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Increased Notch 1 Expression and Attenuated Stimulatory G Protein Coupling to Adenylyl Cyclase in Osteonectin-Null Osteoblasts

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Osteonectin, or secreted protein acidic and rich in cysteine, is one of the most abundant noncollagen matrix components in bone. This matricellular protein regulates extracellular matrix assembly and maturation in addition to modulating cell behavior. Mice lacking osteonectin develop severe low-turnover osteopenia, and *in vitro* studies of osteonectin-null osteoblastic cells showed that osteonectin supports osteoblast formation, maturation, and survival. The present studies demonstrate that osteonectin-null osteoblastic cells have increased expression of Notch 1, a well-documented regulator of cell fate in multiple systems. Furthermore, osteonectin-null cells are more plastic and less committed to osteoblastic differentiation, able to pursue adipogenic differentiation given the appropriate signals. Notch 1 transcripts are down-regulated by inducers of cAMP in both wild-type and osteonectin-null osteoblasts, suggesting that the mutant osteoblasts may have a defect in generation of cAMP in response to stimuli. Indeed, many bone anabolic agents signal through increased cAMP. Wild-type and osteonectin-null osteoblasts generated comparable amounts of cAMP in response to forskolin, a direct stimulator of adenylyl cyclase. However, the ability of osteonectin-null osteoblasts to generate cAMP in response to cholera toxin, a direct stimulator of G_s, was attenuated. These data imply that osteonectin-null osteoblasts have decreased coupling of G_s to adenylyl cyclase. Because osteonectin promotes G protein coupling to an effector, our studies support the concept that low-turnover osteopenia can result from reducing G protein coupled receptor activity. (*Endocrinology* 148: 1666–1674, 2007)

BONE IS A highly dynamic organ, continuously remodeling in response to mechanical and metabolic stresses. The bone matrix, which includes type I collagen, noncollagen matrix components, and hydroxyapaptitie, provides a multidimensional environment that supports structural and physiological needs of the skeleton. In addition, the bone matrix provides for the developmental needs of the cells in this habitat, such as osteoblasts, osteocytes, osteoclasts, stromal cells, and cells in the hematopoietic niche (1–4). Thus, alterations in bone matrix composition have the potential to alter the behavior of the cells interacting with this support network.

Osteonectin, also called SPARC (secreted protein acidic and rich in cysteine) or BM-40, is one of the most abundant noncollagen matrix components in bone (5). This extracellular matrix glycoprotein is highly expressed in conditions of active matrix remodeling or cellular stress. As a member of the matricellular protein family, it and other family members, including thrombospondins and tenacins, function as modulators of cell behavior as well as structural components of the matrix (6). Osteonectin has a high affinity for type I collagen, and there is evidence that osteonectin regulates collagen fibril structure and composition (7). In addition, cells can take in osteonectin from their environment, and it can be located in the cytoplasm and translocated to the nucleus (8–10). Furthermore, osteonectin has been shown to colocalize with tubulin in *Xenopus* embryos and integrin-linked kinase in murine fibroblasts (11, 12). A receptor for osteonectin has yet to be described, suggesting that its ability to interact with transmembrane and intracellular components could provide a means for regulating gene expression.

Although osteonectin-null mice are viable and fertile, they develop cataracts, skin fragility, increased amounts of adipose tissue, and they have accelerated cutaneous wound healing, phenotypes associated with defective extracellular matrix composition and organization (7, 13–15). Our laboratory has focused on analyzing the skeleton of osteonectin-null mice. We found that the mutant mice develop severe low turnover osteopenia, resulting from decreased numbers of osteoblasts and osteoclasts and an attenuated bone formation rate (16). *In vitro* studies of osteonectin-null osteoblastic cells demonstrated that osteonectin supports osteoblast formation, maturation, and survival. Osteonectin-null osteoblasts cultured in osteoblast differentiation medium make fewer mineralized nodules, are less responsive to stimulation with PTH, and have a greater number of adipocytic cells, compared with wild-type osteoblasts. In these cultures expression of osteocalcin mRNA, a marker for mature osteoblasts, is decreased, whereas adipisin mRNA, a marker for mature adipocytes, is increased (17). Concurrently, osteonectin-null cells have differential expression of genes and proteins associated with the control of adipocytic differentiation, in-

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Abbreviations: CSL, C-promoter binding factor 1/suppressor of hairless/LAG-1 or RBP-Jk; EIA, enzyme immunoassay; FBS, fetal bovine serum; GRK, G protein-coupled receptor kinase; HES, hairy enhancer of split; IBMX, isobutrylmethylxanthine; NICD, Notch 1 intracellular domain; rh, recombinant human; SBE, phosphorylated mothers against decapentaplegic binding element.

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cluding the transcription factors CCAAT/enhancer-binding protein-δ and Δ1/2 FBJ murine osteosarcoma viral oncogene homolog B (17). However, these changes in gene expression occur later, *i.e.* after 2–3 wk of *in vitro* differentiation. Because commitment to osteoblastic or adipocytic differentiation should occur before the expression of such markers, we sought to identify genes whose expression was differentially regulated in osteonectin-null and wild-type osteoblastic cells early in culture, genes that may be important in regulation of cell fate.

One gene fitting this description is Notch 1, a transmembrane receptor that interacts with its cell-associated ligands, members of the Serrate/Jagged/Delta family. Notch proteins are cell fate regulators expressed in virtually all metazoans (18). It is suggested that Notch signaling plays an important role in the regulation of both adipogenesis and osteogenesis (19–24). Earlier studies showed that murine osteoblasts express transcripts for Notch 1 and 2 and the ligands Jagged 1 and Delta 1 (25). In the present studies, we examine Notch expression in wild-type and osteonectin-null osteoblasts, revealing potential mechanisms by which osteonectin could modulate the differentiation of skeletal cells.

