Conditional Deletion of Gremlin Causes a Transient Increase in Bone Formation and Bone Mass*

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Gremlin is a glycoprotein that binds bone morphogenetic proteins (BMPs) 2, 4, and 7, antagonizing their actions. Gremlin opposes BMP effects on osteoblastic differentiation and function in vitro and in vivo, and its overexpression causes osteopenia. To define the function of gremlin in the skeleton, we generated grem1 (grem1) conditional null mice by mating mice where grem1 was flanked byloxP sequences with mice expressing the Cre recombinase under the control of the osteocalcin promoter. grem1 null male mice displayed increased trabecular bone volume due to enhanced osteoblastic activity, because mineral apposition and bone formation rates were increased. Osteoblast number and bone resorption were not altered. Marrow stromal cells from grem1 conditional null mice expressed higher levels of alkaline phosphatase activity. Gremlin down-regulation by RNA interference in ST-2 stromal and MC3T3 osteoblastic cells increased the BMP-2 stimulatory effect on alkaline phosphatase activity, on Smad 1/5/8 phosphorylation, and on the transactivation of the BMP/Smad reporter construct 12×SBE-Oc-pGL3. Gremlin down-regulation also enhanced osteocalcin and Runx-2 expression, Wnt 3a signaling, and activity in ST-2 cells. In conclusion, deletion of grem1 in the bone microenvironment results in sensitization of BMP signaling and activity and enhanced bone formation in vivo.

Bone morphogenetic proteins (BMPs) are important determinants of cell fate and play a central role in the regulation of osteoblastogenesis and endochondral bone formation (1).

BMPs, in conjunction with Wnt, induce the differentiation of mesenchymal cells toward the osteoblastic lineage and enhance the pool and function of mature osteoblasts (2–4). Upon ligand binding, BMPs initiate a signal transduction cascade activating the mothers against the decapentaplegic (Smad) or mitogen-activated protein kinase signaling pathways (1, 5–8). In osteoblastic cells, Wnt binding to specific receptors and co-receptors leads to the stabilization of β-catenin and its translocation to the nucleus, where it associates with nuclear factors to regulate transcription (4, 9, 10).

The effects of BMPs and Wnt are controlled by a large group of secreted polypeptides that prevent BMP or Wnt signaling by binding BMPs or Wnt, or their receptors/co-receptors, precluding ligand-receptor interactions (1, 4, 11, 12). The binding affinity and selectivity of secreted antagonists for specific BMPs varies, and selected antagonists can have dual BMP and Wnt antagonistic activity (1, 13–16).

Gremlin and its rat homolog, down-regulated by v-mos (drm), are secreted glycoproteins with a molecular mass of 20.7 kDa (17–19). Gremlin 1 (grem1) is a member of the differentially screening-selected gene aberrative in neuroblastoma (dan)/cerberus family of genes, and gremlin was originally identified as a dorsalizing agent, with BMP antagonistic activity, in Xenopus embryos (1, 17). Gremlin binds and prevents the activity of BMP-2, -4, and -7. Gremlin is expressed by stromal cells surrounding certain neoplastic cells, and it is considered to play a role in cell survival and possibly tumorigenesis (19, 20). Homozygous null mutations of the grem1 gene in mice result in serious developmental limb, metanephric, and lung abnormalities, leading to absent kidneys and intrauterine or newborn lethality (21, 22). The patterning of distal limb skeletal elements is tightly regulated by the reciprocal interactions between BMPs, fibroblast growth factors (FGFs) 4 and 8, and Sonic hedgehog (SHH) (21). By inhibiting BMP action, gremlin allows for FGF 4/8 expression, which in turn promotes SHH expression in the posterior limb bud, which is required for proper limb patterning and development.

Later in skeletal development, after the pattern of skeletal elements has been established, grem1 is expressed by osteoblasts (23). Transgenic mice overexpressing gremlin under the control of the osteocalcin promoter exhibit severe osteopenia secondary to decreased bone formation (24), consistent with the role of gremlin as a BMP antagonist. Gremlin binds and inhibits BMP signaling and activity in cells of the osteoblastic lineage, and tempers Wnt signaling (24). This is in accordance...
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with the dual, BMP and Wnt, inhibitory activity reported for other members of the dan/cerberus family of genes (1, 15, 16).

