Bone morphogenetic proteins (BMPs) induce osteoblast differentiation and bone formation. Smads, a group of functionally and structurally related intracellular effectors, mediate signaling initiated by BMPs and regulate cell definite commitment. Previously, we showed that Smad1 activates osteopontin and osteoprotegerin gene expression by dislodging Hoxc-8 from its DNA binding sites. A domain of Smad1, termed Smad1C, was characterized as interacting with Hoxc-8 and then crippling its DNA-binding ability. Ectopic expression of Smad1C is able to bypass BMP signaling in the induction of osteoblast differentiation and bone formation in vitro. To test the function of Smad1C on osteogenesis in vivo, we generated transgenic mice in which Smad1C expression was induced with doxycycline and localized in bone by using a tetracycline-inducible expression system (Tet-on) modified with a bone-specific gene promoter, type I collagen α1. The mice expressing Smad1C showed increased skeletal bone mineral density compared with their littermates. Bone histomorphometric analysis of mouse tibiae showed that Smad1C significantly increases trabecular bone area and length of trabecular surface covered with osteoid and up-regulates bone marker gene (OPN, Cbfa1, Col I α1, BSP, ALP) expression in vivo. Moreover, stromal cells isolated from mice expressing Smad1C displayed a higher potential for differentiating into osteoblasts than the other mice. These results indicate that Smad1C mimics BMPs in the induction of osteogenesis in vivo. Most important, using a high throughput screening assay based on mimicking Smad1C’s displacement of Hoxc-8 binding to DNA, we identified chemical entities that exhibit bone anabolic activity in cell and bone organ cultures, suggesting the possibility that the compounds may be used as bone anabolic agents to treat bone pathologies.

Osteoporosis is a public health care problem that affects approximately 30 million people in the United States alone. It is an aging-related disease of bone loss, and the problem is becoming more serious as the elderly population increases. Despite recent successes with drugs that inhibit bone resorption, there is a clear need for anabolic agents that will substantially increase bone formation in people who have already suffered substantial bone loss.

The transforming growth factor-β superfamily of secreted polypeptide growth factors exerts extensive control over a broad spectrum of cellular processes including cell growth, differentiation, mobility, and apoptosis. In addition to transforming growth factor-β, the superfamily consists of bone morphogenetic proteins (BMPs) and activins that share many structural and functional similarities (1–5). Among them, BMP-2, -4, and -7 have been demonstrated to be the most potent osteotropic factors in the promotion of bone formation both in vivo and in vitro (6–8). BMPs trigger signaling in their target cells through interaction with their receptors (BMP receptor I and II) and then by activating kinase activity of the receptors. Activated receptors subsequently transfer signaling by phosphorylating a series of mediators, such as Smads, which among others represent a family of structurally and functionally related molecules. According to their hierarchy in the signaling cascade, the Smads are classified into three groups: BMP receptor-regulated Smads (R-Smads), including Smad1, -5, and -8, and transforming growth factor-β-activin receptor-regulated Smad2 and -3; co-Smad, or Smad4, which heterooligomerizes with activated R-Smads; and inhibitory Smads (1-Smads), Smad6 and -7, which, as negative feedback modulators, antagonize the signals (1, 2, 6, 7). On phosphorylation by type I receptors, R-Smads associate with co-Smad, Smad4, to form a complex and then translocate to the nucleus where the complexes regulate corresponding gene transcription via recruiting transcription co-activators or co-repressors such as p300/CBP, TGFβ, C-Ski, and SnoN (9–12).

BMPs are potent growth factors that induce osteoblast differentiation and bone formation. We have been searching for novel mechanisms in the BMP signaling pathway that could be used as bone anabolic drug targets. We found that Smad1 activated osteopontin gene transcription by means of dislodging Hoxc-8 from the promoter in response to BMP signaling (13, 14). Hoxc-8 belongs to a highly conserved hox gene family and expresses in limbs, backbone rudiments, the neural tube of mouse mid-gestation embryos, and in the cartilage and skeleton of newborns (15–18). Skeletal abnormalities have been

