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Role of the Wnt signaling pathway in bone and tooth

Masato Tamura[1], Eiji Nemoto[2], Mari M. Sato[1], Aiko Nakashima[1], Hidetoshi Shimauchi[2]

[1]Department of Biochemistry and Molecular Biology, Graduate School of Dental Medicine, Hokkaido University, N13, W7, Sapporo, Japan, [2]Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba, Sendai, Japan

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1. ABSTRACT

Signaling by the Wnt plays a central role in many processes during embryonic development and adult homeostasis. At least 19 types of Wnts, several families of secreted antagonists and multiple receptors have been identified. Two distinct Wnt signaling pathways, the canonical pathway and the noncanonical pathway have been described. Functional studies and experimental analysis of relevant animal models confirmed the effects of Wnt on regulation of developing mineralized tissue formation and adult homeostasis. In osteoblasts, the canonical Wnt pathway modulates differentiation, proliferation and mineralization, while it blocks apoptosis and osteoclastogenesis by increasing osteoprotegerin. Functional crosstalk between Wnt and bone morphogenetic protein signaling during osteoblastic differentiation has been reported. Recently, non-canonical Wnt signaling was shown to play a role in bone formation. The Wnt signaling pathway also plays an important role not only in tooth formation but also in differentiation and proliferation of cementoblasts and odontoblasts in the tooth. This present review provides an overview of progress in elucidating the role of Wnt signaling pathways in bone and tooth and the resulting possibilities for therapeutic potential.

2. INTRODUCTION

The Wnt secreted proteins of molecular weight ~40kd are a family of glycosylated-lipid-modified proteins which are powerful regulators of embryonic development, cell differentiation, proliferation and migration (1, 2). At least 19 Wnts have been identified in human and mouse. Two types of Wnt proteins have been identified, one class of which comprises the beta-catenin-dependent canonical Wnts such as Wnt1, Wnt2, Wnt3, Wnt3a, Wnt7a, Wnt7b, Wnt8a, Wnt8b, and Wnt10a. The other class is the so-called “noncanonical” Wnts such as Wnt4, Wnt5a, Wnt5b, Wnt6 and Wnt11 which act independently of or inhibit beta-catenin signaling (3). However, their specific function remains to be determined. Functional redundancy of some Wnt proteins has been described in double knockout mice (4). Single knockout mice of Wnt2b, Wnt5b, Wnt6, Wnt8b and Wnt16 resulted in no observable phenotype (5).

Canonical Wnt signaling is initiated by binding of the Wnt ligand to receptor molecules of the 7-transmembrane domain-spanning Frizzled (Fz) family (Fz1-10) and lipoprotein receptor-related proteins (LRP) 5 and 6. In turn, non-canonical Wnt ligands interact with the alternative Wnt receptors such as receptor tyrosine kinase

