Fibroblast Growth Factor-2 Isoform (Low Molecular Weight/18 kDa) Overexpression in Preosteoblast Cells Promotes Bone Regeneration in Critical Size Calvarial Defects in Male Mice

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Repair of bone defects remains a significant clinical problem. Bone morphogenetic protein 2 (BMP2) is US Food and Drug Administration–approved for fracture healing but is expensive and has associated morbidity. Studies have shown that targeted overexpression of the 18-kDa low-molecular-weight fibroblast growth factor 2 isoform (LMW) by the osteoblastic lineage of transgenic mice increased bone mass. This study tested the hypotheses that overexpression of LMW would directly enhance healing of a critical size calvarial bone defect in mice and that this overexpression would have a synergistic effect with low-dose administration of BMP2 on critical size calvarial bone defect healing. Bilateral calvarial defects were created in LMW transgenic male mice and control/vector transgenic (Vector) male mice and scaffold with or without BMP2 was placed into the defects. New bone formation was assessed by VIVA-computed tomography of live animals over a 27-week period. Radiographic and computed tomography analysis revealed that at all time points, healing of the defect was enhanced in LMW mice compared with that in Vector mice. Although the very low concentration of BMP2 did not heal the defect in Vector mice, it resulted in complete healing of the defect in LMW mice. Histomorphometric and gene analysis revealed that targeted overexpression of LMW in osteoblast precursors resulted in enhanced calvarial defect healing due to increased osteoblast activity and increased canonical Wnt signaling. (Endocrinology 155: 965–974, 2014)
isoform. In rodents, there are 2 HMW isoforms of 21 and 22 kDa and a LMW 17.5-kDa FGF2 isoform that is exported from cells (6, 7). We previously reported that global overexpression of all isoforms of human FGF2 protein resulted in dwarfism in mice (8) and decreased bone mineral density (BMD) and bone mass (9). However, targeted overexpression of LMW in the osteoblastic lineage resulted in increased BMD and bone mass in mice (10). In contrast, targeted overexpression of the nuclear HMW isoform in osteoblastic lineage resulted in dwarfism, decreased BMD and bone mass, and hypophosphatemia in mice via modulation of FGF23 and Klotho (11).

Although FGF2 has been shown to be important in bone healing (12, 13), very few studies on the effects of FGF2 on mouse calvarial defect healing have been performed (14–16) and only one had a critical size defect model (14). Furthermore, there have been no reported in vivo studies examining targeted overexpression of FGF2 and very low concentrations of exogenous bone morphogenetic protein 2 (BMP2) in the experimental model of healing of a critical size defect. The first aim of this study was to test the hypothesis that targeted overexpression of LMW in the osteoblastic lineage would directly enhance healing of calvarial defects in the mice. Second, we determined whether overexpression of LMW would be additive or synergistic with low-dose administration of BMP2 on calvarial bone defect healing.

In this study, we also examined the putative role of Wnt signaling in the repair process mediated by LMW and BMP2. We recently reported that BMP2 enhanced in vitro OB differentiation and mineralization of OB progenitors obtained from neonatal LMW mice and that modulation of BMP2-specific Smads (Smad1/5/8) signaling was not the mechanism for this enhancement because BMP2 treatment simultaneously increased Smad1/5/8 in OBs from Vector and LMW mice (17). Therefore, in this study, we examined the potential role of modulation of Wnt signaling in the enhanced defect healing in LMW mice treated with BMP2 because Wnt proteins are a family of secreted glycoproteins that play important roles in skeletal development (18) as well as postnatal bone formation (19, 20). To further support examination of the Wnt signaling pathway, increased bone formation in LMW mice has been reported to be associated with reduced expression of the Wnt antagonist secreted frizzled receptor 1 (sFRP1) (10). In addition, an assay of Wnt/β-catenin–mediated transcription showed increased T-cell factor (TCF) luciferase activity in bone marrow stromal cells from LMW mice (10). Furthermore, previous studies showed that the BMP2 and Wnt/β-catenin signaling pathways cooperatively regulate OB differentiation and bone formation (21).

