Notch 1 Overexpression Inhibits Osteoblastogenesis by Suppressing Wnt/β-Catenin but Not Bone Morphogenetic Protein Signaling*

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Notch proteins are transmembrane receptors that control cell-fate decisions. Upon ligand binding, Notch receptors undergo proteolytic cleavage leading to the release of their intracellular domain (NICD). Overexpression of NICD impairs osteoblastogenesis, but the mechanisms are not understood. We examined consequences of the constitutive activation of Notch 1 in ST-2 cells. Notch opposed the effects of bone morphogenetic protein (BMP)-2 and Wnt 3a on alkaline phosphatase activity (APA). BMP-2 induced the phosphorylation of Smad 1/5/8 and the transactivation of a BMP/Smad-alkaline phosphatase activity (APA). BMP-2 induced the phosphorylation of Smad 1/5/8 and the transactivation of a BMP/Smad-alkaline phosphatase activity (APA). BMP-2 induced the phosphorylation of Smad 1/5/8 and the transactivation of a BMP/Smad-alkaline phosphatase activity (APA). BMP-2 induced the phosphorylation of Smad 1/5/8 and the transactivation of a BMP/Smad-alkaline phosphatase activity (APA). Notch-ligand interaction results in the proteolytic cleavage at the juxtamembrane portion of the Notch receptor and release of the intracellular domain (NICD), which translocates to the nucleus (4, 5). Therefore, NICD is the constitutively active domain of the Notch receptor after ligand activation. The NICD subsequently interacts with members of the Epstein-Barr virus latency C promoter binding factor 1/recombination signal-binding protein of the Jk immunoglobin gene (CBF1/RBP-Jk), Suppressor of Hairless and Lag-1 (CSL) family of transcription factors. This interaction converts the transcriptional repressor CSL proteins into transcriptional activators. Activation of Notch signaling induces, preferentially, Hairy Enhancer of Split (HES) 1 or 5 (6, 7), and HES-related repressor protein 1–3 gene expression (8). Previously, we reported that the constitutive overexpression of NICD in ST-2 stromal and MC3T3 osteoblastic cells results in suppression of osteoblastic maturation (9). However, the mechanisms were not explored, and others have reported contradictory results (10, 11).

Mesenchymal cells can differentiate into cells of various lineages, including osteoblasts, myoblasts, chondrocytes, and adipocytes (12, 13). Bone morphogenetic proteins (BMPs) and Wnt are important signals determining the fate of immature cells into cells of the osteoblastic lineage (14–19). BMP receptor binding leads to signaling mothers against decapentaplegic (Smad) 1, 5, and 8 phosphorylation (20), and in association with Smad 4, to their nuclear translocation and regulation of gene transcription (21–25). BMPs also signal by activating the mitogen-activated protein (MAP) kinases, p38, Jun-N-terminal kinase (JNK), and extracellular regulated kinase (ERK) in cells of the osteoblastic lineage (26–28).

Wnt can activate four distinct signaling pathways, but in osteoblasts the Wnt/β-catenin canonical pathway is utilized (29, 30). In the absence of Wnt, axin, adenomatous polyposis coli, β-catenin, and glycogen synthase kinase 3β (GSK3β) form a complex in which β-catenin is phosphorylated by GSK3β leading to β-catenin degradation after ubiquitination. The binding of Wnt proteins to their specific Frizzled transmembrane receptors and to the low density lipoprotein receptor-related protein 5/6 co-receptors leads to the inhibition of GSK3β and the stabilization and nuclear translocation of β-catenin. In the nucleus, β-catenin associates with members of the lymphoid enhancer binding factor/T cell-specific factor (LEF/TCF) family of transcription factors and regulates gene transcription (31–33). Among the Wnt proteins, Wnt 1, 3, 3a, 7a, and 8 activate the canonical Wnt/β-catenin pathway (34). Wnt 3 and 3a have similar sequences and functions in osteoblastic cells and are commonly used to test Wnt effects on the canonical pathway in these cells (35–37). Cross-talk between the Wnt and the Notch signaling pathways can occur at various levels, and often, but not always, NICD opposes Wnt/β-catenin signaling (38–44).

