THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 5, Issue of January 30, pp. 3662–3670, 2004
Printed in U.S.A.

Notch Signaling and ERK Activation Are Important for the Osteomimetic Properties of Prostate Cancer Bone Metastatic Cell Lines*

Received for publication, July 25, 2003, and in revised form, October 28, 2003
Published, JBC Papers in Press, November 5, 2003, DOI 10.1074/jbc.M308158200

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Prostate cancer bone metastases are characterized by their ability to induce osteoblastic lesions and local bone formation. It has been suggested that bone metastatic prostate cancer cells are osteomimetic and capable of expressing genes and proteins typically expressed by osteoblasts. The ability of preosteoblasts to differentiate and express osteoblastic genes depends on several pathways, including Notch and MAPK. Here we show that *notch1* expression is increased 4–5 times in C4-2B and MDA PCa 2b cells (osteoblastic skeletal prostate metastatic cancer cell lines) when compared with nonskeletal metastatic cell lines (LNCaP and DU145). Notch1 ligand, *dll1*, is expressed only in C4-2B cells. Immunohistochemical studies demonstrate that Notch1 is present in both human clinical samples from prostate cancer bone metastases and the C4-2B cell line. To determine whether prostate cancer bone metastases respond to osteogenic induction similar to osteoblasts, C4-2B cells were cultured in osteogenic medium that promotes mineralization. C4-2B cells mineralize and express *HES-1* (a downstream target of Notch), an effect that is completely inhibited by L-685,458, a Notch activity inhibitor. Furthermore, osteogenic induction increases ERK activation, *runx2* expression, and nuclear localization, independent of Notch signaling. Finally, we show that Notch and ERK activation are essential for Runx2 DNA binding activity and osteocalcin gene expression in C4-2B cells in response to osteogenic induction. These studies demonstrate that prostate cancer bone metastatic cell lines acquire osteoblastic properties through independent activation of ERK and Notch signaling; presumably, both pathways are activated in the bone microenvironment.

Prostate carcinoma most commonly metastasizes to the lymph nodes and to bone with a 70–80% frequency as determined by autopsy studies. A unique characteristic of skeletal metastases of human prostate cancer is their ability to induce osteoblastic lesions, characterized by new woven bone formation and variable degrees of osteoclastic bone resorption (1). It has been proposed that factors produced by prostate cells, both normal and neoplastic, have the potential to stimulate new bone formation by effects on both osteoblasts and osteoclasts. Included in this group are parathyroid hormone-related peptide (2), transforming growth factor-β (3), urokinase-type plasminogen activator (4), bone morphogenetic proteins (5, 6), and endothelin-1 (7). The production of these factors by prostate cancer cells, accompanied by the release of growth factors during osteolysis, is proposed to result in stimulating new woven bone formation by increasing the differentiation of osteoblasts in the bone. Interestingly, there is increasing evidence that prostate cancer bone metastases express genes and proteins typically associated with osteoblasts, including RANK ligand, osteoprotegerin (8), sialoproteins, osteopontin (9), Runx2 (Runt-related transcription factor 2) (10), and osteocalcin (11). This phenomenon has been attributed to a "unique" ability of prostate cancer cells to acquire "osteoblast-like properties" upon metastasizing to the bone microenvironment, thus enabling them to grow and thrive in this highly restrictive environment (10, 11).

Cell fate determination, mediated by local cell-cell contact, plays a critical role during development of multicellular organisms. The Notch signaling pathway is an evolutionarily conserved mechanism utilized by organisms, ranging from worms through humans, involved in fate determination of various cell lineages. Notch belongs to the family of epidermal growth factor-like homeotic genes, which encode transmembrane proteins with variable numbers of epidermal growth factor-like repeats in the extracellular region. In vertebrates, four Notch genes have been described, *notch1*, *-2*, *-3*, and *-4*; these are highly related to each other and to the *Drosophila* Notch and *C. elegans lin-12* (12, 13). There are also multiple Notch ligands in vertebrates that are homologous to the *Drosophila* ligands, Delta and Serrate (14). Delta homologs are called "Delta-like" ligands (Dll1 and -4), and the Serrate homologs are called "Jagged" (Jagged-1 and -2). Activation of Notch upon ligand binding is accompanied by proteolytic processing by γ-secretase that releases the intracellular domain of Notch from the membrane. The Notch intracellular domain then translocates into the nucleus and associates with the CSL (CBF-1 (RBP-Jκ)/Su(H)/Lag-1) family of DNA-binding proteins to form a transcriptional activator (15). Most of the Notch target genes encode transcription regulators, which, in turn, modulate cell fate by affecting the function of tissue-specific basic helix-loop-helix transcription factors or through other molecular targets, such as activating protein-1 (16), NF-κB (17), and most importantly, HES-1 (Hairy and Enhancer-of-split-1) (18). Since Notch ligands are predominantly cell membrane-associated, Notch signaling is thought to mediate interactions between contiguous cells at many sites in the human body, including bone, where it has been shown recently that Notch1 activation by Dll1 stim-

* This work was supported by the National Institutes of Health Grants R01 AR43225 and P30 AR 46031 (to J. M. M.) and by the Howard Hughes Medical Institute (to S. A. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
Prostate cancer cell lines used in these studies

Cell line	Characteristics	Reference
C4–2B	Forms osteoblastic bone metastases *in vivo*Derived from LNCaP cells	22
LNCaP	Isolated from lymph nodes metastases.Metastasize to lymph nodes *in vivo*	47
MDA PCa 2b	Isolated from osteoblastic bone metastases.Forms osteoblastic bone metastases *in vivo*	48
DU145	Isolated from brain metastases	49

