Noggin Suppression Enhances in Vitro Osteogenesis and Accelerates in Vivo Bone Formation

Several investigations have demonstrated a precise balance to exist between bone morphogenetic protein (BMP) agonists and antagonists, dictating BMP signaling and osteogenesis. We report a novel approach to manipulate BMP activity through a down-regulation of the potent BMP antagonist Noggin, and examined the effects on the bone forming capacity of osteoblasts. Reduction of noggin enhanced BMP signaling and in vitro osteoblast bone formation, as demonstrated by both gene expression profiles and histological staining. The effects of noggin suppression on in vivo bone formation were also investigated using critical-sized calvarial defects in mice repaired with noggin-suppressed osteoblasts. Radiographic and histological analyses revealed significantly more bone regeneration at 2 and 4 weeks post-injury. These findings strongly support the concept of enhanced osteogenesis through a down-regulation in Noggin and suggest a novel approach to clinically accelerate bone formation, potentially allowing for earlier mobilization of patients following skeletal injury or surgical resection.

Skeletal homeostasis involves a dynamic interplay of multiple factors, each influencing the metabolic activity of local bone-forming cells. Alteration in the capacity of these cells to deposit a mineralized extracellular matrix occurs not only in disease states and aging, but also during bone repair and natural growth. A multitude of cytokines with the capacity to regulate recruitment and differentiation of osteoprogenitors have already been identified (1). Skeletal cells themselves synthesize several growth factors that can act in an autocrine fashion, including bone morphogenetic proteins (BMPs), which induce osteogenic differentiation of mesenchymal cells and enhance subsequent bone deposition by osteoblasts (2–5).

BMPs have been found to regulate processes as disparate as embryonic dorso-ventral patterning, neuronal differentiation, cardiomyogenesis, thymocyte differentiation, and cranial suture fusion (6–10). It is their osteoinductive ability, however, that BMPs are most commonly identified with, and this property has been exploited clinically to accelerate bone generation and repair (6, 11, 12). BMPs execute their pro-osteogenic effect by promoting dimerization of specific receptors, resulting in phosphorylation of either Smad1/5 or p38 MAPK intermediates and ultimate modulation of transcriptional activity (13). The level of signaling activity can be tempered by a variety of intracellular and extracellular antagonists. Well described mechanisms by which cells naturally regulate the BMP pathway include ligand-competiting pseudoreceptors, inhibitory Smads, and cytoplasmic binding proteins inducing ubiquitination and degradation of both Smads 1 and 5 (14–18).

Several structurally unique extracellular BMP antagonists have also been described, each of which bind to BMPs and interfere with their ability to induce receptor dimerization (19). Of the many extracellular proteins capable of down-regulating BMP signaling, however, studies have identified noggin to be most closely involved in the osteoinductive role of BMPs (20, 21). In response to BMP-2, -4, or -6, osteoblasts have been shown to dramatically upregulate noggin, suggesting a protective role in negative feedback fashion to limit excessive exposure of cells to BMP signaling (22). In addition, several reports have demonstrated the inhibition of BMP signaling by exogenous application of noggin to significantly impair bone formation both in vitro and in vivo (23–25). These investigations collectively highlight the capacity of noggin to downregulate BMP activity and inhibit subsequent bone deposition by osteoblasts. In contrast, the potential ability to promote endogenous BMP signaling and subsequent osteogenesis through the suppression of noggin remains poorly defined. As an aging population increasingly faces skeletal-related problems, such a novel approach could prove advantageous and more cost-effective in future clinical strategies for the treatment of bone deficiencies.

In this study, we evaluated the effects of noggin suppression on osteoblast differentiation and extracellular matrix mineral deposition. Using restriction enzymes, multiple noggin-directed siRNA constructs were generated and validated using quantitative real-time RT-PCR (QRT-PCR) and Western blot analysis (26). Down-regulation of noggin in MC3T3-E1 preosteoblasts and primary mouse calvarial osteoblasts both resulted...
Noggin Suppression Enhances Bone Formation

in enhanced expression of osteogenic differentiation markers and bone nodule deposition. Furthermore, differences in mineralization by osteoblasts expressing noggin-directed siRNA constructs could be detected as early as 12 days. Confirmation of these findings was obtained using Cre-mediated DNA recombination in floxed noggin osteoblasts. Finally, we showed that early reossification of in vivo critical-sized calvarial defects 2 and 4 weeks following injury can be accelerated by RNAi-mediated gene suppression using osteoblasts expressing noggin-specific siRNA constructs. These observations provide strong evidence for the ability of BMP signal manipulation, through novel down-regulation of a potent BMP antagonist, to enhance bone formation in vitro and in vivo. By suppressing noggin, endogenously produced BMP agonists may be left relatively unopposed to drive the differentiation of osteoprogenitors, thereby leading to more rapid repair of bone defects.

