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PERSPECTIVES

Wnt Signaling in Bone

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Abstract

Extensive research over the past 6 years has established canonical Wnt signaling as an essential modulator of bone formation. Results from human genetic investigations, translational studies of knockout and transgenic mice, and *in vitro* experiments have provided evidence that Wnt signaling regulates essentially all aspects of osteoblast physiology. This review will highlight more recent findings regarding the role of canonical Wnt signaling in osteoblast coupling to osteoclastogenesis, the control of osteoblast apoptosis, and the modulation of mechanosensory perception. The roles of the secreted antagonists Dickkopf and sclerostin in bone formation, and the interactions between parathyroid hormone signaling and Wnt pathway components, will be summarized. Finally, this *Perspective* will examine the status and issues of Wnt pathway targets for drug discovery. *BoneKEy-Osteovision*. 2007 March;4(3):108-123.
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Introduction

Wnts are a large family of secreted proteins that regulate many fundamental biological processes (1-4). Over the past half-decade, components of Wnt signaling pathways have also been shown to play important roles in the control of bone formation. Since this topic has been frequently reviewed in recent years (5-16), this *Perspective* will focus on some topics that have received less attention.

Overview of Wnt Signaling

Wnts bind to a membrane receptor complex comprised of a Frizzled (FZD) G-protein coupled receptor (GPCR) and a low-density lipoprotein (LDL) receptor-related protein (LRP) (2-4). Wnt binding activates one of several intracellular signaling pathways (1-4). The best understood of these is the canonical Wnt/β-catenin pathway that signals through LRP-5/6 and leads to inhibition of glycogen synthase kinase (GSK)-3β and the stabilization of β-catenin. β-catenin translocates to the nucleus and activates lymphoid-enhancer binding factor (LEF)/T cell-specific transcription factors (TCFs) (1-4;17). Because Wnt signaling controls many essential physiologic processes, there are numerous extracellular and intracellular proteins that modulate its function (1-3). The extracelluar regulators include secreted proteins like Wnt inhibitory factors (WIFs), secreted frizzled-related proteins (SFRPs) and Dickkopfs (DKKs) (18-20) as well as *SOST*/sclerostin, Wise and connective tissue growth factor (CTGF) (9;21). These proteins bind Wnts (WIFs and SFRPs), bind to FZD receptors (SFRPs) or interact with LRPs (DKKs, *SOST*/sclerostin and CTGF).

The Role of Canonical Wnt Signaling in Osteoclastogenesis

β-catenin signaling has been reported to control not only osteogenesis (22-24), but also osteoclastogenesis. Holmen *et al*. studied the role of osteoblastic β-catenin signaling during postnatal murine bone acquisition by conditionally deleting either β-catenin or adenomatous polyposis coli (APC) from mature osteoblasts using the osteocalcin (OC) promoter to drive cre

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expression (25). Micro-computed tomography (CT) and histological analysis of long bones demonstrated that in the Δβ-catenin mice both trabecular and cortical bone volume were reduced. Osteopenia was correlated with a decrease in osteoblast differentiation and matrix mineralization, as well as an increase in osteoclast differentiation and activity that resulted from down-regulation of osteoblastic osteoprotegerin (OPG) expression and up-regulation of receptor activator of nuclear factor-κB ligand (RANKL) expression. On the other hand, the Δ-APC mice, which had elevated osteoblastic β-catenin levels, exhibited an osteopetrotic phenotype that resulted primarily from reduced osteoclast differentiation and activity as a result of up-regulation of osteoblastic OPG expression and down-regulation of RANKL expression.

Links between osteoblastic β-catenin signaling, OPG expression and osteoclastogenesis have also been obtained by Glass *et al*., who utilized Cre-lox technology to delete portions of the β-catenin gene in murine osteoblasts (26). In addition, these authors showed that deletion of TCF-1 in mice produced a low bone mass phenotype as a result of diminished OPG expression. Surprisingly, the authors did not observe significant changes in osteoblastogenesis or bone formation in any of these transgenic or knockout mouse models. In a report evaluating the molecular events associated with Wnt-3a action on mouse C3H10T1/2 pluripotent mesenchymal stem cells, Jackson *et al*. also found that OPG expression was up-regulated following activation of the β-catenin pathway (27). Thus, the canonical Wnt pathway seems to regulate both bone formation and bone resorption via cells of the osteoblast lineage.