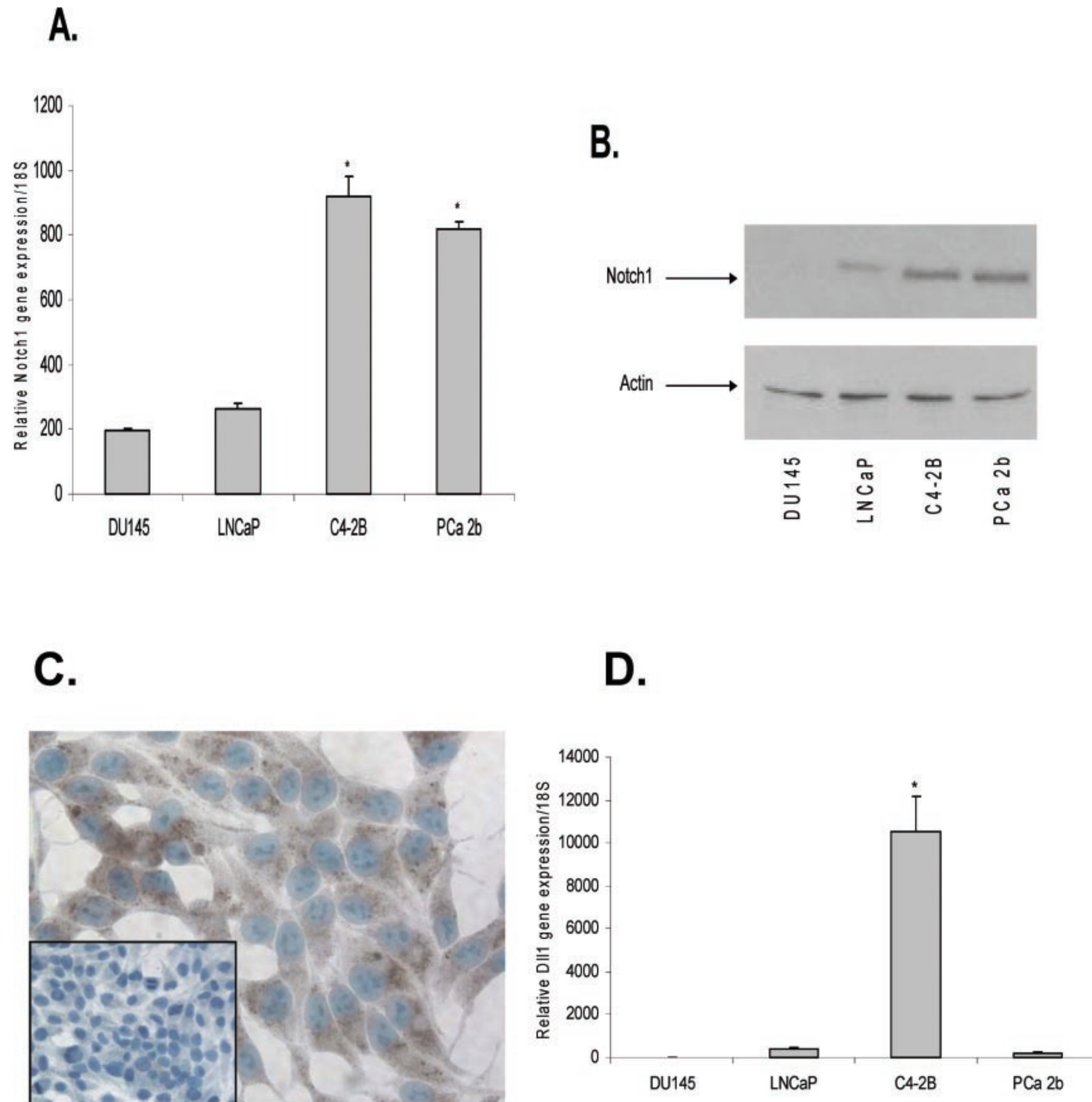


FIG. 1. **Osteoblastic human prostate cancer metastatic cell lines express Notch1 and Dll1.** Cells (DU145, LNCaP, C4-2B, and PCa 2b) were cultured for 10 days in regular medium. RNA and total protein were extracted at the end of the study. *A* and *D*, semiquantitative real time RT-PCRs were performed using primers for *notch1*, *dll1*, and 18 S rRNA. Values were obtained from three experiments and represent the mean ± S.E. of Notch1 or Dll1 mRNA expression relative to 18 S rRNA expression; *, $p \leq 0.01$. *B*, protein extracts (30 μg/lane) were separated by 7% SDS-PAGE. Immunoblots were developed using specific antibodies directed against Notch1 and actin. The autoradiograph is representative of three experiments. *C*, immunohistochemical staining in C4-2B cells was performed using goat polyclonal antibody against Notch1 (*brown*), counterstained with hematoxylin (*blue*) (×400 magnification). Negative control is shown in the *lower left inset* (×100 magnification).

ulates the differentiation of osteoblasts (19).

Osteoblasts, the bone-forming cells, are derived from mesenchymal stem cells after osteogenic differentiation. Under *in vitro* osteogenic conditions, isolated human mesenchymal stem cells (hMSC)[1] form mineralized aggregates or nodules and increase their expression of alkaline phosphatase and osteocalcin, which are important markers of osteoblast differentiation (20). Many of the osteoblast-related genes are modulated by Runx2 binding to a specific DNA element in the regulatory region of these target genes. Runx2 is an essential transcription factor for the differentiation of osteoblasts from mesenchymal precursors and the regulation of bone matrix deposition by differentiated osteoblasts (21). This suggests that Runx2 regulates osteoblast gene expression and function at multiple levels. Recent studies have demonstrated that prostate cancer cells, similar to mature osteoblasts, are capable of expressing and secreting osteocalcin upon metastasizing to bone (11). In addition, it has been shown that the osteoblastic C4-2B pros-

[1] The abbreviations used are: hMSC, human mesenchymal stem cells; ERK, extracellular signal-regulated kinase; OSE-2, osteoblast-specific element 2; MAPK, mitogen-activated protein kinase.

tate cancer cell line (LNCaP derivative cell line) (22) has an increased expression of Runx2 (10), whereas the osteolytic PC3 cell line expresses transcriptionally active Cbaf1 as shown by its ability to bind to OSE2 on the osteocalcin promoter (11). This suggests that Runx2 expression/activity could play a role in the modulation of gene expression in prostate cancer metastases. Interestingly, HES-1 (a downstream target of Notch signaling) can physically interact with Runx2 and potentiate its mediated transactivation in transfected cells (18). Based on this, we hypothesize that the activation of Notch in prostate cancer metastases in the bone microenvironment leads to a cascade of signaling events, which ultimately results in a unique pattern of gene expression similar to that seen in mature osteoblasts.

The aim of the present study was to examine the role of the Notch signaling pathway in the unique osteomimetic properties of prostate cancer bone metastases. We report that osteoblastic prostate cancer cell lines (C4-2B and MDA PCa 2b) specifically express *notch1*, and the Notch ligand, *dll1*, is expressed only in the C4-2B cell line. This expression pattern of Notch receptor and ligand leads to the expression of *HES-1* transcription factor independent of ERK activation. Furthermore, we demonstrate that *in vitro* osteogenic induction of prostate cancer cells increases ERK phosphorylation and Runx2 expression and activation, independent of Notch activation. Finally, we demonstrate that Notch signaling and ERK activation in skeletal prostate cancer metastases is critical for Runx2 DNA binding and osteocalcin expression. Taken together, our results suggest that Notch activation plays a critical role in the ability of prostate cancer metastases to acquire "osteoblast-like" properties.

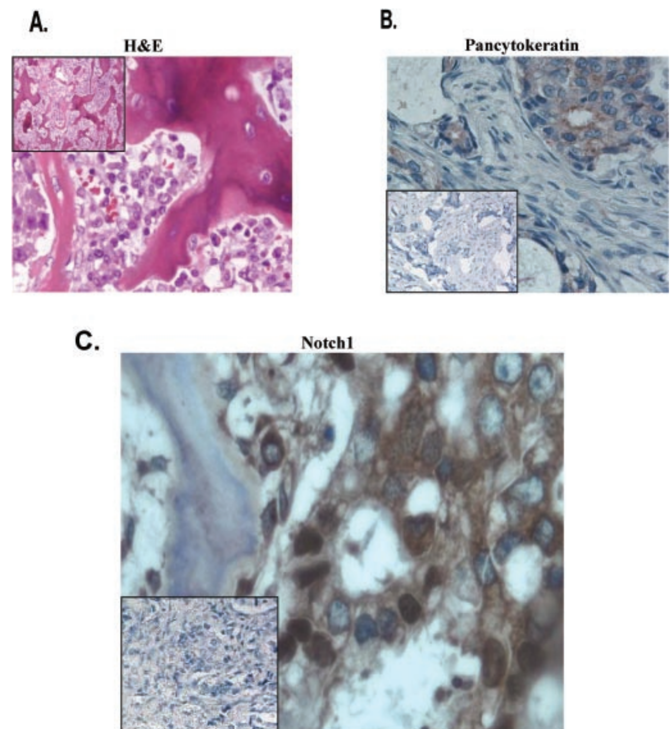


FIG. 2. **Notch1 is expressed in osteoblastic prostate cancer metastasis obtained from human clinical tissue samples.** Human prostate cancer bone metastases were surgically removed, formalin-fixed, and paraffin-embedded. *A*, hematoxylin and eosin staining shows the increase in bone formation (*red*) and metastatic prostate cancer cells. *B*, immunohistochemical staining for pancytokeratin positively stains prostate cancer metastatic cells, since they are of epithelial origin (*brown*), whereas bone stromal cells are counterstained with hematoxylin (*blue*). *C*, immunohistochemical staining for Notch1 (*brown*), counterstained with hematoxylin (*blue*). All pictures were taken at ×400 magnification. Negative controls are shown in the *lower left insets* (×100 magnification).

