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Wnt Signaling and Skeletal Development

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Abstract

Wnt proteins are a family of secreted proteins that regulate many aspects of cellular functions. The discovery that mutations in low-density lipoprotein receptor-related protein 5, a putative Wnt coreceptor, could positively and negatively affect bone mass in humans generated an enormous amount of interest in the possible role of the Wnt signaling pathway in skeletal biology. Over the last decade, considerable progress has been made in determining the role of the canonical Wnt signaling pathway in various aspects of skeletal development. Furthermore, recent evidence indicates the important role of non-canonical Wnt signaling in skeletal development. In this review we discuss the current understanding of the role of Wnt signaling in chondrogenesis, osteoblastogenesis, and osteoclastogenesis.

1. Introduction

In the past 30 years, several important families of signaling molecules have been identified including Wnts, bone morphogenetic proteins (BMPs), Hedgehogs, and fibroblast growth factors (FGFs). These signaling molecules are often involved in both development and disease.

Wnts are secreted cysteine-rich glycoproteins that locally activate receptor mediated signaling pathways. Their function has been the subject of investigation for more than 20 years. The finding that gain and loss-of-function mutations in the Wnt coreceptor lipoprotein receptor related protein 5 (Lrp5) led to high bone mass (HBM) and osteoporosis, respectively, has called the attention of numerous scientists and clinicians to the importance of this signaling pathway in skeletal biology and disease.

The skeleton is composed of two types of tissue, cartilage and bone, which are formed by chondrocytes and osteoblasts, respectively. Bones are formed by two different processes- intramembranous ossification and endochondral ossification. In both cases mesenchymal cells first aggregate to form mesenchymal condensations that prefigure the shape of future skeletal elements. After this patterning event, the cells of the mesenchymal condensations will usually differentiate into chondrocytes that form the template of the future bone. This cartilage template will eventually be replaced by bone through a process called endochondral ossification. Alternatively, some cells of the mesenchymal condensations differentiate directly into

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osteoblasts. This latter case, called intramembranous ossification, occurs in a few skeletal elements including the skull and the lateral halves of the clavicles. When development is complete, most of the skeleton consists of bone. There are two specific cells responsible for the maintenance of constant bone mass: osteoblasts (bone-forming cells), and osteoclasts (bone-resorbing cells). In addition to their bone-forming function, osteoblasts can control osteoclast differentiation. Several positive and negative regulators of osteoclast differentiation have been identified. Osteoblasts secrete both the M-CSF (macrophage colony stimulating factor) and the receptor activator of NF- κ B ligand (RANKL) that are necessary for osteoclast differentiation[1–3]. Osteoblasts also secrete Osteoprotegerin (OPG), which encodes a soluble decoy receptor for RANKL, thereby preventing osteoclast differentiation[4].

In recent years, the volume of Wnt literature has increased rapidly. More and more literature has highlighted the important role of Wnt signaling in essentially all aspects of skeletal development. This review will summarize the role of Wnt signaling in chondrogenesis, osteoblastogenesis and osteoclastogenesis.

2. The Wnt signaling pathway

Wnts are a family of 19 secreted proteins that mediate important biological processes including embryogenesis[5–7]. Wnt binds to a membrane receptor complex composed of one of ten Frizzled (FZD) G-protein coupled receptors and one of two low-density lipoprotein (LDL) receptor-related proteins (Lrps)[6,7]. This binding activates different groups of intracellular signaling pathways based upon the Wnt, FZD, Lrp and cell-types involved.

2.1 β -catenin dependent pathway (Canonical pathway)

β -catenin is an intracellular molecule involved in cell adhesion via its interaction with E-cadherin and α -catenin[8]. It is also the molecular node of the canonical Wnt signaling pathway [6]. In the absence of Wnt ligands, β -catenin is recruited into a “destruction complex” composed minimally of adenomatous polyposis coli (APC) and Axin which facilitate the phosphorylation of β -catenin by casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). This phosphorylation promotes the interaction of β -catenin with β -transducin-repeat containing protein (β -TrCP) and results in the ubiquitylation and proteasomal degradation of β -catenin. When cells receive Wnt signals, the degradation pathway is inhibited, and consequently β -catenin accumulates in the cytoplasm and nucleus. Nuclear β -catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to affect the transcription of target genes[9]. In the absence of Wnt signal, TCF acts as a repressor of Wnt target genes by forming a complex with Groucho[10,11]. Groucho interacts with histone deacetylases (HDAC), which are thought to make DNA refractive to transcriptional activation[12]. Once in the nucleus, it is thought that β -catenin can displace Groucho from TCF/LEF resulting in the recruitment of the histone acetylase CBP/p300 (cyclic AMP response element-binding protein) to activate Wnt target gene transcription [13; Figure 1].

