

Opposite Effects of Notch-1 and Notch-2 on Mesothelioma Cell Survival under Hypoxia Are Exerted through the Akt Pathway

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Abstract

Malignant mesothelioma (MM) is a cancer of the lining of the lungs, heart, and intestine and is known to respond poorly to chemotherapy. Here we show that malignant mesothelial cells have an elevated Notch signaling pathway compared with normal human mesothelial cells. We studied the role of Notch in MM under normoxic and hypoxic conditions, the latter condition best recapitulating the MM microenvironment. Genetic and chemical modulation of the Notch pathway indicated that MM cells are dependent on Notch signaling. More specifically, this signaling was Notch-1 dependent as the result of its negative transcriptional regulation on phosphatase and tensin homologue (PTEN), which led to activation of the prosurvival phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway. Our study also provides evidence that whereas Notch-1 is elevated in the malignant setting, Notch-2 is diminished. This differential expression of the two Notch isoforms benefits cancer cell survival because reexpression of Notch-2 was toxic to MM cells. The mechanism of Notch-2 toxicity to MM cells countered that of Notch-1, as it was the result of positive transcriptional regulation of PTEN and inhibition of the PI3K/Akt/mTOR signaling pathway. These results provide new insight into the role of Notch in MM and suggest that Notch pathway inhibitors may be useful in the treatment of this deadly disease. [Cancer Res 2008;68(23):9678–85]

Introduction

Malignant mesothelioma (MM) is an aggressive tumor of the pleura, pericardium, and peritoneum. MM has been traditionally linked to environmental fiber exposure (asbestos and erionite; ref. 1). More recently, the DNA oncogenic virus SV40 has been linked to MM (1), and the presence of a MM epidemic in parts of Turkey suggests that genetic predisposition may play a major role in MM onset (2, 3). MM is among the tumors with the shortest median survival after diagnosis, with little benefit provided by current chemotherapies (4). It is therefore imperative to better understand the molecular mechanisms underlying MM and to identify novel therapeutic strategies for the treatment of this disease.

Notch signaling is an evolutionarily conserved pathway that regulates critical cellular pathways during development and

postnatal life (5). Humans have four heterodimeric transmembrane receptors (Notch-1 through Notch-4), two Serrate-like (Jagged-1 and Jagged-2), and a Delta-like family of ligands (6). On ligand binding, Notch receptors undergo a number of modifications, including a proteolytic cleavage operated by a γ -secretase complex that ultimately releases the intracellular portion (active form) of Notch receptors (Notch^{IC}, or activated Notch) from the plasma membrane (7). Notch^{IC} translocates to the nucleus where it interacts with CSL (CBF-1 in mammals, Suppressor of Hairless in *Drosophila*, and Lag-1 in *C. elegans*) transcription factors (8). Association of Notch^{IC} with CBF-1 promotes the release of transcriptional corepressors (such as CIR, SMRT, and histone deacetylases) and recruits transcriptional coactivators (e.g., SKIP, MAML-1, and histone acetyltransferases) to the CBF-1 complex. Therefore, Notch association to CBF-1 converts the latter from a transcriptional repressor into a transcriptional activator (9). Target genes of Notch^{IC}/CBF-1 complexes include *HES* (5) and *HEY* genes (10), cyclins (11), components of the nuclear factor- κ B transcription factors (12), and c-Myc (13).

Notch signaling has been increasingly linked to cancer, although the biological effects of Notch activity seem to be tissue specific. In T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), activating mutations of Notch-1 are found in ~50% of cases (14) and shown to predict early treatment response and favorable long-term outcome (15). Conversely, Notch-1 and Jagged-1 overexpression are poor prognostic factors in breast (16) and prostate cancer (17). Overexpression of Notch receptors and ligands has also been described in other solid tumors (such as head and neck, renal, pancreatic, ovarian cancers, etc.) and leukemias (9).

Notch-1 is a tumor suppressor in mouse and human skin (18, 19). Ectopic expression of Notch-1^{IC} led to growth arrest of small-cell lung cancer cell lines (20). In certain tumor types, the role played by specific Notch receptors is still debated. For example, Cre-recombinase-mediated ablation of Notch-1 in mouse hepatocytes caused nodular hyperplasia (21). However, nearly 80% of human hepatocellular carcinoma displayed overexpression of Jagged-1 (22). It should be noted that the effects of Notch signaling are dose and context dependent (23). Additionally, the microenvironment also seems to profoundly affect Notch signaling (especially Notch-1). Hypoxia inducible factor 1 α has been shown to stabilize Notch-1^{IC} from degradation and to greatly increase Notch-1/CBF-1-mediated transcriptional activity in myogenic and neuronal stem cells (24). We have confirmed these effects in lung adenocarcinoma cells, and we have shown that in hypoxic conditions Notch-1 provides essential survival signals to lung adenocarcinoma cells (25). In this regard, it should be underscored that MM is a severely hypoxic malignancy (26).

In a previous study, we showed that Notch-1 activation was required during the process of SV40 transformation of primary

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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human mesothelial cells (HM; ref. 27). Inhibition of Notch-1 activation led to growth arrest of SV40-transformed HM (27). In this study, we analyzed the role of Notch signaling in MM in general (e.g., irrespective of SV40). We found that Notch-1 and Notch-2 have opposite effects on MM cell survival. Notch-1 led to phosphatase and tensin homologue (PTEN) down-regulation, Akt phosphorylation, and increased DNA synthesis, whereas Notch-2 signaling led to PTEN activation, Akt dephosphorylation, and MM cell death. Activation of Notch-2 also led to depressed DNA synthesis and cell proliferation due to p21^{WAF1/Cip1} accumulation.

Materials and Methods

Cell culture, hypoxia, γ -secretase inhibitor, and RNA studies.

Primary HM cultures were obtained and characterized from ascites fluids obtained from noncancerous patients as previously described (28). HM were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Mesothelioma cell lines (ME) were obtained and characterized from primary tumors as previously described (29). ME were grown in DMEM supplemented with 10% FBS. All cells were fingerprinted using the GenePrint fluorescent STR system (Promega). Cells grown in hypoxia were maintained in chambers (Stem Cell Technologies) filled with certified

1% O₂, 5% CO₂, and 94% N₂ (Airgas North Central) at 37°C. Oxygen concentration was measured with MiniOX1 oxygen meters (Mine Safety Appliances Company).