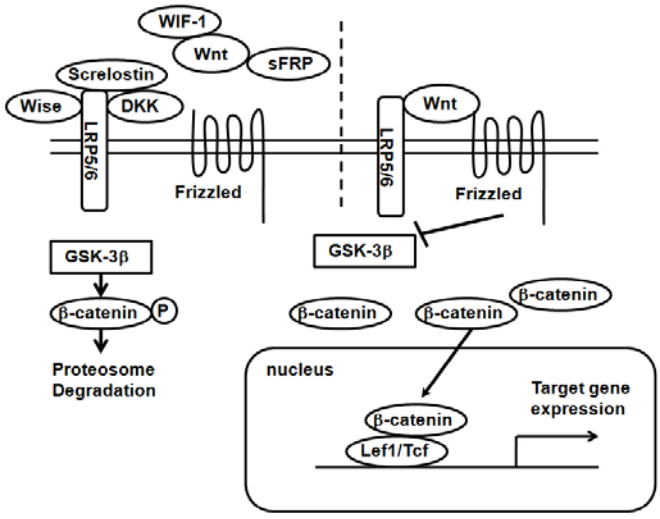

Figure 1. The canonical Wnt signaling pathway. Proteins of the secreted Frizzled-related protein (sFRP) family primarily bind to Wnt proteins, leading to inhibition of the interaction between Wnts and Frizzled (Fz) receptor molecules. Sclerostin (SOST) or Dickkopf (DKK) binds to lipoprotein receptor-related proteins (LRP) 5/6 to inhibit Wnt signaling. Cytosolic accumulation of beta-catenin in response to a canonical Wnt signal is a crucial step in the signaling pathway. Left: in the absence of Wnt ligand, glycogen synthase kinase (GSK)-3beta phosphorylates beta-catenin, inducing rapid degradation of beta-catenin via the ubiquitin/proteasome pathway. Right: Canonical Wnt signaling is initiated by the binding of the Wnt ligand to Fz and LRP5/6. Wnt ligand blocks beta-catenin degradation, allowing transportation to the nucleus, where beta-catenin interacts with the transcription factor Lef1/Tcf and activates canonical Wnt target genes.

Ryk or receptor tyrosine kinase-like orphan receptors (Ror) 2 (6). Several families of secreted Wnt antagonists including secreted frizzled related protein (sFRP), Wnt inhibitory factor-1 (WIF-1), Dickkopf (Dkk), Sclerostin (Sost gene product) and Wise have been identified. Members of the sFRP family and WIF-1 primarily bind to Wnt proteins, thus inhibiting the interaction between Wnts and Fz (7). Sclerostin binds to LRP5/6 to inhibit Wnt signaling (6) (Figure 1). Kremen was originally discovered as a transmembrane protein containing the kringle domain. Both Kremen 1 and its relative Kremen 2 have been identified as high-affinity receptors for Dkk. Dkk binds to LRP5/6 and to Kremen, causing rapid internalization of Kremen-Dkk-LRP complexes and removal of LRP5/6 from the plasma membrane to inhibit canonical Wnt signaling (8). Recently, the secreted protein Wise and its orthologs (Sostdc1, USAG-1, and Ectodin) have been shown to inhibit Wnt signaling by an interaction with LRP6 (9).

The Wnt signaling pathway plays a critical and evolutionarily conserved role in directing cell fates during embryogenesis (2). Postnatally, inappropriate activation of Wnt signaling plays a role in a variety of human cancers (1). The Wnt signaling pathway has been reported to be involved in regulation of bone mass and in bone and tooth formation. In this review, we discuss our current understanding of Wnt signaling and its function in bone and tooth.

3. THE WNT SIGNALING PATHWAY

Canonical Wnt ligands can induce canonical Wnt signaling by forming a tertiary complex with Fz and LRP5/6. According to the model of canonical Wnt action, in cells lacking Wnt signal, glycogen synthase kinase (GSK) -3beta phosphorylates beta-catenin, inducing rapid degradation of beta-catenin via the ubiquitin/proteasome pathway (1). Canonical Wnt signaling causes stabilization of beta-catenin which accumulates in the cytosol and then translocates to the nucleus, where beta-catenin interacts with the transcription factor lymphoid enhancer binding factor 1/T-cell-specific factor (Lef1/Tcf) and transcriptional coactivators (e.g., p300 and cAMP response element-binding protein, p300/CBP) (10) that regulate expression of several canonical Wnt target genes including c-myc and cyclin D1 (11) (Figure 1).

Conversely, noncanonical Wnt signaling is now known to be mediated by at least two mechanisms: i) The Wnt/planar cell polarity (PCP) pathway, a Wnt pathway that signals through the small GTPases, Rho and Rac, to promote changes in the actin cytoskeleton, and ii) The Wnt/Ca^{2+} pathway, a Wnt pathway that promotes intracellular calcium transients to regulate cell movements (12, 13). In the Wnt/Ca^{2+} pathway, the Wnt5a subclass of Wnts triggers intracellular Ca^{2+} release to activate protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (6). Alternatively, Ror2 has been shown to be a receptor for Wnt5a, and binding of Wnt5a to Ror2 can activate c-Jun-N-terminal kinase (JNK) and inhibit canonical Wnt signaling (14). At present, our understanding of noncanonical Wnt signaling lags far behind our knowledge of the canonical Wnt signaling pathway.

