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Regulation of osteogenic differentiation during skeletal development

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1. ABSTRACT

Bone formation during skeletal development involves a complex coordination among multiple cell types and tissues. Bone is of crucial importance for the human body, providing skeletal support, and serving as a home for the formation of hematopoietic cells and as a reservoir for calcium and phosphate. Bone is also continuously remodeled in vertebrates throughout life. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Early development of the vertebrate skeleton depends on genes that control the distribution and proliferation of cells from cranial neural crest, sclerotomes, and lateral plate mesoderm into mesenchymal condensations, where cells differentiate to osteoblasts. Significant progress has been made over the past decade in our understanding of the molecular framework that controls osteogenic differentiation. A large number of morphogens, signaling molecules, and transcriptional regulators have been implicated in regulating bone development. A partial list of these factors includes the Wnt/β-catenin, TGFβ/BMP, FGF, Notch and Hedgehog signaling pathways, and Runx2, Osterix, ATF4, TAZ, and NFATc1 transcriptional factors. A better understanding of molecular mechanisms behind osteogenic differentiation would not only help us to identify pathogenic causes of bone and skeletal diseases but also lead to the development of targeted therapies for these diseases.

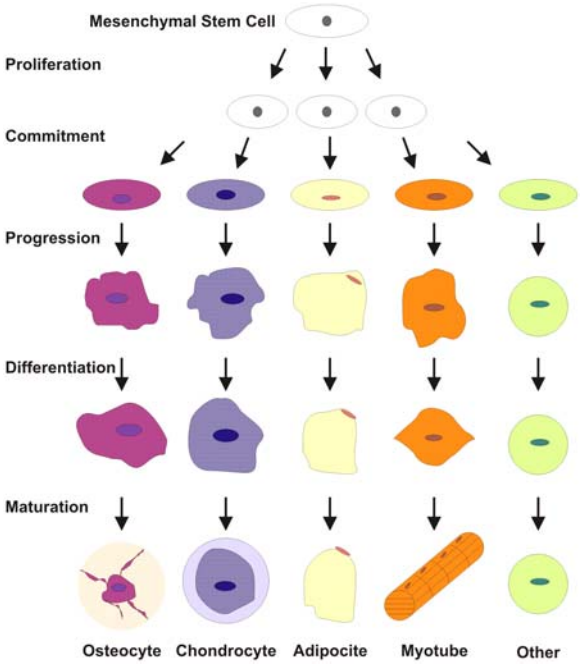

Figure 1. Schematic representation of the lineages derived from mesenchymal stem cell differentiation. See text for details.

2. INTRODUCTION

Osteogenesis during skeletal development results from a well-coordinated sequence of events involving epithelial-mesenchymal interaction, condensation, and differentiation (1-3). Types of bone formation include intramembranous ossification, endochondral ossification, combined endochondral and intramembranous ossification, and heterotopic bone formation. Long bones develop by endochondral ossification, whereas flat bones develop by intramembranous ossification. Bone tissue contains hydroxyapatite and extracellular proteins (1-3). Osteoblasts develop from mesenchymal stem cells (MSCs) (Figure 1). Mature osteoblasts synthesize bone matrix and may further differentiate into osteocytes. Osteocytes maintain structural bone integrity and allow bone to adapt to any mechanical and chemical stimulus. Osteoclasts derive from hematopoietic stem cells. Bone remodeling is carried out by both osteoclasts and osteoblasts (1-3).

Skeletal development involves complex coordination among multiple cell types and tissues. During the development and growth of long bones, a cartilage template surrounded by the perichondrium is first laid down and is subsequently replaced by bone marrow and bone, during a process called endochondral ossification (1-3). Cells in the cartilage template and the surrounding perichondrium are derived from mesenchymal cells, which condense locally. In contrast, many cell types that make up mature bone, in particular the bone marrow are brought in by the vasculature. For the past decade, significant and exciting insights into many aspects of vertebrate skeletal development have been obtained through molecular and genetic studies of animal models and humans with inherited disorders of skeletal morphogenesis, organogenesis, and growth (1-3). Understanding how these signaling pathways and regulatory factors operate during skeletal development through the analyses of genetically altered mice depends on being able to distinguish the effect of these molecules on the different cell types that comprise the skeleton. This review primarily focuses on the complexity of osteogenesis during skeletal development. What emerges is a new paradigm, by which we start to understand the process of normal and abnormal skeletal development (4-16).

3. STEM CELLS AND MESENCHYMAL STEM CELLS

Stem cells are characterized by their ability to self-replicate and to give rise to terminally differentiated cells of multiple lineages (17-22). Although the multi-lineage potential of embryonic stem cells has been well-documented, the ethical, political, and technical issues surrounding their use have encouraged exploration into other sources of stem cells (18, 19, 21). Adult MSCs are nonhematopoeitic stem cells present in the bone marrow and were first described by Friedenstein and Petrakova (23). MSCs provide a source of multipotent stem cells and have the ability to differentiate into cells of connective tissue origins including bone, fat, cartilage, and muscle (24-30) (Figure 1). Although MSCs represent a minimal fraction of nucleated cells in the bone marrow (0.001-0.01%) (25), they can be isolated and expanded easily, and stimulated to differentiate down various lineages in the appropriate culture conditions. Even though MSCs are generally isolated from a bone marrow aspirate of the superior iliac crest of the pelvis in humans (25, 31), MSCs have also been successfully isolated from a number of other tissues including periosteum (32), trabecular bone (33), fat (34), synovium (35), skeletal muscle (36), scalp tissue (37), decidous teeth (38), placenta (39), and umbilical cord blood (40). Although these cells lack tissue specific characteristics, under appropriate external signals they can differentiate into phenotypes distinctly different from their precursor (4, 7, 12).

MSCs have been explored as potential therapies in regenerative medicine (4-7). There has been a precedent of hematopoeitic stem cells use in clinical applications, and transplantation of bone marrow-derived MSCs is relatively new (18, 41, 42). Nonetheless, the potential clinical application of MSCs in bone and musculoskeletal diseases is evident (4-16). For example, successful short-term treatment of osteogenesis imperfecta (OI) has been demonstrated by transplantation of allogenic MSCs without the gene defect into young growing OI patients (43, 44). Furthermore, in orthopaedic applications, the potential use of MSCs is promising as clinical trials have utilized marrow stromal cells in segmental bone defects and articular cartilage defects (6, 7, 16, 45-48).

