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Wnt Signaling as a Therapeutic Target for Bone Diseases

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Abstract

Background—There is a need to develop new bone anabolic agents because current bone regeneration regimens have limitations. The Wingless-type MMTV integration site (Wnt) pathway has emerged as a crucial regulator of bone formation and regeneration.

Objective—Toreview the molecular basis for Wnt pathway modulation and discuss potential strategies that target it and improve bone mass.

Methods—Data in peer-reviewed reports and meeting abstracts are discussed.

Results/Conclusions—Neutralizing inhibitors of Wnt signaling have emerged as promising and feasible strategies. Small molecule inhibitors of GSK3β increase bone mass, lower adiposity and reduce fracture risk. Neutralizing antibodies to Dickkopf 1, secreted Frizzled-related protein 1 and sclerostin produce similar outcomes in animal models. These drugs are exciting breakthroughs, but they are not without risks. The challenges include tissue-specific targeting and consequently, long-term safety.

Keywords

Osteoporosis; GSK-3; Dkk1; Sclerostin; Sfrp1; SOST

1. Introduction

The clinical need to develop new anabolic agents is high because current bone regeneration options have limitations, anti-resorptive therapies have unknown long-term health consequences, and the demand for therapies is rising as the population ages. It is estimated that more than 200 million people worldwide (1 in 3 women and 1 in 12 men over the age of 50 years) suffer from osteoporosis, with 3 to 4 times as many at risk because of low bone mass [1,2]. Osteoporosis-related fractures often begin a downward spiral in health and independence for the elderly. The current standard of care is an anti-resorptive treatment such as bisphosphonates, hormone replacement, and selective estrogen receptor modulators. Although these treatments are effective, controversy exists about their long-term effects on general skeletal health, particularly with regards to repair of microfractures, as well as on breast cancer risk and heart health [3,4]. Teriparatide (Forteo™, parathyroid hormone (PTH) residues 1–34) is the only anabolic drug available at this time. The regulations on Teriparatide use vary by country (e.g., two years of maximum use in the USA) and it is usually only prescribed to patients with established osteoporosis, whom have already

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suffered a fracture, or in whom bisphosphonates are ineffective or contraindicated. Teriparatide is administered daily via subcutaneous injection and stimulates new bone formation, but has several side effects, including hypercalcemia and hypercalciuria. Moreover, high doses of PTH can cause osteosarcomas in rats [5].

New bone anabolic agents could also be used to treat non-union fractures. Approximately one-third of people fracture a bone within their lifetime and 5–10% of those fractures fail to heal normally and result in a non-union, leading to staggering economic consequences [2,6,7]. Bone morphogenic proteins (BMP) 2 and 7 are used respectively to fuse vertebrae and to treat non-union long bone fractures that occur after trauma and for which an allograft is not suitable. BMPs have short half-lives and cannot be delivered systemically, thus, they are not available to treat osteoporosis. New anabolic agents that enhance bone regeneration locally and improve bone density systemically would reduce fracture risk and improve the quality of life for millions of people.

The search for new anabolic therapies is focused on biological pathways that stimulate osteoblast lineage cells to differentiate from a progenitor cell, to proliferate faster and/or to produce more organic matrix proteins. Osteoblasts are derived from mesenchymal progenitor cells in the bone marrow or pericytes. Their maturation process includes consecutive stages of proliferation, matrix production and matrix mineralization (Figure 1). Osteoblasts can ultimately become osteocytes, which are mechanosensory cells within the mineralized matrix, or lining cells that protect the bone surface and form the canopy of a basic multicellular unit (BMU) wherein remodeling occurs [8]. Several signaling pathways, including BMPs, PTH, endothelin, fibroblast growth factors, steroidal hormones, insulin-like growth factors, and prostaglandin agonists, have emerged as positive factors regulating osteoblast maturation, but the Wnt (Wingless-type MMTV integration site) family of ligands has arguably generated the most interest and excitement in recent years. In this report, we review data that highlight the importance Wnt pathways in osteoblast maturation and bone formation. We also discuss the molecular basis, promise and potential limitations of strategies to augment Wnt-dependent bone formation.