4. THE CANONICAL WNT SIGNALING PATHWAY AND BONE

4.1. LRP5

A loss of function mutation in LRP5 was found

to associate with osteoporosis-pseudoglioma syndrome (OPPG), an autosomal recessive disorder (15). A Gly171-to-Val substitution mutation in LRP5 that reduces affinity of LRP5 for Dkk1 is associated with a high-bone-mass phenotype (16). These phenotypes associated with loss of function or substitution mutations of LRP5 indicated that Wnt signaling might be involved in modulating regulation of bone mass and bone formation. Also, the role of LRP5 during skeletogenesis has been investigated using mice. LRP5-/- mice exhibit low bone mass and decreased proliferation and function of osteoblasts (17). Mice over-expressing LRP5G171V in osteoblasts have a phenotype with high bone mass and enhanced strength (18). LRP5-/- mice exhibit increased bone formation in response to intermittent parathyroid hormone (PTH) treatment (19, 20), indicating that LRP5 is not essential for the anabolic bone response to PTH. After *in vivo* loading of the tibia in mice, target gene expression of canonical Wnt signaling are increased (21). There are a further increased in transcriptional response with the LRP5 G171V mice (21), suggesting that LRP5 mediates the response to mechanical loading in bone. Recently, Yadav *et al.* reported that duodenum-derived serotonin inhibits bone formation in an LRP5-dependent manner (22). They mention that serotonin acts on osteoblasts through the serotonin receptor 1B to inhibit their proliferation. LRP5 inhibits expression of tryptophan hydroxylase 1, the rate-limiting biosynthetic enzyme for serotonin in the duodenum. Accordingly, decreasing serotonin blood levels normalizes bone formation and bone mass in LRP5-/- mice, and gut- but not osteoblast-specific LRP5 inactivation decreases bone formation in a beta-catenin-independent manner (22). However, this hypothesis remains to be further investigated.

4. 2. beta-catenin

During embryonic development, beta-catenin is increased in differentiated osteoblasts (23). The role of canonical Wnt signaling during skeletogenesis has been investigated using conditional mutations of beta-catenin. Using Prx1- and Dermo-1Cre mice, beta-catenin was abolished in mesenchymal progenitor cells and this led to early osteoblastic differentiation arrest and increased chondrogenesis, resulting in ectopic cartilage formation (23, 24). Loss of function of beta-catenin in osteoblasts using Col1alpha1-Cre mice led to low bone mass caused by increased numbers of osteoclasts resulting in increased bone resorption (25). These studies indicate enhanced osteoclastogenesis due to down-regulation of osteoprotegerin (OPG) gene expression and up-regulation of the receptor activator of NFkB ligand (RANKL). Also, loss of function of beta-catenin in osteoblasts using osteocalcin-Cre mice led to severe osteopenia with increased osteoclasts (26). These *in vivo* observations indicate that beta-catenin in osteoblasts regulates bone mass via osteoclast formation and function.

4.3. Osteoblasts

In cultured osteoblastic cells, activation of canonical Wnt signaling down-regulates *RANKL* expression (27). We have shown that canonical Wnt signaling induces *OPG* expression in C2C12 cells (28). Deletion and mutation analysis of the murine OPG gene promoter revealed that constitutively active forms of beta-catenin regulate transcription of *OPG* via a promoter region containing two responsive sites (28). These *in vitro* observations also indicate that beta-catenin in osteoblasts regulates bone turnover via osteoclast formation and function.

