Inhibition of the Notch-Hey1 Axis Blocks Embryonal Rhabdomyosarcoma Tumorigenesis

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Abstract

Purpose: Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood and remains refractory to combined-modality therapy in patients with high risk disease. In skeletal myogenesis, Notch signaling prevents muscle differentiation and promotes proliferation of satellite cell progeny. Given its physiologic role in myogenesis and oncogenic role in other human cancers, we hypothesized that aberrant Notch signaling may contribute to RMS tumorigenesis and present novel therapeutic opportunities.

Experimental Design: Human RMS cell lines and tumors were evaluated by immunoblot, IHC, and RT-PCR to measure Notch ligand, receptor, and target gene expression. Manipulation of Notch signaling was accomplished using genetic and pharmacologic approaches. In vitro cell growth, proliferation, and differentiation were assessed using colorimetric MTT and BrdU assays, and biochemical/morphologic changes after incubation in differentiation-promoting media, respectively. In vivo tumorigenesis was assessed using xenograft formation in SCID/beige mice.

Results: Notch signaling is upregulated in human RMS cell lines and tumors compared with primary skeletal muscle, especially in the embryonal (eRMS) subtype. Inhibition of Notch signaling using Notch1 RNAi or γ-secretase inhibitors reduced eRMS cell proliferation in vitro. Hey1 RNAi phenocopied Notch1 loss and permitted modest myogenic differentiation, while overexpression of an activated Notch moiety, ICN1, promoted eRMS cell proliferation and rescued pharmacologic inhibition. Finally, Notch inhibition using RNAi or γ-secretase inhibitors blocked tumorigenesis in vivo.

Conclusions: Aberrant Notch-Hey1 signaling contributes to eRMS by impeding differentiation and promoting proliferation. The efficacy of Notch pathway inhibition in vivo supports the development of Notch-Hey1 axis inhibitors in the treatment of eRMS. Clin Cancer Res; 17(23); 1–10 ©2011 AACR.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma of childhood with approximately 350 new cases diagnosed each year in the United States (1). There are 2 major histologic subtypes of RMS (embryonal, eRMS and alveolar, aRMS) with each subtype having distinct underlying genetic alterations that participate in pathogenesis. Although the precise etiology of RMS remains uncertain, it is commonly believed that tumor cells arise from muscle precursors that fail to differentiate appropriately into skeletal muscle. Current therapy for RMS includes surgery, chemotherapy, and radiation, but despite this aggressive combined-modality approach, patients with metastatic disease have an overall survival rate of less than 20% (2). Improved therapies for RMS may come from targeting signaling pathways that are aberrantly regulated and as a consequence contribute to tumor formation.

The Notch signaling pathway is highly conserved and plays a key role in a variety of important cell fate decisions, including proliferation, differentiation, and apoptosis. Notch signaling is involved in many developmental systems, including but not limited to neurogenesis, angiogenesis, hematopoiesis, and myogenesis (3). Notch signaling members include multiple transmembrane receptors and ligands that function through direct cell surface contact. Mammals possess 4 Notch receptors (Notch1, Notch2, Notch3, Notch4) and 5 Notch ligands (Jagged1, Jagged2, Dll1, Dll3, Dll4; ref. 4). Signaling is initiated when neighboring cells come into direct contact, facilitating ligand-receptor interaction. Activation of Notch receptors by ligand binding is followed by proteolytic cleavage by ADAM10 and γ-secretase, which releases the cytoplasmic component of Notch (intracellular Notch, ICN). ICN then translocates to the nucleus where it cooperates with CSL and Mastermind to form a transcriptional activation complex that promotes expression of target genes involved in cell proliferation.
Translational Relevance
Outcomes for children and adolescents with high risk rhabdomyosarcoma (RMS) remain dismal despite the evaluation of new chemotherapy regimens in controlled clinical trials. To improve survival, the underlying mechanisms of sarcomagenesis must be identified and targeted therapies developed to supplement current cytotoxic regimens. The Notch signaling pathway is critical for skeletal muscle self-renewal and contributes to tumor maintenance in many types of cancer. Our work shows that the Notch-Hey1 axis signaling pathway is aberrantly upregulated in embryonal RMS (eRMS) and that inhibition of this pathway blocks eRMS tumorigenesis through permission of early differentiation and suppression of proliferation. Decreasing the regenerative potential of eRMS cells by inhibition of Notch-Hey1 signaling may offer a novel therapeutic strategy in the treatment of this cancer.

differentiation, and apoptosis. Many targets of Notch signaling have been identified, with the most widely studied being members of the basic helix loop helix (bHLH) family of transcription factors including Hes (Hairy/enhancer of split) and Hey (Hairy/enhancer-of-split related with YRPW motif). Hes and Hey expression varies according to tissue type, and probably causes tissue-specific repression of target genes (5).