Spencer *et al*. have also provided evidence for suppression of osteoclastogenesis by canonical Wnt signaling in osteoblasts (28). Using co-cultures of primary mouse osteoblasts and mononuclear spleen cells, these authors showed that treatment with either Wnt-3a conditioned L-cell medium or the GSK-3β antagonist LiCl inhibited osteoclast formation, as measured by the number of tartrate-resistant acid phosphatase (TRAP) stained cells. In contrast, incubation of human peripheral blood mononuclear cells with Wnt-3a in the presence of RANKL and macrophage-colony stimulating factor (M-CSF) had no effect on osteoclastogenesis and bone resorption, indicating that osteoblasts were required for suppression of osteoclast formation. As expected, treatment of MC-3T3-E1 mouse osteoblasts with Wnt-3a or LiCl reduced RANKL mRNA levels. Moreover, co-transfection of mouse ST2 stromal cells with a human RANKL promoter-luciferase plasmid and β-catenin expression plasmids demonstrated that canonical signaling directly inhibits RANKL expression via TCF/LEF response elements in the promoter.

The secreted Wnt antagonist SFRP-1 has also been shown to regulate osteoclastogenesis *in vitro*, although modulation of Wnt signaling does not appear to be the mechanism for this effect. Hausler *et al*. identified SFRP-1 as a factor expressed by conditionally immortalized murine stromal cell lines that supported osteoclast formation (29). Treatment of murine stromal cell lines and primary calvaria-derived neonatal osteoblasts with prostaglandin E_2 (PGE_2) induced SFRP-1 mRNA expression. In addition, SFRP-1 gene expression was increased by IL-11 treatment, while incubation with dexamethasone, vitamin D, parathyroid hormone (PTH) and IL-1, -4, -10 and -18 had no effect on message levels. Although the authors anticipated that SFRP-1 would stimulate osteoclastogenesis, both an siRNA and a neutralizing antibody to SFRP-1 enhanced osteoclast differentiation. Moreover, treatment of mouse spleen cells with purified rhSFRP-1 protein suppressed RANKL/CSF-1-stimulated osteoclast formation in a dose-dependent manner. Finally, Hausler *et al*. showed that SFRP-1 binds to RANKL in an ELISA format. Thus, in addition to interacting with Wnts, SFRP-1 also has the ability to bind RANKL and block its signaling. Although characterization of *Sfrp1*(-/-) mice did not reveal evidence for an alteration of bone resorption *in vivo* (30),

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when bone marrow cells were differentiated to osteoclasts by incubation with RANKL and CSF-1/M-CSF *in vitro*, the number of TRAP-positive cells was enhanced when compared to cells from wild-type controls. These results suggest that while SFRP-1 can antagonize RANKL *in vitro*, other proteins like OPG may play a more important role in controlling the bone resorbing cytokine *in vivo* (31). However, there may be additional pathophysiological states where SFRP-1 regulation of RANKL may be critical.

The Role of Dickkopf and Sclerostin Proteins in the Regulation of Osteoblast Physiology

Mutations in the *LRP5* gene are associated with marked increases in bone mass and resistance to fractures (7). All of the gain-of-function mutations of *LRP5* identified to date that cause high bone mass (HBM) phenotypes are found in the first β-propeller domain (32). These mutations are scattered throughout the 6 blades of the β-propeller motif; this leads to reduced affinity for DKK-1 and relief from DKK-1 antagonism (33;34). DKK-1 binds to the 3rd and 4th β-propeller motifs of LRP5 and LRP6 and disrupts canonical signaling by targeting LRP internalization and degradation through Kremen (KRN) (20;32). Perhaps not surprisingly, deletion of murine DKK-1 leads to increased trabecular bone volume (TBV) and improved bone strength (35). Although complete loss of DKK-1 is an embryonic lethal trait due to a lack of head development (36), heterozygous mice are viable after birth. Compared to wild-type controls, tibias from *Dkk1*(+/-) animals exhibit increased TBV and elevated trabecular bone formation rate as determined histologically (35). Calvarial-derived osteoblasts from heterozygous mice have reduced DKK-1 mRNA levels, increased canonical Wnt signaling, elevated DNA synthesis and enhanced alkaline phosphatase activity relative to cells from wild-type animals. Thus, partial loss of DKK-1 stimulates osteoblast proliferation, differentiation and activity. These conclusions have been confirmed by studies of transgenic mice that expressed murine DKK-1 in bone using the 2.3 kb rat type 1A1 collagen promoter (37). These animals exhibit reduced TBV, cortical bone area and bone formation rate due to the suppression of osteoblast differentiation and activity. Finally, DKK-1 also appears to play a substantial role in estrogen deficiency-induced bone loss. Wang *et al*. recently reported that ovariectomy of rats results in increased DKK-1 mRNA levels in rat bone and elevated amounts of DKK-1 protein in serum (38). These changes correlate with decreased femoral bone mineral density (BMD), TBV and biomechanical properties. Conversely, systemic treatment of ovariectomized rats with end-capped phosphorothioate DKK-1 antisense oligonucleotides reduced DKK-1 expression in bone and reversed the effects of ovariectomy on bone loss and strength. The authors concluded from these studies that control of DKK-1 signaling is a potential target for therapeutic intervention in postmenopausal osteoporosis.

