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Bone Morphogenetic Proteins

DI CHEN[a], MING ZHAO[b] and GREGORY R. MUNDY[b,*]

[a]*School of Medicine and Dentistry, Department of Orthopaedics, University of Rochester, Rochester, NY 14642, USA;*
[b]*Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX 78229, USA*

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Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor β (TGFβ) superfamily. The roles of BMPs in embryonic development and cellular functions in postnatal and adult animals have been extensively studied in recent years. Signal transduction studies have revealed that Smad1, 5 and 8 are the immediate downstream molecules of BMP receptors and play a central role in BMP signal transduction. Studies from transgenic and knockout mice and from animals and humans with naturally occurring mutations in BMPs and related genes have shown that BMP signaling plays critical roles in heart, neural and cartilage development. BMPs also play an important role in postnatal bone formation. BMP activities are regulated at different molecular levels. Preclinical and clinical studies have shown that BMP-2 can be utilized in various therapeutic interventions such as bone defects, non-union fractures, spinal fusion, osteoporosis and root canal surgery. Tissue-specific knockout of a specific BMP ligand, a subtype of BMP receptors or a specific signaling molecule is required to further determine the specific role of a BMP ligand, receptor or signaling molecule in a particular tissue.

BMPs are members of the TGFβ superfamily. The activity of BMPs was first identified in the 1960s (Urist, M.R. (1965) "Bone formation by autoinduction", *Science* **150**, 893–899), but the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and 4 in the late 1980s (Wozney, J.M. *et al.* (1988) "Novel regulators of bone formation: molecular clones and activities", *Science* **242**, 1528–1534; Luyten, F.P. *et al.* (1989) "Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation", *J. Biol. Chem.* **264**, 13377–13380; Wozney, J.M. (1992) "The bone morphogenetic protein family and osteogenesis", *Mol. Reprod. Dev.* **32**, 160–167). To date, around 20 BMP family members have been identified and characterized. BMPs signal through serine/threonine kinase receptors, composed of type I and II subtypes. Three type I receptors have been shown to bind BMP ligands, type IA and IB BMP receptors (BMPR-IA or ALK-3 and BMPR-IB or ALK-6) and type IA activin receptor (ActR-IA or ALK-2) (Koenig, B.B. *et al.* (1994) "Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells", *Mol. Cell. Biol.* **14**, 5961–5974; ten Dijke, P. *et al.* (1994) "Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4", *J. Biol. Chem.* **269**, 16985–16988; Macias-Silva, M. *et al.* (1998) "Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2", *J. Biol. Chem.* **273**, 25628–25636). Three type II receptors for BMPs have also been identified and they are type II BMP receptor (BMPR-II) and type II and IIB activin receptors (ActR-II and ActR-IIB) (Yamashita, H. *et al.* (1995) "Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects", *J. Cell. Biol.* **130**, 217–226; Rosenzweig, B.L. *et al.* (1995) "Cloning and characterization of a human type II receptor for bone morphogenetic proteins", *Proc. Natl Acad. Sci. USA* **92**, 7632–7636; Kawabata, M. *et al.* (1995) "Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-β receptor", *J. Biol. Chem.* **270**, 5625–5630). Whereas BMPR-IA, IB and II are specific to BMPs, ActR-IA, II and IIB are also signaling receptors for activins. These receptors are expressed differentially in various tissues. Type I and II BMP receptors are both indispensable for signal transduction. After ligand binding they form a heterotetrameric-activated receptor complex consisting of two pairs of a type I and II receptor complex (Moustakas, A. and C.H. Heldi (2002) "From mono- to oligo-Smads: the heart of the matter in TGFβ signal transduction" *Genes Dev.* **16**, 67–871). The type I BMP receptor substrates include a protein family, the Smad proteins, that play a central role in relaying the BMP signal from the receptor to target genes in the nucleus. Smad1, 5 and 8 are phosphorylated by BMP receptors in a ligand-dependent manner (Hoodless, P.A. *et al.* (1996) "MADR1, a MAD-related protein that functions in BMP2 signaling pathways", *Cell* **85**, 489–500; Chen Y. *et al.* (1997) "Smad8 mediates the signaling of the receptor serine kinase", *Proc. Natl Acad. Sci. USA* **94**, 12938–12943; Nishimura R. *et al.* (1998) "Smad5 and DPC4 are key molecules in

*Corresponding author. E-mail: mundy@uthscsa.edu

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mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12", *J. Biol. Chem.* **273**, 1872–1879). After release from the receptor, the phosphorylated Smad proteins associate with the related protein Smad4, which acts as a shared partner. This complex translocates into the nucleus and participates in gene transcription with other transcription factors (Fig. 1). A significant advancement about the understanding of *in vivo* functions of BMP ligands, receptors and signaling molecules has been achieved in recent years.