Materials and Methods

Primary osteoblastic cells

Osteoblastic cells were isolated from parietal bones of neonatal mice by sequential collagenase digestion, as described (26, 27). Wild-type and osteonectin-null mice backcrossed six to eight times into the C57BL/6 genetic background were used (17). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with nonessential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid, and 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA). Subconfluent cultures were trypsinized and replated at 10,000 cells/cm^2, and these first-passage cells were used for experiments. When the cells reached confluence, approximately 1 wk after plating, they were subsequently cultured in osteoblast differentiation medium (DMEM containing 10% FBS, 100 μg/ml ascorbic acid, 20 mM HEPES, and 5 mM β-glycerophosphate). Medium was changed twice a week for up to 3 wk after confluence (17). RNA was isolated from cells once a week, always 3 d after their last medium change.

Wild-type and osteonectin-null osteoblastic cell lines

Wild-type and osteonectin-null osteoblast cell lines (mObI-2; mouse osteoblast immortalized, line 2) were created by transducing primary cultures of osteoblastic cells with replication incompetent retrovirus constitutively expressing the human papilloma virus 16 E6/E7 open reading frame. These cell lines have been previously described, and their characteristics are similar to those of primary osteoblastic cells derived from calvaria of wild-type and osteonectin-null mice (17). Early passage mObI-2 cells were cultured in αMEM containing 20 mM HEPES and 10% FBS. For *in vitro* osteoblast differentiation experiments, this medium was supplemented with 5 mM β-glycerophosphate and an additional 50 μg/ml ascorbic acid and was changed twice a week for up to 4 wk after confluence. RNA was isolated from cells once a week, always 3 d after their last medium change. For adipocytic differentiation experiments, confluent cultures were maintained in an adipocytic differentiation cocktail consisting of αMEM, 20 mM HEPES, 10% FBS, 1 μM dexamethasone (Sigma, St. Louis, MO), 0.1 μM insulin (Calbiochem, La Jolla, CA), and 0.5 mM isobutrylmethylxanthine (IBMX) (Calbiochem) (20). Medium was changed twice a week, and RNA was isolated from cells once a week, always 3 d after their last medium change. For the remaining experiments, confluent cultures were maintained in reduced serum medium (αMEM, 20 mM HEPES, 50 μg/ml ascorbic acid, 1% FBS) for 24 h before treatment with test reagents including cholera toxin (Calbiochem), forskolin (Sigma), dichlorobenzimidazole riboside (Sigma), and recombinant human (rh) TGFβ (rhTGFβ1; R & D Systems, Minneapolis, MN). Reduced serum conditions were used in lieu of serum-free conditions because osteonectin-null osteoblastic cells are sensitive to apoptosis induced by serum deprivation (17).

Wild-type and osteonectin-null osteoblastic cells expressing the Notch 1 intracellular domain (NICD) in the antisense orientation were made using mObI-2 cells. Briefly, a 2398-bp fragment of the Notch 1 cDNA carrying a myc tag at the 3′ end was subcloned in to the retroviral expression vector pLPCX (CLONTECH, Palo Alto, CA) in the antisense orientation (21). Replication-defective retrovirus (vector alone or antisense Notch1) was packaged in Phoenix cells, and virus-containing supernatants were used to transduce wild-type and osteonectin-null mObI-2 cells (17). Pools of puromycin-resistant cells were propagated and analyzed.

Northern blot analysis

RNA was isolated using RNeasy (QIAGEN, Valencia, CA) or Nucleospin RNA II columns (BD Biosciences, Palo Alto, CA), according to the manufacturers' instructions. Equal amounts of RNA (5–10 μg) were denatured and subjected to electrophoresis through formaldehyde-agarose gels, and the RNA was blotted onto Gene Screen Plus as directed by the manufacturer (DuPont, Wilmington, DE). Restriction fragments containing portions of cDNA for osteonectin (provided by M. Young, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD), Notch 1 (provided by U. Lendahl, Karolinska Institute, Stockholm, Sweden), adipsin (American Type Culture Collection, Manassas, VA), and murine 18S rRNA (American Type Culture Collection), myc tag (from pcDNA3.1mycHisA; Invitrogen), and a genomic DNA fragment for rat osteocalcin (provided by J. Lian, University of Massachusetts Medical School, Worcester, MA) were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; NEN, PerkinElmer, Boston, MA) by random-primed, second-strand synthesis (Ready to Go; Amersham, GE Healthcare, Buckinghamshire, UK) (28–32). Hybridizations and post-hybridization washes were carried out as described (17). Autoradiograms were imaged and relative band densities were analyzed using ImageJ software (http://rsb.info.nih.gov/ij/docs/index.html). mRNA levels were normalized to those of 18S.

Immunoprecipitation

Confluent cultures were maintained in reduced serum medium for 24 h before homogenization in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-Cl (pH 8.0)] (26). The protein content of cell lysates was quantified by DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were incubated with goat anti-Notch1 antiserum (M20; Santa Cruz Biotechnology, Santa Cruz, CA) or normal goat IgG overnight at 4 C. Immune complexes were collected on protein G Sepharose (Sigma), washed with radioimmunoprecipitation assay buffer, and eluted by boiling in reducing Laemmli sample buffer. Proteins were fractionated by SDS-PAGE on a 10% gel and silver stained (33). Dried gels were imaged and relative band densities were analyzed using ImageJ software.

Transcription rate analysis

The transcription rate of Notch 1 was estimated in confluent cultures of wild-type and osteonectin-null mObI-2 cells by nuclear run-off assay. Cells were cultured in reduced serum conditions for 24 h before isolation of nuclei. Transcription rate analysis was performed as previously described (34). Briefly, nuclei were isolated from cells by Dounce homogenization in a Tris-Cl buffer containing 0.5% Igepal CA-630 (Sigma). Nascent transcripts were labeled by incubation of nuclei in reaction buffer containing RNAsin (Promega, Madison, WI) and 500 μM each ATP, GTP, and CTP in the presence of 250 μCi [^{32}P]UTP (800 Ci/mM; NEN). RNA was isolated by treatment with DNase I and proteinase K. Linearized plasmid DNA containing approximately 1 μg cDNA sequence of interest was immobilized onto GeneScreen Plus by slot blotting, according to the manufacturers' instructions. Equal counts per minute of [^{32}P]RNA was hybridized to immobilized cDNA. pBS (pBlueScript SK+) was used as a control for nonspecific hybridization, and 18S rRNA was used as a control for loading. Autoradiograms were imaged

and relative band densities were analyzed using ImageJ software. The assay shown is representative of independent two experiments, each yielding similar results.