Null mutations of grem1 nearly always cause embryonic lethality, not allowing for the study of their adult skeletal phenotype (21, 22). In the present study, a conditional grem1 deletion to inactivate gremlin in the bone environment post-natally was used. For this purpose, genetically engineered mice, where the coding sequence of grem1 was flanked by loxP sequences, were created and crossed with transgenic mice expressing the Cre recombinase under the control of the human osteocalcin promoter, and their skeletal phenotype was determined. In addition, mechanisms of gremlin action were explored in vitro, following its down-regulation in ST-2 stromal and MC3T3 osteoblastic cells using RNA interference (RNAi).

EXPERIMENTAL PROCEDURES

Conditional Deletion of grem1—To generate a conditional-null allele of grem1, a segment of exon 2, where the coding sequence of grem1 resides in its totality, was flanked with loxP sequences to allow the excision of the entire open reading frame by Cre recombinase (Fig. 1, A and B). Targeted embryonic stem (ES) cells harboring a loxP-flanked allele for grem1loxP/+ were generated using Velocigene™ technology (25). Briefly, a bacterial artificial chromosome (BAC) containing mouse genomic DNA encompassing grem1 sequences was selected by PCR screen from a 129/Sv mouse BAC library containing ~140 kb of genomic DNA (Release I, BAC id 427a3, Incyte Genomics, Wilmington, DE). To generate the targeting vector, BAC 427a3 was modified using bacterial homologous recombination in a three-step process as follows: 1) a loxP site was introduced in a non-conserved region 335 bp upstream of exon 2, by inserting a LoxP_1_Scel_EM7-Zeo_1_Scel cassette; 2) the I-SceI_EM7-Zeo_1_Scel cassette was removed from the modified BAC by restricting with I-SceI, re-ligating, and selecting for modified BACs that had lost the Zeo cassette while retaining the LoxP_1_Scel sequence upstream of exon 2; and 3) a loxP site was inserted 550 bp downstream of the stop codon of grem1 as part of a flippase recombinase target (FRT)-flanked-phosphoglycerate kinase (PGK)-neomycin phosphotransferase (Neo)-polyA_FRT_LoxP cassette, while simultaneously deleting 66 bp (GATGGCAAACGGGACAGAGGACTGACGCAGGAACG...AAAGGTTCCCAAGGAGCCATTCC-3'), for the wild-type allele (300 bp), and the forward primer, 5'GGTCAATCCGCCGTTTGTTCC-3', for the wild-type allele (328 bp), or the forward primer, 5'GGTGGGTGGGATTAGATTTAGAATA-3', for the targeted loxP allele (696 bp). Genotyping of grem1loxP mice was carried out by PCR using the common reverse primer, 5'AACAGGAGTGGTGCAG-3', and the forward primer, 5'ACGGGACAGAGGACTGACGCAGGAACG...AAAGGTTCCCAAGGAGCCATTCC-3', and reverse primer, 5'TAGTCACGCAACTCGCCGCATC-3', for the targeted loxP allele (500 bp). Deletion of loxP flanked sequences by the Cre recombinase was documented by PCR in DNA extracted from calvariae of 1-month-old mice using the forward primer 5'GGTGATCTGTCAGCTGTC-3', and reverse primer 5'AAACAGGAGTGGTGCAG-3', to create a 670-bp product. Grem1 deletion was confirmed by determination of gremlin mRNA levels by real-time reverse transcription (RT)-PCR in calvarial extracts. Animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

X-ray Analysis and Bone Mineral Density—Radiography was performed on anesthetized or sacrificed mice on a Faxitron x-ray system (model MX 20, Faxitron x-ray Corp., Wheeling, IL). The x-rays were performed at an intensity of 30 kV for 20 s. Bone mineral density (BMD, g/cm²) was measured on anesthetized mice using the PIXImus small animal DEXA system (GE Medical Systems/LUNAR, Madison, WI) (29). Calibrations...
were performed with a phantom of a defined value, and quality assurance measurements were performed prior to each use. The coefficient of variation for total BMD was <1% (n = 9 mice).

**Bone Histomorphometric Analysis**—Static and dynamic histomorphometry was carried out on femurs from experimental and control littermate mice at 1 month and 3 months of age. Mice were injected with calcine, 20 mg/kg, and demeclocycline, 50 mg/kg, at an interval of 2 or 7 days, for 1- or 3-month-old mice, respectively, and sacrificed by CO₂ inhalation 2 days after the demeclocycline injection. Femurs were dissected, fixed in 70% ethanol, dehydrated, and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5 μm thick, were cut on a Microm microtome (Microm, Richard-Allan Scientific, Kalamazoo, MI) and stained with 0.1% toluidine blue, pH 6.4, or Von Kossa. Static parameters of bone formation and resorption were measured in a defined area between 725 μm and 1270 μm from the growth plate, using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in dynamic histomorphometry, mineralizing surface per bone surface, and mineral apposition rate were measured in unstained sections under ultraviolet light, as described (24).

