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REVIEW

Wnt signaling control of bone cell apoptosis

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Wnts are a large family of growth factors that mediate essential biological processes like embryogenesis, morphogenesis and organogenesis. These proteins also play a role in oncogenesis, and they regulate apoptosis in many tissues. Wnts bind to a membrane receptor complex comprised of a frizzled (FZD) G-protein-coupled receptor and a low-density lipoprotein (LDL) receptor-related protein (LRP). The formation of this ligand-receptor complex initiates a number of signaling cascades that include the canonical/beta-catenin pathway as well as several noncanonical pathways. In recent years, canonical Wnt signaling has been reported to play a significant role in the control of bone formation. Clinical studies have found that mutations in LRP-5 are associated with reduced bone mineral density (BMD) and fractures. Investigations of knockout and transgenic mouse models of Wnt pathway components have shown that canonical Wnt signaling modulates most aspects of osteoblast physiology including proliferation, differentiation, function and apoptosis. Transgenic mice expressing a gain of function mutant of LRP-5 in bone, or mice lacking the Wnt antagonist secreted frizzled-related protein-1, exhibit elevated BMD and suppressed osteoblast apoptosis. In addition, preclinical studies with pharmacologic compounds such as those that inhibit glycogen synthase kinase-3β support the importance of the canonical Wnt pathway in modulation of bone formation and osteoblast apoptosis.

Keywords: LDL receptor-related protein, secreted frizzled-related protein, glycogen synthase kinase, osteoblast, bone formation, programmed cell death

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Wnt signaling

Wnts are a large family of 19 secreted carbohydrate- and lipid-modified proteins that mediate vital biological processes like embryogenesis, organogenesis and tumorigenesis [1-4]. These proteins bind to a membrane receptor complex composed of a Frizzled (FZD) G-protein coupled receptor and a low-density lipoprotein (LDL) receptor-related protein (LRP) [2-4]. There are 10 different FZDs as well as 2 LRPs (5 and 6), and ligand binding activates one of several intracellular signaling pathways depending upon the Wnt, FZD receptor and cell-type involved [1-4]. The best characterized of these is the canonical or Wnt/β-catenin pathway that signals through LRP-5 or LRP-6 and initiates activation of disheveled (Dsh), which leads to inhibition of glycogen synthase kinase (GSK)-3β and subsequent stabilization of β-catenin. The stabilized β-catenin protein then translocates to the nucleus, where it binds to and activates lymphoid-enhancer binding factor (LEF)/T cell-specific transcription factors (TCFs) that are members of the high-mobility-group (HMG)-box transcription factor family [1-5]. Additional non-canonical pathways are also activated by Wnts [2, 6]. These include the G-protein mediated Wnt/calcium pathway [7] and the c-Jun NH_2-terminal kinase (JNK) pathway [8], both of which can be controlled by Dsh [6]. Recently, Wnts have also been shown to stimulate adenylyl cyclase and increase cAMP levels via a G-protein coupled mechanism [9, 10]. Although the importance of the canonical pathway in the control of fundamental mammalian processes is thoroughly established, the significance of the non-canonical pathways is less well understood.

Due to the importance of Wnt signaling in the control of key biological processes, it is not surprising that many extracellular and intracellular regulators of the pathways exist [1-3]. The extracelluar regulators include a variety of secreted proteins like Wnt inhibitory factors (WIFs), secreted frizzled-related proteins (SFRPs), dickkopfs (DKKs) [11-13] as well as SOST/sclerostin, Wise and connective tissue growth factor (CTGF) [14, 15]. These proteins bind Wnts (WIFs and SFRPs) or FZD receptors (SFRPs), or

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interact with LRPs (DKKs, SOST, Wise and CTGF).

Wnt signaling control of apoptosis

Activation of canonical Wnt signaling has been associated with either a decrease or increase in apoptosis [16, 17]. Chen *et al.* reported that stable expression of Wnt-1 in Rat-1 cells suppressed apoptosis induced by either vincristine or vinblastine [18]. Suppression of apoptosis by Wnt-1 was demonstrated using assays such as DNA fragmentation, annexin V staining, cytochrome c release and caspase-9 activation and did not involve regulation of JNK or Akt pathways. However, stable expression of a dominant negative TCF blocked the reduction of programmed cell death (PCD) by Wnt-1, demonstrating that canonical signaling was involved in this process.

