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Wnt and the Wnt signaling pathway in bone development and disease

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Abstract

Wnt signaling affects both bone modeling, which occurs during development, and bone remodeling, which is a lifelong process involving tissue renewal. Wnt signals are especially known to affect the differentiation of osteoblasts. In this review, we summarize recent advances in understanding the mechanisms of Wnt signaling, which is divided into two major branches: the canonical pathway and the noncanonical pathway. The canonical pathway is also called the Wnt/β-catenin pathway. There are two major noncanonical pathways: the Wnt-planar cell polarity pathway (Wnt-PCP pathway) and the Wnt-calcium pathway (Wnt-Ca^{2+} pathway). This review also discusses how Wnt ligands, receptors, intracellular effectors, transcription factors, and antagonists affect both the bone modeling and bone remodeling processes. We also review the role of Wnt ligands, receptors, intracellular effectors, transcription factors, and antagonists in bone as demonstrated in mouse models. Disrupted Wnt signaling is linked to several bone diseases, including osteoporosis, van Buchem disease, and sclerosteosis. Studying the mechanism of Wnt signaling and its interactions with other signaling pathways in bone will provide potential therapeutic targets to treat these bone diseases.

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Keywords

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2. INTRODUCTION

Bone is the rigid tissue that functions to move, support, and protect various organs of the body. It is made mostly of collagen and calcium phosphate. Physiological bone turnover can be divided into two temporal phases: modeling, which occurs during development, and remodeling, a lifelong process involving tissue renewal (1). Bone development has two stages (2): intramembranous ossification and endochondral ossification. Intramembranous ossification occurs in the formation of flat bones. This begins with the condensation of mesenchymal stem cells, which then differentiate into osteoprogenitors and become mature osteoblasts. Later, these osteoblasts will either undergo apoptosis or change into osteocytes. Endochondral ossification, which takes place on the long bone, also begins with mesenchymal stem cell condensation. Unlike intramembranous ossification, cartilage is present during endochondral ossification. Osteoblasts and osteoclasts are two major bone cells that affect the remodeling process. There is a balance between osteoclastic bone resorption and osteoblastic bone formation. Most adult skeletal diseases are due to the disturbance of this balance, including osteoporosis, multiple myeloma, and cancer metastases. Therefore, study of the proliferation and differentiation of osteoblasts and osteoclasts can help us to deeply understand these diseases and develop better treatments.

The Wnt family consists of a number of highly conserved genes that regulate gene expression, cell behavior, cell adhesion, and cell polarity, including 19 genes in humans and mice, 7 in *Drosophila*, and 5 in *C. elegans.* The term “Wnt” is derived from the terms wingless and int. The Int oncogenes, including Int1, were first identified in the mouse mammary tumor. In 1987, investigators sequenced *wingless* in *Drosophila* and found it was the homolog of *int-1*(3). In mammals, the complexity and specificity in Wnt signaling are in part achieved through Wnt ligands, R-spondin proteins, and norrin. Receptors on the cell surface and a multi-step process within the cell trigger downstream gene expression.

The production and secretion of Wnt ligands requires lipid modification by the acyltransferase Porcupine (Porcn) followed by the binding of Wntless (Wls), which serves as a Wnt chaperone and facilitates the transport of lipid-modified Wnt to the plasma membrane (4–10). The Wnt pathway is divided into two major branches: the canonical pathway and the noncanonical pathway. The canonical pathway is also called the Wnt/β-catenin pathway (11). There are two major noncanonical pathways: the Wnt-planar cell polarity pathway (Wnt-PCP pathway)(12) and the Wnt-calcium pathway (Wnt-Ca^{2+} pathway)(13). The effect of Wnt ligands, receptors, intracellular effectors, transcription factors, and antagonists on both the bone modeling and remodeling processes have been studied in mouse models (Table 1).

Wnt signaling proteins participate in multiple developmental events during embryogenesis and adult tissue homeostasis. Wnt signals have multiple functions, including mitogenic

stimulation, cell fate determination, and differentiation (1). Wnt signals also affect bone development, especially the differentiation of osteoblasts. It has always been a hot spot of research since the first Wnt family protein was identified. This review summarizes various Wnt signaling pathways and discusses how the Wnt signaling pathway influences bone development and bone diseases.

3. WNT CANONICAL SIGNALING PATHWAY IN SKELETOGENESIS

3.1. Wnt ligands and Wnt agonists in bone

Wnt ligands, which are cysteine-rich proteins of approximately 350–400 amino acids that contain an N-terminal signal peptide for secretion (14), have distinct effects on different phases of bone development, including chondrogenesis, osteoblastogenesis, and osteoclastogenesis. A recent study reported that *Wnt1* mutations were found in four children who have osteogenesis imperfecta (15), a genetic disorder of increased bone fragility, low bone mass, and other connective-tissue manifestations (16). The *Wnt1* knockout mouse model has severe mid- and hindbrain deficiencies (17, 18). *Wnt1* and *Wnt3a* control expression of dorsal genes and suppression of the ventral programs though the Wnt canonical pathway and gliotactin (Gli) activity (19, 20). *Wnt2b* functions with T-box 5b (*Tbx5b)* to initiate forelimb outgrowth and identity through fibroblast growth factor 10 (*fgf10)* (21). *Wnt3a* regulates dorsal mesoderm fate (22) and is required at the earliest stages of limb formation (23, 24) and craniofacial development (25). *Wnt4*, *Wnt6*, *Wnt9a*, and *Wnt16* are required for joint formation (26–28). Conditional expression of Wnt4 during chondrogenesis in $R26^{floxneoWnt4}$*; Col2a1-Cre* mutant mice resulted in dwarfism and an increased number of hypertrophic chondrocytes (29). Yingzi Yang's *et al.* revealed that although *Wnt5a* and *Wnt5b* are the closest Wnt relatives to each other, they exhibit distinct activities in coordinating chondrocyte proliferation and differentiation (30, 31). A recent study showed that *Wnt3a*+/− and *Wnt5a*+/− mice have a low bone mass phenotype (32). Wnt6, Wnt10a, and Wnt10b stimulate osteoblastogenesis and inhibit adipogenesis (33–36). Mutations in *Wnt7a* result in defects in limb development (37–41). *Wnt7b* and *Wnt11* are identified as endogenous ligands regulating chondrocyte and osteoblast differentiation (42–44). *Wnt16* deficiency decreases bone mineral density and increases fracture risk (45).

Norrin, a highly divergent member of the transforming growth factor-beta superfamily, is a kind of Wnt agonist. It exhibits highly-binding-affinity with Frizzled-4(46). Together with low-density lipoprotein receptor-related protein (LRP), Norrin and Frizzled-4 can activate the canonical Wnt signaling pathway (46). Thus, Norrin is related to several inherited disorders, including osteoporosis-pseudoglioma syndrome (47).

The other class of Wnt agonists, the R-spondin family, consists of 4 types of R-spondins (*i.e.* R-spondin1–4) which stimulate β-catenin-dependent signaling. R-spondins were discovered by Kazanskaya *et al.* and identified as a novel family of secreted Wnt agonists (48). All R-spondins contain an N-terminal signal peptide, two furin-like domains, one thrombospondin type 1 domain, and a C-terminal low complexity region enriched with positively charged amino acids (48). R-spondins promote osteoblast differentiation and are highly expressed in skeletal tissues during development and postnatally (49). R-spondin1 promotes osteoblast differentiation and bone formation while blocking osteoclastogenesis (50). R-spondin2

deficiency results in skeletal developmental defects (49). R-spondin3 is required for head cartilage morphogenesis through Wnt/PCP signaling pathway (51). R-spondin4 mutations result in anonychia, which is the absence or hypoplasia of nails on fingers and toes (52).

3.2. Wnt canonical signaling pathway

The Wnt/β-catenin signaling pathway is the best studied of the Wnt pathways. Although Wnt signals through several pathways to regulate cell growth, differentiation, function, and death; it is central to the bone development and homeostasis in adults (53, 54). WNT signaling has been studied primarily in developing embryos, in which cells respond to WNTs in a context-dependent manner, but WNTs also have important functions in adults (55). The current model of how Wnt signals are transduced in the Wnt canonical pathway is shown in Figure 1B. Wnt proteins, following their binding to a frizzled receptor and a Lrp co-receptor (most likely LRP6), activate the canonical Wnt signaling pathway. These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin. This results in the translocation to nucleus of β-catenin, the association of β-catenin with members of the Lef1/Tcf nuclear protein family, and the activation of a specific program of gene expression (See Figure 1B, C)

3.3. Wnt receptors and their inhibitors in bone

3.3.1. Frizzled protein and its antagonists (sFRPs) regulate osteoblastogenesis and osteoclastogenesis—Genetic and biochemical data have demonstrated that the Frizzled (Fz) proteins are primary receptors for the Wnts (56). Frizzled proteins transmit signaling through both β-catenin-dependent and β-catenin independent pathways. All members of the Fz family are characterized by the following features: a putative signal sequence followed by a sequence of 120 amino acids (aa) containing 10 highly conserved cysteine-rich domains(CRD), a highly divergent region of 40–100 aa predicted to form a flexible linker, seven transmembrane segments separated by short extracellular and cytoplasmic loops, and a cytoplasmic tail (57, 58). The CRD appears to be the ligand-binding site of Frizzled proteins. Osteoclastogenesis is promoted independently of osteoblasts in *Fzd8* deficient mice (59). *Fzd9* is required for bone formation (60).

