Wnt Signaling in Bone Development and Disease: Making Stronger Bone with Wnts

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The skeleton as an organ is widely distributed throughout the entire vertebrate body. Wnt signaling has emerged to play major roles in almost all aspects of skeletal development and homeostasis. Because abnormal Wnt signaling causes various human skeletal diseases, Wnt signaling has become a focal point of intensive studies in skeletal development and disease. As a result, promising effective therapeutic agents for bone diseases are being developed by targeting the Wnt signaling pathway. Understanding the functional mechanisms of Wnt signaling in skeletal biology and diseases highlights how basic and clinical studies can stimulate each other to push a quick and productive advancement of the entire field. Here we review the current understanding of Wnt signaling in critical aspects of skeletal biology such as bone development, remodeling, mechanotransduction, and fracture healing. We took special efforts to place fundamentally important discoveries in the context of human skeletal diseases.

The skeleton has many important functions related to human health. Aside from the classical functions of the skeleton in structural support and movement, the bone matrix forms a major reservoir of calcium and other inorganic ions, and bone cells are active regulators of calcium homeostasis. Recent data suggest that bone cells can secrete hormones (e.g., FGF23 and osteocalcin) and likely play a physiologically significant role in regulating phosphate and energy homeostasis. It has emerged that Wnt signaling plays a major role controlling multiple aspects of skeletal development and maintenance. Thus, understanding how the Wnt pathway controls skeletal growth and homeostasis has broad implications for human health and disease.

Cartilage and bone define the skeleton and are produced by chondrocytes and osteoblasts, respectively. During embryonic development, bones are formed by two distinct processes: intramembranous and endochondral ossification (Fig. 1A). A number of cranial bones and the lateral portion of the clavicles are formed by intramembranous ossification. In this process, mesenchymal progenitor cells condense and differentiate directly into bone-forming osteoblasts. The majority of bones in our body are formed by endochondral ossification, during which mesenchymal progenitor cells condense and differentiate first into cartilage-forming chondrocytes to generate an avascular template of the future bone. Chondrocytes in these

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Figure 1. Mechanisms of skeleton formation. (A) Bones can form by either intramembranous or endochondral ossification. Both processes are initiated by the condensation of mesenchymal cells. During intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts and deposit bone. During endochondral ossification, mesenchymal cells differentiate into chondrocytes and first make a cartilage intermediate. Chondrocytes in the center of the bone initiate a growth plate, stop proliferating, and undergo hypertrophy. Hypertrophic chondrocytes mineralize their matrix and undergo apoptosis, attracting blood vessels and osteoblasts that remodel the intermediate into bone. (B) The first histologic sign of synovial joint formation is the gathering and flattening of cells, forming the interzone. Cavitation occurs within the presumptive joint separating the two cartilaginous structures. Remodeling and maturation proceed to give rise to the mature synovial joint. Wnt signaling plays a significant role in controlling almost all aspects of skeleton formation. Osteoblasts (purple); chondrocytes (blue); osteochondroprogenitor cells (brown).

templates undergo a program of proliferation and progressive cellular maturation. Eventually, they exit the cell cycle and become pre-hypertrophic, then terminally differentiating into hypertrophic chondrocytes, which are eliminated ultimately by apoptosis. Hypertrophic chondrocytes produce a matrix that is calcified and functions as a scaffold for new bone formation. Concomitant with chondrocyte hypertrophy, osteoclasts, osteoblasts, and blood vessels mi-

grate in from perichondral regions and remodel this template into bone.

The developing skeletal elements are often segmented to form joints, which are required to support mobility. Synovial joints, which allow movement via smooth articulation between bones, form when chondrogenic cells in a newly formed cartilage undergo a program of dedifferentiation and flattening to form an interzone (Fig. 1B). Cavitation occurs within the flattened

cells, allowing physical separation of the skeletal elements, and the formation of the synovial cavity. Cells and tissues in and around the interzone are remodeled at the same time to form the articular cartilage and other joint structures. Failure to form or maintain joints leads to joint fusion or osteoarthritis, a major skeletal disease.

Following its formation, bone remains a regenerative tissue and is maintained during post-natal life by continuous remodeling. This highly active, homeostatic process is required for its functions and is controlled by three cell types: osteoblasts on the bone surface that deposit new bone matrix; osteocytes embedded in bone that are terminally differentiated from osteoblasts and function as mechanical and metabolic sensors; and the matrix-resorbing osteoclasts (Fig. 2). Osteoblasts are derived from mesenchymal stem cells (MSCs), whereas osteoclasts differentiate from hematopoietic progenitors. Decreased bone mass may be due to reduced osteoblast function or elevated osteoclast activity, and, conversely, increased bone mass may result from increased osteoblast function or decreased osteoclast activity. The precise balance of formation and resorption is critical for maintain-

ing normal bone mass, and alterations in this balance lead to common bone diseases such as osteoporosis and osteopetrosis.

