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PERSPECTIVES

Non-Canonical Wnt Signaling: What Is Its Role in Bone?

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Abstract

Recent findings indicate that Wnt proteins are important for the acquisition of bone mass. The role of these proteins was inferred from the identification of mutations in the Wnt co-receptor low-density lipoprotein receptor-related protein 5 (Lrp5) in patients with heritable skeletal diseases. Mice with conditional deletion of β-catenin in limb and head mesenchyme during early embryonic development exhibit an arrest of osteoblastic differentiation and a lack of mature osteoblasts in membranous bones, demonstrating the importance of Wnt proteins for the development of osteoprogenitors. However, high β-catenin levels in differentiated osteoblasts increase OPG expression and exert a strong negative effect on osteoclast differentiation. In addition, a recent *in vivo* analysis of the role of Lrp5 in osteoblasts gave surprising results: ablation of Lrp5 in osteoblasts had no effect on bone formation. Instead, it seems that the effect of global alteration of Lrp5 on bone metabolism is linked to changes in circulating levels of serotonin. Thus, the direct, cell-autonomous effect of Wnt proteins on osteoblasts stands in need of reconsideration. As described in this *Perspective*, Wnt proteins can activate different types of receptors in osteoblasts and recent studies suggest that non-canonical pathways may play an important role in controlling the development of osteoblast lineage cells and the activity of osteoblasts, suggesting that an understanding of non-canonical signaling in bone cells may lead to new therapeutic strategies for the treatment of osteoporosis. *IBMS BoneKEy*. 2009 March;6(3):107-115.

Wnt Proteins

The Wnt protein family comprises a large number of ligands that affect diverse processes such as embryonic induction, control of cell polarity and specification of cell fate (1). As many as nineteen mammalian Wnt genes are known but for most of them, their specific function remains to be determined. The difficulty in analysis of Wnt proteins resides in their temporally restricted and highly localized expression patterns (2). In addition, functional redundancy for some Wnt genes has been described in double knockout mice. For instance, Wnt1-Wnt3a double knockouts display defects in neural crest development and somite patterning that are not observed in either mutant alone (3;4). Moreover, single knockout of Wnt2B, Wnt5B, Wnt6, Wnt8A, Wnt8B, and Wnt16 produces no observable phenotypes in mice (2). Because of these limitations in Wnt functional analysis, studies have focused mainly on Wnt pathway components that are also complex, but less redundant.

Wnt Receptors

In addition to the nineteen Wnt ligands, the mouse genome contains ten Frizzled (Fzd) receptor genes and two low-density lipoprotein receptor-related protein (Lrp) co-receptor genes, Lrp5 and Lrp6. The seven-pass transmembrane Fzd was the first receptor described to transduce a Wnt signal (5). On the cytoplasmic side, Fzd may interact directly with the Dishevelled protein, a known mediator of Wnt signaling. The single pass transmembrane proteins Lrp5 or Lrp6 are required to transduce the Wnt canonical signal (6). Following Wnt binding, it is thought that Fzd forms a co-receptor complex with Lrp proteins. These co-receptors have a small intracellular domain that contains several potential

phosphorylated protein binding sites (7). The Axin protein, a negative regulator of Wnt signaling, can bind to the cytoplasmic tail of phosphorylated Lrp6, providing a mechanism by which Axin is released from β-catenin for its accumulation in the cytosol (8).

There are other proteins with known Wnt-binding domains that can serve as receptors for Wnt ligands (9). The single pass Ror2, although structurally distinct from Fzd receptors, is involved in other forms of Wnt signaling (see below). In the mouse, Ror2 and Ror1 knockout phenotypes resemble those of *Wnt5a*(-/-) null mice (10). Another well-characterized Wnt receptor is the cell surface atypical receptor tyrosine kinase Ryk that contains a Wnt inhibitory factor (WIF) module in the extracellular domain that can bind Wnt proteins (11). Thus, our recent understanding of Wnt receptors indicates that alternative Wnt signaling can be initiated by distinct receptors.

Wnt Signaling Pathways

Traditionally, the Wnt signaling pathway has been divided into a "canonical" and a "non-canonical" branch, both of which are activated by the binding of Wnt to Fzd transmembrane receptors (1). The canonical Wnt signaling pathway causes the activation of β-catenin-TCF complexes, whereas non-canonical signal transduction uses a multitude of different downstream effectors (12).