The γ -secretase inhibitor MRK-003 was dissolved in DMSO to make 40 mmol/L stock solutions.

Laser microdissection was done on 8 μ m-thick frozen MM and normal lung specimens using a Pixcell II Laser Capture Microdissection System (Arcturus Engineering, Inc.). Normal human pleura (HP) was captured from normal lung biopsies after identifying mesothelial cells using the HistoGene LCM Immunofluorescence Staining Kit (Molecular Devices) with some modifications. Briefly, we used a biotinylated primary antibody specific for mesothelin following the manufacturer's protocol. Cy3-streptavidin was replaced with horseradish peroxidase-conjugated streptavidin, and color was developed in 3' using the 3,3'-diaminobenzidine substrate (Vector). Samples were rapidly dehydrated and processed for laser capture microdissection. MM specimens were stained using the HistoGene LCM frozen section staining kit (Arcturus) and microdissected as described above. We collected ~500 cells for each sample. RNA was extracted using the PicoPure RNA isolation kit (Arcturus) and amplified using the RiboAmp RNA amplification kit (Arcturus; one fourth of the original total RNA preparation was used directly for 18S rRNA normalization). Total RNA was purified from cultured cells using the RNeasy Mini Kit (Qiagen). Reverse transcription and real-time PCR analysis were done as described (25).

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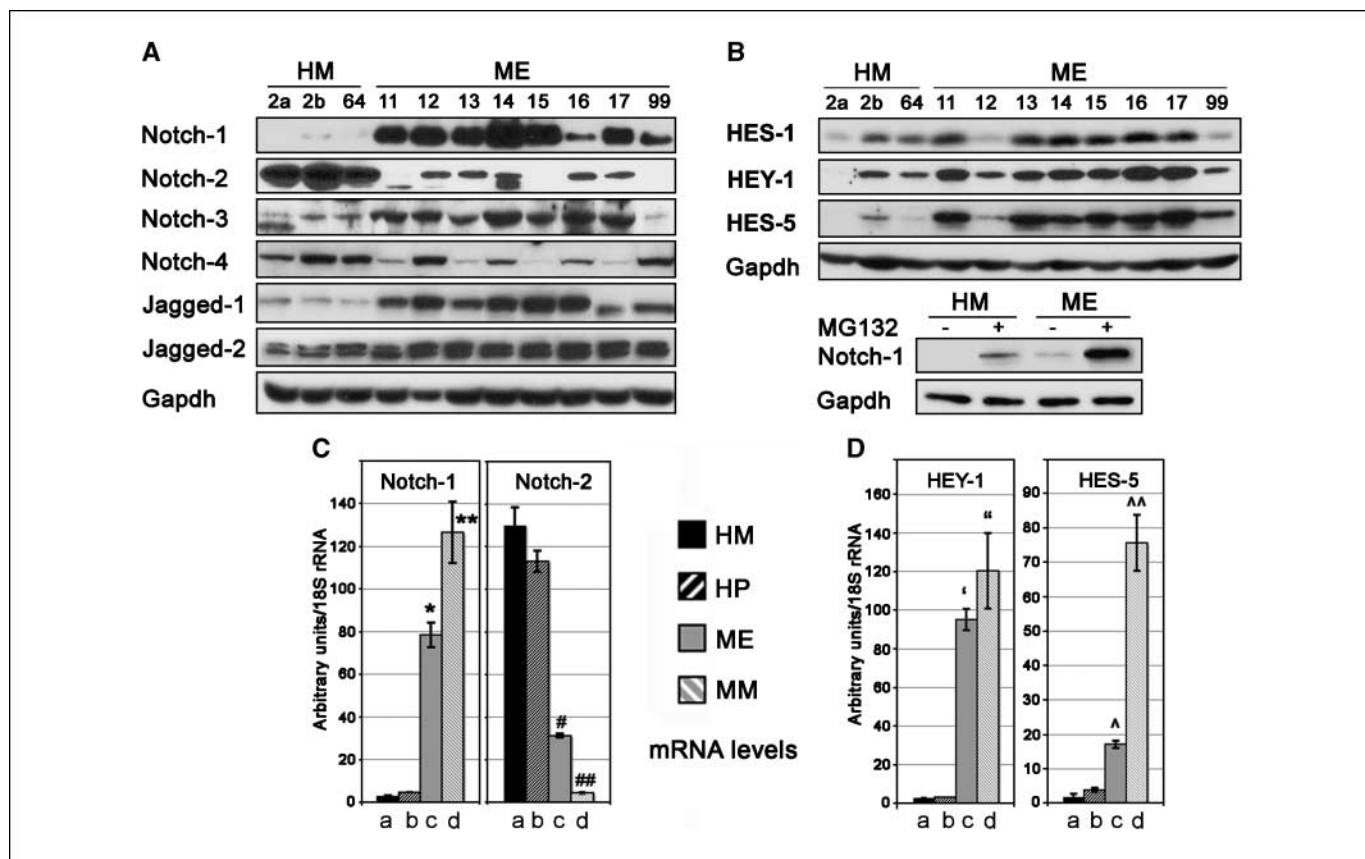


Figure 1. Expression of Notch signaling components in primary HM cultures, ME cells, microdissected HP, and microdissected MM. *A*, Western blot analysis done on three independent HM and eight ME of the indicated gene products. Note up-regulated Notch-1 receptor and Jagged-1 ligand in ME compared with HM. Notch-3 also seems to be up-regulated in ME compared with HM. *B*, *top*, Western blot analysis of the Notch downstream targets HES-1, HEY-1, and HES-5 in HM and ME. Note up-regulation of all three Notch downstream effectors in ME compared with HM. *Bottom*, Western blot analysis of Notch-1^{IC} in HM2b and ME17 in the presence (+) and in the absence (-) of the proteasome inhibitor MG132 (final concentration, 10 μ mol/L). Cell extracts were prepared 24 h after MG132 addition. *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase. *C*, real-time PCR analysis done on RNA extracted from HM (columns a), HP (columns b), ME (columns c), and MM (columns d). Expression of the indicated mRNAs was normalized for 18S rRNA expression of each sample. Columns, average from four independent experiments (four microdissected frozen lungs, four microdissected frozen MM, four HM, and four ME); bars, SD. *, $P > 0.0001$, ME versus HM; **, $P = 0.0004$, MM versus HP; #, $P = 0.0001$, ME versus HM; ###, $P > 0.0001$, MM versus HM (*t* test). *D*, real-time PCR analysis done on RNA extracted from HM, HP, ME, and MM. Expression of the indicated mRNAs was done and normalized as described in *C*. †, $P > 0.0001$, ME versus HM; #, $P = 0.0012$, MM versus HP; ^, $P > 0.0001$, ME versus HM; ^^, $P = 0.0004$, MM versus HP (*t* test).