Materials and Methods

Vector and LMW transgenic mice

We previously reported on the detailed development of the LMW and control/vector transgenic mice (10). In brief, Col3.6-LMW-ires-green fluorescent protein-sapphire (GFPsaph) was built by replacing a chloramphenicol acetyltransferase fragment in previously made Col3.6-CAT-ires-GFPsaph (22) with LMW isoforms of human Fgf2 cDNA (accession number M27968.1). This expression vector concurrently overexpresses LMW and GFPsaph from a single bicistronic mRNA. The Col3.6-ires/ GFPsaph (Vector) construct was also prepared as a control. The construct inserts were released from Col3.6-ires/green fluorescent protein (GFP) or Col3.6-LMW-ires-GFPsaph by digestion with Asel and AflII. Microinjections into the pronuclei of fertilized oocytes were performed at the Gene Targeting and Transgenic Facility at the University of Connecticut Health Center. Founder mice of the F2 (FVBN) strain were bred with wild-type mice to establish individual transgenic lines. The University of Connecticut Health Center Institutional Animal Care and Use Committee approved all animal procedures. Male mice at 2 months of age were used in the present study. GFP expression was seen in bone-forming OBs and osteocytes in these mice (10).

Surgical procedures

Using a diamond-coated trephine bur, bilateral full-thickness 3.5-mm calvarial defects were created in the central, nonsuture-associated area of the left and right parietal bones of LMW mice (n = 5) and Vector mice (n = 4). The dura was left intact. Scaffolds (3.5-mm diameter and 1.5-mm thickness), consisting of 70% type 1 collagen and 30% hydroxyapatite (HEALOS; OrApharma, Inc) were placed in the defects. After placement of the scaffold, the skin was sutured with synthetic absorbable suture (6–0 Vicryl; Ethicon, Inc). In a second set of experiments, subgroups of surgically treated LMW mice (n = 7) and Vector mice (n = 8) received either 0.5 μg/5 μL/scaffold recombinant human (rh) BMP2 or PBS treatment delivered via scaffolds. Escherichia coli–expressed nonglycosylated rhBMP2 was used in these studies and was prepared as described previously (23). The mice were euthanized 4 weeks later. All mice received peritoneal cavity injections of fluorescent bone labels calcein (CA) and xylenol orange (XO) on day 7 and day 2 before euthanasia, respectively.

Radiography

New bone formation at the defect sites was assessed by VIVA-CT, an in vivo micro-computed tomography (μ-CT), of live animals (VIVA-CT) at 4, 6, 10, 16, 24, and 27 weeks postsurgery. A gaussian filter was used to suppress noise. High-resolution scanning in-plane pixel size and slice thickness of 20 μm was performed. The computed tomography scanner’s built-in software was used to make a 3-dimensional reconstruction (TRI/3D-Bon; RATOC). A cylindrical region of interest (ROI) with a diameter of 3.5 mm and 1.5-mm height was selected for analysis. Because the margins of the original defect were recognizable, the ROI was set where the original defect was located. The gray values and the number of voxels with the corresponding gray value were calculated in the ROI. Because the attenuation of the hydroxyapatite (HA) was much lower than that of bone, HA was radiolucent at the scanning resolution. At 4 or 29 weeks after surgery, μ-CT was performed and digital x-rays of the isolated
cranial bone were taken at 33 kV for 3 seconds. In combination with μ-CT, the nonfilled defect area was quantified from the x-ray images.

**Histologic analysis**

At 4 or 29 weeks, the mice were euthanized. Calvaria were harvested and fixed in formalin for 24 hours and immersed in 30% sucrose for 1 day. Then the tissue was frozen embedded and cryosectioned on to Cryofilm type II C tape (Finetec). Then 7-μm sections were taken through the middle of the defect and stored at −20°C until used. Sections were scanned at ×100 for GFP-saph, CA, XO, and bright field using a Zeiss Axioplan 200 inverted microscope with a Zeiss AxioCam color digital camera. The CA and XO were detected with enhanced GFP and a TRITC filter, respectively. The sections were then stained with hematoxylin and eosin (H&E), von Kossa stain, and alkaline phosphatase (ALP), respectively. Bone histomorphometric measurements were made in a blinded, nonbiased manner. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (24). The bone volume (BV), interlabel thickness, and mineral apposition rate (MAR) were measured using OsteoMeasure software (Osteometrics, Inc).

