TGF-β Family Signaling in Mesenchymal Differentiation

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SIGNALING BY TRANSFORMING GROWTH FACTOR-β (TGF-β) FAMILY FACTORS affects many distinct differentiation pathways, including those of the hematopoietic and immune cell lineages, epithelial lineages, and hematogloasts. Similarly, TGF-β family signaling has revealed that it is a major regulatory network that drives lineage selection and differentiation progression in mesenchymal cells. As with other systems, the signaling activity of TGF-β family members correlates with the temporal and spatial regulation of TGF-β family ligand, receptor, and Smad expression, each of which is regulated during mesenchymal cell differentiation. Likewise, the activities of the ligands, receptors, and Smads are also regulated during differentiation. For example, ligand activity is dictated by secreted antagonists or ligand-binding proteins. Receptors are activated through autocrine and paracrine signaling. Smad activity is defined by combinatorial interactions and cross-talk with other signaling pathways, and the Smads then serve to
integrate information from a multitude of signaling pathways that direct the expression and activity of each component of the TGF-β signaling pathway. In this way, the TGF-β family receptors and Smads act as cell-intrinsic regulators of mesenchymal differentiation. In this chapter, we discuss the roles of TGF-β family signaling in mesenchymal differentiation.

MESENCHYMAL DIFFERENTIATION

The mesenchyme consists of loosely associated stellate-shaped cells, which in the trunk and posterior regions of the head derive from mesoderm, and in the face, jaws, and neck, originate from the neural crest (Noden 1986; Couly et al. 1992; Olivera-Martinez et al. 2000; Matsuoka et al. 2005). Neural crest mesenchyme arises from the dorsal edges of the neuroepithelium during neurulation, migrates extensively, and produces a wide variety of cell types. Members of the TGF-β family have essential roles in the epithelial–mesenchymal transformation that underlies the genesis of neural crest as well as the subsequent specification of various lineages (Sela-Donenfeld and Kalcheim 1999; Anderson et al. 2006; Basch and Bronner-Fraser 2006). Similarly, much of the mesenchyme found in the trunk originates as epithelial condensations of paraxial mesoderm (Solursh et al. 1979; Brand-Saberi and Christ 2000; Kalcheim et al. 2006), intermediate mesoderm, or lateral plate mesoderm and relies on members of the TGF-β family for normal development (Dudley et al. 1999; Oxburgh et al. 2004; Gupta et al. 2006; Miura et al. 2006). In contrast, mesoderm of the anterior head (i.e., pre-otic) has an exclusively mesenchymal origin with no epithelial history (Jacobson 1988; Noden 1988).

Neural-crest- and mesoderm-derived mesenchyme populations have many derivatives in common, but they also give rise to distinct cell and tissue types. Both make cartilage, bone, tendon, perivascular smooth muscle, glandular stroma, meninges, adipose tissue, and dermis. Neural crest mesenchyme alone generates peripheral autonomic neurons, sensory neurons, peripheral glia, Schwann cells, calcitonin-producing C cells, melanocytes, odontoblasts, and cementoblasts. Mesodermal mesenchyme uniquely produces skeletal muscle, cardiac muscle, visceral smooth muscle, endothelium, endocardium, and serosa (Noden and Schneider 2006). The progression of neural crest and mesodermal mesenchyme into their terminal cell types involves equivalent developmental mechanisms. TGF-β family members are likely involved in the differentiation of almost all mesenchymal derivatives. Here, we focus on the chondrogenic (cartilage), osteogenic (bone), myogenic (muscle), and adipogenic (fat) lineages (Fig. 1).

Mesenchymal cells transition to a committed but undifferentiated “blast” cell type before adopting the terminally differentiated “cyte” phe-
notype. For example, myoblasts express some markers of the myogenic lineage and are mononuclear, whereas terminally differentiated myocytes form multinucleated myofibers and express the full complement of proteins required for contraction. Cells in each lineage are distinguished by the acquisition of unique phenotypes and protein expression profiles that enable the cells to perform their specialized functions.

As the cells differentiate along these lineages, their proliferation decreases and the fully differentiated cells stop dividing. The differentiation along each of these lineages is driven by the expression and activities of defined transcription factors that are regulated by signaling inputs and through interactions with other proteins (Fig. 1). Thus, Runx2, a runt domain transcription factor, functions as a key driver in osteogenic differentiation (Ducy et al. 1997; Komori et al. 1997; Lian et al. 2006). Muscle-specific basic/helix-loop-helix (bHLH) proteins, such as MyoD, form heterodimeric complexes with E proteins and drive the
progression of myogenic differentiation. Their activities are largely dependent on their interactions with MEF proteins, another set of transcription factors that serve as coactivators (Naya and Olson 1999; Berkes and Tapscott 2005). Adipogenic differentiation requires the C/EBPs (CCAAT/enhancer-binding proteins) and PPARγ (peroxisome proliferator-activated receptor γ). C/EBPβ and δ, members of a larger family of transcription factors characterized by a basic leucine zipper, activate the expression of PPARγ in preadipocytes. PPARγ is a nuclear receptor that functions through heterodimerization with another nuclear receptor RXR (retinoid X receptor). PPARγ cooperates with C/EBPα to drive the expression of proteins that characterize the differentiated adipocyte (Lehrke and Lazar 2005; Otto and Lane 2005). Chondrogenic differentiation is controlled by Nkx3.2 and Sox (Sry-type high-mobility-group box) transcription factors, characterized by HMG (high-mobility group) box DNA-binding domains. Nkx3.2 is required for Sox9 expression and it represses the expression of osteogenic transcription factors. Sox9 is essential for the initiation of chondrogenic differentiation during mesenchymal condensation, yet it also has a role in slowing down or preventing terminal conversion of chondrocytes into hypertrophic chondrocytes. L-Sox5 and Sox6, two closely related Sox proteins that lack a transactivation domain, are required for progression during chondrogenesis, presumably in a complex with Sox9 (Lefebvre and Smits 2005).

In addition to these four distinct cell types, mesenchymal cells also give rise to other cell lineages, including fibroblasts, connective tissue cells, and tenocytes. Less is known about the control of mesenchymal cell differentiation into these other lineages, either because marker genes specific for the terminally differentiated cell types have not been identified or because the pluripotency of mesenchymal stem cells has only recently been appreciated. Nonetheless, their importance in developmental processes and cancer progression is now apparent. Mesodermally derived mesenchymal cells can be experimentally directed along several other differentiation pathways, including those that give rise to neuronal, glial, and epithelial cells. The extent to which this occurs in vivo is under study.

TGF-β FAMILY SIGNALING IN MESENCHYMAL STEM CELLS

Mesenchymal stem cells can be found at most if not all sites where differentiated mesenchymal cell types are found and in loose connective tissue interspersed between other tissues. Thus, besides the adipocytes themselves and the cell types associated with its high level of vascularization, adipose tissue contains seemingly undifferentiated mesenchymal
cells that serve as a cell reservoir to generate fully differentiated fat cells. These adipose stromal cells maintain their self-renewal and are fully capable of differentiating along other lineages, depending on the environment and signals to which they are exposed. Conceptually similarly, mesenchymal cells called satellite cells are interspersed between the muscle fibers of differentiated muscle tissue. They can be activated to differentiate into myoblasts and myocytes, yet have maintained their pluripotency. In the peristomeum or perichondrium, which cover the surfaces of bone or cartilage, respectively, undifferentiated mesenchymal cells can undergo progressive differentiation from mesenchymal stem cells into functional osteoblasts or chondrocytes that deposit the bone or cartilage matrix. Finally, the stromal cells that are found in bone marrow normally differentiate into osteoblasts or adipocytes while also maintaining a pool of undifferentiated cells. Because these cells can differentiate into several lineages, they have gained prominence as a potential source for cell-based therapies in various types of tissue repair.

The selection and progression of mesenchymal stem cells along their individual lineages depend on the location of these cells and the signals to which they are exposed, either through direct cell or matrix interactions or through soluble mediators, including the TGF-β family members. Signals that stimulate mesenchymal cell proliferation, such as TGF-β, generally inhibit differentiation, whereas proliferation-inhibitory signals may promote differentiation. The nature and combination of these signals drive the selection of a defined differentiation pathway, through regulation of the expression and function of lineage-specific transcription factors.

Autocrine and paracrine signaling by the TGF-β family is required for the maintenance of mesenchymal stem cells in an undifferentiated state, for differentiation lineage specification and for the progression of differentiation along that lineage. Although the expression of the receptors and Smads during development and tissue differentiation has not yet been fully characterized, both undifferentiated and differentiated mesenchymal cells express a diverse set of receptors, as well as the receptor-activated Smads and Smad4, thus providing the cells with the potential to respond to signaling by multiple TGF-β family members. Throughout each stage of differentiation, cells express and respond to several TGF-β family members, resulting in an intricate web of autocrine and paracrine signaling. Accordingly, the TGF-β family signaling mediators and the Smad transcription factors are an integral part of the cell-intrinsic engine that regulates selection and progression of differentiation. The role of TGF-β family signaling in the maintenance and function of mesenchymal stem cells is further discussed in Chapter 20.
TGF-β FAMILY SIGNALING IN OSTEOBLAST DIFFERENTIATION

Regulation of Osteogenic Differentiation by Bone Morphogenetic Proteins

Consistent with the expression of bone morphogenetic protein (BMP) and TGF-β receptors in mesenchymal cells and osteoblasts, BMPs TGF-βs, growth and differentiation factors (GDFs), and activins regulate osteogenic differentiation (Fig. 2). Several BMPs are expressed by cells of mesenchymal origin throughout intramembranous and endochondral ossification of bone (see Chapter 22). In vivo studies demonstrate essential but redundant functions of the BMPs in the skeleton. BMP-2, -4, and -6 are most commonly expressed by osteoblast cell lines. BMPs induce bone formation, as apparent from their ability to stimulate ectopic bone formation following injection at nonskeletal sites (Wozney et al. 1988). At the cellular

Figure 2. TGF-β and BMP control of osteoblast differentiation. TGF-β enhances recruitment and proliferation of mesenchymal cells during osteogenesis, but it inhibits terminal osteoblast differentiation. TGF-β activates Smad3 to bind and repress Runx2 function. The Smad3-Runx2 complex recruits class II histone deacetylases (HDAC4/5) to Runx2-target genes, including Runx2 itself. BMP stimulates mesenchymal precursor proliferation and commitment to the osteoblast lineage. BMP induces Dlx5 expression, which then induces Runx2 transcription to promote the progression of osteoblast differentiation. In response to BMP, Smad1 and Smad5 bind Runx2 to enhance its transcriptional activity.
level, BMPs promote mesenchymal cell commitment to osteogenic fate and osteoblast differentiation. BMPs also induce the most terminal event in osteoblast differentiation: osteocyte apoptosis (Hay et al. 2004).

