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Review

Signaling pathways governing osteoblast proliferation, differentiation and function

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Summary. Osteoblasts are bone forming cells that are responsible for bone growth and remodeling. They are derived from bone marrow mesenchymal stem cells through a series of processes including commitment, osteoprogenitor expansion, terminal differentiation and cell death. Osteoblastogenesis and bone formation are regulated by hormones, growth factors, cytokines, mechanical loading and aging. Osteoblasts can sense these external cues, transduce the signals through various signaling pathways and regulate the expression of specific genes, to determine the cell fate. In this review, we aim to update our current understanding of the signaling pathways that control different steps of osteoblast homeostasis, with special focus on how signaling events control cell fate through regulating gene expression.

Key words: Osteoblast, BMP, Wnt, PTH, MAPK

Introduction

The skeleton makes up about 20% of our body weight. Its major functions include mechanical support and motility, protection of internal organs, storage and metabolism of Ca^{2+} and phosphate, and regulating hematopoiesis (Rodan, 1992; Manolagas and Jilka, 1995). In the human lifetime, bones experience slow prepubertal growth, rapid pubertal growth, balanced remodeling, and finally bone loss (Riggs et al., 2002). These stages are controlled by changes in hormones, growth factors, mechanical loading, nutrition and other unidentified factors.

Bone growth and accrual are carried out by osteoblasts, which are derived from bone marrow mesenchymal stem cells (MSCs) (Yang and Karsenty, 2002; Harada and Rodan, 2003). In addition, bones also contain osteoclasts, which have opposite functions to osteoblasts, bone resorption. Osteoclasts are derived from bone marrow hematopoietic stem cells (HSC) and they share the same precursor with macrophages. Coordinated actions of these two types of cells determine the growth status of bones. At the prepubertal stage, bones grow rather slowly. However, due to the surge of growth hormones during the puberty stage, osteoblasts outperform osteoclasts, and as a consequence, bones show fast growth in both size and mass. Peak bone mass is reached shortly after this pubertal spurt (Riggs et al., 2002). In adults, bone mineral density is kept relatively steady until menopause as a result of balanced bone formation and bone resorption. Even at this stage, bones are quite dynamic, with 15% of the trabecular and 3% of the cortical bones being replaced by newly formed bones each year (Manolagas and Jilka, 1995). It is believed that the remodeling process facilitates the bones to adapt to mechanical loading and replaces the microdamage incurred to bones due to wear and tear. The coordination between bone formation and resorption is maintained by multiple coupling mechanisms between osteoblasts and osteoclasts. Osteoblasts can synthesize and secrete RANKL and M-CSF to stimulate osteoclastogenesis, and osteoprotegerin (OPG), an inhibitor of RANKL, to inhibit osteoclastogenesis (Manolagas and Jilka, 1995; Boyle et al., 2003). The ratio of RANKL to OPG dictates osteoclast differentiation status. Reciprocally, bone resorption releases bone matrix-trapped IGF-I, TGF-ß, and BMPs, which promote osteoblast migration to the resorption sites and hasten osteoblastogenesis. Recently, a study reported that osteoclasts express EphrinB2, while osteoblasts express ephrin receptor EphB4 (Zhao et al., 2006). This bidirectional signaling of Ephrin-Eph receptors could provide another coupling mechanism between these two cell types. At the

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postmenopausal stage, due to estrogen shortage, there is an increase in osteoclastogenesis whereby bone resorption outpaces bone formation, leading to bone loss. In later life, due to aging, both men and women show a decline in the number and vigor of osteoblasts, leading to reduced bone formation and slow healing of bone fractures.

The balance between bone formation and resorption can be disrupted under certain physiological and pathological conditions, e.g., malnutrition, hormone dysregulation, application of glucocorticoid, and gravity loss, which could lead to the development of bone-related diseases. The most common one is osteoporosis, featuring decreased bone mass/density and deterioration of bone microstructure, as well as increased risk of fractures (Khan, 2003). It can be classified into two groups based on its pathogenesis. One is postmenopausal osteoporosis, which is caused by an increase in osteoclastogenesis and bone resorption, due to estrogen deficiency, and it mainly affects women. The other type is senile osteoporosis, which is caused by a decline in the number of osteoblasts and the vitality of osteoblasts. Senile osteoporosis affects both men and women over age 65. In total, osteoporosis affects one in every two women and one in every four men over age 50. It is a major public health problem that demands high medical cost. On the contrary to osteoporosis, osteosclerosis and osteopetrosis show increased bone mass/density and constitute a smaller portion of bone-related diseases. Osteosclerosis is caused by overactivity of osteoblasts and increased bone formation, while osteopetrosis is a result of decreased osteoclastogenesis and weakened bone resorption.

Osteoporosis can be prevented and/or alleviated by either slowing down bone resorption, boosting bone formation, or both (Goltzman, 2002; Canalis et al., 2007). There are some clinically effective antiresorptive drugs, such as estrogen, SERMs (Selective Estrogen Receptor Modulators), and bisphosphonates, which mainly serve to inhibit osteoclastogenesis and bone resorption. However, there is a shortage of anabolic drugs for osteoporosis therapy, with parathyroid hormone (PTH (1-34)) as the only approved anabolic drug by the US FDA. As such, much effort is currently being made in the search for novel anabolic drugs for osteoporosis prevention/therapy. The potential drug candidates should possess one or several of the following activities: promoting MSC renewal, directing MSC to differentiate into osteoblasts, promoting osteoblast/osteoprogenitor expansion; augmenting osteoblast activity; and extending the lifetime of osteoblasts. To achieve this goal, it is essential to understand the process of osteoblast turnover, from its birth to death, especially the signaling pathways that regulate each of these steps.

