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REVIEW

The Notch signaling pathway as a mediator of tumor survival

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The Notch signaling pathway is evolutionarily conserved and responsible for cell fate determination in the developing embryo and mature tissue. At the molecular level, ligand binding activates Notch signaling by liberating the Notch intracellular domain, which then translocates into the nucleus and activates gene transcription. Despite the elegant simplicity of this pathway, which lacks secondary messengers or a signaling cascade, Notch regulates gene expression in a highly context- and cell-type-dependent manner. Notch signaling is frequently dysregulated, most commonly by overactivation, across many cancers and confers a survival advantage on tumors, leading to poorer outcomes for patients. Recent studies demonstrate how Notch signaling increases tumor cell proliferation and provide evidence that active Notch signaling maintains the cancer stem-cell pool, induces epithelial–mesenchymal transition and promotes chemoresistance. These studies imply that pharmacological inhibition of Notch signaling may refine control of cancer therapy and improve patient survival. Gamma secretase inhibitors (GSIs) are drugs that inhibit Notch signaling and may be successful in controlling cancer cell growth in conjunction with standard chemotherapy, but substantial side effects have hampered their widespread use. Recent efforts have been aimed at the development of antibodies against specific Notch receptors and ligands with the hope of limiting side effects while providing the same therapeutic benefit as GSIs. Together, studies characterizing Notch signaling and modulation have offered hope that refined methods targeting Notch may become powerful tools in anticancer therapeutics.

Discovery and characterization of Notch

Notch was discovered nearly 100 years ago in *Drosophila melanogaster* by the observation of a notched phenotype in the wings of flies bearing a mutation in this gene (1). Despite the vital role of Notch signaling in embryonic development, it was not until 1985–1986 that *Notch* was sequenced. Notch was found to consist of 2703 amino acids containing 36 tandem repeats with homology to epidermal growth factor (EGF; Figure 1, inset) (2,3). Further studies determined that Notch was a type I single-pass transmembrane protein with an extracellular domain possessing the EGF repeats and an intracellular portion containing a nuclear localization sequence, an RAM domain, a C-terminal PEST domain, and seven ankyrin repeats that can bind to a DNA-binding protein complex called the Recombination Binding Protein-Jκ (RBP-Jκ) in mammals (4). These early data provided evidence that Notch was a transmembrane receptor, as well as a transcription factor, and facilitated the discovery of proteins that interact with Notch to control gene expression, forming the basis of our understanding of Notch signaling today.

Abbreviations: AML, acute myeloid leukemia; CSC, cancer stem cell; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EGF, epidermal growth factor; EMT, epithelial–mesenchymal transition; GBM, glioblastoma multiforme; GSI, gamma secretase inhibitor; HES, human hairy and enhancer of split; HEY, hairy/enhancer-of-split related with YRPW motif; ICD, intracellular domain; NSCLC, non-small-cell lung cancer; RBP-Jκ, recombination binding protein-Jκ; T-ALL, T-cell acute lymphoblastic leukemia.

Notch signaling

The Notch signaling pathway is highly conserved and regulates cell-fate decisions throughout embryonic development and adult life. In the majority of tissues, Notch maintains an undifferentiated state but there are exceptions, cited below, in which Notch signaling induces differentiation. The canonical and non-canonical Notch signaling pathways have been reviewed in great detail (5–8); here, we provide a brief summary of the canonical Notch pathway (Figure 1).

In mammals, there are four Notch receptors (Notch1–4). After protein translation, Notch undergoes posttranslational processing before it is fully functional. When still intracellular, Notch is cleaved by a furin-like protease at the S1 cleavage site, producing a mature heterodimeric receptor (9,10). The resulting Notch heterodimer is held together non-covalently by calcium that causes autoinhibition of the protein (11,12). Activation of canonical Notch signaling in mammals requires physical contact of the Notch receptor with its ligand from one of two families of ligands, Jagged (Jagged1, 2) and Delta-like ligand (DLL-1, -3, -4) (5) while both receptor and ligand are attached to their respective cell membranes (13,14). Therefore, unlike diffusible signaling molecules that act over long distances, Notch ligands have traditionally been believed to signal only to adjacent cells, influencing gene expression and cell fate decisions in immediate neighbors (15). However, there is recent evidence that the Notch ligand Jagged1 can be secreted and activate Notch signaling without direct cell–cell contact (16). Engagement of the Notch receptor with its ligand induces a conformational change in Notch exposing its S2 site for cleavage by an enzyme called tumor necrosis factor-α-converting enzyme, a member of the A Disintegrin And Metalloproteinase family of proteases (17). The remaining Notch fragment is then accessible to the gamma secretase complex, the enzyme complex that carries out the third and final cleavage of the Notch protein. Presenilin is the catalytic subunit of the gamma secretase complex (18), which also includes nicastrin, Anterior Pharynx-defective-1 and Presenilin enhancer-2 ((19–21), reviewed in ref. 22). The final cleavage event liberates the Notch intracellular domain (ICD) allowing it to translocate to the nucleus and modify gene expression.

The Notch ICD alters gene expression by binding to the DNA-bound CSL protein complex (CBF1/RBP-Jκ in humans, Su(H) in Drosophila, and Lag-1 in *Caenorhabditis elegans*) (23), which we refer to as RBP-Jκ in this review. The RBP-Jκ complex is constitutively set up at a consensus DNA sequence with a core TGRGA element (24–28). When activated Notch ICD is not bound, the RBP-Jκ complex acts as a transcriptional repressor. When Notch ICD binds to the RBP-Jκ complex, it converts the complex from a constitutive repressor to a constitutive activator by displacing the corepressor SMRT/HDAC1 complex from RBP-Jκ, allowing the transcription of Notch target genes (29).