There are two major bone types, cortical and trabecular, which show different anatomical properties (Fig. 2). Cortical (or compact) bone is the solid, densely packed bone that forms the outer layer of most bones and gives strength and rigidity. Trabecular (or cancellous) bone is present mostly in the marrow cavities of long bones and is the dominant bone type in vertebral bodies. Trabecular bone forms a porous, cobweb-like network of trabeculae whose large surface area is thought to facilitate the metabolic activity of bones mediated by osteoblasts and osteoclasts. Trabeculae are sites of active remodeling and will often orient in the direction of mechanical loading, dissipating the energy of loading and adding to bone strength. It is trabecular bone, rather than cortical bone, which is most severely affected in osteoporosis.

The Wnt/ β -catenin pathway plays a major role in controlling skeletal development and homeostasis, which are the focus of this work. We focus not only on differentiation of skeletal cells and formation of skeletal tissues, but also on the

Figure 2. Anatomy of bone. Cortical and trabecular bone represent the two major forms of bone. Osteoblasts (dark purple) are present on the surface and form new bone. Osteocytes (brown) are terminally differentiated osteoblasts that have become embedded in bone and communicate information to one another and to cells on the surface to regulate bone homeostasis. Osteoclasts (blue) are of hematopoietic origin and catabolize bone. A major function of Wnt/ β -catenin signaling in osteoblasts is to suppress RANKL and to promote OPG production, thereby inhibiting osteoclast formation.

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role of the Wnt/ β -catenin signaling pathway on bone homeostasis, mechanotransduction, and wound healing, paying particular attention to human and mouse studies.

WNT/ β -CATENIN SIGNALING IN HUMAN SKELETAL DISEASES

The importance of the Wnt/ β -catenin signaling pathway in patterning, development, and maintenance of the human skeleton is underscored by the number of human bone diseases associated with mutations in this pathway (Table 1). Early in skeletal development, Wnt signaling controls pattern formation before skeletal elements are laid down, and abnormal Wnt signaling is associated with defects of the human skeleton. At the more extreme end of the spectrum, loss of WNT3 leads to tetra-amelia, a severe congenital defect in which all four limbs fail to form, as well as craniofacial defects (Niemann et al. 2004). At the milder end of the spectrum, mutations in the Wnt/ β -catenin pathway inhibitor AXIN2 leads to a dominant, familial form of tooth agenesis, in which a large number of permanent teeth fail to form (Table 1) (Lammi et al. 2004).

The first human genetic evidence revealing the importance of the Wnt/ β -catenin pathway in bone formation came from the work of Gong et al. (2001) that identified several loss-of-function (LOF) mutations in the low-density lipoprotein receptor-related protein 5 (LRP5) associated with autosomal recessive osteoporosis pseudoglioma (OPPG) syndrome. Patients suffer from a dramatic reduction in trabecular bone leading to multiple fractures and bone deformities. LRP5 loss-of-function mutations have subsequently been shown to be associated with juvenile osteoporosis (Saarinen et al. 2007). Conversely, gain-of-function mutations of LRP5 lead to the opposite phenotype: autosomal-dominant high bone mass and enhanced bone strength (Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003). Loss-of-function mutations in the closely related LRP6 lead to an autosomal-dominant familial form of coronary artery disease associated with osteoporosis (Mani et al. 2007).

Mutations in the secreted Wnt/ β -catenin signaling antagonist sclerostin (SOST) cause two overlapping diseases: sclerosteosis and van Buchem disease (Balemans et al. 2001, 2002; Brunkow et al. 2001). Sclerosteosis is a rare autosomal-recessive skeletal dysplasia mostly affecting the Afrikaner population of South Africa. It is caused by loss-of-function mutations in the *SOST* gene and is marked by hand malformations, unrestrained bone growth leading to thickened and sclerotic bones, and high bone mass. Bone overgrowth is generalized but most severe in the cranium, leading to increased intracranial pressure and pinching of cranial nerves causing facial palsy and loss of hearing and smell. Interestingly, heterozygous carriers of these *SOST* mutations have increased bone mineral density, suggesting that one affected allele is sufficient to confer a skeletal phenotype and the effects are dominant (Gardner et al. 2005). Most patients with van Buchem disease come from a fishing village in the Netherlands and present with a similar, although milder, sclerosing bone dysplasia affecting mainly cranial bones. Unlike sclerosteosis, van Buchem disease is not caused by mutations in the *SOST* gene coding sequence, but rather by a deletion of an enhancer region downstream causing reduced expression of *SOST*.

