2000

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BONE MORPHOGENETIC PROTEINS INDUCE GENE TRANSCRIPTION AND OSTEOBLASTIC DIFFERENTIATION THROUGH THE INTERACTION BETWEEN SMAD1 AND HOXC-8

by

XIANGLI YANG

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2000

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree PhD Program Pathology

Name of Candidate Xiangli Yang

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Title Bone Morphogenetic Proteins Induce Gene Transcription and Osteoblastic Differentiation through The Interaction Between Smad1 and Hoxc-8

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily that induce bone formation and regeneration, and determine important steps during embryonic development. Downstream signaling of BMPs involves a family of newly identified effector molecules known as Smads. Smad1 undergoes phosphorylation in response to BMP-receptor activation and is then translocated into the nucleus where it modulates transcription of target genes. This study investigated the mechanism by which BMP induces osteoblast differentiation through Smad1-mediated signaling. A yeast two-hybrid approach was employed to identify the DNA binding protein(s) which interacts with Smad1. Hoxc-8, a homeodomain transcription factor that is primarily expressed in mammalian bone tissue, was found to associate with Smad1 in yeast and mammalian cells. Deletional analysis revealed that the interaction involves the N-terminal 87 amino acid residues of Smad1 and the homeodomain of Hoxc-8. A specific Hoxc-8 binding element of Hoxc-8 was located at the 5'-flanking region from -205 to -170 of osteopontin, an important bone marker gene. Hoxc-8 binding element suppressed the promoter activity when linked to a SV40-promoter driven reporter gene. In vitro binding and transient transfection assays demonstrated that Smad1 interacts with Hoxc-8 by inhibiting Hoxc-8 from binding to its

cognate element, hence releasing the repression of gene transcription. Further examination showed that the 87 amino acids of Smad1 that were identified to be sufficient to interact with Hoxc-8 also are able to activate osteopontin and other BMP-responsive gene transcription and to induce alkaline phosphatase activity and mineralization in mesenchymal cells. This dissertation reports a novel interaction between two important transcription factors, Smad1 and Hoxc-8. The findings indicated that Hoxc-8 acts as a transcription repressor that suppresses osteopontin gene expression while the Hoxc-8 binds to the DNA. Smad1 interacting with Hoxc-8 inhibits the Hoxc-8 from binding to its element, resulting in activation of gene expression by derepression that leads to osteoblastic differentiation. Apart from recruiting other DNA-binding proteins to modulate gene transcription, Smad1 also binds directly to DNA and regulates gene transcription. The last part of this dissertation characterizes the Smad1 binding element within the osteopontin promoter.

DEDICATION

To my dearest mother, Yiru He, who had taught me to love and live well. Your love filled my each precious day, and you were and will always be what I live for.

ACKNOWLEDGMENTS

My special thanks first go to the vice chairman of Pathology, the graduate program director, Dr. Tom Lincoln, who helped ease the transition that I have made during my graduate study here at UAB. I count myself to be one of the luckiest students who have the opportunity to pursue their research work in the graduate program of Cellular and Molecular Pathology, a most nurturing and productive environment.

The completion of this research would be impossible without the excellent guidance, generous support, and continuous encouragement that I can always count on receiving from my mentor, Dr. Xu Cao. I would like to thank him particularly for his invaluable advice in helping me to mature as a person.

I am conscious also of a great debt to the members of my dissertation committee, Drs. Tom Lincoln, Joanne Murphy-Ullrich, Charles Prince, and Gerald Fuller, for their intelligent academic advice and scientific insights. They have helped me develop both as a scientist and as a person.

I am grateful to all my colleagues who are or were in Dr. Cao's lab. Among those to whom I am particularly grateful are Xingming Shi, Hassan Selleck, and Shuting Bai for their help in every possible way. My appreciation also extends to all the faculty, students, secretaries, and technicians in the Pathology department who were always willing to help me. They have done more for me than words can say. I shall remember them fondly always.

Many debts of gratitude have also been incurred to my former advisors, Drs. Phillip Ryals and Patrick Higgins, without whom I would never be able to enjoy science as part of life. Their dedication and love of science has been and will always be guidance for my future career.

Finally, the acknowledgement would not be true without my appreciation to my father, Minghu Yang, my two brothers, Bing Yang and He Yang, and my close friends Lingzhen Li, Jack Green, and Dipankar Manna. It is they who give me the love and strength to make it through the frustrations and challenges in my life.

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LIST OF ABBREVIATIONS

Anta-Smads	antagonistic Smads
ALP	alkaline phosphatase
RGD	arginine-glycine-aspartate
BMP	bone morphogenetic protein
BSP	bone sialoprotein
CMV	cytomegalovirus
Col I	type I collagen
Co-Smads	common Smads
CR	conserved region
DMEM	Dulbecco's modified essential medium
DPP	decapentaplegic
EMSA	electrophoresis mobility shift assays
EST	expressed sequence tag
FAST-1	forkhead activating signal transducer-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
GDF	growth differentiation factor
GST	glutathione S-transferase
HBE	Hoxc-8 binding element
HD	homeodomain

HDAC	histone deacetylase complex
HID	Hoxc-8 interaction domain
IGF	insulin-like growth factor
IL	interleukin
Mad	mother against decapentaplegic
M-CSF	macrophage colony-stimulating factor
MH1	Mad-homology domain1
MSC	mesenchymal stem cell
OC	osteocalcin
OCIF	osteoclastogenesis inhibitory factor
ODF	osteoclast differentiation factor
OPG	osteoprotegerin
OPN	osteopontin
PAI-1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related protein
R-Smad	receptor-regulated Smads
RT-PCR	reverse transcriptase polymerase chain reaction
SBE	Smad1 binding element
SHH	sonic hedgehog

Tet	tetracycline
μTF	μ Transcription factor
TGF-β	transforming growth factor-β
TNF-β	tumor necrosis factor-β

INTRODUCTION

Curiosity is the source of my inspiration, nature is the stimulus of my research, and persistence is the assurance of my success.

It is a central precept of modern biology that a multicellular organism is derived through the process of cell division and differentiation. A crucial question concerning the development is how a mass of seemingly identical cells are told to follow certain pathways by which they transform into different cell types, giving rise to diverse tissues, organs, and, eventually, a highly organized living creature. An essential mechanism involved in this process is based on specific stimulus-cell interactions leading to the induction of one cell's fate. Through pioneering work in fruit flies, frogs, rodents, and other eukaryotes, we finally have begun to understand the signals that govern these processes. With the availability of recombinant DNA technology, it is now possible to analyze such signal transduction pathways and study the molecular basis for target gene activation. Detailed knowledge of the molecular controls involved in differentiation is the key to uncovering the essence of diseases like AIDS and cancer.