Transient transfections

Activation of the hairy enhancer of split (HES) 5 promoter was assayed using a construct in which a 3.7-kb fragment of the mouse HES 5 promoter was cloned upstream of the luciferase gene in the vector tk-luc (provided by U. Lendahl) (35). Responsiveness to TGFβ was assayed using 3TP-luc, a reporter construct in which luciferase transcription is driven by a portion of the of the tissue plasminogen activator promoter (provided by J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY) and using phosphorylated mothers against decapentaplegic binding element (SBE)-4-luc, a construct in which luciferase transcription is driven by promoter containing four tandem repeats of the SBE (construct from S. Kern, Johns Hopkins University, Baltimore, MD) (36, 37). Wild-type and osteonectin-null mObI-2 cells were seeded into 12-well plates at 250,000 cells/well. Cells were transiently transfected with 250-1000 ng/well luciferase reporter plus 250 ng/well CMV-βgal (CLONTECH) using FuGENE6 (Roche Diagnostics, Indianapolis, IN) at a ratio of 3 μl FuGENE to 2 μg DNA. At confluence, cells were maintained in reduced serum conditions for 24 h before isolation of cell lysates in reporter lysis buffer (Promega). Luciferase and βgal activities were measured via luminometer using luciferase assay reagent (Promega) and Galacton (Tropix, Bedford, MA), respectively. Assays were performed at least three times, using four wells per experiment. Luciferase activity is expressed as a percentage of βgal activity, and representative experiments are shown. Data are expressed as means ± SEM.

cAMP enzyme immunoassay (EIA)

cAMP levels were determined in cultures that had been maintained in reduced serum medium for 24 h and subsequently treated with 0.5 μM IBMX for 10 min before the addition of cholera toxin (0–100 nM) or forskolin (0–100 mM) for 3 min. Cells were rapidly washed in PBS and lysed in hot 50 mM HCl containing 0.5 μM IBMX and boiled for 5 min before storage at −80 C (38). Cleared lysates were lyophilized and resuspended in assay buffer, and an aliquot of the extract was used to determine cAMP levels with a specific EIA kit, according to the manufacturer's instructions (Biomedical Technologies, Staughton, MA), and an aliquot of the extract was used to determine total protein (Bio-Rad DC assay). Data are expressed as picomoles cAMP per microgram cell protein, and four to six wells were used per experiment.

Data analysis

Statistical differences between wild-type and osteonectin-null cells were calculated by *t* test or ANOVA, where appropriate (KaleidaGraph; Synergy Software, Reading, PA).

Results

Increased Notch 1 in osteonectin-null osteoblasts

In primary cultures of osteoblastic cells from wild-type and osteonectin-null mice, we found that Notch 1 mRNA expression was significantly increased in osteonectin-null cells (Fig. 1, *top panels*). This increase was seen as early as confluence and remained apparent throughout early osteoblastic differentiation. Transcripts for Notch 2, Jagged 1, and Delta 1 were detected, but there was no consistent difference in their expression between control and osteonectin-null osteoblasts (data not shown). As expected, Notch 1 gene expression was also increased in immortalized osteoblastic cell lines from wild-type and osteonectin-null mice (mObI-2 cells), confirming that these cells can be surrogates for primary cultures (Fig. 1, *middle panels*). Immunoprecipitation of Notch 1 from the lysates of confluent mObI-2 cells confirmed that osteonectin-null cells expressed approximately 1.6-fold more Notch 1 than wild-type cells (Fig. 1, *bottom panels*). Nuclear run-off assays showed that Notch 1 transcription was increased approximately 2-fold in osteonectin-null osteoblastic cells (Fig. 2, *top*). Transcription arrest studies, designed to estimate the stability of Notch 1 mRNA, showed that the half-life of Notch 1 mRNA was approximately 2 h in both control and osteonectin-null osteoblasts (Fig. 2, *bottom*). This estimate of Notch 1 mRNA half-life agrees with previous data obtained in the MC3T3 murine preosteoblast cell line (25). Together, these results indicate that Notch 1 expression is increased in osteonectin-null osteoblasts through increased gene transcription.

Members of the HES gene family are frequent downstream targets of Notch signaling, and the HES 5 promoter has been shown to be responsive to Notch signaling in osteoblasts and

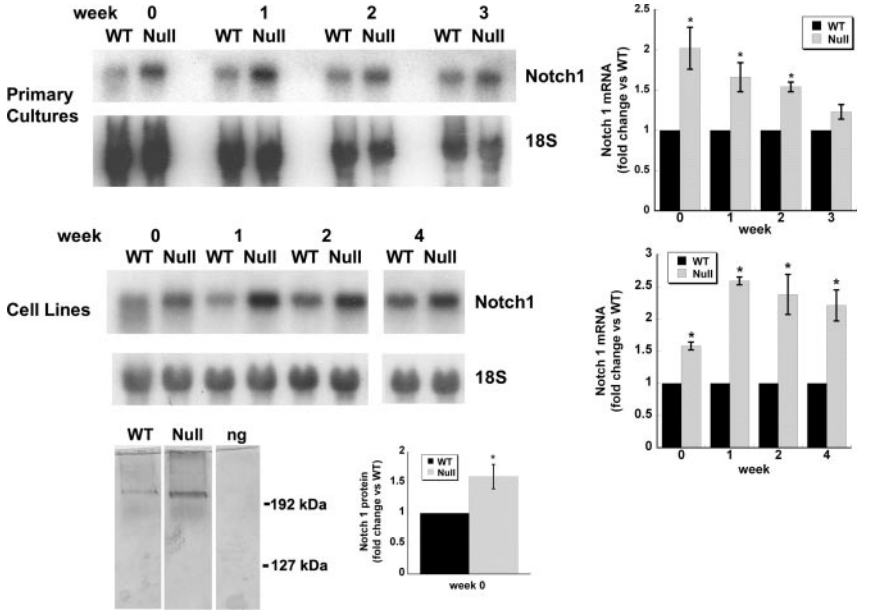

FIG. 1. Increased Notch 1 mRNA and protein in osteonectin-null osteoblastic cells. Northern blot analysis of total RNA isolated from primary osteoblastic cells (*top panels*) or immortalized osteoblastic cells (mObI-2) (*middle panels*) from wild-type (WT) or osteonectin-null (Null) mice. Cells were grown to confluence (0) and then maintained for up to 4 wk in osteoblast differentiation medium. Experiments were performed in triplicate. *Bottom panels*, Confluent cultures of mObI-2 cells were maintained in reduced serum conditions for 24 h before lysis. Full-length Notch 1 protein was immunoprecipitated from cell lysates and visualized by silver staining. Immunoprecipitation with normal goat IgG (ng) was used as a negative control. Data are expressed as mean ± SEM. *, Null significantly different from WT, $P < 0.05$.

