Published in final edited form as:

Bone. 2015 June ; 75: 151–160. doi:10.1016/j.bone.2015.01.008.

Dkk1 haploinsufficiency requires expression of Bmp2 for bone anabolic activity

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Abstract

Bone fractures remain a serious health burden and prevention and enhanced healing of fractures has been obtained by augmenting either BMP or Wnt signaling. However, whether BMP and Wnt signaling are both required or are self-sufficient for anabolic and fracture healing activities has never been fully elucidated. Mice haploinsufficient for Dkk1 (*Dkk1*^{+/-}) exhibit a high bone mass phenotype due to an up-regulation of canonical Wnt signaling while mice lacking Bmp2 expression in the limbs (*Bmp2*^{c/c}; *Prx1*::cre) succumb to spontaneous fracture and are unable to initiate fracture healing; combined, these mice offer an opportunity to examine the requirement for activated BMP signaling on the anabolic and fracture healing activity of Wnts. When *Dkk1*^{+/-} mice were crossed with *Bmp2*^{c/c}; *Prx1*::cre mice, the offspring bearing both genetic alterations were unable to increase bone mass and heal fractures, indicating that increased canonical Wnt signaling is unable to exploit its activity in absence of Bmp2. Thus, our data suggest that BMP signaling is required for Wnt-mediated anabolic activity and that therapies aimed at preventing fractures and fostering fracture repair may need to target both pathways for maximal efficacy.

Keywords

Dkk1; Wnt signaling; Bmp2; BMP signaling; bone homeostasis; fracture healing

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Disclosures

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Introduction

Wnt signaling plays an essential role in the regulation of bone homeostasis and fracture repair (1–3). Activation of Wnt signaling by means of secreted Wnts leads to increased bone formation. This anabolic activity is regulated by the expression of several Wnt antagonists, such as secreted frizzled-related proteins (Sfrps), Wnt-inhibitory factor 1 (Wif1), Dickkopf 1 (Dkk1) and Sclerostin (Sost). Sfrps and Wif1 act as decoy receptors and can antagonize both canonical and non-canonical Wnts whereas Dkk1 and Sost, by binding to the LRP5/LRP6 Wnt receptors, specifically inhibit canonical Wnt signaling.

Several lines of evidence have shown that Dkk1 plays an important role in limb morphogenesis and head induction (4–6) as well as in bone formation and bone disease. For instance, overexpression of Dkk1 in osteoblasts leads to osteopenia (7) and local delivery of DKK1 impairs bone healing (8). Dkk1 haploinsufficiency has been shown to cause an increase in bone formation and bone mass (9) and antibody-mediated neutralization of endogenous DKK1 has been shown to promote fracture healing (10, 11). Thus, new therapeutic approaches for treating bone loss or recalcitrant fractures are exploring the enhancement of Wnt signaling by inactivation of Dkk1 as a means to assure bone mass augmentation and successful fracture repair.

Bone morphogenetic proteins (BMPs) have also been described to have an important role during bone development and fracture repair (12–14). Mice lacking limb skeletal expression of Bmp2 present with spontaneous fractures and lack of fracture repair due to absence of proliferation and subsequent differentiation of periosteal cells into callus forming cells during the early stages of fracture repair (15). In fact, Bmp2 has been described as an early response gene during the fracture healing process, with highest levels of expression reached 24 to 48 hours after the initial injury has occurred (16). Furthermore, recent evidence obtained from small numbers of patients suggests that reduced endogenous BMP signaling may predispose individuals to develop atrophic non-union in patients suffering traumatic fractures (17–19); in fact, BMP treatment has been shown to enhance healing in this population (20–25).

Therefore, of special interest for the development of novel and effective therapeutic approaches to treat bone loss and recalcitrant fractures is the interaction that occurs between Wnt signaling and BMP signaling. Whether BMPs and Wnts are both required or are self-sufficient in the osteoblast differentiation process is still a matter of investigation; understanding this interplay may influence the development of highly effective therapeutic approaches to augment bone mass and favor fracture repair

The existence of a mouse with heterozygous expression of Dkk1 that shows augmented bone formation and bone mass due to the up-regulation of the canonical Wnt signaling (Dkk1^{+/-} mice) (9) and a mouse with conditional inactivation of Bmp2 that shows occurrence of spontaneous fractures and inability to heal bone fractures (Bmp2^{c/c};Prx1::cre mice, hereafter for brevity Bmp2-Prx1 mice) (15) provides us with the opportunity to query whether a hierarchy exists between the Wnt and BMP pathways, and determine if this hierarchy must be maintained for successful therapeutic approaches. Our results indicate that the up-

regulation of canonical Wnt signaling by means of Dkk1 haploinsufficiency is unable to increase bone mass and fracture repair when BMP signaling is absent due to Bmp2 inactivation.