Secreted Frizzled-related proteins(sFRPs), the largest family of Wnt inhibitors, share sequence similarity with the cysteine-rich domain found in the extracellular region of Frizzled (61). sFRPs bind the Wnt ligands through their CRD, thereby preventing their binding to the Frizzled receptor (62). Bodine *et al.* demonstrated that sFRP-1 is an important regulator of osteoblast and osteocyte survival *in vitro* and *in vivo*. They developed a *sFRP1*$^{-/-}$ mouse line and show that deletion of *sFRP-1* not only reduces osteoblast and osteocyte apoptosis, but also potentiates osteoblast proliferation and differentiation, and increases trabecular bone formation (63–65). sFRP1 transgenic mice exhibit blocked bone formation and decreased trabecular bone mass (66). According to Gillespie *et al.*, sFRP-1 also plays a role in receptor activator of NF-kappaB ligand (RANKL)-dependent osteoclast formation (67). sFRP-2 and sFRP-4 are required for limb development (68) and bone formation (69, 70).

3.3.2. Low-density lipoprotein receptor-related proteins regulate osteoblast differentiation through Wnt signaling—The low-density lipoprotein receptor-related protein (LRP) family is a single-pass transmembrane molecule family involved in Wnt signaling. In addition to Wnt/Frizzled interactions, LRP5/6 is required for Wnt signalling in invertebrates (71, 72). The gene *arrow* in *Drosophila* encodes a transmembrane protein that is homologous to LRP5/6 (73). All members of the family contain highly conserved motifs. Most notably, all contain several complement-type and epidermal growth factor (EGF) precursor-like repeats, as well as single transmembrane and cytoplasmic domains (74). LRP5 and LRP6 are 71% homologous and they have overlapping roles during bone mass accrual (75). Investigators proposed that Wnt protein binds to Fz and LRP to form a complex, although it hasn't been observed in *Drosophlia* (76). Extensive evidence indicates the importance of low-density lipoprotein receptor-related proteins in Wnt signaling and the regulation of bone formation. *In situ* hybridization on skeletal elements in developing mice to determine LRP5 is expressed in osteoblast and transducing Wnt signaling (77). The intracellular domain of LRP5 can interact with Axin, stabilize β-catenin, and induce LEF activation (78). Embryos homozygous for an insertion mutation in the LRP6 gene exhibit developmental defects that are a striking composite of those caused by mutations in individual Wnt genes. This indicates a broad role for LRP6 in the transduction of several Wnt signals in mammals (71). Moreover, mice with a targeted disruption of LRP5 develop a low bone mass phenotype. *In vivo* and *in vitro* analyses indicate that this phenotype becomes evident postnatally, and demonstrate that it is secondary to decreased osteoblast proliferation and function in a RUNX-2-independent manner (79). Humans and mice lacking LRP5 exhibit low bone mineral density (BMD). Compound mutants have dose-dependent deficits in BMD, suggesting functional redundancy between LRP5 and LRP6 in bone development (75). Conversely, a gain of function mutation in LRP5(Gly171Val) causes a hereditary high bone mass trait in humans, and transgenic mice expressing this mutation in osteoblasts display greater bone formation and density (80, 81). LRP6 showed similar function as the LRP5 in a spontaneous point mutation mouse model, the ringelschwanz(rs). This model, with a point mutation that leads to an amino acid substitution of tryptophan for the conserved residue arginine at codon 886(R886W) and cannot efficiently transduce signals through Wnt signaling, exhibited delayed ossification at birth and a low bone mass phenotype in adults (82). Disrupting LRP5/6 could affect osteoblastogenesis, then bone formation, and ultimately trigger bone diseases. Mutation in LRP5 causes the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG), whose carriers have reduced bone mass when compared to age- and gender-matched controls (77). Recent studies show that missense mutations in LRP6 can lead to osteoporosis (82, 83). In a duodenal-specific Lrp5 activating mouse model, it was demonstrated that Lrp5 regulates bone mass by affecting serotonin synthesis (84). N-cadherin interacts with LRP5/6 and suppresses Wnt signaling and bone formation, which can be disrupted by a competitor peptide (85). This finding provides a new strategy to promote osteoblast function and bone formation through Wnt signaling. Another member of the LRP family, LRP4, shares structure elements within the extracellular ligand binding domain with LRP5 and LRP6. LRP4 is expressed in bone and is a novel osteoblast expressed Dkk1 and SOST receptor with a physiological role in the regulation of bone growth and turnover (86). LRP4 also can bind Wise, a secreted Wnt modulator and Bone morphogenetic protein (BMP) antagonist.

Recently, a group in the Netherlands identified a new member of the low-density lipoprotein receptor-related protein family: Lrp8 (87). Knockdown of Lrp8 results in a decrease in the level of β-catenin (87). These results indicate that Lrp8 is a novel positive factor of Wnt signaling and may play a role in controlling osteoblast differentiation.

3.3.3. Dkk and Kremen regulate bone mass by modulating Lrp5/6—Among the several known modulators of Lrp5 activity, Dkk proteins are the best characterized secreted Wnt-signaling inhibitors. Dickkopf-1(DKK1), a member of the Dickkopf family, is indispensable for embryonic head induction and limb development in mice (88). Endogenous Dkk1 expression was detected primarily in osteoblasts and osteocytes (89). While *Dkk1* null mice die at birth due to a lack of head structure, *Dkk1* heterozygous mutants *(Dkk1*$^{+/-}$) display increased bone formation and high bone mass phenotype (90). Conversely, the Dkk1 transgenic mouse [collagen, type I, alpha 1 (Col1A1)] showed systemic osteopenia, decreased osteoblastic bone formation, and unaffected osteoclastogenesis (89). Dkk1 is over-produced in human cancer cells while developing osteolytic lesions associated with metastatic bone disease (91–94). Knockdown of Dkk1 expression by end-capped phosphorothioate Dkk1 antisense oligonucleotide (Dkk1-AS) abrogated dexamethasone suppression of alkaline phosphatase activity and osteocalcin expression in MC3T3-E1 preosteoblasts. Exogenous Dkk1-AS treatment alleviated dexamethasone suppression of mineral density, trabecular bone volume, osteoblast surface, and the rate of bone formation in bone tissue and *ex vivo* osteogenesis of primary bone marrow mesenchymal cells (95). Notably, Dkk1 inhibits Wnt signaling by binding to the LRP6 component of the receptor complex, instead of exerting an inhibitory effect by molecular mimicry of Fz or Wnt sequestration like most other Wnt antagonist (96). LRP5 gain-of-function mutations which alter the first epidermal growth factor (EGF)–like domain (*i.e.* LRP5 -propeller 1 region) can prevent DKK1-LRP5 interaction and are the cause of high bone mass (HBM) and mandibular, buccal, and lingual exostoses (80, 91). The *Dkk2*$^{-/-}$ mice represent decreasing bone formation by affecting terminal osteoblast differentiation and mineralized matrix formation (97). This result suggests that Dkk2 plays an opposite role with Dkk1 in osteoblastogenesis. Dkk2 deficiency led to a substantial increase in the number of osteoclasts by delayed mineralization of osteoblasts (97, 98). Dkk1 antagonizes LRP5/6 by competitively binding to LRP with high affinity (99), and its antagonistic function is significantly enhanced by Kremens, which are another type of transmembrane molecule (100). Kremen1 (*Krm1*) and Kremen2 (*Krm2*) are high-affinity Dkk1 receptors that functionally cooperate with Dkk1 to block Wnt canonical signaling (101). Kremen2 forms a ternary complex with Dkk1 and LRP6, and induces rapid endocytosis and removal of the Wnt receptor LRP6 from the plasma membrane. As we described before, the R-spondin (RSpo) family of secreted proteins act as potent activators of the Wnt signaling pathway (102). Although RSpo1 does not directly activate LRP6, it interferes with DKK1/Kremen-mediated internalization of LRP6 through an interaction with Kremen, resulting in increased LRP6 levels on the cell surface (102). *Krm2*, unlike *Krm1*, is predominantly expressed in bone. Specific overexpression of *Krm2* in osteoblasts in transgenic mice(*Col1a1-Krm2*) results in severe osteoporosis (103). Dexamethasone, an agent known to induce osteoporosis, upregulates Dkk1 expression in primary human

osteoblasts and provides a molecular explanation for osteoporosis caused by long-term glucocorticoid use (104).

3.3.4. Other Wnt antagonists—Sclerostin (SOST), which is a member of the cysteine-knot superfamily, has been localized to the chromosome region 17q12-q21 (105). SOST is expressed exclusively in mouse and human bone by osteocytes embedded within the mineralized matrix (106, 107). At first, sclerostin was considered a BMP signaling antagonist because it competed with type I and type II bone morphogenetic protein receptors for binding to BMPs, decreased BMP signaling, and suppressed mineralization of osteoblastic cells (108). However, subsequent studies have shown that it is also a Wnt signaling antagonist by binding LRP5/6(109, 110). Sclerostin binding to the six-bladed β-propeller domain of LRP5/6 is mediated by the central core of sclerostin but not the amino- and carboxyl- terminal flexible arm region (111). Sclerostin also binds to Lrp4 and functions in bone formation and turnover (86, 112). Mouse genetics have demonstrated the link between SOST and bone formation. *In vivo*, $SOST^{-/-}$ mice showed a high bone mass state, and transgenic mice overexpressing SOST exhibited low bone mass and decreased bone strength (108, 113, 114).

Wise shares 38% amino acid identity with sclerostin and appears to be a context-dependent regulator of Wnt signaling; it may inhibit or stimulate Wnt signaling. Data from Itasaki *et al.* shows that Wise is an inhibitor of Wnt signaling by binding to the Wnt co-receptor, lipoprotein-related protein 6, LRP6 and thus competing with Wnt8 for binding to LRP6(115).

