Critical Role of Notch Signaling in Osteosarcoma Invasion and Metastasis

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Abstract

Purpose: Notch signaling is an important mediator of growth and survival in several cancer types, with Notch pathway genes functioning as oncogenes or tumor suppressors in different cancers. However, the role of Notch in osteosarcoma is unknown.

Experimental Design: We assessed the expression of Notch pathway genes in human osteosarcoma cell lines and patient samples. We then used pharmacologic and retroviral manipulation of the Notch pathway and studied the effect on osteosarcoma cell proliferation, survival, anchorage-independent growth, invasion, and metastasis *in vitro* and *in vivo*.

Results: Notch pathway genes, including Notch ligand *DLL1, Notch1* and *Notch2*, and the Notch target gene *HES1*, were expressed in osteosarcoma cells, and expression of *HES1* was associated with invasive and metastatic potential. Blockade of Notch pathway signaling with a small molecule inhibitor of γ secretase eliminated invasion in Matrigel without affecting cell proliferation, survival, or anchorage-independent growth. Manipulation of Notch and HES1 signaling showed a crucial role for HES1 in osteosarcoma invasiveness and metastasis *in vivo*.

Conclusion: These studies identify a new invasion and metastasis-regulating pathway in osteosarcoma and define a novel function for the Notch pathway: regulation of metastasis. Because the Notch pathway can be inhibited pharmacologically, these findings point toward possible new treatments to reduce invasion and metastasis in osteosarcoma.

Osteosarcoma is the third most common cancer in childhood and adolescents and the most common cancer of bone (1). With combination treatment (neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy), the 5-year survival for patients who do not have metastatic disease at diagnosis is 60% to 70% (2–4). However, for patients who present with metastatic disease or whose tumor recurs, outcomes are far worse at <30% and <20% survival, respectively (5). Pulmonary metastasis is the predominant site of osteosarcoma recurrence and the most common cause of death. Unfortunately, survival has not improved for 20 years despite multiple clinical trials with increased intensity, and further gains with refinements in cytotoxic chemotherapy regimens alone are unlikely. Thus, new therapeutic targets and approaches must be sought.

Our knowledge of the mechanistic control of invasion and metastasis in osteosarcoma is limited. Only Ezrin and Fas have been linked mechanistically to osteosarcoma metastasis. Ezrin is a cell membrane–cytoskeleton linking protein that allows the cell to interact with the microenvironment and facilitates signal transduction (6). Impaired Fas signaling may allow osteosarcoma cells to evade host resistance in the lung (7). Thus far, neither Fas nor Ezrin has been a likely target for drug therapy, illustrating the need for new therapeutic targets for osteosarcoma. ERBB2 (Her-2) has been shown to associate with poor clinical outcome of osteosarcoma (8). Other ERBB family member proteins, epidermal growth factor receptor, and nuclear Her4, are also expressed in osteosarcoma (9). Inhibition of ERBB family signaling by CI-1033 (pan-ERBB inhibitor) induces cell growth inhibition and apoptosis (10), suggesting that ERBB signaling is a potential therapeutic target for osteosarcoma.

Notch signaling plays a key role in the normal development of many tissues and cell types through diverse effects on cell fate decision, stem cell renewal, differentiation, survival, and proliferation (11). The Notch signaling pathway includes Notch ligands, receptors, negative and positive modifiers, and Notch target transcription factors. The *Notch* genes (*Notch1-Notch4*), originally identified by homology to a single *Notch* gene from *Drosophila*, encode highly conserved cell surface receptors (12, 13). After activation by ligand binding, the Notch proteins are proteolytically cleaved in two steps by ADAM10 and γ-secretase, after which the intracellular domain of Notch (ICN) is translocated to the nucleus (14). Nuclear ICN interacts

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Received 8/13/07; revised 12/4/07; accepted 2/26/08.

Grant support: Physician Scientist Awards from University of Texas M. D. Anderson Cancer Center (D.P.M. Hughes and P.A. Zweidler-McKay), NIH grant 5K08CA118730-02 (D.P.M. Hughes), Jori Zemel Children's Bone Cancer Foundation and Hope Street Kids Foundation (P. Zhang), and NIH grant 5P30CA016672-32 (flow cytometry core laboratory).

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doi:10.1158/1078-0432.CCR-07-1992

with the transcription factor CSL (15), also known as RBP-Jκ, and the mastermind-like protein, which leads to transcriptional activation of CSL target genes (16). These include the basic helix-loop-helix transcription factors of the *Hes* family and Hes-related repressor proteins, e.g., HES1, HES5, HERP2 (15). Hes family and Herp-related repressor proteins are transcriptional repressors. Independent of CSL activity, intracellular Notch receptors can interact with the Notch target protein DELTEX1 (DTX1; ref. 17), which modulates Notch-mediated transcription and promotes feedback inhibition of Notch pathway signaling.

Notch signaling is aberrantly activated in a variety of human cancers, including T-cell acute lymphoblastic leukemia, lung, colorectal, prostate, and breast carcinomas (18–21). More recently, a tumor suppressor role for Notch signaling has been identified in B-cell malignancies (22), neural crest tumors (23), and skin cancer (24). Therefore, Notch signaling seems to function as an oncogene or a tumor suppressor, depending on the cellular context. Pharmacologic manipulation of Notch signaling is becoming a new strategy for human cancers. γ-Secretase inhibitors (GSI), originally developed for Alzheimer's disease, can inhibit the proteolytic processing of Notch receptors by γ-secretase, which is essential for Notch activation (25), and are being investigated clinically in T-cell leukemia and breast cancer.

Notch signaling is also involved in bone development. Notch-deficient mice have severe skeletal abnormalities (26–28). Strikingly, mutations in the Notch ligand *DLL3* are responsible for "pudgy" mice and spondylocostal dysostosis in humans (29, 30). Furthermore, overexpression of Notch ligand and receptors impairs osteoblastic and osteoclastic cell differentiation from precursor cells (31, 32). Notch receptor expression has been reported in osteosarcoma (33, 34). However, the function of Notch pathway in osteosarcoma has not been established.

To determine the role of Notch signaling in osteosarcoma, we measured the effect of Notch pathway expression on cell proliferation, transformation, and invasion in human osteosarcoma models. Through pharmacologic and direct retroviral modulation of Notch pathway, we found that Notch signaling induces invasiveness and metastasis of osteosarcoma *in vitro* and *in vivo* but does not affect cell proliferation, survival, or tumorigenesis. More importantly, the Notch target gene HES1 is sufficient to induce an invasive and metastatic phenotype in osteosarcoma.

Materials and Methods

Patients. Human tumor cell samples were obtained from malignant effusions removed from patients with widely disseminated osteosarcoma. Both patients consented to have tumor tissue banked for research purposes under a protocol approved by the Institutional Review Board of University of Texas M.D. Anderson Cancer Center.

