Nephroblastoma Overexpressed (Nov) Inhibits Osteoblastogenesis and Causes Osteopenia*

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Nephroblastoma overexpressed (Nov), a member of the Cyr61, connective tissue growth factor, Nov (CCN) family of proteins, is expressed by osteoblasts, but its function in cells of the osteoblastic lineage is not known. We investigated the effects of Nov overexpression by transducing murine ST-2 stromal and MCGT3 osteoblastic cells with a retroviral vector where Nov is under the control of the cytomegalovirus promoter. We also examined the skeletal phenotype of transgenic mice expressing Nov under the control of the human osteocalcin promoter. Overexpression of Nov in ST-2 cells inhibited the appearance of mineralized nodules and decreased alkaline phosphatase activity and osteocalcin mRNA levels. Nov overexpression inhibited the effect of bone morphogenetic protein (BMP)-2 on the phosphorylation of Smad1/5/8; on the transactivation of 12xSBE-Oc-pGL3, a BMP-Smad signaling reporter construct, and of Wnt3 on cytoplasmic β-catenin levels; and on the transactivation of the Wnt/β-catenin signaling reporter construct 16xTCF-Luc. Nov overexpression did not activate Notch or transforming growth factor β signaling. Glutathione S-transferase pulldown assays demonstrated direct Nov-BMP interactions. Nov transgenic mice exhibited osteopenia. In conclusion, Nov binds BMP-2 and antagonizes BMP-2 and Wnt activity, and its overexpression inhibits osteoblastogenesis and causes osteopenia.

Mesenchymal cells can differentiate into cells of various lineages, including osteoblasts, myoblasts, chondrocytes, and adipocytes (1). Bone morphogenetic proteins (BMPs) and Wnt are important determinants of cell fate and induce mesenchymal cells to differentiate toward cells of the osteoblastic lineage (2–4). BMPs interact with type Iα, type Iβ, and type II receptors, and upon ligand binding, they initiate a signal transduction cascade activating the signaling mothers against decapentaplegic (Smad) or the mitogen-activated protein (MAP) kinase signaling pathways (2, 5–8). In osteoblastic cells, Wnt binding to specific transmembrane receptors and co-receptors leads to the stabilization of β-catenin and its translocation to the nucleus where it associates with members of the lymphoid enhancer binding factor/T cell-specific factor (LEF/TCF) family to regulate transcription (9–11).

Whereas BMPs play a central role in the regulation of osteoblastogenesis and endochondral bone formation, in excess they can be detrimental. Consequently the effects of BMPs are controlled by a large group of secreted polypeptides that prevent BMP signaling by binding BMPs, precluding their interactions with specific cell surface receptors (2, 12). Their binding affinity and selectivity for specific BMPs varies, and selected BMP antagonists, such as sclerostin and ectodin, can interact with Wnt co-receptors and block Wnt signaling and activity (2, 13–15). Conversely Wnt antagonists, such as Dickkopf 1, can oppose BMP effects, suggesting coordinated activities between BMP and Wnt (16).

Members of the CCN family of cysteine-rich secreted proteins include cysteine-rich 61 (Cyr61), connective tissue growth factor (CTGF), nephroblastoma overexpressed (Nov), and Wnt-inducible secreted proteins (WISPs) 1, 2, and 3 (17, 18). CCN proteins are highly conserved and share four distinct structural modules: an insulin-like growth factor-binding domain, a von Willebrand type C domain containing the cysteine-rich domain, a thrombospondin-1 domain, and a carboxy-terminal domain (17, 18). CCN proteins are related to certain BMP antagonists, such as twisted gastrulation (Tsg) and chordin, and can have important interactions with regulators of osteoblast cell growth and differentiation (19). CTGF binds to BMP-2 and -4 through its cysteine-rich domain and binds to Wnt co-receptors through its carboxy-terminal domain, and its overexpression modifies BMP and Wnt signaling and effects on osteoblastogenesis (20, 21). CTGF also interacts with TGF-β, enhancing its activity, and Cyr 61 regulates Wnt signaling (20, 22). The structural similarities with BMP antagonists of the Tsg and chordin families and these observations indicate a

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‡ The abbreviations used are: BMP, bone morphogenetic protein; APA, alka-line phosphatase activity; BSA, bovine serum albumin; CCN, Cyr, cytomegalovirus, connective tissue growth factor, Nov; CMV, connective tissue growth factor; CTGF, CBF1/Suppressor of Hairless/Lag1; CTGF, connective tissue growth factor; Cyr61, cysteine-rich 61; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FVB, Fried leukemia virus B sensitive; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HES, hairy and E (spl); JNK, c-Jun NH2-terminal kinase; LEF, lymphoid enhancer binding factor; TCF, T cell-specific factor; LRP, low density lipoprotein receptor-related protein; MAP, mitogen-activated protein; α-MEM, minimum essential medium; NICD, Notch intracellular domain; Nov, nephroblastoma overexpressed; PBS, phosphate-buffered saline; RT, reverse transcription; SBE, Smad binding element; Smad, signaling mothers against decapentaplegic; TGF-β, transforming growth factor β; WISP, Wnt-inducible secreted protein; Tsg, twisted gastrulation.
potential functional relationship between CCN peptides and more classic extracellular BMP antagonists.

Nov shares 50% sequence homology with Cyr 61 and CTGF and is expressed in a variety of tissues, including bone and hypertrophic cartilage, where it enhances TGF-β signaling and chondrogenesis (17, 23, 24). Nov inhibits myoblast cell differentiation possibly by interacting with the extracellular domain of Notch (25). Nov also interacts with integrin receptors and with Connexin 43, a channel protein important in cell–cell communications, skeletal development, and osteoblast function (26–28). However, the function of Nov in skeletal cells is not known.

