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Wnt signaling and cellular metabolism in osteoblasts

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Abstract

The adult human skeleton is a multifunctional organ undergoing continuous remodeling. Homeostasis of bone mass in a healthy adult requires an exquisite balance between bone resorption by osteoclasts and bone formation by osteoblasts; disturbance of such balance is the root cause for various bone disorders including osteoporosis. To develop effective and safe therapeutics to modulate bone formation, it is essential to elucidate the molecular mechanisms governing osteoblast differentiation and activity. Due to their specialized function in collagen synthesis and secretion, osteoblasts are expected to consume large amounts of nutrients. However, studies of bioenergetics and building blocks in osteoblasts have been lagging behind those of growth factors and transcription factors. Genetic studies in both humans and mice over the past fifteen years have established Wnt signaling as a critical mechanism for stimulating osteoblast differentiation and activity. Importantly, recent studies have uncovered that Wnt signaling directly reprograms cellular metabolism by stimulating aerobic glycolysis, glutamine catabolism as well as fatty acid oxidation in osteoblast-lineage cells. Such findings therefore reveal an important regulatory axis between bone anabolic signals and cellular bioenergetics. A comprehensive understanding of osteoblast metabolism and its regulation is likely to reveal molecular targets for novel bone therapies.

Keywords

Wnt; metabolism; mTORC1; mTORC2; glucose; glutamine; fatty acids; osteoblast; bone

Introduction

The mammalian skeleton not only provides support and protection, but also performs endocrine functions. The homeostasis of adult bone mass under healthy conditions is maintained through the exquisite balance of bone resorption by osteoclasts and bone formation by osteoblasts. With aging or under pathological conditions bone resorption dominates over formation, resulting in osteopenia (low bone mass) or in the more severe cases, osteoporosis. Conversely, conditions that favor bone formation over resorption lead to

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high bone mass diseases such as sclerosteosis. Because osteoblasts are the chief cell type producing bone materials, elucidating the mechanisms that regulate osteoblast differentiation and activity is critical not only for understanding bone physiology but also for designing effective bone therapeutics. Extensive studies in the area during the past several decades have mostly focused on endocrine or paracrine signaling as well as transcriptional regulation [1, 2]. Those studies have uncovered the critical roles of growth factors such as Wnt proteins and transcription factors including Runx2, Osterix and ATF4 during osteoblast differentiation [3–8]. However, relatively little is understood about how osteoblasts fulfill their key function of active protein synthesis and matrix secretion, a process highly demanding not only in building blocks but also in energy [9]. Recent studies have discovered that the potent bone anabolic signal Wnt directly reprograms multiple aspects of cellular metabolism integral to osteoblast differentiation and activity. This review summarizes those recent advances.

Wnt Signaling

Wnt proteins are a family of secreted glycoproteins that are critical regulators of osteoblast differentiation and activity in both mice and humans [10–16]. Wnt signals are transduced by a family of seven-pass transmembrane G-protein coupled receptors of the frizzled (Fzd) family and a co-receptor of the arrow/Lrp family (e.g., Lrp5 and Lrp6) or a Ryk or Ror transmembrane tyrosine kinase [17, 18]. The binding of a given Wnt to a Fzd receptor and coreceptor activates multiple distinct intracellular signaling cascades, historically divided into the canonical β -catenin-dependent pathway and noncanonical β -catenin-independent pathways [19]. The best characterized is the canonical Wnt pathway, which results in the stabilization and translocation of β -catenin into the nucleus. β -catenin (encoded by *Catnnb1*) is an important transcriptional co-activator that regulates gene transcription in response to Wnt signaling. Normally, in cells not exposed to ligand, cytoplasmic levels of β -catenin are kept low through interactions with the β -catenin destruction complex [20]. The binding of Wnt to a Fzd receptor complex results in phosphorylation of the Lrp co-receptors and recruitment and tethering of GSK-3 β and Axin to the ligand-receptor complex. This complex is subsequently endocytosed and inhibited through sequestration into multivesicular endosomes resulting in the stabilization and accumulation of cytoplasmic β -catenin [21]. Stabilized β -catenin translocates into the nucleus and interacts with the Lymphoid-enhancing factor/T-cell factor (Lef/Tcf) family of high mobility group (HMG)-type transcription factors to stimulate expression of target genes including *Lef1*, *Tcf7*, *Nkd2* and *Axin2* [22–26]. Additionally, Wnt signaling can activate multiple signaling cascades independent of β -catenin. Here, Fzd seems to function more as a G-protein coupled receptor, activating intracellular cascades involving the GTPases Rho and Rac, the calcium calmodulin dependent kinase 2 (CaMK2), c-Jun N-terminal kinase (JNK) and p38, phospholipase-C, Protein kinase C (PKC), Protein kinase A (PKA), PI3K/AKT, and mTOR [11, 27–33]. The pathway activated by a Wnt ligand is determined by many factors including specific ligand receptor interactions, distinct receptor/co-receptor pairs, or the presence of intracellular proteins that regulate β -catenin activation [34–37]. Wnt signaling is also regulated by a number of secreted extracellular antagonists. These include Dickkopf (e.g., Dkk1 and Dkk2) and Sclerostin (Sost) proteins that bind to the extracellular domains of

