TGF-β Family Signaling in Skeletal Development, Maintenance, and Disease

Tamara Alliston
Department of Orthopaedic Surgery
University of California
San Francisco, California 94143

Ester Piek
Department of Applied Biology
Faculty of Science
Radboud University Nijmegen
6525 ED Nijmegen, The Netherlands

Rik Derynck
Department of Cell and Tissue Biology
University of California
San Francisco, California 94143

The transforming growth factor-β (TGF-β) family is critically involved in the development and maintenance of skeletal tissues. In searches for factors with potent cartilage and bone inductive activities, TGF-β was isolated as cartilage-inducing factor (Seyedin et al. 1987), whereas bone morphogenetic proteins (BMPs) were isolated as factors able to induce cartilage and bone formation (Urist 1965; Luyten et al. 1989). The TGF-β family shows extensive redundancy of ligands, receptors, agonists, and antagonists, yet the diverse temporal and spatial patterns of expression of each pathway component allow these signaling factors to direct skeletal development and homeostasis by regulating patterning, cell-fate determination, cell differentiation, and bone remodeling. The critical roles of each factor are evident in mice with mutations in components of these pathways, whereas in vitro
studies have elucidated the mechanisms for the action of the TGF-β family in mesenchymal and skeletal cells. Because the TGF-β family has a central role in the regulation of mesenchymal differentiation into skeletal cells, its role in osteoblast and chondrocyte development is detailed in Chapter 21. Here we discuss, at the tissue level, the impact of TGF-β family ligands and signaling pathways in skeletal patterning, skeletal development, and skeletal maintenance and metabolism, as well as the effects of their deregulation in many skeletal diseases.

**IMPLICATION AND LOCALIZATION OF TGF-β FAMILY SIGNALING COMPONENTS IN THE SKELETON**

Many members of the TGF-β family, their receptors, and signaling effectors, the Smads, are widely expressed in mesenchymal tissues throughout development and, in particular, at sites of skeletal patterning and bone and cartilage formation. Furthermore, TGF-β family members and their signaling mediators are expressed in differentiating chondrocytes and osteoblasts that deposit cartilage and bone matrix, respectively, and in osteoclasts, whose function is to resorb calcified hypertrophic cartilage or bone matrix. Among the TGF-β family members, the expression and roles of several BMPs, growth and differentiation factors (GDFs), and TGF-βs have been well studied.

**Localization and Function of BMPs and GDFs, Their Antagonists and Receptors**

Since their discovery as inducers of ectopic cartilage and bone formation, BMPs have been studied as key regulators of early embryogenesis, pattern formation, and organogenesis. BMP signaling in skeletal structures is tightly regulated by the localized expression and activities of BMP proteins, their receptors, and extracellular antagonists. BMP-1 to BMP-7 have different but overlapping expression patterns in the developing skeleton (Solloway et al. 1998). Like most TGF-β family receptors, BMP type II receptor (BMPRII) and the type I receptor BMPRIA/ALK-3 are expressed nearly ubiquitously in development (Kawabata et al. 1995); however, the expression of the BMPRIIB/ALK-6 type I receptor is restricted to precartilaginous condensations, developing cartilage and bone (Ishidou et al. 1995). The expression of BMPRIA, BMPRIB, and BMPRII is induced in bone fracture repair (Onishi et al. 1998). In addition, statins, estrogen, and Wnt3a induce BMP expression as part of their osteoblast differentiation promoting activities (Rickard et al. 1998; Mundy et al. 1999; Rawadi et al. 2003). BMP-2 and
BMP-4 also induce their own expression through the osteogenic transcription factor Runx2 (Helvering et al. 2000; Ghosh-Choudhury et al. 2001).

BMP activity is tightly controlled by secreted agonists and antagonists including noggin, sclerostin (SOST), twisted gastrulation (Tsg), chordin, gremlin, follistatin, Dan, Dante, and PRDC (protein related to Dan and Dante), as discussed in Chapter 8. Each of these is expressed in skeletal cells (for review, see Canalis et al. 2003), allowing them to dampen or facilitate BMP binding to their receptors. Remarkably, BMPs induce the expression of their own antagonists, including the secreted noggin, gremlin, and sclerostin and the inhibitory Smad6 and Smad7 (Gazzerro et al. 1998; Takase et al. 1998; Ishisaki et al. 1999; Pereira et al. 2000; van Bezooijen et al. 2005). The expression of BMP ligands, their receptors, and extracellular agonists and antagonists is often regulated through positive or negative feedback loops that respond to other signaling pathways, including fibroblast growth factor (FGF) and sonic hedgehog (Shh), in limb morphogenesis, endochondral bone formation, or pathological conditions. Following binding and activation of BMP receptor complexes, the BMPs activate Smad-mediated and non-Smad signaling pathways, which are critical for BMP action in skeletogenesis. BMP-activated signaling through Smad1, Smad5, and Smad8 is antagonized by Tob, c-Ski, menin, and Smad6 and Smad7 (Berk et al. 1997; Hayashi et al. 1997; Imamura et al. 1997; Nakao et al. 1997; Yoshida et al. 2000; Sowa et al. 2004), whose expression patterns are again regulated during skeletal development or in skeletal cell differentiation. Together, this complex network regulates the temporal and spatial expression and amplitude of the BMP activity that controls skeletal patterning, as well as the organization of growth plates and postnatal maintenance of bone mass.

GDF-5, -6, and -7, also referred to as cartilage-derived morphogenetic proteins (CDMP-1, -2, and -3) (Chang et al. 1994; Storm et al. 1994), were first identified in a screen for BMP-related genes possibly corresponding to the brachypodium mouse locus (Storm et al. 1994). Null mutations in Gdf5 cause the brachypodium phenotype, characterized by reduced length of long bones in the limbs and altered patterning of segments in the digits, indicating crucial roles for GDF-5 in the patterning of the appendicular skeleton, chondrogenesis, and longitudinal bone growth (Storm et al. 1994; Storm and Kingsley 1996, 1999). This is consistent with the striking localization of GDF-5 expression to sites of joint formation (Merino et al. 1999b). GDFs can induce osteogenic differentiation in vitro (Erlacher et al. 1998b; Yeh et al. 2005) and endochondral bone formation when implanted intramuscularly (Hotten et al. 1996) and are involved in bone formation in vivo (Mikic et al. 2002).
Expression of TGF-βs and Their Receptors

The three TGF-βs—TGF-β1, TGF-β2, and TGF-β3—signal through receptor complexes of TGF-β type II receptor (TβRII) and TβRI, whereas the type III receptor, betaglycan, facilitates the presentation of TGF-β ligands to the heteromeric receptor complex. Although the three ligands function similarly at the cellular level, mice with targeted inactivation for each of the corresponding genes exhibit unique phenotypes, thus reflecting their different spatiotemporal distributions and roles. For example, rescue of the severe inflammatory phenotype in TGF-β1-null mice revealed defects in bone quality, detailed in Chapter 21 (Shull et al. 1992; Kulkarni et al. 1993; Geiser et al. 1998). Deletions of TGF-β2 or TGF-β3 cause perinatal lethality resulting from defective palatogenesis and other developmental malformations, in part due to impaired epithelial–mesenchymal transdifferentiation and cell proliferation (Kaartinen et al. 1995; Proetzel et al. 1995; Sanford et al. 1997). TGF-β2-null mice also possess defects in axial, appendicular, and craniofacial skeleton patterning (Sanford et al. 1997).

The unique and overlapping expression patterns of the TGF-β ligands throughout skeletal development are also regulated by feedback loops involving TGF-β family members and other signaling pathways. Some discrepancies between the mRNA and protein expression patterns may reflect the regulation of TGF-β storage in cartilage or bone matrix (Alliston and Derynck 2000). The interactions of TGF-β with extracellular matrix proteins allow for high levels of TGF-β storage, especially TGF-β1, in bone (Pelton et al. 1991). All three TGF-β isoforms are expressed in mesenchyme, and their expression increases during mesenchymal condensation. TGF-β3 is expressed at high levels in early development of cartilage rudiments for ribs and vertebrae, whereas TGF-β1 and TGF-β2 expression is very low (Pelton et al. 1990, 1991). Later in development, TGF-β3 levels are reduced and TGF-β1 and TGF-β2 levels are increased (Pelton et al. 1990). Often, TGF-β3 levels are highest in the soft tissues associated with skeletogenesis, whereas TGF-β2 is up-regulated at sites of new mineralization.

In cartilage, TGF-β3 is expressed at higher levels than the other TGF-βs in the perichondrium (Pelton et al. 1990, 1991). In the growth plate, TGF-β1 and TGF-β3 are primarily expressed in the proliferative and hypertrophic zones, whereas TGF-β2 is expressed in all zones of the growth plate, with its highest levels in the hypertrophic and mineralizing zones (Sandberg et al. 1988; Millan et al. 1991; Thorp et al. 1992; Horner et al. 1998). The periosteum of long bones expresses high levels of TGF-β1, whereas the periosteum of intramembranous bone expresses high levels
of TGF-β2 (Pelton et al. 1989). TGF-β1 is the predominant isoform expressed by osteoclasts. All three isoforms are expressed by osteoblasts. Vitamin D3, parathyroid hormone (PTH), and estrogen induce the expression of TGF-β by osteoblasts in culture (Oursler et al. 1993; Streuli et al. 1993; Wu et al. 1999), although the effects in cultured osteoblasts may not be indicative of in vivo mechanisms.

TβRI and TβRII are also expressed throughout the developing skeleton (Horner et al. 1998), but their expression is reduced or lost in hypertrophic chondrocytes and mineralized osteophyte tissue, respectively (Horner et al. 1998). The presumed reduction of TGF-β responsiveness in osteophytes is consistent with an osteoarthritic phenotype in mice that express a dominant-negative TβRII, or mice null for the expression of Smad3. Expression of the TGF-β-responsive Smads is also regulated in skeletal tissues (Sakou et al. 1999). Smad2 is preferentially expressed in proliferating chondrocytes, whereas Smad3 is expressed at higher levels in mature chondrocytes and Smad4 is expressed throughout the growth plate. The inhibitory Smad6 and Smad7 are expressed by the most mature chondrocytes.