EXPERIMENTAL PROCEDURES

Cell Harvest and Culture—All experiments were approved by the Administrative Panels on Laboratory Animal Care and Biohazard Safety Committee at Stanford University. MC3T3-E1 preosteoblasts were purchased from ATCC (Manassas, VA). Primary osteoblasts were harvested from 5-day-old CD-1 mice as previously described (27). Briefly, calvaria were harvested and parietal bones were trimmed under 2.5 magnification to within 0.5 mm of each fibrous border. Dura mater and pericranial tissue were removed with the assistance of a dissecting microscope and resulting bone was digested in media containing Dispase II and collagenase A. Osteoblasts obtained from each digestion cycle were plated on standard 10-cm culture dishes and incubated with standard growth media (α-MEM with 10% fetal bovine serum and 1% PCN/Strep).

Generation of siRNA Constructs—First passage calvarial osteoblasts were processed for mRNA using a Qiagen RNEasy kit (Qiagen, Inc, San Diego, CA) and reverse transcription was performed with a Taqman® Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA). Noggin-specific primers designed through Primer3 (Whitehead Institute) were used to amplify a 614-base pair sequence from osteoblast cDNA. Primer sequences used were (5'-GGCCAGCAGTCTTACA-3') for the forward reaction and (5'-GAACACTTACTCCGGAATGATG-3') for the reverse reaction. Amplification products were separated on a 2% agarose gel for isolation of noggin cDNA using a QIAquick Gel Extraction kit (Qiagen, Inc.). A modified restriction enzyme generated system, as described by Sen et al. (26) was employed to create candidate small interfering RNA (siRNA) constructs. Ecotropic phoenix packaging cells (gift from Garry Nolan, Stanford University) were transfected with 1 μg of vector plasmid using FuGENE6 (3 μl/1 μg plasmid) (Roche) and media containing infection-competent virus was collected 48 h following transfection.

Validation of Noggin siRNA Constructs—Early passage MC3T3-E1 preosteoblasts and first passage primary calvarial osteoblasts were expanded on 12-well tissue culture plates and grown to subconfluence prior to retroviral infection. Hexadimethrine bromide (5 μg/ml) was added to the viral supernatant to augment infection efficiency. Cells were infected four times and following the last infection, puromycin (2 μg/ml) selection was performed for 3 days. Cells were then expanded in standard growth media.

RNAi-mediated noggin transcript suppression was evaluated in MC3T3-E1 pre-osteoblasts. Noggin and GAPDH primers were designed with the assistance of PrimerBank and QRT-PCR was performed using two-step, multiplexed Taqman® 5'→3' exonuclease assay (28). Each sample was evaluated for noggin and GAPDH transcript levels in triplicate using a SYBR Green assay and quantified with an ABI Prism® 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA). Noggin-directed siRNA constructs demonstrating significant transcript suppression by QRT-PCR analysis were evaluated for protein suppression in MC3T3-E1 preosteoblasts and primary calvarial osteoblasts. Cells were incubated with standard radioimmune precipitation assay buffer, and the protein fraction was isolated by 4 °C ultracentrifugation at 55 × g for 15 min. 80 μg of total protein was then loaded from each cell group and separated by 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad). Products were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and probed with goat polyclonal anti-mouse noggin (R & D Systems, Minneapolis, MN) and monoclonal mouse anti-β-actin antibodies (Abcam Inc., Cambridge, MA) overnight at 4 °C. Incubation with a donkey anti-mouse horseradish peroxidase-linked secondary antibody and enhanced chemiluminescence was used to detect for protein (Amersham Biosciences). siRNA constructs demonstrating greater than 50% noggin transcript reduction and, more importantly, suppression of translated protein were considered efficacious and employed in subsequent studies on differentiation.

Evaluation of BMP Signaling—The effects of noggin suppression on endogenously produced BMP activity were evaluated by QRT-PCR analysis of the signaling intermediates Smad1 and 5. Primers were designed with the assistance of PrimerBank (29). Transcript levels in cells expressing noggin-directed siRNA constructs, a control siRNA, or cells undergoing vehicle only sham infection were compared with baseline Smad expression in unperturbed MC3T3-E1 preosteoblasts. For reference, Smad transcript levels were also determined in a separate group of cells cultured in standard growth media supplemented with 1 ng/ml rhBMP-4 (R & D Systems) for 24 h.