Lrp5 or Lrp6 and interfere with their interaction with Wnt proteins [38–41]. In addition, the secreted frizzled related proteins (sFRPs) bind directly to Wnt ligands and thus inhibit the formation of Wnt-Fzd complexes [42–44]. Overall, Wnt signaling is tightly controlled at multiple levels to ensure its proper activity during normal development and tissue homeostasis.

Wnt signaling in bone

The importance of Wnt signaling during bone formation has been well documented [45]. The original discovery came from human genetic studies where inactivating mutations in the Wnt coreceptor *Lrp5* results in osteoporosis pseudoglioma syndrome while gain-of-function mutations causes osteosclerosis [15, 46, 47]. Moreover, mutations in either coding or regulatory sequences of *Sost* cause high bone mass in sclerosteosis or Van Buchem disease, respectively [48–50]. Subsequent genome-wide association studies in humans strongly support a role for Wnt signaling regulating bone mineral density (BMD) [51–54]. More recently, exome sequencing in humans identified multiple mutations in *Wnt1* associated with early onset osteoporosis and osteogenesis imperfecta [55, 56]. In addition, missense mutations in *Wnt16* are associated with decreased forearm and hip BMD and increased fracture risk [57].

In keeping with those findings in humans, genetic studies in mice have established a causal relationship between Wnt signaling and bone formation. Mice lacking *Lrp5* either globally or in osteoblasts are characterized by osteopenia whereas expression of mutant *Lrp5* alleles associated with human high-bone-mass syndromes increases bone mass in mice [58–60]. Activation of Wnt signaling through deletion of *Sfrp1*, *Sost* or a single allele of *Dkk1* increases osteoblast number and activity [61–63]. In addition, multiple Wnts including Wnt1, Wnt7b, Wnt10b and Wnt16 and the Frizzled receptors Fzd7 and Fzd9 have been shown to regulate bone formation [10, 11, 57, 64–66]. Moreover, targeted deletion of *Gpr177* (also known as Wntless – *Wls*, required for the secretion of all Wnt ligands) inhibits bone formation in mice [67, 68]. It is worth noting that the bone phenotypes resulting from loss of individual Wnt ligands or Fzd receptors are less severe than those reported in *Gpr177* knockout mice, indicating significant functional redundancy amongst Wnt ligands and Fzd receptors. This viewpoint is consistent with the fact that osteoblast-lineage cells express multiple Wnt ligands during development [13, 69].