Notch receptors regulate osteoblastic cell fate, but the mechanisms involved are poorly understood (9, 45–47). The purpose of this study was to investigate mechanisms responsible for the inhibitory effect of NICD overexpression on osteoblastogenesis and examine interactions

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2 The abbreviations used are: NICD, Notch intracellular domain; BMP, bone morphogenetic protein; APA, alkaline phosphatase activity; Smad, signaling mothers against decapentaplegic; MAP, mitogen-activated protein; GSK, glycogen synthase kinase; HES, hairy enhancer of split; TLE, transducin-like enhancer of split; CBF, Epstein-Barr virus latency C promoter binding factor; RBP-Jk, recombination signal binding protein of the Jk immunoglobin gene; CSL, CBF1/RBP-Jk, Suppressor of Hairless and Lag-1 (CSL) family of transcription factors. This interaction converts the transcriptional repressor CSL proteins into transcriptional activators. Activation of Notch signaling induces, preferentially, Hairy Enhancer of Split (HES) 1 or 5 (6, 7), and HES-related repressor protein 1–3 gene expression (8). Previously, we reported that the constitutive overexpression of NICD in ST-2 stromal and MC3T3 osteoblastic cells results in suppression of osteoblastic maturation (9). However, the mechanisms were not explored, and others have reported contradictory results (10, 11).

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between Notch and BMP signaling in cells of the osteoblastic lineage.

**EXPERIMENTAL PROCEDURES**

**Vectors and Packaging Cell Lines**—A 2389-bp DNA fragment containing the murine NICD cDNA, with a Myc epitope tag on the amino-terminal end (kindly provided by J. Nye, Chicago, IL) was cloned into either pcDNA 3.1 (Invitrogen) for use in acute transfection experiments or cloned into the retroviral vector pLPCX (Clontech, Palo Alto, CA) for the creation of transduced cell lines. In both vectors, a cytomegalovirus (CMV) promoter directs the constitutive expression of NICD (48). pLPCX contains a packaging signal under the control of a Moloney murine leukemia virus 5’ long terminal repeat and a puromycin resistance gene under the control of the CMV’ promoter. pLPCX and pLPCX NICD were transfected into Phoenix packaging cells (ATCC, Manassas, VA) by calcium phosphate/DNA co-precipitation and glycerol shock, and cells were selected for puromycin resistance, as described (9). Retrovirus-containing conditioned medium was harvested, filtered through a 0.45-µm membrane, and used to transduce ST-2 cells.

**Cell Culture**—ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice (Dr. S. Harris, San Antonio, TX), were grown in a humidified 5% CO2 incubator at 37 °C in minimum essential medium (α-MEM, Invitrogen), supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). ST-2 cells were selected because of previously characterized osteogenic properties and responsiveness to Notch (9, 49). For the creation of cell lines, ST-2 cells were transduced with pLPCX vector or with pLPCX NICD by replacing the culture medium with retroviral conditioned medium from Phoenix packaging cells, in the presence of 8 µg/ml Polybrene (Sigma) followed by incubation for 16–18 h at 37 °C. The culture medium was replaced with fresh α-MEM, and cells were grown, trypsinized, replated, and selected for puromycin resistance. Cells transduced with pLPCX vector or pLPCX NICD were plated at a density of 10^5 cells/cm^2 and cultured in α-MEM supplemented with 10% fetal bovine serum until reaching confluence (2–4 days). To study the effect of NICD on alkaline phosphatase activity (APA), confluent ST-2 cells were transfected to α-MEM containing 10% fetal bovine serum, 100 µg/ml ascorbic acid, and 5 mM β-glycerophosphate (Sigma), and cultured for 3 days in the presence or absence of recombinant human BMP-2 (Wyeth Research, Collegeville, PA), Wnt 3a (R and D, Minneapolis, MN), or a glycogen synthase kinase (GSK)3 inhibitor (CHIR 99021, Chiron, Emeryville, CA), as indicated in the text and legends. To study the effects on Smad or MAP kinase phosphorylation, confluent cells were serum-deprived overnight and treated with BMP-2 for 15 min to 6 h. To study effects on β-catenin levels, confluent cells were treated with or without GSK3β inhibitor CHIR 99021 for 4 h.

