Wnt signaling in bone formation and its therapeutic potential for bone diseases

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Jeong Hwan Kim, Xing Liu, Jinhua Wang, Xiang Chen, Hongyu Zhang, Stephanie H. Kim, Jing Cui, Ruidong Li, Wenwen Zhang, Yuhan Kong, Jiye Zhang, Wei Shui, Joseph Lamplot, Mary Rose Rogers, Chen Zhao, Ning Wang, Prashant Rajan, Justin Tomal, Joseph Statz, Ningning Wu, Hue H. Luu, Rex C. Haydon and Tong-Chuan He

Abstract: The Wnt signaling pathway plays an important role not only in embryonic development but also in the maintenance and differentiation of the stem cells in adulthood. In particular, Wnt signaling has been shown as an important regulatory pathway in the osteogenic differentiation of mesenchymal stem cells. Induction of the Wnt signaling pathway promotes bone formation while inactivation of the pathway leads to osteopenic states. Our current understanding of Wnt signaling in osteogenesis elucidates the molecular mechanisms of classic osteogenic pathologies. Activating and inactivating aberrations of the canonical Wnt signaling pathway in osteogenesis results in sclerosteosis and osteoporosis respectively. Recent studies have sought to target the Wnt signaling pathway to treat osteogenic disorders. Potential therapeutic approaches attempt to stimulate the Wnt signaling pathway by upregulating the intracellular mediators of the Wnt signaling cascade and inhibiting the endogenous antagonists of the pathway. Antibodies against endogenous antagonists, such as sclerostin and dickkopf-1, have demonstrated promising results in promoting bone formation and fracture healing. Lithium, an inhibitor of glycogen synthase kinase 3β, has also been reported to stimulate osteogenesis by stabilizing β catenin. Although manipulating the Wnt signaling pathway has abundant therapeutic potential, it requires cautious approach due to risks of tumorigenesis. The present review discusses the role of the Wnt signaling pathway in osteogenesis and examines its targeted therapeutic potential.

Keywords: Wnt signaling, bone formation, osteoporosis, fracture healing, bone tumors

Introduction

The Wnt signaling cascade was first discovered in the 1980s from mouse breast tumors induced by the mouse mammary tumor virus [Nusse and Varmus, 1982]. Since then, the Wnt signaling pathway has been investigated extensively and has been characterized as one of the most essential signaling cascades in multiple biological processes. The Wnt signaling pathway plays a critical role in embryonic development, postnatal development and adult tissue homeostasis. To coordinate these complex processes, the Wnt signaling regulates cell proliferation, cell fate determination, cell differentiation and cell polarity [Logan and Nusse, 2004]. A greater interest in the Wnt signaling pathway arose when the adenomatous polyposis coli (APC) protein was discovered to interact with β catenin in familial adenomatous polyposis (FAP) syndrome [Rubinfeld *et al.* 1993; Su *et al.* 1993]. The oncogenic association between APC and β catenin in FAP led to subsequent discoveries of associations between Wnt signaling and other human pathologies, ranging from cancers to metabolic disorders and developmental defects [Clevers and Nusse, 2012].

In recent years, the role of the Wnt signaling pathway in bone biology has gained considerable attention. Specific human pathologies of bone such as osteoporosis pseudoglioma syndrome, sclerosteosis and van Buchem's disease have been associated with aberrant Wnt signaling; these discoveries have triggered further research of the Wnt signaling pathway in bone biology [Monroe *et al.* 2012]. Understanding the role of Wnt signaling in bone disorders has not only helped elucidate the pathogenesis of those disorders but has also led to the development of potential therapeutic avenues to treat the disorders [Hoeppner *et al.* 2009; Wagner *et al.* 2011]. In particular, the development of neutralizing antibodies targeting endogenous Wnt

Correspondence to:
Tong-Chuan He, MD, PhD
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, 5841 South Maryland Avenue, MC 3079, Chicago, IL 60637, USA
tche@uchicago.edu

Xing Liu, MD, PhD
Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA

The Children's Hospital of Chongqing Medical University, Chongqing, China

Stephanie H Kim, BS,
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA

Jinhua Wang, MD, PhD, Hongyu Zhang, MD, PhD, Jing Cui, MD, Ruidong Li, MD, Wenwen Zhang, MD, Yuhan Kong, MD, Jiye Zhang, MD, Wei Shui, MD and Ningning Wu, MD
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA
Chongqing Medical University, Chongqing, China
Xiang Chen, MD, PhD
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA
The Affiliated Tangdu Hospital of the Fourth Military Medical University, Xi'an, China
Jeong Hwan Kim, BA, Joseph Lamplot, BS, Mary Rose Rogers, MSc, Justin Tomal, BS, Joseph Statz, BS, Hue H Luu, MD and Rex C Haydon, MD, PhD
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA
Chen Zhao, MD and Ning Wang, MD
Molecular Oncology Laboratory, Department of Orthopaedic Surgery, University of Chicago Medical Center, Chicago, IL, USA
School of Laboratory Medicine and the Affiliated Southwest Hospital, Third Military Medical University, Chongqing, China
Prashant Rajan, BS
Miami University, Oxford, OH, USA

