Published in final edited form as:

Clin Cancer Res. 2010 June 15; 16(12): 3141–3152. doi:10.1158/1078-0432.CCR-09-2823.

Targeting Cancer Stem Cells through Notch Signaling

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Abstract

The cellular heterogeneity of neoplasms has been at the center of considerable interest since the “cancer stem cell hypothesis”, originally formulated for hematologic malignancies, was extended to solid tumors. The origins of cancer “stem” cells (CSCs) or tumor-initiating cells (TICs) (henceforth referred to as CSCs) and the methods to identify them are hotly debated topics. Nevertheless, the existence of sub-populations of tumor cells with stem-like characteristics has significant therapeutic implications. The stem-like phenotype includes indefinite self-replication, pluripotency and, importantly, resistance to chemotherapeutics. Thus, it is plausible that CSC, regardless of their origin, may escape standard therapies and cause disease recurrences and/or metastasis after apparently complete remissions. Consequently, the idea of selectively targeting CSCs with novel therapeutics is gaining considerable interest. The Notch pathway is one of the most intensively studied putative therapeutic targets in CSC, and several investigational Notch inhibitors are being developed. However, successful targeting of Notch signaling in CSC will require a thorough understanding of Notch regulation and the context-dependent interactions between Notch and other therapeutically relevant pathways. Understanding these interactions will increase our ability to design rational combination regimens that are more likely to prove safe and effective. Additionally, to determine which patients are most likely to benefit from treatment with Notch-targeting therapeutics, reliable biomarkers to measure pathway activity in CSC from specific tumors will have to be identified and validated. This article summarizes the most recent developments in the field of Notch-targeted cancer therapeutics, with emphasis on CSC.

Cancer “stem” cells and treatment resistance

Despite decades of search for an elusive “magic bullet”, the pharmacological treatment of cancer still relies heavily on traditional chemotherapy, which is being slowly supplemented by targeted agents. Incremental improvements are being made, but treatment resistance remains a major cause of morbidity and mortality.

For patients, clinicians and cancer biologists, the most frustrating feature of malignancies is their inherent adaptability. In the clinic, this adaptability translates into drug-resistant disease recurrence and metastasis, often after clinical and even pathological complete responses.

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One possible explanation for the biological plasticity of cancers is their cellular heterogeneity. In recent years, a distinct cellular hierarchy has been identified in several hematopoietic and solid tumors. Many cancers appear to contain a small population of pluripotent “tumor initiating cells” or “cancer stem cells” (CSC) [1-14]. The CSC hypothesis states that CSC possess some of the biological properties of normal stem cells, including indefinite self-replication, asymmetric cell division, and resistance to toxic agents, due in part to elevated expression of ABC transporters. Normal tissue stem cells are characterized by very slow proliferation rates and this is generally assumed to be the case for CSC. However, it is not established that all cancer cells with stem-like markers always proliferate slowly in vivo. Additionally, CSC proliferation rates may depend on microenvironment, type of oncogenic mutations, stage of malignancy and other variables. The origin of CSC is the subject of considerable debate. One popular version of the “CSC hypothesis” proposes that CSC originate from the transformation of normal tissue stem cells, and give rise to other cancer cells through a process of aberrant differentiation that resembles that of normal tissues but evades physiological regulatory mechanisms [11]. CSCs from various primary tumors or cell lines do express tissue stem cells markers, including CD133 (prominin-1), nestin, c-kit, sox2, Oct4, and musashi-1 (Figure 1A) [5,10,15-18]. On the other hand, it is also conceivable that “stemness” is a phenotype that can be acquired through transformation or modulated by extracellular signals. Stemness can be restored in normal fibroblasts by expression of Oct4, Sox2, Nanog, and LIN28 [19], or Oct3/4, sox2, Klf4, and c-Myc [20]. Physiological de-differentiation has been described in vertebrate and invertebrate non-neoplastic systems [21]. For example, spermatogonia in the *Drosophila* testis can restore male germline stem cells by a JAK-STAT-dependent de-differentiation process [22,23]. A well-known process of partial de-differentiation in epithelial cancers is epithelial-mesenchymal transition (EMT) [24]. This consists of loss of epithelial-specific cytokeratins and E-cadherin, and acquisition of mesenchymal markers such as vimentin and N-cadherin. Transcription factors such as Twist, Snail or Slug and TGF- β family secretory factors can induce EMT. A model supported by recent evidence from the Weinberg group [25] is that “stemness” can be re-acquired by cancer cells when they undergo EMT, a process associated with an invasive and metastatic phenotype. An intermediate possibility between the notion that CSC derive exclusively from normal tissue stem cells and the notion that stemness can be acquired by any cell is the suggestion that CSCs may originate from few cell populations, including tissue stem cells and immature progenitors capable of short-term self-replication. Dontu *et al.* [26] suggested that estrogen receptor (ER α)-negative breast cancers and poor-prognosis ER α -positive cancers (presumably Luminal B) arise from ER α -negative primitive mammary stem cells, while less aggressive ER α -positive cancers (presumably luminal A) arise from ER α -positive intermediate progenitors.