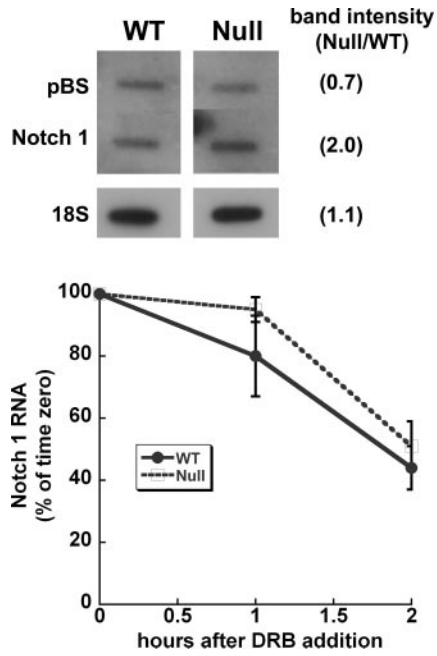

FIG. 2. Notch 1 transcription is increased in osteonectin-null osteoblastic cells. *Top panel*, Nuclear run-off assay was used to estimate the transcription rate of Notch 1 in confluent cultures of wild-type (WT) and osteonectin-null (Null) mObI-2 cells. Hybridization to pBlueScript SK+ (pBS) served as a negative control, whereas hybridization of 18S served as a control for equal RNA loading. This experiment was performed twice, and a representative experiment is shown. *Bottom panel*, Wild-type and osteonectin-null mObI-2 cells were treated with 75 μM dichlorobenzimidazole riboside to arrest transcription, and Notch 1 mRNA levels were quantified by Northern blot analysis and densitometry. Experiments were performed in triplicate. The percent of Notch 1 mRNA (mean ± SEM) remaining after transcription arrest is shown, and the half-life of Notch 1 mRNA in wild-type (*solid line*) and osteonectin-null (*broken line*) osteoblasts was not significantly different.

other cell types (21, 35). Transient transfection of mObI-2 cells showed that expression of the HES 5 promoter-luciferase reporter construct was modestly increased in osteonectin-null osteoblasts transfected with 0.25 μg/well of luciferase reporter DNA, indicating augmented Notch signaling (Fig. 3, *top panel*). Not surprisingly, this endogenous Notch signaling was limiting in nature. A significant difference in promoter response between wild-type and osteonectin-null osteoblasts could not be detected when cells were transfected with greater than 0.25 μg/well HES 5-luciferase, suggesting squelching of trans-acting factor activity with the introduction of additional promoter DNA.

Notch and TGFβ signaling pathways have shown functional synergism in other cell systems (39). To determine whether an interaction between these pathways could be detected in bone cells, wild-type and osteonectin-null osteoblastic cells were transfected with the HES-5 promoter-luciferase construct and treated with 0–1 ng/ml TGFβ for 24 h. TGFβ increased HES 5 promoter activity in a dose-dependent manner in both cell types, confirming cross-talk between Notch and TGF signaling pathways in osteoblasts (Fig. 3, *middle panel*) (39). Interestingly, higher doses of TGFβ (up to 5 ng/ml) were no more effective at stimulating HES 5 promoter activity than 1 ng/ml (data not shown). Osteonectin has been reported to modify TGFβ signaling in other cell systems (40, 41). To determine whether this might be the case in osteoblastic cells, a TGFβ-responsive promoter construct in which a portion of the tissue plasminogen activator promoter drives expression of luciferase (3TP) was transfected into wild-type and osteonectin-null cells. Confluent cultures were stimulated with 0, 0.1, or 1 ng/ml TGFβ for 24 h. The activity of the 3TP promoter was stimulated by TGFβ in a dose-dependent manner, and a significant difference in responsiveness between wild-type and osteonectin-null cells was not detected. Similar data were obtained with SBE4, a TGFβ-responsive construct in which four tandem repeats of the SBE are used to drive luciferase expression (construct from S. Kern; data not shown) (36). These results confirm that classical targets of TGFβ signaling are not affected by endogenous Notch signaling and indicate that the absence of osteonectin does not affect TGFβ signaling in osteoblasts (39).

To determine whether down-regulation of Notch 1 expression could augment osteoblastic differentiation in either wild-type or osteonectin-null osteoblastic cells, we created cell lines expressing a portion of the mouse Notch 1 cDNA and a myc epitope tag in the antisense orientation. Northern blot analysis using a probe for the myc epitope showed that the construct was expressed in both wild-type and osteonectin-null cells, with no expression in cells transduced with vector alone (Fig. 4, *top panel*). When immunoprecipitated from lysates of confluent cultures, Notch 1 protein was undetectable in both wild-type and osteonectin-null cells expressing the antisense construct (Fig. 4, *middle panel*). Cells transduced with vector alone or antisense Notch were cultured for 3 wk after confluence in osteoblastic differentiation medium and then stained with alizarin red to visualize mineralized matrix. The antisense Notch constructed dramatically decreased mineralized matrix formation in both wild-type and osteonectin-null cells. These data confirm that appropriate Notch signaling is required for osteoblastic differentiation (25).