In the absence of Wnt, the rapid turnover of newly synthesized β-catenin is controlled through sequestration of free cytoplasmic β-catenin by the scaffolding complex. This complex consists of Axin, APC, CK1 and the serine/threonine kinase GSK3 that phosphorylates β-catenin for its proteasomal degradation. Binding of Wnt to Fzd triggers the recruitment of Axin to the intracellular domain of the co-receptor Lrp. This effect is associated with the disruption of the scaffolding complex and accumulation of unphosphorylated β-catenin that will then associate with members of the TCF and LEF family of transcription factors in the nucleus (13).

Binding of Wnt to Fzd also triggers the association of Dishevelled to the intracellular domain of Fzd and activates several non-canonical pathways. Initial evidence for the existence of a β-catenin-independent Wnt pathway came from studies in *Drosophila*, where non-canonical Wnt signaling was shown to be required for the establishment of planar cell polarity (PCP), a process in which cells adopt a distinct orientation relative to the plan of the tissue in which they reside (14;15). Downstream effectors of the PCP pathway include small Rho-like GTPases and JNK kinases (16). At the biochemical level, the events in non-canonical Wnt signal transduction have not yet been fully characterized. In *Drosophila*, the combined actions of flamingo, strabismus, prickle and diego proteins result in a complex that differentially affects Dishevelled and Fzd PCP signaling in R3 and R4 photoreceptor cells (17). Some of the vertebrate homologs of these proteins (CELSR (epidermal growth factor-like laminin A G-repeat homology domain-like EGF LAG seven-pass G-type receptor), VANGL (Van Gogh-like), Inversin and Diversin) have also been implicated in non-canonical Wnt signaling (18-21). An additional non-canonical branch is the poorly characterized Wnt-Ca^{2+} pathway, in which signaling of Wnt-Fzd complexes through heterotrimeric G proteins and phospholipase C triggers the release of calcium from intracellular storage.

In addition to these poorly defined non-canonical transduction pathways activated by Fzd receptors, increasing evidence indicates that other membrane receptors that contain known Wnt-binding domains also trigger signaling pathways independently of β-catenin. Ryk possesses a WIF domain that binds Wnt proteins with high affinity (22). In *Drosophila*, the Ryk ortholog Derailed (Drl) binds to Wnt5 to promote commissural axon guidance and proper salivary gland migration, possibly through the activation of members of the Src family of tyrosine kinases (23-25). Ryk was

first discovered in a screen for protein tyrosine kinase in the mouse. The name Ryk comes from the aberrant intracellular domain of this receptor (Related to protein tYrosine Kinase) that has been shown to activate the MAPK pathway.

The characteristics and function of this receptor in mammals have recently been reviewed (9). Essentially, Ryk expression is detected in almost every adult tissue. Ryk is also expressed in the embryo, but the highest levels of expression are observed in structures that differentiate late in development from a single monolayer, such as hair follicles. *Ryk*(-/-) mice have craniofacial defects with a cleft palate and shortened limbs (26). Interestingly, Ephb2/Ephb3 double mutant mice have a similar cleft palate phenotype and a commissural axon path finding defect, consistent with the path finding phenotype in *Drosophila* in Drl mutants. Co-immunoprecipitation of Ryk with Ephb2, 3 and 7, and tyrosyl phosphorylation of Ryk by Ephb2 and 3 but not 7, has been reported (26). This observation indicates that Ryk can associate with and be activated by Ephb2 and 3. As described in *Drosophila*, Wnt proteins have also been shown to bind to Ryk. Wnt1 and Wnt3a directly bind to Ryk through the Wif domain. Surprisingly, this binding was found to be associated with activation of a Wnt-TCF luciferase reporter in transfected 293T cells. Furthermore, it was also shown by co-immunoprecipitation that Ryk binds to Fzd 8 (27), suggesting that Ryk may form a ternary complex with Wnt and Fzd. The observation that Ryk can activate both the MAPK pathway through an intracellular domain and the canonical Wnt pathway through the formation of a ternary complex with Fzd and Wnt proteins, probably through activation of Dishevelled, indicates that Ryk can activate different pathways, possibly through binding of different ligands and by recruiting different intracellular mediators.