**Transient Transfections**—To determine changes in BMP-2 signaling, a construct containing twelve copies of a Smad 1/5 response element, linked to the osteocalcin basal promoter, and cloned upstream of a luciferase reporter gene (12×SE-BE-OC-pGL3, M. Zhao, San Antonio, TX) was tested in transient transfection experiments. To determine changes in β-catenin transactivating activity, a pTOPFLASH reporter construct containing three copies of the LEF-1/TCF-4 binding sequences upstream of a minimal c-fos promoter and a luciferase reporter gene (J. Kitajewski, New York, NY) or a construct containing sixteen copies of the LEF-1/TCF-4 recognition sequence, cloned upstream of a minimal thymidine kinase promoter and a luciferase reporter gene (16×TCF-luc, J. Billiard, Wyeth Research) were tested in transient transfection experiments. The activity of pTOPFLASH and 16×TCF-luc was tested in the absence or presence of the GSK3β inhibitor CHIR 99021 or in the absence or presence of a co-transfected β-catenin expression construct, mutated at Ser-37, and cloned into pcNeo (pcNeo-S37-FLAG, P. J. Morin, Baltimore, MD) or control vector. Transient transfections were conducted in transduced cell lines cultured to 70% confluence and transiently transfected with the indicated constructs using FuGene6 (3 µl of FuGene/2 µg of DNA) according to the manufacturer’s instructions (Roche Applied Science). In one experiment, wild-type ST-2 cells were co-transfected with pcDNA 3.1 or pcDNA NICD and 12×SBE-OC-pGL3, as stated in the text. Co-transfections with a β-galactosidase expression construct (Clontech, San Jose, CA) were used to control for transfection efficiency. For 12×SBE-OC-pGL3, cells were exposed to the FuGene-DNA mix for 16 h and transferred to serum-containing medium for 8 h. Cells were then cultured in medium supplemented with 0.1% fetal bovine serum for 20 h, treated with or without BMP-2, and harvested. For pTOPFLASH or 16×TCF-luc activity, cells were exposed to the FuGene-DNA mix for 16 h, transferred to serum containing test or control medium for 48 h, and harvested. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

**RNAi**—To down-regulate hes-1 and Groucho1/TLE1 and -2 gene expression, a 21-mer double-stranded RNA composed of sense GCG-CCAAAAUGCCUUCUCUt and antisense GAGGAGCGAAAAC-AGCCGTC sequences for HES-1, of sense GGGCUUUACACUAUU-UULUt and antisense AAAAUUAUGUGUAAGAGCCTg sequences for Groucho1/TLE1, and of sense GGGAUUACUCACUCCUt and antisense AGGAGUGAGUAAUCCCTg sequences for Groucho2/TLE2 were obtained commercially (Ambion, Austin, TX). A 19-bp silencing (si) scrambled RNA composed of sequences with no homology to known mouse or rat sequences was used as a control. HES-1, Groucho1/TLE1, or scrambled siRNA, all at 20 nM, were transfected into PC3 confluent ST-2 cells, transduced with pLPCX or pLPCX NICD, using siLentFect lipid reagent, in accordance with the manufacturer’s instructions (Bio-Rad). Following a 24-h recovery period, transfected cells were treated with BMP-2 or Wnt 3a and analyzed for APA 72 h later. In selected experiments, wild-type ST-2 cells were transfected with HES-1, Groucho1/TLE1 or -2, or scrambled siRNA, allowed to recover for 6 h following RNAi, and transfected with pcDNA NICD or pcDNA 3.1, with a Wnt 3 expression vector (Upstate Biotechnologies, Lake Placid, NY) or pCNeo-S27-FLAG or control constructs, and with 16×TCF-luc. Alternatively, silenced transfected cells were tested with GSK3β inhibitor, CHIR 99021, as stated in text and legends.

To ensure adequate RNAi, total RNA was extracted and HES-1, Groucho1/TLE1, and Groucho2/TLE2 mRNA levels determined by real-time reverse transcription-PCR (50, 51). For this purpose, 1–5 µg of RNA was reverse-transcribed in the presence of 5’-ATTCTTGGCC-CCTTGGCGCTTTT-3’ primer for HES-1, 5’-CGGCGTTAGGGTTGT-GTGATCTTG[FAM]G-3’ for Groucho1/TLE1 and 5’-CGCCACCTACTA-CACTAGGCTTCT-3’ for Groucho2/TLE2 and murine Moloney leukemia virus reverse transcriptase (Invitrogen) and amplified in the presence of 5’-GACTTTTCACGGCCCTTGAGCACAAGANN[FAM]C-3’ primer for HES-1, 5’-GCCAAATGCAGCCTGTTCCTT-3’ for Groucho1/TLE1 and 5’-CGGATTGACCCGACAACACTACT-[FAM]G-C for Groucho2/TLE2 and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54 °C for 45 cycles. Gene copy was estimated by comparison with a standard curve constructed using HES-1 cDNA (R. Kagayama, Kyoto, Japan), Groucho1/TLE1 cDNA (R. Shiodas, Boston, MA), or Groucho2/TLE2 cDNA (J. Downing, Memphis, TN), and corrected for glyceraldehyde-3-phosphate dehydrogenase copy number. Glyceraldehyde-3-phosphate dehydrogenase
was reverse-transcribed using 5’-AGCTTCCCGTTCAGCTCTGG-3’ primer and amplified in the presence of 5’-CAGCTCTGGAAAGCTGTGGCG[FIAM]G-3’, and copy number was estimated by comparison with a standard curve. Reactions were conducted in a 96-well spectrophotometric thermal iCycler (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step.