EXPERIMENTAL PROCEDURES

Cell Culture and Osteoblastic Differentiation—The human prostatic cell lines (Table I) DU145, LNCaP, and MDA PCa 2b were purchased from the American Type Culture Collection (Manassas, VA). The C4-2B cell line was purchased from UroCor, Inc. (Oklahoma City, OK). In addition, we used hMSC provided by the University of Alabama National Institutes of Health-funded Research Core Center for Musculoskeletal Disease. DU145 and LNCaP were maintained in RPMI 1640 containing 10% fetal bovine serum. MDA PCa 2b was maintained in Kaighn's modification of Ham's F-12 medium supplemented with 25 ng/ml Cholera toxin, 10 ng/ml epidermal growth factor, 0.005 mM phosphoethanolamine, 100 pg/ml hydrocortisone, 45 nM selenious acid, 0.005 mg/ml insulin, and 20% fetal bovine serum. The C4-2B cell line was maintained in T medium (80% Dulbecco's modified Eagle's medium, 20% F-12 medium, 3 g/liter $NaCO_3$, 5 μg/ml insulin, 13.6 pg/ml triiodothyronine, 5 μg/ml transferrin, 0.25 μg/ml biotin, 25 μg/ml adenine) supplemented with 5% fetal bovine serum. The hMSC were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cultures were supplemented with 100 units/liter penicillin G and 100 μg/ml streptomycin. For osteogenic induction, regular medium was supplemented with 10 mM β-glycerophosphate and 50 μM ascorbic acid-2-phosphate (23).

RNA Extraction and RT-PCR—Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen). The yield and purity of RNA was estimated spectrophotometrically using the A_{260}/A_{280} ratio. The quality of RNA was examined by gel electrophoresis. One microgram of RNA was reverse transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for the PCRs. These were carried out in a final volume of 25 μl containing 0.2 mM dNTPs, 120 nM each primer, and 1 unit of *Taq* DNA polymerase. TaqMan real-time quantitative RT-PCR analysis was performed using the relative standard curve method with SYBRGreen (TaqMan PCR detector 5700; PerkinElmer Life Sciences). The expression of 18 S rRNA was used as control. The sequences for the specific primers used in this study were as follows: *notch1*, forward primer (5′-CACTGTGGGCGGGTCC-3′) and reverse primer (5′-GTTGTATTGGTTCGGCACCAT-3′) (24); *dll1*, forward primer (5′-TGTGTGACGAACACTACTACGGAG-3′) and reverse primer (5′-GTGAAGTGGCCGAAGGCA-3′) (24); *HES-1*, forward primer (5′-AGGCGGACATTCTGGAAATG-3′) and reverse primer (5′-CGGTACTTCCCCAGCACACTT-3′); *runx2*, forward primer (5′-GATGACACTGCCACCTCTGACTT-3′) and reverse primer (5′-AAAAAGGGCCCAGTTCTGAAG-3′); osteocalcin, forward primer (5′-CCCCTGCTTGTGACGAGCTA-3′) and reverse primer (5′-AATAGTGATACCGTAGATGCGTTTGT-3′); 18 S rRNA, forward primer (5′-CGCCGCTAGAGGTGAAATTCT-3′) and reverse primer (5′-CGAACCTCCGACTTTCGTTCT-3′).

Whole Cell Protein Extraction—At the end of the study, cells were washed with chilled phosphate-buffered saline and centrifuged at 800 × *g* for 5 min at 4 °C and then resuspended in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10% glycerol). A mixture of protease and phosphatase inhibitors consisting of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.1 mM β-glycerophosphate was added to the lysis buffer. Samples were then centrifuged at 14,000 rpm for 30 min at 4 °C, and the supernatant protein concentration was measured using the Bio-Rad DC protein assay (25).

Western Blot Analysis—Whole cell or nuclear extracts were loaded (30 μg/lane) onto an SDS mini-PAGE system. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Corp.), using a Bio-Rad wet transfer system. Protein transfer efficiency and size determination were verified using prestained protein markers. Membranes were then blocked with Blotto B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature and subsequently incubated overnight with antibodies directed against Notch1, ERK, phosphorylated ERK, Runx2, and actin (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an ECL detection kit (Amersham Biosciences).

Calcium Measurement—Cells were lysed with double distilled H_2O, accompanied by three freeze/thaw cycles. Calcium content was measured using a calcium detection kit (Arsenazo III; Sigma).

Nuclear Protein Extraction—C4-2B cells were washed with chilled phosphate-buffered saline and centrifuged at 800 × *g* for 5 min at 4 °C. Nuclei were then isolated by detergent lysis of the cells using an

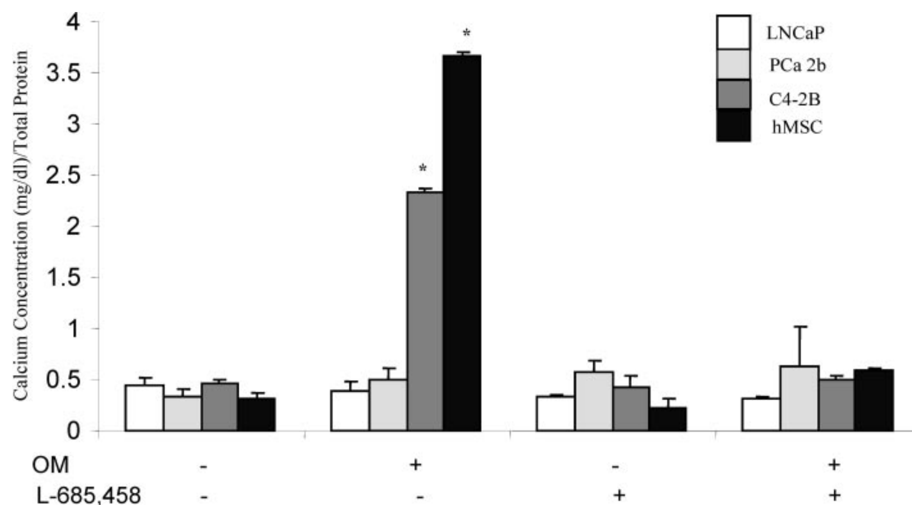


FIG. 3. **Notch signaling is critical for matrix mineralization.** Cells, as indicated, were cultured in regular medium for 4 days (LNCaP, PCa 2b, and C4-2B) or 7 days (hMSC). Cells were then either maintained in the same medium (*OM* (−)) for 10 days or cultured in osteogenic medium ((*OM*) (+)) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Cells were either treated with 100 nM L-685,458 (+), for the duration of osteogenic induction (14 days) to inhibit Notch signaling or vehicle alone (−). At the end of the study, cells were lysed, and calcium content was measured. Values were obtained from three separate experiments, each repeated in triplicate, and represent the mean ± S.E. of calcium content relative to total protein; *, $p \leq 0.02$.

