Musculoskeletal Pathology

Inhibition of Notch Signaling Induces Neural Differentiation in Ewing Sarcoma

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Cells from Ewing sarcoma exhibit cellular features and express markers, suggesting that the tumor is of neuroectodermal origin. Because Notch signaling regulates the differentiation of neuroectodermal cells during development, we examined the role of Notch signaling in Ewing sarcomas. We found that Ewing sarcomas express Notch receptors, ligands, and the Notch target gene *HES1*. To determine the functional implications of Notch signaling, we expressed tetracycline-regulated constitutively active, dominant-negative (DN), or wild-type Notch-1 receptors in two Ewing sarcoma cell lines, or we treated the cell lines with a γ -secretase inhibitor. Expression of the constitutively active Notch-1 reduced proliferation and expression of the DN Notch-1 reduced apoptosis *in vitro*. However, there was only a small difference in the volume of tumors that formed when the cell lines expressing these constructs were implanted in nude mice. Xenograft tumors derived from the cell lines expressing DN Notch-1 exhibited a neural phenotype. Treatment with a γ -secretase inhibitor caused similar changes as expression of the DN construct. Notch signaling plays a role in cell differentiation, proliferation, and apoptosis in Ewing sarcoma, but its inhibition is only associated with a small change in tumor growth potential. (Am J Pathol 2007, 170:1686–1694; DOI: 10.2353/ajpath.2007.060971)

Ewing sarcoma belongs to the Ewing sarcoma family of tumors, which also includes primitive neuroectodermal tumors. These tumors share common genetic and histological features.¹ Although the cell of origin of these tumors is unclear, they exhibit cellular features and express markers of neuroectodermal origin. For example, cells exhibit dendritic formation, expression of the neuronal marker catechol

acetyltransferase, and expression of neuron-specific enolase.^{2,3} This phenotype was noted in early studies that originally described Ewing sarcoma as a metastatic neuroblastoma⁴ and has been supported by recent data from large-scale gene profiling studies.⁵ Eighty-five percent of cases harbor the t(11;22)(q24;q12) chromosomal translocation that generates a fusion of the 5' segment of the *EWS* gene with the 3' segment of the ETS family gene *FLI-1*. This results in the generation of a novel protein (EWS/FLI-1) that regulates the expression of genes not normally modulated by either *EWS* or *FLI-1*.^{6,7} Aberrant modulation of these genes influences downstream events and presumably induces cellular transformation.

Studies in *Drosophila* highlighted a role for Notch signaling in central nervous system and wing development. Notch mediates its effects in neural development via direct cell-cell interactions. For example, in a process termed lateral inhibition, Notch signaling allows initially equivalent cells in the same environment to respond differently to developmental signals, allowing a limited number of cells to differentiate to a specific cell type, with adjacent cells differentiating to a different cell type. In *Drosophila* central nervous system development, cells with less Notch activation adopted a neuronal fate, whereas adjacent cells, with more Notch activation, an epidermal fate.⁸ Where this lateral inhibition signaling failed, adjacent cells differentiated along the same neuronal path at the expense of the epidermal fate, resulting in excess neural differentiation.⁹

A role for Notch signaling in Ewing sarcoma is suggested by data showing that NIH3T3 cells expressing the EWS/FLI-1 fusion protein have high levels of expression of the *MFNG* gene.¹⁰ *MFNG* acts to modify epidermal growth factor-like motifs of the transmembrane protein Notch. These motifs are involved in ligand binding and Notch activation. FNG protein induced modifications alter the affinity of some ligand/Notch receptor interactions. Following ligand activation, Notch is cleaved, resulting in the release of the intracellular domain.^{11,12} There are four