Materials and Methods

Animals

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the Harvard Medical Area Institutional Animal Care and Use Committee. Mice carrying floxed Bmp2 alleles (Bmp2^{c/c}) were crossed with mice heterozygous for Dkk1 (9)(Dkk1^{+/-} mice) to obtain Dkk1^{+/-};Bmp2^{c/c} mice; these mice were subsequently bred to heterozygous Prx1::cre mice (26) to obtain the following littermates: 1) Bmp2^{c/c} mice heretofore referred to as wild-type (WT) mice; 2) Dkk1^{+/-} mice; 3) Dkk1^{+/-}; Bmp2^{c/c};Prx1::cre mice identified as Dkk1^{+/-};Bmp2-Prx1 mice; and 4) Bmp2^{c/c};Prx1::cre mice identified as Bmp2-Px1 mice.

Mice were born at the expected Mendelian ratios. Tail biopsies were collected for genotyping by PCR as described by Tsuji et al. (15) and by Morvan et al. (9).

Dkk1 haploinsufficient mice were kindly provided by Dr. Roland Baron (HSDM) upon permission by Dr. Heinrich Westphal (NIH/NICHD).

Quantification of gene expression by nanoString nCounter technology

Femurs of 15-week-old mice (n=6) were used for this analysis. Each group/genotype consisted of the following: WT mice (2 females and 4 males); Dkk1^{+/-} mice (4 females and 2 males), Dkk1^{+/-};Bmp2-Prx1 mice (4 females and 2 males); and Bmp2-Px1 mice (5 females and 1 males). Femurs were collected and bone marrow was flushed out after removal of the epiphyses. Total RNA was extracted utilizing Trizol Reagent (Life Technologies Inc., Grand Island, NY, USA) following the manufacturer instructions. Molecular counts for Axin2, Cebpa, Ctnnb1, Dkk1, Fzd7, Lef1, Lrp5, Lrp6, Notch2, Opg, Sfrp1, Sfrp2, Sfrp3/FrzB, Sfrp4, Sfrp5, Sost, Wif1, Wnt3, Wnt3a, Wnt5a, and Wnt10b genes (see supplementary Table 1 for accession numbers and targeted sequences) were obtained using nanoString nCounter (nanoString Technologies Inc., Seattle, WA, USA), following the manufacturer protocol (27–31). Statistical analysis was performed using unpaired Student's t-test and results are shown as means ± S.D. Statistical significant difference is indicated as * (p < 0.05).

Micro-computed tomography

Femurs of 15-week-old mice (n=6–7) were used for this analysis. Each group/genotype consisted of the following: WT mice (2 females and 5 males); Dkk1^{+/-} mice (4 females and 2 males), Dkk1^{+/-};Bmp2-Prx1 mice (5 females and 2 males); and Bmp2-Px1 mice (4 females and 3 males).

The long axis of each intact femur was aligned with vertical axis of the specimen tube that rotates within the *ex vivo* micro-computed tomography scanner (μCT40, Scanco Medical, Brüttisellen, Switzerland). While immersed in phosphate buffered saline, the central portion

of all mid-shafts and the metaphyseal region of distal femur were scanned separately using the energy settings of 70 kVp and 145 μ A with 1000 projections per 360° rotation and an integration time of 300 ms to provide images with 12 μ m voxels (isotropic). Following reconstruction, the outer cortex was contoured and the structural parameters computed using standard scripts provided by Scanco. Bone was segmented from air and soft tissue at a threshold of 350 per mille (800 mgHA/cm³) and with a Gaussian noise filter (support of 2 and variance of 0.8). As for the metaphysis, the trabecular compartment was contoured from 0.36 mm to 1.52 mm above the growth plate. Bone was segmented from air and soft tissue at a threshold of 215 per mille (426 mgHA/cm³) and with a Gaussian noise filter (support of 2 and variance of 0.2). Trabecular parameters were computed using the Scanco software. Because the μ CT is calibrated against a hydroxyapatite (HA) phantom, the mean attenuation of all the bone voxels (except surface ones to avoid partial volume effects) provided the tissue mineral density in units of equivalent mineral density.