These models, schematically represented in Figure 1B, may not be mutually exclusive and may apply to different malignancies, different subtypes or stages of the same malignancy. A common element in the different versions of the CSC hypothesis is the concept of a cellular hierarchy in solid tumors and hematological malignancies cancers similar to that of normal tissues. CSC are thought to be capable of asymmetric cell division that maintains the CSC population and produces pluripotent “progenitor-like” cells. These in turn give rise to the “bulk” tumor cells through proliferation and aberrant “differentiation” (Figure 1C). Because of their ability to regenerate all cell types in a tumor, CSC, and possibly progenitors, are thought to have higher tumorigenic potential than “bulk” tumor cells.

A factor that complicates the testing of this model in human tumors is the fact that the identification of CSC as opposed to “bulk” tumor cells in clinical specimens relies on markers that are different for different malignancies and are not universally accepted [2,27]. A widely used functional test involves the measurement of limiting dilution tumorigenicity in immunodeficient NOD/SCID mice, with CSC being highly tumorigenic in very small

numbers, unlike “bulk” tumor cells. However, the tumorigenicity of human cancer cells in mice depends heavily on the degree of immunodeficiency. When melanoma cells were injected into more permissive NOD/SCID IL-2 receptor γ chain knockout mice (“NOG” mice), that lack NK cells, the fraction of tumorigenic cells appeared to be in the order of 25-27%, inconsistent with the rarity of tumorigenic cells identified in NOD/SCID models [28,29]. These observations indicate that the role of immune surveillance, both innate and adaptive, in defining CSC requires careful investigation, and have led some investigators to question the CSC hypothesis. In addition to the immune system, an additional complicating factor is the role of cellular microenvironment, or CSC “niches”, where CSCs survive inside the primary tumor or at distant sites [30]. Particularly in view of the fact that the stem-like phenotype may be inducible by paracrine signals such as TGF- β , Wnt and Hedgehog and signals transmitted by cell-cell contact such as Notch, the importance of microenvironment in determining the biological properties of CSCs should not be underestimated. That said, clinical evidence supporting the existence of CSCs and their role in treatment resistance has emerged, particularly in breast cancer. Li et al. showed that tumorigenic cells with stem-like markers are selected by neo-adjuvant chemotherapy [31]. Creighton et al. have recently shown that breast cancer cells surviving in patients after treatment with either docetaxel or letrozole have gene expression signatures characteristic of stem-like and EMT phenotypes [32]. Taken together, the evidence available today suggests that cells with a stem-like phenotype are found in several human malignancies, and that, to the extent that they exist according to current hypotheses, these cells are not adequately targeted by currently used cancer therapeutics. It is reasonable to hypothesize that a complete eradication of these cells will be necessary to attain long-lasting remissions or cures. A strategy that is receiving considerable attention is to target CSC through evolutionarily ancient pathways that control self-renewal and cell fate decisions in undifferentiated, pluripotent cells [27]. Many such pathways have been identified (Figure 1A). Most of these are evolutionarily conserved and have multiple developmental roles, as well as intricate cross-talk interactions. Among them, Wnt [33], Hedgehog [34], and Notch [14,35] are the focus of intense developmental therapeutics efforts. This article will focus on Notch signaling as a putative therapeutic target in CSC.

Notch signaling: mechanistic complexity with potential therapeutic implications

The Notch pathway is a short-range communication system in which contact between a cell expressing a membrane-associated ligand and a cell expressing a transmembrane receptor sends the receptor-expressing cell (and possibly both cells) a cell fate regulatory signal. This signal takes the form of a cascade of transcriptional regulatory events that affects the expression of hundreds of genes, and has profound, context-dependent phenotypic consequences. Several recent articles discuss the biochemical features of the pathway [36-38] and its possible roles in cancer [39-49].