**Alkaline Phosphatase Activity**—Transduced ST-2 cells cultured to confluence were treated with BMP-2, Wnt 3a, or GSK3β inhibitor CHIR 99021 for 72 h and harvested. APA was determined in 0.5% Triton X-100 cell extracts by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol and measured by spectrophotometry at 405 nm after 10 min of incubation at room temperature according to the manufacturer’s instructions (Sigma). Data are expressed as nanomoles of p-nitrophenol released per minute per microgram of protein. Total protein content was determined in cell extracts by the DC protein assay in accordance with manufacturer’s instructions (Bio-Rad).

**Northern Blot Analysis**—Total cellular RNA was isolated using the RNeasy kit per the manufacturer’s instructions (Qiagen). Equal amounts of RNA were loaded on a formaldehyde-agarose gel following denaturation. The RNA was blotted onto GeneScreen Plus-charged nylon (PerkinElmer Life Sciences). A 1.6-kb Wnt-induced secreted protein (WISP) cDNA and a 0.7-kb 18 S ribosomal RNA cDNA (both from ATCC) were purified by agarose gel electrophoresis. DNAs were labeled with [α-32P]dCTP (50 μCi at a specific activity of 3000 Ci/mmol, PerkinElmer Life Sciences) using Ready-To-Go DNA labeling beads (-dCTP kit, Amersham Biosciences) in accordance with the manufacturer’s instructions. Hybridizations were carried out at 42 °C for 16 h, followed by two post-hybridization washes at room temperature for 15 min in 1 × SSC, and a wash at 65 °C for 20–30 min in 1 × SSC. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak, Rochester, NY), employing Cronex Lightning Plus (PerkinElmer Life Sciences) intensifying screens. Northern analyses are representative of three samples.

**Western Blot Analysis**—To determine the level of phosphorylation of Smad 1/5/8, and of the MAP kinases p44 ERK1, p42 ERK2, JNK 1, and 2/3, and p38, the cell layer of ST-2 cells transduced with pLPCX NICD or pLPCX was washed with cold phosphate-buffered saline and extracted in TNE lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% P40, or pLPCX was washed with cold phosphate-buffered saline and measured by spectroscopy at 405 nm after 10 min of incubation at room temperature according to the manufacturer’s instructions (Sigma). Data are expressed as nanomoles of p-nitrophenol released per minute per microgram of protein. Total protein content was determined in cell extracts by the DC protein assay in accordance with manufacturer’s instructions (Bio-Rad).

**Results**

In accordance with our previous observations, BMP-2 and Wnt 3a increased alkaline phosphatase, an earlier marker of osteoblastic differentiation in ST-2 cells (54). As the culture progresses, ST-2 cells exposed to BMP-2 express additional osteoblastic gene markers, and form mineralized nodules, and these are suppressed by Notch overexpression (9, 49). In accordance, NICD overexpression opposed the dose-dependent effect of BMP-2 and Wnt 3a on APA in ST-2 cells (Fig. 1). To elucidate the mechanism of Notch action in ST-2 cells, we analyzed the effect of NICD overexpression on downstream events of BMP-2 signaling. The signaling pathway used by BMPs is cell line-dependent, and in differentiated osteoblasts BMP-2 signals by activating Smad 1/5/8, and the effect was maximal after 15 min and was sustained for 6 h (data not shown). BMP-2 at 0.3–3.3 nM for 20 min caused an equal increase in phosphorylated Smad 1/5/8, but BMP-2 caused a dose-related increase on the transactivation of the BMP/Smad-dependent 12×SBE-Oc-pGL3 construct (Fig. 2, A and B). The lack of dose dependence on Smad phosphorylation is possibly due to the lesser sensitivity of Western blot analysis in the detection of modest changes. NICD overexpression did not modify either the level of phosphorylated Smad 1/5/8 or the transactivation of the BMP/Smad-dependent 12×SBE-Oc-pGL3 construct in the presence or absence of BMP-2 (Fig. 2, A and B). NICD overexpression did not modify the effect of BMP-2 on Smad 1/5/8 phosphorylation from a 15-min to a 6-h period of study (data not shown).