Melkonyan *et al.* identified a family of factors, which they termed secreted apoptosis-related proteins (SARPs) [19]. These proteins, SARP-1, -2 and -3, are more commonly known as the Wnt antagonists SFRP-2, -1 and -5, respectively. Stable expression of SARP-1/SFRP-2 in MCF-7 human breast adenocarcinoma cells reduced the cytotoxic effects of TNF-α and ceramide, while expression of SARP-2/SFRP-1 sensitized the cells to these apoptotic agents. The ability of these secreted proteins to modulate cell death correlated with control of canonical Wnt signaling. Expression of SARP-1/SFRP-2 in MCF-7 cells increased β-catenin protein levels, while expression of SARP-2/SFRP-1 had the opposite effect and reduced β-catenin.

Although in MCF-7 cells SFRP-1 accelerates apoptosis induced by cytotoxic agents, this Wnt antagonist has the opposite effect on human periodontal ligament fibroblasts (PDLFs). Han and Amar showed that SFRP-1 mRNA and protein levels were high in PDLFs, but were low in human gingival fibroblasts (GFs) [20]. Ceramide treatment of PDLFs induced SFRP-1 expression, while this cytotoxic agent had no effect on SFRP-1 production in GFs. Co-treatment of GFs with ceramide and SFRP-1 protein increased viability and reduced apoptosis, while the addition of SFRP-1 had no effect on PDLFs, which already expressed the protein and were resistant to ceramide treatment. Conversely, treatment of PDLFs with an siRNA to SFRP-1 sensitized the cells to ceramide, while the siRNA had no effect on GFs, which did not express the protein. Thus, the ability of SFRP-1 to regulate apoptosis is cell context dependent.

Bone remodeling

Bone is formed and maintained by teams of osteoblasts and osteoclasts working together in a coordinated manner. This process is known as remodeling, which is the mechanism for replacing immature, damaged or aged bone with new lamellar bone [21, 22]. Osteoblasts are bone-forming cells that synthesize and mineralize the skeleton [23, 24], while osteoclasts are the cells that resorb the calcified tissue [25].

Osteoblasts develop from bone marrow-derived multipotent mesenchymal stem cells of the colony forming unit-fibroblast (CFU-F) lineage that also give rise to fibroblasts, myoblasts, adipocytes, and chondrocytes [23, 24]. The earliest osteoprogenitor cells express the bone-specific transcription factor Runx2, which is required for differentiation of these cells into mature osteoblasts [26, 27]. Osteoprogenitors undergo further differentiation to pre-osteoblasts that still proliferate [23, 24], and these cells then develop into mature osteoblasts that no longer divide, but instead synthesize the bone matrix [23, 24]. Mature osteoblasts have one of three fates upon completion of the synthetic phase of the remodeling cycle [23, 24]: they can become osteocytes upon entrapment within the mineralized matrix; they can evolve into inactive lining cells that protect the bone matrix from osteoclasts; or they can undergo apoptosis, which appears to be the fate of up to 80% of the cells [21, 28]. The role of apoptosis in this process is probably to eliminate excess osteoblasts that are not needed as either lining cells or osteocytes. Some of the key proteins that osteoblasts express are type I collagen (TIC), alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OC) [23, 24].

Osteoclasts are multinucleated giant cells that originate from bone marrow-derived hematopoietic stem cells of the monocyte/macrophage lineage [25]. These cells produce resorption pits when attached to bone and express important marker proteins such as the calcitonin receptor, tartrate-resistant acid phosphatase and the vitronectin receptor [23]. The earliest identifiable progenitor of the osteoclast is the granulocyte and macrophage colony-forming unit (CFU-GM) cell. When these cells bind the cytokines receptor activator of nuclear factor-κB ligand (RANKL), colony stimulating factor (CSF)-1/monocyte-colony stimulating factor (M-CSF), and either interleukin (IL)-1 or tumor necrosis factor (TNF)-α, they undergo differentiation and subsequent fusion to become mature multinucleated osteoclasts [25]. Of these factors, RANKL and CSF-1/M-CSF are thought to be essential for this process. Both of these cytokines are expressed by bone marrow stromal cells (BMSCs) and osteoblasts, and at least part of the osteoclastogenic activities of IL-1 and TNF-α are mediated by up-regulation of RANKL and CSF-1/M-CSF in these cells. Consequently, BMSCs and osteoblasts are thought to be required for the differentiation and activity of osteoclasts [21]. In addition to expressing bone-resorbing cytokines

such as RANKL, cells of the osteoblast lineage also produce osteoclastogenic antagonists like osteoprotegerin (OPG). OPG is a soluble TNF-α receptor that binds to and blocks the ability of RANKL to signal to osteoclast lineage cells [25].

