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NEMO-LIKE KINASE INHIBITS OSTEOBLASTOGENESIS BY SUPPRESSING BONE MORPHOGENETIC PROTEIN AND WNT CANONICAL SIGNALING

Stefano Zanotti[1,2] and **Ernesto Canalis**[1,2,*]

[1]The Department of Research, Saint Francis Hospital and Medical Center, Hartford, CT, USA

[2]The University of Connecticut School of Medicine, Farmington, CT, USA

Abstract

The bone morphogenetic protein/Signaling mothers against decapentaplegic (BMP/Smad) and the WNT signaling pathways regulate the commitment of mesenchymal cells to the osteoblastic lineage. Nemo like kinase (Nlk) is an evolutionary conserved kinase that suppresses Smad transactivation and WNT canonical signaling. However, it is not clear whether these effects of Nlk have any consequence on the differentiation of mammalian cells. To study the function of Nlk during the commitment of ST-2 bone marrow stromal cells to the osteoblastic fate, Nlk was downregulated by RNA interference (RNAi), following transfection of a specific small interfering (si)RNA. Nlk downregulation increased alkaline phosphatase and osteocalcin expression and sensitized ST-2 cells to the effects of BMP2 and WNT3 on alkaline phosphatase mRNA expression and activity. Accordingly, Nlk downregulation enhanced the effect of BMP2 on the transactivation of the BMP/Smad reporter construct 12xSBE-Oc-pGL3, and on the levels of phosphorylated Smad1/5/8, whereas it did not affect the transactivation of the transforming growth factor-β/Smad reporter pSBE-Luc. Nlk downregulation sensitized ST-2 cells to the effects of WNT3 on the transactivation of the WNT/T-cell factor (Tcf) reporter construct 16xTCF-Luc, whereas it did not affect cytosolic β-catenin levels,. To understand the function of Nlk in cells committed to the osteoblastic lineage, Nlk was suppressed by RNAi in primary calvarial osteoblasts. Downregulation of Nlk increased alkaline phosphatase and osteocalcin transcripts and sensitized osteoblasts to the effects of BMP2 on alkaline phosphatase activity and Smad1/5/8 transactivation and phosphorylation. In conclusion, Nlk suppresses osteoblastogenesis by opposing BMP/Smad and WNT canonical signaling.

Keywords

Nlk; ST-2; Osteoblasts; Wnt; BMP; Smad

INTRODUCTION

Mesenchymal cells can differentiate into osteoblasts, chondrocytes and adipocytes, and their commitment to each lineage is regulated by specific signals. Bone morphogenetic proteins (BMP) and WNT play a central role in the differentiation of mesenchymal cells toward the osteoblastic lineage [1–4]. BMPs are members of the transforming growth factor (TGF)β family of peptides. Binding of BMPs to specific cell surface receptors results in the phosphorylation of serine residues located in the C-terminus of the signaling mothers against

Address correspondence to: Ernesto Canalis M.D., Department of Research, Saint Francis Hospital and Medical Center, 114 Woodland Street, Hartford, CT 06105-1299, Tel: (860) 714-4204, Fax: (860) 714-8053, ecanalis@stfranciscare.org.

decapentaplegic (Smad)1/5/8, whereas binding of TGFβ to cognate receptors induces the phosphorylation of the C-terminus of Smad2/3. Phosphorylated Smad1/5/8 or Smad2/3 translocate to the nucleus in association with Smad4 to regulate gene transcription [5]. BMPs also activate the mitogen activated protein kinase (MAPK) signaling pathway to regulate specific cellular events. MAPK also phosphorylate specific serine residues located in the linker region of Smad1, resulting in its nuclear export and suppression of BMP dependent Smad transactivation [6–8]. Similarly, MAPK phosphorylate serine residues present in the linker region of Smad2/3, suppressing TGFβ/Smad transactivation [9, 10]. In cells of the osteoblast lineage, Wnt signals primarily through the WNT canonical signaling pathway. In this pathway under basal conditions, glycogen synthase kinase (GSK)3β phosphorylates β-catenin and induces its ubiquitination and proteasomal degradation Binding of WNT to the Frizzled receptor and low density lipoprotein receptor-related protein (Lrp)5/6 co-receptors leads to the inhibition of GSK3β and the stabilization of cytosolic β-catenin, which translocates to the nucleus. There, β-catenin associates with members of the T cell factor (Tcf) family of transcription factors, to regulate gene transcription [11].

Nemo-like kinase (Nlk) is an evolutionary conserved enzyme that is active as a homodimer in the cell nucleus, and is related to the MAPK family [12, 13]. *Nmo*, the ortholog of *Nlk* in *Drosophila*, suppresses the transactivation of Mothers against decapentaplegic (Mad), the ortholog of Smad, by phosphorylating the N-terminus of Mad and inducing its nuclear export [14, 15]. Although *nmo* antagonizes WNT canonical signaling in *Drosophila* during wing development, the mechanism of this process is not known [16]. In *Caenorhabditis Elegans* and *Xenopus Laevis* Nlk phosphorylates and Tcf for ubiquitinylation and proteasomal degradation, and as a consequence WNT canonical signaling is inhibited [17–21]. Suppression of BMP/Smad and WNT canonical signaling by Nlk has been reported in mammalian immortalized cell culture models and human cancer cell lines; however, it is not known whether these effects are biologically relevant or occur in untransformed mammalian cells [14, 19–21].