Wnt-inhibitory factor-1(WIF-1) is a secreted protein that binds to Wnt proteins and inhibits their activities (116). The deduced 379-amino acid WIF-1 secreted protein contains an N-terminal signal sequence, a 150-amino acid WIF domain, 5 epidermal growth factor (EGF; 131530)-like repeats that are similar to those of tenascin, and a C-terminal hydrophilic domain of approximately 45 amino acids (116). WIF-1 is present in fish, amphibians, and mammals, and is expressed during Xenopus and zebrafish development in a complex pattern that includes paraxial presomitic mesoderm, notochord, branchial arches and neural crest derivatives. *In vitro*, WIF-1 binds to Drosophila Wingless and Xenopus Wnt8 produced by Drosophila S2 cells.

3.4. Dishevelled and Axin proteins relay Wnt signals from receptors to downstream effectors

Dishevelled (Dvl-1, -2, and -3 in mammalian, Dsh in Drosophila) is composed of an amino-terminal DIX domain, a PDZ domain in the middle, and a carboxy-terminal DEP domain (117). Dsh can interact with Fz directly (118) through the conserved the motif (Lys-Thr-X-X-X-Trp) located two amino acids after the seventh transmembrane domain in Fz (119). Investigators have identified PAR-1 as a Dsh-associated kinase. PAR-1 potentiates Wnt activation of the β-catenin pathway. Suppressing endogenous PAR-1 function inhibits Wnt signaling through β-catenin in mammalian cells, and Xenopus and Drosophila embryos. PAR-1 seems to be a positive regulator of the β-catenin pathway.

Similar to Dsh, cytosolic protein Axin (a scaffolding protein controlling beta-catenin stability) interacts with LRP. Wnts stimulate phosphorylation of LRP on the Pro-Pro-Pro-(SerTrp)Pro[PPP(S/T)P] motif, which creates an inducible docking site for Axin (78, 120, 121). The Dvl and Axin proteins each contain a conserved DIX domain in their sequences (122). Though their DIX domain, Dvl-1 directly binds to Axin and Dvl-1 inhibits Axin-promoted GSK-3β-dependent phosphorylation of β-catenin and APC. Furthermore, deletion of the DIX domains of Dvl-1 and Axin destroys their abilities to accumulate and to degrade β-catenin (123). Possibly, Wnt binding of Fz and LRP promotes direct interactions between Axin and Dvl through their domains, reconfiguring the protein complex that regulates the level of β-catenin in the cell (11).

3.5. Wnt signaling in cytoplasm

In the absence of Wnt ligands, a master complex comprising APC, GSK-3β, Axin, and Casein kinase I (CKI) phosphorylates cytoplasmic β-catenin, marking it for ubiquitination and subsequent proteasomal degradation (124). Wnt ligands binding to the membrane co-receptors (LRP5/6 and Frizzled) inhibit this complex, allowing nuclear translocation of dephosphorylated β-catenin, where it activates a large number of context-dependent target genes (125).

The *Apc* (*adenomatous polyposis coli*) tumor suppressor gene is involved in the initiation and progression of colorectal cancer (126). Conditional homozygous *Apc* mutation mice died perinatally showing greatly impaired skeletogenesis. The majority of the precursor cells lacking APC-mediated control of β-catenin level failed to differentiate into chondrocytes or osteoblasts (127). Also, APC is suggested to regulate the function of chondrocytes, osteoblasts, and osteoclasts though catenin-cadherin interactions. Conditional knockout of Apc with the osteocalcin promoter disclosed dramatic defects in bone development, a significant accumulation of bone matrix, disturbance in bone architecture, rapidl rate of bone formation, and lack of osteoclasts (128). Conditional knockout of Apc with the Col2a1 promoter is embryonic lethal and it causes the majority of the precursor cells lacking Apc to fail to differentiate into chondrocytes or osteoblasts (127). Mice carrying osteoblast-specific deletion of both the Apc and β-catenin genes display growth and survival characteristics similar to those lacking only the β-catenin gene, suggesting that the severe phenotype induced by loss of Apc is due to dysregulation of β-catenin signaling (128).

Axin acts as a scaffold in the Axin-APC-GSK3β-CKI complex to assemble β-catenin substrate and kinases (GSK3β and CKI)(129). Axin has several domains. The RGS (Regulators of G protein signaling) domain interacts with APC(78). The DIX domain can interact with Dishevelled as discussed before. There are two vertebrate Axin genes, which act as negative regulators (130). Axin1 is constitutively expressed, but Axin2 (Axil) is a direct target of the Wnt pathway and mediated through Tcf/LEF factors. This suggests that Axin2 participates in a negative feedback loop, which could serve to limit the duration or intensity of a Wnt-initiated signal (131). Mice with deletion of *Axin1* exhibit defects in axis determination and brain patterning during early embryonic development (132). *Axin2*$^{-/-}$ mice display enhanced expansion of osteoprogenitors, accelerated ossification, stimulated expression of osteogenic markers, and increased mineralization (133). *Axin2*-null mice

exhibit a phenotypic defect resembling craniosynostosis in humans (133). Recently, another group revealed that disruption of *Axin2*$^{-/-}$ expression not only played a critical role in intramembranous bone formation, but also accelerated chondrocyte maturation and influenced the endochondral bone formation (134).

Glycogen synthase kinase 3(GSK3) has two highly conserved isoforms α and β originally identified in 1980(135). In the Wnt pathway, GSK3β is recruited to form a complex via interaction with Axin, where it phosphorylates three serine(S)/threonine(T)residues(S33, S37, T41) at the amino-terminal region of β-catenin (130, 136). These phosphorylated S/T residues are critical for its recognition by the F-box β-Trcp (130). Hyperphosphorylated β-catenin is subjected to ubiquitylation by the F-box β-Trcp E3 ligase complex followed by degradation via the 26S proteasome (137). Hoeflich *et al.* found that lithium treatment, which inhibits GSK-3, can inhibit transactivation of NF-κB (a key transcription factor of osteoclasts) without affecting degradation of I-κB and translocation of NF-κB to the nucleus (138). Thus, NF-κB is regulated by GSK-3 at the level of the transcriptional complex (138). *GSK3α*$^{-/-}$*;GSK3β*$^{+/-}$ mice exhibit a dwarfism phenotype with significantly shortened long bones and vertebra, while *GSK3α*$^{+/-}$ and *GSK3β*$^{+/-}$ mice display normal skeleton development (139).

Casein kinase Ialpha (CKIα) is another Axin-associated kinase, whose phosphorylation of β-catenin is required for subsequent phosphorylation of β-catenin by GSK3(140). Wnt signaling inhibits GSK3β, but not CKIα phosphorylation of β-catenin (130, 141). Therefore, CKIα may represent a node at which other signaling pathways regulate β-catenin protein (130, 141).

Rac1, a Rho-family small GTPase, can accumulate β-catenin via $G\alpha_{q/11}\beta\gamma$ signaling involving phosphatidylinositol-3 kinase(PI-3K)(142). The role of Rac1 depends on the phosphorylation of β-catenin at Ser191 and Ser605, an event chiefly mediated by c-Jun NH2-terminal kinase 2(JNK2) in the stromal cell line ST2 (142). Mutations of these residues significantly affect β-catenin nuclear accumulation in response to Wnt (142).

SRY-box containing gene 9 (Sox9) is an intrinsic transcription factor that is inhibited by the Wnt canonical signaling pathway (143). It can antagonize Wnt/β-catenin signaling in chondrocyte differentiation in two distinct mechanisms: the Sox9 N-terminus is necessary and sufficient to promote β-catenin degradation, whereas the C terminus is required to inhibit β-catenin transcriptional activity without affecting its stability (143).

There are generally two pools of β-catenin: one is associated with cadherins while the other is “free” in the cytosol/nucleus. The latter pool is involved in gene transcription regulation (137). Phosphorylated β-catenin is specifically recognized by beta-transducin repeat containing protein (β-Trcp), an F-box/WD40-repeat protein that also associates with S-phase kinase-associated protein 1 (Skp1), which is an essential component of the ubiquitination apparatus (144). Mutations at the critical phosphoserine residues of β-catenin results in the loss of recognition by β-Trcp and in the accumulation of β-catenin (144). Inhibition of endogenous β-Trcp function by a dominant negative mutant stabilizes β-catenin and

activates the Wnt canonical pathway (144). Activating mutations in the human β-catenin gene have been found in human colon cancer and melanomas (145).