Smad5 and phosphorylated Smad Western blot analysis was performed on 50 μg of protein from total cell lysate harvested by incubation of cells in standard radioimmune precipitation assay buffer with 0.5% phosphatase inhibitor and 0.5% protease inhibitor mixture mix (Sigma). Separation was performed on a 7.5% Tris-glycine SDS-polyacrylamide gel (Bio-Rad), and membranes were probed with either monoclonal rabbit anti-phospho-Smad1/5 (Cell Signaling Technology, Danvers, MA) or goat polyclonal anti-Smad5 antibodies. Detection was performed by enhanced chemiluminescence using the appropriate horseradish peroxidase-linked secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Histological Assessment of Differentiation—MC3T3-E1 preosteoblasts and primary calvarial osteoblasts were cultured in osteogenic differentiation medium (ODM, α-MEM, 10% fetal bovine serum, 1% PCN/Strep, 250 μM ascorbate-2-phos-
phate, and 10 mM β-glycerophosphate). Histological assessment of osteoblast differentiation was performed by Alizarin Red staining (30). All quantified data were obtained in triplicate to facilitate statistical analysis.

Gene Expression Analysis—To confirm any differences observed in histologic staining, quantitative gene expression analysis was performed every 3 days beginning at day three of osteogenic differentiation and continuing until day 21. Primers for Runx2, OP, and OC were designed with the assistance of PrimerBank (29). QRT-PCR was performed with transcript levels normalized to GAPDH. Comparison for each gene was performed relative to undifferentiated cells. Each reaction was run in triplicate to provide for statistical comparison between groups.

Cre-dependent Noggin Recombination—Primary calvarial osteoblasts were harvested from a noggin conditional null mice with the noggin allele flanked by loxP sites. Cells were then cultured in growth media containing 50 MOI of Cre-expressing adenovirus (Ad-CMV-Cre, Vector Biolabs, Philadelphia, PA) or empty adenovirus for 48 h. Evaluation of Cre-mediated DNA recombination was performed by Western blot analysis for noggin, as described above. Flow cytometric analysis was performed on an LSR model 1a (Becton Dickinson) running CellQuest Pro. Quantification of data was obtained with FlowJo v6.4.4 (Treestar, Inc., Ashland, OR). Osteoblasts were cultured in ODM for 21 days and extracellular matrix mineralization was determined using Alizarin Red staining, with comparison made to control osteoblasts harvested from the same mice with no adenoviral infection.

Critical-sized Defect Repair—Primary calvarial osteoblasts (1 × 10⁶) infected with noggin-directed siRNA constructs, control siRNA, or undergoing sham infection were resuspended in 20 μl of growth media and seeded onto 4-mm apatite-coated polyactic co-glycolic acid scaffolds (31). Cells were allowed to adhere for 24 h prior to implantation into animals. Critical-sized calvarial defects were created in the right parietal bone of 60-day-old skeletally mature CD-1 mice, as previously described (n = 9 for each group) (32). Seeded scaffolds were placed into the defect, and the skin was sutured closed. Three animals for each group were sacrificed at 2, 4, or 8 weeks for radiographic and histological analyses. Image acquisition was performed on a CTI MicroCAT II (CTI Molecular Imaging, Inc., Knoxville, TN) at an x-ray voltage of 80 kVP and an anode current of 450 μA. A resolution of 40 μm was obtained, with 144 steps over a 360° rotation. X-ray data reconstruction and analysis was performed using Amira v4.0 (Mercury Computer Systems, San Diego, CA) and GEHC Microview v2.0.0029 (GE Healthcare, London, ON). Percentage healing in the region of the defect was evaluated using Image J (NIH, Bethesda, MD).

Following radiographic imaging, calvaria were harvested and fixed in 4% paraformaldehyde for 24 h. Specimens were then decalcified in 19% EDTA, processed, and embedded into paraffin wax for sectioning. Regeneration in the region of the defect was evaluated by pentachrome staining of 8-μm thick sections. Aniline blue staining was also performed on every fifth slide (n = 10 slides evaluated per specimen) through the region of the defect and quantification of bone content was performed using Image J. All stained slides were imaged using a Zeiss AxioPlan microscope.

Statistical Analysis—All experiments were performed in triplicate. A Student’s t test or one-way analysis of variance between groups with Tukey’s Multiple Comparison Test on GraphPad Prism software (GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego, CA) was used for statistical analyses. A *, p value < 0.05 was considered statistically significant.
Evaluation of RNAi-mediated Noggin Suppression—RNA interference was employed to induce a reduction in the level of noggin transcript and protein. Using a modification of the restriction enzyme generated siRNA system, five individual noggin-directed siRNA constructs were obtained and confirmed by sequencing (Fig. 1a). An additional 13 constructs were found to be in the reverse orientation on sequencing and were not further evaluated. QRT-PCR analysis of noggin in MC3T3-E1 preosteoblasts following infection and puromycin selection demonstrated a strong reduction in transcript level (siRNA1 89.6 ± 1.5%; siRNA2 87.9 ± 5.2%; siRNA3 51.5 ± 5.3%; siRNA4 72.3 ± 3.3%; and siRNA5 55.5 ± 4.7% all *, p < 0.001) when compared with control, vehicle only sham-infected cells (Fig. 1b). MC3T3-E1 preosteoblasts infected with a control GFP-targeted siRNA construct demonstrated no significant change in noggin level when compared with controls.

