TARGETING NOTCH3 SIGNALING IN LUNG CANCER

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ORIGINAL PUBLICATIONS

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3. Zhuang G., Song W., Amato K., Hwang Y., Lee K., Boothby M., Ye F., Guo Y., Shyr Y., **Lin L**., Carbone D.P., Brantley-Sieders D.M., and Chen J. Cancer-associated Epha3 mutations attenuate tumor-suppressive effects of normal EphA3 in lung cancer. (In revision)
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Dedicated to

My father, Zengtai Lin and my mother, Meiyu Meng

Who enormously contributed to this work from the very beginning.

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LIST OF ABBREVIATIONS

ADAM	polyacrylamide geletrophoresis
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CSL	CBF-1/RBPj κ/Su(H)/Lag-1
DLL1	Delta-like 1
DLL3	Delta-like 3
DLL4	Delta-like 4
DNMAML	dominant negative mastermind-like 1
DOS	Delta and OSM-11-like protein
DSL	Delta-Serrate-LAG2
EGFR	epidermal growth factor receptor
Erk	extracellular signal-regulated kinase
FBS	fetal bovine serum
FRET	fluorerescence resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GSI	gamma-secretase inhibitor
GST	glutathione-S-transferase
HCl	hydrochloric acid
HD	heterodimerization domain
HES	hairy and enhancer of split
HEY	hairy and enhancer of split related genes
HNSCC	head and neck squamous cell carcinoma
IGF	insulin growth factor
KD	Knockdown
LNR	Lin12-Notch repeats
mAb	monoclonal antibody
MBP	mannose binding protein
MNNL	Module at the N-terminus of Notch Ligand
NaCl	sodium chloride
NECD	Notch extracellular domain
NICD	Notch intrecellular domain
NLS	nuclear localization sequence
NMR	Nuclear magnetic resonance
NRR	negative regulatory region
NSCLC	non-small cell lung cancer
PEST	proline/glutamic acid/serine/threonine-rich motifs
RAM	RBPj κ associated module
SCLC	small cell lung cancer
TACE	tumor necrosis factor-α-converting enzyme
TAD	transactivation domain
T-ALL	T-cell cute lymphoblastic leukemia
TMA	tumor microarray
VSMC	vascular smooth muscle cell

CHAPTER I

INTRODUCTION

Notch got its name from a mutant strain of *Drosophila melanogaster* with notches at the end of their wing blades in 1917. In the mid-1980s, the group of Artananis-Tsakonas and Young cloned the *Notch* gene. These discoveries opened a door to a widening understanding of the molecular mechanism of Notch signaling, as well as physiological and pathological processes that are controlled or influenced by Notch signaling. Over the last two decades, Notch signaling has emerged as an important signaling pathway in the regulation of various oncogenic phenotypes in multiple types of human cancers, which makes Notch signaling an attractive target in various cancers. The roles of Notch signaling in human cancers are quite divergent and context dependent. Notch signaling has oncogenic effects in T-ALL, breast cancer, pancreatic cancer, ovarian cancer and lung cancer, while they have been found to be tumor suppressive in skin cancer. In lung cancer, Notch3 is considered as an oncogene and contributes to maintenance of cancer stem cells. Targeting Notch3 signaling could thus represent a novel therapeutic approach for the treatment of lung cancer patients.

Mechanisms of Notch signaling

Molecules involved in Notch signaling

Notch receptors are a class of single pass Type I transmembrane protein. There are 4 Notch receptors, known as Notch 1-4 in mammals, 1 Notch receptor in *Drosophila*,

whereas *C.elegans* have 2 Notch receptors with redundant functions (1). The number and names of the components involved in Notch signaling differ among *C.elegans*, *Drosophila* and mammals (Table 1.1). The extracellular domains of Notch receptors contains 29-36 tandem epidermal growth factor (EGF)-like repeats. Notch1 and Notch2 contain 36 EGF-like repeats, Notch3 consists 34 EGF-like repeats, and Notch4 only has 29 EGF-like repeats. Some of EGF-like repeats mediate ligand-receptor interaction. Trans activation, in which ligands presented by signaling sending cells activates Notch signaling in signaling receiving cells, is mediated by EGF-like repeat 11-12 of *Drosophila* Notch and human Notch1 (3, 4), On the other hand, cis inhibition, when ligand and receptors presented in the same cell inhibiting Notch signaling, is potentially mediated by repeats 24-29 identified in *Drosophila* Notch (5). Most of the EGF-like repeats requires calcium for receptor-ligand interaction (6, 7). The EGF repeats are followed by a unique negative regulatory region (NRR) consisting of three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD), which participates in preventing activation of receptors in the absence of ligand binding (8-10) (Fig 1.1). Notch receptors are first synthesized in precursor form as 300-350 kD polypeptides. During maturation, Notch polypeptides are cleaved by a furin-like convertase within an unstructured loop protruding from the HD subdomain, which is called S1 cleavage. The cleaved Notch polypeptides, then hold together by a non-covalent bond between the N- and C- terminal halves of the HD domain to present at cell surface as a heterodimer composed of NECD (Notch extracellular domain) and NTMIC (Notch transmembrane and intracellular domain) (11-13). The Notch intracellular domain consists of a RAM domain (RBPj κ associated module), which forms a high affinity binding module of 12-20 amino acids centered around a conserved WxP

motif (14), seven ANK repeates flanked by two NLS domain (nuclear localization sequence), TAD (transactivation domain), and a conserved PEST region (proline/glutamic acid/serine/threonine-rich motifs), which mediates protein degradation of Notch intracellular domain.

Most Notch ligands are also Type I transmembrane proteins (Figure1.1). Canonical Notch ligands fall into two classes, depending on whether they are homologous to the *Drosophila* prototypes Delta and Serrate. There are three Delta-like proteins, named Delta-like1 (DLL1), Delta-like 3 (DLL3) and Delta-like 4 (DLL4) and two homologues of Serrate, named Jagged1 (JAG1) and Jagged2 (JAG2) in mammals. Both Delta and Serrate ligands consist of an N-terminal MNNL (Module at the N-terminus of Notch Ligand) domain, followed by a DSL domain (Delta-Serrate-LAG2) and a variable number of EGF-like repeats (both calcium binding and non-calcium binding) in their extracellular portion. In some ligands, including all Serrate ligands and DLL1, the DSL domain is linked to two variant EGF like repeats, which are also referred to as the DOS domain (Delta and OSM-11-like protein) (15). Jagged family ligands are distinguished from Delta-like ligands by the presence of more EGF like repeats and an additional cysteine-rich domain homologous to the von Willebrand Factor C module linked to transmembrane region. The DSL domain and first few EGF-like repeats of Delta and Serrate ligands are essential to mediate receptor-ligand interaction.

Table 1.1. Core components of the Notch pathway in worms, flies, and mammals

Component&Funtion	*C. elegans*	*Drosophila*	*Mammals*
Receptors	LIN-12, GLP-1	Notch	Notch1-4
Ligands			
DSL/DOS		Delta, Serrate	DLL1, Jagged1 and 2
DSL-only	APX-1, LAG-2, ARG-2, DSL1-7		DLL3 and 4
DOS Co-ligands	DOS1-3, OSM7,11		DLK-1, DLK-2/EGFL9
Non-canonical			DNER, MAGP-1 and 2, F3/Contactin1, NB-3/Contactin6
Nuclear Effectors			
CSL DNA-binding transcription factor	LAG-1	Su(H)	RBPj κ/CBF-1
Transcriptional Co-activator	LAG-3	Mastermind	MAML1-3
Transcriptional Co-repressors		Hairless, SMRTR	Mint/Sharp/SPEN, NCoR/SMRT, Kyot2
Receptor Proteolysis			
Furin convertase(S1 cleavage)	?	?	PC5/6, Furin
Metalloprotease(S2 cleavage)	SUP-17/Kuzbanian, ADM-4/TACE	Kuzbanian, Kuzbanian-like, TACE	ADAM10/Kuzbanian, ADAM17/TACE
γ-secretase(S3/S4 cleavage)	SEL-12. APH-1, APH-2, PEN-2	Presenilin, Nicastrin, APH-1, PEN-2	Presenilin1 and 2, Nicastrin, APH-1a-c, PEN-2
Glycosyltransferase modifiers			
O-fucosyl-transferase	OFUT-1	OFUT-1	POFUT-1
O-glucosyl-transferase		RUMI	RUMI
Beta1,3-GlcNAc-transferase		Fringe	Lunatic, Manic and Radical Fringe
Endosomal Sorting/Membrane Trafficking Regulators			
Ring Finger E3 Ubiquitin ligase (Ligang endocytosis)	Y47D3A.22	Mindbomb 1-2, Neuralized	Mindbomb, Skeletrophin, Neuralized 1 and 2
Ring Finger E3 ubiquitin ligase (recepor endocytosis)		Deltex	Deltex 1-4

HECT domain E3 Ubiquitin ligase (receptor endocytosis)	WWP-1	Nedd4, Su(Dx)	Nedd4, Itch/AIP4
Negative regulators		Numb	Numb, Numb-like, ACBD3
Neuralized Inhibitors		Bearded, Tom, M4	
Other endocytic modifiers		sanpodo	
NICD Degradation			
F-Box Ubiquitin ligase	SEL-10	Archipelago	Fbw-7/SEL-10
Cononical Target bHLH Repressor Genes	REF-1	E(spl)	HES/ESR/HEY

*adapted from Kopan and Ilagan, 2009(2).

Activation of Notch signaling is mediated by a sequence of proteolytic processes (Fig 1.2). Upon ligand binding, Notch receptors are cleaved by an ADAM metalloproteases at site 2, which is located around 12 amino acids before the TMA and deeply buried within the NRR. It is still ambiguous what enzymes mediate S2 cleavage from different studies, such as on one hand, ADAM17/TACE is able to cleave Notch receptors *in vitro* (16), and on the other hand, TACE deficient mice do not have a Notch phenotype (17). In contrast, Kuzbanian/ADAM10/Sup-17 function is required for Notch activity among *C.elegans*, *Drosophila* and mouse (18-21). The S2 cleavage of Notch extracellular domain leads to a membrane-tethered intermediate, which is shed by a γ-secretase complex, a multi-component member of a growing family of intramembrane cleaving proteases, within the TMD (22). After S3 cleavage, released Notch intracellular domain (NICD) translocates into the nucleus where it binds to DNA-binding protein CSL (CBF-1/RBPj κ/Su(H)/Lag-1) via its RAM domain, and converts CSL from a transcriptional repressor into a transcriptional factor to initiate downstream target genes transcription via recruiting the coactivaor Mastermind/Lag-1 (14). The typical Notch target genes are bHLH (basic helix-loop-helix) transcriptional repressor proteins Hes (hairy and enhancer of split) and Hey (hairy enhancer-of-split related with YRPW motif). Besides interacting with CSL, full-length Notch, membrane tethered Notch or NICD also can function in a CSL-independent manner by crosstalking with other signaling pathways. However, the mechanisms of CSL-independent Notch signaling are not clear.

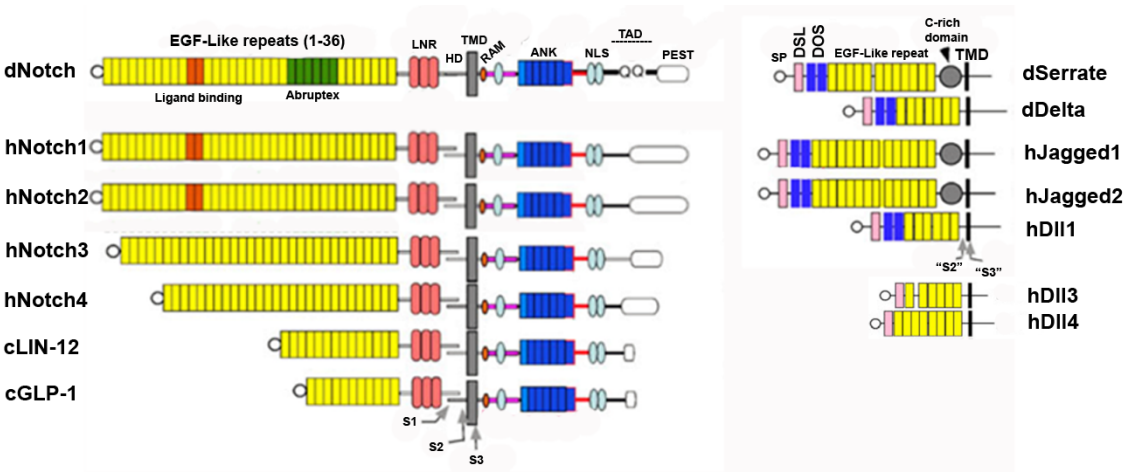

Figure1.1. Notch Receptors and Ligands. Notch receptors are type I transmembrane proteins that composes of multiple EGF-like repeats in their extracellular domain. *Drosophila* Notch has 36 EGF-like repeat, while human paralogs (hNotch 1-4) contains variable number of (36-29) EGF-like repeats. *C.elegans* has 2 Notch receptors, which contains fewer EGF-like repeats. EGF-like repeats are followed by the negative regulatory region (NRR) including three cysteine-rich Lin12-Notch repeats and a heterodimerization domain (HD), transmenbrane domain (TMD), a RAM domain (RBPj κ association module), nucleus localization sequences (NLS), a seven ankrin repeat domain (ANK), a transactivation domain (TAD) and a conserved proline/glutamic acid/serine/threonine-rich motifs (PEST). Notch1 contains a strong TAD, and Notch2 is with a weak TAD, but no TAD is present in Notch3 and Notch4. EGF-like repeat of 11 and 12 (red) are considered as a requirement for ligand interaction in dNotch and hNotch1, 2. dNotch EGF-like repeat 24-29 (green), also called *Abruptex* region mediates cis-inhibition of receptor and ligand interaction. Notch ligands can be classified into two group based on their domain composition. DSerrate and hJagged1, 2 contains N-terminal , DSL domain (Delte-Serrate-LAG-2), DOS domain (Delta and OSM-11-like proteins), EGF-like repeats, C-rich domain (Cysteine-rich domain, also called vWF domain), and a transmembrane domain (TMD). While dDelta and hDll1, 3, 4 has less EGF-like repeats and lack of C-rich domain, and hDll3, 4 also lack of DOS domain. Both receptors and ligands can be cleaved by ADAM metalloproteases (S2) and γ-secretase complex (S3). Modified from Kopan, 2009 (2).

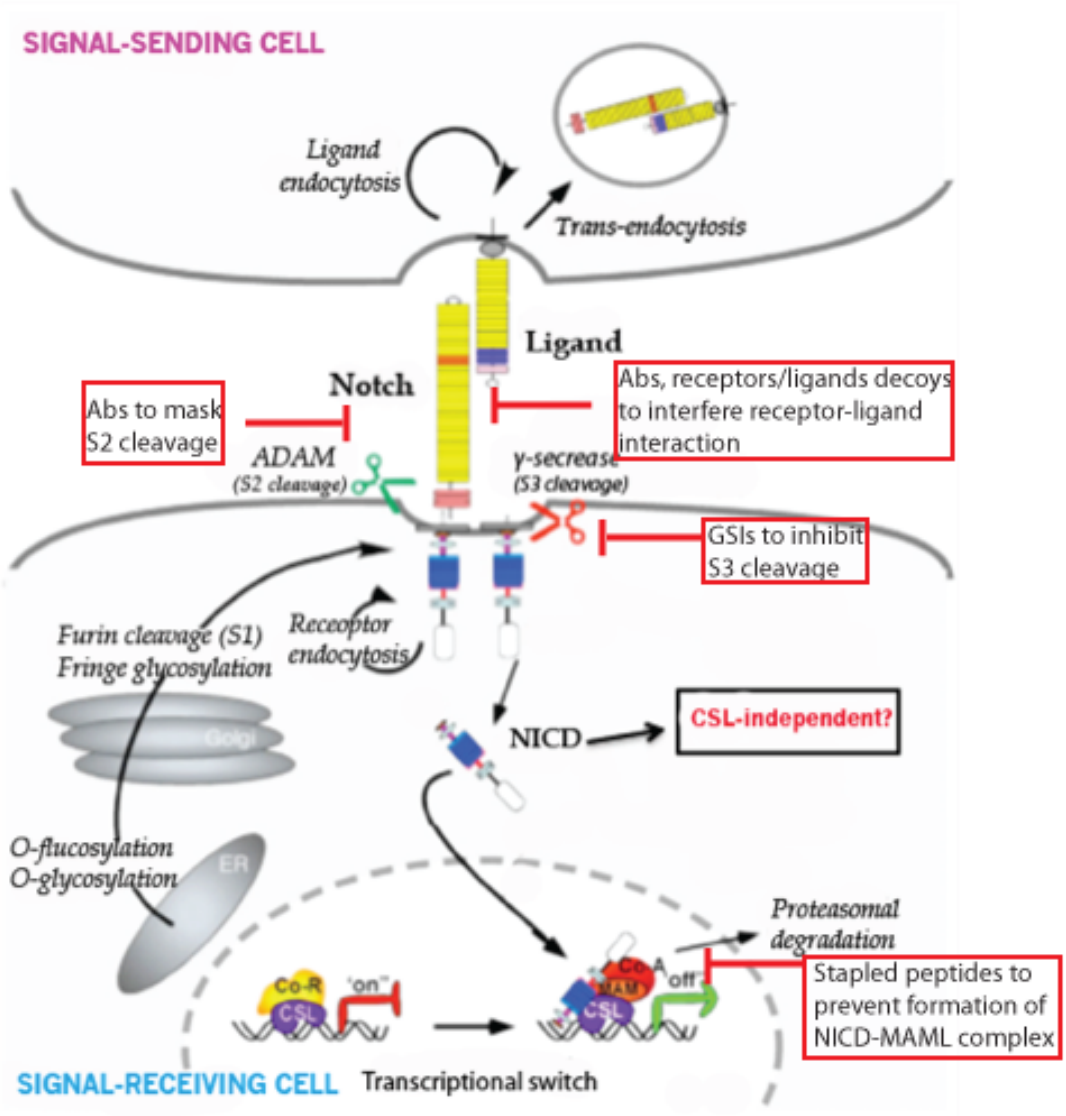

Figure1.2. The core Notch signaling pathway and therapeutic strategies to interfere with Notch signaling. Translated full-length Notch receptor proteins are glycoslated by the enzymes O-fucose and Rumi. and then the mature receptors are presented to the cell surface as a heterodimer after proteolytic cleavage by furin at site 1(S1). In the glycosyltransferase Fringe expressing cells, the O-fucose is extended by Fringe to alter the specificity of Notch receptor to be activated by different ligands. Upon the ligand binding in neighboring cell to generate mechanical force generated by ligand endocytosis exposes the S2 cleavage, the receptors are cleaved by ADAM metalloproteases at site 2 (S2). Juxtamembrane Notch cleaved at S2 produces membrane-anchored Notch extracellular fragment, which is then recognized and cleaved by the γ-secretase complex at Notch transmembrane domain site 3 (S3) to released Notch intracellular domain (NICD). NICD then translocates into nucleus where it converts DNA-binding protein CSL from a transcriptional repressor into a transcriptional factor by recruiting transcriptional coactivator Matermind (MAM) to initiate gene transcription. In contrast, NICD can function in a CSL-independent manner by cross-talking with other signaling pathways. Multiple strategies to therapeutically block Notch signaling are currently developed and tested in preclinical and clinical studies. Blocking strategies are highlighted with a red box, and consist of Abs and receptor/ligand decoys to interfere receptor-ligand interaction, Abs to mask S2 cleavage of the receptors mediated by ADAM protease , GSIs to inhibition S3 cleavage mediated by γ-secretase complex, and stapled peptides to prevent formation of NICD-MAML complex. Modified from Kopan, 2009(2).

Knowledge of receptor-ligand interaction

Domains mediating receptor-ligand interaction

Notch receptors and ligands interaction were first identified using cell aggregation assay. Using this strategy, Rebay et al., showed that EGF-like repeats 11-12 of *Drosophila* Notch receptor is necessary and sufficient to promote aggregation of Notch receptor expressing cells with both Delta- and Serrate-presenting cells (23). A series of evidence supports the conclusion that in mammals, the same region of Notch1 is also necessary for binding of Delta-like and Jagged ligands. Knock-in mice bearing mutated *Notch1* lacking EGF-like repeats 8-12 in place of wild-type receptors phenocopy *Notch1* null phenotypes (24). The surface receptor expression levels of mutant Notch1 were not detectably altered, implying that EGF-like repeat 8-12 are required for Notch1 activity. And biotinylated human Notch1 EGF-like repeats 11-13 are able to aggregate DLL1 expressing cells *in vitro* (3), indicating that EGF-like repeats 11-13 are sufficient to bind to DLL1 ligand. Moreover, biochemical studies using purified proteins have demonstrated that EGF-like repeats 11-14 of human Notch1 bind to a fragment of DLL1 containing the DSL domain and the first three EGF-like repeats. This is a calcium dependent interaction with an estimated Kd of 130uM as measured by equilibrium surface plasmon resonance (6). The ligand binding studies of other Notch receptors have been very limited, however, it is widely assumed that the same region of mammalian Notches, 2, 3 and 4 is also responsible for ligand interaction. This is supported by the finding that EGF-like repeats 1-15 of Notch2 binds to a soluble mouse Jagged1-Fc protein as determined by a solid phase-binding assay using protein fragments derived from mammalian expression systems (25). Furthermore,

deletion of EGF-like repeats 10-11 of human Notch3 (which corresponds to EGF-like repeats 11-12 of *Drosophila* Notch and mammalian Notch1) prevents its binding to Jagged1 and reduces Jagged1 induced CBF-1 reporter activity (26).

The structure of a fragment of human Notch1 containing EGF-like repeats 11-13 was solved both by solution NMR approach (3) and by X-ray crystallography (27). In the X-ray structure, three EGF-like repeats adopt an elongated conformation, with an interdomain orientation defined by the coordination of a calcium ion between two adjacent repeats and by the packing of a tyrosine residue from one repeat against an isoleucine residue from the next repeat. Based on the structure of NMR, models for the entire ectodomain of Notch receptors have been proposed invoking rigidity at inter-repeat linkers, which contain consensus sequences for calcium coordination, and different degree of intrinsic flexibility at linkers lacking a predicted calcium-binding site. The X-ray structure of a region of human Jagged1 including its DSL domain, and first 3 EGF-like repeats was also solved, in which the four-domain fragment is found in a rod-like conformation as well. The combinatorial analysis of this structure using a multiple sequence alignment identified conserve residues and a surface patch on the DSL domain as a potential site for binding to Notch receptors. Mutation of residues at this interface interferes with the formation of receptor-ligand complex, and causes loss-of-function to various degrees in transgenic flies (27). Docking studies with the structure of Notch1 EGF-like repeats 11-13 to the structure of Jagged1 DSL domain and first 3 EGF-like repeats suggests that the contact interface between Notch1 and Jagged1 lies along an extended surface (27). However, the nature of

this interface remains unknown due to unavailability of the structure of their interacting complex.

Glycosylation of receptors

Notch receptors are large glycoproteins, in which many EGF-like repeats can be modified by two kinds of O-glycosylation, O-fucose and O-glucose (28). O-fucose glycans modulate the binding affinity of Notch binding to DSL Notch ligands, while O-glucose glycans facilitate juxtamembrane cleavage of Notch to promote intramembrane cleavage and activation of Notch signaling (29). Loss of *OFUT1,* a gene encoding O-fucosyltransferase 1 protein, phenocopies Notch loss-of-function in *Drosophila* and mice, indicating that O-fucose modification is required for production of functional Notch receptors (30). However, later studies demonstrated that non-fucosylated Notch receptors can still go to the cell surface, interact with ligands and activate signaling, and the phenotype of *OFUT1* loss is mainly due to lack of its activity of ER chaperone, which is not dependent on its fucosylation function (31). This suggests that fucosylation during Notch signaling is only required for Fringe-dependent glycosyltransferase activity to extend O-fucose, but not O-fucosyltranferase activity of Ofut. The Fringe gene was identified via the screen in *Drosophila* for novel genes that regulate Notch signaling (32). Later on, Fringe was shown to be required for Notch signaling at the wing margin and at other tissue boundaries in *Drosophila* (33, 34). In the ligand binding regions of *Drosophila* Notch receptor, this process can affect ligand specificity such that Fringe-mediated adding of a single N-acetylglucosamine on EGF like repeat 12 of *Drosophila* Notch enhances binding to Delta and suppresses binding to Serrate *in vivo* and *in vitro* (35). Mammalian homologues of Fringe, named Lunatic, Manic, and Radical Fringe, were identified with

conserved functions in *Drosophila* (36). Disrupting *LFNG* in mice result in defective somitogenesis leading to pronunced skeletal aberrations (37, 38). The study demonstrated that Notch1 in mammals carries two unusual glycans, one began with fucose linked to Serine or Threonine located between the second and the third cysteine of an EGF like repeat in the consensus $C^2X_{4-5}S/TC^3$, and the other began with glucose linked to Serine and Threonine between the first and the second cysteine of an EGF like repeat with the consensus C^1XSXPC^2 (39, 40).

Endocytosis of receptors and ligands

Endocytosis of Notch receptors and ligands has been shown to be essential for the activation of Notch signaling in both signal-sending and signal-receiving cells. Early studies revealed the presence of Delta in intracellular vesicles in *Drosophila* embryos and imaginal discs (41), implying a endocytotic process of Delta ligand. Moreover, *in vivo* structure-function analysis of specific point mutant alleles of Delta ligand demonstrated that certain EGF-like repeats and certain intracellular lysine residues are indispensable for its endocytosis and proper signaling (42). Work from many laboratories conducted in different model organisms indicates that two distinct RING-containing E3 ligase families, Mind bomb (Mib) 1 and 2, and Neuralized (Neur1 and 2 in mammals), directly mediate DSL ligand endocytosis (43-46). In mice, neur1 and neur2 are dispensable for normal development. However, mice defective in neur1 and neur2 or mib2 do not have *Notch*-null phenotype (47). The single gene disruption of *Mib1* recapitulates the pleiotropic Notch mutant phenotype in mouse embryo (48, 49). Ubiquitinated DSL ligands are potentially recognized by an ubiquitin binding protein Epsin. Loss of function of Epsin in signal sending cells shows Notch loss-of-function phenotype in flies and mice, indicating that

espin mediated trafficking of ubiquitinated DSL ligands is important for activation of Notch signaling.

Endocytosis of DSL ligands is necessary for Notch signaling activation through two potential mechanisms, one is the "ligand activation" model, in which ligands undergo endocytosis and subsequent trafficking back to the surface, following modifications or some changes in the cell, which makes Delta a more effective ligand to send signals. The "ligand activation" hypothesis has been proposed to contain clustering of ligands, trafficking into lipid microdomain, proteolytic cleavage, and other posttranscriptional modifications (45, 50, 51). However, the exact mechanism of this "ligand activation" model is unclear. The other one is the " pulling force" theory, in which endocytosis of ligands facilitates S2 cleavage and removal of Notch extracellular domain. In support of this theory, structural studies have elucidated that the S2 cleavage site of Notch receptors is buried deep within the heterodimerization domain and protected by three LNR domains, suggesting that a physical pulling force is required to expose this site for accessibility of the ADAM protease (52-54). In addition, most secreted forms of DSL ligands act in a dominant-negative fashion to prevent ligand-mediated activation of Notch (55, 56). However, when soluble ligands are crossed-linked, immobilized, or clustered, they can activate Notch signaling in cultured cells (57, 58). This evidence supports the theory that tension and force generated from the interaction between the receptors and ligands is necessary for Notch activation.

Receptor endocytosis

Although NICD is polyubiquitinated and degraded in a proteasome-dependent manner in the nucleus, a substantial amount of intact Notch is present in endosomes (59). Intact Notch receptors are constitutively endocytosed and recycled to the cell surface (60) or degraded in the lysosome (61). Studies from multiple groups indicate that endocytosis of Notch receptors could control their availability for ligand binding and for S3 cleavage. Production of NICD may occur on lysosomal membranes bypassing the requirement for ligand interaction as well as S2 cleavage (62). Disruption of lysosomal degradation of Notch receptor leads to accumulation of Notch receptor in endosomes and ectopic activation of Notch signaling in a ligand-independent manner (63-65). Several components in the pathway of lyosomal degradation of Notch have been reported to be associated with tumor progression, indicating that ligand-independent constitutive activation of Notch signaling in endosomes is important in certain cancers (66).

Notch signaling in development

A short history of Notch

Homozygous mutations of *Notch* result in lethal phenotypes via neurogenic aberrations, where cells intended to differentiate into epidermis, switch fate and give rise to neural tissue (70, 71). Moreover, Notch is involved in many other developmental steps in *Drosophila*, such as bristle formation (72), and maintenance of muscle founder cells (73). The studies of the nematode *Caenorhabditis elegans* increased our knowledge of Notch signaling, in which Notch signaling plays important roles in specifying cell fate decisions as well. Unlike *Drosophila*, *C. elegans* has two Notch homologues, LIN-12 and GLP-1. Both can substitute for each other when expressed in the appropriate tissue (1). In contrast,

LIN12 is important for gonad development in later stage of growth (74), whereas GLP-1 regulates blastomere specification in the early *C.elegans* embryo (75).

Ablations of Notch components in genetically engineered mice

The function of the mammalian Notch signaling has been investigated using mice with genetically engineered gene knockouts of Notch components. These studies prove the importance of Notch signaling in the development of mammals.

Notch1: *Notch1* null mice are embryonic lethal, and embryos die before embryonic day 11.5. Lack of Notch1 causes a delayed and disorganized somitogenesis in the first half of gestation and enhanced neurogenesis by regulation of neural stem cell differentiation. Increased cell death is also observed, but is not considered to be the main cause of developmental defects (76-78). Further study of knock-in mice with a single point mutation at intramembranous processing site of Notch1, V1744G recapitulates the *Notch1* null pheynotypes, suggesting that intramembranous processing of Notch1 is indispensable for embryonic viability and early development (79).

Notch2: By replacing ankyrin repeats of Notch2 receptor with a beta-galactosidase, a mutant Notch2 mouse was created. Notch2 mutated mice die before E11.5, suggesting an indispensable function of ankyrin repeats in Notch2. In contrast to Notch1 mutant, *Notch2* mutant mice did not show disorganized somitogenesis, while increased cell death is also observed (80). Using a hypomorphic allele rather than a real null allele of *Notch2*,

McCright et al. demonstrated a perinatal lethality due to dysfuntion of kidney, and defects of heart and eye vasculature (81).

Notch3: *Notch3* deficient mice are viable and fertile. There is no apparenten redundant with the Notch1 gene during early embryogenesis (82). Later study elucidated that *Notch3*$^{-/-}$ adult mice show dysfunctional arteries in mice due to dysregulation of arterial differentiation and immaturation of vascular smooth muscle cells (vSMC) (83).

Notch4: Notch4 null mice are also viable and fertile. However, embryos homozygous for mutations of both *Notch4* and *Notch1* genes displayed a more severe phenotype than *Notch1* homozygous mutant embryo with severe defects in angiogenic vascular remodeling (84).

Jagged1: *Jagged1* disrupted mice die prior to E11.5 from hemorrhage early during embryogenesis, displaying defects in remodeling of embryonic and yolk sac vasculature. Heterozygous *Jagged1*$^{+/-}$ mice display an eye phenotype similar to that in Alagille's syndrome, but do not exhibit other characteristics of the disease (85). Moreover, double heterozygote mutants *Jagged1*$^{+/-}$ *Notch2*$^{+/-}$ exhibit more severe phenotypes than the single mutants (81).