**Expression Analysis of the β-Galactosidase Reporter Gene**—Whole mount LacZ/β-galactosidase gene expression was analyzed in 3-day-old femurs and tibiae from grem1ΔLacZ and grem1ΔΔ− controls (31). Femurs and tibiae were harvested from 3-day-old mice, and fixed in a 0.4% glutaraldehyde for 4°C overnight. Tissues were rinsed with phosphate-buffered saline and incubated in LacZ staining solution 4 h at 37°C, decalcified in Decal-Stat (Decal Co., Tallman, NY) 24 h at 4°C, and embedded in cryomatrix (Thermofisher, Waltham, MA). 5-μm sections were cut on a cryostat and counterstained with eosin and visualized by microscopy.

**Bone Marrow Stromal Cell Cultures**—Femurs from grem1ΔLacZ and grem1ΔΔ− controls were aseptically removed from 4-week-old mice, after CO₂ asphyxiation, and stromal cells were recovered by centrifugation, as described previously (24). Cells were plated at a density of 5 × 10⁵ cells/cm² and cultured in minimum essential medium (α-MEM, Invitrogen) containing 15% fetal bovine serum (Atlas Biologicals, Norcross, GA) at 37°C in a humidified 5% CO₂ incubator. When cells reached confluence (6–7 days of culture), the medium was changed to α-MEM supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, and 5 mM β-glycerophosphate (Sigma-Aldrich). Cells were cultured for an additional 10- to 16-day period, and serum was deprived overnight, treated with BMP-2 (Wyeth, Collegeville, PA) for 24 h, and analyzed for alkaline phosphatase activity and gremlin mRNA expression.

**Culture of Cell Lines and RNA Interference**—ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice, and MC3T3-E1, osteoblastic cells derived from mouse calvariae, were plated at a density of 10⁶ cells/cm², and grown in a humidified 5% CO₂ incubator at 37°C in α-MEM, supplemented with 10% fetal bovine serum (32, 33). To down-regulate gremlin expression in vitro, a 19-mer double-stranded small interfering (si) RNA targeted to bp 884–902 of grem1 mouse DNA sequence was obtained commercially, and a 19-mer silencing scrambled RNA composed of sequences with no homology to known mouse or rat sequences was used as a control (Ambion, Austin, TX) (34, 35). Gremlin or scrambled siRNA, all at 20 nM, were transfected into sub-confluent ST-2 or MC3T3 cells using siLentFect lipid reagent, in accordance with manufacturer’s instructions (Bio-Rad, Hercules, CA) (36). To ensure adequate down-regulation, total RNA was extracted in cells 24–96 h following the transfection of siRNAs, and gremlin mRNA levels were determined by real-time RT-PCR. To test for effects on osteoblastic function, transfected cells were allowed to recover for 24 h, and treated with recombinant human BMP-2 or Wnt 3a (R&D Systems, Minneapolis, MN) for 72 h in the presence of 5 mM β-glycerophosphate and ascorbic acid (Sigma-Aldrich) and analyzed for alkaline phosphatase activity. In one experiment, ST-2 cells were allowed to recover for 24 h, treated with BMP-2 for 24 h, and analyzed for osteocalcin and runt-related transcription factor (Runx-2) mRNA expression. To test for effects on BMP or Wnt signaling, cells were allowed to recover for 24 h, transfected with BMP/Smad or Wnt/β-catenin reporter constructs, and treated with BMP or Wnt, as described under “Transient Transfections.” Alternately, cells were allowed to reach confluence, serum-deprived, and treated with BMP-2 for 20 min to test for effects on Smad1/5/8 phosphorylation by Western blot analysis.