Differentiation from MSC to osteoblasts

MSCs are residents of the bone marrow, with the potential of differentiating into adipocytes, myoblasts, fibroblasts and chondrocytes, in addition to osteoblasts. Osteoblasts and chondrocytes share the same progenitor (Manolagas and Jilka, 1995; Karsenty, 2008). The process of differentiation into different lineages is controlled by growth factors/cytokines and the expression of lineage-specific transcription factors. For example, differentiation into myoblasts is controlled by the expression of MyoD, whereas differentiation into adipocytes is controlled by transcription factor PPAR. On the other hand, differentiation into chondrocytes is controlled by SOX5/6/9, whereas osteoblast differentiation is controlled by transcription factors Runx2 and Osterix (Fig. 1). Runx2 and Osterix are both necessary and sufficient for osteoblast differentiation and for *in vivo* bone calcification. Mice deficient of either Runx2 or Osterix show no mature osteoblasts or calcified bones (Ducy et al., 1997; Komori et al., 1997; Nakashima et al., 2002). Furthermore, forced expression of either of them enhances or initiates the osteoblast differentiation program *in vitro*. Osterix stimulates osteoblast bone forming activity and there is also evidence suggesting that its protein levels determine the differentiation stages of osteoblasts (Wang et al., 2006). Adding to the complexity, Runx2 activity can be regulated by other transcription factors through protein-protein interaction. For example, Stat1, Schnurri 3, and Msx2 have been found to inhibit the transcription activity of Runx2 (Karsenty, 2008). Therefore, Runx2 and Osterix possibly act as a node in integrating various signals, through different pathways, to regulate osteoblast differentiation and activity.

Besides Runx2 and Osterix, there are a few other transcription factors that have been demonstrated to play critical roles in osteoblast turnover. These include the AP-1 family, Atf4 (activating transcription factor 4), and ß-Catenin (Karsenty, 2008). AP-1 is composed of two members of Fos (Fos, FosB, Fra-1, Fra2), Jun (Jun, JunB, JunD), and sometimes Atf (Wagner and Eferl, 2005). Genetic studies show that osteoblast-specific expression of Fra-1 or ΔFosB, a splicing variant of FosB, in transgenic mice leads to osteosclerosis due to enhanced osteoblastogenesis and bone formation (Jochum et al., 2000; Sabatakos et al., 2000). In contrast, mice with osteoblast-specific deletion of Fra-1 show osteopenia with a defect in osteoblast differentiation, but no effect on the number of osteoblasts (Eferl et al., 2004). Similarly, mice lacking JunB also show osteopenia with a defect in osteoblasts (Kenner et al., 2004). Atf4 is shown to control the expression of collagen type I and osteocalcin, and it is believed to regulate bone mineralization of mature osteoblasts (Yang et al., 2004). This is evident through Atf4 knockout mice, which show delayed bone formation and low bone mass.

Differentiation into various lineages is mutually exclusive and competition exists among them, with the most studied competition being between adipocytes and osteoblasts. Mouse genetic studies have shown that

forced expression of PPAR in mouse promotes adipocyte differentiation at the sacrifice of osteoblasts (Lecka-Czernik et al., 2002; Jeon et al., 2003). On the other hand, overexpression of Fra-1 or FosB leads to enhanced osteoblastogenesis and increased bone formation at the expense of adipocyte formation (Jochum et al., 2000; Sabatakos et al., 2000).

The maturation of MSC to functional osteocytes is an ordered process that consists of at least five steps: MSC commitment to osteo-chondroprogenitors (which express Runx2 and Collagen 2α), commitment to osteoprogenitors (which express Osterix), expansion of osteoprogenitors, maturation of osteoblasts (which express osteocalcin and collagen 1), and apoptosis of osteoblasts (Harada and Rodan, 2003; Rodda and McMahon, 2006) (Fig. 1). These stage-specific markers are useful in determining the differentiation status. They are also used to generate stage-specific Cre transgenic mice, which can be used to genetically delete the gene of interest at specific stages, to analyze its function in osteoblast turnover. While osteoblast differentiation has been extensively studied, signaling pathways that maintain the stemness of MSC, osteoblast expansion, and osteoblast survival are less well understood.

Osteoclasts and bone resorption

Osteoclasts are derived from bone marrow HSC and they share the same precursor as the macrophages (Boyle et al., 2003). PU.1, AP-1, NF-κB, and NFATc are the transcription factors essential for osteoclastogenesis. Among the growth factors/cytokines that control osteoclastogenesis are M-CSF and RANKL, which play positive roles in the expansion of the precursors and osteoclast differentiation, OPG, which has a negative role in osteoclastogenesis, and IL-1, which also promotes osteoclastogenesis. Osteoclastogenesis and bone resorption have been explored in a number of reviews and will not be the focus of this paper.

Signaling pathways that govern osteoblast turnover

Bone growth and bone formation are regulated by hormones, growth factors, and cytokines, e.g., IGF-I, BMPs, FGFs, Wnt, Notch, PTH, as well as nutrition, mechanical loading, and aging (Bonewald and Johnson, 2008). Some are involved not only in rapid bone growth but also in adult bone remodeling. We will summarize the signaling pathways that are activated by these factors, present evidence that these pathways are involved in regulating osteoblast turnover, and discuss the possible mechanisms by which these pathways regulate osteoblast turnover. We will then discuss the interwined crosstalk among these pathways and, finally, we will touch on the major challenge in understanding the mechanisms of osteoblast turnover.

The BMP-Smad pathway

BMPs are initially isolated from demineralized bones and possess the ability to induce ectopic bone growth when implanted in muscle (Wang et al., 1988; Wozney et al., 1988). To date, more than a dozen BMPs have been identified, which constitute a subfamily of the TGF-ß superfamily (Feng and Derynck, 2005; Massague et al., 2005). Unlike TGF-ß, which uses Smad2/3 as signal transducers, BMPs use Smad1/5/8 (B-Smads). BMPs can elicit the canonical BMPR-Smad pathway, and the non-canonical MAPK pathway and PI-3K-Akt pathway (Derynck and Zhang, 2003; ten Dijke and Hill, 2004). BMPs bind to the cell surface BMP receptor type I and type II, both of which are Ser/Thr kinases. The ligand binding first activates BMPRII, which leads to phosphorylation and activation of BMPRI, enabling BMPRI to phosphorylate the C-terminal SXS motif of Smad1/5/8. SXS-phosphorylated B-Smad forms a complex with Smad4 (co-Smad for both BMPs and TGF-ß) and they accumulate in the nucleus, where they bind to the Smad binding elements (SBEs) or GC-rich

