TGF-β Family Signaling in Mesenchymal Differentiation

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Mesenchymal stem cells (MSCs) can differentiate into several lineages during development and also contribute to tissue homeostasis and regeneration, although the requirements for both may be distinct. MSC lineage commitment and progression in differentiation are regulated by members of the transforming growth factor-β (TGF-β) family. This review focuses on the roles of TGF-β family signaling in mesenchymal lineage commitment and differentiation into osteoblasts, chondrocytes, myoblasts, adipocytes, and tenocytes. We summarize the reported findings of cell culture studies, animal models, and interactions with other signaling pathways and highlight how aberrations in TGF-β family signaling can drive human disease by affecting mesenchymal differentiation.

Mesenchymal stem cells (MSCs) are multipotent cells that have the ability for self-renewal and the capacity to progress into several cell lineages, including osteoblasts, chondrocytes, myoblasts, adipocytes, and tenocytes (Friedenstein et al. 1970, 1976; Grigoriadis et al. 1988; Pittenger et al. 1999; Horwitz et al. 2005; Augello and De Bari 2010; Worthley et al. 2015). They contribute to tissue differentiation and regeneration, including maintenance of tissue homeostasis and function, adaptation to altered metabolic or environmental requirements, and repair of damaged tissue (Friedenstein et al. 1970; Grigoriadis et al. 1988; Pittenger et al. 1999; Charge and Rudnicki 2004; Augello and De Bari 2010). MSCs have been isolated from fetal tissues, adult bone marrow, and most connective tissues, including adipose tissue, dental tissues, and skin, as well as from peripheral blood, synovial fluid, and the perivascular compartment (Friedenstein et al. 1970, 1976; Pittenger et al. 1999; Tang et al. 2004; Bartsch et al. 2005; Wagner et al. 2005; Crisan et al. 2008; Morito et al. 2008; Riekstina et al. 2008; Huang et al. 2009a; Ab Kadir et al. 2012; Raynaud et al. 2012). MSCs can, in a first step, commit to specific cell lineages and then, in a second step, progress in differentiation along these lineages. These steps are initiated and regulated through interactions with other cells, in response to mechanical signals, and by extracellular signaling factors. Together, these

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interactions and signals promote or suppress the expression of cell lineage-specific transcription and survival factors that regulate expression of genes important for the specific cell functions of this lineage (Grigoriadis et al. 1988; Pittenger et al. 1999; Langley et al. 2002; Javed et al. 2008; Karalaki et al. 2009; Wang and Chen 2013; Worthley et al. 2015). For instance, MSC-derived preosteoblasts express early markers of the osteoblast lineage, including type I collagen (encoded by *Col1A1* and *Col1A2*) and alkaline phosphatase (encoded by *Alpl*), whereas terminally differentiated osteoblasts express genes such as *Bglap*, encoding osteocalcin (Ocn), and show the capacity to form a mineralized extracellular matrix (Fakhry et al. 2013). Interestingly, and potentially of therapeutical interest, MSCs may also transdifferentiate in culture into cells of ectodermal and endodermal lineages, and express markers of neuronal cells, hepatocytes, or pancreatic cells (Safford et al. 2002; Kanafi et al. 2013; Wang et al. 2014). Furthermore, MSCs can support hematopoietic cells in the bone marrow microenvironment, and exert anti-inflammatory and immunomodulatory effects through interactions with the immune system (Haynesworth et al. 1996; Aggarwal and Pittenger 2005; Li et al. 2005; Carrade et al. 2012; Franquesa et al. 2012; Svobodova et al. 2012).

The commitment of MSCs to certain mesenchymal lineages, and their progression in differentiation along these lineages, is controlled by specific transcription factors. For instance, osteogenic lineage commitment is induced by the expression of runt-related transcription factor 2 (Runx2), a master transcription factor of osteoblastogenesis (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997; Komori 2010a). Runx2 promotes differentiation of MSCs into preosteoblasts and expression of genes during early stages of osteoblast differentiation, while it inhibits MSC commitment to the adipocyte lineage (Komori 2010b). Further osteoblast differentiation and maturation is then driven by the expression of the transcription factor osterix (Osx, encoded by *Sp7*), resulting in increased alkaline phosphatase activity and mineralization (Nakashima et al. 2002; Komori 2006). Runx2 is not crucial to promote differentiation into mature osteoblasts, and its expression is reduced later during differentiation (Maruyama et al. 2007; Komori 2010b).

The commitment of MSCs to the adipocyte lineage is induced by expression of the CCAAT/enhancer binding proteins (C/EBPs) β and δ (encoded by *Cebpb* and *Cebpd*, respectively) (Cao et al. 1991; Otto and Lane 2005). To allow DNA binding, C/EBPβ requires "activation" by phosphorylation by extracellular signal-regulated kinase (Erk) mitogen-activated protein kinase (MAPK), and glycogen synthase kinase-3β (GSK3β), and, consequently, induces expression of C/EBPα and peroxisome proliferator-activated receptor-γ (PPARγ) (encoded by *Cebpa* and *Pparg*, respectively) (Wu et al. 1996; Rosen and MacDougald 2006; Tang and Lane 2012). PPARγ and C/EBPα together regulate genes that are important for the adipocyte phenotype and drive progression of adipocyte differentiation (Tang and Lane 2012). Although PPARγ and C/EBPα are expressed throughout the differentiation process, C/EBPβ expression is down-regulated at later stages (Chen et al. 2016). Interestingly, the key osteogenic and adipogenic transcription factors Runx2 and PPARγ inhibit each other's expression, and PPARγ also inhibits chondrogenesis (Zhang et al. 2006; Isenmann et al. 2009; Valenti et al. 2011).

MSC differentiation to the chondrogenic lineage requires expression of the key chondrogenic transcription factor SRY-box 9 protein (Sox9, encoded by *Sox9*), a member of the "high-mobility group box" transcription factor family (Lefebvre and Smits 2005; Quintana et al. 2009). In addition, the transcription factor NK3 homeobox 2 (Nkx3.2, encoded by *Nkx3-2*) maintains Sox9 expression by blocking the expression of inhibitors of *Sox9* transcription, and Sox9 and Nkx3.2 can induce each other's expression (Zeng et al. 2002; Kozhemyakina et al. 2015). At later stages of differentiation, Sox5 and Sox6, together with Sox9, promote progression to chondrocyte differentiation, but Sox9 expression is reduced in late-stage hypertrophic chondrocytes (Akiyama et al. 2002; Ikeda et al. 2004; Lefebvre and Smits 2005; Kozhemyakina et al. 2015; Liu and Lefebvre 2015).

The key transcription factors for myogenic differentiation are Myf5, Mrf4, MyoD, and myogenin, members of the MyoD family of myogenic regulatory factors (MRFs), which act in cooperation with myocyte enhancer factor (MEF) proteins (Weintraub et al. 1991; Rudnicki et al. 1993; Naya and Olson 1999; Sabourin et al. 1999; Berkes and Tapscott 2005). Myf5, Mrf4, and MyoD are essential for myogenic lineage commitment (Rudnicki et al. 1993; Kassar-Duchossoy et al. 2004), and myogenin together with Mrf4, MyoD, and MEF2 family members, which induce the expression of late muscle-specific genes, drive the progression of myogenic differentiation (Hasty et al. 1993; Naya and Olson 1999; Myer et al. 2001; Berkes and Tapscott 2005).

The key transcription factors that control commitment of MSCs to the tenocyte lineage, and drive progression in differentiation are incompletely understood. Scleraxis (Scx) is a key transcription factor involved in tenocyte lineage selection, and activates the expression of tendon-related genes, while inhibiting osteogenic, chondrogenic, and adipogenic differentiation (Shukunami et al. 2006; Li et al. 2015). However, the exact roles of other transcription factors associated with tendon development, including Six1, Six2, Eya1, Eya2, and Mohawk, have to be elucidated in future studies (Aslan et al. 2008; Jelinsky et al. 2010; Onizuka et al. 2014).

Multiple members of the transforming growth factor-β (TGF-β) signaling family modulate MSC lineage selection and progression of mesenchymal differentiation into specified cells, by controlling the expression and activities of these key transcription factors (Minina et al. 2001; Langley et al. 2002; Huang et al. 2007b; Neumann et al. 2007; Lee et al. 2011; Dorman et al. 2012). TGF-β family signaling is initiated by extracellular ligands that bind at the cell-surface to specific tetrameric transmembrane receptor complexes, consisting of two type II and two type I receptors (Feng and Derynck 2005; Chaikuad and Bullock 2016; Heldin and Moustakas 2016). Ligand binding to the receptor complex induces phosphorylation of the type I receptor I kinase domains by the type II receptors, resulting in the activation of intracellular signaling mediators (Shi and Massagué 2003; Feng and Derynck 2005; Hata and Chen 2016). TGF-β family ligands include TGF-β1, β2, and β3, bone morphogenetic proteins (BMPs), activins, and growth and differentiation factors (GDFs), including myostatin (GDF-8). TGF-β1 and TGF-β3 bind primarily to the TGF-β receptor type II (TβRII), which then activates the ALK-5/TβRI type I receptor (gene name *Tgfbr1*), whereas TGF-β2 requires binding to betaglycan (also called TGF-β type III receptor) or cooperative binding to TβRII and ALK-5/TβRI to activate the TβRI (Chaikuad and Bullock 2016). Activins bind to the activin type II receptors ActRII (also known as ActRIIA) and ActRIIB, which induce phosphorylation of ALK-4 (ActRIB, gene name *ACVR1B*), whereas BMPs bind to BMPRII, ActRII, and ActRIIB, which activate ALK-2 (gene name *Acvr1*), BMPRIA (ALK-3, gene name *Bmpr1a*), and BMPRIB (ALK-6, gene name *Bmpr1b*) (ten Dijke et al. 1993; Massagué 1998; Lux et al. 1999). GDFs interact with several of these type II receptors and induce activation of ALK-2, BMPRIA, BMPRIB, or, in the case of myostatin, ALK-4 or TβRI. The activated type I receptor phosphorylates and thereby activates specific Smad proteins in the canonical signaling pathway, which translocate into the nucleus to control target gene transcription (Feng and Derynck 2005; Hata and Chen 2016; Hill 2016). TGF-βs and activins induce phosphorylation of Smad2 and Smad3 by TβRI or ALK-4, whereas BMPs, acting through ALK-2, BMPRIA, or BMPRIB, activate Smad1, 5, and 8 (de Caestecker 2004; Chaikuad and Bullock 2016; Hata and Chen 2016; Xu et al. 2016). These phosphorylated Smads form complexes with the common co-Smad Smad4, translocate into the nucleus, and form either activating or inhibitory transcriptional regulatory complexes (Hill 2016). The inhibitory Smad6 and Smad7 inhibit these signaling cascades, and their expression is stimulated in response to TGF-β family signaling, thus providing a negative feedback loop (Hayashi et al. 1997; Imamura et al. 1997; Miyazawa and Miyazono 2017). Additionally, ligand–receptor binding also activates non-canonical intracellular signaling cascades, such

as the Erk1 and Erk2 MAPK, c-Jun amino-terminal kinases (JNKs), and p38 MAPK pathways, as well as the phosphatidylinositol 3-kinase (PI3K)–Akt pathway (Zhang 2009).

In this review, we focus on the effects in cell culture and the in vivo roles of TGF-β family signaling in mesenchymal lineage commitment and differentiation into osteoblasts, chondrocytes, myoblasts, adipocytes, and tenocytes.

TGF-β FAMILY SIGNALING IN OSTEOBLAST DIFFERENTIATION

The skeleton functions in physical movement, regulates mineral homeostasis, and secretes endocrine factors (Oldknow et al. 2015). Bone is constantly remodeled in a tightly regulated sequence, coupling bone resorption with bone formation to maintain bone mass (Sims and Vrahnas 2014). Osteoclasts derive from a myeloid lineage and are responsible for bone resorption, whereas osteoblasts mature from a mesenchymal lineage and accomplish bone formation. Osteoblastogenesis occurs in three stages: proliferation, matrix maturation, and mineralization (Huang et al. 2007a). This differentiation process depends on the transcription factors Runx2 and Osx (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997; Nakashima et al. 2002). Osteoblast development is characterized by the expression of a set of gene expression markers, including alkaline phosphatase early in osteoblast differentiation, and osteocalcin and osteopontin at later stages of differentiation (Huang et al. 2007a). Osteoblasts can progress to become osteocytes, which are enveloped in mineralized bone, have mechanosensory and metabolic functions, and regulate bone remodeling (Bonewald 2011; Nakashima et al. 2011; Komori 2013; Sims and Vrahnas 2014). TGF-β family members, including BMPs, TGF-βs, activins, and inhibins regulate differentiation from early bone marrow stromal cells (BMSCs) to mature matrix-secreting osteoblasts and osteocytes (Fig. 1A,B).

BMPs and Osteoblast Differentiation

In cell culture, most BMPs signal through BMPRII and ALK-2, BMPRIA, or BMPRIB to promote osteoblast differentiation (Fig. 1B) (ten Dijke et al. 1994; Chen et al. 1998; Ebisawa et al. 1999; Fujii et al. 1999; Jikko et al. 1999; Suzawa et al. 1999). BMP-2 and -6 potently stimulate, whereas BMP-4 and -7 moderately stimulate osteoblast differentiation, apparent by increased expression and activity of alkaline phosphatase in early osteoblast progenitors, and expression of osteocalcin and osteopontin in differentiated osteoblasts (Yamaguchi et al. 1991; Hughes et al. 1995; Kawasaki et al. 1998; Gori et al. 1999; Cheng et al. 2003; Friedman et al. 2006). Although BMP-2 does not regulate extracellular matrix protein secretion by osteoblasts, it stimulates mineral deposition into the matrix (Yamaguchi et al. 1991; Fromigué et al. 1998; Gori et al. 1999), leading to a higher number of mineralized bone nodules (Chen et al. 1997; Haÿ et al. 1999). BMP-7 also induces highly calcified bone nodules (Chen et al. 2001; Chaudhary et al. 2004), possibly by increasing inositol 1,4,5-trisphosphate (IP_3) receptor levels, which increase calcium mobilization and deposition (Bradford et al. 2000). Unlike other BMP ligands, BMP-3 (also called BMP-3A) and BMP-3b (GDF-10) repress osteoblast differentiation, resulting in decreased expression of osteoblastic markers, bone nodule formation, and mineralization (Kokabu et al. 2012; Matsumoto et al. 2012). Whereas BMP-3A seems to signal through ActRIIB, BMP-3b potentially functions through the ActRII and ALK-4 receptors (Kokabu et al. 2012; Matsumoto et al. 2012). BMP ligands regulate osteoblast differentiation through multiple intracellular pathways, including signaling through Smad1, 5, and/or 8, but also noncanonical signaling pathways such as Erk1/2 MAPK, p38 MAPK, and JNK (Fig. 1A). Smad1, 5, and/or 8 signaling promotes osteoblast differentiation. For instance, increased expression of Smad1 in mesenchymal progenitor cells enhances BMP-2-induced expression of alkaline phosphatase (Ju et al. 2000), whereas bone-specific *Smad1* inactivation results in reduced BMP signaling and delayed bone development in mice (Wang et al. 2011). In addition, blocking Smad5 activation prevents BMP-2-induced alkaline phosphatase and osteocalcin expression (Nishimura