Regulation of Notch 1

Notch is implicated in the commitment of preadipocytes to undergo adipogenic differentiation (19, 20). To determine whether the expression of Notch 1 in wild-type and osteonectin-null osteoblastic cells could be modulated by culture under adipogenic conditions, confluent cultures of mObI-2 cells were maintained for up to 3 wk in medium containing IBMX, dexamethasone, and insulin (42). Parallel cultures in which cells were exposed to osteoblastic differentiation medium were also examined (Fig. 5). As expected, Notch 1 mRNA was elevated in osteonectin-null osteoblastic cells cultured in osteoblast differentiation medium and osteocalcin mRNA was not detected. Osteocalcin mRNA was evident in wild-type cells after 2 and 3 wk *in vitro* differentiation, indicating osteoblastic maturation of the normal cells. In contrast, after 1 wk exposure to adipogenic stimulus, Notch 1 mRNA levels were similar in wild-type and osteonectin-null cells and significantly increased, compared with to wild-type cells grown in osteoblastic medium. After 2 and 3 wk of culture in adipogenic medium, Notch 1 mRNA was higher in wild-type cells, compared with osteonectin-

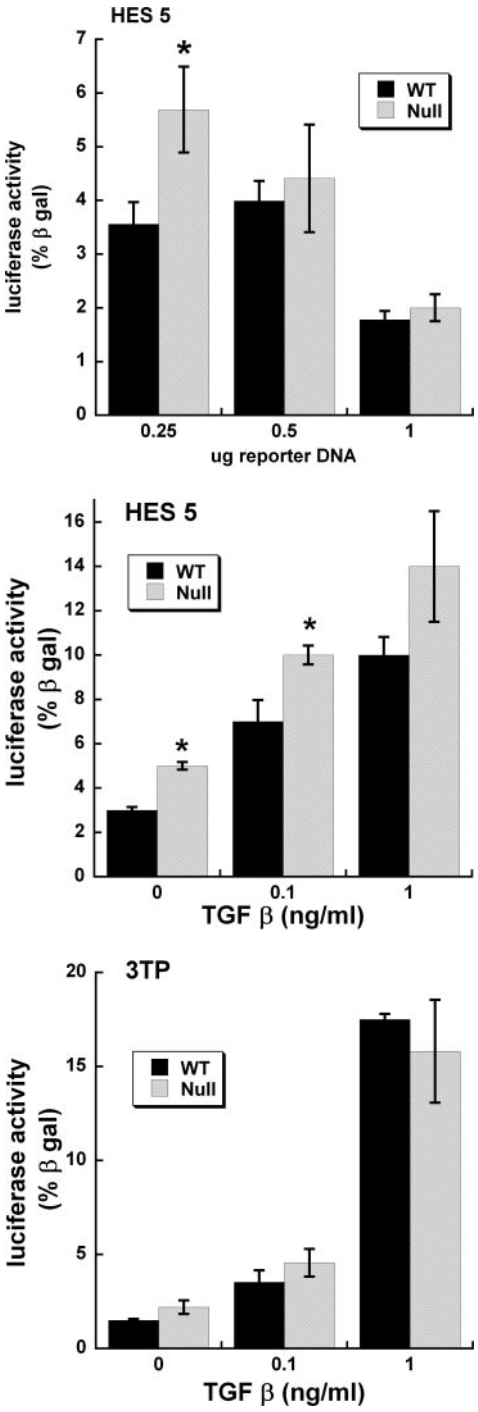

FIG. 3. Increased Notch signaling in osteonectin-null osteoblastic cells. Wild-type (WT) or osteonectin-null (Null) mObI-2 cells were transiently cotransfected with luciferase reporter constructs and a constitutively active β-galactosidase (β gal) reporter. Confluent cultures were treated with or without 0–1 ng/ml TGFβ for 24 h before cell harvest and analysis, and luciferase activity is expressed as a percentage of β gal activity, normalizing for differences in transfection efficiency. *Top panel*, Notch signaling was assessed by transfection with increasing amounts (0.25–1 μg) of HES 5 promoter-luciferase construct. *Middle panel*, Cross-talk between Notch and TGFβ signaling pathways was assessed in cells transfected with the HES 5 promoter-luciferase construct (0.25 μg/well) and treated with doses of TGFβ. *Bottom panel*, TGFβ signaling was assessed in cells transfected with the TGFβ-responsive reporter construct 3TP (0.25 μg/well) and treated with doses of TGFβ. Data are expressed as mean ± SEM. *, Null significantly different from WT, $P < 0.05$.

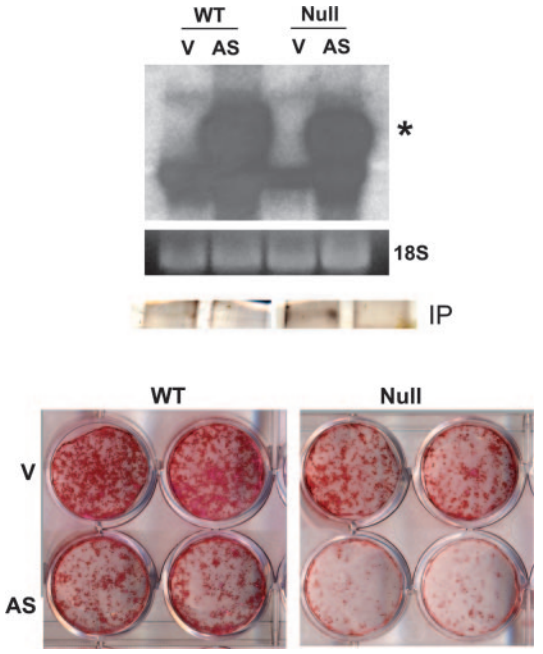

FIG. 4. Antisense Notch 1 decreases osteoblastic differentiation. Wild-type (WT) or osteonectin-null (Null) mObI-2 cells stably transduced with vector alone (V) or antisense Notch 1-myc construct (AS) were cultured for up to 3 wk after confluence in osteoblast differentiation medium. *Top panel*, Northern blot analysis of RNA from confluent cultures of cells, probed with cDNA corresponding to the myc tag on the transgene. *, Band for the transgene RNA, whereas the faster mobility bands are nonspecific. Ethidium bromide staining for 18S rRNA indicates comparable RNA loading. *Middle panel*, Immunoprecipitation (IP) of Notch 1 protein from lysates of confluent cultures. Notch 1 was not detectable in lysates from cells expressing antisense Notch. These data are representative of two cultures. *Bottom panel*, Alizarin red staining for mineralized matrix in cells cultured for 3 wk after confluence in osteoblast differentiation medium. A second independent experiment gave similar results.

null cells. At these time points, osteocalcin transcripts were observed in wild-type cells, even in presence of the adipogenic differentiation stimulus. Adipsin mRNA was not detectable in wild-type cells at any time during culture, whereas transcripts for this adipocytic marker were seen in osteonectin-null cells after 2 and 3 wk of culture. These data indicate that the wild-type osteoblastic cells are committed to osteoblastic differentiation, a program that cannot be overcome by an adipogenic stimulus that, paradoxically, resulted in the enhancement of a marker of osteoblastic differentiation. In contrast, osteonectin-null osteoblastic cells appear to be more plastic and less committed and able to pursue adipogenic differentiation given the appropriate signals.