The purpose of this study was to investigate the direct effects of Nov on the differentiation and function of cells of the osteoblastic lineage and on bone remodeling in vivo. For this purpose, we transduced ST-2 stromal and MC3T3 osteoblastic cell lines with a retroviral vector expressing Nov under the control of the cytomegalovirus (CMV) promoter and created transgenic mice expressing Nov under the control of the osteocalcin promoter. We determined the cellular and skeletal phenotypes and mechanisms responsible for the effects observed.

**EXPERIMENTAL PROCEDURES**

**Vectors and Packaging Cell Lines**—For the creation of transduced cell lines, nov was cloned into the retroviral vector pLPCX (Clontech) where a CMV promoter directs the constitutive expression of the gene of interest. 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. A 1,065-bp DNA fragment containing the murine nov coding sequence with a FLAG epitope tag on the amino-terminal end (American Type Culture Collection (ATCC), Manassas, VA) was cloned into pLPCX vector or pLPCX-Nov were plated at a density of 10^4 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS, 100 μg/ml ascorbic acid, and 5 mM β-glycerophosphate (Sigma-Aldrich) and cultured for 1 day to 4 weeks in the presence or absence of recombinant human BMP-2 (Wyeth Research, Collegeville, PA), Wnt 3a (R&D Systems, Minneapolis, MN), or TGF-β (PeproTech, Rocky Hill, NJ) as indicated in the text and legends. In one experiment, wild type ST-2 cells were cultured under the same conditions, and recombinant human Nov protein (PeproTech) was added in the presence and absence of BMP-2 as indicated in the text and legend.

**Cytochemical Assays, Alkaline Phosphatase Activity, and Cell Viability**—To determine mineralized nodule formation, cells were fixed with 3.7% formaldehyde and stained with 2% alizarin red (Sigma-Aldrich) (30). Alkaline phosphatase activity (APA) was determined in cell extracts by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol and measured by spectroscopy at 410 nm after 10 min of incubation at 25 °C according to the manufacturer’s instructions (Sigma-Aldrich). Data are expressed as nanomoles of p-nitrophenol released/minute/ microgram of protein. Total protein content was determined in cell extracts by the DC protein assay in accordance with the manufacturer’s instructions (Bio-Rad). To estimate the number of viable cells, mitochondrial dehydrogenase activity was measured using the Cell Titer 96 Aqueous One cell proliferation assay (Promega, Madison, WI) in accordance with the manufacturer’s instructions. Metabolically active cells were estimated by their ability to reduce the tetrazolium compound 3-(4,5’-dimethyl-thiazol-2-yl)-5(-3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt to a formazan product, which was quantified at an absorbance of 490 nm. Data are expressed in arbitrary units of absorbance at 490 nm.

**Real Time Reverse Transcription (RT)-PCR**—Total RNA was extracted, and Nov, osteocalcin, and WISP 1 mRNA levels were determined by real time RT-PCR (31, 32). For this purpose, 1–10 μg of RNA were reverse transcribed using the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer’s instructions and amplified in the presence of 5’-GAACCAACAGACTGGCATTCGATG(FAM)TC-3’ and 5’-AACTCTTCTCCTCCTTCGTA-3’ primers for Nov, 5’-CACTTACGGCGCTATCCCTGGTGAAGT(FAM)G-3’ and 5’-CCAGCAACTCTCCTCCCTA-3’ primers for osteocalcin, 5’-TACGCTCTGAACTTTGGTCCTCTCA-3’ and 5’-CGAAC- TTAGTATGACACGGGCTT(FAM)G-3’ primers for Pompe disease dehydrogenase (GAPDH) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54–60 °C for 45 cycles. Gene copy number was estimated by comparison with a standard curve constructed using Nov (ATCC), osteocalcin (I. Lian, University of Massachusetts, Worcester, MA), or WISP 1 (ATCC) DNAs and corrected for GAPDH (R. Wu, Cornell University, Ithaca, NY) copy number (33, 34). Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step.