As discussed in Chapter 7, TGF-β is secreted as an inactive complex of the mature TGF-β dimer noncovalently interacting with the amino-terminal part of the TGF-β propeptide (latency-associated peptide or LAP), which in turn can bind to one of the latent TGF-β-binding proteins (LTBPs). Among the four isoforms of LTBPs, all but LTBP-2 bind to the TGF-β propeptide. These LTBPs facilitate the deposition and storage of TGF-β in the bone matrix. Although all four LTBPs are broadly expressed and regulated, LTBP-3 expression is high in bone, and LTBP-2 is abundantly expressed in chondrogenic condensations (Shipley et al. 2000). The expression of LTBP-1 is regulated by PTH (Kwok et al. 2005). The release of active TGF-β from these complexes is also regulated, as discussed in Chapter 7. In addition to proteases such as plasmin, the acidic microenvironment created by osteoclasts during bone resorption effectively releases active TGF-β (Oreffo et al. 1989; Oursler 1994; Dallas et al. 2002). Therefore, several levels of regulation determine the availability of active TGF-β in the skeleton, including the expression of TGF-β ligands, receptors, and LTBPs; deposition and activation of TGF-β; and presentation of ligands to TGF-β receptor complexes.

Localization of Activins, Inhibins, Follistatin, and Activin Receptors

The activins are involved in skeletal development and metabolism. Activins and activin receptors are expressed in prechondrogenic limb condensations
and developing limbs during chondrogenesis (Roberts et al. 1991; Feijen et al. 1994; Merino et al. 1999a). The activin \( \beta_A \) subunit (encoded by the \( \text{Inhba} \) gene) and ActRII (type II activin receptor) are expressed in proliferating but not hypertrophic chondrocytes in the growth plate. ActRII is highly expressed in newborn rat skeletal tissues and fracture calluses in vivo, particularly in osteoblasts at sites of active proliferation and early intramembranous and endochondral bone formation (Shuto et al. 1997). Activin receptors are also expressed during ectopic bone formation induced by implantation of the demineralized bone matrix (Funaba et al. 1996).

Inhibin, a heterodimer consisting of an inhibin \( \alpha \) with a \( \beta_A \) or \( \beta_B \) subunit (see Chapters 4 and 27), may compete with activin for binding to ActRII and with BMP for binding to ActRII or BMPRII (Lewis et al. 2000), indicating that, also in the context of skeletal development and homeostasis, inhibin may act as a physiological antagonist of activins and BMPs. Activin function is regulated by binding to the extracellular antagonist follistatin, which also binds some BMPs including BMP-2, BMP-4, and BMP-7, thus preventing ligand binding to their receptors (Nakamura et al. 1990; Iemura et al. 1998; Abe et al. 2004; Thompson et al. 2005). Follistatin is expressed in developing limb digits, and its expression is regulated by activin, particularly in zones of digital ray formation at sites that mark the positions of developing tendons (Merino et al. 1999a). Follistatin is expressed by osteoblasts in culture and at sites of endochondral bone formation in vivo and its expression can be induced by BMP-2 (Hashimoto et al. 1992; Funaba et al. 1996; Kearns and Demay 2000; Abe et al. 2004).

**ROLES OF TGF-\( \beta \) FAMILY IN SKELETAL PATTERNING**

TGF-\( \beta \) family signaling has key roles in the patterning of the cranial, axial, and appendicular skeletons, with different TGF-\( \beta \) family members shown to be functionally important in different skeletal elements. The cross-talk of TGF-\( \beta \) family signals with other signaling pathways, including those activated by Shh, Wnt, PTH-related protein (PTHrP), and FGFs, results in synergies and antagonisms that restrict or define the sizes and shapes of the bones and cartilage structures as well as the joints.

**Determination of Left–Right Patterning and Development of Axial Skeleton**

Before limb development, members of the TGF-\( \beta \) family act to specify embryonic germ layers, establish body axes, and initiate left–right
patterning (Chang et al. 2002). Defects in these fundamental aspects of embryogenesis can result in skeletal manifestations. The activin-related nodal is required for mesoderm induction and left–right patterning (Schier and Shen 2000). Because the transcription factor Zic3 is required for nodal expression, Zic3−/− mice exhibit axial skeleton malformations because of insufficient nodal expression and signaling in early embryogenesis (Purandare et al. 2002). Nodal signals through a Smad2-dependent pathway. Although Smad2+/− and Nodal−/− mice are embryonic-lethal, the double heterozygous Smad2+/−;Nodal+/− mice are viable, but they die perinatally with defective left–right patterning and craniofacial malformations (Nomura and Li 1998).

Normal development of the axial skeleton requires specification of the dorsal midline by regulated BMP signaling. Uninhibited BMP signaling in the dorsal mesoderm during somitogenesis causes abnormal development of the vertebrae and ribs of the axial skeleton (Hogan 1996; Dale and Jones 1999; Schier 2001). For example, targeted deletion of the secreted BMP antagonist noggin results in severe rib and vertebral malformations and other skeletal abnormalities (Wijgerde et al. 2005). The axial skeletal defects of Noggin−/− mice can be rescued by heterozygous deletion of Bmp4 (Wijgerde et al. 2005). Axial skeletal defects are also observed in Bmp5−/−;Bmp7−/− mice (Dudley et al. 1995; Luo et al. 1995; Solloway and Robertson 1999).

Regulation of Limb Development by BMP Signaling

BMP signaling is required to establish the limb buds, which give rise to the appendicular skeleton (Wan and Cao 2005). Two signaling centers—the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA)—communicate to establish a gradient of FGF, Shh, BMP, GDF, and Wnt signaling that collectively defines the sizes, shapes, axes, and locations of the joints within the appendicular skeleton. Active BMP signaling is also required to establish the AER, as shown by loss of AER formation and abnormal limb development in mice with a conditional BMPRIA deletion in the limb ectoderm (Ahn et al. 2001).

Once the AER is established, BMP activity must be tightly regulated. The AER expresses BMP-2 and BMP-4, which direct the dorsal–ventral patterning of the limb mesoderm, establish boundaries to limit the size of the AER, and eventually contribute to AER regression (Pizette and Niswander 1999; Ahn et al. 2001; C.K. Wang et al. 2004). The AER also secretes FGFs, whereas the ZPA produces Shh (Fig. 1). These FGFs and Shh reinforce the continued expression of each other through...
a positive feedback loop that supports the growth and maintenance of the AER and ZPA. BMPs antagonize this positive feedback loop and consequently limit the expansion of the AER and ZPA (Niswander and Martin 1993; Laufer et al. 1994). Insufficient and excessive levels of BMP activity in the developing limb bud both result in deregulated appendicular skeletogenesis. Insufficient BMP signaling leads to abnormal expansion of the AER and ZPA, which in turn results in syndactyly, polydactyly, lack of interdigital apoptosis, and severe limb malformation (C.K. Wang et al. 2004). In contrast, excessive BMP signaling, due to loss of gremlin, is the cause of the naturally occurring limb deformity (ld) mouse mutation (Zuniga et al. 2004). Gremlin, a secreted BMP antagonist, is expressed in the developing limb and represses BMP signaling to allow maturation of ZPA and AER signaling centers. In gremlin-null mice, the ZPA and AER disintegrate prematurely, resulting in impaired limb outgrowth and digit and bone agenesis (Khokha et al. 2003).

The same signals that define the ZPA and AER also define the cell populations in the developing limb that undergo apoptosis. BMP-2, BMP-4, and BMP-7 are expressed in the interdigital mesenchyme, where
they promote apoptosis through Smad and p38 mitogen-activated protein (MAP) kinase-dependent pathways (Francis et al. 1994; Lyons et al. 1995; Yokouchi et al. 1996; Zou and Niswander 1996; Laufer et al. 1997; Macias et al. 1997). FGF signaling counteracts the apoptotic BMP signals and thereby inhibits apoptosis (Buckland et al. 1998). Cross-talk with the Wnt pathway has been implicated in the induction of limb mesenchyme apoptosis, although the precise mechanisms remain unclear (Soshnikova et al. 2003). The syndactyly observed in the absence of BMP signaling resembles that observed in transgenic mice with Noggin expression from the Msx2 promoter (they also have reduced BMP signaling in the limb mesenchyme) and results from inhibition of mesenchymal apoptosis (C.K. Wang et al. 2004).

In addition to the role of gremlin in limb outgrowth, other secreted BMP antagonists have been implicated in limb development, including noggin, chordin, Tsg, follistatin, and Dan (see Chapter 8). Each of these is expressed in unique temporal-spatial patterns in the developing limb and has specific binding affinities for select TGF-β family ligands. Noggin binds BMP-2, BMP-4, GDF-5, GDF-6, and GDF-7 with picomolar affinity (Re'em-Kalma et al. 1995; Zimmerman et al. 1996; Chang et al. 1999) and is expressed in the limb mesenchyme and in cartilage condensations where it regulates joint specification (Brunet et al. 1998). As part of the cross-talk between FGF and BMP signaling, noggin expression is induced by FGFs (Warren et al. 2003). A signaling feedback loop between BMP and noggin controls the pattern of BMPRIB expression, which is thought to control digit size and shape (Merino et al. 1998). As mentioned, Noggin^{−/−} mice have severe malformations of the axial and appendicular skeleton (Brunet et al. 1998; McMahon et al. 1998). The observation that these axial malformations, but not their appendicular defects, are rescued by reduction of BMP levels, suggests that noggin may antagonize GDF signaling in the appendicular skeleton (Wijgerde et al. 2005).

**Skeletal Malformations in Mice Lacking BMP Ligands or Antagonists**

GDF and BMP interactions in skeletal patterning are also apparent in Gdf5^{−/−} and Bmp5^{−/−} mice (Kingsley 1994; Storm et al. 1994). Inactivation of Bmp5, which is mutated in the “short ear” mutant mouse, causes defects in appendicular and axial skeletal morphogenesis (Kingsley 1994). Although the GDF-5 and BMP-5 expression patterns do not overlap, Gdf5^{−/−};Bmp5^{−/−} mice exhibit sternum malformations that are more severe than those in either single mutant line alone (Storm and Kingsley 1996).
This synergy of GDF-5 and BMP-5 between two independent cell populations illustrates a principle of TGF-β family signaling in skeletal development. Despite extensive redundancy, the specific expression patterns and unique functions of each family member are required for the intricate patterning, development, and maintenance of the skeleton (Storm and Kingsley 1996).

The same principle helps to explain the phenotypes of Bmp5<sup>−/−</sup>, Bmp6<sup>−/−</sup>, and Bmp7<sup>−/−</sup> mice. Bmp6<sup>−/−</sup> mice exhibit delayed chondrocyte hypertrophy and sternum malformations (Solloway et al. 1998). BMP-6 is coexpressed with BMP-2 and BMP-7 in long bones, which largely compensates for loss of BMP-6. In sternocostal joints, however, BMP-6 is expressed without BMP-2 and BMP-7, explaining the sternal malformations observed in Bmp6<sup>−/−</sup> mice (Solloway et al. 1998). Deletion of BMP-7, which is expressed in bone (Lyons et al. 1995), results in lethal defects in kidney development as well as polydactyly and abnormal rib development (Dudley et al. 1995; Luo et al. 1995). As in Bmp6<sup>−/−</sup> mice, defects in Bmp7<sup>−/−</sup> mice occur at sites that exclusively express BMP-7, suggesting that enough redundancy exists at other locations to compensate for loss of BMP-7 (Dudley and Robertson 1997). In addition, Bmp5<sup>−/−</sup>;Bmp7<sup>−/−</sup> mice have skeletal defects that are much more severe than in either single knockout, illustrating the functional redundancy of BMP-5 and BMP-7 (Dudley et al. 1995; Luo et al. 1995; Solloway and Robertson 1999). Similar compensation among BMPs is observed when crossing Bmp7<sup>−/−</sup> mice with Bmp4<sup>+/−</sup> mice (Katagiri et al. 1998).

