vivo and in vitro (Sjolund et al., 2008). Taken together, these findings suggest that Notch activity is broadly implicated in a range of human cancers.

These findings have stimulated interest in exploring Notch signaling inhibition as a potential treatment strategy for human malignancy. Global inhibition of Notch, using gamma-secretase inhibitors (GSIs) combined with 13-cis retinoic acid, a promoter of differentiation, led to enhanced tumor growth inhibition, differentiation, and migration of neuroblastoma cells (Ferrari-Toninelli et al., 2009). However, GSIs can be associated with significant gastrointestinal toxicities, limiting their use in the clinical setting. Antibodies that selectively target Notch 1 and Notch 2 receptors have demonstrated that individually blocking either receptor alone can reduce the adverse gastrointestinal effects seen with dual blockade (Wu et al., 2010).

The Notch ligand Dll4 is highly expressed in the vasculature, and appears to play an important role in tumor angiogenesis in a number of systems, suggesting that it might be a promising target. Dll4 blockade led to markedly increased tumor vascularity, enhanced angiogenic sprouting, and branching. Paradoxically, this increased vascularity was non-productive and led to poor perfusion, a seven-fold increase in hypoxia, and decreased tumor growth in multiple tumor models. This effect on tumor growth was seen even on tumors that were highly resistant to anti-VEGF inhibitors (Noguera-Troise, et al., 2006; Ridgway et al., 2006). Jagged1-induced activation of Notch enhances the proliferation of neuroblastoma cells. Recent studies from Genentech have raised concerns for the long-term inhibition of the Notch pathway using a Dll4 antibody. Chronic Dll4 blockade led to thymic atrophy and ulcerating subcutaneous tumors in mouse models (Yan et al., 2010).

The effect of Notch inhibition may have distinct effects on different tumor types. Inactivation of RBP-J, the common transcription factor for all four Notch receptors, led to tumor regression in some models but progression in others (Hu et al., 2009). Tumors engineered to express a soluble form of the Notch1 receptor, an antagonist of ligand-dependent Notch signaling, inhibited vascular endothelial growth factor-induced angiogenesis in skin and affected tumor viability in various tumor models (Funahashi et al., 2008). Thus, the utility of Notch pathway inhibitors is likely to be tumor type-dependent.

Notch functions in tumor angiogenesis may be partially mediated by the hypoxic environments present in most cancers. A major mechanism by which cells adapt to low oxygen tension is through the regulation of hypoxia inducible factor-1 alpha (HIF-1α). HIF-1α interacts with the Notch1 intracellular domain to augment responses to hypoxia downstream of Notch (Gustafsson et al., 2005; Sainson and Harris, 2006). In

addition, Dll4 expression by endothelial cells may be directly upregulated by hypoxia, possibly via HIF-1α and hypoxia response elements in the Dll4 promoter (Diez et al., 2007). These findings suggest a role for Notch in the adaptation of tumors to hypoxia.

Notch and metastasis

Metastatic disease remains the major cause of cancer-related death. The "seed and soil" theory proposes that the outcome of metastasis depends on the crosstalk between neoplastic cells and the specific organ microenvironments. In order for tumor cells to develop malignant potential, numerous hurdles must be overcome, including local invasion, intravasation, survival in the circulation, extravasation, and colonization. Thus, the ability to metastasize requires a series of sequential rate-limiting steps that tumor cells must attain. Prior studies suggest that hypoxia can accelerate acquisition of these behaviors by tumor cells. Thus, one possible negative consequence of anti-angiogenic therapy is the potential for eliciting malignant progression of tumors by imposing hypoxia. Consistent with this notion, preclinical studies suggest that blocking primary tumor growth with inhibitors of VEGF and platelet derived growth factor (PDGF) may elicit resistance in these tumor cells and may paradoxically promote increased invasiveness and metastasis (Ebos et al., 2009; Paez-Ribes et al., 2009). By effectively inhibiting neovascularization within tumors, it is possible that anti-angiogenesis may lead to clonal selection of tumor cells by upregulating genes responsible for survival and invasion. The interaction between Notch signaling and hypoxic regulation of gene expression raises the possibility that Notch proteins may function in this phenomenon. Recent evidence suggests that Notch may play a role in the establishment of stem cell populations that allow for the creation of metastatic niches (McGovern et al., 2009).