The focus of this study was to identify the downstream molecules involved in signal transduction by bone morphogenetic protein and to understand the molecular events underlying the process of osteoblastic differentiation. Molecular and biochemical dissection of the local regulators will provide insights into the genes responsible for skeletal morphogenesis and will assist in the development of anabolic drugs for curing bone diseases such as osteoporosis.

Bone

Bone is a metabolically active and highly organized connective tissue that consisits of a loose mineral lattice of hydroxyapatite [$3Ca_3(PO_4)_2 \bullet (OH)_2$] and amorphous calcium phosphate crystals interspersed in an organic matrix (Watrous and Andrews 1989). The matrix is composed of a variety of extracellular proteins, of which 90% is collagen (almost exclusively type I) and the remaining 10% is a spectrum of noncollagenous proteins (Mikulski and Urist 1977). Bone protects soft tissues, serves as a mechanical support for movement, and determines the attributes of body size and shape for vertebrates. Bone acts also as a reservoir for calcium and phosphate and as a site for mineral homeostasis (Prockop 1998). Bone is unique among body tissue not only because of its physicochemical structure, but also because of its exceptional ability for growth, remodeling, and regeneration. The constant remodeling of bone tissue in response to proper signals provides us a model system to investigate the general molecular events that lead to morphogenesis of body tissues.

Bone Cells

There are four types of cells in bone: osteoblasts, osteoclasts, bone lining cells, and osteocytes, as shown in Fig. 1 (Marks and Popoff 1989). Osteoblasts arise from local osteoprogenitor cells, which originate from multipotential mesenchymal stem cells (MSCs). Osteoblasts are fully differentiated cells organized in continuous layers on the periosteal and endosteal surfaces of bone. They are bone-forming cells with the capacity to synthesize and regulate the deposition and mineralization of the extracellular matrix constituents of bone. Newly formed bone that is unmineralized is described as osteoid.

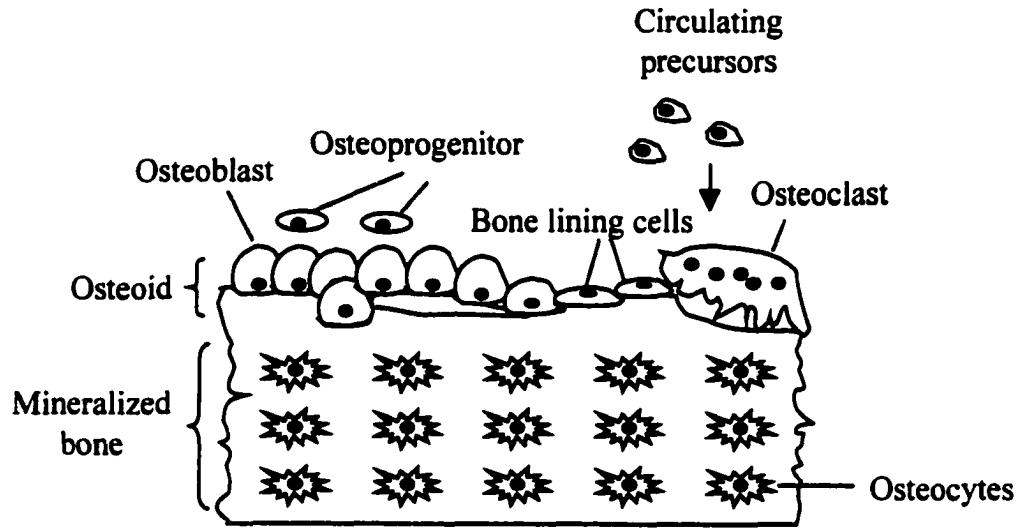

Figure 1. Four types of bone cells showing their origins and location. Osteoblasts originate from osteoprogenitors and are always found in clusters of cuboidal cells along the bone surface. They are responsible for the production of the matrix constituents. Osteoclasts are from circulating hematopoietic monocyte progenitors and are giant multinucleated cells. They are usually found in contact with a calcified bone surface and are responsible for bone resorption. The metabolically inert osteocytes are found embedded deep within the bone, whereas the lining cells form a layer on the surface of the bone (based on Marks and Popoff 1989).

Osteoclasts originate from hematopoietic cell lineage and form by the fusion of mononuclear precursors (Marks and Walker 1981; Suda et al.1996). Osteoclasts are large, multinucleated cells and are responsible for bone resorption. They dissolve the bone mineral and the organic matrix by secreting massive acid and specialized proteinases. The degraded products are then removed by transcytosis (Blair 1998). Bone lining cells are flat, elongated, inactive cells with reduced metabolic activity. The osteocytes are mature osteoblasts that have become embedded within the mineralized bone matrix during the process of bone growth. To a limited extent, osteocytes are capable of synthesizing and resorbing matrix. It has been suggested that osteocytes may be mechanosensory cells that play an important role in the functional adaption of bone (Aarden et al. 1994).

Skeketal Development

Bone tissue is formed during embryonic development by either a direct intramembranous or an indirect endochondral ossification. The embryonic mesenchymal lineages that arise from ectoderm or mesoderm give rise to different skeletal parts (Aubin and Kahn 1998), whether by the intramembranous or endochondral route (Fig. 2). Intramembranous ossification is a process in which a group of mesenchymal precursor cells within a highly vascularized area of connective tissue undergo division and differentiates directly into bone-forming osteoblasts. After matrix deposition and mineralization, blood vessels incorporated between woven bone trabeculae (spongy bone) will form the hematopoietic bone marrow. This process is responsible for the development of the flat bones of the skull and the addition of bone on the periosteal or outer surface of long bones

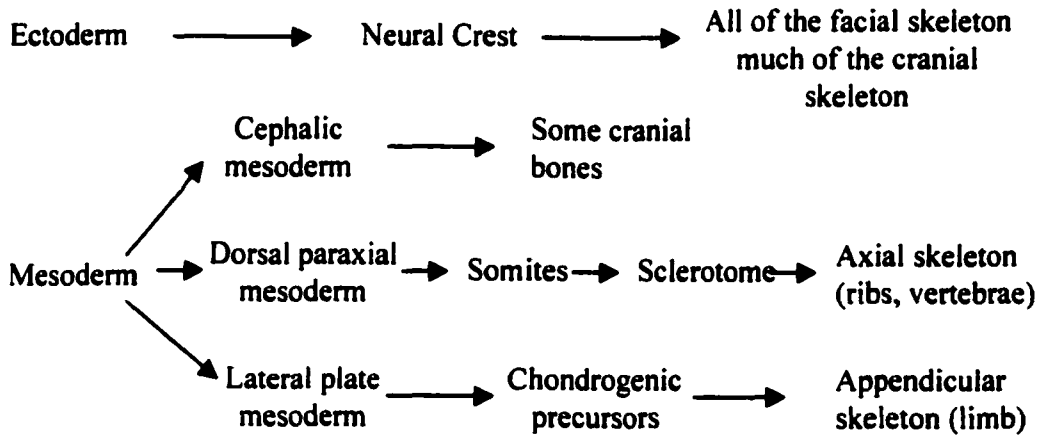

Figure 2. Embryonic origins of different bones of the skeleton. Both ectoderm and mesoderm contribute to the skeleton. Cranial neural crest has the capacity to form the cartilage, bone, connective tissue, and tooth odontoblasts of the facial skeleton (based on Aubin and Kahn 1998).