While DKK-1 is an osteoblast antagonist, DKK-2 plays a different role in bone formation. Li *et al*. examined the role of DKK-2 in osteoblast physiology and bone formation by deleting the gene in mice (39). Since DKK-2 is an extracelluar antagonist of LRP-5 and –6 (1-3), the authors anticipated that loss of this gene would lead to increased bone formation. However, evaluation of the *Dkk2*(-/-) mice showed that these animals were osteopenic. An analysis of long bones from knockout mice by peripheral quantitative computed tomography (pQCT) and static histomorphometry demonstrated a decrease in trabecular and cortical bone mineral content (BMC), as well as a reduction in TBV and trabecular number. In addition, deletion of DKK-2 led to an increase in osteoid surface in the absence of a corresponding elevation in osteoblast number or osteoblast surface. Moreover, *Dkk2*(-/-) mice exhibited a reduced mineral apposition rate (MAR), a measurement of osteoblast activity, as determined by dynamic histomorphometry. Thus, loss of DKK-2 appears to cause a defect in terminal

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osteoblast differentiation and matrix mineralization. These *in vivo* analyses were confirmed by *in vitro* studies of bone marrow-derived and neonatal calvarial-derived osteoblast cultures, which demonstrated that deletion of DKK-2 resulted in delayed cellular differentiation and matrix mineralization, even though canonical Wnt signaling was elevated in cells from the *Dkk2*(-/-) mice. When the authors analyzed the expression of DKK-2 mRNA as a function of osteogenic differentiation, they observed that the levels of this secreted Wnt antagonist increased with advancing osteoblast development. Furthermore, when the authors over-expressed DKK-2 in bone marrow-derived and neonatal calvarial-derived osteoblast cultures obtained from wild-type mice, they detected an enhancement of matrix mineralization as determined by xylenol orange staining. Therefore, while elevation of canonical Wnt signaling may be required for pre-osteoblast proliferation as well as the initiation and/or progression of the osteoblast through cellular differentiation, suppression of the pathway by antagonists like DKK-2 appears to be important for terminal differentiation and matrix mineralization.

Like DKK-1, DKK-3 is also an osteoblast antagonist, but suppression of Wnt signaling does not appear to be the mechanism by which this occurs. Aslan *et al*. used murine C3H10T1/2 mesenchymal stem cells (MSCs) that were engineered to express human bone morphogenetic-2 (BMP-2) in a tetracycline-regulated system and Affymetrix GeneChip mircroarrays to identifiy transcripts involved in bone formation (40). The cells were implanted into mice and allowed to form bone *in vivo*. Histological analysis demonstrated that the cells recapitulated endochondral bone formation under the control of BMP-2. Among the genes that were differentially expressed during this process was *Dkk3*, which peaked at the matrix deposition phase of cartilage and bone formation. Although a member of the DKK family, DKK-3 does not appear to block Wnt signaling (41). When DKK-3 was engineered using the tetracycline-regulated system into C3H10T1/2 mesenchymal cells that expressed BMP-2, it inhibited *in vitro* cell division and osteogenesis as measured by alkaline phosphatase activity. Micro-CT of bone formed by cells in mice *in vivo* demonstrated that bone volume was reduced in MSCs that expressed DKK-3 relative to the BMP-2 controls. Thus, as with DKK-1, DKK-3 also diminishes osteoblast proliferation and differentiation.

Another secreted Wnt antagonist, *SOST*/sclerostin, has also been reported to bind to and antagonize LRP-5 and LRP-6 by interacting with the receptors' 1st and 2nd β-propeller motifs (42). Sclerostin is a member of the DAN cysteine knot-containing family of secreted proteins and has been shown to block both BMP and Wnt signaling (21). Other members of the DAN family also modulate these pathways (21;43). *SOST* was identified as the gene that is inactivated in the bone dysplasia sclerosteosis, a HBM disease that predominantly affects the Afrikaner population of South Africa (44). Sclerostin is highly expressed in osteocytes (45;46). When compared to non-transgenic controls, mice that express human *SOST* driven by the mouse osteocalcin gene 2 (*OG2*) promoter/enhancer exhibit reduced BMC of the lumbar vertebrae and femur, as measured by PIXImus, as well as reduced TBV, osteoblast surface and bone formation rate as determined histologically (46). These results indicate that *SOST*/sclerostin inhibits osteoblast proliferation, differentiation and activity, which are also blunted by DKK-1. Although sclerostin was initially described as a BMP-6 binding protein that suppressed osteoblast proliferation and differentiation by antagonizing BMP signaling (46), more recent work demonstrates that it probably inhibits bone formation by blunting the canonical Wnt pathway through interaction with LRP-5 and –6 (42;47;48). This discrepancy was recently clarified by van Bezooijen *et al*. (48). Using Affymetrix GeneChip microarray technology and bioinformatics analysis of mouse KS483 MSCs treated with human BMP-4 in the presence and absence of human sclerostin, van Bezooijen *et al*. showed that the transcription profiles were inconsistent with