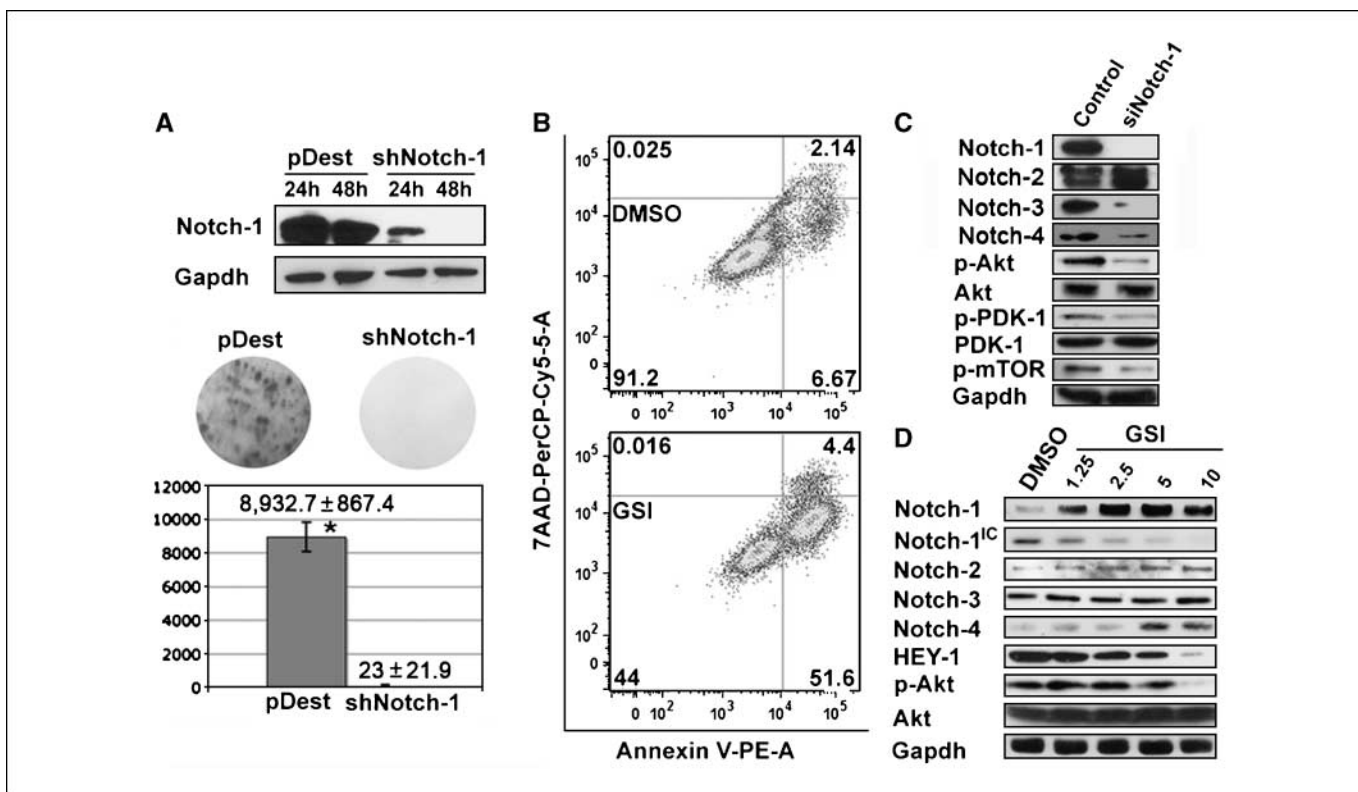


Figure 2. Down-regulation of Notch-1 in ME (obtained either through RNA interference or chemical inhibition) leads to cell death both in normoxia and hypoxia. **A**, representative down-regulation of Notch-1 expression obtained using a lentiviral vector that expresses shNotch-1 on stimulation with doxycycline. *Top*, Western blot analysis of Notch-1 in shNotch-1 and control cells (pDest) 24 and 48 h after doxycycline addition. *Middle*, crystal violet staining of either shNotch-1 and control cell cultures 5 d after doxycycline treatment. *Bottom*, quantification of four independent experiments (10,000 alive cells were plated in each 100-mm dish before doxycycline stimulation); bars, SD. *, $P > 0.0001$. The experiments were done in a standard incubator (21% oxygen, 5% CO₂). **B**, representative Annexin V/7AAD staining of a ME treated either with DMSO (*top*) or with 5 μmol/L of the γ-secretase inhibitor (GSI) MRK-003 in DMSO (*bottom*). All cells were cultured under hypoxia. **C**, Western blot analysis of the indicated gene products of ME14 treated either with a control siRNA (*Control*) or with a siRNA targeting the Notch-1 mRNA (*siNotch-1*). The analysis was done 48 h after transfection. All cells were cultured under hypoxia. **D**, Western blot analysis of the indicated gene products of ME16 treated with the specified concentrations of the γ-secretase inhibitor MRK-003. Analysis was done 24 h after MRK-003 treatment. All cells were cultured in hypoxia.

Briefly, Quantitative real-time PCR was done with SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7300 thermal cycler (Applied Biosystems). For each sample, a serial dilution of cDNA template was measured in triplicate. Non-reverse transcription reactions served as controls. All measurements were normalized for 18S rRNA. Comparison between groups was analyzed by Student's *t* test, with $\alpha < 0.05$.

Plasmids and lentiviral systems. Sequences of oligonucleotides used in this study are provided in Supplementary Table S1. A list of antibodies used in this study is reported in Supplementary Table S2. Notch-1^{IC} and Notch-2^{IC} cloned into pcDNA3.0 (Invitrogen) have previously been described (25). The same inserts were subcloned into pLenti4/TO/V5-DEST to generate the Notch-1^{IC}-DEST and Notch-2^{IC}-DEST to obtain doxycycline-inducible expression of either Notch-1^{IC} or Notch-2^{IC} in lentiviral vectors (the backbone system was the ViraPower T-Rex Lentiviral System; Invitrogen).