2. Wnt Signaling Pathways

Wnts are cysteine-rich, secreted glycoproteins that activate cell surface receptor-mediated signaling pathways to control of gene expression, cell fate determination, proliferation, and migration. Wnts are required for embryogenesis, organogenesis, postnatal development, and regeneration of adult tissues including lymphocytes, skin, colon, hair follicles, and bone [9]. In humans and mice, there are 19 Wnts that bind to receptor complexes containing one of 10 Frizzled (Fzd) receptors and sometimes one of two low-density lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors, theoretically creating at least 380 potential receptor combinations. Complicating the issue further, Wnts also bind to receptor-like tyrosine kinase (Ryk) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) receptors. Wnt ligands can thus stimulate multiple signaling cascades, including the “canonical” β-catenin pathway, the planar cell polarity (PCP) pathway, calcium (Ca^{2+}), protein kinase A, Src, and c-Jun N-terminal kinase (JNK) pathways [10] (Figure 2A). Wnts have historically been classified as “canonical” (e.g., Wnt 1, 3a, 8, 10b) on the basis of their ability to inhibit glycogen synthase kinase (GSK)3 phosphorylation of β-catenin and its subsequent degradation or as “non-canonical” (e.g., Wnt 4, 5a, 11) if they do not affect β-catenin levels. These simplistic classifications are now being challenged by studies demonstrating that some Wnts (including Wnt1, 5a and 11) stimulate several of the above-mentioned signaling pathways in context-dependent manners that can depend on which receptor is available and active [10,11]. Filling our gap in understanding of how Wnts bind to specific receptors will be an

important step in understanding tissue-specific responses and will be crucial for the identification new targets for intervention.

3. Wnt Signaling Stimulates Bone Formation

The role of Wnt signaling in bone formation gained significant recognition in 2001 when Gong and colleagues reported loss-of-function mutations in the LRP5 co-receptor cause the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (OPPG), which is characterized by low bone mass, ocular defects, and a predisposition to fractures [12]. These findings were recapitulated in germline *Lrp5* knockout mice, which developed a low bone mass phenotype similar to patients with OPPG due to decreased osteoblast proliferation [13]. *Lrp6* hypomorphic mice are also osteopenic and mice lacking *Lrp5* and one copy of *Lrp6* have additive reductions in bone mass [14]. Other groups identified a mutation in LRP5 at amino acid G171 in individuals with high bone mass and remarkable skeletal strength [15,16]. Transgenic mice overexpressing the G171V mutation in preosteoblasts using the Col1a1(3.6) promoter recapitulated the high bone mass phenotype and had significantly stronger bones than wildtype animals [17,18]. It was recently demonstrated that expression of the gain-of-function G171V mutation in more mature osteoblasts using the Col1a1(2.3) promoter did not affect bone density [19]. The latter study also demonstrated that *Lrp5*-deficiency in the duodenum, rather than in osteoblasts, decreased osteoblast proliferation indirectly via gut-derived serotonin binding its osteoblast receptor, 5-hydroxytryptamine (Htr1b), and activating the cAMP responsive element binding protein (CREB) transcription factor [19]. These data suggest that *Lrp5* deficiency causes bone loss in a Wnt-independent manner, but does not rule out a crucial role for Wnt signaling in osteoblasts. Lrp6 or another co-receptor might be more crucial for Wnt signaling in osteoblast lineage cells, particularly in immature osteoblasts and in progenitor cells.

Since the LRP5 discoveries earlier this decade, many studies have documented a role for Wnt pathway components in bone formation, regeneration and repair. Table 1 summarizes the bone phenotypes associated with genetic altered expression of Wnt signaling pathway components. The overarching conclusion derived from these studies is that activation of the Wnt pathways facilitates osteoblast specification from mesenchymal progenitors and enhances bone mass and strength, while suppression causes bone loss. The interesting and perplexing caveat is that several mechanisms are responsible for altered bone mass. For example, LRP5 appears to regulate osteoblast numbers and proliferation [13], perhaps in a Wnt-independent fashion [19], while β-catenin regulates osteoprotegerin (OPG) production in mature osteoblasts and affects bone resorption without affecting osteoblast numbers [20]. In progenitor cells, β-catenin activation facilitates osteoblast differentiation at the expense of chondrocyte development [21–24], while Wnt5a and Wnt10b increase bone volume by suppressing PPARγ2 activity to block adipogenesis and promote osteoblast lineage maturation [25–27]. These genetic studies, as well as ones showing that Wnt pathway activation enhances osteoblast and osteocyte survival in vitro [18,23,28] and that Wnt pathways are active in bone regeneration sites (reviewed in [29]), strongly support crucial roles for Wnts pathways in bone mass accrual. However, recent data suggest that more needs to be done to understand how cells at different stages of maturity respond to Wnts.