Jagged2: Mice with DSL-domain deletion mutant of *Jagged2* die at birth with severe craniofacial and limb malformations. The mutant homozygotes exhibit cleft palate and fusion of the tongue with the palatal shelves, and digit fusion of fore-and hindlimbs (86).

DLL1: *DLL1* null mice die around day 12 of embryonic development, exhibiting severe segmentation defects and loss of the ability to maintain the integrity of the somites (87), which is similar to that with *Notch1* deficiency.

DLL3: *DLL3* knockout mice are viable but have a shortened body and short tail. Gene targeting of *DLL3* results in severe axial skeletal defects with highly disorganized vertebrae, costal defects, which are similar to the phenotypes of spondylocostal dysplasis in humans, and delayed and irregular somite formation (88).

DLL4: Homozygous deletion of *DLL4* is lethal before E9.5 and only small portion of heterozygous mice are viable. The incompletely penetrant haploinsufficiency depends on the genetic background of mice. $DLL4^{+/-}$ embryos show defects in vascular structures and reduction of the caliber of the dorsal aortae. DLL4 ligand alone is required in a dosage-sensitive manner for normal arterial patterning in development, suggesting that DLL4 may be a suitable target for intervention in arterial angiogenesis (89).

RBPj κ: *RBPj κ* null mutant mice showed embryonic lethality before 10.5 days of gestation and exhibited severe growth retardation as early as 8.5 days of gestation. Developmental defects include incomplete turning of the body axis, microencephaly, abnormal placental development, anterior neuropore opening and deficient somitogenesis (90).

Notch signaling in mammlian lung development

Lung development in the fetus origdinates from a primitive foregut endodermal bud surrounded by mesenchyme to become a highly branched tracheobronchial tree with bunch of specialized cell types, including ciliated and goblet cells in the proximal airway, Clara cells in the mid-sized and smaller airway, interspersed neuroendocrine and basal cells, and type I and II alveolar cells, plus endothelial cells, smooth muscle, neurons, chondrocytes and fibroblasts (91). Quantitative expression analyses from the developing mouse lung elucidate an increased expression of Notch1-4, DLL1 and Jagged1 mRNA from E11.5 into adulthood (92, 93). Notch1 is expressed in the distal lung endoderm as early as E11.5 and persists through fetal development, but is not expressed at high levels in fetal lung mesenchyme surrounding the primitive epithelium (92, 94). Both Notch2 and Notch3 are expressed in lung mesenchyme. Notch2 does not appear to be expressed in epithelial cells, whereas Notch3 can be detected in epithelial cells. Notch4 expression is endothelial specific. None of the four Notch receptors is found to be expressed in neuroendocrine cells (94). Expression of Notch ligands can be identified in the mesenchymal and neuroendocrine cells. Jagged1 is expressed in lung mesenchyme and prominently in lung vessels, while Jagged2 expression appears to be limited to the most peripheral lung mesenchyme at E13 (94). DLL4 expression in the lung is confined to endothelial cells, potentially to interact with Notch4 (95). In the lung, DLL1 mRNA expression increases in abundance from E12 to E18 (92), and is strikingly limited to neuroendocrine cells, first confirmed at E14.5 (94, 96). Notch target gene Hes1 mRNA has been detected in mouse lung of early pseudoglandular stage at E12, then its expression progressively increases until birth and also remains detectable in adult lung. Hes1 protein expresses in non-endocrine

airway epithelial cells, which express Notch1 and Notch3 as well. Moreover, most Hes1 expressing cells in the distal airway epithelium are destined to become Clara cells, suggesting that Notch signaling activity appears to predetermin the Clara cell lineage in the lung. Another Notch target gene Hes5 is not detectable in whole fetal lung (92). HeyL is expressed in lung vasculature (97), Hey1 mRNA is dominantly expressed in adult lung, and Hey2 expression is much lower than Hey1 (98).

A growing body of evidences elucidates the importance of Notch signaling in the development of respiratory systems. A target of Notch signaling, *Hes1*-deficient mice show hyperplasia of pulmonary neuroendocrine cells (PNECs) and a decreased number of Clara cells, indicating the precursors of Clara and ciliated cells are separated from PNEC precursors through lateral inhibition feedback loop mediated by Notch signaling (92, 99). Forced expression of a constitutively activated form of Notch1 receptor (N1ICD) in lung epithelial cells promoted mucous metaplasia and significantly reduced the number of ciliated cells (100). Conditional deletion of *OFUT1*, a glycosyltransferase required for Notch signaling, or *RBPj κ*, an essential DNA binding partner of all Notch receptors, in the endoderm promoted ciliated cell expansion and caused absence of secretory Clara cells (101). By preventing γ-secretase cleavage of Notch receptors, Tsao et al. demonstrated that disruption of Notch signaling remarkably expands the population of distal progenitors, altering morphogenetic boundaries and preventing formation of proximal structures (102). By deletion of RBPj κ in lung mesenchymal and mesothelial cells, Kopan's group has demonstrated that RBPj κ is required for the recruitment and specification of arterial vascular smooth muscle cells (vSMC) and for regulating mesothelial epithelial-

mesenchymal transition (EMT). vThey also conclude that primary roles for canonical Notch signaling in lung development are in selection of Clara cell fate and in vSMC recruitment (103). Forced expression of Notch3 intracellular domain (N3ICD) in peripheral epithelium resulted in perinatal lethality with phenotypes of altered lung morphology and delayed lung development. In N3ICD transgenic mice, metaplasia of undifferentiated cells in the terminal airways was observed, and the majority of the epithelial cells are undifferentiated, with some maturation of type II pneumocytes but no type I alveolar cells (104). These data strongly suggest the importance of Notch signaling in lung development.

The Notch signaling in human cancers

In the last two decades, evidence has accumulated on the deregulation of Notch receptors, ligands, modulators and downstream targets in an extensive number of solid tumors and subsets of hematopoietic malignancy. Notch signaling can have opposing roles in human tumorigenesis dependent of the cancer types. Even in the same type of cancer, the role of Notch signaling is complicated and highly dependent on the spatial and temporal context of Notch activation as well as other signaling pathways in the cells (Table 1.2).

Notch genes as oncogenes

Notch signaling plays an oncogenic role in various human cancers (Table 1.2). Here we mainly emphasized on Notch signaling in T-cell acute lymphoblastic leukemia (T-ALL) and solid tumors including breast cancer, pancreatic cancer, ovarian cancer and colorectal cancer. The oncogenic role of Notch signaling in lung cancer will be discussed separately.

T-ALL

The oncogenic role of Notch was first identified in human T-ALL, in which a t(7;9)(q34; q34.3) chromosomal translocation has been identified in a subset of these leukemias. The gene at the chromosome 7 locus fused to the T-cell-receptor- β (TCR β) promoter/enhancer is very similar to *Drosophila* Notch so as to get its name TAN1 (translocation-associated Notch homologue), which later became known as human Notch1. This translocation results in constitutive expression of active form of Notch1, N1ICD (105, 106). Indeed, Notch1 signaling drives hematopoietic progenitor cells into the T-cell lineage (107), and mice reconstituted with hematopoietic progenitor cells expressing TAN1 proteins develop T-cell leukemia (108). In contrast, loss of Notch1 in bone marrow progenitor cells showed a cell autonomous blockage in T cell development at an early stage (109). Subsequently, Weng et al identified activating *Notch1* mutations in 56% of T-ALL patients. Sequencing of T-ALL cell lines and patient samples revealed that the majority of *Notch1* mutations are located in two regions, the HD, which causes ligand-independent activation of signaling, and PEST domain, which results in prevention of NICD from proteosome degradation (110, 111). Notch1 has been found can cooperate with c-Myc (112), E2A-PBX1 (113) and Ikaros (114) to induce T-ALL. Consistently, forced expression of the Notch ligand DLL4 also results in the development of T-cell leukemia/lymphoma (115, 116). Notch signaling not only plays important roles for initiation of T-ALL, but also is required for maintenance of T-ALL that continued growth and survival of Notch1-transformed lymphoid cell lines require nuclear access and transcriptional coactivator recruitment by Notch1 (117). In addition to Notch1, other Notch isoforms can also be oncogenic in T-ALL. Rohn et al. showed that recombinant feline leukaemis virus (FeLV) provirus isolated from FeLV-

induced lymphomas in cats contained active Notch2 sequence, suggesting a correlation between Notch2 and leukaemogenesis (118). Dysregulation of Notch3 signaling has been also proposed to be important in T-ALL due to the fact that forced expression of N3ICD in T cells results in multi-organ infiltration of T lymphoblasts and death at 10-14 weeks of age, and Notch3 has been shown to be highly expressed by T-ALL cells and reduced level of Notch3 was found to correlate with disease remission, indicating that Notch3 may be able to induce T-cell leukemia similar to Notch1 (119, 120). Furthermore, Masiero et al. showed that Notch3 promotes survival of T-ALL cells through regulating MKP-1 levels (121). In addition to the mutation of *Notch1* in T-ALL, around 20% of T-ALL patients harbor inactivating mutations of an E3 ubiquitin ligase *FBW7* (F-box and WD repeat domain containing 7), which could potentially function as a tumor suppressor by upregulating the expression of NICD (122, 123).

Breast cancer

The first evidence describing function of Notch signaling in solid tumor came from the observation that integration of the mouse mammary virus (MMTV) into the *Notch4* locus results in a truncated Notch4 mRNA named int3 representing a gain-of-function mutation, and results in the formation of mammary tumors (124-128). This phenomenon was confirmed by studies of transgenic mice with activated form of Notch4 under control of MMTV long term repeat or the whey acidic protein (WAP) demonstrating that 100% of female mice developed mammary tumors (129, 130). Besides Notch4, similar gain-of-function Notch1 truncations were also found by MMTV integration, which further accelerated *MMTV-Nue* induced mammary tumors (131). *MMTV-N1ICD* transgenic

female mice developed lactation-dependent papillary tumor by their third pregnancy, which were non-invasive and regressed upon gland involution, but progressed to invasive adenocarcinomas in subsequent pregnancies. In addition, microarray analysis of N1ICD and c-myc induced tumors reveals a high profile similarity, further suggesting that *c-myc* is a direct target of Notch1 in mammary tumors (132). Notch signaling in mouse mammary tumorigenesis also showed relevance to human breast cancer development. Activated forms of Notch1 and Notch4 have been identified in several human breast cancer cell line (133, 134). Notch3 has been shown to play an important role in the proliferation of ErbB2-negative breast cancer cell lines (135). Further, high levels of Jagged1 and Notch1 expression correlate with poor survival and they are independent prognostic markers in human breast cancer (136, 137). In addition, increased accumulation of N1ICD and Hes1 in ductal carcinoma in situ (DCIS) compared with normal breast tissue can predict reduction of recurrence time 5 years after surgery (138). Interestingly, Notch2 appears to antagonize signals induced by other three Notch receptors in breast cancer cells (139). Consistently, high level of Notch2 expression in breast tumors correlated to higher chance of survival, whereas high level of Notch1 expression seems to be associated with a poorer outcome (140). In primary breast cancer, Weijzen et al. showed increased expression of Notch1 in four breast cancer tumors overexpressing H-Ras, which revealed that Notch1 is a downstream target of oncogene H-Ras and implied that Notch1 is essential for H-Ras transformed breast tumor (141). This scenario is further supported by the study that in the majority of double transgenic mice expressing both v-Ha-ras under control of MMTV promoter and Deltex1, a negative regulator of Notch signaling, palpable mammary tumors were not detectable (142). Additionally, another Notch negative regulator Numb has been

identified to be lost in approximately 50% of human breast cancer (143). In estrogen receptor (ER)-negative breast cancer cells, activation of Notch signaling leads to direct transcriptional up-regulation of the apoptosis inhibitor and cell cycle regulator survivin to enhance cell proliferation, and treatment with a γ-secretase inhibitor (GSI) induced apoptosis and inhibited local and metastatic mammary tumor growth in mice (144). In contrast, in ER positive breast cancer, Rizzo et al. demonstrated that estrodiol could inhibit Notch activity, and Notch inhibition fostered the effects of tamoxifen (145). Taken together, these studies strongly indicated the oncogenic role of Notch signaling in breast cancer.

Pancreatic cancer

Recently, there is increasing evidence to link Notch directly to pancreatic cancer. Various Notch receptors, ligands and downstream target genes have been shown to be expressed in pancreatic intraepithelial neoplasia (PanIN) as well as in pancreatic ductal adenocarcinoma (PDAC) tissue of mice and human (146, 147). Plentz et al. analyzed the responsiveness to a GSI (MRK003) in more than 400 human cancer cell lines, and found that remarkably 50% of 26 PDAC cell lines tested were sensitive to the inhibitor, suggesting that Notch signaling is essential in PDAC (148). In many genetically engineered mouse models for PDAC, expression of Notch1 and its downstream effector Hes1 are increased in PanIN and fully developed PDACs (149, 150). Simultaneous expression of activated form of Notch receptor with an oncogenic form of KRAS in either pancreatic progenitors or mature acinar cells leads to developing of PanIN lesions earlier than expression of either genes alone, suggesting that Notch and KRAS synergize to initiate pancreatic tumorigenesis (149).

Ovarian cancer

Notch3 amplification was identified in around 19% of human ovarian cancer patients, and inactivation of Notch3 by both GSI and Notch3-specific siRNA suppressed cell proliferation and induced apoptosis in the cell lines with Notch3 amplification (151). Recently, a comprehensive and integrated genomic analyses of 489 ovarian tumors revealed that Notch signaling pathway is highly activated in ovarian cancer patients, in which Notch signling was altered in around 22% of ovarian cancer samples, further suggesting importance of Notch signaling in human ovarian cancer (152). Moreover, Notch3 protein expression was significantly associated with advanced stage, lymph node, and distant metastasis of ovarian cancers (153). In addition, Notch3 expression is associated with recurrent postchemotherapy high-grade serous carcinoma (HGSC) and knockdown of Notch3 in the ovarian cancer cell line OVCAR3, which expressed abundant Notch3, made it resensitized to chemo-agents carboplatin (154). Jagged1 has been identified as the primary Notch3 ligand in ovarian cancer cells and Jagged1/Notch3 interaction constitutes a juxtacrine loop promoting proliferation and dissemination of ovarian cancer cells within the intraperitoneal cavity (155). Besides *Notch3* amplification, an activated form of Notch1 is also frequently expressed in human ovarian specimens as well as ovarian cancer cell lines, and depletion of Notch1 results in growth inhibition of ovarian cancer cells (156). Consistently, transfection of the activated form of Notch1 leads to a proliferative and colony formation advantage of ovarian cancer cells (157). Overall, these data suggest that Notch1, Notch3 and Jagged1 are oncogenic in ovarian cancer.

Colon cancer

The coordination of Wnt and Notch signaling pathway is essential for regulation of colonic progenitor cell division and differentiation (158), which suggests that these may also play an important role in intestinal tumorigenesis. Indeed, Fre et al. had demonstrated that Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine (159). Moreover, it has been shown that Notch ligand Jagged1 is transcriptionally regulated by Wnt/β-catanin pathway in colorectal cancer cells, and expression of N1ICD partially reverts the effects of blocking Wnt/β-catanin pathway in tumors implanted subcutaneously in nude mice. Crossing *APC (Min/+)* with *Jagged1* hemizygous mice is sufficient to remarkably reduce the size of the polyps arising in the *APC* mutant background, implying that Jagged1 mediated Notch signaling is an essential modulator of tumorigenesis induced by nuclear β-catenin (160). In addition, Notch signaling has been shown to suppress transcription of Kruppel-like factor (KLF4), which could inhibit cell proliferation, in colon cancer cells and treatment of *APC (Min/+)* mice with GSIs resulted in a 50% reduction in the number of intestinal adenomas, and increase of Klf4 expression (161). In human colorectal cancer, the vast majority of 130 colorectal cancer tissues expressed Notch downstream effector Hes1 mRNA (162), and somatic mutations of *FBXW7* gene were found frequently in human colorectal cancer as well (163). Most recently, Guilmeau et al. also demonstrated that in human small intestinal and colonic epithelium, Jagged1 expression was restricted to enteroendocrine cells or was undetectable, respectively, but was elevated in about 50% of human colon tumors (164). In short, these studies support the notion that many of colorectal tumors, like the APC (Min/+) derived tumor, may respond to anti-Notch or anti-Jagged1 therapy.

Table 1.2. Multiple roles of Notch signaling in solid tumors

Tumor Type	Oncogenic	Tumor suppressive	Tumor progression	Tumor maintenance	Drug resistance
Lung	√	√	√		
Breast	√	√	√	√	√
Colorectal	√		√		
Cervical	√		√		√
Pancreatic	√		√		√
Liver		√	√		√
Medulloblastoma			√	√	
Glioblastoma		√	√	√	√
Prosate		√	√		
Melanoma			√	√	
Head and neck					√
Oral SCC	√	√			
Skin		√			

Adopted from Ranganathan et al., Nature Review Cancer, 2011.

Notch genes as tumor suppressors

Studies on skin cancers have provided evidences that Notch signaling can also be a tumor suppressor. *Notch1*-deficient mice develop spontaneous basal-cell-carcinoma-like tumors over time (165). Additionally, in the same study the authors had shown that *Notch1* deficiency in the skin facilitates chemical-induced carcinogenesis. To determine what mechanism underlies the tumor suppressive property of Notch1 in skin, Nicolas et al. demonstrated that the absence of Notch1 in skin results in a downregulation of Waf1, which leads to increased sensitivity to chemical-induced carcinogenesis. Furthermore, absence of Notch1 in the mouse epidermis leads to aberrant expression of a Sonic Hedgehog downstream effector Gli2, which plays important roles in the regulation of basal-cell carcinoma. Also they found that in the skin of *Notch1*-deficient mice, Wnt pathway is re-activated, which is also associated with basal-cell carcinomas (165). Consistent with mice data, reduced expression level of Notch1, Notch2 and Jagged1 were found in human basal-cell carcinoma (166).

In prostate cancer cells, overexpression of a constitutive activated form of Notch1 inhibits proliferation of various prostate cancer cells (167). And ectopic activation of Notch inhibits cell proliferation concomitantly with an induction of PTEN level, indicating that Notch positively regulates expression of PTEN (168). In liver cancer, Notch1/Jagged1 were frequently low expressed in hepatocellular carcinoma and correlated with the high expression of β-catenin suggesting that downregulation of Notch1/Jagged1 signaling may sustain tumor progression (169).

Notch ligand DLL4 has emerged as an attractive target for tumor angiogenesis. However, chronic inhibition of DLL4 by a DLL4 antibody resulted in pathological activation of endothelial cells, disrupts normal organ homeostasis and induces vascular neoplasmas (170), which not only arises the safety concern of chronic DLL4 blockade, but also implicates that DLL4-Notch signaling may have tumor suppressive function in vascular neoplasmas.

In a recent study, Aifantis's group deleted the Nicastrin (*Ncstn*) gene, which is a member of the γ-secretase complex and one of the few non-redundant members of the pathway in haematopoitic cells, resulted in the development of a myeloproliferative/ myelodysplastic process, reminiscent of human chronic myelomonocytic leukaemia (CMML). Moreover, triple deletion of *Notch1*, *Notch2* and *Notch3* phenocopied the *Ncstn*-deficient phenotypes, suggesting that Notch signaling also can play tumor-suppressive roles within the hematopoietic system. Consistently, they identified novel somatic inactivating Notch pathway mutations, including *NCSTN*, *APH1*, *MAML* and *Notch2*, in a subset of patient with CMML, which further confirmed the tumor suppressive role of Notch signaling in CMML (171).

Notch in tumor progression

Notch signaling in cancer stem cell (CSC)

Many cancers seem to contain a small population of pluripotent tumor initiating cells, also called cancer stem cells (CSCs) (172). The cancer stem cell hypothesis states that CSCs

possess some of the biological properties of normal stem cells, such as indefinite self-replication, asymmetric cell division, and resistance to cytotoxic agents. Signaling pathways essential for embryonic development and cell fate decision are considered to be important in maintenance of CSCs. Notch signaling has been well studied for its roles in CSCs in breast cancer, medulloblastoma, glioma, and lung cancer. In breast ductal carcinoma in situ (DCIS), the ability to form mammospheres is significantly reduced by GSIs or a Notch4 neutralizing antibody, suggesting that Notch4 signaling is important in maintenance of CSCs of DCIS (138). In mammospheres of human breast cancers, Sansone et al. demonstrated that autocrine IL-6 signaling maintains CSCs is through induction of Notch3 signaling to promote a hypoxia-resistant phenotype (173). Indeed, p66Shc-Notch3 pathway has been reported to be an essential factor to maintain the hypoxia-resistant phenotype of human breast cancer mammospheres (174). In medulloblastoma and glioblastoma, GSIs selectively deplete CD133-high cells, which is commonly used as a marker of CSCs (175, 176). Moreover, Wang et al. showed that inhibition of Notch pathway with GSIs renders the glioma stem cells sensitive to radiation by enhancement of radiation-induced cell death and impairment of clonogenic survival of glioma CSCs but not non-stem glioma cells, which was further confirmed by abrogation of Notch1 or Notch2 receptors (177). In pancreatic cancer, activation of Notch signaling promotes epithelial-mesenchymal transition (EMT), which is consistent with CSCs phenotype and contributes to drug resistance (178, 179). In lung cancer, Sullivan et al. used Aldehyde dehydrogenase (ALDH) activity as an indicator to select stem cells of lung adenocarcinoma, and further demonstrated that inhibition of Notch signaling via a GSI or Notch3 shRNA significantly decreased percentage of ALDH-positive lung cancer stem cells, implicating importance of

Notch signaling, especially Notch3 in the maintenance of lung CSCs (180). And Yang et al. using mouse adenocarcinoma cells from *KRAS* and *TP53* mutant mice, demonstrated that CD133-positive cells have higher Notch receptors and ligands expression compared with CD133-negative cells, indicating the importance of Notch signaling in the maintenance of stemness of lung adenocarcinoma (181). By using neutralizing antibody against human DLL4 to treat mouse xenograft tumors, which does not cross react with mouse DLL4, Hoey et al. identified that inhibition of DLL4 in tumor cells substantially reduced colon cancer stem cell frequency (182). These data suggest that therapeutic targeting Notch signaling could be used in clinic to target CSCs, so as to reverse chemo- or radio-resistance of many kinds of cancers.

Notch in EMT and metastasis

The processes of Epithelial-Mesenchymal Transition (EMT) is a unique process by which epithelial cells undergo remarkable morphologic changes characterized by a transition from epithelial cobblestone phenotype to elongated mesenchymal phenotype leading to increased motility and invasion (183). The same signaling pathways govern the processes of EMT in development as well as tumor metastasis (184). A hallmark of EMT from an *in situ* to an invasive carcinoma is loss of expression of the adhesion molecule E-cadherin, the expression of which is inversely correlated with cancer grade and patient survival (185, 186). Zinc finger transcriptional repressors Snail and Slug bind to E boxes located proximal to the transcription start site of the *E-cadherin* gene to suppress transcription of E-cadherin (187). During development, mice with a targeted mutation in the Notch1 receptor or Rbpj κ effector exhibits severely attenuated cardiac snail expression, abnormal

maintenance of intercellular endocardial adhesion complex, and abortive endocardial EMT *in vivo* and *in vitro*. Consistently, overexpression of N1ICD in immortalized porcine aortic endothelial cells induces both EMT and oncogenic transformation, and leads to induction of snail and repression of VE-cadherin. In the study, N1ICD activation of the Snail promoter is dependent of Rbpj κ, but the snail promoter itself lacks Rbpj κ binding sites, suggesting that Notch regulates Snail promoter activation may be indirect (188).

In human cancers, Notch signaling drives EMT by upregulation of Snail, Slug and ZEB as well as interaction with TGF β signaling. Lendahl's group had demonstrated that Notch signaling is required to convert the hypoxic stimulus into EMT, increased motility, and invasiveness by upregulation of Snail (189). Moreover, it had been reported that another EMT inducer Slug is a downstream target gene of Notch as well and is upregulated in Jagged1 and Notch1 positive human breast cancers (190). Pancreatic cancer cells that are gencitabine-resistant (GR) acquired an EMT phenotype as evidenced by elongated fibroblast morphology with downregulation of E-cadherin, and upregulation of ZEB1 (191, 192). Notch2 and its ligand Jagged-1 are remarkably upregulated in GR cells, and attenuation of Notch signaling by siRNA led to partial reversal of the EMT phenotype associated with decreased expression of vimentin, ZEB1, Slug and Snail, suggesting a role of Notch signaling in the acquisition of EMT in pancreatic cancer (178). TGF-β signaling is involved in the promotion of EMT by extensive communication with other signaling pathways, including Notch. TGF-β-induced EMT was attenuated by silencing Hey1, Jagged1, or treatment with GSIs, suggesting the roles of Hey1, Jagged1/Notch in mediating TGF-β signaling-induced EMT (193).

Metastasic disease is the major cause of cancer-associated death. The roles of Notch signaling in the regulation of cancer invasion and metastasis have also been documented. In 154 resected human prostate cancers, high Jagged1 expression is associated with increases in metastases and tumor recurrence, with the pro-metastatic property of Jagged1 thought to be mediated by the induction of EMT (194). In osteosarcoma, osteosarcoma cell lines with the ability to metastasize have higher levels of Notch 1, Notch 2, DLL1 and Hes1 compared to normal human osteoblasts and non-metastatic osteosarcoma cell lines. When invasive osteosarcoma cells are treated with GSIs, invasiveness is abrogated. In a novel orthotopic murine xenograft model of osteosarcoma pulmonary metastasis, blockade of Hes1 expression and Notch signaling eliminated spread of disease from the tibial primary tumor (195). Recently, Sonoshita et al. demonstrate that in their Colon26 transplantation model, Notch receptors are expressed on cancer cells, whereas the ligands are found on stromal cells. The activation of Notch signaling is mainly found in cancer cells in the vicinity of blood vessels in primary tumors, while in metastatic lesions, activated Notch signaling are either in micrometastasis or in the outer rim of large metastasis next to stromal cells, suggesting that Notch activation by tumor-stroma interaction is critical for cancer cell intravasation. Inhibition of Notch signaling by GSIs repressed tumor invasion driven by loss of Aes/Grg5, suggesting that Aes/Grg5 suppressing colon cancer metastasis is mainly by preventing Notch signaling and Notch mediated local invasion and intravasation (196). Their results suggest that inhibition of Notch signaling can be a promising strategy for prevention and treatment of colon cancer metastasis. Tumor-derived Jagged1 had been shown to promote osteolytic bone metastasis of breast cancer by engaging Notch signaling in bone cells, in which Jagged1 enhances

tumor growth by stimulation of IL-6 release from osteoblasts and directly activates osteoclast differentiation. Indeed, GSIs treatment reduces Jagged1-mediated bone metastasis by interfering with the Notch signaling in stromal bone cells, providing a rationale for targeting Notch signaling for breast cancer bone metastasis (197).

Notch in tumor angiogenesis

Components of the Notch signaling pathway have been identified in endothelial cells *in vitro* and *in vivo* during embryonic development and tumor angiogenesis (198). Of the Notch receptors, endothelial cells express Notch1 and Notch4; among the DSL ligands, endothelial cells express DLL1, DLL4, Jagged1 and Jagged2 (199-201). Other key signaling components, including Rpbj κ (202), Hey1, Hey2 (203), Maml (204) are expressed in endothelial cells. Functional studies using gene knockout in mice, mutagenesis and knockdown in zebrafish, and biochemical analysis in cultured endothelial cells have shown that Notch signaling plays a fundamental and crucial role during tumor angiogenesis, which suggests that Notch signaling could be an attractive target for tumor angiogenesis.

In endothelial cells, Notch signaling plays a central role in the cell specification, proliferation, cell motility and cell adhesion. Mosaic analysis of endothelial cells deficient in Notch signaling in mice and zebrafish illustrated that Notch is required cell autonomously for stalk cell specification by actively suppressing the tip cell phenotype (205). In mice, mosaic endothelial *Cre* recombination of a floxed *Notch1* allele showed that the majority of *Notch*-null endothelial cells adopt tip cell properties. Conversely,

ectopic activation of Notch signaling in the mouse retina by injection of the Jagged1 peptide leads to reduced tip cell formation and filopodia extension (206). Studies in several mouse tumor models supports the hypothesis that tip-stalk specification regulated by Notch signaling controls the branching frequency of tumor blood vessels as well. Transplantable tumors in DLL4 heterozygous hosts show significantly increased sprouting angiogenesis. Furthermore, inhibition of Notch signaling by GSIs, DLL4 or Notch1 blocking antibodies results in similar phenotypes. Analysis of tumor growth reveals that the increased vascularization upon inactivation of DLL4/Notch signaling paradoxically causes reduced tumor growth due to unproductive angiogenesis (207). Similarly, increased endothelial Notch signaling triggered by DLL4 expressing tumor cells resulted in reduced vascular branching and density, but enhanced vessel diameter, perfusion, and hence promoted tumor growth (208, 209). Overall, these studies support the concept that Notch coordinated the balance of tip and stalk cell numbers, which is required for effective vascular patterning and function in angiogenesis. However, the exact mechanism for Notch suppresses tip cell formation is not fully understood.

Notch in drug resistance

Cancer cells usually develop resistance to chemotherapeutic and targeted therapeutic agents mainly by activation of survival signaling or by inhibition of apoptosis. Notch signaling is a major regulator of cell survival and has been shown to be very important in drug resistance of multiple cancers. For example, in colorectal cancer, Notch signaling and PI3K-AKT signaling are activated by the treatment with the chemotherapeutic agent oxaliplatin, and inhibition of Notch signaling by GSIs resensitizes cells to oxaliplatin

(210), and combination of GSI and cisplatin elicits a striking induction of cell death (211). Furthermore, blockade of Notch signaling by GSIs also enhance taxane/ taxol-induced mitotic arrest and apoptosis of colon cancer cells both *in vitro* and *in vivo,* indicating that GSIs could be used in combination with these chemotherapeutics to overcome resistance colon cancers (212). Upregulation of Notch1 was found in cisplatin-resistant HNSCC patients and cisplatin resistance of HNSCC was reduced upon inhibition of Notch signaling by GSIs (213). In addition, Notch3 was upregulated and contributed to doxorubicin resistance in human hepatocellular carcinoma (HCC) via regulation of p53 expression and DNA damage machinery, implying that inhibition of Notch3 signaling in combination with chemotherapy could conceivably provide a novel approach for HCC (214). In Trastuzumab, a HER2 neutralizing antibody, resistant HER2-positive breast cancer, Notch activity is upregulated when cells are treated with trastuzumab and treatment with a combination of trastuzumab and a GSI further induced apoptosis in these cells (215). In addition, treatment with tamoxifen in estrogen receptor (ER)-positive breast cancer cells activated Notch pathway so as to activate survival signaling pathway, in which Notch-1 increased the transcription of ERα -responsive genes in the presence or absence of estrogen via a novel chromatin crosstalk mechanism, suggesting that combined inhibition of Notch and estrogen signaling has synergistic effects in ERα -positive breast cancer models (216).

