Review

Signaling pathways regulating the specification and differentiation of the osteoblast lineage

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ABSTRACT

Tissue engineering is an approach to the regeneration of tissues that uses a combination of cell sources, signaling factors and scaffolds. Among these three components, signaling factors for bone regeneration have not yet been established, and it is necessary to better understand osteoblast progenitors as a target cells. Several lines of evidence have revealed that, during bone formation, mesenchymal cells are specified and differentiate into osteoblasts through several stages of precursors. The osteoblast lineage is defined by the expression of stage-specific transcription factors. The specification and differentiation are organized by a variety of signaling pathways including hedgehog (Hh), Wnt, Notch, bone morphogenetic protein (BMP) and transforming growth factor-beta (TGFβ). In this review we integrate the known functions of these signaling pathways and discuss future tasks to gain a better understanding of the signaling network in osteogenesis for tissue engineering.
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1. Introduction

Mammalian bones have three distinct origins; the paraxial mesoderm, the lateral plate mesoderm, and the neural crest. The paraxial mesoderm gives rise to the axial skeleton. The lateral plate mesoderm gives rise to the appendicular skeleton and the neural crest gives rise to cranial skeleton. There are two modes of bone formation; intramembranous and endochondral bone formation. In intramembranous ossification, mesenchymal cells condense and directly differentiate into osteoblasts to deposit bone matrix, whereas in endochondral ossification, after the condensation of mesenchymal cells, a cartilage mold is formed and subsequently the mold is replaced by bone tissue. Bones derived from the paraxial mesoderm and lateral plate mesoderm are formed mainly through the endochondral process. In the initial phase of the process, two distinct cell populations arise after the condensation of mesenchymal cells: cartilage-forming chondrocytes and perichondrial cells [1].

SRY box-containing gene 9 (Sox9) has been shown to be essential for the mesenchymal condensations and subsequent cartilage formation [2,3]. Perichondrial cells constitute the perichondrium, a thin layer of fibroblastic cells surrounding the cartilage mold. During the process, a population of the perichondrial cells is specified into the osteoblast lineage; they differentiate into osteoblasts through several stages of precursors, contributing to the formation of the bone collar, a predecessor of cortical bones and being a source of primary spongiosa [1,4].

Runt-related transcription factor 2 (Runx2) and osterix (Osx) are two essential transcription factors for osteoblast differentiation. Genetic studies have shown that Osx is downstream of Runx2 [5–7]. The sequential osteoblast differentiation is characterized by the expression of stage-specific transcription factors: Sox9-positive mesenchymal cells, called osteo-chondroprogenitors, give rise to Runx2-positive osteoblast precursors. The precursors differentiate into Osx-positive osteoblast precursors, followed by their maturation to bone forming osteoblasts [8,9]. A variety of signaling pathways have been shown to function at specific and/or multiple

stages in the differentiation cascade. Among them, hedgehog (Hh), Wnt, BMP, and Notch pathways have been shown to play pivotal roles. Here, we review these signaling pathways and discuss how these pathways form a network to regulate ossification.

2. Signaling pathways in the osteoblast lineage

2.1. Hh signaling

Mammals have three homologs of drosophila hedgehog: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Among them, Ihh has been shown to be essential for endochondral ossification. Ihh is expressed in prehypertrophic chondrocytes and secreted to specify a certain cell population in the perichondrium into osteoblast precursors [10,11]. In *Col2a1-Gal4;UAS-Ihh* and *Col2a1-Cre;Patched1*$^{-/-}$ (Ptch1; a Hh ligand receptor functioning as a repressor of the signaling) mouse embryos, bone collar formation is enhanced by the mesenchymal cell-specific gain-of-function of Hh signaling [11,12].

Regarding the loss of function, bone collar is absent following *Ihh* ablation and following the chondrocyte- and perichondrium-specific ablation of *Smoothened* (*Smo*; a receptor of Hh ligand functioning as a transducer of Hh activation) using a *Col2a1-Cre* driver [10,11]. Importantly, neither *Runx2* expression nor bone collar formation has been shown in these mutants. These data suggest that Ihh acts as a switch to initiate a program of osteoblast lineage in the Runx2-negative mesenchymal cell state and generate the Runx2-positive osteoblast precursors. In addition, in the absence of Ihh signaling, cells in the perichondrium seem to adopt a chondrocyte fate, as following two lines of evidence; by the layer of immature chondrocytes that surround the hypertrophic chondrocytes in *Ihh*$^{-/-}$ mice [10], and by the existence of ectopic chondrocytes in *Smo*$^{-/-}$ perichondrial cells in chimeric mice [11].