4. MESENCHYMAL STEM CELL LINEAGES

4.1. Myogenic lineage

Vertebrate muscle development involves the

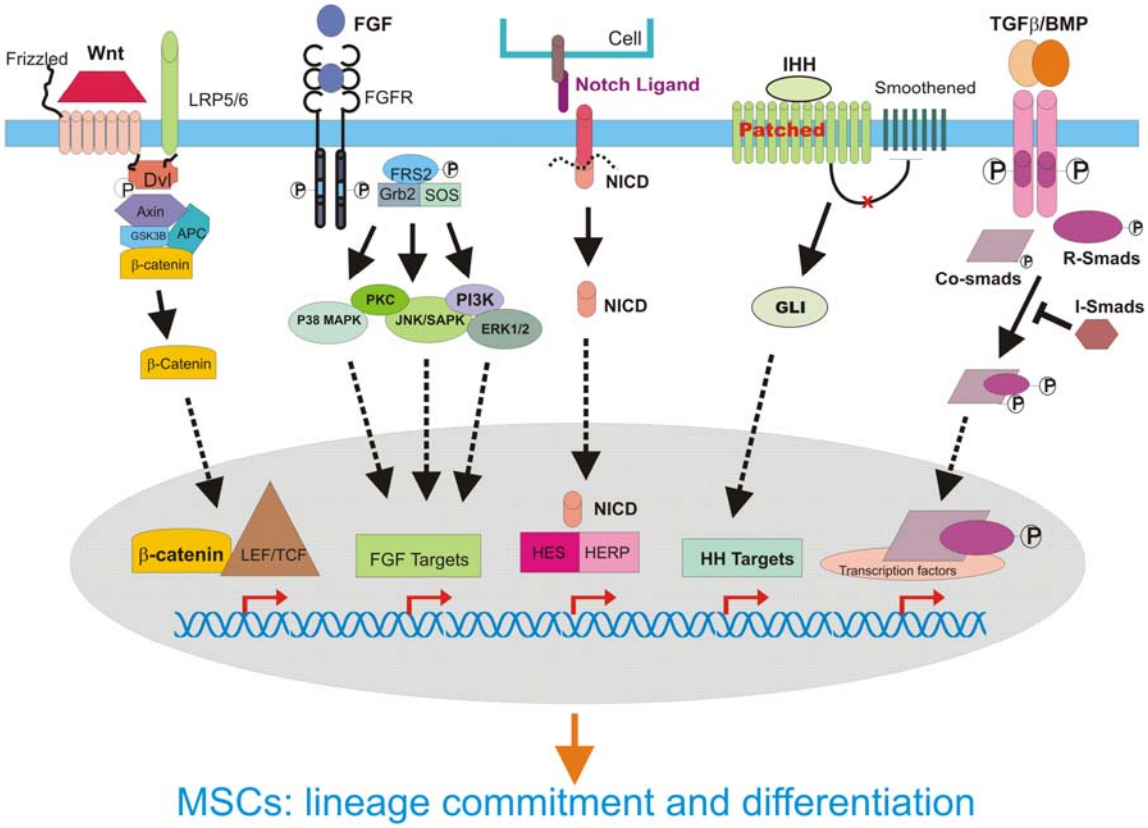

Figure 2. Schematic representation of the major signaling pathways that play an important role in regulating osteogenic differentiation during skeletal development. These include Wnt/β-catenin, TGFβ/BMP, Notch, IHH, and FGF signaling pathways. See text for details.

transition of an uncommitted mesoderm to myotube through a process that is governed by a plethora of genes, morphogens, and cell signaling molecules (49-51). Briefly, uncommitted mesoderm from the somites gives rise to a population of committed, dividing muscle precursor cells called myoblasts (52, 53). The commitment of mesoderm to myoblasts is regulated by a variety of extracellular signals including the MyoD family of basic helix-loop-helix transcription factors, Hedgehog proteins, the Wnt family proteins, and BMPs (54) (Figure 2). For example, the double Myf5/MyoD mutants lack the precursor myoblast population of cells and therefore fail to develop skeletal muscle (55). Following the transition from mesoderm to myoblast, the myoblasts stop dividing and begin to express muscle-specific genes to terminally differentiate into myocytes, and this process depends on the MyoD family as well as the Mef2 family (56). Additionally, recent results suggest that the FGF family of transcription factors plays a role in myoblast proliferation arrest and subsequent expression of muscle-specific genes (57). Ultimately, the terminally differentiated myocytes fuse with one another to form multinucleate myofibers that will make up skeletal muscle (49-51).

4.2. Adipogenic lineage

Adipose tissue was historically regarded as an inactive energy store and a source of insulation. However, over the past three decades there has been an increased interest in adipogenesis and adipocyte function (58). Adipocytes, cells designed for the storage of fat, are either found in loose connective tissue, or may make up the main cell type as in adipose tissue (59). There are two forms of adipose tissue, brown adipose tissue, which is found primarily in newborn mammals and some hibernating animals, and white adipose tissue. Whereas brown adipose tissue is found only in small amounts in adult humans, white adipose tissue makes up 20-25% of total body weight in healthy adult humans and provides energy, insulation and mechanical protection (58, 59).

The development of an adipocyte from a multipotent stem cell occurs through a series of molecular steps that are characterized into two phases (60, 61). The first phase, determination, is the commitment of a multipotent MSC to a preadipocyte or adipoblast (Figure 1). This preadipocyte has lost the ability to differentiate into any other cell type. However it is phenotypically the same as its precursor. The second phase is differentiation in which the preadipocyte becomes a mature adipocyte and gains the capacity to perform lipolysis, synthesize lipids and triglycerides, and secrete adipoctye specific factors. Whereas some preadipoctye cell lines such as the 3T3-L1, 3T3-F442A, or Ob17 cell lines demonstrate an exponential growth phase, followed by a post-confluence mitosis,

called "clonal expansion," other cell lines, such as human preadipocytes, differentiate without mitotic clonal expansion. The mitotic clonal expansion allows for an increase in the proportion of differentiated fat cells. However the necessity of mitotic clonal expansion for adipoctye differentiation remains controversial (60).

4.3. Chondrogenic lineage

Cartilage is a form of supporting structure that comes in three main types: hyaline cartilage, fibrocartilage and elastic cartilage (62, 63). Parallel to adipogenesis, cartilage formation occurs with the differentiation of MSCs into precursor cells called chondroblasts (62, 64-67) (Figure 1). Chondrogenic differentiation can be stimulated *in vitro* when MSCs are grown under conditions that include a 3-dimensional culture format, a serum-free nutrient media, and the use of exogenous cytokines and growth factors, specifically a member of the TGF-β super-family (12). These precursor cells undergo a series of mitotic divisions and secrete an extracellular material, glycosaminoglycan, thereby trapping each chondroblast into a cartilaginous matrix. Subsequently, cells undergo a second series of mitotic divisions to form mature cartilaginous cells called chondrocytes, which maintain the cartilaginous matrix. Chondrocyte differentiation proceeds from the center outward so that cells located in the center differentiate into chondrocytes, whereas cells at the periphery elongate and flatten to form a less differentiated layer of cells, perichondrium, which has the potential to differentiate into mature cartilage. Interactions between the chondrocytes and the perichondrium shape both chondrogenesis and osteogenesis. In long bone development, the cartilage and surrounding perichondrium are eventually replaced by bone and bone marrow, a process called endochondral ossification. The process of endochondral ossification is dependent upon the blood vessels which will ultimately supply many of the cells necessary for mature bone and bone marrow (68).