Notch target genes

Notch signaling is highly context and cell type dependent, although certain genes are consistently upregulated by activated Notch across many tissue types. The best studied examples are the Drosophila proteins hairy and enhancer of split, homologues to human hairy and enhancer of split (HES) (30) and hairy/enhancer-of-split related with YRPW motif (HEY) families (31) (Table I). HES and HEY proteins share several characteristic features, including a basic helix-loop-helix domain, a WRPW motif, and an "orange domain" ((32), reviewed in ref. 33). HES and HEY act as direct repressors of transcription by binding directly to DNA

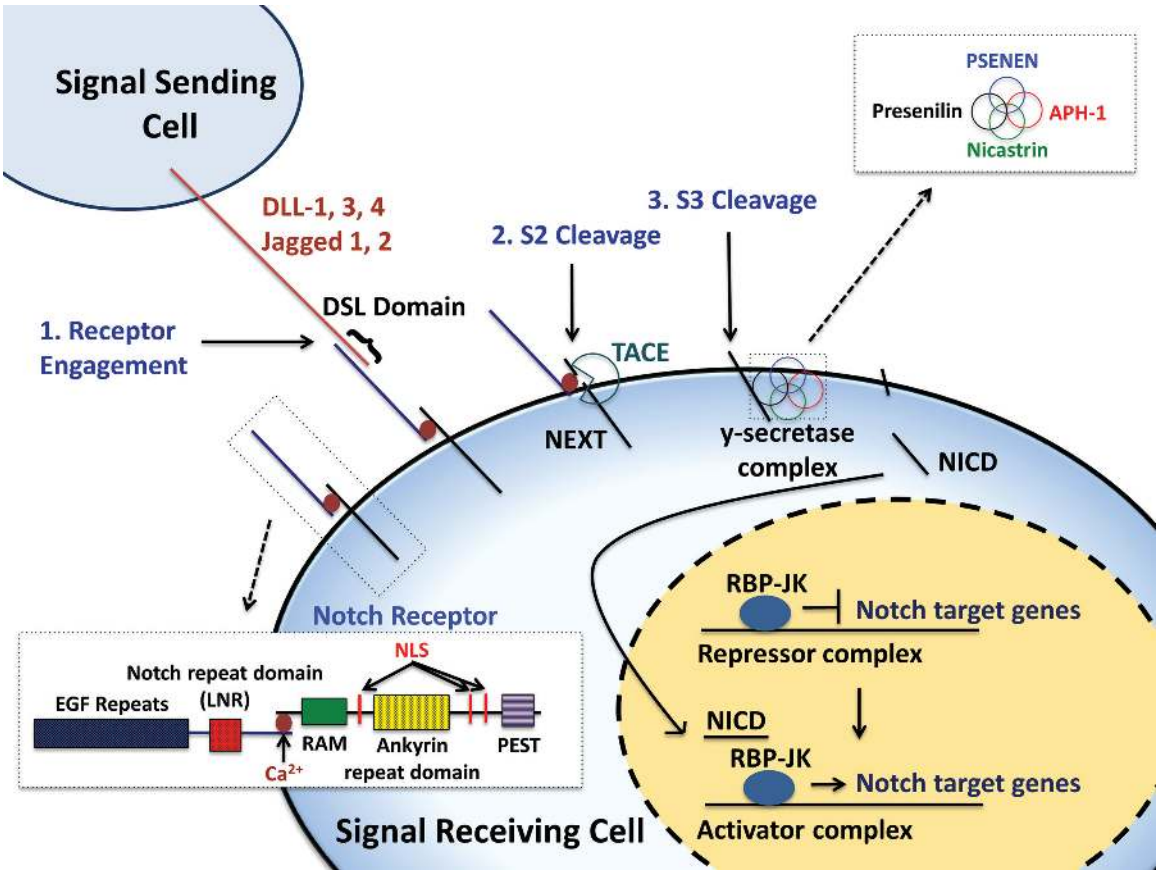

Fig. 1. Diagram of the Notch signaling pathway. A mammalian signaling cell expresses one of the five Notch ligands. Engagement of this ligand with one of the four Notch family receptors causes cleavage of the receptor at the S2 cleavage site by TACE. The remaining Notch receptor undergoes further cleavage at the S3 site by the gamma secretase complex, freeing the Notch ICD. The Notch ICD translocates to the nucleus where it binds to the RPB-Jκ protein complex and converts the complex from a repressor to an activator of Notch target gene transcription. Inset is a schematic of the Notch protein. The EGF repeats are responsible for engaging the ligand; LNR is a negative regulator of Notch protein activity; the RAM domain enhances interaction between Notch ICD and RPB-Jκ; Ankyrin repeats mediate interaction with the RPB-Jκ; PEST domain is rich in proline, glutamate, serine and threonine residues and is involved in degradation of the Notch ICD. DLL, delta-like ligand; PSENEN, Presenilin enhancer-2; APH-1, Anterior Pharynx-defective-1; DSL, Delta-Serrate-Lag2; NLS, nuclear localization signal; TACE, tumor necrosis factor-α-converting enzyme; NEXT, Notch extracellular truncation; NICD, Notch intracellular domain; RPB-Jκ, recombination binding protein J-kappa; RAM, RAM23 domain; PEST, proline (P), glutamine (E), serine (S) and threonine (T)-rich domain.

Table I. Known Notch target genes

Gene	System/tissue in which target was identified	Reference
HES1	Coculture of HeLa and QT6 (quail) cells	(30)
HEY1	Discovered in *D. melanogaster*, where it is known as enhancer of split	(31)
Cyclin D1	HEK293T cells, RKE cells	(39)
NRARP	Xenopus embryos	(40)
NF-kB	Bone marrow progenitor cells, T-ALL mouse model	(41)
p21	Keratinocytes	(42)
pre-Ta	T-lymphocytes	(43)
c-myc	T-ALL, breast cancer	(28,44,45)
IGF1-R	NSCLC	(46)
Survivin	NSCLC	(47)
Slug	Embryonic cardiac cushion	(48)
Nanog	Mouse model of mammary transformation	(83)

through their basic helix-loop-helix and WRPW domains (32). In humans, the genes repressed by HES and HEY are responsible for lineage commitment decisions. Among the majority of cell lineages reported thus far, Notch signaling maintains an undifferentiated state (34–36), although there are exceptions in which Notch promotes differentiation, such as in keratinocytes (37).

Many Notch target genes have been identified (reviewed in ref. 38), including cyclin D1 (39), NRARP (40), NF-κB (41), p21 (42) and pre-Tα (43). Several are particularly important because of their role in cancer, including MYC (28,44,45), IGF1-R (46), survivin (47) and snail homolog 2, commonly known as SLUG (48). Henceforth, we will examine the function of Notch in cancer and how fundamental Notch-driven processes of development and self-renewal have been reappropriated in carcinogenesis, tumor progression and cancer cell survival.

Notch in cancer

Although earlier Notch studies focused primarily on the pivotal role of Notch signaling in development and tissue homeostasis, recent research has been directed at elucidating the role of Notch in cancer. These studies have provided important insights into how overactivation of stem-cell pathways can enhance malignant characteristics of cells. Here, we survey the role of the Notch pathway in individual cancers by highlighting key examples (Table II). We then look at how Notch signaling promotes specific phenotypes across cancer types and conclude with a summary of Notch-based therapeutic strategies.