**Real-time Reverse Transcription-PCR**—Total RNA was extracted from calvariae or cell cultures and mRNA levels determined by real-time RT-PCR (37, 38). For this purpose, 1–10 μg of RNA was reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen), according to the manufacturer’s instructions and amplified in the presence of 5′-CGGTAGCCGCACTATCATCAAC[FAM]G-3′ and 5′-GTGAACTTCTTGGGCTTGCAGA-3′ primers for gremlin; 5′-CACCTACGCGCTACCTTTGGTAAGT[FAM]G-3′ and 5′-CCCAGCACAATCCTCCTCTCA-3′ primers for osteocalcin; 5′-CACAGGGGACAGCTCCACACTCTCTC[G]FAM[G]-3′ and 5′-CACGGGCAGGCTTGTGTTTGG-3′ for Runx2; 5′-CACCCTGTTGAGCATCCTTTCCGAG[FAM]G-3′ and 5′-TGCTGTTGAAAGAAGGCAACAC-3′ for FGF-4; 5′-CAGGCTCTGGAAAGGCTGTGGCCG[FAM]-G-3′ and 5′-AGGTTCCGAGGTCAGCTTGG-3′ primers for glyceraldehyde-3-phosphate 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 gremlin cDNA (Regeneron Pharmaceuticals) and corrected for gapdh (R. Wu, Ithaca, NY) copy number (17, 39). Reactions were conducted in a 96-well spectrophotometric thermal cycle (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step.

**Alkaline Phosphatase Activity**—Alkaline phosphatase activity (APA) was determined in cell extracts by the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol, and measured by spectrophotometry at 405 nm after 10 min of incubation at 25°C, according to manufacturer’s instructions (Sigma-Aldrich). Data are expressed as nanomoles of p-nitrophenol released per
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minute per microgram of protein. Total protein content was determined in cell extracts by the DC protein assay in accordance with manufacturer’s instructions (Bio-Rad).

Transient Transfections—To determine changes in BMP-2 signaling under conditions of gremlin RNAi, a construct containing 12 copies of a Smad 1/5 consensus sequence linked to an osteocalcin minimal promoter and a luciferase reporter gene (12×SBE-Oc-pGL3, M. Zhao, Antonio, TX) was tested in transient transfection experiments (40). To determine changes in Wnt/β-catenin transactivating activity, a construct containing 16 copies of the lymphoid enhancer binding factor/T-cell specific factor (Lef-1/Tcf-4) recognition sequence, cloned upstream of a minimal thymidine kinase promoter and a luciferase reporter gene (16×TCF-Luc, J. Billiard, Wyeth Research), was tested (41). ST-2 or MC3T3 cells were transiently transfected using FuGENE-DNA mix for 16 h and transferred to serum-free medium for 6 h. Cells were then treated with BMP-2 or Wnt 3a for 24 h and harvested. Luciferase and β-catenin transactivating activity were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT). Luciferase activity was corrected for β-galactosidase activity.

Western Blot Analysis—To determine the level of phosphorylation of Smad 1/5/8, the cell layer of ST-2 cells was washed with cold phosphate-buffered saline and extracted in cell lysis buffer (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors, as described before (24). Protein concentrations were determined by DC protein assay, and 20 μg of total cellular protein was fractionated by gel electrophoresis in 10% polyacrylamide gels under reducing conditions, transferred to Immobilon P membranes (Millipore, Billerica, MA), which were blocked with 3% bovine serum albumin in phosphate-buffered saline. Membranes were exposed to a rabbit polyclonal antibody, which recognizes Smad 1, 5, and 8 phosphorylated at the last two serine residues (Cell Signaling Technology) or exposed to a monoclonal antibody to unphosphorylated Smad 1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution. Blots were then exposed to anti-rabbit or anti-mouse IgG antisera conjugated to horseradish peroxidase (Sigma-Aldrich) and developed with a chemiluminescence detection reagent (PerkinElmer Life Sciences).

Statistical Analysis—Data are expressed as means ± S.E. Statistical significance was determined by Student’s t test or analysis of variance.