Deletion of follistatin, chordin, and Tsg in mice also results in skeletal malformations. Follistatin, which antagonizes activin and BMP signaling, is expressed in proliferating chondrocytes and osteoblasts, and its expression is regulated by BMP (Funaba et al. 1996). Exogenous application of follistatin to developing limb buds blocks digit formation, whereas Fst<sup>−/−</sup> mice have various skeletal and nonskeletal abnormalities (Hashimoto et al. 1992; Matzuk et al. 1995b). Tsg is expressed by osteoblasts and can form a complex with chordin, short gastrulation (Sog), and BMP, which then acts as a more efficient BMP antagonist than any component alone (Canalis et al. 2003). However, Tsg can also promote BMP signaling by facilitating the degradation of chordin by tolloid-like proteases (Blader et al. 1997; Piccolo et al. 1997). The phenotype of Tsg<sup>−/−</sup> mice reflects both roles of Tsg. Its role as a BMP antagonist may contribute to the osteoporotic phenotype in the appendicular skeleton, whereas its BMP promoting function may be important for embryonic head development (Zakin and De Robertis 2004). Chordin binds BMPs but not other TGF-β family members. Chordin<sup>−/−</sup> mice exhibit cranio-
facial malformations similar to those seen in DiGeorge syndrome (see further below). Chordin is expressed by chondrocytes and acts as a particularly important regulator of chondrocyte maturation. The malformations of endochondral bone in chordin-null mice result from its action on chondrocytes rather than on osteoblasts (Zhang et al. 2002; Bachiller et al. 2003).

TGF-β and Activin Signaling in Skeletal Patterning

Many components of the TGF-β pathway have been evaluated in mice by targeted deletion, including the three TGF-β ligands, TβRI and TβRII, Smad2, Smad3, Smad4, LTBP-3, and LTBP-4. Because of their critical roles in early development, deletions of the genes encoding TβRI, TβRII, or Smad4 cause early embryonic lethality, precluding analysis of these proteins in skeletogenesis. Among the mice with targeted gene inactivation who survive through skeletogenesis, inactivation of each individual gene for an effector of the TGF-β–Smad pathway (except LTBP-4) results in a bone phenotype. Additional mouse models that delete or overexpress components of the TGF-β pathways in a tissue-specific manner have been particularly informative of the role of TGF-β family signaling molecules in skeletogenesis.

For example, tissue-specific deletion of TβRII under the control of the Col2a1 (α1(II) procollagen gene) promoter showed that TGF-β responsiveness of Col2a1-expressing cells is required for normal patterning of the axial skeleton (Baffi et al. 2004). Mutant mice exhibit several defects in the vertebrae and intervertebral discs. This is most likely due to loss of TβRII expression in the sclerotome, which expresses Col2a1 and gives rise to the axial skeleton. Interestingly, chondrocyte differentiation is normal in these mice, which highlights the importance of the perichondrium in mediating the effects of TGF-β on chondrocytes (Baffi et al. 2004). TGF-β2 participates in patterning of the digits. TGF-β2 is expressed in condensing mesenchyme that gives rise to the digits (Pelton et al. 1989). At that stage, TGF-β prevents apoptosis of interdigital cells. Increased TGF-β expression can promote the formation of extra digits based on its chondrogenic activity (Ganan et al. 1996).

TGF-β family signaling also instructs the formation of the bones and cartilages of the jaw, head, and neck from cranial neural crest cells (Wan and Cao 2005). Deletion of ALK-2, a type I receptor for BMPs, in the neural crest results in craniofacial defects, cleft palate, and hypomorphic mandible formation (Dudas et al. 2004b). Studies in mice and in humans have revealed a particularly critical role for TGF-β signaling in
palate formation. Human mutations in \textit{TGFBR1} and \textit{TGFBR2} genes (encoding TβRI and TβRII, respectively) are associated with craniosynostoses and cleft palate, as well as several soft-tissue anomalies (Loeys et al. 2005). Members of the TGF-β family signaling network that are required for palatogenesis include TGF-β3, TβRI/ALK-5, TβRII, and Smad2, as revealed from mouse models. For example, cleft palates in \textit{Tgfb3}−/− mice are rescued by activation of the ALK-5 receptor or by targeted overexpression of Smad2 in the medial edge epithelium of the palate (Dudas et al. 2004; Cui et al. 2005). In addition to cleft palate, targeted deletion of TβRII also results in calvarial agenesis and other skull defects. These defects were shown to result from impaired cell proliferation and defective signaling interactions with other cranial neural-crest-derived tissues, such as the dura mater (Ito et al. 2003). Homozygous or heterozygous deletion of the MH1 domain of Smad2 results in early embryonic lethality (Nomura and Li 1998). Smad2+/− mice die later in development than their homozygote-null littermates and exhibit mandibular agenesis or hypoplasia. LTBP-3-null mice also have craniofacial malformations, as well as progressive osteosclerosis and osteoarthritis (Dabovic et al. 2002). The high bone mass phenotype of LTBP-3-null mice is consistent with a reduction in TGF-β signaling due to insufficient storage or stabilization of TGF-β in the bone matrix (Dabovic et al. 2005).

Finally, deletion of activin or activin receptor IIB (ActRIIB) in mice also results in defects in skeletal patterning. Activin βA-deficient mice exhibit cleft palates (Matzuk et al. 1995c), whereas ActRIIB-deficient mice exhibit a disruption of axial patterning as specified by the Hox code, resulting in homeotic transformations of vertebrae, as well as other major nonskeletal abnormalities (Oh and Li 1997). Exogenous application of activins in the interdigital space during development induces formation of extra digits, through functional interactions with other members of the TGF-β family, Wnts and FGFs (Merino et al. 1999a).

ROLES OF TGF-β FAMILY IN BONE REMODELING

Signaling by TGF-β family proteins regulates the differentiation and function of the bone-matrix-depositing osteoblasts (discussed in Chapter 21) and of the bone-matrix-resorbing osteoclasts, as well as the cross-talk between both cell types, which controls bone remodeling and homeostasis. Osteoclasts are derived from the monocyte/macrophage lineage and, when fully differentiated, are large multinucleated cells
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They secrete proteases and create an acidic microenvironment that mediates resorption of the organic and mineral components of the bone matrix. Several critical factors are required for the differentiation of the progenitor cells into this highly specialized cell type (Fig. 2). Among these, macrophage colony-stimulating factor (M-CSF) promotes the proliferation of monocyte/macrophage precursors and induces osteoclast differentiation. In addition, the transmembrane and soluble forms of RANK ligand (RANKL), expressed by osteoblasts, bind to the RANK receptor on osteoclasts to activate signaling through the NF-κB pathway. RANK signaling is required for osteoclast differentiation, function, and survival. Osteoblasts also secrete a soluble inhibitor of osteoclast differentiation, osteoprotegerin (OPG), which acts as a “decoy” receptor for RANKL. OPG inhibits activation of the RANK receptor. A balance of these osteoclast promoting and inhibitory signals allows calibration and coordination of bone deposition and bone resorption (Takahashi et al. 2002).
Direct Actions of TGF-β on Osteoclast Function and Differentiation

Manipulation of TGF-β family signaling by deletion or overexpression of their signaling mediators often results in changes in bone mass resulting from altered communication between the osteoblasts and the osteoclasts. Because osteoclasts depend on osteoblast signals for their differentiation, function, and survival, the effects of TGF-β family members on osteoblasts often elicit effects on both bone deposition and bone resorption that result in changes in bone mass.

TGF-β itself is a major regulator of osteoclast function that acts on osteoclasts directly and indirectly through osteoblasts. Although the importance of TGF-β signaling is clear, the literature on this topic seems confusing. This complexity reflects the cross-talk between osteoblasts and osteoclasts, the dose-dependent activities of TGF-β on both the osteoblasts and osteoclasts, and the unique actions of TGF-β at each stage of the osteoclast life cycle.

Because osteoclasts express TGF-β1 and TGF-β receptors, they can respond directly to TGF-β signaling (Kaneda et al. 2000). During bone resorption, osteoclasts release active TGF-β from the bone matrix through the combined activities of the matrix metalloproteinases MMP-2 and MMP-9, which proteolytically release active TGF-β from the latent complex, and the acidic microenvironment that directly activates the TGF-β ligand (Oreffo et al. 1989; Oursler 1994; Dallas et al. 2002). The release of active TGF-β by osteoclasts is a key factor in the progression of osteolytic tumor metastases, as described later in this chapter.

The direct effects of TGF-β on osteoclast differentiation and function depend on the stage of osteoclast differentiation. TGF-β can inhibit the recruitment of osteoclast precursors to bone in fetal bone cultures (Pfeilschifter et al. 1988). However, following infiltration of osteoclast precursors into fetal bones, TGF-β enhances bone resorption by stimulating the proliferation and differentiation of osteoclast precursors (Tashjian et al. 1985; Shinar and Rodan 1990; Dieudonné et al. 1991). Although the mechanism is as yet uncharacterized, the stimulatory effect of TGF-β on the differentiation of monocyte/macrophage precursors into osteoclasts is so potent that many studies include TGF-β in the culture medium to potentiate osteoclast differentiation. One possible underlying mechanism is the induction by TGF-β of SOCS, a negative regulator of the proinflammatory Jak-Stat pathway (Fox et al. 2003). SOCS overexpression drives the differentiation of the monocyte/macrophage precursors into the osteoclast rather than the macrophage lineage. Other investigators have suggested that the stimulatory effects on early osteoclast differentiation require activation...
of the MAP kinase pathway by TGF-β (Karsdal et al. 2003). TGF-β also induces the expression of RANK, the receptor for RANKL, which promotes osteoclast differentiation (Yan et al. 2001). In contrast, TGF-β inhibits later stages of osteoclast differentiation and function. Accordingly, long-term treatment with TGF-β results in reduced expression of RANK and other osteoclast markers and impaired bone resorption (Karsdal et al. 2003).