Notch in leukemia

Aberrant Notch signaling was first identified in human T-cell acute lymphoblastic leukemia (T-ALL) by Reynolds *et al*. through analysis

Table II. Findings of key studies defining the role of Notch in cancer

Cancer	Finding	Reference
Hematopoietic malignancies		
T-ALL	*Notch1* gene identified as one of the genes in the reproducible translocation t(7;9)(q34;q34.3) previously identified in T-ALL	(50)
	Approximately 50% of T-ALL cases have altered Notch signaling	(51)
AML	In AML, Notch1, Jagged1 and Delta1 expression correlated with poorer overall and relapse-free survival	(53,54)
Solid tumors		
Breast cancer	Overexpression of Notch1 ICD blocks p53-mediated damage response and prevents apoptosis	(58)
	Notch1 plays a role in CSC character; Notch4 is required for tumor initiation	(85)
Lung cancer	Approximately 30% of NSCLC cases had Notch1 activation; cells were dependent on these mutations for survival	(62)
	Notch3 is overexpressed in 40% of NSCLC, contributes to inhibition of apoptosis	(68,70)
	High Notch1 expression correlates with poor prognosis in NSCLC	(72)
Glioma	Notch1, DLL-1 and Jagged1 overexpressed in glioma	(73)
	Notch1 is a predictor of poor survival in glioma	(75)

of chromosomal translocations. They initially discovered a recurrent translocation t(7;9)(q34;q34.3) in a subset of T-ALL cases (49). The locus on chromosome 9 involved in the translocation was later found to be a homologue to the Drosophila *Notch1* gene (50). Reynolds *et al*. subsequently discovered that aberrant Notch1 activation was present in as many as 50% of human T-ALL cases (51), suggesting that Notch1 might act as an oncogene. Weng *et al*. reported that the Notch gain-of-function mutations clustered nearly exclusively in either the PEST domain, which is responsible for stability of activated Notch ICD, or within the heterodimerization domain, believed to enhance gamma secretase cleavage, thereby activating Notch (51). In a similar study of precursor T-cell lymphoblastic leukemia/lymphoma assessing Notch-activating mutations in cell lines and primary tumors from mouse models of precursor T-cell lymphoblastic leukemia/lymphoma, Lin *et al*. found that 68% and 59%, respectively, had Notch-activating mutations in either the PEST or the heterodimerization domains (52). Together, these findings highlighted a potential key role of Notch signaling in leukemogenesis, but exactly how activated Notch1 stimulated cell proliferation and/or survival was unclear.

Since Notch is a transcription factor, effort has been focused on identifying its target genes that may drive leukemogenesis. By microarray and functional analyses, the Aster laboratory found that the gene that encodes MYC, a proto-oncogene that drives increased proliferation and downregulates apoptosis, is a direct Notch1 target in T-ALL (28). The authors further demonstrated that the prosurvival effect of Notch signaling on leukemia cells was driven, at least partly, by MYC, because MYC inhibitors interfered with the effects of Notch activation, and overexpression of MYC rescued the effects of Notch inhibition (28). Others have demonstrated NF-κB as a key downstream target of Notch1 and mediator of Notch-1-induced transformation in T-ALL (41).

Recent studies assessed the prognostic implications of Notch in other leukemia subtypes. Xu *et al*. in their study of acute myeloid leukemia (AML) found that *Notch1*, *Jagged1*, and *DLL-1* expressions were each independent factors associated with poor prognosis as measured by overall and relapse-free survival (53). This finding was confirmed by a similar, more recent study in AML (54). Continued research aims to unravel the Notch pathway and its interactions with other signaling pathways that play a key role in leukemogenesis, such as the WNT, SHH and AKT/PI3K pathways. Recent accumulating data lead us to conjecture that Notch works as a hub enabling crosstalk among many of these oncogenic pathways.

Breast cancer

The first solid tumor in which Notch was implicated in molecular carcinogenesis was breast cancer. The discovery that sparked investigation into function of Notch in breast cancer identified an insertion site for the mouse mammary tumor virus (55). The gene overexpressed by mouse mammary tumor virus insertion was subsequently identified as a novel Notch family member, Notch4 (56,57). As was the case for leukemia, subsequent work has focused on discovery of Notch target genes driving breast cancer and on the functional significance of activated Notch signaling in breast cancer. Interestingly, MYC was found to be a Notch target gene in breast cancer, and its expression was required for Notch1 ICD-driven mammary tumorigenesis in mice (44). Using human breast cancer cell lines and primary samples, Stylianou *et al*. demonstrated an accumulation of Notch1 ICD in breast cancer cells compared with normal tissue (58). They probed how Notch signaling contributes to carcinogenesis by treating cells with inducers of apoptosis and found that in cell lines stably overexpressing Notch1 ICD, the TP53-mediated DNA-damage response pathway was blocked, preventing cells from completing their apoptotic program (58). This demonstrated that Notch overexpression also participates in breast carcinogenesis through inhibition of apoptosis.

In addition to mechanistic studies, several reports have shown that overexpression of *Notch1* and *Jagged1* correlates with poor prognosis in patients with breast cancer. By *in situ* hybridization of RNA, Reedijk *et al*. demonstrated that high levels of *Notch1* and *Jagged1* expressions were correlated with poorer overall 10-year breast cancer survival (59). Later studies validated this finding, showing that *Jagged1* expression was correlated with poor breast cancer survival (60) and was associated with a basal phenotype and recurrence in lymph node-negative breast cancer (61). These studies bolster the mechanistic data supporting the critical role of Notch signaling in breast cancer.

Lung cancer

Notch signaling has similarly been studied for its role in lung cancer. The work by Westhoff *et al*. provided one of the earliest pieces of direct evidence for dysregulation of Notch signaling in non-small-cell lung cancer (NSCLC). Induction of Notch signaling by either Notch1 upregulation or Numb downregulation was observed in 30% of primary human NSCLCs (62). Further, cultures of primary NSCLC tumors that harbored gain-of-function Notch mutations were selectively killed in the presence of the gamma secretase inhibitors (GSIs) MRK-003 and *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT), demonstrating that these tumor cells were dependent on Notch signaling for survival and that Notch mutations are driver mutations in NSCLC (62). Conversely, another study demonstrated that overexpression of Notch1 in NSCLC inhibited cell growth, induced cell cycle arrest, limited colony formation *in vitro* and prevented tumor formation *in vivo* (63), although the sample size for the *in vivo* work was small. The conflicting data on the role of Notch signaling in lung cancer may be reconciled through the idea that Notch1 signaling drives proliferation within the lung cancer stem cell (CSC) population (64), or that NSCLCs with Notch alterations are more dependent on Notch signaling than those without aberrant Notch signaling. Another reason for the above discontinuities

in experimental findings may be due to the tumor microenvironment. For example, other research on Notch in NSCLC showed that the downstream effects of Notch1 depend on oxygen concentration (65). Independent studies have shown that under hypoxic conditions, Notch1 stimulates NSCLC tumor growth through direct upregulation of IGF1-R (46) and survivin (47), both of which regulate cell proliferation and survival. These latter studies underscored the importance of the tumor microenvironment in studies focused on therapeutics targeting Notch in NSCLC.