**Immunofluorescent staining**

The sections were washed in 1× PBS/1% fetal bovine serum (FBS) and permeabilized with 0.25% Triton X-100 in 1× PBS/1% FBS for 10min. After being rinsed with 1× PBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody at room temperature for 1 hour. The sections were washed in 1× PBS/1% FBS, sections were incubated with biotinylated goat anti-rabbit secondary antibody at room temperature for 1 hour. Nuclear counterstaining was with 4′,6-diamidino-2-phenyindole. Tissue sections were mounted with UltraCruz Mounting Medium (Santa Cruz Technology).

**mRNA isolation and gene expression**

Total RNA was extracted from the parietal bones using TRIzol reagent. For real-time quantitative RT-PCR analysis, RNA was reverse-transcribed by the SuperScript First-Strand Synthesis System. Quantitative PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN) on a MyiQ instrument (Bio-Rad Laboratories Inc). The primer sequences for the genes of interest are shown in Supplemental Table 1 published on The Endocrine Society’s Journals Online web site at http://end.endojournals.org. β-Actin was used as an internal reference for each sample. mRNA was normalized to the β-actin mRNA level and is expressed as the fold change relative to the Vector-PBS group.

**Statistical analysis**

Experimental values are reported as means ± SE. The t test or ANOVA followed by the least significant difference for post hoc multiple comparisons was used. SPSS software was used for statistical analysis, and the results were considered significantly different at a value of *P* < .05.

**Results**

**Macroscopic observation**

The mice exhibited no gross change in appearance, feeding, or weight loss during the 29-week period of postsurgical follow-up. No complications were observed. All animals reached the planned study time points of 4 or 29 weeks. At sample harvest, the scaffolds were clearly visible, and all were located in the defect (data not shown).

**Time course VIVA-CT comparative analysis of calvarial defect healing**

To assess the healing process in living mice, we measured the rate of healing of the defects by VIVA-CT of the same mice at 4, 6, 10, 16, 24, and 27 weeks as shown in Figure 1A. At all time points there appeared to be greater healing of the defects in LMW mice. Quantitative analysis of the VIVA-CT images was also performed. As shown in Figure 1B, BV was greater in LMW mice than in Vector mice at all time points, indicating that the 18-kDa overexpression enhances bone defect repair as early as 4 weeks. There was a further significant increase in BV at 27 weeks compared with that at 4 weeks in LMW mice. BV in the ROI was significantly increased at 27 weeks compared with that at 4 weeks in Vector mice.

**Radiologic and histomorphometric assessment of bone-filling area of the calvarial defect**

Digital radiography was performed to visualize the bone-filling area of the defects of the calvaria after euthanization of the mice at 29 weeks postsurgery. Representative x-ray images are shown in Figure 2A. The nonbone-filling area is smaller in LMW mice (note arrows). Quantified analysis of the nonfilling area of the calvarial defects from x-ray images is shown in Figure 2B. In both

**Figure 1.** Time course VIVA-CT analysis of calvarial defect healing in Vector and LMW mice. A, Representative VIVA-CT images of the calvarial defect healing. Arrows show greater healing in the LMW mice. B, BV was quantified from the VIVA-CT images. At all time points, the BV was greater in LMW mice than in Vector mice. *P < .05, Vector vs LMW mice; #, *P < .05, compared with 4 weeks of the corresponding genotype. Values are means ± SE for 4 defects/group.
Figure 2. Radiographic analysis of calvarial defect healing in Vector and LMW mice. A, Representative x-ray images. At 29 weeks postsurgery, the non–bone-filling area is smaller in the LMW mice (arrows). B, Quantified analysis of the nonfilling area of the calvarial defect from x-ray images. *, P < .05, Vector vs LMW mice. Values are means ± SE for 4 to 5 defects/group.