RESULTS

Characterization of grem1-conditional Null Mice—Flanking withloxP sites the segment of exon 2 encoding for gremlin was considered sufficient to generate a conditional null allele of grem1, because the entire coding sequence resides in this exon. Furthermore, this design was considered to be advantageous, because the deletion would mimic the grem1 gene null alleles created previously, and the size of the deletion imparted by the Cre recombinase would be small, increasing the probability of excision (21, 22, 42). To monitor for the deletion of grem1 in bone tissue, deletion of theloxP-flanked region and suppressed levels of gremlin transscripts were documented in calvarial extracts from grem1 conditional null mice (grem1loxP/loxP). The mating scheme involved crossing homozygous osteocalcin Cre transgenics in a heterozygous null grem1 background (grem1loxP/LacZ-Oc-Cre/Oc-Cre) with homozygous grem1loxP/loxP mice. Consequently, deletion of theloxP-flanked region was detected in calvarial extracts from the experimental cohort, grem1loxP/LacZ, and in extracts from littermate controls, grem1loxP/loxP (Fig. 1C). No deletion of grem1 sequences was detected in wild-type mice. Gremlin mRNA levels in calvarial extracts from 1-month-old grem1loxP/LacZ conditional null mice were almost undetectable and markedly suppressed in relationship to those measured in littermate controls (Fig. 1D). LacZ staining of 3-day-old femurs and tibiae confirmed that gremlin is expressed during endochondral bone formation (Fig. 1E).

In accordance with their reported normal phenotype, grem1loxP/LacZ haplo-insufficient mice did not exhibit a skeletal phenotype, as determined by bone histomorphometric analysis (Table 1) (22). Furthermore, grem1loxP/loxP homozygous mice were not different from wild-type controls, indicating that the 66-bp deletion engineered in the3′-untranslated region, and the presence ofloxP sequences and selection cassette did not cause a skeletal phenotype. Consequently, grem1loxP/loxP heterozygous littermate mice were considered appropriate controls for grem1loxP/LacZ conditional null mice. 1-month-old grem1loxP/LacZ conditional null mice appeared visually normal, had normal weight, and contact radiography did not reveal any obvious skeletal abnormalities (not shown). BMD was increased by 4–6% in grem1loxP/LacZ conditional null mice, but it was not statistically different from the BMD obtained in grem1loxP/loxP control littermates (Table 2).

Bone histomorphometric analysis of femurs from 1-month-old male grem1loxP/LacZ conditional null mice revealed a 40% increase in trabecular bone volume, secondary to an increase in trabecular thickness and to a lesser extent in trabecular number (Table 3 and Fig. 2). The increase in bone volume observed was not associated with changes in osteoblast number. Osteoblast number/perimeter and osteoblast surface were not different from controls. Changes in trabecular bone volume were not associated with changes in bone resorption, because osteoclast number and eroded surface were normal. Fluorescence microscopy of grem1loxP/LacZ conditional null male mice revealed increased mineral apposition and bone formation rates, indicating that the increased bone volume was secondary to an increase in osteoblast function. The skeletal phenotype of grem1loxP/LacZ conditional null mice was transient and not observed in 3-month-old mice (data not shown). This was attributed to either a transient nature of the phenotype, which was evident in growing but not in mature mice, or to a possible decline in the activity of the osteocalcin promoter, used to direct the Cre recombinase, which is highest at 1 month of age (43, 44).

To investigate the impact of gremlin on osteoblastic cell differentiation and function, marrow stromal cells from grem1loxP conditional null and control mice were cultured
markedly suppressed in relationship to control cells (Table 4). As the culture progressed, there was an increase in alkaline phosphatase activity in control cells. The conditional deletion of grem1 caused an increase in alkaline phosphatase activity and enhanced the effect of BMP-2 (Table 4). The increase in basal activity probably represents sensitization to the effect of endogenous BMPs.

**In Vitro Down-regulation of grem1 Expression**—To determine mechanisms involved in the effect of gremlin on osteoblastic function, ST-2 stromal cells and MC3T3 osteoblastic cells were examined under conditions of gremlin down-regulation by RNAi. This resulted in suppression of gremlin transcripts by about 90% in ST-2 cells and by 60 to 90% in MC3T3 cells, when compared with basal levels of expression (Table 5, Fig. 3). To elucidate the mechanism of gremlin action, we analyzed the impact of gremlin down-regulation on downstream events of BMP-2 signaling and activity in ST-2 stromal and MC3T3 osteoblastic cells. In accordance with the skeletal phenotype observed, gremlin down-regulation enhanced the effect of BMP-2 on the transactivation of the Smad 1/5 dependent 12×SBE-Oc-pGL3 reporter construct in ST-2 cells. Consequently, the effect of BMP-2 on the transactivation of the 12×SBE-Oc-pGL3 reporter was 2.5 to 7 fold greater in the context of gremlin down-regulation.