Notch1 contributes directly to lung carcinogenesis. Allen *et al*. recently developed a transgenic mouse model in which activated Notch1 was overexpressed in the alveolar epithelium (66). The mice developed alveolar hyperplasias and after a long latency developed pulmonary adenomas, suggesting that Notch1 activation leads to dysregulated expansion of lung epithelial cells but is not sufficient to induce carcinomas. When Allen *et al*. crossed these mice with those conditionally overexpressing MYC in the alveolar epithelium, adenocarcinomas formed. The authors suggested that cooperation of MYC with Notch1 led to a shift in the ratio of apoptotic and proliferating cells that allowed progression from adenomas to adenocarcinomas (66).

Notch3 also appears to be a key player in NSCLC. As a postdoctoral fellow in David Carbone's laboratory, Dang *et al*. initially mapped a rare t(15;19) translocation in lung cancer to the highly expressed Notch3 gene and subsequently showed that Notch3 is overexpressed in 40% of NSCLC tumors (67,68). Dang *et al*. later reported that suppression of Notch3 results in loss of the malignant phenotype in both *in vitro* and *in vivo* models (69). They further demonstrated crosstalk between the Notch3 and the EGF receptor-mitogen-activated protein kinase pathways resulting in the inhibition of apoptosis through expression of the gene that encodes the antiapoptotic protein BIM (70). They went on to characterize two regions within the Notch3 extracellular domain EGF receptor-like repeats that may be responsible for the distinct effects of Notch3 versus those of Notch1 in NSCLC (71). More work is necessary to fully elucidate the roles of both Notch1 and Notch3 signalings and their interactions in lung cancer.

Clinically oriented studies have highlighted that Notch signaling also impacts survival in lung cancer patients. A recent study by Donnem *et al*. assessed the prognostic impact of Notch ligands and receptors in NSCLC and found that high Notch1 expression was statistically significantly associated with poor outcomes in lung adenocarcinoma (72). They did not characterize the mechanism underlying these correlations, but their findings reinforced the critical role that the Notch pathway plays in NSCLC, as was the case in breast cancer and leukemia. Their work suggests that inhibiting Notch1 signaling may be a useful strategy for the treatment of NSCLC.

Glioma

Purow *et al*. published a sentinel study in 2005 that identified *Notch1*, *DLL-1* and *Jagged1* in a screen for genes overexpressed in glioma cell lines and primary human gliomas (73). When they blocked Notch signaling by knockdown of *Notch1*, *DLL-1* or *Jagged1* expression, there was an increase in cell death. Furthermore, when they orthotopically injected glioma cells with knocked-down Notch1 or DLL-1 expression into mice, survival was significantly prolonged (73), suggesting that Notch signaling drives glioma cell growth. A recent study identified a brain-specific microRNA, miR-524-5p, that behaves as a tumor suppressor in glioma by negatively targeting *Jagged1* and the Notch target gene, *HES1*. Restoration of miR-524-5p expression increased cell proliferation and invasion both *in vitro* and *in vivo* (74), demonstrating the complexity of the mechanisms regulating Notch signaling during carcinogenesis. Notch is also a prognostic factor in glioma. *Notch1* expression is correlated with glioma progression, and high protein expression of Notch1 is an independent predictor of poor survival in patients with glioma (75), reinforcing the central role of the Notch pathway in glioma.

Overactive Notch signaling has been found in a multitude of other cancers, such as head and neck squamous-cell carcinomas, medulloblastoma, colorectal cancer, pancreatic cancer and melanoma, which we will not detail (reviewed in ref. 76). Increased Notch signaling is most often correlated with increased malignancy or poorer overall survival. However, it should be noted that in some cancers, such as skin squamous-cell carcinoma, Notch signaling is correlated with differentiation and growth arrest (77). Nicolas *et al*. were the first to describe an increased incidence of skin cancer development in Notch1 knockout mice (77). This parallels the role of Notch in normal skin development, where Notch1 upregulates the cell cycle regulator p21, promotes cell cycle arrest in proliferating keratinocytes and helps to initiate terminal differentiation (37). It is not known if loss of Notch function correlates with prognosis in patients with squamous-cell carcinoma of the skin. The differential function of Notch signaling in different tissues and tumors that arise from them underlies the complexity of targeting Notch as a therapeutic strategy.

Notch as a mediator of tumor survival

The well-established correlation between increased Notch signaling and negative clinical outcomes across many cancer types can partly be explained by the function of direct Notch target genes as inducers of proliferation. There has been a recent emphasis on further elucidating the mechanisms responsible for the negative effects of dysregulated Notch signaling on patient survival. There is mounting evidence that the Notch pathway confers a survival advantage on tumors through maintaining CSCs, participating in epithelial–mesenchymal transition (EMT) and increasing chemoresistance. Below, we explore how Notch signaling drives these prosurvival mechanisms in cancer, summarized in Figure 2.

Notch: mediator of CSC survival

Notch signaling regulates cell fate decisions, maintains tissue stem cells and mediates self-renewal and repair in normal tissues after injury (34,35,78,79). Recent evidence has suggested that Notch signaling also regulates self-renewal and survival of CSCs, which are believed to be responsible for tumor initiation and repopulation after chemotherapy or radiation. For example, in a study by Gal *et al.*, CD34+/CD38− AML CSCs expressed significantly higher levels of the Notch ligand Jagged2 compared with the non-CSC CD34+/CD38+ population, and when treated with the GSI DAPT, colony formation ability of the CSCs was reduced (80). Recently, utilizing the Tal1/Lmo2 mouse model of T-ALL, Tatarek *et al*. found that GSI-mediated inhibition of Notch reduced or eliminated the leukemia CSCs and extended survival of animals (81). Dontu *et al*. highlighted the importance of Notch signaling in mammary stem cell fate determination and proposed that dysregulation of Notch signaling in normal mammary stem cells contributes to mammary carcinogenesis (82).

