Notch signaling in lung diseases: focus on Notch1 and Notch3

Dandan Zong, Ruoyun Ouyang, Jinhua Li, Yan Chen and Ping Chen

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***Abstract*:** Notch signaling is an evolutionarily conserved cell–cell communication mechanism that plays a key role in lung homeostasis, injury and repair. The loss of regulation of Notch signaling, especially Notch1 and Notch3, has recently been linked to the pathogenesis of important lung diseases, in particular, chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis, pulmonary arterial hypertension (PAH), lung cancer and lung lesions in some congenital diseases. This review focuses on recent advances related to the mechanisms and the consequences of aberrant or absent Notch1/3 activity in the initiation and progression of lung diseases. Our increasing understanding of this signaling pathway offers great hope that manipulating Notch signaling may represent a promising alternative complementary therapeutic strategy in the future.

Keywords: lung disease, Notch1, Notch3, therapy, γ-secretase

Introduction

Notch is a highly conserved signaling pathway involved in the regulation of cell-fate acquisition and differentiation in several systems [Kopan and Ilagan, 2009]. It was initially discovered to be responsible for the specific phenotype displayed as 'notches' at the wing blades of *Drosophila melanogaster*. Nowadays, it is clear that the Notch-signaling pathway influences cell-fate decisions, such as survival or apoptosis, proliferation and differentiation, and maintains stem-cell quiescence and identity [Bi and Kuang, 2015]. To date, in mammalians, there are four Notch receptors (Notch1-4) and five ligands named Jag1, Jag2 (homologs to Serrate), Delta-like (Dll)1, Dll3 and Dll4 [Fleming, 1998]. Both the receptors and the ligands are single-pass transmembrane proteins with extracellular domains, transmembrane and intracellular domains. The extracellular domain of the receptor is composed of a series of 29–36 epidermal growth-factor-like (EGFR-like) repeats. The EGFR-like domains are the ligand-interacting part of the Notch receptors. Following a single transmembrane domain, the intracellular domain consists of the RBPj association module, the nuclear localization signal, ankyrin repeats and degradation signals (glutamine-rich repeat (OPA)/ proline/glutamic acid/ serine/threonine-rich motifs (PEST) domain) [Bigas *et al.* 2013]. The pathway is initiated on the binding of a Notch receptor to a ligand located on a neighbor cell. Once receptor–ligand interactions occur, the Notch molecules in the target cells are processed by two successive proteolytic cleavages [Wakabayashi *et al.* 2015]. The first cleavage begins extracellularly, close to the transmembrane domain, and is mediated by metalloproteases of the ADAM family. The second cleavage proceeds within the transmembrane domain and is mediated by γ-secretase, which is a multiple protein complex consisting of Presenilin, Nicastrin, Aph1a (anterior pharynx defective 1 homolog) and Psenen (presenilin enhancer 2 homolog) proteins. At the completion of this process, the Notch-intracellular domain (NICD) translocates into the nucleus and interacts with RBPJκ/CSL, a transcriptional repressor. Upon interaction with the NICD, RBPJκ/CSL is converted into a potent transcriptional activator [Ayaz and Osborne, 2014]. This transcriptionally active complex induces the expression of basic-helix-loop-helix (bHLH) family genes such as Hairy and enhancer of split (Hes) family genes (i.e. Hes1, 3, 5 and 7) and Hes-related with a YRPF motif (Hey) family genes (i.e. Hey1, Hey2 and HeyL) [Chen *et al.* 2014]. Both the Hes and Hey proteins execute most biological processes and partially underlie the target specificity of the

Correspondence to:
Ping Chen, MD, PhD
Department of Respiratory Medicine, The Second Xiangya Hospital of Central South University, 139 Renmin Middle Road, Changsha, Hunan 410011, People's Republic of China
pingchen0731@sina.com

Dandan Zong, MD
Ruoyun Ouyang, MD
Jinhua Li, MD
Yan Chen, MD, PhD
Department of Respiratory Medicine, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

different Notch-receptor paralogs. The cell-cycle promoter CyclinD1, the proliferation-related gene c-MyC, the antiapoptotic gene Bcl2, the gene for Notch-regulated ankyrin repeat protein, Deltex1, the pre-T-cell receptor gene, $p21^{cip1/waf1}$ and HER2 have also been identified as Notch target genes [Wakabayashi *et al.* 2015; Takebe *et al.* 2014].

The genomic sites at which Notch activates transcription vary from cell to cell and vary quite likely among different Notch paralogs. Thus, Notch signaling can occur in a variety of circumstances based on the presence of the five different ligands in the microenvironment, the expression of metalloproteinase and γ-secretase complex enzymes, as well as the expression of the four Notch receptors, yielding a large number of potential variations on the Notch signaling [Hernandez Tejada *et al.* 2014]. Little is known about how Notch ligands interact with various Notch receptors, but it does appear that preferences for certain Notch–ligand partnerships exist *in vivo* [Andrawes *et al.* 2013]. One of the best-described examples is that the Notch1–Dll4 interaction is a key regulator of angiogenesis [Hellström *et al.* 2007]. However, a strong endothelial expression of the JAG1 ligand antagonises DLL4-Notch1 signaling during sprouting angiogenesis [Benedito *et al.* 2009]. DLL1 is an essential Notch ligand in the vascular endothelium of large arteries to activate Notch1 and maintains arterial identity [Sörensen *et al.* 2009]. The untypical ligand DLL3 can interact with, but does not activate, Notch [Schuster-Gossler *et al.* 2016]. The interaction of Dll1-Notch2 has been reported to play an important role in marginal zone B-cell development [Descatoire *et al.* 2014]. Notch2 and Jagged1 are necessary for appropriate bile-duct development [Geisler *et al.* 2008]. In adult airways, Jag2-Notch3 has been reported to contribute to restrict the expansion of p63+ cells *in vivo* [Mori *et al.* 2015]. A coordinated activation of Dll4/Notch4 plays a key role in the abnormal remodeling of tumor vessels [Zhang *et al.* 2016]. Despite the fact that these combinations of Notch receptors and ligands are common, it is still unclear whether these preferences are based solely on spatial and temporal differences in expression patterns or if there are underlying intrinsic differences in the affinity among various ligand–receptor complexes [Andrawes *et al.* 2013].