Genetic studies in mice have highlighted the importance of β -catenin in mediating Wnt signaling in bone formation. Multiple studies demonstrate that β -catenin is required for bone formation and acts at multiple stages of osteoblast differentiation to regulate both osteoblast and osteoclasts [12, 13, 16, 70–74]. The direct target genes of β -catenin during osteoblast differentiation are not fully elucidated, but β -catenin together with Tcf1 has been shown to stimulate *Runx2* transcription directly [75]. In mature osteoblasts, *Opg*, encoding an anti-osteoclastogenic factor, is known to be a direct target of β -catenin [71]. While the importance of β -catenin in bone is well established, the contribution of β -catenin-independent Wnt signaling to bone formation is becoming increasingly clear. For example, Wnt7b can stimulate osteoblast differentiation through activation of PKCdelta [11]. Multiple Wnt proteins also activate the serine threonine kinase mammalian target of rapamycin

complex 1 (mTORC1) which promotes protein synthesis and bone formation [29, 76, 77]. The stimulatory effect of mTORC1 in bone formation is further supported by genetic studies that either abolish or enhance mTORC1 activity in the mouse [78, 79]. In addition, Wnt activates mTORC2 that is required for the optimal bone accrual in response to mechanical loading or an anti-sclerostin neutralizing antibody [80–82]. Because the mTOR pathways are central to nutrient sensing and metabolic regulation, Wnt signaling has emerged as an important mechanism for modulating cellular metabolism in osteoblasts.

Glucose Metabolism in Osteoblasts

Glucose is the primary energy source for most mammalian cell types. Glucose is transported into the cell via the Glut family of facilitative glucose transporters. The Gluts transport glucose down a concentration gradient independent of ATP [83, 84]. Inside the cell, glucose is phosphorylated by hexokinase (Hk) to form glucose-6-phosphate (G6P). G6P can be converted to glycogen for storage or metabolized by disparate pathways including hexosamine biosynthetic pathway (HBP), pentose phosphate pathway (PPP) and glycolysis [85]. The HBP is used to produce uridine diphosphate N-acetylglucosamine (UDPGlcNAc) for protein glycosylation. The PPP is important to generate NADPH and ribose-5-phosphate important for nucleotide synthesis. Glycolysis occurs in the cytosol and produces 2 molecules of pyruvate, 2 ATP and 2 reducing equivalents in the form of NADH per glucose molecule. Pyruvate can be converted into lactate by the enzyme lactate dehydrogenase (Ldh) independent of oxygen. This reaction regenerates oxidized NAD (NAD⁺) that is necessary for further glycolysis. Alternatively, pyruvate can be decarboxylated to form acetyl-CoA by the enzyme pyruvate dehydrogenase (Pdh). Pyruvate oxidation in the tricarboxylic acid (TCA) cycle produces the most ATP per glucose molecule through oxidative phosphorylation (OXPHOS). Importantly, TCA intermediates are often extracted from the cycle (cataplerosis) and used for lipid and amino acid biosynthesis, redox regulation and epigenetic regulation of gene expression [86–88]. The TCA cycle intermediates are replenished through metabolism of amino acids or fatty acids, a process known as anaplerosis. Thus, glucose is not only an important energy source but also a critical provider of building blocks for biosynthetic reactions.

Glucose is an important nutrient for osteoblasts. Osteoblasts express the glucose transporter Glut1 and rapidly consume glucose in response to a variety of signals [81, 89–94]. Recent work has revealed a feed-forward mechanism between Glut1 and Runx2 expression, highlighting a critical role for glucose metabolism in osteoblast differentiation [93]. Mature osteoblasts are known to possess numerous mitochondria and exhibit active OXPHOS [95–97]. Interestingly, however, aerobic glycolysis appears to be the dominant mode of glucose utilization in osteoblasts. For example, early studies using both bone slices and isolated osteoblasts reveal that osteoblasts rapidly take up glucose and metabolize it primarily into lactate [98–101]. More recent studies have confirmed aerobic glycolysis as a predominant mode of glucose metabolism in primary calvarial osteoblasts despite increased OXPHOS as the cells further differentiate to form mineralized nodules in response to ascorbic acid and β -glycerophosphate [96, 102]. Functionally, stimulating glycolysis through activation of Hif1 α signaling in preosteoblasts increases bone formation *in vivo*, indicating that reprogramming glucose metabolism is sufficient to promote osteoblast differentiation [103]. Moreover,