Western blot analysis was next performed to determine the capacity of each noggin-directed siRNA construct to suppress noggin protein. Noggin levels were not affected by infection with a control siRNA when compared with cells treated with vehicle only sham infection. And while each of the noggin-targeted siRNA constructs induced a reduction in the level of transcript, only two constructs, siRNA1 and siRNA2, resulted in elimination of detectable protein (Fig. 1c). Noggin protein could still be observed when MC3T3-E1 preosteoblasts were induced to express siRNA3, siRNA4, or siRNA5. Therefore, only siRNA constructs 1 and 2 were selected for further analyses.

Noggin Suppression Enhances Smad Expression and BMP Signaling—The effect of noggin reduction on BMP signaling was first evaluated by QRT-PCR analysis of Smad1 and Smad5 transcription. As expected, relative to untreated MC3T3-E1 preosteoblasts, transcript levels for Smad1 or Smad5 did not change significantly following infection with a control siRNA or vehicle only sham infection (Fig. 2a). Cells expressing either of the two noggin-targeted siRNA constructs, however, demonstrated significant up-regulation of both Smad1 and Smad5 (siRNA1: Smad1 1.29 ± 0.05, Smad5 1.54 ± 0.07; siRNA2: Smad1 1.27 ± 0.08, Smad5 1.50 ± 0.03; all *, p < 0.05), demonstrating that noggin reduction enhances mRNA levels of these BMP signaling intermediates (Fig. 2a). Similar results were observed when untreated MC3T3-E1 preosteoblasts were cultured in the presence of exogenous rhBMP-4 (1 ng/ml) for 24 h (Smad1 1.35 ± 0.06, Smad5 1.67 ± 0.20; both *, p < 0.05).

As BMP ligand-receptor interactions culminate in phosphorylation of Smad intermediates, Western blot analysis of phospho-Smad1/5 protein was next performed. Similar to cells

### RESULTS

Evaluation of RNAi-mediated Noggin Suppression—RNA interference was employed to induce a reduction in the level of noggin transcript and protein. Using a modification of the restriction enzyme generated siRNA system, five individual noggin-directed siRNA constructs were obtained and confirmed by sequencing (Fig. 1a). An additional 13 constructs were found to be in the reverse orientation on sequencing and were not further evaluated. QRT-PCR analysis of noggin in MC3T3-E1 preosteoblasts following infection and puromycin selection demonstrated a strong reduction in transcript level (siRNA1 89.6 ± 1.5%; siRNA2 87.9 ± 5.2%; siRNA3 51.5 ± 5.3%; siRNA4 72.3 ± 3.3%; and siRNA5 55.5 ± 4.7% all *, p < 0.001) when compared with control, vehicle only sham-infected cells (Fig. 1b). MC3T3-E1 preosteoblasts infected with a control GFP-targeted siRNA construct demonstrated no significant change in noggin level when compared with controls.

Western blot analysis was next performed to determine the capacity of each noggin-directed siRNA construct to suppress noggin protein. Noggin levels were not affected by infection with a control siRNA when compared with cells treated with vehicle only sham infection. And while each of the noggin-targeted siRNA constructs induced a reduction in the level of transcript, only two constructs, siRNA1 and siRNA2, resulted in elimination of detectable protein (Fig. 1c). Noggin protein could still be observed when MC3T3-E1 preosteoblasts were induced to express siRNA3, siRNA4, or siRNA5. Therefore, only siRNA constructs 1 and 2 were selected for further analyses.

**Noggin Suppression Enhances Smad Expression and BMP Signaling**—The effect of noggin reduction on BMP signaling was first evaluated by QRT-PCR analysis of Smad1 and Smad5 transcription. As expected, relative to untreated MC3T3-E1 preosteoblasts, transcript levels for Smad1 or Smad5 did not change significantly following infection with a control siRNA or vehicle only sham infection (Fig. 2a). Cells expressing either of the two noggin-targeted siRNA constructs, however, demonstrated significant up-regulation of both Smad1 and Smad5 (siRNA1: Smad1 1.29 ± 0.05, Smad5 1.54 ± 0.07; siRNA2: Smad1 1.27 ± 0.08, Smad5 1.50 ± 0.03; all *, p < 0.05), demonstrating that noggin reduction enhances mRNA levels of these BMP signaling intermediates (Fig. 2a). Similar results were observed when untreated MC3T3-E1 preosteoblasts were cultured in the presence of exogenous rhBMP-4 (1 ng/ml) for 24 h (Smad1 1.35 ± 0.06, Smad5 1.67 ± 0.20; both *, p < 0.05).

