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Targeting the Wnt Signaling Pathway to Augment Bone Formation

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Abstract

Recent discoveries in humans and mice have revealed that the Wnt (Wingless and Int-1) signaling pathway is responsible for a complex array of functions in maintaining bone homeostasis. The Wnt proteins are key modulators of mesenchymal lineage specification and regulate most aspects of osteoblast physiology and post-natal bone acquisition by controlling the differentiation and activity of osteoblasts and osteoclasts. Initial reports have indicated that activators of Wnt signaling are potent promoters of osteogenesis; however, systemic hyperactivation of the canonical Wnt pathway could potentially accelerate neoplastic transformation and subsequent tumor growth. Alternatively, recent investigations of natural soluble antagonists of Wnt signaling in bone suggest the possibilities of bone-specific therapies targeting the negative regulators of Wnt pathway, especially sclerostin. With this new knowledge, novel pharmacologic interventions that alter Wnt signaling are being evaluated for the management of osteoporosis. In this article, we briefly describe the Wnt signaling elements, their characterized role in bone, and summarize the current knowledge on the potential to enhance bone formation through the manipulation of Wnt signaling antagonists.

Introduction

The Wnt (Wingless and Int-1) family of secreted proteins regulates many aspects of cell growth, differentiation, and death during embryogenesis and adult life [1]. The first member of the Wnt gene family, *Wnt1*, was first reported in 1982 to be activated in breast cancer in the mouse [2]. However, it took almost two decades to obtain evidence that Wnt signaling plays a crucial role in mammalian bone homeostasis [3,4]. Two excellent reviews have broadly covered the increasing number of factors involved in the Wnt signal transduction pathways [5,6]. Wnt proteins signal through canonical (β -catenin-dependent) and noncanonical mechanisms (Fig. 1). The activity of the canonical pathway is mediated through β -catenin, which is inactivated in the absence of Wnt ligands by an oligomeric complex that consists of glycogen synthase kinase 3β (GSK- 3β), axin, casein kinase 1 (CK1), adenomatous polyposis coli (APC), and Disheveled. The binding of Wnt proteins to Frizzled (Fz) receptors and its coreceptor, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), stabilizes cytoplasmic β -catenin protein, which in turn translocates to the nucleus and activates the transcription of target genes via transcription factors including lymphoid enhancer-binding factor (LEF) and T cell factors (TCF). The noncanonical Wnt pathways also require Fz receptors and in vertebrates has been characterized to include at least three intracellular cascades: the protein kinase C (PKC) pathway (Wnt/ Ca^{2+}), the Rho family guanosine-5'-triphosphate (GTP)ases pathway, and the Jun N-terminal kinase (JNK) cascade.

Role of Wnt Proteins in Bone Formation

Canonical and noncanonical pathways are involved in coordinating proper bone development, formation and growth, both pre- and postnatally [7,8]. The well-documented role of canonical Wnt signaling in human bone is related to loss or gain-of-function mutations in LRP5, causing osteoporosis-pseudoglioma syndrome [3], or a high bone density syndrome [4], respectively. The abnormal phenotype of high bone mass results from increased Wnt/ β -catenin signaling. Canonical Wnt signaling supports osteogenic differentiation from precursor lines and stem cell lines. During in vivo bone development, canonical Wnt signaling prevents osteoblasts from differentiating into chondrocytes [9•] and targets Runx2 for osteoblast differentiation [10]. Postnatally, overexpression of Wnt10b in transgenic mice increases bone mass [11]. Interestingly, β -catenin signaling in differentiated osteoblasts has been shown to negatively control osteoclast formation and bone resorption through an increase in osteoprotegerin production by osteoblasts [12].