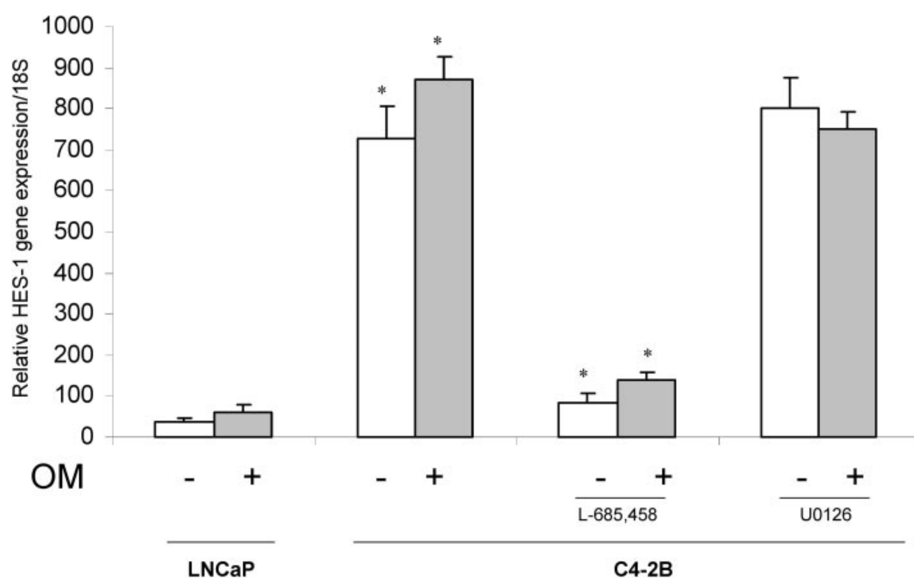


FIG. 4. **HES-1 expression (Notch signaling-dependent) is increased in C4-2B cells and is independent of ERK activation.** LNCaP and C4-2B cells were cultured in regular medium for 4 days. Cells were then either maintained in the same medium (*OM* (−)) for 10 days or cultured in osteogenic medium (*OM* (+)) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Twenty-four hours before harvesting, C4-2B cells were treated with 1 μM L-685,458 to inhibit notch signaling or (10 μM) U0126 to inhibit ERK activation. At the end of the study, RNA was extracted, and semiquantitative RT-PCR reactions were performed using primers for *HES-1* and 18 S rRNA. Values were obtained from three experiments and represent the mean ± S.E. of *HES-1* mRNA expression relative to 18 S rRNA expression; *, $p \leq 0.01$.

Nonidet P-40 lysis buffer containing 10 mM Tris, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.56 M sucrose. Nuclei were then treated with a hypotonic solution containing 10 mM HEPES, 1.5 mM $MgCl_2$, and 10 mM KCl followed by a 30-min incubation at 4 °C in an extraction buffer containing 20 mM HEPES, 20% glycerol, 600 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA. Nuclei were finally centrifuged at 14,000 rpm for 30 min at 4 °C, and the supernatant protein concentration was measured by the Bio-Rad DC Protein Assay. All solutions in this procedure contained a mixture of protease and phosphatase inhibitors similar to that used in the whole cell protein extraction.

Electrophoretic Mobility Shift Assay—Four micrograms of nuclear extracts were incubated for 20 min at room temperature with a ^{32}P-labeled oligonucleotide containing the human Runx2 consensus sequence. The oligonucleotide sequence used as a probe was 5′-CGCAGCTCCCAACCACATATCCTCT-3′ (26) (top strand), derived from the human osteocalcin promoter (−141 to −165), and contained an OSE2 motif (AACCACA) (27). Oligonucleotide with mutation in the Runx2 binding site was also used to confirm binding specificity. The mutant Runx2 sequence is 5′-CGCAGCTCCCAgaCACATATCCTCT-3′ (top strand). The double-stranded Runx2 probe was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase according to standard protocols. DNA-protein complexes were then resolved by 5% native polyacrylamide gels. Gels were dried and exposed to x-ray film at −80 °C with an intensifying screen. For the supershift experiment, nuclear extracts (10 μg) were incubated with HES-1 antibody (Santa Cruz Biotechnology) in binding buffer for 45 min at room temperature. ^{32}P-labeled oligonucleotide containing the human Runx2 consensus sequence was then added, and the mixture was further incubated for 30 min at room temperature.

Immunohistochemistry and Human Tissue Samples—Human bone samples from four patients were obtained from the Surgical Pathology Department of the University of Alabama at Birmingham Hospitals and Clinics with the approval of the Institutional Review Board. Tissues were formalin-fixed and decalcified in EDTA before processing on a VIP tissue processor followed by paraffin embedding. Sections were cut at 5 μm for immunostaining. C4-2B cells were cultured on glass coverslips for 4 days and then formalin-fixed.

Tissues were deparaffinized and rehydrated, followed by antigen retrieval using 10 mM citrate buffer, pH 6. Endogenous peroxidase activity was quenched using 1% hydrogen peroxide. Samples were then blocked for 1 h in Fc receptor blocker (Innovex Biosciences, Richmond, CA). Anti-Notch1 (Santa Cruz Biotechnology) or pancytokeratin (Biogenex) was diluted in Fc Blocker solution and applied to the sections for overnight incubation at 4 °C. Biotin-conjugated secondary antibodies were then used, followed by incubation with avidin-biotin enzyme reagents. Finally, specimens were incubated in peroxidase substrate for 30 s. Tissues were counterstained in Gill's heamatoxylin for 10 s, dehydrated, mounted, and coversliped. Negative controls were processed alongside the examined tissue, but rabbit IgG was used instead of the primary antibody. At least 10 randomly selected microscopic fields were examined using a ×10 and ×40 objective. Photos were taken using a SPOT digital camera.

Statistical Analysis—All statistical analyses were performed using the Microsoft Excel data analysis program for *t* test analysis or using SPSS statistical analysis program for analysis of variance with the Bonferroni test. Experiments were repeated at least three times unless otherwise stated. Values were expressed as a mean ± S.E.