Multiple exogenous cytokines and growth factors play a role in promoting chondrogenesis and there is overlap of these factors in osteogenic differentiation (Figure 2). The Smad signaling pathway (69) and the MAPK signaling pathways (70) are the two main effectors of TGFβ cytokines in chondrogenic differentiation (64-67). Not only do the TGFβ cytokines simulate chondrogenesis through the MAPK pathway, the Fibroblast Growth Factor (FGF) family of cytokines also stimulates chondrogenic differentiation through MAPK signaling, leading to activation of Sox9 (64-67). Parathyroid Hormone-related peptides and Indian Hedgehog appear to cross talk with the FGF-activated cascades, thereby also demonstrating a role in chondrogenesis (71, 72). Finally, the Insulin-like Growth Factor-I (IGF-I) stimulates chondrogenesis (73) via the Inositol 3-Kinase signaling pathway (74), and furthermore appears to have a synergistic effect with TGFβ1 (32) or FGF-2 (75), suggesting a myriad of crosstalk amongst the pathways (76).

The interplay between chondrogenesis and the conversion of perichondrium to periosteum are highly regulated and appear to demonstrate overlap in their signaling. Specifically, factors such as FGF and bone morphogenetic proteins (BMPs) (71, 77, 78) that regulate chondrocyte proliferation, maturation and hypertrophy also appear to regulate the conversion of perichondrium to periosteum (68). For example, the Indian Hedgehog signaling pathway, which is implicated in chondrocyte differentiation, also appears to regulate the conversion of perichondrium into periosteum (79).

4.4. Osteogenic lineage

Bone is a dynamic tissue with the potential for continuous remodeling and regeneration throughout life (1-3). During skeletogenesis, bone formation can occur through two different pathways, either intramembranous ossification (flat bones of the skull) or endochondral ossification (long bones) (1-3, 6, 80). Whereas intramembranous ossification occurs through direct condensations of MSCs, in endochondral ossification MSCs first form a cartilage precursor that is subsequently replaced by an ossified matrix (1, 81-83).

The continuous remodeling of bone occurs through a dynamic process of breakdown and rebuilding mediated by two key regulators, hematopoietically derived osteoclasts and mesenchymal derived osteoblasts (1-3). Bone mass in adults is maintained by a local balance between osteoclastic bone resorption and osteoblastic activities that are mediated via various signaling molecules, such as morphogens, hormones, growth factors, cytokines, matrix proteins, and transcription factors (5-7, 82-85).

Committed pre-osteoblasts reside near the bone surface and express alkaline phosphatase enzyme, an early marker of osteogenic differentiation (7, 83, 84). Once the preosteoblast differentiates into an active mature osteoblast, it undergoes a phenotypic change; large nucleus, enlarged Golgi, extensive endoplasmic reticulum, to support the secretion of bone matrix proteins such as type I collagen (1). Subsequently, the cell becomes terminally differentiated into an osteocyte and these cells can be distinguished by their location in the lacunae as well as their extensive filapodia processes that allow for connections with adjacent cells (1). Mature osteocyte provides mechanical support and regulates mineral deposition. The stages of osteoblast differentiation, namely proliferation, matrix maturation and mineralization, are paralleled by the expression of osteoblast phenotypic markers reflecting the stages of differentiation (1) (Figure 1). Whereas the proliferation stage demonstrates upregulation of genes associated with the cell cycle, the maturation phase demonstrates expression of the early osteogenic marker, alkaline phosphatase, and finally, mineralization is characterized by expression of late markers of differentiation such as osteocalcin and osteopontin (1). In this review, we will provide a detailed overview of the major signaling pathways and transcriptional factors that regulate osteogenesis.

4.5. Reciprocal relationship between osteogenesis and adipogenesis

It has long been hypothesized that osteoblast and marrow adipocyte lineages may have a close but reciprocal

relationship (86-88). Changes in the balance between osteogenesis and adipogenesis have been postulated as contributing factors to physiologic and pathologic conditions, such as ageing and osteoporosis (86, 89-95). A recent study indicated that aging activates adipogenic and suppresses osteogenic programs in marrow stem cells (95). Clinically, a decrease in bone volume in age-related osteoporosis is usually accompanied by an increase in marrow adipose tissue (89-93, 94). Conversely, patients with progressive osseous hyperplasia have heterotopic bone formation within adipose tissue (96).

While the roles of BMPs in skeletal development have been well recognized (97), little is known about the role of BMP signaling in adipose development (7, 98). Mice lacking the BMP-2 regulated zinc finger protein Schnurri-2 exhibited a significant reduction of in white fat mass (99). The possible role of PPARγ2 in BMP-induced osteogenic and adipogenic differentiation of MSCs is rather intriguing. PPARγ2 is readily up-regulated by osteogenic BMPs in pre-osteoblast progenitor cells (100). PPARγ2 insufficiency was shown to enhance osteogenesis (101), whereas the PPARγ agonist rosiglitazone causes bone loss in mice (102, 103) and bone was targeted by the antidiabetic compound rosiglitazone (103, 104), consistent with the recent findings about the increased bone loss and fracture risk for type 2 diabetes mellitus patients using rosiglitazone (105). Nonetheless, Runx2 null mice exhibited both impaired bone formation and reduced adipogenesis (106), suggesting that osteogenic and adipogenic differentiation is closely related (98).

4.6. Plasticity and directed differentiation of mesenchymal stem cells

The central dogma in stem cell biology has been that stem cells isolated from a particular tissue can renew and differentiate into lineages of the tissue they reside in. Stem cell plasticity usually refers to the ability of adult stem cells to acquire mature phenotypes that are different from their tissue of origin (107-109). It has been a long-standing concept that organ-specific stem cells are restricted to making the differentiated cell types of the tissue in which they reside (107). This character of restriction fundamentally distinguishes organ-specific stem cells from embryonic stem (ES) cells. However, recent findings have challenged the notion of lineage-restriction in organ-specific stem cells (107-109). It has been shown that adult stem cells from one tissue or organ can be induced to differentiate into cells of other organs, either *in vitro* or after transplantation *in vivo (*107-109). For example, adult neural-cell cultures containing neural stem cells could differentiate into blood cells (110). There have been a series of studies that reported bone-marrow-to-brain, bone MSCs-to-brain, bone-marrow-to-liver, skin-to-brain, brain-to-heart and other such stem cell differentiations (111), suggesting that organ-specific stem cells may overcome their intrinsic restrictions and "trans-differentiate" into other tissue types upon exposure to a novel environment (107-109, 112).