2.2 β -catenin independent pathway (Non-canonical pathway)

The β -catenin dependent pathway has been well studied in the past 20 years. However, an increasing body of research concerns non-canonical, β -catenin independent Wnt pathways. Potential mechanisms of the non-canonical Wnt signal transduction are very diverse, including signaling through calcium flux, JNK, and both small and heterotrimeric G proteins[14]. The pathway of β -catenin independent Wnt signaling is an unresolved issue. Different authors have classified the pathway variously as Wnt/calcium signaling[15], Wnt/PCP signaling[16], Wnt/JNK signaling[17], and Wnt/Rho signaling[18]. It was previously thought that certain Wnts signal through a β -catenin dependent pathway and others signal through a β -catenin

independent pathway. However, it was found that the same Wnt can activate either a β -catenin dependent pathway or β -catenin independent pathway depending on the cellular and/or functional context. It is expected that additional noncanonical Wnt signaling pathways will be identified in the future.

2.3 Regulation of Wnt signaling

Wnt signaling is tightly regulated by members of several families of secreted antagonists. Wnt signaling requires interaction with frizzled receptors (Fz) and the presence of a single-pass transmembrane molecule of the Lrp family (Lrp5 or 6). Interaction between Wnts and frizzled receptors are inhibited by members of the secreted frizzled-related protein (Sfrp) family and Wnt inhibitory factor 1 (WIF-1). In terms of the latter requirement, Dkk1 binds to Lrp with high affinity and to another class of transmembrane molecules, the Kremens. Dkk1 promotes the internalization of Lrp and makes it unavailable for Wnt reception by forming a complex with Lrp and Kremen[19]. Lrp coreceptor activity is also inhibited by members of sclerostin (SOST gene product).

Wnt signaling in the nucleus is controlled by a number of protein partners. Chibby is a nuclear antagonist that binds to the C terminus of β -catenin[20]. Another β -catenin binding protein, ICAT, can block the binding of β -catenin to TCF[21] and also can lead to the dissociation of complexes between β -catenin, LEF, and CBP/p300[22,23]. TCF can be phosphorylated by the mitogen-activated protein (MAP) kinase-related protein kinase NLK/Nemo[24]. The phosphorylation of TCF/LEF by activated Nemo is thought to diminish the DNA-binding affinity of the β -catenin/TCF/LEF complex, thereby affecting transcriptional regulation of Wnt target genes[24,25].

3. Wnt signaling in chondrogenesis

During endochondral bone formation, a cartilage mold is formed and later replaced by bone. After the formation of the mesenchymal condensations, cells in the center differentiate into proliferating chondrocytes. In the developing cartilage, proliferating chondrocytes differentiate to form first prehypertrophic and then hypertrophic chondrocytes[26]. This is a tightly regulated process, which ensures proper longitudinal growth of long bones[27]. The proliferative chondrocyte region can be further divided into two zones (Zone I and Zone II) [28]. Zone I corresponds to the epiphysis of developing long bones. The cells in this zone are round and divide less frequently[29,30]. Zone II cells are flattened and gradually increase in size the closer they are to the hypertrophic zone. Zone II cells are highly proliferative chondrocytes that exit the cell cycle and undergo hypertrophy[27,31]. Dysregulation within either of the Zones leads to severe skeletal dysplasia in both mice and humans[32]. A number of signaling pathways have been implicated in the regulation of chondrogenic differentiation and hypertrophy including Sox9, parathyroid hormone-related peptide, Indian hedgehog, bone morphogenetic protein, transforming growth factor- β , and the Wnt signaling pathways[33]. The effects of Wnt signaling on chondrogenesis are complex, as results indicate both stimulation and inhibition of chondrogenesis[34–37]. This suggests that the regulation by Wnt signaling may depend on the context of the cell or stage of differentiation (Figure 2). Much information was gained by using chick chondrocytes. Because chick chondrocytes have unique properties not found in those of mammals[38,39], this review will only focus on the data gained from mammalian cells.

3.1 Loss-of-function data

Chondrocyte-specific inactivation of β -catenin using *Col2a1-Cre* transgene leads to decreased chondrocyte proliferation and delayed hypertrophic chondrocyte differentiation[40]. Interestingly, the chondrocyte-specific β -catenin inactivation mice have very similar

phenotypes compared to those of chondrocyte-specific Sox9 overexpression transgenic mice [40]. Sox9 expression in chondrocytes is increased in chondrocyte-specific β -catenin inactivation mice [40]. In another conditional β -catenin knockout mice model, the delayed chondrocyte maturation phenotype was not reported. In this model, Col2a1-Cre transgene activity was not only detected in chondrocytes, but also in part of perichondrium/periosteum where osteoblasts normally differentiate [41]. Authors reported ectopic cartilage formation in short, thick, and bowed long bones [41]. In the same study, *Dermo1-Cre* mice (Cre is active in the mesenchymal progenitor cells, giving rise to both osteoblasts and chondrocytes regardless of the mechanism of ossification) were used for β -catenin conditional knockout. It was shown that ectopic cartilage formation was observed in both the developing calvarium where bone formed through intramembranous ossification and the long bones [41]. In another loss-of-function model, *Dermo 1-Cre* transgene was used to delete the β -catenin gene in the mesenchymal precursors of both chondrocytes and osteoblasts [42]. It was shown that there is a significant delay in chondrocyte maturation in conditional knockout embryos [42]. β -catenin was similarly deleted early in embryonic development in the limb and head mesenchyme by using the *Prx-1 Cre* mice. In line with another group's work [41], authors showed that the periosteal cells differentiate into chondrocytes in the absence of β -catenin and there is ectopic cartilage formation in the region of the membranous parietale and interparietale bones [43]. It was also reported that chondrocyte maturation was affected in this mice model [43], which is consistent with previous observations [42].