Biomechanical evaluations

Following μ CT analysis, femurs (15-week-old mice, n=6–7) were loaded to failure in a three point bending configuration to determine differences in biomechanical properties. Each hydrated femur was placed on the lower support points with the anterior side down (i.e., bending about the medial-lateral plane), and loaded at 3.0 mm/min. Forces and displacements were simultaneously recorded from a 100 N load cell (Honeywell, Morristown, NJ) and a LVDT (Dynamight 8841, Instron, Canton, OH), respectively, at 50 Hz. Because the femur lengths and anterior-posterior thickness varied among the genotypes, the span varied among the groups. Thus, the biomechanical properties included the rigidity (3*stiffness/span) and the peak moment (force*span/4) as well as the post-yield deflection (normalized displacement after yielding) and post-yield work-to-fracture (area under the moment vs. normalized displacement curve after yielding). Yielding occurred when the secant stiffness was 15% less than the initial stiffness (slope of the initial linear portion of the force vs. displacement curve), and the normalized displacement, accounting for differences in span, was computed as 12*deflection/span². Material properties of modulus and strength of the cortex were also estimated using standard beam theory (32). The previously described μ CT scans provided the moment of inertia and the distance between the neutral axis of bending and the outermost point in the anterior–posterior direction (c_{MIN}).

Incidence of radial fractures using X-rays

The same mice utilized for the micro-computed tomography and the biomechanical evaluations were utilized to study the incidence of the radial fracture (n=6–7). After harvesting the femurs, X-rays of the upper limbs were taken using Micro50 (Microfocus Imaging, now Faxitron Bioptics LLC, Tucson, AZ, USA) at 50 kV for 100 seconds. Presence of fracture in the distal radius was detected visually and recorded (presence/absence).

Creation of femur fractures and examination of the fracture healing

Unilateral fractures were produced in the right femurs of 8–10 week-old mice using a method previously described (15). Each group/genotype consisted of the following: WT

mice (1 female and 4 males); $Dkk1^{+/-}$ mice (1 female and 3 males), $Dkk1^{+/-};Bmp2-Prx1$ mice (1 female and 2 males); and $Bmp2-Prx1$ mice (2 females and 3 males).

At 5, 10, and 20 days post-fracture 8–10 week old mice were anesthetized and X-rays were taken using Micro50 (Microfocus Imaging, now Faxitron Bioptics LLC, Tucson, AZ, USA) at 50 kV for 100 seconds.

For histological examination, at the indicated time points, femurs were fixed in 4% paraformaldehyde, decalcified in Tris buffer containing 10% EDTA, and embedded in paraffin. Sections (5 μ m) were stained with 0.1% toluidine blue using standard procedures. Digital images were obtained using a Zeiss AxioImager MI Microscope fitted with an AxioCam HRC digital camera and Zeiss AxioVision imaging software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Frequency of bone bridging

The same mice utilized for the creation and analysis of the femur fractures were utilized to evaluate the frequency of the bone bridging (n=3–5). Upon radiological and histological examination 20 days post fracture, the presence of a mature bone collar around both fracture ends was deemed as bone bridging and the presence of fibrotic tissue between the fracture ends and the lack of bone collar around the fracture ends was deemed as nonunion. Frequency of bone bridging in each group was obtained by counting the number of bridging occurrences.

Results

In absence of *Bmp2* mice heterozygous for *Dkk1* lack the high bone mass phenotype

To test whether up-regulation of canonical Wnt signaling by means of *DKK1* haploinsufficiency requires expression of *Bmp2* to increase bone mass, we crossed mice heterozygous for *Dkk1* ($Dkk1^{+/-}$ mice) with mice lacking expression of *Bmp2* in limbs ($Bmp2-Prx1^{cre}$ mice) to obtain WT, $Dkk1^{+/-}$, $Dkk1^{+/-};Bmp2-Prx1$, and $Bmp2-Prx1$ littermates. The quantitative gene expression analysis confirmed significantly lower levels of expression of *Dkk1* in bones of $Dkk1^{+/-}$ mice versus WT mice (Fig. 1A). Lack of expression of *Bmp2* induces a substantial up-regulation of *Dkk1* transcripts that was significantly reduced in bones of mice lacking expression of *Bmp2* and haploinsufficient for *Dkk1* ($Dkk1^{+/-};Bmp2-Prx1$ mice) (Fig. 1A).

Computed tomography analysis of the trabecular bone microarchitecture of the $DKK1^{+/-}$ femurs showed the expected increase in bone volume fraction (BV/TV) when compared to femurs of WT mice (9) (Fig. 1B and C). In contrast, femurs of the $Dkk1^{+/-};Bmp2-Prx1$ littermates revealed a significant decrease in bone volume (BV), indicating a smaller metaphyseal compartment and in segmented bone surface (BS) when compared to femurs of $Dkk1^{+/-}$ mice (Fig. 1B and C). Decrease was significant when parameters were compared to WT mice as well; no statistically significant differences were seen between femurs of $Dkk1^{+/-};Bmp2-Prx1$ mice and femurs of $Bmp2-Prx1$ mice.