Recent research has shed some light on how Notch may influence invasive behaviors of cancer cells. The skeletal system is recognized as the most common site of metastasis in breast cancer. The final step in the metastatic establishment in the bone of breast cancer cells involves osteolytic outgrowth within the bone. Breast cancer cells expressing Jagged1 interact with osteoblasts, resulting in an increase in active IL6 and TGFß, which promote tumor outgrowth. Inhibition of Jagged 1, using GSIs, led to a decrease in bone metastasis by disrupting the Notch pathway in stromal bone cells (Sethi et al., 2011). This suggests a mechanism whereby Notch signaling functions in providing a host microenvironment that is conducive to engraftment of disseminated tumor cells.

Epidermal-to-mesenchymal transition (EMT) occurs in normal development, and may be partially or wholly recapitulated in the malignant progression of tumors. During normal developmental EMT, epithelial cells alter their gene expressions prior to migration. Aberrant reactivation of EMT has been proposed as an important mechanism in tumor metastasis, allowing polarized epithelial cells to acquire a fibroblast-like phenotype permissive for intravasation and metastasis (Hanahan and Weinberg, 2011). Hypoxia may be one signal that promotes activation of EMT programs. Notch signaling has been implicated in EMT by modulating TGFß-induced downregulation of E-cadherin, a cell-cell adhesion protein (Timmerman et al., 2004; Zavadil et al., 2004). Jagged1-mediated Notch activation leads to upregulation of Snail and Slug, transcriptional repressors critical for regulating EMT, which then repress E-cadherin in various tumor models (Chen et al., 2010; Leong et al., 2007). In vitro, inhibition of Notch signaling abolished hypoxia-induced EMT, motility, and invasion (Sahlgren et al., 2008). In an osteosarcoma model, inhibition of Notch, using a GSI, led to decreased invasion in Matrigel without affecting proliferation or survival (Zhang et al., 2008). In a rhabdomyosarcoma model, inhibition with GSIs led to a decrease in mobility and invasiveness of tumor cells without appreciable effect on cell cycle or apoptosis (Roma et al., 2011). These studies suggest a role for Notch signaling in regulating behaviors related to EMT and metastasis.

This role may be related to a complex cross talk between Notch and other metastasis-related signaling pathways. The human transcriptional regulator Amino-terminal Enhancer of Split (Aes), an endogenous metastasis suppressor, inhibits Notch by sequestering and inactivating Notch transcriptional effectors within the nucleus. Deletion of Aes in a murine model of colon cancer led to Notch activation, which caused marked tumor invasion and intravasation that was suppressed by Notch signaling inhibition. The use of Compound E, a potent GSI, also suppressed metastasis to the liver in this model. In vitro data indicates that loss of Aes enhances the ability of tumor cells to migrate across the endothelium, which is a key step in metastasis (Sonoshita et al., 2011). These results implicate Notch signaling as playing an essential role in this step of hematogenous metastasis.

Conclusion

Notch signaling is critical to physiologic angiogenesis and is implicated in tumor angiogenesis and metastasis. Recent studies have begun to illuminate the complex interactions by which Notch functions to preserve vascular integrity. Notch may also be important in tumor cell behaviors that promote entry into the circulation and establishment of distal metastases. Taken together, these functions suggest that Notch may play an important role in responses of some tumors to microenvironmental stress. Hence, Notch activation may contribute to resistance of cancers to anti-angiogenic agents, and effective targeting may enhance the ability to use this approach in treating refractory malignancies. The recognition that Notch signaling exerts context-dependent effects suggests that in seeking this goal, it

will be critical to characterize effects of inhibiting this pathway in differing tumor systems.

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