**Figure 1.** Effect of NICD overexpression on APA in ST-2 stromal cells. ST-2 cells transduced with control vector or NICD were cultured to confluence and treated with BMP-2 at 1–10 nM, or Wnt 3a at 0.8–8.1 nM for 72 h. APA was quantitated in total cell extracts from control cells (white bars) or NICD overexpressing cells (black bars), and is expressed as nanomoles of p-nitrophenol/min/μg of total protein. Data represent means ± S.E.; n = 6, * significantly different from control vector cells; p < 0.05. **Notch and Cell Differentiation**
phosphorylation of c-JNKs, p38, or ERK1/2, except for a modest and transient stimulation on P-ERK, and NICD overexpression did not modify the response to BMP-2 (Fig. 3). These results indicate that, although NICD overexpression prevents BMP-2 effects on osteoblastogenesis, it does not modify BMP signaling, suggesting that Notch interacts with other factors necessary for BMP downstream events and biological activity.

To explore other possible mechanisms involved in the effects of Notch 1 on stromal cell differentiation, we tested whether NICD overexpression modified the canonical Wnt/β-catenin signaling pathway, which plays a role in osteoblastogenesis and acts in a coordinated fashion with BMP signaling (30). NICD overexpression decreased the activity of the Wnt/β-catenin-dependent pTOPFLASH reporter construct (Fig. 4A) and the cytoplasmic levels of free β-catenin (Fig. 4B) in subconfluent and confluent cultures, respectively. NICD overexpression also suppressed mRNA levels of the Wnt-dependent WISP1 gene, and following a 2- to 3-week exposure to NICD, WISP1 transcripts were not detectable (Fig. 4C).

To investigate further the mechanisms involved in the inhibition of the Wnt/β-catenin canonical pathway by Notch, we attempted to rescue Wnt signaling by transfecting a stable mutant β-catenin resistant to GSK3β-mediated phosphorylation and degradation or by adding an inhibitor of GSK3β.Transient transfections of the stable mutant β-catenin expression pCiNeo-S37-FLAG construct enhanced the transactivation of the Wnt/β-catenin-dependent pTOPFLASH reporter in the presence or absence of NICD overexpression. However, the basal and the stable β-catenin-induced effects were opposed by NICD overexpression (Fig. 5A). Cell exposure to the GSK3β inhibitor CHIR 99021 at 3 μM for 4 h resulted in an increase in free β-catenin levels in control and NICD-overexpressing cells (Fig. 5B). The GSK3β inhibitor at 3 and 6 μM for 48 h increased the transactivation of the transiently transfected pTOPFLASH construct, but NICD opposed the effect (Fig. 5C). The effect of the GSK3β inhibitor at 3 μM on the transactivation of the pTOPFLASH construct was comparable to that of stable β-catenin, and their effects were opposed by NICD by 30–50%. At 6 μM, the GSK3β inhibitor caused a greater increase on the transactivation of the reporter construct, but the absolute suppression of transactivation by NICD was similar when tested in the presence of the GSK3β inhibitor at 3 or 6 μM. Consequently, the relative suppression of pTOPFLASH transactivation was more pronounced when NICD was tested in the presence of the GSK3β inhibitor at 6 μM. Treatment of ST-2 control cells with the GSK3β inhibitor at 1 to 6 μM for 3 days increased alkaline phosphatase activity in the presence or absence of NICD, and the effect was opposed by NICD overexpression (Fig. 5D). These results indicate that Notch opposes Wnt signaling and that normalization of β-catenin levels is not sufficient to reverse completely the

**FIGURE 2.** Effect of NICD overexpression, in the absence or presence of BMP-2, on Smad 1/5/8 phosphorylation and on the transactivation of the BMP/Smad-dependent 12×SBE-Oc-pGL3 construct in ST-2 stromal cells. A, ST-2 cells transfused with control vector (−) or NICD (+) were cultured to confluence, serum-deprived overnight, and treated with BMP-2 at 0.3–3.3 nM for 20 min. Total cell lysates were resolved by 12% SDS-PAGE, transferred to Immobilon P membranes, and incubated with an antibody to phosphorylated Smad 1/5/8, or to unphosphorylated Smad 1, and proteins detected by a chemiluminescence detection system. B, ST-2 cells transfused with control vector (white bars) or NICD (black bars) were transiently transfected with 12×SBE-Oc-pGL3 or pGL3 plasmid (not shown) and a CMV/β-galactosidase reporter construct. After 16 h, cells were serum-deprived overnight and treated with BMP-2 at 0.03–3.3 nM for 24 h. Data shown represent luciferase/β-galactosidase activity for control or NICD-overexpressing cells. Values are means ± S.E.; n = 6. *, significantly different between BMP-2-treated and untreated cells; p < 0.05.