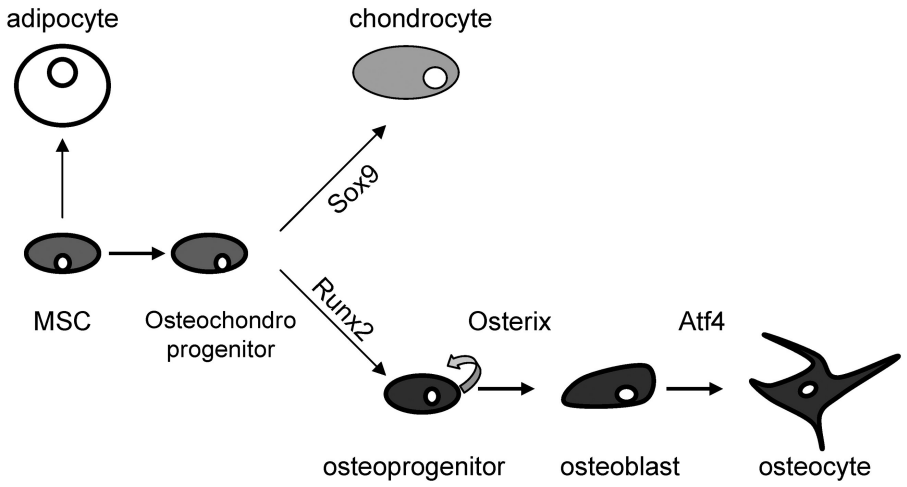

Fig. 1. Differentiation of mesenchymal stem cells to mature osteocytes and other cell lineages, and trascription factors involved in those processes.

sequences to regulate transcription of BMP target genes (Fig. 2).

A surprising finding in BMP/TGF-ß-Smad signaling is that there exist negative regulators at each step of the BMP-Smad pathway (Derynck and Zhang, 2003; Itoh and ten Dijke, 2007). There are a number of BMP antagonists in the extracellular matrix that bind to BMPs and inhibit their activity, e.g. noggin, chordin, gremlin. At the receptor level, a decoy BMPR can inhibit BMPs from binding to their receptors (Onichtchouk et al., 1999). Inhibitory Smads, Smad6 and Smad7, exert their effect on B-Smads signaling by interfering with the interaction of B-Smads with BMPRI, Smad4, and/or binding to SBE of target genes. Smad signaling can also be inhibited by interacting with Ski and Sno in the nucleus. In addition, B-Smads can be quickly degraded by the ubiquitin-proteosome system, which is mediated by Smurf1/Smurf2, the E3 ubiquitin ligases (Lee et al., 2003; Dupont et al., 2005). Furthermore, B-Smads can be negatively regulated by modification in the linker region that inhibits their nuclear entry (Fuentealba et al., 2007; Sapkota et al., 2007).

BMP-Smad signaling is essential for early embryonic development of mouse. Individual deletion of many of the signaling molecules in this pathway leads to embryonic lethality, preventing the analysis of their physiological function (Li, 2008). However, this problem can be circumvented by the Cre-LoxP system. Crossing transgenic mice that express the recombinase Cre under the control of osteoblast stage-specific promoters, e.g., collagen type Iα, Osterix, osteocalcin, or Dermo, with mice carrying the floxed allele of the BMP-Smad signaling molecule, has clearly demonstrated a positive role for BMP-Smad pathway in bone growth and remodeling, except BMP3, which plays a negative role (Rodda and McMahon, 2006). Furthermore, genetic deletion of BMPR or B-Smads in other tissues revealed a tumor suppressor activity for BMP-Smad pathway (Li, 2008).

In vitro and *in vivo* studies have established that BMP-Smad signaling regulates several aspects of osteoblast turnover. These include osteoblast differentiation from MSC, osteoprogenitor cell expansion, osteoblast bone formation activity, and its coupling to osteoclasts. It has been shown that BMPs can induce osteoblast differentiation from MSC and increase the vigor of osteoblasts in *in vitro* mineralization (Cao and Chen, 2005; Miyazono et al., 2005). Deletion of BMP2 and 4 results in mice with defective bone development and osteoblast function (Winnier et al., 1995). Mice with conditional deletion of BMPRIA in osteoblasts or overexpression of noggin in osteoblasts show osteopenia, with a decrease in the number of osteoblasts and a defect in osteoblast differentiation and mineralization (Mishina et al., 1995; Wu et al., 2003). While the cause of the reduction in the number of osteoblasts is unclear, the differentiation defect is believed to be caused by a down-regulation of the expression of Runx2 and Osterix. Both *in vitro* and *in vivo* data show that inhibition of the BMP-Smad signaling greatly impedes the expression of Osterix and Runx2 (Miyazono et al., 2004; Wang et al., 2007). It is

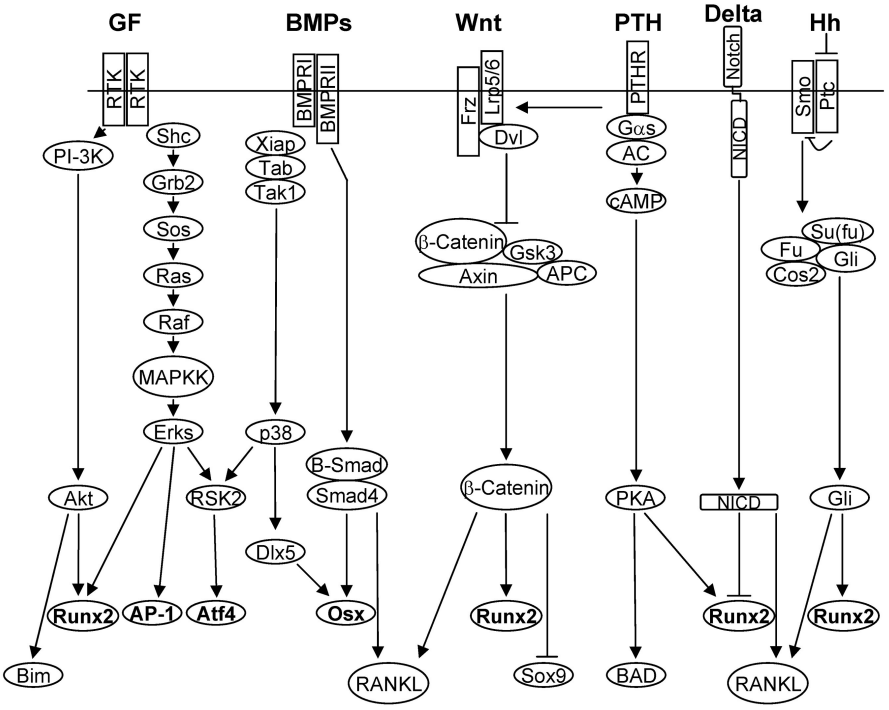

Fig. 2. The major signaling pathway governing osteoblast homeostasis.

worth noting that Osterix induction by BMPs is more dramatic and lasts much longer than Runx2 in cell-based studies. Moreover, Runx2 has been found to interact with Smad1 and Smad5 and this interaction enhances the expression of some osteoblast-specific genes (Zhang et al., 2000). However, there is a lack of evidence to support that Runx2 and Osterix are direct target genes of R-Smads. So far, one study shows that Osterix induction by BMPs is mediated by Dlx5, a homeodomain-containing transcription factor that plays an important role in bone development (Ulsamer et al., 2008).