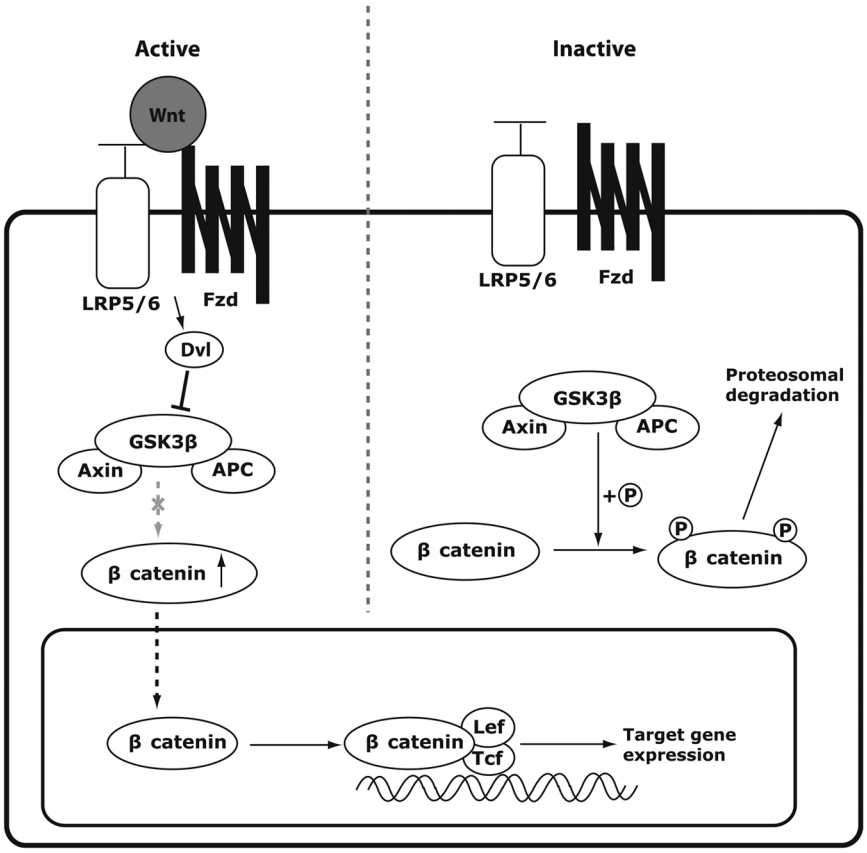

Figure 1. Diagrammatic representation of the canonical Wnt signaling pathway. In the presence of the Wnt ligand, the ligand binds to frizzled (Fzd) receptor and its coreceptor low-density lipoprotein receptor-related protein (LRP)-5/6. Disheveled (Dvl) is subsequently activated, which inhibits glycogen synthase kinase 3β (GSK3β) from phosphorylating β catenin. The cytoplasmic level of β catenin consequently rises, and β catenin translocates into the nucleus to bind with transcriptional factors T-cell factor (Tcf)/lymphoid enhancer-binding factor (Lef-1), upregulating the target gene expression. In the absence of the Wnt ligand, GSK3β constitutively phosphorylates β catenin which leads to ubiquitination and proteosomal degradation of β catenin. Thus, the target gene expression level remains low.

antagonists, such as sclerostin and dickkopf (Dkk) proteins, has shown considerable promise in early clinical trials. Other potential therapeutic targets are also currently undergoing preclinical investigation [Rachner *et al.* 2011]. The present review discusses the role of the Wnt signaling pathway in bone biology and osteogenic disorders and potential therapeutic targets in the Wnt signaling pathway for bone disorders. Finally, some of the oncogenic risks associated with therapeutics targeting the Wnt signaling pathway will be examined.

Overview of the Wnt signaling pathway

Canonical and noncanonical Wnt signaling pathways

The Wnt ligands are a group of 19 secreted glycoproteins that activate their cell surface receptors to induce specific intracellular signaling cascades controlling gene expression. The Wnt signaling pathway can be divided into the canonical and the noncanonical pathways. The canonical pathway mediates signaling through the stabilization of β catenin, whereas the noncanonical pathways work independently of β catenin. This review primarily focuses on the canonical Wnt signaling pathway as the canonical pathway has been better characterized regarding its role and therapeutic potential in bone disorders.

The hallmark of canonical Wnt signaling is the stabilization of β catenin in the cytoplasm upon activation (Figure 1). Without the Wnt ligands, cytoplasmic β catenin is phosphorylated by a protein degradation complex composed of glycogen synthase kinase 3β (GSK3β), axin and APC. GSK3β phosphorylates β catenin, tagging β catenin

for ubiquitination and proteosomal degradation [Rao and Kühl, 2010]. However, when the canonical Wnt ligands, such as Wnt 1, 3a, and 8, are present, the ligands bind to the frizzled (Fzd) receptors and one of the coreceptors, either low-density lipoprotein receptor-related protein (LRP)-5 or LRP6, causing phosphorylation of the intracellular protein disheveled (Dvl). Phosphorylated Dvl inhibits GSK3β from phosphorylating β catenin in the cytoplasm. As a result, β catenin is stabilized and translocated into the nucleus. β catenin forms a transcriptional complex with T-cell factor (Tcf)/lymphoid enhancer-binding factor (Lef) to regulate the expression of target genes such as cyclin D1, axin2, c-Myc and peroxisome proliferator-activated receptor (PPARδ) [Logan and Nusse, 2004; MacDonald *et al.* 2009; Rao and Kühl, 2010].

Two major noncanonical Wnt pathways are the calcium-dependent pathway and the planar cell polarity pathway. The calcium-dependent pathway is important in embryonic development, cell migration and cancer progression [De, 2011; Kühl, 2004]. It is initiated by Wnt ligands binding to their Fzd receptors followed by the activation of G protein and a signal cascade releasing intracellular calcium from the endoplasmic reticulum. The released calcium activates downstream mediators, such as protein kinase C, calcineurin and calcium calmodulin-dependent protein kinase II, which in turn activate transcription factors, such as nuclear factor κB, nuclear factor of activated T cells and cAMP response element binding protein [De, 2011; Rao and Kühl, 2010]. The cell polarity pathway is another noncanonical pathway important in cell planar polarity, migration, motility and division [Vladar *et al.* 2009]. This pathway regulates cytoskeletions by activating GTPases such as rho, rac and cdc42 through Fzd receptors and ultimately downstream kinases such as c-jun NH2 kinase [Rao and Kühl, 2010; Sugimura and Li, 2010]. However, the details of the noncanonical pathways are not well characterized in comparison to the canonical pathway.

Complexity of the Wnt signaling pathway

Given the many roles that Wnt signaling plays in development and stem cell maintenance, it is not surprising that Wnt signaling is under complex regulation. For instance, the binary classification of Wnt signaling into the canonical and the noncanonical pathways may be an oversimplistic view of reality. Classically, it has been viewed that the noncanonical Wnt ligands, such as Wnt 5a and Wnt 11, act through the noncanonical pathway while the canonical ligands, Wnt 1, 3a and 8, signal through the β-catenin signaling pathway [Rao and Kühl, 2010; van Amerongen *et al.* 2008]. However, cross activation of the counterpart pathway does not seem uncommon. For example, Wnt 5a, a classical noncanonical ligand, has been shown to activate the canonical β-catenin pathway in the presence of Fzd 5 [He *et al.* 1997] or Fzd 4 and LRP 5 [Mikels and Nusse, 2006]. In addition to cross activation, there are at least 19 Wnt ligands, 10 Fzd receptors and 2 LRP coreceptors that have been identified. Furthermore, Wnt ligands can also bind receptor tyrosine kinase and receptor tyrosine kinase-like orphan receptor 2 [Hoeppner *et al.* 2009; van Amerongen *et al.* 2008]. This multiplicity of Wnt ligands and receptors implies that numerous potential ligand–receptor combinations exist. In fact, the Wnt signaling pathway does not function as a single linear cascade of events. Rather, it appears to work within a tight regulatory network specific to the context of the receptors present and active on the cell membrane at the onset of activation [van Amerongen *et al.* 2008]. There is ample room for spatial and temporal regulation under the given context of the cell and its environment. Understanding the rich, complex regulation of Wnt signaling will prove to be crucial as we continue to explore potential therapeutic interventions within this signaling cascade.