Role of apoptosis in bone cell physiology and pharmacology

Many physiologic and pharmacologic agents that affect bone remodeling do so in part by controlling bone cell apoptosis [28]. Estrogens and bisphosphonates induce osteoclast PCD but have the opposite effect on osteoblasts and osteocytes, which may partly explain the ability of these agents to suppress bone turnover in diseases like osteoporosis. On the other hand, intermittent treatment with PTH suppresses apoptosis of osteoclasts, osteoblasts and osteocytes. Pharmacologic (i.e., micromolar) levels of glucocorticoids do not appear to control osteoclast PCD, but induce apoptosis of osteoblasts and osteocytes, and this is thought to be the mechanism by which these steroids cause bone loss. Androgens also do not appear to regulate osteoclast apoptosis, but suppress the death of osteoblasts and osteocytes. Similarly, other bone anabolic agents like insulin-like growth factors (IGFs) reduce osteoblast and osteocyte PCD.

Regulation of bone formation by canonical Wnt signaling

The first evidence for the involvement of canonical Wnt signaling in bone formation came from human genetic studies of osteoporosis pseudoglioma (OPPG) syndrome and high bone mass (HBM) phenotypes [29, 30]. Gone *et al.* [31] reported that numerous nonsense and frame-shift mutations throughout the coding region of the human Wnt co-receptor LRP-5 cause OPPG syndrome. These loss-of-function mutations lead to dramatic reductions in trabecular bone volume (TBV), premature fractures and skeletal deformities that resulted from diminished bone accrual. In addition, developmental eye defects also occurred from the loss of LRP-5 function [31].

On the other hand, a gain-of-function mutation in LRP-5 has the opposite effect and causes a HBM trait in humans [32-34]. Two groups, Little *et al.* [35] and Boyden *et al.* [36], found the same G171V mutation in two families that had HBM. This mutation resides in the first β-propeller motif of the extracellular domain of LRP-5 and leads to an increase in hip and spine aerial bone mineral density (aBMD) as determined by dual-energy X-ray absorptiometry (DEXA). Radiographs showed that the bones of HBM individuals have increased trabecular BMD and cortical thickness but are otherwise normal in shape [37]. Additional gain-of-function mutations of human LRP-5 (G171R, A214T, A214V, A242T, T253I and D111Y) also lead to HBM phenotypes like endosteal hyperostosis, Van Buchem disease, autosomal dominant osteosclerosis and osteopetrosis type I [38].

An interesting observation is that all of the gain-of-function mutations identified to date that cause HBM phenotypes are found in the first β-propeller domain of LRP-5 [39]. These mutations are scattered throughout the 6 blades of the β-propeller motif, and this leads to relief from DKK-1 antagonism [36]. DKK-1 binds to the 3rd and 4th β-propeller motifs of LRP-5 and LRP-6 and disrupts canonical signaling by inducing LRP internalization and degradation through kremen [13, 39]. Another secreted Wnt antagonist, SOST/sclerostin, has been reported to bind to and antagonize LRP-5 and LRP-6 by interacting with the receptors' 1st and 2nd β-propeller motifs [40-42].

Role of wnt signaling in the control of bone cell apoptosis

LRP-5 knockout mice and transgenic mouse models of LRP-5 mutations have allowed us to understand the mechanisms by which canonical Wnt signaling controls bone formation. Kato *et al.* showed that deletion of murine LRP-5 reduced vertebral trabecular bone volume (TBV) when compared to LRP-5$^{+/+}$ controls as determined by histomorphometry [43]. LRP-5$^{-/-}$ mice also had tibial fractures as assessed by radiographs. Dynamic histomorphometric analysis of the vertebrae from LRP-5$^{-/-}$ mice demonstrated that deletion of LRP-5 decreased the mineral apposition rate (MAR) indicating that osteoblast function was inhibited by the loss of the gene. Furthermore, deletion of LRP-5 reduced osteoblast numbers in long bones, and this was correlated with a decrease in calvarial osteoblast proliferation as determined by bromodeoxyuridine (BrdU) labeling. However, osteoblast apoptosis and differentiation were not altered by loss of LRP-5.

