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WNT Signaling in Bone Development and Homeostasis

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Abstract

The balance between bone formation and bone resorption controls postnatal bone homeostasis. Research over the last decade has provided a vast amount of evidence that WNT signaling plays a pivotal role in regulating this balance. Therefore, understanding how the WNT signaling pathway regulates skeletal development and homeostasis is of great value for human skeletal health and disease.

1. Overview of Bone Homeostasis

The balance of two activities maintains bone homeostasis: bone formation by mesenchymal stem cell (MSC)-derived osteoblasts, and bone resorption by hematopoietic stem cell (HSC)-derived osteoclasts. Under pathologic conditions, this balance is disrupted. High osteoclast activity or low osteoblast activity leads to low bone mass (osteopenia), while low osteoclast activity or high osteoblast activity leads to high bone mass (osteopetrosis). Several signaling pathways are important for maintaining this delicate balance including those activated by WNT, BMP, PTH/PTHrP, Notch, and Hedgehog. In this review, we focus on the role of Wnt signaling in this process. We will first briefly describe the cellular mechanisms of Wnt signal transduction and then discuss the observations associated with alterations of this pathway in human skeletal disease that focused attention on this pathway. This will be followed by the discussion of studies utilizing genetically engineered mouse models to gain insights into the specific requirements for β -catenin, WNT ligands, and WNT receptors in osteoblast differentiation and function. We conclude with a discussion of the current status of therapeutic development of WNT-based strategies to treat human diseases associated with low bone mass.

2. Overview of Bone Development

Bone is formed by either intramembranous ossification or endochondral ossification (Figure 1). During intramembranous ossification, mesenchymal progenitors differentiate directly into osteoblasts¹. This process underlies the formation of cranial bones, a portion of the mandible, and the clavicle. Intramembranous ossification is initiated by a condensation of mesenchymal stem cells. As the condensation enlarges, osteoblast differentiation occurs within the interior and osteoid matrix is secreted. The matrix is then mineralized, encasing

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osteoblasts within the matrix to form osteocytes. Osteoblasts also then line the bone underneath the periosteum. This matrix is then vascularized to support long term cell survival.

The key difference between intramembranous ossification and endochondral ossification is that in the latter bone forms as a result of replacement of a previously deposited cartilaginous template^{2,3}. This is the process by which the long bones of the body form. After formation of the template (composed of hyaline cartilage), chondrocytes in the area that will become the primary ossification center begin secreting alkaline phosphatase which promotes calcification of the cartilaginous matrix and then undergo apoptosis⁴. The bone collar then forms in the mid-portion of a future long bone via a process similar to intramembranous ossification. The bone collar then facilitates the penetration of blood vessels, providing a path for the introduction of pre-osteoblasts into the area which then begin depositing osteoid and subsequently mineralize the matrix in the primary ossification center. In addition, the distal ends of each long bone (epiphyses) form secondary ossification centers by a process similar to that outlined for the primary ossification center. The cartilage that remains between the primary and secondary ossification centers is referred to as the epiphyseal plate and continues to deposit new cartilage matrix to serve as a scaffold for additional endochondral ossification allowing the bone to grow in length. The hyaline cartilage that remains on the distal surface of the bone is referred to as articular cartilage.

3. WNT/ β -catenin Signaling

Wnts are a family of 19 secreted glycolipoproteins in mammals and can broadly be classified as signaling through β -Catenin-dependent (canonical WNT signaling) or β -Catenin-independent (noncanonical WNT signaling) pathways. WNT pathways play fundamentally important roles in regulating cell fate decision, cell polarity, and cell proliferation; and alterations in components of this pathway are among the most common events associated with human disease⁵.

The first mammalian *Wnt* gene, *Int1* (later referred as *Wnt1*), was identified in 1982 as an insertion target of mouse mammary tumor virus (MMTV) associated with the formation of mammary gland tumors⁶. Shortly thereafter, the *Drosophila Wingless* gene was recognized as a close homolog of *Int1*⁷. The extensive characterization of the genetic requirements for proper specification of *Drosophila* embryonic segment polarity allowed for identification of downstream components of this pathway. In 1991, a new nomenclature was proposed to rename the original *Int1* as *Wnt1* (a combination of *Wingless* and *Int1*) and served as the basis for the naming of all other genes in this family⁸.