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mammalian Notch proteins that share strong structural homology. In addition to the epidermal growth factor-like repeats, there is an intracellular domain composed of six cdc/ankyrin repeats. These are protein-protein interaction motifs involved in binding cytoplasmic effector molecules. Downstream of the cdc repeats are transcriptional activation domains (in the case of Notch 1 and 2) and PEST sequences, which are involved in Notch protein turnover.^{13–15} Mammalian Notch ligands include Delta-like 1, 3, and 4 and Jagged 1 and 2.^{16,17} The intracellular effectors, CBF-1/RBP-Jk, Deltex, and Mastermind proteins, bind to the intracellular domains of Notch, forming a transcription activating complex.^{12,18–20} This up-regulates a variety of target genes, including Hairy/Enhancer of Split-1 (*HES-1*).²⁰ A recent gene profiling study found that *MFNG* is also regulated by the EWS/FLI-1 fusion protein in Ewing sarcoma cell lines. In addition, although it was found that the cell line expressed the various *NOTCH* genes, the level of expression of these genes was not regulated by EWS/FLI-1.²¹

There is variability in the degree to which Ewing tumors exhibit a neural phenotype.^{22,23} Notch signaling could be a factor responsible for the regulation of the neural phenotype in Ewing tumors. Given the development of pharmacological agents that modulate Notch signaling, modulating this pathway has potential therapeutic implications. As such, we investigated Notch signaling in Ewing tumors.

Materials and Methods

Primary Tumors, Cell Lines, and Expression Studies

Ten Ewing sarcoma tumors were studied for evidence that the Notch signaling pathway is active. Samples were from primary bone lesions obtained from diagnostic biopsies performed before the initiation of any therapy. Tumors were cryopreserved as soon as possible after surgery for subsequent expression studies. All of the samples harbored the EWS/FLI1 transgene as detected using polymerase chain reaction (PCR) and harbored a EWS exon 7–FLI1 exon 5 junction.²⁴ Two cell lines derived from Ewing sarcoma tumors were used in this study, the RD-ES and SK-ES-1 (HTB-166 and HTB-86 lines from the American Type Culture Collection, Manassas, VA). The expression of Notch 1 and 2, ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2), the Notch modifier *MFNG*, and the Notch-regulated target gene *HES-1* was determined using reverse transcriptase (RT)-PCR. The expression level of the neural marker neural-specific enolase was also examined using quantitative RT-PCR in grafted cell lines. Previously described primers and conditions^{25–30} were used on RNA isolated from the cryopreserved tissues or the cell lines.

Generation of Constitutively Active and Dominant-Negative Notch-1 Constructs

To study the effect of Notch signaling in the Ewing sarcoma cell lines, dominant-negative (DN) and constitu-

tively active (CA) forms of the Notch-1 receptor were generated from the full-length wild-type Notch-1 cDNA. The DN-Notch1 was generated by deleting a portion of the intracellular domain (nucleotides 5360–7582), removing the cdc/ankyrin repeats, and producing a predicted inactive receptor. The CA construct was generated by deleting a portion of the extracellular domain (nucleotides 1–5329), removing the epidermal growth factor-like repeats and, as such, the extracellular binding capacity of the receptor, allowing for predicted autonomous activation. A FLAG-tag sequence was attached to the start codon of each Notch-1 construct. The constructs were initially subcloned into the pcDNA3 expression vector and then transfected into the RD-ES cell line along with a Notch signaling reporter construct, in which the *Hes-1* promoter was linked to the Firefly luciferase gene, to confirm their ability to alter Notch signaling activity in a Ewing sarcoma cell line. The reporter construct was transfected along with a β -galactosidase expression construct as a control for transfection efficiency. β -Galactosidase activity and luciferase activity were measured as previously reported, and Gli transcriptional activity was represented by luciferase activity normalized for β -galactosidase. Luciferase activity driven by the *Hes-1* promoter elements increased threefold with transfection of the CA construct compared with the wild-type Notch-1 construct and with expression of the DN construct transcriptional activation declined to 28% of activity compared with cells expressing the wild-type receptor, thus confirming the predicted function of these constructs.