The above data clearly indicated the indispensable role of canonical Wnt signaling in chondrogenesis. While it is not clear which Wnts are involved in this process, there is sparse information available regarding the roles of some individual Wnts. Wnt5a is expressed in the chondrocytes of developing long bones in mice [44]. Chondrocyte hypertrophy was significantly delayed in *Wnt5a*^{-/-} mice. In addition, the proliferation in Zone I was slightly increased [44]. By contrast, the proliferation in Zone II was reduced, indicating that Wnt5a is required for chondrocyte proliferation where it is expressed [44]. While the underlying molecular mechanism still needs to be elucidated, it is clear that Wnt5a plays important roles in chondrogenesis. In *Wnt9a*^{-/-} mice, there is no obvious abnormality in chondrogenesis [45]. However, ectopic cartilaginous material was detected in the interfrontal and sagittal suture regions separating frontal and parietal bones, and in the elbow joint [45]. *Wnt4*^{-/-} mice do not display ectopic cartilage formation [46]. However, mice double mutants for *Wnt9a* and *Wnt4* developed ectopic cartilage in two additional major joints, the ankle and knee [45]. It suggests that Wnt4 and Wnt9a are dispensable for chondrogenesis but are essential to maintain joint integrity.

3.2 Gain-of-function data

Two groups used a similar approach to direct constitutively active β -catenin expression in chondrocytes: the Col2a1 promoter [40,47]. It was consistently shown that constitutively active β -catenin in chondrocytes leads to severely compromised cartilage formation [40,47]. Activation of β -catenin in limb and head mesenchyme repressed the expression of Sox9, a factor essential for chondrogenesis, thereby preventing mesenchymal cells from differentiating into skeletal precursors [40]. Col2a1-Wnt5a overexpression caused a delay in chondrocyte differentiation before hypertrophic development and the region of undifferentiated chondrocytes was lengthened [44]. This is caused by the prevention of chondrocytes from entering Zone II [44]. Chondrocyte differentiation in Col2a1-Wnt5b mice was similarly delayed before chondrocyte hypertrophy although the mechanism differs (promotes entrance to Zone II and prevents cell cycle withdraw of Zone II chondrocytes) [44].

The transgenic approach clearly indicated the negative role of constitutively active β -catenin in chondrogenesis. However, an adenovirally expressed constitutively active form of lymphoid

enhancer factor-1 (LEF-1), the main nuclear effector of the Wnt canonical pathway in undifferentiated mesenchymal cells, chondrogenic cells, and primary chondrocytes, promotes chondrogenic differentiation in C3H10T1/2 and hypertrophy in C3H10T1/2, ATDC5, and primary chondrocytes[48]. It was also shown that the Wnt canonical pathway increases the Sox9 mRNA level in C3H10T1/2, ATDC5, and primary chondrocytes[48]. The discrepancy between the *in vitro* and transgenic studies may be partially explained by the different modes of expression of signal modulators (constitutively and transiently, respectively).

In summary, available data suggests that the Canonical Wnt signaling pathway is necessary for the chondrocyte maturation and the lineage determination of osteo-chondrogenic progenitor cells. It has previously been suggested that chondrocytes and osteoblasts derive from a common precursor[49]. Signaling through the β -catenin pathway is likely to act as a chondrogenic repressor. However, it is unknown whether observed effects are solely due to a Wnt-dependent pathway since β -catenin can also be stabilized by other mechanisms[50,51]. We can conclude that Wnt signaling needs to be delicately controlled during development as chondrogenesis will be delayed by both excessive and deficient Wnt signaling (Figure 2).

4. Wnt signaling in osteoblastogenesis

4.1 *In vivo* studies

4.1.1 β -catenin—The molecular node of the canonical Wnt signaling pathway is β -catenin. There is more and more evidence indicating the critical role of β -catenin in osteoblast differentiation and function (Figure 2).