Other research has provided evidence that Notch plays a pivotal role in breast CSCs. Simmons *et al*. showed that constitutive Notch1 ICD expression increased the rate of tumorsphere formation in murine mammary tumor cells, a characteristic associated with CSCs. They identified the embryonic stem-cell marker Nanog homeobox as a direct Notch1 target in mammary tumor cells leading them to postulate that Notch1 mediates CSC features in breast cancer (83). A recent study demonstrated that MEL-18, a polycomb protein, abrogates breast CSC growth at least partly by preventing Notch signaling (84). Reduction of MEL-18 expression by shRNA led to an expansion of CSCs, as defined in this study as side population cells or cells that were ESA+/CD44+/CD24−, and increased colony formation *in vitro* and tumor initiation *in vivo*. MEL-18 blockade enhanced expression of the Notch ligand, Jagged1, leading to activation of the Notch pathway. Inhibition of Notch reduced the CSC population induced by MEL-18 knockdown (84). Together, these studies confirmed that the Notch pathway may be a key mediator of CSC maintenance in breast cancer. Confirmation of these studies by quantifying tumor initiation after modulating Notch signaling within the isolated breast CSC population is needed.

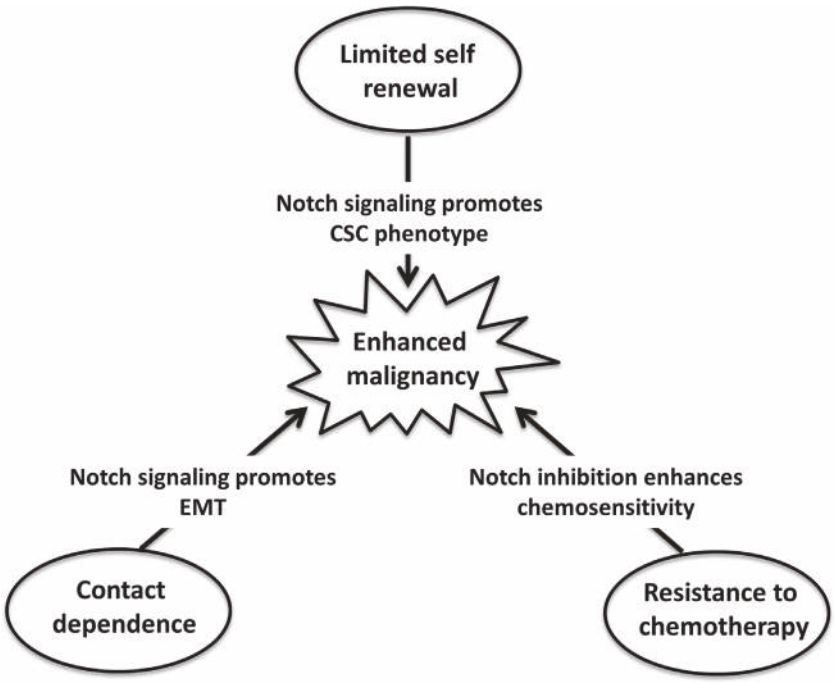

Fig. 2. Three major characteristics by which Notch promotes tumor survival: CSC character, resistance to chemotherapy and EMT. See text for detailed discussion.

There is also evidence that Notch4 participates in maintaining breast CSCs. A study on breast cancer indicated that putative breast CSCs with the ESA+/CD44+/CD24low phenotype had an 8-fold higher level of Notch4 signaling activity, as measured by protein levels of the activated form of Notch4 ICD (85). Inhibition of Notch by GSI treatment (DAPT, dibenzazepine or MK-0752), as well as knockdown of Notch4 expression, decreased the number of ESA+/CD44+/CD24low breast CSCs in adherent monolayers. When Numb, an endogenous Notch inhibitor, was overexpressed in the MDA-MB-231 breast cancer cell line, xenograft tumor initiation was abolished. Then, using doxycycline-inducible Notch1 and Notch4 shRNA-expressing cell lines, the group found that tumors grew *in vivo* at a slower rate in cells with Notch1 knockdown and did not grow with Notch4 knockdown (85). These data suggested that although Notch1 plays a role in breast CSCs, Notch4 is required for tumor initiation, supporting the evidence that Notch signaling contributes to CSC self-renewal.

Recent work has identified glioma CSCs (86,87) and focused on the role of Notch signaling in this population (88). Independent studies demonstrated that CSCs in glioma have enriched Notch signaling compared with bulk tumor cells (89,90). Fan *et al*. tested the functional effects of Notch signaling in glioblastoma multiforme (GBM) cell lines cultured as neurospheres and found that treatment of neurospheres *in vitro* by the GSI's GSI-18 or MRK-003 caused a reduction in clonogenic ability. Inhibition of Notch by GSIs also caused a dose-dependent decrease in CD133, nestin, BMI-1 and OLIG2 levels, which are putative CSC markers in glioma. The group then demonstrated that pretreatment of neurospheres with GSI-18 completely inhibited growth of tumor xenographs. To more closely mimic clinical GBM cases, the group orthotopically implanted glioblastoma neurospheres into mice and after tumor formation, intracranially implanted GSI-18. GSI treatment blocked radiologically detectable tumors at 6 weeks and prolonged survival of animals significantly (91). Other work has validated the importance of Notch signaling in glioblastoma and further defined the understanding of Notch interactions at a molecular level. Zhu *et al*. cocultured human brain microvascular endothelial cells with neurospheres and found that this increased CSC self-renewal and GBM tumor growth. Abrogation of Notch signaling by RNAi prevented CSC self-renewal and growth in both *in vitro* and *in vivo* models (92). A similar study using GBM three-dimensional "explant" cultures of primary GBM samples demonstrated that the loss of endothelial cells significantly reduced Notch signaling, decreasing neurosphere formation in a manner mimicking pharmacological Notch inhibition. Further, this study found that inhibition of Notch before radiation treatment substantially decreased proliferation and self-renewal of cells within tumor explants when compared with explants receiving radiation treatment alone (93). These findings support the role of Notch signaling in maintaining glioma tumor growth through maintenance of CSCs and underscore the importance of the tumor microenvironment. Similar studies have shown that Notch signaling is essential for the maintenance of the putative CSC population in medulloblastoma (94).

Evidence for Notch's role in CSC maintenance has also been shown in colon cancer (16,95), ovarian cancer (96), lung cancer (97–99), and hepatocellular carcinoma (100). Recent validation of the "CSC hypothesis" by three independent groups (101–103) using novel lineage tracing strategies demonstrated that one or several CSCs can give rise to an entire tumor or repopulate a tumor after treatment with chemotherapy or radiation. Due to the mounting evidence that Notch signaling enhances CSC self-renewal, these findings support the idea that targeting the Notch signaling pathway in conjunction with chemotherapy may limit disease recurrence and achieve lasting cancer control.