No changes in BV/TV were observed between $Dkk1^{+/-};Bmp2-Prx1$ mice and the $Dkk1^{+/-}$ mice and both mice had higher values when compared to WT mice. The higher BV/TV observed in $Dkk1^{+/-};Bmp2-Prx1$ is partly due to the lack of bone expansion observed in the absence of Bmp2 (Fig. 1B and C and (33)) that results in a highly packed bone trabeculae in a smaller total volume. Trabecular thickness and number, 2 micro-architectural parameters, were higher in $Dkk1^{+/-};Bmp2-Prx1$ mice versus WT mice but were not significantly different when compared to the values seen in $Dkk1^{+/-}$ mice. These parameters were equally altered in Bmp2-Prx1 mice (Fig. 1B and C), with no statistically significant difference between $Dkk1^{+/-};Bmp2-Prx1$ mice and Bmp2-Prx1 mice, indicating that lack of Bmp2 is indeed responsible for the observed reduction of the cross sectional area that accounts for the increment to these values.

Micro-computed tomography analysis of the cortical morphology of the femur diaphysis from $Dkk1^{+/-}$ mice reveals a significant reduction of the medullary area (Ma.Ar) and an increase of the cortical area fraction (Ct.Ar/Tt.Ar) when compared to values observed in WT mice. When compared to the femurs of $Dkk1^{+/-}$ mice, the diaphysis for $Dkk1^{+/-};Bmp2-Prx1$ littermates showed a significant alteration of all the parameters that measure cortical bone morphology (Fig. 1B and D) with a reduction of the total cross sectional area (Tt.Ar), the cortical bone area (Ct.Ar), and the medullary area (Ma.Ar). The significant increase of the cortical area fraction (Ct.Ar/Tt.Ar) and the cortical thickness (Ct.Th) are once again due to lack of cross sectional expansion attributable to the absence of Bmp2 (33). In fact, no difference for all the parameters of the cortical bone morphology was observed between $Dkk1^{+/-};Bmp2-Prx1$ mice and Bmp2-Prx1 mice.

Morphological analysis (femur length) showed no differences between femurs of WT mice and femurs of $Dkk1^{+/-}$ mice (Fig. 2A). The biophysical and biomechanical evaluation also showed no differences in the parameters related to bone structure and biomechanical properties such as I_{min} (Moment of Inertia), Rigidity, Yield Moment, and Maximum Moment between femurs of WT mice and femurs of $Dkk1^{+/-}$ mice (Fig 2A). Parameters that express a measurement of the tissue properties that are nominally independent of the bone structure, such as Ct.TMD (Cortical Tissue Mineral Density), Yield Strength, and Bending Strength, were found to be also unchanged. Post Yield Deflection and Post Work-to-fracture which express a measurement of the bone brittleness were also unchanged.

Our analyses however show a profound and significant difference for all the morphological, biophysical and biomechanical parameters tested between femurs of $Dkk1^{+/-};Bmp2-Prx1$ and $Dkk1^{+/-}$ mice. Length was significantly reduced in $Dkk1^{+/-};Bmp2-Prx1$ and the bone structure and biomechanical parameters such as I_{min} , Rigidity, Yield Moment, and Maximum Moment (Fig 2A) were also found significantly decreased. Tissue properties parameters independent of the bone structure, such as Ct.TMD (Cortical Tissue Mineral Density), Yield Strength, and Bending Strength, were found increased whereas Post Yield Deflection and Post Work-to-fracture, the inverse of bone brittleness, were found significantly decreased. However, no significant differences were observed between femurs of $Dkk1^{+/-};Bmp2-Prx1$ and femurs of Bmp2-Prx1 for all the morphological, biophysical and biomechanical parameters tested, indicating that lack of expression of Bmp2 is responsible

for the differences between the femurs of $Dkk1^{+/-};Bmp2-Prx1$ mice and the femurs of $Dkk1^{+/-}$ mice.

The biomechanical evaluations are summarized in two graphs plotting the moment of inertia versus the Peak moment or the Rigidity (Fig 2B and C respectively). They illustrate equal distribution of the $Dkk1^{+/-};Bmp2-Prx1$ femurs and the $Bmp2-Prx1$ femurs, highlighting the substantial structural defect of the femur diaphysis of these mice.

When we measured the incidence of spontaneous fractures in $Dkk1^{+/-};Bmp2-Prx1$ mice (Fig. 3A and B) we found that 100% of them presented with fractures 15 weeks after birth. As expected (15), we also found a 100% incidence of fractures in $Bmp2-Prx1$ mice. $Dkk1^{+/-}$ and WT littermates did not show any sign of spontaneous fractures. This data indicate that the structural defects associated to lack of expression of *Bmp2* are responsible for a physical weakness of the bone and that this weakness is not ameliorated by *Dkk1* haploinsufficiency.