While an important role for BMP signaling in osteoblastogenesis and activity has been well established, recent studies revealed a surprising role of BMP-Smad signaling in osteoclastogenesis (Kamiya et al., 2008a,b). Inducible deletion of BMPRIA in osteoblasts during embryo or postnatal stages leads to increased bone mass in mouse. Although these mice do show reduced bone formation, interestingly, they also show a decrease in osteoclastogenesis due to compromised synthesis of RANKL and increased synthesis of OPG. The decreased bone resorption appears to be a dominant event in bone remodeling in these mice.

MAPK pathway downstream of growth factors and cytokines

MAPKs are classified into Erks, JNKs, and p38 MAPKs. In general, Erks are mainly activated by mitogens, such as growth factors IGFs and FGFs, while JNKs and p38 MAPKs are mainly activated by cytokines, such as TGF-ß and BMPs, and various kinds of stress, e.g., oxidative stress and genotoxic stress (Kolch, 2005; Murphy and Blenis, 2006). BMPs have been shown to activate all three classes of MAPKs (Moustakas and Heldin, 2005).

The best studied Erk activators in osteoblast function are FGFs and IGFs, which bind to their cell surface receptor tyrosine kinases (RTK), leading to RTK dimerization and subsequent activation (McKay and Morrison, 2007). These RTKs phosphorylate themselves, as well as adaptor proteins such as Shc and Grb2, which form a complex with RTKs. The phosphorylation sites on Grb2 are recognized by Sos, which bring Ras to the proximity of the plasma membrane, leading to Ras activation. Ras can then stimulate the downstream Raf (MAPKKK), MEK1/2 (MAPKK) and Erk1/2 (MAPK). This allows Erks to phosphorylate transcription factors such as c-Jun and c-Fos to regulate transcription of target genes in the nucleus. This pathway in general has a pro-proliferation function. Further downstream, Erks and p38 MAPKs can also phosphorylate and activate some of the other Ser/Thr kinases. These include RSKs (ribosomal S6 kinases) and MKs (MAPK kinase activated kinases) (Anjum and Blenis, 2008) (Fig. 2). These downstream kinases can in turn phosphorylate their own substrates to regulate cell function. Insulin-like factors (IGF-I and -II) also activate the MAPK pathway with a similar mechanism to FGFs, except that there is an additional IRS (IGF receptor substrates) downstream IGF-IR and upstream of the Shc-Grb2-Ras-Erk. In addition, RTKs can also activate the PI-3 kinase-Akt pathway, which has an anti-apoptotic activity (Fig. 2).

IGFs are synthesized in the liver in response to growth hormone. They can reach blood circulation and act on many organs, including bones. In fact, IGFs are important mediators of growth hormone during pubertal bone growth. They are also synthesized and secreted by osteoblasts and exist substantially in the bone matrix (Niu and Rosen, 2005). However, the function of IGFs in bone growth and remodeling is still debatable as contradictory results have been obtained from different studies. *In vitro* studies have shown that IGFs, as well as FGFs, promote proliferation and inhibit differentiation of MSC and osteoblasts. One possible mechanism by which RTK signaling hampers osteoblast differentiation is by inhibiting the Wnt pathway (Mansukhani et al., 2005; Ambrosetti et al., 2008). FGF-FGFR signaling is found to up-regulate the expression of Sox2, a transcription factor that represses osteoblast differentiation (Mansukhani et al., 2005). An increase in Sox2 is found to repress Wnt signaling, as well as the expression of Wnt target genes. Contrary to cell-based studies, mouse genetic studies indicate that IGF-I plays a necessary role in bone growth and accrual. Mice with overexpression of IGF-I in osteoblasts show increased bone mass and volume, and increased bone formation rate. Surprisingly, the number of osteoblasts is not affected, suggesting that enhanced differentiation might be accountable for the phenotype (Zhao et al., 2000). Moreover, osteoblast-specific deletion of IGF-IR, as well as universal deletion of IGF-I, leads to decreased bone mass and decreased bone formation in mouse (Liu et al., 1993; Zhang et al., 2002). Similarly, disruption of IGF-I in liver also leads to low bone mass and reduced bone formation rates in mouse (Yakar et al., 1999). A noteworthy point is that the number of osteoblasts is not significantly altered in these mouse models, which is contrary to the *in vitro* cell based studies. How do IGFs regulate osteoblast differentiation *in vivo*? One clue comes from a study showing that in cooperation with BMP2, IGF-I could induce the expression of Osterix, through the MAPK pathway (Celil and Campbell, 2005). However, the conflicting results obtained from the *in vitro* and *in vivo* studies await further clarification.

Similarly, while many studies have demonstrated a role for Erk activation in osteoblast differentiation and proliferation, conflicting results have also been reported. In some studies, activation of Erks is found to stimulate OB differentiation in cell cultures, while other studies reported the opposite results. Mouse genetic studies have shown that Erks are required for osteoblast differentiation and bone formation. Transgenic mice expressing constitutively active Erks have increased bone formation and increased osteoblast proliferation and differentiation, while the dominant negative Erks show the opposite results (Ge et al., 2007). The role of

Erks in controlling osteoblast differentiation is likely to be mediated by Runx2, as Erks could phosphorylate Runx2 and this phosphorylation is associated with enhanced transcription activity (Ge et al., 2007).