The potential plasticity or trans-differentiation of adult stem cells would have practical implications in the possible use of stem cell based therapies (108, 109). On one hand, the plasticity of adult stem cells may offer opportunities to repair or engineer multiple or different tissue/cell types using limited sources of stem cells. On the other hand, the transplanted stem cells may generate heterologously differentiated cell types, some of which may comprise unwanted tissue/cell types. Transdifferentiation could clinically be limited by the number of cells that can be introduced into an organ without removal of resident cells, as the transplanted stem cells could in theory repopulate whole organs from a few starting cells. A more urgent issue regarding the possible use of stem cell therapies is that pluripotent stem cells have a propensity to become transformed or tumor-like cells (113-115). Thus, in order to develop any safe and effective stem cell-based therapies, it is essential to fully understand the molecular regulatory circuits governing stem cell differentiation, plasticity/trans-differentiation, and/or dedifferentiation (115-117).

5. MAJOR SIGNALING PATHWAYS CONTROLING OSTEOGENESIS

5.1. Wnt signaling

The Wnt pathway plays an important role in embryonic development, tissue induction and axis polarity (118-127). Abnormal Wnt signaling has been associated with human diseases ranging from cancer to degenerative diseases (128-130). The Wnt family consists of a large number of secreted glycoproteins (118-120). The canonical Wnt/β-catenin pathway plays an important role in bone development (131) (Figure 2). Activation of this pathway occurs when the Wnt ligand binds to the 7-transmembrane domain-spanning Frizzled receptor and LRP5/6 co-receptors (118-120, 127). Ligand binding leads to phosphorylation of the Disheveled protein, which, via its interaction with Axin, Frat-1, and APC tumor suppressor, thereby prevents GSK3β from phosphorylating β-catenin (118-120, 127). Unphosphorylated β-catenin is stabilized and avoids degradation by β -TrCP, allowing it to translocate to the nucleus (118-120, 127, 132). In the nucleus, this transcriptional co-activator interacts with transcription factors LEF/Tcf-4 to activate expression of downstream genes such as c-Myc, cyclin D1, PPARδ, WISPs, CTGF, Cyr61 and other targets that promote cell proliferation, tissue expansions and cell fate determination (127, 133-140).

Increasing evidence suggests that Wnt signaling may play an important role in skeletal development and osteoblast differentiation (141-144). For example, several Wnt genes are expressed in the developing limb and are implicated in mesenchymal chondrogenesis (145, 146). The Wnt co-receptor LRP5 has been demonstrated to participate in bone mass regulation (147). Loss-of-function mutations in human LRP5 are associated with osteoporosis-pseudoglioma syndrome, which is characterized by low bone mineral density and skeletal fragility (131). Conversely, activating mutations in the N-terminus of human LRP5 are associated with a high bone density phenotype (148, 149). Additionally, the low bone mineral density phenotype is further exacerbated when loss of function in LRP5 is coupled to loss of an Lrp6 allele,

suggesting that both the LRP5 and LRP6 co-receptors are important in the Wnt signaling effect on bone mass (150). Consistent with this, disruption in the LRP inhibitors such as Dkk1 (148) and Sclerostin (151) allows for unphosphorlyated β-catenin to stimulate osteogenesis, thereby demonstrating the important role of the LRP5/6 co-receptors in bone formation. Consistent with this model, overexpression of the Wnt antagonist Dkk1 is associated with the presence of lytic bone lesions in patients with multiple myeloma (152), and Wnt/β-catenin signaling is frequently activated in human osteosarcoma (153). Nonetheless, a recent study has demonstrated that Dkk2 may play a role in late stages of osteoblast differentiation into mineralized matrix (154). Wnt3a was shown to promote proliferation and suppress osteogenic differentiation of adult mesenchymal stem cells (155). However, it has been shown that Wnt/β-catenin signaling in osteoblasts may coordinate postnatal bone acquisition by controlling the differentiation of both osteoblasts and osteoclasts (156), and that Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during skeletogenesis (157, 158). Wnt10b null mice displayed decreased trabecular bone, suggesting that Wnt10b may be an endogenous regulator of bone formation (159). It has been reported that stabilization of β-catenin in differentiated osteoblasts results in high bone mass, while its deletion from differentiated osteoblasts leads to osteopenia, through Osteoprotegerin-mediated bone resorption rather than bone formation (160), suggesting that Wnt/β-catenin signaling promotes the ability of differentiated osteoblasts to inhibit osteoclast differentiation. A recent study shows that non-canonical Wnt signaling through G protein-linked PKCδ activation promotes bone formation (161). Interestingly, Wnt14 has been shown to play a pivotal role in inducing synovial joint formation in the developing appendicular skeleton (162).

5.2. TGFβ/BMP superfamily

TGFβ superfamily consists of over 30 secreted dimeric polypeptide factors that play critical roles in regulating a diverse set of cellular functions including proliferation, differentiation, and embryonic development (6, 7, 163-170) (Figure 2). TGFβ family consists of three isoforms TGF-β1, TGF-β2 and TGF-β3, which are highly conserved in mammals, and each polypeptide factor shares a cluster of conserved cysteine residues (163). Mutations in the TGFβ signaling pathway result in various disorders generally affecting the skeletal, muscular, and cardiovascular system and tumor development (85, 97, 171, 172). Camuratie-Engelmann Disease is a human hereditary disease caused by a mutation of the ligand-encoding gene TFGβ1 (173). Clinically, patients present with hyperostosis and sclerosis of the skulls and the diaphysis of the limbs, but may also exhibit reduced adipose tissue, muscle hypotrophy, and bone marrow dysfunction (174, 175). Fibrodysplatic Ossificans Progressive is caused by a specific and recurrent point mutation in the TGFβ Type I receptor resulting in skeletal malformations and progressive extraskeletal ossification (176, 177). Additionally, targeted deletion of the TGFβ2 gene in mice resulted in several skeletal abnormalities including size defects in the limb, rib and spine thereby demonstrating the role of a single locus defect in both axial and appendicular skeletal development (178).