Regulation of the Wnt signaling pathway

The endogenous regulators of the canonical Wnt signaling pathway can be largely divided into extracellular and intracellular antagonists based on their sites of action (Figure 2). Examples of extracellular inhibitors include sclerostin, Dkks, Wnt inhibitory factor 1/2 (Wif-1/2) and secreted frizzled-related proteins (SFRPs). Most extracellular antagonists are secreted factors that interact with either Wnt receptors or Wnt ligands. For example, sclerostin binds the coreceptor LRP5/6 to competitively inhibit the binding of Wnt ligands to LRP5/6 [Li *et al.* 2005; Semënov *et al.* 2005]. Dkk-1 also forms a complex with LRP6 and another transmembrane protein, Kremen1/2 (Krm1/2). This complex formation leads to the internalization and degradation of LRP, lowering the availability of LRP for Wnt ligand binding [Bafico *et al.* 2001; Mao *et al.* 2002]. Meanwhile, SFRPs and Wif-1 directly bind Wnt ligands to prevent their binding to Fzd and LRP5/6 [Hsieh *et al.* 1999; Kawano and Kypta, 2003].

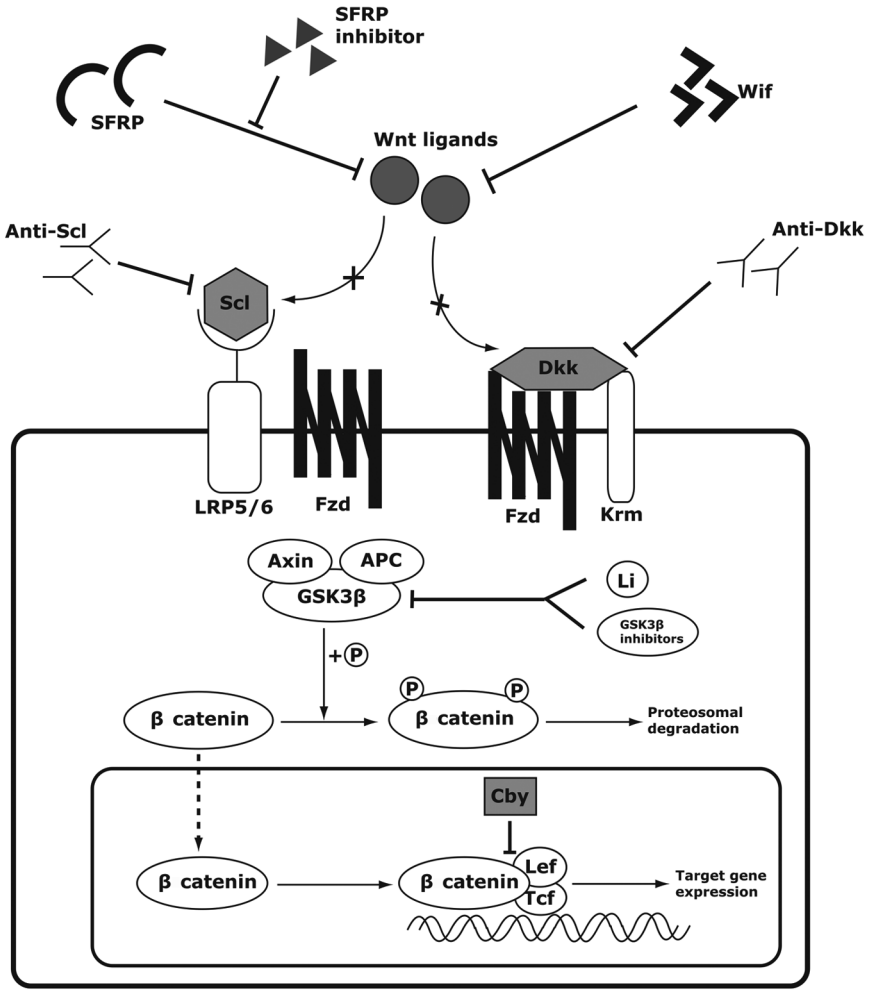

Figure 2. Potential therapeutic targets of the Wnt signaling pathway. In the presence of sclerostin or dickkopf (Dkk) proteins, the ligands cannot bind their receptors to activate the Wnt signaling. The inhibitory effects of sclerostin and Dkk proteins can be neutralized by their respective antibodies, thus activating the Wnt signaling pathway downstream. Secreted Frizzled-related proteins (SFRPs) and Wif inhibit the Wnt signaling by binding the Wnt ligands, but SFRP inhibitors such as diphenylsulfonyl sulfonamide can inhibit SFRPs from binding the Wnt ligands. Glycogen synthase kinase 3β (GSK3β) normally targets β catenin for degradation by phosphorylation. However, lithium or other pharmacological GSK3β-inhibiting compounds can inhibit the GSK3β activity, thus allowing β catenin to accumulate in the cytoplasm and the nucleus expressing the target genes. Chibby (Cby) is an endogenous antagonist, which interrupts the binding of β catenin to transcriptional factors T-cell factor (Tcf)/lymphoid enhancer-binding factor (Lef-1).

However, the intracellular antagonists disrupt the Wnt signaling cascade in the cytoplasm or nucleus. GSK3β, axin and APC form the degradation complex which tags β catenin for degradation in the inactive state [Kimelman and Xu, 2006]. Chibby (Cby) works inside the nucleus to antagonize the interaction between β catenin and Tcf/Lef-1 [Takemaru *et al.* 2003].

These endogenous antagonists deserve attention because they highlight the key points of regulation in the Wnt signaling pathway and help us to better understand the diseases caused by aberrant Wnt signaling. Thus, the endogenous antagonists have become popular targets of potential therapeutic approaches which manipulate the Wnt signaling pathway to treat bone disorders.

Mesenchymal stem cells and the Wnt signaling pathway

Wnt-mediated osteogenic differentiation of mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent progenitors that retain the capacity to differentiate

into multiple types of tissues, including bone, cartilage, fat, tendon and muscle [Bianco *et al.* 2008]. MSCs carry vast therapeutic potential in regenerative medicine because a substantial amount of these cells can be harvested from various locations of the patient's body, especially bone marrow [Zhang *et al.* 2012]. Induction of MSCs along the osteogenic lineage may serve as an effective therapy to promote bone formation in osteogenic disorders.

Commitment of MSCs into a single cell lineage is regulated by a variety of growth factors [Zhang *et al.* 2012], yet the current understanding of this process is still limited. Nonetheless, it is relatively well established that the Wnt signaling pathway plays an important role in promoting the osteogenic differentiation of MSCs [Cawthorne *et al.* 2012; Krishnan *et al.* 2006]. It has been shown that β catenin promotes the progression of MSCs from osteoblastic precursor cells into more mature osteoblasts while suppressing differentiation into adipogenic and chondrogenic lineages [Case and Rubin, 2010; Glass *et al.* 2005]. Specifically, the canonical Wnt pathway inhibits the expression of the major adipogenic inducers PPARγ and CCAAT/enhancer binding protein α to suppress adipogenic differentiation while upregulating the osteogenic regulators Runx2, Dlx5 and Osterix [Bennett *et al.* 2005; Kang *et al.* 2007]. In addition, the noncanonical Wnt pathway has also been shown to induce osteogenic differentiation, albeit it through a different mechanism. The noncanonical ligand Wnt 5a was shown to suppress PPARγ through the inactivation of chromatins rather than through β-catenin action [Takada *et al.* 2007]. Though the interplay between the two independent mechanisms induced by Wnt ligands is still unclear, it is evident that Wnt signaling regulates the osteogenic differentiation of MSCs.