4. Therapeutic Strategies Targeting the Wnt Pathways

Given the plethora of data showing that Wnt pathway activation promotes bone formation, it has become an attractive target in the search for therapies that increase systemic (e.g., osteoporosis) and focal (e.g., critical size defects and non-union fractures) bone formation. Two basic therapeutic strategies for enhancing bone regeneration through the Wnt signaling pathways exist: adding agonists or blocking naturally occurring antagonists. Recombinants

Wnts are difficult and expensive to purify because they are glycoproteins and only palmitoylated forms are active [30]; thus, the former approach is cost-prohibitive. The alternative strategy of inhibiting natural antagonists is a more feasible approach. This is currently being explored by neutralizing secreted inhibitors of Wnt pathways with antibodies or by inactivating intracellular enzymes (e.g., GSK3β) that reduce β-catenin activity with small molecules (Figure 2).

4.1. GSK3β Inhibitors

GSK3β is a crucial regulator of the Wnt-β-catenin pathway. It is a serine-threonine kinase that phosphorylates the amino-terminus of β-catenin, as well as adenomatous polyposis coli (APC) and Axin, members of β-catenin destruction complex, in the absence of a Wnt signal to initiate the degradation of β-catenin (Figure 2B) (reviewed in [9]). Because GSK3β is a kinase, modulating its activity with small molecules is a promising strategy for increasing bone mass. The rationale is blocking the ability of GSK3β to phosphorylate β-catenin would stabilize β-catenin and allow it to translocate to the nucleus where it can interact with lymphoid enhancer-binding factor (Lef)/T-cell factor 7 (Tcf7) transcription factors and regulate the expression of genes involved in bone regeneration. As predicted, several GSK inhibitors increase bone density.

Lithium is a well-characterized GSK3β inhibitor that stimulates osteoblast differentiation in vitro and bone regeneration in vivo. Clement-LaCroix and colleagues [31] administered lithium chloride (LiCl) for four weeks to *Lrp5* knockout mice, which have significantly reduced bone mass [13]. LiCl restored trabecular bone mass to near wild-type levels in the *Lrp5*-deficient animals. LiCl also increased bone mass in animals with senile osteoporosis and ovariectomy-induced osteoporosis; moreover, it enhanced bone densities in wildtype animals. Increased bone mass was associated with reduced adiposity, suggesting that GSK3β inhibition might be acting on bone marrow-residing progenitor cells. In other studies, LiCl enhanced callus formation and fracture healing when administered to mice four days following trauma [32] and prevented myeloma-induced bone disease [33]. Lithium might also be effective at increasing bone mass in humans. Oral lithium is a pharmacologic agent that has been used for over 50 years to treat bipolar patients [34]. Epidemiological studies indicate decreased risks fracture risks [35], and reduced bone turnover in lithium users [36], although it has not been determined that this is a direct effect on bone cells or Wnt target genes and other epidemiological surveys dispute the protective effect of LiCl on fractures [37].

The orally active, small molecule GSK3α/β dual-inhibitor, 603281-31-8, is 500 times more selective for GSK3β than other kinases, and was also reported to increase bone mass [38–40]. It significantly increased mineral apposition rate, bone mineral density, trabecular area, trabecular thickness, and trabecular number in ovariectimized mice within 60 days [39]. Vertebral strength was also improved [39]. It is of interest, 603281-31-8 reduced adipogenesis in ovariectimized mice and elevated mRNA levels of osteoblast products, including biglycan, type 1 collagen, osteocalcin, alkaline phosphatase, osteonectin, and runt-related transcription factor 2 (Runx2), and increased the OPG: Receptor activator of NF-kB ligand (RANKL) ratio [40]. The authors concluded that the increased bone mass observed with the GSK3α/β inhibitor is probably mediated by an increase in bone formation with a small effect on bone resorption.

The observations that GSK3β inhibition improves bone mass in various rodent models and that lithium reduces fracture risk in humans demonstrate that targeting this enzyme is a promising anabolic therapy, at least for short-term use. While the long-standing use of lithium to treat bipolar disorders suggests that is relatively safe, further studies are necessary to confirm the protective effect of lithium on fractures. It is important to note that GSK3 is

not a specialized repressor of β-catenin as it participates in many other intracellular signaling pathways, some of which can be oncogenic [41]. Cancer risk is not elevated in humans treated with lithium; however, 80% of mice expressing constitutively active β-catenin developed benign tumors in the ribs (osteomata) [20]. Perhaps a greater concern for skeletal health is that long-term lithium treatment is associated with increased PTH secretion and hypercalcemia (discussed further in [37]). For GSK3β inhibition to be an efficacious long-term anabolic therapy for bone regeneration in osteopenic or osteoporosis patients, ideal strategies would enable GSK3β inhibition to occur exclusively in osteoblast lineage cells. Finally, it is interesting that in addition to being a negative regulator of β-catenin, GSK3β is a positive regulator of Wnt signaling when it is at the membrane where it phosphorylates Lrp6 [42]. Thus GSK3β inhibitors probably promote signaling downstream of β-catenin but might make the cells less responsive to autocrine or paracrine Wnt signals emanating from cell surface receptors.