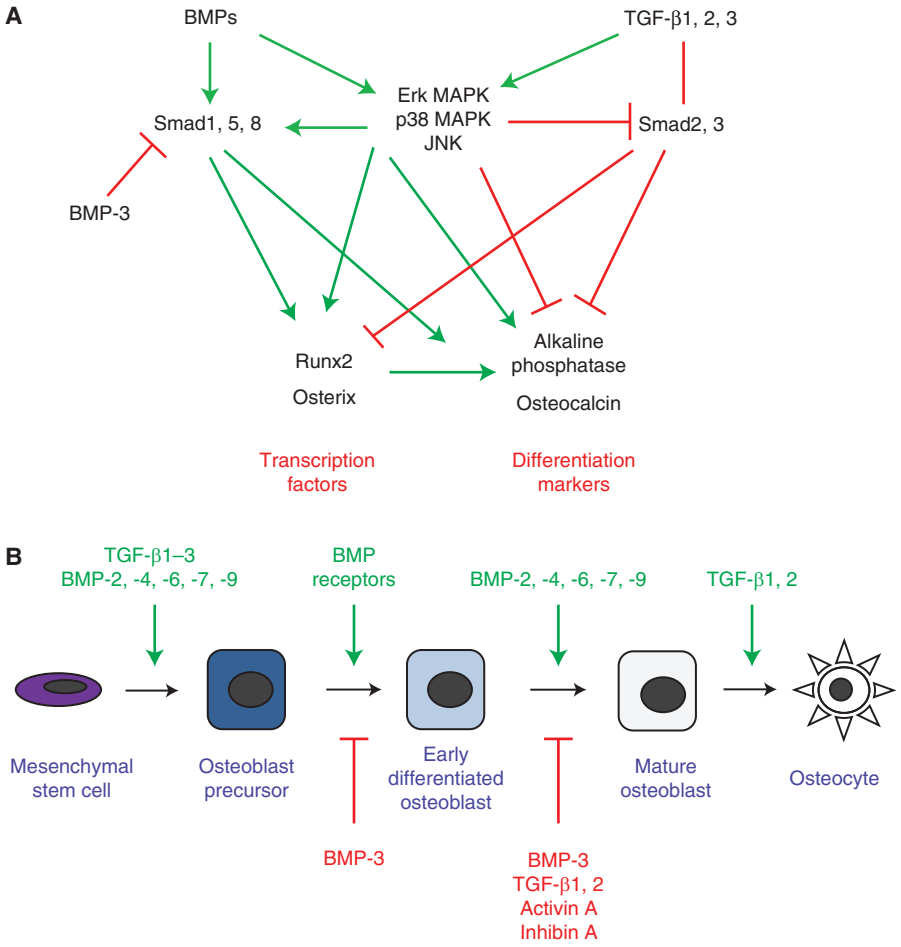

Figure 1. TGF-β family signaling in osteoblast differentiation. (*A*) Major intracellular and transcriptional targets of TGF-β and bone morphogenetic protein (BMP) signaling in osteoblastic differentiation. (*B*) Osteoblasts originate from mesenchymal stem cells. Signaling induced by TGF-β family ligands can inhibit or stimulate lineage selection and progression in differentiation. BMPs, with the exception of BMP-3, mostly promote progression of osteoblast differentiation, whereas activins and inhibins inhibit differentiation, and the TGF-β ligands affect certain stages of osteoblast differentiation. MAPK, mitogen-activated protein kinase; JNK, c-Jun amino-terminal kinase.

et al. 1998). BMP-4 induces activation of Smad1, 5, and 8 in osteoblastic cell lines, whereas BMP-6 and -7 induce activation of Smad1 and 5, but not Smad8, to promote alkaline phosphatase activity, suggesting differential regulation by individual BMPs (Ebisawa et al. 1999; Aoki et al. 2001). In contrast, the inhibitory BMP-3b blocks BMP-2-induced phosphorylation of Smad1, 5, and 8, and expression of osteoblast genes (Matsumoto et al. 2012). BMP-2 and -7 also regulate osteoblast differentiation by activating the Erk1/2 MAPK pathway (Lou et al. 2000; Xiao et al. 2002), and BMP-2 also acts through the p38 MAPK and JNK pathways. Blocking p38 MAPK or Erk MAPK signaling reduces BMP-2-induced *Alp* and *Ocn* expression, whereas inhibiting JNK activation primarily decreases osteocalcin expression (Galléa et al. 2001; Lai and Cheng 2002; Guicheux et al. 2003). In addition, BMP-2-induced

activation of p38 MAPK and JNK enhances Smad1 activation and canonical BMP signaling (Nöth et al. 2003; Liu et al. 2011).

At the transcriptional level, BMP-induced Smad signaling targets genes encoding key osteoblastic differentiation factors such as Runx2 and Osx (Fig. 1A) (Lee et al. 2003). Interestingly, osteoblast precursors cultured from *Runx2*$^{-/-}$ mice do not fully differentiate into osteoblasts, even in the presence of BMP-2, although they still induce alkaline phosphatase and osteocalcin expression (Liu et al. 2007). On the other hand, in cells overexpressing Runx2, anti-BMP-2, -4, and -7 antibodies prevent Runx2 from stimulating *Ocn* transcription, suggesting that BMP signaling is necessary for Runx2 transcriptional activity (Phimphilai et al. 2006). BMP-2 likely does not directly activate Runx2, but rather induces phosphorylation and acetylation by MAPK signaling, modifications necessary for Runx2 to form a complex with phosphorylated Smad1, 5, and/or 8 to target osteoblast gene promoters (Afzal et al. 2005; Javed et al. 2008, 2009; Jun et al. 2010). BMP-2 also regulates *Osx* expression through Smad1 activation, and indirectly through other transcription factors including the homeobox protein Msx2, the homeobox protein Alx3, and the DNA-binding protein inhibitors ID-1, -2, and -3, and Runx2- and p38 MAPK-dependent pathways (Ogata et al. 1993; Lee et al. 2003; Peng et al. 2004; Matsubara et al. 2008; Ulsamer et al. 2008; Matsumoto et al. 2013).

BMP signaling during osteoblast differentiation is repressed by BMP inhibitors, and depends on cross-talk with other signaling pathways, including those activated by Wnt, TGF-β, fibroblast growth factor (FGF), Notch, and tumor necrosis factor-α (TNF-α) (Luo 2017). For example, noggin and gremlin antagonize BMPs by preventing their interaction with their receptors. Furthermore, increasing noggin expression in preosteoblastic cells inhibits BMP-2-induced differentiation (Wu et al. 2003), and decreasing noggin expression increases the phosphorylation of Smad1, 5, and 8, the activity of alkaline phosphatase, and osteocalcin and Runx2 expression (Gazzerro et al. 2007; Wan et al. 2007). During BMP-stimulated osteoblast differentiation, noggin and gremlin expression is enhanced in a feedback loop that inhibits BMP signaling (Gazzerro et al. 1998; Abe et al. 2000; Pereira et al. 2000). Activated Wnt–β-catenin signaling increases BMP-2-induced osteoblast differentiation and is required for the induction of alkaline phosphatase expression by BMP-2 (Rawadi et al. 2003), whereas deletion of β-catenin inhibits the response to BMP-2 (Mbalaviele et al. 2005; Salazar et al. 2008; Zhang et al. 2009). Also, Wnt–β-catenin signaling increases the expression and secretion of BMP-2 (Qiu et al. 2010), whereas BMP-2 can antagonize Wnt–β-catenin signaling by increasing the expression of the Wnt inhibitors sclerostin and dickkopf-1 (Dkk1) (Kamiya et al. 2010). TGF-β can antagonize BMP-2-induced osteoblast differentiation by inhibiting the activation of Smad1, 5, and 8, and promote BMP-2 activity by repressing noggin expression (Galléa et al. 2001; de Gorter et al. 2010). Furthermore, studies in cell culture suggest that FGF-2 is required for BMP-2-induced nuclear accumulation of Runx2 and Smad1, 5, and 8 (Sabbieti et al. 2013), which is supported by the observation that BMP-2 does not induce differentiation of *Fgf2*$^{-/-}$ osteoblast precursor cells (Hanada et al. 1997; Naganawa et al. 2008). Also, Notch signaling increases BMP-2-induced alkaline phosphatase activity and bone nodule formation (Nobta et al. 2005), and by silencing the expression of Hairy/enhancer-of-split related with YRPW motif protein 1 (Hey1), a mediator of Notch signaling, reduces BMP-9-induced osteoblast differentiation (Sharff et al. 2009). In addition, TNF-α acts through either the JNK pathway to inhibit activation of Smad1, 5, and/or 8, and decrease alkaline phosphatase and osteocalcin expression, or the NF-κB pathway to prevent BMP-2-induced *Runx2* expression by blocking Smad complexes from binding to DNA (Eliseev et al. 2006; Singhatanadgit et al. 2006; Mukai et al. 2007; Billings et al. 2008; Yamazaki et al. 2009; Hirata-Tsuchiya et al. 2014). Menin, a protein implicated in multiple endocrine neoplasia type I, also interacts with BMP signaling by binding to Smad1 and 5, and enhances BMP-2-induced osteoblast differentiation (Sowa et al. 2003a).

Most BMPs and their receptors are indispensable for development. Insight into the roles of BMPs in osteoblast differentiation in vivo has mainly come from mouse models in which a gene of interest was conditionally inactivated (conditional knockout; cKO) or overexpressed. Mice have been generated in which either BMP-2 or BMP-4 expression was specifically inactivated in the limb using Cre recombinase expressed from the paired mesoderm homeobox protein 1 (*Prx1*) promoter that is expressed in embryonic limb bud mesenchyme (*Prx1*-Cre mice). Both models with inactivation of BMP-2 or BMP-4 show normal skeletal development. However, compound inactivation of both *Bmp2* and *Bmp4* in the limb almost completely abolishes bone formation, suggesting functional redundancy of BMP-2 and BMP-4, and the requirement of at least one of the two BMPs for normal osteogenesis (Bandyopadhyay et al. 2006; Tsuji et al. 2008). Interestingly, the *Bmp4 Prx1*-Cre cKO mice recover normally from fractures, whereas the corresponding *Bmp2 Prx1*-Cre cKO mice are unable to initiate fracture healing, suggesting that BMP-2 is necessary for normal differentiation of mesenchymal cells in bone repair (Bandyopadhyay et al. 2006; Tsuji et al. 2008). On the other hand, transgenic mice overexpressing BMP-4 under the control of the early osteoblast-specific *Col1a1* promoter develop severe osteopenia and increased osteoclast numbers, suggesting a role for BMP-4 in bone resorption (Okamoto et al. 2006). BMP-4 and -7 may have overlapping functions in the development of the ribs, sternum, and digits, as apparent from the skeletal phenotype of $Bmp4^{+/-}$; $Bmp7^{+/-}$ mice (Katagiri et al. 1998). $Bmp3^{-/-}$ mice have 50% more trabecular bone than wild-type mice, whereas BMP-3 overexpression under the *Col1a1* promoter results in low bone mass and spontaneous fractures in utero, confirming the repression of osteoblast differentiation by BMP-3 seen in cell culture (Daluiski et al. 2001; Gamer et al. 2009). Mice with conditional *Bmpr1a* deletion in mature osteoblasts, by Cre recombinase-mediated recombination from the osteocalcin 2 promoter (*Og2*-Cre mice) show decreased bone formation rate, bone size, bone volume per total volume (BV/TV), and osteoblast marker expression at one month after birth; however, by 10 months these mice show increased BV/TV and bone mineral density (BMD) (Mishina et al. 2004). Disruption of *Bmpr1a* in immature osteoblasts using Cre recombinase expression from the *Col1a1* promoter in mice results in an increased bone mass and BMD at late gestation, weaning, and adult stages (Kamiya et al. 2008a,b). Compared with wild-type controls, these mice develop increased tibial trabecular bone volume and decreased osteoclast numbers in response to mechanical loading (Iura et al. 2015). These ostensibly contradictory outcomes may be explained by considering that BMP signaling may promote the expression of sclerostin and Dkk1 in osteoblasts. These two proteins alter the balance of functional expression of RANKL (receptor activator of NF-κB ligand) and osteoprotegerin (OPG) such that osteoclastogenesis and bone resorption are reduced to a greater extent than osteogenesis, thus resulting in increased overall bone mass (Mishina et al. 2004; Kamiya et al. 2008b, 2010). Disruption of *Bmpr1a* using Cre recombinase expression from the *Dmp1* promoter, which drives expression of dentin matrix protein 1 in more mature osteoblasts and osteocytes, results in a dramatic increase of trabecular bone mass in association with reduction of sclerostin expression (Kamiya et al. 2016; Lim et al. 2016). The similar phenotype of mice with immature osteoblast-specific disruption of *Acvr1* (encoding ALK-2) using Cre recombinase driven by the *Col1a1* promoter further supports the notion that BMP signaling in osteoblasts plays a dual role in promoting osteoblast differentiation to produce bone matrix and supporting osteoclastogenesis to resorb bones (Kamiya et al. 2011). Interestingly, inactivation of *Bmpr1a* using either *Dmp1*- or *Sp7*-induced Cre recombinase expression in mature osteoblasts and osteocytes, or immature osteoblasts, respectively, results in similar increases in bone mass with increased osteoblast numbers despite reduced osteoblast activity (Lim et al. 2016). However, contrary to the mice with osteoblast-specific *Bmpr1a* inactivation from the *Col1a1* promoter, these mice do not show

changes in osteoclast numbers or bone resorption (Lim et al. 2016). Additionally, mice with *Bmpr2* inactivation under the control of the *Prx1* promoter also develop increased bone mass and BMD by 9 weeks of age, but in this case this is the result of increased osteoblast activity with no change in osteoblast numbers (Lowery et al. 2015). This phenotype may be due to the use of the ActRII and/or ActRIIB receptors by the BMPs in the absence of BMPRII as type II receptor. In contrast, global inactivation of *Bmpr1b* expression does not result in overt bone phenotypes, suggesting common and unique functions of the three type I receptors BMPRIA, BMPRIB, and ALK-2 in osteoblasts (Baur et al. 2000; Yi et al. 2000). Mice that express a cytoplasmically truncated, dominant-negative form of BMPRIB from the *Col1a1* promoter have reduced bone mass, suggesting that signaling by BMPRIB plays an important role in physiological osteogenesis (Zhao et al. 2002). Osteoblast-specific *Smad1* inactivation using Cre recombinase from the *Col1a1* promoter in mice results in osteopenia, providing in vivo evidence for the importance of Smad signaling in osteoblast differentiation (Wang et al. 2011). In addition to regulation of bone mass, BMP signaling also controls bone quality in conjunction with mechanosensing mechanisms (Iura et al. 2015). There are many unanswered questions about the functions of BMP signaling in vivo, and future studies will help us to further understand the context-dependent functions of BMP signaling in osteoblasts.

In humans, BMPs have been implicated in disorders affecting limb development. Mutations in the *BMP2* or *BMPR1B* gene result in autosomal dominant brachydactyly type A2, characterized by hypoplastic middle phalanges of the second and fifth fingers (Dathe et al. 2009). A mutation in the noggin gene (*NOG*) that prevents normal binding of noggin to BMPs leads to brachydactyly type B, manifested by extreme shortening or complete loss of the distal portions of fingers and toes (Lehmann et al. 2007). A study of a large cohort of Dutch men and women found no increased risk for osteoporosis in subjects with common *BMP2* polymorphisms that result in Ser37Ala and Arg190Ser substitutions (Fiori et al. 2006).