In skeletal myogenesis, activation of the Notch signaling pathway has been shown to inhibit the progression of muscle stem cells to committed progenitors and promote the proliferation of progenitors prior to terminal differentiation into myotubes (6). These muscle progenitor cells, called satellite cells, retain the ability to proliferate and differentiate in response to stimuli such as tissue damage and growth signals. By inhibiting muscle differentiation and promoting the proliferation of satellite cell progeny, the Notch signaling pathway functions to preserve and expand this subpopulation of muscle stem cells (7).

Aberrant Notch signaling has been implicated in a variety of pediatric cancers including T-cell leukemia and lymphoma, osteosarcoma, glioma, and medulloblastomas (8), with evidence of overexpression of multiple Notch signaling pathway ligands, receptors, and target genes in a number of malignancies (9). In contrast, the Notch signaling pathway functions as a tumor suppressor in some type of malignancies including B-cell leukemia (3). The role of Notch signaling in tumorigenesis likely varies in different cell types and depends in part on tissue contexts (3, 10).

The oncogenic role of Notch in tumorigenesis can be targeted by inhibiting the proteolytic cleavage of the Notch receptors using small molecule inhibition. γ-Secretase is a large protease complex that releases the intracellular domain of the Notch receptor following ligand-receptor activation. This step in Notch signaling is pivotal in the activation of this signaling cascade, and over the past decade, small molecule inhibitors of γ-secretase have been developed and studied for their potential to inhibit Notch signaling. A phase I/II study evaluating the efficacy of Notch inhibitors in children with relapsed or refractory T-cell leukemia, lymphoma, CNS or solid tumors, recently opened (clinicaltrials.gov, NCI NCT01088763).

Given the critical role of Notch signaling in skeletal myogenesis and the oncogenic role of Notch signaling in other cancers, we hypothesized that aberrant regulation of the Notch pathway may present novel therapeutic targets in the treatment of RMS. On the basis of prior observations that the Notch target Hes1 was active in aRMS (11), and that the Notch pathway was important for RMS cell invasion in vitro (12), we turned our attention to investigating the role of Notch signaling in eRMS tumorigenesis, with focus on the Notch target Hey1, and the possibility that Notch pathway inhibition might have efficacy in blocking eRMS tumorigenesis in vivo.

Materials and Methods

Generation of cell lines and constructs
Early passage normal human skeletal muscle myoblasts (HSMM cells, Lonza) were grown in defined media (SkGM-2 Bullet Kit). Human RMS cell lines (RD, SMS-CTR, Rh28, and Rh30) were a gift from Dr. Tim Triche of The Children’s Hospital of Los Angeles and were grown in RPMI-1640 (GIBCO) with 10% FBS. Cell line authentication was confirmed using STR analysis (Promega PowerPlex 1.2) with the assistance of the Fragment Analysis Facility at Johns Hopkins Genetic Resources Core Facility (Supplementary Table S1). Using 9 STR markers, we found 100% identity of our RD cell line compared with a published STR profile (13) and the American Type Culture Collection (ATCC) database and 88.9% identity of our Rh30 cell line compared with published STR profiles (13). There are no published STR profiles for the SMS-CTR and Rh28 cell lines; however, these cell lines had unique profiles when compared with the ATCC comprehensive database. The NOTCH1 shRNA sequence was a gift from Dr. Chris Counter (Duke University), and annealed shRNA oligos (Supplementary Table S2) were ligated into the pSUPER-retro-GFP/neo plasmids. RD and SMS-CTR cells were stably infected with NOTCH1 shRNA or empty vector and selected with 400 μg/mL neomycin (Gibco). HEY1 shRNA sequences (Open Biosystems, Supplementary Table S2) were ligated into the pLKO.1/puro (Addgene 8453) plasmids. RD and SMS-CTR cells were stably infected with HEY1 shRNA or empty vector and selected with 1 μg/mL puromycin (Sigma). ICN1 overexpression virus was a gift from Jordan Blum (Duke University). RD and SMS-CTR cells were stably infected with ICN1 or empty vector and confirmed by YFP expression.