Regulation of the Cross-talk between Osteoblasts and Osteoclasts by TGF-β

Other effects of TGF-β on osteoclasts derive from its actions on osteoblasts. TGF-β regulates osteoblast expression of M-CSF, RANKL, and OPG, which have key roles in osteoclast differentiation. At low doses, TGF-β treatment generally results in increased M-CSF expression and prostaglandin production and an increase in the relative expression of RANKL to OPG, resulting in promotion of osteoclastogenesis (Karst et al. 2004). In contrast, high TGF-β levels repress M-CSF and RANKL expression while increasing OPG expression (Murakami et al. 1998; Thirunavukkarasu et al. 2001). Because high levels of TGF-β do not inhibit osteoclastogenesis in pure osteoclast cultures, the inhibitory effects of TGF-β at high doses are mediated by osteoblasts and thus may serve as a negative feedback loop to limit bone resorption. The high levels of TGF-β released during bone resorption switch osteoblasts to an osteoclast-inhibitory pattern of gene expression.

The cross-talk between osteoblasts and osteoclasts is well illustrated by the phenotypes of mice with increased or decreased autocrine TGF-β signaling in differentiating osteoblasts. Increased TGF-β expression in osteoblasts leads to increased bone deposition by osteoblasts, yet progressive bone loss. Bone loss is due to an increase in bone resorption that outweighs bone deposition (Erlebacher and Derynck 1996). Conversely, decreased TGF-β responsiveness of the osteoblasts due to transgenic expression of a dominant-negative TβRII mutant in osteoblasts causes a progressive increase in bone mass and trabecular bone volume (Filvaroff et al. 1999). This increase primarily results from decreased bone resorption. Therefore, autocrine TGF-β signaling by osteoblasts directs not only bone matrix deposition, but also bone resorption by osteoclasts. Consistent with these findings, mice that lack the TGF-β-inducible transcription factor TIEG1 have increased numbers of osteoblasts with reduced expression of differentiation markers. TIEG1-null mice exhibit decreased osteoclastogenesis, in part, because of reduced RANKL and increased OPG expression (Subramaniam et al. 2003).
2005). These findings further illustrate the cross-talk between osteoblasts and osteoclasts and the role of TGF-β in this communication.

Estrogen is well known to inhibit osteoclast activity and bone resorption. Several reports suggest that TGF-β is downstream from estrogen signaling in bone resorption. First, estrogen induces the expression of TGF-β in osteoblast cultures (Oursler et al. 1991; Robinson et al. 1996; Yang et al. 1996). Antibodies against TGF-β repress the osteoclast-inhibitory effect of estrogen in osteoblast cocultures (Hughes et al. 1996). Second, TGF-β has been implicated in the proapoptotic effects of estrogen on osteoclasts (Hughes et al. 1996). Finally, injection of TGF-β into the marrow cavity decreases the extent of ovariectomy-induced bone loss (Beaudreuil et al. 1995). Additional work is needed to better understand the role of TGF-β and other intermediates in the bone-protective effects of estrogen.

Roles of BMPs in Bone Remodeling

Osteoclasts can also respond directly to BMP signals, consistent with their expression of BMPRIA (but not BMPRIB), BMPRII, Smad1, Smad5, and Smad4. Thus, BMP treatment of highly purified primary osteoclast cultures results in phosphorylation of the BMP-specific R-Smads in osteoclast precursors (Okamoto et al. 2006) and increased resorptive activity (Kaneda et al. 2000). In addition, BMP limits the progression of bone resorption by inhibiting expression of collagenase-3, which digests collagens I and II (Varghese and Canalis 1997; Zhao et al. 1999). Although osteoclasts can respond directly to BMPs, most known actions of BMPs on osteoclasts are indirectly mediated by the osteoblasts. For example, BMP-induced osteoblast maturation results in increased expression of the osteoclast differentiation factors M-CSF (Ghosh-Choudhury et al. 2006) and RANKL (Okamoto et al. 2006). This indirect effect of BMPs on osteoclasts is evident in mice, in which BMPRIA is conditionally deleted in osteoblasts (Mishina et al. 2004). These mice exhibit a low bone mass due to insufficient osteoblast differentiation and function, which is rescued within 6 months because of insufficient bone resorption. The osteoblast-specific Bmpr1a deletion results in reduced osteoclast number and function due to insufficient expression by osteoblasts of RANKL, which is required for osteoclast differentiation, function, and survival. A similar reduction in bone mass is observed when noggin is overexpressed in osteoblasts (Devlin et al. 2003). The osteoblast number is normal in noggin-overexpressing mice, but osteoblast function is reduced, resulting
in reduced osteoclast number and activity. However, a direct effect of noggin on osteoclast BMP signaling cannot be excluded (Wu et al. 2003). Therefore, BMPs have an indirect permissive role in osteoclast differentiation and function by promoting osteoblast differentiation and function. In addition to this permissive role, BMP inhibits osteoclast differentiation and function by inducing the expression of OPG in osteoblasts (Wan et al. 2001). Thus, BMP-activated Smad1 displaces the transcriptional repressor, Hoxc8, to activate transcription from the OPG promoter (Wan et al. 2001). The inhibitory Smad6, whose expression is also induced by BMPs, binds Hoxc8 to prevent Hoxc8 displacement from DNA by Smad1 (Bai et al. 2000).

Effects of Activins on Bone Remodeling

Activin A (inhibin-βA dimer) is secreted by bone marrow cells and, in addition to regulating erythroid differentiation (Meunier et al. 1988; Shiozaki et al. 1992; Yamashita et al. 1992), is thought to regulate osteoclast differentiation of bone marrow macrophages. Activin A can promote osteoclastogenesis in bone marrow cultures as well as in calvarial organ cultures in the absence of exogenous RANKL and M-CSF (Sakai et al. 1993; Gaddy-Kurten et al. 2002). Conversely, inhibin blocks osteoclast differentiation in bone marrow cultures, but this effect cannot be overcome by excess activin or BMP-2, possibly because inhibin acts on osteoclastogenesis by alternative pathways (Gaddy-Kurten et al. 2002). Follistatin, which binds and thereby inactivates activin, as well as an anti-activin A antibody, inhibits osteoclastogenesis in cocultures of bone marrow cells and primary osteoblasts, suggesting that activin A produced by osteoblasts regulates osteoclastogenesis (Murase et al. 2001).

Activin also synergizes with RANKL and M-CSF to stimulate osteoclastogenesis in bone marrow macrophages by increasing osteoclast lineage commitment (Murase et al. 2001; Koseki et al. 2002; Sugatani et al. 2003). However, the terminal differentiation, survival, and activation of osteoclasts are not affected by activin (Koseki et al. 2002; Sugatani et al. 2003). The mechanism by which activin synergizes with RANKL is not fully understood, but enhanced nuclear levels of phosphorylated NF-κB, elevated expression of RANK or JunB (a component of the AP-1 transcription complex), and increased phosphorylation of p38 MAP kinase, Erk MAP kinase, and Smad2 may all contribute to potentiation of osteoclastogenesis (Murase et al. 2001; Koseki et al. 2002; Sugatani et al. 2003). Although JNK MAP kinase is essential for osteoclastogenesis (Wagner
and Karsenty 2001), it does not appear to be activated by the combination of RANKL, M-CSF, and activin in bone marrow macrophages (Koseki et al. 2002; Sugatani et al. 2003).

**ROLES OF TGF-β FAMILY IN TOOTH MORPHOGENESIS**

The development of the tooth represents a striking example of how mesenchymal and epithelial cells communicate to define each other’s differentiation. The outer layer of the fully developed tooth is enamel, deposited by ameloblasts that are of epithelial origin. Inside the enamel is dentin, which is deposited by the mesenchymally derived odontoblasts. The center of the tooth contains the pulp, which is analogous to the bone marrow cavity and consists of mesenchymal and endothelial cells and contains the nerve endings. TGF-β family signaling regulates tooth patterning and development.

Among the BMPs, BMP-2, BMP-4, and BMP-7 are expressed in specific patterns in the enamel knot and the dental mesenchyme. As in limb bud patterning, BMPs interact with Shh and FGFs to pattern teeth (Hogan 1996). BMP induces the expression of the transcription factor Islet1 at the sites of incisor formation. Islet1 then induces BMP expression, thus mediating a positive feedback loop that promotes incisor development while repressing molar development. Islet1 represses the expression of Barx1, the transcription factor that promotes molar development. Accordingly, antagonism of BMP activity by noggin promotes Barx1 expression and molar formation (Tucker et al. 1998; Mitsiadis et al. 2003). In addition, conditional deletion of Bmpr1a in surface epithelium arrests tooth morphogenesis (Andl et al. 2004).

GDF-5, GDF-6, and GDF-7 are expressed during odontogenesis in vivo and can be detected in dental follicle tissue at the root-forming stage (Morotome et al. 1998; Sena et al. 2003), suggesting that GDFs may have a role in the formation of the dental attachment, including the periodontal ligament. The human bone disorder angel-shaped phalangeoepiphyseal dysplasia (ASPED) is caused by heterozygous loss-of-function mutations in GDF5 and, among other skeletal defects, is characterized by dental abnormalities including abnormally positioned teeth, premature loss of teeth, and multiple caries (Holder-Espinasse et al. 2004), indicating that GDF-5 has an important role in positioning, development, and/or maintenance of teeth.

TGF-β-activated Smad2 and Smad3 are expressed in temporally and spatially regulated patterns in the developing tooth (Xu et al. 2003) in both the enamel epithelium and dental mesenchyme. In mandible
explant cultures, Smad2 antisense oligonucleotides cause hyperproliferation of ameloblasts and advanced tooth development. In contrast, Smad7 antisense oligonucleotides inhibit tooth development and apoptosis of the enamel depositing epithelium (Ito et al. 2001).

An essential role for activin signaling in tooth patterning and development has been revealed in mice with targeted gene inactivation (Matzuk et al. 1995a,b,c; Ferguson et al. 1998). Mice defective in activin A expression by deletion of the Inhba gene show impaired development of incisors and mandibular molars but normal development of maxillary molars. A similar phenotype is also observed with low penetrance in mice that lack ActRII (Ferguson et al. 2001) or when soluble extracellular ActRII domains are administered during tooth development of maxillary and mandibular explant cultures (Ferguson et al. 2001). In addition, Smad2+/–, but not Smad3–/–, mice exhibit alterations in tooth development (Ferguson et al. 2001), further suggesting the importance of activin signaling in tooth development. Activin expression in the mesenchyme, which is maintained by FGF-8 secreted from the oral epithelium (Ferguson et al. 1998), is required before tooth bud formation and enables incisor and mandibular molar tooth germs to progress beyond the bud stage.

