Notch signaling from tumor cells: A new mechanism of angiogenesis

Notch signaling is an evolutionarily conserved pathway and plays key roles in embryonic vascular development and angiogenesis. Multiple components of the Notch pathway are expressed in vasculature, and mice deficient for a variety of these components display embryonic lethality with vascular remodeling defects. Alteration of Notch signaling in various endothelial cells generates profound effects on angiogenesis in vitro. New evidence shows that Notch signaling from tumor cells is able to activate endothelial cells and trigger tumor angiogenesis in vitro and in a xenograft mouse tumor model. Selective interruption of Notch signaling within tumors may provide an antiangiogenic strategy.

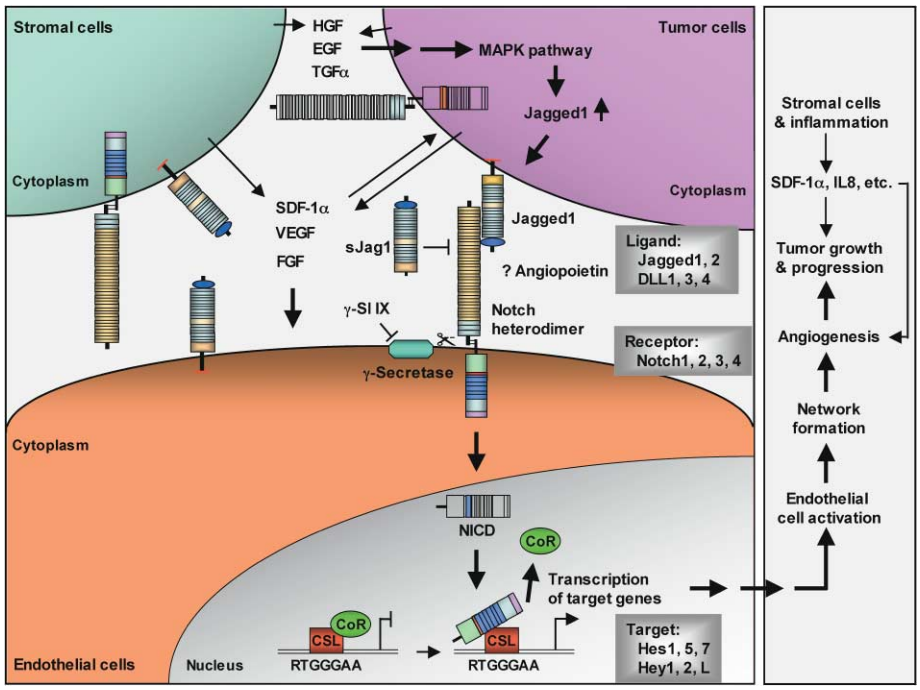
Angiogenesis is critical for tumor growth and is a complex process involving matrix breakdown, endothelial sprouting from preexisting vessels, endothelial proliferation, migration, differentiation to form tubes, and recruitment of pericytes/smooth muscle cells (SMCs). Interactions between tumor cells, endothelial cells (ECs), and stromal cells are crucial for tumor angiogenesis. Various angiogenic molecules produced by either tumor cells or tumor stromal cells can directly bind to their cognate receptors on ECs and thus initiate angiogenesis. For instance, vascular endothelial growth factor (VEGF) secreted by tumor cells and fibroblast growth factor (FGF) generated by tumor fibroblasts are classical proangiogenic factors. Thus, a paracrine regulation of angiogenesis by secreted proteins is a well-recognized mechanism.

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism, affecting many differentiation processes and cell-fate determination during embryonic and postnatal development. Four Notch receptors (Notch1–Notch4) and five DSL (named for *D*elta and *S*errate from *Drosophila* and *L*ag-2 from *C. elegans*) ligands (Jagged1, Jagged2, delta-like1 [DLL1], DLL3, and DLL4) have been described in mammals. Activation of Notch signaling is mediated by interactions of bordering cells via cell-to-cell contact of the membrane-associated Notch receptor and ligand (Figure 1).