4.2. Secreted Inhibitors of Wnt Pathways

An alternative approach to targeting an intracellular component of anabolic Wnt pathways is to take aim at an extracellular antagonist(s). With more than 20 therapeutic monoclonal antibodies now used clinically to block a variety of signaling pathways and treat various conditions including cancer, inflammatory disease, macular degeneration and transplant rejection, it is highly feasible to augment Wnt signals by neutralizing natural suppressors with immunotherapy. Secreted Wnt inhibitors utilize two general mechanisms to antagonize Wnt signaling (Figures 2B and 2C). Dickkopf (Dkk)1, sclerostin, and Wnt modulator in surface ectoderm (Wise) bind to the Lrp5/6 co-receptors and competitively inhibit Wnts from associating with Lrp5/6. Conversely, secreted Frizzled-related proteins (Sfrps), Cerberus, and Wnt inhibitory factor-1 (Wif-1) interact directly with Wnts and/or Fzd receptors to hinder functional interactions between the ligand and receptor (reviewed in [9]).

4.2.1. Dickkopf (Dkk) 1—Dkk1 suppresses Wnt signaling by forming a ternary complex with Lrp5/6 and Kremen (Krm)1/2 (Figure 2C) [43, 44]. Disruption of the interaction between Lrp5/6 and Dkk1 prevents internalization and cell surface depletion of the putative Wnt co-receptors, Lrp5/6. Missense mutations in Lrp5 that cause high bone phenotypes and analogous changes in Lrp6 prevent Dkk1 from associating with the receptors [45, 46]. Molecular genetic studies in murine models support the negative role of the Dkk1/Krm complex in bone formation. Transgenic mice expressing Dkk1 from a type 1 collagen promoter had fewer osteoblasts and severe osteopenia [47], whereas deletion of a single *Dkk1* allele increased bone mass without affecting bone resorption measures [48]. Studies with a hypomorphic *Dkk1* mouse model demonstrated that just a 25% reduction in Dkk1 levels is sufficient to increase trabecular and cortical bone mass [49]. Deletion of both *Krm1* and *Krm2* also increased bone mass without significant changes in bone resorption markers [50]. Recently, the homeodomain transcription factor muscle segment homeodomain homeobox homolog 2 (Msx2) was shown to inhibit Dkk1 expression and transgenic overexpression of Msx2 from a broadly expressed promoter increased bone volume through enhanced canonical Wnt signaling [51]. Finally, suppression of Dkk1 by RNA interference alleviated osteoporosis caused by glucocorticoids and estrogen-deficiency [52, 53]. Together, these data strongly support the hypothesis that inhibition of the Dkk1/Krm complex is a promising strategy for promoting bone formation.

Several groups have tested Dkk1-neutralizing antibodies in various animal models and observed promising effects on bone density. Diarra and colleagues found that anti-Dkk1 reversed bone destruction in a tumor necrosis factor-induced rheumatoid arthritis model [54] by dose-dependently increasing bone formation rates, osteoblast numbers and OPG levels, while reducing osteoclast numbers. Interestingly, an increase in osteophytes (an

osteoarthritis characteristic) was also noted. Meanwhile, Yaccoby *et al* tested humanized Dkk1-neutralizing antibodies in a SCID-rab mouse model of multiple myeloma because serum DKK1 levels are elevated in myeloma patients with osteolytic disease [55,56]. In this model, anti-Dkk1 reduced the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts, increased the numbers of osteocalcin-positive osteoblasts, and reduced the number of myeloma cells on the subcutaneously implanted tumor-bearing bones. The Dkk1-neutralizing antibody also increased the bone mineral density of mouse femurs that did not contain tumors. The authors concluded that inhibiting Dkk1 promotes bone formation, reduces osteolysis, and suppresses multiple myeloma expansion [55]. These conclusions were verified by testing anti-Dkk1 in the 5T2MM murine model of multiple myeloma [57]. In a recent meeting presentation, Glantschnig and colleagues showed that subcutaneous administration of humanized anti-Dkk1 monoclonal antibodies dose-dependently increased bone mineral density within four weeks in six week-old mice and augmented bone mass within eight weeks in ovariectomized mice [58].