Consequently, BMP is a critical growth factor in osteoblast differentiation, just as Runx2 is a critical transcription factor. Several signaling pathways exert bone anabolic or catabolic effects by impacting the expression and activities of BMPs and Runx2 (Ducy 2000; Canalis et al. 2003). Importantly, BMPs and Runx2 also regulate each other’s expression and activity at several levels. In this way, BMP and Runx2 serve as upstream and downstream “anchors” in the pathway that drives osteoblast differentiation.

Regulation of BMP Expression and Activity during Osteogenesis

The expression of BMP-2 and BMP-4 in osteoblasts is activated by Runx2, which integrates signals from several pathways that promote or antagonize osteoblast differentiation. Among these, parathyroid hormone (PTH), TGF-β, and BMPs impact BMP expression, whereas BMP expression is also induced by estrogen, statins, Wnt3a, and other signaling pathways (Gitelman et al. 1995; Rickard et al. 1998; Helvering et al. 2000; Ghosh-Choudhury et al. 2001). Sonic hedgehog (Shh) induces the activity of Gli2, which binds and activates transcription from the BMP-2 promoter (Zhao et al. 2006). Once expressed, BMP activity is regulated by various secreted extracellular matrix proteins (see Chapter 22). Some of these, such as crossveinless-2, promote BMP activity, whereas others, such as gremlin and noggin, antagonize BMP activity (see Chapter 8). Modifications in glycosylation or sulfation of proteoglycans can also affect BMP activity (Klüppel et al. 2005; Jiao et al. 2007).

BMP Signaling and the Transcriptional Control of Osteoblast Differentiation

Several transcription factors are sequentially expressed during BMP-induced osteoblast differentiation and are required for cell maturation. Among these, the expression of Msx2 and Dlx5 is rapidly induced in response to BMP without the need for new protein synthesis. BMP-activated Smad1 can bind to the Msx2 promoter (Brugger et al. 2004) in cooperation with homeobox transcription factors or in a complex with Lef1 in response to Wnt signaling (Hussein et al. 2003). Msx2 is expressed early in skeletogenesis and is important for early events in limb bud patterning and for promoting osteoblast commitment. Accord-
ingly, direct target genes of Msx2 include Pax3 and osteocalcin (Kwang et al. 2002; Bidder et al. 1998). However, when overexpressed, Msx2 can repress terminal differentiation. Msx2^{−/−} mice exhibit skeletal patterning defects (Satokata et al. 2000).

BMPs also directly activate Dlx5 expression independently of new protein synthesis, but the mechanism of this induction remains to be determined. Dlx5 is specifically expressed in differentiating osteoblasts and binds the Runx2 promoter to confer BMP-inducible Runx2 expression (M.H. Lee et al. 2005). Ablation of Dlx5 expression prevents BMP-induced expression of osterix, another osteogenic transcription factor (Lee et al. 2003b), although a direct role for Dlx5 in osterix transcription has not been described (Lee et al. 2003a; M.H. Lee et al. 2005). Overexpression of Dlx5 in osteoblasts promotes the progression of differentiation, whereas reduction of Dlx5 levels prevents it (Lee et al. 2003b). Accordingly, Dlx5^{−/−} mice exhibit skeletal abnormalities due to defective osteoblast differentiation (Acampora et al. 1999).

Like Msx2, Id proteins promote osteoblast progenitor proliferation but inhibit terminal differentiation. BMP activates Smads to induce Id transcription, which is important for undifferentiated mesenchymal cells to select an osteoblast fate (Ogata et al. 1993; Ying et al. 2003; Peng et al. 2004). Id1 also promotes BMP action by repressing the inhibition of BMP expression by Twist-1, a bHLH transcription factor (Hayashi et al. 2007). The expression of the winged helix transcription factor FoxC2 (Mfh1) is induced by BMP early in the developing limb bud and in osteoblasts and chondrocytes (Yang et al. 2000; Nifuji et al. 2001).

In contrast to Msx2 and Dlx5, BMPs do not directly activate the expression of osterix and Runx2; rather, their induction requires newly synthesized transcription factors, such as Dlx5 (Lee et al. 2003b). The induction of Runx2 and osterix expression by BMPs further promotes osteoblast differentiation, because these transcription factors directly activate the expression of osteoblast genes, such as those encoding osteopontin, osteocalcin, or osteonectin (Ducy et al. 1997; Nakashima et al. 2002). Furthermore, the activity of Runx2 is enhanced by Smad-dependent and non-Smad mechanisms in response to BMP. The BMP-responsive Smad1, Smad5, and Smad8 can bind Runx2 in response to BMP, and Runx2 mutants that do not interact with these Smads compromise the osteogenic activity of Runx2 (Zhang et al. 2000; Bae et al. 2007). Such a Runx2 mutation was identified in humans with cleidocranial dysplasia, which results from heterozygous loss of Runx2 function, illustrating that the functional interaction of Smads with Runx2 is critical for Runx2 function in vivo (Zhang et al. 2000). BMP-activated mitogen-activated
protein (MAP) kinase signaling also increases Runx2 function, through phosphorylation of its transactivation domain (Xiao et al. 2000).

Menin, a tumor suppressor that is mutated in multiple endocrine neoplasia 1 (MEN1), also promotes osteogenic fate selection but inhibits terminal differentiation. Early in osteogenic differentiation, menin binds Runx2 in complex with BMP-activated Smad1 and promotes the expression of alkaline phosphatase, an early osteoblast marker. Later in differentiation, menin no longer binds Runx2, but it can repress Runx2 function by forming inhibitory complexes with TGF-β-activated Smad3 (Sowa et al. 2004). Ablation of menin antagonizes BMP-induced osteoblast differentiation (Sowa et al. 2004). The skeletal role of menin cannot be discerned from Men1−/− mice that die at embryonic day 12.5 with abnormalities in craniofacial development (Bertolino et al. 2003).

The transcriptional repressors Tob and c-Ski repress the osteogenic function of BMP-activated Smad1, Smad5, and/or Smad8. Tob binds the transactivation domains of Smad1, Smad5, and Smad8, thereby reducing their function and altering their cellular localization (Yoshida et al. 2000). Tob expression is induced by BMP in osteoblasts, thus forming a negative feedback loop that blunts Smad-dependent BMP signaling. Tob−/− mice have increased bone mass due to increased osteoblast number and activity and increased BMP-induced ectopic bone formation, relative to wild-type littermates (Yoshida et al. 2000). Similarly, c-Ski binds Smad4 to repress its activities and blocks BMP-induced osteoblast differentiation. Ski−/− mice have skeletal abnormalities, including absent cranial bones (Colmenares et al. 2002).

The E3 ubiquitin ligases Smurf1 and Smurf2 are expressed in osteoblasts and facilitate the degradation of the BMP-activated Smads, as well as Runx2 (Zhao et al. 2003). Smurfs regulate BMP signaling through a number of mechanisms, most of which inhibit BMP signaling and osteoblast differentiation. BMP induces acetylation of Runx2 by CBP/p300, which protects Runx2 from Smurf1-mediated degradation. In contrast, deacetylation of Runx2 by histone deacetylase (HDAC) 4 facilitates Runx2 degradation by this pathway (Jeon et al. 2006). Runx2 binding to Smad6 also increases Smurf1 degradation of Runx2 (Shen et al. 2006). Smurf1 binds Smad1 when the linker region of Smad1 is phosphorylated in a MAP kinase-dependent manner (Sapkota et al. 2007). The Smurf1-Smad1 interaction inhibits Smad1 nuclear translocation and targets Smad1 for proteosomal degradation. In addition, Smurf1 inhibits BMP signaling by targeting MEKK2 for degradation. BMP activates MEKK2 to phosphorylate JNK, which increases the expression and activity of JunB. JunB cooperates with Runx2 to activate BMP-inducible gene
expression. Smurf1 therefore negatively regulates not only Smad signaling, but also non-Smad signaling induced by BMP. Consequently, Smurf1−/− mice exhibit increased bone mass due to increased osteoblast number and differentiation (Yamashita et al. 2005). Overexpression of Smurf1 has the opposite effect, resulting in reduced bone mass due to loss of osteoinductive BMP signals (Zhao et al. 2004).

Wnt signaling has emerged as a major pathway in osteogenesis. The Wnt and BMP pathways intersect at several levels. The induction of BMP expression by Wnt signaling partly explains the activities of Wnt in osteogenesis (Fischer et al. 2002). In turn, BMPs induce the expression of Wnts, LRP5 and β-catenin, two key components of Wnt signaling, and the formation of Tcf/Lef complexes with Smad4 (Fischer et al. 2002; Hussein et al. 2003; Rawadi et al. 2003; Nakashima et al. 2005). The secreted antagonists, noggin and sclerostin, inhibit both Wnt and BMP signaling (Winkler et al. 2003; Ellies et al. 2006). Sclerostin, encoded by the Sost gene, binds and inhibits the lipoprotein-receptor-related protein 5 (LRP5) to antagonize Wnt signaling. Sclerostin can also bind noggin, but the sclerostin-noggin complex is a weaker BMP antagonist than either protein alone. Sclerostin inhibits osteoblast proliferation and differentiation and promotes apoptosis of osteoblasts in vitro (Winkler et al. 2003; Sutherland et al. 2004; van Bezooijen et al. 2004), but the extent to which sclerostin antagonizes BMP-induced osteoblast differentiation varies among cell lines.

Although less osteogenic than BMPs, GDF-5, -6, and -7 can promote osteogenic differentiation of several mesenchymal (osteoprogenitor cell lines, as well as adipose- and bone-marrow-derived mesenchymal stem cells in vitro (Nishitoh et al. 1996; Erlacher et al. 1998; Gruber et al. 2001; Yeh et al. 2005; Zeng et al. 2007), mainly through activation of Smad5. GDF-5 stimulates osteogenic differentiation of bone-marrow-derived mesenchymal stem cells in hydroxyapatite ceramic implants in vivo and has direct effects on the structure and composition of cortical bone matrix. The stimulatory effects of GDF-5, -6, or -7 on endochondral bone formation (Storm et al. 1994; Hotten et al. 1996; Erlacher et al. 1998; Tsumaki et al. 1999; Yoshimoto et al. 2006) are primarily due to their ability to promote recruitment, condensation, and chondrogenic differentiation of mesenchymal stem cells, as described below.