The Notch pathway is involved in multiple aspects of vascular development and angiogenesis. Major components of the Notch pathway existing in vasculature consist of three receptors (Notch1, -3, and -4), three ligands (DLL4, Jagged1, and Jagged2), and three downstream targets (Hey1, -2, and -L). Mice deficient for a variety of these components, including Notch1, Notch1/Notch4, Jagged1, DLL1, DLL4, Hey1/Hey2, and presenilins (PS1 and PS2) resulted in embryonic lethality with vascular remodeling defects (for review, see Iso et al., 2003). In human, mutations in the Jagged1 and Notch3

Figure 1. Crosstalk between tumor cells, endothelial cells, and stromal cells is modulated by the Notch pathway and stimulates tumor angiogenesis

The tumor-associated growth factors HGF, EGF, and TGFα, produced by tumor cells and/or stromal cells, induce Jagged1 expression in tumor cells (probably in endothelial cells as well) through MAPK pathway. Jagged1 on tumor cells binds to Notch receptor(s) on endothelial cells. Upon ligand binding, the Notch intracellular domain (NICD) is released from the endothelial cell plasma membrane by the γ-secretase-dependent proteolytic cleavages of Notch receptor. The NICD then translocates to the nucleus, where it interacts with the CSL (named for mammalian *C*BF1/RBP-Jκ, *Drosophila Su*(H), and *C. elegans L*ag-1) transcription repressor to activate the transcription of target genes. The basic-helix-loop-helix proteins (bHLH) Hairy/Enhancer of Split (Hes1, -5, and -7) and Hes-related proteins (Hey1, -2, and -L) are the best-characterized downstream targets. The Notch signaling from the tumor cells is able to activate endothelial cells and thus initiate tumor angiogenesis. Accordingly, the neovasculature is able to stimulate the tumor growth and progression. The γ-secretase inhibitor IX (γ-SI IX) and soluble Jagged1 (sJag1) can protect the proteolytic cleavage by inhibiting the γ-secretase activity and block the interaction of Jagged1 and Notch, respectively, and therefore prevent the endothelial activation. Notch cross-signaling between tumor cells, stromal cells, and endothelial cells is likely to regulate the interaction of Notch ligands on tumor cells with receptors on endothelial cells, and vice versa. Tumor-associated stromal fibroblasts and inflammation stimulate tumor angiogenesis and tumor growth and progression. VEGF secreted by tumor cells and FGF generated by tumor fibroblasts promote tumor angiogenesis.



genes cause the autosomal dominant disorders Alagille syndrome and CADASIL, respectively, and both display abnormal vascular phenotypes. Haploinsufficiency of DLL4 also resulted in embryonic lethality from severe vascular defects in mice (Gale et al., 2004). The only other angiogenic pathway for which haploinsufficiency is reported is for VEGF knockout mice. Remarkably, EC-specific knockin of Notch4 active form and knockout of either Notch1 or Notch1/Notch4 produced similar phenotypes (Uyttendaele et al., 2001), suggesting that either excessive upregulation or downregulation is detrimental to vascular development, and thus, a narrow range of optimal expression seems to be essential.

The role of the Notch signaling in angiogenesis has been evaluated by manipulating the expression of different components in ECs. Overexpression of Hey1 in human capillary ECs blocked angiogenesis in vitro; however, reduction of Hey1 expression by antisense oligonucleotides also blocked the endothelial network formation. Constitutive activation of Notch signaling by expressing N1ICD or Hes1 inhibited human iliac artery EC (HIAEC) proliferation but increased the endothelial survival and network formation on matrigel and vessel-like cord formation in a 3D collagen angiogenesis model, whereas blocking Notch signaling by expressing a dominant-negative form of CSL decreased the network and cord formation in the 3D model (Liu et al., 2003). However, overexpression of N4ICD or CSL in human dermal microvascular EC (HMEC-1) inhibited the endothelial migration and sprouting in vitro and angiogenesis in the chick chorioallantoic membrane in vivo (Leong et al., 2002). Activation of the Notch pathway by Jagged1, N1ICD, and N4ICD inhibited proliferation of human umbilical vein ECs (HUVECs) and human aortic ECs (HAECs) (Noseda et al., 2004), while Jagged1 was able to induce microvessel-like structures in vitro. Overexpression of the dominant-negative form of Jagged1 (sJag1) in HUVEC inhibited the endothelial network formation. However, addition of an antisense Jagged1 oligonucleotide to bovine microvascular ECs (BMECs) enhanced the FGF-dependent invasion and tube formation of BMECs. Thus, although Notch has critical roles in vivo in the embryonic vascular development, in vitro it appears that function of the Notch pathway in ECs is dependent on the endothelial type, and activation of Notch signaling is likely to inhibit endothelial proliferation.