(Thorogood 1993). In contrast, endochondral bone formation involves the conversion of an initial cartilage template into mineralized bone and is responsible for generating most bones of the skeleton (Marks and Hermey 1996). In such bones, the condensed embryonic mesenchyme transforms into cartilage. After vascular invasion, chondrocytes in the cartilage subsequently undergo a program of hypertrophy, calcification, and cell death. At either end of growing bones, chondrocytes become organized into a structure called the epithyseal growth plate cartilage. The growth plate is responsible for longitudinal skeletal growth. This layer of proliferating cells and expanding cartilage matrix becomes entirely calcified and remodeled by the end of the growth period. Osteoblasts and osteoclasts are then recruited to gradually replace the cartilage scaffold with bone matrix and maintain a constant skeletal mass in the adult (Krane 1981).

The development and maintenance of bone tissue during embryogenesis and throughout the lifetime of vertebrates is very complex and incompletely understood. Recent molecular genetic approaches show that mammalian skeletal patterning and bone growth and development are under tight genetic control (Ducy and Karsenty 1998). The pattern of expression of such genes is often restricted to particular places and times during development. Some genes, such as homeobox (hox) genes, specify early mesenchymal lineages, skeletal primordial shape, and the identity of individual skeletal elements (Krumlauf 1993, 1994; Karsenty 1998). Table 1 lists some hox genes that affect skeletal patterning. Other genes, such as growth and differentiation factors and hormones, further refine bone shape and size by influencing mesenchymal conden-sations and/or by affecting subsequent differentiation events (Hogan 1996).

Table 1. *Hox gene mutations that affect sekletal development*

Genes	Phenotypes	References
hoxa-2-6, 9-11	Deletion of a cervical vertebrae, and homeotic transformations of the cervicothoracic vertebrae	(Maconochie et al. 1996; Aubin et al. 1998)
hoxb-4-6	Alterations in regional identity, and anterior transformations of vertebrae	(Horan et al. 1995; Pollock et al. 1992)
hoxc-4,8,9	Defects in thoracic vertebra (t2 to t11) and homeotic transformation of skeletal segments	(Boulet and Capecchi 1996; Le Mouellic et al. 1992)
hoxd-3,4,11,13	Shortening of the long bones, small digit primordia, and impaired skeletal mass	(Goff and Tabin 1997; Zakany and Duboule 1996)

Modeling and Remodeling--Coordination of Osteoblast and Osteoclast

Modeling is the formation of bone on surfaces where bone has not been previously removed by osteoclastic resorption (Frost 1991). Bone modelling is the process associated with growth and re-shaping of bones during childhood and adolescence. In the human life cycle, the first two decades are devoted to the development of the skeleton by modeling, in which bone formation necessarily precedes and exceeds bone resorption. During the next three decades and beyond, the adult skeleton is maintained by remodeling. Remodeling describes the lifelong process whereby skeletal tissue is continually resorbed and replaced in order to maintain skeletal integrity, shape, and mass. The remodeling cycle begins with a localized resorption by osteoclasts that is succeeded by a precisely equal formation of bone by osteoblasts at the same site (Parfitt 1994), which results in removing and replacing a fraction of adult skeleton each year. Bone remodeling is an integral part of the calcium homeostatic system. After the fifth decade, the formation of the remodeling sequence fails to keep pace with resorption, which results in osteoporosis,

a decrease in the skeletal mass and the connectivity of trabecular bone. Osteoporosis reduces skeletal strength and increases the risk of fracture over time (Boonen et al. 1995).

Modeling and remodeling provide the shape, architecture, and normal mass of bone. In the normal adult skeleton, the two steps of a remodeling cycle depend on interactions between osteoblasts, osteoclasts, and constituents of the bone matrix, and are tightly controlled or coupled, with no net change in bone mass. The coupling of resorption and formation allows the skeleton to react to injury by increasing the number of remodeling units at the site of injury, which leads to increased bone turnover without affecting total bone mass. The regulatory mechanisms of the bone resorption and formation coupling are under intensive investigation. It is widely accepted that a variety of factors, from systemic hormones and cytokines to local regulatory factors, must act in concert to regulate the continuous coupling (Watrous and Andrews 1989). Fig. 3 illustrates the coupling mechanisms understood thus far.

The osteoblast provides much of the local control of formation and maintenance of the skeleton because it not only produces bone matrix but also plays an important role in mediating osteoclastic activity. Many of the primary stimulators of bone resorption, such as parathyroid hormone (PTH), PTH-related protein (PTHrP), interleukin (IL)-1, IL-6, tumor necrosis factor-β (TNF-β), vitamin D, and prostaglandins, act on osteoblasts. Osteoblasts receive the appropriate signals to release soluble mediators, such as macrophage colony-stimulating factor (M-CSF), or to express a membrane-bound osteoclastic differentiation factor (ODF) that induces the osteoclastic activity. Osteoblasts can also express a soluble molecule, osteoprotegerin (OPG), to block osteoclastogenesis, providing a feedback regulatory loop (Yasuda et al. 1998). The cytokines and growth factors,