As BMP ligand-receptor interactions culminate in phosphorylation of Smad intermediates, Western blot analysis of phospho-Smad1/5 protein was next performed. Similar to cells

### FIGURE 2.

**Noggin suppression enhances BMP signaling and osteogenic differentiation.** a, expression of siRNA1 or siRNA2 or exogenous application of 1 ng/ml rhBMP-4 enhanced levels of mean transcripts ± S.D. of both Smad1 and Smad5 in MC3T3-E1 preosteoblasts (*, p < 0.05). b, Western blot analysis for phospho-Smad1/5 demonstrated increased levels of this signaling intermediate when cells were either infected with noggin-directed siRNA constructs or exposed to rhBMP-4. However, no change was observed in total Smad5 protein levels. c, Alizarin Red staining of MC3T3-E1 preosteoblasts revealed increased extracellular matrix mineralization when noggin was suppressed. Quantification revealed significantly more staining when cells were infected with either siRNA1 or siRNA2 (mean absorbance ± S.D.; *, p < 0.01) (d).
Noggin Suppression Enhances Bone Formation

treated with rhBMP-4, suppression of noggin was enough to induce a detectable increase in phospho-Smad1/5 relative to control cells (Fig. 2b). Total Smad5 protein level was not observed to change with either rhBMP-4 or infection with noggin-directed siRNA constructs. Together, these findings indicate that a reduction in the level of noggin enhances the signaling activity of endogenously produced BMPs, as reflected by the increase in phosphorylated Smad intermediates.

Reduction of Noggin Promotes Osteoblast Differentiation—With noggin suppression promoting phosphorylation of BMP signaling intermediates, the consequences of noggin reduction on the osteogenic differentiation capacity of MC3T3-E1 preosteoblasts were subsequently evaluated. Cells were either induced to express a noggin-directed siRNA construct, a control siRNA construct, or underwent vehicle only sham retroviral infection. Following 21 days of culture in ODM, histological assessment was performed using Alizarin Red staining. Notable qualitative differences were observed in the degree of osteoblast differentiation, with cells expressing noggin-directed siRNA constructs 1 or 2 depositing more mineralized extracellular matrix relative to either control siRNA expressing or vehicle only sham-infected controls (Fig. 2c). Quantification of staining demonstrated a statistically significant increase in the amount of bone nodule formation observed from cells with noggin suppression relative to controls (*, p < 0.01) (Fig. 2d). No significant difference was observed between siRNA1 and siRNA 2 or between control siRNA infected and vehicle only sham-infected cells.

Gene expression analysis was performed to confirm histological findings. Importantly, noggin expression was observed to increase in control siRNA and sham-infected MC3T3-E1 preosteoblasts when cultured in ODM over 21 days (Fig. 3a). In contrast, cells expressing either siRNA1 or 2 demonstrated continued suppression of noggin transcripts below the level found in undifferentiated cells at all time points (both *, p < 0.05) (Fig. 3a).

Expression levels for the early osteoblast transcription factor Runx were also evaluated. While control siRNA and sham-infected MC3T3-E1 preosteoblasts demonstrated maximal up-regulation following 9 days of differentiation relative to undifferentiated cells, expression of Runx peaked 3 days earlier in noggin-suppressed cells (*, p < 0.01) (Fig. 3b).

FIGURE 3. Noggin suppression promotes expression of osteogenic differentiation markers. a, following 3 weeks of culture in osteogenic differentiation media, MC3T3-E1 preosteoblasts expressing siRNA1 or siRNA2 continued to demonstrate significant noggin suppression (mean expression ± S.D.; *, p < 0.05). Maximal mean expression ± S.D. of Runx (b), an early marker of bone differentiation was noted 3 days earlier in noggin-suppressed cells (red and blue bars) when compared with differentiated control cells (white and yellow bars) (*, p < 0.05). Significant differences in OP expression, an intermediate marker, were noted at days 6 and 9, and again at days 18 and 21 (mean expression ± S.D.; *, p < 0.05) (c). A shift toward earlier up-regulation was again noted in noggin-suppressed cells (red and blue bars). OC expression, a late marker for bone differentiation was greatest in noggin-suppressed cells beginning at day 9 (mean expression ± S.D.; *, p < 0.05) (d). All transcripts levels were expressed relative to undifferentiated control cells.
An intermediate marker of osteoblast differentiation, $\text{OP}$, was similarly examined by QRT-PCR analysis. Relative to undifferentiated cells, control siRNA and sham-infected MC3T3-E1 preosteoblasts demonstrated greatest expression following 12–15 days of osteogenic differentiation (Fig. 3c). Cells expressing noggin-targeted siRNA constructs; however, reached similar levels 3 days earlier. Evaluation at each time point between control and noggin-suppressed cells revealed significant differences in $\text{OP}$ expression at 6 and 9 days and again at 18 and 21 days of differentiation as expression of this intermediate marker tapered (*, $p < 0.05$). Lastly, transcript levels for the late marker of osteogenic differentiation, $\text{OC}$, were investigated by QRT-PCR. When compared with undifferentiated cells, MC3T3-E1 preosteoblasts expressing siRNA1 or 2 were found to significantly upregulate $\text{OC}$ expression as early as 9 days (Fig. 3d). In contrast, significant up-regulation in control siRNA or sham-infected cells could not be detected until 3 days later. Comparing cells at each time point, noggin suppressed MC3T3-E1 preosteoblasts demonstrated significantly more $\text{OC}$ transcripts than control siRNA or sham-infected cells beginning at day 9 and continuing through day 21 (*, $p < 0.05$). As transcription of $\text{OC}$ is limited to differentiated osteoblasts, these findings parallel observations made histologically by Alizarin Red staining. In general, our gene expression analysis after 21 days of differentiation therefore underscores the increased mineralization and bone nodule deposition seen.