A short hairpin targeting Notch-1 (shNotch-1) was obtained by annealing complementary DNA oligonucleotides (25); the resulting double-stranded DNA was ultimately cloned in pLenti4/TO/V5-DEST to obtain a tetracycline-inducible lentiviral system conditionally expressing shNotch-1.

We generated four stable, tetracycline-inducible ME expressing the genes of interest under the control of the tetracycline regulator as follows. First, cells were transduced with the tetracycline regulator lentivirus and selected with blasticidin (3 μg/mL); then transduced with Notch-1^{IC}, Notch-2^{IC}, or shNotch-1; and finally selected with zeocin (200 μg/mL) according to the manufacturer's instructions. Cells transduced with the empty pLenti4/TO/V5-DEST lentiviral vectors (abbreviated pDest in most figures) were used as the controls. In different sets of experiments, we artificially down-regulated Notch-1 and Notch-2 expression using commercially available small

interfering RNAs (siRNA; Santa Cruz Biotechnologies). The nonspecific siRNA (negative control) was commercially available (Santa Cruz Biotechnologies) and is described in detail in Supplementary data. Constitutively active Akt (NH₂-terminal myristoylatable Akt) cloned into the pUSEamp(+) expression plasmid (abbreviated as pUSE in the figures) was purchased from Upstate (Millipore). Transient transfections were done using an electroporator (Gene Pulser II, Bio-Rad) under the following parameters: 300 kV, 975 μF capacity; 1 μg of plasmid DNA/10⁶ cells. Efficiency of transfection was >95%.

Flow cytometry and protein studies. Cells handled in different experimental settings were analyzed by flow cytometry on Annexin V-phycoerythrin (BD Pharmingen)/7-aminoactinomycin D (7-AAD; Sigma) staining, propidium iodide staining (Sigma), or bromodeoxyuridine (BrdUrd) incorporation (FITC BrdU Flow kit, BD Pharmingen). Fluorescence-activated cell sorting analysis was done on a BDFACSCanto instrument (Becton Dickinson) measuring 30,000 events for each sample. Western blot analyses were done as described (25).

Results

Both mesothelioma cell lines and specimens have elevated Notch-1 expression compared with their normal counterparts. We first measured the expression levels of Notch receptors, ligands, and downstream Notch targets in eight ME compared with three HM. Western blot analyses showed that all ME had increased Notch-1, Jagged-1, and, to a lesser extent, Notch-3 expression levels compared with HM (Fig. 1A). According to the pattern of

expression of Notch receptors and ligands, ME displayed increased expression of the Notch downstream targets HES-1, HEY-1, and HES-5 compared with HM (Fig. 1B, top). Treatment of ME with the proteasome inhibitor MG132 led to an increase of Notch-1^{IC} expression, suggesting activation followed by degradation (30) in these cells compared with HM (Fig. 1B, bottom). ME seemed to have significantly decreased (or abolished) Notch-2 expression compared with HM (Fig. 1A). Notably, we detected Notch-2 immunoreactivity in only one of 25 frozen MM specimens analyzed, whereas the same samples tested positive for Notch-1^{IC} staining, indicating that in MM samples Notch-2 protein is poorly or not expressed (Supplementary Fig. S1). These results were confirmed at the mRNA level. ME and MM cells had substantially higher levels of Notch-1 mRNA compared with HP, whereas the opposite was measured with Notch-2 mRNA (Fig. 1C). Based on HEY-1 and HES-5 (Fig. 1D) results, Notch signaling is elevated (>10-fold) in ME and MM compared with HP, and Notch-1 seems to play a predominant role in this activation. The RNA analysis also confirmed that our ME cells were a good *in vitro* model for MM cells because the various mRNA expression patterns seemed to be very similar in ME and MM cells.

Notch-1 inhibition (either through genetic manipulation or by using a chemical inhibitor) causes ME cell death in both normoxia and hypoxia. To further evaluate the role of Notch-1 expression in mesothelioma tissues (ME and MM), we studied the biological effects of artificial inhibition of Notch-1 signaling in ME. We used a tetracycline-inducible lentiviral system that, on doxycycline administration, expressed a short hairpin RNA targeting the Notch-1 mRNA (shNotch-1). Notch-1 knockdown caused complete ME cell death both in normoxia (Fig. 2A) and under hypoxia (data not shown). Treatment of ME cells with the γ -secretase inhibitor MRK-003 also induced cell death; however, the effects seemed to be greater under hypoxia. This is reflected by a 6.72 ± 0.58 -fold increase of Annexin V staining of various ME treated with MRK-003 (5 $\mu\text{mol/L}$) cultured under hypoxia (Fig. 2B) compared with a 3.22 ± 0.49 -fold ($P < 0.01$) increase in cell death observed in normoxia (these numbers reflect averages and SDs of three experiments; data not shown). Both artificial down-regulation of Notch-1 and MRK-003 treatment seemed to depress the Akt pathway activity. Artificial down-regulation of Notch-1 using a siRNA targeting Notch-1 under hypoxia caused decreased phos-

phorylation of phosphoinositide-dependent kinase 1 (PDK-1), Akt, and the downstream effector mammalian target of rapamycin (mTOR; Fig. 2C). Down-regulation of Notch-1 through siRNA caused increased expression of Notch-2 and decreased expression of Notch-3 and Notch-4. This suggested that Notch-1 may negatively regulate Notch-2 while positively regulating Notch-3 and Notch-4 expression in ME. MRK-003 led to a dose-dependent accumulation of the transmembrane (uncleaved) form of Notch-1 and a progressive decrease of Notch-1^{IC} and HEY-1 expression (all indicators of effective Notch-1 inhibition; Fig. 2D). These effects were paralleled by loss of Akt phosphorylation on Ser⁴⁷³ (Fig. 2D). The increase in transmembrane (uncleaved) Notch-1 after MRK-003 treatment was most pronounced compared with the other Notch receptors (Fig. 2D). This suggested that Notch-1 was the major Notch signaling receptor stabilized by the γ -secretase inhibitor MRK-003 in ME. MRK-003-induced cell death was substantially (~50%) rescued in ME transfected with a constitutively active form of Akt (myrAkt; Supplementary Fig. S2A). This rescue was paralleled by reactivation of Akt signaling as indicated by increased phosphorylated mTOR expression in myrAkt-transfected cells (Supplementary Fig. S2B). Similar observations were made when we down-regulated Notch-1 in ME cells with a siRNA. ME cells transfected with siRNA targeting the Notch-1 mRNA displayed decreased Notch-1 protein expression (Supplementary Fig. S2C, lanes b and d) and reduced viability under hypoxia (Supplementary Fig. S2D, column b). Cotransfection of the same cells with myrAkt still resulted in decreased Notch-1 expression, but viability was restored in these cells (Supplementary Fig. S2D, column d). These observations further confirmed that Notch-1 provides survival signals to ME under hypoxia mainly through the Akt signaling pathway, and that a depressed level of Akt activation contributes to ME cell death on Notch-1 down-regulation, obtained through either genetic manipulation or chemical inhibition.