Cell culture and experimental reagents. Human osteosarcoma cell lines OS187, COL, and KRIB were described previously (9, 35). Human osteosarcoma cell line SAOS2 was purchased from American Type Culture Collection, and its metastatic subline LM7 was derived at our institution from SAOS2 cells by seven serial passages as metastatic pulmonary nodules in immunodeficient mice (36). Normal human osteoblast cells were purchased from Cambrex Bio Science and cultured in osteoblast basal medium (Cambrex) supplemented with 10% fetal bovine serum (Hyclone), 1% osteoblast growth supplement (Cambrex), and 1% penicillin/streptomycin. Osteosarcoma cell lines OS187, SAOS2, LM7, and KRIB were cultured in complete DMEM (Invitrogen), supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. COL cells were cultured in complete DMEM containing 1% insulin-transferrin-selenium A (Invitrogen). All cells were incubated in a 5% CO_2 atmosphere at 37°C. GSI XXI (compound E, (*S*,*S*)-2-[2-(3,5-difluorophenyl)-acetylamino]-*N*-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide) was purchased from Calbiochem. All other chemicals and solutions were from Sigma-Aldrich, unless otherwise indicated.

Reverse transcription–PCR and quantitative real-time PCR analysis. TRIzol Reagent (Invitrogen) method was used for RNA isolation. First-strand cDNA was synthesized from total RNA with oligo(dT) primers (Invitrogen) as described (37). The cDNA products were used for reverse transcription–PCR and quantitative PCR analysis. The primer sequences are listed in Supplementary Table S1. Human actin was used as an internal control. Real-time PCR analysis was done using the iCycler iQ quantitative PCR system (Bio-Rad) using 2× SYBR Green PCR Master Mix (Bio-Rad) following manufacturer's protocol. Data were analyzed according to the comparative C_t method and were normalized to actin expression in each sample (9).

Cell proliferation, anchorage-independent growth, and cell cycle analysis. Cell growth was measured daily with Vi-Cell cell viability analyzer (Beckman Coulter). Anchorage-independent growth was examined by colony formation in soft agar. Briefly, cells were suspended in DMEM containing 0.4% agar and 10% fetal bovine serum and plated onto a bottom layer containing 0.8% agar. The cells were plated at a density of 1×10^3 per well in a 24-well plate, and colonies were counted 14 d later. Each condition was analyzed in triplicate, and all experiments were repeated thrice. For cell cycle analysis, cells were fixed in ethanol and stained with 50 μg/mL propidium iodide in saline overnight. Alternatively, DRAQ5 (Alexis Biochemicals) was added to live cells to a final concentration of 5 μmol/L for 10 min. Propidium iodide and DRAQ5 were measured using a FACSCaliber flow cytometer (BD) and analyzed with FlowJo software (Tree Star, Inc.).

Invasion assay. The invasion ability of osteosarcoma cells with or without treatment was tested by BD Matrigel Invasion Chamber (8-μm pore size; BD Biosciences) according to manufacturer's protocol. Briefly, 5×10^4 osteosarcoma cells in 0.5 mL of serum-free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with DMEM with 10% fetal bovine serum as a chemoattractant. After incubation, the cells in the upper chamber were removed, and the cells outside of the bottom membrane were stained with HEMA3 stain set (Fisher Diagnostics). Preliminary studies determined 48-h incubation optimal, whereas too few cells had migrated by 24 h. Therefore, all studies presented here were assessed at 48 h, as recommended by the manufacturer. Cell migration was quantified by direct microscopic visualization and counting.

Western blot analysis. Cells were lysed in lysis buffer (1% Triton X-100, 150 mmol/L NaCl, and 20 mmol/L Na_2PO_4) with Complete Protease Inhibitor Cocktail (Roche Diagnostics). The protein concentrations were determined using the Bradford protein assay (Bio-Rad). The total cell lysates were separated using SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 4% bovine serum albumin in 1× TBS-T buffer for 30 min and then incubated with rabbit anti-HES1 antibody (1:1,000; Chemicon) for 2 h. Horseradish peroxidase–conjugated anti-rabbit IgG (1:3,000; GE Healthcare) was used as the secondary antibody, and the chemiluminescent signals were detected by Immobilon Western detection system (Millipore).

Retroviral transduction. To modulate Notch signaling in tumor cells, the *ICN1* (amino acid 1760-2555), *dominant-negative mastermind* (*dnMAM*, amino acid 13-74), or full-length *HES1* was inserted into MSCV-based retroviral vector MigR1 coexpressing GFP as an expression marker (kind gifts from Jon Aster and Warren Pear; refs. 22, 38). Retroviral transduction procedures were described previously (22).

After transduction, cells were either analyzed for cell cycle profiles of $GFP^{+/-}$ cells in the whole population or sorted for >99% GFP^{+} cells for subsequent assays, as indicated in individual experiments.

In vivo *orthotopic mouse model of osteosarcoma lung metastasis, radiographic, and histologic analysis.* Animal experiments were approved by the University of Texas M. D. Anderson Cancer Center Committee on Use and Care of Animals. Human osteosarcoma cells (2×10^5 in 20 μL of the cell suspension) were injected into left tibia of 5-wk-old NOD/SCID/IL2R γ-deficient mice (The Jackson Laboratory). Tumor size was measured with calipers weekly. To examine the osteolytic lesions, radiographs were taken with a Faxitron MX-20 X-ray machine (Faxitron X-ray). Six weeks after injection, the mice were sacrificed. Serial sections of lung were prepared and stained with H&E. Metastatic nodules in lungs were quantified by direct microscopic visualization and counting of a single lung section.

Statistics and supplementary data. Triplicate samples were analyzed in each experiment, and experiments were repeated thrice, unless otherwise indicated. Mean, SD, and *P* values based on the twp-tailed *t* test were calculated with Excel X (Microsoft). The supplementary data, including seven supplementary figures and one supplementary table, can be found with this article online.

Results

The Notch pathway is present in osteosarcoma cells and correlates with tumor metastatic phenotype in osteosarcoma cells. To assess the status of the Notch pathway in osteosarcoma, we examined Notch pathway gene expression by semiquantitative and quantitative PCR in normal human osteoblasts and four human osteosarcoma cell lines: OS187, COL, SAOS2, and its metastatic subline SAOS2-LM7 (LM7). We measured expression of human Notch ligand *DLL1*, receptors *Notch1-Notch4*, and downstream targets *HES1*, *HES5*, *HERP2*, and *DTX1* genes (Fig. 1A and B). Notch pathway genes are present in normal osteoblasts and osteosarcoma cells. OS187, COL, and LM7 cells have strong expression of *DLL1*, *Notch1-Notch3*, and downstream target genes *HES1* and *HERP2*. The expression of Notch signaling members shows correlation with metastatic phenotype in SAOS2 and its metastatic cell line LM7 (Fig. 1A and B). The metastatic subline LM7 has strong *DLL1*, *Notch1-Notch2*, and *HES1* and weak *HERP2* expression but not *Notch4* expression, whereas parental SAOS2 has only *Notch4* but not *DLL1* and *Notch1-Notch3* expression. We also assessed tumor samples from two patients with widely metastatic osteosarcoma and malignant effusions. *Notch1*, *Notch2*, and *Notch4*, *HES1*, and *HERP2* were expressed in metastatic patient samples. These results suggested that Notch may be active and associated with metastasis in osteosarcoma.

