Novel In Vitro Assay Models to Study Osteogenesis and Chondrogenesis for Human Skeletal Disorders

Takenobu Katagiri

Abstract Bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs) are involved not only in the physiological development of skeletal tissues but also in the pathological conditions in the tissues. Osteogenesis and chondrogenesis during skeletal formation can be studied in vitro using cell lines and primary cultured cells, which are able to differentiate into osteoblasts and chondrocytes in response to BMP/GDF signaling. These in vitro model systems have been applied to the examination of molecular mechanisms of skeletal disorders related to BMPs/GDFs. Moreover, these in vitro model systems are useful for the development of novel treatments for the disorders.

Keywords Osteoblast differentiation • Chondrocyte differentiation • Mesenchymal cells • In vitro model systems

Osteogenic members of the transforming growth factor -β (TGF-β) family, such as bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs), regulate skeletal development during embryogenesis and tissue regeneration in various vertebrates [1, 2]. Optimal activity of BMPs and GDFs is required for normal skeletal tissue because inadequate activity or overactivity causes skeletal disorders in humans and other animals (please see other chapters for details). As Marshall R. Urist originally reported [3], new bone formation is induced by osteogenic BMPs and GDFs in vivo. It has been reported that the implantation of a pharmacological dosage of BMPs or GDFs induces chondrocytes and osteoblasts in the implants within a week [4–8], suggesting that those osteogenic ligands directly regulate differentiation of chondrocytes and osteoblasts from progenitor cells. In this chapter, I will describe in vitro assay models to study molecular mechanisms underlying skeletal disorders in humans.

T. Katagiri
Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Sa-tama 350-1241, Japan
e-mail: katagiri@saitama-med.ac.jp

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1 Phenotypic Markers of Skeletal Tissue-Forming Cells

1.1 Osteoblasts

Osteoblasts are unique and specialized cells that form bone tissue in vivo [9–11]. They are believed to develop from undifferentiated mesenchymal cells during embryonic development. The bone-forming osteoblasts secrete typical organic components of bone matrices, such as type I collagen, bone sialoprotein, osteonectin, and osteocalcin [9–11]. Moreover, osteoblasts regulate the accumulation of minerals, such as hydroxyapatite (calcium phosphate) crystals, in the organic bone matrix (osteoid) by removing pyrophosphate with high alkaline phosphatase (ALP) activity [9]. Osteoclast-dependent bone resorption is indirectly regulated by osteoblasts via expression of receptor activated nuclear factor-kB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG) as well as macrophage colony-stimulating factor (M-CSF) [10, 12, 13]. These differentiations and functions of osteoblasts are regulated by various extracellular stimuli, including a calcium hormone, parathyroid hormone (PTH), various cytokines, and growth factors. Runx2 and Osterix are transcription factors abundantly expressed in osteoblasts. Thus, the expression levels of these phenotypic markers are examined to study osteoblast differentiation in vitro.

1.2 Chondrocytes

Although chondrocytes, similar to osteoblasts, are also derived from undifferentiated mesenchymal cells, they are specialized cells that form cartilage tissue, which is a template before bone formation in endochondral ossification [11, 14–16]. Commitment of chondrocyte differentiation in progenitor cells is regulated by critical transcription factors, such as SOX9, SOX5, and SOX6 [11, 14–16]. Type II collagen and aggrecan are abundantly secreted by proliferating chondrocytes in growth plates. Terminally differentiated hypertrophic chondrocytes express type X collagen, metalloproteinase-13 (MMP-13), and ALP and induce a transition from cartilage to bone tissues [11, 14–16].

2 C2C12 Myoblasts for BMP/GDF Research

2.1 C2C12 Myoblasts

The murine myoblast cell line C2C12 was originally established from regenerating thigh muscle for studying the molecular mechanisms of myogenesis [17]. Indeed, C2C12 myoblasts proliferate as mononuclear cells expressing MyoD, a skeletal muscle-specific transcription factor, and differentiate into myocytes expressing proteins for muscle contraction such as myosin heavy chain and troponin T (Fig. 1).