The expression of several genes known to be essential for tooth development, including Barx1, Msx1, Pax9, and Dlx2, is unaffected in activin βA-deficient tooth germs (Ferguson et al. 1998). In contrast, the expression of the homeobox gene Irx1 and follistatin is lost in dental epithelium of activin βA-deficient mice, including in the maxillary molar epithelium. Because maxillary molars of activin βA-deficient mice develop normally, Irx1 and follistatin are not required for development of these teeth (Ferguson et al. 1998, 2001). Follistatin is involved in early patterning of tooth development and is expressed in the dental epithelium of all teeth adjacent to and in a complementary pattern to mesenchyme-expressed activin (Matzuk et al. 1995b; Ferguson et al. 1998). Follistatin is also a critical regulator of tooth crown morphogenesis, enamel knot formation, and patterning of tooth cusps (Wang et al. 2004b). Follistatin integrates the antagonistic signaling between activin and BMP-4 in the differentiation of dental epithelium into ameloblasts. Activin induces the expression of follistatin on the lingual surface of rodent incisors, where it inhibits ameloblast differentiation induced by BMP-4 that is secreted by odontoblasts. Its expression is down-regulated during BMP-4-induced ameloblast differentiation in the labial epithelium of the developing tooth. Follistatin thereby regulates asymmetric distribution of enamel, which is only deposited on the labial side of the rodent incisor by differentiated ameloblasts (Wang et al. 2004a).
ROLES OF TGF-β FAMILY IN HUMAN SKELETAL PATTERNING DEFECTS

Mutations in GDF5 and BMPR1B Cause Chondrodysplasias and Brachydactyly

Consistent with the roles of TGF-β family signaling in mammalian patterning, discussed above, various skeletal patterning defects in humans have been associated with mutations in genes encoding TGF-β family ligands or their signaling mediators (see Table 1). Homozygous mutations in GDF5, also known as CDMP1, provide the basis for the acromesomelic chondrodysplasia Hunter-Thompson type (CHTT), Grebe type (CGT), and DuPan syndromes in humans (Thomas et al. 1996, 1997; Faiyaz-Ul-Haque et al. 2002a,b), whereas heterozygous mutations in GDF5 can cause brachydactyly types A2 (BDA2) and C (BDC), symphalangism (SYM1), and ASPED (Polinkovsky et al. 1997; Holder-Espinasse et al. 2004; Seemann et al. 2005; Kjaer et al. 2006). Homozygous loss of BMPR1B is implicated in a novel subtype of acromesomelic chondrodysplasia that is accompanied by genital defects (Demirhan et al. 2005), whereas heterozygous missense mutations in BMPR1B cause BDA2 (Lehmann et al. 2003). Chondrodysplasias are generally characterized by appendicular bone dysmorphogenesis, showing a proximal–distal gradient in severity, whereas brachydactylies involve shortening and sometimes the loss of some phalanges. ASPED highly resembles BDC and is further characterized by angel-shaped phalanges, hip dysplasia, and dental abnormalities (Holder-Espinasse et al. 2004).

The broad phenotypic variations within and between the various chondrodysplasias and brachydactylies are partly due to the unique but overlapping expression patterns and the differential effects of each mutation in GDF5 and BMPR1B. Missense mutations in GDF5 or BMPR1B can cause more severe phenotypes than loss-of-function mutations, due to dominant-negative effects by the mutant proteins. For example, CGT is caused by a mutation in the cystine knot of mature GDF-5 (CDMP(C400Y)) that results in impaired secretion and bioavailability not only of GDF-5, but also of other TGF-β family members with which it can heterodimerize (Thomas et al. 1997). In BDA2, missense mutations of BMPR1B cause dominant-negative inhibition of receptor kinase activation, receptor endocytosis, or transphosphorylation by BMPRII (Lehmann et al. 2003, 2006). In contrast, other TGF-β family ligands or receptors may partly compensate for the homozygous loss-of-function mutations in GDF5 or BMPR1B, as observed in CHTT (Thomas et al. 1996), thus resulting in a less severe phenotype.
Table 1. Overview of mutations in genes encoding TGF-β family ligands or their signaling mediators, identified in various skeletal disorders, for which the molecular mechanism causing the disorder has been elucidated

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Mutation</th>
<th>Functional consequence</th>
<th>References</th>
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<tbody>
<tr>
<td>Chondrodysplasia Grebe type</td>
<td>heterozygous Cys400Tyr mutation in cystine knot of GDF-5</td>
<td>impaired secretion of (mutant) GDF-5 and of TGF-β family heterodimerization partners</td>
<td>Thomas et al. (1997)</td>
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<td>(CGT)</td>
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<tr>
<td>Brachydactyly type C (BDC)</td>
<td>heterozygous Cys400Tyr, Arg438Cys, or Cys498Ser mutation in cystine knot of GDF-5</td>
<td>mutations affect normal folding of GDF-5 and result in early degradation</td>
<td>Everman et al. (2002);</td>
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<td>Polinkovsky et al. (1997)</td>
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<tr>
<td>Brachydactyly type A2 (BDA2)</td>
<td>heterozygous Ile200Lys mutation in glycine-serine (GS) domain of BMPRIB</td>
<td>dominant-negative inhibition of BMPRIB kinase activity</td>
<td>Lehmann et al. (2003)</td>
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<tr>
<td>BDA2 or BDC/proximal</td>
<td>heterozygous Arg486Trp or Arg486Gln mutation in NANDOR domain of BMPRIB</td>
<td>dominant-negative inhibition of BMPRIB endocytosis or transphosphorylation by BMPRII</td>
<td>Lehmann et al. (2003, 2006)</td>
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<tr>
<td>symphalangism (SYM1)-like</td>
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<tr>
<td>BDA2</td>
<td>heterozygous Leu441Pro mutation in receptor interaction interface of GDF-5</td>
<td>mutant GDF-5 has lost affinity for BMPRIA and BMPRIB</td>
<td>Kjaer et al. (2006);</td>
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<td></td>
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<td></td>
<td>Seemann et al. (2005)</td>
</tr>
<tr>
<td>DuPan syndrome</td>
<td>homoyzgous Leu441Pro mutation in receptor interaction interface of GDF-5</td>
<td>mutant GDF-5 has lost affinity for BMPRIA and BMPRIB</td>
<td>Faiyaz-Ul-Haque et al. (2002a)</td>
</tr>
<tr>
<td>SYM1, multiple synostosis</td>
<td>heterozygous Arg438Leu mutation in receptor interaction interface of GDF-5</td>
<td>increased affinity for BMPRIA, functionally resembling BMP-2</td>
<td>Dawson et al. (2006);</td>
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<tr>
<td>syndrome (SYNS1)</td>
<td></td>
<td></td>
<td>Seemann et al. (2005)</td>
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<tr>
<td>SYNS1</td>
<td>Trp217Gly mutation in noggin</td>
<td>abolished secretion and dimerization of functional noggin dimers</td>
<td>Gong et al. (1999);</td>
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<td></td>
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<td>Marcelino et al. (2001)</td>
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Table 1. (Continued)

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<thead>
<tr>
<th>Disorder</th>
<th>Mutation</th>
<th>Functional consequence</th>
<th>References</th>
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<tbody>
<tr>
<td>SYM1</td>
<td>Gly189Cys or Pro223Leu mutation in noggin</td>
<td>reduced secretion and dimerization of functional noggin dimers</td>
<td>Gong et al. (1999); Marcelino et al. (2001)</td>
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<tr>
<td>Cleidocranial dysplasia (CCD)</td>
<td>heterozygous CCDαA376 mutation, resulting in truncated Runx2 lacking part of carboxy-terminal activation domain</td>
<td>impaired Smad signaling due to inability of mutant Runx2 to interact with receptor-activated Smads</td>
<td>Otto et al. (2002); Zhang et al. (2000)</td>
</tr>
<tr>
<td>Osteoarthritis (OA), osteoporosis</td>
<td>Leu10Pro polymorphism due to T→C transition in TGF-β1 signal peptide</td>
<td>CC genotype correlates with increased TGF-β1 serum levels in Japanese population; TT genotype correlates with increased TGF-β1 serum levels in European population</td>
<td>Hinke et al. (2001); Langdahl et al. (2003); Yamada (2000, 2001)</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>C-509→T polymorphism in promoter region of TGFBI gene</td>
<td>TT genotype correlates with increased TGF-β1 serum levels</td>
<td>Grainger et al. (1999); Langdahl et al. (2003); Yamada (2001)</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>single-nucleotide polymorphism rs15705 in highly conserved motif of 3'UTR of BMP2 mRNA</td>
<td>increased decay of BMP2 mRNA due to altered affinity of specific proteins for SNP sequence</td>
<td>Fritz et al. (2006); Styrkarsdottir et al. (2003)</td>
</tr>
<tr>
<td>Fibrodysplasia ossificans progressiva (FOP)</td>
<td>heterozygous Arg206His mutation in GS domain of BMP type I receptor ALK-2</td>
<td>unstable GS domain likely resulting in increased ALK-2 activation</td>
<td>Shore et al. (2006)</td>
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<tr>
<td>Condition</td>
<td>Mutation Details</td>
<td>Phenotypic Effect</td>
<td>References</td>
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<td>Camurati–Engelmann disease (CED)</td>
<td>$TGFB1$ missense mutations in exon 4, coding for region in LAP that is responsible for homodimerization</td>
<td>Increased secretion of bioactive TGF-$\beta_1$ and enhanced TGF-$\beta$ signaling</td>
<td>Janssens et al. (2003); Kinoshita et al. (2000); Saito et al. (2001)</td>
</tr>
<tr>
<td>CED</td>
<td>Leu10-12 duplication in exon 1, encoding signal peptide of TGF-$\beta_1$</td>
<td>Impaired secretion and intracellular retention of TGF-$\beta_1$, resulting in increased TGF-$\beta$ signaling</td>
<td>Janssens et al. (2003)</td>
</tr>
<tr>
<td>CED</td>
<td>Tyr81His missense mutation in exon 1 of $TGFB1$, coding for amino-terminal sequence of LAP</td>
<td>Impaired secretion of TGF-$\beta_1$ and enhanced intracellular TGF-$\beta$ signaling</td>
<td>Janssens et al. (2003)</td>
</tr>
<tr>
<td>Sclerosteosis</td>
<td>Loss-of-function, nonsense, splice-site mutations in $SOST$</td>
<td>Loss of function of $SOST$ protein, resulting in unopposed Wnt signaling downstream from BMP signaling during bone formation</td>
<td>Balemans et al. (2001); Brunkow et al. (2001); van Bezooijen et al. (2007)</td>
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<tr>
<td>van Buchem disease</td>
<td>Homozygous 52-kb deletion downstream from $SOST$</td>
<td>Down-regulation of $SOST$ gene expression, resulting in unopposed Wnt signaling downstream from BMP signaling during bone formation</td>
<td>Balemans et al. (2002); van Bezooijen et al. (2007)</td>
</tr>
<tr>
<td>Osteopoikilosis</td>
<td>Total $LEMD3$ deletion or heterozygous loss-of-function mutations in $LEMD3$</td>
<td>Nonsense-mediated decay of $LEMD3$ or truncated $LEMD3$ lacking carboxy-terminal domain, resulting in impaired antagonism of Smad activity</td>
<td>Hellemans et al. (2004); Menten et al. (2007)</td>
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</table>
Mutations in the receptor interaction interface of mature GDF-5 cause skeletal malformations by affecting the affinity or specificity of GDF-5 for its receptors. For example, heterozygous or homozygous point mutations in GDF5 involving the exchange of Leu441 for proline (L441P) result in BDA2 or the more severe DuPan syndrome, respectively (Faiyaz-Ul-Haque et al. 2002a; Seemann et al. 2005; Kjaer et al. 2006). The L441P mutant has lost affinity for BMPRIA and BMPRIB and is functionally strongly impaired (Seemann et al. 2005; Kjaer et al. 2006). Two different GDF5 point mutations, replacing Arg438 by leucine (R438L) (Seemann et al. 2005; Dawson et al. 2006) or Glu491 by lycine (E491K) (Wang et al. 2006), were identified in families with proximal symphalangism (SYM1) or multiple synostosis syndrome (SYNS1), limb disorders that were only known to be caused by mutations in the NOG gene (see below). The R438L mutant has normal affinity for BMPRIB, but it has increased affinity for BMPRIA and functionally resembles BMP-2, resulting in loss of receptor specificity and the SYM1 and SYNS1 phenotypes (Seemann et al. 2005).