To generate inducible constructs, the CA, DN, and wild-type Notch-1 were subcloned into a tetracycline-regulated bidirectional expression vector, pBI (Clontech Laboratories, Inc., Mountain View, CA), which was modified to also express the destabilized enhanced green fluorescent protein gene (d2EGF) in the opposite direction. The newly generated expression constructs (pBIpd2EGF:Notch1, pBIpd2EGF:DN-Notch1, and pBIpd2EGF:CA-Notch1) were used in conjunction with the Tet-Off gene expression system (Clontech Laboratories) to allow for tight regulation of expression via addition of tetracycline or its derivative, doxycycline.

Generation of Transfected Ewing Sarcoma Cell Lines

The two Ewing sarcoma-derived cell lines, RD-ES and SK-ES-1, were initially transfected using lipofection with the Tet-Off expression vector and selected for expression of the G418 sulfate resistance gene. G418-resistant clones were then isolated and expanded to generate lines expressing pBIpd2EGF:Notch1, pBIpd2EGF:DN-Notch1, or pBIpd2EGF:CA-Notch1. Cells were maintained with doxycycline in the media to inhibit transgene activation, but media was changed to media without doxycycline to activate the transgene. Doxycycline was removed from the media, and cell sorting was used to select a population of transfected cells that express GFP. These cells were grown in media with and without doxycycline, and fluorescence

microscopy was used to detect GFP in the living cells in the absence of doxycycline to confirm tight regulation of transgene activation.

Proliferation and Apoptosis Studies

Bromodeoxyuridine (BrdU) was administered in the media for 12 hours, after which cells were fixed in methanol and stained for BrdU incorporation as previously reported.³¹ The percentage of nuclei positively staining for BrdU was counted over 10 high-powered fields, to give an average percent incorporation for each cell expressing each construct. Apoptosis was measured using propidium iodide and annexin V fluorescent cell sorting as previously reported.^{32,33} The percentage of cells taking up annexin V was measured as a percentage of cells undergoing apoptosis. Proliferation and apoptosis experiments were performed in each cell line expressing each of the constructs with and without doxycycline nine times in independent experiments, and results of these replicates are given as means with 95% confidence intervals.

Xenograft Mouse Experiments

Cells (5.0×10^6) derived from the two Ewing cell lines expressing one of the three constructs were resuspended in phosphate-buffered saline and Matrigel (VWR, West Chester, PA) at a 1:1 ratio and injected into two subcutaneous locations on the backs of 4-week-old male nude BALB/c mice. Twelve mice for each cell line expressing each construct (for a total of 72 mice) were studied. For each group of mice, half were treated with doxycycline, which was administered incorporated into their food pellets (200 $\mu\text{g}/\text{ml}$) and added to their water (5% sucrose and 1 $\mu\text{g}/\text{ml}$ doxycycline). Mice were then examined for tumor growth by measuring tumor size with calipers every 5 days for 35 days. At the time of sacrifice, activation of the tetracycline-regulated construct was confirmed by observing GFP in the tumor under fluorescent light. Following sacrifice, the tumors were dissected free from the surrounding tissues, weighed, and prepared for RNA, protein, and histological evaluation. To verify regulation of the Notch transgenes, total protein lysate was prepared from the tumors and Western analysis performed using an antibody to the FLAG tag, using an anti-FLAG antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). RNA was also prepared, and expression of HES-1 gene and neuron-specific enolase from the xenograft tumors was analyzed using real-time PCR. Excised tumors were fixed in 10% formaldehyde in phosphate-buffered saline (formalin) and then sectioned for subsequent hematoxylin and eosin staining to visualize general morphological features of Ewing cells under each experimental condition. Immunohistochemistry was performed on sections from these tumors using antibodies to S100 and neuron-specific enolase, both markers of neural differentiation, and using an antibody to vimentin.