Together, our findings indicate that the anabolic effects of *Dkk1* haploinsufficiency are not permitted in the absence of *Bmp2*.

Mice heterozygous for *Dkk1* and lacking expression of *Bmp2* in limbs cannot repair bone fractures

To test whether mice heterozygous for *Dkk1* and lacking expression of *Bmp2* in limbs can repair bone fractures we induced standardized stable fractures in femur diaphysis of 15 week old WT, $Dkk1^{+/-}$, $Dkk1^{+/-};Bmp2-Prx1$, and $Bmp2-Prx1$ littermates (Figs. 4 and 5).

Serial X-rays (Fig. 4) showed lack of fracture repair in $Dkk1^{+/-};Bmp2-Prx1$ and $Bmp2-Prx1$ mice, as no callus formation was evident at 10 and 20 days post-fracture (p.f.). $Dkk1^{+/-}$ mice, on the contrary, exhibited a normal healing pattern as timing of the callus formation and bone bridging was similar to WT (Fig. 4). When we examined the early healing response 5 days p.f. by means of histological analyses (Fig. 5), we found no evidence of osteo-chondral differentiation of periosteal cells in $Dkk1^{+/-};Bmp2-Prx1$ and $Bmp2-Prx1$ mice. Histological analyses of samples at 20 days p.f. (Fig. 5) showed lack of bone bridging (nonunions) in these mice with 0% frequency of bone bridging (Fig. 5B) whereas WT and $Dkk1^{+/-}$ presented with the formation of a bone bridge between the two segments (Fig. 5A) with 100% frequency of bone bridging (Fig. 5B).

Our experimental findings indicate that absence of *Bmp2* alters the ability of repairing fractures in $Dkk1^{+/-};Bmp2-Prx1$ mice and that expression of *Bmp2* is required in *Dkk1* haploinsufficient mice during fracture repair.

Under conditions of *Bmp2* deficiency *Dkk1* haploinsufficiency normalizes most Wnt inhibitors and reduces expression of *Notch2*

To test whether under conditions of *Bmp2* deficiency *Dkk1* haploinsufficiency is able to alter the expression of genes of the Wnt pathway, we measured the gene expression of 20 genes in femurs of the same 15-week-old littermate mice utilized for the morphological and biomechanical analysis (n=6)(Fig. 6).

Statistical analysis of gene expression between Bmp2-Prx1 and WT mice showed that in absence of Bmp2 there is a significant up-regulation of several Wnt inhibitors (Fig. 6A) along with an up-regulation of canonical Wnt receptors, of β -catenin (Ctnnb1), and of Wnt10b (Fig. 6B). Wnt target genes such as Axin2, and Opg also showed a significant up-regulation in Bmp2-Prx1 (Fig. 6A and C). This data indicate that in absence of Bmp2, Wnt signaling is up-regulated and that such up-regulation is accompanied by an overexpression of Wnt inhibitors.

When we looked at the gene expression levels in mice with Dkk1 haploinsufficiency under conditions of Bmp2 deficiency (Dkk1^{+/-};Bmp2-Prx1 mice), we detected no statistical significant difference with WT mice for all the genes that are up-regulated in the Bmp2-Prx1 mice with the exception of β -catenin (Ctnnb1) (Fig. 6B) and Sfrp4 (Fig. 6A), which are still up-regulated in Dkk1^{+/-};Bmp2-Prx1 mice. This data indicate that under conditions of Bmp2 deficiency Dkk1 haploinsufficiency is able to influence Wnt signaling by reducing to normal levels the expression of all but one (Sfrp4) the Wnt inhibitors evaluated and by maintaining, at least in 15-week-old mice, a certain level of upregulation of Wnt signaling, as indicated by the significant over-expression of β -catenin (Ctnnb1).

Furthermore, in bones of 15-week-old Dkk1^{+/-} mice Cebpa and Notch2 are both down-regulated when compared to WT controls (Fig. 6D). This indicate that, at least in 15-week-old mice, the anabolic effects of Dkk1 haploinsufficiency is mediated by a down-regulation of the Cebpa-controlled adipogenesis (34) and by a down-regulation of the Notch2-controlled osteogenic inhibition (35). Importantly, Dkk1 haploinsufficiency under conditions of Bmp2 deficiency is able to down-regulate the expression of Notch2, bringing it to levels comparable to those observed in WT mice (Fig. 6D).

Discussion

Increasing bone mass and fostering fracture repair in pathophysiological conditions remains a significant clinical challenge. Researchers focus on signaling pathways regulating bone metabolism and bone healing to find an effective pharmacological aid to help the clinician overcome these challenging situations. Many factors have been tested, and among these BMPs and Wnts have both been identified as signals that can be modulated to assure successful bone augmentation and fracture healing.