Elevation of cAMP by the phosphodiesterase inhibitor IBMX is sufficient to down-regulate Wnt10b expression, a step required for the initiation of adipogenesis in adipogenic precursors (42). We wondered whether cAMP levels played a role in the regulation of Notch 1 mRNA. To test this idea, wild-type and osteonectin-null osteoblastic cells were treated with IBMX for up to 48 h (Fig. 5). We found that IBMX decreased Notch 1 mRNA levels in both cell types after 24 or 48 h, suggesting that cAMP decreases Notch 1 mRNA in osteoblastic cells.

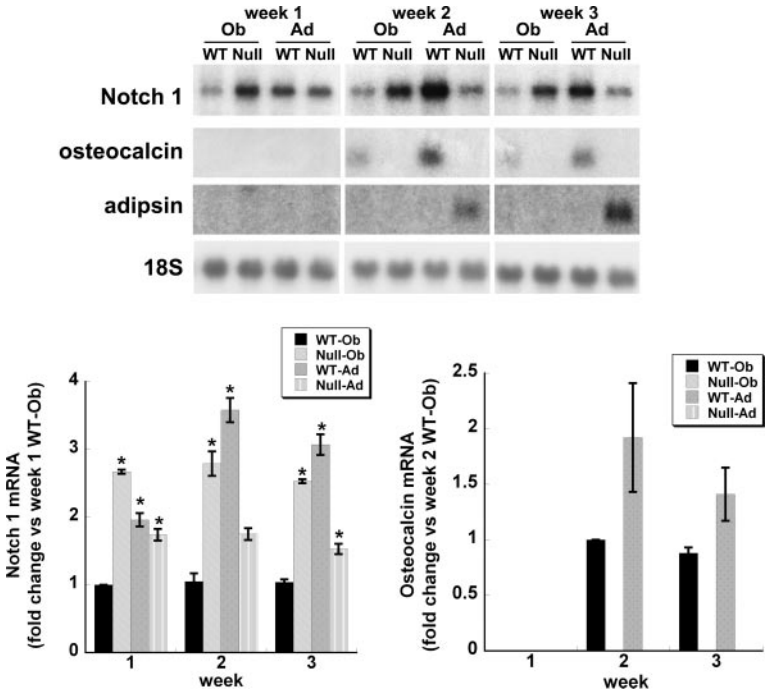

FIG. 5. Analysis of gene expression in wild-type and osteonectin-null osteoblastic cells cultured under osteogenic or adipogenic differentiation conditions. Wild-type (WT) or osteonectin-null (Null) mObI-2 cells were grown to confluence and maintained in osteoblastic (Ob) or adipogenic (Ad) medium for up to 3 wk. RNA was isolated and analyzed by Northern blot for expression of transcripts for Notch 1, osteocalcin (marker of mature osteoblasts) and adipsin (marker of mature adipocytes). Experiments were performed in triplicate, and data are expressed as mean ± SEM. *, Notch expression significantly different from wk 1 wild-type Ob, $P < 0.05$. Osteocalcin mRNA levels in wild-type cells tended to increase in cells exposed to adipogenic medium, but the changes were not statistically significant. Adipsin mRNA levels in osteonectin-null cells cultured in adipogenic medium were 1.6 ± 0.1-fold higher at 3 wk, compared with 2 wk ($P < 0.05$).

Attenuated coupling of G_s to adenylyl cyclase in osteonectin-null osteoblasts

Because cAMP can decrease Notch 1 mRNA and osteonectin-null osteoblasts have increased levels of Notch 1, we considered the hypothesis that the mutant osteoblasts may have a defect in the generation of cAMP in response to physiological stimuli. Ligand activation of G_s coupled receptors, such as those for PTH or prostaglandins, results in increased cAMP due to the stimulation of adenylyl cyclase activity (43, 44). Therefore, we used two pharmacological agents, forskolin and cholera toxin, to test the coupling of G_s to adenylyl cyclase (Fig. 6). Forskolin is a cell-permeable diterpene that directly stimulates adenylyl cyclase activity (45). Cholera toxin is internalized via clatherin-coated pits and catalyzes the ADP-ribosylation of the α-subunit of G_s, resulting in reduced GTPase activity and a subsequent activation of the α-subunit (46). Cultures of wild-type and osteonectin-null osteoblasts were treated with increasing doses of cholera toxin or forskolin for 3 min, and cAMP levels in the cell lysates were determined by EIA (38). Untreated wild-type and osteonectin-null osteoblasts had similar amounts of cAMP, likely due to the fact that they were serum-deprived before the start of the experiment, thus lacking stimulators of cAMP. Forskolin stimulated cAMP production in wild-type and osteonectin-null cells in a dose-dependent manner, and both cell types had a similar response. In contrast, only the highest dose of cholera toxin caused a significant increase in cAMP levels in wild-type cells, whereas the osteonectin-null cells failed to respond. This lack of dose response to cholera toxin in the wild-type cells likely reflects a slight delay in the internalization of the compound so that only the highest dose had an effect at the 3-min time point. However, it is clear that the ability of the osteonectin-null osteoblasts to generate cAMP in the presence of cholera toxin was blunted. These data imply a defect in the coupling of G_s to adenylyl cyclase in the osteonectin-null osteoblasts (38). To confirm the attenuated response of osteonectin-null osteoblasts to cholera toxin,