The phenotype of the global *Nlk* inactivation in mice is influenced by the genetic composition. In a C57BL/6 inbred strain, *Nlk* inactivation causes prenatal lethality, whereas Nlk inactivation in a mixed C57BL/6;129/Sv background is not developmentally lethal. *Nlk* null mice are characterized by increased bone marrow adipocytes and a reduced number of hematopoietic cells, but it is not known whether the inactivation of *Nlk* causes a skeletal phenotype has not been reported [22]. Nlk regulates mesenchymal stem cell lineage specification and prevents differentiation toward the adipocytic lineage by suppressing the transactivation of peroxisome proliferator-activated receptor (PPAR)γ, and this explains the increased adipocyte number in the bone marrow of *Nlk* null mice [23]. The role of Nlk in bone remodeling is not clear. Nlk overexpression suppresses osteoblastic differentiation of ST-2 stromal cell lines, and Nlk is required for the increased expression of osteoprotegerin that follows *in vitro* mechanical loading of C2C12 cells [24, 25]. However, mechanisms of Nlk action in skeletal cells have not been explored.

In the present study, we explored the effects of Nlk on the commitment of mesenchymal cells to the osteoblastic fate and on the function of differentiated osteoblasts, and the mechanisms involved. For this purpose we examined the consequences of Nlk downregulation by RNA interference (RNAi) on BMP/Smad and WNT signaling, and on the differentiation and function of cells of the osteoblastic lineage.

MATERIALS AND METHODS

Cell Culture

ST-2 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), cloned stromal cells isolated from the bone marrow of BC8 mice, were plated at a density of 10^4 cells/cm^2 in α-Minimum Essential Medium (α-MEM; Life Technologies, Carlsbad, CA), supplemented with 20 mM HEPES and 10 % fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Calvarial osteoblasts were isolated from parietal bones of 3- to 5-day old wild type tropism to Friend leukemia virus mice by sequential collagenase digestion, as previously described [26]. Osteoblasts were plated at a density of 1.5×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with non essential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO) and 10 % FBS. Cells were cultured in a humidified 5 % CO_2 incubator at 37°C

RNA interference (RNAi)

To downregulate Nlk expression, a 19-mer double stranded small interfering (si)RNA targeted to the murine Nlk mRNA sequence was obtained commercially, and a scrambled 19-mer siRNA with no homology to known mouse or rat sequences was used as control (Life Technologies) [27, 28]. Nlk or scrambled siRNA, both at 20 nM, were transfected into sub-confluent osteoblastic cells, using siLentFect lipid reagent, in accordance with manufacturer's instructions (BioRad, Hercules, CA), and cells were allowed to recover for 24 h. To ensure adequate downregulation, total RNA was extracted in parallel cell cultures 96 h following the transfection of the siRNAs, and Nlk mRNA levels determined by reverse transcription - quantitative real time polymerase chain reaction (RT-qPCR).

Transient Transfections

To determine the effects of Nlk downregulation on BMP2 or TGFβ transactivation, osteoblastic cells were transfected either with a construct containing 12 copies of a Smad1/5/8 binding element (SBE) linked to an osteocalcin minimal promoter (12xSBE-Oc-pGL3; M. Zhao, University of Texas, San Antonio, TX), or a construct containing 4 copies of a Smad3 binding element upstream of a simian virus 40 minimal promoter (pSBE-Luc; R. Derynck, University of California, San Francisco), both cloned upstream of *luciferase* [29, 30]. To investigate the effects of Nlk downregulation on WNT canonical signaling, ST-2 cells were transfected with a construct containing sixteen copies of the *Tcf4* consensus sequence linked to a minimal thymidine kinase promoter (16xTCF-luc; J. Billiard, Wyeth Research, Collegeville, PA), cloned upstream of *luciferase* [29]. To induce activation of WNT canonical signaling, 16xTCF-Luc was co-transfected with a WNT3 expression construct (Millipore, Billerica, MA), or with pcDNA 3.1, as a control. Following recovery from transfection of the siRNA, cells were transiently transfected with reporter constructs using FuGENE6 at a ratio of 3 μl of FuGENE for 2 μg of DNA, according to manufacturer's instructions (Roche, Indianapolis, IN). A construct where the cytomegalovirus (CMV) promoter directs β-galactosidase expression (Clontech, San Jose, CA) was used to control for transfection efficiency. Cells were allowed to recover for 16 h and transferred to serum free medium for 6 h, and cells transfected with 12xSBE-Oc-pGL3 or pSBE-Luc were exposed respectively to BMP2 or TGFβ for 24 h in serum free medium. Cell lysates were harvested 48 h after transfection of the reporter constructs. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT) and luciferase activity corrected for β-galactosidase activity.

Alkaline Phosphatase Activity

To investigate the effects of Nlk downregulation on alkaline phosphatase activity, cells were cultured in the presence of 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate (Sigma-Aldrich), and treated either with recombinant BMP2 (Wyeth Research) or WNT3a for 72 h and harvested. Alkaline phosphatase activity was assessed in 0.5 % Triton X-100 cellular extracts by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol, and measured by spectroscopy at 405 nm following 10 min of incubation at room temperature, according to manufacturer's instructions (Sigma-Aldrich). Data are expressed as nanomoles of p-nitrophenol released per minute per microgram of protein. Total protein content was determined by the DC protein assay in accordance with manufacturer's instructions (Bio-Rad).