Neutralizing antibodies against Wnt inhibitors such as Dkk1 and Sost appear to be particularly promising for therapies aimed at reducing spontaneous fractures and fostering fracture repair (10, 11, 36, 37). However, whether these potential therapies remain effective in situations in which other bone metabolic pathways are altered remain to be clarified. Here we examine how bone homeostasis and bone healing function in conditions of altered BMP and Wnt signaling. Our studies show that enhancement of canonical Wnt signaling by means of Dkk1 haploinsufficiency is unable to exert its beneficial effects on bone mass augmentation in the absence of Bmp2 expression and is unable to overcome the inability of bone lacking expression of Bmp2 to heal fractures. Our studies provide support for the idea that BMPs and Wnts are dependent during bone formation and fracture healing and highlight

the requirement for intact BMP signaling as a key mediator for bone mass augmentation and fracture repair when a canonical Wnt signaling enhancing therapy is attempted.

The micro-computed tomography analysis of the trabecular bone microarchitecture and of the cortical bone structure of femur diaphyses of $Dkk1^{+/-}$ mice reveals a significant increase of the parameters associated to the trabecular bone mass as well as a significant reduction of the medullary area (Ma.Ar) and an increase of the cortical area fraction (Ct.Ar/Tt.Ar) when compared to values observed in WT mice. These findings are compatible with the expected anabolic effects of previously described in mice haploinsufficient for Dkk1 (9)(Fig.1). However, our findings indicate that Dkk1 haploinsufficiency does not significantly change the morphological (length), biophysical, and biomechanical properties of femoral mid-shaft (Fig. 2). Yet, our data is not in contrast with the findings of Morvan et al. (9) who reported a significant increase of Maximal Load, Stiffness, and Energy in the proximal tibia (trabecular and cortical bone) of $Dkk1^{+/-}$ mice versus proximal tibia of WT mice. They tested the proximal tibia in compression and therefore their measurements are indicative of the strength of the tibial trabecular bone which is increased because of the increased bone mass of the trabecular bones, a finding that we reported above. In contrast, in performing biophysical and biomechanical evaluations, we tested the properties of femur mid-shafts (cortical bone) in three point bending assays.

Interestingly, our evaluations of Post Yield Deflection and Post Work-to-fracture show that the cortical bone is more brittle for Bmp2-deficient mice regardless of Dkk1 haploinsufficiency. The measured values are comparable to those found in mouse models of osteogenesis imperfecta (38) indicating that, in the absence of Bmp2, bone is not properly generated and both structural alterations and tissue alterations are responsible for the observed spontaneous fractures. Altogether, these findings indicate that the anabolic effects of Dkk1 haploinsufficiency are not permitted in the absence of Bmp2.

We examined the incidence of fracture repair and not the variation in terms of volume and strength of the calluses formed upon fracture. Evaluating the effects of DKK1 haploinsufficiency on the enhancement of fracture repair was beyond the scope of our observational study. However, the fact that none of the fractures induced in the $Dkk1^{+/-}$;Bmp2Prx1-cre mice healed (lack of osteo-chondral differentiation 5 days p.f. and lack of callus bridging 20 day p.f.) indicates that Bmp2 signaling is required during fracture repair and that up-regulation of canonical Wnt signaling by means of Dkk1 haploinsufficiency is not able to bypass such requirement.

In mice embryos, expression of Dkk1 starts at E6.5 and is evident in the mesenchymal domains throughout the limb buds formation (39). Since, Prx1-driven cre recombinase dependent Bmp2 inactivation occurs at E9.5–10.5 in limb buds (26), we may assume that in our $Dkk1^{+/-}$;Bmp2-Prx1 mice the up-regulation of canonical Wnt signaling preceded or was at least simultaneous to the inactivation of Bmp2 signaling. Thus, despite its earlier or simultaneous up-regulation, Wnt signaling is unable to exert its anabolic effects in the absence of limb-specific Bmp2 expression.

It is possible that Dkk1 haploinsufficiency is not able to fully up-regulate Wnt signaling and thus the inability to compensate for lack of Bmp2 signaling of our Dkk1^{+/-};Bmp2-Prx1 mice may be due to such partial up-regulation. Another possibility may be related to the fact that Dkk1 haploinsufficiency only up-regulates canonical Wnt signaling and that up-regulation of non-canonical Wnt signaling may overcome and compensate for the lack of expression of Bmp2. Future studies will need to address these possibilities. Nevertheless, we can conclude with confidence that the up-regulation of Wnt signaling achieved by means of Dkk1 haploinsufficiency is unable to compensate for lack of Bmp2.