In mammalian lungs, all Notch ligands and receptors are transcriptionally expressed [Post *et al.* 2000]. They were increasingly linked to a variety of lung diseases. Over the past decade, the role of Notch receptors in the pathogenesis of lung diseases has been subjected to extensive examination. Of them, two Notch receptors have been implicated: Notch1 and Notch3. Here, we mainly review the role of Notch1 and Notch3 in various lung diseases, such as chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis, pulmonary arterial hypertension (PAH), lung cancer and lung lesions in some congenital diseases.

The role of Notch1/Notch3 in chronic obstructive pulmonary disease

Notch1. Mucus hypersecretion has been established as a pathologic characteristic of smoking-related lung diseases, especially COPD [Wang *et al.* 2012b]. The altered balance of ciliated and secretory cells, particularly the increase in mucous (goblet) cells, contributes to the hypersecretion [Curran and Cohn, 2010]. Notch signaling has been identified as a major regulator of goblet-cell fate [Guseh *et al.* 2009; Boucherat *et al.* 2012]. In explanted embryonic lungs, the addition of a Notch ligand or an expression of a constitutively active form of a Notch1 receptor increased MUC5AC-containing mucous cells, whereas a γ-secretase inhibitor (GSI) reduced the mucous cells [Guseh *et al.* 2009]. Boucherat and colleagues reported that the expression levels of activated Notch1 and the effector gene Hey2 are enhanced in the areas of goblet-cell metaplasia along the airway epithelium and in the submucosal glands in COPD patients [Boucherat *et al.* 2012]. The *in-vivo* administration of a GSI attenuates goblet cell metaplasia in a Hoxa5 mutant mouse model. Conversely, in a postnatal mouse lung, Notch signaling directly repressed MUC5AC transcription in lung epithelial cells [Tsao *et al.* 2011] (Table 1). Moreover, disruptions of Notch signaling resulted in an aberrant postnatal airway phenotype characterized by marked goblet-cell metaplasia, decreased Clara cell number and increased ciliated cells. These studies indicate that different thresholds of activation of Notch signaling may determine whether a cell will become a secretory cell or a nonsecretory cell. This mechanism is likely to be in place not only in development but also in aberrant responses of the mature epithelium of the environmental agents that result in airway epithelial metaplasia [Shi *et al.* 2013]. The regulation of Notch signaling may become a potential therapeutic approach to restrain goblet-cell differentiation and mucus

Table 1. Involvement of Notch1/Notch3 in chronic obstructive pulmonary disease.

Reference	Specimen Source	Change of Notch1/Notch3	Biological function
Guseh *et al.* [2009]	Lung tissue from Rosa-NotchIC-IRES-GFP mice	Notch1↑	Increased mucous cells, decreased ciliated cells in the airway and prevented the differentiation of alveolar cell types
Boucherat *et al.* [2012]	Lung tissue from *Hoxa5*$^{-/-}$ mice	Notch1↑	Induced goblet-cell differentiation and mucus overproduction
Tsao *et al.* [2011]	Lung tissue from *Pofut1*^{cTb3} mice	Notch1↓	Increased goblet cells and ciliated cells, decreased Clara cell number
Tilley *et al.* [2009]	Lung tissue from COPD patient	Notch3↓	Notch3 downregulated in airway epithelium
Dang *et al.* [2003]	Lung tissue from SP-C-N3IC transgenic mice	Notch3↑	Inhibited type I pneumocyte differentiation, induced abnormalities of lung morphogenesis and perinatal lethality

COPD, chronic obstructive pulmonary disease.

hyperproduction in the airways of patients with COPD.

Recent data described abnormal apoptotic events as one of the important mechanisms involved in the destruction of pulmonary tissue in COPD. Our previous research also confirmed that endothelial cell apoptosis was closely related to cigarette smoke [Peng *et al.* 2013; Yang *et al.* 2015; Kang *et al.* 2015; Chen *et al.* 2012a]. Over the past decade, Notch signaling has been highlighted in cell-fate determination, including apoptosis [Dang, 2012]. The genetic deletion of Notch1 results in abundant apoptotic cell death [Limbourg *et al.* 2005], whereas Notch1 overexpression protects cells from apoptosis [Qin *et al.* 2011]. Previous studies have suggested that oxidative stress can cause cellular apoptosis *via* both the extrinsic cell-death receptor pathway and the intrinsic mitochondrial-cell-death pathway [Sinha *et al.* 2013]. Thus, oxidative stress and apoptosis can interact and overlap with each other in the overall pathogenesis of COPD. Notch signaling has recently been reported to prevent the production of reactive oxygen species [Small *et al.* 2014; Cai *et al.* 2014]. Notch signaling maintains low oxidative stress in cells, and the inhibition of Notch using GSI results in the enhanced generation of reactive oxygen species. These results suggest that Notch signaling may be involved in cell apoptosis induced by cigarette smoke through regulating oxidative stress.

Notch3. A recent analysis of the airway transcriptome in human subjects has shown that all of the key functional components in the Notch signaling are widely expressed in the airway epithelium [Tilley *et al.* 2009]; and several of them, such as Notch3, Dll1, Hes, and Hey genes, are downregulated in healthy smokers and smokers with COPD. These changes raise the possibility that Notch signaling may contribute to the aberrant differentiation profile of the airway in COPD patients [Shi *et al.* 2009]. Notch3 plays a role in regulating the alveolar epithelium. The constitutive expression of Notch3 in the peripheral epithelium results in altered lung morphology, with a failure of the type I pneumocytes to differentiate from the type II pneumocytes [Dang *et al.* 2003] (Table 1). These observations may explain why, in emphysema, enlarged alveoli are mainly covered by type I-differentiated pneumocytes and why type II-pneumocyte proliferation is minimal [Chilosi *et al.* 2012]. However, how Notch3 exerts its function in COPD is still lacking evidence. Further studies should focus on the role of Notch in the pathogenesis and the progression of the disease.

The role of Notch1/Notch3 in asthma

Notch1. Asthma is a chronic inflammatory disorder of the airways and involves several inflammatory cells (such as eosinophils, mast cells, T lymphocytes and neutrophils), structural cells (such as airway smooth-muscle cells and airway epithelial cells) and multiple mediators that result in characteristic pathophysiological changes [Xu *et al.* 2011]. An imbalance between the T-helper type (Th) 1 and Th2 cell levels where Th2 is predominant plays a central role in the development and the progression of several

Table 2. Involvement of Notch1/Notch3 in asthma.