Although it has long been recognized that bone mass and predisposition to osteoporosis are highly heritable, the mechanism and genes involved have been poorly defined. Several recent genomic screens (both genome-wide association studies as well as more targeted screens) have identified polymorphisms in or close to Wnt/ β -catenin signaling components that correlate with bone mineral density and further strengthen the importance of this pathway in human bone physiology. Polymorphisms in LRP5, LRP6, GPR177 (Wntless), SFRP1, *SOST*, and *RSPO3* have all been reported to be associated with bone parameter variations in humans (Uitterlinden et al. 2004; van Meurs et al. 2006; Sims et al. 2008; Duncan et al. 2011). Studies using mouse genetics have revealed causative and fundamentally important roles of Wnt/ β -catenin signaling in skeletal biology and further established in vivo experimental models to

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Table 1. Human and mouse genetic analysis of Wnt signaling in skeletal biology

Human gene	Human disease	Skeletal phenotype	References
LRP5	Osteoporosis-pseudoglioma syndrome	Low bone mass	Gong et al. 2001
LRP5	Juvenile osteoporosis	Low bone mass	Gong et al. 2001; Saarinen et al. 2007
LRP5	Osteopetrosis	High bone mass	Boyden et al. 2002; Little et al. 2002; Van Wesenbeeck et al. 2003
LRP6	Osteoporosis	Low bone mass	Mani et al. 2007
SOST	Sclerosteosis, Van Buchem disease	High bone mass, bone overgrowth	Balemans et al. 2001, 2002; Brunkow et al. 2001
AXIN2	Tooth agenesis	Fewer permanent teeth	Lammi et al. 2004
WNT3	Tetra-amelia	Absence of all four limbs	Niemann et al. 2004
Mouse allele	Skeletal phenotype		References
<i>β-Catenin conditional LOF</i>			
Dermo1-cre	Reduced osteoblastogenesis, ectopic chondrogenesis, survival to birth		Day et al. 2005; Hu et al. 2005
Prx1-cre	Reduced osteoblastogenesis, ectopic chondrogenesis, survival to birth		Hill et al. 2005
Colla1(2.3)-cre	Normal osteoblastogenesis, increased osteoclastogenesis and bone resorption, reduced bone mass, normal life span		Glass et al. 2005
Osteocalcin-cre	Reduced osteoblastogenesis, increased osteoclastogenesis, reduced bone mass, survival to 5 wk		Holmen et al. 2005
DMP1-cre	Normal osteoblastogenesis, increased osteoclastogenesis, reduced bone mass, survival to 3 mo		Kramer et al. 2010
<i>β-Catenin conditional GOF</i>			
Prx1-cre	Reduced differentiation of osteoblasts and chondrocytes		Hill et al. 2005
Col2-cre	Reduced chondrocyte differentiation, impaired joint formation		Guo et al. 2004
Colla1(2.3)-cre	High bone mass, reduced osteoclastogenesis		Glass et al. 2005
<i>APC conditional LOF</i>			
Osteocalcin-cre	High bone mass, increased osteoblastogenesis, reduced osteoclastogenesis, survival to 2 wk		Holmen et al. 2005
LRP5 KO	Postnatal low bone mass, reduced osteoblastogenesis		Kato et al. 2002; Fujino et al. 2003; Holmen et al. 2004
<i>LRP5 conditional LOF</i>			
Prx1-cre	Reduced bone mass in limbs, normal bone mass in spine		Cui et al. 2011
Villin-cre	Reduced bone mass		Yadav et al. 2008
Villin-cre	Normal bone mass		Cui et al. 2011
Colla1(2.3)-cre	Normal bone mass		Yadav et al. 2008
DMP1-cre	Reduced bone mass		Cui et al. 2011
LRP5 GOF	Increased bone mass, bone formation rate, bone strength		Babij et al. 2003; Yadav et al. 2008; Cui et al. 2011
Villin-cre	Increased bone mass		Yadav et al. 2008
Villin-cre	Normal bone mass		Cui et al. 2011

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Table 1. *Continued*

Mouse allele	Skeletal phenotype	Reference
DMP1-cre	Increased bone mass	Cui et al. 2011
LRP6 KO	Interacts genetically with Lrp5 to reduce bone mass	Holmen et al. 2004
Ringelschwanz (Rs) (LRP6 LOF hypomorph)	Delayed ossification, reduced bone mass	Kokubu et al. 2004
Crooked tail (Cd) (LRP6 GOF hypomorph)	Delayed ossification	Carter et al. 2005
Axin2 KO	Increased osteoblastogenesis and maturation, craniosynostosis	Yu et al. 2005
Dkk1 Het	Increased bone formation	Morvan et al. 2006
Doubleridge (Dkk1 LOF hypomorph)	Increased bone mass	MacDonald et al. 2007
Sfrp1 KO	Increased trabecular bone mass, increased osteoblastogenesis	Bodine et al. 2004
Sfrp3 KO	Stiffer bone with increased cortical bone thickness and density	Lories et al. 2007
Sost KO	Increased bone formation	Li et al. 2008
Gsk3 β Het	Increased bone formation	Kugimiya et al. 2007
Wnt10b KO	Reduced bone mass	Bennett et al. 2005
Fzd9 KO	Reduced bone formation	Albers et al. 2011
Naked cuticle 1 and 2 KO	Craniofacial abnormalities	Zhang et al. 2007
Lef1 Het	Reduced bone mass in females, decreased osteoblast function	Noh et al. 2009
Tcf1 KO	Reduced bone mass, increased osteoclastogenesis	Glass et al. 2005

investigate the pathological mechanisms of human mutations in Wnt pathway components during human bone formation and homeostasis (see below).