Along with the canonical Wnt pathway, there is now increasing evidence for noncanonical Wnt signaling pathways influencing intracellular events responsible for skeletal development and differentiation. Tu et al. [13] have reported the role of Wnt-PKC signaling in osteoblastogenesis in vitro corresponding with PKC homozygous mutant mice exhibiting a deficit in embryonic bone formation. Also, mice deficient in the G proteins α_q and α_{11} —required for Wnt-induced PKC activation in osteoprogenitors—have bone defects in the craniofacial skeleton. The anabolic effect of parathyroid hormone (PTH) on bone formation has been in part attributed to its stimulation of noncanonical Wnt4 signaling promoting osteoprogenitor differentiation and osteoblast development, primarily through the protein kinase A pathway [14]. Noncanonical Wnt4 activation of p38 mitogen-activated protein kinase (MAPK) also has been reported to enhance osteogenic differentiation of mesenchymal stem cells (isolated from human craniofacial tissues) and to promote bone formation in rodent models [15]. Similarly, noncanonical Wnt5a signaling was reported to potently transdifferentiate adipoprogenitors into osteoblasts in vitro by suppressing peroxisome proliferator-activated receptor (PPAR)- γ , a key transcription factor for adipogenesis, and inducing Runx2, a key transcription factor for osteogenesis [16]. Earlier in vivo data also supported the role of Wnt5a in osteoblastogenesis with the observation of decreased trabecular bone mass in the femurs of Wnt5a^{+/-} mice [17]. Loss-of-function mutation of Wnt5a in these animals resulted in truncation of the proximal skeleton and absence of distal digits. Finally, noncanonical Wnt5a has been shown to prevent the apoptosis of osteoblast progenitors and differentiated osteoblasts, comparable to antiapoptotic effects of canonical Wnt1 and Wnt3a [18].

The Wnt Antagonists

As with other growth factors, Wnt signaling can be antagonized by secreted or intracellular inhibitors. The known secreted Wnt signaling inhibitors are sFRPs (secreted frizzled-related proteins), Wif1 (Wnt inhibitor factor), Cer1 (Cerberus), and dickkopfs (Dkks). sFRPs, Wif1, and Cer1 block Wnt function by binding and sequestering the molecule. In contrast, Dkks act through Kremens, the Dkks receptor, to internalize the LRP5/6 coreceptor to prevent formation of the Wnt-Fz-LRP5/6 complex [19]. One other Wnt signaling antagonist is sclerostin, a soluble factor secreted by osteocytes that competitively binds to LRP5.

The intracellular Wnt signaling inhibitors consist of APC, axin, GSK-3 β , CK1 α (all participate in the multiprotein complex that phosphorylates β -catenin for the degradation pathway), Nkd (naked cuticle), hTrCP (transducin repeat-containing proteins), ANKRD6 (ankyrin repeat domain 6), and NLK (Nemo-like kinase). Of this group, GSK-3 β inhibitors

have been studied for many years in other clinical areas, such as in the treatment of bipolar disease. Their effects on bone have only recently been investigated [20].

The importance of Wnt signaling in establishing bone phenotypes and targeting Wnt regulators has been reviewed by Baron and Rawadi [21]. Several members of the Wnt signaling pathway, including LRP5/6, Dkk2, sclerostin, and sFRP1, have been shown to have significant association with bone mineral density (BMD) variation in humans [22•]. Aberrant activation of the Wnt signaling pathway is associated with several human cancers [23] and the oncology literature has contributed significantly to our understanding of the pleiotropic activities of this pathway and its inhibitors. Thus, a highly specific approach that is context dependent would be warranted to use this pathway in therapeutic intervention to avoid untoward effects. Here, we focus on current findings on the most relevant key inhibitory components of Wnt signaling specifically in regard to bone formation.

Dickkopfs

Dkks are secreted regulators of Wnt signaling, with four members identified in the human genome [24]. Dkk1 interferes with Wnt signaling by directly binding to LRP5/6 [25]. It also binds to Kremen 2, another transmembrane receptor, resulting in the internalization of LRP from the cell surface so that the coreceptor is unavailable to interact with Wnt ligands [19]. Surprisingly, Dkk2 and Dkk3 do not antagonize Wnt signaling. Dkk2 was reported to have a role in terminal differentiation of osteoblasts and mineralized matrix formation [26]. Increased Dkk1 and Dkk2 expression is necessary for late-stage osteoblast differentiation in mesenchymal stem cell-like KS483 cells [27]. Dkk2-null mice have increased osteoid on trabecular and endocortical bone surfaces but impaired mineralization, leading to an osteopenic phenotype [26]. In mice, Dkk1 is an essential regulator of distal limb patterning.

Overexpression of Dkk1 induces osteopenia in mice [28], while deletion of a single allele of the *Dkk1* gene leads to an increase in bone formation and bone mass [29]. Dkk1 and sFRP2, another Wnt antagonist, are highly expressed in the myeloma cells and are associated with increased RANKL (receptor activator for nuclear factor- κ B ligand) expression, osteoclastogenesis, and bone loss adjacent to the myeloma cells in vivo [30–33]. Elevated Dkk1 levels in prostate and breast cancer bone metastasis have been reported to be responsible for development of osteolytic bone lesions in these diseases [34,35].