In accordance with the skeletal phenotype observed, gremlin down-regulation enhanced the effect of BMP-2 on alkaline phosphatase activity and the effect of BMP-2 at 0.1 and 0.3 nM on the phosphorylation of Smad 1/5/8 in ST-2 cells (Fig. 3). To elucidate the mechanism of gremlin action, we analyzed the impact of gremlin down-regulation on downstream events of BMP-2 signaling and activity in ST-2 stromal and MC3T3 osteoblastic cells. In accordance with the skeletal phenotype observed, gremlin down-regulation enhanced the effect of BMP-2 on the transactivation of the Smad 1/5 dependent 12×SBE-Oc-pGL3 reporter construct in ST-2 cells. Consequently, the effect of BMP-2 on the transactivation of the 12×SBE-Oc-pGL3 reporter was 2.5 to 7 fold greater in the context of gremlin down-regulation.

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for 10 and 16 days after confluence and treated, or not, with BMP-2 for the last 24 h of culture. Deletion of the loxP flanked region was documented by PCR in DNA from cell extracts (not shown). Gremlin mRNA levels in cells from grem1Δ/Δ were endogenous BMPs secreted by osteoblastic cells (45–47). BMP-2 at 3 nM overcame the sensitization caused by gremlin RNAi, possibly representing the effect of a maximally stimulatory dose of BMP-2 on Smad phosphorylation (48). In
TABLE 1
Femoral static and dynamic bone histomorphometry of grem1 heterozygous (grem1+/LacZ) mice and of grem1+/+ and wild-type controls

Bone histomorphometry was performed on femurs from 1-month-old male grem1Δ/Δ heterozygous or grem1Δ/Δ and wild-type littermate controls. For static histomorphometry, sections were stained with toluidine blue, and for dynamic histomorphometry unstained sections were analyzed by fluorescence microscopy. Values are means ± S.E.; n = 5–8.

|               | Wild type | Grem1Δ/Δ | Grem1Δ/Δ/LacZ |
|---------------|-----------|----------|---------------|
| Bone volume/total volume (%) | 7.4 ± 0.7 | 7.1 ± 0.7 | 7.2 ± 0.8 |
| Trabecular number (1/mm) | 6.9 ± 0.5 | 7.1 ± 0.5 | 6.9 ± 0.5 |
| Trabecular thickness (μm) | 10.7 ± 0.6 | 9.8 ± 0.3 | 10.0 ± 0.5 |
| Osteoblasts/10,000 μm (100 μm) | 46 ± 3 | 50 ± 5 | 47 ± 2 |
| Osteoblast surface/bone surface (%) | 23.8 ± 11 | 23.2 ± 19 | 23.8 ± 11 |
| Osteoclasts/10,000 μm (100 μm) | 9.9 ± 0.6 | 9.6 ± 0.6 | 10.0 ± 0.6 |
| Eroded surface/bone surface (%) | 23.1 ± 1.0 | 22.0 ± 1.2 | 22.5 ± 1.2 |
| Mineral apposition rate (μm/day) | 1.12 ± 0.11 | 1.13 ± 0.05 | 1.15 ± 0.05 |
| Bone formation rate/bone surface (μm²/μm²/day) | 0.037 ± 0.011 | 0.029 ± 0.005 | 0.034 ± 0.005 |

TABLE 2
Weight and BMD (g/cm² × 10⁴) in 1-month-old grem1Δ/Δ control conditional null male mice and littermate control (grem1+/Δ) controls

Values are means ± S.E.; n = 9.

| Weight | Total BMD | Vertebral BMD |
|--------|-----------|---------------|
| g      | g/cm² × 10⁴ |              |
| Grem1Δ/Δ | 14.2 ± 0.7 | 352 ± 6  |
| Grem1Δ/Δ | 15.3 ± 0.8 | 367 ± 6  |

TABLE 3
Femoral static and dynamic bone histomorphometry of grem1Δ/Δ control conditional null male mice and grem1Δ/Δ control

Bone histomorphometry was performed on femurs from 1-month-old male grem1Δ/Δ control conditional null mice and littermate grem1Δ/Δ controls. For static histomorphometry, sections were stained with toluidine blue, and for dynamic histomorphometry unstained sections were analyzed by fluorescence microscopy. Values are means ± S.E.; n = 5–8.