NOG Mutations Cause Symphalangism and Synostoses Syndromes

Heterozygous mutations in the NOG gene, encoding the secreted BMP antagonist noggin, form the basis for various patterning disorders affecting joint development, including SYM1, SYNS1, tarsal/carpal coalition syndrome (TCC), and autosomal dominant stapes ankylosis. SYM1, SYNS1, and TCC are generally characterized by the absence of joint cartilage and fusion of carpal and tarsal bones and phalanges (Gong et al. 1999; Dixon et al. 2001). Patients with congenital stapes ankylosis suffer mainly from conductive hearing loss and share several skeletal features with SYM1 and SYNS1 patients but do not display symphalangism (Brown et al. 2002). As these disorders start in childhood and continue through adult life, noggin seems to be essential to preserve joints during adult life, and loss of BMP antagonism results in joint fusion.

The NOG mutations identified in SYM1, SYNS1, and TCC are predominantly single missense substitutions of evolutionary conserved amino acids (Gong et al. 1999; Debeer et al. 2004). Heterozygous NOG mutations identified in congenital stapes ankylosis are predicted to disrupt the cysteine-rich carboxy-terminal domain of noggin (Brown et al. 2002). Deletions of the carboxy-terminal domain of noggin have also been reported in a family with SYM1 and in a family with SYNS1 (Takahashi et al. 2001). The different phenotypes associated with similar or identical NOG mutations (Dixon et al. 2001; Takahashi et al. 2001; Brown et al. 2002) might be explained by allelic differences in noggin modifier genes.
Functional analysis of the SYM1 and SYNS1 noggin mutants revealed that secretion and dimerization of the SYNS1 mutant is abrogated, whereas the two SYM1 mutations reduce secretion of the noggin dimer (Marcelino et al. 2001), possibly explaining the more severe phenotype of SYNS1. The mutants did not interfere with secretion of the wild-type noggin dimer. Moreover, the noggin mutants are able to interact with GDF-5 and antagonize BMP signaling in Xenopus embryos (Marcelino et al. 2001), suggesting that reduced secretion rather than functional impairment of noggin contributes to development of SYM1 and SYNS1.

Noggin is broadly expressed during development, and it is interesting that heterozygous mutations of NOG mainly affect joint morphogenesis without affecting other tissues. This may result from the functional redundancy by other antagonists or differences in dosage requirements for BMPs in various tissues.

Functional Haploinsufficiency of Runx2 Causes Cleidocranial Dysplasia

Smad proteins physically interact with the osteoblast transcription factor Runx2, and their cooperation is important for osteoblast differentiation (see Chapter 21). Thus, the balance of cooperation with interacting Smads defines the Runx2 function. Heterozygous loss-of-function mutations in RUNX2 have been identified in cleidocranial dysplasia (CCD) (Otto et al. 2002), a bone disorder involving defective endochondral and intramembranous bone formation that results in multiple skeletal abnormalities such as persistently open or delayed fontanelle closure, additional cranial plates, rudimentary clavicles, and short stature, all indicative of defective patterning. Runx2<sup>+/−</sup> mice demonstrated that Runx2 is required for maturation of osteoblasts and bone formation, whereas the Runx2<sup>−/−</sup> phenotype or radiation-induced mutation of Runx2 results in symptoms that closely resemble those observed in CCD patients (Mundlos et al. 1997; Otto et al. 1997).

The RUNX2 nonsense mutation CCDαA376, identified in three unrelated CCD patients (Otto et al. 2002), encodes a truncated Runx2 protein that lacks a large part of its carboxy-terminal activation domain. This truncated protein exerts a dominant-negative effect on wild-type Runx2, likely through its Runt domain, which retains the ability to bind DNA and interact with CBFβ (also known as PEBP2β). The CCDαA376 Runx2 protein fails to interact with BMP or TGF-β receptor-activated Smads and displays severely impaired transactivation activity. Moreover, whereas overexpression of Runx2 in BMP-2-treated C2C12 myoblasts activates alkaline phosphatase expression, overexpression of CCDαA376
Runx2 does not (Zhang et al. 2000). Thus, the phenotypic manifestations of CCD might be explained in part by impaired Smad signaling due to functional haploinsufficiency of the osteoblast-specific transcriptional coregulator Runx2. Furthermore, mice that overexpress TGF-β2 in bone also exhibit phenotypic features of CCD, consistent with the ability of TGF-β to repress Runx2 function (Erlebacher and Derynck 1996; Alliston et al. 2001).

**THE TGF-β FAMILY IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS**

Rheumatoid arthritis and osteoarthritis are joint diseases characterized by destruction of articular cartilage and bone, fibrosis, and loss of joint function. Osteoarthritis is additionally characterized by sclerosis of subchondral bone and formation of osteophytes, that is, cartilage outgrowths at the joint margins undergoing endochondral ossification. Rheumatoid arthritis is a chronic autoimmune disease that primarily results in inflammation of the synovial membrane of peripheral joints. Synovial cells proliferate and develop into an invasive lesion that infiltrates cartilage and bone. Already at early stages of rheumatoid arthritis, chondrocytes synthesize reduced levels of proteoglycans that causes loss of cartilage matrix. Biomechanical, metabolic, and genetic factors, rather than inflammation, are thought to contribute to osteoarthritis, primarily affecting articular cartilage. However, elevated expression of the proinflammatory cytokine interleukin 1 (IL-1) is often detected in the synovium of joints of osteoarthritis. The pathology of osteoarthritis reflects attempted repair of the damaged extracellular matrix by chondrocytes, overruled by matrix-degrading enzymes produced by chondrocytes.

Several TGF-βs, BMPs, and GDFs are expressed at elevated levels in synovial tissue of patients with rheumatoid arthritis and osteoarthritis, although their roles in the progression of these diseases are poorly understood (Fava et al. 1989; Lafayatis et al. 1989; Lories et al. 2003; Nakase et al. 2003). TGF-β is thought to protect against the development of rheumatoid arthritis through its potent antiinflammatory activity, thereby inhibiting macrophage and T-cell proliferation (Lafayatis et al. 1989; Lotz et al. 1990). Overexpression of dominant-negative TβRII or TGF-β1 in murine cells indicated that functional TGF-β signaling in lymphoid cells protects against rheumatoid arthritis (Chernajovsky et al. 1997; Schramm et al. 2004). Furthermore, TGF-β counteracts the destructive activity of IL-1 on articular cartilage through down-regulation of IL-1 receptor expression (Redini et al. 1993; Takahashi et al. 2005).
Endogenous TGF-β signaling is also involved in the development of osteoarthritis (Serra et al. 1997; Yang et al. 2001; Li et al. 2006; Tchetina et al. 2006). A missense mutation in the linker region of Smad3 has been detected in a patient with osteoarthritis and was shown to confer elevated serum levels of the matrix-degrading metalloproteinases MMP-2 and MMP-9 (Yao et al. 2003). An additional link is provided by the small extracellular matrix protein asporin, a leucine-rich proteoglycan that is abundantly expressed in articular cartilage of patients with osteoarthritis and binds TGF-β, thus inhibiting TGF-β’s chondrogenic activity. A human Asp14 allele is overrepresented in individuals with osteoarthritis and confers a greater inhibition of TGF-β signaling than wild-type asporin (Kizawa et al. 2005). Blocking endogenous TGF-β activity in murine models of osteoarthritis by injection of soluble TβRII ectodomain or expression of the LAP of TGF-β1 or Smad7, or deletion of histone deacetylase 4, revealed a protective role for TGF-β in articular cartilage damage and adverse effects of TGF-β signaling on osteophyte formation (Scharstuhl et al. 2002, 2003; Vega et al. 2004), suggesting a dual role for TGF-β in the pathogenesis of osteoarthritis.

TGF-β protects against cartilage destruction by inducing differentiation in synoviocytes, resulting in increased cartilage matrix protein expression (Lafyatis et al. 1989). Moreover, prolonged exposure to TGF-β sensitizes normal articular chondrocytes to TGF-β-induced proteoglycan synthesis (van Beuningen et al. 1994; Gelse et al. 2001), consistent with the stronger induction by TGF-β of proteoglycan synthesis by osteoarthritic cartilage than by healthy cartilage (Lafeber et al. 1993). Excessive TGF-β signaling can cause joint space narrowing due to cartilage overgrowth. Whereas BMP-2 and BMP-9 induce proteoglycan synthesis in normal articular cartilage, they do not restore cartilage loss in a mouse model of arthritis, due to their inability to counteract IL-1 activity (Glansbeek et al. 1997). GDFs are able to reconstitute proteoglycan content in matrix-depleted cartilage explants and stimulate articular chondrocytes of osteoarthritis to synthesize proteoglycans (Erlacher et al. 1998a; Bobacz et al. 2002).

Endogenous TGF-β and BMP contribute to pathogenesis of osteoarthritis by inducing osteophyte formation and synovial thickening, which can be reduced by overexpression of Smad6 and Smad7 as seen in experimental models of osteoarthritis (Scharstuhl et al. 2003; Blaney Davidson et al. 2006). Synovial macrophages contribute significantly to TGF-β-induced osteophyte formation and produce factors, possibly BMP-2 and BMP-4, that, in concert with TGF-β, can induce cartilage formation by mesenchymal cells in vitro (van Lent et al. 2004).