Ror is another alternative Wnt receptor that binds Wnt proteins through a cystein-rich domain also present in Fzd. In the mouse, two homologues of this transmembrane tyrosine kinase have been identified and as mentioned above, the knockout phenotype closely resembles that of Wnt5a mutant mice (28;29). Consistent with this finding, Ror2 mediates Wnt5a signaling that causes inhibition of β-catenin/TCF signaling by a mechanism that remains to be investigated (30). In *Xenopus* embryos and cultured cells, Ror2 influences convergence and extension movements possibly through activation of the c-Jun N-terminal kinase. Ror2-deficient mice have a clear PCP defect in the inner ear. Recently, it was reported that collagen triple helix repeat containing 1 (Cthrc1), a secreted glycoprotein that promotes cell migration by reducing the deposition of the collagen matrix, acts as a key co-factor for formation of Wnt/Fzd/Ror2 complexes and for activation of the PCP pathway (31). Thus, in addition to Wnt/Lrp/Fzd and Wnt/Ryk/Fzd, the Wnt/Ror2/Fzd complex is the third system by which Wnt proteins activate either canonical or non-canonical pathways.

Wnt Signaling in Bone Cells

Role of Canonical Signaling

The role of canonical Wnt signaling during early skeletogenesis has been investigated using conditional loss- and gain-of-function mutations of β-catenin. Loss-of-function mutations of β-catenin were introduced into osteo-chondrogenic progenitor cells using *Prx1*- and *Dermo-Cre*, and resulted in early osteoblast differentiation arrest, increased chondrogenesis, and ectopic cartilage formation (32-34). Conversely, gain-of-function mutations of β-catenin in osteo-chondrogenic progenitors using *Prx1* resulted in the arrest of chondrogenesis (33). In addition, adenoviral Cre-mediated ablation of β-catenin in calvarial mesenchymal progenitor cell cultures inhibited osteoblast differentiation and allowed chondrocyte differentiation, demonstrating that Wnt/β-catenin signaling acts cell-autonomously to promote osteo-chondrogenic progenitor cell differentiation into the osteoblast lineage (33). Loss- and gain-of-function mutations of β-catenin in

osteoblasts using *Col1α1-Cre* revealed an additional function of Wnt signaling in bone. Overexpression of β-catenin resulted in a negative effect on bone resorption and osteoclast differentiation. Mutant mice displayed a decrease in osteoclast numbers and a dramatic increase in bone mass as well as altered tooth development (35). Osteoclast differentiation was affected in both loss- and gain-of-function β-catenin mutants due to deregulation of the osteoprotegerin gene. Osteoprotegerin was proposed to be a direct target of Wnt signaling in osteoblasts, regulated by Tcf1 (35;36). Thus, in osteoblasts, the canonical Wnt/β-catenin pathway acts in a non-cell-autonomous manner to regulate bone resorption. This observation contrasts with the idea that Lrp5/6 is a key molecule in osteoblasts for mediating activation of the Wnt/β-catenin pathway for the regulation of bone mass (37). This concept was challenged very recently; starting from a microarray analysis in *Lrp5*(-/-) mice, research demonstrated that Lrp5 controls bone formation via the synthesis of serotonin in the duodenum (38). Serotonin produced by enterochromaffin cells directly inhibits the proliferation of osteoblasts via the Htr1b receptor and CREB. In this new study, the bone analysis was limited to the axial skeleton, and whether this connection also applies to the regulation of the appendicular skeleton remains unclear. Indeed, a previous study documented that chronic administration of serotonin in rats enhances the cortical thickness of long bones measured by μ-CT whereas trabecular bone volume was decreased (39).