β-catenin through Wnt signaling plays a very important role in skeletal development by regulating chondrogenesis, osteoblastogenesis, osteoclastogenesis, and limb patterning. First, β-catenin regulates chondrocyte differentiation (26, 146, 147). The transgenic mouse of β-catenin under Col2A1 promoter control reveals that cartilage formation and endochondral ossification were greatly reduced (26). In the *β-catenin*$^{c/c}$, *Dermo-1-Cre* mice, the long bones were greatly shortened, thickened, and bowed, and cartilage formation was ectopic due to the ectopic chondrocyte differentiation at the expense osteoblasts (148). Detailed *in vivo* and *in vitro* loss- and gain-of-function analysis reveals that β-catenin activity is necessary and sufficient to repress the differentiation of mesenchymal cells into Runx2- and Sox9-positive skeletal precursors (146). These results suggest that β-catenin is required for the suppression of chondrocyte differentiation and the allowance of osteoblast formation during both intramembranous and endochondral ossification (148). Recent studies of inducible cartilage-derived β-catenin uncovered that β-catenin also affects chondrocyte maturation, primary and secondary ossification center development, and perichondral bone formation (149, 150). Furthermore, β-catenin could affect osteoblast differentiation (146, 147). Global inactivation of β-catenin results in early embryonic death. Conditionally inactivatable β-catenin mice expressing cre under the control of the osteocalcin promoter displayed striking reductions in both the trabecular and cortical bone compartments (128). Study of the calvarial osteoblasts of the conditional knockout mice *in vitro* revealed that β-catenin is not required for the initial commitment of cells to the osteoblast lineage, but that it appears to be essential for the performance of more mature osteoblast (128). Interestingly, Long *et al.* found that conditional knockout of *β-catenin* in osterix expressing osteoblasts promotes osteoblast formation and suppresses bone resorption (151). This finding indicates a complicated role for *β-catenin* in bone homeostasis. Recent studies demonstrate that BMP-2 acts synergistically with β-catenin to promote osteoblast differentiation. The Wnt autocrine loop mediates the induction of alkaline phosphatase and mineralization by BMP-2 in pre-osteoblastic cells (152). Additionally, alterations in β-catenin signaling in osteoblasts brought about by each mutation leads to marked disturbances in osteoclast differentiation (128, 148), as evidenced by the dramatic increase in osteoclast numbers and severe osteopenia in β-catenin conditional knockout mice (128). Stabilizing expressed β-catenin in mice could cause osteopetrosis through osteoclast defects (153, 154). Constitutive activation of β-catenin in osteoclast cells causes severe osteopetrosis (154). Dosage-dependent inhibition of β-catenin expression shows an opposite phenotype of mice. β-catenin heterozygosity in osteoclast lineage causes osteoporosis while β-catenin deletion in osteoclasts causes osteopetrosis (154). Other studies have shown that the osteoprotegerin (*OPG)* gene, a major inhibitor of osteoclast differentiation, may be a direct transcriptional target for complexes containing the β-catenin protein (153, 155). Mesenchymal β-catenin has multiple roles during limb patterning (156). Abnormal expression of mesenchymal β-catenin causes limb truncation and apical ectodermal ridge (AER) defects (156). *In vitro*, osteoblasts lacking the β-catenin gene exhibited impaired maturation and mineralization with elevated expression of the osteoclast differentiation factor, RANKL, and diminished expression of the RANKL decoy receptor, osteoprotegerin (128). According to these

findings, we know that β-catenin regulates bone development during different phases and that abnormal β-catenin may cause bone diseases (*e.g.* osteoporosis and osteopetrosis).

3.6. Wnt signaling in nucleus

In vertebrates, β-catenin acts astranscriptional activator, which is needed to overcome target gene repression by Groucho/TLE proteins and to permit promoter activation as the final consequence of Wnt signaling (157). The vertebrate transcription factors T cell factor (TCF) and lymphocyte enhancer binding factor (LEF) interact with β-catenin and mediate Wnt signaling (158). XTcf-3, also known as transcription factor 7-like 1 (T-cell specific, HMG-box), is a maternally expressed Xenopus homolog of the mammalian (high-mobility-group) HMG box factors Tcf-1 and Lef-1. N-terminal deletion of XTcf-3 (delta N) abrogates the interaction with β-catenin, as well as the consequent transcription activation (159). *Tcf1* is one of the two *Tcf* genes expressed in osteoblasts. Mice lacking *Tcf1* exhibit a low bone mass phenotype that is caused by a secondary increase in bone resorption, as indicated by the increased number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts (153) Mice lacking *Tcf1* also exhibit dwarfism caused by inhibition of chondrocytes differentiation (160). Constitutive expressing β-catenin binding and cooperation element, Lef1ΔN, in osteoblasts increases trabecular bone mass (161). In $Lef^{-/-}Tcf1^{-/-}$ double knockout mice, embryos have defects in limb bud development and paraxial mesoderm differentiation (162). *Opg* expression is decreased in $Tcf1^{-/-}$ osteoblasts, indicating that TCF1 regulates osteoclast differentiation through *Opg* (153).

Runx2 (runt homology domain transcription factor 2) is the major transcription factor for osteogenesis (163). It determines the osteoblastic differentiation at the early stage and inhibits it at the late stage (164). Wnt-dependent gene expression increases during the early phase of osteoblast differentiation *in vitro,* is enhanced by prostaglandin activation of the transcription factor Runx2, and is specifically suppressed in Runx2 antisense-depleted osteoblasts (165). Runx2 can form a complex with Lef or TCF, which then binds the composite binding site in the *fgf18* promoter, a direct target of Wnt canonical signaling and an essential regulator of bone development (166).

4. NONCANONICAL WNT SIGNALING PATHWAY PROMOTES BONE FORMATION

For a long time it was thought that all Wnt signaling was mediated through β-catenin. However, research now proves that Wnt also signals through β-catenin-independent mechanisms, known as the noncanonical pathway, to regulate vertebrate development (167). Like the canonical Wnt pathway, which plays an important role in bone development and diseases, the noncanonical Wnt pathway also participates in bone formation. Noncanonical pathways can be divided in two major subpathways (Figure 1E): the Wnt-planar cell polarity pathway (Wnt-PCP pathway)(12) and the Wnt-calcium pathway (Wnt-Ca^{2+} pathway)(13). In the Wnt-PCP pathway, *Wnt5a* regulates limb morphogenesis (168), chondrogenesis (169–171) and osteoblastogenesis (172) with receptor tyrosine kinase-like orphan receptor (Ror) proteins. Moreover, the Wnt-PCP pathway also regulates osteoclastogenesis. Wnt5a-Ror2 signals activates JNK and recruits c-Jun on the promoter of the gene encoding RANK

(Figure 1F) (173, 174). In the Wnt-Ca^{2+} pathway, Wnt5a binds to the Frizzled receptor, which leads to a short-lived increase of 1,4,5-triphosphate(IP3), 1,2 diacylglycerol (DAC), and Ca^{2+} with PLC, triggers NFκB and NFAT activation, and regulates osteoclastogenesis (175). In a recent study, investigators found that Wnt-Lrp5 signaling may induce mTORC2-AKT signaling activity and trigger glycolytic enzymes in bone cells to promote bone formation (176). This finding indicates that Wnt signaling may regulate bone homeostasis cooperate with glucose metabolism.

5. NETWORK BETWEEN WNT AND OTHER BONE DEVELOPMENT PATHWAYS

5.1. Crosstalk between the Parathyroid hormone (PTH) pathway and Wnt signaling

Parathyroid hormone (PTH) is an 84-amino-acid polypeptide hormone functioning as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis (177). However, the mechanisms of PTH's anabolic effect on bone are not fully studied.

5.1.1. PTH pathway induces osteoblast differentiation through Wnt/β-catenin signaling—Substantial data suggests that PTH can influence Wnt signaling in different phases and then bone development (Figure 1D). Also, PTH treatment can increase the expression of the Wnt protein, wnt4 (178). PTH also can decrease the expression of Wnt inhibitors such as Sost by directly inhibiting Sost transcription, which leads to an increase in Wnt signaling (178–180). A recent study showed that in osteoblastic MC3T3-E1 cells, the up-regulation of expression levels of osteoblast differentiation markers when treated with hPTH(1–34) were blocked by knocking down β-catenin expression (181). Transgenic mice expressing a constitutively active PTH receptor exclusively in osteocytes exhibit increased bone mass and bone remodeling, as well as reduced expression of the osteocyte-derived Wnt antagonist SOST, increased Wnt signaling, increased osteoclast and osteoblast numbers, and decreased apoptosis (182). In postmenopausal woman, intermittent PTH can reduce the circulating sclerostin levels (183). Moreover, the effects of PTH on the canonical Wnt signaling pathway can up-regulate the receptor complex proteins (FZD-1or LRP6) and decrease the antagonist (Dkk-1)(184). Although PTH treatment reduces Dkk1 expression, the over-expression of Dkk1 does not attenuate the anabolic response to PTH *in vivo* (185, 186). In addition, *in vitro* and *in vivo* evidence suggests direct crosstalk of PTH1R and Wnt signaling pathway (187). Binding of PTH to PTH1R induces association of the PTH–PTH1R complex with the extracellular domain of the Lrp6 Wnt co-receptor in absence of the Wnt ligand binding. This results in rapid phosphorylation of Lrp6 by PKA, which is activated by the cAMP signaling pathway downstream of PTH–PTH1R. Phosphorylated Lrp6 recruits Axin and thereby targets the β-catenin degradation complex to the cell membrane. A recent study identified a Dvl-binding motif in the PTH receptor (PTH1R), which activates the β-catenin pathway by directly recruiting Dvl independent of Wnt or LRP5/6(188). These studies suggest that PTH-induces osteoblast differentiation mainly through activation of the Wnt canonical pathway.

5.1.2. Wnt/β-catenin signaling controls chondrocyte hypertrophy and maturation through the PTH pathway—Wnt/β-catenin signaling regulates initiation of

chondrocyte hypertrophy by inhibiting parathyroid hormone-related protein (PTHrP) signaling activity, but it does not regulate PTHrP expression (189). In addition, Wnt/β-catenin signaling regulates chondrocyte hypertrophy in a non-cell autonomous manner and growth differentiation factor 5 (Gdf5)/Bmp signaling may be part of the downstream pathway (189). Furthermore, Wnt/β-catenin signaling also controls final maturation of hypertrophic chondrocytes, but such regulation is PTHrP signaling-independent (189). In long bone development, *Wnt5a* is required for longitudinal skeletal outgrowth and both *Wnt5a* and *Wnt5b* regulate the transition between different chondrocyte zones independently of the Indian hedgehog(Ihh)/PTHrP negative feedback loop(30).