Down-regulation of Notch signaling by GSI suppresses osteosarcoma cell invasion but has no effect on cell proliferation and tumorigenesis. To test if the Notch pathway contributes to osteosarcoma pathogenesis, a small molecule GSI was used to inhibit Notch signaling pharmacologically in osteosarcoma cells. In OS187 cells, the Notch target gene HES1 was down-regulated at both the mRNA and protein levels by GSI at 0.1 and 0.3 nmol/L concentrations and abolished at 1 nmol/L concentration (Fig. 2A), suggesting that Notch signaling is blocked by GSI at subnanomolar concentrations, as expected with this GSI.

We then assessed proliferation, tumorigenesis, and invasiveness *in vitro* under GSI treatment. GSI did not affect proliferation in any osteosarcoma cell lines tested (Supplementary Fig. S1A-D) nor did it affect *in vitro* tumorigenesis of these cell lines as measured by colony formation in soft agar (Supplementary Fig. S2A-D).

We also measured the effects of GSI on the tumor cell invasion *in vitro*. We seeded the cells in the Matrigel transwells for 24 and 48 hours. We found that very few cells can invade through the Matrigel at 24 hours. After 48 hours, tumor cells can invade through the Matrigel well. We also assessed the growth of cells on Matrigel for 24 and 48 hours and found no difference in total cell number between the GSI treatment and control group (data not shown). Therefore, all Matrigel experiments were assessed at 48 hours. Our results show that GSI significantly suppressed the invasiveness of osteosarcoma cell line OS187 in a dose-dependent manner (Fig. 2B). SAOS2 cells have low metastatic potential and weak invasiveness. In contrast, LM7, the metastatic subline of SAOS2, showed strong invasiveness *in vitro* compared with parental SAOS2 cells, and GSI reduced the invasiveness of LM7 to levels similar to SAOS2 (Fig. 2C). In one cell line, COL, GSI treatment did not affect invasiveness (Fig. 2D). However, *HES1* expression in this line did not also change with GSI treatment, even up to 20 μmol/L (data not shown), suggesting that *HES1* expression is sustained in the absence of Notch receptor cleavage in this particular cell line.

Manipulation of Notch signaling controls the invasiveness of osteosarcoma cell lines. γ-Secretase can regulate multiple proteins, including Notch receptors, ERBB4, CD44, and cadherins (39), by cleaving the transmembrane domain of

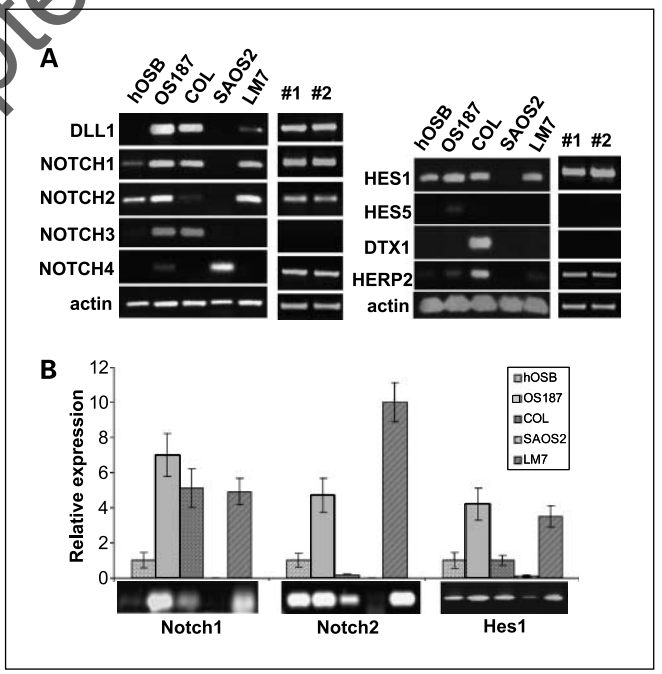

Fig. 1. Notch and HES1 expression are correlated with metastatic phenotype in osteosarcoma cells and tumors of osteosarcoma patients. *A,* reverse transcription – PCR analysis of Notch pathway genes (ligand *DLL1*, receptors *Notch1-Notch4*, and downstream targets *HES1, HES5, HERP2* and *DTX1*) in normal human osteoblast cells (*hOSB*), four human osteosarcoma cells, and two metastatic osteosarcoma tumor specimens (1 and 2). OS187 and COL are primary metastatic osteosarcoma cell lines. SAOS2 is a nonmetastatic osteosarcoma cell line, and LM7 is a metastatic subline of SAOS2. *B,* top, real-time PCR quantification of *Notch1, Notch2*, and *HES1* in human osteoblastic and osteosarcoma cells. All C_t values of osteosarcoma cell lines are normalized to actin first. Comparison is made to the human normal osteoblast sample, which is defined as a relative expression of 1. Bottom, gel image shows the quantitative product of the PCR reaction.