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simple BMP antagonism. Employing reporter gene assays to measure BMP and Wnt signaling in osteoblastic cell lines, the authors showed that sclerostin does not antagonize BMP activity, but does block Wnt activity. Moreover, both sclerostin and DKK-1 inhibited the ability of BMP-4 to activate the canonical Wnt pathway. From these studies, van Bezooijen *et al*. concluded that sclerostin suppresses bone formation by antagonizing Wnt signaling that is activated by BMPs and Wnts. As with DKK-1, HBM mutations of LRP-5 also appear to prevent sclerostin from binding and blocking the canonical pathway (42;43;47).

The Role of Wnt Signaling in the Control of Bone Cell Apoptosis

Initial evidence for the involvement of canonical Wnt signaling in osteoblast physiology came from human genetic studies of osteoporosis pseudoglioma (OPPG) syndrome and HBM phenotypes that associated LRP-5 with bone formation (49;50). Knockout and transgenic mouse models of these LRP-5 mutations have allowed us to understand the mechanisms by which canonical Wnt signaling controls bone formation. Kato *et al*. showed that deletion of murine *Lrp5* reduced vertebral TBV when compared to *Lrp5*(+/+) controls as determined by histomorphometry (51). *Lrp5*(-/-) mice also had tibial fractures as assessed by radiographs. Dynamic histomorphometric analysis of the vertebrae from the *Lrp5(*-/-) mice demonstrated that deletion of *Lrp5* decreased the MAR, indicating that osteoblast function was inhibited by loss of the gene. Furthermore, deletion of *Lrp5* reduced osteoblast numbers in long bones from *Lrp5(*-/-) mice, and this was correlated with a decrease in calvarial osteoblast proliferation as determined by bromodeoxyuridine (BrdU) labeling. However, osteoblast apoptosis and differentiation were not altered by loss of LRP-5.

In contrast to the osteopenic/osteoporotic phenotype of the loss-of-function *Lrp5* knockout mice (51), our group showed that *Lrp5* gain-of-function transgenic mice have HBM (52). These animals were developed by targeting expression of human LRP-5 with a G171V mutation to bone using the 3.6 kb rat type I collagen promoter. The transgenic mice have increased bone formation, but the mechanisms for this effect are different from those that lead to decreased bone formation resulting from loss of LRP-5. Transgenic *Lrp5*^{G171V} mice have increased trabecular volumetric BMD (vBMD) in the distal femur as measured by pQCT. In addition, cortical bone thickness is also increased in the transgenic mice. Histological analysis of the femurs indicated that total bone area is increased in the transgenic mice, while the mineralizing surface is also elevated. However, MAR is not significantly increased in the *Lrp5*^{G171V} mice, indicating that osteoblast activity is not affected by the mutation. Alkaline phosphatase (ALP) staining of calvaria is also elevated in the transgenic mice, while TUNEL (terminal dNTP transferase-mediated dUTP nick end-labeled) staining demonstrated that osteoblast and osteocyte apoptosis is reduced. Thus, increased bone formation in *Lrp5*^{G171V} mice appears to result from elevated osteoblast/osteocyte numbers due to decreased cell death.

We discovered that the secreted Wnt antagonist SFRP-1 played a role in osteoblast physiology during a series of transcription profiling experiments that sought to elucidate the molecular events associated with human osteoblast differentiation and bone formation (53). Basal SFRP-1 mRNA levels were observed to increase during human osteoblast (HOB) differentiation from pre-osteoblasts to pre-osteocytes, and then decline in mature osteocytes. This expression pattern correlated with levels of cellular viability, such that pre-osteocytes, which had the highest levels of SFRP-1 mRNA, also had the highest rates of cell death. In addition, expression of SFRP-1 mRNA was induced following PGE_2 treatment of pre-osteoblasts and mature osteoblasts that have low basal SFRP-1 mRNA levels. In contrast, SFRP-1 expression was observed to be down-regulated following transforming growth factor (TGF)-β1 treatment of pre-osteocytes,

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which have high basal mRNA levels. Consistent with this observation, treatment of pre-osteoblasts and mature osteoblasts with PGE_2 increased apoptosis, while treatment of pre-osteocytes with TGF-β1 decreased cell death. Likewise, overexpression of SFRP-1 in HOB cells that express low levels of the gene accelerated the rate of cell death. Therefore, these results implied that SFRP-1 is a key modulator of human osteoblast and osteocyte survival.