TGF-β and Osteoblast Differentiation

The effects of TGF-β1, β2, and β3 on osteoblastic cells are context-, time-, and dose-dependent, and differentially affect certain stages of osteoblast differentiation (Morikawa et al. 2016). Despite some conflicting findings, most experiments suggest that TGF-β promotes proliferation, early differentiation of osteoblast progenitor cells, and matrix production, while inhibiting later differentiation and matrix mineralization (Fig. 1B) (Chen and Bates 1993; Breen et al. 1994; Harris et al. 1994; Janssens et al. 2005). TGF-β ligands act through canonical Smad2 and Smad3 signaling, as well as noncanonical pathways, to regulate osteoblast differentiation (Fig. 1A). Smad2 overexpression in osteoblasts suppresses expression of *Runx2* but does not seem to affect Runx2 transcriptional activity. In contrast, increased Smad3 expression reduces *Runx2* expression and Runx2 transcriptional activity at early stages of differentiation, but increases *Runx2* expression at later stages (Li et al. 1998; Alliston et al. 2001; Kaji et al. 2006). Activated Smad3 inhibits osteoblastic lineage commitment, yet promotes the progression of osteoblast differentiation at earlier stages and increases the expression of alkaline phosphatase, type I collagen, and proteins involved in matrix mineralization (Alliston et al. 2001; Kaji et al. 2006). Noncanonical TGF-β signaling through the Erk MAPK pathway inhibits alkaline phosphatase expression, but promotes collagen synthesis (Sowa et al. 2002; Arita et al. 2011), while signaling through both Erk1 and/or Erk2 and p38 MAPK leads to suppression of osteocalcin expression (Karsdal et al. 2002). As a feedback mechanism, TGF-β can also inhibit transcription of *Smad3* through the Erk and JNK MAPK pathways (Sowa et al. 2002). In addition, TGF-β may control the progression of osteoblasts to osteocytes by preventing apoptosis of terminally differentiated cells through the Erk MAPK pathway (Karsdal et al. 2002). Consistent with the inhibitory role of TGF-β in early osteoblast differentiation,

inhibition of the TβRI kinase activity increases alkaline phosphatase expression in BMSCs and C2C12 myoblast cells cultured in osteogenic media (Maeda et al. 2004). In addition, TGF-β can also activate a negative feedback loop to inhibit its own signaling via Runx2 to suppress expression of TβRI and thereby reduce the TGF-β responsiveness (Kim et al. 2006).

As in other tissues, TGF-β activation in the skeleton is highly regulated, requiring active TGF-β release from latent complexes and associated proteins that sequester TGF-β in the extracellular matrix. Bone is unique in that more TGF-β is secreted without being attached to latent TGF-β binding proteins (LTBPs) than in other tissues (Dallas et al. 1994). The small leucine-rich proteoglycans biglycan and decorin bind and likely sequester active TGF-β in the extracellular matrix, which is supported by the finding that BMSCs from mice with inactivated biglycan and decorin expression show a higher ratio of active to latent TGF-β than wild-type controls, with impaired osteoblast differentiation (Afzal et al. 2005). Intracellular regulation of TGF-β maturation by E-selectin ligand-1 (ESL-1), a Golgi protein that inhibits TGF-β bioavailability, is required for normal bone development, as mice lacking ESL-1 expression develop severe osteopenia with increased bone resorption and reduced mineralization because of higher TGF-β activity (Yang et al. 2013).

TGF-β signaling interacts with other signaling pathways to affect osteoblast differentiation. In particular, signaling cross-talk between the BMP- and TGF-β pathways seems to play a crucial role (Fig. 1A) (Maeda et al. 2004). For example, BMP-2 can repress TGF-β signaling by repressing the expression and promoting the intracellular relocation of TβRII (Centrella et al. 1995; Chang et al. 2002). In addition, all three TGF-βs can activate the *Sost* gene, which encodes sclerostin, resulting in inhibition of Wnt signaling in bone (Loots et al. 2012). TGF-β can also stabilize β-catenin through activation of Smad3 and the PI3K pathway (Loots et al. 2012). Furthermore, the observation that TGF-β no longer inhibits BMSC differentiation when β-catenin expression is silenced suggests that TGF-β and Wnt signaling synergize to inhibit osteoblast differentiation (Zhou 2011). In addition, Wnt signaling can increase *Tgfbr1*, but not *Tgfbr2* expression in a β-catenin-independent pathway, thus increasing responsiveness to TGF-β (McCarthy and Centrella 2010). Parathyroid hormone (PTH) signaling interacts with TGF-β signaling by increasing the levels of Smad3, which in turn stabilizes β-catenin and thus enhances TGF-β-induced expression of type I collagen in osteoblasts (Sowa et al. 2003b; Inoue et al. 2009). Furthermore, TβRII phosphorylates the PTH receptor 1 (PTH1R), leading to endocytosis of both receptors, and consequently reduced TGF-β and PTH signaling (Qiu et al. 2010). Inactivating TβRII expression in osteoblasts leads to increased PTH1R levels, resulting in a high trabecular/low cortical bone mass phenotype (Qiu et al. 2010). TGF-β also regulates the expression of many other growth factors. For instance, in BMSCs TGF-β increases transcription of the genes encoding fibroblast growth factor 2 (FGF-2), insulin-like growth factor I (IGF-I), and the extracellular matrix-associated protein connective tissue growth factor (CTGF), which all contribute to collagen matrix production (Kveiborg et al. 2001; Sobue et al. 2002; Arnott et al. 2008). Moreover, TNF-α acts through NF-κB to prevent TGF-β from activating Smad2 and Smad3, similar to its role in inhibiting activation of Smad1, 5, and 8, suggesting an inhibitory function of TNF-α in bone (Mukai et al. 2007).

Modulating the expression of TGF-β signaling components in mouse models has further shown its complex roles in osteoblast differentiation and osteogenesis in vivo. *Tgfb1*$^{-/-}$ mice, for example, show a remarkable absence of mature osteoblasts and reduced ALP activity, but normal osteoclast numbers and activity (Geiser et al. 1998). These mice have normal bones early in development, but show by 3 months of age a reduced growth and significant bone loss, consistent with impaired osteoblast differentiation (Geiser et al. 1998; Atti et al. 2002). Further studies show that TGF-β, released from bone matrix during osteoclastic bone resorption, induces migration of BMSCs to sites of bone resorption, thereby "coupling"

bone resorption with bone formation (Pfeilschifter et al. 1990; Hughes et al. 1992; Tang et al. 2009).

Tgfb2$^{-/-}$ mice have reduced bone size and ossification, as well as limb and rib defects by E18.5, and die perinatally from multiple developmental defects, indicating the importance of TGF-β2 in bone patterning and development (Sanford et al. 1997). In contrast, *Tgfb3*$^{-/-}$ mice have normal skeletons (Dünker and Krieglstein 2002). Increased expression of biologically active TGF-β2 in differentiated osteoblasts under control of the *Bglap* promoter results in a dramatic reduction of bone volume with frequent fractures by 1 month of age (Erlebacher and Derynck 1996), and by 7 months severely reduced trabecular bone and thin unmineralized cortical bone. Bone of these mice shows increased osteoclastic resorption, osteoblast activity, osteoprogenitor cell number, and osteocyte density, suggesting that TGF-β2 regulates both osteoclast activity as well as osteoblast differentiation (Erlebacher and Derynck 1996). However, mice overexpressing a dominant-negative form of TβRII have increased bone mass, potentially also because of osteoblast-mediated reduction of osteoclast activity despite normal osteoclast numbers (Filvaroff et al. 1999). *Smad3*$^{-/-}$ mice show a phenotype similar to that of *Tgfb1*$^{-/-}$ mice, with reduced bone volume, normal osteoblast, and osteoclast numbers, but impaired osteoblast function, resulting in a decreased bone formation rate (Borton et al. 2001). These *Smad3*$^{-/-}$ mice also show an increased osteocyte density, similar to mice that overexpress TGF-β2 in mature osteoblasts under control of the *Bglap* promoter (Borton et al. 2001). Pathologically, increased TGF-β signaling also contributes to the phenotype in osteogenesis imperfecta (OI), a genetic bone dysplasia characterized by brittle bones and increased susceptibility to fractures (Grafe et al. 2014). Mouse models of dominant OI (due to heterozygous mutation in *Col1a2*) and recessive OI (due to lack of cartilage-associated protein CRTAP that is involved in post-translational modifications of type I collagen; *Crtap*$^{-/-}$ mice) both show phenotypes similar to models with increased TGF-β signaling, and treating these mice with a pan-anti-TGF-β antibody improves the bone phenotype (Grafe et al. 2014).

In humans, some TGF-β1 polymorphisms associate with osteoporotic phenotypes. One study found an association between osteoporosis with increased bone turnover and a single-base deletion in intron 8 of *TGFB1* that likely affects splicing (Langdahl et al. 1997). Another study found a polymorphism, a T-C polymorphism in the fifth intron, 20 bases upstream of exon 6 that is less common in osteoporotic patients and associates with higher bone mass (Langdahl et al. 2003). Many different mutations in the proregion of TGF-β1, also known as latency-associated peptide (LAP), can cause Camurati-Engelmann disease, an autosomal dominant bone dysplasia that causes osteosclerosis and increased fracture risk (Kinoshita et al. 2000; Campos-Xavier et al. 2001; Wu et al. 2007). Mice generated with these mutations recapitulate the bone dysplasia and show increased levels of active TGF-β1 in the bone marrow, raising the possibility that the mutations affect the ability of TGF-β1 to be sequestered in the extracellular matrix (Tang et al. 2009), and inhibition of TβRI in these mice rescues the bone phenotype and prevents fractures. These findings suggest that modulating TGF-β signaling, and thus altering osteoblast differentiation, could represent a compelling treatment approach for certain bone diseases.

Activins, Inhibins, Follistatin, and Osteoblast Differentiation

The roles of activins and inhibins in osteoblast differentiation have been less well characterized but these TGF-β-related ligands modulate the effects of BMP and TGF-β ligands. For instance, activin A, a homodimer of inhibin β_A chains, acts in a similar way as TGF-β in osteoblastic cell culture, and increases proliferation but inhibits differentiation of early osteoprogenitor cells (Fig. 1B) (Centrella et al. 1991; Hashimoto et al. 1992; Ikenoue et al. 1999). Activin A also inhibits mineralization, at least in part by inhibiting the expression of the transcription factor homeobox protein Msx2, even in late osteoblast

differentiation (Eijken et al. 2007; Alves et al. 2013). However, when overexpressed noggin inhibits BMP signaling, activin A rescues the progression of osteoblast differentiation, suggesting that it acts through multiple pathways in osteoblastogenesis (Gaddy-Kurten et al. 2002). Mice with inactivated *Inhba*, which encodes inhibin $\beta_{A,}$ have severe craniofacial defects, whereas inactivation of *Acvr2*, which encodes the activin type II receptor ActRII, does not affect skeletal development in most but not all mice. These results suggest that activins may act through a different type II receptor to enact their effects on bone (Matzuk et al. 1995a). Inhibin A, a heterodimer of inhibin α and inhibin β_A, also inhibits osteoblast differentiation, reduces alkaline phosphatase activity in early osteoblasts, and suppresses mineralization by mature osteoblasts (Fig. 1B) (Gaddy-Kurten et al. 2002). The finding that inhibin represses osteoblastogenesis even when activin is added suggests that inhibin does not act by competing with activin for the same receptor, but instead might signal through a distinct inhibin-specific receptor (Gaddy-Kurten et al. 2002). Furthermore, inhibin-mediated repression of osteoblastogenesis cannot be rescued with BMP-2, indicating that the inhibitory effect of inhibin is dominant over BMP-2 activity (Gaddy-Kurten et al. 2002). The glycoprotein follistatin, encoded by *Fst*, binds activins and inhibins and prevents their interaction with their receptors (Nakamura et al. 1990; Harrison et al. 2005; Gordon and Blobe 2008). It is expressed only at very low levels at all stages of osteoblast differentiation, and exogenous follistatin can block activin A functions (Funaba et al. 1996; Gaddy-Kurten et al. 2002). In vivo studies are required to determine if follistatin plays a role in osteogenesis.

TGF-β FAMILY SIGNALING IN CHONDROCYTE DIFFERENTIATION

BMPs and Chondrogenesis

BMP signaling is critical during each stage of chondrogenesis. BMP ligands are expressed in a defined spatiotemporal pattern in the precartilagious mesenchyme, the perichondria, and the growth plates. In particular, *Bmp2* and *Bmp4* are highly expressed by prehypertrophic and hypertrophic chondrocytes in the growth plates (Feng et al. 2003; Nilsson et al. 2007). Inactivating both *Bmp2* and *Bmp4* in limb bud mesenchyme results in defective skeletal development (Bandyopadhyay et al. 2006). Inactivating *Bmp2* only in chondrocytes, using Cre-mediated recombination from the *Col2a1* promoter results in similarly severe skeletal defects, which suggests that BMP-2 is a key ligand for growth plate function (Shu et al. 2011). Disrupting the expression of either BMP type I receptor, through inactivation of *Bmpr1a*, *Bmpr1b*, or *Acvr1*, in the chondrogenic lineage has minor consequences for morphogenetic phenotypes (Baur et al. 2000; Yi et al. 2000; Ovchinnikov et al. 2006; Rigueur et al. 2015). In contrast, compound inactivation of *Bmrp1a* and *Bmrp1b* substantially diminishes the size of cartilage primordia by increasing apoptosis (Yoon et al. 2005). These striking results underscore redundancies in some functions of BMP signaling through BMPRIA and BMPRIB during chondrogenesis. Compound inactivation of either *Bmpr1a* and *Acvr1* or *Bmpr1b* and *Acvr1* causes subtle cervical vertebrae abnormalities, suggesting that BMP signal transduction through ACVR1/ALK-2 has a minor role during chondrogenesis (Rigueur et al. 2015). Neural crest-specific deletion of *Bmpr1a* causes early lethality owing to cardiac malfunction (Stottmann et al. 2004; Nomura-Kitabayashi et al. 2009). However, neural crest-specific deletion of *Acvr1* results in craniofacial defects, including mandibular hypoplasia with hypoplastic Meckel's cartilage (Dudas et al. 2004). In contrast, Meckel's cartilage persists in $Nog^{-/-}$ mice that lack expression of the BMP inhibitor noggin (Wang et al. 2013b). Inactivation of *Bmpr1a* expression in the chondrogenic lineage after birth halts long bone growth and reduces Sox9 expression (Jing et al. 2013). Interestingly, the growth plates in these mutants are replaced by bone-like tissue suggesting that BMP signaling through BMPRIA prompts chondrogenic differentiation by regulating *Sox9* expression.

Chondrocyte-specific inactivation of *Smad1*, *Smad5*, or *Smad8* individually results in viable mice. However, compound inactivation of *Smad1* and *Smad5* results in severe chondrodysplasia that mimics the phenotype of chondrocyte-specific *Bmpr1a*$^{-/-}$;*Bmpr1b*$^{-/-}$ compound mutants (Retting et al. 2009). This stands in contrast to the phenotype of chondrocyte-specific disruption of *Smad4* using *Col2a1*-Cre; these mice have disorganized growth plates and shorter bones, but live for at least several months after birth (Zhang et al. 2005). These observations raise the possibility that Smad4 has only a limited role in mediating the BMP–Smad signaling pathway in chondrocytes. Transcriptional intermediary factor-1γ (TIF1γ; also known as TRIM33) binds Smad2 and Smad3 and allows these complexes to exert distinct functions from Smad4 complexes with Smad2 or Smad3 (He et al. 2006; Xi et al. 2011). Accordingly, conditional compound inactivation of *Smad4* and *Tif1g* results in a more severe phenotype than inactivation of the individual genes, and results in cleft palate, as seen because of epithelium-specific *Tgfb3* inactivation (Lane et al. 2015), suggesting that BMP–Smad signaling through TIF1γ/TRIM33 represents an arm of the pathway that does not require Smad4.