WNT/ β -CATENIN SIGNALING IN OSTEOBLAST DIFFERENTIATION AND MATURATION

The mouse has proven to be a productive animal model for the genetic dissection of Wnt/ β -catenin signaling in bone biology (Fig. 3), and work from a number of groups has shown that Wnt/ β -catenin signaling regulates multiple aspects of skeletal development, controlling the differentiation and function of MSCs, chondrocytes, osteoblasts, and osteoclasts. During skeletogenesis, conditional removal of β -catenin in mesenchymal progenitor cells leads to decreased

osteoblast differentiation (Day et al. 2005; Hill et al. 2005; Hu et al. 2005; Rodda and McMahon 2006). Cells surrounding the developing bones are capable of expressing early markers of the osteoblast lineage (i.e., Runx2), but fail to express Osterix, a marker of osteoblast commitment. Indeed, removal of β -catenin in osteoblast progenitor cells promoted chondrogenesis in vitro and ectopic chondrocyte differentiation at the expense of osteoblasts in vivo (Day et al. 2005; Hill et al. 2005). This suggests that, in vivo in an osteochondral progenitor cell, high Wnt/ β -catenin signaling promotes osteoblast differentiation and inhibits chondrogenesis. Indeed, ectopic activation of Wnt/ β -catenin signaling suppresses chondrogenic differentiation from mesenchymal progenitors in culture (Rudnicki and Brown 1997; Day et al. 2005; Reinhold et al. 2006). However, the effect of Wnt/ β -catenin signaling on osteoblast formation appears to

Figure 3. Genetic dissection of skeletal cells using mouse genetics. Schematic diagram of the differentiation of chondrocytes and osteoblasts from a common osteochondral progenitor cell. Overlaid are the corresponding mouse Cre-recombinase lines that allow for the in vivo dissection of signaling pathways. Regulation of cell differentiation by Wnt/β-catenin signaling is indicated (green for activation and red for inhibition). (Figure created from data from Day et al. 2005, Hill et al. 2005, Hu et al. 2005, Rodda and McMahon 2006, Zylstra et al. 2008, and Bonewald 2011.)

depend on the developmental stage of the cells. In vitro activation of the Wnt/β-catenin signaling pathway in MSCs promotes their proliferation and inhibits osteoblastic differentiation (Boland et al. 2004; de Boer et al. 2004; Cho et al. 2006; Regard et al. 2011). Once MSCs have been committed to the osteoblast lineage, Wnt/β-catenin signaling promotes their growth and differentiation, but can also block their terminal differentiation into mature osteoblasts (Kahler and Westendorf 2003; Kahler et al. 2006, 2008; Rodda and McMahon 2006; Eijken et al. 2008). This stage-dependent function of Wnt/β-catenin signaling in bone development is also manifested in human diseases. For instance, fibrous dysplasia (FD) caused by activated Gαs protein is due to inhibition of osteoblast differentiation, and maturation resulted from up-regulated Wnt/β-catenin signaling in MSCs and/or early osteoblasts (Regard et al. 2011).

WNT/β-CATENIN SIGNALING IN CARTILAGE AND SYNOVIAL JOINT FORMATION

Removal of β-catenin from chondrocytes disrupts normal endochondral ossification and leads to neonatal lethality (Akiyama et al. 2004; Day et al. 2005). Mutant knockout mice displayed delayed formation of hypertrophic chondrocytes, reduced chondrocyte proliferation and survival, delays in blood vessel invasion, ossification, and cleft palates. Thus, β-catenin inhibits chondrogenesis, but, once cartilage has formed, it promotes maturation and survival of chondrocytes in the growth plate. Removal of the Wnt antagonist secreted Frizzled-related protein 1 (sFRP1) in mice led to a diminished growth plate and increased calcification in the hypertrophic zone, and accelerated differentiation of hypertrophic chondrocytes (Gaur et al. 2006). A similar phenotype is seen in *Axin2*-null mice

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that were runted and showed evidence of accelerated chondrocyte maturation (Dao et al. 2010). Constitutive activation of β -catenin in chondrocytes and skeletal mesenchyme also caused neonatal lethality with severe chondrodysplasia (Akiyama et al. 2004; Guo et al. 2004; Hill et al. 2005). Activation led to small, hypoplastic skeletal elements with disorganized growth plates. It should be noted that although Wnt/ β -catenin signaling plays a major role in promoting chondrocyte differentiation, maturation, and survival (Mak et al. 2006), chondrocytes still mature eventually (despite a significant delay), and most chondrocytes do survive, suggesting there are other pathways in cartilage that can support chondrocyte hypertrophy and survival independently of Wnt/ β -catenin signaling.

Wnt signaling has also been shown to play an inductive role in synovial joint formation. Topgal, a transgenic LacZ readout of endogenous Wnt/ β -catenin signaling, marks sites of joint formation (Guo et al. 2004). Wnt4, Wnt9a (formerly known as Wnt14), and Wnt16 are expressed early in the development of interzones and can function as markers of synovial joint formation (Guo et al. 2004). However, inactivation of both Wnt4 and Wnt9a leads to only limited joint fusion (Spater et al. 2006), suggesting that removing more Wnt ligands (such as Wnt16) may be required to address the importance of Wnts in this process. β -Catenin is required for the development of synovial joints, because its removal before interzone formation led to a reduction in markers of synovial joint formation and resulted in joint fusions (Guo et al. 2004; Spater et al. 2006). Importantly, removal of β -catenin from the joint region following interzone formation led to a very mild joint phenotype, indicating that Wnt/ β -catenin signaling is particularly important early in joint induction (Fig. 3) (Koyama et al. 2008).