Drug treatments
For in vitro work, GSI-X (Calbiochem) was diluted in dimethyl sulfoxide (DMSO) and added to culture media at desired concentrations. Cells were treated with GSI-X or
equal volumes of DMSO for vehicle control. For in vivo work, GSI-XII (Calbiochem) was suspended in DMSO at 5 mg/mL concentration. Mice were treated with GSI-XII versus DMSO control via intraperitoneal injection at a dose of 5 mg/kg daily for 21 days.

**Immunoblotting**

Cells were lysed in Tris/RIPA buffer with standard protease inhibitors and passed through a 21 g needle to shear DNA. Protein concentration was measured by the DC assay (Bio-Rad). A range of 60 to 100 µg of lysate was resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane and immunoblotted with primary monoclonal antibodies anti-NOTCH1 (Santa Cruz), anti-HEY1 (Abcam), or actin SC-8462 (Santa Cruz). Membranes were reacted with a secondary HRP-labeled goat anti-donkey (Santa Cruz), goat anti-rabbit antibody (Invitrogen-Zymed), or goat anti-mouse antibody (Invitrogen-Zymed) and developed using chemiluminescence (Amersham).

**MTT assays**

The MTT assay is a surrogate measure for cell number based on quantitation of the conversion of yellow methylthiazolyldiphenyl-tetrazolium bromide to purple formazan by the mitochondrial enzyme succinate dehydrogenase. In this work, the MTT assay was used to measure cell growth after genetic or pharmacologic interventions (14). For genetic interventions, cells were stably infected, selected based on antibiotic resistance or FFP positivity, then plated in 96-well flat-bottomed plates at specific cell densities (5,000 cells/well for RD cells and 7,500 cells/well for SM5-CTR cells) and cultured as described above. For pharmacologic interventions, cells were plated at specific cell densities. On day 0, the media was replaced with fresh media supplemented with drug versus vehicle control. Cells were treated for a total of 48 hours with fresh drug replaced every 24 hours. At indicated time points, the culture media was supplemented with 1 mg/mL MTT for 3 hours at 37°C, media removed, cells solubilized with DMSO, and absorbance measured at 540 nm.

**BrdU incorporation**

Cells were grown at the described densities in 96-well plates for 24 hours, then proliferation measured using the Cell Proliferation ELISA BrdU kit (Roche). Cells were labeled with BrdU for 3 hours at 37°C and assayed according to the manufacturer’s protocol. Absorbance was measured at 370 nm.

**Differentiation assays**

To assess their ability to acquire biochemical and morphologic markers of skeletal muscle differentiation, eRMS cells were cultured in DMEM-F12 supplemented with 2% horse serum (“fusion” medium) for 72 hours. Control cells were cultured in standard RPMI-1640 with 10% FBS (“growth” medium). Exposure to fusion medium, so-called because of its ability to induce individual myoblasts to fuse membranes with neighboring cells and generate elongated multinucleate myotubes indicative of terminal differentiation, is an established method used previously by our laboratory (15) to assess the ability of cells in culture to differentiate down the myogenic lineage. Biochemical and morphologic evidence of differentiation was assessed using myogenin expression by PCR, and imaging by phase contrast microscopy using a Nikon DS-Fi1 camera, respectively.

**RT-PCR**

Total cellular RNA was isolated using the RNA-Bee kit (TEL-TEST). Following spectrophotometric quantitation, 2 µg was subject to reverse transcription using the Omniscript RT kit (QIAGEN) with Oligo-dT primers (Life Technologies Invitrogen). Standard PCR using primer sets for NOTCH1-4, DLL1, 3, and 4, JAGGED1, JAGGED2, HES1, HEY1, myogenin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Supplementary Table S3; ref. 16) was done, with product separated on 2% agarose. GAPDH and water controls were included to verify equal RNA and specificity of cDNA input, respectively.