However, information about Notch signaling in tumor angiogenesis is limited. It is known that DLL4 is upregulated in the vasculature of human xenografted tumors in mice, and in human breast cancers and kidney cancers (Mailhos et al., 2001), and DLL4 reporter seems to be preferentially induced in the vasculature of mouse Lewis lung carcinoma (Gale et al., 2004). Recently, it has been demonstrated that DLL4 expression is upregulated about 9-fold in clear cell renal cell carcinoma and is correlated with VEGF expression. Reduction of basal DLL4 level in ECs by siRNA led to the inhibition of multiple endothelial functions in vitro including proliferation, migration, and network formation, implying the potential role of this pathway in cancer (Patel et al., 2005).

Wang and colleagues now provide new information that Notch signaling from tumor cells can trigger the Notch activation of neighboring ECs and consequently promote tumor angiogenesis (Zeng et al., 2005, this issue of *Cancer Cell*) (Figure 1). These authors demonstrate that Jagged1 is expressed in head and neck squamous cell carcinoma (HNSCC) cells and is induced through MAPK pathway by HGF, EGF, and TGFα, all of which, together with their receptors, are associated with HNSCC development and progression. Cocultivation of human dermal microvascular ECs (HDMECs) with HGF-treated or Jagged1-transformed SCC9 tumor cells (SCC9/Jag1) was able to activate Notch signaling in HDMECs and stimulate the endothelial network formation on matrigel. Coimplantation of HDMEC and SCC9/Jag1 in SCID mice showed that Jagged1-transformed tumor cells promoted tumor angiogenesis and tumor growth. Investigation of 102 cases of patient tumor tissues demonstrated that the level of Jagged1 expression was positively correlated with blood vessel density and associated with HNSCC development. Thus, this study showed the juxtacrine effects of tumor cells on endothelium, which may also be important if tumor cells contribute directly to vessel walls with endothelial cells.

However, there are several issues worthy of further consideration. Wang and colleagues have shown that overexpression of N1ICD in HDMEC is able to promote endothelial network formation. Given that all four Notch receptors are expressed in ECs (Patel et al., 2005), we wonder if other Notches are also involved in the binding of Jagged1 on SSC tumor cells. Jagged1 is also expressed in ECs (Figure 1). Thus, whether the Jagged1 signaling from tumor cells affects endothelial Jagged1 function or differs from endothelial Jagged1 signaling needs to be addressed. It will be interesting to investigate which downstream targets in ECs are responsible for the function. From the above evidence, Notch signaling is likely to involve differentiation rather than proliferation, and thus interactions with angiopoietins will need to be examined. Moreover, tumor-associated stromal fibroblasts and inflammation stimulate tumor angiogenesis and growth (Sparmann and Bar-Sagi, 2004; Orimo et al., 2005). Therefore, Notch cross-signaling between tumors cells, stromal cells, and ECs is likely to regulate the interaction of Notch ligands on tumor cells with receptors on ECs.

Nevertheless, the findings of Wang and colleagues highlight that tumor cells express Notch ligands that are able to stimulate tumor angiogenesis and tumor growth. Specific interruption of the Jagged1 signaling within human tumors may provide a potential novel antiangiogenic therapy. Interaction with conventional pathways may be important. VEGF and bFGF were able to induce the expression of DLL4, Notch1, and Notch4 in HUVEC (Liu et al., 2003; Patel et al., 2005), and Notch1, Notch4, and Hey1 can downregulate VEGFR2 expression. The latter observation raises possible risks for Notch inhibition, in that VEGFR2 signaling may go up. Therefore, a combined approach, e.g., interruption of Notch signaling combined with VEGF inhibitors, may be required.