Reference	Specimen Source	Change of Notch1/ Notch3	Biological function
Zhou *et al.* [2015]	Active lung T cells from asthmatic mouse model	Inhibit Notch1 with GSI	Decreased IL-4 and IL-5 expression and increased IFN-γ expression
Fang *et al.* [2007]	$CD4^+$ T cells from $DNMAML^{f/f}DO11.10$ (B10.D2) mice	Forced expression of N1ICD	Promoted IL-4 expression
Kang *et al.* [2009]	Bronchoalveolar lavage cells from asthmatic mouse model	Inhibit Notch1 with GSI	Decreased in Th2 cytokine production and increased in Th1 cytokine secretion
Minter *et al.* [2005]	$CD4^+$ T cells from C57BL/6 mice	Inhibit Notch1 with GSI	Inhibited IFN-γ and Tbx21 expression
Radke *et al.* [2009]	Eosinophils	Inhibit Notch with GSI	Enhanced viability, decreased actin polarization, and diminished chemokinesis of eosinophils
Kang *et al.* [2005] Kang *et al.* [2007]	Umbilical cord blood cells	Inhibit Notch with GSI	Induced eosinophil differentiation
Liu *et al.* [2015]	BAL cells from OVA exposed mice	Inhibit Notch1 with GSI	Inhibited eosinophil accumulation within allergic airways
Anastasi *et al.* [2003]	Spleen, lymph nodes, and pancreas from Notch3-transgenic mice	Notch3↑	Enhanced generation of Treg cells
Kared *et al.* [2006]	$CD4^+CD25^+$ cells from NOD mice	Activate Notch3 by mobilized Lin–Sca-1+c-kit+ HPC	Promoted the expansion of Treg Cells both *in vivo* and *in vitro*
Maekawa *et al.* [2003]	$CD4^+$ T Cell from BALB/c mice	Forced expression of N3ICD	Promoted naïve T cells differentiation toward the Th1 phenotype

GSI, γ-secretase inhibitor; N1ICD, Notch1 intracellular domain; N3ICD, Notch3 intracellular domain; HPC, hematopoietic progenitor cell, BAL, bronchoalveolar lavage; OVA, ovalbumin; IL, interleukin.

forms of asthma [Kallinich *et al.* 2007]. The Notch pathway is confirmed to be a signaling mechanism involved in the development, differentiation and activation of T cells [Zhou *et al.* 2015; Zhang *et al.* 2013]. The introduction of an activated allele of Notch1 into CD4+T cells led to the specific and direct upregulation of a developmentally regulated Gata3 transcript, which acts in concert with Notch signaling to synergistically activate the IL-4 expression and the Th2 cell responses [Fang *et al.* 2007]. Consistent with this, the GSI treatment of bronchoalveolar lavage cells stimulated *via* T-cell receptor (TCR) or non-TCR pathways led to a decrease in Th2 cytokine production with a concomitant increase in Th1 cytokine secretion [Kang *et al.* 2009]. These studies suggest that blocked Notch1 signaling may benefit diseases associated with the excessive production of Th2 cytokines. However, a different opinion has been proposed; that Notch1 can influence T-bet, a Th1-specific T box transcription factor, by regulating Tbx21. The administration of GSI substantially impeded the Th1 polarization both *in vivo* and *in vitro* [Minter *et al.* 2005] (Table 2). Although the evidence supporting a role for Notch in the Th1-cell differentiation cannot be discounted, the evidence so far has been more convincing for a role of Notch in the Th2-cell differentiation than for a role in Th1-cell differentiation [Amsen *et al.* 2009]. Further studies are needed to clarify the exact role of Notch1 in the pathogenesis of asthma and explore whether or not the interference of Notch1 signaling is a feasible treatment option for asthma.

Eosinophils are key effector cells in the pathogenesis of allergic disease and are recruited from the circulation to inflammatory tissues in response to allergic stimuli [Zhang *et al.* 2015]. Studies on

asthmatic patients have shown that eosinophil numbers are significantly increased in bronchoalveolar lavage fluid, sputum and endobronchial biopsies in response to airway hyperresponsiveness [Gaurav *et al.* 2014]. Evidence of Notch-receptor activation and the subsequent transcription of the Notch-responsive gene Hes1 were observed in granulocyte-macrophage colony-stimulating factor (GM-CSF) - stimulated eosinophils [Radke *et al.* 2009]. Notch signaling regulates the terminal differentiation and subsequent effector phenotypes of eosinophils, partly through the modulation of the extracellular signal-regulated kinase pathway [Kang *et al.* 2005, 2007]. GSI treatment induces the differentiation of eosinophils lacking effector functions *in vitro*. In mice *in vivo*, the eosinophil accumulation within allergic airways was impaired following the systemic treatment with GSI or the adoptive transfer of eosinophils treated *ex vivo* with a Notch inhibitor [Liu *et al.* 2015] (Table 2). In summary, the remarkable effect of GSI on eosinophil differentiation implied a multipronged therapeutic value of this protein in the treatment of asthma. The continued attention to the study of Notch signaling in asthma will be crucial for generating new ideas for asthma prevention and treatment.

Notch3. Recent reports have suggested that other CD4+ T-cell subsets may play a role in asthma, including Th17 cells and CD4+CD25+ Treg cells [Shi *et al.* 2011]. Reduction with or without defects in Treg cells have been detected in asthma patients [Xu *et al.* 2012]. The administration of Treg cells can reduce existing inflammation and prevent the subsequent development of airway remodeling [Kearley *et al.* 2008], suggesting that Treg-cell-mediated immunological regulation plays a protective role in asthma. A constitutively active Notch3 intracellular domain (Notch3-ICD) in transgenic mice enhances the generation of Treg cells [Anastasi *et al.* 2003]. In addition, Treg-cell expansion required cell-to-cell contact and Notch3 signaling, which was mediated selectively through the Notch ligand Jag2 expressed by the multipotent hematopoietic progenitor-cell subset [Kared *et al.* 2006]. Thus, Notch3 signaling may be involved in the development of asthma through regulating the generation and the expansion of Treg cells. Besides Treg cells, the overexpression of Notch3-ICD in activated CD4+T cells also promoted Th1, which is associated with the enhanced expression of T-bet [Maekawa *et al.* 2003] (Table 2). This is further confirmed that the enhanced Notch3 level is beneficial to the development of asthma and that the manipulation of this pathway may be particularly effective in the treatment of asthma.