**FIGURE 3.** Effect of NICD overexpression in the absence and presence of BMP-2 at 3. 3 nM on the phosphorylation of MAP kinases, ERK1/2, JNK1–2/3, and p38 in ST-2 stromal cells. ST-2 cells transfused with control vector (−) or NICD (+) were cultured to confluence, serum-deprived overnight, and treated with BMP-2 for 15 min to 6 h. Total cell lysates were resolved by polyacrylamide gel electrophoresis, transferred to Immobilon P membranes, and incubated with antibodies to phosphorylated (P-) or unphosphorylated ERK1/2, JNK1–2/3, p38, and proteins were detected by a chemiluminescence detection system.

**FIGURE 4.** Effect of NICD overexpression on the transactivation of the Wnt/β-catenin-dependent pTOPFLASH construct, on cytoplasmic β-catenin levels, and on Wnt induced secreted protein (WISP) 1 mRNA expression in ST-2 stromal cells. A, subconfluent ST-2-transduced cells with control vector (white bars) or NICD (black bars) were transiently transfected with pTOPFLASH construct, and a CMV/β-galactosidase reporter construct and harvested after 48 h. Data shown represent luciferase/β-galactosidase activity for control and NICD cells. Bars are means ± S.E.; n = 6. *, significantly different from control cells; p < 0.05. B, for β-catenin determinations, cytoplasmic cell lysates from confluent ST-2 cells transfused with control vector (−) or NICD (+) were resolved by polyacrylamide gel electrophoresis, transferred to Immobilon P membranes, and incubated with an antibody to β-catenin for the detection of cytoplasmic free β-catenin levels, using a chemiluminescence detection system. Coomassie Blue staining of the gel demonstrated equal protein loading (not shown). C, ST-2 cells transfused with control vector (−) or NICD (+) were cultured to confluence (0) or for 1–3 weeks post-confluence. Total RNA was extracted, subjected to Northern blot analysis, hybridized with 32P-labeled WISP1 and 18S cDNA, and visualized by autoradiography.
inhibitory effect of Notch on Wnt activity, indicating possible additional mechanisms responsible for the effect of Notch on Wnt actions. In subsequent experiments, a 16×TCF-luc, instead of the pTOPFLASH reporter construct, was used due to the higher basal and stimulated activity of the 16×TCF-luc reporter, which contains 16 instead of 3 TCF/LEF-1 binding sequences upstream of the luciferase gene. When compared in the same experiment, co-transfections of stable β-catenin or Wnt 3 and addition of the GSK3β inhibitor at 3 μM increased the transactivation of pTOPFLASH by 2.7- to 3.5-fold and of 16×TCF-luc by 4- to 36-fold. NICD overexpression decreased the basal and stimulated transactivation of pTOPFLASH by 25–50% and of the 16×TCF-luc construct by 60–80%.