Decades of work have defined the components of the WNT/ β -catenin pathway⁹, which is summarized briefly here (Figure 2). In the absence of Wnt, glycogen synthase kinase-3 (GSK-3) phosphorylates β -catenin, marking it for ubiquitin-dependent proteolysis. When Wnt binds to a member of the Frizzled family of seven-transmembrane receptors and either LRP5 or LRP6^{10,11}, GSK-3 activity is down-regulated. Inactivation of GSK-3 increases β -catenin in the cytosol and nucleus, allowing β -catenin to interact with TCFs and LEF¹². These complexes modulate the transcriptional activity of target promoters¹². Frizzled-

related proteins (sFRPs), as well as other secreted inhibitors like Dickkopf (DKK) and Sclerostin (SOST), regulate signaling at the level of the Wnt/Frizzled/Lrp interaction^{13–19}, while many proteins including Axin/Axin2 and APC control the pathway intracellularly^{11, 12}. Non-canonical pathways induced by Wnt ligands include those that activate Protein kinase C, Protein kinase A, mTOR, PI3K/AKT, and the planar cell polarity pathway which modulates cytoskeletal structure via regulation of small GTPases such as RHOB. These pathways can be induced by interaction of WNTs with Frizzled-receptors, putatively without the co-engagement of LRP5/6, but can also involve alternative receptors such as the receptor tyrosine kinase, ROR2²⁰.

4. Wnt/ β -catenin Signaling in Human Skeletal Diseases

Wnt signaling was first linked to skeletal development in 1994, when *Wnt3a*-deficient embryos were found to exhibit axial defects²¹. However, the identification of loss-of-function mutations in *LRP5* in human osteoporosis pseudoglioma (OPPG), a rare syndrome associated with dramatic reductions in bone mass, intensified interest in this area²². Shortly after this, two groups independently reported that point mutations in *LRP5*, which prevent Sclerostin (SOST)- and Dickkopf (DKK1-mediated) binding and inhibition, caused high bone mass in two different families^{23, 24}. Subsequent studies have identified numerous mutations in *LRP5* to be associated with alterations in human bone mass^{25, 26}. In addition, loss-of-function mutations in the highly related *LRP6* gene were also associated with osteoporosis in humans²⁷. Finally, loss of function mutations in *LRP4*, an antagonist of Wnt/ β -catenin signaling which shares homology with *LRP5/6*, were identified as causes of sclerosteosis in human patients²⁸.

Additional insight into the role of WNT signaling in human disease was obtained from studies of two related human conditions associated with high bone mass. Sclerostin (SOST), a Wnt antagonist, was found to be causally dysregulated in Sclerosteosis and Van Buchem's Disease, rare high-bone-mass genetic disorders^{29–32}. SOST is a Wnt/ β -catenin signaling antagonist which binds LRP5 and LRP6 to prevent their association with Wnt ligands³³. Recent work has definitively shown that interactions of SOST with both LRP5 and LRP6 are necessary for full activity^{34, 35}. Given that SOST is primarily expressed in osteocytes³³, inhibiting SOST to prevent it from normally downregulating LRP5 and LRP6 to treat osteoporosis may cause less unintended side effects^{31, 36} because blocking SOST function would not be expected to affect regulation of any tissues besides the skeleton.

Several other examples of Wnt pathway-associated mutations causing skeletal phenotypes in humans have emerged. For example, heterozygous nonsense mutation in *AXIN2* that leads to Wnt signaling activation causes both severe permanent tooth agenesis and colorectal neoplasia³⁷, and a homozygous *WNT3* loss-of-function mutation causes defects in limb formation and craniofacial and urogenital development³⁸. Finally, several high-profile recent reports have linked mutations in the *WNT1* gene to Osteogenesis Imperfecta^{39–42}, a disease characterized by the presence of brittle bone which is easily fractured.

5. Mouse Models to Assess the Role of β -catenin in the Osteoblast Lineage

Mouse models using different cell-specific promoters to manipulate Wnt components have been generated and characterized⁴³. We focus on insights generated by assessment of genetically engineered mice with alterations in β -catenin (encoded by the *Ctnnb1* gene), WNT ligands, and WNT receptors.