**Tumor xenografts**

Under institutional IACUC-approved protocols, and as done (17), 10 million cells/cell line were resuspended in Matrigel (BD Biosciences) and injected subcutaneously into the flanks of SCID/beige mice in quadruplicate for genetic knockdown studies or in replicates of 6 for γ-secretase inhibitor studies. In drug studies, mice were treated with GSI-XII (or DMSO vehicle) via intraperitoneal injection for 21 days as previously reported, with drug suspended in DMSO to increase solubility (9). Mice were monitored biweekly, and tumor volume was estimated by external caliper measurements and calculated as [(width)² × length]/2. Mice were sacrificed at time points described in the text, at maximal tumor burden, or if showing a decline in health. Following sacrifice, tumors were dissected, weighed, and portions of the tumors were snap-frozen in liquid N₂ for RNA and protein evaluation or fixed in formalin for histologic evaluation.

**Immunohistochemistry**

Human RMS tissue microarrays were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute; other investigators may have received specimens from the same subjects. Staining for Notch1 (Santa Cruz) was done with the assistance of the University of Florida Department of Pathology, Immunology, and Lab Medicine Pathology Core. The tissue array was examined and scored by 2 independent observers who were blinded to the annotated histologic subtype. For each tissue core, positivity for Notch1 was determined on the basis of discrete nuclear staining and a semiquantitative score of the percentage of positive cells (0 = no nuclear staining, 1 = <25% staining, 2 = 25-50% staining, 3 = >50% staining). Paraffin-embedded tumor xenografts were sectioned and stained with H&E to assess tumor morphology. Ki67 (Thermo Scientific) to assess for proliferation, and HEY1 (Abcam) to assess inhibition of Notch signaling. A
pathologist with experience in the evaluation of pediatric sarcomas (R.C.B) evaluated slides.

Statistical analysis

Data are presented as means and SEs of the mean. Comparisons between groups were done using t tests and 2-way ANOVA. Differences were considered statistically significant at the *, P < 0.05; **, P < 0.01; and ***, P < 0.001 levels.

Results

The Notch-Hey1 axis pathway is upregulated in human eRMS cell lines and tumor samples

To determine whether the Notch signaling pathway is upregulated in human RMS, we first analyzed 4 human RMS cell lines and examined a previously published cohort of human RMS tumors for expression of Notch pathway receptors, ligands, and target genes. RT-PCR analysis of RNA extracted from 2 human eRMS (RD and SMS-CTR) and 2 human aRMS cell lines (Rh28 and Rh30) compared with nontransformed primary human skeletal muscle myoblasts (HSMMs) and in silico analysis of microarray expression data of primary human RMS tumors from a publicly available database (18) showed upregulation of multiple Notch signaling members (Supplementary Fig. S1). Although the upregulation of many pathway members suggested that Notch signaling is likely important for both RMS subtypes, most intriguing was that the Notch target HEY1, a transcriptional repressor downstream of total cellular Notch signaling and a critical inhibitor of myogenesis, was upregulated in eRMS compared with aRMS cells. Thus in this work, we focus on the eRMS subtype, with emphasis on the Notch-Hey1 axis. First, we verified by semiquantitative RT-PCR and immunoblot that HEY1 mRNA and protein are upregulated in human eRMS cell lines compared with nontransformed HSMMs and aRMS cell lines (Fig. 1A). Second, in silico analysis of microarray expression data of primary human RMS tumors from a publicly available database (18) showed that relative HEY1 mRNA expression was higher in eRMS compared with aRMS tumors (Fig. 1B). Furthermore, immunohistochemical analysis of nuclear Notch1 staining of a human RMS tumor tissue microarray which included 14 human skeletal muscle controls, 42 eRMS tumors, and 93 aRMS tumors. Also shown are representative sections of Notch1 staining from each of the skeletal muscle, eRMS, and aRMS subgroups, magnification 40×. See Materials and Methods for explanation of statistical analyses.