Notch signaling in lung cancer

Lung cancer is the leading cause of cancer-related deaths in the United State. The five-year survival rate for patient with lung cancer remains low-15%-and has not changed significantly during the past 30 years (217). Lung cancer can be classified as non-small

cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC representing the majority of lung cancers. Although lung cancer is strongly associated with tobacco exposure, there is an increasing NSCLC morbidity in patients who have never smoked. In the past two decades there has been a growing body of evidence underscoring the importance of developmental pathways in tumorigenesis, such as Notch, Wnt and Hedgehog. Two Notch paralogs have been implicated in lung cancer tumorigenesis, Notch1 and Notch3. The role for Notch1 in NSCLC has been controversial. Forced expression of constitutively activated Notch1 inhibited growth of the lung adenocarcinoma cell line A549 in culture and suppressed tumor growth in an *in vivo* xenograft model (218). However, under hypoxia condition, Notch1 provides a critical survival signal to NSCLC cells. Moreover, inhibition of Notch1 signaling in NSCLC cells either through genetic downregulation or using a GSI resulted in apoptosis only under hypoxia condition (219). Notch1 activating mutations has been found in 10% of NSCLC patients. These mutations correlated with a worse prognosis in patients with wild-type p53. Moreover, downregulation of Numb, a negative regulator of Notch signaling was detected in approximately 30% of NSCLC. Primary cells from patients with either *Notch1* mutation or low expression of Numb are more sensitive to GSIs (220). A recent study conducted by Bocchetta's group demonstrated that Notch1 signaling in hypoxic NSCLC microenvironment appears to promote cancer cell survival by dual mechanisms: inhibition of PTEN expression and positive regulation of IGF-1 and its receptor, IGF-1R (221). The connection between Notch1 signaling and hypoxia has been independently shown in A549 cells by Chen et al, in which they also demonstrated that Notch1 signaling upregulates of survivin expression in lung cancer synergistically with HIF-1 alpha (222). These data

suggest that Notch1 appears to be an attractive target in hypoxic tumor regions, which are thought to promote resistance to standard chemotherapy. In addition, a recently study has linked Notch1 and EGF signaling in NSCLC, in which the authors demonstrated a novel molecular circuitry in NSCLC where ADAM17 up-regulates EGFR expression through the activation of Notch1, and that proliferation, survival and colony formation of Notch1 deficient NSCLC cells were insensitive to EGF stimulation. Indeed, there is a significant correlation between Notch1 and EGFR overexpression in NSCLC specimens, but not in normal lung (223). Given their functions in the survival of NSCLC, both ADAM17 and Notch1 constitute promising targets for the treatment of NSCLC.

Notch3 has been considered as an oncogene in NSCLC as well. In an early study from our group, a chromosomal translocation t(15;19) was initially identified in an aggressive lung cancer metastatic to mediastinum and bone arising in a 34-year-old woman without a history of smoking or a family history of cancer. The cell line isolated from this patient, HCC2429 harbors this translocation and causes high expression of full-length Notch3 mRNA. Using Northern blot hybridization, our group also identified seven out of 44 NSCLC cell lines expressed the Notch3 mRNA (224). Furthermore, immunohistochemistry analysis showed that 80 out of 207 resected lung cancers expressed Notch3. The function of Notch3 in NSCLC has been demonstrated by expression of a dominant negative form of the Notch3 receptor lacking the intracellular portion of the protein in NSCLC HCC2429 and H460, which caused reduced growth and increase growth factor dependency of these two cell lines (225). The oncogenic role of Notch3 in NSCLC is further demonstrated by the treatment of a GSI, MRK003 in NSCLC, in which Konishi

et al. demonstrated that treatment with the MRK003 in NSCLC cell lines HCC2429 and H460 resulted in induction of apoptosis and decrease of colonies in soft agar. *In vivo* treatment of MRK003 in xenograft mouse model generated with HCC2429 and H460 cell lines significantly suppressed tumor growth, while the effect was very limited with A549 cells. Genetic inhibition of Notch3 in HCC2429 cell by siRNA converts the cell to be GSI-insensitive, indicating that Notch3 but not Notch1 is the right target of GSI in the inhibition of NSCLC tumor growth (226). EGFR signaling pathway plays a very pivotal role in NSCLC progression. The link between Notch3 and EGFR has been proposed as well, which emphasizes importance of Notch3 in NSCLC. Firstly, expression of Notch3 in NSCLC tumors was significantly correlated to the expression of EGFR (225). Secondly, abrogation of Notch3 in HCC2429 cells resulted in an increase of proapoptotic, BH3-only polypeptide Bim, which is a well-known mediator of gefitinib toxicity in NSCLC with activating mutations of the EGFR (227). Treatment of MRK003 along with erlotinib in xenograft mouse model generated with H460 cells remarkably reduced tumor sizes and increased Bim expression compared with either agent alone(228).

Notch ligands Jagged1 and Jagged2 play roles in lung cancer progression as well. Kurie and colleagues have shown that Jagged1 and Jagged2 have distinct biological functions in lung cancer, in which depletion of Jagged1 but not Jagged2 induced apoptosis of HCC827 cells harboring an EGFR mutation, whereas depletion of Jagged2 but not Jagged1 enhanced the ability of HCC827 cells to chemoattract THP-1 human monocytes, suggesting the novel role of Jagged2 in the promotion of antitumor immunity in lung cancer (229). Subsequently, the same group demonstrated that Jagged2 but not Jagged1

promoted EMT process and metastasis of mouse lung adenocarcinoma cells through suppression of miR-200 expression (181).

In contrast, Notch signaling seems to function as a tumor suppressor in SCLC. In primary SCLC, mammalian achaete scute homologue 1 (Mash1) protein is highly expressed. Mash1 transcription is suppressed by Notch target gene Hes1. During lung development, Notch1 and Hes1 are highly expressed in non-neuroendocrine airway epithelial cells, whereas Mash1 expression is restricted to pulmonary neuroendocrine cells, implicating that Notch signaling seems to control pulmonary epithelial cell fate by upregulation of Hes1, whereas suppresses the neuroendocrine cell fate by repressing Mash1 (92, 230). Intriguingly, activated form of Notch1 and Notch2 cause cell cycle arrest in series of SCLC cell lines by upregulation of WAF, KIP1 and negatively regulating Mash1(231, 232).

CBF-1 independency of Notch signaling in cancer

CBF-1 dependent canonical Notch signaling plays an important role in diverse cellular processes and various human diseases, including cancer. Studies conducted on *Drosophila* mutants provided some clues for existence of CBF-1-independent non-canonical Notch signaling, because the *Su(H)* mutants examined do not exhibit the same phenotypes as *Notch* mutants (233, 234). In CBF-1 independent non-canonical Notch signaling, cleaved NICD could interact with components of other signaling pathways then activates downstream signaling. CBF-1-independent non-canonical Notch signaling has also been demonstrated to be involved in the regulation and maintenance of various cancers. In a breast cancer mouse model, *MMTV-Notch4/Int3* transgenic mice develops mammary

tumors independent of CBF-1 activity as mice with targeted deletion of *Rbpj κ* (eg. CBF-1) in *Notch4/Int3* overexpressing background also developed mammary tumors at a frequency similar to *Notch4/Int3* transgenic mice, suggesting that Notch4-induced mammary tumor development is independent of CBF-1 activity (235). In human breast cancer, KLF4-induced cellular transformation requires Notch1. However, inhibition of canonical Notch signaling by blocking either CBF-1 or MAML1 did not suppress cellular transformation mediated by KLF4, implying that KLF4 contributes to breast tumor progression by activating synthesis of Notch1 and promoting Notch signaling through a CBF-1 independent non-canonical Notch signaling (236). In RK3E cells, a baby rat kidney cell line immortalized by Adenovirus early antigen 1A (E1A), Notch1 receptor lacking the CBF-1 interacting RAM domain is able to translocate into the nucleus and induces neoplastic transformation (237, 238). In HPV-driven human cervical cancer, Jagged1 is preferentially upregulated in human cervical tumors and its expression correlates with the rapid induction of PI3K-mediated EMT. However, expression of dominant negative CBF-1 failed to abrogate EMT-driven motility and PI3K-mediated phosphorylation of Akt in HaCaT-Jagged1 cell, suggesting that Jagged1-Notch-PI3K oncogenic functions can be independent of CBF-1 transcriptional activity (239). Furthermore, Perumalsamy et al. have elucidated that CBF-1-independent non-canonical Notch signaling is involved in the inhibition of apoptosis in mammalian cells and cancerous cells via the activation of mTOR-Akt kinase through membrane-tethered N1ICD (240). In addition, the NICD has been shown to activate the expression of YY1 target gene c-myc in cancer cells through directly interaction with YY1 transcription factor on c-myc promoter region independent of CBF-1 (241). One of the major Notch target genes, Hes1, can be activated by other signaling

pathways as well such as Sonic Hedgehog, independent of Notch in various cancers (242, 243). Stockhausen et al. have demonstrated that the rapid activation of Hes1 in neuroblastoma cells through TGF α-mediated Ras/MAPK pathway is independent of Notch receptor cleavage (244) providing the indication that Notch/CBF-1-independent activation of Hes1 may be also involved in the activation or maintenance of cancers.

Roles of DSL ligands independent of Notch activation

Although the primary role of DSL ligands is to activate Notch signaling, DSL ligands can also function independent of Notch activation. Previous studies indicate that DSL ligands are able to undergo proteolytic cleavage and initiate signaling events in the ligand-expressing cells. For example, forced expression of Jagged1 can transform rat kidney epithelial cells through its intact PDZ-ligand motif. This independent of Notch activation as PDZ-domain of Jagged1 did not affect the ability of JAGGED1 to initiate Notch signaling in neighboring cells, suggesting the existence of bidirectional Notch-DSL signaling (245). Moreover, DSL ligands undergo processing that is similar to the processing of Notch receptors using the same proteolytic machinery to release of the ICD, indicating the biological functions of DLL ICD (246, 247). In addition, Jagged1 ICD (J1ICD) has been shown to activate AP1-mediated transcription, whereas NICD has an antagonistic effect on the AP1-mediated activation produced by J1ICD (246). Consistently, the ICD of the DLL1 ligand is able to induce growth arrest and senescence through the induction of p21 expression, which can be overcome by the NICD as well (248). Although, this *in vitro* evidences supports the existence of reverse signaling of Notch ligands, the physiological

relevance of reverse signaling of DSL ligands and its role in tumorigenesis is still not determined.

Therapeutic agents targeting Notch signaling

Based on the many effects of Notch signaling exhibited in multiple human cancers this pathway represents an attractive target for potential therapeutic benefit, which may result in suppression of cell proliferation, induction of cell death, depletion of CSCs, inhibition of cell proliferation, induction of cell death, depletion of CSCs, inhibition of tumor angiogenesis, and induction of differentiation. There are multiple strategies that have been considered to target Notch signaling: 1). Neutralizing antibodies (Abs) or receptor/ligand decoys against individual Notch receptor and ligands to interfere with receptor-ligand interaction; 2). Receptors specific blocking Abs targeting NRR region to mask S2 cleavage site thereby blocking ADAM-protease-mediated cleavage of receptors; 3). Various γ-secretase inhibitors (GSIs) with different selectivity and efficacy preventing S3 cleavage of receptors by γ-secretase complex; 4). Stapled peptides interfering with the formation of NICD-MAML transcriptional complex. These strategies to target Notch signaling are currently being tested in preclinical studies as well as in clinical trials (Table 1.3).

Receptor/Ligand neutralizing antibodies/decoys

The use of neutralizing Abs or decoys for Notch ligands DLL4 has been shown to be effective in blocking tumor angiogenesis. The expression of DLL4 is restricted to endothelial cells during development, and knockout of a single allele of DLL4 results in an embryonic lethal phenotype due to defect in vasculogenesis. DLL4 upregulation is also

found in tumor vasculature (249). Studies targeting DLL4 ligands by a soluble DLL4-Fc or a DLL4 neutralizing antibody show substantial reduction of tumor growth in xenograft models (209, 250, 251). The mechanism of DLL4 antitumor effect appears to be the promotion of non-productive angiogenesis by increased sprouting of endothelial tip cells (206). Therefore, inhibition of DLL4 in suppression of tumor angiogenesis leading to hyperproliferation of non-functional tumor vessels to inhibit tumor growth is in a manner distinct from traditional anti-angiogenesis therapies (207). In addition to its function on tumor angiogenesis, inhibition of DLL4 can also reduce colon cancer tumor growth via depletion of CSCs by using DLL4 blocking antibody against human DLL4, which does not cross react with mouse DLL4 (182). So far, both the feasibility and efficacy of DLL4 Abs or decoys in the treatment of cancer have yet to be demonstrated in clinical trials. Recently, Yan's group at Genentech reported that chronic inhibition of DLL4 results in severe disruption of normal tissue homeostasis and leads to vascular neoplasma in multiple organs, which has raised a significant safety concern for using DLL4 Abs to treat human cancers (170). Besides inhibition of DLL4, Kitajewski's group had demonstrated that ectopic expression of soluble Notch1 receptor (Notch1 decoy) could reduce signaling stimulated by the binding of three distinct Notch ligands to Notch1 and inhibit morphogenesis of endothelial cells overexpressing Notch4. In their study, Notch1 decoy did not affect tumor cell growth *in vitro*, instead, it inhibited tumor angiogenesis in an *in vivo* xenograft model (252). In addition, expression of dominant negative form of Notch3 in lung cancer cell line remarkably reduced colonies in soft agarose (225). These studies suggest the feasibility of blocking receptor-ligand binding for targeting Notch signaling in cancers.

Receptors antibodies masking S2 cleavage

Proteolytic resistance of Notch prior to ligand binding depends on the structural integrity of the NRR in the receptor. The crystal structure of Notch1 NRR in its autoinhibited conformation provides evidence that antibodies masking NRR might stabilize the "off" conformation of the receptor even in the presence of ligands activation (54). Li et al. had generated antibodies against whole extracellular domain of Notch3 and found out that the strongest inhibitory Abs are specific for the extracellular NRR but not to the ligand binding domain of Notch3 receptor. Those inhibitory antibodies can revert phenotypes conveyed to 293T cells by Notch3 signaling, such as increased cellular proliferation, survival, and motility (253). However, they did not test for anti-tumor activity of those antibodies. Nevertheless, their study provides evidence that targeting NRR of Notch receptors may represent a viable strategy to block ligand-induced Notch signaling. Recently, Siebel's group at Genentech developed blocking antibodies against Notch1 and Notch2 NRR by screening a phage display library. Those antibodies can cross-react with human and mouse sequences. They have demonstrated that selective blockade of Notch1 inhibits tumor growth in pre-clinical models through inhibition of cancer cell growth in T-ALL and deregulation of angiogenesis in solid tumors. Inhibition of Notch1 plus Notch2 causes severe intestinal toxicity as is observed with GSIs treatment, while inhibition of either receptor alone reduces or avoids this effect, demonstrating that inhibition of individual Notch receptors may have advantages over pan-Notch inhibitor, such as GSIs (254). In contrast, Aste-Amezaga et al. developed Notch1 antibodies against either ligand binding regions (LBD) or NRR derived from cell-based and solid-phase screening of a phage display library. They observed that both classes of antibodies are specific for Notch1, bind

Notch1 on the surface of human tumor cell lines, and inhibit ligand-induced expression of Notch target genes in cell lines expressing wild-type Notch1 receptors, while antibodies against NRR are more potent than those against LBD. On the other hand, antibodies against NRR appear to be much less effective in inhibiting Notch1 activation in T-ALL cells than GSIs (255). Ultimately, it is difficult to make conclusions about the relative efficacies of these approaches, as their anti-tumor effects have not been tested *in vivo*.

Gamma secretase inhibitors

One of the emerging strategies for inhibiting Notch signaling is to suppress the S3 proteolytic cleavage of Notch receptors mediated by γ-secretase complex. γ-secretase is a large intramembrane aspartylprotease complex composed of a catalytic subunit (presenilin-1, presenilin-2) and accessory subunits (Pen-2, Aph1 and nicastrin). Originally, GSIs were developed to treat or prevent Alzheimer's disease to prevent release of the amyloid β-peptide, the precursor of amyloid plaques found in the brain of Alzheimer's disease (256). Since GSIs are also able to prevent activation of Notch signaling, several GSIs have been tested for antitumor activity. An early GSI, IL-X (cbz-IL-CHO) was shown to have antineoplastic activity in Ras-transformed fibroblasts. Furthermore, tripeptide GSI (z-Leu-leu-Nle-CHO) was reported to suppress tumor growth in Kaposi sarcoma both *in vitro* and *in vivo* (257). Another GSI, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) has been demonstrated to reduce growth of medulloblastoma cells and xenograft tumors (243) as well as induce G_0-G_1 cell cycle arrest and apoptosis in a T-ALL animal models (258). Furthermore, treatment of a tripeptide GSI, MRK003 in a Notch3 dependent NSCLC cell line leads to suppression of cell proliferation and induction of

apoptosis both *in vitro* and *in vivo* (226). These exciting results strongly indicate a potential clinical application of GSIs in the treatment of human cancers. Currently, several GSIs produced by different pharmaceutical companies have launched preclinical study or clinical trails for T-ALL and solid tumors (Table 1.3). For example, the compound MK-0752 from Merck & Co., Inc is currently under a phase I clinical trial for the treatment of T-ALL and advanced breast cancer (Clinical Trial ID: NCT00106145); Roche's GSI RO4929097 is in phase I/II clinical trail as a single agent in treating young patients with relapsed or refractory solid tumors, CNS tumors, lymphoma, and T-cell leukemia (Clinical Trail ID: NCT01088763); Moreover, combination of RO4929097 and Gemcitabine Hydrochloride is in phase I clinical trail with advanced solid tumor as well (Clinical Trail ID: NCT01145456). However, the major challenges of using GSIs in the cancer treatment is its side effects, especially toxicity in the gastrointestinal tract by induction of goblet cell differentiation, which probably results from chronic blockade of both Notch1 and Notch2 processing in the intestine (259, 260). The toxicity and lack ofspecificity of GSIs in the inhibition of Notch signaling may limit its clinical application. Firstly, pan-Notch signaling pathway is known to be essential in cellular physiology in normal tissue, including intestine (261), hematopoiesis (262) and maintenance of arterial smooth muscle (263), suggesting the possibility that inhibition of γ-secretase may cause dysfunction of multiple organs. Secondly, there are many substrates of γ-secretase in addition to Notch receptors and ligands, such as ErbB4 (264) and CD44 (265). Thirdly, GSIs may target other proteases other than γ-secretase, which participate in various physiological conditions, so GSIs may have other side effects *in vivo*. However, it may be possible to overcome the toxicity of GSIs. One approach could be to decrease the dose of GSIs in hope

that partially inhibition of γ-secretase activity could sufficiently suppress Notch signaling in cancer cells without significantly impairing its function in normal tissue; Alternatively, intermittent dosing of GSIs with 3 consecutive days followed by a rest period of 4 days could help to decrease the gut toxicity (266); Also, combination with other agents might increase the efficacy of GSIs while decreasing its toxicity (266, 267).

Stapled peptides interfering NICD/MAML interaction

A dominant negative fragment of MAML1 has been reported to antagonize canonical Notch signaling thereby suppress cell proliferation when expressed in T-ALL cell lines (117, 268). Based on this notion, Moellering et al. have reported the design of synthetic, cell-permeable, stabilized alpha-helical hydrocarbon stapled peptide SAHM1 that target a critical protein-protein interface in the Notch transactivation complex. Direct antagonism of the Notch transcriptional program by SAHM1 results in a Notch-specific anti-proliferative effects in vitro and in a mouse model of Notch1-drived T-ALL while exhibiting less severe gut toxicity than GSIs (269).

Overall, there are many approaches to target Notch signaling in cancer. And the anti-tumor activities of existing anti-Notch agents are under investigation in cancer cell lines and pre-clinical mouse models, which would be applied to anti-tumor therapies and benefit cancer patients later.

Table 1.3. Notch-targeting agents

Agents	Mechanism	Targets	Companies/ institutes	Development phase
GSIs	Inhibition of S3 cleavage by γ-secretase	All 4 Notch receptors, Notch ligands and multiple other γ-secretase substrates		
MK0752			Merck	Phase I
RO4929097			Roche	Phase I/II
PF-03084014			Pfizer	Phase I
LY450139			Eli Lilly	Phase I
BMS-unknown			Bristol-Myers Squibb	Phase I
GSM	Inhibition of S3 cleavage by γ-secretase	Selective for specific γ-secretase substrates	Myriad	Failed
Notch mAbs	Interference with ligand-induced Notch activation	Specific for individual Notch receptors		
Notch1 mAb		Against NRR masking S2 cleavage	Roche/Genentech Merck	Preclinical
Notch2 mAb		Against NRR masking S2 cleavage	Roche/Genentech	Preclinical
Notch3 mAb		Receptor-ligand interaction/ Against NRR masking S2 cleavage	Aveo Tanox	Preclinical
Ligand mAbs	Interference with ligand-receptor interaction	Specific for individual Notch ligands		Preclinical
DLL4 mAb/decoy		Block DLL4 receptor binding	Roche/Genentech Regeneron	Preclinical
Transcription complex inhibitors	Interference Notch induced gene transcription	All four Notch receptors, potentially other nuclear transcriptional factors		
MAML-stapled peptide	Interference with Notch interaction with MAML1	All four Notch receptors, potentially other nuclear transcriptional factors using coactivator MAML1	Harvard University	Preclinical

clinical mouse models, which would be applied to anti-tumor therapies and benefit cancer patients later.

Summary

Notch signaling is pivotal in embryonic development, as knockout of several Notch components resulted in embryonic lethal phenotypes. During the last decade, a growing body of literature demonstrates that Notch signaling also plays pleiotropic roles in human cancer by promotion of cellular proliferation, suppression of apoptosis, induction of EMT, facilitation of tumor angiogenesis, maintenance of cancer stem cells and prevention of cell differentiation, which makes Notch signaling an attractive target in the development of cancer therapies. Our group first linked Notch3 to lung cancer tumorigenesis. Inhibition of Notch3 signaling by RNA interference or by treatment with a GSI in lung cancer cell lines suppressed tumor progression *in vitro* and *in vivo* xenograft tumor model. There are four key strategies to target Notch signaling: mAbs, receptors/ligands decoys to interfere receptor-ligand interaction; mAbs to mask S2 cleavage site; GSIs to block S3 cleavage; and stapled peptides to prevent formation of Notch-CBF-1-MAML complex. Due to the lack of specificity and severe side effects of GSI, reagents that specifically inhibit Notch3 signaling may be more efficacious for the treatment of lung cancer. Therefore, the goal of this thesis is to develop agents to specifically target Notch3 signaling in lung cancer.

CHAPTER II

TARGETING SPECIFIC REGIONS OF THE NOTCH3 LIGAND-BINDING DOMAIN INDUCES APOPTOSIS AND INHIBITS TUMOR GROWTH IN LUNG CANCER

The work presented in this chapter is published with the same title in the *Cancer Research*, Jan 2010 [70].

Abstract

Like many signaling pathways in development, the Notch receptor pathway plays an important role in cancer pathobiology when it is dysregulated. Potential ligand-binding sites within the epidermal growth factor (EGF)-like repeats of Notch1 have been identified, but the ligand-binding domains in Notch3, which is implicated in lung cancer, are not known. In screening a library of 155 peptides representing all 34 EGF-like repeats in Notch3, we discovered two distinct ligand-binding regions involving the 7-10 and 21-22 repeats that are distinct from the putative ligand-binding domain of Notch1. In cell-based assays, peptides from these regions induced apoptosis and reduced expression of the Notch3-dependent gene Hey1. They also bound directly to the Notch ligand Jagged1, suggesting that their mechanism of action involves disrupting interactions between Notch3 and Jagged1. Recombinant Fc fusion peptides engineered for *in vivo* testing showed that the Notch3 peptides defined could trigger apoptosis and suppress tumor growth in tumor xenograft assays. These findings rationalize a mechanistic approach to lung cancer treatment based on Notch3 receptor-targeted therapeutic development.

Introduction

Notch3 is a type I transmembrane receptor belonging to a family of proteins essential for cellular differentiation and embryonic development. In mammals, there are four Notch receptors (Notch1-Notch4) and two families of ligands, Jagged (Jagged1 and Jagged2) and Delta-like (DLL1, DLL3, and DLL4). Binding of the ligand to the extracellular domain (ECD) of the Notch receptor triggers two successive proteolytic cleavages and untethers the Notch intracellular domain (ICD) from the cytoplasmic membrane. The Notch ICD is then translocated to the nucleus, binds to the transcription factor CSL, and induces expression of target genes. These genes include the hairy-enhancer of split (Hes) and hairy and enhancer-of-split related with YRPW motif (Hey) families.

Activation of the Notch pathway depends on the interaction of the ECD between the ligand and the receptor with subsequent release of the activated ICD. Notch3 is a large protein containing 2,321 amino acids with a predicted molecular mass of 243.66 kDa. The Notch3 ECD, a region containing the ligand recognition site, is estimated to be 210 kDa. Identifying the part within the large ECD important for receptor-ligand interaction will help to better understand the biology of Notch3 signaling and therapeutic design. Using deletion mutants and point mutations of *Drosophila* Notch and mammalian Notch1, the identified ligand-binding site seems to involve epidermal growth factor (EGF)-like repeats 11–12 (4, 6). However, given the functional diversity and the variation in tissue distribution among the different Notch family members, we hypothesized that the targetable ligand recognition sites on Notch3 receptor differ from those of other family members.

Notch3 is overexpressed in ~40% of resected non–small cell lung cancers, and its suppression results in loss of the malignant phenotype both *in vitro* and *in vivo* (225, 226). In both development and cancer, Notch has been shown to cross-talk with oncogenic pathways such as the EGF receptor/ras/ mitogen-activated protein kinase pathway (131, 141, 225, 270). Thus, targeting this pathway represents a rational strategy in the treatment of patients with lung cancer. One approach currently being explored in clinical trials is blocking the essential proteolytic processing of Notch receptors with γ-secretase inhibitors. The efficacy of this class of compounds needs exploring, but the relative lack of target specificity suggests that new more specific strategies targeting this pathway should be pursued.

In this study, we identify the domains within Notch3 ECD important for ligand recognition and binding. Using a high throughput system and a Notch3 peptide library, we discovered two previously unknown regions; EGF-like repeats 7-10 and 21-22, important for Notch3 activation. In addition, we showed that interfering peptides and recombinant proteins mimicking these regions can abrogate Notch3 activation, induce apoptosis, and inhibit tumor growth *in vivo*. The findings of the present study not only give novel insights into Notch3 signaling but also establish a foundation on which targeted therapy can be developed.

Materials and Methods

Peptide library. The peptide library consisted of 155 synthetic peptides. Their sequences were 5 to 15 amino acids in length and spanned nearly the entire Notch3 ECD. Each peptide represented a unique extracellular site on the ECD, with peptide 1 representing the NH2 terminus and peptide 155 representing the COOH terminus of the last EGF-like repeat. They were synthesized by SynPep and diluted in deionized H_2O to bring the concentration to ~10 mg/mL of peptide in 1× PBS. The peptides were biotinylated using E-Z Link Biotin BMCC (Thermo Fisher Scientific, Inc.) in PBS at a molar ratio of approximately 1 to 2 moles of biotin per mole of synthetic peptide for immunofluorescence staining and pull-down assays.

Cell culture and inhibitor. The Notch3-expressing lung cancer cell line HCC2429 was established as previously described (224). HEK293T and HeLa cells were obtained from the American Type Culture Collection and maintained in DMEM with 10% FCS. MRK003 was provided by Merck, Inc. & Co., and its formulation was described previously (271).

Apoptosis screen of peptide library. Both HCC2429 and HeLa cells were seeded onto 384-well plates at 3,000 in 50 μL per well. Twenty-five microliters of Annexin V-Alexa Fluor 680 (Invitrogen, Inc.), diluted 1:2,200 in RPMI 1640 and 10 μL of peptide (diluted to 0.1 mg/mL in RPMI 1640), were added. After an overnight incubation, the treated cells were analyzed with a FMAT 8100 HTS System fluorescent plate reader (Applied Biosystems).

Each peptide was assayed in quadruplicate.

Notch3 deletion mutants. With the Notch3 ECD as template, inverse PCR was used to generate deletions of Notch3 EGF-like repeats 7-10 and EGF 21-22. The ECD containing the deletions was confirmed by DNA sequencing, cloned into pcDNA4, and religated to Notch3 intracellular fragment to generate full-length Notch3 deletion mutants.

In vitro pull-down assay. HEK293T cells were transfected with hemagglutinin (HA)-tagged Jagged1 (provided by Dr. Artavanis-Tsakonas, Harvard Medical School, Boston, MA) using Lipofectamine 2000. The cells were lysed in NP40 buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40 plus 50 mmol/L protease inhibitors]. One microgram of biotin-labeled peptides and streptavidin-conjugated magnetic beads (Promega) was used to pull down HA-tagged Jagged1. The resulting proteins were resolved on SDS-PAGE and detected with an anti-HA antibody. For the Fc fusion protein binding assay, 5 μg of Fc fusion protein and protein A agarose beads (Sigma-Aldrich, Inc.) were used.

Immunofluorescent staining assay. HCC2429 and HEK293T cells were plated on glass chamber slides. After 24 h, the cells were rinsed twice in PBS and fixed in 4% paraformaldehyde and treated with 1 mL of biotin-labeled peptides and 0.5 μg/mL of Alexa Fluor 488–labeled streptavidin (Invitrogen). TO-PRO3 (Invitrogen) was used for nucleus staining. The cells were then examined under confocal fluorescence microscopy.

Antibodies. Notch3 and HA-targeted Jagged1 were detected using a rabbit Notch3 antibody (Orbigen, Inc.) and an anti-HA monoclonal antibody (HA-7; Sigma-Aldrich), respectively, at 1:1,000 dilution. The goat anti-human IgGhorseradish peroxidase antibody (Santa Cruz Biotechnology, Inc.) and the mouse anti–β-tubulin monoclonal antibody (AA2; Millipore) at 1:5,000 dilution were used to detect human Fc fusion protein and β-tubulin, respectively. Fc fusion protein expression. The peptide DNA sequences were cloned into the NH_2 terminus of pFUSE-hIgG1-Fc2 and pFUSE-mIgG1-Fc2 vectors (Invivogen). These vectors produce secreted fusion protein in mammalian cells. The plasmids were then transiently expressed in HEK293E, and the proteins were purified from culture medium with a protein A/G column (GE Healthcare Life Sciences). The eluted Fc fusion proteins were equilibrated with PBS buffer using a HiTrap desalting column (GE Healthcare Life Sciences).

Real-time PCR. Total RNA was extracted from HCC2429 or HeLa cells 24 h after peptide treatment or transfection with deletion mutants using the Qiagen RNase Mini kit. RNA was reverse transcribed with the SuperScript II First-Strand Synthesis kit (Invitrogen) and quantitated using the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad) and QuantiTect SYBR Green reverse transcription-PCR (RT-PCR) kit (Qiagen). Annealing temperature for PCR was 58°C with the following primers: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGCACCACCAACTGCTTAGC-3′ (sense) and 5′-GGCATGGACTGTGGTCATGAG-3′ (antisense); Hey1, 5′-AGATGACCGTGGATCACCTG-3′ (sense) and 5′-TGTTGAGAGCGAAACCAGTC-3′ (antisense); and Hes1, 5′-AGAAGGCGGACATTCTGGA-3′ (sense) and 5′-

GAGTGCGCACCTCGGTATTA-3′ (antisense). The threshold cycle value (Ct) was determined with iCycler Optical system interface software. Mean Ct of Hey1 or Hes1 was calculated from triplicate measurements and normalized with the mean Ct of the gene GAPDH as internal control.

Apoptosis assay. HCC2429 cells were treated with peptides or Fc fusion proteins for 24 h and maintained in serum-free RPMI 1640. Percent apoptosis was determined using the Annexin V-FITC Apoptosis Detection kit (Calbiochem) and a FACSCalibur Flow Cytometer (Beckman Coulter, Inc.).

In vivo tumorigenicity. HCC2429 cells (1 × 106) suspended in 50% Matrigel (BD Biosciences) were injected s.c. into hind limbs of athymic 4- to 6-wk-old female nude mice (nu+/nu+). When the tumors were palpable, the mice were treated with Fc control or with a single loading dose of recombinant protein at 15 mg/kg followed by dose of 10 mg/kg every 3 d. The tumor size was measured every 3 d with a caliper. Tumor volume was calculated with the formula: volume = (length) $(\text{width})^2/2$.