**Western Immunoblot Analysis**—To detect Nov-FLAG peptide, culture medium from cells transduced with pLPCX or pLPCX-Nov was precipitated with 15% trichloroacetic acid and fractionated by polyacrylamide gel electrophoresis in 12.5%
acrylamide gels under non-reducing conditions (35). Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA), blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and exposed overnight to 2 μg/ml mouse monoclonal antibody FLAG-M2 raised to FLAG fusion proteins (Sigma-Aldrich). Blots were exposed to anti-mouse IgG conjugated to horseradish peroxidase (1:10,000) and developed with a chemiluminescence detection reagent (PerkinElmer Life Sciences). Nov-FLAG was identified by migration at the expected molecular masses of 48, 38, and 18 kDa (36). To determine the level of phosphorylation of Smads 1/5/8, Smad 2, and the MAP kinases ERK 1/2, JNK 1 and 2/3, and p38, the cell layer of ST-2 cells transduced with pLPCX or pLPCX-Nov was washed with cold PBS and extracted in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton, and 1 mM EDTA) (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors as described previously (37, 38). Protein concentration was determined by DC protein assay, and 25–70 μg of total cellular protein were fractionated by polyacrylamide gel electrophoresis in 12.5% acrylamide gels under reducing conditions and transferred to Immobilon P membranes. For Smad 1/5/8, the membranes were blocked with 3% BSA in PBS and exposed to a rabbit polyclonal antibody, which recognizes Smads 1, 5, and 8 phosphorylated at Ser-463/465 (Smads 1 and 5), and Ser-426/428 (Smad 8) (Cell Signaling Technology), or exposed to a rabbit polyclonal antibody to unphosphorylated Smad 1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1,000 dilution (39, 40). For Smad 2, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, 0.1% Tween, pH 7.6, and exposed to rabbit polyclonal antibodies to either phosphorylated (Ser–465/467) or to unphosphorylated Smad 2 (Cell Signaling Technology) at a 1:1,000 dilution (39, 40). To estimate MAP kinase activation, membranes were blocked with 3% BSA in PBS and exposed to rabbit polyclonal antibodies to phosphorylated p44 ERK 1 (Thr–183/185) and p42 ERK 2 (Thr–202/204), JNK 1 and 2/3 (Thr–183/Tyr–185), or p38 (Thr–180/Tyr–182) or to rabbit polyclonal antibodies to corresponding unphosphorylated proteins (all at 1:1,000 dilution; all from Cell Signaling Technology) (41). To determine β-catenin levels, the cell layer was extracted in 10 mM Tris, 140 mM NaCl, 5 mM EDTA, and 2 mM dithiothreitol buffer at pH 7.6 in the presence of protease inhibitors, and the cytoplasmic fraction was separated by ultracentrifugation as described previously (42). 100 μg of total cytoplasmic protein were fractionated by gel electrophoresis in 7.5% polyacrylamide gels and transferred to Immobilon P membranes.

The membranes were blocked with 3% BSA in PBS and exposed to a 1:500 dilution of a monoclonal antibody to unphosphorylated β-catenin or a polyclonal antibody to human actin (both from Santa Cruz Biotechnology). All blots were exposed to anti-rabbit or anti-mouse IgG antisera conjugated to horseradish peroxidase and developed with a chemiluminescence detection reagent (PerkinElmer Life Sciences).

**Transient Transfections**—To determine changes in BMP-2 signaling, a construct containing 12 copies of a Smad 1/5 consensus sequence linked to an osteocalcin minimal promoter and a luciferase reporter gene (12xSBE-Oc-pGL3; M. Zhao, University of San Antonio, Health Sciences, Antonio, TX) was tested in transient transfection experiments (43). To determine changes in TGF-β signaling, a construct containing four copies of the Smad 3 consensus sequence linked to a minimal SV40 promoter and a luciferase reporter gene (pSBE4-Luc; R. Derynk, University of California, San Francisco, CA) was used (44). To determine changes in Wnt/β-catenin transactivating activity, a construct containing 16 copies of the Lef1/Tcf-4 recognition sequence cloned upstream of a minimal thymidine kinase promoter and a luciferase reporter gene (16xTCF-Luc; J. Billiard, Wyeth Research) was tested (45). To determine changes in Notch 1 signaling, a construct containing 12 copies of a CBF1/Suppressor of Hairless/Lag 1 (CSL) response element, linked to the β-globin basal promoter (12xCSL-Luc; L. J. Strobl, Munich, Germany) or a 354-bp fragment of the hairy and E (spl)-1 (HES-1) promoter (U. Lendahl, Stockholm, Sweden), both cloned upstream of a luciferase reporter gene, was tested (46–49). Wild type or transduced ST-2 or MC3T3-E1 cells were transiently transfected using FuGENE 6 (3 μl of FuGENE/2 μg of DNA) according to the manufacturer’s instructions (Roche Applied Science) with 12xSBE-Oc-pGL3, pSBE4-Luc, 16xTCF-Luc, 12xCLS-Luc, or HES-1 promoter. A CMV-directed β-galactosidase expression construct (Clontech) was used to control for transfection efficiency. In selected experiments testing for effects on Wnt/β-catenin transactivating activity, cells were co-transfected with a β-catenin expression construct mutated at Ser–37 cloned into pCiNeo (pCiNeo-S37-FLAG; P. J. Morin, Baltimore, MD), a Wnt 3 expression construct cloned into pUSEamp (Upstate Biotechnology, Lake Placid, NY), or control vectors (45, 50). In selected experiments testing for effects on Notch signaling, cells were co-transfected with a Notch intracellular domain (NICD) expression construct cloned into pcDNA 3.1, pcDNA-NICD (41). Cells were exposed to the FuGENE-DNA mixture for 16 h, transferred to serum containing test or control medium for 48 h, and harvested. For 12xSBE-Oc-pGL3 and pSBE4-Luc activity, cells were exposed to the FuGENE-DNA mixture for 16 h and transferred to serum-free medium for 8 h. Cells were then treated with BMP-2 or TGF-β for 24 h and harvested. In selected experiments testing for effects on TGF-β signaling, cells were co-transfected with a TGF-β receptor type I or II expression construct (Clontech) and exposed to 100 ng/ml mouse recombinant TGF-β1 and then tested in transient transfection experiments (43). 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quantified by DC protein assay, and stored at \(-80^\circ\)C. For GST pulldown assays, GST or GST-Nov fusion proteins were mixed in radioimmune precipitation assay lysis buffer (Santa Cruz Biotechnology) containing protease inhibitors with or without purified BMP-2, Wnt 3a, or low density lipoprotein receptor-related protein (LRP)-6/Fc (R&D Systems) at 4 \(^\circ\)C for 16 h. Glutathione-Sepharose 4B was added, and samples were mixed for 30 min at 25 \(^\circ\)C. The resin was washed with radioimmune precipitation assay lysis buffer, and proteins were eluted with 10 \(\mu\)M reduced glutathione. Samples were suspended in non-reducing sample buffer, boiled, resolved on 12\% polyacrylamide gels, transferred to Immobilon P membranes, blocked with 3% BSA, and exposed to anti-human BMP-2/4 monoclonal antibody, anti-mouse Wnt 3a antibody, or anti-human Fc\(\gamma\)RI/CD64 antibody (all at 2 \(\mu\)g/ml) for 16 h at 4 \(^\circ\)C (R&D Systems). Blots were exposed to anti-mouse or anti-rat IgG antibodies conjugated to horseradish peroxidase and developed with chemiluminescence (PerkinElmer Life Sciences).