The zinc finger transcription factors Gli1, Gli2 and Gli3 are thought to mediate transcriptional responses to Hh input. Gli1 is a transcriptional activator. Gli2 is suggested to function primarily as a transcriptional activator, and Gli3 as a transcriptional repressor [13]. Which Gli proteins play important roles in the Ihh-mediated function to specify progenitors into an osteoblast lineage? Impairment of the early osteoblast differentiation in *Ihh*$^{-/-}$ embryos is partially rescued by the disruption of *Gli3* [14,15], whereas it is completely rescued in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$;*C2ΔNGli2* embryos, in which *ΔNGli2* (an N-terminally truncated form of Gli2, as a constitutively active form of Gli2) is exogenously expressed in *Col2a1*-positive cells [16].

In addition, we recently reported that, in addition to Gli2 and Gli3, Gli1 is involved in the Ihh signaling-mediated specification of the osteoblast lineage [17]. Gli1 overexpression induces osteoblast marker genes *in vitro* and *Gli1*$^{-/-}$ shows impaired bone collar formation. Moreover, *Gli1*$^{-/-}$;*Gli2*$^{-/-}$ mice show more severe phenotypes of impaired bone formation than the single knock-out mice [17]. These data suggest that all Gli members function cooperatively in osteogenesis. A question that has not been answered is whether all members of the Gli family function independently of each other or act collectively at a particular stage in this context.

In despite of the significance of the Ihh function in embryonic skeletal development, limited number of studies addressed the roles in the postnatal skeleton. Ohba et al. reported that adult *Ptch1*$^{+/-}$ mice show high bone mass due primarily to enhanced osteoblast differentiation [18]. The mature osteoblast-specific ablation of *Ptch1* using *osteocalcin-Cre* driver leads to increases in both osteoblastogenesis and osteoclastogenesis, resulting in decreased bone mass and osteopenia [19]. Maeda et al. reported that the chondrocyte-specific ablation of *Ihh* in newborn mice using *Col2a1-CreER* mice results in impaired growth plate formation, and

in the loss of trabecular bone continuously in older mice [20]. In addition, administration of a Hh inhibitor in young mice leads to a decrease in bone mass and the disruption of bone structure through suppressed osteoblast differentiation [18,21].

Regarding the Gli family, the Osx-positive cell-specific activation of *Gli2* using *Osx-GFP::Cre* driver, which is BAC transgenic mouse line expressing a GFP::Cre fusion protein under the regulation of the *Osx* promoter, exhibits severe osteopenia due to a marked decrease in osteoblast number and function, although bone resorption is not affected [22]. Taken together, the above findings indicate that Hh signaling is necessary for proper bone formation. However, the excessive activation of Hh signaling is likely to lead to osteopenia through enhanced osteoclastogenesis and/or indirect actions of osteoclastogenesis.

2.2. Wnt signaling

A number of the Wnt family proteins are expressed in skeletal tissue and regulate endochondral ossification through canonical and non-canonical Wnt signaling pathways [23]. In the canonical Wnt pathway [24,25], Wnts bind to Frizzled receptors and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) in vertebrates [26–28], which leads to stabilization of beta-catenin (β-catenin) and activation of the transcription of target genes *via* lymphoid enhancer binding factor-1 (Lef-1) and T cell factors (Tcf1, 3, 4).

The amplitude of the signaling is fine-tuned in part via negative feedback mechanisms that involve the secreted molecule Dickkopf 1 (Dkk1) [29], a direct transcriptional target of canonical Wnt signaling [30,31]. Dkk1 antagonizes the pathway by interfering with the interaction between LRP5/6 and Wnt [32–34]. Non-canonical Wnt pathways include a planar cell polarity pathway, a Ca^{2+}/protein kinase A pathway, and a protein kinase C-dependent pathway [35].