GDF-8, also known as myostatin, is a distinct member of the GDF subfamily that negatively regulates skeletal muscle growth, as discussed further below. In addition, GDF-8/myostatin suppresses osteogenic differentiation of bone-marrow-derived mesenchymal stem cells during mechanical loading by suppressing expression of osteogenic factors, including BMP-2 (Hamrick et al. 2007).
Regulation of Osteogenic Differentiation by TGF-β

TGF-β is a critical regulator of bone formation and remodeling, as illustrated from the dramatic alterations in bone mass in mice with altered TGF-β signaling (see Chapter 22). However, the interpretation of the bone phenotypes in these mice is often difficult, because bone mass reflects the net effects of TGF-β action on several different cell types. TGF-β regulates each stage of chondrocyte, osteoblast, and osteoclast differentiation. Therefore, understanding the role of TGF-β in bone requires understanding its effects on the differentiation and function of each cell type. For example, injection of TGF-β under the periosteum of calvariae or long bones increases bone formation (Noda and Camilliere 1989; Marcelli et al. 1990). In long bones, this result reflects the ability of TGF-β to promote chondrocyte commitment and proliferation, followed by replacement of this cartilage with bone. However, the stimulatory effect of TGF-β in calvaria may instead be due to stimulation of osteoblast progenitor proliferation.

Although TGF-β has many important roles in osteogenesis, it does not regulate the commitment of mesenchymal stem cells to the osteoblast lineage. BMPs are potent agonists for commitment of mesenchymal stem cells to an osteogenic lineage. A large body of work on osteoblast differentiation has been done using C2C12 myoblasts. TGF-β increases the expression of early osteoblast markers in these cells (Katagiri et al. 1994), perhaps as a consequence of its inhibition of myoblast differentiation. TGF-β opposes the induction of Dlx5 expression by BMP in C2C12 cells and promotes JunB and Runx2 expression (Lee et al. 2003b). However, TGF-β by itself fails to convert C2C12 cells into fully differentiated osteoblasts.

TGF-β stimulates the proliferation of osteoblast progenitors but not of differentiated osteoblasts. This is consistent with the elevated numbers of osteoblasts and osteocytes in transgenic mice with increased TGF-β expression in differentiating osteoblasts (Erlebacher and Derynck 1996). TGF-β induces degradation of the Cdk inhibitor p57Kip2 to stimulate osteoprogenitor proliferation (Urano et al. 1999). Additionally, the inhibition of TGF-β-induced osteoblast proliferation by prostaglandin synthetase inhibitors (Ghayor et al. 2005) suggests the involvement of a prostaglandin-dependent mechanism. TGF-β is also a chemotactic for osteoprogenitor cells (Pfeilschifter et al. 1990), which explains the high number of such cells at sites of TGF-β injection or at fractures where active TGF-β is released from bone and platelets.

The effects of TGF-β on osteoblast differentiation depend on the cell source. TGF-β induces alkaline phosphatase expression, an early osteoblast marker, in bone marrow stromal cells and C2C12 myoblasts,
but it inhibits differentiation of primary osteoblasts and cell lines that differentiate into osteoblasts in culture. When inhibiting differentiation, TGF-β inhibits the expression of both early and late osteoblast differentiation markers, including alkaline phosphatase, Runx2, and osteocalcin, as well as matrix mineralization (Centrella et al. 1994; Alliston and Derynck 2000; Alliston et al. 2001; Maeda et al. 2004).

TGF-β also regulates osteocyte apoptosis. Smad3−/− deficiency confers an increase in osteocyte apoptosis, which may contribute to the reduced bone formation and osteopenic phenotype of Smad3−/− mice (Borton et al. 2001). A similar bone phenotype in mice that lack caspase 3, a critical protease in the apoptotic pathway, suggests a linkage to changes in TGF-β signaling (Miura et al. 2004). The osteoblasts of these mice show increased TGF-β type I receptor (TβRI) and Smad2 expression, reduced Runx2 levels, and increased levels of cell cycle inhibitors such as p21Cip1, all of which suggest increased TGF-β signaling. The reduced proliferation and differentiation of osteoblasts result in reduced bone matrix production and decreased osteoclast differentiation due to low RANK ligand levels (Miura et al. 2004).

The regulation of osteoblast proliferation, differentiation, and apoptosis by TGF-β illustrates the critical role of TGF-β as a pacesetter for the progression through the osteoblast life cycle. Insufficient or increased TGF-β signaling can alter the pace of osteoblast differentiation, resulting in defective control of bone mass. Because osteoclast differentiation is tightly linked to the control of osteoblast differentiation by TGF-β (see Chapter 22), the effects of TGF-β on bone mass may additionally result from altered bone resorption. For example, mice that overexpress TGF-β in bone exhibit increased osteoclast activity secondary to increased osteoblast proliferation, resulting in an osteoporotic phenotype (Erlebacher and Derynck 1996). TGF-β is indeed critical for the homeostatic balance between osteoclast and osteoblast activity to maintain normal bone mass.

Bone mass, architecture, and matrix quality are critical determinants of the structural role of bone and its resistance to fracture. In addition to controlling bone mass, TGF-β signaling also regulates the mechanical properties and composition of the bone matrix. The bone mass, which depends on the actions of TGF-β on many cell types, does not correlate strictly with the level of TGF-β signaling. In contrast, the quality of the bone matrix correlates with the level of TGF-β signaling in osteoblasts. Thus, elevated TGF-β signaling leads to production of bone matrix with inferior material properties, whereas reduced TGF-β signaling increases the material quality of bone matrix, independently of changes in bone mass and architecture (Balooch et al. 2005).
How TGF-β controls the mechanical properties and composition of bone matrix remains unknown. This may relate to the regulation by TGF-β of the expression of several bone matrix proteins, including osteocalcin, alkaline phosphatase, collagen I, osteonectin, and osteopontin (Centrella et al. 1988; Noda et al. 1988; Wrana et al. 1988; Noda 1989; Tschan et al. 1993). Each of these may influence the deposition and organization of the protein matrix and the mineral in bone. Furthermore, TGF-β stimulates the expression of a lysyl oxidase that posttranslationally modifies collagen I (Feres-Filho et al. 1995) and induces the expression of Ank, a transmembrane protein that is involved in pyrophosphate transport (Sohn et al. 2002). Mutations in Ank have been associated with bone mineralization defects in humans. Several studies have implicated TGF-β in the etiology of osteoporosis, but the results have been inconclusive (Langdahl et al. 1997; Yamada et al. 1998, 2000; Langdahl et al. 2003). Some mutations in the TGFB1 gene correlate with fracture incidence in individuals with normal or low bone mass but not in those with elevated bone mass. This may reflect the ability of TGF-β to control bone mass independently of bone matrix quality.

Transcriptional Control of Osteoblast Differentiation by TGF-β

An important aspect of the regulation of osteoblast differentiation by TGF-β centers on its effects on Runx2. In addition to regulating genes associated with osteoblast differentiation, Runx2 induces the expression of the TβRI receptor (Ji et al. 1998), and this induction is modulated by other signaling effectors that are important in bone remodeling, including glucocorticoids and estrogen (Chang et al. 1998; McCarthy et al. 2003). Because TGF-β activates Smad-dependent and non-Smad pathways that differentially affect Runx2 function, Runx2 and TβRI act in a regulatory feedback loop to control the progression of osteoblast differentiation.

TGF-β-activated Smads can interact with Runx proteins to enhance transcription at target promoters, including the IgCα promoter (Hanai et al. 1999; Pardali et al. 2000; Zhang and Derynck 2000). In contrast, TGF-β-activated Smad3 binds and represses the transactivation function of Runx2 at the Runx2 and osteocalcin promoters. Because Runx2 can activate transcription of its own promoter, Smad3 inhibits the expression of Runx2 and other Runx2 target genes. This repression occurs by recruiting HDAC4 and HDAC5, which function as transcriptional corepressors, to the Smad3-Runx2 complex, resulting in inhibition of osteoblast differentiation by autocrine or exogenous TGF-β. Conversely, reduced Smad3 or HDAC4/5 activities decrease or prevent the inhibition of osteoblast differentiation by TGF-β (Fig. 3) (Kang et al. 2005).
This mechanism of repression of Runx2 by the TGF-β-regulated Smad3–HDAC complex is operative in vivo. Indeed, mice that overexpress TGF-β in osteoblasts exhibit a cleidocranial dysplasia phenotype, similar to that observed in Runx2<sup>+/−</sup> mice (Erlebacher and Derynck 1996; Komori et al. 1997; Otto et al. 1997). In addition, osteoblasts and chondrocytes from Smad3<sup>−/−</sup> mice are unable to slow the progression of terminal differentiation. This defect results in premature chondrocyte hypertrophy and growth plate mineralization and consequent chondrodysplasia (Borton et al. 2001; Yang et al. 2001). A similar phenotype is observed in mice deficient in HDAC4, which exhibit chondrodysplasia as well, due to failure to delay terminal differentiation (Vega et al. 2004). In bone, Smad3 deficiency causes premature osteocyte apoptosis, resulting in osteopenia (Borton et al. 2001). A similar osteopenic phenotype is observed in mice that overexpress Runx2 in differentiated osteoblasts (Geoffroy et al. 2002).

Additional interactions of Smads with Runx proteins may involve relocalization of both proteins to the same intranuclear foci through association of Runx2 with a nuclear matrix (Zaidi et al. 2002). Furthermore, TGF-β can induce p300-dependent acetylation of Runx proteins that prevents association of Runx with Smurf ubiquitin ligases and Runx
degradation (Jin et al. 2004). Loss of Smad3 in dermal fibroblasts causes these cells to undergo osteogenic differentiation, due to loss of repression of Runx2 by Smad3 and reduced expression of Msx2, a negative regulator of Runx2 function (Hjelmeland et al. 2005).

The Erk and p38 MAP kinases, which are activated by growth factor and inflammatory pathways, as well as by TGF-β (see Chapter 14), phosphorylate Runx2 and enhance its transactivation function. Changes in the relative levels of activation of Smad signaling versus Erk MAP kinase may critically affect the activity of Runx2. Phosphorylation of Runx2 by Erk MAP kinase is required for the induction of matrix metalloprotease-13 expression by TGF-β (Selvamurugan et al. 2004). TGF-β signaling and Erk MAP kinase signaling also intersect through their ability to activate AP-1 transcription complexes, such as c-Jun/c-Fos, that in turn bind and function as coactivators of Runx2 (Selvamurugan et al. 2002). Combined BMP and TGF-β signaling may further affect the balance of activation versus repression of Runx2 (K.S. Lee et al. 2002), because BMPs activate not only different Smads, but also MAP kinase signaling. Inhibition of TGF-β signaling with a TβRI inhibitor greatly enhances BMP-induced osteoblast differentiation of C2C12 myoblasts (Maeda et al. 2004). The delicate balance of Smad and non-Smad signaling may explain the ability of TGF-β to promote some aspects of osteoblast differentiation while inhibiting others. These subtleties of regulation may underlie the reported differences in the ability of TGF-β to regulate Runx2 expression.

For example, TGF-β represses Runx2 expression in osteoblasts but induces it in C2C12 myoblasts (Lee et al. 2000; Alliston et al. 2001). However, BMP can activate Runx2 expression in both cell types.