Figure 1. Notch signaling is upregulated in eRMS cell lines and human tumors. A, semiquantitative PCR (top) and immunoblot (bottom) analysis of the Notch target gene Hey1 in human skeletal muscle myoblasts (HSMMs) in 2 eRMS cell lines and 2 aRMS cell lines, compared with GAPDH and Actin controls, respectively. B, relative Hey1 mRNA expression in human RMS samples as mined in silico from (18). C, immunohistochemical analysis of nuclear Notch1 staining of a human RMS tumor tissue microarray which included 14 human skeletal muscle controls, 42 eRMS tumors, and 93 aRMS tumors. Also shown are representative sections of Notch1 staining from each of the skeletal muscle, eRMS, and aRMS subgroups, magnification 40×. See Materials and Methods for explanation of statistical analyses.
expression is low in fusion-positive aRMS, but high in eRMS (Fig. 1B). Finally, an RMS tissue microarray containing human eRMS, aRMS, and normal skeletal muscle cores was probed for activated Notch1 protein expression, using standard immunohistochemistry, as a surrogate for Hey1 protein expression. As expected in tissues of skeletal muscle origin (19), there was diffuse membrane and cytoplasmic staining of Notch1 in all samples. However, nuclear Notch1 staining, which represents the transcriptionally active form of Notch1, while absent in skeletal muscle and moderately expressed in aRMS, was highly expressed in eRMS (Fig. 1C). Taken together, these data suggest that the Notch pathway is preferentially upregulated in human eRMS cell lines and tumor tissue, and that the Notch effector Hey1 may be playing a vital role in eRMS tumorigenesis.

Genetic suppression of Notch signaling blocks eRMS cell growth in vitro

Given the critical role for Notch signaling in skeletal myogenesis, and the prevalence of the Notch target Hey1 in eRMS cell lines and tissue, we next investigated whether genetic suppression of this pathway would block eRMS tumorigenesis. To this end, we stably expressed an shRNA to NOTCH1 in RD and SMS-CTR eRMS cells. Using immunoblot and semiquantitative RT-PCR, we confirmed knockdown of Notch1 and also concomitant knockdown of Hey1 (Fig. 2A). Then, using a standard MTT assay, we showed that compared with empty vector controls, suppression of Notch1 in both cell lines inhibited cell growth over time (Fig. 2B). Because Hey1 is downstream of Notch1, we predicted that its knockdown would phenocopy Notch1 loss. To this end, we generated an shRNA to HEY1 and showed knockdown of Hey1 at both the protein and mRNA levels in RD and SMS-CTR eRMS cells (Fig. 2C). Following this, we measured cell growth over time and noted an inhibition of eRMS cell growth in cells expressing the HEY1 shRNA (Fig. 2D and Supplementary Fig. S2).

To gain insight into the mechanism of eRMS cell growth inhibition in response to Notch pathway suppression, we investigated 2 cellular processes that are predicted to be supported by Notch signaling and also contribute to tumorigenesis: prevention of differentiation and promotion of proliferation. For the former, we cultured eRMS cells under conditions (“fusion” media; see Materials and Methods) that would stimulate differentiation down the myogenic lineage, then assessed for biochemical and morphologic evidence of differentiation. Given the Notch pathway’s role in impeding skeletal muscle differentiation, we predicted that Hey1 knockdown would at minimum induce expression of promyogenic skeletal muscle transcription factors such as myogenin, and at most induce multinucleate myotube formation indicative of terminal differentiation. We found that compared with cells with an empty vector, eRMS cells with an shRNA to HEY1 indeed showed increased myogenin, which was enhanced in fusion media (Fig. 2E). Hey1 knockdown also caused a change in cellular morphology from round to spindle-shaped, with intercellular protrusions reminiscent of myotubes, also most obvious in fusion media (Fig. 2F). However, Hey1 knockdown did not result in a homogenous population of multinucleate myotubes, indicating that under these conditions, Hey1 loss was not sufficient to induce terminal differentiation.

To investigate the effect of Notch pathway inhibition on proliferation, we evaluated the impact of Notch1 and Hey1 knockdown on RD and SMS-CTR BrdU uptake in vitro. Although cells expressing empty vector showed no change, those with an shRNA to NOTCH1 or HEY1 showed decreased BrdU incorporation (Fig. 2G), indicating an inhibition of proliferation. There was no evidence of increased apoptosis in these experiments as measured by caspase cleavage in vitro and TUNEL staining in vitro (data not shown). In summary, genetic suppression of Notch signaling inhibited the growth of human eRMS cell lines, with some contribution from stimulation of differentiation, but more profoundly due to inhibition of cell proliferation.