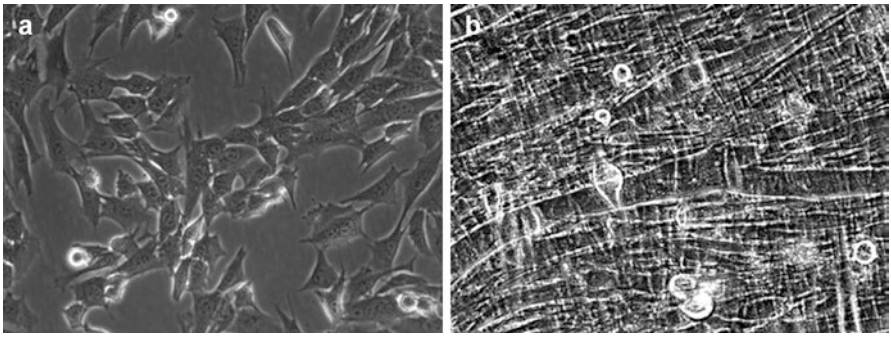

Fig. 1 Murine C2C12 myoblasts. C2C12 cells cultured for 1 (**a**) and 14 (**b**) days in vitro. They proliferate as mononuclear cells and form multinucleated myotubes after differentiation

The cells fuse together and form multinucleated myotubes in vitro (Fig. 1). However, C2C12 myoblasts are also widely used for studying BMP signaling and BMP-induced osteoblastic differentiation.

2.2 Osteoblastic Differentiation of C2C12 Myoblasts by BMPs

In the early 1990s, just after molecular cloning of several BMPs, in in vitro assay systems that reflect the bone-inducing activity of BMPs in vivo were developed [18–21]. These systems allowed the evaluation of biological activity of each recombinant BMP produced, which was needed to study intracellular signal transduction of BMPs. Among the cells examined, C2C12 myoblasts showed the high response to BMP-2, which was evaluated by the induction of ALP activity in vitro [20]. Moreover, the expression of other phenotypic markers related to osteoblast differentiation, such as osteocalcin secretion and PTH receptor, were also induced by the treatment with BMP-2 in C2C12 cells [20]. In contrast, the C2C12 myoblasts treated with BMP-2 were suppressed to express markers related to skeletal muscle differentiation, such as myogenin, myosin heavy chain, and troponin T, and they remained as mononuclear cells [20]. Although BMP-2 induced the expression of type II collagen mRNA and small droplets stained with oil red-O staining, it was still unclear whether chondrogenesis and/or adipogenesis occurred in the cells.

It has been reported that TGF-β1 does not induce heterotopic ossification in vivo [22]. In C2C12 cell cultures in vitro, TGF-β1 has been shown to inhibit myogenesis, but it does not induce any markers related to osteoblast differentiation in vitro [20]. Other inhibitors of myogenesis, such as fibroblast growth factors (FGFs), suppress myogenesis of C2C12 myoblasts but do not induce the markers of osteoblastic differentiation, suggesting that the inducing capacity of osteoblastic differentiation of C2C12 cells is limited for the osteogenic members of the TGF-β family. In addition, all-trans-retinoic acid has been shown to induce ALP activity in C2C12 and another

mesenchymal cell line, C3H101/2 clone 8, but it does not induce other markers related to osteoblast differentiation [20]. Together, these findings suggest that C2C12 cells can reflect the osteogenic and non-osteogenic activities of the members of TGF-β family in vitro.

The differentiation capacity of C2C12 cells into osteoblastic cells in vitro has been applied to evaluate osteogenic activity of 14 types of human BMP (BMP-2 through BMP-15), which were individually overexpressed in the cells using adenovirus vectors [23, 24]. In the assay, not only BMP-2 but also BMP-4, BMP-6, BMP-7, and BMP-9 have been found to induce ALP activity [23, 24]. After transplantation of these C2C12 cells expressing each BMP in mice, BMP-2, BMP-6, BMP-7, and BMP-9 have been found to induce heterotopic ossification in vivo [24]. It is possible that the failure of BMP-4 to induce heterotopic ossification in vivo was due to rapid diffusion of the ligand.