Figure 1. Evidence that Notch signaling is active in Ewing tumors. RT-PCR data showing expression of *NOTCH-1*, *NOTCH-2*, *DLL-1*, *DLL-2*, *DLL-3*, *JAG-1*, *JAG-2*, *MFNG*, and the Notch target gene *HES-1* in five individual primary Ewing tumors. All of the lesions expressed the Notch-regulated target gene *HES-1*. The two cell lines examined in this study also expressed all of the ligands, receptors, and the target gene *HES-1*.

γ -Secretase Inhibitor Studies

To determine whether a pharmacological modulator of Notch signaling would have similar effects as expression of the DN construct, the same Ewing tumor cell lines were treated with dimethyl sulfoxide carrier alone or the γ -secretase inhibitor X (Calbiochem, San Diego, CA) at 0.1 $\mu\text{mol}/\text{L}$ for 3 days.³⁴ RNA was extracted for analysis of *HES-1* expression. Cells were examined for proliferation using BrdU incorporation and grown on coverslips for immunohistochemical analysis using the same antibodies as for the xenograft tumors.

Real-Time PCR

Real-time quantitative PCR was undertaken using 28S rRNA (28S) as the control gene. PCR primer pairs for human 28S rRNA,⁸ neural-specific enolase,³⁵ and *HES-1*³⁶ were taken from previous publications. Validation curves were performed for the primer sets using RNA diluted to 1:5, 1:10, 1:50, 1:100, and 1:1000. The ΔCt method was used for setting up the experiment and

Figure 2. Notch signaling regulation in Ewing sarcoma cell lines. **A:** Immunofluorescence detection of GFP, expressed by the bidirectional tetracycline-regulated expression construct, showing that doxycycline is able to tightly regulate expression in the cell lines. Expression driven by the tetracycline-off construct was suppressed in the presence of doxycycline (DOX). **B:** Western analysis using an antibody to FLAG showing regulated expression of the three Notch-1 constructs by doxycycline. “+” lanes are loaded with protein extracts from cells treated with doxycycline, and lanes labeled “–” are loaded with protein extracts from cells treated without doxycycline. **C:** Activation of the HES-1 luciferase reporter showing increased Notch transcriptional activation with expression of the constitutively active Notch signaling activation to more than twice the level of cells in which the construct was not active, whereas expression of the dominant-negative construct resulted in activation of the construct at roughly half of the level observed in control cells. The control cell data are pooled data from cells in which the constructs are not expressed. CAN1 is the constitutively active construct, DNN1 is the dominant-negative construct, and WTN1 is the wild-type construct. Data are given as relative luminescent units and 95% confidence intervals and are provided for each cell line. Statistically significant differences ($P < 0.05$) are present when the 95% error bar does not cross the mean of the comparison data. Data for both cell lines are presented.

analysis of the data. An arbitrarily designed threshold was set at 0.2 for all analysis, whereas the baseline cycles were set for all analysis from 3 to 10 cycles for 28S and from 3 to 30 cycles. The threshold cycle Ct was determined using the analysis software SDS 2.1 (Applied Biosystems). The result was analyzed using the relative quantitative $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Means, standard deviations, and 95% confidence intervals were calculated, and comparisons between groups were made using Student's *t*-test.

Results

Ewing Sarcomas Express Notch Receptors and Ligands

Expression analysis using RT-PCR was used to test for the expression of *NOTCH-1*, *NOTCH-2*, *DLL-1*, *DLL-3*, *DLL-4*, *JAG-1*, *JAG-2*, *MFNG*, and *HES-1* in 10 cryopreserved cases of Ewing sarcomas harboring *EWS/FLI1*. Each case expressed at least one of the Notch receptors, at several of the ligands, and all expressed *MFNG*. In addition, all of the tumors expressed the Notch target gene *HES-1*. This pattern of expression of ligands, recep-