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The abbreviations used are: BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; Tet-on, tetracycline-inducible system; α1p, type I collagen; rTGF, reverse tetracycline-responsive transcriptional activator; NLS, nuclear localization sequence; Dox, doxycycline; RT-PCR, reverse transcription-PCR; BMD, bone mineral density.
observed in mice with Hoxc-8-null mutation. Mapping the domains interacting with Hoxc-8, we discovered that two regions located on the MH1 and MH1 linker boundary, respectively, were involved (19). One of them, termed Smad1C, spanning from the COOH terminus of MH1 to the linker region (amino acids 145–278) imitates BMP-activated Smad1 in the stimulation of osteopontin gene expression. Ectopic expression of Smad1C linked with a nuclear translocation sequence in 2T3 osteoblast precursor cells stimulated osteoblast differentiation-related gene expression and led to mineralized bone matrix formation (14, 20).

In this study, we examined the function of Smad1C on osteoblast differentiation and bone formation in vivo. Using a modified tetracycline-inducible system (Tet-on), we have created transgenic mice in which Smad1C expression localizes specifically in bone in the presence of doxycycline. The mice expressing Smad1C displayed elevated bone mineral densities and enhanced trabecular bone formation. Most important, we attempted to search for small molecules that could mimic Smad1 in respect to the inhibition of Hoxc-8 binding to the target gene promoter and result in stimulation of bone formation (21, 22). Using a high throughput screening assay, we have identified compounds that displace Hoxc-8 from DNA binding and induce osteoblast differentiation and bone formation in organ culture.

**EXPERIMENTAL PROCEDURES**

**Transgene Construction and Generation of Transgenic Mice—**A Tet-on system with pTet-on and pTRE was purchased from Clontech. The promoter of type I collagen (α1p) from ~2300 to +115 was released from collagen type I dominant-negative BMP receptor type IB (23) and inserted upstream of the reverse tetracycline-responsive transcriptional activators (rtTA) to replace the cytomegalovirus promoter in pTet-on. The construct was termed α1p-tet-on. The Smad1C fragment with FLAG tagged on the NH2 terminal and NLS (nuclear localization sequence), which was isolated from pCMV5B-FLAG-Smad1C-NLS, on the COOH terminus was cloned downstream of the tetracycline-responsive promoter of pTRE, and the construct was named pTRE-Smad1C. Polyadenylation signals of the transgenes (α1p-rTATa), α1p, rTATa, and SV40 were released from the vector α1p-tet-on with digestion of XhoI and SapI unique restriction sites at the 5′ and 3′ ends of the transgene; whereas the integrity of the polyadenylation signals of transgenes carrying the tetracycline-responsive promoter Smad1C and SV40 were liberated from the vector pTRE-Smad1C via EcoRI and BamHI digestion. The transgenic DNA fragments were microinjected into mouse (C57BLAKSNI) eggs and surgically transferred to recipients by standard techniques. Tail tips were cut from pups at 2 weeks old and digested in homogenizing solution (10 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA, 1% SDS, and 0.5 mg/ml proteinase K) at 55 °C for 12 h. Genomic DNA was then extracted with phenol/chloroform. Genotyping of transgenic mice was carried out by PCR. Specific primers were designed for amplification of rTAT and FLAG-Smad1C-NLS. To amplify rTAT, the forward primer was 5′-AGATGACTGGATCTTACCC-3′ and the reverse primer was 5′-AGATGACTGGATCTTACCC-3′. To amplify FLAG-Smad1C-NLS, the forward primer was 5′-GGAGCTCAAGGACGAGTGACA-3′, corresponding to the sequence of FLAG, and the reverse primer was 5′-TTGCTGTCCTCTTAAGACACC-3′, corresponding to the sequence of NLS to distinguish the transgene from endogenous Smad1. The transgenic founders were then back-crossed with C57Bl/6, and the offspring of α1p-rTATa were bred with the offspring of Smad1C mice to activate expression of Smad1C specifically in bone. To induce Smad1C expression, 200 μg/ml doxycycline (Dox) was given in drinking water bottles containing 2.5% sucrose and wrapped with aluminum foil. The water containing Dox was changed every 3 days to avoid precipitation.