In order to confirm these *in vitro* observations, we characterized a knockout mouse model (30). These mice expressed the *LacZ* gene in place of exon 1 of *Sfrp1* so that promoter activity could be measured by β-galactosidase staining. Loss of *Sfrp1* in mice increased distal femur TBV as determined by micro-CT. But in contrast to the *Lrp5*^{G171V} transgenic mice, loss of *Sfrp1* had no effect on cortical bone parameters. An interesting observation about the *Sfrp1(*-/-) mice was that prior to 13 weeks of age, there was no difference in trabecular vBMD of the distal femur between wild-types and knockouts, as determined by pQCT. As the mice aged, however, the *Sfrp1*(+/+) animals lost trabecular bone, while the *Sfrp1(*-/-) mice gained trabecular bone. Thus, deletion of *Sfrp1* delays and enhances the onset of peak bone mass and suppresses senile bone loss.

As with the *Lrp5(*-/-) mice, deletion of *Sfrp1* also affected osteoblast activity. Dynamic histomorphometric analysis of proximal femurs from *Sfrp1*(+/+) and *Sfrp1*(-/-) female mice showed that deletion of *Sfrp1* increased MAR, indicating that osteoblast activity was increased by loss of the gene. In addition, as in *Lrp5*^{G171V} mice, deletion of SFRP-1 also suppressed apoptosis. TUNEL staining of calvaria from female mice demonstrated that loss of SFRP-1 led to an increase in calvarial thickness and a decrease in osteoblast and osteocyte programmed cell death (PCD). In contrast to the *Lrp5(*-/-) mice, deletion of *Sfrp1* also affects osteoblast differentiation. When bone marrow from *Sfrp1*(+/+) and *Sfrp1*(-/-) female mice was differentiated to osteoblasts in culture by incubation with ascorbic acid, β-glycerolphosphate and dexamethasone, the number of ALP+ cells was increased by deletion of *Sfrp1*. Analysis of the differentiating cultures for *LacZ* expression showed that osteoblast development and matrix mineralization also increased as *Sfrp1* promoter activity became elevated, suggesting that control of Wnt signaling by *Sfrp1* modulates osteoblast differentiation and function. In addition, evaluation of the bone marrow cultures from knockout mice by TUNEL staining showed that cellular apoptosis was suppressed when compared to cultures from wild-type controls. Finally, as in *Lrp5*(-/-) mice, deletion of *Sfrp1* also affects osteoblast proliferation. Measurement of DNA synthesis in cultures derived from newborn *Sfrp1*(+/+) and *Sfrp1(*-/-) calvaria showed that osteoblast proliferation increased in the knockout cells during the proliferative phase. When *Sfrp1(*-/-) cultures reached confluence and proliferation ceased, however, the rate of DNA synthesis returned to normal, indicating that the proliferation-differentiation transition was not altered by loss of *Sfrp1*. Therefore, deletion of *Sfrp1* enhances osteoblast proliferation, differentiation and function, while it suppresses osteoblast and osteocyte apoptosis.

Consistent with a negative effect of SFRP-1 on osteoblasts and osteocytes, Wang *et al*. recently reported that pharmacologic doses of glucocorticoids also increase osteoblast *Sfrp1* expression and decrease bone formation in rats (54). These authors showed that high concentrations ($\geq 10^{-6}$M) of dexamethasone up-regulated *Sfrp1* mRNA and protein levels in primary rat MSCs *in vitro* and that this correlated with suppression of osteogenesis, cytosolic β-catenin protein levels and nuclear Runx2 activation as well as increased apoptosis. Steroid treatment of rats *in vivo* increased SFRP-1 protein levels in trabecular osteoblasts and osteocytes, and this correlated with increased bone cell apoptosis as well as decreased femoral TBV and bone strength. Confirmation that SFRP-1 was involved in these processes was

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obtained using siRNA *in vitro* as well as purified protein *in vitro* and *in vivo*.

Additional evidence that Wnts prevent osteoblast apoptosis *in vitro* was recently reported by Ameida *et al*. (55). Using murine C2C12, OB-6 and MC-3T3-E1 cells, the authors showed that treatment with both canonical (Wnt-3a) and noncanonical (Wnt-5a) Wnts suppressed programmed cell death. In addition, while canonical Wnt signaling appeared to play a role in controlling cell survival, additional signaling pathways like Src/Erk (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)/AKT were also involved in this process.