Genetic studies indicate that a certain threshold level of the Wnt signaling is required for multiple stages of osteoblast lineages. The limb bud mesenchyme-specific ablation of *Ctnnb1* (which encodes β-catenin) using *Prx1-Cre* driver [36] and a mesenchymal progenitor cell-specific ablation of *Ctnnb1* using *Dermo1-Cre* driver [37,38] result in greatly diminished osteoblast marker expressions, such as those of *collagen I*, *Osx*, and *osteocalcin*, whereas the initiation of the osteoblast differentiation and Runx2 expression are not affected in these mice. These data indicate that canonical Wnt signaling is required for osteoblast precursors to differentiate into an Osx-positive stage. In mice with the *Osx*-positive cell-specific ablation of *Ctnnb1* using the *Osx-GFP::Cre* driver, cells fail to progress to mature osteoblast stages characterized by high osteocalcin expression, although *Runx2* and *Osx* were expressed [9]. Stabilization of β-catenin in Osx-positive cells using the *Osx1-GFP::Cre* results in robust increase of the proliferation of osteoblast precursors, but the attenuation of their terminal differentiation to osteocalcin-positive osteoblasts [9]. These findings indicate that canonical Wnt signaling needs to be kept within a physiological range in order to positively promote osteoblastogenesis. In addition, when *Ctnnb1* is removed using the *Dermo1-Cre*, *Prx1-Cre*, *Col2a1-Cre* or *Osx1-GFP::Cre* drivers, ectopic chondrocytes are observed in the perichondrium at the expense of osteoblast differentiation during endochondral ossification [9,36,37]. Notably, perichondrial cells in mice with the *Osx*-positive cell-specific ablation of *Ctnnb1* express *Ptch1*, a readout of Hh signaling activation, indicating that Hh signaling is activated in the cells. These data raise the possibility that osteoblast precursors still have the bipotential ability to differentiate into not only osteoblasts but also chondrocytes even after they have already committed to an osteoblast lineage by Hh signaling.

The importance of canonical Wnt signaling in postnatal bone formation is supported by genetic evidence. In humans, LRP-5 loss-of function mutations are linked with the osteoporosis-pseudoglioma syndrome [39], while the gain-of function mutations are linked with a familial high bone density phenotype [40]. Similarly, in mice, $Lrp\text{-}5^{-/-}$ [41] and $Wnt10b^{-/-}$ mice [42] exhibit reduced bone mass. Reduction of $Lrp\text{-}6$ in $Lrp5^{-/-}$ mice is shown to further reduce bone mass [43]. Conversely, mice lacking the secreted Frizzled related protein which is a Wnt antagonist, develop more bone mass in the postnatal life [44]. As for non-canonical pathways, G protein-coupled phosphatidylinositol and PKCΔ activation are involved in the Wnt-mediated osteoblast differentiation [45]. Non-canonical Wnt5a induces osteoblast differentiation in vitro, while $Wnt5a^{+/-}$ shows bone loss in adult mice [46].

2.3. Notch signaling

Notch signaling functions in the communication between neighboring cells and is involved in the cell fate determination. Notch receptors (Notch1-4 in mammals) are single-pass transmembrane proteins which are composed of functional extracellular (NECD), transmembrane (TM) and intracellular (NICD) domains. Upon Notch ligands, such as Jagged1, 2 and Delta-like 1, 3, 4 in mammals bound to the Notch receptors, the receptors undergo proteolytic cleavage, resulting in released of NICD from the plasma membrane and translocated into the nucleus [47]. In the nucleus, NICD interacts with a transcription factor of the CSL family (RBP-Jκ/CBF-1 in mammals) to activate the transcription of target genes, including those of the Hes/Hey family, which are transcription repressors containing a conserved basic helix-loop-helix (bHLH) domain [48]. A functional γ-secretase complex containing either presenilin 1 (PS1) or 2 (PS2) as the catalytic subunit [49–51] is required for the intramembrane cleavage and release of the NICD.

Notch signaling plays important roles in bone formation; however, genetic studies have yielded somewhat controversial results. Regarding gain of function, NICD transgenic mice driven by the 2.3-kb type I collagen promoter exhibit increased bone volume with growth retardation [52]. In contrast, overexpression of NICD driven by the 3.6-kb type I collagen promoter leads a decrease in bone volume through a decrease in osteoblast number [53]. Regarding loss of function, the limb bud mesenchyme-specific ablation of PS1 and PS2 or Notch1 and Notch2 using Prx1-Cre driver do not overtly affect the skeletal morphogenesis in the embryo, but markedly enhance trabecular bone mass in adolescent mice [54]. Notably, the number of mesenchymal progenitors is decreased in the bone marrow of the mice with high bone mass, supporting a model in which Notch signaling normally suppresses osteoblast differentiation from progenitor cells. As a result, these mice develop severe osteopenia as they aged. The osteoblast-specific ablation of PS1 and PS2 using Col1a1-Cre driver is associated with age-related osteoporosis, which in turn results from increased osteoblast-dependent osteoclastic activity due to decreased osteoprotegerin mRNA expression [52]. Taken together with several line of evidence in the molecular study [51–54], Notch signaling is suggested to function to maintain a pool of mesenchymal progenitors. Bone formation and bone homeostasis controlled by Notch signaling are possibly modulated by other signaling factors, including BMP, Wnt, Runx2 and Osx.