**Extraction of Total RNA and mRNA from Bone—**To determine expression of the transgene as well as the bone marker gene, femurs and tibiae were isolated from mice wrapped with aluminum foil, and immediately inserted in liquid nitrogen. Bones were pulverized using a hammer in the presence of liquid nitrogen. The frozen powder was placed in TRIzol reagent (Invitrogen) and homogenized with a cell homogenizer. Total RNA was extracted according to the manufacturer’s instruction. The mRNA was isolated from total RNA using the Oligotex mRNA Mini Kit (Qiagen, Inc., CA).

| Genes | Forward (5′-3′) | Reverse (5′-3′) |
|-------|----------------|----------------|
| Colla1 | TCTGGTCCTCTGTGCTCCC | CTTCCCCATCATGACTTC |
| OPG  | GATGACTGGASCACCTCTGA | ACGAAATGCTGAGACCTC |
| BMP  | TSCACCCGATGCATCATTAGC | GCATTCAGGCTAGCAGA |
| ALP  | GCTCAACACCATGAGCCA | ACACCCCGAGCTGAGCGAG |
| β-actin | AACACCCCGAGCTGAGCAG | GTGTGGGCTAGAGCTTTACGG |

| Primer sequences for each gene | Revertant primer sequences for each gene |

**Analysis of the Expression of Transgenes and Osteogenesis-related Genes Using Real-time RT-PCR—**To examine the expression of rTAT and Smad1C, real-time RT-PCR (Access RT PCR System; Promega Co., Madison, WI) was performed using mRNA isolated from bone following the protocol provided by the manufacturer. The primers used for RT-PCR were the same as those used in the genotyping described above, whereas PCR were carried out using mRNA samples to confirm no DNA contamination. As a control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified from the RNA samples by RT-PCR. The forward primer was 5′-CTCATGACCAATGCTCATG3′, and the reverse primer was 5′-GGGATCTGCTCCTAAATGCAA3′. To examine physiological change in the expression of bone marker genes in mice expressing Smad1C versus their littermates, a relative RT-PCR quantification based on the ratio of the target gene to the housekeeping gene (β-actin) was used. The reverse transcription reaction was carried out following the manufacturer’s protocol (Stratagene, Co., La Jolla, CA). Primer sequences for each gene are listed in Table I.

**Real-time PCR** was performed in a LightCycler apparatus using LC-Start Reaction mix SYBR green I (MJ Research, Co., Reno, NV) as described (24). Briefly, a cDNA standard for each gene was prepared by PCR amplification run to saturation (35 PCR cycles), using corresponding CDNA and primers. Different amounts of cDNA standard were used in the reactions to establish a standard curve to calibrate the amount of the identical gene in samples. After PCR amplification, a melting curve was constructed to ensure identity of the product. To ensure that the correct product was amplified in the reaction, all samples were further separated by 2% agarose. The relative expression of the target gene was calculated by dividing its amount by the quantity of β-actin from the same sample.

**Osteogenesis Induction of Stromal Cells Isolated from Transgenic Mice—**Bone marrow cells were obtained from 3-month-old adult transgenic mice by flushing the lumen of the femur shaft with 1.5 ml of Dulbecco’s modified Eagle’s low glucose medium supplemented with antibiotics and 10% fetal bovine serum. Cells were then cultured in modified Eagle’s low glucose medium supplemented with antibiotics and 10% fetal bovine serum at 37 °C in 5% CO2. After a 4-h incubation, non-adherent cells were removed by replacing the medium (25, 26). The adherent cells were cultured for 48 h and then cultured in the medium with osteogenic supplements (100 mM dexamethasone, 0.05 mM l-ascorbic acid-2-phosphate, and 10 mM glycerophosphate) plus 2 mg/ml doxycycline for 15 days.

**Staining Cells with Immunofluorescence-conjugated Antibodies—**To determine the Smad1C expression at the protein level, osteoblasts differentiated in vitro, as described above, were immunoblotted with anti-FLAG monoclonal antibody as described previously (27). Briefly, cells were fixed with 3% paraformaldehyde and permeabilized with 0.01% Triton X-100 in phosphate-buffered saline and subsequently blotted with monoclonal antibodies against FLAG (Sigma). The cells were then incubated with the second antibody conjugated with fluorescein isothiocyanate against mouse IgG.