Keywords: Bone morphogenetic proteins; Transforming growth factor β; Bone; Fibrodysplasia ossificans progressiva

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BIOLOGICAL FUNCTIONS OF BMPs

Bone morphogenetic proteins (BMPs) have been implicated in a variety of functions. BMPs induce the formation of both cartilage and bone. BMPs also play a role in a number of non-osteogenic developmental processes. Neural induction represents the earliest step in the determination of ectodermal cell fates. In vertebrates, BMPs act as signals of epidermal induction (Muñoz-Sanjuán and Brivanlou, 2002). BMP-2 directs the development of neural crest cells into neuronal phenotypes (Christiansen *et al.*, 2000), while BMP-4 and 7 specifically induce a sympathetic adrenergic phenotype. BMPs give direction to somite development by inhibiting the process of myogenesis. In the limb bud, BMP-2 interacts with the fibroblast growth factor 4 and sonic

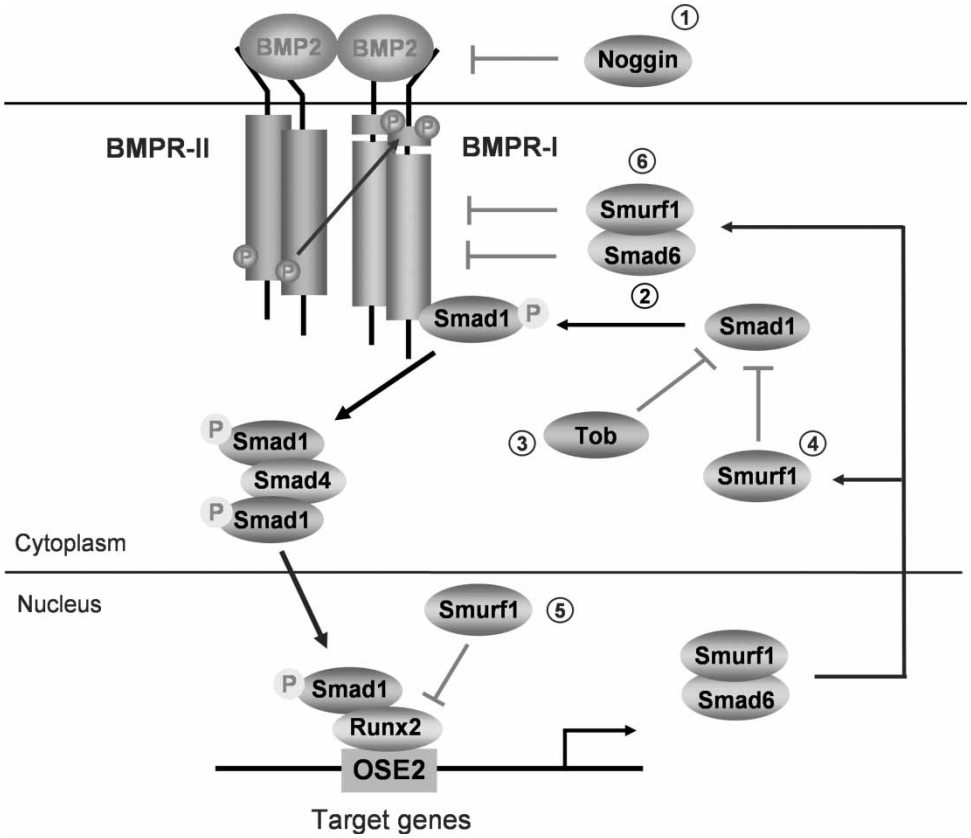

FIGURE 1 BMP signaling and its regulation. BMP signals are mediated by type I and II BMP receptors and their downstream molecules Smad1, 5 and 8. Phosphorylated Smad1, 5 and 8 proteins form a complex with Smad4 and then are translocated into the nucleus where they interact with other transcription factors, such as Runx2 in osteoblasts. BMP signaling is regulated at different molecular levels: (1) Noggin and other cystine knot-containing BMP antagonists bind with BMP-2, 4 and 7 and block BMP signaling. Over-expression of noggin in mature osteoblasts causes osteoporosis in mice (Devlin *et al.*, 2003; Wu *et al.*, 2003). (2) Smad6 binds type I BMP receptor and prevents Smad1, 5 and 8 to be activated (Imamura *et al.*, 1997). Over-expression of Smad6 in chondrocytes causes delays in chondrocyte differentiation and maturation (Horiki *et al.*, 2004). (3) Tob interacts specifically with BMP activated Smad proteins and inhibits BMP signaling. In Tob null mutant mice, BMP signaling is enhanced and bone formation is increased (Yoshida *et al.*, 2000). (4) Smurf1 is a Hect domain E3 ubiquitin ligase. It interacts with Smad1 and 5 and mediates the degradation of these Smad proteins (Zhu *et al.*, 1999). (5) Smurf1 also recognizes bone-specific transcription factor Runx2 and mediates Runx2 degradation (Zhao *et al.*, 2003). (6) Smurf1 also forms a complex with Smad6, is exported from the nucleus and targeted to the type I BMP receptors for their degradation (Murakami *et al.*, 2003). Over-expression of Smurf1 in osteoblasts inhibits postnatal bone formation in mice (Zhao *et al.*, 2004).

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hedgehog, inhibits limb bud expansion and induces the formation of chondrocyte and osteoblast precursors (Niswander and Martin, 1993; Wall and Hogan, 1994).