5.2. Crosstalk between the Indian hedgehog pathway and Wnt signaling

During normal development, Indian hedgehog (Ihh) signaling appears to act as a switch within a specific population of inner perichondral mesenchyme to initiate a program of bone formation (190, 191). Ihh is the only member of the hedgehog family of secreted molecules that is expressed in chondrocytes during endochondral bone formation. Ihh is synthesized by prehypertrophic chondrocytes and by early hypertrophic chondrocytes. $Ihh^{-/-}$ mice have normally shaped skeletal elements at the condensation stage, but subsequently dramatic abnormalities of bone development appear. Investigators indicate that the proliferative effect of Ihh is likely due to a direct action of this molecule on non-hypertrophic chondrocytes (192). Failure to activate this switch results in cells adopting an alternative chondrocyte pathway of development. Ihh is involved in the differentiation of osteoblast progenitors into runt-related transcription factor 2 (Runx2)-positive osteoblast precursors (Figure 2).

Because the expression of *Wnt7b* and *Tcf1* in the perichondrium is lost in the *Ihh* mutant, it was proposed that *Ihh* may signal upstream of Wnt signaling(42). Loss of function of Wnt9 could temporally and spatially downregulate *Ihh* signaling in the appendicular skeleton and ultimately lead to a delay by 1 day in chondrocyte and osteoblast maturation, as well as shorten the proximal long bone(28). Wnt5a cooperates with Ihh to trigger degradation of NK3 homeobox 2 (Nkx3.2), an early-stage chondrogenic factor, and represses chondrogenesis (193). Along with the fact that β-catenin and Lef1 associate with the *Ihh* promoter *in vivo*, this data suggests that Wnt9a-dependent regulation of *Ihh* is probably mediated via the canonical/β-catenin pathway. This is further supported by the observation that *Ihh* expression levels in humeri of Wnt9a;β-catenin double heterozygous animals were slightly reduced and that, depending on the cre-deleter line, *Ihh* expression varies from downregulation to temporary loss or delayed expression in the skeletal elements of mice lacking β-catenin activity (28, 42, 147). Besides Indian hedgehog, Wnt signaling interacts with sonic hedgehog to regulate tooth spatial patterning (194, 195). Wnt and sonic hedgehog (SHH) signaling antagonize each other to regulate patterning through Shh antagonist Gli3 expression (19, 196–199) and Wnt antagonist Sfrp1 and Sfrp2(200–202).

5.3. Crosstalk between the TGF-β/BMP pathway and Wnt signaling

BMPs, members of the transforming growth factor beta (TGF-β) superfamily, are potent osteogenic agents that stimulate maturation of mesenchymal osteoprogenitor cells to osteoblasts (203). BMPs transduce signals by binding to heteromeric complex of type 1 and type 2 serine/threonine kinase receptors (type 1 receptors are divided into three kinds,

BMPR1A, BMPR1B and ActR1) (204). Smads are the major signal transducers for the serine/threonine kinase receptors (205). There are three classes of Smads: receptor-regulated Smads (R-Smads) that can be TGF-β/BMP activated, common partner TGF-β/BMP mediator Smads (Co-Smads), such as Smad 4; and inhibitory Smads (I-Smads). Upon ligand stimulation and activation by type II receptors, type I receptors phosphorylate R-Smads, which in turn form complexes with Co-Smads (206). The R-Smad/Co-Smad complexes then translocate into the nucleus and regulate transcription of target genes by interacting with various transcription factors and transcriptional co-activators or co-repressors. The third class of Smads, I-Smads, negatively regulates signaling by the R-Smads and Co-Smads. Runx2 and R-Smads physically interact with each other upon activation of BMP signaling, and cooperatively regulate the transcription of target genes, leading to the osteoblast differentiation of mesenchymal progenitor cells (207–209). BMP induces Runx2 expression in mesenchymal progenitor cells through the action of R-Smads (210), and R-Smads in turn interact with Runx2 to further induce osteoblastic differentiation.

There are several ways that Wnt and BMP signaling pathways interact with each other and influence bone development. First, BMPR1A signaling upregulates the expression of sclerostin, which is the SOST gene product and acts as a downstream effector of BMPR1A, leading to an inhibition of canonical Wnt signaling and a decrease in bone mass by upregulating osteoclastogenesis through the RANKL-OPG pathway (211). Moreover, GSK3/Wnt regulates BMP/Smad1 signal termination (212). Smad1, an R-Smad, contains mitogen-activated protein kinase (MAPK) and GSK3 phosphorylation sites in its linker region (213). GSK3 phosphorylation is required for the polyubiquitination of Smad1 (213). BMP signaling triggers sequential Smad1 phosphorylation by BMPR, MAPK, and GSK3 and then polyubiquitination (213). Once Smad1 is targeted for degradation, it is transported to the centrosome where the triply phosphorylated and polyubiquitinated Smad1 is degraded by proteasomes (213). This process may be regulated by Wnt signaling. Wnt3a protein inhibits Smad1 phosphorylation by GSK3 and stabilizes $pSmad1^{Cter}$, which is a Smad1 C-terminal phosphorylated by BMPR(213). Thus, the inhibitory phosphorylation of the MAPK and GSK3 sites regulate the duration of the Smad1/5/8 signal (212). In this way, BMP determines the intensity of the Smad1/5/8 response, while FGF decreases and Wnt increases its duration (Figure 1A) (212). Furthermore, BMP-2 antagonizes Wnt signaling in osteoblast progenitors by promoting an interaction between Smad1 and Dvl-1 that restricts β-catenin activation (214). Treatment with Wnt3a (but not BMP-2) stimulated Lef1-mediated transcriptional activity, whereas co-stimulation with both Wnt3a and BMP-2 markedly reduced Wnt3a-induced reporter activity (214). Without stimulation, Dvl-1 and Smad1 are co-immunoprecipitated and form a complex through the linker region of Smad1(214). Wnt3a treatment transiently disrupted the Dvl-1/Smad1 interaction coincident with nuclear accumulation of β-catenin (214). In contrast, when cells were exposed to both Wnt3a and BMP-2, there was an enhanced accumulation of the Dvl-1-Smad1 complex and a decreased nuclear accumulation of β-catenin (Figure 1B) (214). In addition, canonical Wnt signaling can be activated by BMP-2 during osteoblast differentiation (215). When primary calvarial osteoblast cells were treated with BMP-2, there was an increase in the expression of Wnt Ligands (*i.e.* Wnt7a, Wnt10b, Wnt11, and Wnt13) and Wnt Receptors (*e.g.* Fz3, Fz10, and Lrp6)(215). Additionally, Axin regulates TGF-β signaling by promoting the degradation of

Smad7 (216) and regulating the stability and transcriptional activity of the Smad3 co-response with GSK3β (217, 218). Axin, Arkadia, and Smad7 formed a ternary complex with their protein-protein interactions (216). Then Axin acts as a scaffold to facilitate Arkadia-mediated polyubiquitination of Smad (an I-Smad), regardless of TGF-β signaling, and leads to Smad7 degradation (216). A study in 2001 showed that Axin physically interacted with Smad3 through its C-terminal region located between the β-catenin binding site and the Dishevelled-homologous domain (218). Axin colocalized with Smad3 in the cytoplasm *in vivo* and the transcriptional activity of TGF-β was enhanced by Axin (218). Recent research draws an inverse conclusion about the role that Axin plays in TFG-β signaling. It was shown that Axin facilitates GSK3β-mediated phosphorylation of Smad3 at Thr66, which triggers Smad3 ubiquitination and degradation, while reduction in the expression or activity of Axin/GSK3β leads to increased Smad3 stability and transcriptional activity without affecting TGF-β receptors (217). Since the physiological level of Axin protein is usually extremely low and this study relies on loss-of-function assays, the role of Axin in Smad7 degradation remains debatable (217). Axin may negatively regulate TGF-β signaling by ubiquitination and degradation of Smad3 with GSK3β (217).

The abovementioned findings indicate a complicated crosstalk between Wnt and TGF-β/BMP signaling. In skeletal bone formation, activation of Wnt signaling determined osteoblast progenitor commitment, otherwise mesenchymal precursors differentiate into chondrocytes or adipocytes (146, 148, 219). BMP signaling indirectly promotes chondrogenesis by blocking Wnt signaling (220). The proliferation of osteoprogenitors is promoted by Wnt signaling and the maintenance of their precursor status (220). TGF-β/BMP signals stimulate those cells to become mature osteoblasts (220–222). Hence, TGF-β/BMP and Wnt signals have opposing effects on osteoprogenitors and cooperative effects in osteoblasts since both the BMP and Wnt pathways promote further osteoblast differentiation as indicated by expression of alkaline phosphatase (ALP) and mineralization (Figure 2) (220).

6. WNT INVOLVEMENT IN SKELETAL DISEASES

Given the important and diverse biological functions of Wnt signaling, it is not surprising that defects or deregulation of Wnt signaling leads to various human skeletal diseases. Table 2 provides a list of human diseases that are caused by Wnt signaling disorders. *WNT3* is required at the early stages of human limb formation. Tetra-amelia, a rare human genetic disorder characterized by complete absence of all four limbs and other anomalies, is reportedly caused by *Wnt3* loss-of-function mutations (25). A *WNT5A* mutation has been found in patients with Robinow syndrome, which is characterized by short-limbed dwarfism and abnormalities in the head, face, and external genitalia (223). Mutations of WNT7A cause Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (AARRS), which is a rare autosomal recessive disorder characterized by severe malformations of upper and lower limbs with severely hypoplastic pelvis and abnormal genitalia, and Fuhrmann syndrome, which is a syndrome consisting of bowed femurs, aplasia or hypoplasia of the fibula, and poly-, syn-, and oligodactyly (37). Studies of two families with distinct limb malformation disorders indicate that the R292C mutation of WNT7A causes AARRS while the A109T mutation of WNT7A causes Fuhrmann syndrome in humans (37). In 2011, Balwi *et al.*

determined that the G204A mutation of WNT7A also causes AARRS (224). Mutations in WNT10A cause odontoonychodermal dysplasia (OODD), which is described as an ectodermal dysplasia with dystrophic nails, misshapen teeth (*e.g.* peg-shaped incisors), erythematous lesions of the face, and the thickening of palms and soles which showed hyperhidrosis (225–228). A study of 44 human osteosarcoma samples indicated that *WNT10B* expression correlated with survival rate (229). Split-hand/split-foot malformation (SHFM), which is a rare limb development characterized by variable degrees of median clefts of hands and feet, can result from a homozygous missense mutation of *WNT10B* (230–232).