Different from the growth plate cartilage, articular cartilage coats the ends of long bones. Although growth plate cartilage matures and turns over quickly, articular cartilage undergoes minimal turnover and is essentially maintained as a permanent tissue. Pathways that control the maintenance of joints and articular cartilage are

particularly important for the treatment of osteoarthritis (OA), which is characterized by inappropriate hypertrophy of articular chondrocytes and degradation of extracellular matrix leading to loss of articular cartilage. Much of the available data suggest that activation of Wnt/ β -catenin signaling leads to loss of a stable chondrocyte phenotype and destruction of cartilaginous tissues. Genome-wide analysis in human has shown an association between a functional single nucleotide polymorphism (SNP) in secreted Frizzled-related protein 3 (sFRP3, also called FRZB), a Wnt antagonist (Loughlin et al. 2004). Inactivation of *sfrp3* in mice resulted in grossly normal cartilage development; however, mutant mice showed increased cartilage damage in models of OA (Lories et al. 2007). In addition, conditional activation of β -catenin postnatally in articular chondrocytes leads to an OA-like phenotype, suggesting that Wnt/ β -catenin signaling continues to be important in maintaining synovial joints (Zhu et al. 2009).

WNT/ β -CATENIN SIGNALING IN OSTEOBLAST FUNCTION

Wnt/ β -catenin signaling remains important throughout life, playing a central role in controlling bone mass. In mature osteoblasts in vivo, the Wnt/ β -catenin signaling pathway continues to be important, and a major function appears to be in the control of osteoclast formation. Deletion of β -catenin in osteoblasts using the Cre recombinase driven by either the human Osteocalcin or Collagen 1 α 1 (2.3 kb) promoters caused severe low bone mass with strikingly increased osteoclastogenesis, whereas constitutive activation of β -catenin under the same conditions resulted in dramatically increased bone deposition with reduced osteoclast formation (Glass et al. 2005; Holmen et al. 2005). Consistent with these, Wnt7b^{-/-} and Wnt10b^{-/-} mice have produced low bone mass phenotypes (Bennett et al. 2005; Tu et al. 2007). Because it is known that multiple Wnts and Fzds are expressed in the developing bone, it is likely that functional redundancy is masking the relative in vivo importance of these factors. It is likely that work with mice carrying conditional deletions

in either the Porcupine or Wntless/GPR177/Evi genes, which are both specifically required for secretion of Wnt ligands from Wnt-producing cells (Hausmann et al. 2007; Lorenowicz and Korswagen 2009), may help to address these questions (Fu et al. 2011; Stefater et al. 2011).

Bone formation has to be coupled to bone resorption in a delicate balance in order to maintain a healthy bone. This is achieved through an interaction between osteoblasts and osteoclasts. Osteoclast differentiation is regulated by at least three genes expressed in osteoblasts: macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor κ -B ligand (RANKL) and osteoprotegerin (Opg). RANKL is expressed on the surface of osteoblasts and stimulates osteoclast maturation through interactions with its receptor, RANK, which is expressed at the surface of osteoclast precursors. Opg is a secreted decoy receptor for RANKL that functions to inhibit osteoclastogenesis by disrupting RANKL–RANK interaction (Fig. 2). Both RANKL and Opg expression can be controlled by Wnt/ β -catenin signaling. Opg has been shown to be a direct target of β -catenin transcriptional activation, and loss of β -catenin leads to decreased Opg expression and increased osteoclast activity (Glass et al. 2005; Holmen et al. 2005). β -Catenin-deficient osteoblasts have also been documented to express higher RANKL expression (Holmen et al. 2005). Conversely, activation of Wnt/ β -catenin signaling by stabilizing β -catenin increased Opg expression and bone formation (Glass et al. 2005). In addition, activating the pathway by removing adenomatous polyposis coli (Apc) in osteoblasts showed reduced RANKL expression and elevated Opg levels (Holmen et al. 2005). Lef1 and Tcf1 are major downstream mediators of Wnt/ β -catenin signaling in osteoblasts, because their loss is associated with reduced bone mass and, in the case of Tcf1, increased osteoclastogenesis (Glass et al. 2005; Noh et al. 2009).