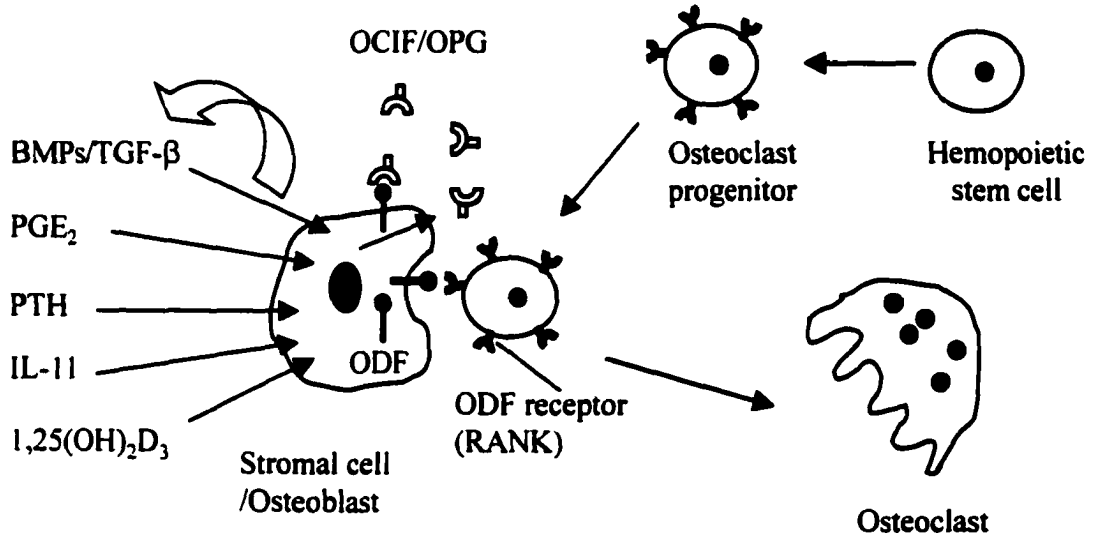

Figure 3. A hypothetical model of the coupling between osteoclastic resorption and osteoblastic formation. Systemic factors, such as PGE2, IL-11, or $1,25(OH)_2D_3$ or the local regulators like BMPs or TGF-β stimulate osteoblast (Stromal cells) to synthesize the osteoclast differentiation factor (ODF), a newly discovered TNF family member (Simonet et al. 1997), which can induce osteoclast differentiation and maturation through interacting with its receptor, RANK (receptor activator of NF-κB). Some local factors secreted by osteoblasts, such as BMPs, might also induce a soluble form of the ODF receptor, osteoprotegerin (OPG), which can compete with RANK to interact with ODF and inhibit osteoclastic differentiaiton (based on Takahashi et al. 1999).

especially transforming growth factor-β (TGF-β), released from matrix during bone resorption act as a feedback loop and trigger the formation and activation of osteoblasts to synthesize and deposit new bone (Dziak 1993).

Osteoblastic Differentiation

In response to proper stimulation, MSCs undergo proliferation and differentiate into preosteoblasts and then into mature osteoblasts. A model of the initiation and progression of osteoblastic differentiation is shown in Fig. 4. The existence of MSCs for nonhematopoietic cells in bone marrow was suggested by transplantation experiments (Friedenstein 1976; Friedenstein et al. 1987; Luria et al. 1987; Mardon et al. 1987). Friededstein's initial observations were extended by a number of investigators during the 1980s. These studies revealed that the MSCs are multipotent and can readily be induced to yield a variety of mesenchymal cell types, including osteoblasts, chondroblasts (Owen 1988; Beresford 1989), myotubes (Wakitani et al. 1995), adipocytes (Dennis and Caplan 1996), tendon cells (Caplan 1994), and fibroblasts (Grigoriadis et al. 1988).

Osteoblastic differentiation is a cascade of events that includes chemotaxis of MSCs to the sites of new bone formation, proliferation of MSCs, differentiation of MSCs into osteoblasts, synthesis and secretion of bone matrix proteins, and mineralization of extracellular matrix. Toward the end of the mineralization period, the osteoblasts become either flat lining cells or osteocytes. It is clear that osteoblasts can express all of the genes necessary for bone formation, but osteoblasts at different stages may express only a subset of molecules from the potential osteoblastic repertoire (Yamashita et al. 1996b). Although there is a correlation between the expression of a marker and the stage of cell

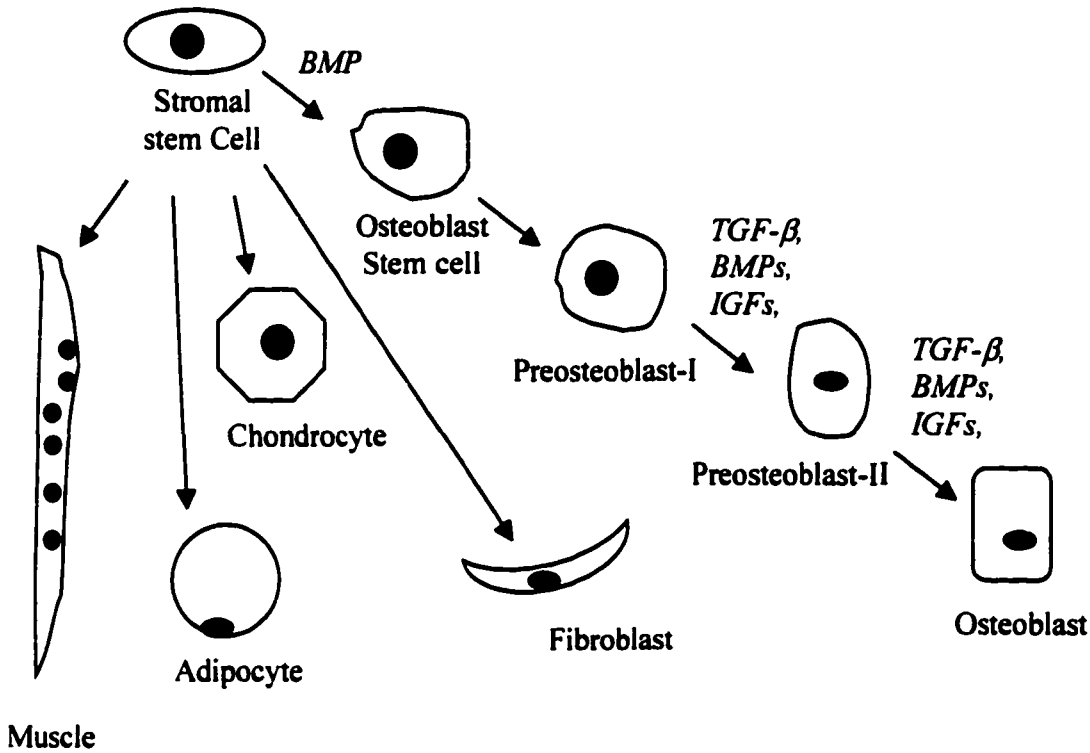

Figure 4. A model illustrating growth factor effects on stages of osteoblast cell differentiation. Osteoblastic lineage cells originate from multipotential mesenchymal stem cells, which can also give rise to chondrocyte, adipocyte, fibroblast, and muscle cells. It is hypothesized that growth factors may act on different target cells of the osteoblast cell lineage. BMPs stimulate pleuripotent mesenchymal stem cell proliferation and initiation of differentiation of these cells into chondrocyte and osteoblasts. In addition, BMPs act on early osteoprogenitor cells. Insulin-like growth factor (IGF) and TGF-β also stimulate synthesis of osteoblast differentiation marker genes in vitro, suggesting they may stimulate osteoblast differentiation by acting on early or late osteoblast progenitors (modified from Mohan and Baylink, 1996).