Crosstalk with other signaling pathways during the osteogenic differentiation of mesenchymal stem cells

It is important to understand that crosstalk between the Wnt pathways and other signaling pathways exists to regulate the osteogenic differentiation of MSCs. For example, bone morphogenic proteins (BMPs) have been shown to either enhance or antagonize the osteogenic differentiation induced by Wnt signaling [Chen *et al.* 2012; Lin and Hankenson, 2011]. BMPs, particularly BMP2, 6 and 9, are major osteogenic growth factors that induce osteogenic differentiation in MSCs [Luu *et al.* 2007]. Studies have demonstrated that Wnt signaling and the BMP signaling pathways have common targets which induce the osteogenic differentiation of MSCs; one such target is connective growth tissue factor [Luo *et al.* 2004; Si *et al.* 2006]. Furthermore, functional Wnt signaling was demonstrated to be required for the BMP-induced osteogenic differentiation of MSCs. Wnt 3a enhanced the osteogenic effects of BMP9 while β-catenin knockdown or overexpression of a Fzd antagonist, FrzB, inhibited the osteogenic effects of BMP9 [Tang *et al.* 2009]. Similarly, conditional β-catenin knockout or Dkk-1 overexpression inhibited BMP2-induced ectopic bone formation [Chen *et al.* 2007b]. It was suggested that BMP2 stimulates LRP5 expression and downregulates β-Trcp, leading to stabilization of β catenin and its signaling to promote osteogenic differentiation [Zhang *et al.* 2009].

Interestingly, Wnt5a, a classical noncanonical Wnt, was recently reported as a critical component of BMP2-mediated osteogenic differentiation [Nemoto *et al.* 2012]. Others have also shown that BMPs can downregulate Wnt signaling in osteogenic differentiation via sclerostin and Dkk-1. Knocking out BMP receptor type 1 in osteoblasts led to downregulation of sclerostin and Dkk-1 and an increased bone mass phenotype in mice [Kamiya *et al.* 2008, 2010]. As an explanation for the Wnt-antagonizing effects of BMP, it was suggested that Smad1 may form a complex with Dvl, thereby sequestering Dvl from the canonical Wnt pathway [Liu *et al.* 2006]. However, these seemingly conflicting findings on the crosstalk between BMPs and Wnts remain unresolved.

In addition to BMPs, the Wnt signaling pathway crosstalks with multiple signaling pathways, including the Notch signaling and Hedgehog (Hh) pathways. Activation of the Notch pathway has been shown to inhibit the osteogenic differentiation induced by the Wnt/β-catenin signaling. Overexpression of Notch intracellular domain, both *in vivo* and *in vitro*, led to the downregulation of Wnt signaling and impaired osteoblastogenesis [Deregowski *et al.* 2006; Sciaudone *et al.* 2003; Zanotti *et al.* 2008]. The Hh pathway has been reported to work upstream of Wnt signaling in a sequential manner to induce osteogenic differentiation of MSCs. Inhibition of Wnt signaling was shown to reduce Hh-induced osteogenic activity both *in vitro* [Rawadi *et al.* 2003] and *in vivo* [Mak *et al.* 2006]. It has been suggested that

Hh signaling regulates the early stages of osteogenic differentiation of MSC followed by the Wnt signaling further downstream [Lin and Hankenson, 2011; Mak *et al.* 2006; Rodda and McMahon, 2006].

Wnt signaling also crosstalks with inflammatory signaling processes during bone formation. Notably, tumor necrosis factor (TNF)-α has been shown to induce Dkk-1, an endogenous regulator of Wnt signaling, thus blocking osteoblast differentiation [Diarra *et al.* 2007; Heiland *et al.* 2010]. Overexpression of TNFα in mice leads to joint destruction without proper bone repair, mimicking rheumatoid arthritis. However, neutralizing Dkk-1 with anti-Dkk-1 antibodies in TNFα transgenic mice inhibited the joint destruction and resulted in osteophyte formation, indicative of active bone repair [Diarra *et al.* 2007]. In other words, the intricate balance between bone formation and bone resorption is maintained by the crosstalk between the Wnt signaling and the TNFα-induced inflammatory process.

Knowledge of the crosstalk between Wnt and other signaling pathways continues to expand. Recently, it was shown that the Wnt signaling pathway reciprocally regulates progranulin growth factor in frontotemporal dementia [Rosen *et al.* 2011; Wexler *et al.* 2011]. Meanwhile, progranulin, also known as proepithelin, is one of the newly identified growth factors that promotes chondrogenic differentiation of MSCs and endochondral ossification [Feng *et al.* 2010; Wu *et al.* 2011]. More details of the crosstalk between Wnts and progranulin in bone formation will need to be elucidated by further investigation.

MicroRNA (miRNA) represents another category of elements that interact with Wnt signaling to regulate osteogenic differentiation. Numerous miRNAs have been reported to either promote or inhibit the osteogenic differentiation of MSCs. Different miRNAs intervene at different locations and stages of osteogenic differentiation, interacting with extracellular growth factors and intracellular transcriptional factors such as Runx2 and osterix [Vimalraj and Selvamurugan, 2012]. Several miRNAs have been reported to specifically interact with Wnt signaling molecules to affect osteogenesis. miR-27 inhibits APC, thus activating canonical Wnt signaling to promote bone formation [Wang and Xu, 2010]. miR-335-5p was shown to downregulate Dkk-1, thus enhancing osteogenic differentiation [Zhang *et al.* 2011].

Overall, a complex regulatory network exists between the Wnt signaling pathway and other signaling pathways during osteogenic differentiation. A complete characterization of all the interactions between Wnts and these other pathways are far from complete. Nevertheless, a better understanding of the intricate crosstalks between Wnt and other signaling pathways in osteogenesis will prove to be essential in discovering therapeutic interventions to effectively manipulate these signaling pathways and treat osteogenic disorders.

The Wnt signaling pathway in osteogenic pathologies

Since Wnt signaling plays such a critical role in the osteogenic differentiation of MSCs, it is not surprising that aberrations in the Wnt signaling pathway may lead to failures of proper bone formation. Indeed, studies of animal models and human pathologies have shown that aberrant Wnt signaling results in various osteogenic disorders. The effects of these altered Wnt signaling pathways not only highlight the crucial role that the Wnt signaling plays in normal bone physiology but also point to the potential of the Wnt signaling pathway as a target for the development of therapeutic targets in treating bone disorders (Figure 2).