Reverse Transcription - Quantitative Real Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted and changes in mRNA levels quantified by RT-qPCR [31, 32]. For this purpose, 0.5–1 μg of total RNA was reverse-transcribed using SuperScript III Platinum Two-Step RT-qPCR kit (Life Technologies), according to manufacturer's instructions, and amplified in the presence of specific primers (Table 1) and Platinum Quantitative PCR SuperMix-UDG (Life Technologies) at 60°C for 45 cycles. mRNA copy number was estimated by comparison with a standard curve constructed using alkaline phosphatase, Nlk (both from American Type Culture Collection, ATCC, Manassas, VA), and osteocalcin (J.B. Lian, University of Massachusetts, Worcester, MA) cDNAs, and corrected for Rpl38 (ATCC) expression [33, 34]. PCR reactions were conducted in a 96-well spectrofluorometric thermal iCycler or in a CFX96 real time PCR detection system (BioRad). Fluorescence was monitored during every PCR cycle at the annealing step.

Western Blot Analysis

To assess the effects of Nlk downregulation on Smad1/5/8 phosphorylation or cytosolic β-catenin levels, cells were transferred to serum free medium for 24 h and exposed to BMP2 for 20 min, and WNT3a for 6 h, or to control vehicle. To determine the levels of Smad1/5/8 phosphorylation, the cell layer was washed in phosphate buffered saline (PBS) and extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors. To determine cytosolic β-catenin levels, the cell layer was washed in PBS and extracted in 10 mM Tris, 140 mM NaCl, 5 mM EDTA, and 2 mM dithiothreitol buffer at pH 7.6 (all from Sigma-Aldrich), in the presence of protease inhibitors, and the cytosolic fraction separated by ultracentrifugation, as described [35]. Protein concentrations were determined using a DC protein assay kit (BioRad), and 20 μg of protein were fractionated by electrophoresis in 7.5 % polyacrylamide gels (Sigma-Aldrich), and transferred to Immobilon P membranes (Millipore), which were blocked with 3 % bovine serum albumin (Sigma-Aldrich) in PBS. For the detection of phospho-Smad1/5/8, membranes were exposed to a 1:1000 dilution of a rabbit polyclonal antibody to Smad1/5/8 phosphorylated at carboxyl-terminal serine residues (Cell Signaling Technology; Catalog number 95116), or a murine monoclonal antibody to unphosphorylated Smad1 (Santa Cruz Biotechnology, Santa Cruz, CA; Catalog number sc-7965), as a loading control. For the detection of β-catenin, membranes were exposed to a 1:500 dilution of a murine monoclonal antibody to β-catenin (Santa Cruz Biotechnology; Catalog number sc-7963), or to a 1:1000 dilution of a goat polyclonal antibody to actin (Santa Cruz Biotechnology; Catalog number sc-1616), as a loading control. Blots were exposed to anti-rabbit and anti-goat antiserum or anti-mouse IgG, conjugated to horseradish peroxidase (all from Sigma-Aldrich) and developed with a chemiluminescence detection reagent (Perkin Elmer, Waltham, MA).

Statistical analysis

Data are expressed as means ± SEM. Statistical differences were determined by Student's *t* test or by ANOVA with Schaeffe's post-hoc analysis respectively for pairwise or multiple comparisons.

RESULTS

Nlk suppresses commitment of ST-2 cells to the osteoblastic lineage

To test the effects of Nlk downregulation on the commitment of ST-2 bone marrow stromal cells to the osteoblastic lineage, sub-confluent cells were transfected with a siRNA targeted to the murine Nlk mRNA, or with a scrambled siRNA control. RT-qPCR revealed that downregulation of Nlk increased alkaline phosphatase and osteocalcin transcripts (Fig. 1A), suggesting that Nlk inhibits differentiation of ST-2 cells toward the osteoblastic lineage. To explore the mechanisms involved, we examined whether BMP and WNT activities on osteoblastic differentiation of ST-2 cells were modified by the downregulation of Nlk [14, 15, 17–20]. Exposure to BMP2 increased alkaline phosphatase mRNA levels, and Nlk downregulation sensitized ST-2 cells to this effect (Fig. 1B). Although exposure to WNT3a did not increase alkaline phosphatase transcripts, a significant induction of alkaline phosphatase mRNA by WNT3a was observed in the context of Nlk downregulation (Fig. 1B). Accordingly, treatment with BMP2 or WNT3a caused a dose dependent increase in alkaline phosphatase activity, and the effect was enhanced by downregulation of Nlk (Fig. 1C and D). Nlk downregulation increased the basal levels of alkaline phosphatase activity, possibly because it sensitized ST-2 cells to the effects of endogenous BMPs or WNTs. These results indicate that Nlk suppresses the commitment of ST-2 cells to the osteoblastic lineage and the activity of BMP2 and WNT3a.

Nlk inhibits BMP/Smad signaling

To understand the mechanism of Nlk action, we tested whether the downregulation of Nlk enhanced BMP/Smad transactivation by transfecting ST-2 cells with a 12xSBE-Oc-pGL3 reporter construct. Treatment with BMP2 for 24 h induced the transactivation of 12xSBE-Oc-pGL3, and downregulation of Nlk sensitized ST-2 cells to the effect of BMP2 and enhanced the basal activity of 12xSBE-Oc-pGL3 (Fig. 2A and B). To confirm that Nlk enhanced BMP2/Smad signaling, phosphorylated Smad1/5/8 was determined by Western blot analysis. BMP2 treatment for 20', a period of exposure previously shown to have a maximal effect on Smad1/5/8 phosphorylation in ST-2 cells, induced the phosphorylation of Smad1/5/8, and downregulation of Nlk enhanced this effect (Fig. 2C), demonstrating that Nlk decreases Smad signaling in ST-2 cells [36, 37]. We asked whether this effect was specific to BMP/Smad signaling, and we examined the consequences of Nlk downregulation on TGFβ/Smad transactivation following transfection of ST-2 cells with a pSBE-Luc reporter construct. Treatment with TGFβ for 24 h induced the transactivation of pSBE-Luc, and this effect was not modified by downregulation of Nlk (Fig. 3), demonstrating that Nlk specifically suppresses BMP/Smad signaling.