In contrast to the osteopenic/osteoporotic phenotype of the LRP-5 knockout mice [43], our group showed that LRP-5 gain-of-function transgenic mice have HBM [44]. These animals were developed by targeted expression of human LRP-5^{G171V} in bone using the 3.6 kb rat type I collagen promoter. The transgenic mice have increased bone formation, but the mechanisms for this effect are different from those that lead to decreased bone formation by the loss of LRP-5. Heterozygous mice (LRP-5$^{G171V/+}$) have increased distal femur trabecular volumetric BMD (vBMD) as measured by peripheral quantitative computed tomography (pQCT). In addition, cortical bone thickness is also increased in the LRP-5$^{G171V/+}$ mice. Histological analysis

of the femurs indicated that total bone area is increased in the transgenic mice, while the mineralizing surface is also elevated. However, MAR is not significantly increased in the LRP-$5^{G171V/+}$ mice, indicating that osteoblast activity is not affected by the mutation. ALP staining of calvaria is also elevated in the LRP-$5^{G171V/+}$ mice, while TUNEL (terminal dNTP transferase-mediated dUTP nick end-labeled) staining demonstrated that osteoblast and osteocyte apoptosis is reduced. Thus, the primary mechanism for increased bone formation in the LRP-$5^{G171V/+}$ mice appears to be elevated osteoblast/osteocyte numbers due to decreased cell death, which indicates that modulation of this process can have a significant impact on the skeleton [28].

We discovered that SFRP-1 plays a role in osteoblast physiology during a series of transcription profiling experiments that sought to elucidate the molecular events associated with human osteoblast differentiation and bone formation [45]. Basal SFRP-1 mRNA levels were observed to increase during human osteoblast (HOB) differentiation from pre-osteoblasts to pre-osteocytes, and then decline in mature osteocytes. This expression pattern correlated with levels of cellular viability such that the pre-osteocytes, which had the highest levels of SFRP-1 mRNA, also had the highest rate of cell death. In addition, expression of SFRP-1 mRNA was induced following PGE_2 treatment of pre-osteoblasts and mature osteoblasts that have low basal SFRP-1 message levels. In contrast, SFRP-1 expression was observed to be down-regulated following transforming growth factor (TGF)-β1 treatment of pre-osteocytes that have high basal SFRP-1 mRNA levels. Consistent with this observation, treatment of pre-osteoblasts and mature osteoblasts with PGE_2 increased apoptosis, while treatment of pre-osteocytes with TGF-β1 decreased cell death. Likewise, over-expression of SFRP-1 in HOB cells that express low levels of the gene product accelerated the rate of cell death. Together, these results implied that SFRP-1 is key modulator of human osteoblast and osteocyte survival.

In order to confirm these *in vitro* observations, we characterized a knockout mouse model [46]. These mice expressed the *LacZ* gene in place of exon 1 of SFRP-1 so that SFRP-1 promoter activity could be measured by b-galactosidase staining. Loss of SFRP-1 in mice increased distal femur TBV as determined by micro-CT. But in contrast to the LRP-$5^{G171V/+}$ mice, loss of SFRP-1 had no effect on cortical bone parameters. An interesting observation about the SFRP-$1^{-/-}$ mice was that prior to 13 weeks of age, there was no difference in trabecular vBMD of the distal femur as determined by pQCT between wild-type and knockout mice. However, as the mice aged, the SFRP-$1^{+/+}$ animals lost trabecular bone, while the SFRP-$1^{-/-}$ mice gained trabecular bone. Thus, deletion of SFRP-1 delays and enhances the onset of peak bone mass and suppresses senile bone loss.