Physiological roles of BMPs and BMP receptor signaling in normal bone formation have been investigated. Injection of BMP-2 locally over the surface of calvariae of mice induces periosteal bone formation on the surface of calvariae without a prior cartilage phase (Chen *et al.*, 1997). Over-expression of a dominant-negative truncated BMPR-IB in osteoblast precursor 2T3 cells inhibits osteoblast-specific gene expression and mineralized bone matrix formation (Chen *et al.*, 1998). In the transgenic mice in which expression of a dominant-negative truncated BMPR-IB transgene is targeted to the osteoblast lineage using the osteoblast-specific type I collagen promoter, the postnatal bone formation, including bone mineral density, static bone volume and dynamic bone formation rates, is decreased (Zhao *et al.*, 2002). These results demonstrate that BMP receptor signaling plays a necessary role in normal postnatal bone formation.

MUTATIONS IN BMPs AND BMP RECEPTORS

Studies of naturally occurring mutations of BMPs and BMP receptors have shown that BMPs play important roles in several inherited diseases. In mice with short ear mutations BMP-5 gene was disrupted. This mutation in the BMP-5 gene is associated with a wide range of skeletal defects, including reductions in long bone width and the size of several vertebral processes and an overall lower body mass (Kingsley *et al.*, 1992; Mikic *et al.*, 1995). Mutations in growth/differentiation factor-5 (GDF-5, CDMP-1 and BMP-11) gene result in brachypodism in mice (Storm *et al.*, 1994) and chondrodysplasia in humans (Thomas *et al.*, 1996; Thomas *et al.*, 1997). Both BMP-5 and GDF-5 genes are localized on chromosome 2 in mice and on chromosome 20 in humans (Storm *et al.*, 1994). GDF-5 has been shown to bind BMPR-IB specifically (Nishitoh *et al.*, 1996) and null mutations in the BMPR-IB gene causes a similar skeletal phenotype as that observed in GDF-5 mutant mice (Yi *et al.*, 2000).

Fibrodysplasia ossificans progressiva (FOP) is an extremely rare and disabling genetic disorder characterized by congenital malformations of the great toes and by progressive heterotopic endochondral ossification in predictable anatomical patterns. Ectopic expression of BMP-4 was found in FOP patients (Gannon *et al.*, 1997; Xu *et al.*, 2000). Familial primary pulmonary hypertension is a rare autosomal dominant disorder that has been mapped to chromosome 2q33. Monoclonal plexiform lesions of proliferating endothelial cells in pulmonary arterioles are the major phenotype of this disease. These lesions lead to elevated pulmonary artery pressure, right ventricular failure and death. After genotyping multiple families with this disorder, BMPR-II mutations have been found in these patients (Lane *et al.*, 2000; Deng *et al.*, 2000; Newman *et al.*, 2001). Mutations in GDF-9 and GDF-9b genes have been found in patients with premature ovarian failure and polycystic ovary syndrome (Takebayashi *et al.*, 2000). Over-expression of BMP-2, 4 and 5 and BMPR-IA is associated with malignancy of the oral epithelium (Jin *et al.*, 2001) and over-expression of BMP-3 and 2 has been described in prostate cancer cells (Harris *et al.*, 1994).