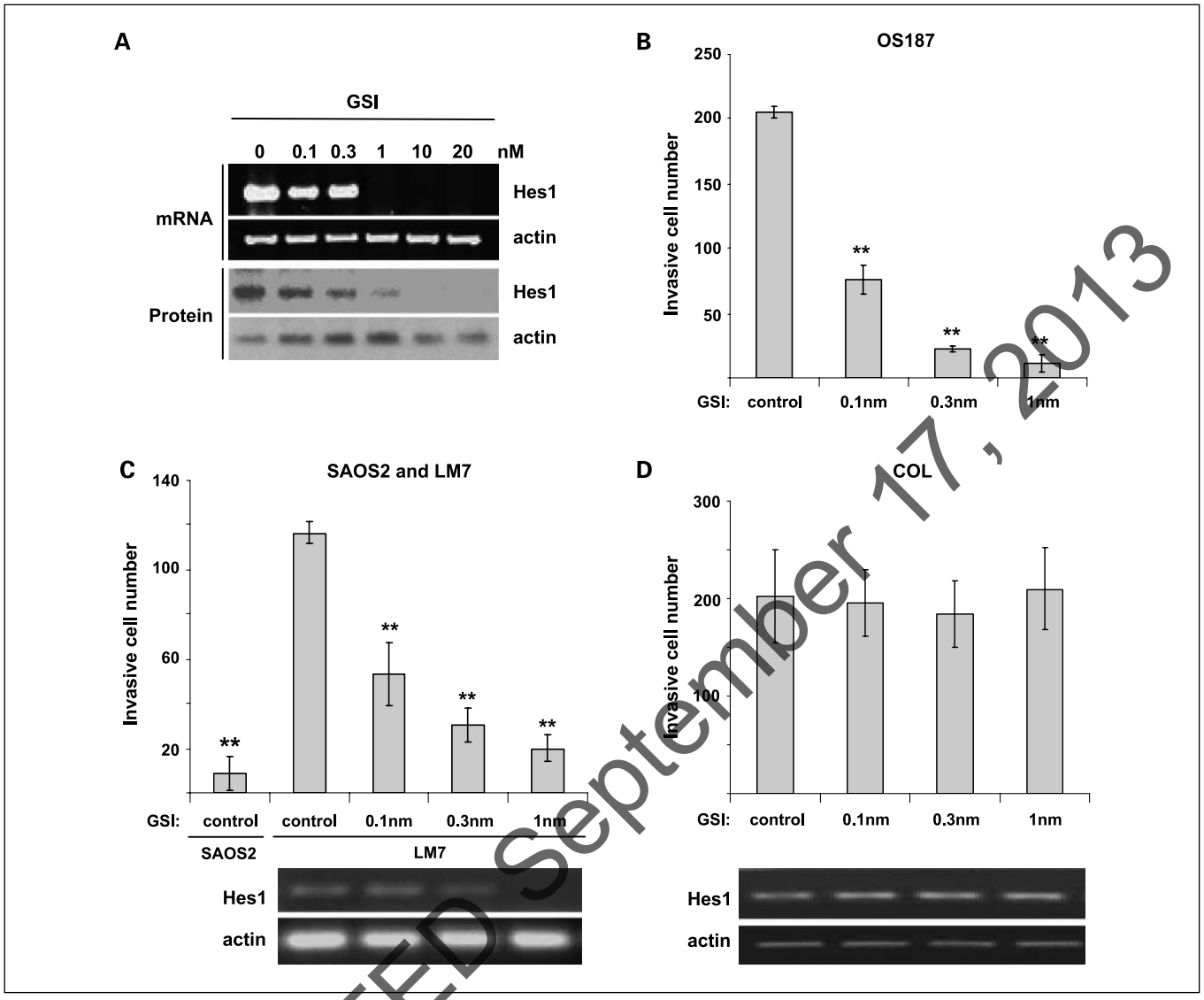

Fig. 2. Effects of GSI on the cell proliferation and *in vitro* invasiveness of osteosarcoma cells. *A,* Notch signaling inhibition by GSI at the indicated concentrations was inferred by HES1 expression at mRNA and protein level. OS187 cells were cultured with GSI for 48 h. *B,* Matrigel transwell assay of OS187 cells (5×10^4 cells per well) treated by GSI. Histograms depict the mean number of transmigrated cells at 48 h from three wells for each treatment. Invasion of OS187 cells was decreased significantly by GSI at 0.1, 0.3, and 1 nmol/L concentration (**, $P < 0.005$). *C,* Matrigel transwell assay of SAOS2 (5×10^4 per well) and LM7 cells (5×10^4 per well) with GSI treatment. LM7 cells showed higher invasive ability than SAOS2 cells (**, $P < 0.005$), and *in vitro* invasiveness of LM7 cells was significantly reduced by GSI at 0.1, 0.3, and 1 nmol/L (**, $P < 0.005$). Top, histogram depicts quantified invasiveness as in *B*; bottom, gel depicts reverse transcription – PCR analysis of *HES1* and *actin* expression in LM7 cells treated with GSI, suggesting the inhibition of Notch signaling. *D, in vitro* invasion assay of COL cells with GSI treatment. Data are depicted as in *C*. Invasiveness and *HES1* expression of COL cells were not affected by GSI.

these proteins. To confirm that the effects of GSI on tumor cell invasion result specifically from Notch inhibition, we manipulated Notch signaling activity in osteosarcoma cells using Notch pathway gene expression constructs. Osteosarcoma cells were transduced with retroviral constructs expressing the constitutively active *ICN1*, the Notch target gene *HES1*, the Notch pathway inhibitor *dnMAM*, or the empty vector (MigR1) to up-regulate or down-regulate Notch signaling. In osteosarcoma cells, manipulation of Notch signaling had no effect on cell proliferation (Supplementary Fig. S3A-D), cell cycle (Supplementary Fig. S4A-D), or *in vitro* tumorigenesis (Supplementary Fig. S5A-D).

However, up-regulation of Notch signaling by *ICN1* increased the *in vitro* invasiveness of OS187 and SAOS2 (Fig. 3A and B) cell lines. Importantly, expression of *HES1* was sufficient to induce this invasiveness. Consistent with these findings, down-regulation of Notch signaling by *dnMAM* decreased the invasion ability of OS187 (Fig. 3A) and LM7 cell lines (Fig. 3C), similar to the effects of GSI. Reverse transcription–PCR showed that *HES1* expression was up-regulated by ICN1 and down-regulated by dnMAM in OS187, SAOS2, and LM7 (Fig. 3A-C) cells. LM7 cells transduced with *ICN1* or *HES1* did not survive after cell sorting in three separate experiments, presumably due to toxicity in these particular cell lines. We also measured additional Notch downstream targets *HES5*, *HERP2*, and *DTX1* in OS187 cells transduced with *ICN1*, *dnMAM*, *HES1*, or vector. We found that neither *HES5*, *HERP2*, nor *DTX1* expression correlated

with the invasiveness of OS187 cell line (data not shown). These data suggest that the effect of Notch signaling on osteosarcoma invasiveness is likely mediated by the expression of HES1.

Transduction with constitutively active* Notch1 *rescues osteosarcoma cells from GSI-mediated inhibition of invasion. We have shown that inhibition of γ-secretase, an enzyme necessary for Notch activation, impeded the invasiveness of osteosarcoma cells *in vitro* (Fig. 2). Retroviral transduction of Notch pathway genes also showed that Notch-mediated HES1 expression was sufficient to enhance osteosarcoma invasion. To confirm that the GSI effect is due to its effect on the Notch pathway, we assessed the ability of ICN1 and HES1 to rescue osteosarcoma invasiveness from GSI treatment. OS187 cells transduced with constitutively active *ICN1* or *HES1* retained invasive potential even in 1 nmol/L GSI, whereas transduction with the empty vector conferred no resistance (Fig. 4A and B). These data show that the loss of osteosarcoma invasiveness induced by GSI can be rescued by Notch-mediated HES1 expression.

***Inhibition of Notch/HES1 signaling suppresses osteosarcoma metastasis* in vivo.** To investigate the effect of Notch signaling inhibition on osteosarcoma tumor metastasis *in vivo*, we developed a novel orthotopic osteosarcoma murine xenograft model using OS187 cells. Intratibial injection of unmodified human OS187 cells in NOD/SCID/IL2Rγ mice induced primary osteosarcoma tumor formation 2 weeks after inoculation. These primary tumors gave rise to microscopically detectible micrometastases in the lungs within 4 to 6 weeks after injection, mimicking the malignant process from early tumor growth to development of lung metastasis. Using this OS187 orthotopic xenograft model, we examined the effects on metastasis of inhibition of Notch signaling and HES1 expression by dnMAM. OS187 cells were transduced with either *dnMAM* or the empty vector before intratibilial injection, and primary tumors were allowed to grow for 6 weeks. Radiographs of the primary tumors showed no significant difference between *dnMAM*-transduced and empty vector–transduced cells in the osteolytic primary lesions formed (Fig. 5A and B). The primary tumors from *dnMAM* and empty vector control cells had similar tumor latency and growth rates (Fig. 5C). However, inhibition of Notch signaling and HES1 expression by dnMAM significantly decreased the number of lung metastases observed ($P < 0.005$; Fig. 5A, B, and D). We counted all visible micrometastases (tumor cluster with >10 cells) in a single 5-μm thick coronal section from the middle of the lungs. The vector control group had an average of 15 metastases per slice of lung, whereas the *dnMAM* group averaged only one small metastatic focus. These results show that blocking Notch and HES1 signaling prevented osteosarcoma invasion and metastasis *in vivo*.