differentiation, the present panel of stage-specific markers is incomplete. Fig. 5 shows the sequential expression pattern of osteoblastic differentiaton-related genes that have been identified thus far. In the early stages of bone development, there is extensive proliferation of progenitors, which express growth-related genes, such as *c-myc* and *c-fos*, *histone* (H4), and *osf*-2. Osteoblastic cells also express hormone and growth factor receptors, such as TGF-β, and BMPs (Abu et al. 1997a, b; Bodine et al.1998; Kondo et al. 1997; Shimizu et al. 1998). Mature osteoblastic cells exhibit several biochemical characteristics that have been used to determine osteoblastic phenotype. The production of type I collagen (Col I) starts with the onset of differentiation and increases with the cell's progression to a mature osteoctytic state (Sakano et al. 1993). The mature osteoblastic phenotype is also characterized by the ability of the cells to synthesize membrane-associated alkaline phosphatase (ALP), osteopontin (OPN), osteonectin, bone sialoprotein, and proteoglycans (Hultenby et al. 1994; McKee et al. 1993; Weinreb et al. 1990). Although most of these markers are not bone specific, they represent major components of osteoblastic expression and development. As the matrix and matrix-maturing proteins are suppressed, new gene products associated with the mineralization phase begin to be expressed, such as osteocalcin (OC; Hoffmann et al. 1994), thus leading to hydroxyapatite accumulation and compete mineralization, which is a feature of late osteoblastic and osteocytic differentiaiton.

Systemic Factors Affecting Osteoblastic Differentiation

Many systemic hormones are key molecules that have significant roles in the coordinate expression and regulation of marker genes during osteoblastic differentiation and

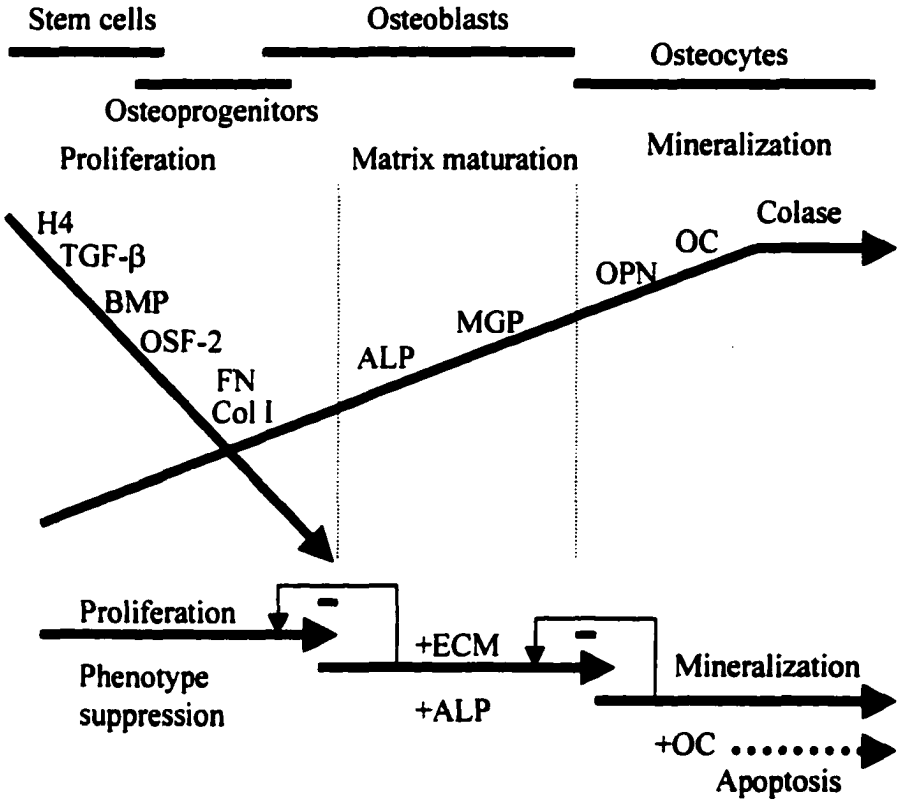

Figure 5. Patterns of gene expression in osteoblastic differentiation. A reciprocal relationship between two distinct groups of genes that predominantly act on one another in a proliferative and postmitotic differentiated stage. The high levels of histone proteins and other genes related to the proliferation gradually approach residual constituent level or are completely suppressed with the onset of osteoblastic differentiation. Expression of *osf-2* gene triggers cascade of phenotype specific genes. The earliest events that signifies the onset of osteoblastic maturation is the up-regulation of type I collagen (Col I), followed by the up-regulation of alkaline phosphatase (ALP). High levels of osteopontin (OPN), osteocalcin (OC), and collagenase(Colase) are characteristic of the mineralized phase of osteoblastic differentation (modified from Stein et al. 1996).

skeletal development. Due to the focus of this study, however, only a subset of widely studied systemic factors is reviewed here.

The most fully studied factors are PTH and PTHrP hormones. PTH is a polypeptide with a molecular weight (MW) of 9.5 kDa and is essential for the maintenance of calcium homeostasis. Through direct action on bone, PTH promotes the release of calcium into the extracellular fluid to activate osteoclastic activity (Fitzpatrick et al. 1996). PTH inhibits the synthesis of osteoblastic marker genes such as *Col* I and reduces the acitivity of ALP (Hall and Dickson 1985). PTHrP shares similarities with PTH at the levels of amino acid content, three-dimensional structure, the classical receptor (Soifer et al. 1992), and gene structure (Philbrick et al. 1996). Similar to PTH, the PTHrP functions as a local paracrine factor in the growth and differentiation of bone cells.

Vitamin D3 [$1\alpha,25(OH)_2D_3$] is one of the most prominent members of secosteroids that are derived from the cyclopentanoperhydrophenanthrene ring system (Finkelman and Butler 1985; Norman and Collins 1996). It functions as a principle factor required for the development and maintenance of bone as well as for the maintenance of normal calcium and phosphorus homeostasis. $1\alpha,25(OH)_2D_3$ directly acts on bone by increasing the availability of calcium and phosphorus for incorporation into bone and by affecting the differentiation of immature progenitors into osteoclasts. It also stimulates the expression of osteoblastic marker genes such as *oc* (Price et al. 1984) and *opn* (Prince and Butler 1987) in osteoblastic cells.

Mature calcitonin is a single-chain peptide of 32 amino acids produced by the parafollicular cells of the thyroid gland (Copp 1992). It acts as a key inhibitor of bone resorption by inducing an acute quiescence of cell motility, which is followed by a grad-

ual retraction of the osteoclasts (Alam et al. 1993). Calcitonin also diminishes osteoclastic activity by inhibiting the release of acid phosphatase, the expression of carbonic anhydrase II, the activity of focal adhesion kinase, and the production of OPN. Because of these activities, calcitonin has been successfully used to treat disorders of bone loss and to reduce the egress of osseous calcium into the blood in hypercalcemic conditions (Becker 1996).