For the production and secretion of Wnt ligands, both Porcupine (Porcn) and Wntless (Wls) have crucial and non-redundant roles as indicated by the severe phenotypes in Porcn and Wls mouse models that are similar to several Wnt knockout mice (6, 233–239). It was recently discovered that mutations in PORCN drive the X-linked dominant syndrome known as focal dermal hypoplasia (FDH) or Goltz syndrome (OMIM: 305600)(6, 234, 240–242).

Wnt agonists, R-spondins, are newly recognized factors in osteoarthritis. A recent study showed that R-spondin1 acts as an anabolic agent for preservation of joint architecture in arthritis by antagonizing DKK1(50). The expression level of R-spondin2 is reduced in osteoarthritis osteoblasts and is at least partially responsible for their reduced Wnt signaling and abnormal mineralization (243). Wnt antagonist Sclerostin is related with several bone diseases. Sclerosteosis, an autosomal recessive sclerosing bone dysplasia is due to the loss of the *SOST* expression (108, 244). Van Buchem disease, an inherited skeletal dysplasia characterized by enlargement of the lower jaw and a thickening of the long bones and the top of the skull, is also caused by the deletion of *SOST*-specific regulatory element in the patients' genome (114, 245, 246). Craniodiaphyseal dysplasia (CCD), which results in facial distortion, is the most severe form of sclerotic bone diseases caused by mutations in *SOST* (247). Plenty of studies demonstrate that DKK1 is attributed to cancer bone metastases, osteolytic lesions, osteopenia, and multiple myeloma (89, 91, 248). SNPs in the sFRP1 intron and 3′-untranslated region were significantly associated with the BMD value of Japanese women (249). Functional polymorphisms within the frizzled-related protein 3 gene (*FZP3*) confer susceptibility for hip osteoarthritis in females (250). Mutations of LRP4 cause Cenani-Lenz syndrome, a rare autosomal recessive disorder characterized by syndactyly and oligodactyly of fingers and toes as well as disorganization and fusion of metacarpals (251, 252). Sclerosteosis2, which presents cortical hyperostosis, syndactyly of fingers, and the shortening and radial deviation of several distal phalanges, is less severe than Sclerosteosis (253, 254). Kneissel *et al.* identified two mutations of LRP4 (*i.e.* R1170W and W1186S) in patients suffering from Sclerosteosis2 (112). Osteoporosis pseudoglioma syndrome (OPPG) is an autosomal recessive disorder characterized by severe juvenile-onset osteoporosis and congenital or juvenile-onset blindness (255). Several mutations of LRP5 were observed in OPPG patients (77, 256–260). Mutations in *LRP5* will not only cause osteoporosis, but also cause high bone mass syndrome (80, 261–264). The binding affinity of Wnt antagonists and LRPs was decreased by LRP5/6 mutations and results in a high-bone-mass phenotype in humans (265–268). Ringelschwanz is caused by a point mutation in *Lrp6* in mice, and it leads to delayed ossification at birth and osteoporosis in adults (82).

Mice lacking *Axin2* exhibit malformation of skull structures, a phenotype resembling intramembranous ossification in humans (133). Axin2 is also linked to normal tooth development since the loss-of-function of Axin2 may cause family tooth agenesis (269). Repression of Wnt canonical signaling in osteocytes contributes to a bone pathology characterized by bone mineralization deficiency and known as renal osteodystrophy or chronic kidney disease-mineral and bone disorder (CKD-MBD) (270).

7. TARGETING WNT SIGNALING TO TREAT BONE DISEASES

Historically, diseases of bone loss have been treated with agents that block bone resorption. However, this type of therapy stimulates only a modest increase in bone mineral density, and osteoporotic patients retain an elevated risk for fracture (271). Wnt signaling has emerged as a key regulator of skeletogenesis. In most cases, Wnt ligands promote bone growth, which leads to the expectation that Wnt signaling factors could be used to treat bone diseases. Wnt canonical signaling offers multiple steps that may be considered as potential drug targets.

Osteosclerosis, an elevation in bone density, is normally detected on an X-ray as an area of whiteness. The pathogenesis of osteosclerosis involves an inactivating mutation in the *SOST* gene. The *SOST* gene encodes a protein Sclerostin that is expressed in various tissues, but is found chiefly on bone cells (osteocytes)(272). In the Wnt signaling pathway, sclerostin acts as an inhibitor by inactivating LRP5. As aforementioned, $SOST^{-/-}$ mice showed high bone mass and transgenic mice overexpressing SOST exhibit low bone mass and decreased bone strength (108, 113, 114). These findings indicate that sclerostin inhibits bone anabolic effects and may be a therapeutic target for osteoporosis. Osteoporosis is a silent disease that makes bone fragile and increases the risk of fracture. Osteoporosis is considered a major public health threat for 44 million Americans, including approximately 30 million women. In a recent first-in-human study, administration of sclerostin monoclonal antibody (AMG 785) to healthy men and postmenopausal women inhibited sclerostin and showed promise for further clinical studies for stimulating bone formation in bone diseases such as osteoporosis (273). A recent study in mice demonstrated that the sclerostin antibody improves skeletal parameters in the osteogenesis imperfecta mouse model (274). This finding provides a new therapy to increase bone mass and reduce fractures in pediatric OI.

Dickkopf-1 (Dkk1) is a soluble inhibitor of Wnt, which disrupts osteoblast differentiation and action (275). In a femoral fractures repair study, the anti-Dkk1 antibody (Dkk1 Ab) influences fracture repair, with prompt activation enhancing repair and inactivation impairing it (276). Femoral fractures were generated in C57BL/6 mice. The mice were treated twice a week with vehicle or Dkk1 Ab initiated immediately postoperatively (Day 0). Day 0 initiation enhanced repair, with significant gains seen for callus area, BMC, BMD, and biomechanical properties. These data suggest that Dkk Ab may have clinical utility in facilitating fracture repair. Multiple myeloma (MM) is associated with the development of osteolytic bone disease, mediated by increased osteoclastic bone resorption and impaired osteoblastic bone formation. In the study of the effect of Dkk1 on the development of osteolytic lesions in the 5T2MM murine model of myeloma, inhibiting Dkk1 prevented the suppression of bone formation and prevented the development of osteolytic bone disease in

myeloma (277). Dkk1 is expressed by murine 5T2MM myeloma cells. After injection of 5T2MM cells into C57BL/KaLwRij mice, anti-Dkk1 treatment prevented 5T2MM-induced suppression of osteoblast numbers and surface. Treatment increased the mineralizing surface by 28%, increased the bone formation rate by 25%, significantly protected against 5T2MM-induced trabecular bone loss, and reduced the development of osteolytic bone lesions. By evaluating the bone anabolic effects of a Dkk1 neutralizing antibody (BHQ880) in MM, we know that Dkk1 inhibits osteoblast activity (278). *In vitro* BHQ880 increased OB differentiation, neutralized the negative effect of MM cells on osteoblastogenesis, and reduced IL-6 secretion. In a severe combined immunodeficiency (SCID)-hu murine model of human MM, BHQ880 treatment led to a significant increase in OB number, serum human osteocalcin level, and trabecular bone. Also, *in vivo* BHQ880 treatment inhibits MM cell growth in the SCID-hu murine model. These studies provide evidence that confirm Dkk1 as an important therapeutic target in myeloma and provide the rationale for clinical evaluation of the Dkk1 antibody to improve bone disease and to inhibit MM growth.

GSK-3β is a crucial regulator of the Wnt canonical pathway and lithium is an inhibitor of GSK-3β (279). Lithium enhances bone formation and improves bone mass in mice, perhaps via activation of the canonical Wnt pathway (279). Activation of β-catenin by lithium treatment has the potential to improve fracture healing, but only when utilized in later phases of repair after mesenchymal cells have become committed to the osteoblast lineage (280). Furthermore, lithium chloride (LiCl) treatment inhibited myeloma bone disease and decreased the tumor burden in bone (281). As a potential clinical treatment to bone diseases, lithium also has the advantage that it has been used safely and effectively for over half a century to treat bipolar illness (282).

In the design of therapeutic drugs for Wnt signaling related bone diseases, there are several advantages in targeting sclerostin, dkk, and GSK-3β. Sclerostin and dkk are characterized as extracellular targets that are suitable for the use of biologics. In addition, the inhibition of GSK-3β or the absence of sclerostin or dkk results in increased bone mass. Sclerostin has the additional advantage of being selectively expressed in bone, which is better than Dkk and GSK-3β (283). Dkk is also highly expressed in bone. Notably, Wnt agonists and R-spondins are extracellular ligands which modulate the Wnt pathway through LRP5 and have great potential as Wnt signaling targets for the design of drugs for osteoarthritis.