**Generation and Analysis of Transgenic Mice**—For the generation of Nov transgenic mice, a 1,065-bp fragment coding for murine Nov was cloned downstream of a 182-bp artificial intron and a 3.8-kb fragment of the human osteocalcin promoter (E. Gardiner, Sydney, Australia) and upstream of polyadenylation sequences and a 3.5-kb fragment of the 3' untranscribed region and flanking DNA of the osteocalcin gene (52). Microinjection of linearized DNA into pronuclei of fertilized oocytes from Freud leukemia virus B sensitive (FVB) inbred mice and transfer of microinjected embryos into pseudopregnant FVB mice were carried out by the transgenic facility at the University of Connecticut Health Center (Farmington, CT). Positive founders, identified by Southern blot analysis of tail DNA, were bred to wild type FVB mice to generate transgenic lines. Intermatings of heterozygous transgenics were used to create a homozygous line. Static and dynamic histomorphometry was carried out on transgenic mice and wild type littermate controls at 1 month of age. Mice were injected with calcine (20 mg/kg) and demeclocycline (50 mg/kg) at an interval of 2 days and sacrificed by CO\(_2\) inhalation 2 days after the demeclocycline injection. Femurs were dissected, fixed in 70% ethanol, dehydrated, and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5-\(\mu\)m thick, were cut on a Microm microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with toluidine blue or Von Kossa. Static parameters of bone formation and resorption were measured in a defined area between 181 and 725 \(\mu\)m from the growth plate using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) (38). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained sections under ultraviolet light using a B-2A set long pass filter, and bone formation rate was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (53). To obtain bone marrow stromal cells, femurs were aseptically removed from 4-week-old mice after CO\(_2\) asphyxiation, and stromal cells were recovered by centrifugation as described previously (38). Cells were plated at a density of 5 \(\times\) 10\(^5\) cells/cm\(^2\) and cultured in \(\alpha\)-MEM containing 15% FBS at 37 \(^\circ\)C in a humidified 5% CO\(_2\) incubator. Half the volume of the culture medium was replaced after 4 days of culture with fresh medium, and when cells reached confluence (6–7 days of culture), the medium was changed to \(\alpha\)-MEM supplemented with 10% FBS, 50 \(\mu\)g/ml ascorbic acid, and 5 mM \(\beta\)-glycerophosphate. To assess changes in Smad 1/5/8 phosphorylation and \(\beta\)-catenin levels, cells were cultured for 10 days after confluence, serum-deprived overnight, and exposed to control medium, BMP-2 for 20 min, or Wnt 3a for 24 h as indicated in text and legends. Cell extracts were processed for the determination of phospho-Smads or \(\beta\)-catenin by Western immunoblot as described for pLPCX- and pLPCX-Nov transduced cells. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

**Statistical Analysis**—Data are expressed as means \(\pm\) S.E. Statistical differences were determined by analysis of variance.

**RESULTS**

To examine the impact of Nov on the ST-2 cell phenotype, ST-2 cells were transduced with the retroviral expression construct pLPCX-Nov and compared with cells transduced with pLPCX vector. Nov overexpression was determined by real time RT-PCR and by Western immunoblot analysis, which confirmed the expression of the 48-kDa full-length and 38- and 18-kDa proteins, likely processed isoforms, as described previously (36) (Fig. 1). Although Nov overexpression was more pronounced at the initiation of the culture, it was sustained for a
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FIGURE 2. Effect of Nov overexpression, in the absence or presence of BMP-2, on the differentiation of ST-2 stromal cells. ST-2 cells transduced with pLPCX or pLPCX-Nov were cultured to confluence (0) or for up to 4 weeks following confluence. In Panels A and B, total RNA was extracted from pLPCX (white bars) and pLPCX-Nov (black bars) cells cultured in the absence of BMP-2 or from pLPCX (slanted stripes) and pLPCX-Nov (horizontal stripes) cultured in the presence of BMP-2 at 1 nM for 4 weeks (Panel A) or 3.3 mM in serum-free medium for 24 h (Panel B) and subjected to real time RT-PCR for the determination of osteocalcin and GAPDH mRNA. Data are expressed as osteocalcin copy number corrected for gaelp expression. In Panel C, a table indicates the number of mineralized nodules formed in pLPCX or pLPCX-Nov cells cultured in the presence of BMP-2 at 1 nM. In Panels D, E, and F, APA was quantified in total cell extracts from pLPCX (white bars) or from pLPCX-Nov (black bars) cultured in the absence or presence of BMP-2 at 3.3 nM for 24 h (Panel D), at 10 nM for 3 days (Panel E), or at 1 nM for 3 weeks (Panel F). APA is expressed as nmol of p-nitrophenol/min/µg of total protein. Bars and data for all panels represent means ± S.E. for three (Panels A and B) or five to six (Panels C–F) observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05.