Wnt/ β -catenin signaling activity is tightly regulated by secreted antagonists, one of which is Dkk1, which also plays a critical role in bone formation. $Dkk1^{-/-}$ mice die in utero because of lack of head formation and also show limb defects with altered cell proliferation and sur-

vival (Mukhopadhyay et al. 2001). $Dkk1^{+/-}$ mice, however, survive and show higher numbers of osteoblasts and increased bone mass, demonstrating that partial loss of Dkk1 is sufficient to generate skeletal phenotypes (Morvan et al. 2006). Indeed, a Dkk1 allelic series including a hypomorphic Dkk1 allele (doubleridge; $Dkk1^d$) showed that bone mass correlates inversely with Dkk1 levels (MacDonald et al. 2007). Consistent with Dkk1 suppressing bone formation in vivo, transgenic mice expressing Dkk1 under control of osteoblast promoters are osteopenic (Li et al. 2006; Guo et al. 2010). In addition, a Dkk1-neutralizing antibody promotes bone formation when injected into mice (Yaccoby et al. 2007). Kremens, the Dkk1 coreceptors, also play physiologically significant roles in bone formation. Ablation of both kremens ($Krm1$ and $Krm2$) increased trabecular bone volume twofold and was associated with increased osteoblast number and function (Ellwanger et al. 2008). $Krm2$ is likely the more relevant gene in bone, because $Krm2^{-/-}$ mice developed a high bone mass phenotype and overexpression of $Krm2$ in bone led to severe osteoporosis with decreased osteoblast and elevated osteoclast differentiation and function (Schulze et al. 2010).

Sost is another secreted Wnt antagonist that is specifically expressed in osteocytes, and its overexpression in bone results in decreased osteoblast numbers and reduced bone formation rate (Winkler et al. 2003). $Sost^{-/-}$ mice show high bone mass in both cortical and trabecular bone compartments due to increased osteoblast numbers and normal osteoclast functions (Li et al. 2008). Sost-neutralizing antibodies have also been shown to promote bone formation and increase bone mass in a rat model of osteoporosis (Li et al. 2009).

Secreted Frizzled-related proteins (Sfrps) bind Wnt ligands and are Wnt pathway antagonists proving to be important in bone formation. Although widely expressed during development, phenotypes of the $Sfrp1^{-/-}$ mice appear to be largely restricted to skeletal tissues (Bodine et al. 2004; Morvan et al. 2006; Li et al. 2008). $Sfrp1^{-/-}$ mice have normal cortical bone but increased trabecular bone associated with

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decreased osteoblast apoptosis and enhanced osteoblast function. Although modest, *Sfrp3*^{-/-} mice show increased cortical bone thickness and density and demonstrated increased stiffness relative to controls (Lories et al. 2007).

Axin2, an intracellular negative regulator of Wnt/ β -catenin signaling, also regulates bone formation. Loss of Axin2 leads to enhanced proliferation and differentiation of osteoblasts, and *Axin2*^{-/-} mice develop craniosynostosis, a premature closure of cranial sutures (Yu et al. 2005). This phenotype can be rescued by reducing levels of β -catenin (Liu et al. 2007). Compound *Axin2*^{-/-}; *Axin1*^{+/-} mice have recently been shown to develop severe craniofacial and axial skeleton defects (Dao et al. 2010), again highlighting the important pro-osteogenic functions of the Wnt/ β -catenin signaling pathway in skeletal development.

Gsk3 β is another important intracellular negative regulator of the pathway. Although *Gsk3 β* ^{-/-} mice die around embryonic days 13.5–14.5 (E13.5–E14.5), *Gsk3 β* ^{+/-} mice survive and show increased bone formation (Kugimiya et al. 2007). Similarly, lithium chloride (LiCl), a Gsk3 β inhibitor, increased bone mass and osteoblast function in mice and rescued the low bone mass phenotype of the *Lrp5*^{-/-} mice (Clement-Lacroix et al. 2005). Consistent with this, LiCl treatment in humans is associated with a decreased incident of fractures (Vestergaard et al. 2005; Wilting et al. 2007). All of these results indicate that Wnt/ β -catenin signaling in osteoblasts regulates both bone formation and resorption.

Despite the important roles of Wnt/ β -catenin signaling in osteoblasts, one should not forget that it also acts directly in osteoclasts (Modarresi et al. 2009; Wei et al. 2011). Wnt/ β -catenin signaling blocks osteoclast differentiation by inhibiting RANKL signaling. Osteoclast differentiation is enhanced in β -catenin heterozygous mice, whereas stabilizing β -catenin in osteoclast precursors inhibits osteoclast differentiation.

Although the functional importance of *Lrp5* in regulating bone mass is clear, the precise mechanism has become controversial. Studies in mouse models confirmed the human find-

ings: Mice globally deficient for *Lrp5* have decreased bone mass, whereas mice carrying the gain-of-function (GOF) *Lrp5*-G171V point mutations under the control of an osteoblast promoter displayed increased bone mass (Kato et al. 2002; Babij et al. 2003; Cui et al. 2011). The controversy originates from the model in which *Lrp5* functions within the enterochromaffin cells of the small intestine to regulate the production of serotonin (Yadav et al. 2008). Increased serotonin secretion is associated with loss of *Lrp5* and reduction in osteoblast activity, leading to lower bone mass. In support of this model, Yadav et al. (2008) found that conditional deletion of *Lrp5* in the intestine led to low bone mass, whereas conditional deletion within the osteoblast had no discernible phenotype. Furthermore, intestinal-specific expression of the gain-of-function *Lrp5*-G171V allele led to increased bone mass, but osteoblast-specific expression of the same allele had no effect (Yadav et al. 2008). However, this model has recently been challenged when the characterization of independently derived mouse models carrying similar alterations in the *Lrp5* locus suggested that *Lrp5* did, as has been expected, function within the osteoblast and not in the intestine to control bone mass (Cui et al. 2011). In addition, recent work has shown that *Lrp5* acts redundantly with *Lrp6* within the osteochondral progenitor cells to mediate commitment to the osteoblast lineage (Joeng et al. 2011). The reasons for these different observations regarding *Lrp5* function are currently unclear and will undoubtedly be the subject of additional investigation.