Nlk suppresses WNT canonical signaling

To assess whether the downregulation of Nlk enhanced WNT canonical signaling, ST-2 cells were transfected with a 16xTCF reporter construct and co-transfected with a WNT3 expression vector, or with pcDNA 3.1 as a control. WNT3 induced the transactivation of 16xTCF-Luc and downregulation of Nlk caused an increase in reporter activity, either in the context of WNT3 expression or under basal conditions (Fig. 4A and B). To determine whether Nlk modified the effects of WNT on cytosolic β-catenin levels, ST-2 cells were exposed to recombinant WNT3a or vehicle control for 6 h, a time of exposure sufficient to

observe accumulation of cytosolic β-catenin in ST-2 cells in response to WNT [38]. WNT3a increased cytosolic levels of β-catenin, and Nlk downregulation did not alter this effect, suggesting that Nlk inhibits WNT canonical signaling in ST-2 cells by alternate mechanisms (Fig. 4C).

Nlk suppresses osteoblast function by inhibiting BMP/Smad signaling

To determine whether Nlk regulated the function of cells committed to the osteoblastic lineage, sub-confluent primary calvarial osteoblasts were transfected with a siRNA specific for Nlk, or with a scrambled siRNA control. In agreement with the results obtained in ST-2 cells, downregulation of Nlk caused an increase in alkaline phosphatase and osteocalcin transcripts (Fig. 5A), and on the effect of BMP2 on alkaline phosphatase activity, (Fig. 5B). Confirming previous observations, WNT3a did not induce alkaline phosphatase activity in primary calvarial osteoblasts (data not shown) [38].

To explore the mechanisms of Nlk action in osteoblasts, we investigated the consequences of Nlk downregulation on BMP/Smad transactivation by transfecting primary calvarial osteoblasts with a 12xSBE-Oc-pGL3 reporter construct. In accordance with the results obtained in ST-2 cells, BMP2 induced the transactivation of 12xSBE-Oc-pGL3, and downregulation of Nlk sensitized the osteoblasts to the effect of BMP2 and enhanced basal levels of Smad1/5/8 transactivation (Fig. 6A and B). In addition, treatment with BMP2 at 3 nM for 20’ induced the phosphorylation of Smad1/5/8 and downregulation of Nlk enhanced this effect (Fig. 6C), confirming that Nlk suppresses Smad signaling in primary calvarial osteoblasts.

DISCUSSION

In the present study we investigated the effects of Nlk downregulation by RNAi on the commitment of ST-2 bone marrow stromal cells to the osteoblastic fate and on osteoblast function. Nlk transcripts were reduced up to 80%, demonstrating that RNAi was an appropriate strategy to study the function of endogenous *Nlk* in osteoblastic cells. We report that downregulation of Nlk induces markers of osteoblast differentiation and function, supporting the concept that Nlk is a suppressor of osteoblastogenesis, and confirming previous observations demonstrating an inhibitory effect of Nlk on osteoblastogenesis and suggest that endogenous Nlk is a suppressor of osteoblastic differentiation and function [24, 25].

Nlk prevents the commitment of mesenchymal stem cells to the adipocytic lineage by modifying chromatin organization and suppressing PPAR-γ transactivation [23]. Our results demonstrated that in mesenchymal cells Nlk suppresses BMP/Smad and WNT canonical signaling. These signaling pathways are critical regulators of the commitment of ST-2 bone marrow stromal cells toward the osteoblastic lineage, and their suppression represents a mechanism for the inhibition of osteoblastic differentiation by Nlk. It is important to note that downregulation of Nlk enhanced the basal levels of BMP/Smad and Tcf transactivation, and this likely represents sensitization to the effects of endogenous BMP2 and WNT3, which are secreted by osteoblastic cells [3].

This is the first report documenting suppression of Smad1/5/8 transactivation by Nlk in mammalian cells. MAPK signaling can induce phosphorylation of specific serines located in the linker region of Smad1, resulting in the nuclear export of Smad1 and suppression of its transactivation [6]. In light of the structural similarity between Nlk and members of the MAPK family, it is conceivable that Nlk opposes BMP/Smad signaling in an analogous fashion to MAPK [6]. Nlk downregulation did not affect TGFβ/Smad transactivation, and

this is not surprising, since the cellular context is critical to determine whether members of the MAPK family recognize the linker region of Smad1/5/8 or Smad2/3 as a substrate [10].