Pharmacologic inhibition of Notch signaling blocks eRMS cell growth in vitro

To complement the genetic inhibition studies above, and reflecting the reality of how patients with RMS would be treated, we next evaluated pharmacologic blockade of Notch signaling in the same eRMS cell lines using GSI-X, a commercially available γ-secretase inhibitor (20). To characterize the effect of GSI-X on Notch activity in eRMS cells in vitro, we first determined the concentration of GSI-X required to functionally inhibit Notch signaling. Using expression of ICN1 and Hey1 as readouts for Notch pathway inhibition, we found that 10 μmol/L GSI-X unequivocally inhibited the Notch pathway (Fig. 3A). We also observed that the same dose exerted a phenotypic effect on eRMS cell lines in vitro (Fig. 3B). Next, GSI-X treatment was compared with DMSO vehicle and found to inhibit the growth of both eRMS cell lines in vitro (Fig. 3C). We found that eRMS cells were more sensitive to γ-secretase inhibition than aRMS cells, with significantly lower IC50 values (Fig. 3D), which may reflect the greater expression of the Notch pathway in eRMS. Finally, to complement our genetic data showing that suppression of Notch1 largely blocked eRMS cell growth by inhibition of proliferation, we evaluated the ability of GSI-X to inhibit BrdU incorporation. In both RD and SMS-CTR cell lines, BrdU incorporation was significantly decreased in the presence of GSI-X (Fig. 3E).

Because similar to other drugs GSI-X could have off-target effects, even at low micromolar dosing, we sought to prove that GSI-X-mediated inhibition of cell growth was specific and could be rescued by amplified Notch signaling. To this end, we stably expressed a constitutively active ICN1 (21) in RD and SMS-CTR eRMS cells, confirmed by immunoblot (Fig. 4A.) Functionality of this constitutively active ICN1 was shown both by its ability to upregulate Hey1 (Fig. 4A) and to stimulate cell growth in vitro (Fig. 4B). Cells expressing ICN1 were rescued from
the increasing doses of GSI-X compared with cells expressing an empty vector, since the IC50 of cells expressing ICN1 increased (Fig. 4C and D). Finally, cells expressing ICN1 had increased BrdU incorporation compared with cells expressing an empty vector (Fig. 4E), again implicating proliferation as the dominant effect of Notch.
signaling in eRMS. In summary, pharmacologic blockade of Notch signaling using the γ-secretase inhibitor GSI-X inhibited RMS cell growth in a manner parallel to genetic suppression.

**Both genetic and pharmacologic inhibition of Notch signaling blocks eRMS tumorigenesis in vivo**

Although the *in vitro* data above suggested that eRMS cell growth could be blocked by Notch inhibition, whether through genetic silencing of single Notch family members such as NOTCH1 or HEY1, or through Notch receptor pharmacologic blockade, the gold standard for assessing the importance of a target in tumorigenesis is through *in vivo* assays. To this end, the eRMS cell lines expressing NOTCH1 shRNA versus empty vectors were evaluated as subcutaneous xenografts in SCID/beige mice (Fig. 5A). Suppression of Notch1 in both RD and SMS-CTR cells blocked tumor growth compared with empty vector, with a more dramatic effect in the RD cells (Fig. 5A, left panel).

To assess the impact of Notch pharmacologic inhibition by GSI-XII, RD cells were injected as xenografts into SCID/beige mice, as above. When palpable tumors developed, the mice were randomly divided into 2 groups and treated with either GSI-XII or DMSO vehicle control daily for 21 days. As predicted by the genetic suppression, mice treated with GSI-XII exhibited decreased tumor growth by volume and tumor weight (Fig. 5B). To confirm that Notch signaling was being inhibited in this xenograft model, tumor lysates were probed for ICN1 and Hey1 protein expression (Fig. 5C). ICN1 downregulation in the GSI-treated samples was evident by immunoblot, and quantitated as a greater than 50% decrease compared with DMSO control. Changes in Hey1 were not as demonstrable due to faint bands and limited...
tumor tissue, although densitometry suggested downregulation in the actual tumors. Finally, given the results of our in vitro work showing that inhibition of eRMS cell growth was due in part to reduced cell proliferation, we examined sections of tumor tissue from the xenografts resulting from either the genetic suppression or pharmacologic inhibition experiments. In both methods of Notch signaling inhibition, the percentage of Ki67 positive cells was significantly decreased (Fig. 5D), further implicating proliferation as a mechanism of Notch-mediated tumorigenesis in eRMS.