Extracellular components of the Wnt signaling pathway in osteogenic pathologies

The most noteworthy example of altered Wnt signaling causing an osteogenic pathology is the inactivating mutation of LRP5 leading to osteoporosis pseudoglioma syndrome (OPPG). OPPG is an autosomal recessive disorder characterized by early-onset osteoporosis, low bone mineral density (BMD) and blindness [Levasseur *et al.* 2005]. A loss-of-function mutation of LRP5 has been linked to the pathogenesis of OPPG through genetic analyses [Gong *et al.* 2001] and later confirmed by animal studies. Inactivating mutations of LRP5 in mice recapitulated the phenotypes of OPPG in human patients [Cui *et al.* 2011; Kato *et al.* 2002], and LRP5 knockout resulted in impaired fracture healing in comparison to wild-type mice [Komatsu *et al.* 2010]. On the contrary, researchers have also identified a gain-of-function missense mutation of LRP5 (G171V) that results in high-bone-mass (HBM) phenotypes in humans [Boyden *et al.* 2002; Little *et al.* 2002]. Transgenic mice with the same G171V mutation again recapitulated the HBM phenotypes, confirming that

an activating mutation of LRP5 leads to HBM [Babij *et al.* 2003].

In contrast to LRP5, there is no well-defined human osteogenic pathology associated with an LRP6 mutation. Nevertheless, genetic analyses and animal studies demonstrate that disruption of LRP6 in the Wnt signaling pathway severely affects osteogenic development. A missense mutation of LRP6 mutation in humans led to osteoporosis with some features of the metabolic syndrome [Mani *et al.* 2007]. In addition, certain polymorphisms of LRP6 were significantly associated with low BMD [Riancho *et al.* 2011]. In mice, an LRP6 null mutation led to neonatal death, severe axial skeleton defects and limb abnormalities, mimicking the phenotypes of various Wnt gene mutations [Pinson *et al.* 2000]. Similarly, a spontaneous missense mutation of LRP6 in mice (named *ringelswanz*) was also discovered to affect development and osteogenic homeostasis. Mice homozygous for *ringelswanz* showed skeletal abnormalities, delayed ossification during development [Kokubu *et al.* 2004] and low bone density as adults due to reduced canonical Wnt signaling [Kubota *et al.* 2008]. Lastly, LRP6 deletion was shown to disrupt bone density synergistically with LRP5 deletion during the osteogenic development of mice, illustrating the critical interplay between LRP6 and LRP5 in skeletal development and bone formation [Holmen *et al.* 2004].

Osteogenic pathologies can also result from the disruption of secreted extracellular antagonists of Wnt signaling. Sclerostin is one of the best characterized Wnt antagonists. As the product of *SOST* gene, sclerostin is secreted by osteocytes during bone remodeling. Sclerostin binds to LRP5/6 to inhibit the Wnt signaling pathway during bone formation, completing a negative feedback loop of osteogenesis [van Bezooijen *et al.* 2005] (Figure 2). Two related pathologies have been linked to specific mutations of *SOST*: sclerosteosis and van Buchem's disease. Both pathologies are autosomal recessive disorders characterized by progressive bone growth, increased bone density, volume and strength. Mutations within the *SOST* gene were shown to be responsible for sclerosteosis [Balemans *et al.* 2001], whereas van Buchem's disease is associated with a 52 kb deletion in the noncoding region containing the enhancer element of *SOST* [Balemans *et al.* 2002; Loots *et al.* 2005]. The phenotypes of both diseases were recapitulated in *SOST* knockout mice which exhibited higher bone mass with increased bone density, volume and strength [Li *et al.* 2008]. As expected, overexpression of *SOST* in mice conversely led to osteopenia [Winkler *et al.* 2003]. The Wnt inhibitory effects of sclerostin can be also reproduced by certain LRP5 mutations. The LRP5 mutations causing HBM, such as the G171V mutant, have been shown to render LRP5 unable to bind sclerostin, thus making it resistant to the inhibitory effects of sclerostin [Ellies *et al.* 2006; Semenov and He, 2006]. Therefore, sclerostin is a key endogenous regulator of the Wnt signaling pathway in bone formation and has abundant potential as a therapeutic target in modulating the Wnt pathway either to promote or reduce bone formation.

Dkk proteins are another example of extracellular antagonists of the Wnt signaling pathway. Dkk-1 has been proposed to form a ternary complex with Krm and LRP5/6 on the plasma membrane, thus preventing LRPs from binding Wnt ligands [Mao *et al.* 2002] (Figure 2). It is controversial whether or not LRP6 is internalized and degraded upon Dkk-1 binding [Li *et al.* 2010b; Mao *et al.* 2002]. Some researchers have argued that LRP6 disrupts the Wnt-induced formation of Fzd-LRP6 complex without internalizing the receptors [Semënov *et al.* 2008;Wang *et al.* 2008]. Regardless, the substantial impact that Dkk-1 has on bone development and homeostasis is evident from genetic and animal studies. Several mutations of LRP5 causing the HBM phenotype in humans were found to have resistance to inhibition by Dkk-1 [Ai *et al.* 2005; Balemans *et al.* 2007; Boyden *et al.* 2002]. This is consistent with findings from mouse models deficient of Dkk-1. A single allele deletion of Dkk-1 in mice showed increased bone mass and greater bone formation activity [Morvan *et al.* 2006], as did mice with hypomorphic alleles (*doubleridge*) of Dkk-1 [MacDonald *et al.* 2007]. However, transgenic overexpression of Dkk-1 in osteoblasts of mice led to osteopenia and skeletal defects [Li *et al.* 2006]. Thus, Dkk-1 plays an important regulatory role in bone tissue development and homeostasis and causes osteogenic disruptions when altered from its physiologic function.

SFRP is another antagonist of the Wnt signaling pathway. SFRP directly binds Wnt ligands to prevent their binding to Fzd and LRP5/6 [Kawano and Kypta, 2003] (Figure 2). A deficiency of SFRP has been shown to disrupt proper osteogenesis in mice. SFRP-1 knockout mice showed higher trabecular bone volume, density and mass

indicative of increased bone formation [Bodine *et al.* 2004; Gaur *et al.* 2005]. More specifically, deleting SFRP-1 enhanced endochondral ossification [Gaur *et al.* 2006] and even led to accelerated fracture healing in animal models [Gaur *et al.* 2009]. This target can potentially serve as a therapeutic avenue for improving fracture healing in humans. Additionally, certain polymorphisms of SFRP3 in humans have been associated with hip osteoarthritis [Loughlin *et al.* 2004], making SFRP an attractive therapeutic target in the treatment of arthritis.

The Wnt ligands themselves can also lead to abnormalities in osteogenesis when overexpressed or mutated. Wnt 10b overexpression in mice caused increased postnatal bone mass compared with wild-type mice [Bennett *et al.* 2005, 2007]. Conversely, Wnt 10b deficient mice exhibited lower bone mass, fewer trabeculae [Bennett *et al.* 2005] and progressive bone loss after 1 month of age [Stevens *et al.* 2010]. In addition, Wnt 7b deficiency led to defective osteoblastogenesis and thus ineffective bone formation in the embryos of mice [Tu *et al.* 2007].