Mature Notch receptors (in mammals Notch-1 through -4) are non-covalent heterodimers consisting of an extracellular subunit (N^{EC}), and a transmembrane subunit (N^{TM} , Figure 2). N^{EC} contains multiple EGF-like repeats and three specialized Lin-Notch repeats (LNR) that form a tight hydrophobic interaction with the extracellular stump of N^{TM} , masking an ADAM (A Disintegrin and Metalloprotease) cleavage site. The region of interaction between the two subunits is called HD (Heterodimerization Domain). Canonical Notch ligands are also transmembrane proteins (Figure 2) with multiple EGF-like repeats, a short cytoplasmic tail and a specialized DSL (Delta-Serrate-Lag2) domain at the N-terminus. Ligand binding triggers dissociation of N^{EC} from N^{TM} , unmasking the ADAM cleavage site (Figure 3A, B). N^{EC} is trans-endocytosed into ligand-expressing cells while N^{TM} is cleaved at the membrane by an ADAM, generating an intermediate called N^{EXT} (Notch Extracellular

Truncation). The latter is further cleaved by γ -secretase, generating an active fragment (N^{IC}) (Notch Intracellular) or NICD (Notch Intracellular Domain, Figure 3A). N^{IC} is transported into the nucleus (Figure 3D) where it binds ubiquitous transcription factor CSL, (CBF-1, Suppressor of Hairless, Lag-1), also known as RBP-jk in mice. N^{IC} binds to CSL and displaces a large co-repressor complex containing SKIP, SHARP, histone deacetylases and other co-repressors. The N^{IC} -CSL complex recruits a co-activator complex containing a Mastermind-like protein (MAML1-3 in mammals) as well as p300 and other chromatin-modifying enzymes, forming the Notch Transcriptional Complex (NTC), and activating transcription [38]. CSL target genes are numerous and include HLH-family negative transcriptional regulators of the HES and HEY family, but also oncogenes such as c-Myc. In the nucleus, N^{IC} is phosphorylated by CDK8, ubiquitinated by Sel10/FWB7 and degraded by the proteasome, thus terminating the signal. Putative non-canonical pathways have been suggested but remain incompletely characterized. Among them, physical interaction of Notch-1 IC with the IKK signalosome [50], with nuclear IKK α [51,52] and with p50 [53,54] and of Notch-3 IC with cytoplasmic IKK α [55] may mediate therapeutically relevant cross-talk with NF- κ B. Physical interaction of Notch-1 IC with p85 PI3-kinase α [56] may mediate non-nuclear cross-talk with AKT, leading to survival signaling [57].

A closer look at this apparently simple signaling pathway reveals a dizzyingly intricate series of mechanisms that finely regulate the timing, intensity and biological consequences of Notch signaling, and are likely to have significant therapeutic implications. The main factors that should be taken into consideration when Notch signaling in the clinical setting are listed below:

1) Paralog-specific effects

Mammals have four Notch paralogs, which differ mostly in the number of EGF repeats and the C-terminal part of N^{IC} . Although in theory they all signal through the same mechanisms, in many systems they have non-overlapping and even opposing effects [58-61]. This is relevant when comparing agents that block all Notch signaling to agents that selectively block a single receptor. In our hands, siRNA knockdown of different Notch receptors in breast cancer cells affects the expression of different gene sets (Pannuti et al., in preparation). This is consistent with data indicating that Notch-2, unlike Notch-1, -3 and -4, may have a positive prognostic significance in breast cancer [62,63]. Moreover, these paralog-specific effects are context-dependent, i.e., they may be different in different diseases and in CSC from different diseases. For example, in mesothelioma [64] Notch-2 inhibits the pro-survival effects of Notch-1, while in medulloblastoma [59], Notch-2 stimulates tumorigenesis and Notch-1 inhibits it. Hence, the need to determine in CSC from each disease and possibly disease subtype what the role of individual Notch paralogs is.

2) Ligand diversity

There are several Notch ligands with different functions. Canonical ligands include the Delta and Serrate/Jagged families (Delta-1, -3 and -4 and Jagged-1 and -2 in humans). These have multiple EGF-like repeats, a DSL (Delta-Serrate-Lag2) domain with or without a DOS (Delta-Osm11) domain. Serrate/Jagged family ligands also have a cysteine-rich (CR) motif (Figure 2). It has been suggested that DSL-only ligands lacking DOS domains may function in tandem with DOS-only putative co-ligands such as DLK1 (reviewed in [38]). Non-canonical ligands such as contactins, DNER and MAGP1-2 have been described [38]. While Notch binding to ligands expressed in trans (on contiguous cells) triggers Notch activation, some DSL-containing ligands can also bind Notch in cis (on the same cell), causing cis-inhibition. This is relevant to efforts to target specific ligands using monoclonal antibodies (mAb).

3) Notch glycosylation

The affinity of Notch receptors for specific ligands is controlled by N^{EC} glycosylation. POFUT-1 decorates N^{EC} with fucose residues, to which Fringe enzymes (in humans Lunatic, Radical or Manic Fringe) add N-acetylglucosamine (Figure 2). This regulates the differential affinity of Notch receptors for their ligands. In mammals, Fringe-modified Notch-1 loses its ability to respond to Jagged ligands, while Notch-2 does not [38]. POFUT-1 also has an unrelated chaperone activity that allows folded Notch to be presented at the membrane. Glucosyltransferase RUMI also modifies Notch in *Drosophila* (reviewed in [38]). Notch glycosylation has not been studied in detail in human cancers or in CSC.