Discussion

Metastasis causes 90% of human cancer deaths (40), and defining the mechanisms controlling metastasis is essential to improving cancer survival. For osteosarcoma, lung metastasis is the major cause of death, because the primary tumor usually can be treated effectively with surgery and chemotherapy. If the mechanisms regulating invasion and metastasis of osteosarcoma can be clearly defined, there are likely to be key elements that can be exploited therapeutically, reducing metastasis and improving survival. The Notch pathway may yield treatment targets for many, but not all, cancers because Notch can act as an oncogene in some malignancies and a tumor suppressor in others (21). Identifying the Notch pathway components that control metastasis is likely to be the key in targeting this pathway for osteosarcoma and other solid tumors.

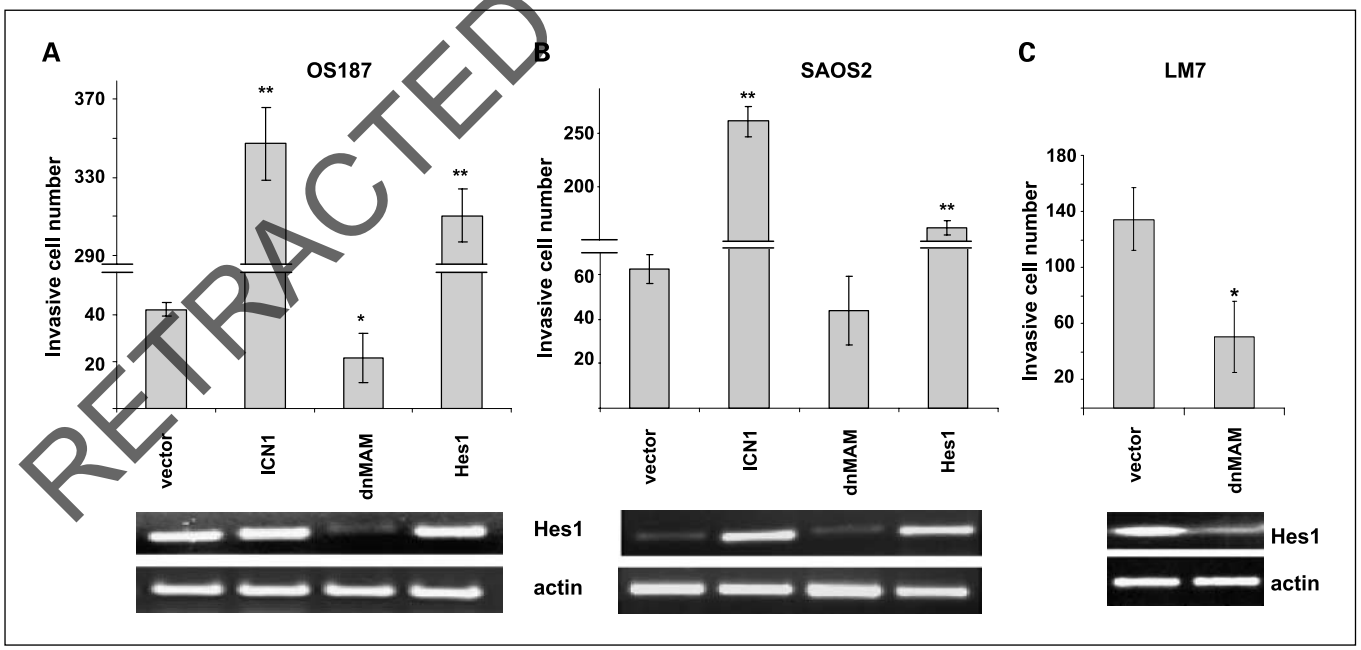

Fig. 3. Effects of manipulation of Notch signaling on osteosarcoma invasiveness *in vitro*. *A-C,* top, relative invasiveness *in vitro* of OS187 cells (1×10^4 per well; *A*), SAOS2 cells (1.5×10^5 per well; *B*), and LM7 cells (5×10^4 per well; *C*) transduced with *ICN1*, *dnMAM*, or *HES1*. Note: the input of cells for each cell line was adjusted to give roughly equal number of invading cells for each vector control sample. Histograms depict the quantified invasiveness. **, $P < 0.005$; *, $P < 0.05$. Bottom, gel depicts PCR analysis of *HES1* and *actin* in the transduced cells.

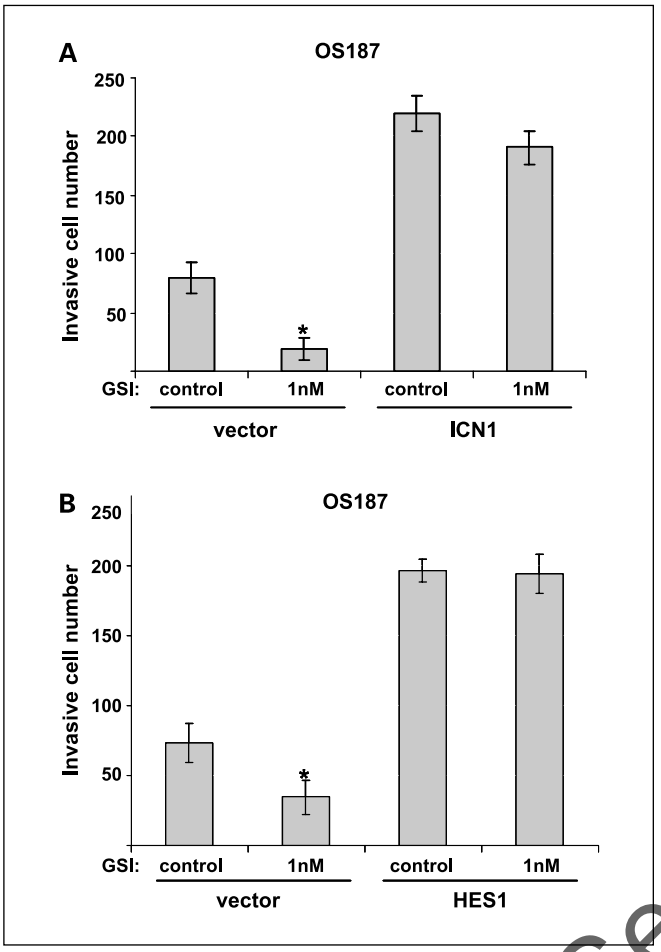

Fig. 4. Effects of GSI (1 nmol/L) on *in vitro* invasiveness of OS187 cells transduced with empty vector, ICN1 (*, $P < 0.05$), and HES1 (*, $P < 0.05$). GSI (1 nmol/L) significantly suppressed invasion of OS187 cells transduced with empty vector. However, OS187 cells transduced with ICN1 (*A*) or HES1 (*B*) are resistant to GSI treatment. Data are displayed as in Fig. 2C.