**Alkaline Phosphatase and Mineralized Bone Matrix Formation Assays—**Bone cell differentiation was monitored using an alkaline phosphatase assay (28) and the von Kossa stain of mineralized bone matrix (29). In brief, cells were fixed with 10% formalin buffered with phos-
RESULTS

Generation of Transgenic Mouse in which Expression of Smad1C, Exclusively in Bone, Is Induced with Doxycycline—To examine the effects of the Smad1C-Hoxc-8 interaction domain (14, 19) on bone formation in mice, using a tetracycline-inducible expression system we made transgenic mice in which Smad1C expression is specifically localized in bone cells and its induction is under the control of doxycycline. To get Smad1C tissue-specific expression, the cytomegalo virus promoter in the pTet-on vector was replaced with bone-specific mouse type I collagen α promoter from −2300 to +115 bp (α1p) (23). Smad1C with FLAG tagged on its NH2 terminus and NLS flanked to the COOH terminal was inserted downstream of the rtTA-regulated promoter (Fig. 1A). Transgenes of α1p-rtTA, including α1p, rtTA, and SV40 polyadenylation signal, and Smad1C with tetracycline-responsive promoter Smad1C and SV40 polyadenylation signal were isolated from corresponding constructs and then injected separately into fertilized mouse eggs. Eleven founders were generated from α1p-rtTA transgene injections and four founders from the Smad1C transgene. All of them transmitted the transgene at the expected Mendelian ratio. The assessment of expression of rtTA in the bone was performed by RT-PCR. At the same time, a PCR assay was carried out using mRNA samples to confirm no DNA contamination. We checked three lines of α1p-rtTA (rtTA-28, rtTA-38, and rtTA-24) and all of them expressed rtTA in bone (Fig. 1B). To obtain mice with both α1p-rtTA and Smad1C, the transgenic mice from rtTA-38 were bred with one line of transgenic mice with Smad1C (Smad1C-II). Genotypes of the offspring were determined using PCR, and mice with different genotypes were fed with doxycycline in sugar water or placebo (sugar water). The expression of Smad1C in the offspring was examined by RT-PCR using specific primers derived from a sequence of Smad1C (165 bp) and from Smad1C and Smad1C-FLAG (115 bp). As can be seen, mRNA of Smad1C is detected only in the binary transgenic mice (α1p-rtTA/Smad1C) fed with Dox. Double-transgenic α1p-rtTA/TRE-Smad1C mice, in the absence of Dox and any single transgenic mice (either α1p-rtTA or Smad1C) fed with doxycycline, do not show any noticeable Smad1C expression. The results of PCR
indicate that there is no DNA contamination in the above RNA samples. To check whether Smad1C expression is restricted to bone, mRNA was isolated from other tissues including lung, liver, spleen, thymus, kidney, heart, and skin and subjected to RT-PCR analysis. No perceptible Smad1C was detected in these tissues (Fig. 1D). To test the protein expression of Smad1C, we isolated stroma cells from transgenic mice, cultured them in osteogenic differentiation medium for 10 days, and immunostained them with monoclonal antibodies against FLAG (Fig. 1E). The protein of Smad1C was detected only in cells with dual transgenes in the presence of Dox.

**Smad1C Stimulates Osteoblast Differentiation in Vivo**—Previously, we observed that the stable expression of Smad1C in 2T3 osteoblast precursor cells stimulated osteoblast differentiation marker gene expression and led to mineralized bone matrix formation, indicating that the interaction between Smad1C and Hoxc-8 mimics BMP signaling and is sufficient to induce osteoblast differentiation (14). To verify the effect of Smad1C on osteoblast differentiation in mice, we isolated bone marrow stromal cells from Smad1C transgenic as well as Hoxc-8 transgenic mice. The alkaline phosphatase activity was examined and deposition of mineralized extracellular matrix containing hydroxyapatite was measured by von Kossa staining. Enhanced alkaline phosphatase activity and mineral stain were observed in cells expressing Smad1C (Fig. 2, middle and lower panels). Cells expressing Hoxc-8 that were exposed to an osteogenic medium for 10 days remained in the undifferentiated stage, whereas the others, especially the cells expressing Smad1C, displayed the most mature osteoblastic morphology characterized as cuboidal and plump (Fig. 2, upper panel).