Although development of skeletal anabolic agents that inhibit Dkk1 is in the early stages, preliminary studies in mice suggest that Dkk1 is an attractive therapeutic target for inducing bone formation. To date, most drug development efforts have focused on creating Dkk1-neutralizing monoclonal antibodies. However, small molecules that disrupt the Dkk1-Lrp5/6 or Dkk1-Krm1/2 interaction offer another possibility given the high bone mass phenotypes observed in humans with LRP5 mutations that prevent it from associating with Dkk1. The expression of Dkk1 in multiple tissues might eventually limit the efficacy of Dkk1-targeted therapies; however, the observation that that just a 25% reduction in *Dkk1* levels is sufficient to increase bone mass suggest that partial inhibition might be sufficient.

4.2.2. Sclerostin—Sclerostin is the product of the *SOST* gene. Loss-of-function mutations in *SOST* cause autosomal recessive sclerosteosis, which is characterized by progressive bone thickening and a hyperostotic skeleton [59]. In addition, a homozygous 52 kb noncoding deletion of a *SOST* enhancer element that drives sclerostin expression in the skeleton was found in patients with Van Buchem disease another autosomal recessive high bone mass disease [60,61]. *SOST* knockout mice have increased bone mineral density, bone volume, bone formation and bone strength [62], whereas transgenic mice overexpressing *SOST* are osteopenic [63]. Sclerostin is a secreted protein that seems to be primarily expressed in bone tissue, predominantly by osteocytes [63,64] also by premature osteoclasts [65]. Mechanical loading and intermittent PTH treatment suppress sclerostin expression in osteocytes [66,67], while RANKL and Macrophage colony stimulating factor 1 (CSF1) reduce its production by preosteoclasts [65]. Like Dkk1, sclerostin binds to Lrp5/6 and antagonizes canonical Wnt signaling [68–70]; however, it binds to different region of LRP5/6 than DKK1 and it does not mediate receptor internalization [69]. Recent reports suggest that it might also signal through Wnt–independent pathways [71,72]. Because inactivating mutations in *SOST* cause high bone mass diseases in humans and animal models and sclerostin expression is seemingly restricted to bone cells, inhibiting sclerostin has emerged an attractive pursuit that is currently progressing through preclinical testing.

A sclerostin-neutralizing monoclonal antibody was developed and evaluated in several bone loss models. In a rodent model of postmenopausal osteoporosis, six month-old female rats were ovariectomized, aged for one year to allow for bone loss, and then treated with anti-sclerostin for five weeks. Anti-sclerostin antibodies reversed the estrogen-deficiency-induced bone loss by increasing bone formation rates without affecting bone resorption parameters and improving bone strength to levels greater than those found in non-ovariectomized rats [73]. The same group reported that anti-sclerostin increased bone formation, bone mineral density and bone strength in cynomologus monkeys after just two months [74]. Finally, in a single injection study, post-menopausal women who received a

subcutaneous dose of anti-sclerostin had increased bone formation markers for at least three weeks following treatment. The single dose of anti-sclerostin was purportedly well tolerated in these women [75]. Taken together, these results indicated that sclerostin inhibition increases bone formation and suggest that anti-sclerostin represents a promising therapy for the anabolic treatment of diseases characterized by bone loss such as osteoporosis.

4.2.3. Sfrp1—Sfrp1 antagonizes canonical Wnt signaling either by interacting with Wnts to prevent them from associating with Fzd receptors or by binding directly to Fzd proteins to form a nonfunctional complex [76]. Despite a broad tissue expression profile, several lines of evidence suggest that inhibition of Sfrp1 stimulates canonical Wnt signaling and promotes bone accrual. *Sfrp1* deficient mice have increased trabecular bone mineral density, volume, and mineral apposition, but no changes in cortical bone density [77]. *Sfrp1* depletion also prevented age-associated bone loss and reduced body fat percentages [77]. Meanwhile, administration of recombinant human SFRP1 decreased proximal femur bone density and trabecular bone volume in rats [78]. Overexpression of SFRP1 in immortalized human osteoblasts suppressed canonical Wnt signaling by 70% and accelerated apoptosis, implicating Sfrp1 as a negative regulator of osteoblast and osteocyte survival [78].