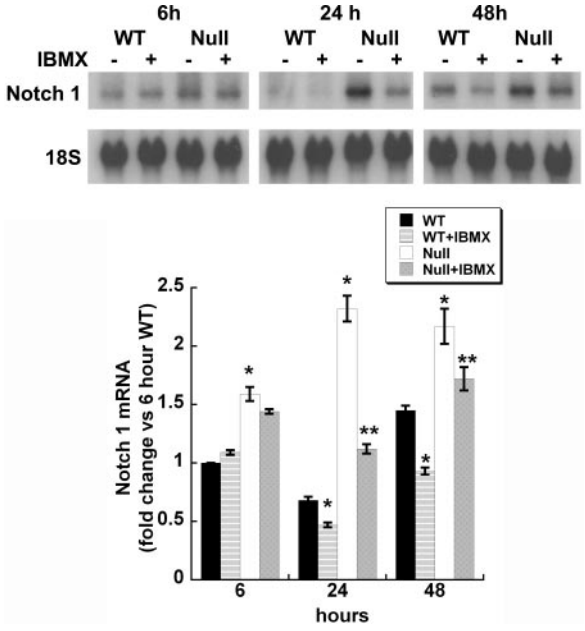

FIG. 6. Down-regulation of Notch 1 mRNA in the presence of IBMX, a phosphodiesterase inhibitor. Wild-type (WT) or osteonectin-null (Null) mObI-2 cells were grown to confluence, transferred to reduced-serum conditions for 24 h, and then treated with 500 nM IBMX for up to 48 h. RNA was isolated and analyzed by Northern blot for expression of Notch 1 transcripts. Treatment of cells with this phosphodiesterase inhibitor results in the accumulation of cAMP. Experiments were performed in triplicate, and data are expressed as mean ± SEM. *, Significantly different from corresponding untreated wild-type cells, $P < 0.01$; **, significantly different from corresponding untreated osteonectin-null cells, $P < 0.05$.

wild-type and osteonectin-null osteoblasts were treated with increasing doses of cholera toxin for 3 h, a time point when regulation of Notch 1 mRNA is clearly evident (our unpublished data). Here cholera toxin treatment decreased Notch 1 transcripts in both cell types in a dose-dependent manner. However, the effect of cholera toxin on Notch 1 was blunted in the osteonectin-null osteoblasts, compared with wild-type. An approximately 50% decrease in Notch 1 mRNA was achieved with 10 nM cholera toxin in wild-type cells, but this level of down-regulation was seen only with 100 nM cholera toxin in osteonectin-null cells, confirming our hypothesis.

Discussion

Notch signaling is known to influence cell fate by inhibiting the development of specific lineages and, in some cases, promoting the development of other lineages in a manner that is highly dependent on cell type and context. Upon interaction with ligand, the NICD is cleaved from the plasma membrane, translocates into the nucleus, and interacts with the C-promoter binding factor 1/suppressor of hairless/LAG-1 or RBP-Jk (CSL). CSL is a DNA binding protein that acts as a transcriptional corepressor with a complex containing histone deacetylase. Upon interaction with NICD, the corepressor complex is displaced from CSL and replaced with an activating complex containing p300 histone acetyltransferase, converting CSL from a transcriptional repressor to a transcriptional activator. Members of the HES family of transcription factors are well-characterized targets of Notch signaling, and their NICD-mediated increase in transcription is thought to initiate a signaling cascade necessary for regulation of gene expression (18, 20). In osteonectin-null osteoblasts, Notch 1 expression was modestly but consistently up-regulated as a result of increased gene transcription, and increased Notch signaling was detected in these cells at a level consistent with the low level of increase in Notch 1 gene expression (Figs. 1–3).

The physiological effect of Notch signaling on osteoblast biology remains controversial. We found that expression of antisense Notch 1 inhibited osteoblastic differentiation in both wild-type and osteonectin-null cells. Our results are consistent with studies showing that Notch 1 small interfering RNA, a dominant-negative form of Notch 1, or a γ-secretase inhibitor (which would prevent cleavage of the Notch receptor) repressed basal and bone morphogenetic protein-2-induced osteoblastic differentiation (23). In contrast, constitutive Notch signaling due to high-level expression of the Notch intracellular domain was also found to inhibit osteoblastic differentiation (21, 22). However, this high-level, constitutive activation of Notch signaling may not be physiologically relevant; *in vivo*, more modest increases (*i.e.* 3- to 5-fold) in Notch 1 and its ligands are observed a bone regeneration model (23). Together, these somewhat conflicting reports suggest that appropriate control of both timing and quantity of Notch signaling is important for normal osteoblast function. In a situation such as wound healing or fracture repair, we hypothesize that Notch signaling may stall osteoblastic maturation in the early phase, allowing for the accumulation of cells in a more uncommitted differentiation state. Later, as matrix deposition and mineralization is required, down-regulation of Notch signaling may promote osteoblastic maturation.

Like osteoblastogenesis, adipogenesis appears to require appropriate timing and quantity of Notch signaling (19, 20). In our study, we found that, compared with osteonectin-null cells, Notch 1 mRNA levels were increased in wild-type cells after 2 or 3 wk of culture in adipogenic medium (Fig. 4). The wild-type osteoblasts maintained in adipogenic medium did not express detectable levels of adipsin transcripts; rather osteocalcin mRNA levels were somewhat higher, compared with cells cultured in osteoblast differentiation medium. These data indicate that the wild-type cells are committed to osteoblastic differentiation and that components of the adipogenic cocktail, likely those that stimulate cAMP (IBMX and insulin), enhance this process. It is possible that the elevated expression of Notch 1 in the wild-type cells may have contributed to the block in adipogenesis through regulation of adipogenic repressors (20). Relative to the wild-type cells, Notch 1 mRNA was decreased in osteonectin-null cells cultured in adipogenic medium, and the mutant cells expressed adipogenic markers, indicating that osteonectin-null cells represent a less committed population able to adopt an adipocytic phenotype (Fig. 5).