**Earlier Mineralization by Calvarial Osteoblasts Is Induced by Noggin Abrogation**—To investigate the effects of noggin reduction on primary calvarial osteoblasts, cells were harvested from wild-type CD-1 mice and retrovirally infected with the noggin-directed siRNA constructs (siRNA1 or siRNA2) to achieve transcript suppression. Confirmation of noggin suppression was obtained by QRT-PCR and Western blot analysis (see supplemental Fig. S1). Osteogenic differentiation was subsequently induced and histological assessment was made starting at 9 days and every 3 days thereafter. Paralleling observations in MC3T3-E1 preosteoblasts, noggin suppression in primary calvarial osteoblasts also enhanced Alizarin Red staining for extracellular matrix mineralization, with qualita-
tive differences detectable as early as 12 days following culture in ODM (Fig. 4a). Spectrophotometric quantification revealed these differences to be significant (Fig. 4b).

To confirm observations made using RNAi-mediated noggin suppression, primary calvarial osteoblasts were also harvested from transgenic mice homozygous for a noggin allele flanked by loxP sites. Cre-mediated DNA recombination was induced in these cells by treatment with an Adeno-CMV-Cre. Western blot analysis following adenoviral infection demonstrated no detectable noggin protein, suggesting successful recombination to have occurred in these cells (Fig. 5a). In contrast, osteoblasts treated with either an empty adenovirus or undergoing sham infection continued to express detectable levels of noggin protein (Fig. 5a). As these mice also carry a GFP reporter activated in the presence of Cre recombinase, flow cytometry was employed to quantify the percentage of cells successfully infected by Adeno-CMV-Cre. Using this modality, 96.4% of cells treated with Adeno-CMV-Cre were GFP-positive and, therefore, also presumably deficient for noggin (Fig. 5b, right column). When these osteoblasts were cultured in differentiation media for 21 days, more Alizarin Red staining was appreciated in cells treated with Cre recombinase relative to other cells undergoing empty adenoviral infection or sham infection (Fig. 5c). Spectrophotometric quantification revealed this increase in Alizarin Red staining for Adeno-CMV-Cre-treated cells to

**FIGURE 5.** Confirmation using floxed-noggin primary calvarial osteoblasts. a, Western blot analysis demonstrated treatment of floxed-noggin primary calvarial osteoblasts with 50 MOI Adeno-CMV-Cre eliminated detectable noggin protein. b, FACS analysis revealed 96.4% of the Adeno-CMV-Cre-treated cells to be positive for a GFP reporter and thus noggin-deficient. c, Alizarin Red staining following 21 days of differentiation showed enhanced mineralization in Adeno-CMV-Cre-treated cells relative to controls. d, spectrophotometric quantification confirmed a significant increase in staining (mean absorbance ± S.D.) with noggin-deficient cells (*, p < 0.001).
Repair of Critical-sized Calvarial Defects Is Accelerated by Noggin Suppression—Given the observation that noggin reduction promotes in vitro osteogenic differentiation, the ability to also accelerate in vivo bone regeneration and repair of calvarial defects using primary osteoblasts was next investigated. Critical-sized parietal bone defects were created in skeletally mature mice, and repair was performed with either infected osteoblasts (siRNA1, siRNA2, or control siRNA) or vehicle only sham-infected controls seeded onto apatite-coated poly(lactic-co-glycolic acid) scaffolds (Fig. 6a). Two weeks following surgery, analysis by microcomputed tomography demonstrated increased radiographic density when defects were repaired with osteoblasts expressing either noggin-directed siRNA1 or siRNA2 (Fig. 6b, top row). Qualitatively more reossification was also appreciated in the region of the defect at 4 weeks when osteoblasts expressing siRNA constructs 1 or 2 were implanted relative to controls (Fig. 6b, middle row). By 8 weeks, nearly complete bony bridging was appreciated in all groups (Fig. 6b, bottom row). These findings support the notion that a reduction in noggin protein can therefore not only promote in vitro osteogenic differentiation, but also accelerate in vivo bone regeneration.