When β -catenin was conditionally deleted from the mesenchymal precursors of both chondrocytes and osteoblasts in Dermo1-Cre mice, mature osteoblasts failed to develop[41, 42]. In the absence of β -catenin, the expression of early osteoblast markers, such as collagen I, osterix, and osteocalcin was greatly diminished[41,42]. A similar phenotype was observed when β -catenin was deleted in limb and head mesenchyme of Prx1-Cre mice with the exception that the early steps of osteoblastogenesis are unaffected and Runx2 expression is initiated. There were no osteocalcin-positive osteoblasts developed in mutant mice and periosteal cells did not express osterix[43]. The above data indicates that without a β -catenin signal osteoblasts can not differentiate to an osterix positive stage. When β -catenin was deleted in osterix positive osteoblasts, those cells failed to progress to mature osteoblasts (characterized by high osteocalcin expression) although collagen I, Runx2, and osterix were observed[52]. In summary, the above data suggest that canonical Wnt signaling is required for both osteoblast early differentiation and terminal differentiation.

Interestingly, continuous expression of a stabilized form of β -catenin in limb and mesenchyme using Prx1-Cre mice also negatively affected osteoblastogenesis[43]. In these mutant mice, the limbs contained only tiny remnants of skeletal elements and there was also a loss of skull bone[43]. The stabilization of β -catenin in osterix positive osteoblast precursor cells led to a marked increase in proliferation and an accelerated progression to mature bone matrix-secreting osteoblasts[52]. However, these cells failed to differentiate into terminal osteocalcin-positive osteoblasts.

Taken together, these findings indicate that canonical Wnt signaling is necessary for osteoblastogenesis (Figure 2). However, canonical Wnt signaling needs to be kept within a physiological range in order to positively promote osteoblastogenesis.

4.1.2 Lrp5—A loss-of-function mutation in the Lrp5 gene of humans causes OPPG (osteoporosis pseudoglioma syndrome), characterized by blindness (due to aberrant vitreo-retinal vascular growth) and osteoporosis (resulting in fractures and deformation)[53]. There

are two murine Lrp5 deficiency models with different targeting strategies[54,55]. Following one model, the first group disrupted exon 6 of the Lrp5 gene mimicking mutations observed in OPPG that result in a truncated receptor. The mutant mice had an early-onset osteoporosis, with delayed ossification and they died within the first month of life as a result of multiple fragility fractures. However, the mutant embryo displayed normal osteogenesis, implying that the defect occurred postnatally[54]. The other group generated Lrp5 $-/-$ mice by disrupting exon 18, which encodes a ligand-binding repeat. A low-bone-mass phenotype was apparent in knockout females, but only after 6 months of age. There was a 30–50% reduction in the thickness of femora, tibiae and parietal bones of these knockout female mice compared with wild-type counterparts[55]. The discrepancies between the bone phenotypes of these two models are puzzling and complicate the interpretation of the results. Nevertheless, they do show the important role of Lrp5 in bone-mass determination. Interestingly, Lithium, which is predicted to act downstream of Lrp5 to activate canonical Wnt signaling, restored bone metabolism and bone mass to near wild-type levels in Lrp5 $-/-$ mice[56].

In contrast to the situation in the loss-of-function mutations, gain-of-function mutations in the human Lrp5 gene cause increased bone mass, initially described in two North American Caucasian families[57,58]. The mutation results in the substitution of a conserved glycine by valine at residue 171, enabling the dysregulated activation of Lrp5-Wnt signaling[58,59]. Affected individuals have elevated bone synthesis but normal bone resorption and bone architecture[58]. Some affected family members also have torus platinus phenotype[58]. A corresponding gain-of-function mouse model was generated, which bears the G171V mutation ascribed to human high bone mass phenotype[60,61]. The Lrp5_{G171V} mice have increased bone mineral density(BMD) as a result of constitutively active receptor function. The high bone mass phenotype of Lrp5_{G171V} mice is due to increased osteoblast activity and survival[60]. Recently, a novel heterozygous missense mutation in the Lrp5 gene was identified in a patient with a high bone mass phenotype. It was shown that a reduced antagonistic effect of Dkk1 on canonical Wnt signaling contributes to the molecular effect of this mutation[62].

4.1.3 Lrp6—To date, no disorders have been associated with Lrp6 genetic variation in humans. Lrp6 $-/-$ mice die at birth[63]. During embryogenesis, their phenotype mimics a combination of various Wnt loss-of-function mutations: midbrain and hindbrain defects in Wnt1 $-/-$ mice, axial skeletal truncation in Wnt3a $-/-$ mice, and limb patterning defects in Wnt7a $-/-$ mice[63]. Lrp6 haplo-insufficiency independently reduces bone mass and further accentuates the low BMD phenotype observed in Lrp5-deficient mice[64]. The hypomorphic Lrp6 mutant mice, called ringelschwanz mutant mice, have a naturally occurring arginine to tryptophan mutation at amino acid 886 that inhibits efficient transmission of canonical Wnt signaling[65]. The ringelschwanz mutant mice exhibit a combination of multiple Wnt-deficient phenotypes, including dysmorphologies of the axial skeleton, digits, and the neural tube[65]. The Lrp6 dysfunction in these mice also leads to delayed ossification at birth and a low bone mass phenotype in adults[65].