Sex steroid hormones, such as estrogen, androgen, testosterone, and progestin, play a major role in mineral homeostasis during reproduction and in bone balance in adults. Estrogen and androgens are important in skeletal maturation and in prevention of bone loss. Ovariectomy leads to a deficit in bone mineral density in adult rats and monkeys (Kalu 1991) caused by changes in cortical bone modeling and net resorption of cancellous bone (Wronski et al. 1988). The skeletal changes caused by ovariectomy can be entirely prevented by pharmacological replacement with estrogen. Thus, ovariectomized rats have been used as a model system in the study of bone loss. Estrogen has been reported to inhibit bone resorption (Turner et al. 1988), possibly by reducing the synthesis of bone resorption stimulators, such as IL-1 and IL-6, and to stimulate cancellous bone formation (Takano-Yamamoto and Rodan 1990).

Glucocorticoid is a steroid hormone that inhibits pituitary secretion of growth hormone, leading to a decrease in the biologic activity of growth factors and loss of their anabolic effect on bone. Glucocorticoids have both anabolic and catabolic effects on bone, depending on the experimental model and the concentration of hormone. Physiological concentrations of glucocorticoids enhance the activity of hormones and growth factors and increase the differentiation of osteoblastic cells and the formation of bone tis-

sue. Glucocorticoids up-regulate some central osteoblastic differentiation-related genes, such as bone sialoprotein (Ogata et al. 1995). In contrast, a high concentration of glucocorticoids can cause a decrease in bone formation (Quarles 1992) and an increase in bone resorption, leading to osteoporosis (Lukert 1992). This is possibly caused by a reduction of the replication in osteoblastic pregenitors (Gronowicz and McCarthy 1995) and by down-regulation of the expression of *Col* I, matrix metalloprotease-13, or interstitial *collagenase*-3 (Delany et al. 1995).

Local Regulators of Bone Cell Formation

Many growth factors are now known to act on osteoprogenitors that divide and differentiate to osteoblasts, possibly by inducing transcription factors. Widespread studies have demonstrated that bone matrix contains at least five different classes of osteoinductive growth factors, including insulin-like growth factors (IGFs), TGF-βs, platelet-derived growth factors (PDGFs), fibroblast growth factors (FGF), and BMPs (Jennings et al. 1987; Mundy 1993; Reddi 1995; Sampath et al. 1990; Wang et al. 1988). All these growth factors can act as local stimulators for bone formation.

With a MW of 7.6 kDa (Jones and Clemmons 1995), IGFs, the most abundant polypeptides, are produced by osteoblasts and released from bone matrix to act as autocrine/paracrine hormones regulating specific bone cell functions. IGFs stimulate proliferation of cells of the osteoblastic lineage by enhancing the synthesis of bone collagen and matrix (Hock et al. 1988) and enhancing the differentiated function of mature osteoblasts (Canalis et al. 1993; Reddi 1994a; Rosen and Donahue 1998).

PDGF, a polypeptide with a MW of 30 kDa, is present in platelets and is expressed by various cell types. It is considered to be both a systemic and a local regulator of cell metabolism (Heldin and Westermark 1987). In skeletal tissue, PDGF is synthesized by bone cells and stimulates osteoblast proliferation, resulting in an increase in the pool of cells of the osteoblastic lineage, which will eventually express the osteoblastic differentiated phenotypes (Franchimont and Canalis 1995).

FGF-1 and FGF-2 are heparin-binding proteins with a MW of 17 KDa (Burgess et al. 1994). They have been shown to stimulate bone cell proliferation (Canalis and Raisz 1980). In the ROS 17/2.8 osteosarcoma cell line, FGF-1 and -2 reduce the expression of some bone marker genes, such as *col I* (Hurley et al. 1992), *alp*, and *oc* (Rodan et al. 1989).

TGF-β is a polypeptide with a MW of 25 kDa and is synthesized by many tissues, including bone. It is a potent multifunctional cytokine that regulates cell growth, stimulates matrix production, and suppresses the immune responses. In bone tissue, TGF-β participates in each stage of the bone remodeling process, including acting as a potent chemotactic factor that recruits osteoblastic precursors to sites of bone formation and as a mitogen that stimulates proliferation of osteoblasts (Pfeilschifter et al. 1990). The major effect of TGF-β on the osteoblasts is to cause them to differentiate into matrix-secreting cells. However, TGF-β inhibits OC production and mineralization of osteoblastic cells (Harris et al. 1994). TGF-β generally inhibits bone resorption by preventing the maturation and the activation of osteoclasts and by inducing the apoptosis of mature osteoclasts (Hughes et al. 1996).

BMP is unique among all the local regulators of bone because it is the only growth factor that promotes de novo bone formation. It can initiate and perpetuate the entire program that leads to the production of terminally differentiated osteoblasts from stem cells in the induction of bone formation (Zheng et al. 1992; Yeh et al. 1997; Yamaguchi et al. 1996). The process of bone formation induced by BMPs shows a strong similarity to the steps seen during normal embryonic bone development. Normal bone development begins with the chemotaxis of progenitors and MSCs to the site of BMP delivery, followed by stimulation of proliferation and initiation of differentiation of MSCs into chondrocytes and osteoblasts (Reddi and Cunningham 1993). The cellular and molecular mechanism of BMP-induced osteoblastic differentiation remains to be clarified and is the focus of this study.

BMPs—discovery. Urist (1965) first observed the osteoinductive activity of BMPs about 34 years ago because of its remarkable ability to induce new bone formation in the interior of an implant of demineralized bone. The search for these mysterious osteoinductive agents led to the discovery of a family of BMPs (Urist and Dowell 1967; Urist and Strates 1971; Urist 1971; Urist et al. 1975). Advances in the isolation of BMPs were made later when Urist and coworkers extracted what appeared to be hydrophobic, low-molecular-weight (MW 25-30 kDa) glycoprotein from demineralized rabbit, bovine, and human bone and from decalcified denin (Urist and Adams 1967; Urist and Strates 1971; Urist et al. 1979). The activity of these agents was found to be non-species specific. Although the osteoinductive properties of BMP were reproducible, the precise composition of proteins and their corresponding gene structure remained elusive. A ma-

jor breakthrough in identification of BMP was made through investigations by several other groups (Wang et al. 1988; Wozney et al. 1988). Wozney and colleagues (1989) identified seven unique BMPs from a protein fraction selected for its ability to induce endochondral ossification in an in vivo extraskeletal system. The protein coding regions for these BMPs were derived from the amino acid sequences of trypsin-digested purified fractions and were termed BMP1-BMP7. To date, about 15 BMPs have been isolated and cloned from the extracellular matrix. BMPs are members of the TGF-β superfamily, with the exception of BMP-1, which is a protease that processes the carboxy-terminal procollagen peptide (Celeste et al. 1990; Kessler et al. 1996).