Our gene expression data indicate that in absence of Bmp2, in both Bmp2-Prx1 and Dkk1^{+/-};Bmp2-Prx1 mice, Wnt signaling is up-regulated, perhaps in an attempt to compensate for the lack of Bmp signaling. However, as supported by our morphological and biomechanical analyses, such up-regulation is not able to exert any bone anabolic effect under conditions of Bmp2 deficiency. This remains true in conditions of Dkk1 haploinsufficiency and Bmp2 deficiency (Dkk1^{+/-};Bmp2-Prx1 mice), when most of the Wnt inhibitors are normalized to WT levels, while β -catenin remains significantly up-regulated and Notch2 is significantly down-regulated.

One explanation for our findings is that the roles of canonical Wnt and canonical BMP signaling are epistatic during osteogenesis, where Bmp2 acts downstream of canonical Wnt. However, other reports suggest that BMP2 acts upstream, not downstream of canonical Wnt signaling. In particular, β -catenin is required for osteoblast differentiation in response to Bmp2 (40), and also for ectopic bone formation observed after intramuscular injection of Bmp2 (41). In fact, Tcf/Lef response element (β -catenin/TCF complex binding site) has been confirmed in Bmp2 promoter and is responsible for Wnt/ β -catenin mediated Bmp2 expression (42). While additional work is needed to clarify the relationship between canonical WNT and canonical BMP2 signaling during bone formation and fracture repair, cumulative observations point to the fact that bone formation is optimal when both of these pathways are active concomitantly.

Conclusions

Our data provide novel evidence that a cooperative interaction between BMPs and Wnts is necessary for bone formation and fracture repair. In normal bone formation and fracture healing, existing BMP signaling is sufficient to allow for treatment with Wnt signaling enhancers. However, reduced expression of Bmp2 is associated with skeletal aging in both mice and humans; the anabolic activity and the gene expression of Bmp2 are decreased in senile animals (43–45), and polymorphism of the Bmp2 gene is one of the possible causes of osteoporosis (46). While not proven to be due to reduced Bmp2 expression, the inability to effectively heal fractures in the aging population is known to be due to a delayed activation of the periosteum, the primary site of Bmp2 responsive skeletal progenitors (47). Furthermore, recent evidence suggests that reduced endogenous BMP signaling may predispose individuals to develop atrophic non-union in patients suffering traumatic fractures (17–19). Taken together, these observations suggest that inadequate bone formation and inadequate fracture healing response in clinical settings may be due in part to insufficient BMP activity. Based on our studies, in clinical conditions associated to low

BMP signaling a canonical Wnt-targeting therapy may not achieve maximum efficacy. Thus, we propose that enhancing BMP signaling is necessary for maximum efficacy in anabolic treatments that target the Wnt pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by grant # K99/R00 DE021069 to Dr. Giuseppe Intini (NIH/NIDCR) and in part by grant # R01AR055904 to Dr. Vicki Rosen (NIH/NIAMS) while Dr. Intini was a postdoctoral fellow in Dr. Rosen's laboratory at the Harvard School of Dental Medicine. Authors thank Dr. Vicki Rosen for releasing the data and permitting publication and the members of the Rosen's laboratory for their assistance and technical support during the execution of the studies. Authors also wish to acknowledge Dr. J. Robert Chang (OsteoInvent Inc., USA) for reviewing and editing the manuscript and for assisting with the interpretation and discussion of the results.

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Highlights

- Mice haploinsufficient for Dkk1 were crossed with mice lacking expression of Bmp2 in limbs.
- We examined the effects of Dkk1 haploinsufficiency in absence of expression of Bmp2 on bone properties.
- We show that Dkk1 haploinsufficiency is unable to exert bone anabolic effects in absence of Bmp2 signaling.

Figure 1. Micro-computed tomography analysis

(A) Quantification of expression of Dkk1 in bones of WT, Dkk1^{+/-}, Dkk1^{+/-};Bmp2-Prx1, and Bmp2-Prx1 mice (n=6; * indicates statistically significant difference (p<0.05)).

(B) Representative tomography images of femur diaphyses (cross-sectional plane) and metaphyses (sagittal plane).

(C) Trabecular bone microarchitecture (n=6–7; * indicates statistically significant difference from WT (p<0.05); # indicates statistically significant difference between Dkk1^{+/-} and Dkk1^{+/-};Bmp2-Prx1 (p<0.05); ¥ indicates statistically significant difference between Dkk1^{+/-} and Bmp2-Prx1 (p<0.05)).

(D) Cortical bone morphology (n=6–7; * indicates statistically significant difference from WT (p<0.05); # indicates statistically significant difference between Dkk1^{+/-} and Dkk1^{+/-};Bmp2-Prx1 (p<0.05); ¥ indicates statistically significant difference between Dkk1^{+/-} and Bmp2-Prx1 (p<0.05)).