4) ADAM redundancy

The ADAM cleavage step can be catalyzed by at least 2 proteases: ADAM17 (also known as TACE, TNF- α converting enzyme) or ADAM10. Recent evidence suggests that ADAM10 is required for ligand-dependent activation of Notch, while ADAM17 participates in ligand-independent receptor activation [65]. This should be kept in mind when considering the use of ADAM inhibitors to block Notch activation.

5) Multiple γ -secretase isoforms and substrates

γ -secretase, which is targeted by several investigational drugs currently being tested in cancer, is a multisubunit transmembrane aspartyl protease that contains presenilin-1 or -2 (the catalytic subunit) plus APH1, Pen2 and Nicastrin. Since there are 2 presenilin isoforms and at least 2 APH isoforms, there can potentially exist at least four different γ -secretase complexes. There is some evidence, reviewed in [38], that these have different biochemical properties and may contribute differently to Notch activation. In addition to cleaving Notch, γ -secretase catalyzes the regulated intramembranous proteolysis (RIP) of many other proteins [66]. These include ADAM10 [67], putative cancer stem cell marker CD44 [66] and circulating tumor cell (CTC) marker EpCAM [68].

6) Multiple forms of N^{IC} and post-translational modifications regulating its stability

It was originally thought that γ -secretase specifically cleaves Notch before V1744. This led to the production of antibodies recognizing the sequence V¹⁷⁷⁴LLS, at the N-terminus of N^{IC}. However, γ -secretase can cleave Notch at several positions in the 1743-1748 region, with a preference for the L1746-S1747 bond [69]. This means that there are potentially several species of N^{IC}, not all of which are detectable by currently available antibodies. These have different half-lives, with the V1744 being the most stable [38]. However, the intracellular stability and transcriptional activity of N^{IC} is regulated by many factors, including hypoxia [70,71], prolyl isomerase Pin1 [72] and Nemo-like kinase (NLK) [73]. Thus, a potential role of alternative N^{IC}s in cancer cannot be ruled out. Cleavage site selection can be affected by the cellular compartment in which cleavage occurs [38]. This is relevant to the choice of methods to detect N^{IC} in clinical specimens. Undetectable staining with a V¹⁷⁷⁴LLS antibody does not automatically imply lack of Notch activity.

7) Cellular trafficking of Notch receptors and ligands

Ubiquitination, endocytosis and endosome sorting regulate the amount of Notch receptors at the membrane and the balance between ligand-mediated receptor activation, ligand-independent receptor activation and degradation [37,38]. DSL-containing Notch ligands are ubiquitinated by Mindbomb or Neuralized E3 ligases, endocytosed, “activated” by a poorly understood process and recycled to the membrane in “active” form (Figure 3A). Upon binding, ligand-N^{EC} complexes are trans-endocytosed into ligand-expressing cells, a dynamin-dependent process that separates N^{EC} from NTM [74]. Mono-ubiquitination and endocytosis of Notch as a pre-requisite to activation have been suggested [75], though this

remains controversial [37,38]. The amount of Notch available at the membrane is controlled by several E3 ubiquitin ligases (Itch/AIP4, Cbl, Deltex, Nedd4) that can direct Notch to either lysosomal degradation or recycling (Figure 3C). Deltex forms a complex with Notch and β -arrestin homolog Kurz, leading to Notch ubiquitination and degradation [76]. In the absence of Kurz, Deltex sorts Notch into late endosomes via Rab5, causing Notch activation [77]. In *Drosophila*, defects in endosome trafficking caused by mutations in ESCRT complex components or in tumor suppressor LGD (Lethal Giant Disc) cause accumulation of Notch in endosomes, where Notch is activated by γ -secretase, causing aberrant cell proliferation. Aquaporin-related anion transporter big-brain (bib), which regulates endosome acidification, is required for Notch signaling [78], perhaps by facilitating the release of cleaved N^{IC} from endosomes [38]. In asymmetrically dividing cells, endocytic mediator Numb, in cooperation with ACBD3, adaptin and Numb-associated kinase (NAK) promotes selective degradation of Notch in one of two daughter cells. This is thought to promote differentiation of the daughter cell that loses Notch. Loss of Numb has been described in breast cancer [79] and asymmetric cell division is a hallmark of “stemness”. Loss of Numb could conceivably result in aberrant retention of “stemness”. Endocytic mediators dynamin and Rab5 are also required in Notch signal-receiving cells. Available evidence indicates that the γ -secretase cleavage step can occur at multiple cellular locations, including at or near the plasma membrane and after endocytosis (reviewed in [38]).