5.1 β -catenin

Ectopic activation of β -catenin in osteochondral progenitors at a stage before full commitment to either the chondrocyte or osteoblast lineage promotes osteoblast differentiation and inhibits chondrogenesis^{44–46}, while conditional inactivation of Wnt/ β -catenin signaling in progenitor cells has the opposite effect^{44, 47–49}. The developing bones with WNT/ β -catenin signaling inactivation were capable of expressing early markers of the osteoblast lineage, but failed to express markers of osteoblast commitment.

Loss of β -catenin in mature osteoblasts and/or osteocytes causes severe osteopenia associated with increased osteoclastogenesis, whereas constitutive activation of β -catenin within osteoblasts impairs osteoclast formation^{50, 51}. Osteoblasts and osteocytes secrete two proteins, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand (RANKL), required for osteoclast differentiation and maturation⁵² (Figure 3). RANKL stimulates osteoclast maturation through interacting with its receptor, RANK, expressed at the surface of osteoclast precursors. The RANKL-RANK interaction is also regulated by another secreted glycoprotein, osteoprotegerin (OPG), which can also be derived from osteoblasts/osteocytes⁵³. OPG is a soluble TNF α receptor that acts as a decoy receptor for RANKL, and it inhibits osteoclast maturation and protects bone from both normal osteoclast remodeling and ovariectomy-associated bone loss⁵⁴. Importantly, elevated OPG levels were associated with ectopic activation of β -catenin within osteoblasts^{50, 51} and OPG was shown to be direct transcriptional target of β -catenin⁵¹, consistent with the elevated osteoclastogenesis seen in mouse models with loss of β -catenin signaling in osteoblasts (Figure 3).

Importantly, understanding the mechanisms of OPG/RANKL interactions has facilitated the development of a novel treatment for osteoporosis, denosumab, which has demonstrated efficacy in increasing bone mass in human clinical trials^{55, 56}.

5.2 WNT Ligands in Bone

WNT requires a lipid modification to facilitate secretion and function, which complicated their purification and structural analysis⁵⁷. This lipid modification, the addition of a palmitoleic acid to a serine residue, is mediated by the ER-resident protein, Porcupine (encoded by the *PORCN* gene), a member of the membrane-bound O-acyltransferase family⁵⁸. Lipid-modified Wnt is then recognized by another ER-resident protein, Wntless/GPR177, which is required to escort Wnt ligands to the cell surface for secretion⁵⁸. Further confirmation of the importance of lipid modification in Wnt function was provided when the crystal structure of the *Xenopus* Wnt8 in complex with cysteine-rich domain (CRD) of

mouse FZD8 was reported revealing that the palmitoleic acid binds to specific pocket on the surface of the Frizzled protein⁵⁹.

Several groups, including our own, have taken advantage of the pivotal role of *Porcupine* or *GPR177* to all WNTs, to address questions related to the requirement for Wnt family ligands derived from a single cell type within bone to mediate bone development and/or homeostasis. Using this approach, mature osteoblasts were shown to be a critical source of Wnt ligands for postnatal skeletal maintenance^{60, 61}. Furthermore, when *Gpr177* was deleted in both osteoblasts and chondrocytes, cartilage development was affected and a more severe skeletal phenotype was observed^{62, 63}. Much work is still needed to elucidate the role of Wnts from different tissues, and finding a way to distinguish the consequence from inhibiting the effects of canonical or noncanonical Wnt would be invaluable.

5.2a Canonical Wnts in bone—Mice homozygous for germline deficient mutations of several Wnt genes exhibit skeletal phenotypes (reviewed in⁶⁴). One such example is that *Wnt10b*-deficient mice have slightly increased trabecular bone at a young age, but progressively lose bone as they age. This phenotype in *Wnt10b*-null mice is presumably due to progressive reduction of osteochondral progenitors^{65, 66}. Consistent with a role for Wnt10b in bone homeostasis, transgenic mice ectopically expressing Wnt10b in brown adipose and bone marrow showed increased bone mass⁶⁵. The recent finding that mutations in WNT1 were causally associated with some types of Osteogenesis Imperfecta also emphasizes the importance of Wnt proteins in normal bone development and homeostasis^{39–42, 67}. In addition, WNT16 was also found to influence bone mineral density, and specifically cortical bone thickness, but more work needs to be done to evaluate which downstream pathways mediate these effects^{68, 69}.