|               | Grem1Δ/Δ | Grem1Δ/Δ/LacZ |
|---------------|----------|---------------|
| Bone volume/total volume (%) | 8.7 ± 0.8 | 12.1 ± 0.8* |
| Trabecular number (1/mm) | 8.3 ± 0.6 | 10.1 ± 0.7 |
| Trabecular thickness (μm) | 10.5 ± 0.6 | 12.1 ± 0.4* |
| Osteoblasts/10,000 μm (100 μm) | 39 ± 2 | 38 ± 3 |
| Osteoblast surface/bone surface (%) | 19.0 ± 0.8 | 19.1 ± 1.0 |
| Osteoclasts/10,000 μm (100 μm) | 10.7 ± 0.7 | 10.8 ± 0.7 |
| Eroded surface/bone surface (%) | 24.2 ± 1.4 | 24.0 ± 1.6 |
| Mineral apposition rate (μm/day) | 0.7 ± 0.08 | 0.9 ± 0.03* |
| Bone formation rate/bone surface (μm²/μm²/day) | 0.010 ± 0.003 | 0.027 ± 0.005* |

* Significantly different from controls, p < 0.05.

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According to the results obtained in ST-2 cells, grem1 down-regulation in MC3T3 cells enhanced the stimulatory effect of BMP-2 on the transactivation of the 12×SBE-Oc- pGL3 reporter by 2 to 7 fold and on alkaline phosphatase activity by about 1.5 fold (Fig. 3B and D). These results indicate that grem1 is an endogenous BMP antagonist that opposes BMP effects on Smad signaling and on osteoblast maturation and function. In accordance with these effects, down-regulation of grem1 in ST-2 cells enhanced the stimulatory effect of BMP-2 on osteocalcin and Runx-2 mRNA expression (Table 6).

To explore additional mechanisms involved in the effects of grem1 oligonucleotides, we tested whether its down-regulation modified Wnt/β-catenin signaling and Wnt effects in ST-2 stromal cells, which are less differentiated and responsive to Wnt 3 than the more mature MC3T3 cells (32, 33, 36). Wnt3a caused a dose dependent increase in...
the transactivation of the Wnt/β-catenin dependent 16XTCF-Luc reporter construct and on alkaline phosphatase activity (Fig. 4A and B). Gremlin down-regulation enhanced the stimulatory effect of Wnt 3a on the transactivation of the Wnt dependent 16XTCF-Luc reporter construct by 2.5 to 3.5 fold and on alkaline phosphatase activity by 1.5 to 2 fold, indicating that gremlin has BMP and Wnt/β-catenin antagonizing activity.

Gremlin down-regulation did not modify the expression of FGF-4 in ST-2 cells, and FGF-4 transcripts were virtually undetectable in MC3T3 cells. In an experiment where gremlin mRNA levels were suppressed by >90% following gremlin RNAi, the expression of FGF-4 was (means ± S.E.; n = 3) 0.75 ± 0.2 fgf-4/gapdh copies in control ST-2 cells and 0.85 ± 0.1 fgf-4/gapdh copies in gremlin-silenced ST-2 cells. Levels of SHH mRNA in ST-2 cells were undetectable (data not shown).

**DISCUSSION**

Our findings demonstrate that the conditional deletion of the BMP antagonist gremlin, in the skeletal environment, causes increased trabecular bone volume secondary to increased bone formation and osteoblastic activity. This is the converse phenotype of that displayed by transgenic mice overexpressing gremlin under the control of the osteoblastic specific osteocalcin promoter, which caused an inhibition of bone formation and marked osteopenia (24). *Grem1* haplo-insufficiency did not cause a skeletal phenotype, and this is in accordance with the lack of observable limb developmental abnormalities or other apparent phenotypic alterations in *grem1* heterozygous mice (21, 22). Although studies in *grem1* null mice have demonstrated a role of gremlin in cell survival during limb and kidney organogenesis, conditional deletion of the *grem1* gene in the post natal bone environment did not result in a change in osteoblast number (22). The skeletal phenotype of *grem1* conditional null male mice was transient and observed at 1 month, but not at 3 months of age. This could be attributed to the decline in the activity of the osteocalcin promoter directing the Cre recombinase (43, 44). However, it is possible that gremlin plays a more significant role during the early phases of skeletal growth, and a lesser effect in the adult skeleton. It is of interest that the conditional deletion of *grem1* did not cause an obvious skeletal phenotype in 1-month-old female mice (data not shown). Recently, we reported marked age-related differences in the trabecular bone structure of C57BL/6 mice and noted a particularly unstable phenotype in wild-type young female mice with