The role of Notch1/Notch3 in pulmonary fibrosis

Notch1. Pulmonary fibrosis is characterized by epithelial-cell dysfunctions, the accumulation of fibroblasts and myofibroblasts and the relentless deposition of an extracellular matrix [Loomis-King *et al.* 2013]. The differentiation of fibroblasts into α-smooth-muscle actin-(α-SMA) expressing myofibroblasts represents a critical step in the pathogenesis of idiopathic pulmonary fibrosis [Garrison *et al.* 2013]. The overexpression of Notch has been shown to facilitate the myofibroblast differentiation from lung fibroblasts [Liu *et al.* 2009], suggesting a potential role of Notch1 in pulmonary fibrosis. Epithelial–mesenchymal transition (EMT), a process during which epithelial cells are converted to mesenchymal cells (such as myofibroblasts), is considered to contribute to pulmonary fibrosis [Chapman, 2011]. It was recently reported that the activation of Notch1 signaling induced EMT, whereas Notch1 silencing reversed the EMT process both *in vitro* and *in vivo* [Namba *et al.* 2010; Shao *et al.* 2015] (Table 3). These results support implication that Notch1 plays an active role in the pathogenesis of pulmonary fibrosis.

Notch3. A possible role of Notch3 in myofibroblast differentiation was postulated in studies in which the knockdown of Notch3 using small interfering RNA (siRNA) effectively reduced the expression of SMA [Chen *et al.* 2012b]. Along this line of evidence, it has been demonstrated that the transforming growth factor β (TGF-β)-induced differentiation of C2C12 cells into myofibroblasts was enhanced by Notch3 [Ono *et al.* 2007]. *In vivo*, myofibroblast differentiation was impaired in Notch2 -/-/Notch3- /- compound-mutant embryos but not in single mutants, suggesting that these receptors function redundantly to induce myofibroblast differentiation [Xu *et al.* 2010]. Similar to Notch1, the role of Notch3 in myofibroblast differentiation is also controversial. Kennard and colleagues showed us that the Notch3 overexpression blocked the TGF-β-induced differentiation of 10T1/2 fibroblasts into myofibroblasts (Table 3) [Kennard *et al.* 2008]. These findings suggest that the effect of Notch on myofibroblast differentiation can be either stimulatory or inhibitory, depending on the cell of

Table 3. Involvement of Notch1/Notch3 in pulmonary fibrosis.

Reference	Specimen Source	Change of Notch1/ Notch3	Biological function
Liu *et al.* [2009]	Lung Fibroblast from C57BL/6 mice	Upregulated Notch1 by FIZZ1	Induced fibroblast α-SMA expression
Namba *et al.* [2010]	A549 cells	Inhibit Notch1 with GSI	Partially inhibited the expression of α-SMA, E-cadherin and SIP1
Shao *et al.* [2015]	MCF-7 and MDA-MB-231 cells and tumor tissues from mice	Inhibit Notch1 with shRNA	Reversed EMT process both *in vitro* and *in vivo*
Chen *et al.* [2012]	Liver tissue from Sprague–Dawley rats and HSC-T6 Cells	Inhibit Notch3 with GSI	Inhibited EMT both *in vitro* and *in vivo*
Ono *et al.* [2007]	C2C12 cells	Inhibit Notch3 with siRNA	Inhibited the expression of α-SMA protein
Xu *et al.* [2010]	Lung tissue from Notch2$^{-/-}$/Notch3$^{-/-}$ double mutant mice	Notch3↓, Notch2↓	Inhibited myofibroblast differentiation
Kennard *et al.* [2008]	C3H/10T1/2 mouse fibroblasts	Forced expression of N3ICD	Inhibited the expression of α-SMA, *SM22*α, and calponin

FIZZ1, Found in inflammatory zone 1; α-SMA, α-smooth muscle actin; GSI, γ-secretase inhibitor; EMT, epithelial-mesenchymal transition; N3ICD, Notch3 intracellular domain; siRNAP, small interfering RNA; HSC, hepatic stellate cell.

origin, the inducer and the specific Notch receptor involved [Xu *et al.* 2010].

The role of Notch3 in pulmonary arterial hypertension

PAH is a disease that affects small pulmonary arteries. The proliferation of smooth-muscle cells in the small peripheral pulmonary arteries is a common characteristic in all forms of PAH [Montani *et al.* 2013]. Notch3 is expressed only in arterial smooth-muscle cells in human vasculature [Thistlethwaite *et al.* 2010]. Several studies have shown that Notch3 was involved in vascular smooth-muscle-cell differentiation and proliferation [Xia *et al.* 2012; Campos *et al.* 2002] and that Notch3 knockout mice displayed vascular smooth-muscle defects associated with postnatal maturation and arterial specification [Domenga *et al.* 2004]. Thus, it is not difficult to understand that Notch3 plays a role in the development of PAH. Recently, Notch3 has been studied in PAH in humans, as well as in rodents, by Li and colleagues [Li *et al.* 2009]. They found that elevated levels of Notch3 expression were found in the lung tissues of the PAH group compared with the control group and the severity of the disease was correlated with the amount of Notch3 protein in the lung. Mice with homozygous deletion of Notch3 did not develop PAH in response to hypoxic stimulation, and PAH could be successfully treated in mice by the administration of GSI. In addition, when exposed to chronic hypobaric hypoxia, C-C chemokine ligand type-2 receptor(CCR2)-deficient mice display a more severe PAH phenotype than wild-type mice with an increased expression of Notch3, implying that the absence of CCR2 results in spontaneous PAH, most likely *via* the dysregulation of Notch3 signaling [Yu *et al.* 2013] (Table 4). Above all, the inhibition of Notch3 signaling might be a novel strategy in the intervention of pulmonary hypertension.