NULL MUTATIONS OF BMPs, BMP RECEPTORS AND SMADS

To determine the roles of BMP ligands, receptors and signaling proteins in embryonic development and in postnatal life, null mutations of BMP ligands, receptors and Smad genes have been created and phenotypic changes in these animals have been extensively studied. Mice deficient for BMP-2 and 4 are nonviable. Homozygous BMP-2 mutant embryos die between embryonic day 7.5 (E7.5) and 10.5 (E10.5) and have defects in cardiac development, manifested by the abnormal development of the heart in the exocoelomic cavity (Zhang and Bradley, 1996). Homozygous BMP-4 mutant embryos die between E6.5 and E9.5 and show little or no mesodermal differentiation (Winnier *et al.*, 1995). BMP-7 deficient mice die shortly after birth because of poor kidney development. Histological analysis of mutant embryos at several stages of development reveals that metanephric mesenchymal cells fail to differentiate, resulting in a virtual absence of glomerulus in newborn kidneys. In addition, BMP-7 deficient mice have eye defects that appear to originate during lens induction. BMP-7 deficient mice have minor defects in the skeleton (Dudley *et al.*, 1995; Luo *et al.*, 1995). BMP-6 deficient mice are viable and fertile, and show no overt defects in tissues known to express BMP-6 mRNA (Solloway *et al.*, 1998). BMP-6 is mainly expressed in hypertrophic cartilage. Since BMP-2 and 6 are co-expressed in this tissue, BMP-2 may functionally compensate the loss of BMP-6 in BMP-6 null mutant mice. Conditional knockout of BMP-2 gene in cartilage with BMP-6 null mutation will be required to answer this question.

Growth/differentiation factor-8 (GDF-8, myostatin) is expressed specifically in developing and adult skeletal muscle. During early stages of embryogenesis, GDF-8 expression is restricted to the myotome compartment of developing somites. At later stages and in adult animals, GDF-8 is expressed in many different muscles throughout the body. GDF-8 null mutant mice are significantly larger than wild-type mice and show a large and widespread increase in skeletal muscle mass (McPherron *et al.*, 1997).

Null mutation of the BMPR-IA gene causes embryonic lethality in mice. Animals die at E9.5. Homozygous mutants with morphological defects are first detected at E7.5. No mesoderm forms in the mutant embryos, suggesting that BMPR-IA is essential for the inductive events that lead to the formation of mesoderm during gastrulation (Mishina *et al.*, 1995). Mice lacking

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BMPR-IB are viable and exhibit defects in the appendicular skeleton. In BMPR-IB deficient mice, proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region are markedly reduced. In adult mutant mice, the proximal interphalangeal joint is absent, and the phalanges are replaced by a single rudimentary element, while the distal phalanges are unaffected. The lengths of the radius, ulna and tibia are normal, but the metacarpals and metatarsals are reduced (Yi *et al.*, 2000). The appendicular defects in BMPR-IB mutant mice resemble those seen in mice homozygous for the GDF-5^{bp-j} null allele of the GDF-5 locus. Since GDF-5 has been shown to play a critical role in cartilage formation and binds BMPR-IB with high affinity (Gannon *et al.*, 1997), these results suggest that BMPR-IB plays a non-redundant role in cartilage formation *in vivo*. BMP ligands may utilize multiple type I BMP receptors to mediate their signaling during cartilage and bone formation. In BMPR-IB and BMP-7 double mutant mice, severe appendicular skeletal defects have been observed in the forelimbs and hind limbs. The ulna is nearly absent and the radius is shortened (Yi *et al.*, 2000). Since BMP-7 binds efficiently to both BMPR-IB and ActR-IA (Alk2) (Macias-Silva *et al.*, 1998), it is conceivable that BMPR-IB and ActR-IA (Alk2) play important synergistic or overlapping roles in cartilage and bone formation *in vivo*.