The Role of Wnt Signaling in the Modulation of Mechanosensory Perception by Bone

Canonical Wnt signaling controls essentially all aspects of osteoblast physiology, including the ability of bone to respond to mechanical stimulation. Johnson, Recker and colleagues were the first to postulate the importance of LRP-5 in the modulation of mechanosensory perception by bone (7;56). In recent years, experimental results have accumulated to support this hypothesis.

Norvell *et al*. showed that fluid shear stress induced the nuclear translocation of β-catenin in MC-3T3-E1 mouse osteoblasts, primary rat calvarial-derived osteoblasts and UMR-106 rat osteosarcoma cells (57). Fluid shear stress was also observed to inactivate GSK-3β while activating a *TCF*-luciferase reporter gene. Using calvarial-derived osteoblasts from TOPGAL transgenic mice that harbor the *LacZ* reporter gene under control of TCF DNA response elements, Hens *et al.* demonstrated that physical deformation upregulated TCF expression as well as a canonical target gene, *cyclin D1* (58). Furthermore, these effects were blocked by treatment of the cells with a soluble form of FZD-4, indicating that mechanical stimulation increased secretion of a Wnt.

These *in vitro* experiments have been supported by *in vivo* studies. Lau *et al*. showed that tibias of C57BL/6J mice respond to mechanical loading, while those of C3H/Hej mice do not (59). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of RNA isolated from loaded tibias demonstrated that Wnt pathway genes like *Wnt-1*, *Wnt-3a*, *Lrp5* and *β-catenin* were up-regulated in C57BL/6J mice, but not in C3H/Hej mice. Moreover, other important pathways like those for bone morphogenetic proteins, insulin-like growth factor-1 and estrogens were also induced in loaded tibias from C57BL/6J mice.

An essential role for LRP-5 in the mechanosensory perception of bone was recently demonstrated by Sawakami *et al*. (60). By loading the ulnas of *Lrp5*(+/+) and *Lrp5*(-/-) mice, these authors found that both the periosteal and endocortical bone formation rates were suppressed by loss of LRP-5. *In vitro* studies using fluid shear stress of calvarial-derived osteoblasts from *Lrp5* wild-type and knockout mice narrowed the mechanism for this defect to ablation of osteopontin/bone matrix gene expression. Additional support for the importance of LRP-5 in the bone anabolic effects of mechanical loading comes from studies of the G171V gain-of-function mutation of *Lrp-5*. Robinson *et al*. examined the gene expression patterns of tibias isolated from non-transgenic and *Lrp5*^{G171V} transgenic mice after a 4-hour regime of 4-point bending mechanical loading (61). RNA levels of known mechanical stress-induced genes like prostaglandin synthase (*COX-2*), prostacyclin synthase (*Ptgis*) and endothelial nitric-oxide synthase (*eNOS*) were up-regulated more prominently in loaded tibias from *Lrp5*^{G171V} transgenic mice than in non-transgenic littermate controls. Likewise, canonical Wnt pathway target genes such as *cyclin D1*, *connexin 43*, *Wnt-inducible secreted protein* (*WISP*)*-2*, *Wnt-10b*, *SFRP-1* and *SFRP-4* were induced to a greater extent in loaded tibias from *Lrp5*^{G171V} transgenic mice than in non-transgenic littermate controls. Confirmation that the canonical Wnt pathway was involved in this process was obtained by loading the tibias

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of wild-type C57BL/6J mice after treatment with a GSK-3β antagonist. When compared to vehicle treated controls, the gene expression pattern of loaded tibias from inhibitor-treated mice resembled bones from *Lrp5*^{G171V} transgenic animals. Collectively, these studies with *Lrp5* loss- and gain-of-function mice establish this canonical Wnt co-receptor as an important component of the mechanosensory system in bone.