Our report specifically identifies *Hes1* as the Notch pathway gene that is critical for osteosarcoma invasion. Recently, Notch signaling was suggested to promote the invasive ability of pancreatic cancer cells through up-regulating vascular endothelial growth factor and matrix metalloproteinase expression *in vitro* (41). The functions defined here are distinct, because Hes1 signaling is not involved in anchorage-independent growth and cell proliferation *in vitro* or tumor latency and growth *in vivo* of osteosarcoma, and vascular endothelial growth factor and matrix metalloproteinases seem unaffected by Hes1 in osteosarcoma (data not shown). Therefore, our results show that invasive behavior can be separated from other cancer characteristics and that HES1 signaling causes invasion and may be responsible for the Notch-mediated metastatic phenotype in osteosarcoma. This essential and specific role of Notch signaling on invasion and metastasis has not been described previously in any tumor types.

Notch signaling can induce expression of several downstream target genes, including *HES1*, *HES5*, and *HERP2* (20). Other Notch downstream targets include *pre-Tα*, *cyclin D1*, *nuclear factor-κB*, and $p21^{Cip1}$ (20). The diversity of Notch functions in different cell lineages is achieved through tissue-specific activation of particular downstream target genes. Our data show that Notch signaling controls invasion and metastasis and that the Notch downstream target HES1 is the effector. Because HES1 is a transcriptional regulator, other functional genes related to invasion and metastasis should be regulated by HES1. There are several known classes of proteins involved in the cell-to-microenvironment interaction and invasive or metastatic process, such as cell-cell adhesion molecules, extracellular proteases, and angiogenesis promoting factors (42). Our efforts to identify HES1-regulated targets have excluded several known metastatic genes, such as cell scaffolding protein *Ezrin*, *cell adhesion molecule intercellular adhesion molecule 1*, *matrix-degrading protease matrix metalloproteinase 9*, and *vascular endothelial growth factor* (data not shown). But our screening for HES1 target genes is still limited. An unbiased high-throughput screening, such as cDNA microarray analysis, will likely be necessary to identify all the relevant genes regulated by HES1 in osteosarcoma.

Invasiveness of cell line COL is resistant to GSI treatment because GSI cannot suppress HES1 expression in this cell line. This result suggests either that Hes1 expression is Notch-independent in COL cells or that these cells have an as-yet uncharacterized activating mutation in the Notch pathway. This mechanism remains to be elucidated.

To study the mechanisms involved in the pathogenesis of human osteosarcoma and evaluate the drug efficacy in preclinical studies, animal models are critical. The KRIB model is a well-established orthotopic osteosarcoma model, in which intraosseous implantation forms local tumor within 4 weeks and subsequently metastasize to the lung in 6 weeks (35). However, KRIB cells were derived from human osteosarcoma cells transformed with oncogenic Ras, a mutation never found in spontaneous osteosarcoma samples (35). The LM7 metastatic model requires i.v. administration and does not form orthotopic tumors (36). Thus, LM7 is unable to examine mechanisms of metastasis relating to release of cancer cells from the primary tumor. The OS187 orthotopic tumor xenograft model is an improved model for several reasons. OS187 cells are human osteosarcoma tumor cells without any experimentally induced genetic modification. This orthotopic model can reproduce all aspects of malignant behavior, from the establishment and growth of primary tumors to the subsequent micrometastatic hematogenous spread to the lungs. It is amenable to genetic and pharmacologic manipulation, which should prove useful for both mechanistic studies and preclinical testing of promising new therapies. Using this model, we showed the importance of Notch pathway signaling for osteosarcoma metastasis.

Therapeutic agents that target Notch signaling are being developed for antitumor effects. Originally, GSIs were developed for potential use in prevention of Alzheimer's disease, in which the proteolytic cleavage of the β-amyloid precursor protein by β-secretase and γ-secretase leads to the accumulation of neurotoxic Aβ peptide in the brain (39). However, the clinical trials showed potent Notch pathway inhibition. With the discovery of aberrant Notch signaling in human cancer, the potential use of GSI for various human malignancies has been explored. Currently, GSI small molecules that target proteolytic step of Notch activation is being evaluated for advanced breast cancer and relapsed or refractory T-cell acute lymphoblastic leukemia (National Cancer Institute Clinical Trial Protocol ID