After endochondral ossification, a small population of chondrocytes at the end of long bone remains as articular cartilage (Kronenberg 2003). How this subpopulation of chondrocytes is destined to become articular cartilage, differently from the chondrocytes in the growth plate, is not well understood; however, BMP and Wnt signaling activities likely contribute to their differentiation phenotype (Tsumaki et al. 1999; Guo et al. 2004; Pacifici et al. 2005; Spater et al. 2006a,b). Wnt signaling at the interzone, the site of the future joint, is essential for articular cartilage development (Hartmann and Tabin 2001; Guo et al. 2004; Spater et al. 2006a,b). Furthermore, noggin is highly expressed just proximal to the distal proliferating zone (DPZ) to "insulate" BMP signaling from more proximal regions of the growth plate (Ray et al. 2015). This expression pattern of noggin may explain how chondrocytes in the most distal part of cartilage primordia are exposed to a high ratio of Wnt/BMP signaling and specify to become articular cartilage.

TGF-β-activated kinase 1 (TAK1) initiates p38 MAPK and JNK signaling in response to TGF-β, yet is also involved in signaling responses to other types of ligands (Cui et al. 2014). Chondrocyte-specific inactivation of *Tak1* results in chondrodysplasia, characterized by delayed formation of secondary ossification centers and absence of elbow and tarsal joints (Shim et al. 2009; Greenblatt et al. 2010a). These mutant mice also have defective cartilage proliferation and maturation (Gunnell et al. 2010). Inactivation of *Tak1* in developing limb mesenchyme results in widespread joint fusions (Gunnell et al. 2010). During both embryogenesis and postnatal development, TAK1 signaling promotes the expression of three Sox transcription factors (i.e., Sox5, Sox6, and Sox9) that are essential for the organization of growth plates and articular cartilage development (Gunnell et al. 2010; Gao et al. 2013). In addition to the expected reduction of p38 MAPK and JNK activation, these *Tak1*$^{-/-}$ mice show decreased Erk MAP kinase activity and decreased activation of the BMP-responsive Smad1, 5, and 8 (Shim et al. 2009). Similarly, decreased Smad1, 5, and 8 activation is observed following osteoblast-specific or neural crest-specific inactivation of *Tak1* (Greenblatt et al. 2010b; Yumoto et al. 2013), suggesting that TAK1 controls both BMP-activated Smad and non-Smad pathways in multiple cell types. However, because BMP or TGF-β ligands are not the only ones that initiate TAK1-mediated signaling, these phenotypes do not necessarily result from alterations in BMP- or TGF-β signaling only.

More evidence that BMP signaling plays pivotal roles in chondrogenesis comes from the identification of gene mutations that result in fibrodysplasia ossificans progressiva (FOP). FOP is a rare, autosomal dominant disease characterized by ectopic ossification in soft tissues following even minor trauma. *ACVR1* mutations have been identified in all patients diagnosed so far (Shore et al. 2006; Kaplan et al. 2012). The mutation in *ACVR1* that results in R206H substitution is believed to affect the interaction of the type I receptor with FKBP12,

and confers increased basal signaling activity (Shore et al. 2006). Thus, the R206H substitution in ACVR1/ALK-2 enhances chondrogenesis in micromass culture (Shen et al. 2009b), and chimeric mice with the R206H substitution develop ectopic ossification on blunt injury (Chakkalakal et al. 2012). ACVR1 with the R206H substitution can also respond to activin ligands that normally antagonize BMP signaling through ACVR1 (Hatsell et al. 2015). Administration of an activin A-blocking antibody to mice that express ACVR1 with the R206H substitution prevents formation of FOP-like lesions, which strongly suggests that a broadened ligand specificity because of the mutation contributes to the pathogenesis of FOP (Hatsell et al. 2015). Another substitution, Q207D, renders ACVR1 constitutively active, and conditional transgenic mice that express the Q207D mutant receptor in skeletal muscles, activated by intramuscular injection of Cre recombinase-expressing adenovirus, develop ectopic ossification in combination with inflammation (Fukuda et al. 2006; Yu et al. 2008). Ligand antagonists of the nuclear retinoic acid receptor-γ (RAR-γ) are known for their antichondrogenic action (Pacifici et al. 1980), and administration of RAR-γ agonists was shown to block heterotopic ossification in the Q207D mouse model (Shimono et al. 2011). Together, these observations reinforce the idea that chondrogenic differentiation promoted by aberrantly increased BMP signaling in progenitor cell populations is a critical step for heterotopic ossification.

Enhanced BMP–Smad signaling through BMPRIA in neural crest cells leads to an increase of p53-mediated apoptosis in developing nasal cartilage, resulting in abnormal nasal cavity morphogenesis leading to perinatal lethality (Hayano et al. 2015). In this model, increased levels of p53 protein are observed without increases of *p53* gene expression, but are accompanied by decreased MDM2–p53 complex formation and increased complex formation of p53 with Smad1, 5, and 8 (Hayano et al. 2015). MDM2 acts as an E3 ligase promoting proteasomal degradation of p53 (Momand et al. 1992; Kussie et al. 1996; Lai et al. 2001). Together with the observation that association of activated Smad1 with p53 prevents MDM2-mediated p53 degradation (Chau et al. 2012), these results raise the possibility that increased BMP–Smad signaling not only increases the nuclear levels of activated Smad1, 5, and 8, but additionally prevents the MDM2–p53 interaction that leads to activation of apoptotic pathways in chondrocytes at nasal cavity.

During early embryogenesis, the relative timing of Sonic hedgehog (Shh) and BMP signals defines the fate selection of lateral plate mesoderm toward either a chondrogenic or presomitic mesoderm (PSM) fate. Thus, sequential exposure of lateral plate mesoderm to Shh followed by BMP-4 robustly induces chondrogenesis, whereas simultaneous exposure of both Shh and BMP blocks chondrogenesis (Murtaugh et al. 1999, 2001). Shh signaling activates *Sox9* expression through activation of Gli2 and Gli3 at the lateral plate mesoderm and at the same time induces the expression of Nkx3.2, which blocks the expression of the GATA4, 5, and 6 transcription factors (Zeng et al. 2002; Daoud et al. 2014). On the other hand, BMP signaling in the PSM blocks Shh-mediated induction of *Nkx3-2* and *Sox9* through induction of the expression of the GATA4, 5, and 6 transcription factors that suppress *Nkx3-2* expression and the expression of Gli transcription factors dependent on the zinc finger protein FOG1 (friend of GATA protein 1, also known as ZFPM1) (Daoud et al. 2014). These results suggest that Shh signaling installs competence in lateral plate mesoderm for BMP-induced chondrogenesis by inducing Nkx3.2 expression.

BMP signaling, in conjunction with other signaling pathways, regulates the size and organization of the growth plate. A feedback loop between Indian hedgehog (Ihh) produced in the prehypertrophic and hypertrophic zones and parathyroid hormone related protein (PTHrP) produced in the resting zone plays a critical role in maintaining the columnar height of the growth plate (Fig. 2) (Kronenberg 2003). FGF signaling inhibits proliferation of chondrocytes, whereas BMP signaling stimulates chondrocyte proliferation and differentiation, and inhibits apoptosis of hypertrophic chondrocytes (Fig. 2)

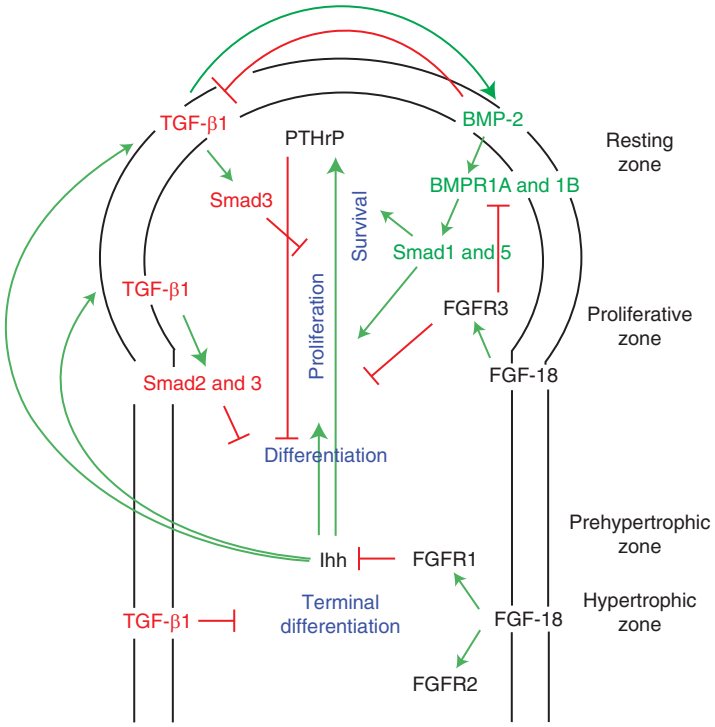

Figure 2. TGF-β family signaling in chondrocyte differentiation during growth plate development. Regulation of endochondral ossification involves a feedback loop between parathyroid hormone related protein (PTHrP) and Indian hedgehog (Ihh) controlling chondrocyte differentiation, whereas fibroblast growth factor (FGF) signaling represses chondrocyte proliferation. Bone morphogenetic protein (BMP)-2, expressed within perichondrium, promotes the survival and proliferation of chondrocytes through the type I receptors BMPRIA and BMPRIB and subsequent Smad1 and Smad5 activation. FGF-18 signaling through the fibroblast growth factor receptor 3 (FGFR3) receptor further refines chondrocyte proliferation by repressing BMP receptor activity. Ihh expressed in the prehypertrophic zone induces the expression of both TGF-β1 in the perichondrium and PTHrP in the resting zone, respectively. PTHrP participates in this intricate feedback loop by inhibiting differentiation until the proliferating chondrocytes enter the prehypertrophic zone. TGF-β1 represses terminal chondrocyte differentiation in the hypertrophic zone. It is suggested that TGF-β1 induces BMP-2 expression, which then inhibits further TGF-β1 expression.

(Minina et al. 2001, 2002). BMP signaling induces *Ihh* expression, and Ihh signaling induces BMP expression, forming a positive feedback loop (Minina et al. 2002). Accordingly, Cre-recombinase-mediated, cartilage-specific conditional inactivation of *Smad1* and *Smad5* from the *Col2a1*-promoter results in reduced *Ihh* expression in the hypertrophic zone (Retting et al. 2009). Activation of BMP signaling in the perichondrium together with activation of hedgehog signaling prompts osteogenic differentiation, whereas BMP signaling alone induces chondrogenic differentiation (Hojo et al. 2013). Pharmacological inhibition of hedgehog signaling results in reduced Smad and p38 MAPK activation in response to BMP-2, and suppresses BMP-2-induced chondrogenesis in micromass culture (Mundy et al. 2015), suggesting that Hedgehog signaling may repress BMP signaling. As with the PSM, the timing of these two signaling stimuli is critical for lineage specification of the cells in perichondrium.

FGF signaling inhibits *Ihh* expression in the growth plate (Minina et al. 2002), and increased FGF signaling is observed in growth plates in both *Bmpr1a*$^{-/-}$;*Bmpr1b*$^{-/-}$ and *Smad1*$^{-/-}$;

Smad5$^{-/-}$ compound mutant mice (Yoon et al. 2005; Retting et al. 2009). These results suggest that diminished BMP signaling in the growth plate leads to imbalanced cross talk between BMP, FGF, and Ihh signaling. Hyperactivated FGF receptor 3 (FGFR3) promotes degradation of BMPRIA through the E3 ubiquitin ligase Smurf1 (Smad ubiquitination regulatory factor-1), thus inhibiting BMP-induced chondrogenesis (Qi et al. 2014). These findings lend credence to the notion that shortened growth plates, found in achondroplasia due to single gain-of-function amino acid substitutions in FGFR3, such as K644E, may result from reduced BMP signaling. Indeed, BMP-2 treatment of metatarsals of *Fgfr3K644E* mice show increased hypertrophic zone length (Qi et al. 2014).

Growth Differentiation Factors and Joint Formation

The TGF-β family proteins named "growth and differentiation factors" (GDFs) also play critical roles during endochondral ossification and joint formation. *Gdf5*, which encodes GDF-5/BMP-14, is expressed in precartilaginous mesenchyme and the perichondrium of proximal structures of the limb buds in E12.5 mouse embryos. At later stages, *Gdf5* expression localizes to the sites of joint formation. *Gdf6*, which encodes GDF-6/BMP-13, and *Gdf7*, which encodes GDF-7/BMP-12, are also expressed in a subset of developing joints (Wolfman et al. 1997; Settle et al. 2003). The spontaneous mutation *brachypodism* in mice shows abnormal skeletal patterns that are attributed to mutations in *Gdf5* (Storm et al. 1994). *Gdf5*$^{-/-}$ mice show shorter appendicular bones although axial bones are unaffected (Storm et al. 1994; Storm and Kingsley 1996), and abnormal joint formation in the synovial joints of the limb leading to abnormal fusion between particular skeletal elements (Storm and Kingsley 1996). In humans, *GDF5* mutations are at the basis of hereditary diseases, such as acromesomelic chondrodysplasia, Hunter–Thompson type (CHTT) and chondrodysplasia, Grebe type (CGT) (Thomas et al. 1996, 1997). These diseases are characterized by shortening of the appendicular skeleton and abnormal joint development resembling the skeletal abnormalities in *Gdf5*$^{-/-}$ mice. CHTT is due to a missense mutation in *GDF5* resulting in total loss of function, whereas CGT is due to a C400Y substitution in GDF-5 that affects dimerization of BMP/GDF ligands (Thomas et al. 1996, 1997). Overexpression of *Gdf5* in the chick limb results in larger size of cartilage condensation (Francis-West et al. 1999), suggesting a potential role of GDF-5 in skeletal growth. Chondrocyte-specific expression of *Gdf5* in mice also prompts mesenchymal condensations caused by increased cell adhesion and proliferation (Tsumaki et al. 1999, 2002). Increased expression of *Gdf5* in chondrocytes restricts expression of joint markers, and promotes overgrowth of cartilage, and thus may cause fusion of adjacent skeletal elements and loss of joints.

Gdf6$^{-/-}$ mice show skeletal phenotypes that are similar to, but distinct from, those of *Gdf5*$^{-/-}$ mice. *Gdf6*$^{-/-}$ mice display fusions between specific carpal bones in the wrists and between talus and the central tarsal bones in ankle, coincident with high expression of *Gdf6* (Settle et al. 2003). In *Gdf6*$^{-/-}$ mutants, the process to subdivide larger skeletal precursors into individual skeletal elements does not take place (Settle et al. 2003). *Gdf6*$^{-/-}$ mice also show loss of coronal sutures, which separate frontal bones from parietal bones in the skull (Settle et al. 2003). In control embryos, the frontal and parietal bones are visible at E14.5 as separate ossification centers; however, one continuous bone is found in the *Gdf6*$^{-/-}$ embryos (Clendenning and Mortlock 2012). Suture width is reduced in *Gdf6*$^{+/-}$ embryos, and sutures are absent, accompanied with increased alkaline phosphatase activity, in *Gdf6*$^{-/-}$ embryos (Clendenning and Mortlock 2012). *Fgfr2* is highly expressed in proliferating osteoprogenitors, and its expression is down-regulated as differentiation progresses (Iseki et al. 1999). However, the expression of *Fgfr2* that is normally observed in coronal sutures is repressed in the *Gdf6*$^{-/-}$ embryos (Settle et al. 2003). These results suggest that GDF-6 represses and prevents osteogenic differentiation through expres-

sion of *Fgfr2* to maintain the suture mesenchyme undifferentiated.