Figure 3. Notch signaling regulates cell proliferation and apoptosis. **A:** BrdU incorporation in cell lines expressing the constitutively active, dominant-negative, and wild-type Notch-1 constructs. Percentage of cells over 10 high-powered fields showing positive staining and 95% confidence intervals are shown. Expression of the dominant-negative construct reduced cell proliferation in the RD-ES cell line. Treatment with the γ -secretase inhibitor also reduced cell proliferation but not to as great a degree as with expression of the dominant-negative construct. **B:** Apoptosis rate as percentage of cells undergoing apoptosis and 95% confidence intervals. Expression of the dominant-negative Notch constructs decreased cell apoptosis rate in both cell lines, whereas expression of the constitutive active construct in the SK-ES-1 cell line also resulted in a decrease in cell apoptosis. Treatment with the γ -secretase inhibitor did not alter the apoptosis rate.

tors, and *HES-1* suggests that Notch signaling is activated in Ewing tumors. Representative RT-PCR data are shown in Figure 1. We also examined the expression of the ligands, receptors, and transcription factors in two Ewing sarcoma cell lines, RD-ES and SK-ES-1, for use in *in vitro* studies. Both of these cell lines expressed the Notch receptors, ligands, and *HES-1*. There was not a substantial difference in expression between these two cell lines.

Notch Signaling Can Be Regulated in Ewing Sarcoma Cell Lines

To determine whether Notch signaling would regulate the neoplastic behavior in Ewing sarcoma, we generated tetracycline-regulated constitutively active, dominant-negative, and wild-type Notch-1 constructs. When transfected into the two Ewing sarcoma cell lines, doxycycline was able to regulate expression of the Tet-Off constructs, as demonstrated by regulation of the GFP driven by the bidirectional tetracycline-regulated expression construct in the cell lines (Figure 2A). Western analysis using an antibody to the FLAG epitope showed tetracycline-regulated expression of the three Notch-1 constructs and that all were expressed at similar levels (Figure 2B). Expression of the Notch target gene *HES-1* increased with expression of the constitutively active Notch signaling activation to more than twice the level of cells in which the

construct was not active, whereas expression of the dominant-negative construct resulted in expression of *HES-1* at roughly half of the level observed in control cells. This regulation was verified by both quantitative RT-PCR and activation of the *HES-1*-regulated luciferase reporter construct (Figure 2C).

To determine whether pharmacological regulation of Notch signaling would have a similar effect as expression of the Notch constructs, we treated the cell lines with γ -secretase inhibitor X (Calbiochem). The γ -secretase inhibitor suppressed Notch signaling as demonstrated by decreased expression of *HES-1* to half of control levels.

Notch Signaling Regulates Cell Proliferation in Ewing Cell Lines

Cell lines expressing the constitutively active, dominant-negative, and wild-type Notch-1 constructs were examined for proliferation by measuring BrdU incorporation. Treatment of the Ewing cell lines with doxycycline alone did not alter cell proliferation. Expression of the dominant-negative construct reduced cell proliferation in the RD-ES-1 cell line, but not in the SK-ES-1 cell lines (Figure 3A). Treatment with the γ -secretase inhibitor also resulted in a lower proliferation rate in the RD-ES cell line and a trend toward a lower proliferation rate in the SK-ES-1 cell line. There was an increase in proliferation in both cell lines when expressing the constitutively active construct.

Notch Signaling Dysregulation Inhibits Apoptosis in Ewing Cell Lines

Expression of the dominant-negative Notch constructs decreased cell apoptosis rate in both cell lines (Figure 3B). Surprisingly, expression of the constitutively active construct in the RD-ES cell line also resulted in the same decrease in cell apoptosis. Treatment of the Ewing cell lines with doxycycline alone did not alter apoptosis rate. Treatment with the γ -secretase inhibitor caused a trend toward a lower apoptosis rate in the cell lines. Taken together, this shows that Notch signaling plays a role regulating cell apoptosis, although the results varied depending on the cell line examined and on the method used to alter Notch signaling activity.