Hif1 α activation has been shown to promote bone healing partly through reprogramming of glucose metabolism, highlighting the clinical implications of understanding metabolic regulation in osteoblasts [104]. Recent studies have demonstrated that aerobic glycolysis is directly stimulated in response to osteogenic signals such as PTH and Wnt [105, 106]. For example, Wnt rapidly increases Glut1 and Hk2 protein levels to increase glucose consumption. Wnt further promotes aerobic glycolysis by upregulating Ldha and Pdk1 to favor lactate over acetyl-CoA production from pyruvate (Figure 1). Consistent with these observations, osteoblasts from mice expressing the human *Lrp5* high bone mass mutation show increased glucose consumption and expression of glycolytic enzymes. Conversely, *Lrp5*^{-/-} mice have decreased glycolytic enzyme expression and lower serum lactate levels. Mechanistically, Wnt can induce glycolysis independent of β -catenin activity, instead through mTORC2 signaling [81]. It should be noted that β -catenin has been shown to induce transcription of *Pdk1* in cancer cells, indicating an additional mechanism for Wnt to stimulate aerobic glycolysis [107]. Direct inhibition of mTORC2 signaling by deletion of *Rictor* reduced both physiological bone formation and Wnt induced bone formation in response to an anti-sclerostin neutralizing antibody [80, 108]. It is important to note that increased aerobic glycolysis contributes to Wnt-induced osteoblast differentiation *in vitro* as Ldha or Pdk1 knockdown impaired induction of osteoblast differentiation marker genes [106]. Likewise, dichloroacetate, which inhibits Pdk1 and promotes glucose metabolism through the TCA cycle, reduced bone formation *in vivo* in response to Hif1 α overexpression or anabolic PTH treatment [91, 109]. Overall, both Wnt and PTH stimulate aerobic glycolysis as a mechanism to promote bone anabolism.

The reasons for osteoblasts to prefer aerobic glycolysis are not fully understood at present. From the bioenergetics viewpoint, aerobic glycolysis is a less efficient means of producing ATP compared to metabolism through the TCA cycle and OXPHOS. Cancer cells display a similar metabolic reprogramming which is postulated to provide nucleotides, amino acids and lipids needed to support cell division [110]. Mature osteoblasts however generally exhibit little proliferation *in vivo* [111]. Increased aerobic glycolysis may help reducing reactive oxygen species or generate more amino acids to support protein synthesis in osteoblasts. Moreover, glycolytic changes could directly exert epigenetic regulation to influence osteoblast differentiation. We have recently demonstrated a link between increased aerobic glycolysis and gene suppression in response to Wnt. Increased aerobic glycolysis limits the amount of citrate exiting the TCA cycle, resulting in decreased nuclear levels of both citrate and acetyl-CoA. This leads to a large scale decrease in histone acetylation and suppression of adipogenic or chondrogenic transcription factors, thus favoring osteogenic differentiation over the alternative fates in the multipotent progenitors [112]. Further studies are warranted to elucidate the full mechanism whereby aerobic glycolysis promotes the osteoblast phenotype.

Amino Acid Metabolism in Osteoblasts

Amino acids are not only the building block of proteins, but also an important energy source. The cellular amino acid pool is derived from multiple sources including import of extracellular amino acids, degradation of intracellular protein and *de novo* synthesis. Based on their mode of catabolism, amino acids can be categorized as glucogenic or ketogenic or

both. Whereas ketogenic amino acids are broken down into acetyl-coA or acetoacetate, glucogenic amino acids are broken down into either pyruvate, or different TCA intermediates including oxaloacetate, α -ketoglutarate, fumarate and succinyl-coA. Thus, amino acids can contribute directly to ATP production via the TCA cycle and OXPHOS. Indeed, amino acid catabolism through the TCA cycle is required in many contexts including cancer cell proliferation, pluripotent progenitor maintenance and differentiation [113–117].