Activation of JNK and p38 MAPKs by cytokines, especially TGF-ß and BMPs, is mediated by the Tak1-Tab1 complex. It has been shown that TGF-ß receptors could recruit Tak1-Tab1 through Xiap and/or TRAF molecules (Fig. 2). Tak1 is the MAPKKK for JNK and p38 MAPK. How do p38 MAPKs regulate osteoblast function and turnover? A number of studies have shown that inhibition of p38 MAPK with inhibitors or dominant negative p38 MAPK diminishes osteoblast differentiation, accompanied by a down-regulation of Osterix, but not Runx2 (Li et al., 2002b; Guicheux et al., 2003; Hu et al., 2003). Moreover, p38 MAPK is found to be required for maximal Osterix induction, at the basal level or in response to BMP stimulation. When Osterix is overexpressed, the effects of p38 MAPK inhibition on osteoblast differentiation are diminished (Wang et al., 2007). Recent studies also show that BMPs could stimulate Osterix expression through Dlx5. p38 MAPK could phosphorylate Dlx5 at Ser34 and Ser217 to enhance the transcription of Osterix (Ulsamer et al., 2008). These findings indicate that p38 MAPKs might be mediating the effects of BMPs on osteoblast activity. On the other hand, cell based studies show that p38 MAPK does not affect proliferation of MSC cells, calvarial osteoblasts, or transformed cells lines. Though much *in vitro* studies have been conducted, *in vivo* evidence for p38 MAPK in bone formation is still lacking.

In addition to directly modifying and/or controlling the expression of transcription factors involved in osteoblast differentiation, both p38 MAPK and Erks are known to activate RSK2, which is a disease gene for the development of Coffin-Lowry Syndrome (CLS), an X-linked mental retardation disorder associated with skeletal abnormalities (Hunter, 2002). Mouse genetic studies have confirmed that RSK2 is required for osteoblast differentiation and function. One of the RSK2 substrates is transcription factor Atf4. Further studies show that Atf4 is required for osteoblast differentiation (Yang et al., 2004). Therefore, MAPKs might regulate osteoblast differentiation through the downstream MAPK activated kinases.

AKT Pathway

Another important pathway downstream of RTK is PI-3K-Akt, which has a prominent anti-apoptotic activity, as previously mentioned. PTEN (phosphatase and tensin homolog) negatively regulates the intracellular levels of phosphatidylinositol-3, 4, 5-trisphosphate and acts as a negative regulator of Akt signaling. Akt can also be activated by BMPs in osteoblasts (Ghosh-Choudhury et al., 2002). Universal deletion of Akt1 results in a mouse line featuring osteopenia, which is accompanied by a defect in osteoblast differentiation and an increase in osteoblast apoptosis (Kawamura et al., 2007). In accordance, conditional deletion of Pten in osteo-chondroprogenitors (with Collagen type II-Cre (Col2α1-Cre)) and mature osteoblasts (with osteocalcin-Cre) results in mouse lines featuring osteosclerosis, with osteoblasts showing hastened differentiation and compromised apoptosis. Inactivation of the Akt pathway also leads to a down-regulation of Runx2, which might account for compromised differentiation, and an up-regulation of proapoptotic protein Bim, which might be a cause of enhanced osteoblast apoptosis (Ford-Hutchinson et al., 2007; Liu et al., 2007). Furthermore, it is also found that Akt plays a positive role in IGF-I induced expression of Runx2 (Fujita et al., 2004).

Wnt-ß-Catenin pathway

Wnt family has at least 19 members and they play important roles in embryogenesis, postnatal development, and tumorigenesis (Moon et al., 2004; Clevers, 2006). They function by binding to the cell surface receptor Frizzled (Frz), a 7 transmembrane protein, together with the co-receptors LRP5/6 (low density lipoprotein receptor-related protein), to activate the downstream GSK3-ß-Catenin pathway (He et al., 2004). Under resting conditions, APC (adenomatous polyposis coli), GSK3 (glycogen synthase kinase 3), Axin, and ß-Catenin form a signaling complex. GSK3 can phosphorylate ß-Catenin, leading to a proteosome-dependent degradation of ß-Catenin. Upon Wnt stimulation, Dishevelled (Dvl), Axin and Frat-1 are involved in the inactivation of GSK3 and disruption of the signaling complex, leading to ß-Catenin stabilization. ß-Catenin then accumulates in the nucleus, where it can form a complex with transcription factors TCF/LEF, displacing a transcription repression complex and subsequently turning on Wnt target genes (Fig. 2). Among many target genes, Myc, cyclin D, c-Jun, Fra-1, BMP4, FGF18, Runx2, Twist and osteocalcin might play a role in osteoblast turnover. In addition to the canonical pathway, Wnt can also activate non-canonical pathways such as JNK and PKC. Wnt pathway is also negatively regulated by extracellular proteins that can bind Wnt or LRP5/6. For example, Sost and DKK can bind LRP5/6 and inhibit the activation of Wnt pathway (Bafico et al., 2001; Li et al., 2005; Semenov et al., 2005).

A critical role for the Wnt pathway in bone development and homeostasis has been demonstrated by human and mouse genetic studies, as well as cell based studies. Wnt pathway regulates many aspects of osteoblast activities, including commitment of MSC, osteoblast progenitor amplification, terminal differentiation and mineralization, and cell death (Krishnan et al., 2006; Glass and Karsenty, 2007; Bonewald and Johnson, 2008). Patients with loss-of-function mutation of LRP5 show severe osteoporosis, while the opposite is true for patients with constitutive active LRP5 (Gong et al., 2001; Boyden et al., 2002).

Genetically engineered mice recapitulate these human disorders. Mice deficient for LRP5 show reduced bone mass and reduced proliferation of osteoblasts, while transgenic mice overexpressing active LRP5 (G171V) show an increase in osteoblast activity and number (Kato et al., 2002; Babij et al., 2003). Further studies have indicated that the Wnt pathway regulates osteoblastogenesis and bone formation through the canonical pathway. Inhibition of GSK3 with lithium or other inhibitors is found to stimulate osteoblastogenesis in cultures and in mouse models (Kulkarni et al., 2006). The generation of stage-specific conditional ß-Catenin knockout mouse lines has greatly advanced our understanding of the physiological function of the Wnt-ß-Catenin pathway in osteoblast turnover. For example, in MSC, deletion of ß-Catenin by Dermo-Cre reveals that it is required for early osteoblast commitment, as the mice showed enhanced chondrogenesis and decreased osteoblastogenesis (Rodda and McMahon, 2006). This could be explained by the finding that the Wnt pathway represses the expression of Sox9, a transcription factor required for chondrogenesis (Akiyama et al., 2004). In Osterix-Cre mice, there is reduced calcification of bones and a lack of mature osteocalcin-expressing osteoblasts, while ectopic expression of ß-Catenin using Osterix promoter gives rise to opposite results (Rodda and McMahon, 2006).