Finally, TGF-β also induces the expression of the transcription factor TIEG/TIEG1 (TGF-β-inducible early gene 1), which recruits HDACs to repress transcription of target promoters. Mice that are defective in TIEG1 expression have increased numbers of osteoblasts but reduced expression of differentiation markers (Subramaniam et al. 2005).

Role of Activins in Osteogenic Differentiation

Activins have also been implicated in osteoblast differentiation and bone remodeling. Activin A is abundant in bone matrix (Ogawa et al. 1992) and is secreted by osteoblasts during bone matrix deposition and released during bone resorption, as shown in calvarial organ cultures (Sakai et al. 2000a). Periosteal injection of activin A in newborn rats increases the thickness of the periosteum and the bone matrix layers, and local application of activin A in a fracture model promotes endochondral ossification, resulting in increased callus mass and mechanical strength of the
healing bone (Sakai et al. 1999, 2000b). Activin synergizes with BMP to induce bone formation when implanted subcutaneously in a synthetic matrix (Ogawa et al. 1992) and stimulates osteoblast differentiation of bone marrow stromal cells when BMP signaling is antagonized by noggin. Activin A can stimulate collagen synthesis and proliferation in osteoblast-enriched cultures from fetal parietal bone. In contrast, activin A inhibits osteoblast differentiation in calvarial osteoblast cultures (Hashimoto et al. 1992; Ikenoue et al. 1999). Thus, as with TGF-β, the physiological effects of activin on osteoblast differentiation may depend on the type of osteoprogenitors and the local microenvironment.

In bone marrow stromal cell cultures, inhibin impairs the differentiation of mesenchymal stem cells along the osteogenic lineage and matrix mineralization (Gaddy-Kurten et al. 2002). Excess activin or BMP-2 fails to overcome the suppression of osteoblast differentiation by inhibin, suggesting that inhibin may regulate osteoblast differentiation via other pathways. In this system, follistatin, which blocks activin signaling, interferes only with the later stage of differentiation, coinciding with activin A secretion by osteoblasts (Gaddy-Kurten et al. 2002). In bone formation after implantation of demineralized bone matrix, follistatin expression is down-regulated during the conversion from cartilage to bone, and injection of follistatin near the implant delays endochondral ossification (Funaba et al. 1996). This down-regulation may enable activin to stimulate the progression from chondrogenesis to osteogenesis. Follistatin not only counteracts the bone-inducing activities of activin but also antagonizes the induction of bone nodule formation in osteoblasts by BMP-2 (Hashimoto et al. 1992; Funaba et al. 1996; Abe et al. 2004).

TGF-β FAMILY SIGNALING IN CHONDROGENIC DIFFERENTIATION

Studying the regulation of chondrocyte differentiation by TGF-β family members has been hampered by problems associated with recapitulating in cell culture the in vivo differentiation cascade of chondrocytes. Nevertheless, organ cultures and extensive mouse model studies have brought insight to the roles of TGF-β family signaling in chondrocyte differentiation. BMPs, GDFs, TGF-βs, and activins have critical roles in chondrogenic differentiation and maturation.

Regulation of Chondrocyte Differentiation by BMP Signaling

BMP signaling is important at each stage of chondrogenesis from commitment to the chondrogenic lineage to inhibition of apoptosis in termi-
nally differentiated hypertrophic chondrocytes. Each BMP is expressed in a defined spatiotemporal pattern in the precartilaginous mesenchyme and perichondrium and/or throughout the growth plate. Although several mice with mutations in the BMP pathway have cartilage phenotypes, the roles for the individual BMPs, either directly in chondrocyte differentiation or indirectly in skeletal patterning, have been difficult to define. Inactivation of both \( Bmp2 \) and \( Bmp4 \) in limb bud mesenchyme reveals that a threshold of BMP signaling is required for chondrogenesis. However, several aspects of chondrocyte differentiation occur normally in these mice, in contrast to the profound disruption of osteoblast differentiation (Bandyopadhyay et al. 2006). A more severe cartilage phenotype was observed upon inactivation of both the \( Bmpr1a \) or \( Bmpr1b \) genes, which encode the BMP type I receptors specifically in cartilage. These mice exhibited chondrodysplasia, illustrating a direct effect of BMP signaling in cartilage (Yoon et al. 2005). The dysplasia results from growth plate disorganization, reduced chondrocyte proliferation, and increased chondrocyte apoptosis. The cartilage in mice with deletion of only one receptor is normal, suggesting functional compensation between the two type I BMP receptors (Yoon et al. 2005). Targeted deletion of ALK-2, a type I receptor for BMPs, in neural crest cells of mice results in multiple craniofacial defects, including mandibular hypoplasia with delayed growth of Meckel’s cartilage and cleft palate (Dudas et al. 2004). These and other mice with cartilage malformations due to mutations in the BMP pathway illustrate the importance and redundancy of BMP signaling from chondrocyte commitment through terminal differentiation.

BMPs cooperate with Shh signaling to potentiate the commitment of presomitic mesenchymal cells to a chondrogenic lineage (Murtaugh et al. 1999, 2001; Hatakeyama et al. 2004). In the absence of Shh, BMPs induce these cells to form lateral plate mesenchyme (Murtaugh et al. 1999). Thus, Shh installs a state of competence in presomitic mesenchymal cells to undergo BMP-induced chondrogenesis by inducing the expression of the homeobox transcription factor Nkx3.2. Nkx3.2 is required for BMPs to activate the expression of the chondrogenic transcription factor Sox9 (Zeng et al. 2002), which promotes the selection of the chondrogenic lineage and represses the expression of the osteoblast transcription factor Runx2 (Lengner et al. 2005).

Once cells are committed to the chondrogenic lineage, BMPs stimulate the progression of chondrocyte maturation in part through the expression of Sox9. Accordingly, BMPs stimulate proliferation, matrix production, and cell maturation in metatarsal organ cultures and chondrocyte cultures (Hatakeyama et al. 2004). Sox9 antisense RNA blunts BMP-induced expression of types II and X collagen, resulting in inhibition of both matrix pro-
duction and chondrocyte differentiation. BMP-4 and GDF-5 also induce the expression of another transcription factor, Barx2, which cooperates with Sox9 to promote chondrogenesis (Meech et al. 2005).

By regulating the expression of chondrocyte transcription factors and their target genes, BMPs control the size and organization of the growth plate. BMPs stimulate chondrocyte proliferation and differentiation and inhibit hypertrophic chondrocyte apoptosis, thus increasing the length of the proliferative and hypertrophic zones (Minina et al. 2001, 2002; Tsumaki et al. 2002). In contrast, secreted BMP-binding proteins such as noggin and twisted gastrulation (Tsg) antagonize these activities and restrict BMP activity to refine growth plate size and structure (Minina et al. 2001; Tsumaki et al. 2002; Schmidl et al. 2006) (see Chapter 22).

Other signaling pathways intersect with BMP signaling to control chondrogenesis. As in limb bud development (see Chapter 22), BMP and fibroblast growth factor (FGF) antagonize each other in chondrocyte proliferation, in part through antagonistic regulation of Indian hedgehog (Ihh) expression. BMPs induce Ihh expression, whereas FGF inhibits it. Ihh also induces BMP expression, forming a positive feedback loop to promote the proliferation of chondrocyte precursors and to inhibit terminal differentiation (Minina et al. 2002). Many, but not all, of the functions ascribed to Ihh depend on BMP signaling (Krishnan et al. 2001; Murtaugh et al. 2001; Zeng et al. 2002).

Role of GDFs in Chondrogenesis and Joint Formation

GDFs have also been implicated in chondrocyte differentiation and cartilage development, yet they have revealed themselves to be regulators of joint specification and formation. Cell culture experiments demonstrate that GDF-5 promotes cartilage formation in micromass cultures of mouse embryonic limb bud mesenchyme by enhancing chondroprogenitor aggregation (Hatakeyama et al. 2004). In ATDC5 cells, GDF-5 also promotes initiation of chondrogenesis and signals through the BMPRIB/ALK-6 that involves activation of the p38 and Erk MAP kinase pathways. Sustained activation of p38 MAP kinase activity is required for GDF-5-induced cartilage nodule formation and collagen type II expression, but not for induction of alkaline phosphatase activity, a determinant of terminal chondrogenic differentiation (Nakamura et al. 1999).

Consistent with its effects on chondrocyte differentiation, Gdf5 expression has been localized in 12.5-day-old mouse embryos to precartilaginous mesenchymal condensations and the perichondrium of proximal skeletal structures in the limb. At later stages, however, Gdf5 is
expressed at sites of joint formation and particularly in interzone regions (Storm et al. 1994; Storm and Kingsley 1996; Wolfman et al. 1997; Merino et al. 1999b; Buxton et al. 2001), where articular cartilage, synovial membranes, tendons, and ligaments are formed following segmentation. GDF-5 is one of the earliest known markers of joint formation during embryonic development. GDF-6 and GDF-7 are also expressed in embryonic skeletal condensations, but their expression is restricted to different subsets of developing joints (Wolfman et al. 1997; Settle et al. 2003).

Gdf5–/– mice have impaired development of a subset of bones and joints, whereas most sites in the skeleton are unaffected (Storm and Kingsley 1996). Gdf5–/–;Bmp5–/– mice display more severe skeletal defects than either single knockout and reveal a role for GDF-5 in the formation and segmentation of the sternum (Storm and Kingsley 1996). Gdf6–/– mice show fusion of bones in wrists and ankles due to a failure of the larger skeletal condensations to subdivide into individual skeletal elements and a loss of the coronal suture of the skull. In addition, the middle ear bones of Gdf6–/– mice are undersized, have altered shapes, and are incompletely attached due to reduced cell proliferation along the articular surfaces (Settle et al. 2003). Gdf5–/–;Gdf6–/– mice lack more bones and joints than observed in any of the single knockouts. Reduced size and incomplete ossification of some bones, including the ulna, result in bowing of the long bones. With incomplete penetrance, these mice have alterations in the cartilage composition of intervertebral joints, which despite the normal vertebrae, result in spinal curvature that resembles human idiopathic scoliosis (Settle et al. 2003). Although GDF-7 is also expressed in joint interzones, Gdf7–/– mice lack apparent skeletal abnormalities (Lee et al. 1998; Settle et al. 2001). The analyses of these and other knockout mice identify GDF-5, -6, and -7 as a TGF-β family subclass involved in establishing the boundaries between skeletal elements, thereby controlling both cartilage formation and joint development.