4.1.4 SOST—The Sost gene product sclerostin is also a secreted Wnt antagonist, which has the ability to bind BMPs to inhibit their signaling. However, recent studies demonstrate that sclerostin can inhibit the canonical Wnt signaling pathway by interacting with Lrp5 and 6 [66,67]. Importantly, sclerostin is almost exclusively expressed in osteocytes[68]. The HBM Lrp5 variant (Lrp5_{G171V}) exhibits reduced[69] or abolished[66] SOST binding. It is proposed that Lrp5 HBM mutations render Lrp5 more resistant to SOST inhibition[69]. Genetic analysis showed that sclerosteosis results from loss of function of SOST gene product[70,71]. Transgenic mice overexpressing SOST exhibit low bone mass due to a significant reduction in osteoblast activity and bone formation[72].

4.1.5 Dickkopfs (Dkks)—The Dkk family encodes secreted proteins and, in vertebrates, includes four main members (Dkk1, 2, 3, 4). The hallmark of the Dkk1/2/4 family is their ability to modulate Wnt signaling. Dkks specifically affect the canonical Wnt signaling pathway by binding and modulating Wnt coreceptors of the lipoprotein receptor-related protein 5/6, which are indispensable for routing the Wnt signal to the β -catenin pathway. Dkk1 $-/-$ embryos lack head structures anterior of the midbrain[73]. Dkk1 $+/-$ mice are viable and show an increase in BMD with an increase in bone formation parameters and no change in bone resorption[74]. Conversely, transgenic mice overexpressing Dkk1 in bone develop osteopenia [75]. The decrease in bone mass results from a 50% decrease in osteoblast numbers and is reflected by a 45% reduction in serum osteocalcin[75]. Treatment of cultured MC3T3-E1 cells with recombinant human Dkk1 causes suppression of osteoblast matrix mineralization[75]. Tumor-produced endothelin-1 can stimulate osteoblasts to form new bone. It is mediated by Wnt signaling that is activated through suppression of Dkk1[76]. This data supports the notion that Dkk1 negatively controls Wnt signaling to regulate physiological levels of bone mass. Surprisingly, Dkk2 $-/-$ mice show low bone mineral density and osteopenia due to a major defects in mineralization[77]. The osteoid surface is increased in mutant mice without a significant change in osteoblast number. It is proposed that Dkk2 is involved in osteoblast terminal differentiation.

4.1.6 Secreted frizzle receptor proteins (Sfrps)—Sfrps are another class of Wnt antagonists. Expression of Sfrp-1 increased with advancing osteoblast differentiation and peaked in the preosteocyte stage[78]. Sfrp-1 $-/-$ mice exhibit increased trabecular bone mineral density and volume. They also exhibit an increased mineral apposition rate. However, cortical bone is unaffected[79]. The increased trabecular bone mass of Sfrp-1 $-/-$ mice is observed in adult animals after 13 weeks of age in both sexes[79]. It is suggested that loss of Sfrp-1 reduces osteoblast and osteocyte apoptosis. Changes in bone resorption are not observed in vivo[79]. These findings demonstrate that Sfrp-1 deficiency activates Wnt signaling in osteoblasts, leading to enhanced trabecular bone formation in adults. Absence of a cortical bone phenotype in Sfrp-1 $-/-$ mice may reflect a predominant role of Sfrp-1 in bone remodeling, which is more pronounced in trabecular than in cortical bone[79].

4.1.7 Axin2—Axin1 and Axin2 are negative regulators of the canonical Wnt signaling pathway and suppress signal transduction by promoting degradation of β -catenin. Axin1 $-/-$ mice exhibit defects in axis determination and brain patterning during early embryonic development[80]. Axin2 is expressed in the osteogenic fronts and periosteum of developing sutures during skull morphogenesis. Axin2 $-/-$ mice show malformations of skull structures - a phenotype resembling craniosynostosis in humans[81]. Premature fusion of cranial sutures occurs at early postnatal stages in Axin2 $-/-$ mice[81]. Inactivation of Axin2 enhances expansion of osteoprogenitors, accelerates ossification, stimulates expression of osteogenic markers and increases mineralization[81]. Axin2 deficiency promotes osteoblast proliferation and differentiation through modulation of the disparate roles of β -catenin in these two processes [82]. As a transcriptional co-activator, β -catenin promotes cell division by stimulating cyclin D1 in osteoprogenitors[82]. As an adhesion molecule, β -catenin promotes cell-cell interaction in mature osteoblasts. The Axin2 $-/-$ phenotype can be alleviated by the haploid deficiency of β -catenin[82].