The pathogenesis of glucocorticoid (GC)-induced osteoporosis may also result from increased expression of *Dkk1* that suppresses osteoblastic differentiation through the Wnt pathway [36]. We performed a microarray on whole bone extracts from mice that were treated with GCs for 56 days and found that Wnt antagonists, including Dkk1, sclerostin, and Wif1, were upregulated from day 28 to day 56 [37]. Thus, suppression of Wnt signaling may be responsible for some of the pathogenesis of prolonged suppression of bone formation after GC administration. Concurrent treatment of GC-treated mice with PTH for 28 days reversed the elevation of Dkk1, sclerostin, and Wif1 [37]. Wang et al. [38] reported that deleting Dkk1 expression in bone tissue with Dkk1 antisense oligonucleotide attenuated GC-induced osteoblast apoptosis [38] and prevented osteoclastogenesis and bone loss in ovariectomized rats [39]. *Dkk1* also has been reported to have a role in the regulation of bone and cartilage metabolism in degenerative and inflammatory joint diseases. Inhibition of Dkk1 by administering anti-Dkk1 antibody prevented the bone-destructive process in a mouse model of rheumatoid arthritis by augmenting bone formation [40]. Also, anti-Dkk1 treatment in mice implanted with human multiple myeloma cells suppressed bone resorption and increased BMD above the pretreatment levels [41]. These data support the role of Dkk1 in the regulation of bone mass and suggest that targeting this protein may be an effective intervention to augment bone formation in treatment of postmenopausal osteoporosis, GC-induced osteoporosis, and possibly other secondary causes of osteoporosis.

Sclerostin

Sclerostin is the product of the *Sost* gene, which was originally identified as the gene that, when mutated, causes sclerosteosis, a rare condition of progressive bone dysplasia characterized by a generalized skeletal overgrowth [42]. Sclerostin is expressed mainly by osteocytes after they become embedded in a mineralized matrix [43]; it also limits further bone formation by osteoblasts [44]. Sclerostin expression also has been reported in bone-resorbing osteoclasts [45] to be the mechanism in which bone apposition is inhibited in the vicinity of resorption. The physiologic role of sclerostin in suppression of bone formation supports its potential in the development of therapeutic strategies for osteoporosis.

Several investigators have suggested mechanisms for inhibiting osteoblast function with sclerostin. Sclerostin was shown to antagonize bone morphogenetic protein (BMP) signaling, suppress mineralization of osteoblastic cells, and promote the apoptosis of human osteoblastic cells [46]. Sclerostin binds only weakly to BMPs and does not inhibit direct BMP-induced responses. Sclerostin is known to inhibit Wnt signaling on osteoblastic cells by binding to Wnt coreceptors LRP5/6. Sclerostin competes with Dkk1 for binding to LRP5 to inhibit Wnt signaling and can be displaced from LRP5 by Dkk1 [47]. Sclerostin has decreased binding to LRP5 mutants associated with a high bone mass phenotype in mice and humans. Such mutations make LRP5 somewhat resistant to the inhibition of Wnt signaling [48].

Sclerostin inhibits osteoblast differentiation and plays a key role in controlling bone formation and bone mass throughout life. Transgenic mice that overexpress *Sost* exhibit low bone mass and bone strength as a result of a significant reduction in osteoblast activity. Concordantly, targeted deletion of the sclerostin gene in mice resulted in high bone mass and increased bone strength [49•]. Sclerostin production by osteocytes was reported to rapidly decrease with PTH or parathyroid hormone-related protein treatment [50], and with mechanical loading [51]. Recent clinical data with sclerostin neutralizing antibody in postmenopausal women demonstrated an increase in bone formation markers and BMD with a simultaneous reduction in serum type I collagen C-telopeptides, a serum marker of bone resorption [52]. Because sclerostin expression is essentially limited to bone, developing antisclerostin antibodies may possibly affect only the skeleton and, by reducing inhibition of Wnt signaling, increase bone formation.