Notch-2 signaling contrasts the Notch-1 prosurvival signals in ME cells. In contrast to the elevated levels of Notch-1 in ME and MM, we observed reduced (or suppressed) Notch-2 expression in both ME and MM samples compared with their normal counterparts (Fig. 1; Supplementary Fig. S1). To understand the biological effects elicited by Notch-2 signaling in ME, we reexpressed constitutively active Notch-2^{IC} using both transient transfection and controlled induction of Notch-2^{IC} with an inducible lentiviral

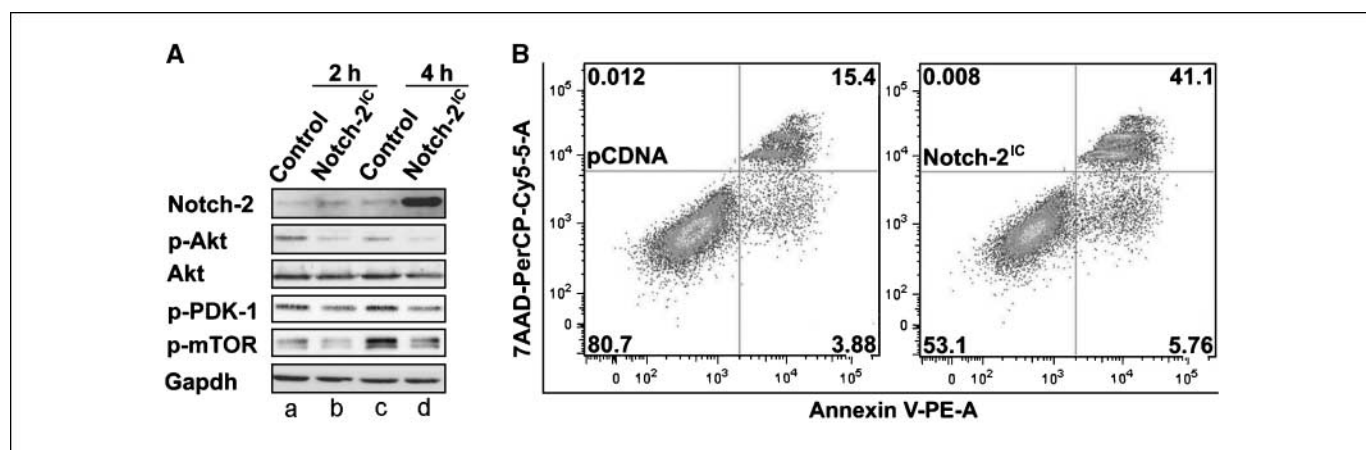


Figure 3. Reintroduction of Notch-2^{IC} causes ME cell death through depression of the Akt/mTOR signaling pathway. **A**, Western blot analysis of the indicated gene products of ME16 transfected with a control plasmid or with a plasmid expressing Notch-2^{IC}. In the experiments presented here, ME were cultured in normoxia. The same experiments done under hypoxia gave identical results. **B**, Annexin V/7AAD staining of ME16 transfected with either a control plasmid (pCDNA) or a plasmid expressing Notch-2^{IC} (Notch-2^{IC}).

system. These two expression strategies were evaluated to account for possible differences in the levels of expression of Notch-2^{IC}. Notch-2^{IC} expression using low concentrations (0.2 μg/mL) of doxycycline yielded a modest increase in protein expression levels in Notch-2^{IC} cells compared with controls (Fig. 3A, compare lanes *a* and *b*). Nevertheless, Notch-2^{IC}-expressing cells showed a 3-fold reduction of phosphorylated Akt 2 h after induction, an event paralleled by a reduction in phosphorylated mTOR (Fig. 3A). These levels of Notch-2^{IC} induction were sufficient to elicit a 2.4-fold increase in cell death 24 h after induction (Fig. 3B). These observations indicated that Notch-2 signaling induced ME cell death, possibly through depression of Akt activity. To test this hypothesis, we designed the following experiment. ME transduced with either a control lentivirus (pDest cells; Fig. 4A, lanes *a-d*) or a tetracycline-inducible lentiviral vector expressing Notch-2^{IC} (Notch-2^{IC} cells; Fig. 4A, lanes *e-h*) were transiently transfected with either a control plasmid (pUSE; Fig. 4A, lanes *a, b, e, and f*) or a plasmid expressing a constitutively active Akt (myrAkt; Fig. 4A, lanes *c, d, g, and h*). These cells were then exposed to either 0.5 μg/mL doxycycline or medium alone (+ and – symbols in Fig. 4A). Twenty-four hours after treatment, the expression levels of Notch-2, phosphorylated mTOR, and total Akt were measured by Western blot analysis (Fig. 4A). In parallel, the different cell populations were assayed for apoptosis using Annexin V/7-AAD staining (Fig. 4B). The results showed that cells expressing elevated levels of Akt (corresponding to the cells transfected with myrAkt) had increased phosphorylation of mTOR compared with controls only if Notch-2^{IC} was not induced (Fig. 4A, compare lanes *c* and *d* with lanes *a* and *b*). Induction of Notch-2^{IC} efficiently depressed Akt phosphorylation (Fig. 4A, compare lanes *e* and *f*). Coexpression of Notch-2^{IC} and myrAkt resulted in intermediate levels of mTOR phosphorylation (Fig. 4A, lane *h*). As expected, the cells that displayed the highest levels of apoptosis were those expressing Notch-2^{IC} in the absence of myrAkt (Fig. 4B, column *f*). On the other hand, expression of myrAkt in the presence of Notch-2^{IC} rescued cells from Notch-2^{IC}-induced programmed cell death (Fig. 4B, column *h*) although it did not fully restore the level of phosphorylation of the Akt downstream target mTOR. These results confirmed that Notch-2 signaling induced programmed cell death in ME, and that this effect was largely mediated through modulation of Akt activity because Notch-2^{IC} expression depressed Akt signaling and the introduction of a constitutively active Akt mutant reversed the proapoptotic effects induced by Notch-2^{IC} under hypoxia. Secondary mechanisms of Notch-2^{IC}-mediated promotion of apoptosis in ME could derive from prolonged phosphorylation of c-jun NH₂-terminal kinase and increased expression of Bax (Supplementary Fig. S3).