In summary, inhibition of Notch signaling, whether through genetic knockdown of one Notch receptor, or pharmacologic inhibition with a γ-secretase inhibitor, blocked eRMS tumorigenesis in vivo, suggesting that the Notch pathway may be a useful therapeutic target in patients with eRMS.

Discussion

In this work, we show using in vitro and in vivo, genetic and pharmacologic approaches, that the Notch signaling
pathway is upregulated in the embryonal variant of RMS (eRMS), and that inhibition of the Notch pathway blocks eRMS tumorigenesis. Emphasis is placed on the role of the Notch target Hey1, known to be important in skeletal myogenesis. Previously published work showed a role for Notch signaling in aRMS tumorigenesis, as the Notch target Hes1 was found to allow evasion of differentiation and promote proliferation in the RhJT aRMS cell line (11, 22), and Notch inhibition reduced both eRMS and aRMS invasiveness in vitro (12). Thus our study focuses on the role of Notch signaling in eRMS tumorigenesis, establishes a role for Hey1 in eRMS, and shows the efficacy of Notch inhibition in an in vivo preclinical model.

An oncogenic role for Notch was first identified in human T-cell ALL during the 1980s. Since then, aberrant Notch signaling has been implicated in a variety of pediatric and adult malignancies including breast, pancreatic, bone, and brain (3, 8). Whether Hes or Hey predominates as the ultimate Notch effector depends upon cellular context, as for example Hes1 is important in osteosarcoma (23, 24), while Hey1 is key in glioblastoma (5, 25). The oncogenic role of Notch signaling seems related to its role in normal tissue maintenance, where it functions in many cell types to prevent terminal differentiation and maintain populations of undifferentiated progenitor cells including pancreatic, breast, and...
neural stem cells (26–28). In select types of cancer, these same properties of the Notch signaling pathway function to support a population of cancer stem cells, as described in both brain (29) and pancreatic cancer (26).

In the case of skeletal muscle, the Notch-Hey1 axis is critical for the proliferation and preservation of muscle stem cells, termed satellite cells. This seems to occur through both prevention of differentiation and promotion of proliferation of satellite cells and their progeny. For example, constitutive expression of Hey1 prevents myoblast differentiation by repressing promyogenic transcription factors such as Mef2C and myogenin (30, 31), while inhibiting Notch signaling recruits satellite cells to undergo differentiation and induces myotube hypertrophy, thus reducing satellite cell number (7). Notch-Hey1 signaling also promotes proliferation of satellite cells, allowing expansion and maintenance of a reserve population (6, 32). Although not addressed in the current study, it is possible that dysregulated Notch signaling is similarly preventing differentiation and promoting the proliferation of RMS tumor-propagating cells.

Our findings suggest that Notch signaling supports RMS tumorigenesis predominantly through promotion of proliferation. We found that inhibition of Notch signaling via

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**Figure 5.** Inhibition of Notch signaling decreases RMS tumorigenesis in vivo. A, tumor growth of RD and SMS-CTR eRMS cells stably expressing either vector or Notch1 shRNA assessed in vivo as subcutaneous xenografts in SCID/beige mice. B, tumor growth over time (left) and harvested tumor weights (right) of RD cells injected as subcutaneous xenografts in SCID/beige mice and treated with either DMSO vehicle control or GSI-X. C, immunoblot analysis of ICN1 and Hey1 in protein lysates from xenograft tumors treated with either DMSO vehicle control or GSI-X. These blots were quantitated using densitometry.
genetic and pharmacologic techniques decreased eRMS proliferation *in vitro* and *in vivo*, while overexpression of the Notch pathway using a constitutively active Notch1 receptor resulted in increased eRMS proliferation *in vitro*. Most importantly, pharmacologic inhibition of the Notch pathway using a γ-secretase inhibitor decreased proliferation and tumor growth *in vivo*. A lesser, but still apparent role for Notch signaling in eRMS tumorigenesis is through the prevention of myogenic differentiation. When Hey1 expression was knocked down using shRNA, myogenin expression increased, reflecting more commitment to the myogenic lineage. This is consistent with the known role of Hey1 in binding the myogenin promoter and blocking its transcription (31), and also consistent with previous published data showing that both dnHes1 and GSI treatment increase myosin heavy chain mRNA, another marker of commitment to the myogenic lineage, in the presence of fusion medium (11). Because we did not see overt evidence of terminal differentiation in our *in vivo* xenograft studies, it seems that Notch pathway inhibition is insufficient to induce terminal differentiation in eRMS, a finding consistent with our *in vitro* Hey1 knockdown data, previous analyses of Notch inhibition in skeletal muscle (30, 33), and the known difficulty in driving RMS cells *in vitro* to terminal differentiation (15, 17). However, even in the absence of terminal differentiation, this modest effect may have therapeutic consequences, particularly if Notch inhibition in eRMS promotes a transition from self-renewal to differentiation fates.