Secreted frizzled-related proteins

sFRPs are among the extracellular proteins that control Wnt signaling. There are at least seven members in vertebrates (sFRPs 1 to 5, crescent, and sizzled) and contain the frizzled-related cysteine-rich domain, which can bind to Wnts and Fz. But, they lack the intracellular sequence found in the Fz receptors. sFRPs antagonize Wnt function by binding to the Wnt molecule, thereby preventing receptor activation. There is evidence to support the ability of sFRPs to inhibit canonical and noncanonical Wnt signaling [53].

The discovery and the name of Frzb [54] (later named sFRP3) highlights its association with bone development. sFRP3 was shown in vitro to suppress osteoblast proliferation in a dose-dependent manner. However, it increased osteoblast differentiation, suggesting a role for sFRP3 in a noncanonical Wnt pathway—in addition to its function as a decoy receptor for Wnt [55]. sFRP3 and sFRP2 were both shown to be upregulated during mesenchymal stem cell osteogenesis [56]. The observation that *sFRP3* gene expression is significantly suppressed in osteosarcoma cells suggested a tumor-suppressive role for this gene [57]. Also, two polymorphisms in the *sFRP3* gene (rs288326 and rs7775), reported to attenuate the ability to antagonize Wnt signaling, are associated with an increased risk of hip osteoarthritis in elderly Caucasian women and multiple site osteoarthritis in other

populations [58]. Similarly, high serum levels of sFRP3 in women were associated with a modest reduction in hip osteoarthritis [59]. The sFRP3 inhibition of Wnt signaling in chondrocytes increases transcription of matrix metalloproteinases, which degrade the articular cartilage matrix (Klein, Unpublished data). These findings suggest that sFRP3 affects osteoblasts and chondrocytes and requires more research to explore the Wnt signaling in bone and joint.

sFRP1 is also an important negative regulator of osteoblasts and osteocytes; overexpression in human osteoblasts accelerates the cell death rate [60]. Adult mice deficient in sFRP1 show increased trabecular bone accrual and resistance to age-related bone loss, suggesting that without sFRP1 there is an increase in Wnt signaling and a decrease in osteoblast/osteocyte apoptosis. Bodine et al. [61] showed that the sFRP1 knockout mice also had reduced bone anabolic responses to PTH treatment.

Our research group has determined that in sFRP1 transgenic mice (sFRP1-Tg), overexpression of sFRP1 results in osteopenia with lower osteoblastogenesis and bone formation with males more severely affected than females [62]. In addition, we observed that sFRP1-Tg mice also had reduced bone anabolic responses to human PTH (1-34) treatment [62]. sFRP1 overexpression appears to counteract the PTH-induced increases in osteoblast differentiation and activity.

Along with osteoblastogenesis, sFRP1 affects osteoclastogenesis. sFRP1 can directly bind RANKL [63] and/or inhibit the fusion of mononuclear cells [64] thereby inhibiting osteoclast formation. Despite broad tissue distribution, ablation of sFRP1 in mice did not affect blood and urine markers of bone resorption and most nonskeletal tissues [65]. A small molecule inhibitor (diarylsulfone sulfonamide) that can bind and inhibit sFRP1 was recently shown to stimulate bone formation [66], suggesting inhibition of sFRP1 may be a potential target to stimulate Wnt signaling to increase bone formation.

sFRP4 is ubiquitously expressed, is present on bone cells, and is reported to suppress the proliferation of osteoblasts through inhibition of Wnt signaling [67]. Transgenic mice overexpressing sFRP4 in osteoblasts exhibited low bone mass caused by a decrease in bone formation [68]. sFRP4 is also a circulating phosphatonin in normal human serum that antagonizes renal Wnt signaling. Increased serum sFRP4 levels in tumor-induced osteomalacia are reported to promote phosphaturia and hypophosphatemia, [69], suggesting a function for sFRP4 in homeostasis of serum phosphate and vitamin D.

Other Wnt Antagonists

Wif1

Wif1 plays a role in bone biology as a negative regulator of bone mass. In situ hybridization of Wif1 found strong expression in osteoblasts and endosteal lining cells. Microarray profiling of murine osteoblastic cells lines stimulated by BMP2 supported that *Wif1* and *sFRP2* were two of the most strongly upregulated genes during terminal osteoblasts differentiation [70]. Transgenic overexpression of *Wif1* decreases BMD and increases susceptibility to bone fractures in mice. *Wif1* expression was increased in mice after 4 weeks of treatment with GCs, suggesting that it may participate in the pathogenesis of the prolonged inhibition of bone formation by GCs [37]. Thus, it appears that antagonism of Wif1 may have therapeutic potential in osteoporosis treatment.