The role of Notch1/Notch3 in lung cancer

Notch1. Lung cancer, a major killer cancer that accounts for millions of deaths every year worldwide, is a heterogeneous disease group, divided into two major categories: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) [Agalioti *et al.* 2014]. NSCLC makes up approximately 85% of lung cancers. SCLC comprises small parts of the total of lung cancer cases [Zhou, 2014]. Recently, there has been an increasing interest in Notch in lung cancer research. Abnormalities in the Notch-signaling system are considered to play a role in the tumorigenesis of bronchiogenic carcinoma [Zhou *et al.* 2013]. A recent meta-analysis conducted by Yuan and

Table 4. Involvement of Notch1/Notch3 in pulmonary arterial hypertension.

Reference	Specimen Source	Change of Notch1/Notch3	Biological function
Xia *et al.* [2012]	Primary human coronary artery smooth muscle cells	Activate Notch3 by cocultured with human coronary artery endothelial cells	Induced the expression of α-SMA and calponin
Campos *et al.* [2002]	Vascular smooth muscle cells	Forced expression of N3ICD	The growth rate of the cells was retarded during the subconfluent phase and failed to decelerate at postconfluence
Domenga *et al.* [2004]	Tissue from Notch3$^{-/-}$ mice	Notch3↓	vSMC coat was thinner than in wild-type arteries; Arterial myogenic responses are defective; Postnatal maturation stage of vSMC is deficient
Li *et al.* [2009]	Lung tissue from PAH patient Lung tissue from *Notch3*$^{-/-}$ mice	Notch3↑ Notch3↓	The severity of PAH correlated with the amount of Notch3 protein. Notch3 knockout mice were resistent to the development of PAH.
Yu *et al.* [2013]	Lung tissue from *Ccr2*$^{-/-}$ mice	Notch3↑	Displayed a more severe PAH phenotype than wild-type mice

α-SMA, α-smooth muscle actin; N3ICD, Notch3 intracellular domain; vSMC, vascular smooth-muscle cells; PAH, pulmonary arterial hypertension.

colleagues indicated that Notch signaling is a valuable biomarker for predicting the progression of NSCLC, and that the higher expression of Notch signaling (mainly Notch1 and Notch3) was associated with a greater possibility of lymph node metastasis, higher tumor-node metastasis (TNM) stages and poor survival of NSCLC patients [Yuan *et al.* 2015]. However, Notch1 function in lung cancer exhibits properties suggesting both tumor promotion and inhibition depending on the tumor cell type and the survival environment. For example, Notch1 is suspected to have a growth-promoting function on NSCLC, but it plays a tumor-suppressive role in SCLC [Eliasz *et al.* 2010; Sriuranpong *et al.* 2001]. Notch1 was detected to suppress tumor proliferation under normoxia; however, under hypoxia (a condition that more closely reflects tumor physiology), it had a converse role in tumor promotion [Chen *et al.* 2007].

The expression of Notch1 protein in the lung adenocarcinoma group and squamous cell carcinoma was significantly higher compared with the normal lung group [Zhou *et al.* 2013]. The high level of Notch1 in NSCLC may be explained by a recent study that the loss of NUMB, an inhibitor of Notch signaling, was detected in about 30% of NSCLC cases, which leads to increased Notch activity [Westhoff *et al.* 2009]. In the same study, a Notch1-activating mutation was found in about 10% of the clinical NSCLC cases. The expression of activated N1ICD in the pulmonary epithelium of mice induced lung adenomas, which progressed to generate adenocarcinoma when combined with the overexpression of MyC, which also develops lung tumors following a prolonged latency period [Allen *et al.* 2011]. The activation of Notch1 by ADAM17 and the subsequent regulation of the EGFR expression are required for the tumorigenicity of NSCLC cells [Baumgart *et al.* 2010]. After Notch1 ablation *in vivo*, there is a dramatic decrease in tumor initiation and burden in a mouse model of lung adenocarcinoma, demonstrating that Notch1 is implicated in the initiation, proliferation and survival of NSCLC models in preclinical studies [Licciulli *et al.* 2013]. The overexpression of Notch1 has been shown to inhibit apoptosis in lung adenocarcinoma [Wael *et al.* 2014]. Inhibiting Notch signaling by GSI could induce apoptosis in lung squamous cell carcinoma cells through a caspase-dependent and caspase-independent manner [Cao *et al.* 2012]. The downregulation of the Notch pathway was correlated with the upregulation of miR-34a, which can inhibit NSCLC cell proliferation, induce apoptosis and inhibit the invasion in NSCLC [Ji *et al.* 2012]. Notch signaling not only activates cell proliferation and antagonises apoptosis, but it is also involved in the invasion and metastasis of lung

cancer. EMT is an important process leading to cancer cell metastasis [Thiery *et al.* 2009]. The present study investigates the hypothesis that EMT could be induced by Notch activation *via* the mediating expression of various EMT-related genes, which are associated with cancer cell resistance to therapy and metastasis [Matsuno *et al.* 2012]. Meanwhile, the inactivation of Notch signaling by a GSI could reverse the EMT process [Xie *et al.* 2012]. In addition, the silencing of Notch using siRNA resulted in a mesenchymal–epithelial transition, which was associated with the impaired invasion and the anchorage-independent growth of NSCLC [Xie *et al.* 2013]. Notch1 can also promote the invasion of lung cancer cells by regulating the MMP9 expression. The elevated expression of MMP9 induced by DLK1, an important factor associated with tumor invasion, could be significantly decreased by inhibiting Notch signaling using GSI [Li *et al.* 2014] (Table 5).

Regarding SCLC, the overexpression of Notch1 resulted in the inhibition of SCLC growth and the suppression of the neuroendocrine (NE) tumor phenotype [Sriuranpong *et al.* 2001]. Thus, Notch1 signaling is suppressed in SCLC. Inactivating mutations in Notch family genes has been observed in 25% of human SCLC cases [George *et al.* 2015]. The activation of Notch signaling in a preclinical SCLC mouse model strikingly reduced the tumor number, abrogated the neuroendocrine gene expression and extended the survival of the mutant mice [George *et al.* 2015], suggesting a tumor-suppressor role of Notch in SCLC. Moreover, Notch1 can affect the invasion and metastasis of SCLC by controlling EMT. The induction of Notch1 in SCLC cells resulted in the suppression of EMT markers and inhibited the expression of gamma-laminin 2-chain alpha, which contributes to cell motility and invasion [Hassan *et al.* 2014] (Table 5).