These examples of osteogenic abnormalities associated with aberrant Wnt signaling illustrate potential targets for therapeutic interventions in treating osteogenic disorders. Both inhibition of endogenous antagonists and mimicry of the functions of endogenous agonists of the Wnt signaling should be pursued as potential therapeutic approaches for osteogenic disorders.

Intracellular mediators of the Wnt signaling pathway in osteogenic pathologies

Similar to their extracellular counterparts, intracellular mediators of the Wnt signaling pathway can cause aberrant osteogenesis when altered from their physiologic states. For example, the functional activity of β catenin directly affects the osteogenic differentiation of MSCs. Conditional deletion of β catenin in early osteogenic progenitors prevented terminal differentiation into osteoblasts and instead resulted in chondrocyte formation [Day *et al.* 2005; Hill *et al.* 2005; Rodda and McMahon, 2006]. Conversely, stabilization of β catenin led to high bone mass and premature ossification without chondrocyte formation [Glass *et al.* 2005; Rodda and McMahon, 2006]. Interestingly, the osteopenic state caused by β-catenin inactivation was accompanied by osteoclast activation while stabilization of β catenin was associated with decreased osteoclasts [Holmen *et al.* 2005]. This suggests that β catenin is involved in the regulation of both osteoblasts and osteoclasts, thus controlling overall bone formation.

Deficiency of Axin-GSK3β-APC complex or Tcf/Lef-1 can also lead to the disruption of normal osteogenic development. Axin2 deletion in mice has been shown to cause overactivation of the canonical Wnt signaling pathway. Phenotypes of Axin2-deficient mice include increased bone mass, increased osteoblast proliferation, increased chondrocyte maturation and craniosynostosis [Dao *et al.* 2010; Yan *et al.* 2009; Yu *et al.* 2005]. Similarly, as expected from its physiologic interaction with β catenin, haploinsufficiency of GSK3β in mice resulted in increased bone formation with enhanced Runx2 activity [Kugimiya *et al.* 2007]. However, alteration of APC has been shown to both promote and disrupt osteogenesis. APC deletion in osteoblasts led to increased bone mass through overactivating the β-catenin-dependent Wnt signaling [Holmen *et al.* 2005]. Another study showed that conditional deletion of APC in Col2a1-expressing cells increased β-catenin level as expected, but caused skeletal defects from the failure of proper osteogenic and chondrogenic differentiation [Miclea *et al.* 2009]. Thus, it appears that a tight regulation of β-catenin level by APC is necessary before commitment into the osteogenic lineage for proper osteogenesis. Lastly, Tcf/Lef-1 transcriptional factors have also been shown to be essential for proper osteogenesis. *Lef-1*$^{+/-}$ mice showed significantly reduced bone volume during the early years of life [Noh *et al.* 2009] while *Tcf*$^{-/-}$ mice exhibited increased bone resorption, thus leading to a lower bone mass phenotype [Glass *et al.* 2005].

When the Wnt signaling pathway is altered in progenitor cells, proper osteogenic differentiation is compromised leading to osteogenic pathologies/abnormalities. Thus, it is conceivable that the mediators of Wnt signaling may be appealing targets for potential therapeutic interventions that aim to either restore proper osteogenic signaling or manipulate Wnt signaling in favor of bone formation.

Potential therapeutic targets in the Wnt signaling pathway

Given the numerous findings showing that the Wnt signaling pathway regulates osteogenesis,

this pathway has arisen as an attractive therapeutic target for treating several osteogenic disorders. Common osteogenic disorders of interest include osteoporosis, fracture healing, nonunion fractures and critical-sized defects. These disorders represent either systemic or local failures of proper bone formation. Thus, inducing the Wnt signaling pathway to promote bone formation is a plausible therapeutic approach to treat these disorders. As reviewed in the previous sections, Wnt signaling contains multiple levels of regulation that can be manipulated to modulate the pathway of osteogenesis: Wnt receptors, secreted Wnt antagonists and intracellular mediators (see Figure 2 and Table 1). Therefore, researchers have been working to develop drugs that target specific components of Wnt signaling to induce osteogenesis. Recently, a few agents have shown promising results and are currently undergoing clinical trials. Other drugs continue to be investigated at the preclinical stages.

Targeting the extracellular antagonists

Inhibition of the extracellular antagonists with monoclonal antibodies has been a popular approach to modify the Wnt signaling pathway and promote bone formation. Sclerostin-neutralizing antibody is one of the most prominent examples. Given the physiologic role of sclerostin as a negative regulator of the Wnt pathway, neutralization of sclerostin is expected to upregulate the Wnt signaling pathway, promoting bone formation (see Figure 2 and Table 1). Indeed, studies have shown that subcutaneous injection of sclerostin-neutralizing antibody markedly increased bone formation in ovariectomized rats after 5 weeks of treatment [Li *et al.* 2009]. In fact, the anabolic effect of antisclerostin antibody was so effective that it not only reversed bone loss by estrogen deficiency, but it actually increased the bone mass compared with nonovariectomized rats. The bone-forming effects of antisclerostin antibody were similarly observed in aged male rats after 5 weeks of treatment [Li *et al.* 2010a] and in ovary-intact cynomolgus monkeys after 2 months [Ominsky *et al.* 2010]. Moreover, the osteogenic effect of antisclerostin antibody was persistent regardless of mechanical loading [Agholme *et al.* 2011b] or presence of fracture [Ominsky *et al.* 2011]. In humans, polymorphisms of the SOST promotor have been associated with osteoporosis, further making antisclerostin antibody an appealing therapeutic agent for treating osteoporosis [Huang *et al.* 2009]. Recently, a phase I clinical trial of antisclerostin antibody was completed. Single injections of antisclerostin antibody into healthy men and postmenopausal women were well tolerated and resulted in a dose-dependent increase in bone formation markers and a dose-dependent decrease in bone resorption markers over the 85-day study period [Padhi *et al.* 2011]. Currently, a phase II trial is ongoing, and it investigates the efficacy of antisclerostin antibody in comparison to alendronate and teriparatide as treatment for osteoporosis in postmenopausal women with low BMD [ClinicalTrials.gov identifier: NCT00896532].