To address additional interactions between Notch and Wnt, we explored the possible signals utilized by Notch in ST-2 cells. In preliminary experiments, we determined that Notch induces HES-1, a transcriptional inhibitor known to recruit Groucho1/TLE1 or -2 co-repressors and known to inhibit the activity of LEF-1, which associates with β-catenin in the nucleus to regulate transcription (57, 58). To determine the role of HES-1 and its co-repressors Groucho1/TLE1 and -2 in Notch effects in ST-2 stromal cells and in the inhibition of Wnt signaling by Notch, the consequences of silencing HES-1 or Groucho1/TLE1 and -2 were examined. HES-1 and Groucho1/TLE1 and -2 siRNAs were transfected into ST-2 cells, and HES-1 mRNA levels were suppressed by 45–80% (Fig. 6A), and Groucho1/TLE1 (Fig. 7A) and -2 (not shown) were suppressed by 80–90%. The effect was sustained for a 24- to 96-h period. Stable β-catenin, Wnt 3, and the GSK3β inhibitor enhanced the transactivation of the 16×TCF-luc reporter, although transfection of a Wnt 3 expression vector resulted in lesser transactivating activity than that achieved with the transfection of stable β-catenin (Figs. 6A and 7B). The reason is possibly because of different levels of expression or because the endogenous β-catenin induced by Wnt 3 is less stable than the transfected stabilized β-catenin. Silencing of HES-1 (Fig. 6B) or Groucho1/TLE1 (Fig. 7B) enhanced the effect of stable β-catenin, Wnt 3, and the GSK3β inhibitor on the transactivation of the 16×TCF-luc reporter. The effect was opposed by NICD, but the degree of transactivation of the 16×TCF-luc construct in Notch overexpressing cells silenced with either HES-1 or Groucho1/TLE1 was at least comparable to control cultures (Figs. 6B and 7B). Groucho2/TLE2 siRNA did not modify the basal or induced transactivation of 16×TCF-luc (data not shown). HES-1 RNAi increased the effect of BMP-2 and Wnt 3a on APA, indicating that HES-1 suppressed the effect of BMP-2 and Wnt 3a on APA (Fig. 6C). NICD overexpression decreased the effect of BMP-2 and Wnt 3a on APA, but in the context of HES-1 RNAi, the levels were equal or slightly higher than control vector transfected cells. Silencing of Groucho1/TLE1 increased the effect of BMP-2 on APA in control and NICD-transduced cells but did not modify the effect of Wnt 3a on basal or NICD suppressed APA (Fig. 7C). The results indicate that HES-1 and Groucho1/TLE1 regulate the inhibitory effects of Notch on Wnt signaling, but only HES-1 contributes to the effect of Wnt 3a on APA.

**DISCUSSION**

The present study confirms that overexpression of NICD in ST-2 stromal cells inhibits the activity of BMP-2 and Wnt 3a on osteoblastic differentiation. To analyze the mechanism of action of Notch, we tested its effect on BMP-2 and Wnt canonical signaling pathways. In contrast to other stromal cell lines, in ST-2 cells BMP-2 induced the phospho-
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FIGURE 6. Effect of HES-1 RNA interference on the actions of NICD overexpression on APA and on the transactivation of the Wnt/β-catenin-dependent 16×TCF-luc construct in ST-2 stromal cells. A, ST-2 cells were transfected with HES-1 or control scrambled siRNA (control) for 24–96 h. The copy number of HES-1 was determined by real-time RT-PCR and the results expressed as the ratio of HES-1 to glyceraldehyde-3-phosphate dehydrogenase mRNA, n = 2–8. ST-2 cells were transiently transfected with control vector and scrambled siRNA (white bars) or with NICD and scrambled siRNA (black bars), or with control vector and HES-1 silencing (dark gray bars), or with NICD and HES-1 siRNA (light gray bars) and with a stable β-catenin or Wnt 3 expression construct and with a 16×TCF-luc and a CMV/β-galactosidase reporter construct, and harvested after 48 h. For GSK3β suppression, transiently transfected ST-2 cells were treated 16 h after transfection with GSK3β inhibitor at 3 μM for 24 h and harvested. Bars are means ± S.E., n = 4. *, significantly different between control pcDNA 3.1 and NICD transduced cells; p < 0.05. +, significantly different between scrambled and HES-1 siRNA transfected cells; p < 0.05. C, ST-2 cells transduced with control vector (white bars) or NICD (black bars) were transiently transfected with HES-1 silencing (dark and light gray bars) or scrambled RNA (white and black bars), and treated with BMP-2 or Wnt 3a protein for 72 h. APA was quantitated in total cell extracts and is expressed as nmol of p-nitrophenol/min/μg of total protein. Data represent means ± S.E., n = 4. *, significantly different between vector control and NICD transduced cells; p < 0.05. +, significantly different between scrambled and HES-1 siRNA transfected cells; p < 0.05.