RESULTS

Given the importance of Notch activation in determining cell fate, we examined *notch1* gene expressions in two human osteoblastic prostate cancer cell lines (C4-2B and MDA PCa 2b)

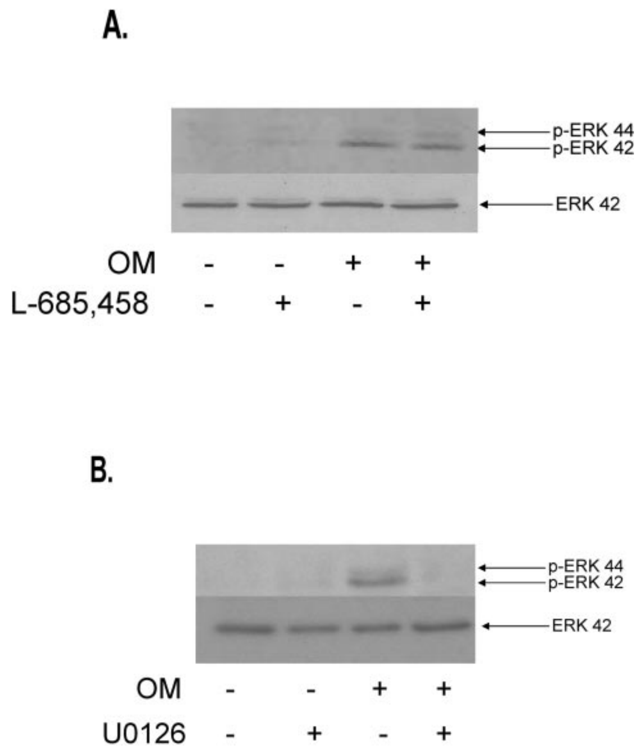


FIG. 5. **Osteogenic induction increases ERK activity in C4-2B cells independent of Notch signaling.** LNCaP and C4-2B cells were cultured in their regular medium for 4 days. Cells were then either maintained in the same medium (*OM* (−)) for 10 days or cultured in osteogenic medium (*OM* (+)) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Twenty-four hours before harvesting, C4-2B cells were treated with 1 μM L-685,458 (*A*) or 10 μM U0126 (*B*). At the end of the study, cytosolic protein was extracted and separated by 10% SDS-PAGE (30 μg/lane). Immunoblots were developed using specific antibodies directed against ERK 42 and phosphorylated ERK (*p-ERK*). The autoradiographs are representative of three separate experiments.

and compared them with brain (DU145) and lymph node (LNCaP) metastatic prostate cancer cell lines (Table I). Fig. 1*A* demonstrates that *notch1* gene expression is increased 5-fold in osteoblastic (C4-2B and PCa 2b) *versus* nonosteoblastic (LNCaP and DU145) prostate cancer metastatic cell lines. This increase in gene expression is accompanied by an increase in the amount of Notch1 protein (Fig. 1*B*), suggesting that Notch1 is specifically expressed in osteoblastic prostate metastases. Immunohistochemical studies further demonstrate the expression and distribution of Notch1 in C4-2B cells (Fig. 1*C*). Interestingly, Dll1 Notch ligand is only detected in the C4-2B cell line (Fig. 1*D*). The presence of both Notch1 and Dll1 in the same cell suggests that Notch signaling can be successfully activated in C4-2B cells without the need to co-culture them with Notch ligand-expressing cells. The expression of other Notch receptors (Notch2, -3, and -4) and ligands (Dll4 and Jagged-1 and -2) were also examined but showed no significant correlation between skeletal and nonskeletal metastatic cell lines (data not shown).

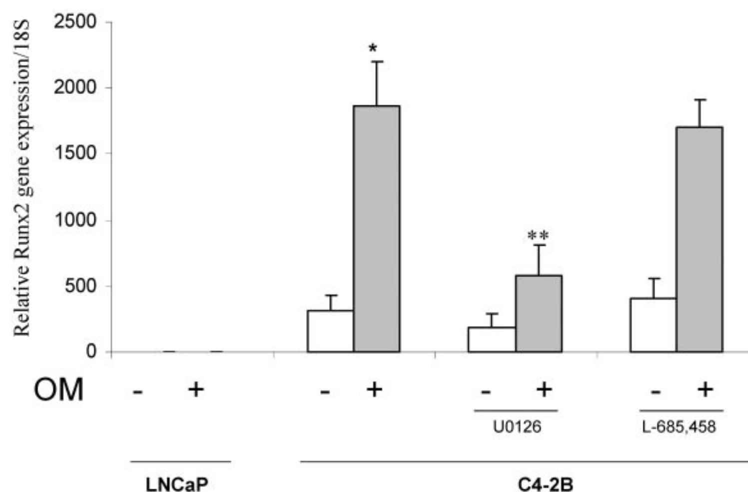
In order to examine the expression of Notch1 in prostate cancer bone metastases *in vivo*, human clinical samples from patients who developed bone metastases from a primary prostate tumor were obtained from the Surgical Pathology Department of the University of Alabama at Birmingham Hospitals and Clinics. Hematoxylin and eosin staining (Fig. 2*A*) confirms the osteoblastic nature of the prostate cancer metastases, which is demonstrated by the degree of trabacular bone present in the tissue. Immunohistochemical staining for pancytokeratin confirms the epithelial origin of the metastatic cancer cells and clearly distinguished them from the bone mesenchymal cells (Fig. 2*B*). Similar to C4-2B cells, human osteoblastic prostate cancer metastatic cells express Notch1 (Fig. 2*C*). Although most of the positively stained cells show an extranuclear distribution, Notch1 is also detected intranuclearly.

To examine the ability of prostate cancer metastases to function similar to osteoblasts and to form mineralized bone, we cultured LNCaP, MDA PCa 2b, and C4-2B cells with and without osteogenic medium, consisting of normal culture medium, supplemented with ascorbic acid and β-glycerophosphate. This method of osteogenic induction has been previously characterized and is widely used for inducing osteoblast differentiation *in vitro* (23). Cells were also treated with 100 nM Notch inhibitor (L-685,458), known to be a potent γ-secretase inhibitor (28), to determine the role of Notch signaling in bone formation by metastatic prostate cancer cells. After 10 days of osteogenic induction, the calcium content in cultures was measured. Fig. 3 demonstrates that both LNCaP cells (which do not express Notch1) and MDA PCa 2b cells (which do not express Dll1) fail to increase calcium deposition in culture. On the other hand, C4-2B cells, which form osteoblastic bone metastases *in vivo* (22) and express both Notch1 and Dll1, increase calcium deposition 5-fold when cultured in osteogenic medium. This increase is completely abolished when Notch inhibitor (L-685,458) is added. We also used hMSC as a positive control, since these cells develop into osteoblasts when cultured in osteogenic medium (23). We demonstrate a 7-fold increase in calcium content when hMSC are induced to develop into osteoblasts, by the presence of osteogenic medium. Interestingly, Notch inhibitor is also effective in blocking this mineralization.

Osteogenic induction induces the osteoblastic differentiation of preosteoblasts by increasing the activity of MAPK, which ultimately leads to an increase in Runx2 activation and DNA binding (28). In addition, osteoblastic cell differentiation is also positively regulated by Notch1 (19). Therefore, we tested the effect of osteogenic induction on Notch signaling by examining the expression of *HES-1*, a known downstream target of Notch signaling, in C4-2B cells. Furthermore, to determine the specific role of both Notch and ERK signaling in *HES-1* expression, we pharmacologically inhibited Notch signaling with L-685–458 or ERK signaling with U0126 (29). As expected, LNCaP cells, which lack Notch1 receptor, did not express *HES-1* (Fig. 4). In contrast, C4-2B cells, which express both Notch1 and its ligand, expressed *HES-1*, regardless of osteogenic induction. The inhibition of Notch signaling by γ-secretase inhibitor successfully inhibited *HES-1* expression, whereas the ERK inhibitor, U0126, had no effect.