BMPs and TGF-β superfamily. There are more than 43 members of the TGF-β superfamily that have been identified in species including sea urchin, *Caenorhabditis elegans, Drosophila, Xenopus*, zebrafish, chicken, mouse, and human (Wozney and Rosen 1998). The secreted and biologically active forms of members in the TGF-β superfamily are homo- or heterodimeric. Although the sequences of members of the TGF-β superfamily vary considerably, all are very similar in structure. All members of the TGF-β superfamily are synthesized as large precursor molecules consisting of an N-terminal signal peptide, a prodomain, and a C-terminal region of 110-140 in amino acids (Fig. 6). The prodomain can be cleaved at an RXXR site to release the C-terminal peptide, which is also called the mature domain. In most cases, the mature region contains 7-9 cysteine residues that are important for correct folding of these molecules. For example, TGF-β2 and BMP-7 each has a cysteine knot formed by three internal disulfide bridges between highly conserved cysteine residues in each subunit (Griffith et al. 1996). A fourth inter

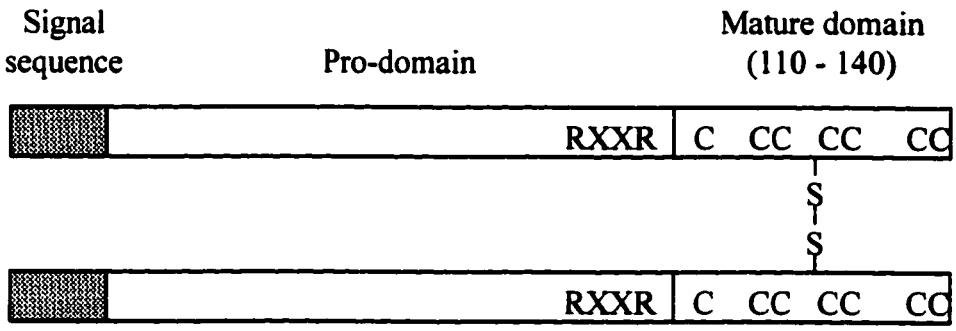

Figure 6. Structure of TGF-β related proteins. The members of TGF-β superfamily are synthesized as precursors with a signal sequence, pro-domain, and a C-terminal mature doamin. Precursor molecules can be cleaved at the dibasic proteolytic site, RXXR, to release the mature domain. In most cases, a single cycteine within the nature region is involved in forming a disulfide bone (-S-S-) in homo- or heterodimers (based on Hogan et al. 1994).

molecular disulfide bond stabilizes the dimer. However, the precise mechanism of the dimerization of the C-terminal mature region in vivo is largely unknown.

Comparison of the C-terminal mature regions of TGF-β superfamily proteins suggests that the members can be classified into a number of subgroups. The phylogenetic relationships between them are shown in Fig. 7. Members of TGF-β family act on target cells during embryonic development and at various times throughout a species' life span to simulate or inhibit cell division and differentiation. Among them, BMP2, BMP4, and BMPs5-7, and growth differentiation factors (GDFs) 5-7 have been shown to be fundamentally important regulators of skeletal tissue formation and repair.

BMPs—function. BMPs hold pleiotropic functions that range from extraskeletal and skeletal organogenesis during embryonic development (Bellows et al. 1986; Holley Kingsley 1994; and Ferguson 1997). The expression of BMP has been observed in nearly all developing organs, such as brain, heart, liver, lung, skin, hair follicles, placenta, and skeletal elements (Wall and Hogan 1995; Reissmann et al. 1996; Zhao and Hogan 1996; Urist 1997; Wawersik et al. 1999). BMPs also play a vital role in the specification and patterning of other tissues or organs, such as eye, kidney (Luo et al. 1995; Jena et al. 1997; Furuta and Hogan 1998), testis (Zhao et al. 1996), and heart (Zhang and Bradley 1996).

Null mutation of a gene, either naturally occurring or genetically manipulated, usually gives rise to detectable phenotypes, which are useful for assigning a specific function to each individual gene. The naturally occurring mutation of *bmp*-5 in mice shows a loss of specific cartilage condensations and other defects in the skeletal structure