Notch1 exerting its biological effect on lung cancer depends on oxygen concentrations. Recently, Notch1 has been reported as markedly upregulated under hypoxic conditions [Chen *et al.* 2007]. The inhibition of Notch1 signaling, either using a GSI or through Notch1-RNA interference, led to NSCLC cell death, specifically under hypoxia. The reintroduction of active Notch1 rescued the pro-apoptotic effects of GSI. On the other hand, Notch inhibition in normoxic lung adenocarcinoma cells had no effect on lung adenocarcinoma cell survival. These results suggest that the survival of NSCLC cells under hypoxia is highly dependent upon Notch1 signaling. Donnem and colleagues showed that Notch1 is an independent prognostic factor in resected NSCLC through its correlation with the vascular endothelial growth-factor A and that the mutual overexpression could well reflect a higher level of hypoxia in these neoplasms [Donnem *et al.* 2010]. Moreover, in the subset of NSCLC patients without TP53 mutations, the level of activated Notch1 correlates with poor clinical outcomes [Westhoff *et al.* 2009]. This may be explained by the observation that Notch1 can suppress p53-mediated NSCLC cells' apoptosis [Licciulli *et al.* 2013] (Table 5). Notch1 ablation induces p53-dependent apoptosis as a consequence of increased p53 stability. Thus, high Notch1 activation in NSCLC may result in a worse prognosis and treatment resistance. These results suggest a potential role for inhibiting Notch1 activity as a new therapeutic approach for NSCLC.

Immune therapy is already established as a central component of many cancer-treatment regimens, including lung cancer, for its low toxicity [Dougan and Dranoff, 2009]. Given that T cells can recognize specific antigens with their large repertoire of TCRs, it is proven to be an effective and safe adoptive immunotherapy [Wang *et al.* 2012a]. Notch signaling is confirmed to play a role in the modulation of T-cell differentiation and immune responses. Activated DLL1–Notch signaling can induce robust tumor antigen-specific T-cell effector and memory responses, enhance T-cell infiltration into the tumor, while decreasing Treg differentiation, and dramatically slow tumor growth [Biktasova *et al.* 2015; Huang *et al.* 2011]. These results suggest that the stimulation of DLL1–Notch signaling may be a potential therapeutic utility in cancer-treatment settings. An approach to generally inactivate Notch signaling *via* the inhibition of γ-secretase is currently being evaluated as a possible anticancer strategy for tumors with an acquired Notch gain of function [Egloff and Grandis, 2012]. GSIs have numerous possible targets, but their antineoplastic effects are thought to be mostly due to Notch inhibition, primarily Notch1, observed in several studies [Nguyen *et al.* 2015]. GSI administration after radiation significantly improved the radiation resistance induced by Notch activity in Notch-expressing lung cancer [Mizugaki *et al.* 2012] (Table 5). However, there is still a lack of studies about GSI in the treatment of NSCLC. It follows that a broad number

Table 5. Involvement of Notch1/Notch3 in lung cancer.

Notch receptor	Reference	Specimen Source	Change of Notch1/ Notch3	Biological function
Notch1	**Westhoff *et al.* [2009]**	Cancerous tissue from NSCLC patient	Notch1↑	Involved in the pathogenesis of NSCLC and correlated with poor clinical outcomes in the NSCLC patients without TP53 mutations
	Allen *et al.* [2011]	Lung tissue from transgenic mice	Forced expression of N1ICD	Induced lung adenomas and generated adenocarcinoma when combined with overexpression of MyC
	Baumgart *et al.* [2010]	NCI-H520, NCI-H292, NCI-H358, NCI-1650, NCI-1975 and NCI-2170 cells	Activated Notch1 by ADAM17	Involved in the pathogenesis of NSCLC
	Licciulli *et al.* [2013]	Lung tissue from *Notch1*$^{tfox/flox}$ mice and A549, H460, H522, H441, H727 cells	Notch1↓ *in vivo*; Inhibit Notch1 with siRNA *in vitro*	Notch1 downregulation inhibited the initiation, proliferation and survival of NSCLC and induced p53-dependent apoptosis
	Wael *et al.* [2014]	H69, H69AR, HI668, A549, H2170 and SBC-3	Inhibit Notch1 with shRNA or forced expression of N1ICD	Notch1 has an inhibitory effect on cell growth and NE differentiation in SCLC, and has a tumor inhibitory effect on ADC cells, but not SCC cells.
	Cao *et al.* [2012]	Cancerous tissue from NSCLC patient and human lung SCC cell line	Notch1↑ in vivo; Inhibit Notch1 with GSI *in vitro*	Notch 1, 2 are positively correlated with lymph node metastasis; Notch1 inhibition induced cell apoptosis
	Ji *et al.* [2012]	A549 and H1650 cells	Inhibit Notch1 by miR-34a	Inhibit NSCLC cell proliferation, induce apoptosis and inhibit invasion
	Xie *et al.* [2012]	PC9 cells and PC9/AB2 cells	Inhibit Notch1 with siRNA or forced expression of N1ICD	Notch1 activation promoted EMT in PC9 cells; Notch1 inhibition reversed EMT and restored sensitivity to gefitinib in PC9/AB2 cells.
	Xie *et al.* [2013]	PC9, NCI-H1650, and gefitinib-acquired resistant PC9/AB2 and NCI-H1650 cell lines	Forced expression of N1ICD; Inhibit Notch1 with siRNA or GSI	Notch1 activation promoted EMT in gefitinib-acquired resistant PC9/AB2 and NCI-H1650 cell lines; Notch1 inhibition resulted MET and restored sensitivity to gefitinib in PC9/AB2 and NCI-H1650 cell lines.
	Li *et al.* [2014]	H520, H1299 and A549 cell lines	Activated Notch1 by delta-like 1 homolog	Involved in the invasion of lung cancer
	Sriuranpong *et al.* [2001]	DMS53 and NCI-H209 cells	Forced expression of N1ICD	Inhibited SCLC cell growth and hASH1 expression
	George *et al.* [2015]	Cancerous tissue from SCLC patient and preclinical SCLC mouse	Forced expression of N1ICD in preclinical SCLC mouse	Inactivating mutations in Notch family genes has been observed in 25% of human SCLC; Notch1 activation inhibited the proliferation of SCLC tumours and the expression of neuroendocrine gene.
	Hassan *et al.* [2014]	H69AR, SBC3, H69 and H1688 cells	Inhibit Notch1 with siRNA or forced expression of N1ICD	Notch1 activation inhibited EMT and invasion of SCLC.
	Chen *et al.* [2007]	A549 and H1755 cells	Activated Notch1 by hypoxia	Involved in the pathogenesis of lung adenocarcinoma
	Donnem *et al.* [2010]	Cancerous tissue from NSCLC patient	Notch1↑	Notch-1 expression was independently associated with poor prognosis in adenocarcinomas; Coexpression of Notch-1 and VEGF-A indicated a particularly poor prognosis in NSCLC.
	Biktasova *et al.* [2015]	H157, H460, HCC15, HCC1437, HCC1264, HCC2469, Lewis lung carcinoma cells and D459 cells	Activated Notch by Dll1	Increased T-cell infiltration into tumors, elevated tumor antigen-specific T-cell effector and memory responses, decreased the number of regulatory T cells and limited tumor vascularization
	Huang *et al.* [2011]	Lewis lung carcinoma cells and D459 cells	Activated Notch by Dll1	Augmented T cell function and dramatically slowed tumor growth
	Mizugaki *et al.* [2012]	HCC2429, H460, A549 and H1395 cells	Inhibit Notch1 with GSI	Inhibited tumor growth, induced cell apoptosis and prevented Notch-induced radiation resistance