Ectopic expression of Gdf5 indicates that GDF-5 is not sufficient to initiate joint formation and induce joint-specific marker gene expression. Rather, ectopic Gdf5 expression in developing chicken limbs (Francis-West et al. 1999; Buxton et al. 2001), implanting GDF-5 protein on beads in chicken limbs (Merino et al. 1999b; Storm and Kingsley 1999), and targeted GDF-5 expression from the Col11a2 promoter in transgenic mice (Tsumaki et al. 1999, 2002) show that Gdf5 stimulates progression of chondrogenesis by promoting expansion of mesenchymal condensations due to increased cell adhesion and proliferation, increased recruitment of mesenchymal cells, and at later stages, increased chondrocyte proliferation and cartilage formation. Moreover, overexpression of Gdf5 restricts
expression of joint markers. The resulting cartilage overgrowth obliterates proper joint formation and thus causes fusion of adjacent joints (Francis-West et al. 1999; Merino et al. 1999b; Storm and Kingsley 1999).

It has been suggested that GDF-5 may only control cartilage development and that defects in joint formation as observed in Gdf5<sup>−/−</sup> brachypodism mice result indirectly from impaired cartilage formation or reduced limb mobility (Francis-West et al. 1999; Merino et al. 1999b). Others proposed that GDF-5 primarily stimulates mesenchymal condensation and cartilage formation and restricts joint formation to proper locations and, at later stages, promotes segmentation of skeletal precursors (Storm and Kingsley 1999; Baur et al. 2000). The latter scenario is supported by the Gdf6<sup>−/−</sup> and Gdf5<sup>−/−</sup>;Gdf6<sup>−/−</sup> phenotypes (Settle et al. 2003). The loss of the coronal suture in Gdf6<sup>−/−</sup> mice supports the notion that GDFs have a direct role in joint formation, since the coronal suture forms in the absence of a cartilage intermediate (Settle et al. 2003).

GDF-5 and -6 signal preferentially through a BMPRII receptor complex with BMPRIIB and have only weak affinity for BMPRIA/ALK-3 (Nishitoh et al. 1996; Erlacher et al. 1998; Nickel et al. 2005). Bmpr1b is expressed in early cartilage condensation and later in the epiphyseal chondrocytes adjacent to the joint (Kawakami et al. 1996; Zou et al. 1997; Yi et al. 2000). Expression of a constitutively activated BMPRIIB in the chick limb bud causes extensive mesenchymal condensation followed by expansion of the primordial cartilage, resulting in a phenotype similar to that seen upon overexpression of Gdf5, strongly suggesting a prominent role of GDF-5 and/or GDF-6 in defining the cartilage anlagen (Zou et al. 1997). Conversely, deletion of Bmpr1b in mice results in an appendicular skeleton phenotype (Baur et al. 2000; Yi et al. 2000) that is very similar to that observed for loss of Gdf5 expression. Initial condensations form but they lack proper cell adhesion and fail to form digits. In addition, prechondrogenic proliferation and chondrogenic differentiation are impaired. Expression of GDF-5, as well as of the zinc-finger transcription factors Gli2 and Gli3, molecular markers of joint formation, are no longer restricted to the joint interzones in the developing limbs in Bmpr1b<sup>−/−</sup> mice. Their expression is expanded and prolonged and correlates with the region that undergoes excessive apoptosis, intrinsic to digit segmentation and joint formation (Baur et al. 2000; Yi et al. 2000). Unlike Gdf5<sup>−/−</sup> mice, Bmpr1b<sup>−/−</sup> mice do not show reduced length of the skeletal elements, suggesting that GDF-5 might control skeletal growth through additional type I receptors such as BMPRIA or ALK-2/ActRI. The limb phenotype of Bmpr1b<sup>−/−</sup>;Gdf5<sup>−/−</sup> mice highly resembles the single-knockout phenotypes, consistent with the notion that GDF-5 largely signals through BMPRIIB in limb development. The synergistic malfor-
mations in the carpal and tarsal bones of these mice suggest that GDF-5 and BMPRIB act through complementary pathways in the regulation of digital arch segmentation (Baur et al. 2000; Yi et al. 2000).

In addition to the GDFs and their receptors, the BMP inhibitor noggin is expressed during, and is involved in, joint specification. Noggin interacts with GDF-5 and inhibits the effects of coimplanted GDF-5 on chick autopod digit development in vivo (Merino et al. 1999b). Noggin–/– mice show various skeletal abnormalities with a striking absence of joints, correlating with loss of Gdf5 expression in prospective joint regions. Initial segmentation of limb cartilage condensations occurs normally in Noggin–/– mice but is followed by increased recruitment of precursor cells, resulting in cartilage overgrowth, likely due to unopposed BMP activity (Brunet et al. 1998). Ectopic expression of BMP-2, -4, or -7 in chick limb buds suppresses Gdf5 expression and results in limb phenotypes similar to that observed for Noggin–/– mice (Macias et al. 1997; Merino et al. 1999b). Noggin is, however, insufficient for the induction of joint formation because increased Noggin expression only inhibits cartilage development and does not affect joint formation (Capdevila and Johnson 1998; Pathi et al. 1999; Pizette and Niswander 2000). In humans, mutations in Noggin are also associated with disorders that affect joint formation, including proximal symphalangism and multiple synostosis syndrome (Gong et al. 1999).

ERG, a member of the ets gene family of transcription factors, is expressed in developing synovial joints and articular cartilage in a spatiotemporal pattern very similar to that of GDF-5. Transgenic expression of ERG under control of the Col2a1 promoter in mouse embryos results in largely cartilaginous skeletons. ERG prevents chondrocyte maturation and hypertrophy and instead favors formation of joint- and articular-like cartilage (Iwamoto et al. 2007). GDF-5 rapidly and effectively induces ERG expression in mouse autopods and chick embryo explants, and ERG may be a downstream mediator of GDF-5-regulated joint formation (Iwamoto et al. 2000, 2007).

In the segmentation of early skeletal elements, GDF signaling is complemented by the responses to Wnt14 (Hartmann and Tabin 2001; Guo et al. 2004). Wnt14, Wnt4, and Wnt16 are expressed in developing interzones before cartilage segmentation. Ectopic expression of Wnt14 or an activated β-catenin in early differentiating chondrocytes enhances expression of Gdf5, reverses chondrocyte differentiation, and induces ectopic joint formation in the mouse limbs. Loss of β-catenin expression in mesenchymal condensations or early differentiating chondrocytes results in joint fusion and correlates with strongly impaired Gdf5 expression. Thus, in contrast to the activities of GDFs and noggin, the Wnt-β-catenin signaling pathway is necessary and sufficient to induce
early steps of synovial joint formation and may do so through cross-talk with GDF signaling pathways.

Regulation of Chondrocyte Differentiation by TGF-β

TGF-β is a potent regulator of chondrogenesis both in vitro and in vivo, as apparent from its initial identification as a “cartilage-inducing factor” (Seyedin et al. 1986, 1987). Mesenchymal stem cells, perichondrial cells, and chondrocytes express and respond to TGF-β, and cartilage matrix contains low levels of plasmin that can activate latent TGF-β stored in cartilage matrix. Elevated plasmin levels following injury promote chondrogenic proliferation and wound repair by releasing high levels of active TGF-β. Injection of TGF-β under the periosteum, or at sites of cartilage wounds or tibial fractures, results in increased chondrocyte proliferation, differentiation, and formation of cartilage (Joyce et al. 1990; Beck et al. 1991; Critchlow et al. 1995; Pedrozo et al. 1999). Conversely, antisense TGF-β3 oligonucleotides inhibit chondrogenesis in Meckel’s cartilage explant cultures (Chai et al. 1994). The induction of chondrogenesis in vivo by TGF-β results from its ability to induce chondrocyte commitment, recruitment, proliferation, and differentiation.

TGF-β can induce commitment of mesenchymal cells to the chondrogenic lineage (Seyedin et al. 1985; Kulyk et al. 1989; Denker et al. 1995). One of the first steps in chondrogenic differentiation in vivo is mesenchymal condensation, which requires cell contact with extracellular matrix and other cells. In cell culture, the cell–cell contact required for chondrogenic differentiation can be provided by culturing cells in micromass conditions. TGF-β can compensate for the absence of normal cell or matrix contacts to stimulate chondrogenic differentiation of subconfluent cultures. Furthermore, in one system, TGF-β does not promote chondrogenic commitment when cells are cultured on plastic or collagen I, but it has full chondrogenic activity in Matrigel. These effects may be due in part to the effect of TGF-β on N-cadherin expression. N-cadherin is required for the cell contacts that drive mesenchymal condensation and chondrogenic commitment. TGF-β activates Wnt7a expression in bone marrow mesenchymal cells, and Wnt7a signaling stabilizes β-catenin protein, a potent activator of N-cadherin expression (Tuli et al. 2003). In this way, TGF-β may potentiate the formation of cell–cell contacts required for commitment of mesenchymal cells to the chondrogenic lineage.

The effect of TGF-β on chondrocyte proliferation depends on the experimental system. At early stages, TGF-β promotes chondrocyte proliferation and matrix production in a Smad3-dependent manner (Alvarez and Serra 2004). In the growth plate of metatarsal bone cultures, how-
ever, TGF-β inhibits chondrocyte proliferation. As with other effects of TGF-β on the growth plate, TGF-β inhibits chondrocyte proliferation indirectly through the perichondrium. A reduction in Smad3 activity in the perichondrium prevents the antiproliferative effects of TGF-β in growth plate chondrocytes (Alvarez and Serra 2004).

Activation of chondrocyte differentiation and extracellular matrix protein expression by TGF-β requires both Smad and non-Smad signaling. TGF-β-activated Smad3 binds Sox9 and activates its transcriptional activity. The Sox9-Smad3 complex recruits CBP/p300 to the Col2A1 promoter to activate collagen II expression (Furumatsu et al. 2005). TGF-β induction of collagen II expression also requires TAK1, a member of the MAPKKK family that is activated by TGF-β (Qiao et al. 2005). In undifferentiated ATDC5 chondroprogenitor cells, TGF-β-induced aggrecan expression requires Smad2 and p38 and Erk MAP kinase signaling. The relative contribution of p38 and Erk MAP kinase signaling increases with progression of chondrogenic differentiation. At later stages, inhibition of these non-Smad pathways confers a greater decrease in TGF-β-induced aggrecan expression (Watanabe et al. 2001). Thus, the balance of these signaling pathways and their roles in gene expression may vary with differentiation.

Whereas TGF-β promotes the early events in chondrogenesis, it inhibits hypertrophic chondrocyte differentiation and “stabilizes” the pre-hypertrophic chondrocyte phenotype (Kato et al. 1988; Carrington and Reddi 1990; Ballock et al. 1993; Dieudonné et al. 1994). Several studies have shown that loss of TGF-β signaling results in advancement of hypertrophic differentiation and mineralization, a reduction of proteoglycan synthesis, and degradation of the articular cartilage surface (Carrington and Reddi 1990; Serra et al. 1997; Borton et al. 2001; Yang et al. 2001). In mice, overexpression of a dominant-negative version of the TGF-β type II receptor (TβRII) or deletion of Smad3 both result in premature chondrocyte hypertrophy and growth plate mineralization. The loss of cartilage integrity results in an osteoarthritis-like phenotype (Serra et al. 1997; Yang et al. 2001; Vega et al. 2004). Thus, the stabilization of chondrocyte differentiation by TGF-β is essential for normal cartilage and growth plate development and maintenance of healthy permanent articular cartilages.