8. SUMMARY AND FUTURE DIRECTIONS

The relationship between Wnt signaling components and human bone diseases or skeletal abnormalities observed in mutant mice revealed the importance of Wnt signaling in bone development. Defects in Wnt ligands and its agonists have resulted in bone development disorders, joint formation deficiency, or osteoporosis (284). Mutation in Wnt cell surface receptor LRP5/6 leads to various kinds of bone diseases. The extracellular antagonist sclerostin is related to several bone diseases, including sclerosteosis, van Buchem disease, and CCD. The mutation of other antagonists (*e.g.* Dkk and WIF) results in altered bone density. Furthermore, the Wnt signaling pathway has networks with other bone development signaling pathways such as the PTH pathway, the Indian hedgehog pathway, and the TGF-β/BMP-Smad pathway. Through this network, Wnt signaling regulates bone remodeling and

mesenchymal stem cell fate determination. After decades of studying Wnt signaling, a picture is formed of how Wnt ligands bind to cell surface receptors and trigger intracellular responses and the transcription of downstream genes. However, many important questions regarding this pathway remain unresolved (*e.g.* molecular structure of Wnt pathway components and their mechanism of interaction, the complicated network between the canonical Wnt pathway, noncanonical Wnt pathway, and other pathways in bone).

Because of the important role of Wnt signaling in bone development and diseases, researchers have designed several drugs based on this pathway. Preclinical studies with agents designed to inhibit SOST, Dkk1, and GSK-3β hold promises in treating bone diseases. However, potential problems exist with the long-term use of GSK-3 inhibitors since GSK-3 inhibitors would be expected to mimic the overexpression of Wnt signaling and, therefore, may become oncogenic (285). Another approach to the Wnt pathway has been to focus on extracellular mediators such as Sclerostin, which is selectively and highly expressed in bone. Targeting Sclerostin has great promise for treating osteoporosis and for fracture repair, but the kinetics of bone formation changes over time remain to be studied (283). The Wnt pathway has many ligands, antagonists, and intracellular proteins that influence bone development and diseases. Thus, there are many potential drug targets in Wnt signaling that may be useful in treating bone diseases. Future research will include determining which target is the best to use in clinical therapy. In any drug discovery program, issues of safety are paramount, especially in the treatment of chronic bone diseases that will likely involve long-term therapy (271). When considering how best to direct drug discovery in the Wnt canonical pathway, identification and screening upstream in the pathway is more promising than targeting β-catenin and downstream events (271). For instance, Wnt 10b, LRP5, and sFRP1 all have no negative side effects such as familial exudative vitreoretinopathy (286) and irregular skin thickness (287). R-spondins are potential drug targets as well.

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Abbreviations

GSK	glycogen synthase kinase-3β
SOST	sclerostin
DKK1	Dickkopf-1
Tbx5b	T-box 5b
fgf10	fibroblast growth factor 10
LRP	low-density lipoprotein receptor-related protein
Dsh	Dishevelled
APC	Adenomatous Polyposis Coli

sFRPs	Frizzled protein and its antagonists
Fz	Frizzled
aa	amino acids
CRD	cysteine residues
TCF	factors T cell factor
LEF	lymphocyte enhancer binding factor
EGF	epidermal growth factor
BMD	bone mineral density
rs	ringelschwanz
OPPG	osteoporosis-pseudoglioma syndrome
BMP	Bone morphogenetic proteins
HBM	high bone mass
Krm1	Kremen1
Krm2	Kremen2
RSpo	R-spondin
WIF-1	Wnt-inhibitory factor-1
Apc	adenomatous polyposis coli
CKI	Casein kinase I
RGS	Regulators of G protein signaling
S	serine
T	threonine
PI-3K	phosphatidylinositol-3 kinase
JNK2	c-Jun NH2-terminal kinase 2
HMG	high-mobility-group
Runx2	(runt homology domain transcription factor 2
DAC	diacylglycerol
PTHrP	parathyroid hormone-related protein
PTH	Parathyroid hormone
Ihh	Indian hedgehog
R-Smads	receptor-regulated Smads
Co-Smads	common partner Smads
I-Smads	inhibitory Smads

ALP	alkaline phosphatase
AARRS	Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome
OODD	odontoonychodermal dysplasia
SHFM	Split-hand/split-foot malformation
CCD	Craniodiaphyseal dysplasia
FZP3	frizzled-related protein 3 gene
CKD-MBD	chronic kidney disease-mineral and bone disorder
MM	Multiple myeloma
SCID	severe combined immunodeficiency
Sox9	SRY-box containing gene 9
β-Trcp	beta-transducin repeat containing protein
Skp1	S-phase kinase-associated protein 1
OPG	osteoprotegerin
AER	apical ectodermal ridge
RANKL	receptor activator of NF-kappaB ligand
XTcf-3	transcription factor 7-like 1 (T-cell specific, HMG-box)
TRAP	Tartrate-resistant acid phosphatase
Gdf5	Growth/differentiation factor 5
Nkx3.2	NK3 homeobox 2
SHH	sonic hedgehog
TGF-β	transforming growth factor beta
MAPK	Mitogen-activated protein kinases
Gli	Gliotactin
Col1A1	collagen, type I, alpha 1
Dkk1-AS	antisense oligonucleotide

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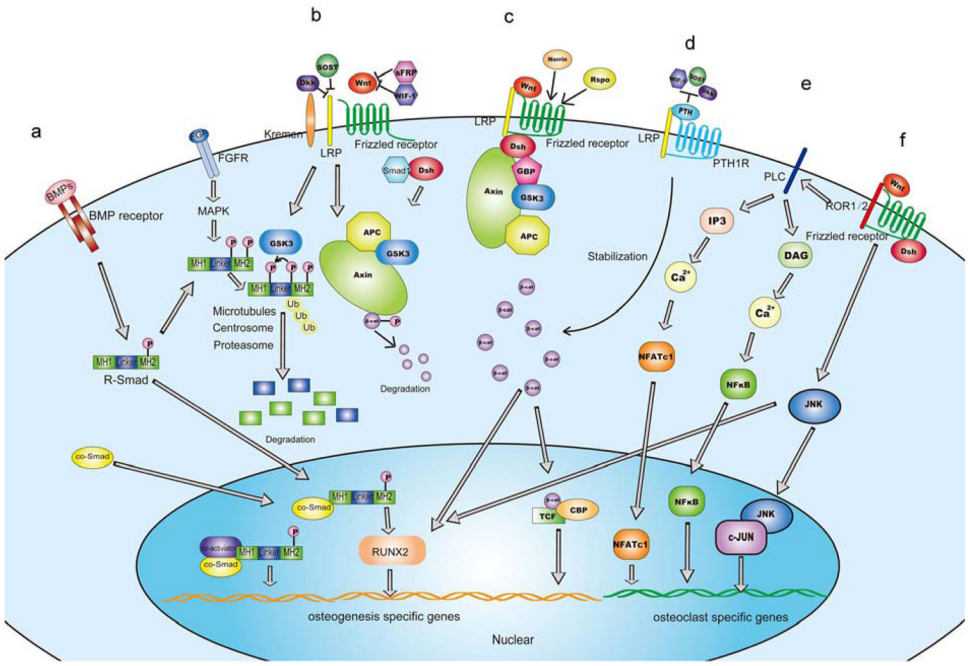

Figure 1.

The Wnt pathway and its interactions with other pathways in bone. (a)Wnt signaling and the TGF-β/BMP-Smad pathway influence each other during bone development. The Wnt pathway participates in R-Smad degradation and so does the Bmp pathway in β-catenin degradation. (b. c)The Wnt canonical pathway: Wnt proteins, following their binding to a frizzled receptor and a Lrp co-receptor (most likely LRP6), activate the canonical Wnt signaling pathway. These receptors transduce a signal to several intracellular proteins that include Dishevelled (Dsh), glycogen synthase kinase-3β(GSK-3), Axin, Adenomatous Polyposis Coli(APC), and the transcriptional regulator, β-catenin. This results in the translocation to nucleus of β-catenin, β-catenin's association with members of the Lef1/Tcf family of nuclear proteins, and activation of a specific program of gene expression. (d) Wnt interacts with PTH1R, decreases wnt antagonists, Sost, WIF1 and Dkk expression, and sustains β-catenin stabilization. (e, f) The Wnt noncanonical pathway. The Ca^{2+} pathway and PCP pathway affect osteoblastogenesis and osteoclastogenesis.

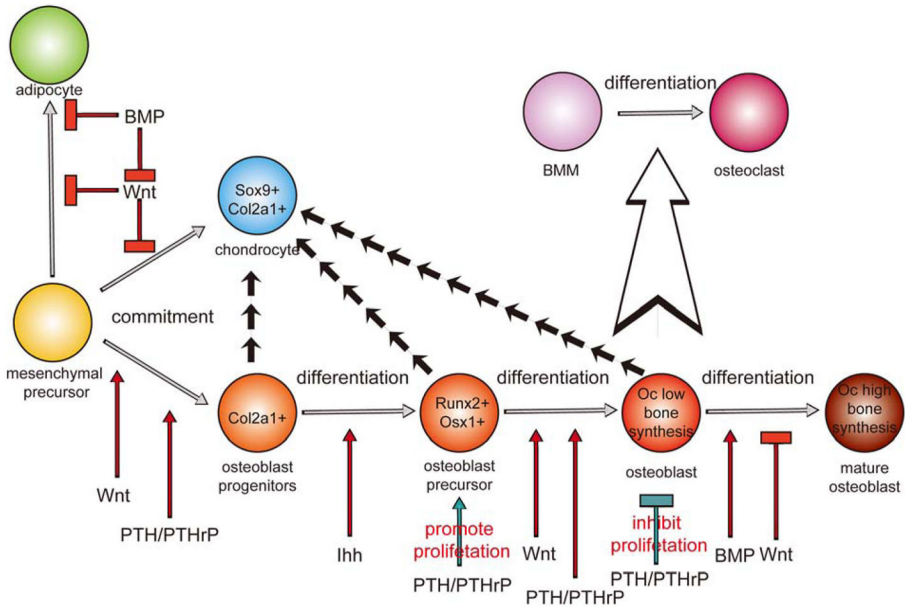

Figure 2.