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Gankyrin: An intriguing name for a novel regulator of p53 and RB

The RB and p53 tumor suppressors lie at the heart of cancer biology, and inactivation of both pathways is seemingly essential for tumor development. Previous studies identified gankyrin as a component of the 26S proteasome that is consistently overexpressed in liver cancer and promotes cell transformation by binding RB. In the current issue of *Cancer Cell*, Fujita and colleagues (Higashitsuji et al., 2005) show that gankyrin also binds MDM2 and facilitates its destruction of p53. These important findings implicate gankyrin as a dual-purpose negative regulator of RB and p53, thereby identifying gankyrin as a rational cancer therapeutic target.

Persistent proliferation and enhanced cell survival are hallmarks of aggressive tumor cells. Two key proteins that suppress abnormal cell proliferation are the RB and p53 tumor suppressors. RB acts as a brake to block cell cycle progression through its ability to repress E2F, a transcription factor that activates genes essential for the S phase of the cell cycle (for review, see Sherr, 2004). p53 also blocks cell proliferation in part by inducing $p21^{CIP1}$, a protein that binds and inhibits the cyclin/CDK complexes, which are required for progression through the cell cycle. Additionally, p53 induces cell death by activating the expression of genes involved in apoptosis (for review, see Vogelstein et al., 2000). Therefore, not surprisingly, most cancer cells have inactivated both of these factors either directly or indirectly to sever their signaling pathways.

Recently, Fujita and colleagues (Higashitsuji et al., 2000) identified gankyrin as a gene that was consistently overexpressed in human liver cancers. Gankyrin is highly conserved throughout evolution (~40% identity to yeast Nas6P) (Hori et al. 1998) and is localized on human chromosome Xq22.3 in a region where DNA gains are frequently detected in kidney and colon carcinomas. Whether gankyrin expression is deregulated in cancers other than liver tumors is not yet known. Gankyrin contains two special domains consisting of ankyrin repeats and the RB-recognition motif LxCxE (^{178}LACDE^{182}), and derives its name from these features. "Gann" is the Japanese word for cancer, and ankyrin is the functional domain involved in protein-protein interactions.

Gankyrin was initially purified and characterized by Tanaka and coworkers (Hori et al., 1998) as the p28 component of the regulatory subunit of the 26S proteasome, which is an ATP-dependent protease responsible for the degradation of proteins. The observation that gankyrin binds RB, but not p107 or p130 (RB related proteins), in vitro and in vivo when ectopically expressed provided an initial glimpse into the role of gankyrin in tumorigenesis (Higashitsuji et al., 2000). Consistent with these findings, enforced expression of gankyrin in immortalized mouse fibroblasts and human tumor cells conferred growth in soft agar, and this transformation phenotype was dependent on the ability of gankyrin to bind RB (e.g., the LxCxE point mutant E182A is inactive). Interestingly, a splice variant of gankyrin is produced that lacks the LACDE motif, which should render it inactive in targeting RB directly. However, the physiologic role of this variant and how gankyrin gene expression is regulated in general has not yet been explored. Gankyrin facilitates the phosphorylation and degradation of RB (Figure 1), suggesting that increased expression of gankyrin promotes tumorigenicity by targeting RB to the proteasome. Yet gankyrin disrupts RB function by other means as well. Gankyrin also binds cyclin-dependent kinase 4 (CDK4) resulting in a gankyrin-CDK4-Cyclin D ternary complex (Li and Tsai, 2002). In so doing, gankyrin competes with INK4A, an inhibitor of cyclin kinases, for binding to CDK4. Based upon these findings, gankyrin appears to indirectly activate CDK4, resulting in the hyperphosphorylation of RB and concomitant deregulation of E2F1-mediated transcription and cell cycle progression (Figure 1). Taken together, these studies suggest that gankyrin deactivates the RB tumor suppressor pathway at multiple levels, including by direct binding to RB and by ensuring its inactivation through the maintenance of CDK4 kinase activity.

In the present study in *Cancer Cell* by Fujita and colleagues (Higashitsuji et al., 2005), gankyrin is now shown to bind to MDM2, an E3 ubiquitin ligase that negatively regulates p53. Compelling data are provided showing that this interaction occurs naturally between endoge-