The inhibition of terminal chondrocyte differentiation by TGF-β relies on the intersection with other signaling pathways in the perichondrium. Ihh/Shh, TGF-β, and PTH-related protein (PTHrP) inhibit terminal chondrocyte differentiation through connected pathways. Ihh and Shh stimulate the expression of TGF-β2 by the perichondrium. The action of TGF-β on the perichondrium, in turn, induces PTHrP expression, and PTHrP then acts on growth plate chondrocytes to inhibit hypertrophic differentiation and mineralization. TGF-β is required for Shh/Ihh regu-
lation of PTHrP expression (Serra et al. 1999; Alvarez et al. 2002). Whereas PTHrP is a critical effector of TGF-β action on chondrocytes, TGF-β also inhibits terminal differentiation through PTHrP-independent pathways. The activities of TGF-β are also linked to those of FGF. TGF-β induces the expression of FGF-18 by the perichondrium, and TGF-β and FGF synergize to inhibit differentiation. Accordingly, inhibition of FGF signaling prevents the inhibition of chondrocyte hypertrophy by TGF-β (Mukherjee et al. 2005). However, FGF can inhibit chondrocyte hypertrophy when the perichondrium is removed, even though TGF-β cannot, suggesting that FGF is a downstream target of TGF-β that acts directly on chondrocytes.

The balance between TGF-β and BMP signaling is an important determinant of chondrogenesis. TGF-β family ligands are stored in cartilage matrix bound to proteoglycans. Chondroitin-4-sulfotransferase 1 (C4st1) is one of the enzymes required for proteoglycan production. Inactivation of the gene for C4st1 in mice results in severe dwarfism and other skeletal defects that occur due to impaired chondrocyte proliferation and apoptosis and abnormal growth plate organization (Klüppel et al. 2005). Impaired proteoglycan production alters the normal balance of BMP and TGF-β signaling, such that mutants have decreased BMP and increased TGF-β signaling in growth plates. Some aspects of this phenotype resemble those in mice with a conditional deletion of Smad4 in chondrocytes, which also exhibit dwarfism and impaired growth plate organization due to deficits in both TGF-β and BMP function (Zhang et al. 2005).

The role of TGF-β in cartilage degeneration seen in osteoarthritis remains unclear. Although injection of TGF-β into osteoarthritic joints increases proteoglycan synthesis and cartilage repair (Glansbeek et al. 1998), other studies suggest that osteoarthritic chondrocytes have increased TGF-β responsiveness that contributes to the progression of the disease. Further research is needed to understand the role of TGF-β in normal joint maintenance and its disruption in osteoarthritis (see also Chapter 22).

Role of Activins in Chondrogenic Differentiation

Activins also have a role in cartilage development. Exogenous activin A enhances chondrogenesis in limb bud micromass cultures by increasing the size of precartilaginous condensations and cartilaginous nodules, resulting from increased synthesis of neural cell adhesion molecule (NCAM) and tenascin, which are involved in mesenchymal cell adhesion (Jiang et al. 1993). Activin A was also reported to inhibit differentiation using a similar model system (Chen et al. 1993). Injection of activin A
near demineralized bone matrix increases cartilage formation. Furthermore, implanted activin beads induce ectopic chondrogenesis and digit formation in the interdigital limb mesenchyme. This induction is preceded by increased Bmpr1b expression, resulting in activation of BMPRIB by local BMPs, which initiates chondrogenesis and induces activin βA (inhibin βA subunit) and TGF-β2 expression, thereby ensuring outgrowth of the digits. Implantation of beads with follistatin, which neutralizes activins, into the tips of growing digits when activin βA is expressed blocks chondrogenesis and subsequent digit formation (Merino et al. 1999a). Ectopic digits formed by implantation of TGF-β1 beads lacked joints when follistatin beads are coimplanted (Merino et al. 1999a). These observations strongly support roles for follistatin and activin in chondrogenesis, digit formation, and joint development.

Gene inactivations in mice have also revealed important roles for activin signaling in craniofacial development. Activin βA-deficient (or Inhba−/−) mice show craniofacial defects including abnormalities in their secondary palates, cleft palate, and lack of lower incisors and mandibular molar teeth (Matzuk et al. 1995c; Ferguson et al. 1998). In addition, activin type II receptor (ActRII)-deficient mice display with low penetrance skeletal and facial defects that include mandibular hypoplasia and defects in Meckel’s cartilage, in addition to those seen in Inhba−/− mice (Matzuk et al. 1995a). Follistatin-null mice display skeletal abnormalities including defects in the ribs and reduced number of lumbar vertebrae, in addition to delayed development of lower incisors and palate defects that are also seen in activin knockouts (Matzuk et al. 1995b). Activin A was also able to induce craniofacial cartilage in cultures of undifferentiated presumptive Xenopus laevis ectoderm, as revealed by goosecoid expression that is restricted to cartilage of the lower jaw in vivo (Furue et al. 2002).

**TGF-β FAMILY SIGNALING IN MYOBLAST DIFFERENTIATION**

Skeletal muscle cells differentiate from uncommitted mesenchymal cells through a complex set of differentiation steps that involves commitment to the myoblast lineage, progression of differentiation with expression of muscle-cell-specific proteins, and fusion of myoblasts into multinucleated myocytes or myofibers. Essential for the terminal differentiation into myocytes is the withdrawal from the cell cycle with concomitant growth arrest. Myoblast differentiation is driven by parallel and sequential activities of myogenic bHLH transcription factors, that is, MyoD, myogenin, MRF4, and Myf5, which function as heterodimers with a distinct class of ubiquitously expressed bHLH proteins in the E-protein family. The MRF-E protein heterodimers then bind to a conserved DNA sequence, “CANNTG,”
also known as the E box, which is located in regulatory regions of many muscle-specific genes (Murre et al. 1989; Davis et al. 1990; Lassar et al. 1991). MyoD and Myf5 are expressed in proliferating myoblast cells during embryonic development or in culture systems well before activation of myogenic differentiation. Satellite cells that represent muscle stem cells or progenitors are interspersed with the myofibers and are mobilized to undergo myogenic differentiation into myofibers upon injury (Collins 2006). Similar to the other mesenchymal differentiation lineages, myogenesis is regulated by members of the TGF-β family. TGF-βs, myostatin/GDF-8, and BMPs act as potent inhibitors of the progression of myogenic differentiation, although TGF-β may also have a stimulatory role in the generation of myoblasts, presumably at early stages of differentiation.

### TGF-β and Myostatin in Skeletal Muscle Differentiation

TGF-β is a potent inhibitor of myoblast differentiation. Adding TGF-β to myoblast cultures, for example, C2C12 myoblasts, blocks the progression of differentiation and thus the expression of proteins known to be associated with myogenic differentiation as well as the formation of multinucleated myofibers (Olson et al. 1986). On the other hand, several observations reveal a stimulatory role for TGF-β in myoblast generation or early differentiation. Blocking TGF-β signaling by expressing a dominant-negative version of TβRII blocks myogenic differentiation (Filvaroff et al. 1994). In addition, inhibition of TGF-β activity inhibits myotome induction, whereas TGF-β together with FGF induces myotome formation (Stern et al. 1997). The expression of TGF-β within muscle tissues during early embryonic development is also consistent with its involvement in muscle pattern formation. These and other observations led to the suggestion that the high level of TGF-β in the first wave of myofiber formation inhibits the fusion of late but not early myoblasts and that the onset of secondary myofiber formation is triggered by a decrease in local TGF-β levels (Cusella-De Angelis et al. 1994). Currently, TGF-β is thought to promote the expansion and maintenance of the cells that give rise to muscle cells and to provide competence for progenitors to initiate myogenic differentiation (Filvaroff et al. 1994).

Myostatin/GDF-8 is expressed in the myotome layer during development and then is primarily expressed in muscle cells (see also Chapter 33). Targeted gene inactivation confers an increased muscularity in mice, associated with increased cell proliferation and muscle cell hypertrophy (McPherron and Lee 1997). In cattle, spontaneous mutations in the coding region of myostatin have been associated with increased muscle mass, designated as “double muscling” (McPherron et al. 1997), whereas a muta-
tion in the human GDF8 gene has also been shown to correlate with gross muscle hypertrophy in children (Schuelke et al. 2004). Consistent with these phenotypes, myostatin inhibits the proliferation and differentiation of myoblasts (Langley et al. 2002; McCroskery et al. 2003; Wagner et al. 2005). In addition, myoblasts and satellite cells, cultured from myostatin-null mice, proliferate and differentiate more rapidly, suggesting that the increased muscle mass in myostatin-null mice might result in part from the removal of autocrine inhibition of satellite cells by myostatin (McCroskery et al. 2003; Wagner et al. 2005). Thus, myostatin should be considered as an endogenous and autocrine inhibitor of muscle generation.

Complementary to the studies in which the myostatin gene was inactivated, overexpression and administration of myostatin in vivo supports the role of myostatin as an inhibitor of muscle cell differentiation and muscle tissue generation. Indeed, systemic release of myostatin results in muscle and fat loss, as is observed in cachexia (Zimmers et al. 2002). Furthermore, myostatin administration resulted in muscle loss concomitant with activation of genes involved in ubiquitin-mediated protein degradation, as observed in various forms of muscle wasting (McFarlane et al. 2006). Additional observations that myostatin levels increase as muscles undergo atrophy and in association with cachexia (Gonzalez-Cadavid et al. 1998; Carlson et al. 1999; Dasarathy et al. 2004) raise the possibility that myostatin may contribute to muscle wasting associated with different pathologies. Accordingly, inhibitors of myostatin have been considered for therapeutic applications for conditions that confer muscular degeneration and wasting. Indeed, increased muscle mass and reduced muscle wasting are apparent in model systems, in which inhibitors of myostatin are administered (Bogdanovich et al. 2002; Tobin and Celeste 2005).