An interesting question raised by our study is the reason for the redundancy of Notch pathway members in RMS. A likely explanation is the importance of this signaling pathway in skeletal myogenesis and the necessity for multiple receptors and ligands to allow for amplification of signal and autocrine/paracrine signaling. However, it is also possible that each ligand/receptor has independent, disparate effects in addition to the canonical Notch signaling pathway, and the pattern of upregulation may contribute in other ways to the oncogenic effect in RMS. Finally, although not part of the current investigation, there may be upstream influences promoting such strong Notch upregulation in eRMS. Possible drivers of Notch signaling may include oncogenic Ras, which has been reported to activate Notch signaling (34), and mutations of which have been described in eRMS tumors and cell lines (35).

Another interesting finding of this study is the differential expression and activation of the Notch pathway in eRMS versus aRMS. Previous work by Roma and colleagues showed the broad upregulation of Notch signaling in RMS tumors, and while they did not report a dramatic upregulation in eRMS subtype, their data suggest a trend to increased Notch target gene expression in eRMS versus aRMS (12). In the current work, we specifically show that the eRMS subtype is more sensitive to inhibition by γ-secretase inhibitors than aRMS. The reason for this difference in Notch expression and sensitivity between the 2 subtypes is unclear, however at least 2 possibilities exist. First, it is possible that upregulation of the Notch pathw...
signaling pathway is driven by an eRMS-specific mechanism. As mentioned above, several studies have shown that dysregulation or mutational activation of Ras signaling may contribute to eRMS pathogenesis (35–37), and the Notch signaling pathway is a putative downstream effector of oncogenic Ras (34). Alternatively, dissimilar cells of origin of eRMS and aRMS may be responsible for the difference in Notch signaling. It has previously been suggested that eRMS might arise from satellite cells (36, 38, 39), and these cells have upregulated Notch signaling when compared with other muscle precursor cells (32). Therefore, eRMS tumors may arise from a cell of origin which already has upregulated Notch signaling.

Despite the clear impact of Notch pathway inhibition in these preclinical experiments, the feasibility of Notch inhibition in humans with cancer may be challenging. γ-Secretase inhibitors, while currently in phase I and II testing, are known to cause gastrointestinal toxicity resulting from goblet cell differentiation and hyperplasia (40, 41). Thus approaches to abrogate this toxicity, including the use of steroids and intermittent schedules of γ-secretase inhibitors, or additional pharmacologic approaches to inhibit the Notch pathway may be necessary (42–44).

In summary, new therapies are needed in the treatment of RMS. However, further improvements in cure rates may not be possible with the addition of new cytotoxic chemotherapy regimens alone. Instead, new treatment strategies that target aberrantly regulated signaling pathways in RMS may be necessary. Decreasing the regenerative potential of RMS cells by permitting differentiation and inhibiting proliferation may represent an alternative approach to treating RMS. The Notch-Hey1 signaling pathway is known to prevent differentiation, promote proliferation, and preserve stem cells in normal skeletal muscle. Our study shows that the Notch-Hey1 axis plays a similar role in eRMS tumorigenesis and with the in vivo preclinical data firmly supports the development and evaluation of Notch-Hey1 axis inhibitors in the treatment of eRMS.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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