Figure 3. Histomorphometric analysis of calvarial defect healing in Vector and LMW mice. A, Representative fluorescent scanning images of the calvarial defects. High-magnification fluorescent scanning (Figure 3A) showed that OBs (blue, shown by arrowhead) were depositing new bone indicated by CA (green) and XO (red) labeling. ALP-positive OBs as well as ALP-negative fibroblasts filled the defect area. New bone formation occurred outward from the calvarial defect margin as well as in isolated bone islands surrounded by ALP-positive tissue (Figure 3B). H&E staining did not show any chronic inflammatory reaction such as lymphocyte, macrophage, or plasma cell infiltration (Figure 3C). Consistent with x-ray findings, von Kossa staining (Figure 3D) showed that most new bone formed at the defect edges with a few small islands of bone in the center of the defect. The unfilled defect area was smaller in the LMW group than in the Vector group. As shown in Figure 3E, there was increased distance of CA and XO double mineral labeling in the LMW than in the Vector mice. Quantitative histomorphometry analysis showed that the interlabel thickness (Figure 3F) and MAR (Figure 3G) were significantly increased in LMW mice by 70% and 73%, respectively, compared with those in Vector mice (8.38 ± 0.66 μm vs 4.86 ± 0.50 μm and 1.68 ± 0.30 μm/d vs 0.97 ± 0.19 μm/d).

Radiographic, μ-CT and histomorphometric analysis of the effect of BMP2 treatment on calvarial defect healing in Vector and LMW mice

Because the defect was not completely healed in LMW mice, we examined the effect of a low concentration of BMP2 on the healing process at 4 weeks in both genotypes as shown in representative x-ray images (Figure 4A) and μ-CT images (Figures 4, B and C). Consistent with the 29-week data, digital x-ray and μ-CT analysis at 4 weeks showed enhanced but incomplete calvarial defect healing in LMW mice relative to that in Vector mice. BMP2 treatment alone at a low dose resulted in incomplete healing of the defect compared with that for PBS treatment in Vector mice. Interestingly, healing of the defect in BMP2-treated Vector mice was still less than that observed in PBS-treated LMW mice. However, BMP2 treatment of LMW mice completely healed the defect. As shown in Figure 4D, in the presence of LMW, BMP2 almost completely filled the defect area. As shown in Figure 4E, newly formed BV in the calvarial defect area was quantified from μ-CT images. BV was significantly increased in LMW mice compared with that in Vector mice. BMP2 significantly increased BV in Vector mice, but the increase was smaller than that observed in LMW mice treated with PBS. Histomorphometric analysis was performed on excised calvaria to further assess the effects of 4 weeks of BMP2 treatment on defect healing in both genotypes. As shown in Figure 5A and Supplemental Figure 2, H&E and von Kossa staining showed that most newly mineralized tissue formed in the center of the defect in the Vector-BMP2 group. In contrast, most newly mineralized tissue formed at the defect edges with a few small islands of bone in the center of the defect.
in the LMW-PBS group. However, most newly mineralized tissue formed at both the defect edges and the center of the defect in the LMW-BMP2 group. Representative images of GFP, CA, and XO double labeling are shown in Figure 5B and Supplemental Figure 3. As shown in Box 1, the central area of the defect, GFPsaph-positive OBs (blue, shown by arrowhead) were actively depositing mineralized bone shown by CA (green) and XO (red) labeling in the groups with BMP2 treatment (Vector-BMP2 and LMW-BMP2). On the other hand, images in Box 2 show active bone formation at the edge of the defect with LMW (LMW-PBS and LMW-BMP2). Evaluation of the bone regenerative capacity by histomorphometry is shown in Figure 5, C and D. Dynamic histomorphometry showed that BMP2 treatment significantly increased BV and MAR in both Vector and LMW mice. However, in the presence of LMW, BMP2 further increased the bone volume compared with PBS-treated mice from each genotype (P < .05).

**Effect of BMP2 treatment on fibroblast growth factor receptors and bone marker genes expression in calvarial defect healing in Vector and LMW mice**