Gdf5$^{-/-}$;*Gdf6*$^{-/-}$ mice have a more severe phenotype of bone size and joint formation than either single mutant (Settle et al. 2003). Many limb bones are much smaller or completely missing. The vertebral column of these double mutant mice has a reduction of alcian blue-stained extracellular matrix, suggesting a reduction in cartilaginous extracellular matrix, whereas no overt phenotype is observed in the vertebral column of either mutant (Settle et al. 2003). *Gdf7* is also expressed in joint interzones, but unlike *Gdf5*$^{-/-}$ or *Gdf6*$^{-/-}$ mice, no overt morphological skeletal phenotypes are found in *Gdf7*$^{-/-}$ mutant mice (Settle et al. 2001). Although the tibial growth plates of *Gdf7*$^{-/-}$ mice have a histologically normal columnar structure, their proliferation rate is higher than that of control mice (Mikic et al. 2008). This distinguishes *Gdf7*$^{-/-}$ mice from *Gdf5*$^{-/-}$ mice that show a reduced proliferation rate of hypertrophic chondrocytes in the tibial growth plate (Mikic et al. 2004).

GDF-5, like other BMP ligands, interacts with type I and type II receptors to induce activation of Smad proteins (Nishitoh et al. 1996; Nohe et al. 2004). GDF-5 dimers interact with BMPRIA or BMPRIB, albeit preferentially with BMPRIB (Nickel et al. 2005). *Bmpr1b* is expressed in early cartilage condensations, and is later defined in the digital rays of hands or feet that outline the future digits pattern, and colocalizes with *Gdf5* (Kawakami et al. 1996; Zou et al. 1997; Degenkolbe et al. 2014). When *Gdf5* expression concentrates in joint interzones, starting at E13.5 in mice, those domains are flanked by *Bmpr1b* expression (Degenkolbe et al. 2014). In contrast, *Bmpr1a* is expressed in direct proximity in the interphalangeal regions and surrounding limb epithelium. The limb phenotype of *Bmpr1b*$^{-/-}$;*Gdf5*$^{-/-}$ mice highly resembles that of *Gdf5*$^{-/-}$ mutants (Baur et al. 2000; Yi et al. 2000), further reinforcing the idea that BMPRIB is the primary receptor for GDF-5 during limb development.

The activities of GDF-5 are counteracted by ligand antagonists, such as the BMP antagonist noggin, encoded by *Nog* (Merino et al. 1999a). *Nog* is expressed during chondrogenesis and joint specification, and its expression domains overlap with those of *Bmpr1b* (Degenkolbe et al. 2014). *Nog*$^{-/-}$ mice show skeletal abnormalities that include the absence of joints. After the initial condensations of limb mesenchyme, *Nog*$^{-/-}$ mice show increased recruitment of mesenchymal precursors that subsequently results in overgrowth of cartilage and fusion of neighboring skeletal elements (Brunet et al. 1998). In chick embryos, ectopic expression of BMPs suppresses *Gdf5* expression, suggesting that increased BMP signaling in *Nog*$^{-/-}$ limb bud may lead to decreased GDF-5 levels, which is at the basis of the similar phenotypes of *Gdf5*$^{-/-}$ limbs (Macias et al. 1997; Merino et al. 1999a).

TGF-β, Chondrogenesis, and Osteoarthritis

TGF-β has potent chondrogenic inductive ability both in cell culture and in vivo. Bovine bone extracts were shown to induce chondrocyte differentiation of embryonic rat muscle mesenchymal cells, and this chondrogenic activity was then identified as TGF-β (Seyedin et al. 1983, 1986). TGF-β ligands and receptors are broadly expressed in skeletal systems, and TGF-β plays a pivotal role during mesenchymal condensation (Kulyk et al. 1989; Tuli et al. 2003; Song et al. 2007). TGF-β further stimulates the expression of cartilage-specific extracellular matrix proteins such as type II collagen and aggrecan (Denker et al. 1995; Blaney Davidson et al. 2007; Shen et al. 2014). TGF-β does not promote chondrogenic differentiation when bone marrow mesenchymal cells are cultured on plastic or type I collagen, but strongly promotes chondrogenic differentiation of cells cultured in Matrigel (Tuli et al. 2003). In the latter case, TGF-β induces Wnt7a expression leading to N-cadherin expression that increases cell–cell contacts that are required for chondrogenic differentiation.

Injection of TGF-β underneath the periosteum results in increased chondrocyte proliferation, differentiation, and formation of cartilage (Joyce et al. 1990; Critchlow et al. 1995;

Pedrozo et al. 1999). During endochondral bone formation, the perichondrium is a critical site of TGF-β1 signaling. TGF-β1 treatment of metatarsal bone cultures results in partial reduction of chondrocyte proliferation and chondrogenic differentiation, measured by collagen X expression (Alvarez et al. 2001). These inhibitory effects of TGF-β1 are diminished when the perichondrium is removed before culture (Alvarez et al. 2001). Perichondrium produces and secretes several other growth factors that control chondrocyte differentiation, such as Ihh and Shh, and PTHrP (Kronenberg 2003). Ihh and Shh induce perichondrial TGF-β2 expression, and TGF-β2 induces PTHrP expression in the perichondrium, which then inhibits differentiation into hypertrophic chondrocytes (Lanske et al. 1996; Vortkamp et al. 1996; Serra et al. 1999). However, the inhibitory effect of TGF-β1 on longitudinal bone growth is PTHrP-independent (Serra et al. 1999).

The severe bone defects in mice deficient for *Tgfb2* or *Tgfb3* underscore the important roles of these TGF-β isoforms during skeletogenesis (Dünker and Krieglstein 2000). Expressing a dominant-negative form of TβRII (*dnTgfbr2*) in skeletal tissues promotes terminal differentiation of chondrocytes in the growth plate (Serra et al. 1997) and hypertrophy of the articular chondrocytes in the superficial zone, concomitant with loss of proteoglycan, leading to progressive cartilage degradation as seen in osteoarthritis (Serra et al. 1997). Global disruption of *Smad3* leads to chondrocyte hypertrophy of articular chondrocytes in the superficial zone and spontaneous joint degeneration (Yang et al. 2001). Treatment of bones from *Smad3*-deficient mice with TGF-β1 in culture results in partially impaired differentiation and inhibition of cell proliferation (Alvarez and Serra 2004). These findings suggest that Smad3 is the major signaling mediator of TGF-β-induced inhibition of chondrocyte proliferation in growth plate and articular cartilage. Several lines of evidence from mice with tissue-specific inactivation of *Tgfbr2* further support the essential roles of TGF-β signaling in normal cartilage development and maintenance of the both growth plate and articular cartilages. Targeted inactivation of *Tgfbr2* in undifferentiated limb bud mesenchyme reduces chondrocyte proliferation and accelerates hypertrophic differentiation, but delays terminal differentiation into hypertrophic chondrocytes (Seo and Serra 2007; Spagnoli et al. 2007). In contrast, inactivation of *Tgfbr2* specifically using *Col2a1*-Cre in chondrogenic cells results in defects in the axial skeleton without altering chondrocyte differentiation (Baffi et al. 2004). Cre-mediated inactivation of *Tgfbr2* specifically from the *Col10a1* promoter in hypertrophic chondrocytes leads to delayed conversion of proliferating chondrocytes into hypertrophic chondrocytes and subsequent terminal differentiation (Sueyoshi et al. 2012). These results suggest that the function of TGF-β signaling depends on the differentiation state of the chondrocytes, and that TGF-β promotes terminal chondrocyte differentiation (Fig. 2).

Postnatal inactivation of *Tgfbr2* using the tamoxifen-inducible *Col2a1-CreERT2* cassette in chondrogenic cells results in increased *Runx2*, *Mmp13* (encoding matrix metalloproteinase 13), *Adamts5* (encoding a disintegrin and metalloproteinase with thrombospondin motif, ADAMTS 5), and *Col10* expression in articular cartilage (Chen et al. 2007; Zhu et al. 2008; Shen et al. 2013). These mice show articular cartilage degradation at three months, and loss of the entire articular cartilage with extensive osteophyte formation, resembling osteoarthritis, by six months (Shen et al. 2013). In these mice, the osteoarthritis phenotype is alleviated by compound inactivation of *Mmp13*, whereas treatment with the MMP13 inhibitor CL82198 also decelerates the progression of the osteoarthritis phenotype (Shen et al. 2013). These observations are consistent with the attenuation of articular cartilage degeneration upon *Mmp13* inactivation in a mouse model for medial meniscus destabilization, and provide a potential therapeutic strategy for human osteoarthritis (Little et al. 2009; Wang et al. 2013a; Shen et al. 2014; Ha et al. 2015).

TGF-β and BMP signaling interact functionally with each other during chondrogenic differentiation and growth plate development. Chondrocyte-specific expression of dominant-

negative form of TβRI from the *Col2a1* promoter results in an elongated growth plate, expanded prehypertrophic zone, and increased chondrocyte proliferation (Keller et al. 2011), supporting the idea that TGF-β signaling is critical for terminal differentiation of chondrocytes. Interestingly, BMP-2 treatment suppresses TGF-β-induced Smad activation, while inducing BMP–Smad signaling, in ATDC5 chondrogenic cells (Fig. 2) (Keller et al. 2011). TGF-β treatment of other cell types, such as C2C12 myoblasts, mouse embryonic fibroblasts, and HepG2 hepatoma cells, also increases BMP signaling (Wrighton et al. 2009). As mentioned already, chondrocyte-specific disruption of *Smad4* with *Col2a1-Cre* results in impaired growth plate organization and dwarfism, but does not cause the lethality that is seen after chondrocyte-specific inactivation of both *Smad1* and *Smad5* (Zhang et al. 2005). It is possible that inactivation of both Smad signaling pathways in the *Smad4*-defective mice somewhat compensates for the loss of each Smad signaling branch to lessen the phenotype, although the loss of Smad4 may merely attenuate Smad signaling. Results using ATDC5 cells suggest that TGF-β and BMP signaling interact in chondrocytes to precisely regulate the length of the growth plates by forming a feedback loop similar to Ihh and PTHrP.

Activins and Chondrogenesis

Compared with TGF-β and BMPs, less information is available on the roles of activins in chondrogenesis. Activin A (inhibin β_A homodimer), added to limb bud micromass cultures, enhances chondrogenesis by increasing the size of precartilaginous condensations and cartilaginous nodules (Jiang et al. 1993). However, another report describes that activin A inhibits chondrogenic differentiation while inhibin A (inhibin α and inhibin β_A heterodimer) stimulates chondrogenesis in limb bud micromass culture (Chen et al. 1993). Implantation of activin-soaked beads into limb mesenchyme induces *Bmpr1b* expression that in turn increases local BMP signaling and subsequently induces the expression of activin A and TGF-β2, which are both necessary for digit elongation (Merino et al. 1999b). In contrast, implantation of follistatin-soaked beads into the tips of growing digits blocks chondrogenesis and digit formation (Merino et al. 1999b).

As mentioned, *Inhba*$^{-/-}$ mice and *Fst*$^{-/-}$ mice show craniofacial abnormalities, including cleft palate (Matzuk et al. 1995b,c). *Acvr2*$^{-/-}$ mice also display craniofacial abnormalities, including mandibular hypoplasia and defective Meckel's cartilage, underscoring the role of activin signaling in chondrogenesis (Matzuk et al. 1995a). Although transgenic mice with chondrocyte-specific increase of activin signaling have not been generated, administration of activin A onto the periosteum of parietal bone in newborn rats results in increased thickness of both the periosteal and bone matrix layers (Oue et al. 1994). Transgenic mice that produce human inhibin α, encoded by *INHA*, display increased bone mass and improved biomechanical properties of the tibia through suppression of activin signaling (Perrien et al. 2007).

TGF-β FAMILY SIGNALING IN MYOBLAST DIFFERENTIATION

Muscle tissue contributes ∼40% to total body mass in the human body (Huard et al. 2002). Skeletal muscle has a variety of physiological functions, including locomotion, protection of underlying structures, metabolic functions, such as modulating blood glucose levels, and paracrine and endocrine functions (Huard et al. 2002; LeBrasseur et al. 2011; Pedersen and Febbraio 2012). The basic structural units of mammalian skeletal muscle are multinucleated myofibers (Huard et al. 2002). They contain sarcomeres consisting of myosin and actin filaments that facilitate the contractile function (Huard et al. 2002). To repair minor lesions caused by normal daily activity and injury after trauma, skeletal muscle tissue is regenerated in a coordinated process in which local myogenic progenitors, termed satellite cells, are activated (Mauro 1961; Kaji et al. 2006; Karalaki et al. 2009). Although normally quiescent and localized between myofibers, satellite cells migrate to the site of damage, where they proliferate

(Charge and Rudnicki 2004; Kaji et al. 2006; Karalaki et al. 2009). Activated satellite cells can then differentiate into myoblasts that can advance to terminal myogenic differentiation to ultimately form new muscle fibers (McLennan and Koishi 2002). In this process, myoblasts fuse with each other or existing myofibers to finally form multinucleated myofibers (Pavlath and Horsley 2003; Charge and Rudnicki 2004; Kaji et al. 2006; Karalaki et al. 2009).

Multiple members of the TGF-β family are involved in coordinating these differentiation processes, including BMPs (Patterson et al. 2010), TGF-β (Massagué et al. 1986; Olson et al. 1986; Filvaroff et al. 1994; Karalaki et al. 2009), and myostatin (Langley et al. 2002; Rios et al. 2004; Amthor et al. 2006; McFarlane et al. 2008; Karalaki et al. 2009). They control the expression of myogenic transcription factors (Vaidya et al. 1989; Martin et al. 1992; Langley et al. 2002), including Pax transcription factors, involved in satellite cell determination and survival (Seale et al. 2000; Olguin and Pisconti 2012), as well as MRFs, which are essential for myoblastic lineage determination and terminal differentiation (Fig. 3A) (Sassoon et al. 1989; Weintraub et al. 1991; Megeney and Rudnicki 1995; Sabourin et al. 1999; Charge and Rudnicki 2004; Beylkin et al. 2006). The final outcomes of TGF-β family signaling on myoblast differentiation is context-dependent, including the cell type and differentiation state (Kollias and McDermott 2008), presence of other regulatory factors (Blachowski et al. 1993; Ewton et al. 1994; Engert et al. 1996; Florini et al. 1996; McLennan and Koishi 2002; Karalaki et al. 2009), and modulation of TGF-β family ligand bioavailability (e.g., through extracellular matrix molecules such as proteoglycans) (Casar et al. 2004; Droguett et al. 2006; Karalaki et al. 2009; Olguin and Pisconti 2012). This section will focus on the effects of BMP-2, TGF-β, and myostatin signaling in myoblast differentiation.