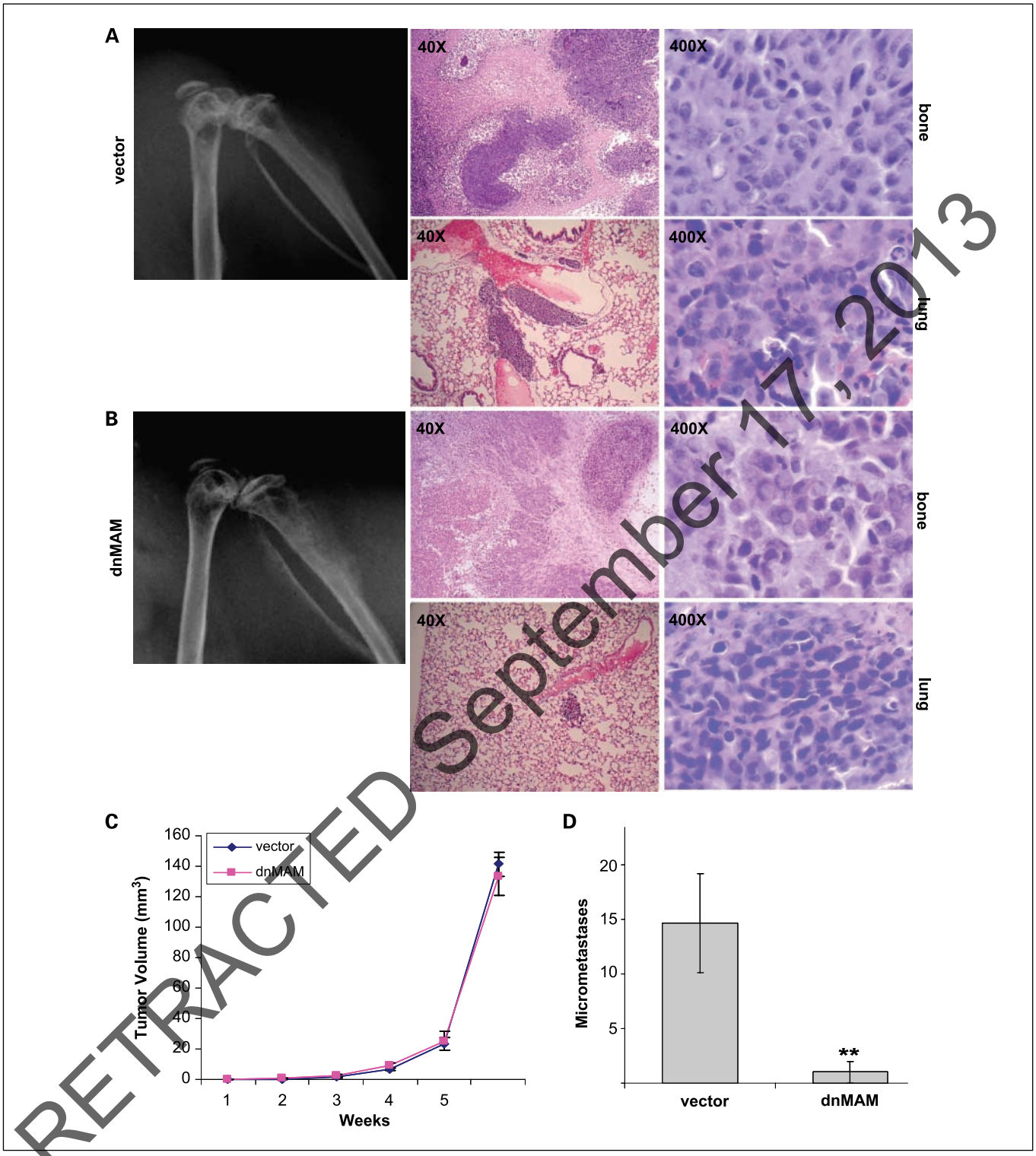

Fig. 5. Effects of manipulation of Notch signaling on osteosarcoma invasiveness and metastasis *in vivo*. *A* and *B,* representative X-ray images (*left*) show the osteolytic lesion in the primary tumor site (tibia) of both vector and dnMAM group. Two representative images (right, 40×; left, 400×) show the H&E staining of orthotopic bone tumor (*top two*) and lung metastasis (*bottom two*) in vector (*A*) and dnMAM (*B*) groups. *C,* average tibial tumor volume of the OS187 orthotopic osteosarcoma xenograft model using cells transduced with dnMAM or empty vector as control. A representative experiment of three is shown. *D,* the average number of metastatic foci formed in lung tissues of vector and dnMAM groups is indicated. **, $P < 0.005$; $n = 3$ per group in each experiment. All tumor clusters with >10 cells were counted as micrometastases.

2004_97NCT00100152).[1] Our results suggest that Notch and HES1 signaling may be a rationale therapeutic target to prevent metastasis for human osteosarcoma patients, and using GSI or ADAMS inhibitors may reduce the rate at which metastatic disease develops. Our data also indicate that Notch pathway activation, especially *HES1* gene expression, should be evaluated

[1] http://www.cancer.gov/clinicaltrials

as a novel marker for the metastatic potential of osteosarcoma in patients.

In conclusion, we reveal a specific and essential role of Notch signaling in promoting osteosarcoma invasiveness and metastasis *in vitro* and *in vivo*. Our report also suggests that HES1 merits investigation as a prognostic marker for osteosarcoma. Furthermore, inhibition of Notch signaling by small molecular inhibitors may have important therapeutic applications for treating and preventing osteosarcoma metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Dr. Eugenie Kleinermen and Dr. Douglas D. Boyd for helpful discussions and Dr. Shufang Jia and Wendy Fang for technical help.

References

1. Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 2006;32:423–36.
2. Provisor A, Ettinger L, Nachman J, et al. Treatment of nonmetastatic osteosarcoma of the extremity with preoperative and postoperative chemotherapy: a report from the Children's Cancer Group. J Clin Oncol 1997;15:76–84.
3. Bacci G, Ferrari S, Bertoni F, et al. Long-term outcome for patients with nonmetastatic osteosarcoma of the extremity treated at the Istituto Ortopedico Rizzoli according to the Istituto Ortopedico Rizzoli/osteosarcoma-2 protocol: an updated report. J Clin Oncol 2000;18:4016–27.
4. Rytting M, Pearson P, Raymond AK, et al. Osteosarcoma in preadolescent patients. Clin Orthopaedics Relat Res 2000;373:39–50.
5. Ferguson W, Goorin A. Current treatment of osteosarcoma. Cancer Invest 2001;19:292–315.
6. Khanna C, Wan X, Bose S, et al. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 2004;10:182–6.
7. Lafleur EA, Koshkina NV, Stewart J, et al. Increased Fas expression reduces the metastatic potential of human osteosarcoma cells. Clin Cancer Res 2004;10:8114–9.
8. Gorlick R, Huvos AG, Heller G, et al. Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 1999;17:2781–8.
9. Hughes DPM, Thomas DG, Giordano TJ, Baker LH, McDonagh KT. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. Cancer Res 2004;64:2047–53.
10. Hughes DPM, Thomas DG, Giordano TJ, McDonagh KT, Baker LH. Essential erbB family phosphorylation in osteosarcoma as a target for CI-1033 inhibition. Pediatric Blood Cancer 2006;46:614–23.
11. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284:770–6.
12. Wharton K, Johansen K, Xu T, Artavanis-Tsakonas S. Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 1985;43:567–81.
13. Kidd S, Kelley MR, Young MW. Sequence of the notch locus of *Drosophila melanogaster*: relationship of the encoded protein to mammalian clotting and growth factors. Mol Cell Biol 1986;6:3094–108.
14. Struhl G, Adachi A. Nuclear access and action of notch *in vivo*. Cell 1998;93:649–60.
15. Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the notch signaling pathway. J Cell Physiol 2003;194:237–55.
16. Jeffries S, Robbins DJ, Capobianco AJ. Characterization of a high-molecular-weight notch complex in the nucleus of notchic-transformed RKE cells and in a human T-cell leukemia cell line. Mol Cell Biol 2002;22:3927–41.
17. Matsuno K, Ito M, Hori K, et al. Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. Development 2002;129:1049–59.
18. Allenspach E, Maillard I, Aster J, Pear W. Notch signaling in cancer. Cancer Biol Ther 2002;1:466–76.
19. Nickoloff B, Osborne B, Miele L. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene 2003;22:6598–608.
20. Leong KG, Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. Blood 2006;107:2223–33.
21. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 2003;3:756–67.
22. Zweidler-McKay PA, He Y, Xu L, et al. Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. Blood 2005;106:3898–906.
23. Kunnimalaiyaan M, Chen H. Tumor Suppressor Role of Notch-1 Signaling in Neuroendocrine Tumors. Oncologist 2007;12:535–42.
24. Proweller A, Tu L, Lepore JJ, et al. Impaired notch signaling promotes *de novo* squamous cell carcinoma formation. Cancer Res 2006;66:7438–44.
25. Seiffert D, Bradley JD, Rominger CM, et al. Presenilin-1 and -2 are molecular targets for γ-secretase inhibitors. J Biol Chem 2000;275:34086–91.
26. Shen J, Bronson R, Chen D, Xia W, Selkoe D, Tonegawa S. Skeletal and CNS defects in presenilin-1-deficient mice. Cell 1997;89:629–39.
27. Swiatek P, Lindsell C, del Amo F, Weinmaster G, Gridley T. Notch1 is essential for postimplantation development in mice. Genes Dev 1994;8:707–19.
28. Conlon R, Reaume A, Rossant J. Notch1 is required for the coordinate segmentation of somites. Development 1995;121:1533–45.
29. Bulman M, Kusumi K, Frayling T, et al. Mutations in the human δ homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. Nat Genet 2000;24:438–41.
30. Kusumi K, Sun E, Kerrebrock A, et al. The mouse pudgy mutation disrupts Δ homologue Dll3 and initiation of early somite boundaries. Nat Genet 1998;19:274–8.
31. Sciavdone M, Gazzerro E, Priest L, Delany AM, Canalis E. Notch 1 impairs osteoblastic cell differentiation. Endocrinology 2003;144:5631–9.
32. Yamada T, Yamazaki H, Yamane T, et al. Regulation of osteoclast development by Notch signaling directed to osteoclast precursors and through stromal cells. Blood 2003;101:2227–34.
33. Schnabel M, Fichtel I, Gotzen L, Schlegel J. Differential expression of Notch genes in human osteoblastic cells. Int J Mol Med 2002;9:229–32.
34. Kawakami T, Siar C, Ng K, et al. Expression of Notch in a case of osteosarcoma of the maxilla. Eur J Med Res 2004;9:533–5.
35. Berlin O, Samid D, Donthineni-Rao R, Akeson W, Amiel D, Woods VL, Jr. Development of a novel spontaneous metastasis model of human osteosarcoma transplanted orthotopically into bone of athymic mice. Cancer Res 1993;53:4890–5.
36. Jia S-F, Worth LL, Densmore CL, Xu B, Duan X, Kleinerman ES. Aerosol gene therapy with PEI: IL-12 eradicates osteosarcoma lung metastases. Clin Cancer Res 2003;9:3462–8.
37. Zhang P, Tan HTW, Pwee K-H, Kumar PP. Conservation of class C function of floral organ development during 300 million years of evolution from gymnosperms to angiosperms. Plant J 2004;37:566–77.
38. Weng AP, Nam Y, Wolfe MS, et al. Growth Suppression of Pre-T Acute Lymphoblastic Leukemia Cells by Inhibition of Notch Signaling. Mol Cell Biol 2003;23:655–64.
39. Pollack S, Lewis H. Secretase inhibitors for Alzheimer's disease: challenges of a promiscuous protease. Curr Opin Investig Drugs 2005;6:35–47.
40. Sporn MB. The war on cancer. Lancet 1996;347:1377–81.
41. Wang Z, Banerjee S, Li Y, Rahman KMW, Zhang Y, Sarkar FH. Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-{κ}B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 2006;66:2778–84.
42. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.