Taken together, our results indicate that Smad1C can mimic BMP signals in the regulation of osteogenesis in the mouse. To further investigate the effect of Smad1C on osteoblast differentiation, the expression level of osteoblast marker genes...
in femoral and tibial bone tissue of transgenic mice was examined with RT-PCR. The relative expression level of each marker gene was determined by dividing the level of marker gene expression with that of \(\beta\)-actin from the same sample. Comparing single and dual transgenic mice without Dox stimulation, the expression of all these marker genes was enhanced to some extent by Smad1C expression whereas Hoxc-8 inhibited the expression (Fig. 3). The phenomena are consistent with the \textit{in vitro} cell differentiation assay (14) and histomorphometric data (Fig. 4.), as well as with a previous study (14). The variation in the level of bone marker gene expression between Smad1C dual transgenic and Hoxc-8 dual transgenic mice suggests that Smad1C enhances bone constitution through binding to Hoxc-8 and then impairing its function.

**Smad1C Enhances Bone Formation in Transgenic Mice**—To examine the possibility of developing a bone anabolic drug using a Smad1C analogue, we investigated the \textit{in vivo} effect of Smad1C on bone. To address this, nine batches of offspring were generated from crossbreeding rtTA-38 and Smad1C-II mice, and six batches of them, starting from 1 month old, were fed with Dox for 2 months. We performed histomorphometric investigations in these transgenic mice after a bone density scan. With von Kossa staining, more densely distributed, thicker trabeculae and better trabecular connectivity were found in sections from the tibiae of Smad1C dual transgenic mice fed with Dox than in those from other control groups (Fig. 4, B and C), and more osteoid-covered trabecular surface was found in toluidine blue-stained sections from this group than others. The plump, cuboidal osteoblasts along the osteoid-covered trabecular surface were also much more easily observed in this group (Fig. 4 A). In order not to disturb the tetracycline-inducible transgene system, we did not perform dynamic bone labeling by using tetracycline or its analogs.

Before performing the histomorphometric assay, mouse BMD was examined using dual energy x-ray absorptiometry. The relative BMD of each double transgenic mouse was determined by dividing its BMD measurement by that of a littermate. The figure represents one of three independent experiments with observations of multiple microscopic fields.
The figure represents one of three independent experiments with 3-fold compared with vehicle control. The thickness of new bone was increased in bone culture treatments.

The result is expressed as the mean of three different experiments. The thickness of new bone was increased in bone culture treated with PGE-5516909 by 2-3-fold compared with vehicle control. The figure represents one of three independent experiments with observations of multiple microscopic fields.

Figure 6. Compound mimics Smad1C in enhancement of osteoblast differentiation and bone formation. A, an alkaline phosphatase assay shows that the compound PGE-5516909 stimulated alkaline phosphatase activity in a dose-dependent manner in C2C12 osteogenic cells. The thickness of new bone was increased in bone culture treated with PGE-5516909 by 2-3-fold compared with vehicle control. The figure represents one of three independent experiments with observations of multiple microscopic fields.

Biomolecules Stimulate Bone Formation

Hoxc-8 in BMP Signaling—Previous studies have suggested that Hox genes play a role in downstream events in BMP signaling (33, 34). It has been suggested that repression may be a general mode of action for Hox proteins, a phenomenon that may be required for maintaining cells in an undifferentiated state during development to prevent premature differentiation of precursor cells (35–37). Hoxc-8, as one of the three members of paralog VIII, is predominantly expressed at a high level in the limbs, backbone, and spinal cord in early mouse embryos (38, 39). Overexpression of Hoxc-8 in skeletal tissue inhibits chondrocyte maturation and stimulates chondrocyte proliferation, and results in an accumulation of progenitors in the hypertrophic area (40, 41). Bending and fusion of the ribs, anterior transformation of the vertebrae, and abnormal patterns of ossification in the sternum were observed in adult Hoxc-8-null mice (40). Our previous work (14, 19) showed that Hox-8 functions as a transcriptional repressor of the osteopontin gene. We further found that Smad1 interacts with Hoxc-8, dislodges Hoxc-8 binding from its element, and specifically activates the osteopontin gene transcription in response to BMP stimulation.