8) Chromatin cross-talk

Finally, though there are many putative CSL responsive elements in the genome, the actual pattern of usage (and thus the gene expression changes affected by Notch activation) appear to depend on the functional interaction of Notch with other nuclear effectors, including for example HIF-1 α [70], and the estrogen receptor ER α [52]. Two NTCs can cooperatively bind to sequence-paired CSL-responsive elements [80], possibly making some promoters differentially responsive to monomeric versus dimeric NTC.

These basic observations have significant clinical implications. Simply determining levels of Notch expression at the protein or even mRNA level is not necessarily indicative of how active the pathway is in a specific cancer cell. The best target genes to use as biomarkers may well vary in different diseases and disease subsets. Investigators developing γ -secretase inhibitors (GSIs) should be mindful of possible selectivity for γ -secretase isoforms, of multiple off-target effects and of the fact that compounds with different chemical properties may affect Notch cleavage at the membrane and/or in different intracellular compartments, at neutral or acidic pH. This may directly affect the pharmacological activity of GSIs *in vivo*.

Targeting Notch in cancer and CSC

Notch signaling has been implicated in a growing number of hematopoietic and solid tumors [39-49]. In most cases, one or more Notch paralogs have oncogenic activity. Inappropriate Notch activation stimulates proliferation, restricts differentiation and/or prevents apoptosis. In the skin, Notch-1 appears to act as a tumor suppressor. Recent evidence suggests that this is an indirect effect. Loss of Notch signaling in the skin causes a barrier defect which causes local inflammation, predisposing to transformation, hyperproduction of thymic stromal lymphopoietin (TSLP), and systemic immunological disturbances [81]. For a general discussion of Notch targeting in cancer, the reader is referred to [35]. This discussion will focus on Notch as a target in CSC.

The strongest evidence to date for a role of Notch in CSC is in breast cancer [6,8,82-84], embryonal brain tumors [85], and gliomas [86,87]. GSIs abolish the formation of secondary mammospheres from a variety of human breast cancer cell lines as well as primary patient

specimens (Grudzien et al., submitted for publication, Figure 4). In breast ductal carcinoma in situ (DCIS), the ability to form multilineage spheroids (“mammospheres”, an indicator of stem-like cells) is dramatically decreased by GSIs, a Notch-4 monoclonal antibody or gefitinib [83]. This suggests cooperation between EGFR and Notch-4 in DCIS “stem cell” maintenance. There is evidence for a feedback loop between Her2/Neu and Notch [88,89], which may maintain CSC in Her2/Neu-overexpressing tumors [90]. Sansone et al. [91] showed that in mammospheres from human breast cancers, interleukin-6 (IL-6) induces Notch-3 signaling, increases expression of Jagged-1 and, through Notch-3, promotes a hypoxia-resistant phenotype. The same group [84] described a p66Shc-Notch-3 pathway as essential to maintain the hypoxia-resistant phenotype of human breast cancer mammospheres. Fan et al. [85] showed that Notch inhibition selectively depletes medulloblastoma CSC as determined by CD133-high status or dye exclusion. The same group has described very similar findings in glioblastoma CSC [86]. Importantly, in gliomas Notch appears to confer radio-resistance to CSC [87]. GSI treatment selectively enhanced radiation-induced death of glioma CSC but not bulk glioma cells. This effect was replicated by Notch-1 or Notch-2 knockdown, and was accompanied by AKT inhibition and reduced Mcl-1 expression. Other malignancies are being actively investigated. A role of Notch, STAT3 and TGF- β in hepatocellular carcinoma CSC maintenance has been suggested [92]. In gemcitabine-resistant pancreatic carcinoma cells, EMT is associated with activation of Notch signaling, potentially linking Notch to the “Weinberg model” of stemness acquisition through EMT [93] and to treatment resistance. Inhibition of Notch signaling through GSIs [86] or Delta-4 mAb [94] decreased the numbers of CSC and/or their tumorigenicity in some preclinical models.