Smad1 null mutant mice die at E10.5 because they fail to connect to the placenta. Smad1 mutant embryos show overgrowth of the posterior visceral endoderm as well as extra-embryonic ectoderm and mesoderm of the chorion. The overgrowth effect on the allantois in Smad1 mutant embryos leads to a dramatic reduction in the size and patterning of this tissue and concomitant failure to form the umbilical connection to the placenta (Tremblay *et al.*, 2001). Homozygous Smad5 null mutant mice die between days 10.5 and 11.5 of gestation due to defects in angiogenesis. The mutant yolk sacs lack normal vasculature and had irregularly distributed blood cells. Smad5 mutant embryos have enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells (Yang *et al.*, 1999). These findings suggest that Smad5 may regulate endothelium-mesenchyme interactions during angiogenesis.

NEGATIVE REGULATION OF BMP SIGNALING

BMPs are potent stimulators on bone formation and on other cellular functions. The activity of BMPs is controlled at different molecular levels: (1) a series of BMP antagonists bind BMP ligands and inhibit BMP functions, (2) Smad6 is member of the Smad family. It binds type I BMP receptors and prevents the binding and phosphorylation of Smad1 and 5, (3) tob is an anti-proliferative protein. It selectively binds Smad1 and 5 and inhibits BMP signaling in osteoblasts and (4) Smad ubiquitin regulatory factor 1 (Smurf1) is an E3 ubiquitin ligase. It interacts with Smad1 and 5 and mediates the degradation of these Smad proteins.

The mutations of the BMP antagonists have shown how important the activity of BMPs is controlled in a given system. For example, proximal symphalangism is an autosomal-dominant disorder with ankylosis of the proximal interphalangeal joints, carpal and tarsal bone fusion and conductive deafness. These symptoms are shared by another disorder of joint morphogenesis, multiple synostoses syndrome. Recently, it was reported that both disorders were caused by heterozygous mutations of the human noggin gene. To date, seven mutations of noggin gene have been identified from unrelated families affected with joint morphogenesis (Gong *et al.*, 1999; Takahashi *et al.*, 2001). Noggin is a secreted polypeptide which binds and inactivates BMP-2, 4 and 7. Co-crystal structures of noggin and BMP-7 show that noggin inhibits BMP signaling by blocking the molecular interfaces of the binding epitopes for both type I and II BMP receptors. The type I and II receptor binding domains on each BMP-7 monomer interact with a specific clip section from each monomer of the dimeric noggin complex (Groppe *et al.*, 2002), thus preventing BMP-7 to bind with BMP receptors. This 3D crystal structure clearly shows how noggin specifically inhibits BMP-2, 4 and 7. A transgenic mouse model has recently been established using the osteocalcin promoter to drive the noggin transgene. The animals develop osteoporosis. Significant reductions in bone mineral density, bone volume and bone formation rates are observed (Fig. 1) (Devlin *et al.*, 2003; Wu *et al.*, 2003).

Sclerostosis is a recessive inherited osteosclerotic disorder caused by mutations in the protein sclerostin. The disease was initially considered to be a variant of osteopetrosis (Truswell, 1958), but subsequent metabolic studies reveal that the disorder is primarily due to increased bone formation, rather than defects in bone resorption (Stein *et al.*, 1983). Recently, it was found that sclerostin is related in sequence to the family of secreted BMP antagonists, which includes Noggin, Chordin, Gremlin and Dan. Sclerostin is expressed in osteoblasts and osteocytes and binds BMP-5, 6 and 7 with high affinity. Expression of sclerostin in multipotent fibroblast C3H10T1/2 cells blocks osteoblast differentiation and over-expression of sclerostin in osteoblasts under the control of the osteocalcin promoter in transgenic mice causes osteoporosis (Winkler *et al.*, 2003). Taken together, these findings provide evidences that activation of endogenous BMP signaling can enhance bone formation, and regulation of the amount of BMP activity in postnatal stage is required for normal bone formation.