Notch: mediator of EMT transition

EMT is a process by which epithelial cells adopt a mesenchymal phenotype, allowing them to lose intercellular adhesion and migrate to new locations (104). In cancer, EMT is proposed to be responsible for metastatic disease, where tumor cells lose adhesion to each other, migrate to different sites and establish disease in distant organs (104). The Notch pathway is a key participant in EMT. For example, in renal tubules, Notch1 overexpression resulted in increased expression of snail homolog 1, a transcription factor that induces EMT, and reduced expression of the epithelial marker, E-cadherin. Inhibition of Notch with the GSI DAPT attenuated transforming growth factor-β-induced EMT (105). In samples from cancer of unknown primary syndrome patients, Notch2 and Notch3 expressions were positively correlated with expression of snail homolog 1 (106). Mechanistic studies in pancreatic cancer demonstrated that overexpression of Notch1 caused migration and invasion, a hallmark of EMT (107). In lung cancer cells, gefitinib-resistant

cells exhibited an EMT phenotype and had significantly increased Notch1 expression compared with parental cells. Knocking down Notch1 reversed their EMT phenotype (108). When Notch1 was overexpressed in the gefitinib-sensitive parental cells, they acquired an EMT phenotype similar to the gefitinib-resistant cells (108), providing strong evidence that Notch1 regulates EMT in lung cancer. Many of the above studies point out the overlapping features of CSCs and cancer cells that have undergone EMT. Strikingly, Notch appears to be a key mediator of both, as well as a mediator of chemoresistance, described below.

Notch: mediator of chemotherapeutic resistance

There has been increasing evidence that the Notch pathway contributes to therapy resistance. A recent study of ovarian cancer demonstrated that overexpression of Notch3 increased resistance to platinum-based chemotherapy and increased the CSC population within tumors (109). Further, use of a GSI, called GSI1, sensitized the cells to cisplatin. A combination of cisplatin and GSI1 had a synergistic effect on cytotoxicity (109), suggesting that inhibiting the Notch pathway sensitizes cancer cells to chemotherapy. A similar study in prostate cancer demonstrated that knocking down expression of Notch1 sensitized the cells to treatment with docetaxel (110). Similar results were found in colon cancer, where chemotherapy with oxaliplatin induced Notch1 signaling, and GSI treatment enhanced chemosensitivity of cancer cells to oxaliplatin (111).

Recent work in glioma demonstrated that the combination of temozolamide and the GSI DAPT similarly sensitized cells. DAPT and temozolamide together decreased neurosphere formation *in vitro* at a similar rate as temozolamide alone, but in the posttreatment follow-up period, the number of neurospheres in the temozolamide-only group increased, whereas there was no recovery in the group treated with both DAPT and temozolamide. Further, the group showed that treatment of tumor xenographs with temozolamide and the GSI LY-411575 extended tumor latency and blocked tumor progression in 50% of mice with preexisting tumors (112). Additional work in glioma demonstrated that Notch promotes not only chemoresistance but also radioresistance. Similar to findings by Hovinga *et al.* (93), Wang *et al*. demonstrated that pretreatment of cells with a GSI to inhibit the Notch pathway sensitized cells to radiotherapy at clinically relevant doses. Furthermore, overexpression of the activated intracellular domain of either Notch1 or Notch2 protected the CSC population against radiation, indicating that Notch signaling is critical to CSC resistance in the face of radiotherapeutic and chemotherapeutic assaults (113). Combined, these studies provide a strong basis for the use of GSIs in combination with traditional therapy options.

The future of Notch as a therapeutic target

Gamma secretase inhibitors

The potential use of Notch inhibitors in conjunction with chemotherapy is an active area of research, incited by the multitude of studies showing that components of the Notch pathway are overexpressed across many cancer types and that Notch signaling enhances prosurvival characteristics. All GSIs that attenuate Notch signaling do so by inhibiting Notch activation, despite their diverse chemical structures (Figure 3) (114–117). Preclinical cancer models have clearly demonstrated that GSIs suppress pancreatic, breast and lung cancer growth (Table III) (114,118,119); however, GSI treatment *in vivo* is associated with significant side effects. In mice, chronic administration of the GSI LY-411575 drastically altered intestinal architecture, increased goblet cell number and mucin secretion, led to epithelial erosion, caused inflammatory cell infiltration into the lamina propria and led to abnormal changes in the thymus and spleen. All of these side effects are consistent with the role of Notch in these organs but are cause for concern in using GSIs in the clinic (115). Furthermore, although intense focus has been on the role of gamma secretase in pathological conditions, it is important to note that this complex is involved in the cleavage of a multitude of proteins (120,121). As such, GSIs are fundamentally non-specific and therefore additional drugs must be developed that more specifically target Notch signaling.

Clinicaltrials.gov lists over 40 clinical trials using GSIs that are in progress or recently completed for T-ALL, breast, colorectal, lung, prostate and pancreatic cancers, with GSI treatment alone or in combination with other drugs (122,123). The majority of the trials are either at a stage where it is too early to know the side effects or have been completed but the side effects have not yet been reported. In one completed Phase 2 clinical trial using GSI Ro4929097 for metastatic colon cancer patients who had received at least two prior lines of systemic chemotherapy, none of the 33 patients experienced a radiographic response, and the median progression-free survival was only 1.8 months, demonstrating that the use of Ro4929097 as a single agent is not effective (124). Some adverse events reflected the toxicities seen in mice. Nine percent of participants reported nausea and 6% reported vomiting specifically related to drug treatment (124). A Phase 1 trial of MK-0752 for adult patients with advanced solid tumors demonstrated that high dosage once per week was more tolerable than a daily dose schedule and demonstrated clinical benefit in about 10% of patients, as measured by stable disease for greater than 4 months (125). As more ongoing trials publish results, the efficacy of GSIs and their side effects will be better quantified. Recent studies have assessed the combined effects of GSIs with glucocorticoids as a means of limiting side effects. Samon *et al*. found that not only did combination treatment have a synergistic antileukemic effect in both cell lines and patient samples but also that *in vivo* the use of glucocorticoids reversed gastrointestinal toxicity by inhibiting goblet cell metaplasia (126). This raises the intriguing possibility that GSIs may be successfully used as adjuvant chemotherapy in combination with glucocorticoids as a means of successfully eradicating the CSC population. In fact, one ongoing clinical trial is currently in progress assessing the safety of GSI Ro4929097 with and without dexamethasone (127), and the results may pave the way for further efficacy studies and ultimately clinical use of this drug combination.