Notch Signaling Regulates the Size of Tumors That Formed When the Cell Lines Are Implanted into Immunodeficient Mice

The cell lines expressing Notch constructs were implanted into nude mice, and half of the mice were treated with doxycycline to suppress expression. Transgene activation was confirmed by detection of GFP and using Western analysis of tumor lysates. All of the cells in the xenografted tumors, except for occasional vascular cellular elements, exhibited GFP expression. We found a high degree of variability in the size of xenograft tumors that formed in each group. Although the RD-ES cell line expressing the dominant-negative construct showed a decreased tumor volume compared with the control, we did not observe a difference in the SK-ES-1 cell line (Figure 4).

Notch Inhibition Causes a More Neural-Like Phenotype

Cells from tumors derived from explanted cell lines expressing the wild-type Notch or the constitutively active construct showed a phenotype that was indistinct from the appearance of control cells that did not express these constructs. Cells from xenograft tumors expressing the dominant-negative Notch construct were larger in size and stained intensely for markers of neural differentiation (Figures 5 and 6). This effect was observed in both cell lines. Treatment of the cells with the γ -secretase inhibitor resulted in an identical change in histological appearance and immunohistochemical staining pattern as for cells expressing the dominant-negative construct. Quantitative RT-PCR showed a doubling of expression of neural-specific enolase in the grafted tumors expressing the dominant-negative construct compared with the other grafted tumors. Notch signaling inhibition in these Ewing cell lines was associated with the adoption of a more neural phenotype

Discussion

Although Ewing tumors express EWS/FLI1, how this causes neoplasia and what factors might modulate the

Figure 4. Notch signaling has an effect on the size of tumors that formed when the cell lines are implanted into nude mice. The cell lines expressing Notch constructs were implanted into nude mice, and half of the mice were treated with doxycycline to suppress expression. Data are given as mean volume and 95% confidence intervals. **A:** Data from the RD-ES cell line, and **B** shows data from the SK-ES-1 cell line. Statistically significant differences ($P < 0.05$) are present when the 95% error bar does not cross the mean of the comparison data. As such, there is a significant difference for SK-ES-1 cells expressing the dominant-negative construct compared with the control cells. The wide variability on grafted tumor size is apparent in the large 95% confidence intervals.

neoplastic phenotype are incompletely elucidated. Our data show that Notch signaling components are expressed in Ewing tumors. Importantly, *HES-1*, a downstream transcriptional target of Notch signaling, was expressed in all of the cases examined. Furthermore, modulating Notch signaling regulates cell proliferation and apoptosis. Notch signaling inhibition causes xenograft tumors from Ewing sarcoma cell lines to adopt a more neural cell-like phenotype. Thus, Notch signaling is one such pathway that modulates the way that tumor cells in Ewing sarcoma behave.

Although it is problematic to use phenotypic or expression data from tumor cells to determine the cell type from which the tumor arises, such data can provide suggestive or supportive evidence. Although the cell of origin of Ewing sarcoma is unknown, its histological appearance, as well as data from gene profiling studies, supports the concept that Ewing sarcomas are derived from a cell of neuroectodermal origin. Recent data show that mesenchymal bone marrow cells expressing EWS/FLI1 transplanted into immunodeficient mice develop a Ewing sar-

Figure 5. Histology reveals an altered cytology in cells with Notch inhibition. Cells from xenograft tumors expressing the dominant-negative Notch construct were larger in size and had more of a neuroblastoma-like appearance than control cell lines or cells expressing the constitutively active construct.