To examine whether C4-2B cells would respond similarly to osteoblasts when cultured in osteogenic medium, we tested the activation of ERK in response to osteogenic induction while inhibiting the activation of either Notch or ERK signaling. Fig. 5, *A* and *B*, demonstrate that osteogenic induction increases ERK phosphorylation in C4-2B cells, as in osteoblasts, without any change in the total ERK levels. Notch signaling does not seem to play a role in this activation, since we show no significant difference in ERK activity when Notch signaling was inhibited for 24 h by 1000 nM L-685,458 (Fig. 5*A*). As expected, U0126 inhibited the activation of ERK in response to osteogenic induction (Fig. 5*B*).

The activity of MAPK in osteoblasts ultimately leads to an increase in Runx2 expression and its activation/nuclear localization (30). Fig. 6 demonstrates that LNCaP cells do not express Runx2, even under conditions of osteogenic induction. In contrast, C4-2B cells express basal levels of Runx2 mRNA and nuclear protein. Upon osteogenic induction, both mRNA and nuclear localization of Runx2 are increased in C4-2B cells. This



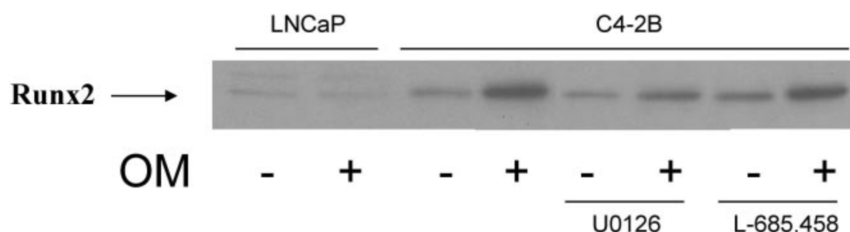


FIG. 6. **Osteogenic induction increases Runx2 expression and activation in C4-2B cells independent of Notch signaling.** LNCaP and C4-2B cells were cultured in regular medium for 4 days. Cells were then either maintained in the same medium (*OM* (−)) for 10 days or cultured in osteogenic medium (*OM* (+)) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Twenty-four hours before harvesting, C4-2B cells were treated with 1 μM L-685,458, to inhibit notch signaling, or 10 μM U0126, to inhibit ERK activation. At the end of the study, RNA and nuclear protein were extracted. *A*, semiquantitative RT-PCRs were performed using primers for Runx2 and 18 S rRNA. Values were obtained from three experiments and represent the mean ± S.E. of Runx2 mRNA expression relative to 18 S rRNA expression. Runx2 expression in C4-2B cultured under osteogenic induction conditions was significantly higher when compared with uninduced cells; *, $p \leq 0.01$. U0126 treatment significantly decreased Runx2 expression when compared with untreated cells (**, $p \leq 0.03$), whereas treatment with L-685,458 had no effect. *B*, nuclear protein extracts (30 μg/lane) were separated by 12% SDS-PAGE. Immunoblots were developed using specific antibodies directed against Runx2. The autoradiographs are representative of three separate experiments.

response is ERK-dependent, since we show that U0126 is able to inhibit both Runx2 gene expression and nuclear localization. However, Notch signaling does not play a role in Runx2 expression and activation in response to osteogenic induction because of the inability of L-685,458 to alter its expression (Fig. 6, *A* and *B*).

Many osteoblast-associated genes, such as osteocalcin (31), are modulated by Runx2 binding to a specific DNA element in the regulatory region of the target genes (21, 32). Fig. 7*A* demonstrates that osteogenic induction of C4-2B prostate cancer cells increases Runx2 binding activity on the OSE-2 of the human osteocalcin promoter (Fig. 7*A*, *lane 3*). This binding is inhibited by L-685,458, the inhibitor for Notch activity (Fig. 7*A*, *lane 5*) or U0126, inhibitor of ERK activity (Fig. 7*A*, *lane 7*). This suggests that the presence of both transcription factors, Runx2 and HES-1, are required for OSE-2 DNA binding in the C4-2B osteoblastic prostate cancer metastatic cell line. Fig. 7*B* confirms that HES-1 is part of this DNA-protein complex, since it was shifted by the addition of HES-1 antibody in C4-2B cells after osteogenic induction with and without U0126 treatment. Not surprisingly, HES-1 antibody failed to produce a shift in cells treated with L-685,458. Furthermore, binding competition experiments were performed in the presence of a 50-fold molar excess of unlabeled normal or mutant OSE-2 oligonucleotides to confirm binding specificity. The addition of 50× OSE-2 oligonucleotide is successful in competing for Runx2 binding, whereas the mutant oligonucleotide fails to compete (data not shown).

Finally, we examined the gene expression of osteocalcin in C4-2B cells, in response to osteogenic induction. The role of both Notch and ERK signaling in this expression were also examined. Osteogenic induction causes a 6-fold increase in osteocalcin gene expression, and the inhibition of either Notch or ERK signaling pathways is sufficient to inhibit osteocalcin expression in response to osteogenic induction in C4-2B cells (Fig. 8). Taken together, our results suggest that when prostate cancer cell lines, which are derived from bone metastases, are exposed to the bone microenvironment and interact with the extracellular matrix and stromal cells, the Notch and ERK pathways are independently activated. This leads to an increase in OSE2 binding activity by both Runx2 and HES-1 and transformation of C4-2B cells to osteoblast-like cells capable of expressing osteoblastic genes, such as osteocalcin (Fig. 9).

DISCUSSION

Prostate carcinoma is the most commonly diagnosed cancer in United States men and is the second leading cause of cancer mortality in men over the age of 50 years in the United States (33). Advanced prostate carcinoma is associated with osteoblastic bone metastases in contrast to the osteolytic bone metastases observed in most other types of cancer. The tissue-specific mechanisms responsible for these unique osteoblastic proper-