Alternatives to GSI-based anti-Notch therapeutics

GSI toxicities spurred the development of more specific Notch pathway inhibitors. A recent strategy has been the development of specific Notch ligand- or receptor-targeting antibodies (Table III). This has been a complex process because of the striking similarities among all four Notch receptors. A recent study combined phage display and X-ray crystallography to identify the unique structural differences between the negative regulatory regions of Notch1 and Notch2. Utilizing antibodies that selectively inhibited each of the Notch receptors, it was found that Notch1 is specifically responsible for T-ALL growth. Further, inhibition of Notch1 or Notch2 alone did not lead to the weight loss previously associated with GSI treatment-induced gastrointestinal toxicity (128). Thus, specific Notch receptor targeting holds promise for inhibiting Notch signaling without the side effects of GSI treatment, although long-term studies of efficacy and side effect profiles are needed. Using phage display, Falk *et al*. generated Notch1- and Notch2-specific antibodies and used them to block both Notch receptors simultaneously. Blocking both Notch1 and Notch2 led normal neural stem cells to differentiate, causing cells to adopt a neuronal cell fate (129). This finding invokes the intriguing possibility that Notch-receptor-specific antibodies could be used therapeutically to induce CSC differentiation, rendering cells unable to self-renew and repopulate a tumor, similar to the function of all-*trans* retinoic-acid for acute promyelocytic leukemia.

Another recent strategy has been the development of antibodies targeting specific Notch ligands. The results of multiple studies have together led to the conclusion that blocking DLL-4 leads to an increase in non-functional vessel formation (reviewed in ref. 130). DLL-4 is a target of vascular endothelial growth factor and acts as a negative regulator of vessel formation; therefore, blocking DLL-4 results in unrestrained vessel formation. However, vessels formed due to DLL-4 blockade are non-functional and do not provide the oxygen and nutrients needed for tumor growth, indicating that DLL-4 blockade may be an effective anticancer strategy (131). A recent DLL-4 targeting antibody named MEDI0639 was shown to inhibit the DLL-4-Notch1 interaction. Although *in vitro* studies on the effects of the DLL-4 antibody were equivocal, *in vivo* studies demonstrated that MEDI0639 treatment

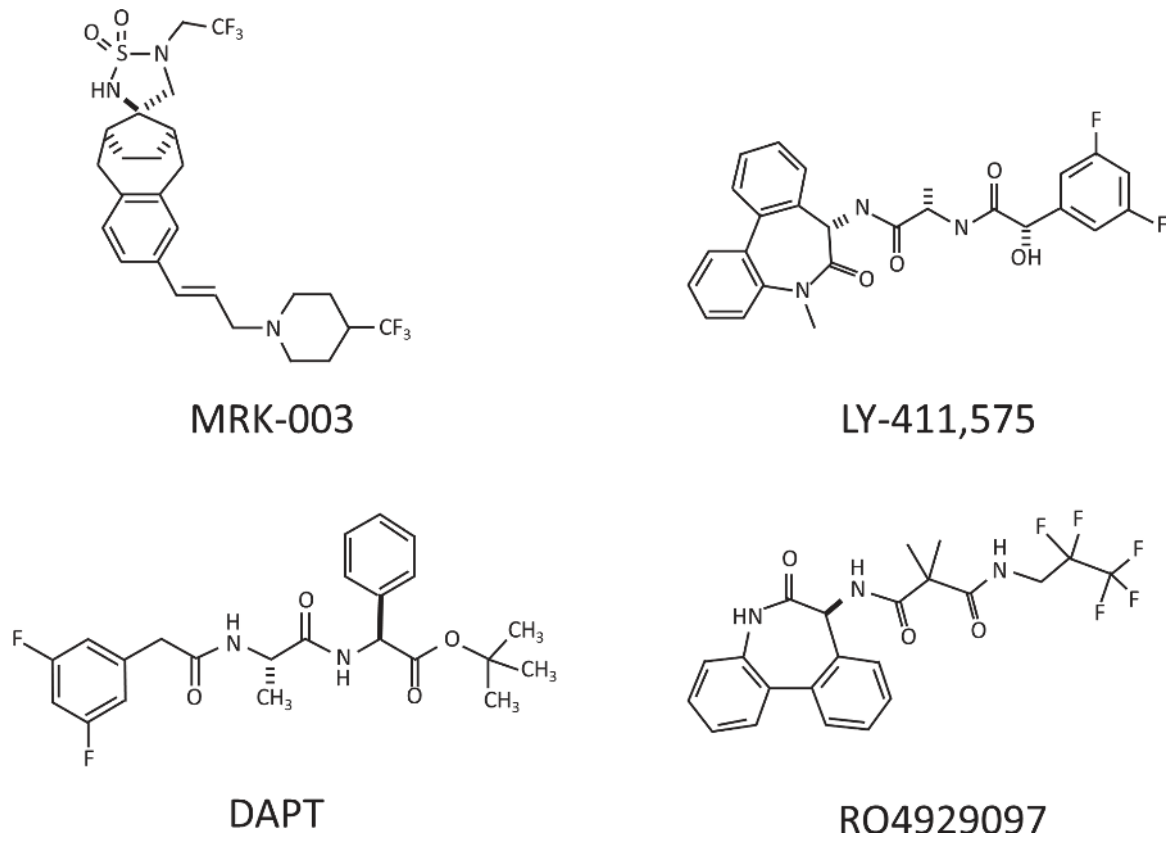

Fig. 3. Common GSIs used in experimental studies and clinical trials: MRK-003, LY-411,575, DAPT and Ro4929097.

led to increased vessel sprouting, but the vessels were not coated with smooth-muscle mural cells, supporting that DLL-4 blockade induced non-functional vessel formation (132). A Phase 1 trial of MEDI0639 is currently recruiting participants to test the safety, tolerability and pharmacokinetics of the antibody for advanced solid tumors (133). Other DLL-4-targeting research efforts assessed the effect of DLL-4 blockade in combination with ionizing radiation. Mice with tumor xenografts were treated with the GSI dibenzazepine alone, dibenzazepine in conjunction with radiation, anti-DLL-4 blocking antibody alone or anti-DLL-4 blocking antibody in conjunction with radiation. Tumor growth was dramatically reduced in the animals treated with an anti-DLL-4 antibody and radiation, compared with either treatment alone. Tumor reduction was attributed to increased vessel density but reduced tumor blood flow, as expected, resulting in extensive tumor necrosis (134).