coma phenotype, suggesting that Ewing tumors are derived from a mesenchymal progenitor cells.³⁷ Because progenitor cells from bone marrow can differentiate into a variety of cell types, including cells exhibiting ectodermal and neural phenotypes, and there are common precursors from which both neuroectodermal cells and mesenchymal cells may derive,^{38–40} data that bone marrow cells can differentiate into tumors with an Ewing sarcoma phenotype are not inconsistent with a neuroectodermal cell phenotype playing a role in the cell of origin. Thus,

this information is consistent with the notion that Ewing tumor cells arise from a neuroectodermal precursor or from stem cells that differentiate down the neuroectodermal lineage. As Notch signaling can modulate the neural phenotype during development, our finding that Notch signaling inhibition causes Ewing sarcoma cells to adapt a more neural cell-like phenotype gives support to the notion that Ewing tumors are derived from a neuroectodermal precursor. In the context of early development, ligands expressed by adjacent cells activate Notch re-

Figure 6. Notch inhibition causes expression of markers of a neural phenotype. Immunohistochemistry of cells from xenograft tumors expressing the dominant-negative Notch construct shows intense staining for markers of neural differentiation.

ceptors to inhibit neural differentiation. In contrast, Ewing sarcoma cells express both ligands and receptors. Nonetheless, inhibition of Notch signaling resulted in the cell adopting a more neural cell-like phenotype. Thus, similar to the situation in early development, Notch signaling inhibition enhances neural differentiation in this neoplastic cell line.

Although these two cell lines harbor the same EWS/FLI1 transcript and express the various Notch signaling components examined at similar mRNA levels, they have slightly different behavior when expressing the various Notch constructs. There are a variety of explanations for this finding: 1) There may be variability in the expression of other modifying genes. 2) Because there is variability in the cell types present in Ewing tumors, they may represent slightly different cell types. 3) There may be secondary changes that occur with subsequent passages in culture. 4) There is patient-to-patient variability in polymorphisms, which change the expression of genes not directly related to the underlying etiology of the tumor. Despite these differences, there are similar overall trends in the behavior to modulating Notch signaling, suggesting a common role for Notch signaling in Ewing sarcoma.

Notch signaling regulates the neoplastic phenotype in a variety of tumor types. In some, it plays a role in tumor initiation, whereas in others, such as in colonic neoplasia,³⁷ it has a secondary effect modulating the neoplastic phenotype. In either case, Notch signaling is emerging as an enticing therapeutic target, due to the development of pharmacological agents that modulate the pathway. Although we found that modulating Notch signaling altered cell proliferation and apoptosis in Ewing sarcoma cell lines, we only observed a small effect on the size of tumors that developed when grafted into nude mice. Although it is possible that the wide variability we found in tumor size masked an effect of Notch signaling, our finding suggests that on balance the effects of Notch signaling do not result in a major change in tumorigenic potential. Recent evidence suggests that some solid tumors are derived from a subpopulation of tumor initiating or "stem" cells. In the case of xenotransplantation, tumor formation may be related to the number of tumor-initiating cells present.^{41,42} Although Notch signaling can regulate cell proliferation and apoptosis in the Ewing cell lines *in vitro* and can modulate stem-like cells in other cancer types,⁴³ it may not substantially alter the number of tumor initiating cells in Ewing tumors, and this may explain the relative lack of a difference to the size of tumors that formed in the mice.

There was a striking difference in histology between tumors from grafted cells expressing the different constructs. Immunohistochemistry showed that cells expressing dominant-negative Notch1 express high levels of neural markers. This change in histological appearance was not associated with a change in the size of xenograft tumors that formed in the immunodeficient mice, suggesting that Notch-regulated neural differentiation is not associated with a major change in tumor cell growth in Ewing sarcoma. Clinical studies show variability in the prognostic effect of neural differentiation on the

outcome in Ewing sarcoma.²³ Our findings are in agreement with these clinical observations in which neural differentiation was not found to be associated with a major change in clinical outcome.²²

In summary, Notch signaling is active in Ewing sarcoma human tumors and cell lines. Its inhibition induces neural differentiation. However, unlike some other tumor types in which Notch signaling is activated, inhibition of Notch signaling only results in a minor change in tumor growth.

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