We now know that the Wnt-ß-Catenin pathway regulates osteoblast turnover, but what are the mechanisms? In MSC-like cells, Wnt not only stimulates the expression of Runx2 and osteocalcin, but also represses C/EBP alpha, PPAR gamma, and Sox9. This may explain why the Wnt pathway favors osteoblast differentiation and inhibits chondrocyte differentiation (Day et al., 2005; Hill et al., 2005). In the aspects of osteoblast proliferation and apoptosis, the Wnt pathway has been found to regulate the expression of Myc and cyclin D, c-Jun and Fra-1, which are known to have pro-proliferation effects, and Wnt activated Erks and Akt pathways are important in regulating the expression of the antiapoptotic protein Bcl2. However, in many cases, there is still a lack of *in vivo* evidence to pinpoint which of these genes is the main contributor.

A recent study suggests that Lrp5 might regulate bone remodeling by controlling the expression of serotonin (Yadav et al., 2008). Lrp5 inhibits the expression of Tph1, a rate-limiting enzyme for serotonin synthesis in enterochromaffin. Lrp5-/- mice show an up-regulation of serotonin, and decreasing the blood levels of serotonin normalizes bone formation and bone mass in these mice. Moreover, gut-specific, but not osteoblast-specific, Lrp5 deletion decreases bone formation, while gut-specific activation of Lrp5, or inactivation of Tph1, increases bone mass. Serotonin acts on osteoblasts through the Htr1b receptor and CREB to inhibit their proliferation. Thus, duodenum-derived serotonin can act as a hormone that inhibits bone formation.

In addition to osteoblast autonomous effects, the Wnt-ß-Catenin pathway also has a secondary effect on osteoclastogenesis. Conditional deletion of ß-Catenin by collagen 1α-Cre or osteocalcin-Cre leads to osteopenia in mouse. In addition to a modest defect in osteoblast differentiation, the knockout mice also showed enhanced osteoclastogenesis and bone resorption, due to an increase in RANKL and a reduction in OPG (Glass et al., 2005).

PTH and PKA

Parathyroid hormone (PTH), an 84 aa peptide hormone, is synthesized and secreted by the parathyroid gland. It mainly acts on bones, kidney and intestines. High levels of PTH lead to an increase in blood Ca^{2+} levels, due to enhanced bone resorption by osteoclasts and enhanced Ca^{2+} absorption by the intestines, due to PTH-induced expression of vitamin D (Swarthout et al., 2002). The effect of PTH on Ca^{2+} release from bones needs osteoblasts, as osteoclasts do not express PTH receptors. The binding of PTH to osteoblast receptors induces the expression of RANKL, which in turn stimulates osteoclastogenesis and bone resorption, leading to the release of Ca^{2+}. On the other hand, Parathyroid hormone-related proteins (PTHrP) are expressed by many cell types and they appear to have a much broader effect on cell proliferation, differentiation and apoptosis. While they do have a similar function in inducing hypercalcimia as PTH, PTHrPs also play a unique role in chondrogenesis, being necessary for chondrocyte proliferation and differentiation (van der Eerden et al., 2003; Kronenberg, 2006). Moreover, osteoblast-specific deletion of PTHrP led to decreased bone mass in mouse, followed by a drop in osteoprogenitor cell proliferation, differentiation, and increased apoptosis (Martin, 2005).

While continuous infusion of PTH leads to enhanced bone resorption, intermittent application of PTH shows anabolic effects in mouse, rat, and human, mainly due to the elevated number of osteoblasts. Intermittent PTH has been reported to stimulate osteoblastogenesis and inhibit osteoblast apoptosis (Rosen, 2004; Jilka, 2007). In postmenopausal women, men, as well as mouse models, injection of PTH (1-34) was found to increase bone mass, bone formation and the number of osteoblasts (Hodsman et al., 2005).

PTH, as well as PTHrP, binds to the seven transmembrane G protein coupled receptors on the cell surface, which activate Gαs and, subsequently, adenylyl cyclase, leading to an increase in the cellular levels of cAMP. cAMP binds to the regulatory subunits of the tetrameric PKA, releasing its inhibitory effects on the catalytic subunits. Activated PKA then phosphorylates its substrates on Ser/Thr residues to regulate their function. One of the best studied substrates of PKA is transcription factor CEBP. In addition, PTH can also activate Gαq, which activates phospholipase C (PLC), and subsequently PKC and MAPKs. *In vivo* studies show that intermittent application of PTH decreases the number of TUNEL positive apoptotic osteoblasts,

accompanied by an increase in the number of osteoblasts. How does PTH inhibit osteoblast apoptosis? Cell based studies suggest that PTH-activated PKA can phosphorylate pro-apoptotic protein BAD, leading to its deactivation (Yamamoto et al., 2007). In addition, it can also stimulate the transcription of anti-apoptotic protein Bcl2 (Yamashita et al., 2008). On the other hand, intermittent application of PTH shows no significant effect on the proliferation of osteoblasts or their progenitors. Instead, PTH was found to repress osteoblast proliferation by up-regulating p21 and p27. The exit from cell cycle is generally believed to facilitate differentiation (Qin et al., 2005). PTH has also been found to up-regulate the protein levels of Runx2 and to enhance the transcription activity of Runx2 (Fig. 2) (Krishnan et al., 2003; Merciris et al., 2007). This might be one of the mechanisms behind PTH's effect on osteoblast differentiation.

Though it may look clear-cut at first glance, the effects of intermittent PTH on bone formation can be more complex. For example, administration of PTH has been reported to up-regulate the levels of IGF-I *in vitro* and *in vivo* (Jilka, 2007). The anabolic effect of PTH is diminished in IGF-I knockout mice, suggesting that IGF-I and its downstream pathways also play an important role in PTH-induced bone formation (Miyakoshi et al., 2001). Moreover, PTH has been found to trigger the activation of the ß-Catenin pathway and regulate osteoblastogenesis through the LRP5/6 pathway. A study reported that PTH-PTHR could recruit LRP6, which promotes LRP6 phosphorylation and the binding of Axin to LRP6, leading to ß-Catenin stabilization (Wan et al., 2008).