BMPs belong to the TGFβ1 superfamily, with more than 30 members identified in mammals and 14 types of human BMPs (6, 7, 179-181) (Figure 2). Many of these signaling proteins, mostly notably BMP2, BMP6, BMP7, and BMP9, are potent inducers of osteogenesis both *in vitro* and *in vivo* (6, 7, 48, 182), and initiate their signaling cascade by binding to the dimeric complex of two transmembrane serine-threonine kinase receptors, termed Type I and Type II (180, 183-185). The activated receptors initiate intracellular signaling by phosphorylating several transcription factors called Smads, and the Smad complex translocates to the nucleus to activate the transcription of various BMP-responsive genes (6, 7, 85, 139, 140, 186) . The Smad family consists of eight mammalian factors including the Receptor-regulated or R-Smads (Smads 1, 2, 3, 5, and 8) that are directly phosphorylated by the Type I receptors, the Co-Smads (Smad 4), and the Inhibitory Smads (Smad 6 and 7) (187). The inhibitory Smads hinder TGFβ signaling by blocking phosphorylation of the R-Smads by the activated Type I TGFβ receptors (165, 188-192). A recent study demonstrated that BMP2 is dispensable for bone formation, but is required for the initiation of fracture healing (193).

5.3. Notch signaling

Notch signaling is important for cell fate decision during development and maintenance of homeostasis in adults (194-201). The Notch gene encodes a single pass transmembrane receptor that is activated by a membrane bound ligand (Figure 2). In mammals, four Notch receptors (Notch 1-4) and five ligands (Delta-like1, Delta-like3, Delta-like4, Jagged 1 and Jagged 2) have been identified (196-201). The interaction of the Notch receptor with its ligand results in proteolytic cleavage of the receptor and release of the Notch Intracellular Domain (NICD) that translocates into the nucleus (202, 203). In the nucleus, NICD binds other transcription factors and up-regulates expression of primary target genes of Notch such as HES (Hairy Enhancer of Split) and HES-related Repressor Protein (HERP) in mammals (196-201, 204).

Notch signaling is critical for somite formation during embryogenesis, and mutations in Notch1 result in embryonic defects in somite segmentation (196-201, 205). Whereas Notch1 knockout results in embryos with delayed and disorganized somitogenesis (206), double knockout of both Notch 1 and Notch 2 resulted in embryos displaying axial truncation with only formation of cervical somites (207). Furthermore, the Spondylocostal Dysostoses are a group of human disorders characterized by vertebral segmentation defects and rib defects (208). Although this disorder can either be inherited or sporadic, it has been demonstrated that the autosomal recessive Spondylocostal Dysostosis was caused by mutations in the gene encoding the Notch ligand DLL3 (209).

Notch signaling also plays a critical role in adult stem cells (210). Notch signaling regulates osteogenesis,

although the mechanisms involved in this pathway are poorly understood (210-212). Notch/TGFβ cross talk between the Notch1 and TGFβ ligand BMP-2 promotes osteogenic differentiation (213), although a recent study demonstrated that Notch1 overexpression inhibits osteogenesis by repressing Wnt/β-catenin but not BMP signaling (214). Nonetheless, a synergy between Notch and BMP-4 inhibit myogenic differentiation (215). Additionally, inhibition of the Notch pathway interferes with adipogenesis *in vitro* (216). Consistent with this finding, mice deficient in Pref-I, a secreted Notch/Delta/Jagged ligand that inhibits adipogenesis, demonstrated skeletal malformations, growth retardation and obesity (217).

5.4. Hedgehog signaling

Hedgehog proteins are secreted factors that are essential for many developmental processes in both vertebrates and invertebrates (218-222). The Hedgehog family of secreted proteins consists of three mammalian orthologs, Sonic Hedgehog, Indian Hedgehog, and Desert Hedgehog, each of which has a unique set of functions in regulating developmental processes (218, 219) (Figure 2). Desert Hedgehog is required for development of peripheral nerves and spermatogenesis, whereas Sonic Hedgehog is important for central nervous system development and skeletal patterning (218, 219). Indian Hedgehog demonstrates many overlapping functions with Sonic Hedgehog but is most important in endochondral bone development (223-226). Hedgehog proteins are synthesized as 45 kDa precursor that are catalytically cleaved to an active 19 kDa N-terminal fragment, which is modified by the attachment of a cholesterol and palmitic acid. Secreted active Hedgehog fragments regulate the cellular activities of neighboring and distant cells by binding to two components on the cell surface, Patched (Ptc), (2 mammalian homologues, Ptc1 and Ptc2) a 12-transmembrane protein, and Smoothened (Smo), a 7-transmembrane protein. In the absence of Hedgehog ligands, Ptc represses the activity of Smo, however, when the Hedgehog ligand binds to Ptc, Smo is released which allows for activation of the transcription factor Cubitus Interuptus (vertebrate orthologs Gli1, Gli2, Gli3) to allow for Hedgehog target gene expression (222, 227).

Sonic Hedgehog and Indian Hedgehog are essential for skeletal development as demonstrated by knockout of Shh or Ihh genes in transgenic mice (218, 219, 222, 228). Whereas Shh-knockout mice demonstrate abnormalities in skeletal patterning (229), Ihh-knockout mice demonstrate reduced chondrocyte proliferation, inappropriate chondrocyte maturation and an absence of osteoblast differentiation in endochondral bones, suggesting the importance of Ihh in endochondral bone development (230). In humans, Brachydactyly Type A-1, shortening or absence of the middle phalanges (231), and Acrocapitofemoral Dysplasia, short stature with short limbs secondary to cone-shaped epiphyses (232), are the skeletal disorders linked to mutations in the Ihh gene. Furthermore, mutations in the downstream transcription factor Gli3 results in a variety of inherited skeletal patterning defects such as polysyndactyly and craniofacial abnormalities (Greig Cephalopolysyndactlyly Syndrome) (233-235). A recent study has demonstrated that Ihh produced by postnatal chondrocytes is essential for maintaining growth plate and trabecular bone (236).

5.5. FGF signaling

Fibroblast growth Factors (FGFs) play an important role in chondrogenesis and osteoblast differentiation (237-240). FGFs consist of 23 structurally-related members that are expressed in almost all tissue types during important developmental stages (241, 242). The FGF ligands are usually between 20-35 kDa and bind to the FGF receptor's extracellular ligand binding domain to induce FGF signaling (242) (Figure 2). The FGF receptors (FGFRs 1-4) contain the extracellular ligand binding domain as well as a highly conserved intracellular signaling domain which contains intrinsic kinase and tyrosine residues (243). Upon ligand binding, the FGF receptors dimerize and subsequently cause autophosphorylation of the intrinsic kinase residues, setting off the FGF signaling cascade (242, 243). Briefly, the FGF Receptor Substrate 2 (FRS2) protein is phosphorylated which subsequently recruits the Grb2/SOS complex to the plasma membrane and activates the MAPK pathways (240). Specifically, the FGF signaling has been shown to activate ERK1/2, p38 MAPK, SAPK/JNK, PKC, and PI3K pathways to transduce cell signaling (240, 243).