rylation of the BMP-2-dependent Smads 1/5/8 but did not activate appreciably MAP kinase signaling, except for a modest and transient stimulation on P-ERK (26–28). The reason for this difference is not known, but it may be related to the degree of cell maturation or to the presence or absence of preformed heteromeric complexes of type I and II BMP receptors on the cell surface prior to BMP binding. BMP binding to preformed BMP type I and type II heteromeric complexes activates Smad signaling, whereas BMP binding to a homomeric receptor leading to the secondary formation of a heteromeric complex activates MAP kinase signaling (20, 59). NICD overexpression did not alter BMP-induced Smad 1/5/8 phosphorylation or the activity of a reporter construct containing twelve copies of a Smad recognition sequence directing luciferase expression, and it did not modify MAP kinase activation indicating that Notch does not regulate BMP-2 signaling in ST-2 stromal cells. In contrast, Notch opposed the Wnt canonical signaling pathway, suggesting that its mechanism of action is specific to Wnt signaling. Because Notch overexpression opposes the effects of BMP-2 and Wnt 3a on APA and osteoblastogenesis, the results indicate that Wnt signaling is required for BMP-2 actions on osteoblastic cell differentiation (9). Because alkaline phosphatase is not a direct target of BMP-2, the results suggest that Wnt signaling is required for a downstream effect of BMP-2 on this marker of osteoblastic function. Sclerostin, the product of the sost gene, plays a role similar to that of Notch, because it was described to antagonize BMP-induced osteoblast differentiation without affecting BMP signaling (60). Furthermore, sclerostin (SOST), like Notch, inhibits Wnt signaling in Xenopus embryos and mammalian cells (61, 62).

Our results are in agreement with previous investigations demonstrating an inhibitory effect of NICD on osteogenesis in C2C12, ST-2, MC3T3, and Kusa mesenchymal cells (9, 47, 63). However, others have reported that Notch overexpression can, under certain conditions, sensitize osteoblastic cells to selective effects of BMP-2 despite an inhibition of osteocalcin transcription (10, 11). The differences in the results observed may be due to the use of different cell lines and culture conditions, or due to the use of adenoviral instead of retroviral vector delivery systems resulting in a transient instead of a continuous activation of Notch signaling. However, it is important to note that an inhibition of osteocalcin transcription by Notch is indicative of at least a partial impairment of osteoblastic function by Notch. Furthermore, in the present studies, Notch opposed Wnt signaling in permanently transduced cells as well as in cells acutely transfected with Notch expression constructs. The discordant effects of Notch on osteoblastogenesis may also reflect a dual role of this factor in osteoblastic cell differentiation. Dual roles for Notch in the differentiation of preadipocytes have been
and with a stable ratio of Groucho1/TLE1 to glyceraldehyde-3-phosphate dehydrogenase mRNA, cho1/TLE1 or control scrambled siRNA (control) for 24–96 h. The copy number of Groucho1/TLE1 siRNA transfected cells were treated 16 h after transfection with GSK3β-nitrophenol/min/μg of total protein. Data represent means ± S.E., n = 6, * significantly different between scrambled and Groucho1/TLE1 siRNA transfected cells; p < 0.05. 

reported, indicating that whereas Notch and its intermediary HES-1 are required for adipogenesis, activation of Notch signaling can inhibit preadipocyte differentiation (64). HES-1 and -5 are essential effectors of Notch activity and mediate its transcriptional events. Following Wnt signaling, Notch decreases β-catenin pathway. Notch 1 transcripts are induced by glucocorticoids, because glucocorticoids oppose the Wnt/β-catenin signaling (67, 71). This may reflect the postulated epistatic relationship between Notch and Wnt signaling, particularly because Groucho1/TLE1 silencing increased the effect of BMP-2 under basal conditions.

It is of interest that Notch 1 and Wnt have opposite effects on osteoblastic cell differentiation, and Wnt and Notch signaling also play opposite roles in adipogenesis (68, 69) and in keratinocytes, where Notch represses Wnt signaling (70). Although interactions between Notch and Wnt/β-catenin often explain the regulatory effects of Notch on cell differentiation, this is not always the case. For instance, overexpression of NICD in intestinal cells results in an inhibition of cell differentiation and amplification of the progenitor cell pool, whereas the loss of Notch signaling results in cell differentiation, and neither event is associated with altered Wnt/β-catenin signaling (67, 71). This may reflect the postulated epistatic relationship between Notch and Wnt signaling in certain cell systems, such as in intestinal precursor cells, where Wnt and Notch have superimposing activities favoring cell proliferation and opposing cell differentiation.

The effects of NICD overexpression on ST-2 cells have similarities to those of glucocorticoids in osteoblasts, because glucocorticoids oppose the Wnt/β-catenin pathway. Notch 1 transcripts are induced by glu-
corticoid in ST-2 stromal and MC3T3 osteoblastic cells, and glu-
corticoid may act in conjunction with Notch suppressing Wnt sig-
naling (54, 72, 73).

In conclusion, our studies demonstrate that Notch overexpression inhibits ST-2 stromal cell differentiation toward an osteoblastic phenotype and opposes Wnt/β-catenin, but not BMP-2, signaling. HES-1 plays an important role in the inhibition of osteoblastogenesis and Wnt/β-catenin signaling by NICD.

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