Statistical analyses. The size of implanted tumors at different time intervals after treatment was compared with that of control treated with mouse Fc. Unless specifically stated, statistical inference in comparative experiments both in vivo and in vitro was obtained using Wilcoxon rank-sum tests. For all statistical comparisons, the differences were considered significant at $P < 0.05$.

Results

Peptide library screening identifies potential ligand binding sites

Notch receptors differ in the number of tandem EGF-like repeats in the ECD. Notch3 contains 34 EGF-like repeats, whereas Notch1 possesses 36. In contrast to Notch1, the ligand-binding site for Notch3 is not as well characterized. Therefore, to identify Notch3-binding sites, we created a peptide library consisting of 155 short peptides sequences, 5 to 15 amino acids in length, spanning the entire 34 EGF-like repeats within the Notch3 ECD. Because inhibition of Notch3 induces apoptosis in tumor cells, the conjugated carrier peptides were then screened for the ability to induce apoptosis. Of the 155 peptides, we identified 15 peptides with reproducible apoptosis-promoting activities in both HCC2429 and HeLa cells (Table 2.1). The effect on apoptosis by these peptides was dose dependent (Fig. 2.1A and B). Interestingly, the locations of these peptides mapped to two discrete regions, EGF-like repeats 7-10 and 21-22 (Fig. 2.1C). The amino acid sequences from Notch3 repeats 7-10 are most similar to Notch1 EGF-like repeats 8-11 with 79% identity (data not shown). When we compared the Notch3 sequence with that from putative Notch1 ligand-binding sites, the EGF-like repeats 11-12, only 40% identity was observed, suggesting that the Notch3-binding domain differs from that of Notch1. An example of the alignment between Notch3 EGF-like repeat 7 and Notch 1 EGF-like repeat 11 is shown in Fig. 2.1D. The highly conserved class II EGF-like repeat is observed in all Notch receptors, and its secondary structure contains a core with a β-pleated sheet, three disulfide bonds, and a series of loops (10). At this time, only the structure of the class II EGF-like domain from human Notch1 is known. Because Notch3 also contains class II EGF-like

repeats, we mapped the Notch3 peptides with proapoptotic-promoting activity to the class II consensus sequence (PDB ID: 2VJ3). Interestingly, the peptide sequences mapped to the loop regions of the EGF-like repeats, suggesting that the loop regions are responsible for ligand interaction. Molecular visualizations of the relative positions of N17 and N132 peptides within an EGF-like repeat are shown in Fig. 2.1E (blue).

Notch3 peptides bind to Jagged1 and inhibit Hey1 transcription

To determine whether the apoptosis induced by the peptides is Notch dependent, we examined the ability of the peptides to bind to Jagged1 and to alter transcription of Notch-dependent genes. Using biotin-labeled Notch3 peptides, we found that the labeled peptides bind to Jagged1- expressing HCC2429 but not HEK293T, which does not express endogenous Jagged1 (Fig. 2.2A). Of the 15 peptides identified, 6 were found to both induce apoptosis and bind to Jagged1. This interaction was subsequently confirmed with the in vitro pull-down studies (Fig. 2.2B). The peptides also inhibited Hey1 transcription with varying potency (Fig. 2.2C). Interestingly, Hes1 transcription was not altered (data not shown). These findings were consistent with our earlier observation that Notch3 preferentially regulates Hey1 and not Hes1 in our lung cancer models (4). The ability of the peptides to induce apoptosis confirmed the findings from the fluorescent screening assay (Fig. 2.2D). Of the 15 peptides identified by FMAT 8100 HTS screen, six induced apoptosis and bound to Jagged1.

Table 2.1. List of peptides, their location, and effect on apoptosis in HCC2429

Peptide ID	Peptide sequences	Effects on apoptosis	EGF-like repeats
N16	CFNTLGGHS	+	7
N17	CVCVNGWTGES	++	7
N22	CVNTQGSFL	+	10
N52	CTCHGGYTGPS	+	21
N65	CREAAAQIGVRLEQL	+	21
N70	CIDLVARYL	+	29
N102	CATAV	++	8
N103	CFHGAT	++	8
N105	CVSNP	+	9
N117	CTFGV	+	16
N130	CDQDIND	+	21
N132	CLNGGS	++	22

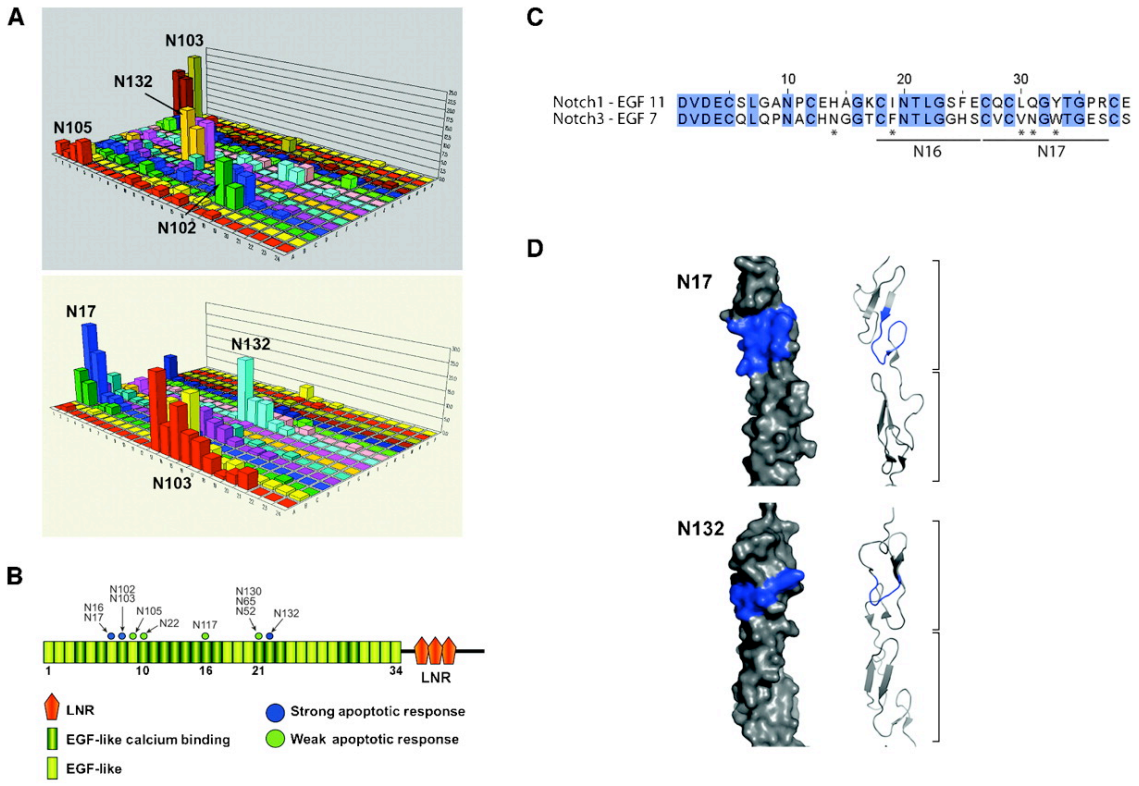

Figure 2.1. Screening of peptides. A. Result from a representative experiment performed on the FMAT 8100 HTS fluorescent plate reader and assayed with Annexin V–Alexa Fluor 680 showing that Notch3 peptides N132, N105, N103, and N102 induced apoptosis. Fifteen of the total 155 peptides induced apoptosis in HCC2429 cells. Top, each peptide was assayed in quadruplicate; bottom, a dose response was noted in which signal intensity was correlated with peptide concentration. B. The peptides with proapoptotic activity mapped to two distinct regions, EGF-like repeats 7–10 and 21–22, within the ECD. Z axis is fluorescence intensity, as a measure of apoptosis. X and Y axes show the location of individual wells on the plate. C. Blue background, alignment of Notch3 EGF-like repeat 7 and Notch1 EGF-like repeat 11 showing 40% identity. Conserved substitution (*) was observed in five residues within these repeats. D. Cartoon and surface representations of an EGF-like domain reconstructed using PyMOL molecular visualization software. Location of N17 (top) and N132 (bottom) sequences based on class II EGF domain consensus sequence is colored in blue, illustrating the putative surface involved in ligand binding.

Notch3 Fc fusion proteins bind to Jagged1 and inhibit Notch3 activation

A major limitation to using peptides for *in vivo* applications is their short biological half-life in the bloodstream. To overcome this limitation, we used Notch3 Fc fusion proteins, in which recombinant protein is fused to the Fc domain of human IgG. Fc-N17, Fc-N16,N17, and Fc-N132 Fc fusion proteins reduced activated Notch3 to differing degrees. Fc-N132 had a greater effect than either Fc- N16 or Fc-N130, suggesting that the inhibiting activity may be related to the sequences themselves and not the length. A similar effect was observed when conditioned media containing secreted Fc fusion protein were used (Fig. 2.3A). Interestingly, although not all Fc fusion constructs affected Notch3 activation, they all retained the ability to bind to Jagged1 (Fig. 2.3B). Notch3 Fc fusion proteins induce apoptosis and inhibit tumor growth *in vivo*. Treatment with purified Fc-N16,N17 and Fc-N132 proteins resulted in inhibition of Notch3 activation to levels resemble those obtained with MRK003 (Fig. 2.4A). This observation confirmed our early peptide data (Fig. 2.2D). To determine the effect of Notch3 Fc fusion proteins *in vivo*, we used a HCC2429 human lung cancer xenograft model. We observed a statistically significant reduction of tumor volume with Fc-N16,N17 and Fc-N132 treatment compared with Fc control after 12 days of treatment. After 16 days, the average tumor volumes with Fc-N16, N17 (0.256 cm^3) and Fc-N132 (0.256 cm^3) treatment showed a 2-fold reduction compared with Fc control (0.612 cm^3; Fig. 2.4B and C).

Deletion of putative ligand-binding sites abrogated Notch3 activation *in vitro*

To determine whether EGF-like repeats 7–10 and 21–22 are necessary for signaling, we created constructs N3Δ7-10, N3Δ21-22 and N3Δ7-10, Δ21-22, similar to the full-length

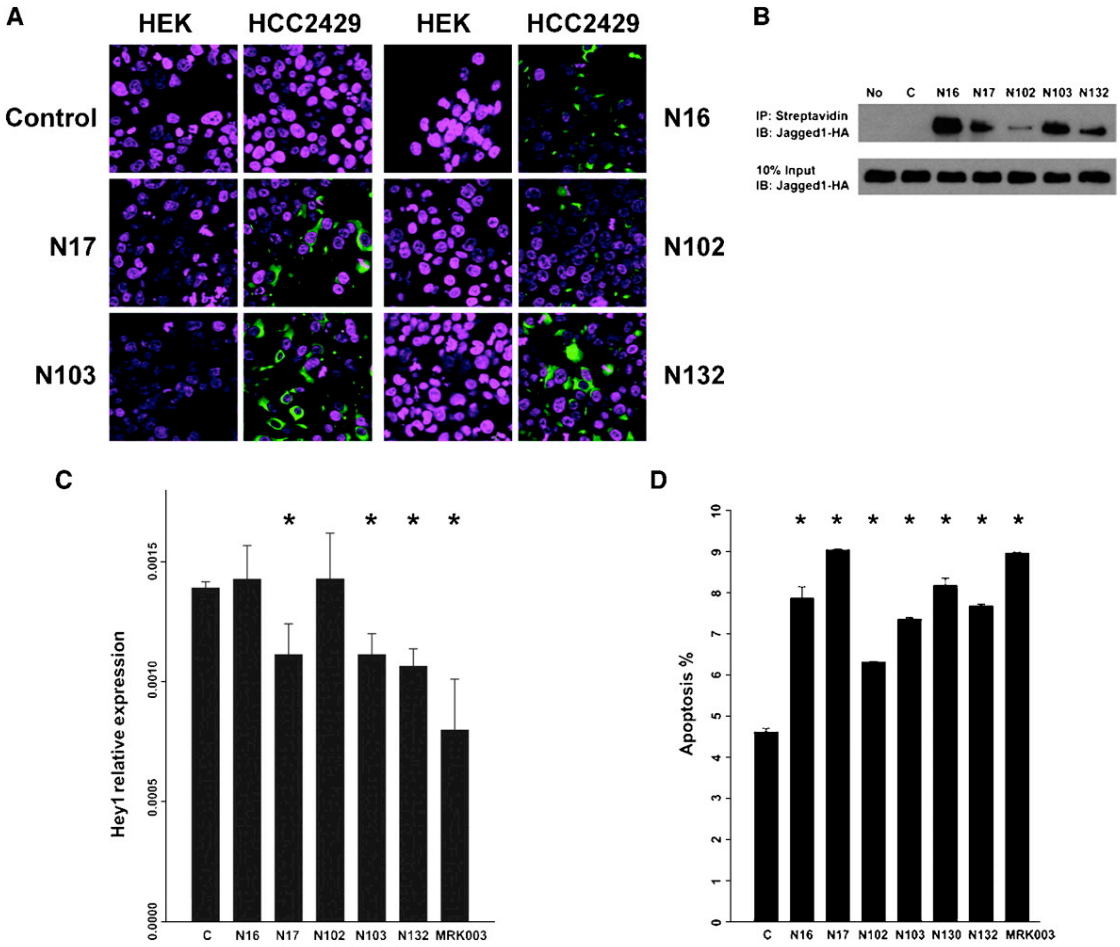

Figure 2.2. Notch3 peptides bound to Jagged1 and inhibited transcription of Notch3-dependent gene Hey1. A. Fluorescent-labeled Notch3 peptides N16, N17, N102, N103, and N132 (green) bind to HCC2429 cells expressing Jagged1 but not to HEK293T cells that do not express endogenous Jagged1. B. Notch3 peptides inhibited signaling through binding to Jagged1. Immunoprecipitation experiment showing that Jagged1 binds to Notch3 peptides but not to control peptide (C). No, no input. C. Treatment of lung cancer cell line HCC2429 with Notch3 peptides reduced transcription of Notch3-dependent gene Hey1 determined by real-time RT-PCR. Note that the N17 peptide exhibited both the highest apoptotic activity and greatest reduction in Hey1 transcription. D. All Notch3 peptides can induce apoptosis in Notch3-expressing HCC2429 cancer cells. Cells treated with a γ-secretase inhibitor MRK003 were used as positive control. *, $P < 0.05$.

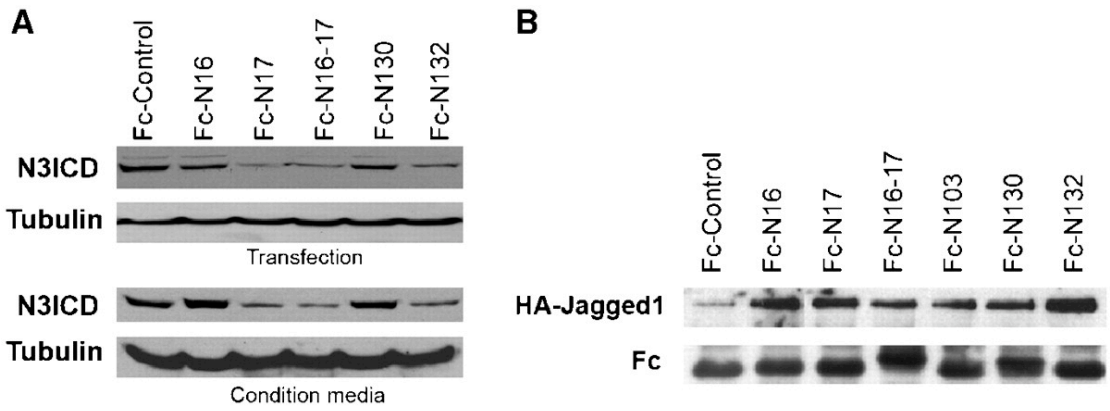

Figure 2.3. Notch3 Fc fusion proteins bind to Jagged1 and inhibit Notch3 activation. A. Transfection of Notch3 Fc fusion expression plasmids Fc-N16, Fc-N16,N17, and Fc-N132 into HCC2429 downregulated expression of Notch3 ICD. Conditioned media from transfected HEK293T also reduced activated Notch3 in HCC2429. Similar to the previous transfection experiment, Fc-N16, Fc-N16,N17, and Fc-N132 can reduce Notch3 ICD level but not Fc-N16 or Fc-N130. B. Consistent with the peptide data, the immunoprecipitation experiment shows that Fc fusion proteins bound to Jagged1 but not to Fc control.

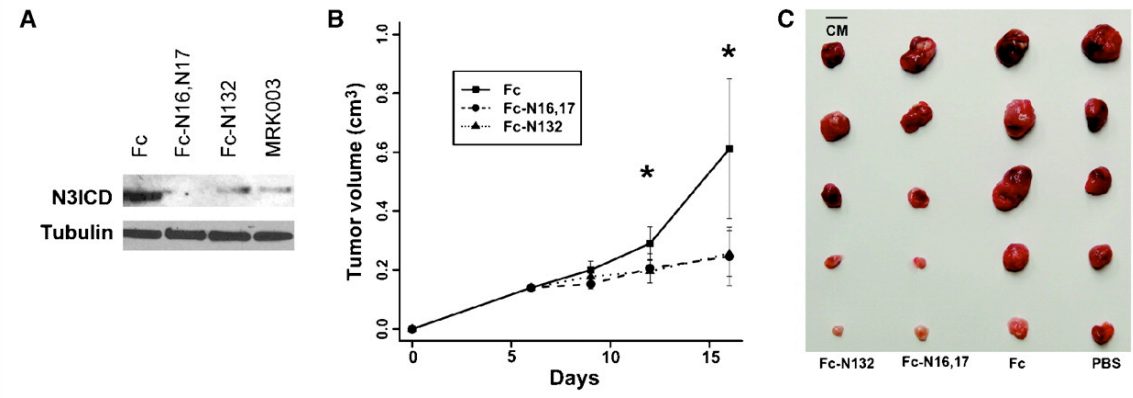

Figure 2.4. Notch3 Fc fusion proteins induced apoptosis and inhibited tumor growth *in vivo.* A. Purified Fc fusion proteins Fc-N16,N17 and Fc-N132 inhibited Notch3 activation compared with control. B. When HCC2429 xenografts were treated with Fc fusion proteins Fc-N16,N17 and Fc-N132, tumor growth was significantly reduced. C. Tumors resected from mice treated with Fc-N132, Fc-N16,N17, Fc control, and PBS. The tumors from Fc fusion protein–treated animals were significantly smaller than those treated with Fc control and PBS. *, $P < 0.05$.

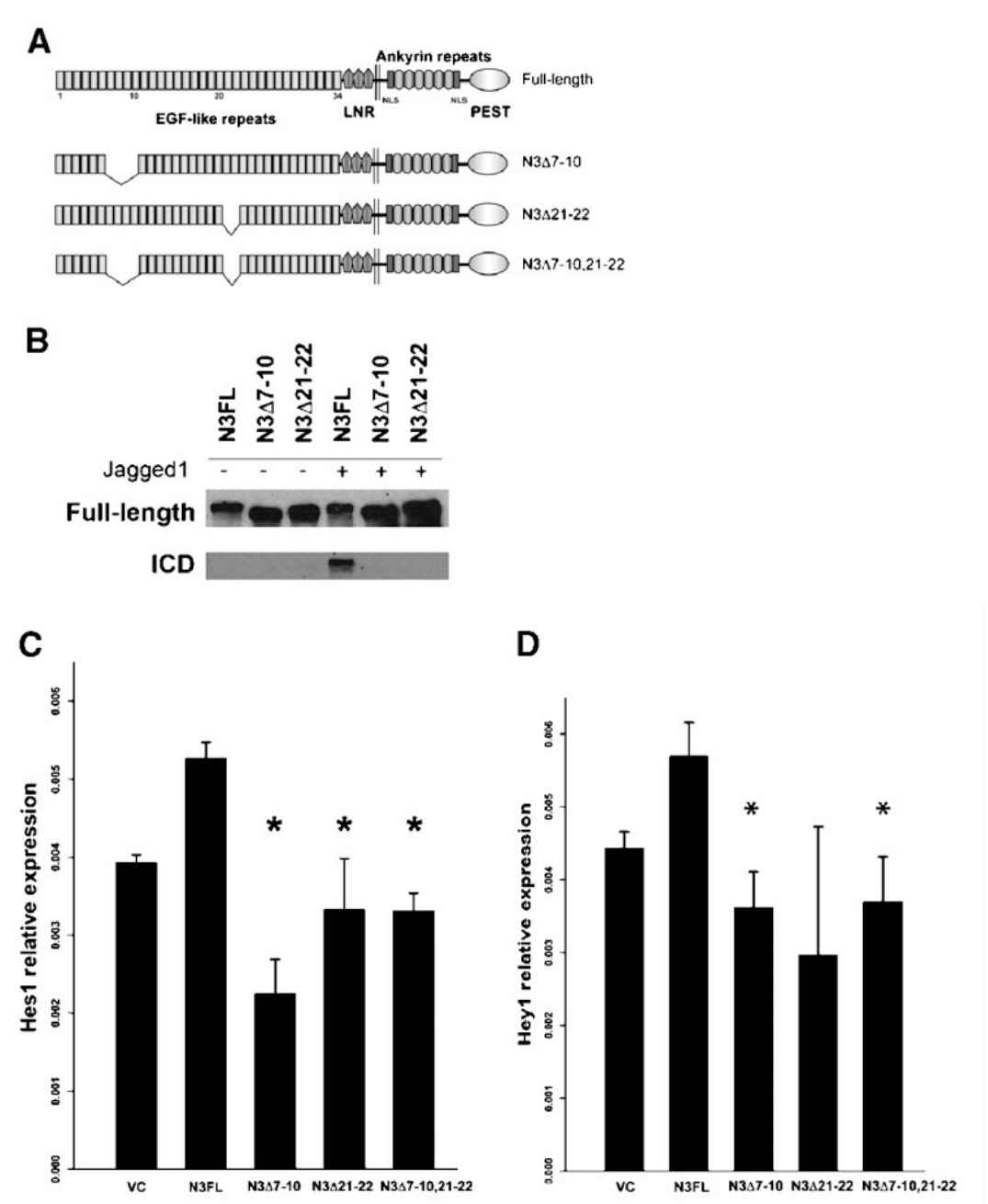

Figure 2.5. Deletions of EGF-like repeats 7–10 and 21–22 reduce Notch3 activity *in vitro.* A. Diagrams of full-length receptors and mutants with deletions of EGF-like repeats 7–10, 21–22, or both. B. Cotransfection of Jagged1 and full-length Notch3 into HeLa cells resulted in induction of activated Notch3 (ICD). In contrast, activated Notch3 was absent in cells transfected with Jagged1 and mutants N3Δ7-10 and N3Δ21-22. Full-length Notch3 induced transcription of Hes1 (C) and Hey1 (D) in HeLa cells in the presence of Jagged1. Deletions of EGF-like repeats 7–10, 21–22, or both resulted in decreased level of Hes1 transcription compared with full-length Notch3. *, $P < 0.05$.

receptor but lacking EGF-like domains 7–10, 21–22, or both (Fig. 2.5A). Similar strategies were used in *Drosophila* to better understand domain functions of *Drosophila* Notch (11). Unlike the native full-length receptor, both N3Δ7-10 and N3Δ21-22 constructs were unable to activate Notch3 cleavage in the presence of Jagged1 (Fig. 2.5B). As in many biological systems, modulation of Notch3-dependent genes is context dependent. In contrast to early findings that Notch3 regulates only Hey1 in lung cancer cell lines, in HeLa cells, transcription of both Hes1 and Hey1 was reduced when Notch3 with deletions of EGF-like domains 7-10, 21-22, or both was used (Fig. 2.5C and D), supporting the hypothesis that these regions are important for Notch activation.

Discussion

Information about the binding site for Notch receptors has been mostly gleaned from studies using Notch deletion mutants in *Drosophila*. Of the 36 EGF-like repeats in Drosophila Notch, repeats 11-12 are sufficient and necessary for interaction with both Delta and Serrate (12). Similar observations have been made for mammalian Notch1, in which the loss of calcium binding EGF-like repeats 11, 12, and 13 has been shown to abrogate receptor function (6).

However, there are differences in structure, tissue distribution, and activation of downstream target genes among the Notch receptors (272). Unlike Notch1, Notch3 contains 34 instead of 36 EGF-like repeats. Notch3 also differs from Notch1 and Notch2 by its lack of a transactivation domain. Structural and functional differences, therefore, can implicate different ligand recognition sites among the four mammalian Notch receptors. In

this study, using a Notch3 peptide library, we discovered two regions within the Notch3 ECD important for ligand binding. Unlike EGF-like repeats 11-13 in Notch1, our findings suggest that the binding site on Notch3 involves EGF-like repeats 7-10, with the strongest functional activity in EGF-like repeats 7-8. This observation differs from that of Joutel and colleagues (273), in which the mutation C428S located on EGF-like repeat 11 abrogated ligand binding. It is possible that this mutation results in a conformational change in the receptor that prevents ligand binding without being within the ligand-interacting surface of the receptor.

The present data show two potential ligand-binding sites, EGF-like repeats 7-10 and 21-22, within the ECD of Notch3. Either site seemed sufficient for receptor activation. Because similar studies have not been carried out for Notch1, it is not known whether Notch3 is the only mammalian Notch receptor with two functional domains. In *Drosophila*, deletion of Notch EGF-like repeats 24-26, a part of the genetically defined *Abruptex* region, results in reduced signaling by the ligand Serrate but not the ligand Delta (274). This observation suggests that all mammalian Notch receptors may possess two ligand domains and that the second binding site is important for regulating ligand specificity. Further studies are needed to test this hypothesis.

The expression of Notch3 in adult mammals is limited to the vascular system. Embryonic deletion of Notch3 in mice results in vascular smooth vessel defects, suggesting that targeting this pathway in cancer will result in antiangiogenic effects (83). Given the role of Notch signaling in maintaining stem cells, it is possible that inhibiting Notch3 could also

have gut toxicity as observed with γ-secretase inhibitors (259). Unlike Notch1 in T-cell acute lymphoblastic leukemia, oncogenic mutation has not been associated with Notch3 (110, 275). By contrast, the dysregulation of the Notch3 pathway in cancer has mostly been associated with overexpression and gene amplification (151, 225). Thus, interfering with ligand-receptor interaction using peptides and recombinant protein constitutes promising strategies targeting this pathway.

Although the effects of the peptides and recombinant proteins may not be specific to Notch3, because other Notch receptors use similar ligands, the strategies used in the present study can potentially be developed for clinical use. Furthermore, the identified functional sites within the receptor could also serve as targets for therapeutic antibodies or chemical peptidomimetic screening and production. Our study, therefore, provides not only insights into the mechanism of Notch3 signaling in lung cancer but also uncovers specific receptor regions whose targeting results in antitumor activity and may serve as the basis for designing specific anticancer therapeutics targeting this pathway.

CHAPTER III

GENERATION OF NOTCH3 NEUTRALIZING ANTIBODIES AGAINST ITS LIGAND BINDING REGIONS

Abstract

The Notch receptors are essential for both normal development and tumorigenesis in many human cancers. Notch3 is expressed in 40% of all lung cancers. Inhibiting this pathway results in reduced tumor growth in lung *in vitro* as well as *in vivo*. Thus, this pathway represents a potentially important target for therapeutic development. Here we report the early results of a strategy to inhibiting Notch3 signaling through the development of Notch3 monoclonal antibodies. Using a Notch3 peptide library, we discovered two regions of the extracellular domain, believed to be the binding regions for the Notch3 ligand, Jagged1. The recombinant proteins representing these two regions, respectively, were used as antigens to immunize mice. We demonstrated that antisera from these mice immunized with portions of the receptor extracellular domain could inhibit Notch3 activation. Further developments of hybridoma clones were screened with ELISA, immunoprecipitation and their ability to inhibit Notch3 cleavage. 12 hybridoma clones were selected, and 4 out of 12 antibodies with IgG1 or IgM isotypes were found to specifically inhibit Notch3 activation but not Notch1. Further testing is on going to validate these findings as well as to determine affinity and anti-tumor activity of these antibodies. Development of monoclonal antibody to specifically inhibit Notch3 ligand binding regions could serve as a strategy for the development of future therapeutics for patients with lung cancer.

Introduction

Notch signaling is a conserved developmental pathway that regulates embryonic development and cellular homeostasis as well as multiple human diseases including cancer (2, 276, 277). Activating mutations of *Notch1* have been found in 50% of T-ALL patients (110) that makes Notch1 as a very attractive target for the treatment of Notch1-drived T-ALL. Moreover, Notch ligand DLL4 is emerging as a critical regulator of tumor angiogenesis, and anti-DLL4 mAbs and decoys are effective in the inhibition of tumor angiogenesis and tumor initiating cells (182, 209, 250). In lung cancer, Notch3 is overexpressed in 40% of resected lung tumors, and inhibition of Notch3 by either siRNA or a γ-Secretase inhibitor in HCC2429 cell induced apoptosis and suppressed tumor growth *in vivo* xenograft tumor models (225, 226).

Activation of Notch signaling is mediated by receptor-ligand interaction. Then receptor get sequential cleavages by ADAM protease and γ-secretase complex to release its intracellular domain, which translocates into nucleus to convert transcriptional repressor CSL to be a transcription factor to initiate gene transcription by recruiting transcriptional coactivators, such as Mastermind-like protein and p300 (2, 276). There are four strategies to block Notch signaling, 1). using mAbs or decoys of receptor/ligand to interfere receptor-ligand interaction; 2), using mAbs making S2 cleavage site to prevent S2 cleavage; 3). γ-secretase inhibitor to block S3 cleavage; 4). stapled peptides or small molecule inhibitors to prevent formation of NICD-CBF-1-MAML complex. Nowadays, pan-Notch inhibitors GSIs, would work to target Notch3 driven cancers. However, the unspecificity and gut

toxicity limit applications of GSIs in clinic (259, 278, 279). Therefore, development of specific inhibitors targeting Notch3 signaling is needed for the treatment of lung cancer.

Owing to their exquisite specificity, antibodies have been in the spotlights as potential therapy for human cancer (280). Anti-cancer mAbs typically have a combination of mechanisms in directing cytotoxic effects to a tumor cell. Most interact with components of the immune system through antibody-dependent cellular cytotoxicity (ADCC) and or complement-dependent cytotoxicity (CDC), and many alter signal transduction within the tumor cell or act to eliminate a critical cell-surface antigen (281). Monoclonal antibodies generally have been correctly viewed as being less toxic than cytotoxic chemotherapy agents for cancer treatment (281). Notch3 monoclonal antibodies raised against whole the extracellular domain of Notch3 including ligand binding regions and NRR of Notch3 have been reported (253). However, the antitumor effects of these antibodies have not been determined. Previously we have identified that Notch3 EGF-like repeats 7-10 and 21-22 are important for receptor-ligand interaction. Given the advantages of monoclonal antibodies in the treatment of cancer, no existing mutations of Notch3 in lung cancer, and overexpression of both Notch3 and ligand Jagged1 in lung cancer, generating monoclonal antibodies against ligand binding regions of Notch3 receptor will be a prominent strategy to target Notch3 in lung cancer.

In this chapter, we reported that we have generated monoclonal antibodies against Notch3 ligand binding regions, which are EGF-like repeat 7-10 and EGF-like repeat 21-22 in its extracellular domain. These antibodies are able to bind to Notch3 expressing cells,

immunoprecipitate full-length Notch3 receptor, and suppressed production of activated form of Notch3 receptor in HCC2429 cells.