Notch-1 and Notch-2 have opposite effects on the Akt signaling pathway because of their opposite regulation of PTEN. Phosphorylation of Akt results from the activation of phosphatidylinositol 3-kinase (PI3K) in response to various growth factors or adhesion molecules. PI3K, in turn, generates the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃), which recruits the kinase PDK-1 to the plasma membrane and stimulates PDK-1-dependent phosphorylation of Akt (reviewed in ref. 31). PIP₃ is the major substrate of multifunctional phosphatase PTEN; therefore, PTEN acts as a major tumor suppressor gene by reducing the cellular amounts of a second messenger that induces Akt activation, which in turn promotes cell survival and proliferation (31). A recent study showed that loss of PTEN leads to resistance to Notch inhibition in some T-ALL cells (32), first suggesting a link between Notch-1 oncogenicity and PTEN activity. We investigated

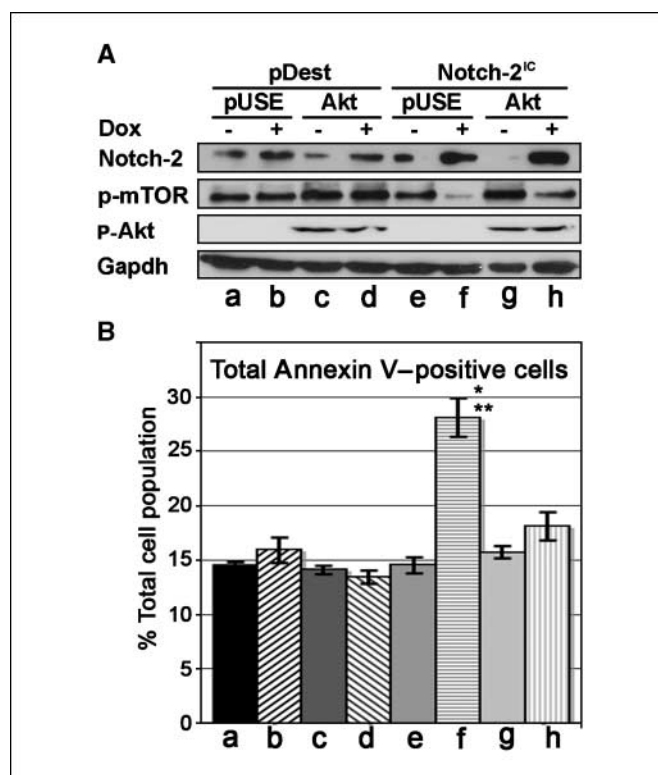


Figure 4. Coexpression of active Akt and Notch-2^{IC} rescues ME from Notch-2^{IC}-induced apoptosis. **A**, Western blot analysis of the indicated gene products of a representative ME transduced with a control lentiviral vector (pDest) or with a lentiviral vector that expressed Notch-2^{IC} on doxycycline (Dox) treatment and transfected with a control plasmid (pUSE) or with a plasmid expressing myristylated, active Akt (see text for details). **B**, Annexin V/7AAD staining of the cells treated as in **A**. Columns, average of total Annexin V-positive cells from three independent experiments; bars, SD. Each column has been identified with the letter corresponding to each lane shown in **A**. *, $P = 0.002$, *f* versus *e*; **, $P = 0.005$, *f* to *e* versus *h* to *g*.

the possibility that Notch-1 and Notch-2 activation could have had opposite effects on Akt phosphorylation due to opposite regulation of the PTEN gene. This was analyzed both at the protein and mRNA levels. We overexpressed Notch-1^{IC} in ME using a doxycycline-inducible lentiviral vector. When we induced Notch-1^{IC}, we observed a 3.1-fold reduction in PTEN protein levels (Fig. 5A, compare lanes *b* and *d*). Doxycycline seemed to nonspecifically up-regulate PTEN in these cells, but this effect was mostly suppressed by Notch-1^{IC} overexpression. We measured the PTEN mRNA levels in ME transiently transfected with a plasmid expressing Notch-1^{IC} cells, and we observed a 2.4-fold reduction of the PTEN mRNA (Fig. 5C). When we decreased Notch-1 expression using a siRNA targeting Notch-1 in the same cells, we observed a 4.4-fold increase of PTEN protein expression (Fig. 5A, compare lanes *e* and *f*). Opposite effects were observed when we expressed Notch-2^{IC} in the same ME cells. Doxycycline-induced expression of Notch-2^{IC} caused a 4.2-fold increase in PTEN expression (Fig. 5B), paralleled by a 3.2-fold increase of the PTEN mRNA levels (Fig. 5D). Notch-2 seemed to regulate PTEN expression also in HM. Artificial down-regulation of Notch-2 using a siRNA targeting the Notch-2 mRNA in HM caused decreased PTEN expression (Supplementary Fig. S4). Combined, the data suggested that the opposite effects observed by Notch-1 and Notch-2 signaling on Akt activation in ME and survival of said cells could have resulted from the opposite

regulation of these two Notch isoforms of the PTEN promoter. To ascertain whether such Notch regulation was direct, we assayed the PTEN promoter region by chromatin immunoprecipitation assay. More specifically, we probed the 2,646-bp-long region between positions -3,033 and -387 of the PTEN promoter (+1 is the PTEN initiation codon) using 11 partially overlapping amplicons derived by the use of 11 oligo pairs (Supplementary Table S1). We coimmunoprecipitated a 215-bp DNA fragment of the PTEN promoter (positions -602 to -387) alongside Notch-1 in ME14 cultured under hypoxia in three independent experiments (Supplementary Fig. S5). Because this DNA fragment contains a CBF-1 consensus sequence, these results suggested direct Notch regulation of the PTEN promoter in ME cells.