2.3 Applications of C2C12 Myoblasts for Signaling Molecules of BMPs/GDFs

The osteoblastic differentiation of C2C12 cells in response to osteogenic BMPs is clear and easily detectable because the basal levels of ALP or osteocalcin expression are quite low in the untreated C2C12 cells. Thus, this cell line has been used to elucidate intracellular signaling molecules of BMPs, and the findings have been expanded to studies of human skeletal disorders related to BMPs later.

2.3.1 Receptors

Osteogenic BMPs and GDFs bind to type II receptors (BMPR-II, ActR-IIA, and ActR-IIB) and type I receptors (ALK1, ALK2, ALK3, and ALK6) [25–31], and they are expressed in C2C12 cells even though ALK1 and ALK6 are quite low [32, 33]. Each BMP and GDF binds to various combinations of the type I and type II receptors that are expressed on target cell plasma membranes and activates intracellular signaling.

Both types of receptors are transmembrane serine/threonine kinase. Although type II receptors are active regardless of ligand binding, type I receptors are inactive and activated by type II receptors via phosphorylation at the glycine/serine rich domain (GS domain) in the intracellular region at the juxtamembrane [25–31]. Type I receptors, rather than type II receptors, determine intracellular signaling pathways. Overexpression of constitutively active forms of type I receptors for osteogenic BMPs and GDFs (ALK1, ALK2, ALK3, or ALK6) in C2C12 cells can inhibit myogenesis and induce osteoblastic differentiation without the addition of exogenous ligands [33–35]. In contrast, overexpression of dominant negative forms of type I receptors, which have the extracellular ligand-binding domain and transmembrane domain but lack a functional intracellular kinase domain, inhibits osteoblastic

differentiation of C2C12 cells even in the presence of ligands [32]. These findings support a hypothesis that type I receptors are downstream effectors of type II receptors.

2.3.2 Transcription Factors

The type I receptors bound to osteogenic ligands phosphorylate intracellular signaling molecules in the cytoplasm and subsequently transduce intracellular signaling. Smad1, Smad5, and Smad9 (also known as Smad8) are known as major substrates critical for downstream signaling [25–31]. The type I receptors phosphorylate two serine residues in the serine-valine-serine (SVS) motif at the carboxyl termini of these Smad proteins [25–31]. The phosphorylation of the carboxyl termini leads to a conformational change of Smad proteins and allows them to form complexes with other transcriptional regulators, such as coactivators and corepressors including Smad4 and RAN-binding domain-containing protein 2, respectively [36–38].

Indeed, co-expression of a constitutively active type I receptor kinase, with Smad1 or Smad5, which is a substrate of the receptor kinase, induces osteoblastic differentiation of C2C12 cells [39–41]. This induction is blocked by addition of a small molecule inhibitor of type I receptor kinases, such as LDN-193189 and LDN-212854, thus supporting that the kinase activity of type I receptors is essential for intracellular signal transduction through Smad proteins [39–41].

Stimulation of cells with a ligand of BMP/GDF members activates multiple intracellular signaling pathways, including Smad1, Smad5, Smad9, phosphatidylinositol-3 kinase, and p38 mitogen activated protein kinase. C2C12 cells have been used to examine the role of each Smad pathway in osteoblastic differentiation. To activate only one phosphorylated Smad without activating the others, the constitutively activated forms of Smad1, Smad5, and Smad9 have been generated by substituting the SVS motif with the DVD sequence in each Smad [36, 38]. The DVD forms of Smad1, Smad5, and Smad9 are recognized by an antibody against phosphorylated Smad1/Smad5/Smad9 [36, 38]. Moreover, they activate transcription of target genes and osteoblastic differentiation of C2C12 cells without addition of ligands or active receptors [36, 38]. However, Smad9 shows weaker transcriptional activity than Smad1 and Smad5 and fails to activate osteoblastic differentiation of the cells, owing to a deletion of a small part of the linker region [38]. Interestingly, expression of Smad9 mRNA has been found to be induced by BMP-4 stimulation in C2C12 cells, similarly to that of an inhibitory Smad, Smad6 [38]. Although Smad6 suppresses BMP receptors, Smad9 suppresses a constitutively active form of Smad1 as a dominant negative Smad [38].