Initial studies in bone explants and calvarial osteoblasts focused on amino acid uptake. These studies defined multiple transport systems in bone, including system A, system L and system ASC, and suggested differences in amino acid transport between adult and fetal bone [118–123]. Amino acid uptake is regulated in osteoblasts, being stimulated by cAMP and various growth factors and hormones [122–128]. More recent studies have implicated individual amino acid transporters in bone biology. For example, the cystine/glutamate antiporter xCT normally suppresses osteoblast differentiation likely through decrease of glutathione production [129–131]. Amino acid uptake is also regulated transcriptionally by the transcription factor Atf4, a critical transcription factor that can be activated by unfolded protein in the endoplasmic reticulum (ER) or amino acid depletion [7, 132, 133]. Atf4 stimulates osteoblast differentiation in part through increasing amino acid import to support collagen synthesis [134–137]. The importance of amino acid import is highlighted by the observation that a high protein diet or amino acid supplementation corrects differentiation defects and bone loss in *Atf4*^{-/-} osteoblasts [134]. Besides direct contribution to protein translation, amino acids also act as a growth signal to activate mTORC1, a critical regulator of osteoblast differentiation and bone formation [29, 79, 138–144]. Thus, amino acids regulate bone formation through multiple mechanisms.

The amino acid glutamine has emerged as an important regulator of osteoblasts. Glutamine is the most abundant free amino acid in circulation and is not only an important oxidative fuel, but also a precursor for the synthesis of non-essential amino acids, nucleotides and the anti-oxidant glutathione. Initial studies in isolated calvaria and long bones demonstrated an active consumption and metabolism of glutamine [145]. Glutamine was later shown to be required in calvarial osteoblasts for matrix mineralization [146]. Decreased glutamine consumption by bone marrow stromal cells has been linked with impaired osteoblast differentiation associated with aging [147]. Moreover, increased glutathione production from glutamine in response to Hif1 α has been shown to improve cell survival and bone repair in a critical-size tibial defect model [148]. In differentiating osteoblasts, glutamine anaplerosis fulfills part of the energetic requirement of bone formation in response to Wnt signaling. Wnt increases glutamine anaplerosis into the TCA cycle by rapidly increasing glutaminase (Gls) protein levels and activity (Figure 2). Strikingly, increased glutamine anaplerosis reduces intracellular glutamine levels, leading to activation of Gcn2. This results in Atf4 activation which stimulates both the uptake and the *de novo* synthesis of amino acids to promote protein synthesis. Mechanistically, this cascade of events is dependent on mTORC1 activity downstream of Wnt. Importantly, pharmacological inhibition of Gls reduces bone formation in the *Lrp5*^{A214V/+} high bone mass mouse model demonstrating a critical role for glutamine anaplerosis to support excessive bone anabolism [135]. Whether Gls and

glutamine anaplerosis are required for physiological bone formation remains unknown as a systematic analysis of the bones is yet to be performed with the *Gls* knockout mice [149].

Fatty Acid Metabolism in Osteoblasts

Lipids are another important carbon and energy source in mammalian cells. Lipids can be synthesized *de novo* or acquired either as free fatty acids that are taken up by cell surface transporters or as lipoprotein particles bound by LDL receptor family members. Once transported into the cell, fatty acids can be metabolized in the mitochondrial matrix through β -oxidation that sequentially cleaves off two carbons as acetyl-coA that enters the TCA cycle. Fatty acids are first transported into the mitochondria by the carnitine shuttle. Here the rate limiting enzyme carnitine palmitoyltransferase 1 (CPT1) generates acyl-carnitine by transferring the acyl group of a long-chain fatty acyl-CoA to the hydroxyl group of carnitine. Acyl-carnitine is then shuttled inside the mitochondria in exchange for carnitine and converted back to acyl-CoA on the inner mitochondrial membrane by CPT2. Inside the mitochondrial matrix, acyl-coA can then undergo β -oxidation. Even chain fatty acids can be completely oxidized into acetyl-CoA whereas odd chain fatty acids ultimately form acetyl-CoA and succinyl-CoA which enter the TCA cycle. Complete oxidation of lipids by β -oxidation yields the most ATP per molecule compared to glucose or amino acids.