Neutralization of Dkk-1 with antibody is another therapeutic approach under extensive investigation (see Figure 2 and Table 1). Although still at the preclinical stage, animal studies have demonstrated promising results. Similar to antisclerostin antibody, anti-Dkk-1 antibodies increase trabecular bone mass and density in mice [Glantschnig *et al.* 2010] and restore bone density in osteopenic mice and rhesus macaques [Glantschnig *et al.* 2011]. The use of anti-Dkk-1 antibody has been shown to be useful in several specific pathologies. First, anti-Dkk-1 antibodies reduced bone loss in a rheumatoid arthritis mouse model [Diarra *et al.* 2007] as well as in systemic inflammatory states [Heiland *et al.* 2010]. Furthermore, antibody-mediated blockade of Dkk-1 stimulated bone formation during healing from traumatic injury, such as fracture repair and orthopedic implant fixation [Agholme *et al.* 2011a; Komatsu *et al.* 2010; Li *et al.* 2011]. Lastly, Dkk-1 blockade has been reported to prevent the suppression of osteoblasts in multiple myeloma mouse models while decreasing osteoclasts, thus reducing osteolytic lesions [Fulciniti *et al.* 2009; Heath *et al.* 2009; Yaccoby *et al.* 2007]. Furthermore, the neutralization of Dkk-1 with antibody helped control the growth of myeloma cells [Fulciniti *et al.* 2009], and active immunization with Dkk-1 was shown to protect mice from developing multiple myeloma [Qian *et al.* 2012]. Currently, a phase I/II clinical trial is ongoing to test the safety and the efficacy of anti-Dkk-1 antibody in combination with zoledronic acid in refractory multiple myeloma [ClinicalTrials.gov identifier: NCT00741377].

In comparison to sclerostin and Dkk-1, therapies targeting SFRP are at early stages of investigation. By screening multiple compounds, one group has identified a compound, diphenylsulfonyl sulfonamide, which inhibits SFRP-1 activity and induces bone formation *ex vivo* [Bodine *et al.* 2009;

Table 1. Potential therapeutic targets in the Wnt signaling pathway and their therapeutic agents.

Component	Physiological role	Therapeutic agent	Mechanism of intervention	Therapeutic effects	References
Sclerostin	Inhibits β-catenin signaling via binding LRP5/6	Anti-sclerostin antibody (AMG 785)	Neutralizes sclerostin	Increased bone mass, density, and strength in OVX rats, aged male rats, and cynomolgus monkeys	[Li *et al*. 2009, 2010; Ominsky *et al*. 2010]
				Phase I clinical trial: Dose-dependent increase in bone formation without adverse events	[Padhi *et al*. 2011]
				Phase II clinical trial: compares AMG 785 with alendronate and teriparatide in postmenopausal women with low BMD	[ClinicalTrials.gov identifier: NCT00896532]
Dkk-1	Inhibits β-catenin signaling via binding (and potentially internalizing) LRP	Anti-Dkk-1 antibody (BHQ 880)	Neutralizes Dkk-1	Reduced bone loss from rheumatoid arthritis in mice	[Diarra *et al*. 2007]
				Enhanced fracture healing and orthopedic implant fixation in mice	[Agholme *et al*. 2011a; Komatsu *et al*. 2010; Li *et al*. 2011]
				Reduced osteolytic lesions in myeloma mouse models	[Fulciniti *et al*. 2009; Heath *et al*. 2009; Yaccoby *et al*. 2007]
				Phase I/II clinical trial: Examines the safety and the efficacy of BHQ 880 in combination with zoledronic acid in refractory myeloma	[ClinicalTrials.gov identifier: NCT00741377]
SFRP	Inhibits β-catenin signaling via binding the Wnt ligands	Diphenylsulfonyl sulfonamide	Inhibits SFRPs	Inhibits SFRP-1 *in vitro* and induces bone formation *ex vivo*	[Bodine *et al*. 2009; Moore *et al*. 2009, 2010]
GSK3β	Inhibits β catenin via targeting β catenin for proteosomal degradation	Lithium	Inhibits GSK3β	Restores bone mass in LRP5 KO mice and increases bone mass in WT mice	[Clément-Lacroix *et al*. 2005]
				Enhances fracture healing and prevents tumor growth in myeloma mouse model	[Edwards *et al*. 2008]
		GSK3β inhibitors		Increases bone mass in normal mice	[Gambardella *et al*. 2011]
				Restores bone loss in OVX mice	[Kulkarni *et al*. 2006, 2007]
β catenin	Binds Tcf/Lef-1 and upregulates target gene expression	Deoxycholic acid	Activates β catenin	Upregulates target gene expression levels of β catenin	[Pai *et al*. 2004]

BMD, bone mineral density; Dkk, dickkopf; GSK3β, glycogen synthase kinase 3β; KO, knockout; Lef-1, lymphoid enhancer-binding factor; LRP, low-density lipoprotein receptor-related protein; OVX, ovariectomized; SFRP, secreted frizzled-related protein; Tcf, T-cell factor; WT, wild type.

Moore *et al.* 2009, 2010] (see Figure 2 and Table 1). No study has explicitly studied the *in vivo* osteogenic activity of SFRP antibodies or inhibitors. However, commercially available polyclonal antibodies to SFRP-1 were demonstrated to reduce inflammation-induced periodontal bone loss and osteoclastogenesis [Li and Amar, 2007]. Thus, the therapeutic potential of antagonizing SFRP in bone formation remains worthy of further investigation.

Targeting the intracellular mediators

Directly manipulating the intracellular mediators of the Wnt signaling pathway is another potential approach to promote osteogenesis. For instance, inhibiting GSK3β from phosphorylating β catenin would stabilize the cytoplasmic level of β catenin, allowing further progression through the Wnt signaling pathway downstream. Lithium, a commonly used medication for bipolar disorder, is a well characterized example of a GSK3β inhibitor (see Figure 2 and Table 1). Animal studies have shown that the administration of lithium chloride for 4 weeks in LRP5 knockout mice restored bone mass to normal levels and increased the bone mass of wild-type mice [Clément-Lacroix *et al.* 2005]. Additionally, mice treated with lithium after bone injury exhibited enhanced fracture healing [Chen *et al.* 2007a]. Furthermore, lithium chloride activated osteoblast differentiation and prevented tumor growth in myeloma mouse models [Edwards *et al.* 2008]. The association between lithium and enhanced bone formation in mouse models is consistent with epidemiological data in humans. It has been shown that lithium use is associated with reduced fracture risks [Vestergaard *et al.* 2005] and increased bone mass and density [Zamani *et al.* 2009]. Yet, a more extensive mechanistic study of lithium and its effect on bone formation is necessary because there are some reports that argue against the pharmacologic benefit of lithium reducing fracture risks [Wilting *et al.* 2007].

Similar effects of GSK3β can be found with the use of pharmacologic interventions to inhibit GSK3β (see Figure 2 and Table 1). An orally active GSK3α/β inhibitor, 603281-31-8, has demonstrated the ability to induce osteoblastogenesis *in vitro* and enhance bone formation with greater bone density, thickness and strength *in vivo* after 60 days [Kulkarni *et al.* 2006]. The compound 603281-31-8 was also able to reverse trabecular bone volume loss from estrogen deficiency in ovariectomized rats and restore the adipogenicity of bone marrow down to the normal level after 60 days of treatment [Kulkarni *et al.* 2007]. The effect of GSK3β inhibition was recapitulated with the administration of another GSK3β inhibitor, AR28, which increased osteogenesis while decreasing adipogenicity in mice after 14 days of treatment [Gambardella *et al.* 2011]. Despite the promising osteogenic benefits of GSK3β inhibitors including lithium and other pharmacologic agents, it is important to note that the GSK3β activity is not limited to bone formation but also involved in other intracellular processes. Thus, caution needs to be taken in overinhibiting GSK3β due to oncogenic risks which will be discussed in the subsequent section.