Notch signaling

Notch signaling is well known for its role in determining cell fate. In mammals, four Notch receptors (Notch 1-4) have been identified. Their ligands include Delta 1, 3, 4, and Jagged 1, 2. Since Notch receptors and their ligands are transmembrane proteins, cell-cell interaction is required for activating Notch signaling cascades (Fiuza and Arias, 2007). Notch proteins can be divided into three parts: an extracellular domain, a transmembrane segment, and an intracellular domain (NICD) (Bray, 2000). Notch-ligand interaction on the extracellular domain leads to the exposure of a metalloprotease site, which can be cleaved by ADAM (a disintegrin and metalloprotease). Subsequent intracellular cleavage mediated by γ-secretase complex, which includes presenilin 1 or presenilin 2, nicastrin, APH1 and PEN2, releases NICD to the cytoplasm (Brou et al., 2000; Bray, 2006). The released NICD translocates into the nucleus and assembles with transcriptional factor CSL (mammal C-promoter-binding factor 1) to regulate the expression of the target genes.

Notch 1 and Notch 2 are generally expressed in osteoblasts, while Notch 3 and 4 are only expressed in selected subsets of the osteoblast lineage (Pereira et al., 2000; Engin et al., 2008). Several studies suggest that Notch signaling is involved in osteoblastogenesis, but its exact role is less well defined. A number of *in vitro* studies support the notion that Notch impairs osteoblastogenesis by suppressing osteoblast differentiation (Deregowski et al., 2006), but other studies suggest that osteoblast differentiation is regulated positively by Notch signaling (Tezuka et al., 2002). Although *in vivo* studies support the hypothesis of Notch opposing osteoblast differentiation, the proposed molecular mechanisms and the observed phenotypes of different lines of transgenic and knockout mice are quite different. In a gain of function study, Engin et al. show that enhanced Notch function results in severe osteosclerosis. Ectopically expressed Notch stimulates the proliferation of early osteoblast precursors, probably through upregulation of cyclin D and cyclin E, but their differentiation into mature osteoblasts is inhibited, leading to the expansion of a pool of immature non-functional osteoblasts. Moreover, NICD is able to bind to Runx2 and repress its transactivation ability, hence disturbing its function on osteoblast maturation (Engin et al., 2008). The Runx2 transcriptional activity may also be downregulated by HEY1 (HES-related with YRPW motif 1), a direct target of Notch. It has been shown that HEY1 abrogates Runx2 transcriptional activity and negatively regulates BMP-induced osteoblast maturation. However, transgenic mice overexpressing NICD reported by Zanottie et al. seem to exhibit osteopenia (Zanotti et al., 2008). They show that overexpression of NICD impairs the process of osteoblastogenesis by suppressing the Wnt/beta-Catenin pathway (Sciaudone et al., 2003; Zanotti et al., 2008). Indeed, Notch impairing the Wnt-ß-Catenin pathway is also supported by other *in vitro* studies from the same research group (Deregowski et al., 2006). The phenotypic differences of the two lines of transgenic mice may be due to the difference in type I collagen promoter fragments.

In the loss of function studies, Engin et al. show that disruption of Notch function in osteoblasts leads to osteoporosis, due to an enhanced osteoclastogenesis and increased bone resorption. It is revealed that Notch signaling in osteoblasts represses osteoclast differentiation by regulating OPG expression (Engin et al., 2008). Providing more support to this notion, Hilton et al. show that Notch signaling in osteoblasts mediates osteoclastogenesis by the production of RANKL and OPG. The RANKL/OPG ratio is higher in the Notch dysfunctional osteoblasts, leading to the increase of osteoclast number in the mutant mice (Hilton et al., 2008). In the same study, Hilton et al. also reported that disruption of Notch function in mice enhances osteoblast differentiation from precursors, suggesting a role of Notch in suppressing osteoblast differentiation to maintain a pool of osteoblast precursors (Hilton et al., 2008). This may be mediated by two Notch targets, HES and HEY, which are able to bind to Runx2, leading to disruption of its transcriptional activity. Although there

are discrepant results from *in vitro* studies, *in vivo* models consistently support an inhibitory role of Notch signaling on osteoblast differentiation.

Other emerging pathways

In addition to those discussed pathways, a major breakthrough of bone and mineral research is the discovery that adipocyte-secreted leptin acts as a regulator of bone homeostasis (Karsenty, 2006). Leptin is capable of acting on a subgroup of neurons in the hypothalamus to activate the sympathetic nervous system, which in turn acts on osteoblasts through ß2-AR (adrenergic receptor) and the PKA-Atf4 pathway to regulate osteoblast activity, and the expression of RANKL. The consequence is such that both bone formation and resorption are regulated by leptin.

Another story comes from studies on mouse lines deficient for Atm, c-Abl, Mdm2, or p53, genes involved in the DNA damage response. While these molecules play critical roles in cell response to genotoxic stress, acting as a barrier for tumorigenesis, they are also found to regulate osteoblast differentiation and bone formation. Mice with c-Abl (Li et al., 2000, 2004), Atm (Hishiya et al., 2005; Rasheed et al., 2006), or Mdm2 (Lengner et al., 2006) deletion show osteopenia/osteoporosis, accompanied by reduced bone formation and a defect in osteoblast differentiation, whereas mice deficient for p53 show osteosclerosis, accompanied by increased bone formation and hastened osteoblast differentiation (Lengner et al., 2006; Wang et al., 2006). In addition, p53-/- osteoblasts also show an enhanced capacity in supporting osteoclastogenesis by up-regulating CSF-1 (Wang et al., 2006). These proteins appear to affect the expression of Runx2 and/or Osterix. There is a possibility that this pathway is linked to the BMP-Smad pathway and/or Wnt-ß-Catenin pathway. To add to this already complicated picture, a recent publication reported that Runx2 also plays a critical role in DNA damage, genome stability and tumorigenesis (Zaidi et al., 2007).