Antibody-based targeting is a relatively young field and questions remain regarding the efficacy of this strategy. It is unclear if anti-DLL-4 antibodies alone would be clinically effective due to the redundancy of Notch ligands and Notch receptors, and if this limitation could be overcome by using multiple antibodies. Multiple antibodies could recapitulate side effects seen with GSI treatment. A recent study of anti-DLL-4 antibody treatment resulted in significant pathological changes in the livers of rats and the formation of vascular neoplasms (135), raising concerns for patients following this treatment regimen. Furthermore, activation of Notch signaling with a chimeric DLL-4 protein led to inhibition of megakaryocytic differentiation, suggesting that if the Notch pathway is inhibited, there could be significant side effects on hematopoiesis (136). Clearly, Notch inhibition-based strategies hold great promise for cancer therapy and also carry potential risk. Future studies aimed at refining our understanding of individual Notch ligands and receptors will aid in the development of safe and effective therapies targeting the Notch pathway.

Investigators have also analyzed the effect of curcumin, a known natural inhibitor of Notch and NF-κB signaling ((137), reviewed in ref. 138) in conjunction with other chemotherapies. Meriva, a curcumin formulation with enhanced bioavailability, overcame oxaliplatin-induced chemoresistance in colon cancer cells *in vitro* and increased oxaliplatin efficacy *in vivo* (139). Curcumin also limits osteosarcoma growth and invasion *in vitro*, and causes cell cycle arrest through inhibition of Notch1 signaling (140). Although curcumin does not selectively inhibit Notch, it may still prove to be a useful compound for Notch inhibition.

Conclusion

Almost 100 years of research has illuminated our understanding of the mechanisms of Notch signaling and how this pathway contributes to normal development, tissue homeostasis and pathophysiological disease processes. Notch signaling in cancer has become a hot area of research as a multitude of studies has shown that Notch contributes to tumor cell proliferation, maintenance of CSCs, EMT and chemoresistance. This spurred investigation into Notch-modulating strategies for use as adjuvant chemotherapy. However, numerous unanswered questions remain. One research focus still in its infancy is that of the downstream targets of Notch signaling that mediate prosurvival characteristics. It is critical to comprehensively and exhaustively define Notch target genes in each cancer so that drugs can be tailored against these effectors to abrogate downstream Notch signaling in cancer cells. Other future studies in this field will be aimed at developing our understanding of the effects of the tumor microenvironment on Notch signaling. To successfully target the Notch signaling pathway as part of a multidrug anticancer strategy, it will essential to fully characterize the microenvironmental factors that modulate Notch signaling. A closely related area for future study is the emerging investigation into molecular biomarkers and cell surface markers that correlate with Notch signaling. As we embark in the era of personalized medicine, identification of a specific and limited patient population that would benefit from therapy targeting the Notch pathway would be a great advance so that patients for whom Notch-inhibiting therapies would be beneficial will be treated, and others will be spared the side effects of an

Table III. Proven effects of Notch-targeting therapies

Treatment	Tissue	Effect	Reference
GSIs			
DAPT	AML-derived stem cells	Inhibition of colony formation	80
GSI, unspecified	Tal1/Lmo2 mouse model of T-ALL	Reduced or eliminated CSCs; increased survival of animals	81
DAPT	Breast cancer cell lines	Reduced number of putative breast CSCs	85
MRK-003, GSI-18	Glioma cell line neurospheres	Reduced clonogenicity; dose-dependent reduction in CSC markers; prolonged survival; reduced tumor-forming potential	91
DAPT	Primary GBM explant cultures	Combination of Notch inhibition and radiation significantly decreased proliferation and self-renewal in tumor explants compared with radiation treatment alone	93
GSI-18	Medulloblastoma	Reduced putative CSC population; decreased proliferation and increased differentiation; induced apoptosis in nestin-positive cells; inhibited engraftment *in vivo*	94
GSI1	Ovarian cancer cell lines	Sensitized cells to cisplatin treatment	109
GSI34	Colon cancer cell lines	Sensitized cells to chemotherapy; synergistic with oxaliplatin, 5-FU and SN-38	111
DAPT	Glioma cell lines	Combination with TMZ prevented recovery of neurospheres compared with recovery in posttreatment period with TMZ treatment alone	112
LY411,575	Glioma xenograph in mice	In combination with TMZ-blocked tumor progression in 50% of mice	112
DAPT, L685,458	Glioma surgical specimen CSCs	Rendered glioma CSCs more sensitive to radiation, enhanced radiation-induced cell death and impaired clonogenic survival	113
MRK-003	Pancreatic ductal adenocarcinoma cell lines and patient-derived xenographs	Inhibition of anchorage-independent growth; reduced number of CSCs; pretreatment of xenograph inhibited engraftment in mice; blocked xenograph growth in 56% of mice	114
MRK-003	Mouse model of ERBB2 breast cancer known to have a high CSC frequency	Inhibited survival of tumorsphere-derived cells *in vitro*; reduced or eliminated CSCs as assessed by failure of serial transplantation; administration to tumor-bearing mice resulted in rapid and lasting tumor regression	118
LSN-411575	KrasG12V-driven NSCLC	Growth inhibition of autochthonous murine tumors; higher frequency of apoptosis in drug-treated versus vehicle-treated cells; mice treated with GSI showed complete blockade of cancer growth	119
Antibodies targeting Notch pathway proteins			
Specific anti-Notch1 antibody	Xenograph lung cancer and melanoma cell lines	Inhibition of tumor growth through inhibition of cancer cell growth without GI toxicity	128
Combination of specific anti-Notch1, anti-Notch-2 and anti-Notch-3 antibodies	Human neuroepithelial stem cells	Induced differentiation of neural stem cells into neuronal cells	129
Anti-DLL-4 antibody (unspecified)	Human umbilical vein endothelial cells alone and cocultured with glioma cells, DLL-4 reporter mice with xenograph tumors	Blocked tumor growth by promoting non-productive angiogenesis	131
Anti-DLL-4 antibody MEDI0639	Human umbilical vein endothelial cells	Reduced number of functional vessels as measured by number of actin-positive smooth-muscle mural cells	132
Anti-DLL-4 antibody (unspecified)	DLL-4+ and DLL-4– tumor xenographs	Fourfold reduction in tumor growth rate when treated with antibody and ionizing radiation in comparison with GSI dibenzazepine and ionizing radiation	134

ineffective drug. Currently, many groups are working to refine both our understanding of the mechanisms by which the Notch pathway increases the malignant phenotype and our ability to regulate Notch signaling in order to develop novel therapeutic strategies targeting this pathway.

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