FIGURE 3. Effect of Nov overexpression, in the absence or presence of BMP-2, on Smad 1/5/8 phosphorylation (Panel A) and on the transactivation of the 12xSBE-Oc-pGL3 construct (Panel B) in ST-2 stromal cells. For Smad 1/5/8 phosphorylation, ST-2 cells transduced with pLPCX or pLPCX-Nov were cultured to confluence, switched to serum-free medium for 20 h, and treated with BMP-2 at 1 nM for 15 min. Total cell lysates were resolved by gel electrophoresis and transferred to Immobilon P membranes, which were incubated with an antibody to phosphorylated Smad 1/5/8 (P-SMAD) or to unphosphorylated Smad 1. For 12xSBE-Oc-pGL3 transactivation, ST-2 cells transduced with pLPCX (white bars) or pLPCX-Nov (black bars) were cultured to subconfluence and transiently transfected with 12xSBE-Oc-pGL3 and a CMV/β-galactosidase expression vector. After 16 h, cells were switched to serum-free medium for 8 h and treated with BMP-2 at 0.3–10 nM for 24 h. Data shown represent luciferase activity/β-galactosidase activity. Bars represent means ± S.E. for six observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05. V, vector; N, Nov.

4-week period. Nov overexpression had no acute effects on cell viability, and 24 h after confluence, arbitrary units of mitochondrial dehydrogenase activity were 0.34 ± 0.01 in pLPCX and 0.40 ± 0.01 (means ± S.E.; n = 6) in pLPCX-Nov cultures. The number of viable ST-2 cells declined over a 4-week culture period in control and Nov-overexpressing cells, and Nov had only modest effects on cell viability (Fig. 1). Cells overexpressing Nov were cultured under osteoblastic differentiating conditions in the presence of 5 mM β-glycerophosphate and 100 µg/ml ascorbic acid in the presence or absence of BMP-2 for up to 4 weeks. Nov overexpression opposed osteoblastic cell maturation and antagonized BMP-2 effects. pLPCX-Nov cells expressed lower levels of osteocalcin transcripts when compared with vectortransduced cells, although the difference was not statistically significant (Fig. 2). BMP-2 increased osteocalcin transcripts, and Nov opposed this effect throughout a 4-week period; this effect was significant after 3 and 4 weeks of culture. To test the acute effect of Nov on osteocalcin mRNA expression, confluent cultures of ST-2 cells transduced with pLPCX or pLPCX-Nov were treated, or not, with BMP-2 for 24 h in the absence of serum. In accordance with the results observed in 4-week experiments, Nov overexpression decreased osteocalcin mRNA levels after 24 h (Fig. 2). In addition, Nov overexpression decreased the formation of mineralized nodules induced by BMP-2 and inhibited the stimulatory effect of BMP-2 on alkaline phosphatase activity after 24 h, 3 days, or 3 weeks of culture (Fig. 2).

To elucidate the mechanism of Nov action in ST-2 cells, we analyzed the effect of Nov 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 Smads 1, 5, and 8 or the MAP kinases ERK 1/2, JNK 1 and 2/3, and p38 (5–8). Previously we have shown that in ST-2 cells BMP-2 induces the phosphorylation of Smads 1, 5, and 8, and the effect is maximal after 15 min, sustained for 6 h, and observed at BMP-2 concentrations of 0.03–3.3 nM (41). In addition, we confirmed that BMP-2, but not TGF-β, induced the transactivation of the BMP/Smad 1/5/8 reporter construct 12xSBE-Oc-pGL3 transiently transfected into ST-2 cells (not shown) (43). In accordance with the cellular phenotype observed, Nov overexpression reduced the effect of BMP-2 on Smad 1/5/8 phosphorylation and on the transactivation of the Smad 1/5-dependent 12xSBE-Oc-pGL3 reporter construct so that the effect of BMP-2 was about 50% lower in pLPCX-Nov cells compared with control vector cells (Fig. 3). BMP-2 did not induce the phosphorylation of MAP kinases p38 or JNK but did increase ERK phosphorylation, an effect that was enhanced and then reduced by Nov overexpression (Fig. 4). Whereas ERK phosphorylation can decrease the nuclear translocation of phospho-Smads and BMP signaling,
this event does not explain the results observed because Nov inhibited Smad phosphorylation in cytosolic extracts (54, 55). Furthermore ERK inhibitors did not modify the suppressive effect of Nov on the transactivation of the BMP/Smad-dependent 12xSBE-Oc-pGL3 reporter construct (Fig. 4). To determine whether the effect of Nov was specific for BMP-2, we investigated Nov actions on TGF-β signaling. TGF-β induces Smad 2/3 phosphorylation, and we confirmed that TGF-β, but not BMP-2, enhanced the transactivation of the TGF-β/Smad 3 reporter construct pSBE4-Luc transiently transfected into ST-2 cells (not shown) (44). We found that Nov overexpression did not modify the TGF-β-induced phosphorylation of Smad 2 or the transactivation of the TGF-β-dependent reporter construct pSBE4-Luc in ST-2 cells (Fig. 5).