Materials and Methods

Production of Antigen. cDNAs encoding (i) the EGF-like repeat 7-10 and (ii) the EGF-like repeat 21-22 of Notch3 was ligated in-frame into pSV282 vector provided by Center for Structural biology at Vanderbilt University. The pSV282-EGFL7-10 and pSV282-EGFL21-22 were transformed into *E. coli* BL21 (DE3) for expression of recombinant protein. Bacterial expressed proteins were purified by Amylose resin (NEB) following manufactory introduction.

Immunization and Fusion. Four A/J (or BALB/c) mice were immunized with the antigen described above. For primary injections, 50 μg of purified antigen were emulsified in 50% phosphate-buffered saline (PBS), 50% Freund's complete adjuvant and injected subcutaneously into the nape of the neck (50%) and intramuscularly to the gluteal muscles (50%). In subsequent booster injections, Freund's incomplete adjuvant was substituted for Freund's complete adjuvant. Fourteen days after each booster injection, serum was collected from each mouse and assayed for reactivity with the antigen by enzyme-linked immunoadsorbent assay (ELISA), other assays. The mouse with the highest level of reactivity was chosen for final boosting by intraperitoneal injection of the antigen diluted in PBS. Fours days after the final immunization, spleen cells were harvested and electro-fused/fused by standard methods with SP2/0 myeloma cells. The products of the fusion

were plated for under selection for fourteen days in semi-solid media (Stemcell Technologies). Resulting colonies were picked and distributed individually into 96-well plates. Hybridomas producing antigen-specific antibodies were initially identified by ELISA, and subsequently verified by other assays. Clones producing antibodies with the desired properties were subcloned to ensure monoclonality and cryopreserved. Selected clones were scaled up and inoculated into one-liter bioreactors (Wilson Wolf Manufacturing Corporation) and grown for 3-4 weeks. mAbs were subsequently purified from the supernatant by affinity chromatography on Protein-G sepharose (GE Life Sciences). Purified MAbs were isotyped and subsequently quantified by SDS-PAGE electrophoresis followed by infrared coomassie staining.

ELISA assay. Flat-bottomed 96-well microtiter plates were coated with the desired antigen at 10 μg/ml in Carbonate-Bicarbonate buffer pH 9.6 (15mM Na2CO3, 30mM NaHCO3, .001% Thimerosal) and incubated for 4 h at 37°C or overnight at 4°C. Coated plates were then washed and blocked with PBST (PBS + 0.1% Tween-20) utilizing an ELx405 Select (Bio-Tek, Winooski, VT). One hundred microliters per well of supernatant or appropriately diluted sera were added and the plates were incubated at 37°C for 1 h before washing three times with PBST. Peroxidase-conjugated goat anti-mouse IgG Fc fragment specific secondary antibody (Jackson Immunoresearch labs) diluted 1:5000 in PBST/1% BSA was added to the wells and incubated 1 h at 37°C. Plates were washed three times in PBST and bound antibodies were detected utilizing the colorimetric substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (Sigma, St Louis, MO) and hydrogen peroxide.

The reaction was allowed to develop for 30 minutes at room temperature and the optical density was determined at 405nM using a Powerwave HT-340 (Bio-Tek, Winooski, VT).

Immunoprecipitation assay. HEK293T cells were transfected with Myc-tagged full-length Notch3 using Lipofectamine 2000. The cells were lysed in NP40 buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40 plus 50 mmol/L protease inhibitors]. One microgram of individual antibodies was used to pull down myc-tagged full-length. The resulting proteins were resolved on SDS-PAGE and detected with an anti-myc antibody (Sigma-Aldrich, Inc.).

Immunofluorecent staining. HCC2429 and H1171 cells were plated on glass chamber slides. After 24 h, the cells were rinsed twice in PBS and fixed in 4% paraformaldehyde and stained with individual antibodies and Alexa Fluor 594-labeled anti-mouse IgG or IgM (Invitrogen). TO-PRO3 (Invitrogen) was used for nucleus staining. The cells were then examined under confocal fluorescence microscopy.

Western blotting. HCC2429 cells (1.5×10^5) were plated in a 6-well plate 24 hours before the treatment, and were treated with 1 microliter of sera or various dosing of purified antibodies in complete RPMI 1640 medium. 24 hours after treatment, cells were lysed with RIPA buffer (50mM Tris, 150 mM NaCl, 0.1 % SDS, 0.5 % Na.Deoxycholate, 1% NP40), then were applied for SDS-PAGE and immunobloted with anti-Notch3 antibody (Cell Signaling Technology), anti-tubulin antibody (AA2; Millipore) and anti-actin antibody (Sigma-Aldrich, Inc.).

Result

Antisera from immunized mice are able to bind to full-length Notch3 and inhibit N3ICD production

Recombinant MPB-tagged proteins containing the sequences of Notch3 EGF-like repeat 7-10 and 21-22 were expressed and purified from in *E. coli* to immunize A/J and BALB/c mice. The antisera were screened for binding and activity using immunoblotting (IB) and cell-based functional assay. For the mice immunized with EGF-like repeat 7-10, the specific antibodies were detected from the antisera of AJ/R mice, while for the mice immunized with EGF-like repeat 21-22, the specific antibodies were detected from the antisera of AJ/O, AJ/L, AJ/R, Balb/R and Balb/RL mice. When treated HCC2429 cells with pre-and post-immunized antisera of, the activated form of Notch3 (N3ICD) was significantly decreased in AJ/O mouse with antigen of EGF-like repeat 7-10, AJ/RL, Balb/O mice with antigen of EGF-like repeat 21-22 (Fig 3.1). Thus, AJ/O, AJ/R, and Balb/O mice were for fusion to make hybridomas.

Monoclonal antibodies are able to bind to Notch3 and inhibit N3ICD production

The resulting hybridomas were first screened with ELISA using purified EGF-like repeat 7-10-GST and EGF-like repeat 21-22-GST. About 50 parental hybridoma were further tested for abilities of Notch3 binding and inhibition. We picked these parental hybridomas: 7G7, 1B1, 2E6, 5F5, 1D9, 3C7 for subcloning based on their performance on ELISA score, Notch3 binding (immunobloting), immunoprecipitation, and cell-based functional assay.

The mAbs from subcloned hybridomas were screened by ELISA. We picked 2 clones with high ELISA scores from each parental hybridoma for further study. We first ran a cell-based functional assay by treating HCC2429 cells with increasing dose of mAbs, and found that 4 (7G7B12, 1B1F1, 2E6C3, 2E6D12) out of 12 mAbs are able to inhibit production of N3ICD (Fig 3.2). mAbs 7G7B12, 1B1F1, 1B1F6, 2E6C2, 2E6D12, 5F5C4, 1D9E4 and 3C7A10 are able to immunoprecipitate full-length Notch3 from the cell lysate of HEK293T overexpression myc-tagged Notch3 (Fig 3.3A). mAbs from 3C7A10, 3C7F1, 1D9A7, 1D9E4, 7G7A11, 7G7B12, 5F5C4, 5F5C6, 2E6D12 are able to bind to HCC2429 cell in which Notch3 expression level is very high, but not H1171 cell which expressed Notch1 receptor instead of Notch3 clones. MAbs 7G7B12 and 1B1F1 inhibit N3ICD production in a dosage dependent manner. Treatment with 1B1F1 mAb does not affect production of N1ICD, whereas higher dosage of 7G7B12 mAb inhibits production of N1ICD as well (Fig 3.3C). However, these mAbs fail to recognize full-length Notch3 protein by immunoblotting, which may due to these mAbs only recognize natural Notch3 protein but not denatured form. We also checked the isotypes of these antibodies. In fact, 1B1F1 is an IgM antibody; 2E6D12 is a mixture of IgM, IgG2a and IgG2b. 7G7B12 shared the same isotype IgG1 with 7G7A11 for that the parental 7G7 is a pure IgG1 antibody (Table 3.1 and 3.2). 1B1F1 is hybrodoma fusions from the mice immunized with EGF-like repeat 7-10, while 7G7B12 mAbs are that with EGF-like 21-22 antigen.

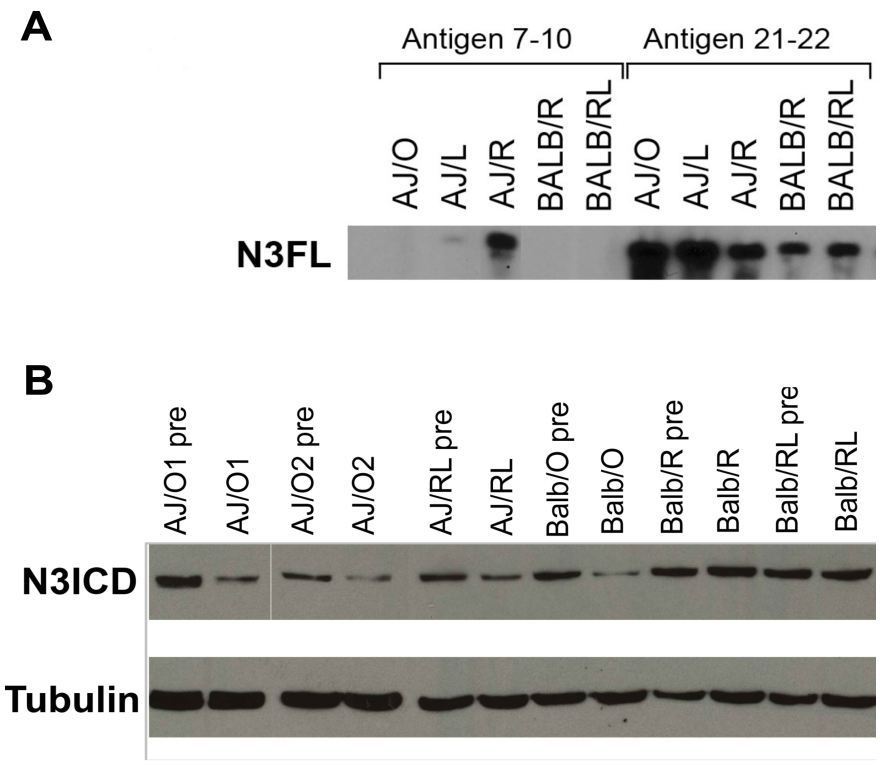

Figure 3.1. A representative screen for Notch3 binding and inhibition of mice antisera. A. Polyclonal antibodies from immunized mice can detect full-length Notch3 (N3FL). B. HCC2429 cells were treated with sera (1 μl) for 24 hours, then analyzed for activated Notch3 (N3ICD), and Tubulin as an internal control.

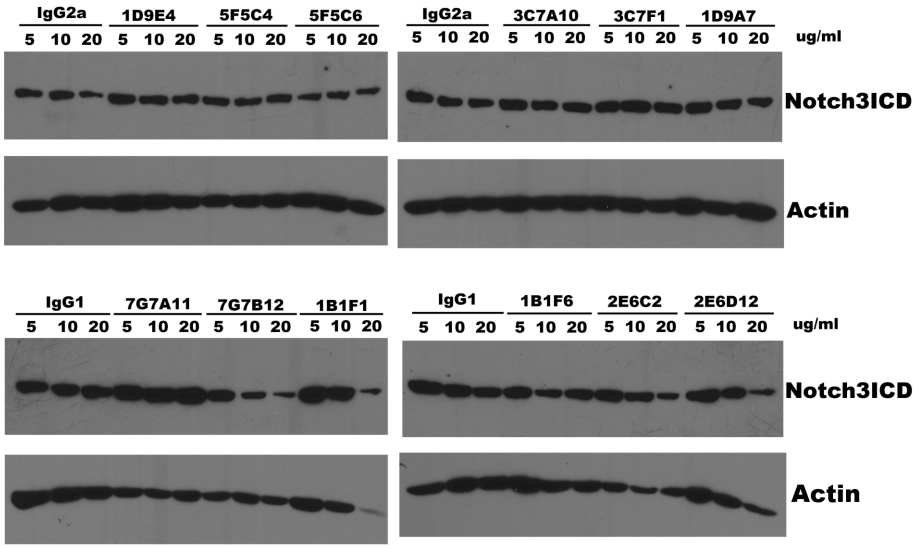

Figure 3.2. 4 out 12 purified mAbs are able to inhibit production of Notch3 ICD. Western blotting showing that mAbs 7G7B12, 1B1F1, 2E6C2 and 2E6D12 are able to inhibit production of N3ICD in a dose dependent manner when treat to HCC2429 cell.

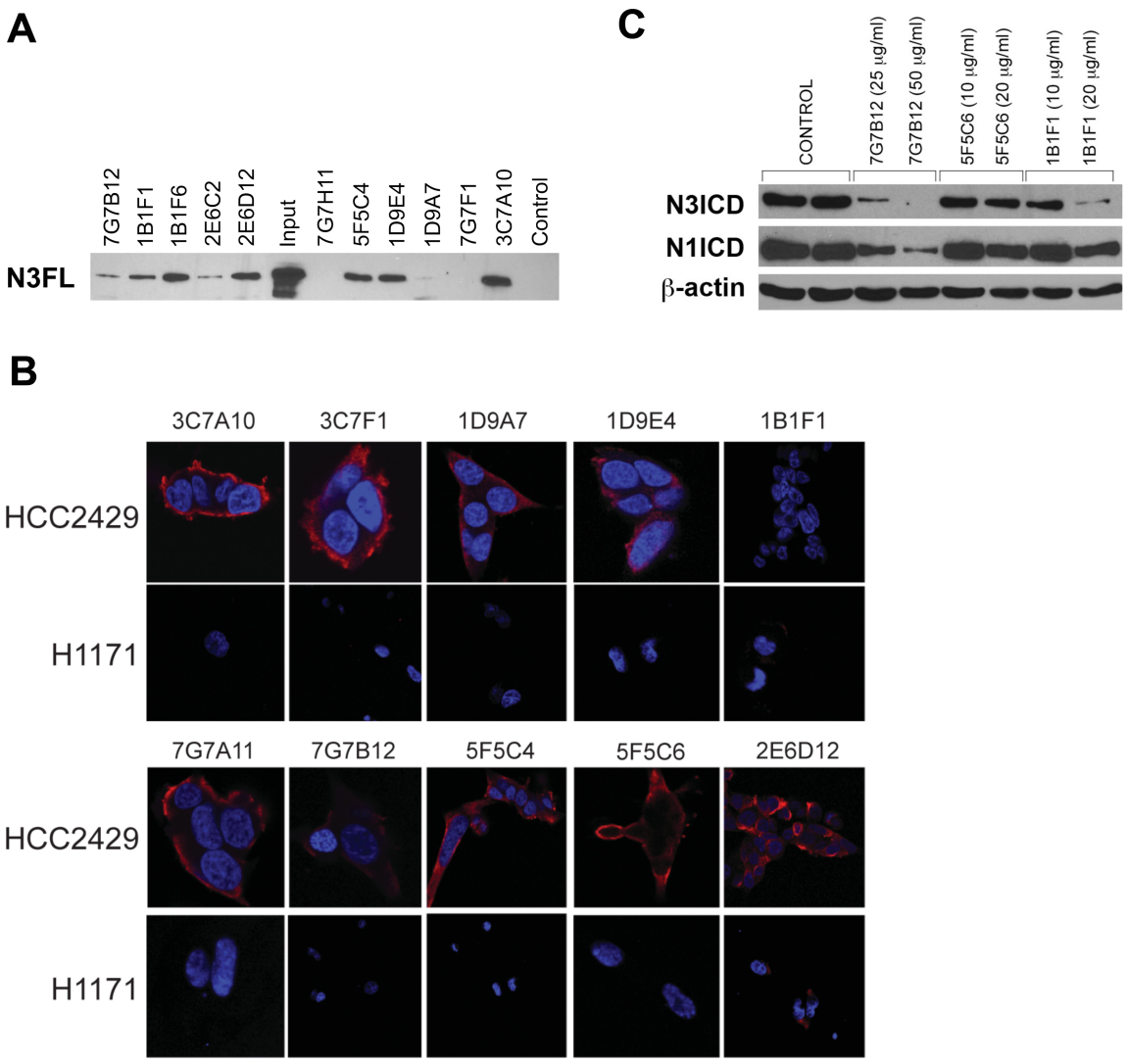

Figure 3.3. mAbs are able to immunoprecipitate Notch3, bind to Notch3 expressing cells and inhibit N3ICD production. A. A representative experiment demonstrating the ability of many mAbs from hybridoma subclones to immunoprecipitate Notch3 in HEK 293 transfected with full-length, myc-tagged Notch3. B. Using immunofluorescence, mAbs bind to Notch3-expressing lung cancer cell line HCC2429 but not in H1171, which expresses only Notch1. C. A representative western blotting experiment demonstrating that some mAbs are able to specifically inhibit Notch3 signaling but not Notch1 (1B1F1) and with higher dose, 7G7B12 can also inhibit Notch1 activation.

Table 3.1 Immunoglobin isotypes of parental hybridoma

Parental pools	Antigen (EGF-like repeats)	Isotypes
3C7	7-10	IgG2a
1B1	7-10	N/A
2E6	21-22	N/A
5F5	21-22	IgG1
1D9	21-22	IgG2b
7G7	21-22	IgG1

Table 3.2 Immunoglobin isotypes of candidate hybridomas clones

	2E6D12	5F5C6	7G7A11	1B1F1
IgG	4.165	4.209	4.221	3.635
IgM	4.292	0.052	0.088	4.02
IgG1	0.05	0.045	4.345	0.055
IgG2a	2.524	0.249	0.06	0.054
IgG2b	2.777	4.234	0.233	0.046
IgG3	0.041	0.041	0.044	0.043
Kappa	4.287	4.07	3.942	4.077
Lamda	0.044	0.122	0.082	0.049

Discussion

In this chapter, we have generated monoclonal antibodies against Notch3 EGF-like repeat 7-10 and 21-22, respectively. Antisera from immunized mice are able to bind to full-length Notch3 protein and inhibit production of N3ICD when treated to HCC2429 cells. AJ/O, AJ/R and Balb/O mice were selected to generate hybridomas fusions. Parental hybridomas pool were screened by ELISA for antigen binding, immunoblotting, immunoprecipitation and cell-based functional assay. Pool of 7G7, 3C7, 1B1, 2E6, 5F5, 1D6 were selected for subcloning process. mAbs 1B1F1 with IgM isotype, 7G7B12 with IgG1 isotype and 2E6D12 with mixed isotypes (both IgM, IgG2a and IgG2b) are able to bind to natural Notch3 protein and suppress production of N3ICD in a dose dependent manner. Our study provides a rationale that antibodies against Notch3 putative ligand binding region, especially the one differs from that of Notch1 EGF-like repeat 21-22, are able to specifically targeting Notch3 signaling.

We tried to study anti-tumor activity of 7G7B12 and 1B1F1 antibodies purified from large-scale bioreactor production. However, the mAbs purified from large-scale production failed to inhibit production of N3ICD when treated to HCC2429 cells as the mAbs purified from small-scale production shown by figure 3.2C. We have not found out the reason why these mAbs lost their capacity of anti-Notch3 when produced in a large-scale bioreactor. We have went back to culture the same batch of hybridomas in a small-scale culture flask, and the mAbs from the same batch of cells but cultured in flasks are able to inhibit N3ICD production. Moreover, we have purified some of mAbs from the large-scale culture medium using a small purification column and found that these mAbs are not able to

inhibit production of N3ICD either, suggesting that either the hybridomas themselves or purification processes is not the cause for lost of function of mAbs. It is possible that our monoclonal antibody core may have a technical problem in the process of antibody production. However, it is also possible that the mAbs affects growth, expansion and antibody production of hybridomas in the bioreactor as inhibition of Notch signaling could induce apoptosis of myeloma cells used to make hybridoma fusions. Currently, we have collaborated with a biotech company to further develop these antibodies, and test their anti-tumor properties by proliferation assay, soft agarose colony formation assay, apopotosis assay and *in vivo* xenograft tumor model.

One of the candidate antibodies, 1B1F1, is an IgM isotype, which can be purified by Protein L column. IgM monoclonal antibodies have been underrepresented in human therapeutic development due to production difficulties and other manufacturing constraints. In our study, we found that the yield of IgM antibody is much lower than that of IgG isotype, around 2 folds less. However, it is still possible to use IgMs mAbs as performed successfully by CMC Biopharmaceuticals of Copenhagen to produce a therapeutic antibody MORAb-028 to target advanced melanoma and other cancers including NSCLC. In addition, a recent paper showed that monoclonal IgM antibodies against PIM-1 protein could suppress tumor growth (282). Therefore, we are further developing and testing the anti-tumor ability of 7G7B12 IgG1 antibody as well as 1B1F1 IgM antibody. If IgM antibody would be very effective in the inhibition of Notch3 signaling and tumor progression, we would either try to use IgM antibody for further development to preclinical

trial or clone the Fab region of IgM to mammalian expressing vectors, such as pIgG, to make IgG.

We have not tested the specificity of the mAbs to other Notch receptors or mouse Notch receptors. As the Figure 3.3 C shown, 7G7B12 mAb suppressed activated forms of both Notch3 and Notch1 receptors, suggesting that 7G7B12 mAb may cross-react with Notch1 as well. However, it is possible that Notch3 regulates production or expression of N1ICD. In fact, in HCC2429 cell, inhibition of Notch3 by siRNA, a GSI or Notch3 peptides, results in decrease expression of Notch3 itself, Notch1 and Notch ligand Jagged1 measured by immunoblotting. Therefore, it is possible that 7G7B12 mAb inhibits Notch3 initially, and then Jagged1 and Notch1 receptor are further attenuated by suppressed Notch3 signaling. Indeed, cell-autonomous role for Notch has been established in endothelial cells during EMT and valve development (188, 283), whereas during *Drosophila* development, the involvement non-cell autonomous effects of Notch signaling is a very important aspect, in which Notch signaling between two populations of cells results in segregation of those cells (277). It is also possible that high dosage of mAbs causes its unspecific binding to Notch1. The identity between Notch3 EGF-like repeats of human and that of mouse are very high. Thus, it is highly possible that these mAbs can cross-react with mouse Notch3. In fact, human mAbs cross-reacting to mouse Notch3 actually mimic the action of antibody-based therapy in human that anti-Notch3 agents suppress both tumor cells and tumor microenvironment, such as endothelial cells and pericytes. Further studies are needed to investigate the specificity of these mAbs against Notch3.

Some mAbs are able to immunoprecipitate full-length Notch3 and bind to Notch3 expressing cells, but are not able to inhibit production of N3ICD. Those mAbs can be further developed to use in immunoprecipitation, immunofluorecent, or flow cytometry assays for studying Notch3 signaling.

CHAPTER IV

JAGGED1 PARADOXICALLY REGULATES LUNG CANCER GROWTH AND MIGRATION THROUGH A CBF-1-INDEPENDENT MECHANISM

Abstract

Many lines of evidence suggest that deregulation of the Notch pathway plays an important role in oncogenesis. Although evidence for Notch receptors in cancer is clear, the role of tumor cell expression of the Notch ligands is less well established. We show that Jagged1 and Notch3, but not Notch1, are overexpressed in NSCLC samples compared with normal lung tissue. Abrogation of Jagged1 in lung cancer cell lines attenuates lung cancer growth both *in vitro* and *in vivo*. Further, our data suggest that Jagged1 activates AKT signaling via cross talk with EGF and/or IGF signaling. Paradoxically, abrogation of Jagged1 in lung cancer cell lines promotes cell migration through upregulation of the integrin β1 protein level, which then promotes focal adhesion and activates FAK-SRC signaling cascades. We also show that inhibition of CBF-1-dependent Notch signaling by DNMAML protein does not recapitulate the phenotype of Jagged1 knockdown in lung cancer tumor growth and migration, suggesting that Jagged1 promotes tumor growth and suppresses migration in lung cancer primarily through a CBF-1-independent mechanism. Overall, these findings suggest that Jagged1 plays paradoxical roles in lung cancer progression through activation of AKT and suppression of integrin β1 protein expression, suggesting that the combination of Jagged1 and Integrin β1 inhibitors may be a reasonable new therapeutic approach for lung cancer.

Introduction

Notch signaling is an evolutionary conserved signaling pathway involved in the regulation of embryonic development and cellular homeostasis (2, 276). There are four Notch receptors, Notch 1-4, and five canonical ligands (Jagged1, 2, Delta-like 1, 3, 4) in mammals. Canonical Notch signaling takes place between two juxtaposed cells in normal development. Notch signaling can be classified into two different pathways, CBF-1-dependent and –independent. Signal regulated CBF-1-dependent Notch signaling is activated upon ligand-receptor binding, inducing Notch receptor sequential cleavages to release the Notch intracellular domain (NICD) from the membrane. NICD then translocates into the nucleus to convert the CBF-1 transcriptional repressor complex into a transcriptional activator complex to initiate gene transcription by recruiting transcriptional coactivators, including mastermind (MAML) (2). In reported CBF-1-independent Notch signaling, NICD interacts with components of other signaling pathways, such as EGFR, Wnt, BMP, NFkB, to activate downstream targets (284). The detailed mechanisms for CBF-1-independent Notch signaling remain unclear.

Our group has identified Notch3 as an oncogene contributing to lung cancer progression. Inhibition of Notch3 signaling by a γ-secretase inhibitor or decoy receptor peptides suppress the growth of lung cancer cell lines both *in vitro* and *in vivo* (224-226, 285). Importantly, inhibition of Notch3 by shRNA results in a significant decrease of the aldehyde dehydrogenase-positive stem cell-like population in lung cancer cell lines, suggesting a role for Notch3 in lung cancer stem cells (286). Published work has also

suggested the importance of Notch1 lung cancer progression. Activating mutations of NOTCH1 and loss of a negative regulator of Notch signaling, Numb are present in NSCLC, and inhibition of Notch1 signaling in primary cells harboring NOTCH1 activating mutations suppressed NSCLC progression (220). Regarding the role of Notch ligands in lung cancer, Jagged1 has been shown to suppress apoptosis in the EGFR mutant lung cancer cell line HCC827 (229). The role(s) of Notch ligands in lung cancer cell lines with different genetic backgrounds, such as KRAS mutations found in 20-30% of NSCLC, is unknown (287). In addition to interactions with mutant oncoproteins in cancer cells, cancers often express high levels of both ligands and receptors, in contrast to juxtaposed cells during normal development, adding to the complexity of Notch signaling in cancer. Additionally, besides regulating tumor proliferation and apoptosis, Notch signaling has been linked to epithelial-mesenchymal transition and metastasis in human cancers (190, 196, 288, 289). However, it is not known if Jagged1-mediated Notch signaling plays roles in lung cancer invasion.

A growing body of evidence also indicates the existence of non-canonical Notch signaling in tumorigenesis. For example, loss of Rbpj κ (i.e., CBF-1) in Notch (N)4ICD transgenic mice does not attenuate N4ICD-induced mammary tumor development, suggesting that Notch4 induced breast cancer progression is through a CBF-1-independent mechanism (235). Although the involvement of the Notch pathway in lung cancer progression is clear, it has not been established whether canonical, CBF-1-independent, or both drive progression of lung cancer.

In this study, we identified that elevated expression levels of Jagged1 and Notch3, but not Notch1, are associated with advanced stages of NSCLC tumors, and that knocking down Jagged1 suppresses lung cancer cell growth both *in vitro* and *in vivo* through activation of the AKT signaling pathway via cross talk with EGF signaling and/or IGF signaling. Surprisingly, abrogation of Jagged1 increases lung cancer cell migration via increased protein expression of integrin β1, which in turn activates the FAK-SRC signaling cascade to promote cell migration. Intriguingly, these processes of lung cancer cell growth and migration are not mainly CBF-1-dependent canonical Notch signaling, as expression of dominant-negative Mastermind does not recapitulate the phenotypes observed with knockdown of Jagged1.

Materials and Methods

Antibodies Antibodies against Jagged1, Notch1, DLL1, DLL3, DLL4, Jagged2, Phospho-FAK, total-FAK, phospho-Src, total-Src, phospho-Erk, total-Erk, phospho-Akt, total-Akt for Western Blot analysis are purchased from Cell Signaling Technology, Danvers, MA. Antibodies against Integrin β1 for both Western Blot analysis and functional blocking are purchased from Millipore, Billerica, MA. An antibody against β-actin is from Sigma-Aldrich, Inc. IHC staining for Notch1 was done using monoclonal antibody (1:100, Cell Signaling Technology, #3608); Notch3 using polyclonal antibody (1:100, Abcam, ab60087-100); and Jagged1 using monoclonal antibody (1:100, R&D, AF1277). FITC-conjugated Phalloidin for F-actin, Phospho-FAK (Tyr925) antibody (Cell Signaling Technology, Danvers, MA) and Alexa594-

conjugated goat anti-Rabbit and TOTO-3 (Invitrogen, Carlsbad, CA) are used in immunofluorecent staining.

Tissue microarray and IHC evaluation. Tissue microarrays were obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Core at Vanderbilt University and analyzed by immunohistochemistry for Notch1, Notch3 and Jagged1. For evaluation, the localized membrane expression was scored with slight modifications to Ariol SL-50 platform based on the percentage of the cells with positive immunohistochemical staining. Staining indices were classified as follow: 0-Negative; 1-weak; 2-moderate; 3-strong. The correlation between expression levels and stages was analyzed using Chi square test.

Quantitative Real-time PCR. Total RNA was extracted using Qiagen RNase Mini kit. RNA was reverse transcribed with SuperScript II First-Strand Synthesis kit (Invitrogen). Annealing temperature for PCR was 58°C with the following primers: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′ TGCACCACCAACTGCTTAGC-3′ (sense) and 5′-GGCATGGACTGTGGTCATGAG-3′ (antisense); Hey1, 5′-AGATGACCGTGGAT CACCTG-3′ (sense) and 5′-TGTTGAGAGCGAAACCAGTC-3′ (antisense); and ITGB, 5’-GTTACACGGCTGCTGGTGTT-3’ (sense) and 5’-CTACTGCTGACTTAGGGATC-3’(antisense). The threshold cycle value (Ct) was determined with iCycler Optical system interface software. Primers for Mean Ct was calculated from triplicate measurements and normalized with the mean Ct of GAPDH as internal control.

Stable expression of Jagged1 short hairpin RNA and DNMAML in lung cancer cell lines. Lung cancer cell lines were obtained from American Type Culture Collection and maintained in RPMI-1640 (Invitrogen) with 10% FBS (hyclone). The lenti-viral based short hairpin RNA plasmid constructs against human JAG1 and corresponding empty vector were purchased (Sigma-Aldrich, Inc). Plasminds were co-transfected into HEK293FT with lenti-virus packaging vectors using FuGENE 6 (Roche). The filtered viral supernatant was resuspended in 3μg/mL polybrene (Sigma-Aldrich, Inc) and added to culture medium of NSCLC lines. After 6 hours, media was replaced with complete medium. After 48 hours, 1μg/ml puromycin (Invivogen) was added to the medium for selection and transfectants were passed serially for 2 weeks to generate Jag1 knockdown (KD) stable cell lines. The migR1-GFP control constructs and migR1-DNMAML-GFP constructs were kindly provided by Dr. Warren Pear (University of Pennsylvania, Philadelphia, PA). Plasmids were transfected into GP2-293 Packaging cell line using FuGENE 6 (Roche). After 24 hours, medium was replaced with RPMI 1640 containing 10% FBS. After 72 hours, infected cells were sorted for GFP positivity by flow cytometry.

Rat Jagged1 stable expression in Jag1 KD lung cancer cell line. The full-length Rat Jagged1 was subcloned from pBOS-SN3T, which was kindly provided by Dr. Geraldine Weinmaster (University of California, Los Angeles, CA) into pCDNA3.1 vectors with G418 selection marker. pCDNA3.1 control vector and pCDNA3.1-rJag1 were transfected into Calu-6 non-target control shRNA cells and Calu-6 Jag1 KD cells. After 72 hours, transfected cells are selected with 500mg/ml G418 for 2 weeks to obtain stably expressing rJag1.