Another emerging pathway is Hedgehog (Hh)-Smo-Gli. Indian hedgehog has been known to play essential role in chondrocyte/osteoblast proliferation and differentiation during endochondral ossification. Recent studies have shown that Hh also plays a positive role in osteoblast proliferation and differentiation, although different knockout mouse lines with altered Hh signaling show dissimilar bone phenotypes (Mundy and Yang, 2008). One study reported that mice with one allele of Patched, a negative regulator of Hh signaling, show increased bone mass and enhanced osteoblast differentiation due to the alleviation of Gli3's inhibitory effect on Runx2 (Ohba et al., 2008), while another study reported that mice with mature osteoblast-specific deletion of Patched show enhanced osteoclastogenesis and bone resorption due to an up-regulation of RANKL, and mice with Smo deleted in mature osteoblasts show the opposite results (Mak et al., 2008). Further investigation will be needed to figure out the true function of Hh signaling in osteoblast turnover.

Interaction among the signaling pathways

MAPK and Smads

As discussed earlier, BMPs could activate MAPKs in a Tak1-dependent manner, in addition to the canonical Smad1/5/8 pathway. Moreover, MAPKs can also be activated by growth factors and cytokines. In fact, crosstalk between MAPK pathway and Smad1 pathway is exemplified in BMP-induced Osterix expression and osteoblast differentiation, where both Smad1 and MAPK play a positive role. On the other hand, MAPKs have been found to play a negative role in Smad1 signaling. BMPs can activate MAPKs, which in turn impede Smad1 activation. MAPKs, as well as GSKs, can phosphorylate a few Ser/Thr residues located in the linker region between MH1 and MH2 of Smad1. Phosphorylation of these sites seems to inhibit Smad1 nuclear transport and/or enhance Smad1 degradation (Fig. 2) (Fuentealba et al., 2007; Sapkota et al., 2007). However, the physiological significance of the interaction between these two pathways needs further investigation.

Wnt and BMPs

A reciprocal regulation between the BMP-Smad pathway and the Wnt-ß-Catenin pathway has been observed in osteoblasts as well as other cell types. BMPs have been found to upregulate Wnt1 and Wnt3a, while Wnt has been shown to up-regulate BMP4 and 6 (Dai et al., 2008; Guo and Wang, 2009). Therefore, these two pathways might cooperate in regulating osteoblast differentiation. A review of the literature revealed more support for the notion that BMP-induced osteoblast differentiation requires activation of the Wnt pathway (Rawadi et al., 2003; Chen et al., 2007; Fujita and Janz, 2007; Qiang et al., 2008). Blocking the Wnt pathway negatively affects BMP-induced osteoblast differentiation. However, there are also studies suggesting that the BMP-Smad pathway might negatively regulate Wnt-ß-Catenin signaling. For example, Smad1 was found to interact with Dvl in MSC, and this interaction seems to inhibit Wnt signaling (Liu et al., 2006) (Fig. 2). *In vivo* studies show that loss of BMP signaling leads to a decrease in Sost, thus enhancing Wnt signaling (Kamiya et al., 2008b). Moreover, in hair follicles, a loss of BMPRIA also leads to elevated levels of Lef1 and ß-Catenin, resulting in expansion of the niche and loss of slow cycling cells (Kobielak et al., 2003).

Multiple pathways to regulate bone formation in response to mechanical stress

It is well established that mechanical loading or gravity stress is important for proper bone remodeling. This is because weight-bearing exercise can promote

osteoblast activity and increase bone formation (Hughes-Fulford, 2004; Robling et al., 2006). Lack of physical activity due to injuries or gravity loss due to space travel can cause osteoporosis. While the mechanisms for the pathogenesis are still nebulous, recent studies indicate that mechanotransduction controls the expression of many genes that are involved in osteoblast proliferation and differentiation. Mechanotransduction involves multiple signaling pathways, including Wnt-ß-Catenin, integrins, Ca^{2+}, PKA, and MAPKs. Some of these claims are supported by *in vivo* studies. For example, mouse studies suggest that both Wnt and Ca^{2+} play a role in mechanotransduction, as the high bone mass Lrp5 (G171V) mutant mice show overadaptation to normal loading forces, and animals treated with Ca^{2+} blocker show up to a 61% decrease in bone formation after mechanical stress (Li et al., 2002a; Akhter et al., 2004). In addition, cell based studies also indicate that the MAPK pathway, activated by growth factors, TGF-ß, and intergrin, and PKA/PKCs activated by G protein coupled receptors, also play important roles in mechanotransduction. These signals are integrated to regulate the expression of proteins such as c-Fos, Egr-1, c-Jun, and Runx2, which are known to be involved in osteoblast proliferation and differentiation (Hughes-Fulford, 2004).

Perspectives

The signaling pathways discussed above have been delineated by studies from various cell types, in which they appear to have cell type-specific effects. Despite the great progress made in the last decade, we are still baffled by questions like i) what activate these signaling pathways? ii) what are the transcription factors and other effector molecules downstream of these signaling pathways? iii) how do they crosstalk, at different stages of osteoblast turnover? To answer these questions, stage-specific knockout mouse lines will be needed. In addition, since growth factors or cytokines could activate multiple pathways, it is necessary to determine which pathway(s) plays a dominant role in regulating osteoblast turnover. This can be solved by generating mouse lines altering two or more genes in different pathways. For example, a mouse line with osteoblast-specific deletion of BMPRIA and an overexpression of constitutively active Erk1 might be able to tell the importance of Erk activation in BMP-controlled osteoblast homeostasis. A mouse line with osteoblast-specific deletion of IGF-IR and osteoblast-specific deletion of Pten might help us to understand the significance of Akt activation in IGF-I mediated osteoblast turnover. Similarly, a mouse line with osteoblast-specific expression of constitutive active Lrp5 mutant and osteoblast-specific deletion of BMPRIA can be used to test the relationship between BMP-Smad and Wnt-ß-Catenin pathways. For cell based studies, *ex vivo* MSC cultures and calvarial osteoblast cultures should be used as they are closer to the *in vivo* situation than transformed cell lines. Moreover, under *in vivo* conditions, cells are immersed in a mixture of various growth factors, cytokines, and are in contact with ECM and other cell types. Thus, it is virtually impossible to establish *in vitro* culture systems that truly mimic the in vivo situation. As such, conclusions obtained from cell-based studies need to be verified by *in vivo* studies. Only after understanding how osteoblast turnover is precisely controlled at each stage can we select the drug targets to screen for anabolic drugs for osteoporosis prevention/therapy.

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