Several other lines of *in vitro* evidence support the hypothesis that canonical Wnt signaling stimulates the development and differentiation of osteoblasts. Stimulation of canonical Wnt signaling using constitutively active forms of beta-catenin induced the activity of alkaline phosphatase (ALP) and also induced mineralization of osteoblastic cells in culture and participated in BMP-2 mediated signal transduction (29). We also observed ALP activity induced by Wnt3a but not Wnt5a in C2C12 cells (30). BMP-2 induction of ALP partially relies on the Wnt expression cascade (31). However, in our experiments using C2C12 cells, BMP-2 did not induce canonical Wnt expression and Lef1/Tcf-dependent transcriptional activation (30). This therefore ruled out the possibility that BMP-2 regulates canonical Wnt expression leading to direct regulation of beta-catenin, at least in C2C12 cells. Our observations suggest that canonical Wnt signaling can participate in non-BMP-2-dependent osteoblastic differentiation processes (30).

4.4. Crosstalk between canonical Wnt and BMP signaling

BMPs regulate the proliferation, differentiation and apoptosis of various types of cells and organs not only in embryonic development but also in postnatal physiological function (32). The canonical Wnt and BMP signaling pathways have been extensively studied in the regulation of early embryonic development and in the control of cell differentiation and proliferation in adult tissues. During osteogenic differentiation of multipotent cell lines, beta-catenin and BMP-2 synergize to promote osteoblastic differentiation such as induction of ALP activity (33). Several studies have reported that these two signaling pathways link to biological responses via the formation of a nuclear transcription factor complex that acts on target genes. *In vitro* studies on C2C12 cells revealed that a combination of BMP-2 and Wnt3a induced expression of late osteoblastic differentiation marker genes such as matrix extracellular phosphoglycoprotein (MEPE) and matrix metalloproteinase (MMP)-13 (30, 34). BMP-2 enhances beta-catenin-induced transcriptional activation of the *OPG* promoter**,** while the *OPG* gene promoter functionally interacts with beta-catenin/Tcf-1 in cooperation with Smad1/4, and these complexes then cooperate to regulate graded expression of *OPG* (28) (Figure 2). There are several studies reporting that TGF-beta signaling results in the C-Smads, Smad4 and Smad3, directly interacting with beta-catenin/Lef1 in the transcriptional activation of Lef1/Tcf-responsive promoters (35, 36). The synergistic effect of Wnt3a and BMP-2 on gene transcription occurred without altering expression of Runx2, suggesting that canonical Wnt's actions are independent or downstream of this osteoblast-specific

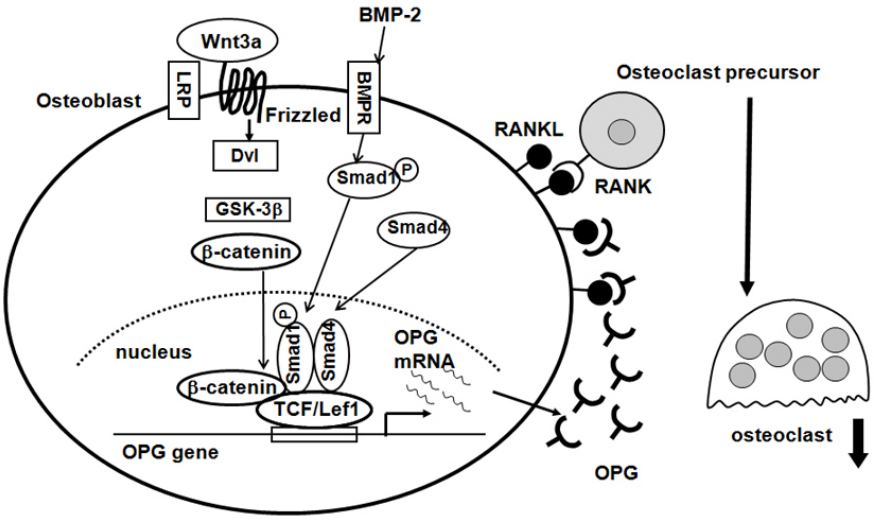

Figure 2. Crosstalk between canonical Wnt and bone morphogenetic protein (BMP)-2 signaling. Canonical Wnt signaling such as via Wnt3a, in combination with BMP-2, regulates *OPG* expression. In response to BMP-2 stimulation via BMPR (BMP receptor), phosphorylated Smad 1 and 4 interact with beta-catenin and TCF/Lef1 on the Wnt/beta-catenin responsive sites of the promoter on target genes such as osteoprotegerin (OPG), then up-regulate beta-catenin-dependent transcriptional activation. Canonical Wnt and BMP-2 signaling in osteoblasts play indirect roles in the regulation of osteoclast formation and differentiation via OPG.

transcription factor. The phenotypic abnormalities observed in Wnt-deficient mice occur in the context of normal expression of Runx2, indicating a role for a Runx2-independent pathway in control of osteoblast differentiation.