WNT/ β -CATENIN SIGNALING IN OSTEOCYTES

Osteocytes represent >90%–95% of all bone cells and communicate through extensive dendritic processes present in canaliculi with one another, with cells on the bone surface, and in the bone marrow (Fig. 2). Osteocytes can recruit osteoclast precursors to stimulate bone resorption and regulate osteoblastic differentiation from a mesenchymal stem cell (Kamioka et al. 2001; Zhao et al. 2002; Heino et al. 2004).

Similar to mice with deletions of β -catenin in osteoblasts, mice with deletion of β -catenin induced by the DMP1-cre (which is highly enriched in osteocytes) showed a severe low bone mass phenotype, which was associated with increased osteoclast activity caused by decreased Opg expression in osteocytes (Kramer et al. 2010).

Mechanical stress leads to the movement of bone fluid through the lacuno-canalicular system, generating shear stress that the osteocyte senses. Osteocytes were first proposed to be mechanosensory cells 20 years ago, when an increased ^3H -uridine level was detected in osteocytes following mechanical loading in vivo (Pead et al. 1988). More recently, it has been shown that, in mice, ablation of osteocytes caused resistance to unloading-induced bone loss, providing the first direct evidence for the role of osteocytes in mechanotransduction (Tatsumi et al. 2007). Several reports have shown that β -catenin signaling is activated as a normal physiological response to mechanical loading in bone (Robinson et al. 2006). In the $\text{Lrp5}^{-/-}$ mice, the osteogenic response to mechanical loading of the ulna was dramatically reduced, with normal recruitment and activation of osteoblasts at the mechanically strained surface (Sawakami et al. 2006). This suggests that $\text{Lrp5}^{-/-}$ osteocytes may have a normal primary response to mechanic loading, whereas the intrinsic osteogenic abilities of osteoblasts and/or osteocytes are reduced in the absence of Lrp5 . Thus, the osteoporotic phenotype of the $\text{Lrp5}^{-/-}$ mouse could be due to inadequate processing of signals derived from mechanical stimulation. Consistent with this model, mice expressing the Lrp5 G171V mutation specifically in bone showed a higher sensitivity to mechanical loading with stronger β -catenin activation, and experienced significantly less bone loss induced by unloading (Akhter et al. 2004; Robinson et al. 2006; Saxon et al. 2011).

Osteocytes may be involved in a feedback signaling system to maintain bone-lining cells in a quiescent state under conditions of low mechanical load (Marotti et al. 1992; Martin 2000; Metz et al. 2003). A candidate to mediate such a feedback mechanism is *Sost*, which is

specifically expressed in mature osteocytes and plays a negative regulatory role in bone formation (van Bezooijen et al. 2004; Poole et al. 2005). *Sost* has been reported to function as both an inhibitor of BMP signaling and as an inhibitor of Wnt/ β -catenin signaling by binding directly to LRP5 and LRP6 (Li et al. 2005; Semenov et al. 2005). Ulmar loading decreased *Sost* expression and increased bone formation rate in a strain dose-dependent manner (Robling et al. 2008). $\text{Sost}^{-/-}$ mice were resistant to mechanical unloading-induced bone loss and failed to show decreased Wnt/ β -catenin signaling during unloading (Lin et al. 2009). Thus, *Sost* may represent an important primary response to mechanical loading. Mechanical loading may regulate bone mass by modulating the expression levels of this Wnt inhibitor.

In vitro models to mimic physiologic shear stress include steady fluid flow, pulsatile fluid flow, or oscillatory flow to generate shear stress on osteoblasts or osteoblast-like cells (Reich et al. 1990; Williams et al. 1994; Hillsley and Frangos 1997; Jacobs et al. 1998). There are limitations for these kinds of in vitro fluid flow shear stress on osteoblast cultures (Bonewald 2011), but they provide valuable information toward understanding the in vivo response to such forces. Consistent with the in vivo findings, in vitro studies also show that β -catenin signaling is required for the response to fluid shear stress (Lau et al. 2006; Robinson et al. 2006; Sawakami et al. 2006). In addition, some in vitro studies suggest that osteocytes support osteoclast formation through a response to fluid shear stress (Kim et al. 2006; You et al. 2008).

WNT/ β -CATENIN SIGNALING IN BONE FRACTURE HEALING

We briefly cover the function of Wnt/ β -catenin signaling in bone fracture healing here because this topic is covered in more detail in Whyte et al. (2012). Adult bone has the ability to regenerate after injury or fracture. In most aspects, fracture healing recapitulates key steps of embryonic bone development (Bruder et al. 1994); however, there are also differences between fracture healing and embryonic bone development.