The direct interaction between Smad1 and Hox protein(s) suggests their functional relationship and the mechanisms in BMP-induced skeletal development. Our mapping data further defined the Hoxc-8 interaction domain spanning from the COOH terminal of MH1 to the linker region (amino acids 145–278). Stable expression of this Smad1 fragment (Smad1C, amino acids 145–278) in 2T3 osteoblast precursor cells stimulated osteoblast differentiation-related gene expression and led to mineralized bone matrix formation. Therefore, various functional domains of both Smad and Hox proteins may be selectively utilized for mediating protein–protein or protein–DNA interaction and for repression or activation of gene transcription, depending on the developmental stage, cell type, and promoter context.

Smad1C, a Hoxc-8-interacting Peptide, Acts as An Anabolic Agent for Bone in Vivo—Based on our previous studies, we examined the biological effect of Smad1C on bone in vivo by taking advantage of the tetracycline-inducible transgene system. This sophisticated system allows precise control of target gene expression in eukaryotic systems via application of small molecules, such as tetracycline or its analogs (e.g., doxycycline). Tetracycline or its analogs and the transactivator proteins exert no pleiotropic effects on eukaryotic cells. Thus, regulation of gene expression by the Tet system is very specific. Only the expression level of the gene of interest is altered.

To express Smad1C in bone, we modified this system by inserting α1p promoter upstream of rtTA, generating transgenic mice with tissue-specific and doxycycline-inducible Smad1C expression. Consistent with our previous in vitro studies, the bone marrow stromal cells from the mice specifically expressing Smad1C in bone displayed enhanced alkaline phosphatase activity and bone mineralization when induced with osteogenic medium. The histomorphometric study further corroborated our in vitro study in that the dual transgenic mice induced with Dox showed enhanced osteoblast differentiation and osteoid formation. However, without information about bone mineralization rate, it is not safe to conclude that the enhanced osteoid formation is caused by more active bone formation. The enhanced osteoid formation may also be caused by lagged bone mineralization, which is a well-known cause of rickets. The more cuboidal and plump osteoblasts along with the more densely populated osteoid largely exclude the possi-
bility of a lagging bone mineralization. The enhanced bone formation in Dox-treated dual transgenic mice is, therefore, attributed to the stimulated osteoblast differentiation by Smad1C. In line with this, the whole body BMD was significantly higher in dual transgenic mice fed with Dox than in other control mice. Thus, the efficacy of Smad1C in enhancing bone formation was thoroughly confirmed at different levels, from in vitro molecular interaction to whole body BMD assessment. Although bone resorption and osteoclast activity in these mice were not observed in this study, our previous study allows us to hypothesize that the prevention of bone resorption may act through a similar mechanism by which Hoxc-8 is displaced from its DNA binding site is likely the major mechanism in enhancing bone formation by BMP (14, 19, 20, 42).

Compounds Mimic Smad1 Effects on Bone Formation—The most compelling therapeutic need in treating common diseases of bone loss is an anabolic agent that can enhance new bone formation and restore bone that has been lost. There are precedents for small molecular weight compounds enhancing bone formation in vivo (32, 41). Here what we found is a very potent peptide by which bone formation and regeneration will be enhanced. To attempt to further move this peptide from bench to bedside, we used a high through put assay to screen 300,000 compounds for the agents that mimic Smad1C activity to induce bone formation. The assay provides a powerful tool for rapidly assessing various factors involved in protein-DNA and protein-protein interactions and for screening large compound libraries for potentially active agents at a given target site (21). The compound PGE-5516909 as a representative of compounds that mimic Smad1 effects on bone formation when added to calvarial bone culture dramatically increased new bone formation. This agent, however, was not sufficiently soluble to be tested in vivo. Nevertheless, small molecules mimicking Smad1C effects on bone formation may have therapeutic application for treating common metabolic diseases of bone loss and healing large bone defects secondary to tumor or trauma, and may merit further investigation as potential bone anabolic agent for clinical use.

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