2.4. TGF-β/BMP signaling

The transforming growth factor-beta (TGF-β) superfamily is a large and diverse group of structurally related secreted growth factors [55,56]. The TGF-β superfamily members bind as dimers to receptor complexes consisting of heterotetrameric combinations of types I and II serine–threonine kinase receptors. Based on the structural characteristics and on the signal transduction pathways that they activate, the members of this superfamily can be subdivided into the TGF-β class and the BMP class.

Upon a ligand binds to the receptor complex, the type II receptor phosphorylates the type I receptor within a glycine/serine-rich (GS) domain, which subsequently activates receptor Smads (R-Smads; Smads 1, 2, 3, 5, and 8). R-Smads then recruit the common partner Smad (Co-Smad; Smad4) and form complex, followed by entering the nucleus. The complex binds to the regulatory elements of target genes through interaction with other transcription factors [57,58]. Ligands of the BMP subclass bind to the type I receptors ALK2, ALK3 (BMPRIA), and ALK6 (BMPRIB), and activate Smads 1, 5, and 8, whereas ligands of the TGF-β subclass bind to receptor complexes containing the type I receptors ALK4, ALK5 (TGF-β RI), and ALK7, and activate Smads 2 and 3. These distinctions, however, are not always exact.

Regarding the expression patterns of BMP signaling components in skeletal tissues, BMP2, 3, 4 and 7 are reported to be expressed in the perichondrium [59–62], and BMP2 and 6 are mainly expressed in hypertrophic chondrocytes in growth plate [63]. The type I BMP receptors are also reported to have characteristic expression patterns in the growth plate. ALK3 is highly expressed in columnar and hypertrophic chondrocytes and perichondrial cells. ALK2 is expressed primarily in round and columnar chondrocytes, while ALK6 expression is observed throughout the growth plate, including the developing articular surface and in the perichondrium [60,64–66]. The type II BMP receptor is also expressed throughout the growth plate [60,64–66]. Thus, as is the case with the ligands, essentially every region of the growth plate expresses at least one type I BMP receptor.

BMPs derive their name from their potent ability to induce ectopic bone formation when subcutaneously implanted in rodents [67]. A number of studies indicate that BMPs have positive impact on the osteoblast differentiation, whereas Noggin, a BMP antagonist, inhibits osteoblast differentiation [68]. Further, a heterozygous gain-of function mutation in the gene encoding ALK2 is identified as a clinical feature of fibrodysplasia ossificans progressiva (FOP; MIM #135100) which is an autosomal dominant disorder manifested as skeletal malformations and progressive extraskeletal ossification [69].

Despite these lines of evidence, our understanding of the role of BMPs through genetic studies has been hampered for mainly two reasons. First, as already mentioned, BMP family members are often expressed in overlapping tissues and may play redundant roles. Second, BMP signaling plays critical roles in cartilage development, so that disruption of the pathway in the skeleton often leads to profound early defects that preclude a precise assessment of osteoblast development [70]. In mice with the limb bud mesenchyme-specific ablation of Bmp2 and Bmp4 using Prx1-Cre driver, normal bone collar formation is observed in the fetal stage, but bone marrow formation and trabecular bone formation are delayed at birth [71]. $Bmp2^{+/-}$; $Bmp6^{-/-}$ mice exhibit a reduction in the trabecular bone volume with impaired bone formation in both fetal and adult stages [72].

On the other hand, overexpression of Noggin driven by the Col1a1 promoter in mice shows increased bone volume with a decreased of both bone formation rate and osteoclast number [73]. In addition, a study of the osteoblast-specific ablation of Bmpr1a using osteocalcin2-Cre driver shows that the response of the osteoblasts is age-dependent: bone volume is decreased in young mice but increased in old mice [74]. An osteoblast-specific ablation of Bmpr1a in adult mice using Col1a1-CreER shows increased bone mass [75]. These data suggest that BMP signaling may not directly

induce bone formation in the fetal stage, but rather may regulate bone homeostasis after birth.