Development and testing of Sfrp1 inhibitors is still in the early stages. Bodine and colleagues screened a large panel of compounds using a cell based reporter gene assay and identified several potential Sfrp1 antagonists [79]. Specifically, a diphenylsulfone sulfonamide was found to prevent Sfrp1-mediated apoptosis in preosteocytes in vitro and stimulate bone formation ex vivo [79]. In other studies, a commercially available Sfrp1 polyclonal antibody suppressed *Porphyromonas gingivalis*-induced periodontal bone loss, reduced osteoclastogenesis, and decreased inflammatory cell infiltration [80]. To our knowledge, the effect of Sfrp1 antibodies or inhibitors on *in vivo* bone parameters has yet to be reported.

For potential therapeutic targeting of Sfrp1 as a bone anabolic agent to advance, the molecular actions of Sfrp1 must be more clearly deciphered. Reports have indicated that some Sfrps interact with each other and may quench one another's activity thus promoting Wnt signaling. Furthermore, in the absence of Wnt ligands, it has been proposed that the interaction between Sfrps and Fzd might be sufficient to activate signal transduction [81]. Given the potential biphasic nature of Sfrps, further basic research is necessary before inhibition of Sfrp1 can emerge as a safe, efficacious bone anabolic agent.

5. Conclusion

Modulation of Wnt signaling pathways has emerged as a promising and feasible strategy to increase bone density. Small molecule inhibitors of the intracellular Wnt-β-catenin pathway regulator GSK3β increase bone mass, lower adiposity and reduce fracture risk. Neutralizing antibodies to secreted inhibitors of Wnt signaling such as Dkk1, Sfrp1 and sclerostin stimulate bone formation in animal models. Although these novel therapies offer much promise, systemic stimulation of Wnt pathways to enhance bone mass has potential risks and the long-term safety of these therapies must be determined. Tissue-specific targeting of new bone anabolic drugs is an important challenge that must be overcome to ensure safe and efficacious treatment of individuals with osteoporosis and/or fractures.

6. Expert Opinion

Since the seminal discoveries earlier this decade that LRP5 controls bone density and strength [12,15,16], other Wnt signaling pathway components have been intensely examined (Table 1). Molecular and genetic studies have identified multiple mechanisms for bone mass

regulation (i.e., osteoblast number, osteoclast maturation and bone resorption, and progenitor differentiation) by Wnt pathways. Several reports demonstrated that Lrp5 regulates bone mass by affecting osteoblast numbers; however, usage of several different conditional promoters indicate that this occurs via Wnt-dependent pathways in progenitor cells and via Wnt-independent endocrine pathways (e.g., gut-derived serotonin) in committed osteoblasts [19]. The affects of Wnts on progenitor cell specification to the osteoblast lineage are convincing. Adipogenesis is increased by the absence of Wnt5a or Wnt10b or by the presence of LiCl or Sfrp1, whereas β-catenin is a crucial factor for specifying osteoblastogenesis over chondrogenesis. However, β-catenin seems to have a different role in mature osteoblasts where it has no effect on osteoblast number but stimulates OPG production to block bone resorption and thereby increase overall bone density. Hypomorphic deletion of *Lrp6* also increases bone resorption by augmenting RANKL expression. In sum, the data suggest that Wnts and the Lrp5/6 co-receptors affect osteoblast differentiation from progenitor cells and the coupling of bone formation with bone resorption.

The disparate mechanisms might be explained by several non-mutually exclusive factors. The first is receptor expression on osteoblastic cells. We know little about how the expression of Wnt receptors (e.g., Lrp5/6, Fzd1–10, Ror2, Ryk) fluctuates on the cell surface during the differentiation of a progenitor cell to a committed osteoblast or during the maturation of a committed osteoblast towards a lining cell or osteocyte. Perhaps Lrp6 or specific Fzds are sufficient to transmit Wnt survival signals and/or regulate gene expression in mature osteoblastic cells. Second, Wnts do not stimulate linear signaling cascades with specialized components. Wnts and their receptors are initiators that mobilize intracellular signaling molecules (e.g., β-catenin, GSK3β), which subsequently amplify and transmit the signal to the nucleus where gene expression programs are controlled. These intracellular molecules are not exclusive to the Wnt pathway and in fact regulate signals from other cell surface molecules, including receptor tyrosine kinases, G-protein coupled receptors, and E-cadherin. Thus, the range of outcomes from Wnt signals will contextually depend on receptor availability, cell maturation status, and other stimuli present in the environment.