Smad6 is another member of Smad family which plays a negative regulatory role in BMP signaling by stably binding to type I BMP receptors. Smad6 interferes the phosphorylation of Smad1 and 5 proteins and the subsequent heteromerization with Smad4 (Fig. 1) (Imamura *et al.*, 1997). Over-expression of Smad6 in

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chondrocytes causes delays in chondrocyte differentiation and maturation (Horiki *et al.*, 2004). Expression of Smad6 is regulated by BMPs. In mouse Smad6 promoter, four overlapping copies of the GCCGnCGC-like motif, which is the binding site for Smad1 and 5, have been identified (Ishida *et al.*, 2000). These findings establish a negative feedback regulation mechanism for BMP signaling. Smad6 knock-in mice shows that expression of Smad6 is largely restricted to the heart and blood vessels. Smad6 mutant mice have multiple cardiovascular abnormalities. Hyperplasia of the cardiac valves and outflow tract separation defects indicate that Smad6 plays an important function in the regulation of endocardial cushion transformation. The development of aortic ossification and elevated blood pressure in Smad6 mutant mice demonstrate that Smad6 also plays a role in homeostasis of adult cardiovascular system (Galvin *et al.*, 2000).

Tob is a member of a novel anti-proliferative protein family including Tob, Tob2, BTG1, BTG2 and BTG3. Tob inhibits BMP-induced, Smad-dependent transcription in osteoblasts through its association with Smad1 and 5 proteins (Yoshida *et al.*, 2000). In Tob knockout mice, BMP-2 signaling is enhanced and the effects of BMP-2 on osteoblast proliferation and differentiation are increased. BMP-2-induced local bone formation is also enhanced in Tob knockout mice (Usui *et al.*, 2002). Bone volume and bone formation rates are increased in adult Tob knockout mice (Fig. 1) (Yoshida *et al.*, 2000).

Another important regulatory mechanism by which the activity of BMP signaling proteins is modulated involves ubquitin-mediated proteasomal degradation. The ubiquitin-proteasome proteolytic pathway is essential for various important biological processes including cell-cycle progression, gene transcription and signal transduction (Hershko and Ciechanover, 1998; Weissman, 2001). The formation of ubiquitin-protein conjugates requires three enzymes that participate in a cascade of ubiquitin transfer reactions: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). The specificity of protein ubiquitination is determined by E3 ubiquitin ligases, which play a crucial role in defining substrate specificity and subsequent protein degradation by 26S proteasomes (Hershko, 1983; Ciechanover *et al.*, 2000).

Smurf1 was identified by the yeast two-hybrid assay by its ability to interact with Smad1 and 5 and mediate the degradation of these Smad proteins (Fig. 1) (Zhu *et al.*, 1999). Since bone-specific transcription factor Runx2/Cbfa1 interacts with Smad1 protein (Hanai *et al.*, 1999) whose degradation is mediated by Smurf1 (Zhu *et al.*, 1999), we have examined the effect of Smurf1 on Runx2 degradation in myoblast/osteoblast precursor C2C12 cells and osteoblast precursor 2T3 cells. We found that Smurf1 mediates Runx2 degradation in a ubiquitin-proteasome-dependent manner (Fig. 1) (Zhao *et al.*, 2003). Smurf1 also binds Smad6 in the nucleus and is exported with Smad6 to the plasma membrane to target the degradation of the type I BMP receptors (Fig. 1) (Murakami *et al.*, 2003). These findings suggest that Smurf1 regulates BMP signaling through targeting to multiple BMP signaling proteins. To determine the role of Smurf1 in bone formation *in vivo*, we have recently generated transgenic mice (Col1a1-Smurf1) in which expression of a Smurf1 transgene is targeted to osteoblasts using the murine 2.3 kb type I collagen promoter. In Col1a1-Smurf1 transgenic mice, trabecular bone volume and bone formation rates are decreased. Osteoblast proliferation and differentiation are inhibited in Col1a1-Smurf1 transgenic mice, suggesting that bone formation defects found in Smurf1 transgenic mice are mainly due to decreased osteoblast proliferation and differentiation (Fig. 2) (Zhao *et al.*, 2004). Consistent with these findings, recent studies demonstrate that bone formation is enhanced in Smurf1 null mutant mice. Bone mineral density is increased in 4- to12-month-old Smurf1 null mutant mice. Trabecular bone volume and bone formation rates are also increased in these mice (Yamashita *et al.*, 2003). These results demonstrate that regulation of BMP signaling proteins may also play an important physiological role in bone formation *in vivo*.