As with the LRP-$5^{-/-}$ mice, deletion of SFRP-1 also affected osteoblast activity. Dynamic histomorphometric analysis of proximal femurs from SFRP-$1^{+/+}$ and SFRP-$1^{-/-}$ female mice showed that deletion of SFRP-1 increased the mineral apposition rate, indicating that osteoblast activity was increased by loss of the gene. In addition, like the LRP-$5^{G171V/+}$ mice, deletion of SFRP-1 also suppressed apoptosis. TUNEL staining of calvaria from female mice demonstrated that loss of SFRP-1 led to an increase in calvarial thickness and a decrease in osteoblast and osteocyte PCD. In contrast to the LRP-$5^{-/-}$ mice, deletion of SFRP-1 also affects osteoblast differentiation. When bone marrow from SFRP-$1^{+/+}$ and SFRP-$1^{-/-}$ female mice was differentiated to osteoblasts in culture by incubation with ascorbic acid, β-glycerolphosphate and dexamethasone, the number of ALP+ cells was increased by deletion of SFRP-1. Analysis of the differentiating cultures for LacZ expression showed that osteoblast development and matrix mineralization also increased as SFRP-1 promoter activity became elevated, suggesting that control of Wnt signaling by SFRP-1 modulates osteoblast differentiation and function. In addition, evaluation of the bone marrow cultures from knockout mice by TUNEL staining showed that cellular apoptosis was suppressed when compared to cultures from wild-type controls. Finally, like the LRP-$5^{-/-}$ mice, deletion of SFRP-1 also affects osteoblast proliferation. Measurement of DNA synthesis in cultures derived from calvaria of newborn SFRP-$1^{+/+}$ and SFRP-$1^{-/-}$ mice showed that osteoblast proliferation increased in the knockout cells during the proliferative-phase. However, when the SFRP-$1^{-/-}$ cultures reached confluence and proliferation ceased, the rate of DNA synthesis returned to normal, indicating that the proliferation-differentiation transition was not altered by loss of SFRP-1. Therefore, deletion of SFRP-1 enhances osteoblast proliferation, differentiation and function, and suppresses osteoblast and osteocyte apoptosis.

Consistent with a negative effect of SFRP-1 on osteoblasts and osteocytes, Wang *et al.* reported that pharmacologic doses of glucocorticoids also increase osteoblast SFRP-1 expression and decrease bone formation in rats [47]. These authors showed that high concentrations ($\geq 10^{-6}$ M) of dexamethasone up-regulated SFRP-1 mRNA and protein levels in primary rat MSCs *in vitro*, and that this correlated with suppression of osteogenesis, reduced cytosolic β-catenin protein levels and blunted nuclear Runx2 activation, as well as increased apoptosis. Steroid treatment of rats *in vivo* increased SFRP-1 protein levels in trabecular osteoblasts and osteocytes, and this correlated with increased bone cell apoptosis as well as decreased femoral TBV and bone strength. Confirmation that SFRP-1 was involved in these processes was obtained from experi-

ments using siRNA *in vitro* as well as purified proteins *in vitro* and *in viv*o.

Additional evidence that Wnts prevent osteoblast apoptosis *in vitro* was recently reported by Ameida *et al.* [48]. Using murine C2C12, OB-6 and MC-3T3-E1 cells, the authors showed that treatment with both canonical (Wnt-3a) and noncanonical (Wnt-5a) Wnts suppressed PCD. In addition, while canonical Wnt signaling appeared to play a role in controlling cell survival, additional signaling pathways like Src/Erk (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)/AKT were also involved in this process.

More recently, Tobimatsu *et al.* reported that PTH 1-34 treatment of mouse MC-3T3-E1 osteoblastic cells also increased β-catenin protein levels, and that this effect occurred through modulation of both the protein kinase A and protein kinase C pathways [49]. Furthermore, the authors showed that treatment of the cells with either PTH or $LiCl_2$, which inhibits GSK-3β and activates canonical Wnt signaling, suppressed apoptosis induced by either dexamethasone or etoposide.

Collectively, these studies demonstrate that as in other cell types, Wnt signaling plays an important role in the control of bone cell apoptosis. Moreover, suppression of PCD is at least part of the mechanism by which this important pathway modulates bone formation. However, it is not yet entirely clear to what extent the effects of canonical Wnt signaling on osteoblast and osteocyte apoptosis contribute to the overall impact of this pathway on bone formation. Therefore, future studies in this area should be directed at trying to dissect out the regulation of cellular apoptosis from other aspects of osteoblast physiology.

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