4.1.8 Wnts—Reporter mouse strains for active canonical Wnt signaling (TOPGAL mice) reveal activity at sites of endochondral and intramembranous bone formation within the perichondrium and osteoblasts[41,83,84]. However, the physiologic sources of Wnts in the bone-forming microenvironment are unknown. Extracellular matrix may release Wnts as it is degraded or remodeled. Wnts can also be produced by osteoblasts. Wnt1, Wnt4, and Wnt14 are detected in calvariae while Wnt3a expression is not detected in calvaria and long bones

[54]. Wnt7b is expressed by potential osteogenic cells in the developing long bone (perichondrial cells flanking the prehypertrophic chondrocytes)[42]. In bone marrow stromal osteoblast cultures, Wnt7b showed a marked increase in its expression after induction of differentiation[85]. An initial report on the Wnt7b $-/-$ E18.5 embryo did not show any clear skeletal phenotype[52]. However, ablation of Wnt7b from skeletal progenitors by using Cre-loxP technique with Dermo1-Cre results in a deficiency in embryonic bone formation, which is at least partly due to a deficit in osterix activation and subsequent osteoblast differentiation [86]. BMP2 induced Wnt1 and Wnt3a expression in C3H10T1/2 cells[87]. Wnt3a, Wnt5a, and Wnt10b are expressed in bone marrow[88].

Wnt10b $-/-$ mice display decreased trabecular bone and serum osteocalcin. Since there is no change in serum TRAP5b, it is likely that Wnt10 $-/-$ mice have a bone formation defect rather than a bone resorption defect[89]. In contrast, bone marrow-targeted Wnt10b overexpression leads to increased bone mass[89]. Wnt10b stimulates osteoblastogenesis and inhibits adipogenesis of biopotential mesenchymal precursors[89].

4.1.9 GSK-3 β —GSK-3 β $-/-$ mice are embryonic lethal at E13.5–E14.5 dpc and no skeletal abnormalities are present until then[90]. Oral administration of LiCl, a GSK3 β inhibitor, improves bone mass in mice due to enhanced bone formation[56]. It also rescues the osteopenia of Lrp5 $-/-$ mice to near wild-type levels[56]. Another orally bioavailable GSK-3 α/β dual inhibitor, 603281-31-8, is able to increase bone formation markers expression, bone mass, and strength in rats[91].

4.2 *In vitro* studies

4.2.1 β -catenin—A few studies addressed the effect of a constitutively active mutant form of β -catenin on osteoblast differentiation in pluripotent mesenchymal cell lines. It was shown that stable β -catenin is able to induce ALP activity in C3H10T1/2 cells by transient transfection [53], enhance osteoblastogenesis (by retroviral infection) in both C3H10T1/2 and ST2 cells [89], and dramatically stimulate osteoblastogenesis by synergizing with BMP-2 in C3H10T1/2 cells via retroviral infection[92]. A study showed that retroviral expression of a stabilized form of β -catenin in C3H10T1/2 cells induced expression of ALP mRNA and protein (but not osteocalcin expression) in non-differentiated medium[93]. Another study showed that constitutively active β -catenin has no effect on basal ALP activity in either C3H10T1/2 or C2C12 cells[92]. Deletion of β -catenin by using adenovirus expressing the Cre recombinase in calvarial osteoblasts resulted in delayed and diminished expression of osteocalcin as well as in significantly reduced mineralization[94].

4.2.2 Wnts

4.2.2.1 Wnt1: Transient transfection of Wnt1 in C3H10T1/2 cell[53,87,95], ST2 cell[87], and C2C12 cells[87] (but not in MC3T3-E1 cells[87]) led to increased ALP activity. Retrovirally infected Wnt1 ST2 and C3H10T1/2 cells underwent rapid osteoblastogenesis when cultured in osteogenic media[89]. Adenovirally infected bone marrow stromal osteoblasts and calvarial osteoblasts with Wnt1 led to enhanced osteoblastogenesis[77].

4.2.2.2 Wnt3a: Wnt3a has been reported to induce ALP activity in C3H10T1/2 cells through transient transfection[53,87], conditional medium treatment[77,95–97], purified protein treatment[96], adenoviral treatment[98], retroviral infection[99]; (in ST2 cells) transient transfection[53,87], conditional medium treatment[86,97]; (in C2C12 cells) transient transfection[87], or conditional medium treatment[97]. Wnt3a can also enhance ALP activity and BSP in conditional medium, enhance osteocalcin expression in ST2 cells in non-differentiation medium, and increase mineralization in differentiation medium[86]. Wnt3a protein dose dependently induced ALP activity in ST2 cells in a serum-free medium[86].

Wnt3a protein is able to stimulate ALP activity in murine embryonic mesenchymal stem cells but not calvaria cells[56]. Wnt3a was thought to be a “canonical” Wnt protein, signaling through a β -catenin-dependent signaling pathway. However, it was shown that Wnt3a signals through the $G_{\alpha_{q/11}}$ subunits of G proteins to activate phosphatidylinositol signaling and PKC δ in ST2 cells. $G_{\alpha_{q/11}}$ - PKC δ signaling is required for Wnt3a-induced osteoblastogenesis in ST2 cells[86]. Although Wnt3a can signal through both the β -catenin and PKC δ pathways, it was determined that Wnt3a activates the PKC δ pathway independently of β -catenin signaling to promote osteoblast differentiation[86]. It was also reported that Wnt3a in conditional medium can enhance mineralization in mouse calvaria cell osteogenic cultures with increased expression of BSP and osteocalcin[77]. However, it was reported that, although Wnt3a-expressing C3H10T1/2 cells have higher ALP activity, they exhibit decreased expression of other bone markers such as BSP, osteocalcin, and osteopontin both at basal level and after osteogenic culture[99]. Wnt3a overexpression modifies neither ALP activity nor mineralization capacity in MC3T3-E1 cells[87].