The role of the canonical Wnt, Ihh, Bmp and PTH/PTHrP signaling pathways in regulating the differentiation of mesenchymal precursors. The Wnt canonical pathway and Ihh, Bmp, PTH/PTHrP pathways control the commitment of mesenchymal precursors and also the differentiation of osteoblasts/osteocytes. Though these processes, they regulate the osteoblastogenesis and bone remodeling.

Table 1

Mouse Model of Wnt ligands and its signaling effectors regulate skeletogenesis

Model	Phenotype							References
	Bone resorption	Bone formation	Chondrocyte differentiation	BMD change	Cortical thickness	Trabecular thickness	Others Features	
Functional Group: Wnt ligands								
Wnt3a+/−	nd	↓	nd	↓	nd	Number (down)	Low bone mass	(32)
Wnt4 transgenic (Col2a1-cre)	nd	↓	Increased hypertrophic chondrocytes	—	nd	nd	Dwarfism	(29)
Wnt5a+/−	nd	↓	nd	↓	nd	Number (down)	Low bone mass	(32)
Wnt5a transgenic (Col2a1-cre)	nd	↓	↓	nd	nd	nd	Short long bones and reduce ossification	(30)
Wnt5b transgenic (Col2a1-cre)	nd	↓	↓	nd	nd	nd	Open skull, short long bones and reduce ossification	(30)
Wnt7 conditional knockout (dermo-cre)	nd	↓	↓	nd	nd	nd	Bone development defects	(44)
Wnt9a/Wnt14−/−	nd	nd	↓	↓	nd	nd	Reduced the length of long bone and lead to ectopic differentiation of cartilage	(28)
Wnt9a/Wnt14 transgenic	Nd	nd	↓	↑	nd	nd	Enhanced ossification and reduced joint formation	(26)
Wnt10b−	—	↓	nd	↓	nd	Number (down)	Low bone mass	(34)
Wnt10b+	—	↓	nd	↓	nd	↓	Osteopenia and has less osteoprogenitors	(36)
Wnt10b transgenic (osteocalcin)	nd	↑	nd	↑	nd	↑	Increase mandibular bone, trabecular bone and delayed incisor development	(35)
Wnt10b transgenic (*FABP4* promoter)	nd	↑	nd	↑	—	↑	Increased bone mass	(34)
Wnt16−/−	nd	nd	nd	↓	↓	—	Low bone mass and increase risk of fracture	(45)
Functional Group: Wnt receptors								
Fzd8−/−	↑	—	nd	↓	nd	↓	Low trabecular bone volume	(59)
Fzd9−/−	nd	↓	nd	↓	nd	Number (down)	osteopenia	(60)

Model	Phenotype							Refere
	Bone resorption	Bone formation	Chondrocyte differentiation	BMD change	Cortical thickness	Trabecular thickness	Others Features	
Lrp4−/−	nd	nd	Disrupted	nd	nd	nd	Polysyndactyly, fused digital bones, and tooth development abnormalities	(288)
Lrp5−/−	—	↓	nd	↓	nd	nd	Low bone mass	(79)
Lrp5+/−	nd	↓	nd	↓	nd	nd	Low bone mass	(79)
Lrp5 conditional knockout (Dmp1-cre)	nd	↓	nd	↓	nd	nd	Low bone mass	(289)
Lrp5 a point mutation (A214V)	—	↑	nd	↑	↑	↑	Increased bone mass, bone strength and bone formation rate	(289, 2
Lrp5 a point mutation (G171V)	—	↑	nd	↑	↑	↑	Increased bone mass, bone strength and bone formation rate	(289, 2
Lrp6 a point mutation (R886W)	nd	nd	nd	↓	↓	Number (down)	Delayed ossification at birth and osteoporosis in adult	(82)
Functional Group: Wnt antagonist								
Dkk1+/−	—	↑	nd	↑	nd	↑	High bone mass	(90)
Dkk1 transgenic (*Col1A1*)	—	↓	nd	↓	↓	↓	Systemic osteopenia	(89)
Dkk2−/−	↑	↓	nd	↓	↓	↓	osteopenia	(97)
Sfrp1−/−	—	↑	nd	—	—	↑	Increase trabecular bone formation	(63)
Sfrp2−/−	nd	↓	↓	nd	nd	nd	Brachydactyly, mild mesomelic shortening and posterior soft-tissue syndactyly	(68)
Sfrp4 transgenic (col1a1)	nd	↓	nd	↓	nd	Number (down)	Low bone mass	(70)
Sost transgenic (human *SOST*)	—	↓	↓	↓	↓	↓	Low bone mass	(108)
Functional Group: Effectors in cytoplasm								
GSK3β−/+	↑	↑	nd	↑	↑	↑	High bone mass	(291)
GSK3α−/−; GSK3β+/−	nd	↓	↓	nd	nd	nd	Dwarfism with significantly shortened long bone and vertebra.	(139)
Axin2−/−	nd	↑	↑	↑	nd	nd	Craniosynostosis	(133, 1

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Model	Phenotype							Refere
	Bone resorption	Bone formation	Chondrocyte differentiation	BMD change	Cortical thickness	Trabecular thickness	Others Features	
Apc−/− conditional knockout (*osteocalcin*)	↓	↑	nd	↑	nd	Nd	Increased bone deposition and a disappearance of osteoclasts	(128)
Apc conditional knockout (Col2a1)	nd	↓	↓	↓	nd	nd	Perinatally lethal; craniofacial abnormalities, short trunk, an incomplete closure of both thoracic and abdominal cavities	(127)
Functional Group: Transcription regulation								
β-catenin conditional knock out (*Prx*)	—	↓	↓	↓	nd	nd	Bone development defect.	(146)
β-catenin conditional knockout (*Dermo1*)	↓	nd	↑	nd	nd	nd	Long bone shortened, thickened, bowed, and ectopic cartilage formation	(148)
β-catenin conditional knockout (Col2a1)	nd	↓	↓	nd	nd	nd	Died shortly after birth. Limbs were shortened and head was dome shaped. Joints between the future tarsal bones were either missing or incompletely formed.	(26, 14
β-catenin conditional knockout (*Col1a1*)	↑	—	nd	↓	nd	nd	Low bone mass	(153)
β-catenin conditional knockout (*Osterix*)	nd	↓	↓	nd	nd	nd	Lack the membranous bone of cranial ossification center and complete loss of bone deposition	(190)
β-catenin conditional knockout (*osteocalcin*)	↑	nd	nd	↓	↓	↓	Occasionally paralysis, consistent with osteoporotic-related fracture	(128)
β-catenin+/− conditional knockout (*PPARγ*)	↑	—	nd	↓	↓	↓	Osteoporosis	(154)
β-catenin conditional knockout (*PPARγ*)	↓	—	nd	↑	↑	↑	Osteopetrosis	(154)
β-catenin transgenic (*Prx*)	nd	↓	↓	nd	nd	nd	Limbs contain only tiny remnants of skeletal elements and skull bones are lost.	(146)

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Model	Phenotype							Refere
	Bone resorption	Bone formation	Chondrocyte differentiation	BMD change	Cortical thickness	Trabecular thickness	Others Features	
β-catenin transgenic (*Col2 A1*)	nd	nd	↓	nd	nd	nd	Perinatal lethal, ectopic joint formation and endochondral ossification	(26)
β-catenin transgenic (*Col1A1*)	↓	—	nd	↑	nd	nd	osteopetrosis	(153)
β-catenin+/− transgenic (*Osterix*)	nd	↑	—	nd	nd	nd	Died at birth, shorter limbs, intense and broader ossification center in long bone.	(190)
β-catenin+/− transgenic (*PPARγ*)	↓	—	nd	↑	↑	↑	Osteopetrosis	(154)
Tcf1−/−	↑	—	nd	↓	nd	nd	Low bone mass	(153)
Tcf1 Dominant negative *(Col2a1)*	nd	↓	↓	nd	nd	nd	Dwarfism, retarded mineralization in limbs, ribs and vertebrae	(160)
Lef1ΔN Transgenic *(Col1a1)*	nd	↑	nd	↑	nd	↑	High bone mass	(161)
β-catenin+/− conditional knockout (*Col1A1*); *Tcf+/−*	↑	—	nd	↓	nd	nd	Low bone mass	(153)

BMD, bone mineral density; ↑ promotion; ↓ inhibition; — not changed; nd, not detected

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Table 2

Human genetic skeletal disease and Wnt signaling

Gene	Nature of miscues	Diseases	References
WNT3	Loss of function	Tetra-amelia	(25)
WNT5A/ROR2	Mutations	Robinow syndrome	(223)
WNT7A	Mutations	Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome(AARRS)	(37, 224)
		Fuhrmann syndrome	(37)
WNT10A	Mutations	Odontoonychodermal dysplasia(OODD)	(225–228)
WNT10B	Mutations	Split-hand/foot malformation(SHFM)	(230–232)
	Expression correlates with survival rate	Osteosarcoma	(229)
SOST	Mutations	Sclerosteosis	(244)
	deletion Sost-specific regulatory element	Van Buchem disease	(114, 245, 246)
	Mutations	Craniodiaphyseal dysplasia(CCD)	(247)
DKK1	Expression is higher	Paget's disease	(292)
LRP4	Mutations	Cenani-Lenz syndactyly syndrome	(251, 252)
	Mutations	Sclerosteosis2	(112)
LRP5	Mutations	Osteoporosis prseudoglioma syndrome(OPPG)	(77, 256–260)
	Mutations	High bone mass syndrome	(80, 261–264)
	Mutations affect binding affinity of Wnt antagonists and LRPs	High bone mass	(265, 266)
Axin2	Loss of function	Family tooth agenesis	(269)
FRP3	Polymorphic SNPs	Higher incidence of osteoarthritis in females	(250)