To explore other possible mechanisms involved in the effects of Nov on stromal cell differentiation, we tested whether Nov modified the activity of the canonical Wnt/β-catenin signaling pathway. Wnt3a caused a dose-dependent increase in alkaline phosphatase activity, and Nov overexpression opposed this
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FIGURE 7. Effect of Nov overexpression on the transactivation of the Notch-dependent 12xCSL-Luc reporter and HES-1 promoter constructs and effect of Nov on the Wnt/β-catenin-dependent 16xTCF-Luc reporter construct in the context of Notch inactivation in ST-2 stromal cells. ST-2 cells transduced with pLPCX (white bars) or pLPCX-Nov (black bars) were cultured to subconfluence. In Panel A, pLPCX or pLPCX-Nov cells were transiently transfected with 12xCSL-Luc and a CMV/β-galactosidase expression vector and with an NICD (+) or control (−) expression construct. In Panel B, pLPCX or pLPCX-Nov cells were transiently transfected with an HES-1 promoter construct. Note the difference in scale used without and with NICD. In Panel C, pLPCX or pLPCX-Nov cells were treated (+) or not (−) with a γ-secretase II inhibitor and transiently transfected with a 16xTCF-Luc and a CMV/β-galactosidase expression vector and with a Wnt 3 expression construct. In all panels, 16 h after the transfection, cells were switched to serum-containing medium for 24 h and harvested. Data shown represent luciferase activity/β-galactosidase activity. Bars represent means ± S.E. for six observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05.

FIGURE 8. Effect of Nov overexpression, in the absence or presence of BMP-2, on the differentiation of MC3T3 cells. For APA (Panels A and B), cell viability (Panel C), and mineralized nodule formation (Panel D), cells transduced with pLPCX or pLPCX-Nov were cultured to confluence (0) or for up to 4 weeks following confluence. In Panels A and B, APA was quantified in total cell extracts from pLPCX (white bars) or from pLPCX-Nov (black bars) cultured in the presence of BMP-2 for 3 days at the indicated doses (Panel A) or for 4 weeks at 1 nm (Panel B). APA is expressed as nmol of p-nitrophenol/min/μg of total protein. In Panel C, cell viability was determined by mitochondrial enzymatic activity and is expressed in arbitrary formazan units read at 490 nm. In Panel D, a table indicates the number of mineralized nodules in pLPCX or pLPCX-Nov cells cultured in the presence of BMP-2 at 1 nm. For the transactivation of 12xSBE-Oc-pGL3 (Panel E), MC3T3 cells transduced with pLPCX (white bars) or pLPCX-Nov (black bars) were cultured to subconfluence and transiently transfected with 12xSBE-Oc-pGL3 and a CMV/β-galactosidase expression vector. After 16 h, cells were switched to serum-free α-MEM for 20 h and treated with BMP-2 at the indicated doses for 24 h. Data shown represent luciferase activity/β-galactosidase activity. Bars and data for all panels represent means ± S.E. for three (Panels D and E) or six (Panels A, B, C, and E) observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05.

Effect of Nov overexpression on the transactivation of the Notch-dependent 12xCSL-Luc reporter and HES-1 promoter constructs and effect of Nov on the Wnt/β-catenin-dependent 16xTCF-Luc reporter construct in the context of Notch inactivation in ST-2 stromal cells. ST-2 cells transduced with pLPCX (white bars) or pLPCX-Nov (black bars) were cultured to subconfluence. In Panel A, pLPCX or pLPCX-Nov cells were transiently transfected with 12xCSL-Luc and a CMV/β-galactosidase expression vector and with an NICD (+) or control (−) expression construct. In Panel B, pLPCX or pLPCX-Nov cells were transiently transfected with an HES-1 promoter construct. Note the difference in scale used without and with NICD. In Panel C, pLPCX or pLPCX-Nov cells were treated (+) or not (−) with a γ-secretase II inhibitor and transiently transfected with a 16xTCF-Luc and a CMV/β-galactosidase expression vector and with a Wnt 3 expression construct. In all panels, 16 h after the transfection, cells were switched to serum-containing medium for 24 h and harvested. Data shown represent luciferase activity/β-galactosidase activity. Bars represent means ± S.E. for six observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05.

To explore additional mechanisms involved in the inhibitory effects of Nov on osteoblastogenesis, Nov-Notch interactions were examined because they were considered relevant to the inhibitory effect of Nov on myogenesis (25). An NICD expression vector transfected into ST-2 cells induced the transactivation of a Notch-dependent 12xCSL-Luc reporter construct containing 12 repeats of a CSL binding site and of a promoter fragment of HES-1, a Notch target gene (Fig. 7). These results confirm that NICD activates the CSL signaling pathway (41). Nov-overexpressing cells decreased the transactivation of the 12xCSL-Luc reporter and the HES-1 promoter construct and opposed the transactivating effect of NICD on both reporter constructs. This indicates that Nov did not enhance but decreased Notch signaling in ST-2 cells. Notch inhibits Wnt but not BMP signal transduction in cells of the osteoblastic lineage, and to ensure that Notch activation did not play a role in the inhibitory effect of Nov on Wnt signaling, the effects of Nov were tested in the context of Notch inactivation (41). For this purpose, we used a γ-secretase II inhibitor, which precludes one of three cleavages required for the release of the intracellular domain of Notch and thus the Wnt-dependent gene WISP 1 by 35–55% (not shown). Nov decreased not only the effect of added Wnt 3a or of a transfected Wnt 3 expression construct on the 16xTCF-Luc reporter but also the effect of the transfected stable β-catenin mutant expression vector PCiNeo-S37-FLAG (Fig. 6) (50). These results suggest interactions between Nov and the canonical Wnt/β-catenin signaling pathway that are beyond receptor ligand activation.