Luciferase assay. NSCLC cells were seeded on 24-well plates (1x105 cells/well) one day before co-transfecting with 400ng 12XCBF-1-reporter construct, 1ug NICD1 plasmid, and 25ng TK-Renilla construct as an internal control. After 24 hours after transfection, luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

MTT cell proliferation assay. NSCLCs (2x104/well) were plated in triplicate in complete medium on 12-well plates for 1-3 days as indicated. Cells were incubated with 550ul (0.5mg/ml) MTT into complete medium for 4 hours, then 500ul of SDS-HCl (0.01M HCl, 10% SDS) is added to each well. Cell proliferation was quantified using High-throughput microplate reader at 560nm.

Soft agar colony formation assay. Cells were suspended in 0.4% soft agarose in RMPI 1640 supplemented with 10% FBS and plated in triplicate over a layer of 0.6% soft agarose in 6-well plates. After 2 weeks, colonies were stained with 0.05% crystal violet and counted.

Cell migration assay. Transwell plates (Corning) were used as instructed by manufacturers. Briefly, cells were cultured in serum-free medium for 24 hours, then $1x10^5$ cells were plated in triplicate in serum-free OPTIMEN medium in upper chamber, and complete medium placed in bottom wells for 24 hours. The migrating cells were fixed in 10% formalin solution, and stained by 0.05% crystal violet. Three microscopic fields

(20X) were photographed and counted per chamber, and results were expressed as mean ± SD of results from replicate wells. For Integrin β1 blocking experiment, 10ug/ml Integrin β1 blocking antibody was added into complete medium placed in bottom wells.

Wound healing assay. H358 cells with non-target shRNA and Jagged1 shRNA were cultured in a 6-well plate. 24 hours later, at 90% confluence, a single wound was generated in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The debris was removed by washing with medium. After 24 and 48 hours, migrated cells were visualized and photographed using an inverted microscope. Each experiment was performed three times independently.

Immunofluorescent staining. 4-well Glass Chamber Slides (Thermo Scientific Inc, Nunc, NY) were coated with 400ul (10ug/ml) overnight at 4°C and rinsed one time with PBS. 5x103 cells were cultured on Fibronectin coated chamber slides overnight, washed one time with PBS, then fixed in 4% paraformaldehyde for 30 minutes. Following fixation, cells were blocked by 2% BSA then stained with primary antibodies (see Supplementary Materials) overnight at 4°C, followed by secondary antibody. Images were recorded by confocal microscopy.

In vivo tumorigenesis. Athymic 4- to 6-week-old nude mice (Jackson Laboratory) were used for the xenograft experiments. 1x106 cells from Calu-6 and H358 cell lines with either non-target shRNA, Jag1 shRNA, migR1 or DNMAML were diluted into 200ul of 1:1 PBS-Matrigel solution (BD Biosciences). Control and experimental cells were

subcutaneously injected into left or right flank of nude mice respectively. When the tumors were palpable, tumor volume was measured every 3 days using a caliper and calculated with the formula: volume = (length) 2 × (width) 2/2. All experimental procedures were performed with approval from the Vanderbilt Institutional Animal Care and Use Committee.

Statistical Analysis. Unless specifically stated, statistical inference in comparative experiments both in vivo and in vitro was performed using the two-tailed Welch's t test. For all statistical comparisons, the differences were considered significant at $p<0.05$. The correlation between Jagged1/Notch1/Notch3 expressions and tumor stages was analyzed by Chi square test.

Results

Jagged1 and Notch3, but not Notch1, are highly expressed in lung cancer tumors

To determine expression of Notch signaling components in lung cancer, we compared their expression levels in adjacent normal lung tissue to that in the involved lung tumor tissue from our microarray dataset of Vanderbilt patients. We have identified that Notch3 is the only receptor among all four Notch receptors to be upregulated at the mRNA level in lung tumors compared with normal lung tissues (Fig. 4.1A). In contrast, several Notch ligands (Jagged1, 2, DLL1, and 3), except DLL4, are overexpressed in 49 lung cancer patient samples compared with 9 adjacent normal lung tissues (Fig. 4.1B). Notch1, Notch3 and Jagged1 have been extensively documented to function as oncogenes in human solid

tumors (135, 164, 210). To further investigate the frequency of Notch1, Notch3 receptors and Jagged1 protein expression in lung cancer patients, we performed an immunohistochemistry analysis using a tissue microarray consisting of 101 NSCLC tissues and 20 normal lung tissues. Indeed, Notch3 and Jagged1, but not Notch1 are frequently highly expressed in NSCLC tumors (Fig. 4.2A). We also classified their expression level based on tumor stages. Both Jagged1 and Notch3 expressions are elevated in more advanced stage tumors, but low in normal tissue with a statistical significance (Fig. 4.2B and C). Notch1 does not appear to correlate with tumor stage, as the levels of Notch1 protein do not increase with advanced stages of tumor progression (Fig. 4.2D).

Abrogation of Jagged1 suppresses NSCLC tumor growth both *in vitro* and *in vivo*

Due to the fact that Jagged1 is overexpressed in NSCLC and literatures indicate that Jagged1 plays an oncogenic role in many cancers (136, 290-292), we hypothesized that Jagged1 is oncogenic in lung cancer and inhibition of Jagged1 will suppress lung cancer tumorigenesis. To define whether the Notch1 and 3 receptors and Jagged1 and 2 ligands are expressed in lung cancer cell lines as in NSCLC tumors, we examined Calu-6, H358, Calu-1, H1993, H2122, H23, H1299 and an immortalized lung epithelial cell line 16HBE. Jagged1 ligand is highly expressed in these lung cancer cell lines, but not in immortalized lung epithelial cell line 16HBE (Fig. 4.3A). Jagged2 ligand is also expressed in these cell lines, but the expression level and frequency is much lower than Jagged1. As expected, Notch1 and Notch3 are expressed in both lung cancer cell lines and 16HBE (Fig. 4.3A). DLL1, DLL3 and DLL4 protein expression was not detected in these cell lines using multiple commercially available antibodies (data not shown). These data suggest that

Jagged1 is the dominant Notch ligand expressed in lung cancer cell lines. To test our hypothesis, we used shRNAs against Jagged1 to stably knockdown Jagged1 in lung cancer cell lines Calu-6 and H358 (Fig. 4.3B). Abrogation of Jagged1 in these cell lines significantly decreased cell growth (Fig. 4.3C, Fig. 4.8A). Importantly, inhibition of Jagged1 in Calu-6 and Calu-1 cell lines also suppressed their ability to form colonies in soft agar (Fig. 4.3D). In vivo, we observed a statistically significant reduction of tumor growth with Jag1 KD in both Calu-6 and H358 cell lines compared with non-targeted control Calu-6 and H358 cells (Fig. 4.3E).

Jagged1 promotes NSCLC growth mainly through a CBF-1-independent mechanism

To investigate if Jagged1 promotes lung cancer growth through CBF-1-dependent canonical Notch signaling, we generated Calu-6 and H358 cell lines stably expressing dominant-negative Mastermind like-1, which encodes the N-terminal Notch-binding domain of

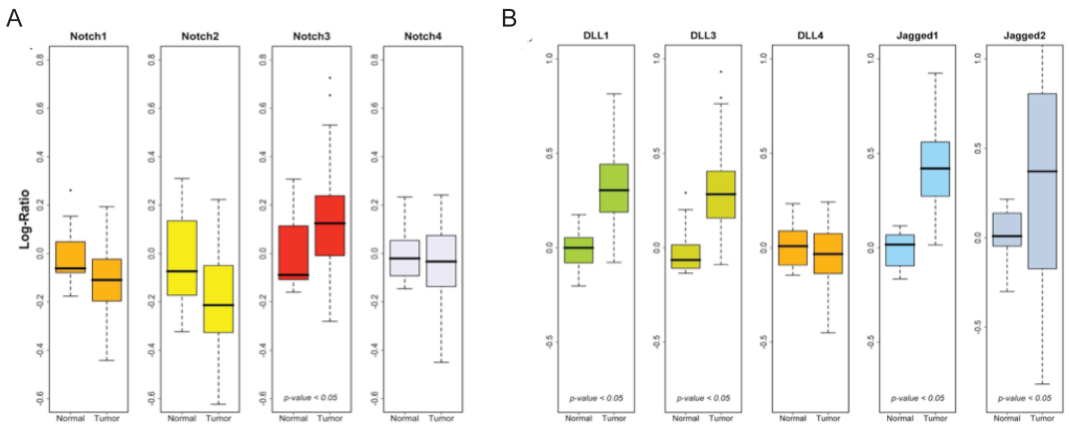

Figure 4.1. Notch3 receptor, Jagged1, Jagged2, DLL1 and DLL3 ligands are upregulated in lung tumors compared with normal lung tissues from microarray analysis. A. Relative expression of Notch 1-4 receptors, B. Notch ligands Jagged1, 2 and Dll 1,3 ,4 in clinical samples of lung cancer, by Affymetrix chip, Human genome U133. Data in log 2 scale and normalized with the average from a pooled normal lung control (BD Clontech) and nine normal lung tissues (10 total samples). The numbers and types of samples are as follows: 19 squamous cell carcinoma, 23 adenocarcinoma, and 7 large cell carcinoma.

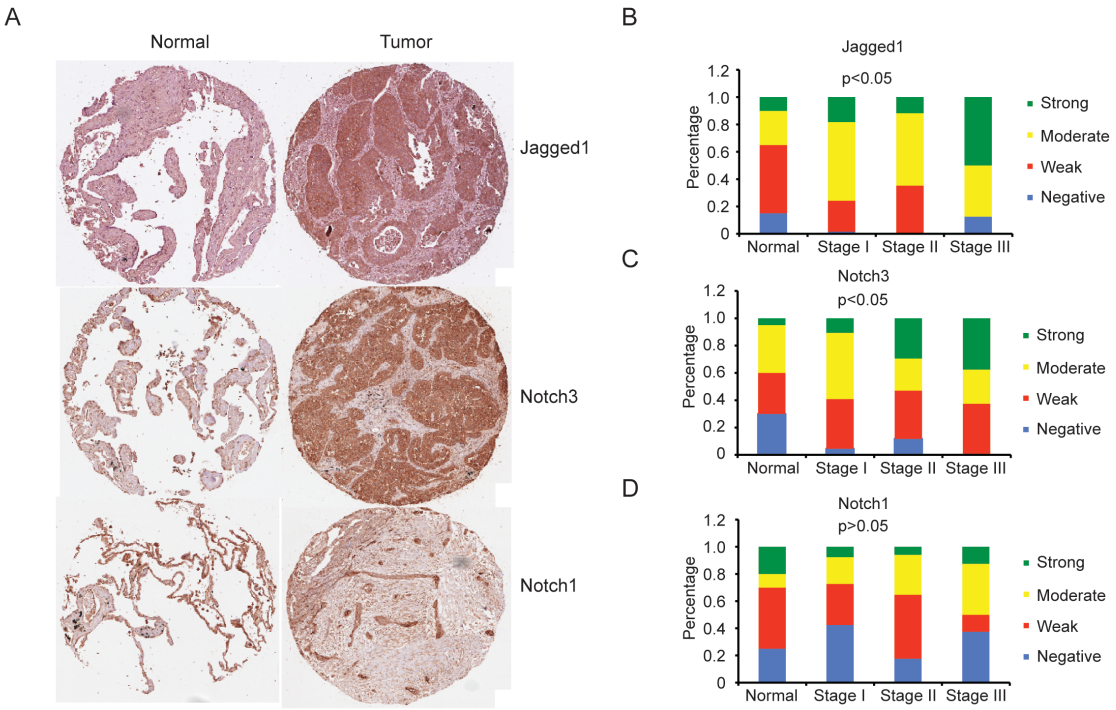

Figure 4.2. Tissue microarray analysis of Jagged1, Notch1 and Notch3 in normal lung and NSCLC samples. A. Representative images of immunohistochemistry. B. Percentage of samples with different Jagged1, C. Notch1, D. Notch3 expression levels in normal tissue and NSCLC classified by different stages. Blue bar: percentage of negative staining; red bar: percentage of weak staining; yellow bar: percentage of moderate staining; green bar: percentage of strong staining. The significant p values indicate significant association between stages and protein expression.

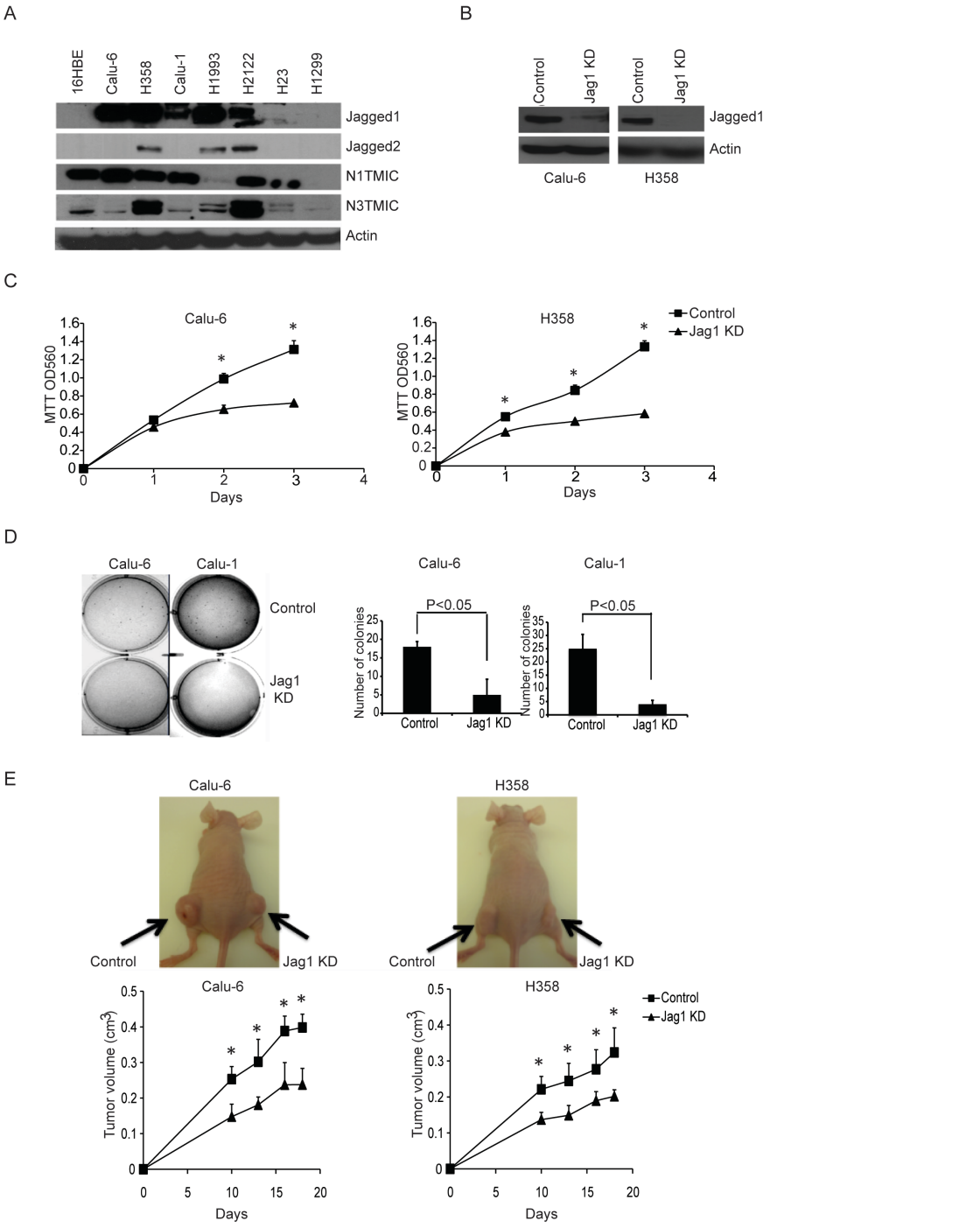

Figure 4.3. Jagged1 regulates lung cancer cell growth both *in vitro* and *in vivo*. A. Western blot showing Jagged1, Jagged2, Notch1 and Notch3 protein expression levels in lung cancer cell lines and immortalized lung epithelial cell 16HBE. B. Western blot showing Jagged1 expression in Calu-6 and H358 cells with non-target control shRNA and shRNA against Jagged1. C. Cell growth curve of Calu-6 and H358 cells with non-target control shRNA and Jagged1 shRNA for 3 days measured by MTT cell proliferation assay. D. Colonies numbers of Calu1 and Calu-6 cells with non-target control shRNA and Jagged1 shRNA using soft agarose colony formation assay. Quantification of colonies numbers is the average colony numbers of 3 respective experiments. E. Tumor growth curve of Calu-6 and H358 cells with non-target control shRNA and Jagged1 shRNA of *in vivo* mouse xenograft tumor model. p values is from Welch's t-test. * indicated $p<0.05$, statistically significant.

MAML1 fused with GFP protein (DNMAML) to suppress Notch-CBF-1-MAML mediated gene transcription (117). Expression of DNMAML in Calu-6 and H358 cells significantly decreased N1ICD activated CBF-1 luciferase reporter (Fig. 4.4A), and reduced canonical Notch target gene Hey1 mRNA expression (Fig. 4.4B) indicating that expression of DNMAML protein in lung cancer cell lines inhibits CBF-1-dependent canonical Notch activity. We observed that inhibition of canonical Notch signaling does not affect Calu-6 cell growth with 10% serum, while cell growth is reduced in media without serum. In H358 cells, grown under conditions with or without serum, there is a slight but statistically significant reduction of cell growth when canonical Notch signaling is inhibited (Fig. 4.4C). Nevertheless, the reduction rates in both cell lines are minimal compared to the effects caused by abrogation of Jagged1. We do not observe any increase of apoptotic cells in DNMAML expressing cells compared with control when grown in either 10% serum or serum free medium (data not shown). In vivo, the xenograft tumor models generated with Calu-6 cells demonstrated no difference in tumor growth by expression of DNMAML protein. While there is a statistically significant reduction of tumor volume by DNMAML expression compared to control in xenograft tumors generated with H358 cells examined before the tumor reached the size of 0.3 cm^3. However, as tumors progressed, they reached the same size independent of DNMAML expression (Fig. 4.4D).

Jagged1 signaling activates AKT in lung cancer via IGF and/or EGF signaling

Given our observations that Jagged1 promotes lung cancer cell growth mainly via CBF-1-independent non-canonical Notch signaling, we set out to investigate the possible

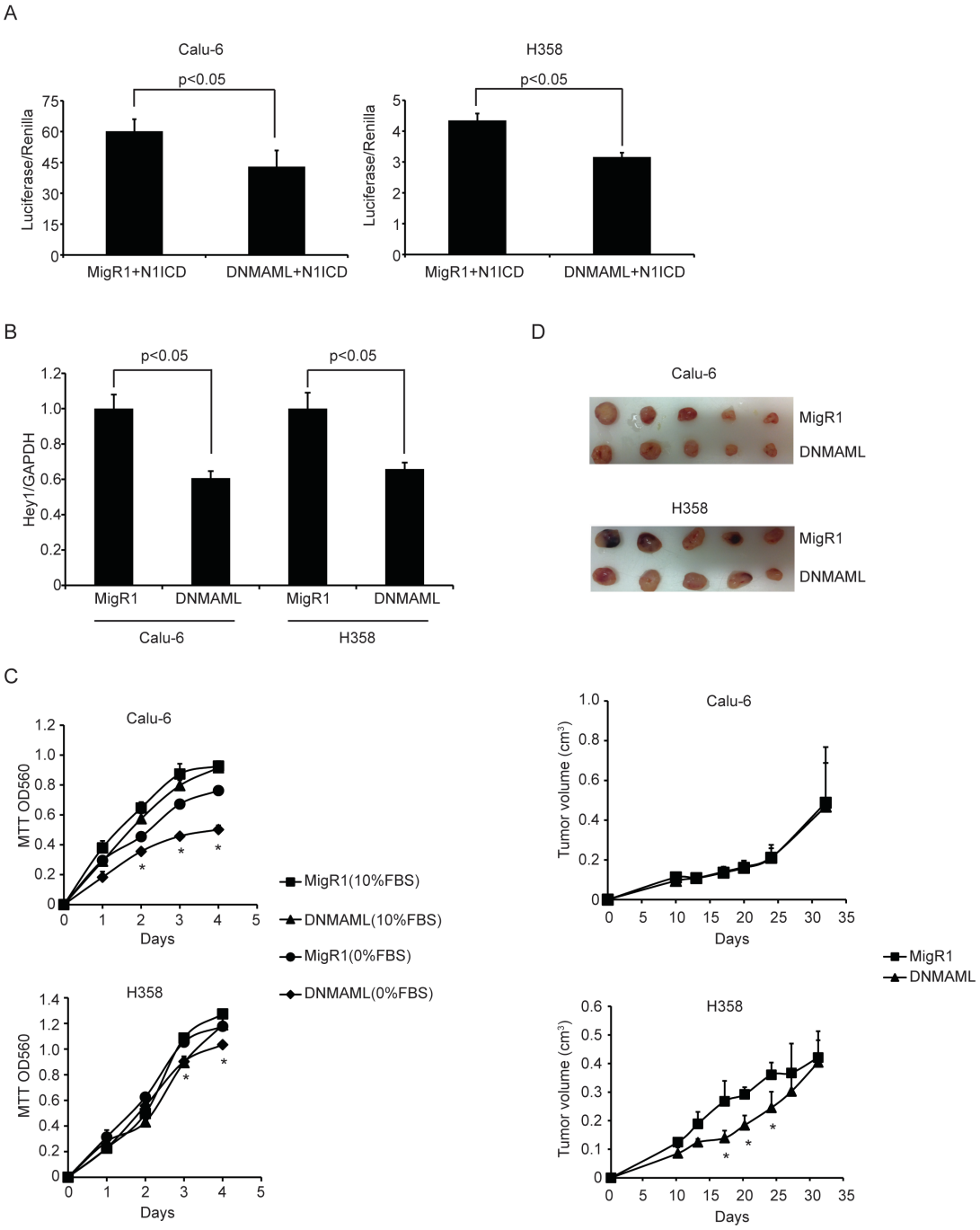

Figure 4.4. Inhibition of canonical Notch signaling by DNMAML slightly inhibits lung cancer progression *in vitro* and *in vivo*. A. Luciferase reporter activities of CBF-1 response element for Calu-6 and H358 cells with migR1 vector control and DNMAML expression induced by N1ICD. B. Relative expression of Hey1 mRNA measured by real-time quantitative RT-PCR for Calu-6 and H358 cells with migR1 vector control and DNMAML expression vectors. C. Cell growth curve of Calu-6 and H358 cells with migR1 vector control and DNMAML expression vectors for 3 days under serum free and 10% FBS medium measured by MTT cell proliferation assay. D. Tumor growth curve of Calu-6 and H358 cells Calu-6 and H358 cells with migR1 vector control and DNMAML expression vectors in mouse xenograft tumor model. p values is from Welch's t-test. * indicated $p<0.05$, statistically significant.

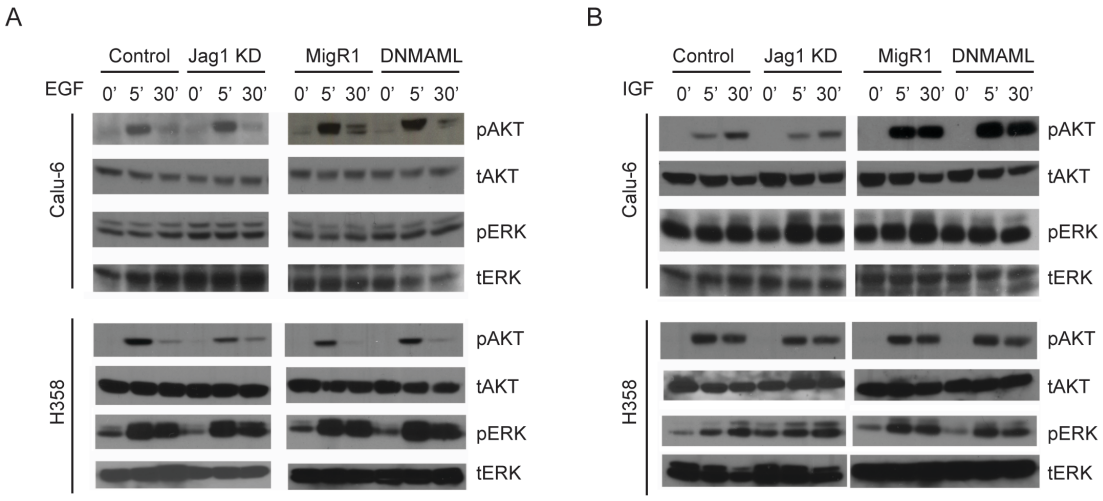

Figure 4.5. Jagged1 signaling regulates AKT pathway through crosstalk to EGF and/or IGF signaling. Western blot showing phospho-AKT, total-AKT, phospho-ERK, and total-ERK expression in Calu-6 and H358 with stable expression of non-target control shRNA, Jagged1 shRNA, migR1 control vector and DNMAML after stimulation of A. 50ng/ml EGF for 0min, 5min and 30min, B. 10ng/ml IGF for 0min, 5min and 30min.

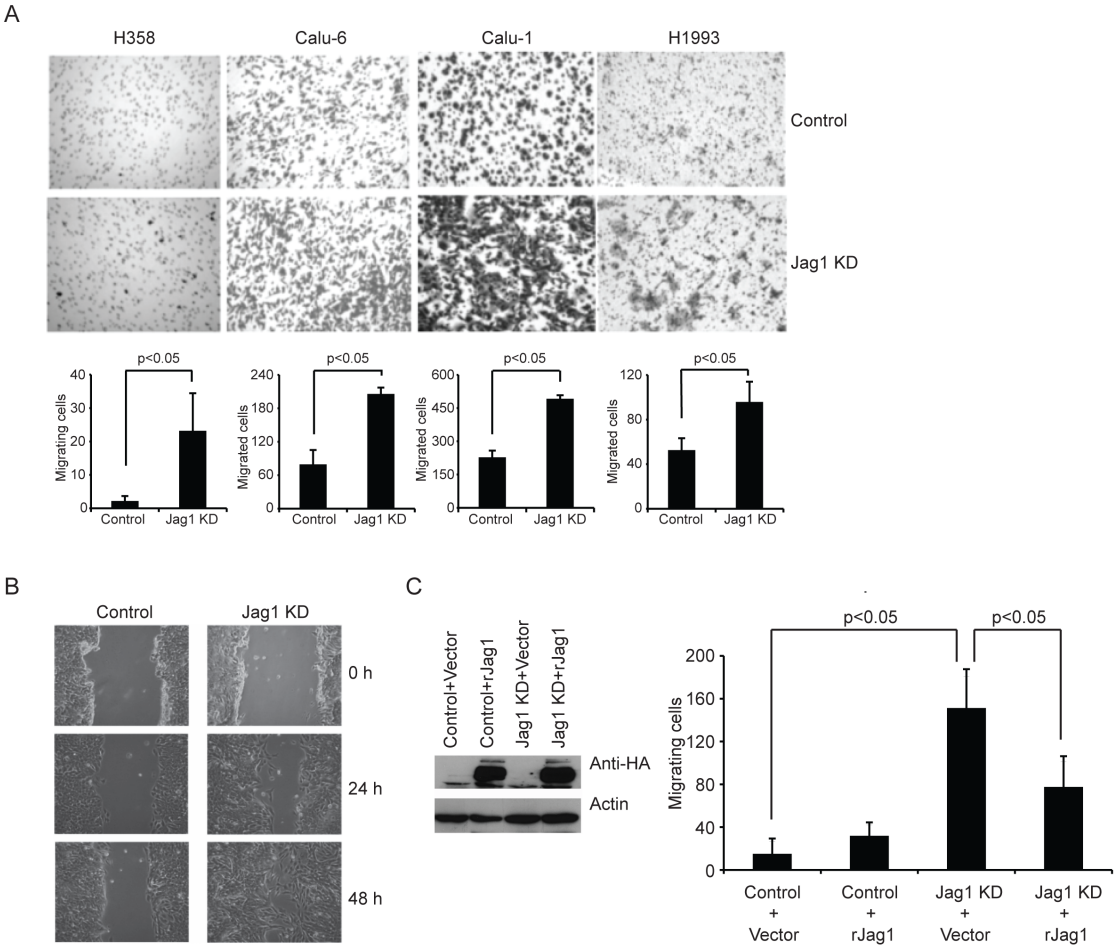

Figure 4.6. Figure 5. Abrogation of Jagged1 promotes lung cancer cell migration. A. Cell migration of H358, Calu-6, Calu-1, and H1993 with non-target control shRNA and Jagged1 shRNA measured by transwell migration assay. B. Ability of H358 cells with non-target shRNA and Jagged1 shRNA to close wound within 48 hours. C. Western blot showing that rat Jagged1 with HA tag is stably expressed in Calu-6 cells. And Cell migration of Calu-6 cells with pCDNA3.1 vector+non-target control shRNA, rat Jagged1 expression vector+non-target shRNA, pCDNA3.1+hJagged1 shRNA, and rat Jagged1 expression vector+hJagged1 shRNA.

mechanism. Based on the report of AKT activation being a key mediator of Notch1 pro-survival effects under hypoxia through IGF1R signaling in lung cancer (293), and of crosstalk between Notch receptor intracellular domain and EGF signaling during cancer progression (225, 226, 294), we tested if Jagged1 can activate AKT and/or ERK signaling by crosstalk with these pathways. We observed that in Calu-6, abrogation of Jagged1 attenuates IGF but not EGF mediated phosphorylation of AKT. However, in H358, abrogation of Jagged1 attenuates both EGF and, to a lesser extent, IGF mediated phosphorylation of AKT. DNMAML expression has no effect on phosphorylation of AKT in either cell line (Fig. 4.5A, B), suggesting that Jagged1 regulates lung cancer growth by activation of AKT through crosstalk with EGF and/or IGF signaling. Phosphorylation of ERK is not affected in either Jag1 KD or DNMAML expressing cell lines (Fig. 4.5A, B), which may be due to the fact that both Calu-6 and H358 cell lines have a KRAS mutation, such that phosphorylation of ERK is constitutively activated.