BMPs and Myoblast Differentiation

BMP-2 inhibits myogenic differentiation, and promotes chondrogenic and osteogenic lineage selection and differentiation (Fig. 3B) (Yamaguchi 1995). In cell culture, BMP-2 prevents myogenic differentiation of C2C12 myoblasts, resulting in an almost complete inhibition of myotube formation (Katagiri et al. 1994; Yamaguchi 1995), and induces osteoblastic differentiation, with increased expression of alkaline phosphatase and osteocalcin (Katagiri et al. 1994; Yamaguchi 1995). Similarly, in C26 osteoblast precursors, which can also differentiate into myotubes, addition of BMP-2 reduces myotube formation while promoting osteoblastic differentiation (Yamaguchi et al. 1991; Yamaguchi 1995). Additionally, BMP-2 inhibits myotube formation of primary murine muscle cells (Katagiri et al. 1994; Yamaguchi 1995). At the molecular level, BMP-2 inhibits the expression of MRFs, and, consequently, myogenic lineage selection (Fig. 3A) (Yamaguchi 1995). For instance, MyoD and myogenin expression normally increase during myogenic differentiation of C2C12 cells, but BMP-2 inhibits their expression (Katagiri et al. 1994). In vivo, administration of BMP-2 into muscles of mice results in ectopic bone formation (Wozney et al. 1988; Yamaguchi 1995), supporting the notion that BMP-2 alters muscle stem cell differentiation toward an osteoblastic lineage. Accordingly, increased BMP signaling is associated with FOP, a genetic disorder with progressive ectopic bone formation in muscle, tendons, and other connective tissues (Shafritz et al. 1996). As described already, FOP patients have mutations in the BMP type I receptor ACVR1/ALK-2 that lead to increased basal downstream receptor signaling (Shafritz et al. 1996; Fiori et al. 2006; Shore et al. 2006; Billings et al. 2008; Kaplan et al. 2009), increased signaling in response to BMP (de Gorter et al. 2010), and additional responsiveness to activins (Hatsell et al. 2015; Hino et al. 2015).

TGF-β and Myoblast Differentiation

TGF-β1, β2, and β3 inhibit myoblast proliferation, differentiation, and myotube formation in culture (Massagué et al. 1986; Olson et al. 1986; Yamaguchi 1995), but, in contrast to BMP-2, do not promote osteoblastic differenti-

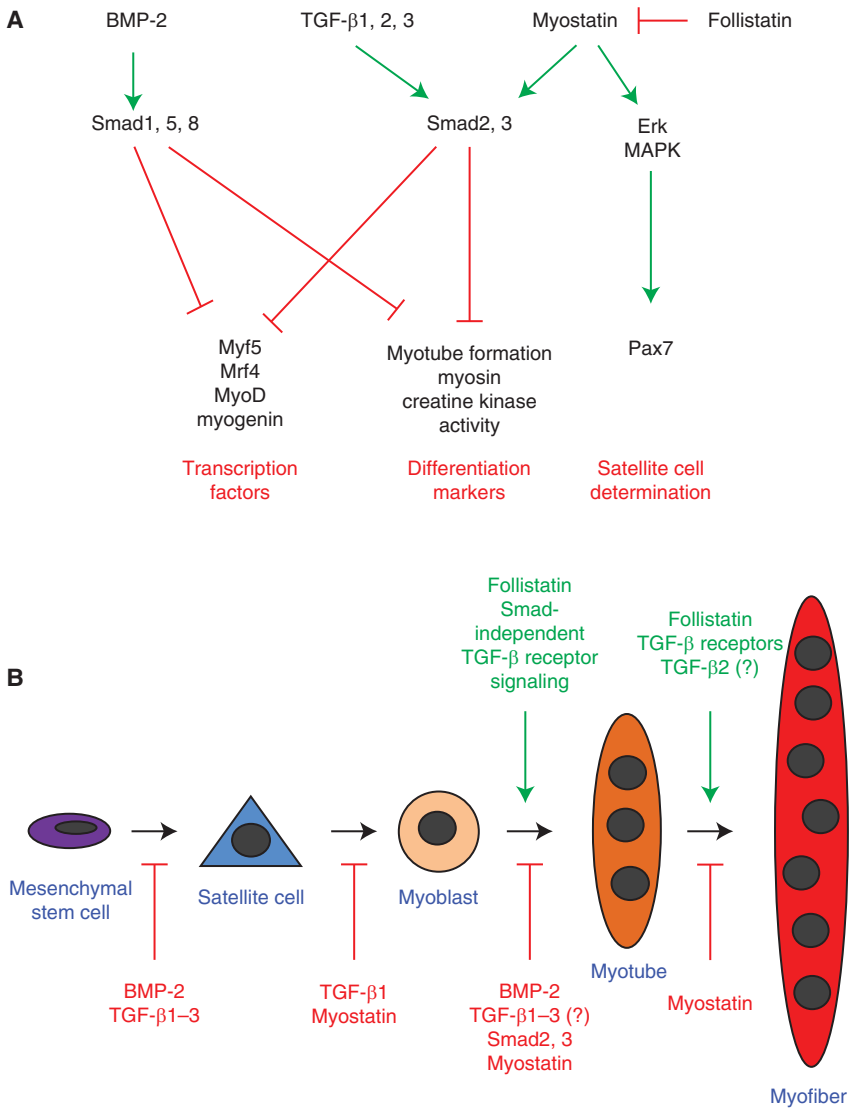

Figure 3. TGF-β family signaling in myoblast differentiation. (*A*) Major intracellular and transcriptional targets of TGF-β and bone morphogenetic protein (BMP) signaling in myoblastic differentiation. (*B*) Satellite cells and myoblasts arise from mesenchymal stem cells. Members of the TGF-β family mostly inhibit myoblast differentiation. However, signaling through TGF-β receptors is required for myotube and myofiber formation. Myostatin inhibits myoblast differentiation, and Smad-independent TGF-β receptor mediated signaling may promote final myoblast differentiation. Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

ation (Fig. 3B) (Katagiri et al. 1994; Yamaguchi 1995; Karalaki et al. 2009). However, while TGF-β can inhibit expression of muscle-specific genes, myotube formation and fusion of myoblasts (Fig. 3A) (Massagué et al. 1986; Olson et al. 1986; Allen and Boxhorn 1987; McLennan and Koishi 2002), it promotes embryonic myoblast differentiation (Cusella-De Angelis et al. 1994). This difference may be explained by intrinsic differences in the embryonic and fetal myoblasts with differential expression of surface molecules and transcription

factors, and different sensitivity to TGF-β and other ligands (Biressi et al. 2007). Pharmacological inhibition of the TβRI kinase and silencing of Smad2 and Smad3 using siRNA increase the expression of myogenin in rat myoblasts, supporting the notion that TGF-β signaling partially inhibits early myoblast differentiation (Droguett et al. 2010). Interestingly, silencing of *Smad2* and *Smad3* also promotes expression of the later differentiation marker myosin and myotube fusion, whereas TβRI inhibition reduces myosin expression and fusion, even when Smad2 and Smad3 expression is silenced (Droguett et al. 2010). Expression of a dominant-negative TβRII also inhibits differentiation and myoblast fusion of C2C12 and rat myoblasts (Filvaroff et al. 1994; Droguett et al. 2010). Together, these findings suggest that TGF-β signaling is required for normal late myoblast differentiation, potentially through Smad-independent mechanisms (Fig. 3B) (Droguett et al. 2010).

The net effects of TGF-β ligands on myoblasts also depend on the presence of other factors such as IGF-I and FGFs (Olson et al. 1986; Cook et al. 1993; Florini et al. 1996; McLennan and Koishi 2002; Karalaki et al. 2009). For example, in primary satellite cells, IGF-I prevents the inhibition of differentiation by TGF-β (Allen and Boxhorn 1989). Additionally, differences of TGF-β receptor expression in myogenic cells in culture may help explain differences in responses. L6 cells, a rodent myoblast cell line, do not express betaglycan and the TGF-β receptor RIIB, an alternatively spliced variant of TβRII that can bind TGF-β2 in the absence of betaglycan, resulting in their inability to respond to TGF-β2 (Lopez-Casillas et al. 1993; Rotzer et al. 2001; McLennan and Koishi 2002).

Mechanistically, TGF-βs inhibit myoblast differentiation by repressing the expression and activities of MRFs, including myogenin and MyoD, through Smad3 activation (Fig. 3A) (Katagiri et al. 1994; Liu et al. 2001, 2004). Thus, inhibition of TβRI signaling using a kinase inhibitor increases expression of MRFs and Pax transcription factors in human embryonic stem cells (Mahmood et al. 2010). Additionally, TGF-β can repress myoblast differentiation by impairing the responsiveness to IGF-I (Schabort et al. 2011), reducing the activity of 2-5A synthetase and double-stranded RNA activated protein kinase (PKR) (Salzberg et al. 1995), as well as reducing miR-24 expression (Sun et al. 2008). Moreover, TβRI expression in rat myotubes is repressed by electrical activity (Ugarte and Brandan 2006), suggesting that muscle activity could promote tissue growth by preventing TGF-β inhibition of myoblast differentiation and fusion.

Despite similar effects on myoblast differentiation in cell culture, TGF-β1, 2, and 3 may have distinct effects on myoblast fusion in vivo (McLennan and Koishi 2002). TGF-β1 levels remain constant during myotube development (McLennan 1993), and *Tgfb1*$^{-/-}$ mice show normal myotube formation and muscle fiber development (McLennan et al. 2000; McLennan and Koishi 2002). In contrast, TGF-β2 is expressed by myotubes in developing and regenerating muscles, and its levels increase during development (McLennan and Koishi 1994, 1997), suggesting a role in late myoblast differentiation and during initiation of myotube formation in vivo (McLennan et al. 2000; McLennan and Koishi 2002). Consistent with this notion, C2C12 cells that express a dominant-negative form of TβRII cannot fuse when injected in vivo (Filvaroff et al. 1994). After injury, TGF-β1 and TGF-β3 are released from damaged muscle tissue and platelets, and stimulate further TGF-β synthesis by myogenic cells within the regenerating muscles (Assoian and Sporn 1986; Husmann et al. 1996; Karalaki et al. 2009). TGF-β is chemotactic for inflammatory cells, such as neutrophils and macrophages, which secrete pro-inflammatory factors including FGF, TNF, and interleukin 1 (IL-1), and thus induces angiogenesis (Husmann et al. 1996; Karalaki et al. 2009). In addition, other signaling factors are released from the extracellular matrix and vasculature, including IGF-I and hepatocyte growth factor (HGF), and together promote wound healing (Koutsilieris et al. 1997; Karalaki et al. 2009). Although HGF and IGF-I initiate proliferation and promote satellite cell differentiation, TGF-β1 can inhibit

satellite cell proliferation and differentiation in a dose-dependent manner (Allen and Boxhorn 1987; Ewton et al. 1994; Bladt et al. 1995; Engert et al. 1996; Dietrich et al. 1999; Yamane et al. 2003; Karalaki et al. 2009). Furthermore, TGF-β1 induces the expression of proteins of the extracellular matrix that surrounds the muscular defect during injury repair, and promotes regeneration of the myofiber basement membrane (Edwards et al. 1987; Streuli et al. 1993; Husmann et al. 1996). By stimulating the expression of these proteins, including collagens and proteoglycans, TGF-β promotes postinjury generation of fibrosis and scar tissue (Massagué et al. 1986; Husmann et al. 1996; Karalaki et al. 2009). As a result, intramuscular application of decorin, which inhibits TGF-β activity in the extracellular matrix, improves muscle healing and prevents muscle fibrosis (Sato et al. 2003; Casar et al. 2004; Karalaki et al. 2009; Ten Broek et al. 2010).

Myostatin and Myoblast Differentiation

Myostatin is a potent repressor of myoblast differentiation required for muscle development, growth and repair, and, thus, of muscle mass (Langley et al. 2002; Rios et al. 2004; Karalaki et al. 2009). Myostatin is expressed by satellite cells and myoblasts during embryogenesis and regeneration of skeletal muscle (Karalaki et al. 2009; Ten Broek et al. 2010), and inhibits myoblast recruitment and differentiation, thereby decreasing the fiber size and number (Fig. 3B) (Kaji et al. 2006; Karalaki et al. 2009; Trendelenburg et al. 2009; Ge et al. 2011). In cell culture, myostatin inhibits proliferation, differentiation, and protein synthesis of rodent myoblasts (Langley et al. 2002; McFarlane et al. 2006; Huang et al. 2007a), and reduces myoblast fusion and creatine kinase activity in human myoblasts (Trendelenburg et al. 2009). Mechanistically, myostatin binds to the type II receptor ActRIIB (and to a lesser extent ActRII) and the type I receptors ALK-4 or TβRI/ALK-5, resulting in Smad2 and Smad3 as well as Erk MAPK activation (Lee and McPherron 2001; Zhu et al. 2004; Yang et al. 2006; McFarlane et al. 2008). Inhibition of Akt signaling through mTOR complex 1 further enhances myostatin-induced Smad activation and potentiates myostatin's inhibitory effects (Bentzinger et al. 2008; Trendelenburg et al. 2009). Myostatin can also inhibit IGF-I signaling through the PI3K–Akt pathway in myoblasts (Huang et al. 2007a), and stimulate the ubiquitin-mediated proteolysis through a FoxO1-dependent mechanism (McFarlane et al. 2006). At the transcriptional level, myostatin increases Pax7 expression, which requires Erk MAPK activation, and represses the expression of Myf5, myogenin and MyoD, thereby inhibiting satellite cells from progressing in myoblastic lineage differentiation (Fig. 3A) (Amthor et al. 2002; Langley et al. 2002; McFarlane et al. 2008; Trendelenburg et al. 2009; Ten Broek et al. 2010). In addition, myostatin induces expression of the cell cycle inhibitor $p21^{Cip1}$ to maintain satellite cell quiescence (McCroskery et al. 2003; Kaji et al. 2006). Mice with inactivated myostatin expression show an extensive increase of skeletal muscle mass, muscle hypertrophy and hyperplasia, and increased myofiber size and number (McPherron et al. 1997; Lee and McPherron 2001; Karalaki et al. 2009). Cattle with a homozygous frameshift mutation that removes a conserved sequence of the myostatin protein show doubling of their muscle mass, further supporting a key role of myostatin in repressing muscle mass (Grobet et al. 1997; Kambadur et al. 1997; McPherron et al. 1997). After injury, myostatin is detected in necrotic muscle fibers, but not in regenerating myotubes during maturation and fusion, consistent with the concept that myostatin expression is repressed during muscle repair to facilitate satellite cell recruitment and proliferation (Bradford et al. 2000; Karalaki et al. 2009).

Follistatin, Smads, and Myoblast Differentiation

Follistatin binds not only to activins and inhibins, but also to myostatin and some BMPs, and can inhibit interactions between these TGF-β ligands with their cell surface receptors (Nakamura et al. 1990; Harrison et al. 2005; Gordon and Blobe 2008). Consequently, follistatin can oppose the inhibitory effects of these ligands on myoblast differentiation and promote differen-

tiation as well as myoblast fusion (Fig. 3A, B) (Lee and McPherron 2001; Iezzi et al. 2004; Pisconti et al. 2006). Follistatin administration also antagonizes the pro-apoptotic effects of BMP-7, but remarkably enhances BMP-7-induced *Pax3* expression during chick limb development (Amthor et al. 2002). Perhaps, for BMPs that bind to follistatin with low affinity such as BMP-7, follistatin could store and present these BMPs to myogenic cells, and thereby in part promote BMP signaling (Amthor et al. 2002).

In vivo, follistatin overexpression under control of the myosin light chain promoter leads to increased muscle mass in mice (Lee and McPherron 2001), and follistatin overexpression under control of the myosin promoter in zebrafish increases muscle growth by inducing myofiber hyperplasia (Li et al. 2011). It is currently unclear if follistatin acts in vivo mainly through inhibition of myostatin or other TGF-β family ligands (Amthor et al. 2004; Olguin and Pisconti 2012). That follistatin not merely acts through inhibition of myostatin is supported by observations that follistatin overexpression in muscle of myostatin-deficient mice further increases muscle mass, whereas heterozygous loss of follistatin results in reduced muscle mass (Gilson et al. 2009; Lee et al. 2010). Modulation of activin A activity as well as myostatin-independent Smad3 and mTOR signaling are also involved in the in vivo effects of muscle mass regulation by follistatin (Lee et al. 2010; Winbanks et al. 2012).