These encouraging results suggest that therapeutic regimens including Notch inhibitors may be used in the clinic to target CSC and reverse or prevent chemo- or radio-resistance. However, for clinicians interested in targeting Notch in CSC, numerous questions remain to be addressed. There are numerous investigational Notch inhibitors to choose from, some of which are already in the clinic (Table 1). At least four chemically distinct GSIs are being developed by pharmaceutical companies. It is unclear whether these drugs are pharmacologically equivalent and, based on our evolving understanding of γ -secretase, it is possible that they may have significant differences in both Notch inhibition and off-target effects. Due to the very nature of their target, GSIs should block the cleavage of all four Notch paralogs, and multiple other γ -secretase substrates. Thus, they are relatively non-selective drugs, although in vivo their dose-limiting toxicity (secretory diarrhea) is due to Notch inhibition in intestinal stem cells. Inhibition of other γ -secretase targets may either contribute to therapeutic efficacy or hinder it, and these off-target effects may be different for different GSIs. Recently, Watters et al. [95] generated a library of murine mammary tumor models from genetically engineered oncogenic mammary stem cells, essentially artificial CSC. These authors treated tumors with a specific GSI (from Merck), and describe a “GSI response signature”, which is enriched for Notch pathway genes but also includes genes from G-protein activated pathways, eicosanoid pathways and others. Which of these effects are secondary to Notch inhibition and which are off-target remains to be established. However, these data provide a useful platform to compare different GSIs, and GSIs to other agents. The discovery that some NSAIDs-related compounds can allosterically modify the substrate specificity of γ -secretase [96] suggests that Notch-selective γ -secretase modifiers (GSM) can be developed, though specificity for individual Notch homologs remains unlikely. Inhibitors of the MAML/CSL/Notch complex formation were a theoretical possibility until recently. Innovative work by Moellering et al. [97] shows that a hydrocarbon-stapled cell-permeable peptide derived from MAML-1 can selectively prevent the assembly of the NTC and has efficacy as a Notch inhibitor in vitro and in vivo. If non-peptide drugs with similar characteristics can be developed, they would likely be pan-Notch inhibitors without the off-target effects of GSIs. Conversely, mAbs to Notch ligands and

receptors offer single-target specificity. Antibodies that “lock” Notch receptors in an inactive conformation by preventing N^{EC}-NTM dissociation are in preclinical development [98]. To date, Notch-1 mAb appear to have similar toxicities compared to GSIs. Delta-4 mAbs are effective against breast CSC in vivo [94]. However, their chronic use can cause vascular neoplasms [99].

GSIs and other small molecules have the advantages of relative ease of administration, oral bioavailability and low cost. Pan-Notch inhibition may or may not be an advantage depending on the relative roles of specific Notch paralogs in individual cancers, and on whether redundancy between Notch paralogs can result in resistance to single-target agents [35]. Off-target effects are not necessarily a disadvantage if they contribute to efficacy. On the other hand, clinical experience so far has shown that GSI must be administered in intermittent dosing regimens to prevent dose-limiting intestinal toxicity. Whether intermittent Notch inhibition is sufficient for effective CSC targeting in patients is an open - and very important - question. If continuous inhibition is necessary for optimal results, systemically delivered GSIs may be at a disadvantage over more specific agents such as anti-ligand mAbs, or may have to be delivered selectively to tumors using innovative pharmaceuticals. Situations in which a specific Notch-ligand pair is involved in CSC self-renewal may be targeted more specifically using a mAb. Tumor-selective mAbs (e.g., bispecific antibodies) would theoretically offer site-specific inhibition that could be used chronically. However, the choice of mAb for each specific indication will depend upon the Notch receptors and ligands that play a predominant role in that malignancy's CSC.

Rational combinations and personalized medicine

Even targeting developmental pathways such as Notch will most likely not give us the elusive “magic bullet”, and will require the development of rational combinations. Such combinations will be made possible only through a thorough understanding of cross-talk between Notch and other developmental and non-developmental pathways that may play roles in CSC in specific malignancies. Our knowledge is rapidly evolving, but there is evidence to support some combinations. The following examples are not meant to be all-inclusive, but these classes of agents are reasonable candidates for combination with Notch inhibitors: 1) Inhibitors of the PI3-kinase-AKT-mTOR pathway [35,87,100]; 2) NF-κB inhibitors [50,51,101], 3) Her2/Neu inhibitors [88,90], platinum compounds [51,102], EGFR inhibitors [83] and Hedgehog inhibitors [103]. In breast cancer, a newly discovered feedback between Notch and ERα [52,104] supports combining Notch inhibitors with anti-estrogens. Anti-estrogen plus GSI and Hedgehog-inhibitor plus GSI combinations are being investigated in ongoing clinical trials. In the case of the Hedgehog inhibitor-GSI combination, anti-CSC effects are being measured specifically.

Ultimately, the best use of Notch inhibitors and other CSC-targeted agents will be in the context of personalized medicine. To that end, we will have to determine: 1) which cancers and specific cancer subtypes contain Notch-dependent CSC; 2) what role do specific components of Notch signaling play in these CSC; 3) what pathways cross-talk with Notch in specific CSCs; and 4) how can one measure Notch activity in CSC from individual patients (e.g., in biopsy material).

The design of clinical trials of CSC-targeted agents will have to consider that anti-CSC effects will not necessarily translate into rapid tumor volume changes. Disease-free or recurrence-free survival will be the most informative endpoints. For situations when this would require prohibitively long follow-ups, it will be important to develop accurate surrogate biomarkers that reflect anti-CSC effects. These may be spheroid formation assays, flow cytometry, molecular tests, or other tests, but post-treatment tumor tissue will be

required in most cases. A question of potentially great interest is whether it is possible to assess CSC numbers or the relative “stemness” of individual tumors by studying circulating tumor cells (CTC) [105-107]. These can be isolated from patient blood by several methods, one of which is FDA-approved. While these trials may be challenging, the payoff may be novel treatments that eliminate or greatly reduce treatment-resistance in a whole range of malignancies.