The Role of Wnt Signaling in the Bone Anabolic Effects of Parathyroid Hormone

In the United States, the only Food and Drug Administration (FDA) approved osteogenic agent for the treatment of osteoporosis is teriparatide or PTH(1-34) (62-64). Although the molecular mechanisms by which PTH increases bone formation are not entirely known, evidence has accumulated for an intersection between PTH signaling and Wnt pathways in osteoblasts. In 1992, Chan *et al*. reported that PTH(1-34) treatment of UMR 106-01 rat osteosarcoma cells increases FZD-1 and –2 mRNA levels (65). More recently, Qin *et al*. observed that PTH elevates SFRP-4 mRNA expression in UMR cells (66). Keller and Kneissel have reported that PTH(1-34) suppresses *SOST*/sclerostin mRNA levels in mouse calvaria, ovariectomized rat femurs and UMR cells (67), while Bellido *et al*. observed that both intermittent and continuous treatment of mice *in vivo* with PTH(1-84) reduced *SOST*/sclerostin expression in vertebral osteocytes (68). In addition, Kulkarni *et al*. have shown that PTH 1-38 up-regulates the expression of LRP-6, FZD-1 and KRN-1 in rat metaphyseal bone and UMR cells, but down-regulates LRP-5 and DKK-1 mRNA levels (69). PTH 1-38 was also observed to increase β-catenin protein levels and TCF signaling in UMR cells (69). More recently, Tobimatsu *et al*. reported that PTH(1-34) treatment of mouse MC-3T3-E1 osteoblastic cells also increased β-catenin protein levels, and that this effect occurred through modulation of both the protein kinase A and protein kinase C pathways (70). Furthermore, the authors showed that treatment of the cells with either PTH or $LiCl_2$, which inhibits GSK-3β and activates canonical Wnt signaling, suppressed apoptosis induced by either dexamethasone or etoposide. Collectively, the results of these studies imply that at least part of the anabolic actions of PTH in the skeleton are mediated via control of Wnt signaling.

In order to address this question further, investigators have begun to examine the osteogenic effects of PTH in knockout and transgenic mouse models of Wnt pathway components. Sawakami *et al*. recently reported that intermittent PTH(1-34) treatment of *Lrp5*(+/+) and *Lrp5*(-/-) mice increased distal femur total areal BMD (aBMD) as measured by PIXImus to a similar extent (60). These authors did not perform additional studies to determine if the anabolic actions of PTH were similar on cortical and trabecular bone.

Iwaniec *et al*. also studied the anabolic effect of PTH in *Lrp5*(+/+) and *Lrp5*(-/-) mice (71). These authors demonstrated that intermittent treatment with PTH(1-34) increased femoral aBMD as well as histological measures of bone formation rate, osteoblast surface and osteoclast surface to a similar extent in vertebrae from wild-type and knockout mice. Using micro-CT, PTH treatment was also seen to increase femoral cortical thickness to the same degree in wild-type and knockout mice. However, PTH treatment had no effect on vertebral TBV of either genotype as measured by micro-CT or histology.

We have also studied the anabolic effects of PTH in *Lrp5*(+/+) and *Lrp5*(-/-) mice (72). As in the 2 previous reports, we found that intermittent treatment with PTH(1-34) increased femoral total vBMD as determined by pQCT to a similar extent in wild-type and knockout mice, and that this was due to an elevation of cortical vBMD. In contrast, while PTH treatment increased trabecular vBMD in the femurs of wild-type mice, it had no effect on cancellous bone of knockout animals. Likewise, PTH treatment elevated the femoral trabecular MAR in wild-type, but not in knockout, mice.

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The reasons for the differences between the results of these three studies of PTH on *Lrp5*(+/+) and *Lrp5*(-/-) mice are not entirely clear. The discrepancies do not appear to be due to the dose or duration of PTH(1-34) treatment, which was similar for the three studies (40-100 µg/kg/day for 4-6 weeks). In addition, two of the studies used mice generated by Lexicon Genetics (71;72), and all three groups found that loss of LRP-5 resulted in low bone mass. The age of the mice in two of the studies (60;72) was also similar (10-12-week-olds), and all 3 studies utilized male animals. Collectively, it appears that PTH increases cortical BMD and thickness independently of LRP-5. However, the anabolic effects of PTH on trabecular bone may or may not require LRP-5. Additional investigations are required to resolve this issue.

Our group has also examined the effects of intermittent PTH treatment on transgenic mice that express the human G171V gain-of-function mutation of *Lrp5* via the type I collagen promoter in bone (73). While treatment of 8-52-week-old male non-transgenic controls with PTH(1-34) increased femoral trabecular vBMD by 12-36%, a 32-61% gain in vBMD was seen in *Lrp5*^{G171V} animals following intermittent dosing of the hormone. Thus, the anabolic effects of intermittent PTH treatment appear to be enhanced when canonical Wnt signaling is elevated in osteoblasts by a gain-of-function mutation of *Lrp5*.

We have also studied the effects of intermittent PTH(1-34) treatment on bone formation in female *Sfrp1*(+/+) and *Sfrp1*(-/-) mice (74). Deletion of the Wnt antagonist SFRP-1 in female mice results in increased distal femur trabecular vBMD after 12 weeks of age (30), and the ability of PTH(1-34) to increase trabecular vBMD in *Sfrp1*(+/+) and *Sfrp1*(-/-) mice declined with advancing age. Moreover, PTH(1-34) increased trabecular vBMD to a similar level in wild-type and knockout mice, and in aged animals, this level was set by deletion of *Sfrp1*, suggesting that bone formation at this age is optimally activated by loss of the Wnt antagonist. Finally, in aged mice, treatment with PTH(1-34) elevated trabecular vBMD in wild-type controls, while it had no effect on this parameter in knockout animals.