Adverse effects of TGF-β may result partly from synergy with IL-1 (Takahashi et al. 2005) and induction of expression of aggrecanase-1 and
urokinase-type plasminogen activator (uPA) in synovial fibroblasts (Hamilton et al. 1991; Yamanishi et al. 2002), possibly contributing to matrix degradation as seen upon increased TGF-β1 expression in antigen-induced arthritic rabbit knees (Mi et al. 2003).

Osteoarthritis seems to protect against osteoporotic fractures (Dequeker et al. 2003), and an inverse correlation has been found between the occurrence of a Leu10Pro polymorphism in the TGF-β1 signal peptide sequence, caused by a T→C base substitution, and the frequency of spinal osteoarthritis and osteoporosis in the Japanese population. The CC genotype correlates with a higher risk for osteoarthritis than the TC or TT genotype, and the C allele is associated with elevated TGF-β serum levels. On the other hand, the T allele is a risk factor for genetic susceptibility to postmenopausal osteoporosis in Japanese women (Yamada et al. 1998; Yamada 2000). Recently, it was shown that the T allele is also associated with increased inflammatory activity, poor long-term disease outcome, and mortality in rheumatoid arthritis of Northern European patients (Mattey et al. 2005).

**TGFB1 POLYMORPHISMS AND OSTEOPOROSIS**

Osteoporosis commonly affects postmenopausal women and the elderly and is characterized by increased fracture risk resulting from reduced bone quality, including low bone mass and deteriorated micro-architecture of the bone. Multiple factors contribute to the etiology of osteoporosis, and the genetic susceptibility of low bone density may result from combined effects of polymorphisms in a number of genes involved in bone homeostasis. Although many polymorphisms in the TGFB1 gene exist, only a few are associated with bone mineral density, osteoporosis, and/or fracture risk. The pathophysiological mechanisms by which these TGFB1 polymorphisms affect bone density and susceptibility to bone fracture are not known. The availability of TGF-β1 is likely altered as a result of altered splicing or stability of the TGF-β1 mRNA, intracellular trafficking and/or efficiency of export of the TGF-β1 preproprotein, or transcriptional activation of the TGFB1 promoter (Hinke et al. 2001; Yamada 2001; Dick et al. 2003; Langdahl et al. 2003). Recently, TGF-β was shown to regulate material properties of bone matrix, but the role of these properties in osteoporosis requires further investigation (Balooch et al. 2005). Polymorphisms in the BMP2 and BMP4 genes have also been linked to low bone mass and osteoporosis (Styrkarsdottir et al. 2003; Ramesh Babu et al. 2005; Fritz et al. 2006).
ECTOPIC BONE FORMATION

Fibrodysplasia ossificans progressiva (FOP), also known as myositis ossificans progressiva, is a rare genetic connective tissue disorder characterized by progressive postnatal endochondral ossification of tendons, ligaments, fascia, and striated muscle. In affected individuals, osteogenic lesions often arise spontaneously, but they can be induced by surgery, trauma, or intramuscular injections. Interestingly, normal skeletogenesis is not affected.

Although the pathophysiology of initiation and development of early FOP lesions is largely unknown, the current hypothesis is that BMP-4 overexpressing lymphocytes infiltrate connective tissue after local soft-tissue injury. The elevated local BMP-4 levels may cause recruitment of mesenchymal stem cells, which in turn produce autocrine BMP-4 and develop into fibroproliferative and, ultimately, osseous lesions (Shafritz et al. 1996; Kan et al. 2004). In support of this hypothesis, BMP signaling is up-regulated in several ways in lymphoblastoid cell lines (LCLs) and fibroblast-like lesional cells from FOP patients (Shafritz et al. 1996; Gannon et al. 1997). Specifically, these cells display increased BMP-4 transcription (Olmsted et al. 2003), increased levels of hyperphosphorylated BMPRIA at the cell surface (de la Pena et al. 2005), increased BMP-inducible MAP kinase activation (Fiori et al. 2006), and decreased expression of BMP antagonists (Ahn et al. 2003). Targeted overexpression of BMP-4 in similar cell types of mice also results in an FOP-like phenotype (Kan et al. 2004). Although these studies suggest that increased BMP signaling contributes to the development of FOP, linkage analysis and mutational screening indicate that the BMP4 or BMPR1A genes are not the genetic cause of FOP. In fact, the genetic defect involves a heterozygous Arg206His mutation in the glycine-serine domain of the BMP type I receptor ALK-2, causing instability of the receptor and possibly resulting in increased activation of ALK-2 (Shore et al. 2006). More research is needed to understand the molecular mechanisms of FOP.

SCLEROSING BONE DISORDERS

Activating Mutations in TGFB1 Cause Camurati–Engelmann Disease

TGFB1 mutations resulting in a mutant signal peptide or the propeptide LAP form the pathological basis for Camurati–Engelmann disease (CED), also referred to as progressive diaphyseal dysplasia. CED is a craniofacial dysplasia, characterized by hyperostosis and sclerosis of the diaphyses of the long bones and base of the skull. Progressive muscle fibrosis is also frequently observed. Most TGFB1 mutations associated with CED are
located in exon 4 (Janssens et al. 2000, 2006; Kinoshita et al. 2000, 2004), which encodes the carboxy-terminal sequence of LAP and is important for its interaction with mature TGF-β1 (see Chapter 7). These mutations result in a significant increase in bioactive TGF-β1 secreted from the cell and correlate with enhanced TGF-β signaling (Saito et al. 2001; Janssens et al. 2003). Other CED-associated mutations in the signal peptide of the TGF-β1 precursor or in the amino-terminal segment of the LAP interfere with intracellular trafficking or secretion. These mutations enhance intracellular activation of the TGF-β pathway (Janssens et al. 2003). Thus, elevated TGF-β1 signaling that favors bone formation over bone resorption appears to underlie the pathophysiological mechanism of CED.

As TGF-β1 is made by many cell types, it is intriguing that CED patients predominantly show hyperostosis and muscle fibrosis without other tissues being affected. Bone abnormalities may prevail in CED patients because osteoblasts, in contrast to many other cell types, also secrete small latent TGF-β complexes lacking LTBP5 (Bonewald et al. 1991; Dallas et al. 1994). LTBP5 facilitate folding and secretion of the TGF-β1 complex and can modulate TGF-β bioavailability (see Chapter 7). Interestingly, LTBP-3-null mice show osteosclerotic bone malformations that affect the long bones and skull (Dabovic et al. 2002). Locus heterogeneity has been described to underlie CED (Hecht et al. 2001; Nishimura et al. 2002), and the identification of another modifying gene or locus linked to CED may provide clues as to why no other tissues are affected by the TGF-β1 mutations.

Loss of Sclerostin, a BMP Antagonist, Leads to Sclerosteosis and van Buchem Disease

Among the BMP antagonists is sclerostin, a member of the DAN family of secreted cystine-knot-containing glycoproteins that is encoded by the SOST gene. Mutations in SOST are the genetic basis for sclerosteosis and van Buchem disease, two progressive craniofacial hyperostotic disorders. Loss-of-function mutations in SOST prevail in sclerosteosis, whereas in van Buchem disease, a homozygous 52-kbp deletion downstream from SOST has been identified, probably harboring regulatory elements that control SOST expression (Balemans et al. 2001, 2002; Brunkow et al. 2001). Loss of SOST expression results in increased osteoblast activity, causing thicker than normal trabeculae and cortical bone, increased bone mineral density, and increased bone strength. This leads to massive bone overgrowth throughout postnatal life and entrapment of cranial nerves and mandibular overgrowth. Tall stature and syndactyly discriminates sclerosteosis from the milder van Buchem disease.
Although sclerostin can bind to various BMPs and interferes with their binding to BMP receptors (Winkler et al. 2003), sclerostin inhibits BMP-stimulated bone formation indirectly through binding to the LRP5 and LRP6 Wnt coreceptors, thus antagonizing downstream Wnt signaling (Li et al. 2005; Semenov et al. 2005; Ellies et al. 2006; van Bezooijen et al. 2007). Excessive postnatal bone deposition in sclerosteosis patients likely results from unopposed Wnt signaling due to loss of sclerostin expression in osteocytes. Underscoring the role of sclerostin in bone homeostasis, mice overexpressing SOST under control of the osteocalcin promoter display an osteopenic phenotype with reduced bone formation and fragile bones, resembling osteoporosis (Winkler et al. 2003).

Loss of LEMD3, a Smad Antagonist, Results in Osteopoikilosis

Osteopoikilosis is a skeletal dysplasia characterized by symmetric but unequal distribution of hyperostotic areas in different parts of the skeleton. Buschke-Ollendorff syndrome (BOS) and melorheostosis are allelic variants of osteopoikilosis with additional abnormalities, including sclerotic skin lesions. Linkage analysis revealed that these disorders are caused by total deletion of LEMD3 (LEM domain-containing 3, also called MAN1) or heterozygous loss-of-function mutations in LEMD3 (Hellemans et al. 2004; Menten et al. 2007). LEMD3 is an integral protein of the inner nuclear membrane and antagonizes Smad activity through interaction of its RNA recognition motif (RRM) region with the MH2 domain of BMP- or TGF-β-activated Smads. This prevents R-Smad–Smad4 interaction and nuclear translocation, resulting in impaired TGF-β- and BMP-induced gene expression (Osada et al. 2003; Hellemans et al. 2004; Lin et al. 2005).

The LEMD3 loss-of-function mutations identified so far result in either decay of mRNA from the mutant allele or expression of truncated LEMD3 protein lacking the Smad-interacting segment (Hellemans et al. 2004, 2006). Expression of loss-of-function mutants fails to reduce TGF-β-induced luciferase reporter activation; moreover, skin fibroblasts from an affected person showed enhanced expression of a TGF-β-responsive gene (Hellemans et al. 2004). These findings suggest that haploinsufficiency of LEMD3 confers increased BMP and/or TGF-β signaling, which in turn leads to hyperostotic bone lesions in osteopoikilosis, BOS, and melorheostosis. As LEMD3 appears to be ubiquitously expressed (Lin et al. 2000), it is unclear at present why a specific bone phenotype is observed in osteopoikilosis.

Other genetic factors may contribute to the additional abnormalities in BOS and melorheostosis, as no somatic mutations in the second
LEMD3 allele occur in skin and bone lesions of these patients (Hellemans et al. 2004, 2006; Mumm et al. 2007). Increased TGF-β signaling has been implicated in the development of skin fibrosis and may contribute to the skin lesions associated with BOS and melorheostosis.