Abrogation of Jagged1 promotes lung cancer cell migration

To test if Jagged1 signaling affects tumor migration, we performed a transwell migration assay in H358, Calu-6, Calu-1 and H1993 cells. Surprisingly, we observed a statistically significant increase of cell migration when Jagged1 signaling is attenuated (Fig. 4.6A). With an additional independent shRNA against in Calu-6 cells, we were able to demonstrate a consistent stimulation of lung cancer cell migration (Fig. 4.8C). Moreover, using a wound-healing assay, we also observed that H358 cells with Jag1 KD shRNA closed the wound faster than control within 48 hours (Fig. 4.6B). To rule out off-target

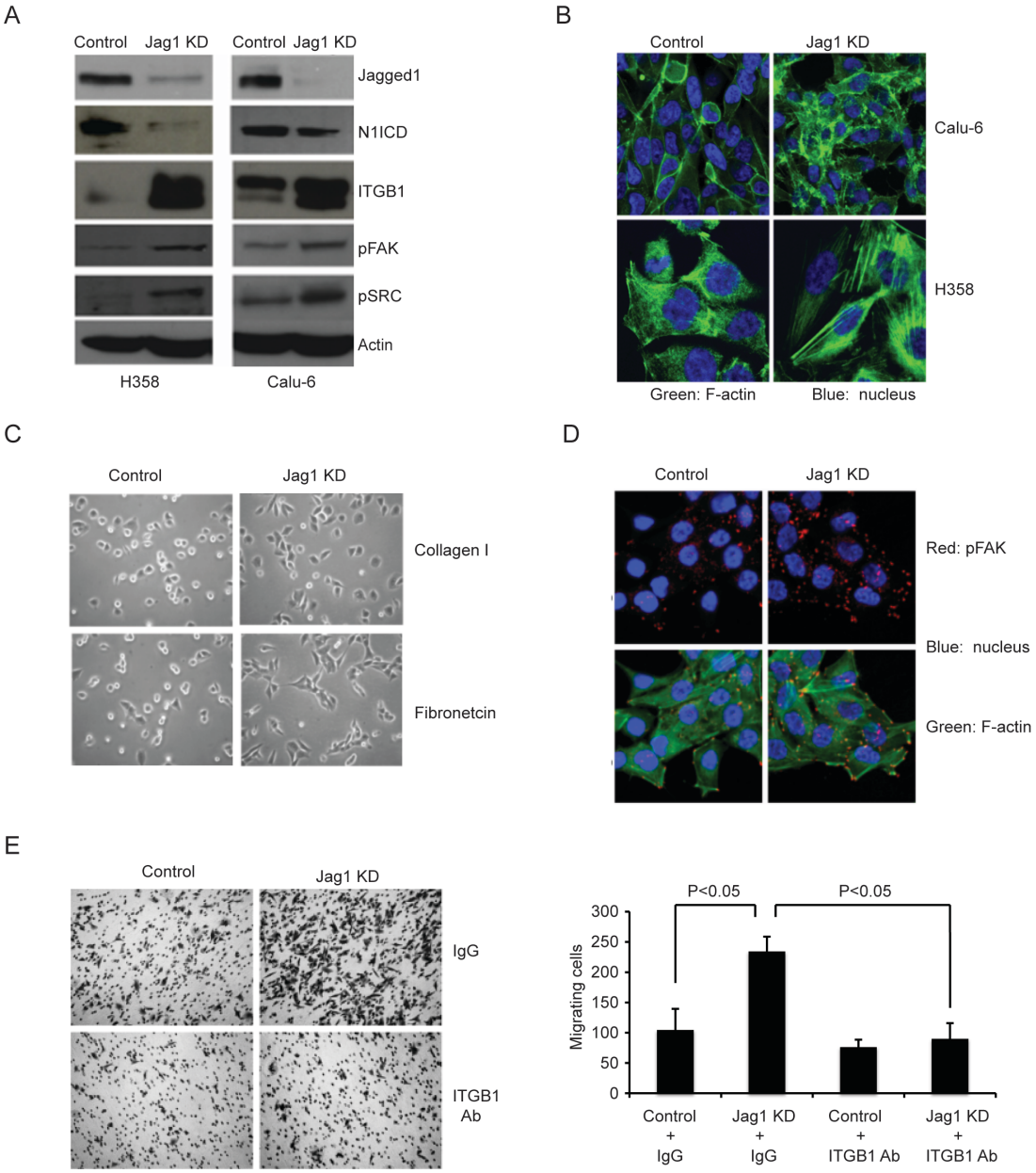

Figure 4.7. Jagged1 regulates lung cancer cell migration is mediated by Integrin β1 signaling. A. Western blot showing that expression of Integrin β1 protein level and its downstream cascade phosphorylation of FAK, and phosphorylation of Src protein in Calu-6 and H358 cells with non-target control shRNA and Jagged1 shRNA. B. Immunofluorescent staining of F-actin in Calu-6 and H358 cells with non-target control shRNA and Jagged1 shRNA on fibronectin coated chambers. C. Cell adhesion and spreading of H358 cells with non-target control shRNA and Jagged1 shRNA on collagen I and fibronectin coated plates. D. Immunofluorescent staining of F-actin and phospho-FAK in H358 cells with non-target control shRNA and Jagged1 shRNA on Fibronecton coated glasses chambers. E. Cell migration of Calu-6 cells with non-target control shRNA and Jagged1 shRNA treated with control IgG and Integrin β1 blocking antibody.

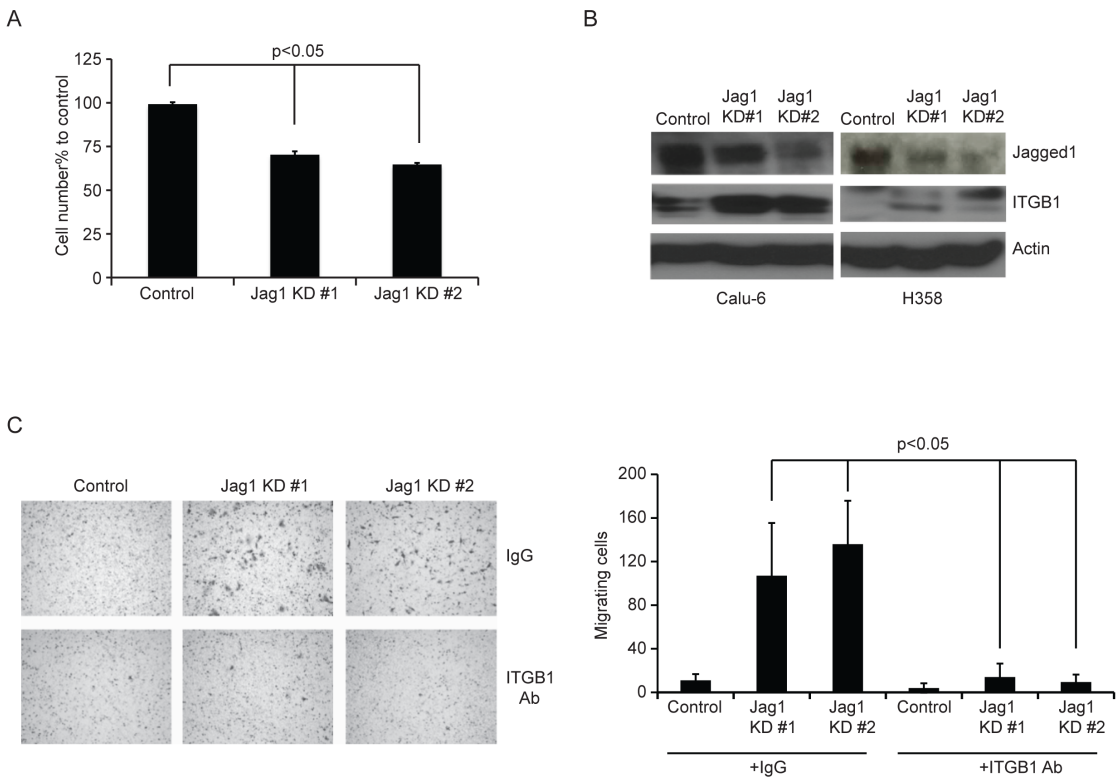

Figure 4.8. Abrogation of Jagged1 by different shRNA showed the same effect in cell growth and migration. A. Cell growth of Calu-6 with non-target control shRNA, Jagged1 shRNA #1 and #2 for 2 days measured by MTT cell proliferation assay. B. Western blot showing that expression of Jagged1 and Integrin β1 protein levels in Calu-6 and H358 cells infected with non-target control shRNA, Jagged1 shRNA #1 and #2. C. Cell migration of Calu-6 cells with non-target control shRNA, Jagged1 shRNA#1 and #2 treated with control IgG and Integrin β1 blocking antibody. Migrating cells were counted and expressed as the mean values (+- S.D) of triplicate wells (bar graphs), with p values from Welch's t-test. * indicates statistically significant $p<0.05$. Jagged1 shRNA #1 is an additional shRNA. #2 is the same shRNA used in all the studies.

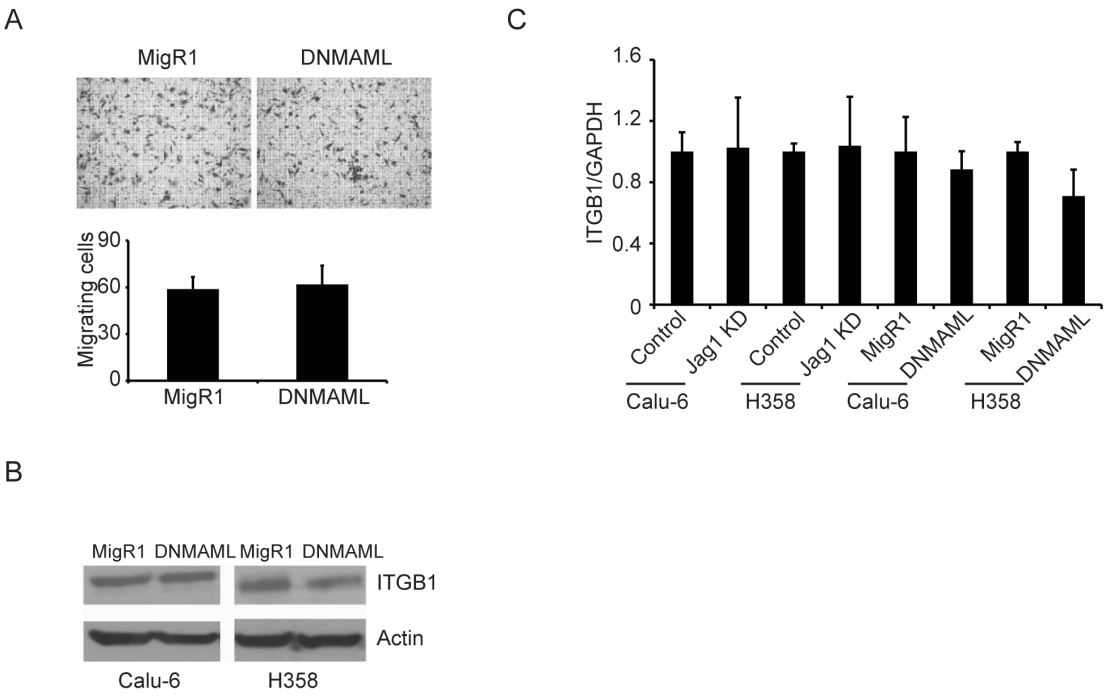

Figure 4.9. Inhibition of canonical Notch signaling does not affect lung cancer cell migration. A. Cell migration of Calu-6 cells with migR1 control vector and DNMAML expression. Migrated cells were counted and expressed as the mean values (+- S.D) of triplicate wells (bar graphs). B. Western blot showing Integrin β1 protein expression level in Calu-6 cells with migR1 vector control and DNMAML expression. C. Relative expression of Integrin β1 mRNA level in Calu-6 cell and H358 cell with non-target control shRNA, shRNA against Jagged1, migR1 control vector and DNMAML expression. Relative Integrin β1 mRNA expression value was normalized by GAPDH mRNA expression.

effects of shRNA, as a cause of the increased migration, we re-expressed rat Jagged1 protein stably in Calu-6 cell with abrogation of human Jagged1, as there is no consensus sequence for this shRNA in rat Jagged1. Stable re-expression of rat Jagged1 protein in Calu-6 cells with human Jagged1 shRNA significantly decreased cell migration compared with Jag1 KD cells (Fig. 4.6C). Stable re-expression of rat Jagged1 protein in Calu-6 cell with non-target control shRNA does not affect cell migration, likely due to the high endogenous level of Jagged1 in Calu-6 cells. Inhibition of CBF-1-dependent canonical Notch signaling by stable expression of DNMAML protein does not affect cell migration in either Calu-6 (Fig. 4.9A) or H358 cell lines (data not shown), suggesting that Jagged1 regulation of lung cancer cell migration is not through CBF-1-dependent canonical Notch signaling.

Jagged1 regulates lung cancer cell migration through integrin β1 signaling

Integrins contribute to cell movement by providing traction to migratory cells through the signals from extracellular matrix (295). Expression of the intracellular domain of Notch1 has been linked to the activation of β1 integrin independent of CBF-1 transcriptional activity, and without affecting integrin expression (296). Indeed, integrin β1 protein level was increased upon Jagged1 knockdown in both cell lines (Fig. 4.7A, Fig. 4.8B), whereas integrin α5 and α1 expression levels are not altered by attenuation of Jagged1 (data not shown). Additionally, downstream integrin signaling cascades including phospho-FAK and phospho-Src are also upregulated (Fig 4.7A). A prominent function of integrins is to mediate the adhesion of cells to their substrate providing a physical link between ECM and cytoskeleton (297). Decreasing expression levels of Jagged1 increased actin stress fibers

and caused a disorganized actin cytoskeleton when cells were cultured on fibronectin-coated chambers (Fig. 4.7B). Increase of adhesion spreading of H358 Jag1 KD cells is observed as well when cells were cultured on collagen I or fibronectin coated plates for 4 hours (Fig. 4.7C). Focal adhesion sites are increased in Jag1 KD H358 cells when cells cultured on fibronectin-coated chambers (Fig. 4.7D). Furthermore, inhibition of integrin β1 by a blocking antibody in Jag1 KD Calu-6 decreased the number of migrating cells to the same level as control (Fig. 4.7E, Fig. 4.8C), indicating that integrin β1 is the essential factor regulated by Jagged1 to induce cell migration. In contrast, stable expression of DNMAML protein did not affect integrin β1 protein levels in either Calu-6 or H358 (Fig. 4.9B). In addition, neither Jag1 KD nor DNMAML expression affected integrin β1 mRNA expression levels (Fig. 4.9C), indicating that the effect of Jag1 KD on integrin β1 protein expression is post-transcriptional.

Discussion

Altered expression level of oncoproteins in tumors compared with normal tissue is often an indication of relative biological importance in tumorigenesis and may help define therapeutic targets in human cancer. Even more strongly, lack of target expression suggests lack of efficacy. Expression of Notch components in human cancer including lung cancer has been previously described, and the Notch ligand Jagged1 has been shown to correlate with poor overall survival in breast cancer (136, 290). Notch3 has been shown to be upregulated in colorectal cancer (298), NSCLC (225), ovarian cancer (153), and cervical cancer (299). Our data further strengthen the finding in lung cancer that Jagged1 and Notch3 are overexpressed, and their expression is correlated with advanced tumor

stages, indicating their oncogenic function in lung cancer. In contrast, Notch1 protein is not upregulated in NSCLC tumors compared with normal lung tissue using clinical samples, suggesting that Notch1 may not be an important target in NSCLC.

Inhibition of Notch3 signaling has been shown previously to suppress growth of lung cancer cells (225, 226, 285). However, in two assayed KRAS mutant cell lines, H356 and Calu-6, knockdown of either Notch1 or Notch3 did not lead to remarkable growth suppression as observed upon knockdown of Jagged1 (data not shown). Lack of an obvious change in growth may be explained by redundant roles of Notch1 and Notch3 on the regulation of lung cancer cell growth in genetic subclasses. Notch3, Notch1, Jagged1 and Jagged2 have been shown to play roles in various lung cancer models with different genetic background (219, 226, 229). Notably, these studies did not investigate the importance of CBF-1-dependent canonical Notch signaling in lung cancer. In this study, we have used DNMAML to suppress canonical Notch signaling. The DNMAML approach is more specific for Notch signaling than knocking down levels of CBF-1, as the DNMAML protein can still bind to NICD, but lacks the C-terminal portion to initiate gene transcription. Our study demonstrates that in particular lung cancer contexts, inhibition of CBF-1-dependent canonical Notch signaling had minimal effects on tumor cell growth in vitro and in vivo, implying that the pro-tumor effects of Notch signaling in these tumors may not be primarily through CBF-1-dependent canonical Notch signaling. Besides targeting Notch receptors and ligands, stapled peptides preventing formation of Notch-CBF-1-MAML transcriptional complex have emerged as a potential therapy to target CBF-1-dependent Notch signaling, such as T-ALL (269). Our study indicates that strategies to

target Notch-CBF-1-MAML transcriptional machinery may not work for inhibition of lung tumor growth. Notch signaling has been found to crosstalk with other signaling pathways independent of CBF-1 (284). We found that Jagged1-mediated Notch signaling is able to activate the AKT signaling pathway through crosstalk with EGF and/or IGF signaling, also independent of CBF-1. Other signaling pathways may also cooperate with Notch signaling as well in a CBF-1-independent manner during lung cancer tumorigenesis. Further investigations will be needed to address this further.

For patients with NSCLC, the biggest threat to survival is metastasis. Several studies indicate Notch signaling promotes EMT and tumor metastasis via regulation of EMT regulators Slug, ZEB and miR200 (181, 190, 288). However, in our study, inhibition of Jagged1 does not affect the process of EMT in lung cancer cells (data not shown). Surprisingly, we found that abrogation of Jagged1 increased motility of lung cancer cells through upregulation of integrin β1 protein expression, which then activates the FAK-SRC signaling cascade. Indeed, integerin α5β1 has been link to NSCLC metastasis by the observation that increased expression of integrins α5β1 is significantly correlated with lymph node metastasis of human NSCLC patients (300). The finding that Jagged1 is responsible for growth promotion but migration suppression appears counter-intuitive. The reasons why Jagged1 has such paradoxical function in tumor progression is unknown, but it is possible that tumor cells surviving from the inhibition of Jagged1 become more aggressive/metastatic via upregulation of the integrin signaling pathway. This result clearly raises the possibility that chronic blockade of Jagged1 in lung cancer could be a double-edged sword. Inhibition of integrin β1 by a blocking antibody reverses the

migration phenotype upon Jagged1 abrogation, indicating that the combination of a Jagged1 inhibiting reagent with an integrin β1 inhibitor could be a good strategy to target lung cancer.

Recently, Yang et al reported that Jagged2 promotes metastasis of transgenic mutant KRAS-driven adenocarcinoma cells through downregulation of miR-200, while Jagged1 does not affect metastasis in this model (181). The fact that we observed a different migration effect of Jagged1 in human lung cancer cell lines may due to the complicated genetic landscape of lung cancer cell lines compared to genetically defined tumor models. Moreover, we performed an analysis of microRNA expression with control and Jag1 KD in Calu-6 cells, but did not see any significant expression changes of the microRNAs observed in the murine study upon Jagged1 inhibition (data not shown). This suggests that in the human lung cancer cell line Calu-6, Jagged1 is not regulating cell migration through miR-200 expression.

Ectopic expression of N1ICD caused down-regulation of multiple matrix-adhesion genes, including integrin β1 in immortalized mammary epithelial cells in MCF-10A (301). It is not clear from our study if Jagged1 regulates cell motility and integrin β1 expression in lung cancer in a Notch receptor-dependent manner. We have tried to assess this aspect using the immortalized lung epithelial cell line 16HBE overexpressing constitutive active N1ICD. However, neither cells migration nor integrin β1 protein expression is altered upon constitutively activation of Notch1 (Fig 4.10A). In addition, by blocking S3 cleavage of Notch receptors using a γ-secretase inhibitor MRK003, we did not observe an increase

of integrin β1 or phospho-FAK protein expression (Fig. 4.10B). These data suggest the possibility that Jagged1 regulates lung cancer migration and integrin β1 protein expression through Jagged1 signaling independent of Notch receptors. Yet, we have not ruled out whether full-length or membrane tethered Notch receptors suppress cell migration and integrin β1 expression. Also, whether the process is regulated through multiple activated Notch receptors is not clear. This question can be addressed when the blocking antibodies for individual Notch receptors become available.

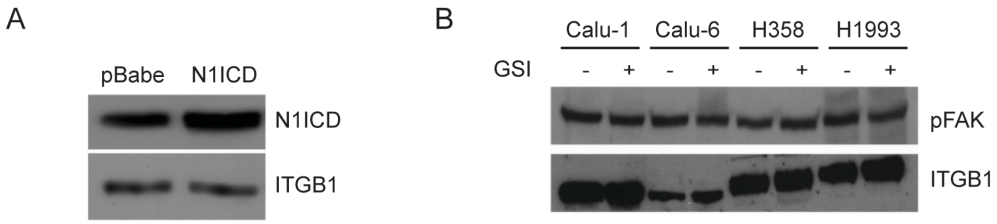

Figure 4.10. Neither overexpression of N1ICD nor treatment with a γ -secretase inhibitor affects Integrin β1 protein expression. A. Western blot showing Integrin β1 expression in 16HBE cells with control pBabe vector and N1ICD expression vector. B. Western blot showing Integrin β1 expression and phospho-FAK expression in Calu6, H358, Calu1 and H1993 cells before and after 10uM gamma-secretase inhibitor treatment for 24 hours.

CHAPTER V

SUMMARY AND DISCUSSION

Summary

Notch signaling is important in the regulation of human physiological and pathological processes including various human cancers such as T-ALL, ovarian cancer, breast cancer, pancreatic cancer and lung cancer, which makes Notch signaling as an attractive target for the treatment of human cancers. In lung cancer, it has been reported by our group that the existence of chromosome translocation and overexpression of Notch3 in patient tumor specimens occurs, further inhibition of Notch3 signaling by RNA interference or with a pharmacological γ-secretase inhibitor results in suppression of tumor progression *in vitro* and *in vivo*. Due to the contradictory roles of Notch1 in lung cancer and nonspecificity and gut toxicity of GSIs, specific inhibitors against Notch3 signaling may have more advantages for lung cancer patients over pan-Notch inhibitors. The goal of the work described in this thesis was the development of therapeutic reagents targeting Notch3 signaling in lung cancer.

We used a high-throughput strategy to screen a peptide library spanning all 34 EGF-like repeats of Notch3 extracellular domain for their ability to induce apoptosis of the lung cancer cell line HCC2429. We also generated neutralizing mAbs against putative ligand binding regions of Notch3 receptor. Moreover, we tested the hypothesis that targeting

Jagged1 or the Notch-CBF-1-MAML transcriptional complex are potentially alternative approaches to block Notch3 signaling in lung cancer.

The work presented here has demonstrated that 1) a rationale for developing anti-Notch3 therapeutic reagents by interfering with receptor-ligand interactions in lung cancer, such as by decoy receptor peptides or neutralizing antibodies; 2) Notch3 EGF-like repeats 7-10 and 21-22 are putative ligand binding regions which differ from that of Notch1; 3) Fc-fusion proteins with peptides representing Notch3 ligand binding regions are able to inhibit Notch3 signaling and suppress lung cancer progression *in vitro* and *in vivo*; 4) mAbs targeting Notch3 EGF-like repeats 7-10 or 21-22 are able to bind to full-length Notch3 and suppress production of the activated form of Notch3 in lung cancer cells; 5) the roles of the Notch ligand Jagged1 in lung cancer cell growth and migration are paradoxical; 6) abrogation of Jagged1 results in promotion of lung cancer cell migration through upregulation of integrin β1 protein level; 7) blocking the CBF-1 dependent canonical Notch pathway has minimal effects on lung cancer growth and migration.

The Fc-fusion proteins and neutralizing mAbs developed in this thesis project have the potential to be further developed and used in lung cancer patients to target Notch3 signaling. Furthermore we provide a rationale for targeting the receptor-ligand interaction of Notch3 signaling in lung cancer. It is noteworthy that inhibition of Notch-CBF-1-MAML in two lung cancer cells has very minimum effects on lung tumor growth. This suggests that the alternative mechanisms of Notch signaling are important in lung cancer.

Lastly, the unexpected pro-migratory effect of chronic inhibition of Notch ligand Jagged1 in lung cancer cells may help guide a strategy for how anti-Jagged1 therapies are used.

Discussion

Our work has raised as many questions as it has answered. Given that Notch is involved in a series of fundamental processes during embryonic development and in adult tissues, the emerging reports of Notch signaling in human cancers highlights the intriguing dual role of a single signaling and complexity of Notch signaling in human cancers of different context. Even in the same context as lung cancer, the roles of different components of Notch signaling are not the same. The divergent roles of Notch signaling raise several following unsolved questions that need to be solved in order to understand Notch signaling in human cancers.

<u>What are the roles of Notch3 versus Notch1 in lung cancer, and why do they behave differently?</u>

The third mammalian Notch, the Notch3 gene was initially described as being expressed in proliferating neuroepithelium (302). Targeted deletion of Notch3 does not result in embryonic lethality as is observed with deletion of Notch1 and Notch2, possibly due to different tissue distribution. The biological functions of Notch3 and Notch1 in human cancers, such as lung cancer are different. In the lung cancer cell line HCC2429, which expresses both Notch1 and Notch3 receptors, deletion of Notch3 by siRNA abrogated the response to GSI treatment, suggesting that Notch3 but not Notch1 contributes to tumorigenesis of HCC2429 cells. Moreover, in HCC2429 cells, Notch3 seems to

preferentially activate Hey1 gene transcription instead of Hes1, as cells retaining Notch3 expression show higher expression of Hey1, whereas no difference in Hes1 is observed between the control and Notch3 knockdown (226). The variation in their downstream targets may explain the biological difference between Notch3 and Notch1 in lung cancer progression.

In lung cancer cells with different genetic backgrounds, such as those harboring a *KRAS* mutation, the situation is quite different. We showed in chapter IV that attenuation of Jagged1 in H358 and Calu-6 cells suppressed their growth *in vitro* and *in vivo*. Originally, we have knocked down Notch1 and Notch3 in both cell lines by two independent shRNAs. However, we did not observe any growth defects upon inhibition of either Notch1 or Notch3 (data not shown). After measuring Notch receptor levels in each knockdown line, we observed that in the Notch1 knockdown cells, Notch3 level is slightly increased compared with control, whereas in the Notch3 knockdown cells, Notch1 level is also increased compared with control, indicating that both Notch1 and Notch3 are potentially important in regulating lung cancer cell growth, and their function in lung cancer progression is redundant. More insights into differential functions of Notch3 versus Notch1 in lung cancer could be assessed when specific inhibitors against individual Notch receptors become available.

Although Notch3 shares a similar basic structure with Notch1 and Notch2, Notch3 displays several structural differences, which would explain the functional difference between Notch3 and Notch1 in cancers. Firstly, Notch3 has a shorter C-terminal region including

the TAD domain, which may explain the weaker transactivation activity of N3ICD when compared to that of N1ICD and N2ICD (303). Secondly, the amino acid identity between Notch3 and Notch1 in the RAM domain is low, which mediates the interaction with CBF-1, is low (272). Both of these differences could possibly explain the altered N3ICD in the regulation of gene transcriptions. Thirdly, there are subtle differences in the transmembrane domain of Notch3 compared to that of other Notch proteins (304), which may mediate differential Notch3 intramembranous cleavage as well as its recruitment to the membrane. Finally, there is a slight differences with respect to its EGF-like repeats as Notch3 lacks EGF-like repeats 2, 3 and 21 in Notch1 and Notch2 (302), which may lead to a change in specificity and affinity with Notch ligands. In the thesis study, we found that Notch3 EGF-like repeat 7-10 and 21-22 are essential to mediate ligand interaction, which is different from that of Notch1, EGF-like repeat 11-12 from published works, which would add a novel difference between Notch3 and Notch1.

<u>How do two regions of Notch3 ECD mediate receptor-ligand interaction?</u>

In the work described in chapter II, we found two ligand binding regions of Notch3 receptor, while in Notch1 only one ligand-binding region was reported. However, it is highly possible that Notch1 also has an additional ligand-binding region in the similar region. Ligand-binding domains of *Drosophila* Notch were identified by a cell aggregation assay, in which cells expressing a series of deletion mutants of Notch lacking different EGF-like repeat were assayed for their abilities to aggregate Serrate/Delte expressing cells. In our study, we used a functional assay to evaluate signaling transduction activities of Notch3 mutants and showed that deletion of EGF-like 7-10 or 21-22 of Notch3 receptor

significantly suppressed production of N3ICD and expression of Notch target genes Hes1 and Hey1. Therefore, it is possible that the aggregation assay only identified minimum regions for ligand binding but not minimum regions for ligand-mediated activation of Notch signaling. It is possible that EGF-like repeats 21-22 of Notch3 or its equivalent EGF-like repeat 25-26 of Notch1 may contribute to secondary ligand-binding site in order to generate a pulling force on both sides of the ligand after the primary binding of EGF-like repeat 7-10 of Notch3 or 11-12 of Notch1. Indeed, deletion of EGF-like repeat 24-26 of *Drosophila* Notch, also defined as *Abruptex* region, has been shown to reduce Notch signaling activated by Serrate but not Delta (274), suggesting an additional region of EGF-like repeat is important for Serrate-mediated Notch signaling. Jagged1 ligands have around 8 more EGF-like repeats than DLL ligands. Thus, it is possible that Jagged ligands bind to two regions of Notch receptors, whereas DLL ligands only bind to one region of Notch receptors. In our study, we used HCC2429 cells that highly express Jagged1 ligands instead of DLL ligands, to screen the peptide library. Thus that secondary binding site identified from this study could just mediate Notch3-Jagged1 interaction, but not that with DLL ligands. More studies need to be done to address the following questions for better understanding of receptor-ligand interaction in Notch signaling: 1). Whether EGF-like repeats 25-26 of Notch1 are important for Notch1-mediated signaling transduction. 2). Whether EGF-like repeats 21-22 of Notch3 only binds to Jagged ligands, but not DLL ligands. 3). Which domain in Jagged ligands are responsible for binding to Notch3 EGF-like repeat 21-22?

It is not clear if individual Notch receptors have a preference for binding to Jagged ligands versus DLL ligands. *In vitro* solid- phase binding assay showed that mouse Jagged1 bound to various soluble Notch receptors including their N-terminal and EGF-like repeats 1-15 with differing affinity as Notch3>Notch2>Notch1 (25), suggesting that there is there is some preferential relationship between Notch3 and Jagged1. Indeed, preferential interaction between Jagged1 and Notch3 are indicated in the context of cancer cell survival and growth (155, 173, 226). Moreover, It has bee showed that different DSL proteins bind to soluble Notch3 or Notch1 proteins including their N-terminal and EGF-like repeat 1-15 with different affinity as DLL1>Jagged2>Jagged1 (305). In fact, binding affinities between receptors and different ligands identified by in vitro solid-phase binding assay may not be representative of the *in vivo* situation as there is a possibility that affinities are modulated by other factors such as Fringe proteins. Fringe modified Notch receptors prefer to bind to DLL ligands instead of Jagged ligands (35). More studies to investigate the existence of preferential interactions between different receptors and ligands using mammalian cell systems would aid the design of therapeutic agents against individual Notch receptors/ligands pairs.

Are *Notch* mutations drivers or passengers in cancer?