Role of Non-Canonical Signaling

As summarized above and in the accompanying figure (Fig. 1), Wnt ligands can activate signals through different membrane receptor systems. *In vivo* and *in vitro* studies indicate that the Lrp/Fzd complex plays an important role in transducing Wnt signals in osteoblastic lineage cells for autonomous and non-autonomous cell responses. Whereas the *in vivo* function of the canonical β-catenin pathway is well-documented (see above), the importance of non-canonical pathways activated by this receptor system in bone biology remains unclear. In cultured osteoblastic cells, however, activation of ERK, Src and Akt by Wnt3a and Wnt5a was first shown to be involved in the prevention of apoptosis induced by the absence of serum growth factors (40). In addition to this observation, our laboratory recently documented that p38α is activated by Wnt3a in mesenchymal cells and is involved in their differentiation into osteogenic cells (41). Interestingly, activation of this MAPK and of ERK by Wnt3a was not blunted by Dkk1, a selective antagonist of the Lrp5/6-induced β-catenin canonical pathway. This observation suggests an important role of Wnt non-canonical pathways for mesenchymal cell differentiation into osteogenic cells. However, the molecular mechanism by which these non-canonical pathways are activated by Wnt ligands remained unclear. Results from a recent study strongly suggest that Fzd receptors are involved in non-canonical signaling through associated G proteins (42). Using cultured osteoblastic cells, research shows that Wnt3a can activate the Gαq/11 subunit of G proteins. This effect was independent of Dkk1 and generated inositol signaling and activation of PKCδ. Biochemical analysis and *in vivo* studies of PKCδ homozygous mutant mice indicate a direct role of PKCδ in embryonic bone formation (42). This research offered the first evidence for an *in vivo* function of Wnt non-canonical pathways in bone metabolism.

In addition to these effects of non-canonical pathways activated by Fzd receptors, recent findings strongly suggest that non-canonical pathways activated by Ror2 can also influence bone development and metabolism. It was first observed that Ror2 is expressed in human osteoblasts and is strongly upregulated during differentiation (43). Also, overexpression of Ror2 in human mesenchymal stem cells (hMSCs) by adenoviral infection induces expression of the osteogenic transcription factors osterix and Runx2 and causes formation of a

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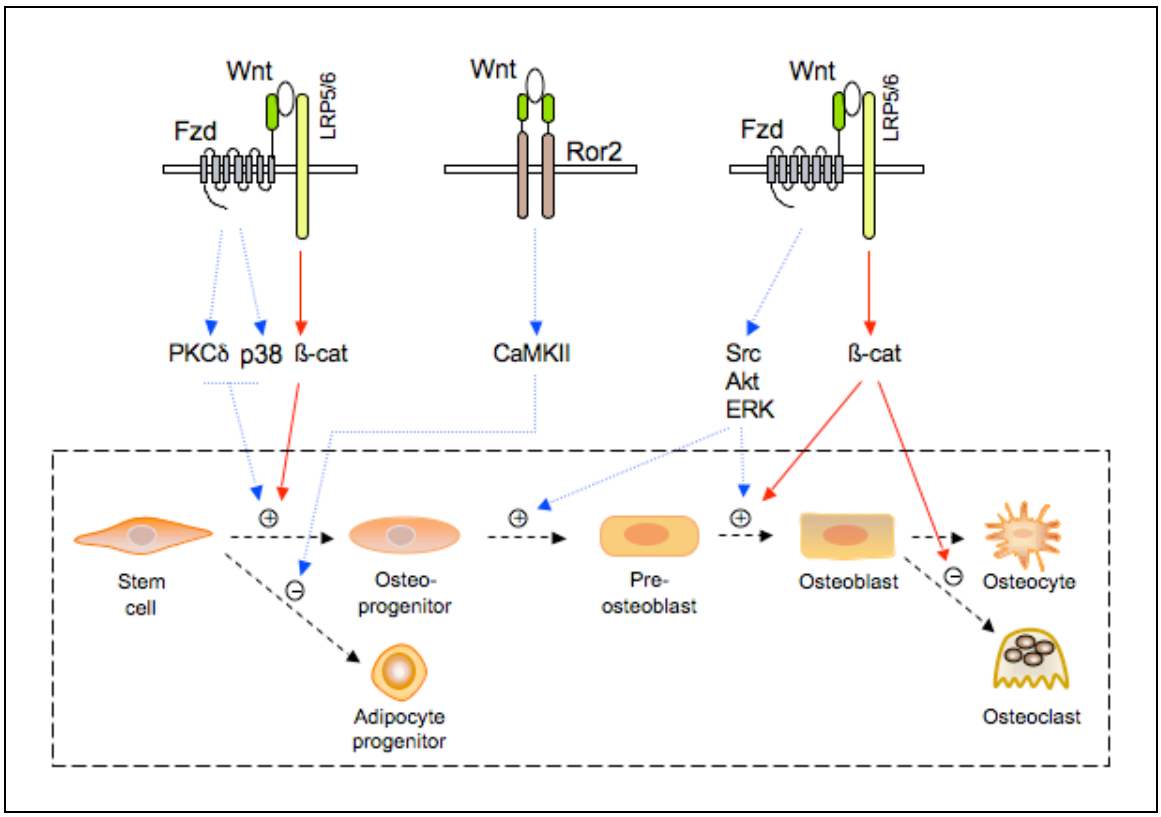