4.5. Sost

Several families of secreted Wnt antagonists have been identified. Mutations of *Sost* in humans are associated with sclerosteosis and van Buchem disease. Knock-down or overexpression of *Sost* in mice led to increased or decreased bone mass, respectively (37, 38). These observations indicate that sclerostin modulates bone mass through altered bone formation. Also, sclerostin is expressed in osteocytes. Several reports indicate that sclerostin mediates the bone response to mechanical unloading by antagonizing canonical Wnt signaling (39). As mentioned below, sclerostin monoclonal antibodies increase bone formation in mice.

5. NON- CANONICAL WNT SIGNALING AND BONE

Several studies have indicated that non-canonical Wnt signaling plays a role in bone biology. The non-canonical Wnt signaling pathway activated by Ror2 can modulate bone development and metabolism. Ror2 and Wnt5a are expressed in osteoblasts and are up-regulated during osteoblastic differentiation (40). Overexpression of Ror2 induces mineralized nodule formation and Runx-2 expression in mesenchymal cells in culture. Ror2 requires tyrosine kinase activity to mediate Wnt5a signaling, and Wnt5a directly modulates Ror2 tyrosine kinase activity. Wnt5a also increases phosphorylation of the Ror2 substrate, 14-3-3beta scaffold protein (41). Inhibition of 14-3-3beta with RNA interference–based short hairpin RNA (shRNA) induces osteoblastic differentiation and new bone formation, indicating that this scaffold protein is a negative regulator of osteogenesis (41). Runx2, osterix and ALP expression were down-regulated in Wnt5a-/- cells (42). These observations indicate that non-canonical Wnt signaling mediated by Wnt5a and Ror2 is a positive regulator of osteoblastic differentiation. The non-canonical Wnt signaling pathway also suppresses peroxisome proliferator-activated receptor (PPAR)-gamma function through chromatin inactivation, thus leading to osteoblastic differentiation (43).

Wnt proteins prevent apoptosis of both osteoblast progenitors and differentiated osteoblasts in a beta-catenin-independent manner involving the Src/ERK phosphorylation pathway and the phosphatidylinositol 3-kinase/AKT signaling pathway (44). On the other hand, Wnt3a signals through the Galpha(q/11) subunits of G proteins to activate phosphatidylinositol signaling and protein kinase (PK) Cdelta in ST2 cells. Galpha(q/11)-PKCdelta signaling is required for Wnt3a-induced osteoblastogenesis in these cells, and PKCdelta(-/-) mice exhibit reduced embryonic bone formation (45). Furthermore, Wnt7b, expressed by osteogenic cells *in vivo*, induces osteoblast differentiation *in vitro* via the PKCdelta-mediated pathway; ablation of Wnt7b in skeletal progenitors results in less bone in the mouse embryo. Taken together, it appears that several non-canonical Wnt signaling pathways are positive regulators of bone formation and part of their function cooperated with canonical Wnt signaling.

6. WNT SIGNALING AND BONE DISEASE

The Wnt signaling pathway has also been associated with diverse diseases including cancer, diabetes, osteoporosis and psychiatric disorders. Recently, a

genome-wide association study and a large scale analysis have demonstrated that LRP5 variants are associated with bone mineral density and fracture risk (46, 47).