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For example, embryonic bone development does not go through an inflammatory response. Fracture healing involves the participation of hematopoietic cells, immune cells, and mesenchymal stem cells that are recruited from the surrounding tissues and the circulation. In classical histological terms, fracture healing can be divided into direct and indirect healing (Dimitriou et al. 2005). The majority of fracture healing is through an indirect healing process, which consists of three main phases: (1) the inflammatory stage, (2) the repair stage, and (3) the remodeling stage (Chen and Alman 2009). A microarray study specifies the process further into five specific physiological events: inflammation, intramembranous ossification, chondrogenesis, endochondral ossification, and remodeling (Hadjiargyrou et al. 2002).

Wnt signaling may be involved throughout the healing process by regulating a wide variety of cell-fate decisions associated with osteogenesis. Notably, the β -catenin signaling pathway is significantly activated in the fracture callus (Hadjiargyrou et al. 2002; Chen et al. 2007). Consistent with this, studies on the Topgal mouse, an in vivo LacZ reporter for Wnt/ β -catenin signaling, showed that LacZ activity was activated specifically at the site of injury (Kim et al. 2007).

Because mouse models with gain of function in Wnt signaling show high bone mass phenotypes, it is not surprising to find that the mice with an activated form of β -catenin in osteoblasts and the $Axin2^{-/-}$ mouse show dramatically enhanced bone healing (Chen et al. 2007; Minear et al. 2010). However, in a fracture-healing model involving both endochondral and intramembranous ossification, Wnt/ β -catenin signaling plays varying roles in different phases of fracture repair. For example, either increases or decreases in Wnt/ β -catenin signaling during early stages of healing inhibit the differentiation of mesenchymal stem cells into osteoblasts. At later stages, Wnt/ β -catenin signaling positively regulates osteoblasts after the undifferentiated cells have committed to the osteoblast lineage (Chen et al. 2007). Both recombinant Wnt3a and LiCl can enhance healing when the treatment starts after the fracture

(Chen et al. 2007; Minear et al. 2010). Similarly, an anti-Sost antibody can enhance bone healing in rats (Agholme et al. 2010). Conversely, Wnt inhibition in the injury site by adenoviral expression of DKK1 can prevent the differentiation of osteoblastic cells (Kim et al. 2007).

Although it is clear that Wnt/ β -catenin signaling plays a crucial role during fracture healing, this does not rule out the possibility that β -catenin-independent pathways are also involved. It has been reported that Wnts that often do not signal via the β -catenin pathway are up-regulated during fracture healing (for review, see Chen and Alman 2009). In addition, periodontal ligament stem cells, a recently identified population of mesenchymal stem cells with bone-regeneration ability, were reported to have a lower osteogenic differentiation in inflammatory microenvironments, which might be due to inhibition of non-canonical Wnt pathways (Liu et al. 2011).

WNT IN BONE CANCER AND METASTASIS

The function of Wnt/ β -catenin signaling within the skeletal microenvironment has drawn the attention of not only academic, but also industry scientists and clinicians targeting this pathway to treat skeletal tumors. DKK1 has been proposed to be an important regulator in prostate cancer bone metastasis because alterations in DKK1 expression are linked to several tumor types with a propensity to grow in bone including prostate carcinoma, breast carcinoma, and multiple myeloma (Forget et al. 2007). Prostate cancer cells overexpressing DKK1 have a higher potential to metastasize into bone and generate osteolytic lesions (Thudi et al. 2010). DKK1 up-regulation is a frequent event in mammary cancer, and increased serum levels of DKK1 are associated with the presence of bone metastasis in patients (Voorzanger-Rousselot et al. 2007; Bu et al. 2008). In multiple myeloma, the serum level of DKK1 is positively correlated with the presence of bone lesions, and anti-DKK1 antibody may be an effective adjunct in the treatment of multiple myeloma-associated bone disease (Tian et al. 2003; Yaccoby et al. 2007; Fulciniti et al. 2009; Heath et al. 2009). Based

on these observations, several pharmaceutical companies have agents at various stages of clinical trials that target this pathway for treatment of bone metastases. For example, antibodies or small molecules that target DKK1 or SOST are in various stages of development for treating multiple myeloma and skeletal metastases (Rey and Ellies 2010).

CONCLUDING REMARKS

In this article, we attempt to summarize the current understanding of Wnt/ β -catenin signaling in skeletal tissues. Work from many groups in the past decade has clearly shown the importance of the Wnt/ β -catenin pathway in both embryonic and postnatal bone formation and homeostasis. Modulating this pathway is widely recognized as an important area of therapeutic development for osteoporosis, fracture healing, and tissue engineering. Moving forward, important questions still remain regarding the precise mechanism(s) by which Wnt/ β -catenin signaling regulates skeletal tissues and how this pathway interacts with other major pathways (e.g., BMP, Hedgehog, FGF, etc.) in the skeleton.

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