Fig. 1. Canonical (continuous lines) Wnt pathways are involved in the commitment of mesenchymal cells into osteoprogenitors, the proliferation of preosteoblasts and the control of osteoclast development through regulation of osteoprotegerin. Non-canonical (dashed lines) PKCδ and p38 pathways are involved in the differentiation of mesenchymal cells into osteoprogenitors. Calmodulin kinase II (CaMKII) inhibits the commitment of mesenchymal cells into adipocyte progenitors in favor of osteoprogenitors. The Src, Akt and ERK pathways protect osteogenic cells from apoptosis. As indicated in the figure and in the text, various membrane receptor molecules contribute to the generation of non-canonical pathways and the regulation of osteoblast cell lineage development.

mineralized matrix (44). It is unlikely that these effects are mediated through canonical pathways since activation of β-catenin by Wnt3a in these cells inhibits their osteogenic differentiation (45). Interestingly, the Ror2 tyrosine kinase substrate 14-3-3β is probably involved in controlling yet unknown downstream non-canonical signaling pathways. Indeed, inhibition of 14-3-3β with specific shRNA induces hMSC osteogenesis *in vitro* and new bone formation *in vivo*, suggesting that this scaffold protein is a negative regulator of osteogenesis (46). Thus, Ror2-induced bone formation is mediated by phosphorylation of 14-3-3β to relieve inhibition of osteogenesis exerted by this scaffold protein. The Wnt ligand(s) that functionally activate Ror2 have recently been investigated. Previous studies had shown that both Wnt3a and Wnt5a bind Ror2 equally well in immunoprecipitation experiments (43). Recently, however, research from the same group documented that only Wnt5a, and not Wnt3a, is able to induce Ror2 dimerization, its autophosphorylation and phosphorylation of its cellular substrate 14-3-3β, as well as osteoblast differentiation and *ex vivo* bone formation (47). Another recent study confirmed that Wnt5a plays an important role in MSC differentiation into osteoblast lineage cells by stimulating their proliferation and expression of genes regulating osteoblast differentiation, including Runx2, osterix and alkaline phosphatase (48).

In line with these recent observations, another important functional role of Wnt5a and Ror2 has been described recently in MSCs. Research demonstrates that Wnt5a suppresses the expression of adipogenic PPAR-γ target genes by activating non-canonical signaling pathways (49). The new work shows that Wnt5a activates Nemo-like kinase (NLK) through a calcium/calmodulin-dependent protein kinase II (CaMKII) α and mitogen-activated protein kinase kinase kinase (MAPKKK) TAK1/TAB2 signaling cascade. Studies found that phosphorylation of SETDB1 (SET domain bifurcated 1) by NLK leads to the formation of a chromatin-associated complex in which CHD7 (chromodomain helicase DNA binding protein 7) serves as a platform on which to assemble ligand-bound PPAR-γ, NLK and SETDB1 on target gene promoters that results in gene silencing. Thus, the CHD7-SETDB1-PPARγ complex activated by Wnt5a, by preventing the adipogenesis cell lineage decision, favors the differentiation of bone marrow mesenchymal precursors into osteoblasts. These *in vitro* data have been corroborated with the phenotype of the corresponding gene-deficient mice. Heterozygous PPAR-γ mice exhibit an increase in bone mass, whereas the number of bone marrow adipocytes is reduced. In contrast, mice heterozygous for Wnt5a exhibit an increase in bone marrow adipocytes with osteopenia (49).

In conclusion, recent studies on Wnts and bone suggest a potential role of non-canonical Wnt proteins for regulation of bone metabolism. Ror2 is probably an important receptor for mediating non-canonical Wnt effects on mesenchymal cell differentiation into either adipocytes or osteoblasts with the Ror2 substrate 14-3-3ß appearing as an interesting therapeutic target for the treatment of osteoporosis. The *in vivo* function of pathways activated by Wnt proteins through the Ryk and Fzd receptors, however, remains to be further investigated.

Conflict of Interest: None reported.

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