5.2b Noncanonical WNTs in bone—Some WNTs preferentially signal through β -catenin-independent mechanisms including Ca^{2+} flux, Protein kinase C activation, and planar cell polarity (PCP) pathway activation⁷⁰. Among noncanonical WNTs, WNT5A is the most extensively studied and plays a critical role in embryonic bone development⁷¹. WNT5A binds to the extracellular cysteine-rich domain (CRD) of ROR2 (Receptor tyrosine kinase-like orphan receptor 2)⁷². A close functional relationship between ROR2 and WNT5A is consistent with the fact that mice deficient for either gene develop similar phenotypes including dwarfism, facial abnormalities, and shortened limbs and tails,⁷³. Recently, osteoblast-derived Wnt5a was shown to enhance osteoclastogenesis⁷⁴ by engaging ROR2 on the surface of osteoclast precursors to activate Jun N-terminal kinase (JNK) which then increased Receptor activator of nuclear factor κB (RANK) expression. However, WNT5A may exert its effects in cell-context dependent manner. For example, a reduction in WNT5A expression was correlated with tail defects and spinal bifida associated with decreased cell proliferation within the ventral caudal region⁷⁵. A role for another noncanonical Wnt pathway required for bone formation, activated by WNT7B has also been demonstrated^{76, 77}. A link between WNT ligands, LRP receptors, and activation of metabolic pathways has also been established and will undoubtedly be the subject of intense study in upcoming years^{77, 78}.

5.3 Wnt Co-receptors in Bone

5.3a. LRP5/6—The finding that both loss-of-function and gain-of-function mutations in *LRP5* and *LRP6* alter bone homeostasis in humans stimulated studies on the role of Wnt signaling in this process. Global inactivation of *Lrp5* in mice recapitulates the phenotypes seen in human OPPG patients^{79–83}, and germline mutations in *Lrp6* in mice also decrease bone mass^{79, 84, 85}. Several recent studies demonstrate that osteoblast- or osteocyte-specific alterations of *Lrp5* and/or *Lrp6* result in significant changes in bone mass^{86, 87}.

It is important to note that an alternative model postulates that LRP5 functions through regulating serotonin secretion from the gut to affect osteoblast activity^{88, 89}. In this model, loss of LRP5 in the enterochromaffin cells of the intestine leads to elevated production and secretion of serotonin. Increased levels of serum serotonin then function via binding to the HTR1B receptor to inhibit osteoblast proliferation. However, data from several other laboratories is not consistent with this model^{87, 90}. The reasons for these discrepancies are currently unclear.

In terms of earlier stages of osteoblast differentiation, inactivation of both *Lrp5* and *Lrp6* in mouse osteochondral progenitors results in a failure in differentiation⁹¹, phenocopying what is seen in embryos in which β -catenin (*Ctnnb1*) is conditionally deleted in the same lineage^{44, 47–49}.

5.3b Other LDL Receptor Family Members—Several other members of the LDL receptor family (Figure 4) have putative roles in skeletal development and bone homeostasis. For example, loss-of-function mutations in *LRP4* are found in patients with bone overgrowth and skeletal abnormalities^{92–95}. LRP4 contains an extracellular motif that interacts with SOST supporting a model by which LRP4 is required to facilitate the activities of SOST^{92, 93, 96}. The interactions of LRP4 with the Wise protein may also contribute to the observed phenotypes⁹⁷. LRP8, also known as Apolipoprotein E Receptor 2 (APOER2), may also play a role in maintaining normal bone as siRNA-mediated knockdown of LRP8 in C2C12 myoblast cells or KS483 pre-osteoblast cells reduced β -catenin-induced transcriptional reporter activity and interfered with osteoblast differentiation in vitro. Finally, there are also reports that the LRP1 protein can modulate the activity of the WNT pathway, although further validation of the importance of this interaction in skeletal development is required^{98, 99}.

6. Therapeutic Considerations

Because Wnt/ β -catenin signaling is elevated in many human tumors, there is significant interest in identifying agents that inhibit the pathway to treat several types of cancer¹⁰⁰. The same properties of WNT/ β -catenin signaling to stimulate proliferation and/or regulate cell differentiation in the context of cancer cells are also what make it an attractive target for activation in the setting of regenerative medicine to treat diseases such as osteoporosis. It will be critical to remain cognizant of these properties in targeting this pathway in both settings.