THERAPEUTIC UTILIZATION OF BMP-2

The osteoinductive capacity of BMP-2 has been demonstrated in preclinical models and evaluated in clinical trials. Many of the animal models used to evaluate the capacity of BMP-2 to heal bone defects have utilized critical-sized defects. In these animal models, bone defects are large enough that they will not heal without a therapeutic intervention. This setting facilitates analysis of the ability of BMP-2 to induce bone. Healing of long bone critical-sized defects by BMP-2 has been demonstrated in species including rats, rabbits, dogs, sheep and non-human primates (Murakami *et al.*, 2002). Gene therapy studies show that bone defects are healed by the implantation of a bioresorbable polymer mixed with bone marrow mesenchymal stem cells to which adenovirus BMP-2 is transferred (Chang *et al.*, 2003). Systemic administration of rhBMP-2 increases mesenchymal stem cell activity and reverses ovariectomy-induced and age-related bone loss in two different mouse models (Turgeman *et al.*, 2002). These results suggest that BMP-2 may be utilized for the treatment of osteoporosis. Recent studies show that rhBMP-2 delivered in an injectable formula with a calcium phosphate carrier or with a liposome carrier accelerates bone healing in a rabbit ulna osteotomy model and a rat femoral bone defect model (Li *et al.*, 2003; Matsuo *et al.*, 2003). Recent clinical studies show that rhBMP-2 can be used as complete bone graft substitutes in spinal fusion surgery. In some circumstances, the efficacy of BMP-2 for inducing successful fusion is superior to that of autogenous bone graft. BMP-2 is shown to be efficacious in several fusion applications, including intervertebral and lumbar posterolateral fusion (Sandhu, 2003). BMP-2 has also

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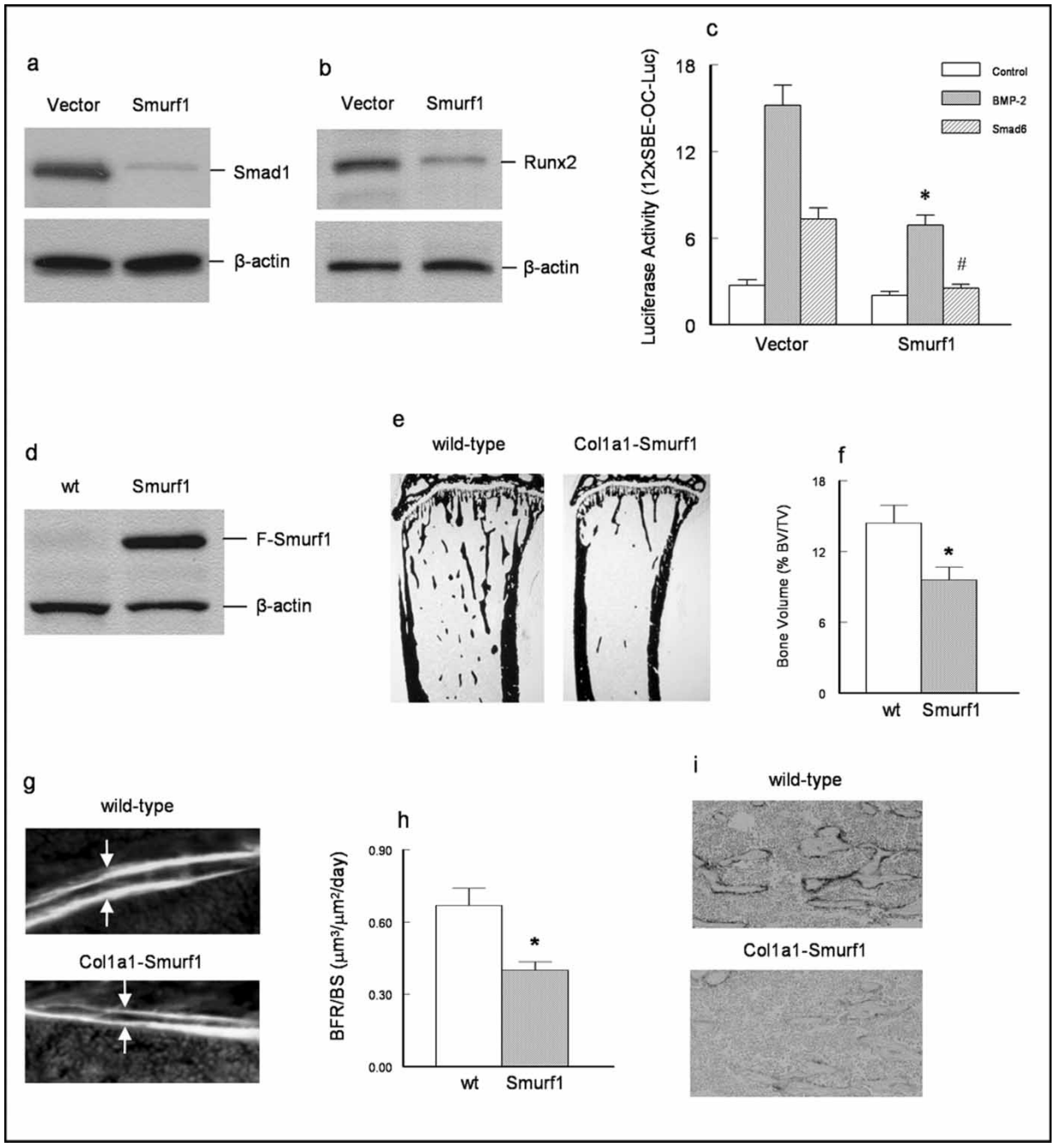