Acknowledgments

This work was supported by NIH grant P01 AG2553101 and DOD IDEA grant W81XWH-04-1-0478.

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Figure 1.

A: schematic representation of an idealized CSC. The figure shows a list, not meant to be all-inclusive, of pathways that modulate the CSC phenotype. CSC exist in the context of “niches” formed by neighboring cells and extracellular matrix ECM). The Hedgehog (Hh), Notch, and Wnt pathways mediate short-range interactions with neighboring cells. Soluble mediators such as TGF- β and the related BMPs, or growth factors such as hepatocyte growth factor (Met ligand), as well as signals from ECM proteins may all participate in regulating the maintenance, self-renewal and differentiation of CSCs. These are characterized by slow replication, ability to generate partially differentiated progenies (pluripotency), highly effective DNA repair, ability to eliminate xenobiotics including chemotherapeutics through ABC family transporters (ABC) and expression of primitive membrane markers (CD133, Met). Transcription factors such as Bmi-1, Musashi, Sox2, Oct4 and others are commonly expressed in putative CSCs. Immune surveillance by the innate and possibly adaptive immune systems also contributes to the CSC microenvironment, with effects that at least in mice are inhibitory. **B: Models of CSC origins.** In the traditional model, CSC originate from the transformation (red dashed arrows) of normal tissue stem cells (TSC), or possibly of progenitor cells with limited self-replication ability that normally generate cells destined for differentiation. In the alternative model proposed by Mani and colleagues, transformation of cells at many stages of the differentiation process can produce CSC through EMT, which restores a stem-like phenotype -and the ability to metastasize- to some cancer cells. Besides EMT, other mechanisms of de-differentiation have been described and may contribute to restoration of stemness in transformed cells. **C: Hierarchical organization of cancers.** Once CSC are formed, they are thought to generate other tumor cells through a process akin to normal tissue differentiation. In a widely accepted model, asymmetric cell division of CSC produces pluripotent “progenitors”, which in turn generate one or more bulk tumor cell types through proliferation and aberrant differentiation. CSC and “progenitors” are more tumorigenic in xenografts and less chemosensitive than bulk cancer cells.

Figure 2. Schematic structure of Notch receptors and ligands

Left: Notch ligands contain EGF-like repeats and a trans-membrane domain (TMD) with a short cytoplasmic tail of variable length. The N-terminus contains a specialized DSL region that structurally resembles EGF repeats [108], and a DOS region consisting of two atypical EGF-like repeats. Jagged-1 and -2 (mammalian homologs of *Drosophila* Serrate) have a longer EGF region than other ligands, and also contain a cysteine-rich motif (CR). Delta homologs in mammals include DSL and DOS-containing ligands (Delta-1) and DSL-only ligands (Delta-3 and -4). It is unclear whether these require a co ligand protein containing a DOS but no DSL (DLK1 and DLK2) to activate Notch. Delta-3 does not activate Notch well in cell culture systems, while Delta-4 does.

Right: A typical mature, full length Notch receptor (N^{FL}), in this case human Notch-1, contains an N^{EC} featuring multiple EGF-like repeats (36 in Notch-1). Of these, repeats 11 and 12 (purple) represent the primary ligand binding site, with repeats 24-29 (purple) playing an accessory role. N^{EC} is glycosylated by Pofut-1 and the Fringe enzymes, which add fucose (Fuc) and N-acetylglucosamine (Glc-Nac) to it. RUMI enzymes (so far only identified in *Drosophila*) add glucose to it. N^{EC} ends with three Lin-Notch repeats (LNR), which fold over the heterodimerization domain (HD), masking the ADAM cleavage site (red) in the N-terminus of N^{TM} . Distal to the HD, N^{TM} contains a transmembrane domain (TMD, shown here with membrane phospholipids surrounding it). The γ -secretase cleavage region is at the cytoplasmic end of the TMD. Moving from N- to C-terminus, we find a RAM (RBP-j κ Activation Motif), a nuclear localization sequence (NLS), seven ankyrin repeats (ANK), and a C-terminal region that contains a PEST (Proline-Glutamic-Serine-Threonine rich) sequence which controls receptor turnover by being phosphorylated by CDK8, leading to ubiquitination by SEL10/Fbw7 and degradation. After ligand-induced subunit separation, the ADAM site is exposed and cleaved by ADAM10 or ADAM17, generating a Notch extracellular truncated (N^{EXT}) intermediate, which is still membrane associated. N^{EXT} is cleaved by γ -secretase, generating N^{IC} .