One example of targeting the pathway for inhibition of tumor cell growth is an ongoing Phase I clinical trial in which a chemical inhibitor of Porcupine is being tested for efficacy in several types of cancer¹⁰¹. There is a strong rationale for these trials. However, clinicians monitoring patients participating in these trials should be cognizant of potential side effects related to the normal functions of WNT signaling in mediating self-renewal of stem cell compartments. For example, in the context of bone biology one would be concerned, given that inhibition of WNT secretion disrupts normal bone homeostasis^{60, 62}, that patients being treated with Porcupine inhibitors might be susceptible to increased bone loss. If this were the case, perhaps this could be partially mitigated by concurrent treatment with agents like bisphosphonates that block bone resorption.

In the case of anabolic therapies designed to treat conditions associated with low bone mass, the potential for agents which activate the WNT pathway to enhance cell proliferation should always be considered. One potentially reassuring aspect for the use of therapies that activate WNT/ β -catenin signaling in anabolic therapies is the experience with lithium chloride (LiCl)¹⁰². A major mechanism for LiCl action is inhibition of GSK3 activity, thus leading to stabilization of β -catenin. LiCl has been used for over 60 years as a treatment for bipolar disorder¹⁰². Despite this widespread use, there appears to be no reported cancer predisposition in these patients. Another way to diminish the potential for side effects in therapies designed to activate Wnt/ β -catenin is to use strategies that limit the targets to specific tissues. In this respect, the specific enrichment of Sclerostin expression from osteocytes is a significant advantage.

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Intramembranous Ossification

Endochondral Ossification

Figure 1. Overview of Bone Formation

During intramembranous ossification, new bone forms directly from a condensation of mesenchymal stem cells. In contrast, endochondral ossification occurs when a cartilaginous template is replaced by mineralized osteoid. In both cases, osteoblasts become encased in mineralized matrix creating osteocytes. Please refer to the text for more detail.

Figure 2. Canonical Wnt Signaling Pathway

In the absence of an upstream ligand, receptor Frizzled (FZD) and co-receptor LRP5/6 are inactive (1). The cytoplasmic β -catenin will be recruited into a "destruction complex" consisting of Axin, APC, casein kinase 1a (CK1) and glycogen synthase kinase 3 (GSK3) (2). This "destruction complex" facilitates the phosphorylation of β -catenin by GSK3 and subsequent ubiquitinylation (Ub) by β -TrCP, an E3 ligase, targeting β -catenin for proteasome-mediated degradation. In this context, the transcription repressor Groucho occupies T-Cell Factor/Lymphoid enhancer factor (TCF/LEF) DNA binding sites (7). This inactive state could also be caused by lack of co-receptors availability due to effectors (DKK1/WISE/SOST) binding to LRP5/6 and preventing its association with Wnt (3), or by other effectors associating with Wnts and blocking their ability to interact with the co-receptor complex (4). When a Wnt ligand engages the receptor complex (5), the C-terminus of LRP5/6 is phosphorylated, creating a binding site for Axin, resulting in inhibition of the destruction complex (6). This inhibits GSK3 activity, allowing cytoplasmic levels of β -catenin to increase (8). β -catenin subsequently translocates into the nucleus and complexes with LEF/TCF proteins and other co-factors to activate transcription of target genes (9).

Figure 3. Regulation of RANKL Signaling

The Receptor activator of nuclear factor $\kappa\beta$ (RANK) is expressed on osteoclasts and upon binding from its cognate ligand, RANKL, stimulates osteoclast differentiation and activation. RANKL is expressed by cells of the osteoblast lineage, which also express another protein referred to as Osteoprotegerin (OPG). OPG binds to RANKL, prevents interaction between RANKL with RANK with the end result being inhibition of osteoclast differentiation and activation. OPG expression can also be activation at the transcriptional level via the binding of β -catenin/TCF complexes to the OPG promoter.

Figure 4. Low-density Lipoprotein (LDL) Receptor Family Members with Putative Roles in Bone Development

The Low-density lipoprotein related receptors are single pass Type I transmembrane proteins which share several structural motifs. These include β -propellers (each containing several individual EGF repeats) and ligand-binding repeats (which mediate interactions with apolipoproteins). The relative sizes of LRP1, LRP4, LRP5/6, and LRP8 are represented. LRP5 and LRP6 form a novel subclass of this family with the cytoplasmic tail of these proteins containing PPPS/TP motifs which are targets of phosphorylation and act to stimulate downstream signaling upon activation by WNT ligands.