(Continued)

Table 5. (Continued)

Notch receptor	Reference	Specimen Source	Change of Notch1/ Notch3	Biological function
Notch3	**Dang *et al.* [2000]**	44 lung cancer cell lines (including HCC2429)	Notch3↑	Notch3 overexpression is associated with a translocation involving 19p, and overexpression is frequent in NSCLC
	Zhou *et al.* [2013]	Cancerous tissue from lung cancer patient	Notch3↑ in NSCLC Notch3↓ in SCLC	Involved in the pathogenesis of bronchogenic carcinoma
	Zheng *et al.* [2013]	$CD24^{+}ITGB4^{+}Notch^{hi}$ cells from $Kras^{G12D}$, $Trp53^{fl/fl}$, *eYFP* mice and primary human NSCLC cells isolated from patient samples	Inhibited Notch3 with shRNA	Attenuate self-renewal and tumor propagation in NSCLC cell lines and primary patient tumors.
	Yi *et al.* [2013]	HCC2429, H460 cells and lung tissue from tumor xenograft model	Inhibited Notch3 by Manic fringe	Inhibited lung cancer cell proliferation and tumorigenesis
	Sullivan *et al.* [2010]	45 NSCLC lines, 7 SCLC lines and cancerous tissue from lung cancer patient	Inhibited Notch3 with shRNA or GSI	Reduced ALDH+ lung cancer cells, commensurate with a reduction in tumor-cell proliferation and clonogenicity.
	Konishi *et al.* [2007]	HCC2429, HCC461, HCC193, HCC95, HCC15, HCC827, HCC44 and HCC78 cells	Inhibited Notch3 with GSI	Reduced tumor cell proliferation, inhibited serum independence, and induced apoptosis
	Haruki *et al.* [2005]	Cancerous tissue from lung cancer patient and HCC2429, H460, BEAS-2B cells	Notch3↑ *in vivo*; Inhibited Notch3 by dominant-negative receptor *in vitro*	Notch3 is overexpressed in 39% of resected NSCLCs; Notch3 inhibition dramatically reduced soft agar colony formation, increased apoptosis, and increased the tumor's dependency on exogenous growth factors
	Shi *et al.* [2014]	Cancerous tissue from NSCLC patient and H292, A549, Calu-3 cells	Notch3↑ in vivo; Inhibited Notch3 with siRNA *in vitro*	Patients with high Notch3 expression had a poorer prognosis; Notch3 inhibition dramatically suppressed the proliferation, migration, invasiveness abilities and prompted apoptosis in NSCLC cells
	Ye *et al.* [2013]	Cancerous tissue from NSCLC patient	Notch3↑	Notch3 overexpression was significantly correlated with TNM stage, lymph node metastasis and shorter overall survival
	Yen *et al.* [2015]	Small cell lung xenograft tumors from mice	Inhibited Notch2/3 by OMP-59R5	Reduced cancer stem cells frequency
	Lin *et al.* [2010]	HCC2429 cells	Inhibited Notch2/3 by Notch3 recombinant Fc-fusion proteins	Induced apoptosis and suppressed tumor growth

NSCLC, non-small cell lung carcinoma; N1ICD, Notch1 intracellular domain; NE, neuroendocrine cell; SCLC, small cell lung carcinoma; ADC, adenocarcinoma; SCC, squamous-cell carcinoma; GSI, γ-secretase inhibitor; EMT, epithelial mesenchymal transition; VEGF-A, vascular endothelial growth factor-A; Dll1, Delta-like 1; TNM, tumor-node metastasis; MyC, proliferation-related gene; ALDH, aldehyde dehydrogenase; SCLC, small cell lung cancer; EMT, epithelial–mesenchymal transition; OMP, olfactory marker protein; Fc, cell-surface protein.

of drugs with sufficient specificity and affinity for the inhibition of Notch receptor could be discovered for lung-cancer therapy.