CANCER METASTASIS TO BONE

Osteolytic Breast Cancer Bone Metastases

Bone provides an attractive microenvironment for the homing and metastasis of particular cancer types. Breast tumor cells preferentially and frequently metastasize to bone where they give rise to osteolytic lesions, causing hypercalcemia, bone pain and fracture, and nerve compression syndromes (Pluijm et al. 2000).

An important mediator of breast-tumor-induced bone destruction is PTHrP (parathyroid-hormone-related protein), which stimulates RANKL expression and inhibits OPG expression by osteoblasts, thereby activating osteoclasts (Guise and Chirgwin 2003). Autocrine PTHrP secretion by the breast tumor cells causes bone destruction that results in release of TGF-β from the bone matrix. In turn, TGF-β promotes progression of osteolytic bone metastases by inducing PTHrP expression by breast tumor cells in a Smad- and p38 MAP kinase-dependent manner (Yin et al. 1999; Kakonen et al. 2002), thus establishing a positive feedback loop for mutual control of PTHrP and TGF-β expression in tumor-induced bone destruction (Guise and Chirgwin 2003).

In addition to stimulating osteoclast activity, breast tumor cells may aggravate osteolytic bone lesions by inhibiting osteoblast differentiation and function. TGF-β produced by the breast cancer cells mediates this inhibition and may furthermore reduce adhesion and induce apoptosis of osteoblasts (Mastro et al. 2004; Mercer et al. 2004). Breast tumor cells metastasized to bone show a prominent increase in expression of the angiogenic connective tissue growth factor (CTGF) and IL-11, an activator of osteoclast differentiation (Kang et al. 2003). TGF-β induces the expression of IL-11 and CTGF (Chirgwin and Guise 2000; Kang et al. 2003, 2005). Smad4 is essential for induction of IL-11 and contributes to the formation of osteolytic bone metastases (Kang et al. 2005; Deckers et al. 2006). Combined overexpression of IL-11 and CTGF together with osteopontin endows breast cancer cells with potent metastatic activity, suggesting mechanisms by which TGF-β may promote breast-cancer-induced bone destruction.
Osteoblastic Prostate Cancer Bone Metastasis

In contrast to breast cancer cells that generally cause osteolytic bone metastases, dissemination of prostate cancer cells to bone typically results in osteoblastic metastases, resulting in bone pain and increased risk of bone fracture due to the poor quality of newly formed bone. The molecular mechanisms that contribute to osteoblastic metastasis are poorly understood, but a role for TGF-β and BMPs is suspected.

Elevated TGF-β expression by prostate cancer cells correlates with poor prognosis (Ivanovic et al. 1995). Prostate tumor cells with increased TGF-β1 expression have lost sensitivity to the growth-inhibitory effects of TGF-β and instead show enhanced tumorigenicity and metastasis (Steiner and Barrack 1992), to which various mechanisms may contribute. Thus, bone-derived TGF-β1 is chemotactic and stimulates invasion of prostate cancer cells in vitro (Festuccia et al. 1999a,b, 2000). TGF-β also induces the expression of proteases by these cells, including prostate-specific antigen (PSA) and uPA, which may not only facilitate tumor invasiveness, but also activate latent TGF-β (Killian et al. 1993; Festuccia et al. 1999a, 2000). TGF-β induces the expression of the \( \alpha_2 \beta_1 \) and \( \alpha_3 \beta_1 \) integrins by prostate cancer cells, thus resulting in increased adhesion to type I collagen (Kostenuik et al. 1997; Festuccia et al. 1999a), which may enable disseminating prostate cancer cells to adhere to bone matrix.

Increased expression of BMP-6 or BMP-7 (Hamdy et al. 1997; Masuda et al. 2003) and reduced expression of BMP-2, Smad4, Smad8, or BMP receptors have been detected in bone metastases of prostate cancer and correlated with poor prognosis (Ide et al. 1997; Kim et al. 2000; Horvath et al. 2004). Proliferation and tumorigenicity of prostate tumor cell lines can be inhibited by increasing BMP signaling (Brubaker et al. 2004; Miyazaki et al. 2004), and reduced Smad and BMP receptor expression during malignant progression might allow prostate tumor cells to escape the growth-inhibitory effects of BMPs. BMPs expressed by prostate cancer cells can induce osteoblasts to form new bone, resulting in osteoblastic lesions (Dai et al. 2005).

FRACTURE HEALING

Fracture healing largely recapitulates embryonic endochondral bone formation and involves repair of broken bone to original quality and function. Upon injury, chemotactic factors including TGF-β and BMPs are released from the bone matrix and platelets. In addition, inflamma-
tory cells, such as monocytes, macrophages, and T cells, that are recruited into the fracture site (Andrew et al. 1994) secrete cytokines and growth factors, including TGF-β1 and BMP-2 (Champagne et al. 2002), that are chemotactic and osteoinductive to mesenchymal precursor cells, pre-osteoblasts, and chondroblasts (Cunningham et al. 1992; Lind et al. 1996). These cells consequently proliferate and undergo chondrogenic and/or osteogenic differentiation to form a callus that fills the fracture gap. Although the osteoinductive capacity of TGF-β is overall weak in comparison to BMPs, TGF-β1 synergizes with BMPs and insulin-like growth factor-1 in fracture healing (Schmidmaier et al. 2003; Centrella et al. 1994), likely by enhancing recruitment and proliferation of progenitor cells to the fracture site. The distinct spatial and temporal expression patterns of various TGF-βs, BMPs, and GDFs during fracture healing (Cho et al. 2002) suggest that they may have unique roles during bone healing. In addition, the expression of distinct BMP type I and type II receptors is up-regulated during fracture healing, and the spatial distribution of these receptors resembles that of BMP-2, BMP-4, and BMP-7 (Ishidou et al. 1995; Onishi et al. 1998).

Although their effects on bone are complex, ectopic application of TGF-β, BMP-2, BMP-4, or BMP-7 in animal models of fracture healing as well as in patients revealed a potent role for these growth factors in regeneration of bone at the fracture site, repair of critical-size defects, improved healing of non-union fractures, and accelerating fracture healing, resulting in increased bone or callus formation and mechanical stability (Joyce et al. 1990; Yasko et al. 1992; Lind et al. 1993; Cook et al. 1994; Nielsen et al. 1994; Bostrom et al. 1996; Friedlaender et al. 2001; Govender et al. 2002; Einhorn et al. 2003; Edwards et al. 2004). Calibration of growth factor dose and timing will improve therapeutic outcomes by targeting specific cell populations at the appropriate stage of differentiation.

THERAPEUTIC POTENTIAL

The role of TGF-β family members in skeletal disorders involves, on the one hand, increased signaling as seen in conditions such as FOP and sclerosing bone disorders, osteosarcomas with expression of BMPs and/or BMPRII, pathological conditions of ectopic ossification affecting the spinal ligament, and traumatic heterotopic ossification as is frequently seen in patients that have had head and neck traumas or total hip replacement. On the other hand, reduced signaling by TGF-β family members can also contribute to development of skeletal disorders, as is the case in slowly healing or non-union fractures.
For those disorders that result from excessive BMP signaling, BMP antagonists such as noggin might restore the balance in BMP activity. Thus, intramuscular gene transfer of Noggin prevents onset and progression of the spondyloarthropathy ankylosing enthesitis (Lories et al. 2005), a chronic inflammatory joint disorder characterized by joint ankylosis due to new bone formation in which BMP-2, BMP-6, and BMP-7 are thought to have a role. Promise holds for deletion mutants of stable osteoinhibitory BMP folding variants, which were even more effective than noggin in inhibiting BMP activity by competing with natural BMPs for binding to the BMP receptors (Weber et al. 2003).

Drugs that selectively enhance the expression of TGF-β or BMPs may provide therapeutic approaches for diseases such as arthritis and osteoporosis. Diacerein and avocado/soya unsaponifiables have been shown to be beneficial both in animal models of osteoarthritis and in patients with osteoarthritis (Maheu et al. 1998; Dougados 2001) and are thought to act by inducing expression of TGF-β1 and TGF-β2 (Boumediene et al. 1999; Felisaz et al. 1999). Isoflavones extracted from Sophorae fructus act like estrogen by binding to estrogen receptors and may be useful in the treatment of estrogen-deficient osteoporosis patients, because these isoflavones were shown to induce expression of TGF-β (Joo et al. 2004).

Combined therapies might be necessary for diseases such as rheumatoid arthritis and osteoarthritis whereby TGF-β has a protective role as well as a pathological role. Thus, application of TGF-β to reduce inflammation and promote cartilage repair, combined with a BMP antagonist to reduce osteophyte formation, might control the disease. BMP-2, BMP-7, and GDF-5 have been shown to be effective in animal models of (osteoc)chondral defects in cartilage repair or intervertebral disc cartilage repair (Mont et al. 2004; Chujo et al. 2006; Masuda et al. 2006). Studies involving in vivo gene therapy of arthritis look promising (Evans et al. 2006), but clinical application will depend on the development of safe and effective vectors. Cell-mediated gene transfer provides an alternative, safer method for gene therapy. Injection of ex-vivo-transduced mesenchymal cells with adenoviral BMP-2 enables repair of articular cartilage defects in rodents, although osteophyte formation was observed (Gelse et al. 2003).

BMPs are attractive molecules for orthopedic applications and are shown to be effective and safe in the healing of critical-size defects and delayed or non-union fractures (Friedlaender et al. 2001; Govender et al. 2002) (see Chapter 33). BMP-2 and BMP-7 have been approved by the U.S. Food and Drug Administration for treatment of open tibial fractures and spinal fusion. BMP-9 and BMP mixtures extracted from bovine bone are under investigation in clinical trials.
For regeneration of cartilage and bone, optimal effects are achieved when BMPs are applied in combination with a carrier that prolongs the residence time and function of BMPs at target sites and allows for a significant reduction in the quantity of BMP required compared to bolus injections (Seeherman and Wozney 2005). In humans, BMPs loaded onto collagen type I carriers are as effective as autogenous bone grafting (Friedlaender et al. 2001; Johnsson et al. 2002) and optimization of dosage, mixture of BMPs added, time course, release dynamics, and composition of matrix carrier may ultimately result in therapeutic application of BMPs that are superior to bone grafts.

When designing experiments to study the role of a particular TGF-β family member in a certain disease process, it should be realized that these growth factors exert pleiotropic effects and may need to synergize with other family members to achieve the desired biological response and that the biological effect triggered by addition of a particular TGF-β family member will depend on growth factor concentration, species, cell type, stage of differentiation, and local presence of additional growth factors. A tempting task for the future will be to reveal optimal growth factor or growth factor antagonist concentrations, combinations, delivery methods, and duration of growth factor and/or antagonist administration to functionally restore the affected tissues in the various skeletal disorders in which TGF-β family members are involved.

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