FIGURE 2 Smurf1 inhibits bone formation in Col1a1-Smurf1 transgenic mice. (a,b) Reduction in Smad1 and Runx2 protein levels in 2T3 osteoblast precursor cells stably transfected with Smurf1 expression plasmid (2T3/Smurf1). The protein levels of Smad1 and Runx2 were detected by Western blot analysis. In 2T3/Smurf1 cells, Smad1 and Runx2 protein levels were decreased. (c) Inhibition of BMP signaling in 2T3/Smurf1 cells. 2T3/vector and 2T3/Smurf1 cells were transfected with BMP signaling reporter construct, 12xSBE-OC-Luc (Zhao *et al.*, 2003), and treated with 50 ng/ml of BMP-2. Expression of Smurf1 inhibited BMP-2-stimulated luciferase activity. Transfection of Smad6 further inhibited BMP-2-stimulated luciferase activity. $*p < 0.05$, $\#p < 0.05$, unpaired *t*-test, compared to the same treatments in 2T3/vector group. (d) Expression of the Flag-tagged Smurf1 transgene in Col1a1-Smurf1 transgenic mice. Primary osteoblasts were isolated from calvariae of transgenic mice and their wild-type littermates. Expression of Flag-Smurf1 protein in osteoblasts of Smurf1 transgenic mice was detected by Western blot analysis using the anti-Flag M2 antibody. (e, f) Trabecular bone volume (BV) is decreased in Col1a1-Smurf1 transgenic mice. Bone volume was analyzed in two separate lines of transgenic mice in a defined area in proximal tibiae of 3-mo-old Smurf1 transgenic mice and wild-type littermates ($n = 10$) and the BV was normalized to tissue volume (TV) (f). In Smurf1 transgenic mice, a 33% decrease in BV was observed compared with their wild-type littermates (e, f). $*p < 0.05$, unpaired *t*-test. (g, h) Bone formation rates (BFR) are decreased in Col1a1-Smurf1 transgenic mice. BFR were measured and calculated in the same area as BV was measured using the OsteoMeasure system (Osteometrics Inc., Atlanta, GA). BFR were significantly decreased (40%) in Smurf1 transgenic mice compared with littermate control mice. $*p < 0.05$, unpaired *t*-test. (i) ALP staining. In Col1a1-Smurf1 transgenic mice, ALP staining in osteoblasts of trabecular bone of proximal tibiae was reduced compared with that in wild-type control mice.

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been shown to induce new dentine formation and has a potential application as a substitute for root canal surgery and BMP-2 is an effective bone inducer around dental implants for periodontal reconstruction (Cochran and Wozney, 1999).

Although a significant acheivment has been made in recent years in understanding the role of BMP signaling *in vivo*, tissue-specific knock-outs of the individual BMP ligands, receptors and signaling molecules are required to further determine the specific roles of BMP signaling in a particular tissue since null mutations of most of BMP ligands, receptors and signaling molecules produce lethal phenotype perinatally. Generation of tissue-specific and inducible conditional knockout alleles for BMP ligands, receptors and signaling molecules would allow us to gain further information about physiological functions of BMP signaling in postnatal and adult animals.

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