Osterix (also known as SP7) is a transcription factor essential for bone formation during embryogenesis in mice. Osterix was identified as a novel transcription factor in C2C12 cells stimulated with BMP [42]. Osterix-deficient mice lack bone formation due to a loss of osteoblast differentiation similarly to Runx2-deficient mice. A premature natural mutation of Osterix/SP7 has been identified in a patient with osteogenesis imperfecta, type XII (MIM: 613849) [43]. Heterozygous

loss-of-function mutation of RUNX2 has been found in patients with cleidocranial dysplasia (MIM: 119600) [44–46].

2.3.3 Early Response Genes of BMP Signaling

Although BMP/GDF proteins are multifunctional growth factors, they activate transcription of common genes within an hour after binding in various types of cells. The inhibitor of differentiation/DNA binding (Id) genes, *Id1*, *Id2*, and *Id3*, have been identified as the early responsive genes of BMP signaling [47, 48]. The regulatory elements in their 5′ enhancer regions have a conserved GGCGCC sequence, which is recognized by a complex of Smad1/5 and Smad4 [47, 48]. The same sequence has been found in the 5′ region of BMP-inducible transcript-1, which is also induced by BMP signaling within an hour [48]. The regulatory regions of the BMP early responsive genes can be placed in front of the luciferase gene to examine quantitatively examine BMP-specific intracellular signaling [47, 48]. However, the direct targets of Smad proteins, which regulate osteoblastic differentiation upstream of Osterix and/or Runx2 in the BMP/GDF signaling pathway, still need to be clarified.

3 Models of Chondrogenesis

Because osteogenic BMPs and GDFs induce cartilage before the induction of bone tissue in vivo, the differentiation of chondrocytes in response to ligands are examined in vitro.

3.1 Skeletal Muscle Cells

The induction of cartilage in skeletal muscle by BMPs in vivo suggests that skeletal muscle contains progenitor cells of chondrocytes. Thus, the minced skeletal muscle has been cultured on the demineralized bone matrix to examine the chondrogenesis in vitro [49]. Histological analysis has identified chondrocytes in the cavities formed in the bone matrix, confirming that the possibility of the presence of chondrocyte progenitor cells in the skeletal muscle [49]. Chondrogenesis-inducing activity in the extracts of the demineralized bone matrix has been examined in vitro using skeletal muscle cells embedded in agarose by monitoring synthesis of cartilage-specific proteoglycans [50]. Recent studies of cell lineage tracing using fluorescent proteins have revealed that the progenitor cells in the skeletal muscle tissue, which differentiate into chondrocytes and/or osteoblasts in response to BMP signaling, are interstitial mesenchymal cells, not satellite cells or endothelial cells [51].

3.2 Embryonic Fibroblasts, Embryonic Stem Cells, and Induced Pluripotent Stem Cells

Embryonic fibroblasts and embryonic stem (ES) cells are used in vitro as sources of pluripotent cells. In these types of cells, chondrogenesis is inducible in pellet cultures in the presence of TGF-β and/or BMPs. Cells prepared from chicken or mouse embryonic limb buds also show the chondrogenic activity in high-density micromass cultures. Recently, induced pluripotent stem (iPS) cells established from patients with skeletal disorders have also been used in chondrogenesis in vitro.

4 Analysis of Skeletal Disorders Related to BMP Activity In Vitro

4.1 Fibrodysplasia Ossificans Progressiva

Fibrodysplasia ossificans progressiva (FOP) (MIM: 135100) is a rare disorder characterized by progressive heterotopic ossification in soft tissues, such as skeletal muscle, tendon, and ligaments, after birth [27, 28, 52]. Although the soft tissues are almost normal at birth, muscle trauma induces an acute heterotopic ossification. The incidence of FOP is estimated to be one in two million, regardless of race, gender, location, or age [52]. The involvement of BMP signaling has been suggested in heterotopic ossification in FOP [53]. Although there is no approved treatment for inhibiting heterotopic ossification in FOP, the in vitro models are useful for studying the molecular mechanisms of the disease and the development of novel treatments.