MORPHOLOGICAL, BIOPHYSICAL, AND BIOMECHANICAL EVALUATION FEMUR

	WT (n=7)	Dkk1 ^{+/-} (n=6)	Dkk1 ^{+/-} ;Bmp2-Prx1 (n=7)	Bmp2-Prx1 (n=7)
Length	15.27 +/- 0.33	15.01 +/- 0.49	12.32 +/- 0.35 *#	12.54 +/- 0.43 *¥
Imin (mm ⁴)	0.14 +/- 0.03	0.13 +/- 0.03	0.05 +/- 0.02 *#	0.05 +/- 0.01 *¥
CI.TMD (mgHA/cm ³)	1295.12 +/- 31.31	1323.14 +/- 24.16	1354.43 +/- 21.95 *#	1345.58 +/- 18.69 *¥
Rigidity (kN mm ⁻¹)	1.46 +/- 0.37	1.43 +/- 0.36	0.67 +/- 0.18 *#	0.60 +/- 0.16 *¥
Yield moment (N mm)	34.00 +/- 4.20	37.02 +/- 6.68	27.71 +/- 8.09 #	26.74 +/- 4.01 *¥
Maximum moment (N mm)	43.16 +/- 8.28	44.69 +/- 7.74	28.82 +/- 8.52 *#	27.89 +/- 3.81 *¥
Yield strength (MPa)	153.44 +/- 28.49	162.39 +/- 47.77	290.31 +/- 46.37 *#	293.41 +/- 45.06 *¥
Bending strength (MPa)	191.17 +/- 20.50	221.77 +/- 59.52	292.42 +/- 55.49 *#	295.39 +/- 42.39 *¥
Post Yield Deflection (mm-1)	0.07 +/- 0.02	0.07 +/- 0.02	0.01 +/- 0.01 *#	0.01 +/- 0.01 *¥
Post work-to-fracture (Nmm/mm)	1.20 +/- 0.40	1.26 +/- 0.06	0.25 +/- 0.22 *#	0.19 +/- 0.13 *¥

* Indicates statistically significant difference from WT (p<0.05)
Indicates statistically significant difference between Dkk1^{+/-} and Dkk1^{+/-};Bmp2-Prx1 (p<0.05)
¥ Indicates statistically significant difference between Dkk1^{+/-} and Bmp2-Prx1 (p<0.05)

Figure 2. Morphological, biophysical, and biomechanical evaluation
(A) Morphological, biophysical, and biomechanical evaluation of femurs (n=6–7; * indicates statistically significant difference from WT (p<0.05); # indicates statistically significant difference between Dkk1+/- and Dkk1+/-;Bmp2-Prx1 (p<0.05); ¥ indicates statistically significant difference between Dkk1+/- and Bmp2-Prx1 (p<0.05)).
(B) Graph plotting the moment of inertia versus the Peak moment.
(C) Graph plotting the moment of inertia versus the Rigidity.

Figure 3. Wrist fractures
(A) Representative pictures of forelimbs. Red arrows point at radiological evidence of wrist fractures.
(B) Incidence of wrist fractures at 15 week of age (n=6–7).

Figure 4. X-ray analysis of fracture healing

X-ray analysis of fracture healing in WT, $Dkk1^{+/-}$, $Dkk1^{+/-}; Bmp2-Prx1$, and $Bmp2-Prx1$ mice ($n=3-5$, 8–10 week old animals). Orange arrows indicate initial bridging of the callus 10 days post fracture (p.f.) and red arrows indicate mature formed callus 20 days p.f.

Figure 5. Histological analysis of fracture healing
(A) Histological analysis of fracture healing in WT, Dkk1^{+/-}, Dkk1^{+/-};Bmp2-Prx1, and Bmp2-Prx1 mice (n=3–5, 8–10 week old animals) 5 days and 20 days post fracture (p.f.). The blue arrows indicate osteo-chondral differentiation of periosteal cells 5 days p.f., the red arrows indicate mature callus 20 days p.f., and the black arrows indicate fibrotic non-unions.
(B) Frequency of bone bridging in WT, Dkk1^{+/-}, Dkk1^{+/-};Bmp2-Prx1, and Bmp2-Prx1 mice 20 days post fracture (n=3–5, 8–10 week old animals).

Figure 6. Gene expression analysis of Wnt signaling

Gene expression analysis in femurs of WT, *Dkk1*^{+/-}, *Dkk1*^{+/-};Bmp2-Prx1, and Bmp2-Prx1 littermate mice (n=6, 15-week-old animals). (A) Wnt inhibitors, (B) Wnt receptors and agonists, (C) Wnt target genes, (D) gene associated to adipogenesis (Cebpa) and osteogenic inhibition (Notch2).