To confirm our radiographic findings, histological analysis was performed to evaluate the extent of bone deposition across the region of the defect. At both 2 and 4 weeks, pentachrome staining revealed more bone formation relative to controls when repair was performed using osteoblasts expressing either noggin-directed siRNA constructs (Fig. 6c). Histomorphometric analysis on aniline blue-stained sections at 2 weeks demonstrated a significant difference in bone content between experimental groups (siRNA1 24.1 ± 4.5%, siRNA2 25.4 ± 5.0%) and controls (vehicle control 3.9 ± 1.4%, control siRNA 5.5 ± 2.1%) (*, p < 0.001) (Fig. 6d). Histomorphometric analysis performed on sections at 4 weeks yielded similar findings (siRNA1 64.7 ± 5.3%, siRNA2 68.6 ± 5.9%, vehicle control 46.1 ± 5.0%, control siRNA 42.9 ± 7.4%; *, p < 0.001) (Fig. 6d). Paralleling observations made on microCT, near complete bony bridging was appreciated in all groups at 8 weeks (siRNA1 92.3 ± 3.6%, siRNA2 89.4 ± 2.7%, vehicle control 86.7 ± 5.5%, control siRNA 90.4 ± 1.9%) (Fig. 6, c and d).

DISCUSSION

The regulation of BMP signaling has been shown to be critical in multiple developmental processes, guiding embryologic patterning and organogenesis (21). Postnatally, BMP antagonists, have also been found to regulate osteoblast differentiation and adult skeletogenesis. In particular, overexpression or exogenous application of noggin has been shown to severely limit in vitro osteogenesis and in vivo bone formation (23–25). From a clinical standpoint, however, the utility of osteogenic repression pales in comparison to the overwhelmingly greater need for bone generation. Over 1 million skeletal-related procedures are performed annually at an aggregate cost exceeding 1.5 billion dollars (33). Many of these procedures deal with bone deficit secondary to surgical resection, congenital defects, trauma, or fracture malunion/nonunion. While current treatment approaches have begun to incorporate cytokine therapy to augment bone formation, the inordinate cost of rhBMP-2 (approximately $5,000-$8,000 per application) has only further contributed to a blossoming of health care expenditures. Given the wealth of evidence suggesting the strong ability for noggin to inhibit BMP signaling and subsequent osteogenic differentiation, a distinct potential thus exists for bone formation to be alternatively enhanced through a reduction of this BMP antag-
Noggin Suppression Enhances Bone Formation

onist. In an aging population with an increasing need for bone regeneration, exploiting such a novel strategy to accelerate in vitro osteogenesis would undoubtedly prove to be an advantageous solution clinically.

In this study, we demonstrated that noggin suppression can indeed accelerate in vitro osteogenesis and in vivo healing of critical-sized calvarial defects. Furthermore, the data in this report extend our understanding of the role of noggin in the postnatal state, revealing that it is not only sufficient to regulate bone formation, but is also critical for proper coordination of osteogenesis. Utilizing restriction enzyme-generated siRNAs, we were able to fashion numerous constructs targeting noggin transcripts for degradation (26). Two of these constructs, siRNA1 and siRNA2, proved capable of eliminating detectable protein by Western blot analysis. Evaluation of resultant BMP signaling in the setting of noggin suppression revealed enhanced activity, similar to effects observed by culturing cells in the presence of rhBMP-4. We observed an up-regulation of transcript for both Smad1 and Smad5 when cells were induced to express either noggin-directed siRNA constructs. This was in concordance with other published reports describing enhanced levels of these intermediates secondary to BMP signaling (34). While total Smad5 protein levels were not observed to change significantly, increased phosphorylated-Smad1/5 protein was noted when noggin was suppressed. Collectively, these data suggest that removal of the BMP antagonist noggin can augment the signaling activity of endogenously produced BMPs. Suppression of noggin eliminates a potential negative-feedback mechanism, resulting in enhanced levels of phospho-Smads to mediate BMP induced changes in nuclear transcription.

With a reduction in noggin, we also observed increased differentiation in MC3T3-E1 preosteoblasts. Following 21 days of culture in ODM, significantly more staining for bone nodule deposition and extracellular matrix mineralization was appreciated. This finding was confirmed in both primary calvarial osteoblasts treated with functional siRNA constructs targeted against noggin or by means of Adeno-CMV-Cre infection of floxed noggin osteoblasts. In addition, a shift toward earlier mineralization was observed, with detectable differences in Alizarin Red staining noted as early as 12 days following culture in osteogenic differentiation media.