Like Wnts, PTH binds to a GPCR and signals through both the adenylyl cyclase/cAMP pathway and the PLC-β/calcium pathway (62). The ability of PTH to activate canonical Wnt signaling in UMR 106 and MC-3T3 cells has been shown to occur through stimulation of adenylyl cyclase (69;70), while regulation of PLC-β may also play a role in this effect in MC-3T3 cells (70). Likewise, we recently demonstrated that deletion of murine SFRP-1 activates the canonical Wnt pathway in bone (75). Thus, loss of SFRP-1 may blunt the ability of PTH to increase trabecular bone formation by optimal elevation of canonical/β-catenin signaling in osteoblasts. Moreover, since SFRP-1 binds Wnts and potentially inhibits both canonical and noncanonical pathways (19), deletion of this Wnt antagonist might also elevate noncanonical signaling such as that which occurs through PLC-β and calcium. In addition, since deletion of SFRP-1 and PTH treatment both result in decreased osteoblast/osteocyte apoptosis (30;53;70;76), loss of SFRP-1 may also blunt PTH action by optimum suppression of programmed cell death.

Collectively, these *in vivo* studies in knockout and transgenic mouse models of LRP-5 and SFRP-1 underscore the complexity of interactions between PTH signaling and Wnt pathway components in bone. Additional studies like these are needed to gain a better understanding of the mechanisms for the bone anabolic actions of PTH and the dependencies and synergies of these effects with Wnt signaling in osteoblasts.

The Potential of Wnt Signaling Targets for Drug Discovery

Since its discovery 6 years ago, the Wnt pathway has been the focus of many clinicians, academic researchers and drug discovery scientists seeking to understand bone formation processes and to identify

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new potential targets and therapies for metabolic bone diseases like osteoporosis (4;6;9;11;77). Some of the challenges that exist in targeting this pathway for therapeutic intervention are that a number of the well-validated targets like LRP-5, DKK-1 and sclerostin are not members of traditionally drugable classes of proteins such as enzymes, hormone receptors and ion channels. Moreover, many of these potential targets, for instance LRP-5 and GSK-3β, have broad tissue distribution, and pharmacologic modulation at these sites may therefore have unwanted side effects like cancer (4). However, existing data on a few of these pathway components, for example LRP-5, imply that while these are expressed by many tissues, intervention at these sites can have a specific and beneficial clinical effect on bone (33;78).

Some proof-of-concept data has already been obtained regarding the potentially beneficial effects of pharmacologic stimulation of canonical Wnt signaling in bone. Treatment of mice with LiCl, which inhibits GSK-3β, increases bone formation and bone mass in osteopenic animals by suppressing osteoblast apoptosis and adipogenesis while enhancing osteogenesis (79). Since lithium has been used for decades to treat bipolar disorder, Vestergaard *et al*. performed a case-control study and observed a reduced relative risk of fracture in patients treated with this drug (80). However, the authors noted that lithium has other pharmacologic effects, such as inhibiting vitamin D action and reducing the sensitivity of the calcium-sensing receptor to calcium, which could explain these results. Therefore, the ovariectomized rat study by Kulkarni *et al*. of an orally active GSK-3α/β antagonist is important, since this also showed that inhibition of this enzyme increased osteoblast activity, TBV, cortical bone area and bone strength (81). GSK-3 inhibitors have been developed for a variety of non-skeletal indications including type 2 diabetes and Azheimer’s disease (82), but as yet none of these compounds have entered clinical development for osteoporosis.

Therapeutic antibodies that neutralize the secreted Wnt antagonists sclerostin and DKK-1 are currently in late preclinical/early clinical development (83;84). Although not as ideal as an orally active small molecule, a bio-therapeutic approach to secreted targets has the advantage of bypassing the technological hurdles of identifying orally active small molecules that disrupt protein-protein interactions. In addition to being a potential therapy for osteoporosis, antagonism of DKK-1 has application for the treatment of other skeletal diseases like rheumatoid arthritis and osteolytic lesions in multiple myeloma (85-87). While therapeutic antibodies are a good starting point at which to modulate Wnt signaling pharmacologically, the ultimate goal of drug discovery is to develop orally active compounds that will be less expensive to produce and easier to administer, thus improving cost-effectiveness and compliance in long term degenerative diseases like osteoporosis.

Conflict of Interest: Dr. Bodine reports that he is a full-time employee of Wyeth Research and owns stock and holds stock options in the company.

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