To investigate the mechanism of the enhanced healing, we examined the expression of Fgf2, Bmp2, Fgfr1, Fgfr2, and OB-related genes, Runx2 and osterix, as well as osteocalcin (OCN), a marker of differentiated OBs in calvaria from both genotypes treated with or without BMP2. Basal Fgf2 mRNA was significantly increased in LMW compared with that in Vector mice. BMP2 treatment significantly increased Fgf2 mRNA expression in LMW mice (Supplemental Figure 4A). BMP2 treatment significantly increased endogenous Bmp2 mRNA expression to a similar extent in both Vector and LMW mice (Supplemental Figure 4B). As shown in Figure 6A, BMP2 treatment significantly increased Fgfr1 and Fgfr2 mRNA expression in Vector mice. Basal Fgfr1 and Fgfr2 mRNA were significantly increased in LMW mice compared with those in Vector mice. Runx2 mRNA was similar in LMW and Vector mice; however, BMP2 caused a significant increase in Runx2 mRNA in LMW mice. Osterix mRNA was similar in Vector and LMW mice; however, BMP2 significantly increased osterix mRNA in LMW mice. OCN mRNA was significantly increased in LMW
mice compared with that in Vector mice; BMP2 treatment significantly increased OCN in Vector mice. However, there was a synergistic increase in OCN mRNA in BMP2-treated LMW mice.

**Effect of BMP2 treatment on Wnt signaling in calvarial defect healing in Vector and LMW mice**

Because we previously reported that increased bone formation in LMW mice was associated with reduced Wnt inhibitor Sfrp1 and increased β-catenin expression in OBs (10), we examined whether enhanced calvarial defect healing in LMW mice was associated with modulation of the components of the Wnt signaling pathway, especially β-catenin in the absence and presence of BMP2. As shown in Figure 7A, Sfrp1 mRNA expression was significantly decreased in LMW mice compared with that in Vector mice. In the presence of overexpression of LMW, BMP2 treatment significantly increased Wnt receptor Lrp5 and Wnt ligand (Wnt10b) mRNA (Figure 7A). There was no increase in Lrp6 mRNA (data not shown). Also shown in Figure 7A, β-catenin mRNA was increased in LMW mice compared with that in Vector mice; BMP2 treatment increased β-catenin in Vector mice, and there was an additive increase in β-catenin mRNA in BMP2-treated LMW mice. Immunohistochemical staining (Figure 7B and Supplemental Figure 5) showed a greater increase in β-catenin protein expression in LMW BMP2-treated calvaria than in LMW PBS-treated calvaria.

**Discussion**

We previously demonstrated that the LMW isoform of FGF2 is a critical determinant of endochondral bone formation (10). The results of the present study demonstrate that LMW overexpression also enhanced bone healing in mouse calvarial defects. Thus, LMW enhances both endochondral as well as intramembranous bone formation. This study also shows that most of the calvarial defect healing occurred within the first 4 weeks postsurgery; therefore, we posit that LMW plays a role in the initial phase of bone regeneration. In support of this, FGF2 is most abundantly accumulated in bone matrix and expressed from the early stages of bone formation (25), and FGF2 is known to improve angiogenesis, which is an important process for early bone formation (26). FGF2 is also known to induce expression of differentiation factors such as BMPs and TGF β (27).

The late stage of bone formation, at which point mineralization is occurring, requires structural proteins such as collagen type 1 and osteocalcin (28). Collagen type 1 and carbonated apatite mineral are major extracellular components of bone; therefore, scaffolds consisting of type 1 collagen and HA, which is similar to bone mineral have been applied to facilitate bone regeneration (1). Type 1 collagen and HA have been reported to not only enhance OB differentiation, but also together to accelerate osteogenesis (29, 30). Our study used a commercially available scaffold consisting of type 1 collagen and HA. However, in the Vector mice, there was no significant healing of the defect by scaffold alone in the absence of growth factor. Very few studies have been performed to evaluate the effect of FGF2 administration in calvarial defect healing. In one of these studies (31), a collagen hydrogel sponge composite containing 0, 3, and 15 μg of FGF2 was implanted in the 4 × 4 × 0.2-mm size rat calvarial defects. New bone formation in both the 3 and 15 μg of FGF2 groups was significantly higher than that in non-FGF2 groups; however, these were noncritical size defects. Interestingly, local application of a single injection of the gelatin hydrogel containing either placebo or 0.8 mg of rhFGF2 accelerated and completed bone healing in tibial shaft fractures (32). However, although FGF2 administration is useful in augmenting the healing of small size defects such as bone fractures, it clearly did not completely heal a critical size calvarial defect as a single agent in the present studies.