In immortalized human cell culture systems, Nlk is activated by the non-canonical WNT Ca^{2+} pathway, resulting in phosphorylation and degradation of Tcf and inhibition of the canonical WNT signaling pathway [18–21]. These studies were critical to demonstrate that mammalian cells possess the machinery necessary for the inhibitory action of Nlk on Tcf transactivation, but did not analyze the biological consequences of this effect. Our results complement these findings by showing that endogenously expressed Nlk opposes the commitment of bone marrow stromal cells to the osteoblastic lineage, possibly by inhibiting the WNT canonical signaling pathway. BMP/Smad and Wnt signaling form a regulatory network where the two signaling pathways modulate each other's transactivation in a tissue specific manner [39]. In C2C12 cells, a pluripotent mesenchymal cell line, some of the effects of BMP2 on lineage specification appear to be mediated by induction of Tcf transactivation [40]. In light of this observation, it cannot be excluded that the stimulation of Tcf transactivation caused by Nlk downregulation is secondary to the stimulation of BMP/Smad activity.

In conclusion Nlk suppresses osteoblastic differentiation and function possibly by inhibiting the BMP/Smad and WNT canonical signaling pathways.

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The abbreviations used are

α-MEM	α-Minimum Essential Medium
AP	Alkaline Phosphatase
ATCC	American Type Culture Collection
BMP	bone morphogenetic protein
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
Fwd	forward
GFP	Green Fluorescent Protein
GSK3β	glycogen synthase kinase 3β
Lrp	low density lipoprotein receptor-related protein
Mad	mothers against decapentaplegic
MAPK	mitogen activated protein kinase
Nlk	nemo-like kinase
PBS	phosphate buffered saline
PPAR	peroxisome proliferator-activated receptor

Rev	reverse
Rpl38	ribosomal protein L38
RT-qPCR	by reverse transcription-quantitative real time polymerase chain reaction
RNAi	RNA interference
SBE	Smad binding element
siRNA	small interfering RNA
Smad	Signaling mothers against decapentaplegic
Tcf	T cell factor
TGF	transforming growth factor

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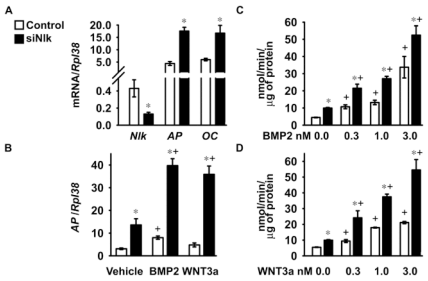

Figure 1.

Effects of Nlk downregulation during osteoblastic differentiation of ST-2 cells. Subconfluent ST-2 cells were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). Cells were exposed for 96 h to recombinant BMP2 or WNT3 at the indicated concentrations, or with vehicle, as a control. In panels A and B, total RNA was extracted and mRNA reverse-transcribed and amplified by RT-qPCR. Data are expressed as *Nlk*, *Alkaline Phosphatase* (*AP*), and *Osteocalcin* (*OC*) copy number, corrected for *Rpl38* expression. Values are means ± SEM, n = 4. In panels C and D, the cell layer was extracted with Triton X-100 and alkaline phosphatase activity quantified. Alkaline phosphatase activity is expressed as nanomoles of p-nitrophenol/min/µg of total protein. Values are means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between vehicle and exposure to BMP2 or WNT3a, $p < 0.05$.

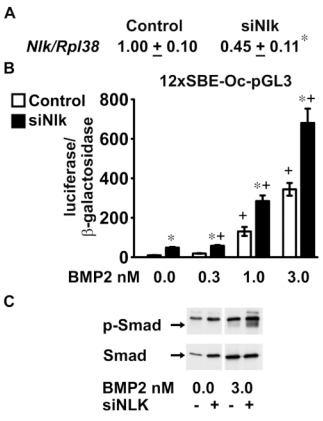

Figure 2.

Effects of Nlk downregulation on BMP/Smad signaling in ST-2 cells. Subconfluent ST-2 cells were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). In panel A, downregulation of *Nlk* mRNA was documented in parallel cultures by RT-qPCR. Data are expressed as fold of *Nlk* expression change between control and siNlk, corrected for *Rpl38* expression. Values represent means ± SEM for 4 observations. In panel B, cells were transiently transfected with a Smad1/5/8 dependent 12xSBE-Oc-pGL3 reporter construct and co-transfected with a CMV/β-galactosidase expression vector to test for transfection efficiency. Cells were allowed to recover for 24 h, treated for 24 h with BMP2 at the indicated concentrations, and harvested. Data is expressed as luciferase/β-galactosidase activity. Bars represent means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between vehicle and exposure to BMP2, $p < 0.05$. In panel C, confluent cells were treated for 20 min either with BMP2 or vehicle control at the indicated. Total proteins were extracted and fractioned by gel-electrophoresis and transferred to Immobilon P membranes, which were probed with antibodies against either phospho-Smad1/5/8, or Smad1.