Gene expression analysis provided complementary data to observations made by histological staining. Though noggin expression increased during differentiation of controls, transcript levels remained low in both groups expressing noggin-directed siRNA constructs. The increase in noggin transcript seen in controls may reflect a rise in BMP signaling as osteoblasts differentiate, serving to negatively regulate the stimulatory effects of BMPs (22). With gene analysis demonstrating significantly less noggin following infection with either siRNA1 or siRNA2, continued effective suppression of this protein was confirmed, even after 21 days of osteogenic differentiation. Analysis of osteoblast differentiation markers reflected changes in the state of cells, with accelerated early osteogenesis and a more terminally differentiated phenotype at late time points in the presence of noggin reduction. Maximal up-regulation of both Runx2 and OP were found 3 days earlier in noggin suppressed cells relative to controls, with Runx2 peaking at day 6 and OP peaking at days 9–12 when cells were induced to express either siRNA1 or 2. These findings suggest a shift toward earlier differentiation when noggin is suppressed. Both Runx2 and OP are known early/intermediate markers of differentiation; it is therefore not unexpected that relative expression may diminish as cells take on a more mature phenotype. Studies evaluating the expression of such markers over a time course in calvarial osteoblasts have also demonstrated Runx2 transcripts to peak around 7 days, before gradually declining over the following 3 weeks (35).

In contrast to these early and intermediate markers, OC expression demonstrated gradual up-regulation beginning at day 9 for noggin-suppressed cells and at day 12 for control siRNA or sham-infected cells, with continued increase noted in all cells through day 21. At each time point beginning at day 9, however, significantly more OC transcripts were found in cells expressing noggin-targeted siRNA constructs. OC demonstrates a strong affinity for deposited bone mineral and generally serves as a sensitive marker for bone formation (36). Investigations have shown OC levels to correlate well with both bone formation rate and calcium accretion (37, 38). Increased amounts of OC transcript therefore strongly support the concept that noggin suppression enhances osteoblast differentiation, and ultimately, osteogenesis. The dramatic increase in OC transcripts by osteoblasts expressing siRNA1 or siRNA2 relative to control siRNA and sham-infected cells is consistent with the increase in terminal differentiation and production of mineralized bone nodules as shown by Alizarin Red staining.

With evidence of accelerated in vitro bone formation detected as early as 12 days in primary calvarial osteoblasts, the potential translational implications of noggin reduction in vivo were therefore investigated. Clinical use of autologous bone grafting has long been in practice, and reports have already defined the ability of murine osteoblasts, when seeded onto apatite-coated polyactic co-glycolic acid scaffolds, to repair critical-sized parietal bone defects after 8–12 weeks (39). Using this model, the ability of noggin suppression to enhance repair of calvarial defects was evaluated both radiographically and histologically over the course of 8 weeks. Interestingly, accelerated osteogenesis was observed at 2 and 4 weeks post-implantation, with as much as a 20% increase in reossification noted on histomorphometric analysis when repair was performed using noggin-suppressed osteoblasts. By 8 weeks, however, near complete bony bridging was appreciated in all groups and no statistical significance could be detected. Retroviral inactivation and diminished RNAi activity at this late time point may have potentially contributed to this observation (40). Production of noggin by the subjacent dura mater and native cells along the edge of the defect may also have further diminished differences in observed healing. Nonetheless, enhanced early bone formation was demonstrated, suggesting more rapid healing to result from the reduction of BMP antagonists. Clinically, accelerated bone formation would be particularly advantageous, allowing earlier mobilization of patients and faster
return to function following skeletal injury or surgical resection.

As a corollary to several studies which have defined a significant role for noggin in the regulation of adult bone formation, our findings have thus demonstrated a novel reduction of this BMP antagonist to promote both in vitro and in vivo osteogenesis. While multiple other BMP antagonists have also been described, many with complementary roles and overlapping functions, it may be entirely plausible that similar effects can be observed through a reduction in these other proteins (41–45). Irrespective of this, we have shown that the novel strategy of noggin suppression is sufficient to drive endogenous BMP signaling and accelerate the generation of bone by osteoblasts. From a clinical perspective, translation of these findings may ultimately depend on the definition of a means by which this strategy can be delivered to sites of need. And beyond the repair bone defects, a multitude of pathologic states, such as osteoporosis and associated osteopenia, in which a chronic imbalance between the expression of BMP agonists and antagonists potentially exists, similar benefit may be derived through the suppression of noggin (25).

Acknowledgments—We thank M. Tataria, C. Chan, and M. Kwan for technical assistance provided in this manuscript.

REFERENCES

Noggin Suppression Enhances in Vitro Osteogenesis and Accelerates in Vivo Bone Formation

Derrick C. Wan, Jason H. Pomerantz, Lisa J. Brunet, Jae-Beom Kim, Yu-Fen Chou, Benjamin M. Wu, Richard Harland, Helen M. Blau and Michael T. Longaker

doi: 10.1074/jbc.M703282200 originally published online July 2, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M703282200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2007/07/05/M703282200.DC1

This article cites 45 references, 13 of which can be accessed free at http://www.jbc.org/content/282/36/26450.full.html#ref-list-1