Effect of Nov overexpression on the transactivation of the Notch-dependent 12xCSL-Luc reporter and HES-1 promoter constructs and effect of Nov on the Wnt/β-catenin-dependent 16xTCF-Luc reporter construct in the context of Notch inactivation in ST-2 stromal cells. ST-2 cells transduced with pLPCX (white bars) or pLPCX-Nov (black bars) were cultured to subconfluence. In Panel A, pLPCX or pLPCX-Nov cells were transiently transfected with 12xCSL-Luc and a CMV/β-galactosidase expression vector and with an NICD (+) or control (−) expression construct. In Panel B, pLPCX or pLPCX-Nov cells were transiently transfected with an HES-1 promoter construct. Note the difference in scale used without and with NICD. In Panel C, pLPCX or pLPCX-Nov cells were treated (+) or not (−) with a γ-secretase II inhibitor and transiently transfected with a 16xTCF-Luc and a CMV/β-galactosidase expression vector and with a Wnt 3 expression construct. In all panels, 16 h after the transfection, cells were switched to serum-containing medium for 24 h and harvested. Data shown represent luciferase activity/β-galactosidase activity. Bars represent means ± S.E. for six observations. *, significantly different between pLPCX and pLPCX-Nov cells, p < 0.05.
not activate the canonical Notch signaling pathway and instead opposes the effects of Notch on CSL signaling.

In accordance with the results obtained in ST-2 cells, Nov opposed BMP activity in MC3T3 cells. Nov overexpression was determined by real-time RT-PCR, and confluent control pLPCX-transduced cells expressed (means ± S.E.; n = 3) 1.6 ± 0.9 nov/gapdh copies, whereas pLPCX-Nov-transduced cells expressed 11.6 ± 1.3 nov/gapdh copies. Confirming the results in ST-2 cells, Nov overexpression suppressed the stimulatory effect of BMP-2 on alkaline phosphatase activity and on the formation of mineralized nodules over a 4-week period, indicating that Nov opposed osteoblast maturation (Fig. 8). Nov overexpression was confirmed in parallel cultures, and it was sustained over a 4-week period. As shown for ST-2 cells, Nov overexpression had a tendency to decline as the culture progressed, and at 4 weeks pLPCX-transduced cells expressed (means ± S.E.; n = 3) 0.9 ± 0.8 nov/gapdh copies, and pLPCX-Nov cells expressed 4.1 ± 2.2 nov/gapdh copies. Nov overexpression in MC3T3 cells decreased the effect of BMP-2 on the transactivation of the transfected BMP/Smad reporter construct 12xSBE-Oc-pGL3. These results confirm that Nov inhibits BMP activity in stromal as well as in more mature osteoblastic cells. Nov overexpression had no effect on MC3T3 cell viability in the initial 2 weeks of the culture period, although a decline was observed toward the final phases of the culture possibly secondary to the suppression of endogenous Bmps (57–59).

To investigate direct interactions between Nov and BMP-2, Wnt 3, and the Wnt co-receptor LRP-6, a GST pulldown assay using a GST-Nov fusion protein or GST alone was conducted. The GST pulldown assay showed that GST-Nov, but not GST alone, selectively bound BMP-2 (Fig. 9) but did not bind Wnt 3a or its co-receptor LRP-6 (not shown). To confirm that direct extracellular interactions between Nov and BMP-2 had biological consequences, BMP-2 was tested for its effects on Smad signaling and alkaline phosphatase activity in ST-2 stromal cells in the absence and presence of added Nov protein. Nov decreased the stimulatory effect of BMP-2 on the transactivation of the 12xSBE-Oc-pGL3 reporter construct by ~25–40% and the effect of BMP-2 on alkaline phosphatase by ~50% (Fig. 10).

To confirm the function of Nov in vivo, transgenic mice were created to express Nov under the control of the osteocalcin promoter. Nov transgenic mice were compared with wild type, sex-matched littermates of 1 month of age, a time of high activity of the osteocalcin promoter (60). In one line studied in detail, heterozygous transgenics expressed ~25–250 times higher levels of Nov transcripts in calvarial extracts than wild type littermates (Fig. 11). Visually transgenic mice did not exhibit obvious skeletal abnormalities and had normal growth and only minor changes in body weight. At 1 month of age, female Nov transgenics weighed (means ± S.E.; n = 5–10) 17.6 ± 0.3 g, and wild type controls weighed 18.6 ± 0.4 g (not significantly different), whereas male Nov transgenics weighed 18.3 ± 0.4 g, and controls weighed 20.6 ± 0.5 g (p < 0.05). Histomorphometric analysis of femurs from 4-week-old male and female heterozygous transgenics revealed a ~30–35% decrease in trabecular bone volume due to a reduced number of trabeculae. The number of osteoblasts and osteoclasts per perimeter and the osteoblast, osteoclast, and eroded surfaces were not different between transgenics and control mice, indicating normal cell number and normal bone resorption in these Nov-overexpressing mice. In contrast, fluorescence microscopy revealed a significant ~25% decrease in mineral apposition rate and a ~50% decrease in bone formation rate, indicating impaired osteoblastic function in Nov transgenics (Fig. 11). The in vivo phenotype correlated highly with the levels of Nov overexpression in bone. A second line of Nov homozygous transgenics expressing 1,000–
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DISCUSSION

Former studies have shown that cells of the osteoblastic lineage express transcripts for Nov and other members of the CCN family of proteins (61). However, the function of Nov in skeletal cells has not been established. In the present study, we demonstrated that overexpression of Nov inhibits osteoblast differentiation and the activity of BMP-2 and Wnt 3 in cells of the osteoblastic lineage. Nov overexpression inhibited basal as well as BMP-2 induced activity. The reason why basal activity was suppressed is probably because cells of the osteoblastic lineage express various BMPs, which act as autologous regulators of osteoblastic function (57–59). This may also explain the decline in cell viability observed in the terminal phases of the culture period in MC3T3 cells. The mechanism of Nov action involved the inhibition of BMP/Smad signaling as well as the inhibition of the Wnt/β-catenin canonical signaling pathway. It is of interest that Nov did not suppress MAP kinase activation and caused a transient stimulation of ERK phosphorylation. Whereas activation of ERK can alter the distribution of phospho-Smads and prevent their nuclear translocation, this event does not seem to explain the results observed because Nov inhibited the phosphorylation of Smads in cytosolic extracts, and ERK inhibitors did not alter the effect of BMP-2 on Smad signaling (54, 55).