In contrast to the data in the cell types described above, Wnt3a was reported to have an adverse effect on ALP expression in human mesenchymal stem cell osteogenic cultures[100–102] - the earlier the treatment with Wnt3a conditional medium, the stronger the inhibition of mineralization[100]. However, one report showed that addition of Wnt3a in conditional medium to mouse bone marrow stromal cell osteogenic culture led to increased mineralization [77]. The differences between these studies might be due to culture regime or species.

4.2.2.3 Wnt7b: It was shown that Wnt7b was produced in differentiating bone marrow stromal cells[77,85]. Wnt7b deficiency in either calvarial cells or bone marrow stromal cells led to reduced osteoblastogenesis[86]. In contrast, overexpression of Wnt7b, either by transient transfection or by viral infection, induced ALP activity in C3H10T1/2 cells[86,103]. Wnt7b overexpression enhanced mineralization in both C3H10T1/2 and ST2 cells in differentiation medium[86]. Interestingly, Wnt7b failed to stimulate canonical Wnt signaling in either C3H10T1/2 or ST2 cells, but activated PKC δ in both cell types[86].

4.2.2.4 Wnt4 and Wnt5a: It was shown that Wnt4 and Wnt5a did not increase ALP activity in C3H10T1/2 and ST2 cells by transient transfection[53]. Retroviral infection of Wnt5a had no effect on osteoblastogenesis in ST2 cells[89]. Our data supported this observation (unpublished). Interestingly, one study showed that transfection of Wnt5a in human mesenchymal stem cells in osteogenic culture resulted in an increase in alkaline phosphatase staining and ALP and osteocalcin expression[101]. Very recently, we observed that Wnt-4 strongly enhanced osteogenic differentiation of MSCs isolated from craniofacial tissues. Using the models of craniofacial and periodontal defects, we found that MSCs genetically engineered to express Wnt-4 exhibited strong bone formation capacities and facilitated the repair of craniofacial bone defects[104]. More importantly, we found that Wnt-4 enhanced osteoblastic differentiation of MSC by activating p38 independent of β -catenin.

4.2.3 GSK-3 inhibitors—Inactivation of glycogen synthase kinase-3 (GSK-3) leads to stabilization, accumulation, and translocation of β -catenin into the nucleus to activate downstream Wnt target genes. LiCl mimicked activation of the canonical Wnt signaling pathway by inhibiting GSK-3 β activity[105]. The LiCl dose dependently induced ALP activity (from 0.1 mM to 20 mM)[95] and ALP mRNA expression in C3H10T1/2[87,93], ST2[87], and C2C12[87] cells. However, it was shown that 4 mM LiCl either had no apparent effect on ALP level (4 mM) in human mesenchymal stem cells[102], or reduced the BMP2-induced osteoblast differentiation (in C2C12 cells and human mesenchymal stem cells)[106] and dexamethasone-induced osteoblast differentiation[100]. LiCl is able to stimulate ALP activity in murine embryonic mesenchymal stem cells but not in calvaria cells[56]. 603281-31-8, a small molecule GSK-3 α/β dual inhibitor, can increase the osteoblast differentiation marker

expression in C3H10T1/2 cells with a biphasic effect[91]. CHIR99021, another GSK inhibitor, can induce osteoblastogenesis in ST2 cells[89].

5. Wnt signaling and osteoclastogenesis

Osteoblast-specific inactivation of β -catenin using $\alpha_1(I)$ collagen-Cre mice led to a decrease in bone mass caused by an increase in the osteoclast number (and subsequent bone resorption) coupled with an unchanged osteoblast number/function[84]. This increase in bone resorption is caused by a decrease in the expression of osteoprotegerin (OPG), an inhibitor of osteoclast differentiation[84]. Similar findings were observed when osteocalcin-Cre mice were used to examine osteoblast-specific inactivation of β -catenin. However, in this *in vitro* model, osteoblasts lacking β -catenin additionally showed impaired maturation and mineralization with elevated expression of the osteoclast differentiation factor receptor activated by nuclear factor- κ B ligand (RANKL)[94]. In co-cultures of mouse spleen cells and osteoblasts, activation of Wnt signaling downregulated RANKL expression, inhibiting formation of tartrate-resistant acid phosphatase-positive multinucleated cells[107].

In contrast, osteoblast-specific activation of β -catenin, using $\alpha_1(I)$ collagen-Cre mice, resulted in high bone mass. These mice had defects in osteoclast differentiation and function due to an increase in the expression of OPG coupled with unchanged osteoblastogenesis[84]. Osteoblast-specific inactivation of APC to constitutively activate canonical Wnt signaling resulted in a dramatic increase in bone deposition and a disappearance of osteoclasts with no obvious change in osteoblast number (*in vitro*)[94].