Notch3. In lung cancers, Notch signaling was originally implicated in an epithelial tumor by the discovery of chromosome 19 translocation causing a massive overexpression of Notch3 [Dang *et al.* 2000]. Notch3 had a stronger positive degree of expression in NSCLC compared with the corresponding nontumor tissue [Zhou *et al.* 2013]. It has been shown that Notch3 is overexpressed in 39% of resected NSCLC cases [Haruki *et al.* 2005]. In a genetically engineered murine model of NSCLC, tumor cells with an induced expression of Notch3 had an increased tumorigenicity [Zheng *et al.* 2013]. Manic Fringe plays a role in tumor suppression in the context of lung cancer [Yi *et al.* 2013]. The reintroduction of Manic Fringe in lung cancer cells can decrease Notch3 protein stability and reduce cell proliferation and tumor growth. Cancer stem cells have been identified in a number of solid tumors, including breast cancer, brain tumors, lung cancer, colon cancer and melanoma [Dawood *et al.* 2014]. ALDH activity was identified as a marker for

lung-cancer cells with stem-cell properties, and the inhibition of Notch3 resulted in a significant decrease in ALDH+ lung-cancer cells, commensurate with a reduction in tumor-cell proliferation and clonogenicity [Sullivan *et al.* 2010]. The suppression of Notch3 results in the loss of the malignant phenotype in both *in-vitro* and *in-vivo* models [Konishi *et al.* 2007; Haruki *et al.* 2005]. The bodies of data described above support a potential role for Notch3 in the carcinogenesis of NSCLC. However, the expression of Notch3 has been reported to be common in NSCLC but not in SCLC [Dang *et al.* 2000]. It has been shown that the expression of Notch3 in SCLC was lower compared with that of the corresponding nontumor tissue [Zhou *et al.* 2013] (Table 5). Nevertheless, studies on Notch3, compared with Notch1, remain small in number, and whether or not Notch3 and Notch1 have a syntrophic effect in SCLC is unknown.

Similar to Notch1, Notch3 was also identified as a prognostic factor for patients with NSCLC. Patients with a high Notch3 expression had a poorer prognosis than those with a low Notch3 expression [Shi *et al.* 2014]. Notch3 overexpression was significantly correlated with TNM stage, lymph node metastasis and shorter overall survival [Ye *et al.* 2013]. Notch3 inhibition dramatically suppressed the proliferation, migration and invasiveness abilities and prompted apoptosis in NSCLC cells [Shi *et al.* 2014]. Thus, Notch3 may be used as a marker to predict the chemotherapy response and the prognosis of advanced NSCLC. Accumulating evidence has indicated that cancer stem cells are inherently resistant to cytotoxic chemotherapy and radiation, and evidence has linked stemness to prognosis and therapy-failure therapies [Yang and Rycaj, 2015]. The blocking of Notch2/3 signaling by OMP-59R5 reduced cancer stem-cell frequency in combination with chemotherapeutic agents in various cancer models, including lung cancer [Yen *et al.* 2015]. In addition, Notch has been shown to crosstalk with oncogenic pathways, such as the EGFR/ras/MAPK pathway, in both development and cancer [Lin *et al.* 2010]. Notch3 inhibition dramatically reduces soft agar colony formation, increases apoptosis and increases sensitivity to EGFR tyrosine kinase inhibition [Haruki *et al.* 2005] (Table 5). From a therapeutic standpoint, Notch3 is a candidate target for therapeutic intervention alone and in combination with growth factor receptor inhibitors. These findings rationalise a mechanistic approach to lung-cancer treatment based on Notch3 receptor-targeted therapeutic development. Nevertheless, further molecular biologic analyses of Notch3, and a longitudinal clinical study in a large population to validate its prognostic value in developing a novel strategy for improving treatment efficiencies of lung cancer will be needed.

The role of Notch1/3 in lung lesions in congenital diseases

Lung development occurs in the embryonic period and can be regulated by various molecules and signaling pathways. If there is something wrong in these molecules and signaling pathways, the lung development may be affected, and the survivors may suffer from additional morbidities of lung diseases. Mutations in Notch-signaling pathway members cause developmental phenotypes that affect the development of many organ systems. Here, we briefly review some lung lesions seen in congenital diseases related to mutant Notch1/3 genes.

Notch1. Adams–Oliver syndrome (AOS) is a rare syndrome characterized by aplasia cutis congenita of the scalp and terminal transverse-limb defects. Pulmonary vascular abnormalities have been described in AOS, including PAH, pulmonary vein stenosis, hypoplastic pulmonary arteries and pulmonary arterio-venous malformation [Lehman *et al.* 2016]. Recently, Stittrich and colleagues found mutations of the NOTCH1 gene in a proportion of an AOS cohort [Stittrich *et al.* 2014]. Southgate and colleagues also identified loss of function or haploinsufficiency of NOTCH1 as the primary cause of AOS and an important genetic factor in AOS with associated cardiovascular complications [Southgate *et al.* 2015]. Mutant NOTCH1 expression was associated with the downregulation of the Notch target genes Hey1 and Hes1, indicating that NOTCH1-related AOS arises through dysregulation of the Notch-signaling pathway.

Notch3. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a dominantly inherited small-artery disease that leads to dementia and disability in midlife. CADASIL is caused by mutations in the extracellular domain of NOTCH3, resulting from the gain or loss of cysteine residues in the EGFR-like repeats [Penton *et al.* 2012]. Recently, it has been reported that

multiple neoplastic lesions were observed in a 62-year-old man who was diagnosed with CADASIL. In the lungs of the patient, carcinoid tumorlet and foci of NE cell proliferation were seen, which were related to the activation of Notch3 [Hassan *et al.* 2015]. This case displays a striking correlation between Notch3 and pulmonary NE neoplasms, highlighting the importance of Notch3 signaling in multiple developmental processes of CADASIL, but the exact mechanism needs to be studied further.

Conclusion

The present investigation indicates that Notch signaling has clearly emerged as a critical pathway in diverse lung disorders. It has been plainly appreciated that aberrant Notch signaling, especially Notch1 and Notch3, contributes to the pathophysiology of human pulmonary disease, such as COPD, asthma, pulmonary fibrosis, PAH, lung cancer and lung lesions in some congenital diseases. In different pulmonary diseases, the change of Notch signaling is inconsistent. For instance, Notch was reported to be downregulated in COPD and SCLC, whereas it was increased in other lung diseases. Moreover, Notch signaling may exert completely opposite effects on lung cancer for the promotion or inhibition, depending on the cell type. Notch signaling was activated in NSCLC, which was closely related to the initiation and survival of the tumor. Conversely, Notch signaling is suppressed in SCLC, and the overexpression of Notch signaling resulted in the inhibition of SCLC growth. Notch signaling could be targeted for the treatment of selected pulmonary malignancies, but the results from the necessary clinical trials to establish the safety and efficacy of this approach are lacking. A number of interventions are already proceeding, and in the years to come, Notch will undoubtedly be an important tool for understanding and treating many pulmonary diseases. Thus, a better understanding of Notch signaling in the lung is likely to be important and provide information central to new treatment approaches.

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