Rheumatoid arthritis is accompanied by synovial inflammation, proliferation of synoviocytes, and cartilage destruction. Osteoarthritic synovial tissues express significantly reduced levels of Wnt5a and Fz5. Compared with normal synovial fibroblasts, cultured RA fibroblast-like synoviocytes express higher levels of interleukin (IL)-6, IL-8, and IL-15. Transfection of normal fibroblasts with a Wnt5a expression vector reproduced this pattern of cytokine expression and stimulated IL-15 secretion (48), while blockade of Wnt5a/Fz5 signaling inhibited rheumatoid synoviocyte activation (49, 50). Functional polymorphisms within sFRP3 confer susceptibility for hip osteoarthritis in females (51). These genetic aspects implicate the Wnt signaling pathway in the pathogenesis of osteoarthritis.

7. WNT SIGNALING IN THE TOOTH

It has been reported that the Wnt signaling pathway plays essential roles in early tooth development. Several Wnt genes, such as Wnt4, Wnt5a, Wnt6, and Wnt10b are broadly expressed in dental epithelium and mesenchyme (52). During tooth development, nuclear beta-catenin is observed in both the dental epithelium and the underlying mesenchyme, and the canonical Wnt signaling pathway is activated at multiple stages of tooth morphogenesis (53). Consistent with an essential role of Wnt/beta-catenin signaling in early tooth development, the inhibition of canonical Wnt signaling either by deleting *Lef1* or overexpressing DKK1 arrests tooth morphogenesis at the late bud stage (54, 55), and oral epithelium expressing constitutively active beta-catenin results in the formation of multiple teeth, following transplantation to the kidney capsule (56).

It has been reported that Wnt5a, a representative non-canonical Wnt, is strongly expressed in murine (57) and human (58) dental papilla mesenchyme. Overexpression of Wnt5a inhibited the proliferation and migration of human dental papilla cells (hDPC) (58) and stimulation with recombinant Wnt5a increased expression of development-related transcription factors, including Runx2 in hDPC, suggesting that Wnt5a may promote the differentiation of hDPC (59). Furthermore, overexpression of Wnt6, another non-canonical Wnt, also up-regulates expression of osteogenesis-related genes while exerting no significant effect on proliferation of hDPC (60), indicating the important role of Wnt6 in odontogenic differentiation. On the other hand, Yamashiro *et al.* reported that Wnt10a, a canonical Wnt, was specifically associated with the differentiation of odontoblasts and that it showed striking co localization with dentin sialophosphoprotein expression in odontoblasts (61). Wnt1, a canonical Wnt, negatively regulates the odontoblast-like differentiation of dental pulp stem cells by the inhibition of ALP activity and the formation of mineralized nodules in dental pulp stem cells (62), suggesting that odontogenesis is elaboratively regulated through spatial and temporal expression of Wnts during odontogenic differentiation.

Unlike odontogenesis, there is little information available on the role of Wnt signaling in the development and homeostasis of periodontal tissue. Cementoblasts share phenotypical features with osteoblasts in terms of the expression of osteogenic genes such as ALP, Runx-2, and type I collagen. It has been reported that the distribution of cells showing Wnt responsiveness is adjacent to the cementum in the periodontal ligament of continuously erupting incisor teeth, and is coincident with the distribution of proliferating cells (63). Furthermore, it has been reported that Wnt3a, a canonical Wnt, inhibits ALP activity and mRNA expression of ALP, bone sialoprotein and osteocalcin in an immortalized murine cementoblast cell line, OCCM-30 (64). Wnt3a also increases expression of cyclin D1, known as a cell cycle regulator, as well as cell proliferation, suggesting that Wnt3a signaling inhibits cementoblast differentiation and promotes cell proliferation (64). Amelogenins, enamel organic matrix proteins, which are produced by ameloblasts, not only play an important role in amelogenesis but have also been implicated in cementogenesis (65). It has been reported that amelogenins can activate Wnt/beta-catenin signaling in human periodontal ligament cells although the mechanism remains uncertain (66).

The above observations indicate that the Wnt signaling pathway plays critical roles in tooth development and suggest that Wnt activation may have potential uses in regenerative therapy of oral tissues.