Notch-1 and Notch-2 have opposing effects on DNA synthesis activity and on proliferation of ME and HM. Having determined that Notch-1 and Notch-2 signaling had opposite regulation of cell survival of ME acting through the PIP₃/PTEN/Akt axis, we then asked whether these two Notch receptors could have affected other biological parameters in HM and ME, with a special focus on proliferative potential. We induced expression of Notch-1^{IC} using our doxycycline-inducible lentiviral system in HM and observed no effect on the rate of apoptosis in these cells (data not shown). On the other hand, we measured a significant increase of DNA synthesis as measured by BrdUrd incorporation assay (Supplementary Fig. S6A), which coincided with increased Akt, PDK-1, and mTOR phosphorylation (Supplementary Fig. S6B). These results suggested that Notch-1 activation in HM could support a protransformation environment. Interestingly, down-regulation of Notch-2 in HM cells obtained with a siRNA targeting Notch-2 also resulted in a substantial increase in DNA synthesis in HM (Supplementary Fig. S7A), and these effects were also paralleled by increased phosphorylation of Akt, PDK-1, and mTOR (Supplementary Fig. S7B). Taken together, these data suggest that in HM, stimulation of Notch-1 expression and/or depression of Notch-2 expression may favor cell transformation.

In ME, Notch-2 reexpression seemed to have opposite effects

compared with Notch-1 induction in HM: Notch-2^{IC}-expressing ME cells were significantly arrested in G₁ (Fig. 6B), an event paralleled by a 3-fold increase in p21 expression levels (Fig. 6A). Moreover, Notch-2^{IC}-expressing ME cells incorporated significantly less DNA compared with controls (Fig. 6C).

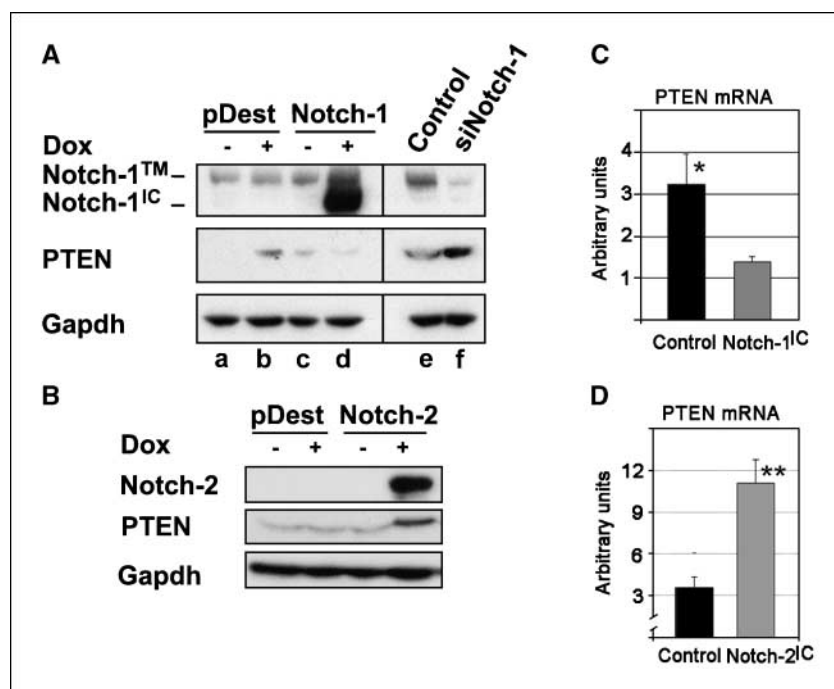
Collectively, our results suggest that Notch-1 plays a pro-oncogenic function in MM, whereas Notch-2 activity seems to have a tumor-suppressive role in this malignancy. It seems that these activities predominantly affect MM cell survival. Additionally, it seems that Notch-1 and Notch-2 affect the proliferation potential of mesothelial cells. It should be noted that mesothelial cells, transformed or not, seem to finely regulate the reciprocal expression levels of Notch-1 and Notch-2. When we induced Notch-1^{IC} expression in HM, the expression level of Notch-2 was depressed (Supplementary Fig. S8A). Artificial up-regulation of Notch-1 and Notch-2 in ME slightly affected Notch-3 expression (more specifically, Notch-1^{IC} overexpression led to ~50% increase in Notch-3 expression, whereas Notch-2^{IC} overexpression led to a 2.4 decrease in Notch-3 expression; Supplementary Fig. S8C and D). The same genetic manipulations did not seem to substantially affect the Notch-4 expression levels.

Discussion

In this study, we show that Notch-1 signaling is required for MM cell survival. This phenomenon is mediated by affecting the PIP₃/Akt/mTOR signaling pathway through regulation of PTEN expression. These results confirm what was observed in T-ALL cells (32) and strongly suggest that the Notch-1 and PI3K/Akt cross talk may be of general validity in cancer and may have far-reaching therapeutic implications.

In contrast to Notch-1, we found that Notch-2 plays a tumor-suppressive role in MM by depressing the PIP₃/PTEN/Akt axis. Such opposing roles of Notch-1 and Notch-2 in cancers are not unprecedented, as it is known that the different Notch receptors display diversity in their functions (33). In breast cancer, there is strong evidence that Notch-1 and Notch-4 are oncogenic, whereas

Figure 5. Notch-1 and Notch-2 have opposite effects on PTEN expression both at the protein and mRNA levels. **A**, Western blot analysis of the indicated gene products of ME14 transduced with either a control lentivirus (*pDest*) or a lentiviral vector that expressed Notch-1^{IC} on doxycycline treatment (*Notch-1*; lanes *a-d*) or ME transfected with either a control siRNA (*Control*) or a siRNA targeting the Notch-1 mRNA (*siNotch-1*; lanes *e* and *f*). **B**, Western blot analysis of the indicated gene products of ME14 transduced with either a control lentivirus (*pDest*) or with a lentiviral vector that expressed Notch-2^{IC} on doxycycline treatment (*Notch-2*). Note clone-specific effects of doxycycline exposure on PTEN expression (compare *A* with *B*). **C**, real-time PCR analysis of the PTEN mRNA measured in ME14 transfected with a control plasmid (*black column*) or with a plasmid expressing Notch-1^{IC} (*gray column*). Bars, SD of four independent experiments (ME14, ME15, ME16, and ME17). **D**, real-time PCR analysis of the PTEN mRNA measured in ME cells transfected with a control plasmid (*black column*) or with a plasmid expressing Notch-2^{IC} (*gray column*). Bars, SD of four independent experiments (ME14, ME15, ME16, and ME17). PTEN mRNA levels were normalized for 18S rRNA expression in each experiment. *, *P* > 0.01; **, *P* > 0.0001. All experiments were done in hypoxia.