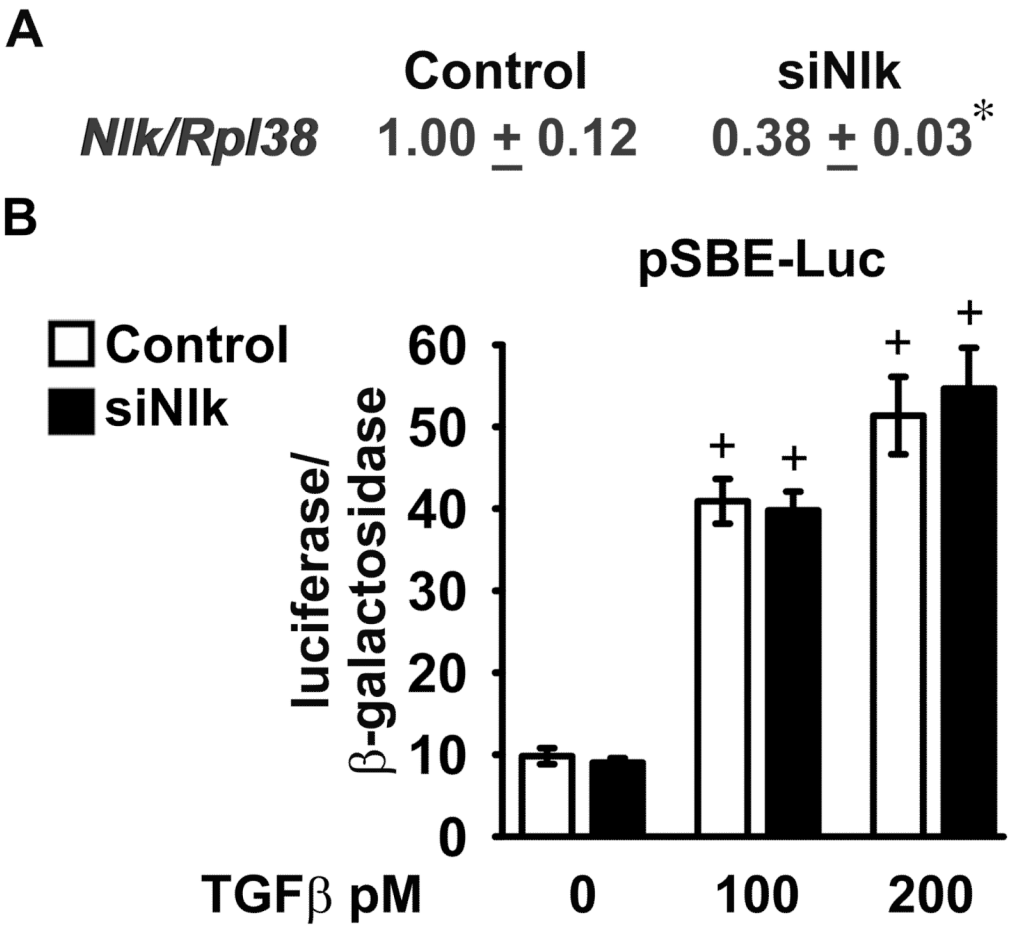

Figure 3.

Effects of Nlk downregulation on TGFβ/Smad signaling in ST-2 cells. Subconfluent ST-2 cells were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). In panel A, downregulation of *Nlk* mRNA was documented in parallel cultures by RT-qPCR. Data are expressed as fold of *Nlk* expression change between control and siNlk, corrected for *Rpl38* expression. Values represent means ± SEM for 4 observations. In panel B, cells were transiently transfected with a Smad2/3 dependent pSBE-Luc reporter construct and co-transfected with a CMV/β-galactosidase expression vector to test for transfection efficiency. Cells were allowed to recover for 24 h, treated for 24 h with TGFβ at the indicated concentrations, and harvested. Data is expressed as luciferase/β-galactosidase activity. Bars represent means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between vehicle and exposure to TGFβ, $p < 0.05$.

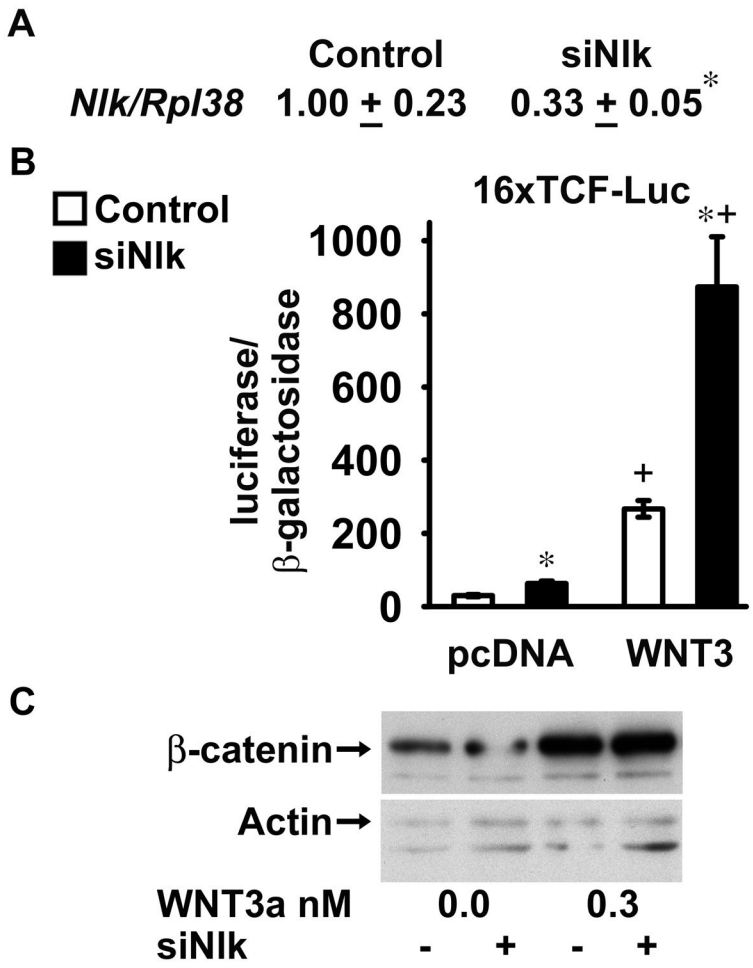

Figure 4.