Wnt pathways have been studied for nearly 30 years. The work begun with the *Drosophila* protein Wingless was quickly linked to human cancers, tissue development and regeneration, and more recently to tissue degeneration [82–84]. Overall, the vast efforts must be considered successful as our understanding of the cellular, biochemical and molecular events were translated into several therapeutic strategies for treating diseases, including systemic bone loss and critical-sized defects. As discussed in this review, modulating Wnt or Lrp5/6 signaling antagonists (e.g., GSK3β, Dkk1, sclerostin, Sfrp1) seems to be the most feasible approach for treating low bone mass or stimulating bone regeneration. The immense literature on Wnt signaling suggests that optimism should be cautiously tempered for a number of reasons. First and foremost is that unregulated activation of Wnt/β-catenin pathways is carcinogenic [84]. So far, the only mouse model that has developed skeletal tumors is one in which β-catenin is overexpressed from the collagen 1a1 (2.3) promoter throughout development [20]. These tumors are benign (osteomata), but occur in 80% of the animals. It has not been determined if these mice are more sensitive to classic tumor initiators, like loss of p53 or pRb. There is no indication of increased tumor risks in patients carrying activating LRP5 or inactivating *SOST* mutations, but these rare inherited diseases affect small cohorts.

In addition to cancer, systemic stimulation of Wnt pathways to enhance bone mass has other concerns. It was recently discovered that germline inactivating mutations in a gene encoding for a Wnt pathway inhibitor, Wilms Tumor on the X (WTX), cause X-linked sclerosing bone dysplasia [85]. Thus, if used during development, molecules that activate Wnt

signaling may cause developmental defects in a variety of tissues, including sclerosis or craniosynostosis in skeletal tissues. In adults, vascular calcification is a risk because Wnts are overexpressed and Wnt/Lrp signaling pathways are activated in calcified vasculature [86,87]. A third risk is hyperparathyroidism and hypercalcemia, which develops in 25% of patients that are treated with LiCl [37]. Fourth, prolonged activation of Wnt-β-catenin signaling pathways in skeletal tissues produces osteoarthritis (OA) symptoms. SFRP3 (FRZB) variants with diminished ability to antagonize Wnts are common in OA patients [88]; whereas DKK1 serum levels are associated with reduced risk of joint space narrowing [89]. Dkk1-neutralizing antibodies produced osteophytes in mouse joints after an inflammatory stimulus [54] and recently it was demonstrated that constitutive overexpression of active β-catenin in mature chondrocytes caused progressive OA and osteophytes [90].

Much work remains to be done in clinical and basic research laboratories to optimize current biologics and develop tissue-specific treatments. Broad, genome-scale functional screens will likely uncover previously unappreciated modifiers of Wnt signaling and might identify new therapeutic targets and their importance to bone biology must be empirically determined. Meanwhile, the receptor specificity of Wnt ligands, receptor expression patterns, and the ligand specificity of secreted antagonists must be defined. An important question to be answered is what is the most efficient cellular target of compounds that target the Wnt pathways. If it is an osteoblast progenitor cell, the effectiveness of Wnt anabolics might be limited in older patients that have fewer bone marrow mesenchymal stem cells than younger people. Proper dosing regimens and delivery vehicles must be determined identified to prevent unwanted side effects. Of the therapies mentioned in this review, the sclerostin antibodies ostensibly hold the most promise because sclerostin seems to predominantly secreted by cells in bone; thus, targeting it would theoretically have few side effects. It is likely that systemic treatments for osteoporosis that revolve around activating the Wnt pathway will be used for short-periods of time, perhaps alternating with the anti-resorptive agents, as is the current practice with the only approved anabolic, PTH.

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Abbreviations

APC	Adenomatous polyposis coli
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
CSF1	Macrophage colony stimulating factor
Dkk	Dickkopf
Fzd	Frizzled
GSK	Glycogen synthase kinase
JNK	c-Jun N-terminal kinase
Krm	Kremen
LiCl	Lithium Chloride
Lrp	Low-density lipoprotein receptor-related protein

OA	Osteoarthritis
OPG	Osteoprotegerin
OPPG	Osteoporosis-pseudoglioma syndrome
PCP	Planar cell polarity
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
RANKL	Receptor activator of NF-kappaB ligand
SCID-rab	Severe combined immunodeficient-rabbit
Scl	Sclerostin
SFRP	Secreted Frizzled-related protein
Wif	Wnt inhibitory factor
Wise	Wnt modulator in surface ectoderm
Wnt	Wingless-type MMTV integration site

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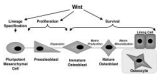

