

Dll4/Notch1 signaling from tip/stalk endothelial cell specification to stroma-dependent lung tumor inhibition: a flavor of Dll4/Notch1 pleiotropy in tumor cell biology

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Abstract: Non-small cell lung cancer (NSCLC) still represents the leading cause of cancer death. Treating this disease with systemic chemotherapy has reached a plateau in effectiveness and is rather toxic to the patients, while molecularly targeted therapies against Epidermal Growth Factor Receptor can lead to resistance. On the other hand, therapies based on tumor angiogenesis inhibition have been recently proposed. Here we will discuss on the pleiotropy of the Dll4/Notch1 cell-to-cell signaling in NSCLC, as alternative target for future therapeutic approaches.

Keywords: Non-small cell lung cancer (NSCLC); tumor angiogenesis; Delta-like 4 (Dll4); Notch



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Lung cancer is still the leading cause of morbidity and death all over the world, representing the 17% of new diagnoses in men and the 23% of total cancer mortality (1). Non-small-cell lung cancer (NSCLC) accounts for about 85% of lung cancers with most of the patients presenting advanced disease at the time of diagnosis, and with a 5-year survival less than 5% (2). Standard therapy, such as systemic chemotherapy, has reached a plateau in effectiveness and causes significant toxicity to the patients (3). However, enhanced understanding of cellular and molecular mechanisms of tumor progression has allowed for the discovery of specific molecular targets and the development of targeted therapies. At this regard, the standard first-line treatment for NSCLC patients that present mutations in the *EGFR* is now the use of EGFR inhibitors (4). Despite these improvements, prognosis remains poor, and alternative strategies for this devastating disease are required.

It has long been established that angiogenesis is a rate-limiting step during tumor growth (5). The VEGF ligands and their cognate receptors constitute the most essential axis of signaling for blood vessel formation in

tumor angiogenesis (6). Current strategies based on the combination of small tyrosine kinase inhibitors acting on VEGF ligands or VEGF receptors with cytotoxic agents have been exploited in cancer patients including those with advanced-stage NSCLC (2). However, tumors that may be initially responsive to available treatments can acquire resistance (2). Redundant angiogenic factors with up-regulation of alternative angiogenic signals, induction of hypoxia, selection of more aggressive tumor cells, recruitment of bone-marrow-derived myeloid cells that favor angiogenesis, modification of vascular pericyte coverage and vessel cooption are the main mechanisms through which resistance takes place (6).

The limited success of molecularly targeted therapies either alone or in combination has spurred the development of alternative approaches (7). Nowadays, based on their antiangiogenic potential, other agents, different from those interfering with the VEGF axis, are under clinical development (8). In this context, genetic and pharmacological studies uncovered a critical role for the Notch cell-to-cell signaling family of transmembrane

receptors and ligands, not only in vessel biology (9,10), but also in tumor angiogenesis (8). Endothelial cells (ECs) express several Notch receptors (Notch1, 4) and ligands (Delta-like 1, 4 and Jagged1). Among these, Notch1 and Delta-like 4 (Dll4) are recognized as key players (9).

The haploinsufficiency of VEGF and Dll4 ligands is a proof of concept of the crucial role played by the bioavailability of VEGF and Dll4 during vessel patterning (11). During angiogenesis, in response to VEGF, vascular cells degrade the surrounding extracellular matrix, proliferate, and migrate to form new blood vessels (10). Vascular sprouting results from the ability of this potent angiogenic factor to increase the expression of membrane-bound Dll4 on specialized ECs, denoted as “tip cells” (9,10,12). As the result of the Dll4 expression and the activation of the Dll4/Notch1 signaling on adjacent ECs the “stalk cell” phenotype is induced, the tip cell behavior is restrained and vascular sprout towards the angiogenic cue limited. Therefore, as elegantly demonstrated by Hellström *et al.* (13), Dll4/Notch1 signaling is required for an appropriate ratio between tip and stalk cells leading to a proper branching formation in response to VEGF.

Moreover, a number of studies strongly support a picture in which Dll4-Notch signaling serves as a negative regulator of VEGF-induced angiogenesis. This implies that VEGF acts as a central driver of angiogenesis, while Dll4/Notch signaling helps to coordinate the response appropriately (10,14,15).

In analogy with developmental process, in experimental tumor models the principle of tip-stalk specification by Notch signaling seems to be crucial in tumor angiogenesis. Therefore based on the crucial role of Dll4/Notch signaling in the selection of tip cells and vascular sprouting, a number of therapeutic approaches targeting this pathway have been proposed (16). Noguera-Troise *et al.* (17), elegantly demonstrated that blockade of Dll4 signaling, either by using selective anti-Dll4 antibody or an Fc-Dll4 fusion protein, led to an increased number of tip cells and an hyperdense immature vascular network in C6 glioma tumor-bearing mice. Unexpectedly Dll4 blockade leads to the so called “non-functional angiogenesis”, resulting in a striking uncoupling of tumor growth from vessel density. Consistent with these data, immunotherapeutic approaches, targeting Dll4 by DNA vaccination significantly attenuated the growth of orthotopically implanted ERBB2+ mammary carcinomas in mice by induction of a disproportionate and poorly perfused vascular bed (18). Overall these data indicate that interference with the Dll4/Notch signaling led to an aberrant vascular patterning unable to support tumor

growth. However, continuous dosing with anti-Dll4 mAb has resulted in new vessel formation and angiomas in different experimental models (19). Thereby, it has been suggested that a more efficacious anti-angiogenesis therapy could be obtained by simultaneously targeting VEGF and Dll4 (20).