Nov is a member of the CCN multigene superfamily of secreted cysteine-rich peptides, which are related to selected BMP antagonists such as Tsg and chordin (17–19). GST pull-down assays confirmed direct protein-protein interactions between Nov and BMP-2 but not between Nov and Wnt 3a or LRP-6. Although Nov could interact with other Wnt receptors or co-receptors, the tempering of Wnt activity by Nov is likely explained by the suppression of BMP-2 activity. Classic BMP antagonists have been shown to decrease BMP and Wnt activity, and Wnt antagonists such as Dickkopf 1 and sclerostin oppose BMP actions (13, 16). This is not surprising in view of the close relationship between BMP-2 and Wnt signaling pathways and their effects in cells of the osteoblastic lineage (62).

It is of interest that whereas Nov opposes BMP and Wnt activity and osteogenesis, various CCN proteins have diverse effects. CTGF can act as an agonist and antagonist of BMP activity. Although CTGF is required for osteoblastogenesis, it can interact with BMP-2 and Wnt co-receptors and under specific experimental conditions alter BMP and Wnt signaling and activity (20, 21). Dual permissive and inhibitory activity is not selective to CTGF, and it is shared by BMP antagonists of the Tsg/chordin family (63–67). RNA inter-
ference experiments revealed that Cyr 61 is necessary for the effect of Wnt 3 on selected parameters of osteogenesis, and Cyr 61 is induced by Wnt 3 (68). Exogenously expressed Cyr 61 also promoted mesenchymal cell migration, but the direct effects of Cyr 61 on osteoblastogenesis were not reported.

Nov impairs myoblast cell differentiation, but the mechanism is controversial (25, 69). Nov has been reported to interact with the extracellular region of Notch, inducing its activation and possibly explaining the inhibitory effect of Nov on myogenesis (25). However, recent work from another group of investigators has failed to demonstrate activation of the canonical Notch signaling pathway by Nov despite a suppressive effect on myogenesis (69). Our results in cells of the osteoblastic lineage are analogous, and we not only failed to demonstrate activation of Notch signaling by Nov, but instead we found that Nov inhibits its Notch signaling. Notch-ligand interaction results in the proteolytic cleavage of the Notch receptor and release of the intracellular domain, which translocates to the nucleus (46, 47). The intracellular domain subsequently interacts with members of the CSL family of transcription factors, leading to the induction of HES-1 and -5 and HES-related proteins (46, 47). Although Notch inhibits osteoblastic differentiation, Nov overexpression did not increase but inhibited the transactivation of a 12xCSL-Luc reporter construct and the transcription of HES-1. The reason why Nov opposes Notch signaling has not been elucidated, but similar results were reported in C2C12 myoblastic cells (69). The difference between the results obtained in myoblasts by one group of investigators and those obtained in ST-2 and C2C12 cells is not clear. It could be due to differences in basal levels of Notch under the culture conditions used or differences between the interactions of the natural ligands, Jagged and Delta, and Notch that might have precluded interactions between Notch and Nov. The mechanisms of the inhibitory effect of Nov on Notch signaling have not been explored and are beyond the scope of this work because a suppression of Notch activity does not explain the inhibitory effect of Nov on osteoblastogenesis (41).

Nov overexpression can enhance the effects of TGF-β on chondrogenesis, but we failed to demonstrate an effect of Nov on TGF-β signaling in ST-2 cells (24). A sensitization of TGF-β action in chondrocytes may play a role favoring chondrocytic instead of osteoblastic differentiation by Nov.

Our in vivo findings demonstrate that a line of transgenic mice overexpressing Nov under the control of the osteocalcin promoter exhibited suppressed bone formation and developed osteopenia. Histomorphometric analysis of femurs from Nov heterozygous transgenic mice revealed reduced trabecular bone volume due to decreased bone formation without a decrease in osteoblast surface. This lack of an effect of Nov on osteoblast number may be a reflection of the osteocalcin promoter used; it is active in mature non-dividing differentiated osteoblasts. Osteoblast-targeted Nov overexpression caused an inhibition of bone formation possibly because of a decrease in osteoblastic function because bone formation and mineral apposition rates were reduced. The results observed in vivo in this line are congruent with those obtained in vitro in osteoblastic cells constitutively overexpressing Nov and with an antagonistic effect of Nov on BMP signaling. The phenotype of Nov transgenics was dependent on the levels of Nov overexpression. A second line of Nov transgenics expressing Nov transcripts that were 1,000–9,000-fold higher than controls was osteopnenic secondary to increased bone resorption, indicating that at levels of marked overexpression an osteoclastic resorptive phenotype was apparent. The increased bone resorption could be secondary to Nov-integrin interactions or to a marked suppression of Wnt signaling, which is known to suppress osteoclastogenesis (26, 70). However, the physiological relevance of phenotypes secondary to extreme levels of Nov overexpression is not apparent. In conclusion, our studies demonstrated that Nov is a novel BMP and Wnt antagonist and that Nov overexpression inhibits osteoblastic cell differentiation and function in vitro and causes osteopenia in vivo.

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