Figure 1. Wnts affect multiple stages of osteoblast-linage maturation

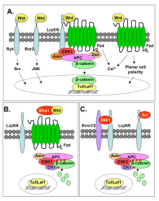

Figure 2. Activation and inhibition of Wnt signaling pathways

A) The “canonical pathway” is stimulated when Wnts bind to Frizzled (Fzd) receptors and low-density lipoprotein receptor-related protein (Lrp)5/6 co-receptors (center). This activates Disheveled (Dsh), which inhibits a cytoplasmic complex composed of glycogen synthase kinase (GSK)3β, Axin, and adenomatous polyposis coli (APC). Cytoplasmic β-catenin levels rise and some β-catenin translocates to the nucleus where it associates with T-cell factor (Tcf)/lymphoid enhancer-binding factor (Lef) transcription factors to regulate gene expression. During non-canonical Wnt signaling (right side of the figure), Wnts bind a Fzd receptor, but the downstream signaling events do not involve GSK3β or β-catenin. Two non-canonical Wnt signaling cascades that have been identified are: 1) Wnt/calcium signaling increases intracellular Ca^{2+} levels and activates protein kinase C and calcium/calmodulin-dependent kinase; and 2) The Wnt/planar cell polarity pathway that signals through Rho/Rac GTPases and c-Jun N-terminal kinase (JNK) to modulate cytoskeletal elements and gene expression. Wnts also bind to receptor tyrosine kinases receptor-like tyrosine (Ryk) and receptor tyrosine kinase-like orphan receptor 2 (Ror2) to activate oncogene (Src) and JNK signaling, respectively (left side of figure). **B**) Secreted frizzled-related proteins (Sfrps) antagonize canonical Wnt signaling by binding the ligands and preventing their association with Fzd receptors. In the absence of Wnt signaling, GSK3β phosphorylates (asterisks) β-catenin, which marks it for ubiquitination and degradation by the proteosome. **C**) Dickkoph (Dkk1) suppresses Wnt signaling by forming a ternary complex with Lrp5/6 and Kremen (Krm)1/2. Sclerostin (Scl) also binds to Lrp5/6, but not Krm1/2, to antagonize canonical Wnt signaling.

Table 1

Summary of Bone Phenotypes in Genetic Models of Altered Wnt Signaling

Gene	Role in Wnt pathway(s)	Bone Phenotype(s)*	References
APC	Downregulates of β-catenin signaling	CKO: Increased BMD	[91,92]
Axin2	Downregulates of β-catenin signaling	CKO: Craniosynostosis; Increased BMD	[93,94]
β-Catenin	Interacts with Lef1/Tcfs to regulate gene expression	CKO: Ectopic formation of chondrocytes	[21–23]
		Tg: Increased ossification, suppression of chondrocytes	[20,24]
Dkk1	Inhibits β-catenin signaling	Het: High bone mass	[47]
		Tg: Low bone mass	[46]
Dkk2	Inhibits β-catenin signaling	KO: Low bone mass	[95]
		Het: Normal	
Fzd9	Receptor for Wnts	KO: Decreased bone formation; Osteopenic	[96]
GSK3β	Inhibits β-catenin signaling by targeting β-catenin for degradation	Het: Increased bone formation	[97]
		Pharmacological inhibition: Increased BMD	[31]
Krm1/2	Potentiate capacity of Dkks to inhibit β-catenin signaling	CKO: Increases BMD	[49]
Lrp5	Co-receptor for Wnts	KO: Decreased BMD	[13]
		CKO (Osteoblasts): Normal	[19]
		CKO (Duodenum): Low BMD	
		GOF: Increased BMD	[15,16]
Lrp6	Co-receptor for Wnts	CKO: Decreased BMD	[98]
Sfrp1	Secreted inhibitor of β-catenin signaling	KO: Increased BMD	[76]
SOST	Inhibits β-catenin signaling	KO: Increased BMD	[61]
		Tg: Osteopenic	[62]
Tcf7 (Tcf1)	Transcription factor activated through interaction with β-catenin	DKO (Tcf7−/−Lef1−/−): Similar to Wnt3a−/− phenotype	[99]
		KO: Low bone mass, increase in bone resorption	[20]
Wnt3a	Activates β-catenin pathway	Het: No change in BMD	[25]
Wnt5a	Activates β-catenin and noncanonical pathways	Het: Reduced BMD, increased adipogenesis	[25]
Wnt10b	Activates β-catenin	KO: Reduced BMD, increased adipogenesis	[26,27]
		Tg: Increased BMD	

*BMD: bone mineral density; CKO: conditional knockout mouse; Het: heterozygous knockout mouse; KO: global knockout; DKO: double global knockout; Tg: transgenic; GOF: gain of function