The role of myostatin as an endogenous autocrine inhibitor of muscle cell differentiation has raised interest in how far inhibition of myostatin activity may promote muscle cell regeneration. The latter is particularly relevant because muscular dystrophies arise from increased muscle degeneration that is not sufficiently compensated for by the generation of new muscle from satellite cells, resulting in depletion of the satellite cell population. In mdx mice, a model for Duchenne’s muscular dystrophy, inhibition of endogenous myostatin activity by intraperitoneal injections of myostatin-blocking antibodies resulted in an increase in body weight, muscle mass, muscle size, and absolute muscle strength, along with a significant decrease in muscle degeneration (Bogdanovich et al. 2002). Furthermore, senescent myostatin-null mice have muscle with increased mass and strength relative to controls. In contrast to wild-type senescent mice, muscles of senescent myostatin-null mice regenerate robustly from both chronic and acute injury. Early markers of regeneration were
enhanced in the absence of myostatin, suggesting a mechanism by which myostatin inhibition may attenuate the dystrophic features found in mdx mice (Wagner et al. 2005). These and other observations strongly suggest that inhibition of myostatin might provide a therapeutic approach for muscular dystrophies. Accordingly, transgenic mdx mice that express a dominant-negative type II receptor for myostatin, that is, ActRIIB, as discussed below, have bigger muscles than their nontransgenic counterparts. Moreover, nondystrophic myoblasts expressing a dominant-negative ActRIIB formed more abundant and bigger dystrophin-positive fibers when transplanted in mdx mice (Benabdallah et al. 2005). Thus, various approaches to inhibit myostatin activity, for example, through administration of antibodies, soluble receptor ectodomain, or follistatin, which binds myostatin to prevent receptor activation, are being explored as possible therapies to enhance muscle regeneration and quality (Tobin and Celeste 2005).

The control of myostatin expression is a subject of ongoing research. One of the factors controlling myostatin expression is MyoD, which was shown to target the regulatory sequences of the myostatin gene to activate its expression (Spiller et al. 2002). Because myostatin inhibits the expression of MyoD (Langley et al. 2002), there is a clear feedback mechanism whereby MyoD, and most likely other myogenic bHLH transcription factors, regulates the expression of myostatin and vice versa. The myostatin gene also contains several glucocorticoid response elements, which explains the ability of dexamethasone to activate myostatin expression (Feldman et al. 2006). Dexamethasone activation of myostatin expression may explain some effects of dexamethasone in vivo. Dexamethasone administration in rats results in gradual loss of skeletal muscle mass, concomitant with increased myostatin expression (Salehian et al. 2006). In addition, myostatin-null mice are protected from glucocorticoid-induced muscle atrophy, when compared with wild-type mice (Gilson et al. 2007). Myostatin expression is also subject to autoregulation through Smad7. Thus, myostatin induces Smad7, which in turn inhibits myostatin expression. Consequently, myotubes from cattle with spontaneous inactivating mutations in the myostatin gene exhibit decreased Smad7 expression and increased expression of the nonfunctional myostatin (Forbes et al. 2006). Finally, the forkhead transcription factor FoxO1, which is expressed in skeletal muscle, also binds to the regulatory sequences of the myostatin gene and activates its expression. FoxO1 may enhance muscle atrophy, in part, by inducing myostatin expression (Allen and Unterman 2007).

Myostatin is expressed as a latent complex, whereby its long propeptide interacts with the mature dimer, preventing it from binding to its
receptor. Activation of this complex may involve members of the BMP-1/Tolloid family of metalloproteinases, because they can cleave the myostatin propeptide in this complex and thereby activate latent myostatin (Wolfman et al. 2003). Accordingly, administration of the myostatin propeptide with its presumed cleavage site mutated into mice results in increased muscle in mice, which is not seen with the native propeptide.

Myostatin exerts its biological activities by binding primarily to ActRIIB, but also to ActRII (Lee and McPherron 2001; Rebbapragada et al. 2003; S.J. Lee et al. 2005). Thus, transgenic expression of a dominant-negative version of ActRII results in a dramatic increase in muscle mass, reminiscent of the myostatin-null phenotype (Lee and McPherron 2001). In response to myostatin binding, these type II receptors function in partnership with ActRIB/ALK-4, the activin type I receptor, or TβRI, the major type I TGF-β receptor (Rebbapragada et al. 2003). Although this would predict that both Smad2 and Smad3 would be activated, only Smad3 was shown to be activated by myostatin in myoblasts (Langley et al. 2002). The mechanisms underlying the inhibition of myogenic differentiation by myostatin are therefore likely to be identical to the Smad3-mediated mechanisms exerted by TGF-β.

TGF-β-activated Smad3 inhibits myogenic differentiation through its association with myogenic transcription factors in response to TGF-β, with consequent interference with transcription complex formation at the regulatory DNA sequences (see Fig. 3). In response to TGF-β, Smad3 directly represses the transcriptional activity of myogenic bHLH transcription factors, such as MyoD and myogenin, which drive myogenic differentiation. Smad3 associates with the bHLH domain of MyoD (or other myogenic bHLH transcription factors) in response to TGF-β. Because this domain is required for association with E protein partners such as E12 and E47, the Smad-MyoD association interferes with the dimerization of MyoD with an E protein. Because the dimerization is required for efficient DNA binding of myogenic bHLH proteins, this interference by Smad3 prevents efficient DNA binding of MyoD, thus preventing transcriptional activation (Liu et al. 2001). Smad3 also represses the function of MEF2, another class of myogenic transcription factors that physically interacts with myogenic bHLH transcription factors and strongly enhances their activity through transcriptional cooperation (Black and Olson 1998). The physical interaction between Smad3 and MEF2 disrupts the association of MEF2 with GRIP1, a coactivator required for MEF2’s activity in myogenic differentiation. Consistent with this physical displacement, TGF-β signaling blocks the GRIP1-induced redistribution of MEF2 to discrete subnuclear loci and GRIP1 recruitment to the myogenin promoter (Liu et al. 2004).
BMPs as Inhibitors of Muscle Cell Differentiation

BMPs also inhibit myogenic differentiation, but through a mechanism that differs from TGF-β-induced inhibition of myogenesis. This is reflected in the differentiation response of the myoblasts to ligand; whereas BMPs concomitantly initiate osteoblast differentiation, TGF-β does not. A most critical difference at the molecular level is that BMPs directly activate expression of Id1, but TGF-β does not (Ogata et al. 1993; Katagiri et al. 1994; Nakashima et al. 2001). As discussed earlier, Id proteins promote early events in osteoblast differentiation. These proteins have an HLH domain, but they lack the basic domain that allows for DNA binding. Therefore, Id heterodimerization with E proteins interferes with the interactions of E proteins with bHLH proteins that are required for binding to DNA (Norton 2000). Consequently, BMP-induced Id1 expression in myoblasts (Katagiri et al. 1994) inhibits the activity of the myogenic bHLH transcription factors, additionally leading to their accelerated degradation (Vinals and Ventura 2004). Consistent with the direct transcriptional activation of Id1 upon BMP stimulation, the Id1 promoter contains Smad-binding sites capable of binding to BMP-activated Smad1/5 and Smad4 (Korchynskyi and ten Dijke 2002; Lopez-Rovira et al. 2002). The induction of Id protein expression by BMPs is not restricted to mesenchymal cells and also occurs in embryonic stem cells (Hollnagel et al. 1999) and neural progenitor cells (Nakashima et al. 2001). In the latter case, BMP-induced Id1 expression redirects the cells from neuronal to astroglial differentiation through inhibition of neurogenic HLH transcription factors (Nakashima et al. 2001), which mechanistically resembles BMP-induced conversion of myoblasts. These findings illustrate a mechanism whereby BMPs can redirect the lineage specification of cells that are already expressing differentiation-specific HLH factors. Consistent with these findings, the inhibitory Smad7, which inhibits Smad2/3 activation in response to myostatin or TGF-β, promotes skeletal muscle cell differentiation (Kollias et al. 2006).

TGF-β FAMILY SIGNALING IN ADIPOCYTE DIFFERENTIATION

Adipogenic differentiation of mesenchymal cells into fully differentiated fat cells or adipocytes that accumulate lipid is driven by the sequential and parallel activities of two types of transcription factors: the C/EBPs and PPARγ. C/EBPβ and δ, members of a larger family of transcription factors, are characterized by a basic/leucine zipper and activate the expression of PPARγ in preadipocytes. PPARγ is a nuclear receptor that functions through heterodimerization with another nuclear receptor RXR
and, in cooperation with C/EBPα, drives the expression of various proteins that characterize the differentiated adipocyte, including those involved in lipid accumulation (Otto and Lane 2005).

Compared to the differentiation of other mesenchymal lineages, much less is known about the regulation of adipogenic differentiation by TGF-β and BMP-related factors, and most of our knowledge is gained from cell culture experiments. BMPs, such as BMP-2, -4, and -7, affect the adipogenic differentiation of undifferentiated mesenchymal cells. Although some reports conclude that BMPs inhibit adipogenic differentiation, others provide evidence for stimulation by BMPs. For example, BMP-2 was shown to inhibit adipocyte differentiation of bone marrow stromal cell lines and 3T3-F442A preadipocytes (Gimble et al. 1995; Gori et al. 1999; Skillington et al. 2002), but BMP-2 and -7 conversely stimulate adipocyte differentiation of multipotential 10T1/2 cells and 3T3-L1 preadipocytes (Ahrens et al. 1993; Rebbapragada et al. 2003). This discrepancy may relate to differences in cell systems and/or culture conditions, for example, relative levels of ligand for PPARγ, the central transcription factor in the adipocyte differentiation pathway, in the culture medium (Sottile and Seuwen 2000). In addition, BMP signaling may be required for early stages of adipogenic differentiation while inhibiting later stages. This possibility is favored by several observations. For example, treatment of the multipotential mesenchymal 10T1/2 cells with BMP-4, followed by induction of growth arrest, leads to adipocyte differentiation at high frequency. Furthermore, subcutaneous injection of the BMP-4-treated cells into nude mice results in the development of adipose tissue (Tang et al. 2004). Additional evidence for a key role of BMP signaling is provided by the analysis of a committed preadipocyte cell line. These cells are under the control of autocrine BMP signaling, and inhibition of this circuitry by noggin blocks adipocyte differentiation (Bowers et al. 2006). These observations may suggest a role for BMP signaling in providing competence to adipocyte differentiation (Bowers and Lane 2007).

The finding that BMPs can stimulate both osteoblast and adipocyte differentiation of the same cell population (Asahina et al. 1996) may suggest a common early differentiation step involving BMP-activated Smads, the identification of which would greatly enhance our understanding of the normal differentiation of bone marrow stromal cells into osteoblasts versus adipocytes. Whether osteoblast or adipocyte differentiation in response to BMPs is favored may depend on the relative ratios of BMP-activated type I receptors or Smads. Thus, it has been proposed that BMPRIIB signaling favors osteoblast differentiation, whereas BMPRIA signaling favors adipogenic differentiation of 2T3 mesenchymal cells (Chen et al. 1998), although the relative roles of the different BMP receptors and
Smads in osteoblast versus adipocyte differentiation remain to be better defined. Downstream from the BMP receptors, the activated complex of Smad1 with Smad4 may cooperate with Schnurri-2, a large zinc-finger transcription factor, to activate the expression of PPARγ. Consistent with the direct induction of PPARγ expression by C/EBP transcription factors, Schnurri-2 was found to interact with Smad1/4 and C/EBPα on the PPARγ promoter. Illustrating the participation of Schnurri-2 in adipocyte differentiation, mice with the gene for inactivated Schnurri-2 have reduced white adipocyte tissue (Jin et al. 2006). Schnurri-2 is a mammalian homolog of Drosophila Schnurri, a transcription factor that functions downstream from Dpp, as discussed in Chapter 17, illustrating a functional conservation of the Dpp/BMP pathway link to Schnurri. The BMPs that function in adipogenic differentiation in vivo remains unclear.