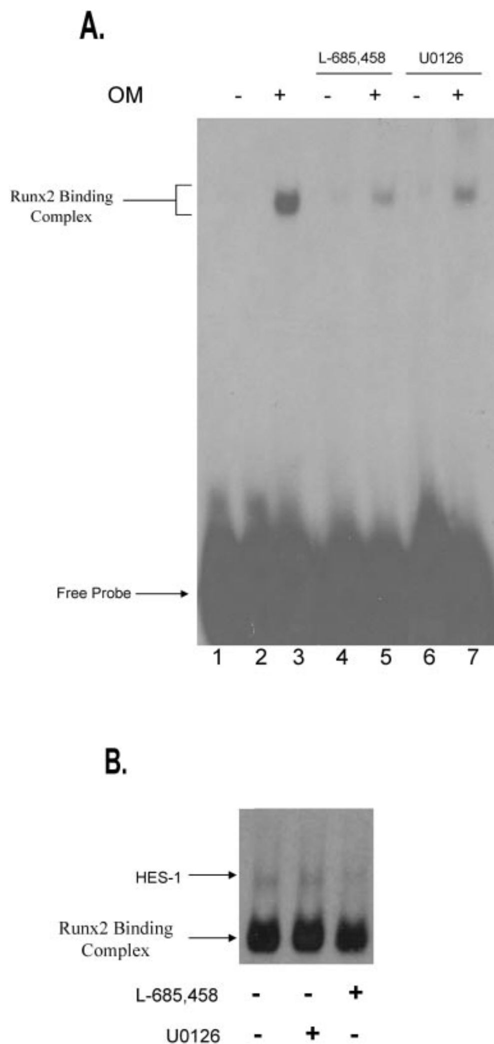


FIG. 7. **Osteogenic induction of C4-2B cells induces osteoblast-specific Runx2 DNA binding activity, a Notch and ERK signaling-dependent response.** C4-2B cells were cultured in regular medium for 4 days. Cells were then either maintained in the same medium (*OM* (−); *lanes 2*, *4*, and *6*) for 10 days or cultured in osteogenic medium (*OM* (+); *lanes 3*, *5*, and *7*) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Cells were treated for 24 h with 1 μM L-685,458 (*lanes 4* and *5*) or 10 μM U0126 (*lanes 6* and *7*). *A*, nuclear extracts (4 μg) were mixed with a ^{32}P-labeled oligonucleotide containing a human consensus OSE-2 sequence (5′-CGCAGCTCCCAACCACATATCCTCT-3′), and an electrophoretic mobility shift assay was performed. *Lane 1*, free DNA. *B*, nuclear extracts (10 μg) were incubated with HES-1 antibody followed by incubation with OSE-2 ^{32}P-labeled oligonucleotide sequence before performing an electrophoretic mobility shift assay. The autoradiograph is representative of three separate experiments.

ties of prostate cancer bone metastases are not known. Recently, it has been demonstrated that Notch1 activation by Notch ligand (Dll1) leads to an increase in osteoblast differentiation (19). It has also been shown that Notch1 is expressed by prostate basal epithelial cells (24). Our findings provide evidence that prostate cancer cells that are capable of metastasizing to bone *in vivo* specifically express Notch1 receptor. Furthermore, through both Notch and MAPK signaling, osteogenic induction of the C4-2B prostate cancer osteoblastic metastatic cell line uniquely increases Runx2 DNA binding activity and osteocalcin gene expression, transforming the cancer cells into a mineralizing osteoblastic phenotype.

Notch signaling plays a role in the development of various tissues and cells including neurogenesis (34, 35) and myogenesis (36). It has been shown that osteoblast differentiation is also Notch-dependent (19), specifically through activation of the Notch1 receptor by Dll1 ligand. Our data demonstrate that cultured prostate cancer cells with the ability to form bone metastases *in vivo* (C4-2B and PCa 2b) have an increase in Notch1 expression compared with nonskeletal metastatic cells (LNCaP and DU145). This suggests that these cells are capable of activating Notch signaling when placed in an environment where Notch ligand is present. In addition, we show that from all of the cells we studied, C4-2B is the only prostate cancer metastatic cell line that expressed the Notch ligand, Dll1. Our data are consistent with the report describing the dynamics of Notch expression during murine prostate development, where it was demonstrated that Notch1 is the predominant Notch receptor expressed by prostate cancer metastatic cell lines (24). In addition, our immunohistochemical staining demonstrates that Notch1 is not only present in cell lines but also present in human clinical samples. The presence of nuclear Notch1 staining suggests that Notch signaling is active in these cells.

The osteomimetic properties of prostate cancer metastases have been previously investigated. Our data support the phenomenon demonstrated by Lin *et al.* (10) whereby C4-2B cells acquire an "osteoblast-like" phenotype in response to osteogenic induction. Additionally, we demonstrate that inhibiting Notch signaling with L-685–458, a potent γ-secretase inhibitor (37), completely suppresses calcium deposition by C4-2B cells. This suggests that Notch activation is essential for acquisition of the osteoblastic phenotype. Other prostate cancer metastatic cells fail to respond to osteogenic induction, most likely because of the lack of expression of Notch1 (LNCaP and DU145) or Dll1 (PCa 2b). Furthermore, it has been reported that in papillomavirus-associated neoplasia, Notch activation plays a role in regulating proliferation and apoptosis (38). The inhibition of γ-secretase by L-685–458 treatment had no effect on proliferation and viable cell numbers as determined by the trypan blue exclusion method (data not shown). On the other hand, we were not able to inhibit ERK activation for the duration of the culture to examine its role in calcium deposition by C4-2B cells because of the cytotoxic effects of the inhibitors.

HES-1 is the best characterized member of the mammalian HES family, and it is has been shown to be an important downstream target of Notch signaling (39). HES genes encode basic helix loop helix transcription factors, where the basic domain is required for DNA binding and the helix-loop-helix domain mediates dimerization with other basic helix loop helix proteins. Our results demonstrate that *HES-1* expression in C4-2B cells is 20-fold higher than in LNCaP cells, suggesting that the presence of both Notch1 and Dll1 is sufficient to activate Notch signaling, independent of osteogenic induction. This is further confirmed by the inhibition of *HES-1* expression when cells were treated with L-685–458 (γ-secretase inhibitor).

Several signaling pathways are involved in osteoblast differentiation, including the ERK/MAPK pathway (30, 40). Our results demonstrate that C4-2B cells, like osteoblasts, increase ERK phosphorylation in response to *in vitro* osteogenic induction. This ERK activation in prostate cancer metastases could also occur in the bone microenvironment. Prostate cancer cells have been shown to activate ERK in response to 1) the autocrine effect of Urokinase-type plasminogen activator (41), 2) the paracrine effect of transforming growth factor-β1 (42), or 3) attachment of prostate cancer cells to the extracellular matrix in the bone microenvironment (43). In addition, there is a possible link between Notch and activation of ERK. It has been shown that the activation of Notch in small cell lung cancer markedly activates ERK (44). Furthermore, it has previously been reported that transformation by Notch in some cells requires active signals from the ERK/MAPK (45). However, this

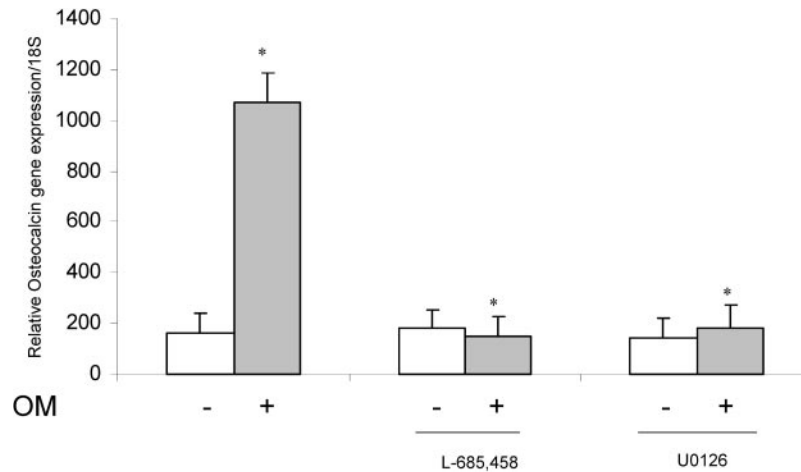


FIG. 8. **Osteogenic induction increases osteocalcin expression in C4-2B cells, a Notch and ERK signaling-dependent response.** C4-2B cells were cultured in regular medium for 4 days. Cells were then either maintained in the same medium (*OM* (−)) for 10 days or cultured in osteogenic medium (*OM* (+)) consisting of regular medium supplemented with β-glycerophosphate and ascorbic acid-2-phosphate. Twenty-four hours before harvesting, C4-2B cells were treated with 1 μM L-685,458, to inhibit notch signaling, or 10 μM U0126, to inhibit ERK activation. At the end of the study, RNA was extracted, and semiquantitative RT-PCRs were performed using primers for osteocalcin and 18 S rRNA. Values were obtained from three experiments and represent the mean ± S.E. of Runx2 mRNA expression relative to 18 S rRNA expression. Osteocalcin expression in C4-2B cultured under osteogenic induction conditions was significantly higher when compared with uninduced cells; *, $p \leq 0.01$. U0126 and L-685,458 treatment significantly decreased osteocalcin expression when compared with untreated cells (*, $p \leq 0.01$).