The role and regulation of lipid metabolism in osteoblasts is understudied. However, it has been reported that bone takes up the second highest amount of postprandial lipoproteins behind liver in the mouse [150]. Moreover, lipid supplementation of serum-free medium was sufficient to support proliferation of osteoblastic cells *in vitro* [151]. Whether this requirement reflects an energetic or synthetic need for lipids was not clear, but another study estimated that fatty acid oxidation provided 40–80% of the energy derived from glucose in rat calvarial osteoblasts [152]. Furthermore, fatty acid oxidation increases during osteoblast differentiation in both murine and porcine models, implicating lipid metabolism in energy production [153, 154]. Interestingly, recent studies have implicated Wnt signaling in the regulation of lipid metabolism in bone. Osteoblasts lacking the Wnt co-receptor *Lrp5* exhibit decreased both expression of lipid metabolism genes and lipid oxidation. Conversely, expression of the *Lrp5*^{G171V} allele or stimulation with the ligand Wnt10b increases lipid metabolism gene expression and stimulates lipid oxidation in bone. Mechanistically, the regulation by Wnt appears to be β -catenin dependent as GSK3 β inhibition or β -catenin overexpression is sufficient to stimulate lipid oxidation in osteoblasts [154]. It appears that Wnt promotes fatty acid oxidation to fuel the TCA cycle and OXPHOS while also stimulating aerobic glycolysis and glutamine anaplerosis. Further studies are warranted to determine the physiological importance of lipid oxidation for osteoblast differentiation and bone formation *in vivo*.

Summary

Here we have highlighted data exploring the role and molecular regulation of cellular metabolism in osteoblasts by Wnt signaling. Recent evidence has indicated that Wnt signaling stimulates aerobic glycolysis, glutamine anaplerosis and β -oxidation of fatty acids in osteoblast-lineage cells. It is likely that β -oxidation and glutamine anaplerosis contribute

to ATP production via OXPHOS to sustain protein synthesis during bone formation. The mechanism through which aerobic glycolysis contributes to the osteoblast phenotype is likely multifaceted and certainly warrants further investigation. Similarly, future studies on the role of the various metabolic pathways during physiological bone formation are necessary. It is tempting to speculate that metabolic dysregulation at the cellular level might be involved in various bone pathologies such as ectopic ossification, vascular calcification, skeletal aging or diabetes-associated bone disorders. The potential link with diabetes is of particular clinical significance as patients with either type I or type II diabetes exhibit increased risk in bone fracture [155, 156]. Although the mechanisms for diabetes-related bone fragility are undoubtedly complex, pharmaceutical targeting of osteoblast metabolism may be a promising direction towards novel therapies to improve bone health in diabetic patients.

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Figure 1. Wnt signaling promotes aerobic glycolysis through mTORC2 activation

Wnt signaling through Frizzled (Fz) and Lrp5/6 induces mTORC2 activity downstream of PI3K-Rac1 signaling whereas mTORC2 activation acutely increases the protein abundance of metabolic enzymes (in red) without changing their mRNA levels. Glc: glucose; Glc-6-P: glucose 6-phosphate; Fruc-6-P: fructose 6-phosphate; Fruc-1,6-P: fructose 1,6-bisphosphate; Pyr: pyruvate; Lac: lactate. Hk2: hexokinase 2. Pfk1: phosphofructokinase 1; Ldha: lactate dehydrogenase A; Pdk1: pyruvate dehydrogenase kinase 1; PDC: pyruvate dehydrogenase complex. TCA: tricarboxylic acid cycle. See original reference for details [106].

Figure 2. Wnt signaling stimulates glutamine catabolism through the TCA cycle

Wnt signaling through Frizzled (Fz) and Lrp5/6 activates mTORC1 in a PI3K-Akt dependent manner; mTORC1 increases the protein abundance of glutaminase (Gls) and stimulates glutamine oxidation for ATP production. The increase in glutamine consumption results in lower intracellular glutamine levels that activate the Gcn2-Atf4 stress pathway and up-regulate the transcription of genes important for protein translation. Gln: glutamine; Glu: glutamate; α-KG: α-ketoglutarate. See original reference for details [76, 77].