GSK-3 β

GSK-3 β is a key enzyme for the phosphorylation that is necessary in β -catenin degradation and for several different cellular pathways including developmental patterning and cell

survival. By inhibiting canonical Wnt pathway, GSK-3 β acts as a negative regulator of osteogenesis and therefore its suppression may enhance bone formation. Treatment with GSK-3 β inhibitors, such as lithium, increased bone mass in rodent models [71,72] and reduced the relative risk of fractures in humans [73]. GSK-3 β has been suggested to inhibit the DNA binding and transcriptional activity of Runx2 through phosphorylation of the protein. Mice deficient in GSK-3 β displayed an increase in the rate of bone formation due to an enhanced transcription of Runx2 [74]. Cleidocranial dysplasia, a consequence of insufficiency in the transcription of Runx2, was successfully rescued or prevented in GSK-3 β –insufficient mice by oral administration of lithium chloride [74]. GSK-3 β inhibition can also abrogate nuclear factor- κ B binding to its target gene promoters [75] and may decrease osteoclastogenesis. In a rat osteopenia model, PTH or a GSK-3 inhibitor administered once daily decreased markers of adipogenesis, and increased markers of osteogenesis, chondrogenesis, and bone formation [20]. Although GSK-3 β suppression may provide a therapeutic approach to treat bone catabolic disorders and osteoporosis, the enzyme's broad involvement in several cellular processes would limit its potential.

Conclusions

The Wnt pathway is a key regulator of skeletogenesis and bone cell differentiation. Upregulation of Wnt ligands and downregulation of Wnt inhibitors stimulates bone formation, but its effects on cell differentiation are not limited to the bone. Therefore, the challenge is to develop therapeutic agents that activate Wnt signaling only in the skeleton. Currently, inhibitors of sclerostin and Dkk-1 seem promising for this purpose. Further investigations regarding the complex interactions of Wnt signaling proteins in the maturation and activity of bone cells will provide additional understanding of this complex pathway and allow for a more informed approach to regulate the Wnt pathway in the treatment of bone diseases.

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Figure 1.

Wnt signaling. In the absence of a Wnt signal, β -catenin is targeted for degradation by a protein complex that contains axin and glycogen synthase kinase 3 (GSK3). Binding of Wnt ligand to the frizzled and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) receptors leads to stabilization of β -catenin and its translocation to the nucleus to activate transcription of target genes. Noncanonical/Wnt signals are transduced through frizzled family receptors and Ror2/RYK coreceptors to Dsh-dependent (Rho family guanosine-5'-triphosphate [GTP]ases and Jun N-terminal kinase [JNK]) or Ca²⁺-dependent signaling cascades. Wnt/ β -catenin signaling has a major role in osteoblast proliferation and differentiation during embryonic development and after birth. Wnt pathway in osteoblasts is also controlled by osterix, an osteoblast-specific transcription factor. Mechanical loading and unloading of bone is perceived by the osteocyte, triggering release or inhibition of

biochemical signals including sclerostin. Negative regulators of Wnt signaling including secreted frizzled-related proteins (sFRP), Dickkopf-1 (Dkk1), Wnt-inhibitory factor-1 (WIF), Cerberus (Cer), Wnt modulator of surface ectoderm (Wise), and bone-specific sclerostin all act extracellularly. The binding of the Wnt antagonist Dkk1 results in the formation of Dkk1-LRP5/6-Kremen complex and endocytosis and degradation of LRP5/6, making it unavailable to interact with Wnt and frizzled. The Wnt inhibitor Wise also binds to the Wnt coreceptors LRP. In contrast to broadly expressed Dkk1, expression of sclerostin is restricted to osteocytes. Sclerostin binds to LRP5 and prevents the formation of a Wnt-LRP-frizzled complex. sFRPs may bind Wnts or frizzleds. sFRP also has been shown to bind to receptor activator for nuclear factor- κ B (RANKL) and prevent osteoclastogenesis. APC—adenomatous polyposis coli; NFAT—nuclear factor of activated T cells; NLK—nemo-like kinase; OPG—osteoprotegerin; PKC—protein kinase C; TCF—T-cell factors.