8. THERAPEUTIC POTENTIAL AND PROSPECTIVE

The above investigations reveal a Wnt-dependent osteogenic mechanism; it is likely that Wnt-signaling-mimetic drugs may be of value for future therapies to improve bone mass in patients with bone disorders. Recombinant Wnt proteins are not easy to purify because they are fatty-acid-modified glycoproteins and only palmitoylated forms are biologically active (67). Therefore, this approach is difficult and cost-prohibitive. An alternative strategy of inhibiting natural antagonists is currently being explored by inactivating intracellular enzymes that reduce beta-catenin activity with small molecules or by neutralizing secreted inhibitors of Wnt signaling pathways with antibodies. Among the intracellular elements of the Wnt signaling pathway, one of those amenable to drug targeting is GSK-3beta. Lithium is a well-characterized GSK-3beta inhibitor that stimulates osteoblast differentiation *in vitro* (68). Administration of lithium chloride (LiCl) increased bone densities in mice, moreover, it increased bone mass in animals with osteoporosis (69). The orally active, small molecule Gsk3alpha/beta dual-inhibitor, 603281-31-8, is more selective for Gsk3beta than other kinases, and has also been reported to increase bone mass, bone mineral density and vertebral strength in mice (70).

Some investigations have tried another strategy to inhibit GSK-3beta using small nucleotide molecules such as siRNA (71) or small guide RNA (sgRNA) (72). We have been developing a unique system for down-regulation of expression of specific genes through utilizing the long form of tRNA 3′

processing endoribonuclease (tRNase ZL) to cut a specific mRNA under the direction of sgRNA (73). Using this system, 2'-O-methyl sgRNA for GSK-3beta can down regulate expression of GSK-3beta by degrading the mRNA, suggesting that sgRNA might be utilized as therapeutic agents to treat diseases (72). However, GSK-3beta is a key regulator of numerous signaling pathways, including cellular responses or cancer pathways involving not only Wnt, but also receptor tyrosine kinases (74). The potential risks and the long-term safety of these therapies must be determined.

Several studies in animals have shown that neutralizing antibodies to Dkk1, sFRP1 and sclerostin increase bone mass (75). Li *et al.* demonstrated that short-term administration of a sclerostin-neutralizing antibody (Scl-AbII) in an aged ovariectomy rat model of postmenopausal osteoporosis resulted in marked increases in bone formation, bone mass, and bone strength (76). Using a mouse model of chronic colitis, an antibody to sclerostin (Scl-AbI) did not reduce the weight loss or histological changes associated with colitis but did prevent inflammation-induced bone loss (77). Recently, the effects of administration of a humanized sclerostin-neutralizing monoclonal antibody (Scl-AbIV) to female cynomolgus monkeys have been reported. In this report, two once-monthly subcutaneous injections of Scl-AbIV were administered. Scl-AbIV treatment had anabolic effects with marked dose-dependent increases in bone formation (78). These studies suggest that sclerostin inhibition represents a promising new therapeutic approach to bone formation for medical conditions such as in fracture healing and osteoporosis.

In conclusion, several recent studies have indicated a role of both canonical and noncanonical Wnt signaling in the regulation of bone and tooth maintenance and turnover. Strategies to modulate the Wnt signaling pathway are therefore very attractive techniques for treatment of skeletal diseases and tooth regeneration. Hopefully, the efforts currently underway to target Wnt signaling will be successful and generate new therapeutics in future.

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Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein; DKK, Dickkopf; Dvl, Dishevelled; Frz, Frizzled; GSK-3beta, glycogen synthase kinase-3beta; LRP, lipoprotein receptor-related protein; LEF, lymphocyte enhancer factor; OPG, osteoprotegerin; PCP, planar cell polarity; PK, protein kinase; sFRP, secreted Frizzled-related protein; TCF, T-cell transcription factors; IL, interleukin; WIF, Wnt inhibitory factor

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Send correspondence to: Masato Tamura, Department of Biochemistry and Molecular Biology, Graduate School of Dental Medicine, Hokkaido University, North 13, West 7, Sapporo, 060-8586, Japan, Tel: 011-81-11-706-4231**,** Fax; 011-81-11-706-4877, E-mail; mtamura@den.hokudai.ac.jp

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