Figure 3. Diagram of Notch activation and canonical signaling mechanisms

A: Ligand activation and functions. In ligand expressing cells, ligands are ubiquitinated (UQ) by E3 ligases Mindbomb and Neuralized, endocytosed and “activated”. “Active” ligands bind Notch receptors, dissociating N^{EC} from N^{TM} . The complex ligand- N^{EC} is trans-endocytosed into the ligand-expressing cell, perhaps providing mechanical energy to separate N^{EC} from N^{TM} . Some ligands expressed in cis can bind Notch on the same cell, causing cis-inhibition. **B: ligand-dependent and -independent activation.** Ligand-induced N^{EC} separation unmasks the ADAM cleavage site (red), which is cleaved by ADAM10 or ADAM17, producing N^{EXT} and a short peptide which is released. N^{EXT} is cleaved by γ -secretase, at the membrane or during endocytosis, generating N^{IC} . The release of N^{IC} from endosomes (or the selection of cleavage site by γ -secretase) may require endosome acidification (H^+) by aquaporin Bib. The stability of N^{IC} is regulated by factors such as Pin-1 prolyl isomerase and NLK kinase. Endocytosis can lead to ligand-independent Notch activation catalyzed by γ -secretase. In the absence of non-visual β -arrestin Kurz, Deltex may lead to Notch endocytosis and activation. **C: control of Notch availability and trafficking.** The amount of Notch available at the membrane is controlled by many endocytosis-recycling mechanisms. Several E3 ligases (Itch, CBL, Nedd4, the Deltex-Kurz complex) can target Notch for degradation. The ESCRT complex and lgd in *Drosophila* (and presumably their homologues in mammals) control Notch degradation, and their loss causes accumulation of Notch in endosomes and ligand-independent activation. In actively dividing cells, Numb/ACBD3 asymmetrically partitions to one daughter cell, causing selective Notch degradation in it. **D: nuclear events.** N^{IC} is transported to the nucleus, where it causes the dissociation of the co-repressor complex including SHARP, SKIP and several other proteins (CoR) from CSL. Notch, CSL and MAML form a tertiary complex [109] which in turn recruits p300 and other coactivators (CoA) to the chromatin and forming the NTC that activates transcription. The NTC can form heterodimers on the chromatin with other NTCs or supramolecular complexes with other transcription factors, modulating the choice of genes regulated by Notch.

Figure 4. Effects of a GSI on human breast cancer secondary mammospheres

Mammospheres were formed ex vivo from a pleural aspirate from a late-stage breast cancer patient. Mammospheres were dissociated and secondary mammospheres were formed and characterized for multilineage differentiation (an indication they contain pluripotent stem-like cells). Secondary mammospheres were treated with vehicle or a panel of GSIs at clinically achievable concentrations (Grudzien et al., submitted for publication).

Mammosphere and isolated cells were enumerated at different times. This example shows phase contrast microphotographs of secondary mammospheres from a clinical isolate treated with vehicle (left) or 10 μ M GSI MRK003 for 7 days (right). GSI completely and irreversibly blocked the formation of new mammospheres, and only isolated cells were observed in culture. These eventually underwent apoptosis.

Table 1

Notch-targeting agents

Agent	Mechanism	Targets	Development phase
GSI (γ-secretase inhibitors): MK0752 (Merck) R04929097 (Roche) PF-03084014 (Pfizer) LY450139 (Eli Lilly) BMS-unknown (BMS)	Inhibition of final Notch cleavage by γ-secretase	All 4 Notch paralogs; Notch ligands; multiple other γ-secretase substrates	Phase 1/2
GSMs (γ-secretase modifiers): MPC-7869 (Myriad)	Inhibition of final substrate cleavage by γ-secretase	Selective for specific γ-secretase substrates	Unsuccessful Phase 3 for MPC-7869, discovery for Notch-targeted GSMs
MAML1 stapled peptide	Interference with Notch nuclear co-activator MAML1	All 4 Notch paralogs, potentially other nuclear transcription factors that use MAML1	Pre-clinical
Notch mAbs	Interference with ligand-induced Notch subunit separation	Specific for individual Notch receptors	Pre-clinical
DLL4 mAbs	Interference with ligand-receptor interaction	Specific for Delta-4 ligand	Pre-clinical
Other ligand mAbs	Interference with ligand-receptor interaction	Specific for other Notch ligands	Pre-clinical
Notch soluble receptor decoys	Interference with ligand-receptor interaction	Relatively specific for Notch paralogs, potential pan-Notch inhibition	Pre-clinical
siRNA, miRNA-based therapeutics	Interference with expression of Notch signaling components	Specific for target mRNAs	Pre-clinical