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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 have 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.

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 the 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 h 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

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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 μA with 1000 projections per 360° rotation and an integration time of 300 ms to provide images with 12 μm voxels (isotropic). Following reconstruction, the outer cortex was contoured and the structural parameters were computed using standard scripts provided by Scanco. Bone was segmented from air and soft tissue at a threshold of 350 per mille (800 mgHA/cm^3) 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/cm^3) and with a Gaussian noise filter (support of 2 and variance of 0.2). Trabecular parameters were computed using the Scanco software. Because the μCT 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 μCT 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 ($3 \times$ stiffness / span) and the peak moment (force $\times$ 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 $12 \times$ deflection / span^2. Material properties of modulus and strength of the cortex were also estimated using standard beam theory [32]. The previously described μCT 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 s. 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-Px1 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 s.

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 μm) were stained with 0.1% toluidine blue using standard procedures. Digital images were obtained using a Zeiss AxioImager MI Microscope fitted with an AxioCam