TGF-βs 1-3, ALK5 and TGF-β RII are expressed in the perichondrium [76,77]. Mice with the mesenchymal precursor cell-specific ablation of ALK5 using *Dermo1-Cre* driver have an abnormally thin perichondrial cell layer and reduced proliferation and differentiation of osteoblasts [78]. In mice with the limb bud mesenchyme-specific ablation of *Tgfbr2* using *Prx1-Cre* driver, the length of the long bones is short due primarily to a decrease in chondrocyte proliferation and the joints are fused [79]. In addition, Yi Tang et al. suggest a model in which active TGF-β1 released during bone resorption coordinates bone formation by inducing the migration of bone mesenchymal stem cells in bone marrow [80]. These data suggest that the signaling plays important roles in the patterning, proliferation and migration of cells in the skeletal tissues including osteoblast precursors, but indirectly regulates osteoblast differentiation.

2.5. Signaling networks among Hh, Wnt and BMP signaling

As described above, Hh, Wnt and BMP signaling play important roles in the osteoblast lineage. In this context, which signaling pathway initiates the differentiation cascade and how do these pathways interact with each other? Although several groups have tried to answer these questions, the signaling networks are still not fully revealed. As mentioned earlier, Hh signaling appears to initiate the osteoblast program by specifying Runx2-negative mesenchymal cells into Runx2-positive osteoblast precursors. However, Hh-mediated Runx2 induction is unlikely to be sufficient for this Hh function, given that the recovery of *Runx2* expression in skeletal cells does not restore bone formation in *Ihh*$^{-/-}$ embryos [81]. This finding suggests that additional factors besides Runx2 mediate the functions of Ihh signaling in the initiation of the osteoblast program.

Regarding the interaction of Hh and Wnt signaling, it has been demonstrated that the nuclear localization of β-catenin (which is a readout of canonical Wnt signaling activation) and the expression of target genes for the Wnt canonical pathway are abolished in the perichondrium in *Ihh*$^{-/-}$ embryos [37,38]. In contrast, the osteoblast differentiation and bone collar formation are normal in mice with *Osx*-positive cell-specific ablation of *Smo* using *Osx1-GFP::Cre* driver [9]. These data suggest that Wnt/β-catenin signaling acts downstream of Hh signaling in osteogenesis, and that Hh signaling does not play an essential role in the late osteoblast differentiation beyond an *Osx*-positive cell state.

BMP signaling has a positive impact on both osteogenesis and chondrogenesis. Although the pathway is unlikely to be involved in the specification of the osteoblast linage, it has a positive effect, in conjunction with Hh signaling, on osteoblast differentiation *in vitro* and *in vivo* [82,83]. We also recently reported that activation of BMP signaling enhances ectopic chondrocyte formation in the perichondrium in the absence of Hh input. Hh signaling suppresses BMP-mediated chondrocyte differentiation *in vitro* [82]. Taken together, these findings lead us to infer that among the osteochondrogenic functions of BMP, osteogenic function is elicited by Hh signaling in the perichondrium under physiological conditions.

3. Future perspective

Osteoblast differentiation is well coordinated by a number of signaling pathways. In this context, the distinct window for each signaling in terms of timing and the threshold level of its activation is important. A major challenge for gaining a better understanding of the 'osteogenic signaling network' is to reveal how these signaling pathways are controlled to act within the proper window.

Recently, Regard et al. identified Gαs as a key regulator of proper osteoblast differentiation maintaining a balance between the Hedgehog and Wnt/β-catenin pathways [84].

It will also be beneficial to identify the molecular mechanisms underlying ligand secretion and diffusion to form the proper gradients for functional outcomes. Another challenge is to identify the gene regulatory network programming the osteoblast lineage. Chromatin immunoprecipitation followed by massively-parallel sequencing (i.e., ChIP-seq) is a powerful tool to extensively detect protein-DNA binding events and histone modifications that highlight the epigenetic state of the genome [85]. This approach may provide insights into the regulatory networks in the osteoblast lineage in terms of the target genes of transcription factors and the stage-specific epigenetic landscape.

As described in this review, many signaling factors have already been identified as osteogenic factors and some of them are thought to be good candidates for skeletal tissue engineering. In order to apply these factors to clinical settings, it will be important to optimize the signaling activity for the target cells by integration of the results of studies of drug delivery systems and scaffolds.

Conflict of interest

All authors declare no conflict of interest.

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