Notch-2 may play a tumor-suppressive role (34). Biologically, there is evidence that Notch-2 has opposite effects compared with Notch-1 and Notch-4 in breast cancer MDA-MB231 cells (34). Examples of opposite effects of Notch-2 and Notch-1 have been discovered in multiple myeloma (35) and embryonal brain tumors (36). Biochemically, there is published evidence that the transcriptional activity of Notch-2 is dramatically lower than that of Notch-1 or Notch-3 (33). Moreover, expression of Notch-2 together with either Notch-1 or Notch-3 inhibits their transcriptional activity (33). The molecular mechanism(s) for these differential effects is not known. It should be pointed out that the postulated trans-activation domain is the least conserved region between Notch homologues (37). It has been proposed that all four Notch homologues bind to CBF-1 (38). It is possible that Notch-2 is not as effective as Notch-1 or Notch-3 either in stabilizing the interaction between Mastermind-like 1 (MAML1) and CSL or in recruiting coactivators such as p300 to the Notch transcriptional complex. Alternatively, it is possible that Notch-2 does not displace corepressors such as SMRT from CSL. The molecular composition of the Notch transcriptional complex including Notch-1 or Notch-2 at the PTEN promoter will have to be determined in future studies.

It is reasonable to suggest that the cell environment will also contribute to the role each Notch receptor will play in any given tumor, and that in certain contexts Notch-1 and Notch-2 can have opposite biological effects. The mechanism of these differing effects is still unclear. It is not clear whether Notch-2 acts by antagonizing Notch-1 (e.g., by competing for coactivators and CBF-1) or the two homologues have independent or opposing effects on certain downstream targets.

Because Notch signaling does not rely on amplification phosphorylation cascades (such as the extracellular signal-regulated kinase signaling pathway), the strength and duration of Notch stimulation can play a critical role. We have shown, as have others, that microenvironmental condition, such as hypoxia, can also alter the biological significance of Notch signaling (24, 25). For these reasons, we have tested our cell systems using different levels of expression, different durations of Notch activation, and different oxygen concentrations. Although quantitatively variable, the results invariably led to the same qualitative conclusions (e.g., Notch-1 plays a pro-oncogenic role in MM, whereas Notch-2 seems to be oncosuppressive in this malignancy).

Chromatin immunoprecipitation assays suggested that Notch-1 could directly regulate PTEN promoter in ME. The fragment we coimmunoprecipitated includes the CBF-1 consensus sequence (TGGGAA) that has previously been shown to be bound by CBF-1 in gel mobility assays *in vitro* (38). Using luciferase assays and reverse transcription-PCR analysis, Whelan and colleagues (39) showed Notch-1 direct regulation of the PTEN promoter mediated by CBF-1 binding in the same region where we immunoprecipitated Notch-1 in chromatin immunoprecipitation assays, thus supporting our data. On the other hand, in T-ALL cells, Notch-1 seems to regulate this promoter indirectly through HES-1 (32). It is possible that these apparent differences may be tissue specific.

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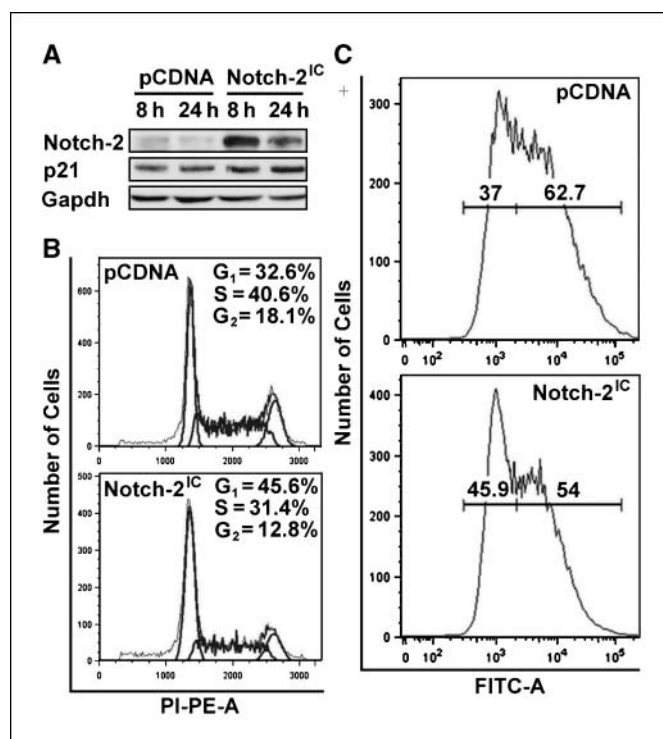


Figure 6. Notch-2 reexpression causes G_1 arrest and decreased DNA synthesis in ME. Representative experiments. **A**, Western blot analysis of the indicated gene products in ME transfected with a control plasmid (*pCDNA*) or with a plasmid expressing Notch-2^{IC} (*Notch-2^{IC}*). **B**, propidium iodide staining of ME 24 h after transfection with the control plasmid (*top*) or with the plasmid expressing Notch-2^{IC} (*bottom*). Percentages of cells in the various phases of the cell cycle are reported in the figure. **C**, BrdUrd incorporation of ME 24 h after transfection with the control plasmid (*top*) or with the plasmid expressing Notch-2^{IC} (*bottom*). All experiments were done in hypoxia.

Notch-1 inhibition using the γ -secretase inhibitor MRK-003 seemed to induce apoptosis of ME most efficiently in hypoxic conditions (Fig. 2B). This may have profound therapeutic implications because MM is highly hypoxic (26). The availability of a drug that specifically targets these malignant cells in hypoxia may prove to be ideal for future therapeutic regimens, and may lead to a more rational design of combination therapies.

Disclosure of Potential Conflicts of Interest

P.R. Strack: ownership interest, Merck Research Laboratories.

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