Nevertheless, the significant pathophysiology of Notch signaling pathway in tumors have spurred clinicians to move from preclinical to the clinical trial by exploiting agents that either hinder Notch receptor cleavages such as gamma-secretase inhibitors (GSIs), or interfere with the Notch ligand-receptor interaction, including humanized monoclonal antibodies (mAbs). Although antitumor activity by GSIs and mAbs administered as single agent in early phases of clinical trials has been observed in different advanced or metastatic cancers, a number of mechanism-based adverse events, particularly gastrointestinal toxicities, emerged (21). To mitigate treatment-related toxicities of such molecularly targeted therapies, alternative approaches based on dose and schedule drug delivery with tolerable safety profile are nowadays under investigation (8).

Despite these advancements in many types of solid tumors, the role of Notch1 signaling in lung tumors is still debated (21,22). Notch1 and Notch2 are frequently expressed in NSCLCs and have been suspected to have a growth promotion function (22). However, recent studies demonstrated a reduced or even undetectable Notch1 expression in NSCLC. This implies a putative Notch1 tumor-suppressive role in these tumors (23,24) and suggests that Notch function in NSCLC is more complex than expected. At this regard apparently discordant effects of over-expressing Notch1 have been reported in a well known model of NSCLC, the A549 cell line. These cells can undergo cell death when Notch1 over-expression was obtained by *in vitro* manipulation but can activate cell survival signals when cultured in hypoxic conditions that mimic tumor microenvironment (23). Although discordant, these observations simply reflect the biological pleiotropy of Notch in developmental processes where Notch signaling outcome basically depends on the specific tissue context, microenvironment and cross-talk with other signaling pathways (21).

As described above, Notch acts as a juxtacrine signaling molecule. Indeed, Notch signaling is initiated by binding of a Notch ligand expressed on one cell to a Notch receptor on a neighboring cell (9). Based on this notion, Ding *et al.* (25) have proposed an alternative strategy to impair NSCLC tumor growth. They hypothesized that the cross-talk between ECs and A549 tumor cells might mimic tip-stalk cell interaction, restraining tumor cell growth. Interestingly,

they showed that ECs over-expressing Dll4, when co-cultured with A549 cells, can suppress tumor cell *in vitro* and *in vivo* proliferation, leading to a marked decrease in the *in vivo* tumor growth. Unexpectedly, instead of inducing non-functional angiogenesis, Dll4 silencing on ECs was associated with enhanced NSCLC cell proliferation and *in vivo* tumor formation. Moreover, although they did not discriminate between A549 and ECs, they showed that Notch1 and its nuclear targets are up-regulated in tumor cells when co-cultured with Dll4 expressing ECs. To sustain the inhibitory pathway, Ding *et al.* (25), also showed a correlation between expression of Notch1 and the oncosuppressive protein PTEN in xenografts derived from tumor cells co-injected with Dll4 expressing ECs. They did not prove formally that PTEN is transcriptionally regulated by Notch1, however these data are in line with the observation that PTEN is a direct Notch1 target in prostate adenocarcinoma cells (26).

It is well known that transcriptional activation of Notch target genes includes either genes involved in cell proliferation/survival and genes related to growth arrest (8). This implies that the outcome of Notch activation widely differs with cellular context and dose, from apoptosis to cell survival, and unrestrained growth to growth arrest. The data by Ding *et al.* (25), although provocative in providing a novel and alternative strategy targeting Notch pathway in NSCLCs, once again might recapitulate the pleiotropy of the Notch1 signaling in tumor cell biology. This suggests that to clarify the mechanisms underpinning Notch1 expression/activation in lung cancer further studies are requested. Moreover, the relevance of Dll4/Notch1 signaling in driving tumor growth inhibition, provided by this study, suggests that particular attention may be devoted to understand the mechanisms regulating stroma/tumor cell direct interaction as well. If validated, these data would provide the rational basis to future efforts in developing novel druggable targets to specifically hinder lung tumor growth. Finally, in light of these data, an additional challenge would be to identify a lung cancer preclinical model that reliably quantify the effects of Dll4/Notch1 in both tumor and stroma cells. Such a model would predict the growth inhibitory potential mediated by high levels of Notch1 expression and eventually, it would be useful to identify patients eligible for this therapeutic approach.

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