**TABLE 6**

|                       | osteocalcin/gapdh | Gremlin RNAi |
|-----------------------|-------------------|--------------|
| Scrambled RNAi        | 1.1 ± 0.4         | 2.1 ± 0.4    |
| BMP-2                 | 0.8 ± 0.5         | 4.1 ± 0.3*   |

|                       | runs-2/gapdh      | Gremlin RNAi |
|-----------------------|-------------------|--------------|
| Control               | 1.1 ± 0.6         | 3.0 ± 0.2*   |
| BMP-2                 | 0.9 ± 0.3         | 4.3 ± 1.2*   |

|                       | grem1/gapdh       | Gremlin RNAi |
|-----------------------|-------------------|--------------|
| Control               | 8.9 ± 3.1         | 0.8 ± 0.1 (p < 0.057) |
| BMP-2                 | 9.0 ± 2.4         | 0.9 ± 0.1*   |

* Significantly different from controls, p < 0.05.
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FIGURE 4. Effect of gremlin down-regulation by RNA interference on Wnt 3a signaling and activity in ST-2 stromal cells. ST-2 cells were cultured to confluence and transfected with gremlin or control scrambled small interfering RNA (siRNA). Down-regulation of gremlin mRNA was documented in parallel cultures by real-time RT-PCR at the completion of the experiment. Gremlin mRNA levels determined in duplicate parallel samples were suppressed from 4.3 to 1.2 (A) and 4.7 to 0.1 (B) grem1/gapdh copies in cells transfected with control scrambled or gremlin siRNA, respectively. A, cells were transfected with 16×TCF-Luc and CMV/β-galactosidase expression vector, 24 h after transfecting the siRNAs. Cells were switched to α-MEM for 6 h and exposed to control medium or Wnt 3a at 0.3 to 2.7 nM for 24 h. Data shown represent luciferase/β-galactosidase activity for cells transfected with scrambled siRNA (white bars) or gremlin siRNA (black bars). B, cells were exposed to control medium or Wnt 3a at 0.8 to 8.1 nM, 24 h after transfecting the siRNAs and cultured for 72 h, and APA was quantified in extracts from cells transfected with scrambled RNA (white bars) or gremlin siRNA (black bars). APA is expressed as nanomoles of p-nitrophenol/min/μg of total protein. Bars represent means ± S.E.; n = 6 observations. *, significantly different between cells transfected with scrambled and gremlin siRNA (p < 0.05).

Conversely, down-regulation of gremlin results in increased Wnt/β-catenin signaling and activity. This sensitization of Wnt/β-catenin signaling could imply direct interactions of gremlin with Wnt or Wnt receptors/co-receptors. Members of the dan/cerberus family of BMP antagonists can inhibit BMP as well as Wnt signaling and activity (13–16). However, direct interactions between gremlin and Wnt and its receptors have not been reported, and therefore the possibility remains that the effect of Gremlin on Wnt signaling is mediated through its suppression of BMP signaling or through as yet undiscovered mechanisms. The sensitization of Wnt signaling by the removal of a BMP antagonist is not surprising, in view of the close relationship between BMP-2 and Wnt effects in cells of the osteoblastic lineage, but the exact mechanisms involved are not fully elucidated, because BMP and Wnt signal through independent pathways.

Although overexpression of various BMP/Wnt antagonists in the skeletal environment can lead to osteopenia, their ultimate physiological role in bone is probably distinct and dependent on their mechanism of action and patterns and levels of expression by skeletal cells (24, 51–53). Gremlin is important in the regulation of BMP activity and skeletal physiology and may have additional functions. Gremlin is expressed by selected cancer-associated stromal cells and has been postulated to favor tumor cell survival and expansion (20). The results observed in mice and cells misexpressing gremlin can be explained by its capacity to bind and block the actions of BMP-2, -4, and -7; however, BMP-independent biological effects of gremlin have not been completely excluded (1, 17). Recent work in endothelial cells has demonstrated pro-angiogenic activity of gremlin and has provided evidence for direct binding of gremlin to endothelial cells and the activation of extracellular regulated kinases (ERK) 1/2 (54). ERK1/2 phosphorylates non-activating sites of Smad, reducing the nuclear accumulation of Smads and their effects on transcription and could contribute to the BMP antagonistic activity of gremlin (55, 56).

In conclusion, gremlin is a physiological antagonist of BMPs in the skeleton, and its deletion or down-regulation sensitizes skeletal cells to the actions of BMP and Wnt, and enhances bone formation in vivo.

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