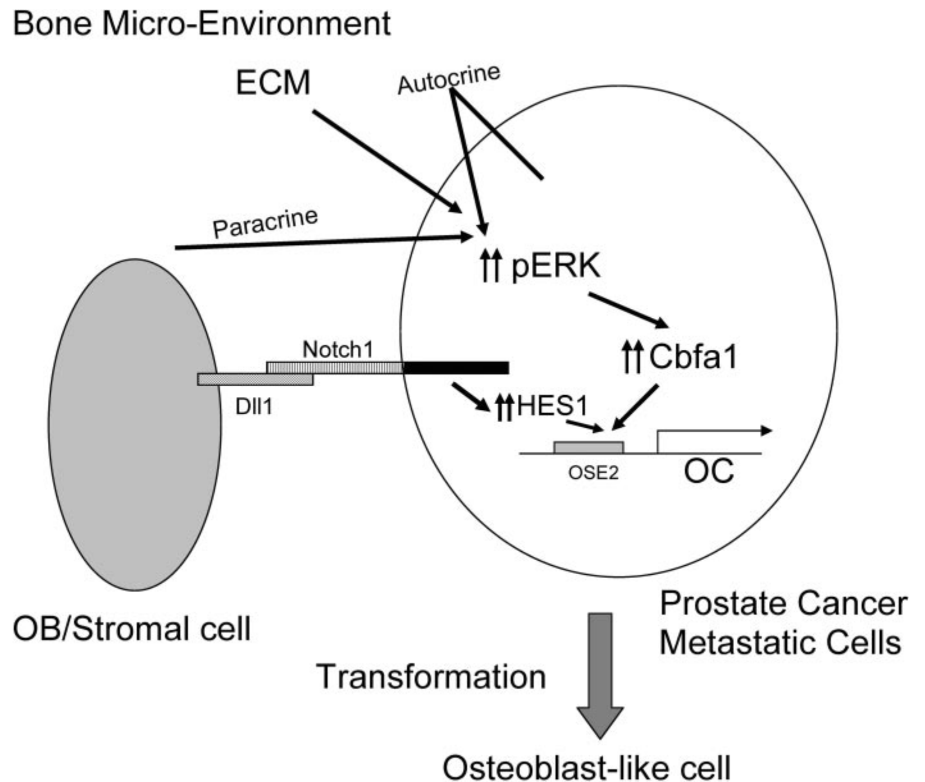


FIG. 9. **A proposed mechanism depicting the osteoblastic transformation of prostate cancer metastases in the bone microenvironment.** The cellular interaction between prostate cancer metastatic cells and osteoblasts (*OB*) or bone stromal cells activates Notch signaling and increases the expression of HES-1. ERK activity increases in the bone microenvironment due to paracrine or autocrine hormones, or attachment to bone extracellular matrix (*ECM*) proteins. This leads to an increase in both Runx2 expression and activity. Together, HES-1 and Runx2 lead to an activation of osteoblast specific genes, such as osteocalcin (*OC*), thereby leading to the transformation of PCa into osteoblast-like cells.

is in contrast to our results, since we demonstrate that ERK activation is Notch-independent due to the absence of a response in ERK activation to L-685–458 treatment. We also demonstrate that Notch activation in C4-2B cells is ERK-independent, since there is no change in *HES-1* expression in response to U0126 treatment. Interestingly, we did not detect any activation of ERK until cells were treated with osteogenic medium. Therefore, we believe that in prostate cancer metastases the concomitant activation of the ERK and Notch pathways while in the bone microenvironment is critical for the successful osteoblastic change of phenotype.

We and others have shown that osteoblastic prostate cancer metastatic cells, like osteoblasts, also express Runx2 in response to osteogenic medium (10), and it has been shown that inhibition of Runx2 expression in C4-2B cells leads to loss of their mineralizing capability (10). These findings are consistent with our results. We demonstrate here that inhibition of ERK activation, but not Notch, decreases Runx2 gene expression and nuclear protein localization. Interestingly, we also show that Runx2 gene expression and nuclear translocation is absent in LNCaP when compared with C4-2B cell line, even in the presence of osteogenic induction. This suggests that the decreased expression of Notch1 and Dll1 in LNCaP cells is not the only explanation for acquiring osteomimetic properties. Other signaling and genetic factors such as ERK activation and Runx2 expression are also involved. The link between ERK and

Runx2 has already been studied in osteoblasts and primary osteoblast cultures (30, 40). The increase in Runx2 expression and activity is known to be essential for normal skeletal development and osteoblast differentiation. Interestingly, it has been shown that HES-1 can antagonize the binding of Runx2 to mammalian transcriptional co-repressors of the Groucho family (46). In addition, HES-1 was shown to potentiate Runx2-mediated transactivation in transfected cells (18). This suggests that the concomitant expression and activation of these two transcription factors, Runx2 and HES-1, in osteoblastic prostate cancer metastatic cell lines can potentiate each other's action. This postulation is supported by our findings that osteogenic induction induces Runx2 DNA binding activity. The lack of OSE-2 binding in the absence of osteogenic induction and in the presence of L-685–458 and U0126 suggests that Runx2-DNA binding is both Notch- and ERK-dependent. In addition, the presence of HES-1 in the protein-DNA complex confirms that this binding is Notch-dependent. Furthermore, we show that osteocalcin expression, which is regulated by Runx2 activation, is also Notch- and ERK-dependent.

Taken together, our data suggest that upon metastasis of prostate cancer cells to the bone microenvironment, an interaction with the extracellular matrix and stromal cells occurs. The Notch and ERK pathways are both activated, leading to an increase in *HES-1* and *runx2* expression. These two transcription factors interact, leading to an increase in the expression of genes, dependent on the activation OSE-2. This ultimately directs the transformation of prostate cancer bone metastatic cells to osteoblast-like cells (Fig. 9).

Despite the importance of the Notch signaling pathway in cell fate determination and the ability of prostatic cancer bone metastasis to induce osteoblastic lesions, Notch signaling in prostate cancer metastasis remains an understudied subject. Our findings provide the first documentation of a novel mechanism to explain the ability of prostate cancer skeletal metastases to induce osteosclerotic lesions. A basis may ultimately be provided for the development of a new target for drug design and therapeutic intervention to combat the debilitating condition of prostate carcinoma bone metastases.

Acknowledgments—We thank Patty Lott in the University of Alabama at Birmingham Histomorphometry and Molecular Analysis Core Laboratory of the Center for Metabolic Bone Disease for help with the immunohistochemistry staining. We also thank Margaret A. McKenna for technical assistance and critical reading of the manuscript.

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