Effects of Nlk downregulation on WNT canonical signaling in ST-2 cells. Subconfluent ST-2 cells were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). In panel A, downregulation of *Nlk* mRNA was documented in parallel cultures by RT-qPCR. Data are expressed as fold of *Nlk* expression change between control and siNlk, corrected for *Rpl38* expression. Values represent means ± SEM for 4 observations. In panel B, cells were transiently transfected with a Tcf dependent 16x-TCF-Luc reporter and co-transfected with a WNT3a expression construct or pcDNA3.1 as control, and before harvesting cells were allowed to recover for 24 h. A CMV/β-galactosidase expression vector was co-transfected to determine transfection efficiency. Data is expressed as luciferase/β-galactosidase activity. Bars represent means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between pcDNA and WNT3, $p < 0.05$. In panel C, confluent cells were treated for 6 h with 100 ng/ml of WNT3a or vehicle control. Total proteins were extracted and fractioned by gel-electrophoresis and transferred to Immobilon P membranes, which were probed with antibodies against either β-catenin, or Actin.

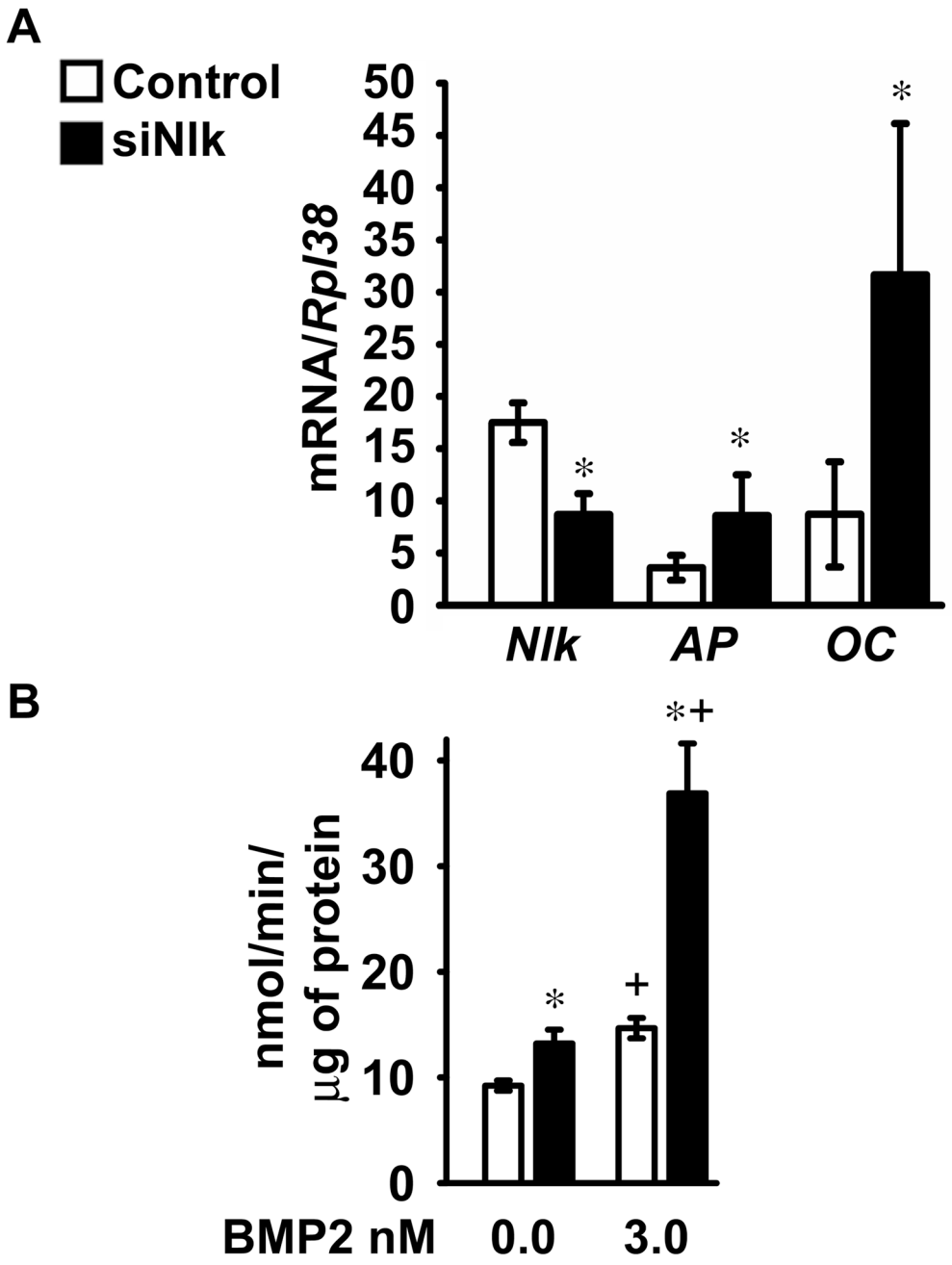

Figure 5.

Effects of Nlk downregulation on osteoblast function. Subconfluent primary calvarial osteoblasts were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). Osteoblasts were exposed for 96 h to recombinant BMP2 at the indicated concentrations, or with vehicle, as a control. In panels A and B, total RNA was extracted and mRNA reverse-transcribed and amplified by RT-qPCR. Data are expressed as *Nlk*, *Alkaline Phosphatase* (*AP*), and *Osteocalcin* (*OC*) copy number, corrected for *Rpl38* expression. Values are means ± SEM, n = 4. In panel C, osteoblasts were treated for 72 h with recombinant BMP2 at the indicated concentrations, or with vehicle control. The cell layer was extracted with Triton X-100 and alkaline

phosphatase activity quantified. Alkaline phosphatase activity is expressed as nanomoles of p-nitrophenol/min/μg of total protein. Values are means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between vehicle and exposure to BMP2, $p < 0.05$.