We demonstrated that Notch 1 mRNA is down-regulated by cAMP (Fig. 6), and it is possible that the elevated Notch 1 expression observed in osteonectin-null cells could be a symptom of a more global problem involving the generation of cAMP in response to stimuli. Our data suggest that the ability of G_s to stimulate adenylyl cyclase was attenuated in osteonectin-null cells, implying a defect in coupling of G_s to adenylyl cyclase (Fig. 7). An age-related decrease in coupling of G_s to adenylyl cyclase has been observed in rodent osteoblasts, and it is intriguing to note that osteonectin-null mice display a phenotype somewhat descriptive of accelerated aging: the mice develop osteopenia, cataracts, skin fragility, and increased adipose deposits (6, 38).

Although osteonectin is an extracellular matrix component, it can also reside inside the cell, displaying a diffuse, punctuate staining pattern. A recent study showed that osteonectin can interact with integrin-linked kinase in fibroblastic cells, promoting fibronectin-induced signaling and supporting fibronectin matrix assembly (12). Integrin-linked kinase activity in osteonectin-null cells could be rescued by delivery of an osteonectin expression construct but could not be rescued by the addition of exogenous rh-osteonectin to the culture medium (12). These data indicate that localization of the osteonectin plays an important role in its activity. Similarly, we previously showed that retroviral delivery of an osteonectin expression construct to osteonectin-null osteoblastic cells rescued defects in osteoblast maturation and cell survival, whereas doses of exogenous rh-osteonectin (generously provided by E. H. Sage, Benaroya Research Institute at Virginia Mason, Seattle, WA) failed to rescue these defects (17 and data not shown). Together, these data suggest that cell-associated and extracellular matrix-associated osteonectin may play distinct roles in the modulation of cell behavior (12).

G protein-coupled receptors and their agonists play are critical for bone remodeling, and there is increased focus on understanding how signaling from these receptors is mod-

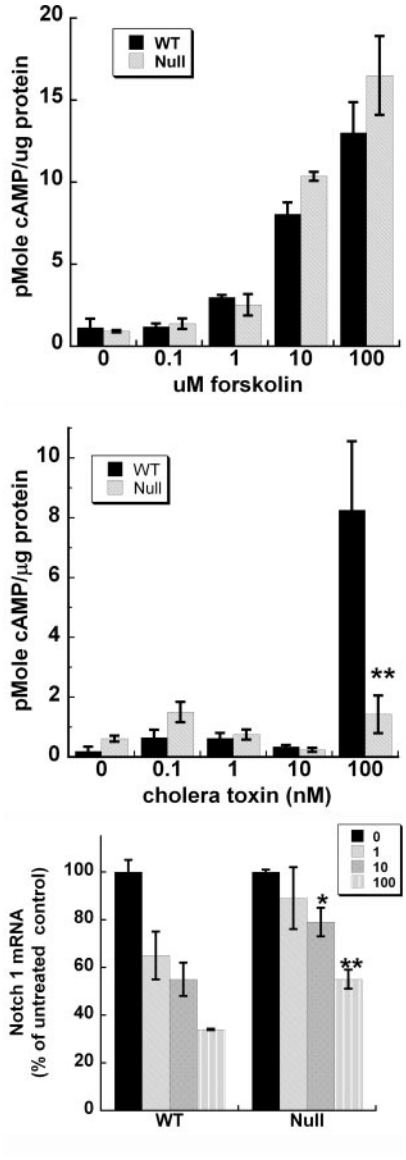

FIG. 7. Differential response of wild-type and osteonectin-null osteoblasts to forskolin and cholera toxin, suggesting a defect in coupling of G_s to adenylyl cyclase. Wild-type (WT) or osteonectin-null (Null) mObI-2 cells were grown to confluence and transferred to reduced-serum conditions for 24 h before treatment with forskolin, a direct activator of adenylyl cyclase, or cholera toxin, a direct activator of G_s. *Top panel*, Cells were treated with or without increasing doses of forskolin for 3 min. Cell lysates were isolated and assayed for cAMP accumulation. *Middle panel*, Cells were treated with or without increasing doses of cholera toxin for 3 min. Cell lysates were isolated and assayed for cAMP accumulation. *Bottom panel*, Cells were treated with or without 0–100 nM cholera toxin for 3 h. RNA was isolated and Notch 1 mRNA levels were quantified by Northern blot and densitometry. Notch 1 mRNA levels in the presence of cholera toxin are expressed as a percentage of that found in untreated cells. Data are expressed as mean ± SEM. *, Null significantly different from WT, $P < 0.05$; **, null significantly different from WT, $P < 0.01$.

ulated in the skeleton. The PTH receptor is of particular interest because therapeutic administration of PTH(1–34) can decrease fracture risk in osteoporotic patients (47). Signaling from the ligand activated PTH receptor is rapidly desensitized due to phosphorylation of the receptor by G protein-coupled receptor kinases (GRKs), members of the serine/threonine kinase family. Phosphorylation of the PTH receptor allows its interaction with β-arrestin2, leading to receptor uncoupling from G proteins, receptor internalization, and activation of phosphodiesterase activity (48–51).

Interestingly, targeted overexpression of GRK-2 in osteoblasts of transgenic mice results in a skeletal phenotype very similar to that of osteonectin-null mice (16, 52). For example, cAMP generation in response to PTH was attenuated in calvaria from GRK-2 transgenic mice, although cAMP response to forskolin, a direct activator of adenylyl cyclase, was similar to that of nontransgenic mice (52). Furthermore, GRK-2 transgenic mice had reduced whole-body bone mineral density, with prominent decreases in trabecular bone volume, osteoblast and osteoclast surface, and bone formation rate, whereas cortical bone mineral density was not significantly affected (52). These observations bear a striking resemblance to our data on the skeletal phenotype of the osteonectin-null mice (Refs. 16, 17, and our unpublished data). Our results support the concept that low-turnover osteopenia can result from reducing G protein-coupled receptor activity via enhanced GRK-2 or attenuation of the coupling of G_s due to the absence of osteonectin. A defect in the ability to generate appropriate levels of cAMP in response to physiological and pharmacological stimuli suggests a mechanism for the decreased osteoblastic maturation and low-turnover osteopenia observed in osteonectin-null mice (16, 17, 52).

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