Notch1 mutations were first identified in T-ALL, and mutagenesis studies indicated that these mutations are activating and drive T-ALL (110). Later, mutations in *FBXW7* ubiquitin ligase, which regulates levels of Notch, cyclinE and other proteins, were found in human colorectal, ovarian, endometrial tumors and T-ALL and B-ALL (306). *Notch* genes are very large genes consisting of up to 34 coding exons and about 30 EGF-like repeats,

which hampers classical DNA sequencing. Thanks to the accelerating technologies for deep sequencing of the cancer genome and cancer transcriptome, more and more unknown mutations have being identified in human cancers including in the *Notch* gene family. Inactivating mutations of *Notch1* were found as a potentially important tumor suppressors in head and neck cancer by two independent groups. This was done by sequencing the exons of all known human genes in tumor DNA and comparing it to the sequence to that of the corresponding normal DNA of the same patient. In both studies, inactivating mutations of *Notch1* were found in 10 to 15% of the head and neck squamous cell carcinomas (HNSCCs), and *Notch1* was the second most frequently mutated gene after *TP53* in HNSCC. Mutations in *Notch2* and *Notch3* genes are found as well with less frequency. In the study conducted by Stransky et al. the authors showed showed that several Notch1 nonsense mutations are predicted to generate truncated proteins lacking the C-terminal ankyrin repeat domain, and that several mutations cluster in highly conserved residues situated within or nearby the putative ligand-binding regions. Agrawal et al. found several mutations in the intracellular domain in addition to truncations and mutations in the ECD. However, those mutations show little overlap. Unlike Notch3 mutations in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) that mutations altered the number of cysteine residues in the extracellular domain leading to its abnormal accumulation in the vessels of patients, most of the nonsynonymous mutations identified in *Notch1* ECD in these two studies are not in cysteine residue, and the consequences of these mutations are hard to predict. While the finding of numerous inactivation mutations in *Notch1* in HNSCCs combined with the observation that *Notch1* null mice developed a skin cancer provide strong evidence that

Notch1 is an important tumor suppressor in HNSCCs, this does not necessarily indicate that they are all " driver mutations" causally associated with the malignant transformation process. Tumor cells are genetically unstable and acquired many mutations including "passenger mutations", which are probably a consequence of malignant transformation but not the cause. Conversely, Agrawal et al. also found inactivating mutation of *FBXW7* in tumors that lack inactivating mutation of *Notch1*. The primary function of Fbxw7 in cancer is to mediate degradation of Notch receptor so as to inactivate Notch signaling. We would expect to see activating mutation of negative regulators of a tumor suppressor gene. Therefore, it is still not well defined that Notch1 is a tumor suppressor in HNSCC. More mechanistic studies in cell lines and animal models are required to demonstrate the exact roles of the mutated Notch1 receptor in HNSCC. It is possible that more and more *Notch* mutations and other unknown mutations will be found crossing multiple human cancers including lung cancer, by next generation sequencing or RNAseq. Development of bioinformatic approaches and more efficient functional assays to assess newly identified mutations will be essential to determine their exact functions as driver mutations or passenger mutations, which would further facilitate designs of targeted therapies against driver mutations in human cancers.

Do Notch inhibitors in cancer function only as anti-angiogenic agents?

Multipe evidences suggest the oncogenic roles of Notch signaling in the tumorigenesis of multiple human cancers. Thus, targeting Notch signaling became an attractive approach, and various pharmaceutical and biotech companies are generating Notch inhibitors, including Roche/Genentech, Roche, Regeneron, Merck, Pfizer and Eli Lilly etc. Most

development has focused on small molecule inhibitors targeting γ-secretase, while more and more companies are switching to more specific inhibitors against individual Notch receptors or ligands. However, individual Notch receptors/ligands inhibitors have been more appreciated by their anti-angiogenic effects in solid tumors, as Notch signaling is highly activated in vasculature and is upstream of VEGF signaling in the regulation of angiogenesis. Wu et al. have screened a panel of nearly 45 cell lines for anti-Notch1 mAb against NRR region, and identified a human colon cancer line MT-3 as a sensitive line in a dose-dependent but ligand-independent manner. It turned out that this cell line has an activating point mutation in the NRR region, which activates Notch1 signaling independent of ligand. They also demonstrate the anti-Notch1 antibody treatment decreased tumor angiogenesis in the xenograft model generated from Calu-6 and HM7 cells which were previously reported to be sensitive to anti-angiogenic therapy. However, growth of these cell lines is not affected by anti-Notch1 mAbs treatment in cell culture. Their data suggest that except for the tumor cells harboring activating mutations of *Notch1* receptor, anti-Notch1 mAbs basically inhibited tumor growth through anti-angiogenesis.

Are anti-Notch therapies only anti-angiogenic agents in tumors without *Notch* mutations? Our data would suggest that this is not the case. In chapter IV of the thesis, using the same cell line Calu-6, we showed that abrogation of Notch ligand Jagged1 suppressed cell growth both *in vitro* and *in vivo*. Indeed treatment with the GSI, MRK003, in the same cell line attenuated cell growth *in vitro* as well (data not shown), suggesting that Notch signaling does play a role in the cell growth of Calu-6 cells. The redundant functions of Notch signaling mediated by different Notch receptors could explain why this cell line can

respond to GSIs and ligand inhibition but not anti-Notch1 mAbs. Blocking Jagged1 or using γ-secretase shuts down all four Notch receptors mediated signaling, while anti-Notch1 mAbs only works to inhibit Notch1 mediated signaling. Since Notch1 is the major player in tumor blood vessel, inhibition of Notch1 is sufficient to attenuate angiogenesis driven by Notch signaling. However, there are many examples in the literature of cancer cell lines responding to pan-Notch inhibitors, such as GSIs, which attenuate tumor progression in vitro and in vivo, suggesting that anti-Notch treatment would work directly on tumor growth as well.

In terms of inhibitors that target specifically each of the Notch receptors, one needs to know which Notch receptor is important in certain human cancers. For example, in lung cancer, Notch3 is a better target than Notch1. We showed in chapter IV that Notch3, but not Notch1 is highly expressed in lung cancer tissues, and that its expression being correlated with advanced stage disease. If multiple Notch receptors are present and functionally redundant in certain cancers, GSIs would be a better choice than inhibitors for individual Notch receptors if side effects of GSIs could be overcome. More studies need to be done to determine which Notch receptors or if all-Notch receptors are important in certain cancers so as to determine which Notch inhibitors should be used.

Can Notch inhibitors be used as single agents for cancer therapy?

Based on the published data regarding the use of Notch inhibitors to treat cancer cells *in vitro* and *in vivo*, cancer cells harboring mutations in genes involved with Notch signaling are sensitive to anti-Notch treatment. Examples of these include T-ALL cells harboring

activating mutations of *Notch1*, primary lung cancer cells with activating mutations of *Notch1* or loss of Numb expression, and HCC2429 cells with *Notch3* translocation (220, 226, 254). Plentz et al. have screened around 400 human cancer cell lines derived from different solid tumors for their responsiveness to a selective GSI, MRK003, and found that 50% of their cohort of 26 PDAC cell lines was sensitive to the inhibitor. Moreover, the GSI completely inhibited tumor development in the genetically engineered model of invasive PDAC (148). These studies suggest that some subtypes of cancer without a genetic alternation of *Notch* would respond to anti-Notch therapy. However, even tumors addicted to EGFR signaling would not respond completely to EGFR tyrosine kinase inhibitor (TKI) or eventually developed resistance via different mechanisms. Moreover responsiveness of inhibitors in real patients is not predictable based on the responsiveness in genetic tumor model or xenograft tumors model due to the genetic complexity of individual patients. The phase I clinical trial analyzing the effects of a GSI in relapsed and refractory T-ALL showed that none of the patients enrolled in this study showed any significant clinical response (307), which correlates with the weak antileukemic effects of GSIs against human T-ALL cells *in vitro*, consistent with existence of some other signaling pathways in synergy with Notch1 signaling in human T-ALL patients. Nevertheless, given the fact that inhibition of Notch1 signaling has a profound effect on the homeostasis of T-ALL (308-310), it has been reported that GSIs are able to reverse glucocorticoid resistance in T-ALL (267). In lung cancer, HCC2429 cells with high Notch3 expression are sensitive to Notch inhibition, whereas other lung cancer cell lines, such as A549 are not sensitive to anti-Notch treatment. At this point, there is no other existing genetic alternation in HCC2429. By comparison, most of lung cancer cells lines harbor various genetic

alternations that are important for cancer cell survivals. Therefore, for the majority of lung cancer patients, Notch inhibitors would not work as a single agent for treatment. Recently, based on the notion that Notch signaling plays important roles in the maintenance of cancer stem cells in many cancers including lung cancer and glioma, anti-Notch therapies open a new window to treat cancer stem cells, which are resistance to conventional chemo- and radiation treatment. Even though the cancer stem cell hypothesis is still under debate, the efficacy of Notch inhibitors to suppress progression of cancer cells with different clonal and tumorigenic capacities provides the rationale that anti-Notch therapy could increase the efficacy of conventional therapies for cancer patients. In addition, in lung cancer, it had been demonstrated that Notch3 and EGFR signaling cooperate together to modulate apoptosis through induction of proapoptotic protein Bim expression. Using a GSI and erlotinib in a xenograft model, tumor inhibition was observed to be enhanced compared with each agent alone, suggesting that anti-Notch therapy could be useful to increase responsiveness of lung cancer patients to EGFR TKI. Thereby, in my opinion, although Notch signaling is very important in multiple cancers, anti-Notch agents might produce little benefit as a single agent in treatment regimen of a majority of cancer patients. Instead, anti-Notch thereby may provide optimal benefit when treating patients using a combination of anti-Notch inhibitors with conventional drugs or other targeted agents.

What are the biomarkers to predict sensitivity to Notch inhibition?

Predictive biomarkers are used to assess the probability that a patient will benefit from a particular treatment (311). Mutations in the genetic region encoding the kinase domain of EGFR predict the sensitivity of lung tumors to the EGFR TKI erotinib (312). Conversely,

distinct mutations in *KRAS* predict that patients with lung cancer will fail to respond to erlotinib (313). In T-ALL, activating mutations of *Notch1* could predict the possibility of the patient responding to Notch inhibitors. However, in solid tumors, such as lung cancer, very few mutations of components in Notch signaling are present. Thus, it becomes important to define the biomarkers that can determine if tumors would be sensitive to Notch inhibitors or not. Nowadays, most studies to determine which cells would be sensitive to Notch inhibitors are based on expression levels of Notch components in tumor cells. For example, high expression levels of Notch receptors, ligands or downstream targets Hes1 or Hey1 are often used as indicators for responsiveness to anti-Notch therapy, such as GSIs. However, due to the complexity of Notch signaling and its roles in the maintenance of cancer stem cells, using expression levels of Notch related proteins as predictive biomarkers for anti-Notch therapy would be very limited to guide personalized cancer therapies. AVEO Pharmaceutical Inc had reported at the AACR annual meeting that active Notch signaling alone did not predict dependence of Notch, but expression of a single Notch target gene HeyL was highly correlated with sensitivity of human cancer cell lines to inhibition of ligand-dependent Notch signaling. Moreover, they showed that pancreatic cancer cell lines harboring a *KRAS* mutation are more sensitive to Notch pathway inhibition. Recently, Sage's group reported that Notch signaling was activated in HCC tumors derived from Rb depletion mice. However, the treatment of a GSI increased the tumor incidence and size (314). One would expect that signaling pathways activated in tumors are most likely to be oncogenic rather than tumor suppressive. However, this study indicates that in liver cancer, Notch signaling is in fact tumor suppressive and high expression of Notch associated proteins in tumors is not sufficient to predict outcomes of

Notch inhibition. Due to the context dependent roles of Notch signaling in human cancers, it will be important to identify predictive biomarkers for responsiveness to Notch inhibitors for each cancer type. In lung cancer, more studies need to be done to firstly, elucidate if high expression of Notch receptors or ligands is enough to indicate importance of Notch signaling among multiple lung cancer cell lines; secondly, to determine the genetic alternations of certain cancer cells that would / would not be inhibited by anti-Notch agents; thirdly, to figure out a way to assess the presence of Notch-associated cancer stem cells from patient biopsy; fourthly, to investigate characteristics or gene signatures of tumors from patients that respond to GSI treatment from clinical trials. Overall, cancer is a complicated disease of genetic complexity and heterogeneity. So it is always important to develop clinical biomarkers to determine which patients are most likely to benefit from specific targeted therapies.

What are the difficulties in developing Notch blocking agents?

We generated neutralizing monoclonal antibodies against Notch3 putative ligand binding regions, EGF-like repeat 7-10 and 21-22, respectively, and we have identified a handful of mAbs that are able to bind to Notch3 receptor. These mAbs have been able to reduce production of N3ICD in HCC2429 cells. However, once we expanded the hybridoma clones to produce large amount of mAbs in a bioreactor, the mAbs lost their abilities to reduce production of N3ICD in the HCC2429 cells. The myeloma cell line SP2/0 used for making the hybridoma expresses the Notch1, Notch2 and Notch3 receptors (data not shown). Indeed, it had been reported that inhibition of Notch signaling by a GSI induces apoptosis of myeloma (315). Thus, it is possible that the mAbs produced by the hybridoma

inhibits its own growth so as to influence the ability to produce antibodies with the correct conformation. There are two approaches used by published studies to generate Notch inhibitory antibodies: making mAbs by hybridomas (253) or screening a phage display library (254, 255). The only blocking antibodies to significantly suppress tumor growth are antibodies generated by Siebel's group at Genentech. They screened a phage display library for phages binding to NRR regions of Notch1 and Notch2 respectively, and generated antibodies to express full-length IgGs by cloning the light chain (VL) and heavy chain (VH) regions into LPG3 and LPG4 vectors, respectively, then transiently expressed antibodies in mammalian cells and purified using protein A. Using this method, they avoided the possibility that Notch neutralizing antibodies affects production and conformation of antibodies themselves. Li et al. used whole Notch3 extracellular domain as an antigen to generate Notch3 blocking antibodies from the hybridomas, and showed that antibodies recognizing both the NRR region and ligand-binding regions are able to block Notch3 signaling. However, they only showed their abilities in blocking ligand-mediated signaling transduction and inhibition of cell proliferation and migration in HEK293T-Notch3 cells, but not their anti-tumor abilities in tumor cells. It has been three years since they reported these anti-Notch3 blocking antibodies, but to our knowledge, no anti-tumor activities of those antibodies were ever reported. Originally, in the collaboration with Dr. Ray Mernaugh we tried to screen the phage display library for antibodies against all the Notch3 EGF-like repeats. The peptides used in the apoptosis screening described in chapter II were originally designed for screening of the phage display library. We found several phage display antibodies that bind to Notch3 peptides. However, these antibodies failed to either immunoprecipitate full-length Notch3 or inhibit

Notch3 signal transduction. It is possible that we took a wrong approach in screening the phage display library using small peptides of Notch3 ECD. Notch3 EGF-like repeat 7-10 and 21-22 proteins should be used for screening of phage display library if our antibodies from hybridomas fail to block Notch3 signaling or suppress tumor growth in lung cancer. Studies published during the thesis research suggested that the NRR region is the better target than the ligand binding regions of Notch1/3 receptors. Therefore, it is also a good strategy to target Notch3 NRR regions by screening a phage display library or making mAbs from hybridomas.

Small molecule inhibitors may interfere with receptor-ligand interaction as well, albeit small molecule inhibitors are rarely used for blocking extracellular protein-protein interaction. In fact, we have tried to screen small molecule inhibitors by fluorescent depolarization assay and Corning EPIC label free technology. However, we could not prove the concept of these assays for further screening. Cell-based assay would be more feasible for screening of small molecule inhibitors against Notch signaling but the readout of the screening would be very critical. CBF-1 reporter activity could be used to screen the inhibitors against canonical Notch signaling. Unfortunately the hits from screening may not only target Notch signaling due to unspecific roles of CBF-1. A FRET assay to assess NICD-CBF-1 interaction is more specific for screening inhibitors against canonical Notch signaling. However, none of these approaches is able to identify specific inhibitors for individual Notch receptor mediated signaling as all the Notch receptors share similar intracellular mechanisms to activate downstream targets. Due to gut toxicity caused by inhibition of both Notch1 and Notch2 signaling, specific inhibitors against individual

Notch receptor mediated signaling would be more useful in the clinic. Fragment-based approaches and structure-based design are prominent steps in cancer drug discovery, such as the discovery of the Bcl-2 inhibitor ABT-737 (316). Indeed, structures of both putative Notch1 ligand binding region and Jagged1 ligand binding region were solved separately, and a computational models of the structures of their complexes were also generated. Therefore, it is possible to design inhibitors to interfere with an individual Notch receptor-ligand interaction based on their structures. More structural studies of ligand binding regions of each Notch receptor, such as Notch3 EGF-like repeat 7-10 and 21-22, and individual ligands as well as their interaction complexes would be very useful for the design of small molecule inhibitors.

What are the mechanisms for non-canonical Notch signaling in cancer?

Our study described in chapter IV demonstrated that CBF-1 dependent canonical Notch signaling is not important in lung cancer cell growth *in vitro* and *in vivo* determined in lung cancer cell lines Calu-6 and H358. In other words, the CBF-1 independent non-canonical Notch signaling has a greater impact in lung cancer (Fig 5.1). We also tried to assess the importance of CBF-1 dependent canonical Notch signaling in a Notch3-dependent cell line HCC2429 by expressing DNMAML protein. In cell culture, cell growth was not attenuated in cell culture, yet an increase in tumor growth was observed in a xenograft tumor model (data not shown). We do not understand why inhibition of canonical Notch signaling in a cell line dependent on Notch3 in fact promotes tumor growth *in vivo*. One possibility may be due to different functions between canonical and non-canonical Notch signalingn in HCC2429 cells. Nonetheless, this result further confirmed that Notch3

mediated tumor progression of another cell line, HCC2429, is primarily through a CBF-1 independent mechanism. Nowadays, there are two accepted methods to shut down CBF-1 dependent canonical Notch signaling: the one is to knock down CBF-1 and the other is to express a dominant negative version of MAML protein. I have tried both strategies to shut down canonical Notch signaling. Knockdown of CBF-1 in these cell lines did not result in a decrease of CBF-1 luciferase reporter activity in the presence or absence of NICD (data not shown). Therefore, we have focused on the method using DNMAML protein, which is actually more specific as it is still able to bind to NICD but fails to initiate NICD-mediated genes transcription mediated by NICD. However, none of these methods is specific for canonical Notch signaling, since both CBF-1 and MAML protein have biological functions besides activation of canonical Notch signaling and SAHM1 may have off-target activity. For example, MAML1 is emerging as a co-activator of other pathways such as NF-κB to regulate cellular survival (317). More experimental tools for specifically attenuating CBF-1-dependent canonical Notch signaling will be meaningful to the Notch field, such as small molecule inhibitors/peptides to specifically saturate the binding domain of individual NICD to CBF-1, or small molecule inhibitors to prevent specific NICD localization to the nucleus.

There are multiple ways of how Notch signaling functions independent of CBF-1. We believe that there would be various unknown mechanisms of how Notch signaling functions independent of CBF-1 to regulate lung cancer tumorigenesis within different genetic backgrounds. NICD could interact with other transcriptional factors in addition to CBF-1, and initiates transcription of non-canonical Notch target genes as NICD lacking its

RAM domain that mediates its binding to CBF-1 is able to translocate into the nucleus and induce neoplastic transformation (237, 238). Alternatively, NICD, membrane-tethered receptor or full-length receptor could crosstalk with other signaling pathways to activate non-canonical Notch targets (Fig 5.1). Further studies to identify the interactome of membrane tethered Notch receptors and the activated form of Notch receptors in both the cytoplasm and nucleus would help reveal the potential mechanisms of how non-canonical Notch signaling impacts lung cancer tumorigenesis.

Are Notch ligands pro-migratory or anti-migratory?

Previous reports indicate the pro-migratory roles of Notch signaling in cancer progression by activating migratory signaling pathways or providing migratory microenvironments. However, the functions of Notch ligands in cell migration are controversial. Lindner et al, elucidated that secreted forms of the extracellular domain of Jagged1, which suppresses Notch signaling, decreases extracellular matrix adhesion and migration of NIH3T3 cells (318). In contrast, a DLL1 ligand has been reported to reduce the motility of 3T3 cells, which is independent of its activity as a Notch ligand (319). Our findings show that knockdown of Jagged1 protein in lung cancer cell lines actually increases cell motility. It is possible that the extracellular regions of Notch ligands, lacking the intracellular domain, are different compared with full-length Notch ligands in the regulation of cell motility. Indeed, evidence indicates that DSL proteins can also undergo proteolytic cleavage, which may elicit an intracellular signaling activity within the ligand expressing cells (246, 247, 320-322). Ectopic expression of Jagged1 can transform rat kidney epithelial cells independent of Notch signaling, which is mediated by the PDZ motif in Jagged1

intracellular domain (245). Additionally, Jagged1 intracellular domain can activate AP-1 mediated transcription as well (246). Therefore, in our study, we could not rule out the possibility that Jagged1 ICD or full-length Jagged1 directly contributes to lung cancer cell growth and migration independent of Notch signaling (Fig 5.1). Further studies are needed to address: firstly, if inhibition of individual or all Notch receptors or together promotes lung cancer migration; secondly, if Jagged1 has its own functions in lung cancer independent of Notch; thirdly, if Jagged1 ICD has any biological functions in lung cancer growth and migration.

What are potential mechanisms for how Jagged1 regulates integrin in cancer?

In chaper V, we found that abrogation of Jagged1 in lung cancer cell lines increased integrin β1 protein expression independent of CBF-1. However, we do not know the mechanism of how Jagged1 regulates integrin β1 protein expression. Since Jagged1 does not regulate integrin β1 mRNA transcription, we tried to identify the intermediates to link expression of integrin β1 with Jagged1 by comparing with the gene expression signatures between control and Jag1 KD Calu-6 cells via microarray analysis. We found several candidate molecules, such as Urokinase-type plasminogen activator (uPA) that is upregulated in Jag1 KD cells compared with control. It had been reported that EGFR

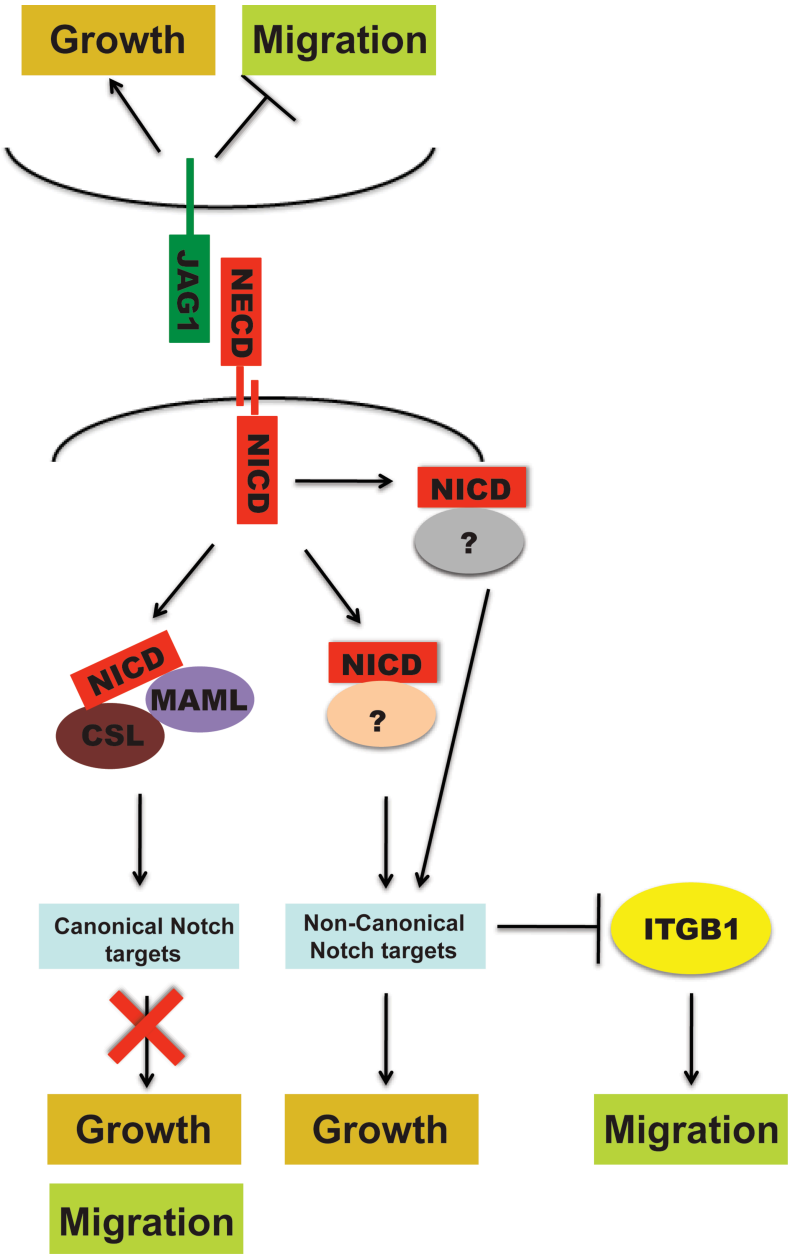

Figure 5.1. Schema of how Jagged1 mediated Notch signaling regulates lung cancer cell growth and migration. CSL-dependent canonical Notch signaling does not regulate lung cancer cell growth and migration. Instead, CSL-independent non-canonical Notch signaling or Jagged1 itself could contribute to promote lung cancer growth and suppress migration by regulating non-canonical Notch target genes.

siganling, integrin β1 and uPA-uPAR signaling can cross talk to promote cell invasion and metastasis (323). Thus, we originally hypothesized that integrin β1protein level is upregulated autonomously by activation of integrin β1 signaling via crosstalk with uPA-uPAR signaling. However, when we inhibited uPA-uPAR signaling by an uPA blocking antibody in control and JAG1 KD Calu-6 cells, neither integrin β1 protein level nor cell migration was altered, suggesting that uPA is not the intermediate factor regulated by Jagged1 to regulate integrin β1 level and cell migration. There are plenty of genes regulated by Jagged1 in Calu-6 cells, which potentially contribute to cell growth, migration and metastasis based on the published literature (Figure 5.2). It is possible that some or one of these genes is responsible to regulate integrin β1 protein expression, or some of these genes cooperatively contribute to the migration phenotypes driven by abrogation of Jagged1. Further studies will be needed to test these possibilities. Moreover, it is also possible that Jagged1 promotes degradation of integrin β1 by directly / indirectly interacting with integrin β1. Identification of Jagged1 interacting proteins would be helpful to test this hypothesis.

Integrins are heterodimeric transmembrane glycoproteins, which mediate cell-cell and cell-matrix interaction (297). Integrin signaling is crucial for embryogenesis, and contributes to the neurogenesis, myogenesis and angiogenesis processes (324, 325) which are also tightly regulated by Notch signaling. Genetic studies have demonstrated that Notch and integrin mutations have related phenotypes in key developmental processes such as vascular development and somitogenesis. In neural stem cells, expression of Notch4 in endothelial cells increased cell adhesion to collagen (326). Hodkinson et al. demonstrated that NICD

could activate integrin-ligand binding activity without affecting the integrin expression level in a CHO cell system (296). Previous reports had also linked Notch signaling and integrin expression in the epidermis. For example, *in vivo* α4β6 integrin expression is decreased in N1ICD transgenic skins, but not altered in RBPj κ (i.e CBF-1) knockout mice (327). Moreover, in mouse epidermal development, integrin expression is decreased in *DLL1*-null keratinocytes but increased in *Jagged1*-null cells (328). Our finding in chapter IV showed that abrogation of Jagged1-mediated Notch signaling resulted in an increase of integrin β1 protein level independent of CBF-1, which is consistent with the *Jagged1*-null phenomena in the epidermis. Therefore, it is possible that Notch may affect integrin expression and activation, modulating important developmental processes by alternating cell-matrix interactions (329). In addition, Notch signaling in cancer cells uses the same mechanism to regulate cell adhesion, migration, and metastasis. We do not quite understand why "smart" tumor cells activated integrin signaling when Jagged1 is attenuated. Jagged ligands have a von Willebrand factor (vWF) type C domain in its extracellular domain, which also exists in the majority of integrin and extracellular matrix proteins. Thus, on the one hand, it is possible that Jagged1 also mediates cell adhesion through its vWF domain. When Jagged1 is abrogated, cancer cells activate integrin signaling to compensate for the lack of Jagged1 as an adhesion molecule. On the other hand, Jagged1-Notch signaling mediates a cell-cell interaction through ligand-receptor interaction. Based on the aggregation assay performed in *Drosophila* cells, loss of Jagged1 on the cell surface results in a decrease of cell-cell adhesion/ interaction. Therefore, it is also possible that when less Jagged1 protein is presented on the cell surface, cancer cells lose cell-cell adhesion so as to activate cell-matrix adhesion via upregulation of integrin β1

protein. Further studies to investigate the roles of Jagged1 in cell adhesion would be useful to address why and how abrogation of Jagged1 leads to upregulation of integrin β1, and possibly identify new functions of Jagged1 and Notch signaling in cancers.

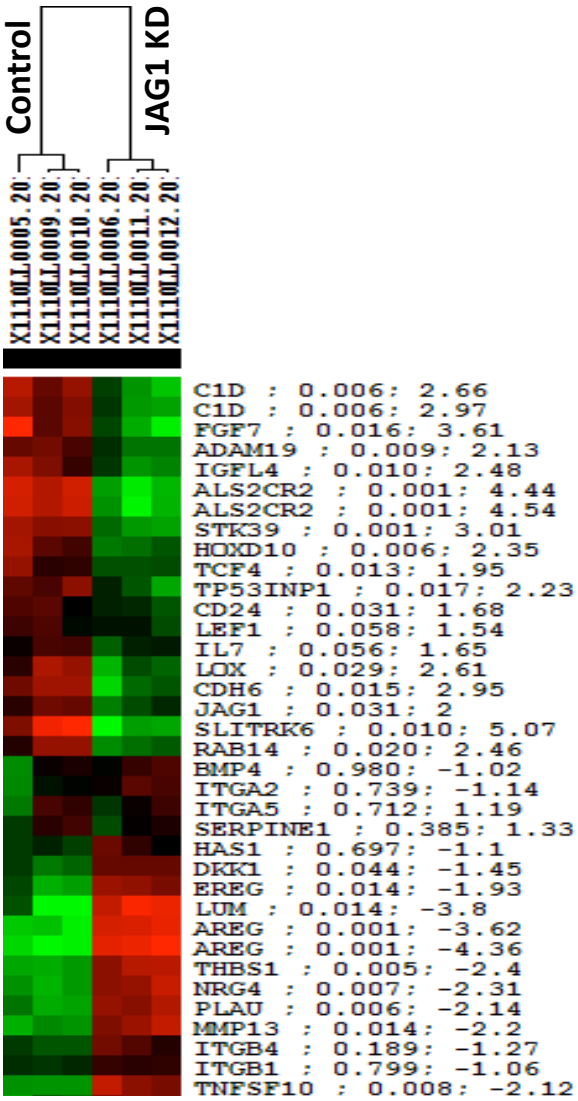

Figure 5.2. Heat-map image for the differentially expressed genes between control and JAG1 KD Calu-6 cells. The selected genes are genes potentially contribute to cell migration and metastasis. Red indicates higher expression level, and green indicates lower expression level.

Concluding remark

Notch3 signaling is important in the regulation of lung cancer growth and maintenance of lung cancer stem cells. Therefore, this thesis has focused on targeting Notch3 signaling in lung cancer. We discovered EGF-like repeat 7-10 and 21-22 as ligand binding regions of Notch3. Based on this discovery, we generated Fc-fusion proteins and monoclonal antibodies to interfere with receptor-ligand interaction. Our studies demonstrate that it is possible to develop potent inhibitors that interfere with receptor-ligand interaction of an individual mammalian Notch receptor, Notch3. This "proof-of-principle" demonstration has significant mechanistic and applied implications. Further development and characterization of these Fc-fusion proteins and mAbs targeting Notch3 is thus likely to have a broad experimental and therapeutic impact. Owing to the complexity of Notch signaling and the complicated genetic landscape of lung cancer, additional studies applying these Fc-fusion proteins and antibodies to more lung cancer cell lines and tumor models or other cancer types such as ovarian cancer would help to further evaluate the efficacy of these agents.

Our studies also demonstrated a CBF-1 independent mechanism of Jagged1 in lung cancer cell growth and migration, which differs from the conventional thought about how Notch signaling regulates lung cancer progression. Our studies reveal a new angle for studying Notch signaling and lung cancer. Further studies investigating the detailed mechanisms of how Jagged1/Notch signaling contributes to lung cancer progression non-canonically would insight into the world of Notch biology, as well as lung cancer physiopathology. With the contribution of the emerging new technologies, experimental tools, and

accumulating knowledge of Notch signaling, it is possible to better understand the biology of Notch signaling in lung cancer, and direct lung cancer therapies using novel Notch inhibitors.

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