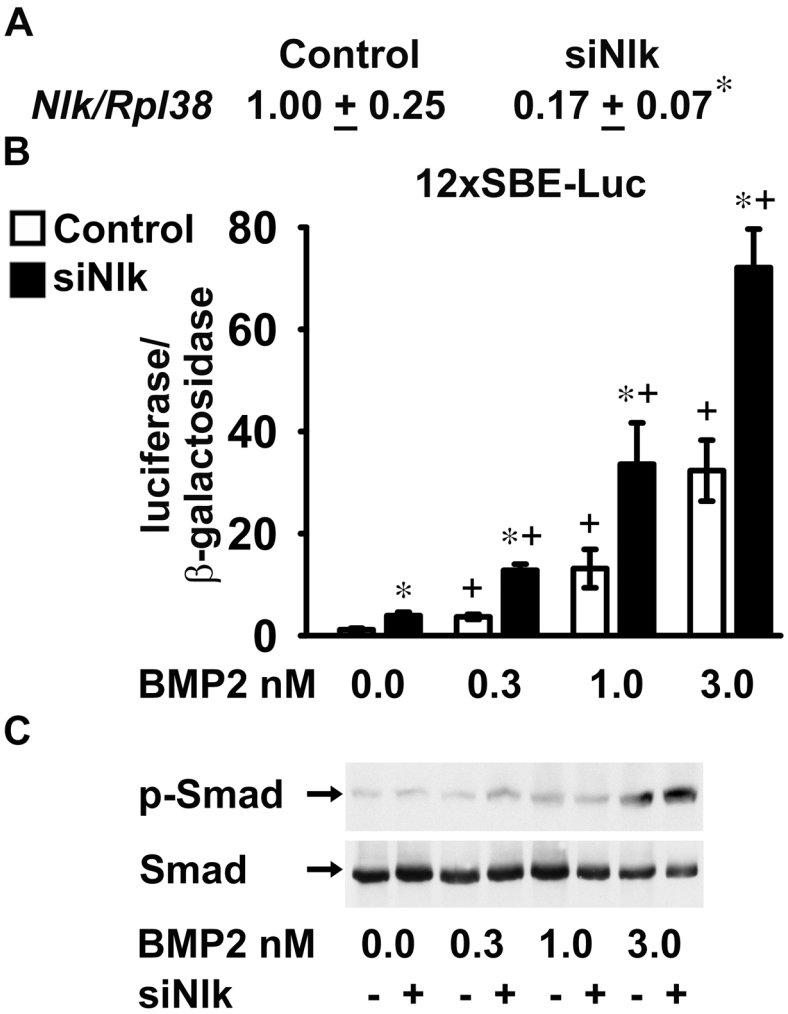

Figure 6.

Effects of Nlk downregulation on BMP/Smad signaling in osteoblasts. Subconfluent primary calvarial osteoblasts were transfected with a small interfering (si)RNA specific for Nlk (siNlk, black bars) or with a scrambled siRNA control (Control, white bars). In panel A, downregulation of *Nlk* mRNA was documented in parallel cultures by RT-qPCR. Data are expressed as fold of *Nlk* expression change between control and siNlk, corrected for *Rpl38* expression. Values represent means ± SEM for 4 observations. In panel B, subconfluent osteoblasts were transiently transfected with a Smad dependent 12xSBE-Oc-pGL3 reporter construct and co-transfected with a CMV/β-galactosidase expression vector to check for transfection efficiency. Cells were allowed to recover for 24 h, treated for 24 h with BMP2 at the indicated concentrations, and harvested. Data shown represent luciferase/β-galactosidase activity. Bars represent means ± SEM for 6 observations. *Significantly different between Control and siNlk, $p < 0.05$. +Significantly different between vehicle and exposure to BMP2, $p < 0.05$. In panel C, confluent osteoblasts were treated for 20 min either with BMP2 or vehicle control at the indicated concentrations. Total proteins were extracted and fractioned by gel-electrophoresis and transferred to Immobilon P membranes, which were probed with antibodies against phospho-Smad1/5/8 or Smad1.

Table 1

Forward (Fwd) and reverse (Rev) primers used for RT-qPCR analysis of mRNA levels.

Gene	Primer	Sequence
Alkaline Phosphatase	Rev	5’-CGGTTAGGGCGTCTCCACAGTAAC[FAM]G-3’
	Fwd	5’-CTTGGAGAGGGCCACAAAGG-3’
Nlk	Rev	5’-CGAAGAGTCCAGCAGCATACGTCTT[FAM]G-3’
	Fwd	5’-GCTGTTGTTGCCCAGGGTTT-3’
Osteocalcin	Rev	5’-CACTTACGGCGCTACCTTGGGTAAGT[FAM]G-3’
	Fwd	5’-CCCAGCACAACTCCTCCCTA-3’
Rpl38	Rev	5’-CGAACCGGATAATGTGAAGTTCAAGGTT[FAM]G-3’
	Fwd	5’-CTGCTTCAGCTTCTCTGCCTTT-3’