BMP2 promotes OB differentiation and has been widely used to promote bone healing in patients; however, there are issues of cost due to large dose requirements, as
well as morbidity associated with ectopic bone formation and excessive bone resorption (1). Because overexpression of LMW did not completely heal the critical size calvarial defect, we investigated whether a very low concentration of BMP2, which alone should not result in healing of a critical size defect in Vector mice, would further enhance healing in LMW mice. Consistent with our hypothesis, a very low concentration of BMP2 did not heal the defect in Vector mice, but completely healed the defect in LMW mice. Similar to our observation, combined administration of rhBMP2 and FGF2 in rats with a parietal defect produced a higher degree of calcification and greater new bone formation than those in the group treated with rh-BMP2 alone (33). However, in that study, there were no groups that received FGF2 as a single agent or were treated with vehicle only (34). The histomorphometric analysis of our study demonstrated that overexpression of LMW increased BV and MAR in LMW mice compared with those in Vector mice and that BMP2 increased BV and MAR in Vector mice to an extent similar to that observed in the non–BMP2-treated LMW mice. However, the addition of BMP2 caused a further significant increase in BV and MAR in LMW mice, suggesting that BMP2 further enhances osteoblastic lineage recruitment and differentiation and mineralization in LMW mice. It is also possible that BMP2 potentiation is mediated by FGF2 bioavailability to the lesion via stimulation of processing/proteolysis of heparan sulfate glycosaminoglycans. Studies by Colin (35) suggested that the heparan sulfate proteoglycan (HSPG)–dependent internalization and catabolism pathway controls the in vivo bioavailability of FGF2. Other studies showed that the glycosaminoglycan chains located in HSPG bind basic FGF, promoting its mitogenic and angiogenic activities (36). Because BMP2 binds immobilized heparin and heparan-like polymers (37, 38), its biological activity may also be modulated by the glycosaminoglycan chains located in HSPG.

To explore the mechanism of enhanced calvarial defect healing in LMW mice in the absence and presence of BMP2 and because transcription factors Runx2 and osteonectin are important in OB progenitor recruitment and differentiation, we examined whether their expressions were differentially modulated. Interestingly both transcription factors were only increased in LMW mice treated with BMP2. However, in this study, these genes were examined at a late stage in the bone-healing process of OB differentiation and mineralization. FGF2 induces OCN (34), and we previously reported that partial or complete disruption of FGF2 expression in mice reduced OCN gene expression that was associated with decreased bone formation in OBs (12). Interestingly, OCN was increased in PBS-treated LMW mice to an extent similar to that observed in BMP2-treated Vector mice. However, BMP2 caused a synergistic increase in OCN in LMW mice treated with BMP2. However, in this study, these genes were examined at a late stage in the bone-healing process of OB differentiation and mineralization. FGF2 induces OCN (34), and we previously reported that partial or complete disruption of FGF2 expression in mice reduced OCN gene expression that was associated with decreased bone formation in OBs (12). Interestingly, OCN was increased in PBS-treated LMW mice to an extent similar to that observed in BMP2-treated Vector mice. However, BMP2 caused a synergistic increase in OCN in LMW mice, suggesting either enhanced OB differentiation and or cross talk between the FGF2 and BMP2 signaling pathways. Although classically BMPs signal via serine/threonine kinase receptors to regulate bone development (1) and maintain adult skeleton by modulating mesenchymal differentiation (1), we previously reported that endogenous FGF2 is necessary for maximal bone formation because BMP2-induced bone formation was markedly impaired in Fgf2-null mice (39). Furthermore, we reported that although BMP2 was able to activate downstream signaling molecules SMAD1/5/8 in OBs, it was unable to promote SMAD/RUNX2 protein nuclear accumulation in OBs lacking endogenous FGF2.
Interestingly, we recently reported, using calvarial OBs from Vector and LMW mice, that BMP2 caused a sustained increase in phosphorylated SMAD1/5/8, RUNX2, and osterix protein in both Vector and LMW mouse calvarial OBs (17), supporting our hypothesis of involvement of other signaling pathways for the enhanced calvarial healing response to BMP2 treatment in LMW mice.