TGF-βs and myostatin exert their inhibitory effects on myoblast differentiation, at least in part, through activation of Smad signaling. Both *Smad3*$^{-/-}$ and *Smad4*$^{-/-}$ mice show defects in satellite cell number and function, myogenic differentiation, and myoblast fusion, and have decreased muscle mass (Ge et al. 2011; Han et al. 2012). Interestingly, *Smad3*$^{-/-}$ mice show increased levels of myostatin in myoblasts, and myostatin inactivation in *Smad3*$^{-/-}$ mice improves the muscle phenotype (Ge et al. 2011). In addition, noncanonical pathways and cross-talk with other signaling pathways may also determine the net outcome of TGF-β signaling on myoblast differentiation in vivo (Luo 2017; Zhang 2017).

TGF-β FAMILY SIGNALING IN ADIPOCYTE DIFFERENTIATION

Adipose tissue can be divided into two groups based on function; white adipose tissues (WATs) store energy, and brown adipose tissues (BATs) are thermogenic. WATs and BATs are found at anatomically distinct positions in the body. WATs are located intra-abdominally where they are also called visceral fat, and subcutaneously, whereas BATs are most commonly located in the neck and supraclavicular regions (Hilton et al. 2015). Cold exposure or β-adrenergic stimulation induces brown adipose-like tissues within WATs, a phenomenon known as browning of WATs. These brown fat-like cells in WATs show comparable levels of uncoupling protein-1 (UCP-1) that are critical for thermogenesis similar to classical brown adipocytes in BATs. Although brown adipocytes in BATs are of the *Myf5*$^{+}$ lineage, these induced brown adipocytes within WATs are *Myf5*$^{-}$ like other cells found in WATs. These cells are now thought to constitute a third type of adipocyte referred to as either a "beige" adipocyte or "brite" (brown-in-white) adipocyte (Fig. 4) (Gesta et al. 2007; Wu et al. 2012, 2013; Harms and Seale 2013).

Undifferentiated MSCs initially differentiate into a preadipocyte stage and then proliferate by mitotic clonal expansion. Hormonal cues stimulate further differentiation by initiating production of C/EBPs β and δ, encoded by *Cebpb* and *Cebpd*, respectively (Wu et al. 1996). These transcription factors then activate the expression of PPARγ2 (encoded by *Pparg2*) and C/EBPα (encoded by *Cebpa*) leading to growth arrest for terminal differentiation and expression of adipocyte marker genes such as FABP4 (fatty acid-binding protein 4)/aP2 and leptin. At this stage, activation of FoxO1 transcription factor expression activates the cell cycle inhibitor p21^{CIP1} (Morrison and Farmer 1999; Nakae et al. 2003).

BMPs and Adipogenesis

Many BMPs play dual roles in osteogenesis and adipogenesis of multipotent cell populations (Asahina et al. 1996; Kang et al. 2009).

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Figure 4. TGF-β family signaling in adipocyte differentiation. Mesenchymal stem cells give rise to adipocytes under the guidance of a complex signaling network. The *Myf5*$^-$ lineage gives rise to beige and white preadipocytes, and the *Myf5*$^+$ lineage gives rise to brown adipocytes. Growth and differentiation factor (GDF)-3 inhibits the differentiation of beige preadipocytes from progenitor cells as well as beige preadipocytes into beige adipocytes. Bone morphogenetic protein (BMP)-4 and -7 promote white and brown preadipocyte differentiation, respectively. TGF-β and myostatin (GDF-8) each inhibit both brown and white preadipocyte formation. Enhancer binding proteins are stimulated by BMP-4 and hormonal cues. These DNA-binding proteins promote gene expression indicative of adipocyte maturation, such as the genes encoding CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor-γ2 (PPARγ2), whereas Smad2 and Smad3 signaling, as well as Smad6 and/or Smad7 in the mesenchymal progenitor reduce expression of these genes. UCP-1, uncoupling protein-1.

C3H10T1/2 cells are fibroblasts established from late stage embryos of the C3H mouse strain. These cells functionally resemble MSCs and are competent to differentiate into adipocytes, osteoblasts, myoblasts, and chondrocytes (Reznikoff et al. 1973). Among the BMP ligands, BMP-4 has proadipogenic action and directs multipotent C3H10T1/2 cells to the adipocyte lineage (Bowers and Lane 2007; Zamani and Brown 2011). Subcutaneous injection of BMP-4-treated C3H10T1/2 cells results in development of adipose tissues (Fig. 4) (Tang et al. 2004). Genetic engineered expression of a constitutively active form of *Bmpr1a* or *Bmpr1b* in C3H10T1/2 cells results in accumulation of lipid and expression of *Fabp4*, which lends further credence to the notion that BMP signaling in multipotent cell populations favors adipogenic differentiation (Huang et al. 2009b). Interestingly, noggin treatment of A33 cells, a committed preadipocyte line derived from C3H10T1/2 cells, blocks adipocyte differentiation (Bowers et al. 2006), suggesting that autocrine BMP signaling enables competence for adipocyte differentiation.

BMP-7 is also involved in adipogenesis in many populations of multipotent mesenchymal cells including BMSCs (Chen et al. 2001). BMP-7 can induce human MSCs to an adipogenic lineage in high-density micromass culture, a condition that normally induces chondrogenic differentiation (Neumann et al. 2007). Treating brown preadipocytes with BMP-7 markedly increases *Ucp1* expression compared with other BMP ligands (Tseng et al. 2008). It is noteworthy that BMP-7 can activate Smad1, 5, and 8 in both brown and white preadipocytes, whereas a robust activation of p38 MAPK is additionally observed in brown preadipocytes (Tseng et al. 2008). These findings indicate roles of BMP-7-induced Smad and p38 MAPK pathway signaling in the regulation of thermogenesis in conjunction with a nuclear coactivator PGC1 (PPARγ coactivator-1). Furthermore, detailed gene expression analyses show that BMP-7 treatment significantly suppresses expression of necdin, PREF1 (preadipocyte factor 1) and Wnt10a, which inhibit brown adipogenesis while increasing the expression of PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16), a transcription factor that directs the brown fat lineage (Seale et al. 2007). $Bmp7^{-/-}$ mice die at birth, and $Bmp7^{-/-}$ embryos show significant reduction in interscapular BAT mass compared with that of littermate controls (Tseng et al. 2008). Tail vein injection of BMP-7 expressing but not BMP-3 expressing adenovirus specifically induces *Prdm16* and *Ucp1* expression in BAT, but not other genes involved in energy metabolisms in other tissues, leading to increased body temperature and decreased body mass of the injected mice (Tseng et al. 2008). These results highlight the indispensable roles of BMP-7 in brown adipocyte differentiation and energy expenditure (Fig. 4).

In contrast to the proadipogenic function of BMP-4 to promote white adipocytes, and of BMP-7 to promote brown adipocytes, BMP-2 treatment of BMSCs promotes osteogenic differentiation and inhibits adipogenesis (Gimble et al. 1995; Pereira et al. 2002). Dose-dependent effects of BMP-2 treatment of C3H10T1/2 cells are observed. Low doses of BMP-2 induce adipogenic differentiation, whereas high doses of BMP-2 promote chondrogenic and osteogenic differentiation (Wang et al. 1993; Asahina et al. 1996). Similar to other BMPs, BMP-2 is also able to induce adipogenesis, yielding white adipocytes from committed preadipocytes such as 3T3-L1 and 3T3-F442A (Ji et al. 2000; Rebbapragada et al. 2003). Treatment of preadipocytes with BMP-2 along with rosiglitazone, a PPARγ agonist, further enhances adipogenesis (Sottile and Seuwen 2000). Of note, forced expression of a constitutively active form of either BMPRIA or BMPRIB in 3T3-F442A cells inhibits adipogenesis and stimulates osteogenesis (Skillington et al. 2002). Differences in culture methods or clonal variation of the cells used in each study may explain such discrepancies; however, an alternative interpretation is that cell fate specification of mesenchymal cells through BMP signaling is highly dose-dependent. The differentiation factor NEL-like molecule-1 (NELL1), a secreted protein that can induce bone formation (Ting et al. 1999), can antagonize adipogenesis of the mouse BMSC

cell line M2-10B4 and human primary BMSCs, when adipogenesis is induced by BMP-2 (Shen et al. 2016). These findings suggest that, in addition to the dose of BMP-2, additional factors may contribute to cell fate determination of BMSCs. BMP-2 induces both Smad and p38 MAPK signaling in C3H10T1/2 cells. Studies using specific inhibitors reveal that the Smad pathway is important for *Pparg* expression, whereas p38 MAPK signaling is critical for PPARγ to control gene transcription (Hata et al. 2003). Upon BMP-2 treatment, Schnurri-2 (Shn2) enters the nucleus and directly interacts with Smad1 and Smad4, and with C/EBPα at the *Pparg2* promoter to induce *Pparg2* expression (Jin et al. 2006). *Shn2*$^{-/-}$ mice show reduced white fat mass with normal brown fat mass, which suggests that BMP ligands (or at least BMP-2) use the BMP–Smad pathway along with Shn2 to regulate white adipogenesis in vivo (Jin et al. 2006). These findings suggest that the effects of BMP-2 exposure are largely determined by the dose administered as well as the adipocyte differentiation state at the time of administration.

Pioneering work using 2T3 mesenchymal cells have led to the hypothesis that BMP signaling through BMPRIA favors adipogenic differentiation, whereas signaling through BMPRIB favors osteoblastic differentiation (Chen et al. 1998). The experimental strategy was to overexpress dominant-negative forms of receptors, because RNA interference technology to silence gene expression in mammalian cells had not been established; thus, the specificity of the signaling pathways blocked by each dominant-negative receptor form has been a concern. Although body weight is slightly reduced in *Bmrp1b*$^{-/-}$ mice, no overt phenotypes are found in adipose tissues (Schulz et al. 2013). Studies in humans reveal that increased expression of BMPRIA is associated with visceral and subcutaneous white fat deposits in obese individuals (Bottcher et al. 2009). In mice, the specific disruption of *Bmpr1a* in *Myf5*$^{+}$ lineage cells results in a significant reduction of BAT (Schulz et al. 2013). Subcutaneous WAT and epididymal WAT that chiefly originate from a *Myf5*$^{-}$ lineage have the expected mass, whereas interscapular WAT and retroperitoneal WAT with subpopulations of cells from the *Myf5*$^{+}$ lineage have reductions in size, showing the importance of BMPRIA-induced BMP signaling in adipogenesis (Schulz et al. 2013). Specific disruption of *Acvr1*/*Alk2* in *Myf5*$^{+}$ lineage results in a similar yet milder phenotype, whereas compound conditional inactivation of *Bmpr1a* and *Acvr1* in *Myf5*$^{+}$ lineage cells results in complete loss of brown adipogenesis. In the mice with inactivated *Bmpr1a* expression in *Myf5*$^{+}$ lineage cells, the severe BAT paucity increases sympathetic input to WAT, and thus enhances beige adipogenesis to promote browning of the WAT (Fig. 4) (Schulz et al. 2013). Although BMP-2 and BMP-4 bind BMPRIA with much higher affinity than BMP-7 (Sebald et al. 2004), the results suggest that BMP-7 binding to BMPRIA is of key importance for brown adipogenesis. These results also reveal a potentially new mechanism to compensate for loss of brown adipocytes, by promoting beige adipogenesis to restore total thermogenic capacity.

TGF-β as a Negative Regulator of Adipogenesis

Unlike the BMPs, TGF-β has inhibitory roles during adipogenesis. TGF-β treatment increases proliferation of 3T3-F442A preadipocytes (Jeoung et al. 1995; Choy et al. 2000), and potently inhibits the adipogenic conversion of 3T3-L1 preadipocytes (Ignotz and Massagué 1985). TGF-β also down-regulates adipose gene expression in fully differentiated TA1 adipocytes (Torti et al. 1989). Transgenic mice expressing TGF-β1 from the rat *Pck1* promoter (of the gene encoding phosphoenolpyruvate carboxykinase 1) in several tissues, including WAT and BAT, show severely reduced WAT and BAT (Clouthier et al. 1997), supporting the notion that TGF-β represses adipogenesis.

During adipogenesis the expression levels of Smad2, 3, and 4 are unchanged in 3T3-F442A preadipocytes, whereas those of Smad6 and Smad7 are severely decreased (Choy et al. 2000). Overexpression of Smad2 or Smad3 inhibits lipid accumulation of 3T3-F442A preadi-

pocytes, with *Smad3* exerting stronger effect. In contrast, a dominant-negative form of Smad3 is able to suppress the inhibitory function of TGF-β signaling on adipogenesis; however, adipogenesis proceeds normally in the presence of dominant-negative form of Smad2 (Choy et al. 2000). These findings support Smad3 as a TGF-β signaling component that inhibits adipogenic differentiation, with Smad2 playing no discernible role in this process (Fig. 4). Smad3, along with Smad4, associates with C/EBPβ and C/EBPδ resulting in decreased *Pparg* expression (Choy and Derynck 2003). Smad6 and Smad7, known as inhibitory Smads, block TGF-β family signaling through Smads. However, overexpressing *Smad6* or *Smad7* in 3T3-F442A preadipocytes yields a strong inhibitory effect on adipogenesis (Choy et al. 2000). In consideration of the finding that inhibitory Smad levels are sharply reduced during adipogenesis, Smad6 and Smad7 may play a distinct role in regulating TGF-β family signaling activity in adipocytes (Fig. 4) (Choy et al. 2000). Because Smad6 inhibits BMP signaling, and Smad7 inhibits both BMP and TGF-β signaling (Hayashi et al. 1997; Imamura et al. 1997), and BMP-4 and BMP-7 stimulate white and brown adipogenesis, respectively (Fig. 4) (Bowers and Lane 2007; Tseng et al. 2008; Zamani and Brown 2011), the inhibitory effect on adipogenesis by Smad6 and/or Smad7 may additionally relate to inhibition of BMP signaling.

Growth Differentiation Factors and Adipogenesis

GDF-3 is primarily expressed in adipose tissue (McPherron and Lee 1993), and a high-fat diet selectively increases *Gdf3* expression in WAT (Witthuhn and Bernlohr 2001). Increased expression of *Gdf3* in mice by adenoviral-mediated gene transfer results in increased body fat with adipocyte hypertrophy when the mice are fed a high-fat diet, but these mice have normal lipid distribution when on a normal fat diet (Wang et al. 2004). In contrast, $Gdf3^{-/-}$ mice show resistance to diet-induced obesity (Andersson et al. 2008; Shen et al. 2009a). Control mice become obese when fed with a high-fat diet, whereas $Gdf3^{-/-}$ mice accumulate less WAT while consuming a similar amount of food to account for their higher metabolic rate (Shen et al. 2009a). Interestingly, the gene expression profile of WAT in $Gdf3^{-/-}$ mice resembles that of BAT, suggesting that loss of GDF-3 leads to browning of WAT and prompts beige adipogenesis (Fig. 4). GDF-3 binds to the activin type I receptors ActRIB/ALK-4 and ActRIC/ALK-7 along with ActRIIB, and induces activation of Smad2 and Smad3 (Chen et al. 2006; Levine and Brivanlou 2006). Because $Gdf3^{-/-}$ mice experience WAT browning only when placed on high-fat diets, this differentiation phenotype differs from the WAT browning that results from *Bmpr1a* inactivation in $Myf5^{+}$ lineage cells (Schulz et al. 2013). These findings suggest that different nutrient conditions regulate GDF-3-induced signaling through ActRIB/C and Smad2 and Smad3 to control beige adipogenesis in WAT.