Both the FGF ligands and FGF receptors have a demonstrated role in osteogenic differentiation (237-240, 244). For example, FGF-2 has been shown to induce alkaline phosphatase activity in rat bone marrow precursor cells, and FGF-2, 4, and 8 have been shown to induce Runx2, an essential transcription factor in osteogenic differentiation (244). Furthermore, FGF-9 can induce osteocalcin expression, a late marker of osteogenic differentiation, and FGF-2, 9, and 18 are important in subsequent matrix mineralization. Despite the demonstrated role of the FGF ligands in osteogenesis, conflicting evidence exists regarding the FGF ligands effect on osteoblast proliferation and the expression of the osteoblast markers, and this discrepancy appears to be a result of the stage-specific effect of the FGF ligands (238, 244).

FGF receptors also play a key role in osteogenic differentiation (238, 244). FGFR1 plays a dominant role during osteogenic differentiation, and FGFR2 appears to function during both osteogenic proliferation and differentiation. Although FGFR3 has been shown to function primarily during endochondral ossification to control chondrocyte proliferation, recent evidence suggests it may also play a critical role in osteogenesis. For example, adult mice lacking FGFR3 are osteopenic and develop osteomalacia (245), while constitutive activation of the FGFR3 due to Gly369Cys homozygous mutation in mice causes a defect in endochondral ossification resulting in an achondroplasia-like phenotype and upregulation of the osteogenic gene markers, osteocalcin, osteopontin, and osteonectin (246). Many of the skeletal dysplasias such as craniosynosotoses, limb abnormalities, and achondroplasia highlight the importance of the FGF receptors in skeletal

development (238, 244). Point mutations in FGF receptors 1-3 result in many craniosynostosis syndromes (Apert, Pfeiffer, Crouzon, Jackson-Weiss, Muenke) characterized by unregulated intramembranous bone formation and therefore premature closure of the cranial sutures (240, 247). Whereas mutations in FGF receptors 1-3 are known to cause skeletal deformities, the role of FGF receptor 4 in osteogenesis is unclear. However, recent evidence suggests that FGF receptor 4 is expressed in rudimentary membranous bone and localized to the osteoblasts, suggesting that it may also be an important regulator of osteogenesis and play a role in intramembranous ossification (248).

5.6. Other signaling pathways

Skeletal homeostasis is maintained by a balance of osteoclastic bone resorption and osteoblastic bone formation, and this system is maintained at its steady state by multiple factors (67). One such important factor is parathyroid hormone-related protein (PTHrP), which has been shown to play a role in endochondral bone formation (249-251). PTHrP is a protein secreted during fetal life by perichondrial cells at the ends of cartilage moulds and by early proliferative chondrocytes (249-251). PTHrP binds to the same G-protein-coupled receptors used by parathyroid hormone (PTH) (252). These PTH/PTHrP receptors (PPRs) are expressed at low levels by proliferating chondrocytes and at high levels by prehypertrophic and early hypertrophic chondrocytes. PTHrP functions primarily to keep proliferating chondrocytes in the proliferative pool. In $PTHrP^{-/-}$ or $PPR^{-/-}$ mutant mice, chondrocytes become hypertrophic close to the ends of bones (253, 254). Conversely, overexpression of PTHrP in chondrocytes or expression of a constitutively active PPR delays the appearance of hypertrophic chondrocytes (255, 256). Further evidence indicates that The interactions of Ihh and PTHrP controls the decision of chondrocytes to leave the proliferative pool through a feedback loop, and hence determines the lengths of proliferative columns in individual bones (257, 258). Interestingly, despite their limited sequence homology, PTH and PTHrP bind and activate the same PTH/PTHrP receptor with almost indistinguishable high affinity. Thus, the PTH/PTHrP receptor mediates both the endocrine actions of PTH and the autocrine/paracrine actions of PTHrP (249, 251). The *in vivo* and clinical studies have demonstrated that PTH (1-34), to a lesser extent PTHrP (1-34), can stimulate bone formation and is being used to treat osteoporosis (259, 260).

It has been shown that osteoclasts express the NFATc1 target gene ephrinB2, while osteoblasts express the receptor EphB4, along with other ephrin-Eph family members (261). Reverse signaling through ephrinB2 into osteoclast precursors suppresses osteoclast differentiation by inhibiting the osteoclastogenic c-Fos-NFATc1 cascade, whereas forward signaling through EphB4 into osteoblasts enhances osteogenic differentiation, and overexpression of EphB4 in osteoblasts increases bone mass in transgenic mice (261), suggesting that ephrin-Eph bidirectional signaling links the differentiation of osteoclasts and osteoblasts and thereby maintains bone homeostasis. Osteoclastic bone resorption increases circulating levels of serum calcium and this process is mediated by parathyroid hormone and 1,25 (OH)2 vitamin D (262). Additionally, parathyroid hormone stimulates growth of osteoprogenitor populations (263), whereas 1,25 (OH)2 vitamin D is a potent regulator of osteoblast phenotypic genes (264). Furthermore, human sex steroids such as estrogen and testosterone demonstrate a multi-functional role in regulating bone mass (265-267). In humans, estrogen inhibits bone resorption by reducing osteoclast number, and is essential for attaining peak bone mass in both males and females (266). Testosterone couples with estrogen to increase bone mass and can also inhibit bone resorption when converted to estrogen by the enzyme aromatase (268).

6. TRANSCRIPTIONAL REGULATION OF OSTEOGENIC DIFFERENTIATION

6.1 Runx2

Runt-related transcription factor 2 (Runx2) has been identified as a master regulator of osteoblast differentiation and chondrogenesis (269-271). Runx2 (a.k.a., Cbfa1, Osf2, and AML3), is a member of the Runx class of transcription factors that contain a highly conserved 128 amino acid motif conferring DNA binding, protein-protein interactions and ATP binding activities (269). The Runx genes have been identified in a many species and in mammals there are three identified Runx genes (i.e., Runx1, Runx2, and Runx3). Runx2 protein heterodimerizes with Cbfβ to induce osteogenesis (272).

The essential role of Runx2 in osteogenesis was demonstrated by Runx2 gene knockout. Runx2 $^{-/-}$ die shortly after birth and demonstrated a cartilaginous skeleton with complete absence of ossification (273). Despite the cartilaginous phenotype in the Runx2-null mice, histologic analysis demonstrated delayed chondrocyte maturation suggesting the importance of Runx2 in chondrogenesis and osteogenesis (274). Additionally, when Runx2 is overexpressed in chondrocytes via the chondrocyte-specific type II collagen promoter, it results in ectopic chondrocyte hypertrophy and endochondral ossification, thereby demonstrating the importance of Runx2 in controlling differentiation of both chondrocytes and osteoblasts (275, 276).