4.1.1 Functional Changes of ALK2/ACVR1 in FOP

In 2006, a recurrent mutation in both familial and sporadic cases of FOP was identified in one BMP type I receptor, ALK2/ACVR1 [54]. The mutation, c.617G>A, causes an Arg to His substitution mutation of ALK2 at position 206 (p.R206H) in the GS domain, which is a phosphorylation site of BMP type II receptors [54]. The mutation changes conformation of the GS domain and affects the interaction between the GS domain and kinase domain.

Functional changes of the mutant ALK2 has been examined in vitro using C2C12 cells. Transient overexpression of ALK2(R206H) in C2C12 cells induces phosphorylation of Smad1/Smad5/Smad9 and activates a BMP-specific luciferase reporter driven by an enhancer region of the early responsive gene of BMP signaling, such as Id1 [39–41]. ALP activity, a typical marker of osteoblastic differentiation of C2C12 cells, is also induced when ALK2(R206H) is co-expressed with Smad1 or Smad5 [39–41]. Moreover, myogenesis of is suppressed in the ALK(R206H)-

expressing C2C12 cells [39, 40]. These BMP activities induced by the mutant ALK2 are blocked by treatment with a small chemical inhibitor against BMP type I receptor kinases [39–41], suggesting that the kinase activity of the mutant ALK2 is increased and phosphorylated Smad1 and/or Smad5 mediate the biological activity of the receptor.

4.1.2 Molecular Mechanisms of the Activation of ALK2 in FOP

To date, more than 10 different mutations in the intracellular region, such as the GS domain and the kinase domain, of ALK2 have been identified from patients with typical or atypical FOP [28, 29]. All of the mutant ALK2 identified activate BMP signaling when they are overexpressed in C2C12 cells, although some mutants show quite weak activity [55]. FKBP12, a small binding protein for an immunosuppressor FK506, has been shown to bind to type I receptors for the TGF-β family and stabilize the inactive state of the kinase [56]. Crystal structures of the cytoplasmic domain of ALK2 and FKBP12 have revealed that the FOP mutations break critical interactions with FKBP12 [57].

In C2C12 cells, co-expression of BMP type II receptor, such as BMPR-II or ActR-IIB but not ActR-IIA, synergistically increases the kinase activity of the mutant forms of ALK2 associated with FOP but not the wild type or associated with heart diseases [55]. This stimulation depends on the kinase activity of the type II receptors. ALK2(Q207D), a constitutively active form created by genetic engineering, is activated even by the kinase activity-deficient type II receptors in C2C12 cells [55]. This suggests that the mutant forms of ALK2 associated with FOP are not constitutively active but require upstream effectors, such as type II receptors and possible ligands [55]. The threonine residue at position 203 in ALK2 is essential for the type II receptor-dependent activation of BMP signaling through regulating the phosphorylation levels of ALK2 by the type II receptors [55]. Moreover, the conserved Thr residues in other BMP type I receptors, such as ALK1, ALK3, and ALK6, are also required for the ligand-induced intracellular signaling [55].

Recently, Activin A, which is a non-osteogenic member of the TGF-β family, has been shown to activate BMP-like activity in cells expressing ALK2(R206H) [58]. Moreover, anti-Activin A antibody suppressed heterotopic ossification in conditional-on knock-in mice of human ALK2(R206H) [58]. These findings suggest that heterotopic ossification in patients with FOP is a ligand-dependent event, and Activin A is responsible for it.

4.1.3 Chondrogenesis Models in Vitro for FOP

Heterotopic skeletal tissues in FOP are formed through an endochondral ossification process, suggesting that mutant forms of ALK2 induce chondrocyte differentiation in progenitor cells in the soft tissues.