In addition, Tcf1 $-/-$ mice have significantly decreased bone mass at 1 month of age[84]. However, there is no change in osteoblast number or function and the main cause of the phenotype is due to the increase in osteoclast number and function[84]. The increased osteoclastogenesis is accompanied by a decrease in the expression level of OPG but an unvarying RANKL expression. Mice doubly heterozygous for Tcf1 and β -catenin have a low bone mass not seen in either of the singly heterozygous mice, with an increase in osteoclast number and a decrease in OPG expression[84].

6. Conclusions and perspectives

The finding that both gain- and loss-of-function mutations in the Lrp5 receptor regulate bone mass in humans highlighted the important role of Wnt signaling in skeletal biology. The information from various genetic mouse models expanded our knowledge about the extensive involvement of this signaling pathway in regulating skeletal development. Various *in vitro* studies further enriched our understanding with regard to how Wnt signaling might regulate skeletogenesis. However, many questions still remain unanswered.

It is proposed that Wnt regulates osteoblastogenesis through the canonical, β -catenin dependent pathway. There is some supporting evidence for this statement. However, the recent finding that Wnt3a and Wnt7b promote bone formation through G protein-linked PKC δ activation challenged this view. It will be necessary to gather more information about the effect of this pathway on *in vivo* bone formation. The detailed molecular mechanism of other “canonical Wnts,” such as Wnt1 and Wnt10b, in the regulation of osteoblastogenesis needs further investigation.

Studies using transgenic and knockout mice have provided tremendous information about the role of Wnt signaling in skeletal development, *in vivo*. Although mice models manipulating the β -catenin exist, the β -catenin dependent pathway may not be the sole or critical pathway for the effect of some Wnts on the skeletal system. It is expected that gain- and loss-of-function mouse models targeting specific skeletal component will greatly help elucidate the role of Wnt

signaling in skeletal development. Because of possible functional redundancy, the development of skeletal conditional double or triple knockout mice models will be necessary

We are starting to understand the role of Wnt signaling in osteoclastogenesis. To date, the majority of the data points to the indirect role of Wnt signaling in osteoclasts via a regulation of the RANKL-OPG pathway by osteoblasts. However, it is largely unknown whether and by what means Wnt signaling affects osteoclasts. Mice models with osteoclast specific overexpression or deletion of the Wnt signaling components would provide valuable information for this.

The molecular mechanisms for the different responses to canonical Wnt signaling in various cells and stages of differentiation need further study. In particular, it's important to know how Wnt signaling promotes or inhibits mesenchymal stem cell differentiation towards osteoblast lineage. The underlying mechanism that is responsible for the inhibition of osteogenic differentiation of MSCs by Wnt1 or Wnt3a is not clear. Although the canonical Wnt signaling plays a critical role in bone development, targeting the canonical Wnt signaling pathway may not be useful for improving the osteogenic potential of MSCs. Targeting non-canonical Wnt signaling to enhance osteogenic differentiation of MSCs needs further study.

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Figure 1. The Canonical Wnt signaling pathway

(a) In the absence of Wnt signal, β -catenin forms a complex with Axin and APC. Axin acts as a scaffold for the protein kinases GSK3 and CK1 α . After phosphorylation by GSK3 and CK1 α , β -catenin is degraded by ubiquitination involving interactions with β -TrCP. In the absence of β -catenin, TCF acts as a repressor of Wnt target genes by forming a complex with Groucho, which interacts with histone deacetylases that are thought to make DNA refractive to transcriptional activation.

(b) When Wnt ligand binds to its receptors Frizzled and LDL receptor related protein 5 or 6, the Frizzled and Lrp receptors recruit Dishevelled (Dsh) and Axin to the membrane, and the destruction complex is disassembled. As a result, the degradation of β -catenin is inhibited and β -catenin accumulates in the cytoplasm and nucleus. Accumulated β -catenin interferes with the interaction between TCF and Groucho and converts TCF from transcription repressor to activator.

Figure 2. Role of canonical Wnt signaling in skeletal development

Mesenchymal stem cells have the ability to differentiate into chondrocytes or osteoblasts, depending on the environmental cues. Canonical Wnt signaling is regulated to control the lineage progression between chondrocyte and osteoblast. If there is inadequate Canonical Wnt signaling, differentiation towards chondrocyte lineage is encouraged. However, the maturation of chondrocytes requires the presence of Canonical Wnt signaling. Canonical Wnt signaling is also required for the progression of osteoblast progenitor cells toward osterix positive osteoblasts and then osteocalcin positive osteoblasts. Canonical Wnt signaling represses osteoclastogenesis by increasing the osteoprotegerin (OPG) expression, therefore the OPG/RANKL ratio. Green plus signs indicate positive effects of Wnt; red circle minus signs indicate inhibitory effects of physiological Canonical Wnt signaling (supported by loss-of-function data).