Myostatin, known as a key negative regulator of skeletal mass, promotes adipogenesis and inhibits myogenesis in C3H10T1/2 multipotent mesenchymal cells (Artaza et al. 2005; Feldman et al. 2006). However, the resulting adipocytes in cell culture are smaller and have a gene expression profile reminiscent of immature adipocytes (Feldman et al. 2006). In contrast, human MSCs cultured with myostatin undergo less adipogenic differentiation, as apparent by decreased expression of key adipogenic genes such as *PPARG* and *CEBPA* (Guo et al. 2008). *CEBPB* expression, which is required for clonal expansion of committed adipocytes (Tang et al. 2003), is not affected by myostatin treatment, suggesting that myostatin-induced inhibition occurs after mitotic clonal expansion during early adipogenic differentiation. These observations are consistent with the inhibition of adipogenic differentiation of 3T3-L1 preadipocytes by myostatin treatment (Kim et al. 2001). These inhibitory effects require Smad3 as in the case of TGF-β (Guo et al. 2008).

$Gdf8^{-/-}$ mice have reduced quantities of adipose tissues, decreased cell size of the remaining adipocytes, and slower metabolic rate than control mice, while maintaining normal

body temperature and food intake (Lin et al. 2002; McPherron and Lee 2002; Hamrick et al. 2006). Overexpression of a dominant-negative form of ActRIIB in skeletal muscle from the *Myl1* promoter (of the gene encoding myosin light chain 1) results in muscle and adipose phenotypes similar to *Gdf8*$^{-/-}$ mice (Lee and McPherron 2001). In contrast, expression of the dominant-negative form of ActRIIB only in adipose tissue from an *Fabp4* (also named *aP2*) promoter in mice does not lead to overt changes in either muscle or adipose tissue (Guo et al. 2009). Moreover, myostatin signaling activates both PPARγ and MyoD expression in adipose-derived stem cells, whereas it represses their expression in muscle satellite cells (Zhang et al. 2015). Taken together, these findings suggest that the reduced adiposity of *Gdf8*$^{-/-}$ mice may be a result of the muscle hypertrophy that increases muscle glucose metabolism and decreases the energy available for lipid storage.

Systemic increase of myostatin levels by injecting myostatin producing cells into athymic nude mice results in a reduced skeletal muscle mass and a dramatic reduction of WATs (Zimmers et al. 2002). Expression of *Gdf8* in adipocytes of mice from an *Fabp4* promoter decreases adipocyte size and increases resistance to diet-induced obesity (Feldman et al. 2006). Muscle-directed expression of *Gdf8* from the *Cmk* promoter (gene encoding muscle creatine kinase) increases epididymal fat pads in mice (Reisz-Porszasz et al. 2003). These results show a sharp contrast with reduction in WAT resulting from systemic increase in myostatin. A plausible explanation is that adipose and muscle tissues have sensitive but varied dose responses to myostatin signaling. An alternative explanation is that overexpressed myostatin competes with other signaling pathways. Thus, the interaction of myostatin with ActRIIB may inhibit the BMP-7 signaling pathway (Rebbapragada et al. 2003), leading to reduced overall adipogenesis that primarily affects BATs (Singh et al. 2014).

BMP-3b, also known as GDF-10, is expressed at much higher levels than either BMP-3 or BMP-2 in both WATs and BATs (Cunningham et al. 1995; Hino et al. 1996, 2012). BMP-3b inhibits osteoblast differentiation of C2C12 myoblasts by binding to ActRIB/ALK-4 and ActRIIB and consequent Smad3 activation (Matsumoto et al. 2012). siRNA-mediated silencing of *Bmp3b* in 3T3-L1 preadipocytes increases adipogenic gene expression, apparent from expression of adiponectin and PPARγ, and increased expression of *Bmp3b* reduces the expression of these genes (Hino et al. 2012). *Bmp3b*$^{-/-}$ mice lack an apparent developmental phenotype, suggesting compensation for the loss of BMP-3b in vivo by other members of TGF-β family (Zhao et al. 1999). Future research (e.g., using high-fat diet and cold exposure) on *Bmp3b*$^{-/-}$ mice will inform investigators of the functions of BMP-3b during adipogenesis.

Activin and Energy Expenditure

The effects of activin on adipogenesis in cell culture are similar to those of TGF-β. Activin A promotes proliferation of human adipocyte precursor cells but inhibits early stages of differentiation (Zaragosi et al. 2010). Activin A-treated 3T3-L1 preadipocytes express less PPARγ and CEBPα than untreated controls (Hirai et al. 2005). Silencing of *SMAD2* in human adipose progenitor cells overrides the effect of activin A treatment to prompt adipogenesis, suggesting that activin A signaling is mediated largely by Smad2 along with C/EBPβ (Zaragosi et al. 2010). In human adipocytes, the expression of *INHBA*, which encodes the activin A monomer, is repressed during adipocyte differentiation, whereas the expression of activin B from the *INHBB* gene remains high (Sjöholm et al. 2006; Carlsson et al. 2009; Zaragosi et al. 2010). A mutant mouse line that has *Inhbb* introduced into the *Inhba* locus allows for activin B expression with the same spatiotemporal pattern of activin A (Brown et al. 2000). These mice have much less WAT, elevated metabolic rates, and increased expression of genes necessary for mitochondrial biogenesis and uncoupling (Li et al. 2009). These results suggest that activin signaling controls mitochondrial energy expenditure, in addition to its function in adipocyte differentiation.

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TGF-β FAMILY SIGNALING IN TENOCYTE DIFFERENTIATION

Clinically, tendon and ligament injuries are some of the most common musculoskeletal injuries ranging from ankle or wrist sprains and strains to Achilles tendon rupture (Boyer et al. 2005; Towler and Gelberman 2006). These injuries include tendinosis, tendinitis, and paratendinitis, and can be traumatic and inflammatory in nature, or stem from overuse or degenerative etiologies (Maffulli et al. 1998; Asplund and Best 2013). Regardless of the specific condition, severe tendon injuries are challenging to manage as restoration of flexibility and tensile strength at tendon–tendon and tendon–bone interfaces are difficult to replicate with surgical procedures that rely on autograft, allograft, or synthetic materials (Olson et al. 1988; Sabiston et al. 1990; Paulos et al. 1992; Jackson and Simon 2002; Towler and Gelberman 2006; Bagnaninchi et al. 2007). Tendon itself is a highly specialized tissue with type I collagen arranged in hierarchical, longitudinal fibril arrays bound into fascicles by the endotenon, which runs contiguously with the epitenon through which the vasculature, innervation, and lymphatics traverse (Kastelic et al. 1978; Fenwick et al. 2002; Clegg et al. 2007). Embryologically, tendons derive from a specialized compartment of the somite, the syndetome (Brent et al. 2003). A specific marker, the basic helix-loop-helix (bHLH) transcription factor scleraxis (encoded by the *Scx* gene), is present in tendon progenitor cells and persists in mature tenocytes (Cserjesi et al. 1995; Schweitzer et al. 2001). Although this unique marker is very useful in tenocyte research, the signaling pathways that lead to tenogenic mesenchymal differentiation are still being elucidated.

TGF-β family members play a role in tenocyte differentiation and maintenance of the tenocyte phenotype. Although the transcription factors that guide tenocyte differentiation significantly overlap with those in other musculoskeletal tissues, most research to date on tenogenic lineage differentiation and tendon healing focuses on differences between these factors. These studies also show that certain members of the TGF-β family are protenogenic, whereas others promote cartilaginous, osteogenic, or myogenic differentiation. Specifically, BMP-2, -4, and -7 are antitenogenic, whereas GDF-7 (BMP-12) is the best studied tenogenic BMP (Lee et al. 2011; Yee Lui et al. 2011; Rui et al. 2012b). GDF-5, -6, and -7 (also known as BMP-14, -13, and -12, respectively) have been shown to induce new tendon formation in animal models, and TGF-β promotes retention of *Scx* expression and maintenance of the tenocyte phenotype (Wolfman et al. 1997; Barsby et al. 2014). Furthermore, Smads are crucial in the gene expression profile resulting from tensile and compressive forces, which are essential for tenocyte formation and regeneration (Maeda et al. 2011).

Similar to myoblast differentiation into myocytes, BMPs play a variety of roles in tenogenesis. BMP-2 promotes tenocyte precursor cell differentiation into osteocytes, chondrocytes, and adipocytes in cell culture with increased production of glycosaminoglycans and expression of aggrecan (Rui et al. 2013; Liu et al. 2014). Furthermore, BMP-2 potently induces osteogenic differentiation of tendon-derived stem cells, which express higher levels of the BMPRIA, BMPRIB, and BMPRII receptors than bone marrow-derived stem cells (Rui et al. 2012a). Although it does not promote tenocyte differentiation, BMP-2 signaling may be important at the tendon–bone junction (Rodeo et al. 1999; Ma et al. 2007; Rui et al. 2012a). Specifically, canonical BMP signaling stimulates ectopic bone formation after tendon transection as there is an increase in downstream Smad1/5/8 phosphorylation and inhibition of BMP signaling mitigates this process (Peterson et al. 2014). Additionally, overexpression in BMP receptor ALK-2 in cells of the scleraxis lineage (thought to mark tendon progenitor cells) leads to ectopic bone at sites of enthesis such as the Achilles tendon (Agarwal et al. 2017). In addition to BMP-2, BMP-4 and BMP-7 were shown to be antitenogenic. In calcifying tendinopathy models, these BMPs induce chondrocyte-like and fibroblast-like differentiation changes in healing tendon (Yee Lui et al. 2011). BMP-7 changes the gene expression

profile of tendon tissue to more closely resemble cartilaginous meniscus tissue (Ozeki et al. 2013). Interestingly, these BMPs are found in clinical samples of tendinopathy, but not in healthy tendon (Rui et al. 2013). Furthermore, tendon-derived stem cells from unhealthy or injured tendon are more sensitive to BMP signaling through Smads to induce nontenogenic differentiation (Lui and Wong 2013).

Although BMP-2, -4, and -7 are antitenogenic, other BMPs directly control the differentiation of tenogenic precursors into tenocytes. GDF-5, GDF-6, and GDF-7 have been shown to promote tendon repair and the formation of new tendon tissue (Wolfman et al. 1997; Yu et al. 2007; Lee et al. 2011), and the tenogenic activity of GDF-7 specifically was shown both in cell culture and in vivo (Lou et al. 2001; Wang et al. 2005; Violini et al. 2009; Lee et al. 2011). Of particular interest is the ability of GDF-7 to induce MSCs of nontendon origin (e.g., bone marrow–derived stem cells) to differentiate into cells with tenocyte characteristics, with higher expression of scleraxis and tenomodulin (Lee et al. 2011). In addition, collagen scaffolds seeded with bone marrow-derived stem cells exposed to GDF-7 improve tendon repair in an in vivo model (Lee et al. 2011).

As tendon plays a unique role in the musculoskeletal system, sustaining dynamic ranges of compression and extension while retaining flexibility, physical forces play an essential role in tenocyte differentiation and function. These forces act in conjunction with effects of other members of the TGF-β family. TGF-β is known to maintain scleraxis expression in tendons in response to mechanical forces (Maeda et al. 2011). Specifically, TGF-β3 promotes *Scx* expression and scleraxis translation, as well as tenocyte differentiation, when combined with tensile and compressive forces (Barsby and Guest 2013; Barsby et al. 2014). TGF-β2 has also been shown to induce tenogenic differentiation of MSCs (Hoffmann et al. 2006; Guerquin et al. 2013). As mentioned, other TGF-β family members including GDF-5, GDF-6, and GDF-7 (those most related to BMPs) can induce new tendon and ligament formation, even at ectopic sites in animal models (Wolfman et al. 1997). Smads, specifically Smad2 and Smad3, are also required in the regulation of tenogenic gene expression that is induced by physical forces acting on tendon tissue (Maeda et al. 2011). Furthermore, Smad8 has been shown to play a role in the tenogenic differentiation of MSCs (Hoffmann et al. 2006).

Although the body of work regarding MSC differentiation to tenocytes is not as robust as that of adipogenic, myogenic, and osteogenic differentiation, it is clear that BMP and TGF-β signaling through Smads plays a leading role. Tendon is a functionally distinct and dynamic musculoskeletal tissue, and its mesenchymal roots are evident in the signaling pathways that share common mediators with other differentiated mesenchymal tissues.

CONCLUDING REMARKS

The effects of TGF-β family signaling in mesenchymal lineage selection and progression in differentiation are complex and time-, dose-, as well as context-dependent. Also, some TGF-β signaling factors differentially affect specific stages during mesenchymal differentiation. For example, TGF-βs promote proliferation and early differentiation of MSCs into osteoblast, chondrocyte, and adipocyte progenitor cells, but inhibit later conversion into mature osteoblasts, chondrocytes, and adipocytes. In contrast, TGF-βs inhibit myoblast proliferation, differentiation, and myotube formation. Similar to TGF-βs, BMP-2 promotes chondrogenic and osteogenic lineage selection and differentiation, and inhibits myogenic differentiation, but, in contrast to TGF-βs, also promotes late osteoblast differentiation and matrix mineralization. We discussed in this review how the final effect of TGF-β family signaling on mesenchymal differentiation also depends on interactions between different TGF-β signaling pathways, and the presence of other signaling molecules such as IGF-I, Wnts, FGFs, and others.

In addition to findings in cell culture, discoveries in animal models and genetic studies in patients during the past decades have greatly extended our understanding of how signaling by the TGF-β family members can drive disease.

This includes diseases caused by mutations in genes encoding ligands or receptors of the TGF-β family as well as disorders caused by other primary mechanisms, in which altered TGF-β family signaling contributes to disease. The first group includes, for example, Camurati-Engelmann disease, with mutations in the sequence encoding pro-TGF-β1 (Kinoshita et al. 2000), brachydactyly type A2 with mutations in the *BMP2* or *BMPRIB* genes, brachydactyly type B resulting from *NOG* mutations (Lehmann et al. 2007; Dathe et al. 2009), acromesomelic chondrodysplasia (Hunter-Thompson type), and chondrodysplasia Grebe type with *GDF5* mutations (Thomas et al. 1996, 1997), as well as FOP resulting from mutations in the ACVR1/ALK-2 receptor (Shore et al. 2006). The second group includes genetic as well as acquired disorders, for instance, OI and osteoarthritis, in which increased TGF-β signaling has been identified as a contributing mechanism in mouse models (Zhen et al. 2013; Grafe et al. 2014). Additionally, genetic polymorphisms in genes encoding TGF-β family proteins or their signaling mediators may modify the susceptibility, course, and severity of genetic, acquired or age-related diseases, for instance for the risk of osteoporosis (Langdahl et al. 1997, 2003). Importantly, preclinical studies suggest that pharmacological targeting of TGF-β family members may be beneficial for the treatment of certain diseases. For instance, inhibition of TGF-β ligands with antibodies in mouse models improve the bone and extraskeletal phenotype in OI and attenuate the degeneration of articular cartilage in osteoarthritis (Zhen et al. 2013; Grafe et al. 2014). As another example, inhibition of TGF-β signaling prevents obesity and diabetes mellitus in mice (Yadav et al. 2011), and inhibition of GDF-3 function may represent an additional target for the treatment of obesity (Shen et al. 2009a). Similarly, myostatin antagonists may be of clinical value for the future treatment of muscle wasting conditions, and potentially in muscular dystrophies (Wagner et al. 2002). In the context of cell therapy, understanding the roles of TGF-β family signaling in MSC differentiation will help to realize the potential of MSCs in therapy, ,(e.g., for the repair of cartilage and other connective tissues) (Mobasheri et al. 2009). Ultimately, these encouraging new avenues have to be studied in the human context to ascertain effectiveness and safety.

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