LMW enhances bone formation via activation of FGF receptors (1) and consistent with our previous in vitro finding (17), we observed that basal Fgfr1 and Fgfr2 mRNA were significantly increased in LMW mice relative to those in Vector mice. It is conceivable that augmented Fgfr1 and Fgfr2 expression contributes to the enhanced LMW calvarial defect healing.

We investigated the role of Wnt/β-catenin in calvarial defect healing in LMW and BMP2-treated LMW mice because Wnt signaling is important in bone formation and the maintenance of bone mass (10, 41). Sfrp1 mRNA was decreased in LMW and in BMP2-treated Vector transgenic calvarial defect healed bone. However, there was no further decrease in LMW mice treated with BMP2, suggesting a common mechanism of reduction. Interestingly, there was an additive effect of BMP2 on Lrp5 mRNA expression, whereas Wnt10b mRNA was only increased in LMW mice treated with BMP2, suggesting that more than one signaling pathway is involved. Our observation that β-catenin is increased in LMW calvarial defect healing is novel because there are no published reports of FGF2 modulating Wnt signaling during bone healing. The synergistic increase in β-catenin in BMP2-treated LMW mice is also interesting and novel. Other investigators reported that BMP2 enhances Wnt/β-catenin signaling (42) and that Wnt and BMP pathways cooperatively control bone formation because Wnt promotes differentiation of mesenchymal progenitors into OB progenitors, whereas BMP signaling stimulates their differentiation into mature OBs. In addition, both pathways mediate further differentiation as demonstrated by increased alkaline phosphatase and mineralization, resulting in increased bone formation (43). Based on the results of the present study, as well as the previously published data from other groups, we propose a model (Figure 8) to demonstrate the mechanism of action of BMP2 and FGF2 on OB precursors and OB populations during fracture repair. The administration of exogenous BMP2 in vivo increased fracture healing and caused an even greater increase in the presence of the LMW FGF2 isofrom. It has already been demonstrated that BMP2 and LMW positively regulate each other’s expression (39, 44). Fakhry et al (44) reported that exogenous LMW increases BMP2 in calvarial OBs. The reverse was also reported because BMP2 increased the expression of LMW (39) (Supplemental Figure 4A). These dual increases drive the pathways to promote OB differentiation. An increase in LMW blocks secreted frizzled receptor protein from inhibiting Wnt10b (Figures 7A and 8). This allows the Wnt ligand to bind the LRP5-frizzled receptor complex, thereby activating the canonical Wnt pathway (Figs. 7A and 8). LRP5 and Wnt10b mRNA were significantly increased in the LMW mice treated with BMP2 (Figs. 7A and 8). Disheveled (DVL), recruited by the LRP5-frizzled receptor complex inhibits the Axin-APC-GSK3β destruction complex from ubiquitinating β-catenin (37). β-Catenin was increased in LMW as well as both BMP2-treated Vector and LMW mice (Figure 7A). Accumulation of β-catenin allows it to translocate across the nuclear membrane and bind the TCF/Leff transcription factors to promote transcription of Runx2, osterix, and OCN (Figs. 6B and 8). Similarly, BMP2 promotes translation of the same differentiation factors by phosphorylating Smad1 through BMP receptors (39).

Figure 8. Model of cross talk between FGF2 and BMP2 bone mineralization pathways. Exogenous BMP2 and overexpression of endogenous LMW increases expression of FGFR1 and FGFR2. This results in repression of sFRP1 and up-regulation of LRPS and Wnt10b. Binding/activation of the Wnt receptor/Wnt ligand blocks activation of the GSK3β destruction complex and ubiquitination of β-catenin. This allows up-regulated β-catenin to accumulate and enter the nucleus, promoting the expression of Runx2, OCN, and osterix through the TCF/Leff transcription factors. BMP2 increases phosphorylation of Smad proteins, which can also bind the TCF/Leff transcription factors further, promoting the differentiation factors and bone mineralization.
Consistent with this report, we previously reported that phosphorylation of Smad1/5/8 was increased in BMP2-treated OBs from LMW mice (17). The up-regulation of Runx2, osterix, and OCN causes the OB precursors to differentiate and begin mineralization of new bone during fracture healing (Figure 8).

In summary, LMW overexpression can enhance bone healing in critical size calvarial defects in mice but requires other factors such as BMP2 to improve the later stage of bone formation in critical size defects.

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