A BMP-related protein that may be of particular relevance for adipocyte differentiation is GDF-3, which in mice is primarily expressed in adipose tissue (McPherron and Lee 1993) and regulated by high-fat-diet feeding (Witthuhn and Bernlohr 2001). Exposure of mice to viral vectors expressing GDF-3 results in increased body fat with prominent adipocyte hypertrophy when these mice are fed a high-fat diet. Furthermore, GDF-3 stimulates PPARγ expression in primary adipocytes and 3T3-L1 preadipocytes in culture (Wang et al. 2004). These observations suggest a physiological role of GDF-3 in adipogenic differentiation and fat formation. Surprisingly, GDF-3 has also been found to function in a totally different context in early development. Human and mouse embryonic stem cells express GDF-3, which in these contexts is thought to function as an extracellular inhibitor of BMP signaling through direct interaction with other BMPs. GDF-3 has a role in maintaining the undifferentiated pluripotent state of human embryonic stem cells and in the ability of mouse embryonic stem cells to differentiate into multiple cell types (Levine and Brivanlou 2006). Other findings, however, provide evidence that GDF-3 acts in early development as a nodal-like ligand that, similar to nodal, binds to activin receptors, thereby requiring Cripto as a coreceptor, and thus should signal through Smad2 (Chen et al. 2006). Clearly, further characterization of the mechanism of action of GDF-3 is required and its role in adipogenic stimulation remains to be defined.

In contrast to the BMPs, TGF-β blocks adipogenic differentiation both in vitro (Ignotz and Massagué 1985; Torti et al. 1989) and in vivo (Clouthier et al. 1997) while it stimulates the proliferation of preadipocytes (Jeoung et al. 1995; Choy et al. 2000). As with mesenchymal cells differentiating into osteoblasts, autocrine and paracrine TGF-β responsiveness thus allows an expansion of the progenitor population, thereby allowing an increased number of cells to differentiate into adipocytes.
Such a scenario would be compatible with the increased expression of TGF-β by obese adipose tissue (Samad et al. 1997; Fain et al. 2005). The inhibition of differentiation by TGF-β is mediated by Smad3. Increased activity of Smad3, but not Smad2, inhibits adipogenic conversion, whereas interfering with Smad3 function enhances and accelerates adipose conversion in culture and confers resistance to inhibition of adipocyte differentiation by TGF-β. Increased expression of Smad6 or Smad7 strongly inhibits adipogenic differentiation and thereby remarkably cooperates with the inhibitory activity of TGF-β, even though these “inhibitory” Smads have been characterized as inhibitors of TGF-β and/or BMP signaling (Choy et al. 2000). Thus, as with osteoblast differentiation, the Smad system, or at least Smad3 and the inhibitory Smads, act as a cell-intrinsic system to regulate progression of differentiation in response to external factors.

Myostatin, a key regulator of skeletal muscle differentiation and muscle mass as discussed above, also regulates adipogenesis. Mice lacking myostatin expression due to targeted gene inactivation develop less adipose tissue (McPherron and Lee 2002), suggesting that myostatin promotes adipose conversion of mesenchymal cells. This would be consistent with the observation that myostatin promotes adipogenic differentiation of 10T1/2 cells (Artaza et al. 2005), although the myostatin-induced differentiation may lead to incompletely differentiated, immature adipocytes (Feldman et al. 2006). On the other hand, myostatin inhibits BMP-induced adipogenic differentiation of 10T1/2 mesenchymal cells and 3T3-L1 preadipocytes (Rebbapragada et al. 2003). In addition, systemic overexpression and release of myostatin in adult mice results in loss of both muscle mass and body fat (Zimmers et al. 2002). Because myostatin acts through two type I receptors, that is, ActRIB and TβRI (Rebbapragada et al. 2003), this inhibition by myostatin is consistent with the inhibition of adipocyte differentiation of 3T3-L1 cells by activin (Hirai et al. 2005) or TGF-β. How this inhibition of differentiation is reconciled with the decreased fat formation in myostatin-deficient mice is unclear but presumably involves metabolic effects of myostatin at the organismal level; perhaps increased muscle mass resulting from myostatin deficiency increases basal metabolic rates, thus reducing the energy available for lipid storage.

Whereas the contribution of non-Smad mechanisms in this context has yet to be elucidated, the strong inhibition by TGF-β is primarily mediated by Smad3 as transcriptional repressor. Epistasis and functional and physical interaction analyses revealed that Smad3 targets the C/EBPs for functional repression through physical interactions (Fig. 4). (Choy and Derynck 2003). Thus, adipogenic conversion of NIH-3T3 cells by ectopic C/EBPα, β, or δ expression is efficiently blocked by TGF-β-Smad3 signaling, which
represses the transcription function of these C/EBPs without decreasing C/EBP protein levels. As C/EBPβ and δ activate PPARγ expression during differentiation, their functional repression prevents adipogenic conversion and the expression of other C/EBP target genes. The repression of C/EBP function by TGF-β-activated Smad3 correlates with the direct association of Smad3 with the transactivation domain of the C/EBPs in response to TGF-β, resulting in repression of their transcription function. Smad3 binding does not interfere with the binding of C/EBPs to their cognate DNA sequence in regulatory promoter regions. In contrast to C/EBPs, Smad3 neither interacts with PPARγ nor affects its transcription function (Choy and Derynck 2003). Thus, as with osteoblast differentiation, the inhibition of adipogenic differentiation by TGF-β is mediated through transcriptional repression by Smad3 that targets critical transcription factors, whose function is essential in the progression of differentiation.

Figure 4. Transcription factor cascade in adipocyte differentiation and inhibition of adipogenesis by TGF-β or myostatin. Mesenchymal progenitor cells, exposed to adipogenic stimuli, activate the expression and function of C/EBPβ and δ, which directly induce transcription of the PPARγ gene. PPARγ2 is the adipocyte-specific isoform of PPARγ and is specifically required for activation of adipocyte genes. It heterodimerizes with RXR and requires activation by unknown endogenous ligand(s) to activate transcription of genes in the adipocyte differentiation program. C/EBPα is then induced and takes over the function of C/EBPβ and δ, maintaining the transcription of PPARγ and cooperating with PPARγ to activate the genes of the adipocyte transcription program. PPARγ and C/EBPα stimulate each other’s expression to create a positive feedback loop and drive adipogenesis. TGF-β or myostatin/GDF-8 binding to their cell-surface receptors results in activation of Smad3. Activated Smad3 physically interacts directly with C/EBPβ, δ, and α and represses their transcription function, thus inhibiting activation of the adipocyte differentiation program.
SUMMARY AND PERSPECTIVE

In this chapter, we aimed to provide an overview of the role of the TGF-β family in mesenchymal differentiation. We focused on the best-characterized lineages of mesenchymal differentiation, specifically the osteoblast, chondrocyte, myocyte, and adipocyte lineages. Among these four lineages, the roles of TGF-β family signaling in osteogenic and chondrogenic differentiation that drive skeletal development and maintenance have received considerable attention, due in part to the highly sensitive and readily observable nature of skeletal development that is apparent from the manipulation of mouse models. Thus, the combination of transgenic and gene inactivation approaches in mice with mechanistic and molecular studies provides us with a substantial body of insight into these processes as detailed next in Chapter 22. In studying the roles of TGF-β family signaling in muscle cell differentiation, the TGF-β family protein, GDF-8, better known as myostatin, has revealed itself as a key regulator of muscle cell differentiation and of muscle mass. These findings have led to a high enthusiasm for the development of myostatin inhibitors for therapeutic approaches toward muscle degenerative diseases or to counteract muscle wasting associated with catabolic conditions. Finally, the roles of TGF-β family signaling in adipocyte differentiation and adipose tissue development remain to be further characterized because relatively little, in comparison to the three other lineages, is known.

In addition to these four specialized differentiation lineages, TGF-β family signaling has key roles in mesenchymal stem cell maintenance and in the direction of lineage specification. TGF-β itself may function primarily to stimulate proliferation and inhibit terminal differentiation. In this way, TGF-β helps to maintain the pools of mesenchymal progenitors and slows the progression of cells to terminal differentiation, which is often characterized by reduced biosynthetic activity or by apoptosis. Other TGF-β family members are more critical in the specification of differentiation along a defined lineage.

In addition to the well-studied osteoblast, chondrocyte, myocyte, and adipocyte lineages, mesenchymal cells also differentiate into other cell types. Fibroblasts found in various types of connective tissues, including tendons, ligaments, and serosa, may represent unique subpopulations of fibroblasts. These fibroblast subpopulations are difficult to distinguish or study due to a lack of differentiation markers and model systems that can recapitulate the normal cell and matrix environment in vitro. Consequently, very little is known about the mechanisms controlling their differentiation. Among the mesenchymal cells found in connective tissues, the fibroblast-like cells that are closely packed and align themselves to
form tendons are increasingly considered as a separate, albeit poorly characterized, differentiation lineage (Towler and Gelberman 2006). Tenocytes likely have a characteristic gene expression profile that sets them apart from other mesenchymal connective tissue cells, including the expression of tenomodulin (Docheva et al. 2005). Tenocyte differentiation is regulated by TGF-β family signaling. Indeed, GDF-5, -6, and -7 induce the formation of tendon and ligament tissue when ectopically implanted in vivo (Wolfman et al. 2003) and are expressed at sites of synovial joint formation, as already discussed. Furthermore, mesenchymal 10T1/2 cells ectopically expressing BMP-2 together with an activated fragment of Smad8 give rise to neotendon formation when implanted in vivo (Hoffmann et al. 2006). These observations suggest unique roles for TGF-β family signaling in tenocyte differentiation and tendon formation.

TGF-β family signaling also regulates the differentiation of smooth muscle cells and cardiomyocytes, topics that have not been elaborated upon in this chapter. Increasing evidence implicates TGF-β proteins and BMPs in the selection of those lineages that are involved in cardiovascular development and in the control of their differentiation. Some of these findings are discussed in Chapter 24. Finally, TGF-β signaling also drives epithelial–mesenchymal transition toward myofibroblasts, as is discussed in Chapter 29. Much additional research is required to define the differentiation of these largely understudied mesenchymal differentiation lineages and the roles of TGF-β family signaling in this process.

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