Murine ES cells that express human ALK2(R206H) under the control of the Tet-off system have been established, and their chondrogenic capacity in vitro has been analyzed [59]. Withdrawal of doxycycline from the culture medium induces the expression of ALK2(R206H), the phosphorylation of Smad1/5 and the expression of markers related to chondrocyte differentiation, such as type II collagen and aggrecan [59]. As expected, a small chemical inhibitor of the BMP type I receptor kinases inhibits these doxycycline-dependent events except for the expression of human ALK2 [59].

Knock-in mice of the ALK2(R206H) mutation have been examined. Although they show the malformation of great toe and the heterotopic ossification in soft tissues, similarly to patients with FOP, these mice die after birth [60]. Embryonic fibroblasts prepared from the knock-in mice show enhanced chondrogenic activity in vitro compared to that of wild-type mice [61]. iPS cells have been established from patients with typical FOP who carry the R206H mutation [62]. The iPS cells show accelerated chondrogenic ability in vitro compared to that of the gene-corrected and rescued iPS cells [63].

4.2 Brachydactyly, Symphalangism, and Multiple Synostosis Syndrome

Among BMPs/GDFs, GDF5 is a key regulator of skeletal development during embryogenesis, especially for digit and joint formation. Loss-of-function and gain-of-function mutations have been identified in a ligand (GDF5), receptor (BMPR-IB/ALK6), and antagonist (Noggin) in patients with skeletal disorders, such as brachydactyly, symphalangism, and multiple synostosis syndrome.

4.2.1 Gain-of-Function and Loss-of Function Mutations in GDF5

Human GDF5 has been shown to be mutated in skeletal malformation syndromes including brachydactyly type C (BDC) (MIM: 113100), which is characterized by the shortening of digits and hypersegmentation of phalanges and the recessive acromesomelic dysplasias of the Hunter-Thompson, Grebe, and DuPan types, which are characterized by short stature, severe limb shortening, and profound brachydactyly.

A mutation of p.L441P in GDF5 has been identified from patients showing short index fingers and variable clinodactyly, similarly to the patients with brachydactyly type A2 (BDA2) (MIM: 112600), which is caused by a mutation in BMPR-IB/ALK6 [64]. Another mutation in GDF5, p.R438L, has been identified in patients with proximal symphalangism (SYM1) (MIM: 185800) and is characterized by a bony fusion between the proximal and middle phalanges in the digits [64]. C2C12 cells express BMPR-IA/ALK3, but they do not express functional levels of BMPR-IB/ALK6. Thus, the cells respond to BMP-2, but they do not respond to GDF5 [64]. GDF5

stimulates chondrogenesis of chicken limb bud cells in micromass cultures [64]. The p.L441P mutant GDF5 seems to be a loss-of-function mutation because it does not show BMP/GDF-like activity in vitro [64]. In contrast, the p.R438L mutant is a gain-of-function mutation, because it has increased in binding affinity to BMPR-IA/ALK3 [64]. The p.R438L mutant GDF5, but not p.L441P, induces ALP activity in C2C12 cells and suppresses myogenesis similarly to BMP-2 [64].

Additional mutations in GDF5, p.N445T/K, and p.W414R have been identified from patients with SYM1 and combined clinical features of brachydactyly type A2 (BDA2) and multiple synostosis syndrome 2 (SYNS2) (MIM: 610017), respectively [65]. These mutant GDF5 are insensitive and resistant to Noggin, similarly to BMP-9 and BMP-10 [65]. Overexpression of the mutant GDF5 or BMP-9 in the micromass cultures of chicken limb bud cells shows high chondrogenic activity [65], suggesting that normal joint formation induced by GDF5 requires a negative feedback through an antagonist, i.e., Noggin.

5 Conclusion

The original bone-inducing activity of BMPs can be reflected, at least in part, in in vitro model systems using cell lines or primary cultured cells. These systems have been applied to the examination of molecular mechanisms of skeletal disorders related to BMPs/GDFs. Moreover, these in vitro model systems are useful for the development of novel treatments for the disorders.

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