Given the important role of Runx2 in controlling skeletal mineralization by stimulating osteoblast differentiation, promoting chondrocyte hypertrophy, and contributing to endothelial cell migration and vascular invasion of developing bones, it is not surprising that Runx2 transcriptional activity is regulated by numerous transcriptional co-activators and co-repressors (271, 277-279). The Runx2 co-repressors include histone deacetylases (HDACs), transducin-like enhancer of split (TLE) proteins, mSin3a, and yes-associated protein (YAP) (278, 279). These co-repressor proteins do interact with Runx2 and prevent it from binding DNA, altering chromatin structure, and/or by possibly blocking co-activator complexes (278). It has been reported that the activity of Runx2/Cbfa1 is modulated by interacting

proteins, such as Smads, C/EBP, and Tcf4/LEF1 (278, 280-282). As discussed later, it has been shown that Rb physically interacts with the Runx2 and may function as a direct transcriptional co-activator of osteoblast differentiation (283, 284). Additionally, Runx2 is phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway, and protein kinase A (PKA), whereas Runx2 activity is enhanced by factors known to stimulate specific signal transduction pathways such as PTH/PTHrP (signals through PKA and PKC pathways) and Smads (285). These functional properties of Runx2 provide novel insights into the requirements for multiple levels of transcriptional control within the context of nuclear architecture to support the convergence of regulatory signals that control tissue-specific gene expression (272, 278, 286).

6.2. Osterix

Osterix is a zinc-finger-containing transcription factor that is expressed in osteoblasts during mouse embryonic development and is essential for bone development (271, 287). Osterix contains a DNA binding domain that consists of three C2H2-type zinc fingers at its C-terminus. In Osterix-null mice, no cortical bone and no trabecular bone were formed through either intramembranous or endochondral ossification (287). Although both Runx2-null mice and Osterix-null mice are characterized by absence of ossification, the phenotypes of these two mutants differed. Whereas Runx2 deficient mice demonstrated delayed chondrocyte maturation (274), the cartilage of the Osterix-null mice was normal. Additionally, Osterix appears to act downstream of Runx2 in osteogenic differentiation, as demonstrated by the fact that Osterix is not expressed in Runx2-null mice, whereas the expression of Runx2 is normal in Osterix-null mice (287).

6.3. ATF4

Activating transcription factor 4 (ATF4) is a basic domain-leucine zipper protein and has been shown to control key aspects of osteoblast biology (271). Coffin-Lowry Syndrome (CLS) is an X-linked mental retardation condition associated with skeletal abnormalities (288). The gene mutated in CLS, RSK2, encodes a growth factor-regulated kinase. RSK2 is required for osteoblast differentiation and function. The transcription factor ATF4 has been shown to be a critical substrate of RSK2 that is required for the timely onset of osteoblast differentiation, for terminal differentiation of osteoblasts, and for osteoblast-specific gene expression, such as the synthesis of type I collagen, and the transcription of the osteocalcin and RANKL genes (288). While the levels and activity of ATF4 are under tight control through mechanisms that include protein stability and phosphorylation, it has been shown that the inhibition of ATF4 can also be achieved through its interaction with the leucine zipper protein FIAT (Factor Inhibiting ATF4-mediated Transcription, a.k.a., gamma-taxilin) (289). FIAT/gamma-taxilin localizes to the nucleus in osteoblasts and dimerizes with ATF4 to form inactive dimers, due to its lack of a DNA-binding domain (289). The interaction of FIAT/gamma-taxilin with ATF4 thus inhibits ATF4-mediated transcription. Transgenic mice overexpressing FIAT/gamma-taxilin show osteopenia and reduced expression of the ATF4 target gene, osteocalcin (289, 290). Since ATF4 is also important for amino acid transport, a low-protein diet decreased bone protein synthesis and normalized bone formation and bone mass in Nf1 (ob) (-/-) mice, while a high-protein diet overcame Atf4 (-/-) and Rsk2 (-/-) mice developmental defects, perinatal lethality, and low bone mass (290), suggesting that ATF4-dependent skeletal dysphasia may be corrected by dietary manipulations.

6.4. TAZ

The transcriptional modulator TAZ is a recently described transcriptional co-activator that regulates mesenchymal stem cell differentiation down osteogenic or adipogenic lineages (271). TAZ binds to 14-3-3 proteins, a conserved family of ~30KDa proteins involved in differentiation, cell cycle progression and apoptosis (291). Furthermore, TAZ which was identified through a proteomic screening is similar to the Yes-associated protein, YAP. Both these proteins contain a WV domain that binds to Pro-Pro-X-Tyr motifs (X is any amino acid). These motifs are also found in the transcriptional activation domains of Runx2 and the adipogenic transcription factor PPARγ thereby suggesting that TAZ and YAP function as transcriptional modulators (292).

TAZ activates Runx2-driven genes *in vitro* during terminal osteoblast differentiation but inhibits adipogenic PPARγ-induced gene transcription and therefore inhibits adipogenic differentiation. TAZ has been shown to play a critical role in osteoblast differentiation in the vertebrate zebrafish model (291). Using the zebrafish TAZ ortholog, demonstrated that TAZ-depleted zebrafish embryos survived up to 8 days following fertilization however demonstrated no bone formation (291). In contrast, the control animals demonstrated extensive skeletal development at 8 days following fertilization (291). Nonetheless, the precise role of TAZ in osteogenesis during skeletogenesis remains to be defined.

6.5. Post-translational Regulation of Osteogenic Differentiation

Post-translational modifications (such as acetylation, ubiquitination, and sumoylation) of regulatory proteins have become increasingly appreciated in regulating cellular processes. Ubiquitination is an emerging mechanism that is implicated in a variety of nonproteolytic cellular functions (293, 294). The attachment of a single ubiquitin (Ub) or poly-Ub (lysine 63) chains to proteins control numerous cellular processes, and yet protein ubiquitination exhibits inducibility, reversibility and recognition by specialized domains (293, 294). The functional activity of TGFβ/BMP signaling can be regulated by E3 ubiquitin ligases, such as the Smad1 ubiquitin ligase Smurf-1, that target specific Smads for proteolytic destruction (295, 296). For example, Smad7 can be modified by acetylation, ubiquitination, and/or sumoylation (296).