Interventions on other downstream intracellular mediators carry abundant therapeutic potential for bone disorders. For example, modulation of the interaction between β catenin and Tcf/Lef-1 is a theoretically plausible approach to control the Wnt signaling pathway. Some compounds have been identified to exert activating or inhibitory effects on the interaction between β catenin and Tcf/Lef-1. For example, deoxycholic acid, a secondary bile acid, has been shown to increase the activation of β catenin and expression of its target genes [Pai *et al.* 2004]. Yet, explicit data on deoxycholic acid promoting bone formation is lacking. Cby, a conserved nuclear protein, has been reported to antagonize β catenin by competing with Lef-1 for binding β catenin [Takemaru *et al.* 2003] (Figure 2). As a result, Cby is considered an important factor that promotes adipogenic differentiation while inhibiting downstream β-catenin signaling [Li *et al.* 2007]. Thus, either utilizing its adipogenic properties or developing an intervention to antagonize Cby could provide another therapeutic avenue to manipulate Wnt signaling.

Tumorigenic risks associated with Wnt-targeted therapies

Despite the therapeutic potential of manipulating the Wnt signaling pathway for osteogenic disorders, caution must be taken. The potential therapeutics involve stimulation of the Wnt signaling pathway to promote osteogenesis. Given that the Wnt signaling pathway is not specific to bone tissue, any overactivation of Wnt signaling inevitably leads to consequences outside of bone. More specifically, promoting the proliferation and renewal of stem cells via activation of the Wnt signaling pathway carries the risk of inducing cancer. In

fact, these oncogenic risks have been well documented with mutations of Wnt signaling mediators such as APC, Axin, GSK3β, β catenin and LRP [Polakis, 2012]. APC mutation is strongly associated with FAP syndrome and colorectal cancers [Kinzler *et al.* 1991; Kinzler and Vogelstein, 1996; Nishisho *et al.* 1991]. Mutations of Axin proteins have also been linked to an increased risk of colorectal cancer [Lammi *et al.* 2004]. Tcf4 is commonly mutated in colorectal cancers as well [Cuilliere-Dartigues *et al.* 2006]. Mutations of β catenin and its associated transcription factors can lead to several types of cancer, including hepatoblastoma, hair follicle tumors, sebaceous tumors and leukemia [Chan *et al.* 1999; Jamieson *et al.* 2004; Polakis 2007; Takeda *et al.* 2006].

Therefore, any therapeutic intervention that modulates the Wnt signaling pathway should take into account the potential oncogenic risks associated with exogenous manipulation of the pathway. Deoxycholic acid is an example of exogenous stimulation of the β catenin, which has been reported to induce tumor progression [Pai *et al.* 2004]. In the context of bone, close attention must be paid to the risk of osteosarcoma development. It has been reported that Wif-1, an endogenous antagonist of Wnt signaling, is downregulated in 76% of human osteosarcoma [Rubin *et al.* 2010]. More specifically, Wif-1 was epigenetically silenced via promotor hypermethylation, thus overactivating the Wnt signaling pathway and promoting the proliferation of tumor cells [Kansara *et al.* 2009]. This is consistent with findings that reveal high β-catenin levels in the cytoplasm and nucleus of osteosarcoma cells indicative of high Wnt signaling activity [Iwaya *et al.* 2003]. Conversely, it is conceivable that any therapeutic intervention inhibiting Wnt signaling can be utilized as an anticancer therapy. Recently, an antibody targeting Fzd receptors, OMP-18R5, was reported to inhibit the growth of human breast, pancreatic, colon and lung tumors in mouse models [Gurney *et al.* 2012]. Nevertheless, most therapeutic interventions being investigated for the treatment of bone disorders aim to stimulate the Wnt signaling pathway to promote bone formation. Future studies need to carefully examine the optimal level of modulation of the Wnt signaling pathway in treating osteogenic disorders so that oncogenic risks are minimized.

In addition to oncogenic risks, therapeutics involving Wnt signaling should take into account several other considerations [discussed in Hoeppner *et al.* 2009]. One particularly relevant point in the context of bone disorders is the potential development of osteoarthritic symptoms. Prolonged exogenous stimulation of the Wnt signaling pathway appears to lead to osteoarthritic symptoms. For example, anti-Dkk-1 antibodies, despite reducing bone loss in the rheumatoid arthritis mouse model, produced osteophytes characteristic of osteoarthritis [Diarra *et al.* 2007]. This observation is consistent with animal studies showing that either upregulating β catenin or inhibiting GSK3β led to osteoarthritic phenotypes with osteophytes [Miclea *et al.* 2011; Zhu *et al.* 2009]. Therefore, when utilizing anti-Dkk-1 antibody to prevent bone loss from rheumatoid inflammation or any other therapeutics to activate Wnt signaling for a prolonged period, a careful calibration of the dose and duration of treatment is required to minimize osteoarthritic side effects.

Conclusion and future directions

Our current understanding of the Wnt signaling pathway in osteogenic disorders has broadened considerably over the past decade. We now not only better understand the pathogenesis of these bone disorders caused by aberrant Wnt signaling, but tremendous therapeutic avenues for treating these bone disorders by targeting the Wnt signaling pathway are being thoroughly explored. Antisclerostin antibody exemplifies translation of our knowledge of the Wnt signaling into clinical application. Other agents, such as anti-Dkk-1 antibody and SFRP inhibitors, will continue to undergo clinical investigation and may soon become available for clinical use.

Nevertheless, a considerable amount of work remains. The oncogenic risks associated with exogenous modulation of the Wnt signaling pathway must always be taken into consideration when developing a therapy that targets any component of the Wnt signaling pathway. Finding the optimal balance between the osteogenic effects and the oncogenic risks of Wnt-targeted therapies will be critical and must be explored carefully and extensively. Furthermore, as Wnt signaling is not limited to bone tissue, finding a method to directly target bone tissues without affecting other tissues may be ideal to maximize therapeutic benefits while minimizing oncogenic risks. Further studies clearly defining the best therapeutic targets in the Wnt signaling pathway will be critical in the development of future treatments. Despite current

obstacles to the development of new Wnt-targeted therapies, targeting the Wnt signaling pathway has emerged as an attractive strategy to treat many of the most common bone disorders and thus holds great promise as a future therapeutic approach.

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Conflict of interest statement

The authors declare no conflicts of interest.

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