Clinical Cancer Research

Retraction: Critical Role of Notch Signaling in Osteosarcoma Invasion and Metastasis

The authors wish to retract the article titled "Critical Role of Notch Signaling in Osteosarcoma Invasion and Metastasis," which was published in the May 15, 2008, issue of *Clinical Cancer Research* (1).

Many years after this article was published, we obtained new information showing that two of the cell lines used in this publication, OS 187 and COL, are not osteosarcoma cell lines. Both lines were first reported by us (2).

Each of these cell lines has now been tested by DNA fingerprint analysis. OS 187 tested identically to the common NCI60 colon cancer line HCT 15, even in a vial from passage 2 in our laboratory that had been frozen in 2002. Injection of OS 187 into the cecum of NOD/SCID/IL-2R$\gamma^{-/-}$ mice yielded a colonic primary with metastasis to the liver (data not shown). Therefore, all of our published results with OS 187 likely reflect the biology of colon cancer. Given the subsequent report by another group that Notch pathway signaling promotes invasion and metastasis of colon cancer (3), the data we reported for OS 187 are not surprising.

The pattern observed for tumor growth after injection of COL cells into the tibia of NOD/SCID/IL-2R$\gamma^{-/-}$ mice (no bone primary, rapid growth of liver tumors, with additional masses arising in skin and retro-orbital spaces) suggested that this cell line likely represented neuroblastoma. We have recently reported the subsequent testing confirming that COL is a neuroblastoma (4).

In this article, OS 187 and COL were included in Figs. 1 and 2. OS 187 was among the cell lines shown in Fig. 3 and is the only cell line shown in Figs. 4 and 5. When these two cell lines are removed from the data presented, two findings reported should be interpreted with caution. First, the only real osteosarcoma cell lines in which Notch was directly manipulated genetically were SAOS2 (with low levels of Notch) and LM7 (a subline of SAOS2 with high levels of Notch). Although transduction of SAOS2 with constitutively active intracellular Notch1 (ICN1) or the Notch target gene *Hes1* increased *in vitro* invasiveness, LM7 was not able to grow after transduction with these constructs, as indicated in the original publication. Thus, we have not proved that osteosarcoma invasiveness can be increased by further upregulating Notch activity in Notch-positive osteosarcoma. Second, because only OS 187 was used *in vivo* in this report, we can no longer say that our publication proves Notch's role in metastasis *in vivo* for osteosarcoma. However, the subsequent confirmation of this function of Notch by other groups (5) renders it likely that this conclusion remains valid for osteosarcoma. Nonetheless, because the data provided from *bona fide* osteosarcoma cell lines are no longer adequate to support the assertions and conclusions of the article, the authors respectfully request its retraction.

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References

1. Zhang P, Yang Y, Zweidler-McKay PA, Hughes DPM. Critical role of notch signaling in osteosarcoma invasion and metastasis. Clin Cancer Res 2008;14:2962–9.
2. Hughes DPM, Thomas DG, Giordano TJ, Baker LH, McDonagh KT. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear expression of Her-4 in primary osteosarcoma. Cancer Res 2004;64:2047–53.
3. Sonoshita M, Aoki M, Fuwa H, Aoki K, Hosogi H, Sakai Y, et al. Suppression of colon cancer metastasis by Aes through inhibition of Notch signaling. Cancer Cell 2011;19:125–37.

4. Hua Y, Gorshkov K, Yang Y, Wang W, Zhang N, Hughes DP. Slow down to stay alive: HER4 protects against cellular stress and confers chemoresistance in neuroblastoma. Cancer 2012;118:5140–54.
5. Engin F, Bertin T, Ma O, Jiang MM, Wang L, Sutton RE, et al. Notch signaling contributes to the pathogenesis of human osteosarcomas. Hum Mol Genet 2009;8:1464–70.

Published online September 17, 2013.
doi: 10.1158/1078-0432.CCR-13-1914

Clinical Cancer Research

Critical Role of Notch Signaling in Osteosarcoma Invasion and Metastasis

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Clin Cancer Res 2008;14:2962-2969.