It has been shown that Smurf1 interacts with Smads 1, 5, 6, and 7, and that a bone-specific

overexpression of Smurf1 leads to the inhibition of osteoblast differentiation and bone formation (297). Furthermore, it has been found that Smad6 interacts with Runx2 and mediates Smurf1-induced Runx2 degradation (298). The physiological role in suppressing the osteogenic activity of osteoblasts has been demonstrated in Smurf1-deficient mice that were born normal but exhibited an age-dependent increase of bone mass due to enhanced and sensitized activities of osteoblasts in response to BMPs (299). Loss of Smurf1 leads to accumulation of phosphorylated MEKK2 and activation of the downstream JNK signaling cascade (299). Further analysis shows that Smurf1 physically interacts with MEKK2 and promotes the ubiquitination and turnover of MEKK2, suggesting that Smurf1-mediated inhibition of osteoblast activity may be through controlling MEKK2 degradation (299). Accordingly, a recent study found that the ERK-MAPK pathway may play an important role in osteoblast differentiation and skeletal development by stimulating Runx2 phosphorylation and transcriptional activity (300). C/EBP homologous protein CHOP is shown to act a dominant-negative inhibitor of C/EBPβ and prevents osteoblast differentiation, and promotes BMP signaling in a cell-type specific manner (301). Human short-stature syndromes gene Shox2 is shown to function as an upstream regulator of Runx2 during long bone development (302). A recent study demonstrates that HOXA10 controls osteogenesis by activating Runx2 in mesenchymal cells and by directly regulating osteoblast phenotypic genes (303).

6.6. Other regulators of osteogenesis

Osteogenesis and bone remodeling are well orchestrated developmental and cellular processes. Although the above signaling pathways and osteogenic regulators may play an important role in these processes, it is conceivable that other factors may also play a significant role in modulating and fine-tuning osteogenesis and bone homeostasis. For example, Fos-family transcription factors (c-fos, fra-1), Jun, homeobox-containing transcription factors Msx1, Msx2, Dlx5, and Dlx6, and other transcription factors including Twist, Knox-20, Sp3 are also involved in osteoblast differentiation and regulate osteoblast differentiation (2, 271).

Several recent studies provide more insights into the complexity of the regulatory circuit for osteogenesis. The zinc finger adapter protein Schnurri-3 was shown to regulate postnatal bone mass by promoting E3 ubiquitin ligase-mediated degradation of Runx2 protein, as Schnurri-3 deficient animals display adult-onset osteosclerosis with increased bone mass (304). Interestingly, Schnurri-2 controls BMP-dependent adipogenesis through interaction with Smad proteins (99). NFATc1 and Osterix form a complex that activates Osterix-dependent transcription (305). While bone formation is inhibited in NFATc1 deficient cells (305), transgenic overexepression of NFATc1 in osteoblasts leads to drastically increased bone formation (306). Furthermore, NFATc1-deficient mice have defects in skull bone formation and exhibit increased osteoclastogenesis despite normal levels of RANKL and Osteoprotegerin (306), suggesting that NFATc1 may regulate bone mass by functioning in both osteoblasts and osteoclasts. Nuclear matrix protein SATB2 interacts with both Runx2 and ATF4 and promotes osteoblast differentiation, as the SATB2 null animals exhibit craniofacial abnormalities (306). Therefore, SATB2 may act as a molecular node in a transcriptional network that regulates skeletal development and osteoblast differentiation. Tumor suppressor PTEN deficient osteoblasts exhibit much more rapid differentiation and greatly reduced apoptosis, while PTEN deficient mice demonstrate a dramatic and progressively increasing bone mineral density throughout life (307). Similarly, osteoblasts deleted for MDM2 exhibit elevated p53 activity, reduced proliferation, reduced levels of Runx2, and reduced differentiation (308). In contrast, p53-null osteoblasts exhibit increased proliferation, increased expression of Runx2 and osteoblast maturation, and increased tumorigenic potential (308). Lastly, it has been reported that loss of Rb but not p107 or p130 blocks late osteoblast differentiation (284). Rb physically interacts with the Runx2 and results in synergistic transactivation of an osteoblast-specific reporter, suggesting that Rb may functions as a direct transcriptional co-activator promoting osteoblast differentiation (283, 284).

7. SUMMARY AND FUTURE DIRECTIONS

There have been remarkable advances in understanding bone and skeletal development at the genetic and molecular levels during the past decade. Several major signaling pathways, such Wnt, BMP, FGF, and Hedgehog signaling, have been shown to play an important role in regulating osteogenic differentiation (Figure 2). At the transcription level, several transcriptional factors have been identified as important regulators of osteogenic lineage commitment and terminal differentiation. These transcriptional factors include Runx2, Osterix, ATF4 and TAZ. For the past few years, emerging findings have indicated that ubiquitination of the osteogenic regulators, especially Runx2, may play a larger than expected role in regulating osteogenic differentiation. Nonetheless, there are numerous challenges ahead us. A deeper understanding of bone and skeletal development comes from knowledge of the molecular components, cell and tissue structure, and biophysical and vascular mechanisms underlying physiologic functions. Many of the currently identified signaling molecules and transcriptional factors that play an important role in osteogenic differentiation consist of multiple family members. How much do the different members contribute to osteogenesis? How can we circumvent their functional redundancy in order to investigate the role of a specific factor? What are the earliest signal (s) that specify and control osteogenesis? What are the fundamental differences between osteogenesis during skeletal development and bone regeneration after injury? What are the critical factors that control the divergence between osteogenic lineage and other lineages during mesenchymal stem cell differentiation? The potential use of MSCs in regenerative medicine is promising given the advances in our understanding of osteogenesis. However, these adult stem cells have to be directed towards certain lineage-specific differentiation. It has been widely reported that multipotent stem cells could

undergo spontaneous transformation in the absence of directed differentiation. Therefore, a better understanding of molecular mechanisms behind osteogenic differentiation would not only help us to identify pathogenic causes of bone and skeletal diseases but also lead to the development of novel and targeted therapies for these diseases.

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Abbreviations: ATF4, Activating transcription factor 4; BMP, Bone Morphogenetic Protein; C/EBP, CCAAT/Enhancer Binding Protein; ERK, Extracellular signal-Related Kinase; FGF, Fibroblast Growth Factor; FGFR, FGF Receptor; Grb2/SOS, Growth factor receptor-bound protein 2/Son of Sevenless; Ihh, Indian hedgehog; LRP5/6, Low-density lipoprotein receptor-Related Protein 5 and 6; MAPK, Mitogen-Activated Protein Kinase; MSC, Mesenchymal Stem Cell; NFAT, Nuclear Factor of Activated T cells; PI3K, Phosphatidylinositol 3 Kinase; PKC, Protein Kinase C; PPAR, Peroxisome Proliferator-

Activated Receptor; Rb, Retinoblastoma tumor suppressor protein; Runx2, Runt-related transcription factor 2; Smad, mammalian homologs of the *Drosophila* Mothers against dpp (Mad); SAPK/JNK, Stress-Activated Protein Kinase/c-Jun N-terminal Kinase; Shh, Sonic hedgehog; Smurf, Smad ubiquitination regulatory factor; TGFβ, Transforming Growth Factor β.

Key Words: Adipogenesis, ATF4, BMP, TGF beta, Bone Formation, Chondrogenesis, Lineage Commitment, Mesenchymal Stem Cells, Osteogenic Differentiation, Osterix, Runx2, Skeletal Development, Wnt, beta-catenin, Review

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