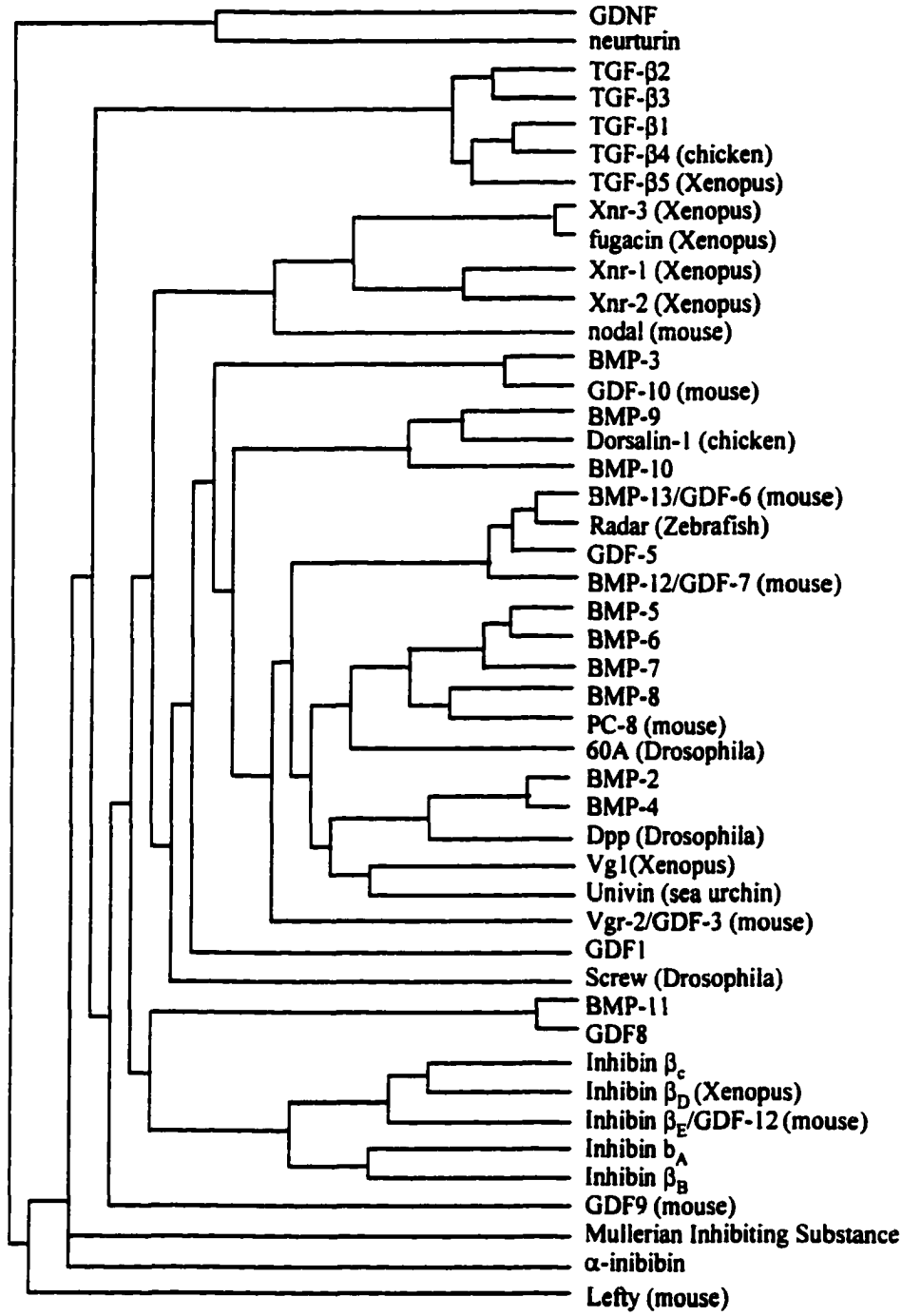

Figure 7. Phylogenetic tree of the members of the TGF-β superfamily. The similarity was calculated by comparison of amino acid identities from the first conserved cysteine residue. This figure was generated using the genetic computer group (GCG) Pileup program (based on Wozney and Rosen, 1998).

of the ears, sternum, and ribs (King et al. 1994; Kingsley et al. 1992), directly implicating the involvement of BMP in bone development. However, targeted disruption of either *bmp*-2 or *bmp*-4 leads to early embryonic lethality or to death at birth (Winnier et al. 1995; Zhang and Bradley 1996), making it difficult to identify the roles of individual BMPs in skeletal formation and repair. Knockout of the *bmp*-2 gene in mice results in defects in heart and amnion formation, all before onset of skeletogenesis, whereas loss of function of the *bmp*-4 in mice leads to the failure of mesoderm formation. The elimination of *bmp*-7 expression gives rise to alterations in the kidney, eyes, ribs, skull, and hind limbs (Dudley et al. 1995; Wilson and Hemmati-Brivanlou 1995). The deletion of *bmp*-6 in mice results in a consistent delay in ossification strictly confined to the developing sternum (Solloway et al. 1998).

The significance of BMP genes in regulating the skeletogenesis is also suggested by studies employing transgenic technology. Overexpression of BMP-2 and BMP-4 through retroviral infection of embryonic limbs during chick limb development results in changes in the size and shape of limb bones, whereas overexpression of BMP-5 results in an overgrowth of the cartilage elements (Duprez et al. 1996). Misexpression of the constitutively active BMP type I receptors causes similar abnormalities in cartilage (Zou et al. 1997), whereas misexpression of dominant-negative forms of BMP receptors leads to shortening of the cartilage elements (Kawakami et al. 1996; Zou and Niswander 1996).

Many members of the BMP family are able to induce ectopic bone and cartilage formation. Although their normal physiological functions have not been completely established, the cumulative data reviewed above suggest that BMPs play an important role in the regulation of skeletogenesis and in the induction of formation, patterning, and re-

pair of particular morphological features in higher animals. Research has now extended to the fields of developmental biology, genetics, and evolution.

BMP receptors. BMP receptors are memebers of a large family of transmembrane serine/threonine kinases that includes the receptors for TGF-βs, activins, and inhibins (Massague and Weis-Garcia 1996). An active receptor is a ligand-induced transient complex of two distantly related receptors, called type I and type II receptors (Massague 1998). The type II receptor is a constitutively active kinase that is autophosphorylated upon ligand binding, whereas the type I receptor kinase is activated when it is phosphorylated by the type II receptor. BMPs bind specifically to type I and type II serine/threonine kinase receptors with dissociation constants in the low nanomolar range (Estevez et al. 1993; Koenig et al. 1994). The binding affinity of BMPs to the receptor is higher when both types of receptors are present (Brummel et al. 1994). Formation of a heteromeric complex between members of the TGF-β superfamily and their respective receptors is one of the early events of signal transduction.

Early studies using cross-linking techniques identified the TGF-β type I receptor as about 65-70 kDa and the type II receptor as about 85-110 kDa (Cheifetz et al. 1986). Vale and colleagues (Donaldson et al. 1992) first cloned a type II activin receptor, which led to the discovery of various type I and type II receptors for TGF-β (Lin et al. 1992; Franzen et al. 1993) and for the BMPs (Yamaji et al. 1994; Rosenzweig et al. 1995; Liu et al. 1995). The two types of receptors have short extracellular domains, a transmembrane domain, and a long cytoplasmic region that mainly consists of the kinase domain (Fig. 8). The type I receptors are characterized by the presence of a highly conserved

cysteine-rich region in their extracellular domain and by a GS box immediately before the intracellular serine/threonine kinase domain. In contrast, type II receptors lack a GS box, have a different pattern of extracellular cysteine, and have a C-terminal tail rich in serine and threonine (Rosenzweig et al. 1995). In addition, the kinase domains of type II receptors contain two short inserts. Mutant forms of both type I and type II receptors that lack the cytoplasmic kinase domain have dominant negative activity (Brand et al. 1993). Mutations of a conserved proline in the second short insert of the daf-1 (type II receptor of *C. elegans*) disrupts its function (Georgi et al. 1990). The type I receptor has the key signal-transducing activity. Mutation of a single amino acid substitution to asparatate or glutamate in a particular residue of the GC box of type I receptors confers constitutive serine-threonine kinase activity (Wieser et al. 1995; Wu et al. 1996). The constitutively active forms of the type I receptor are sufficient to recapitulate the effects of ligand stimulation in a cell line that lacks both types of the TGF-β receptors (Wrana et al. 1994). Additional studies of mutationally activated type I receptors in BMP, activin, and TGF-β signal transduction assays consistently indicate that this receptor is sufficient for signaling. Mutant forms of both negative dominant and constitutively active type I receptors are widely used in research (Charng et al. 1998; Macias-Silva et al. 1998; Nikaido et al. 1999), and both constitutively active forms of type I BMP receptor (ALK3Q233D and ALK6Q203D) were used in this study.

The full set of physiologically significant combinations of ligands and receptors that interact in vivo has not been fully addressed. TGF-β and possibly activin have a non-serine/threonine kinase type III receptor that may facilitate or modulate binding to type I and type II receptors (Lopez-Casillas et al. 1993). The type III is an integral mem-

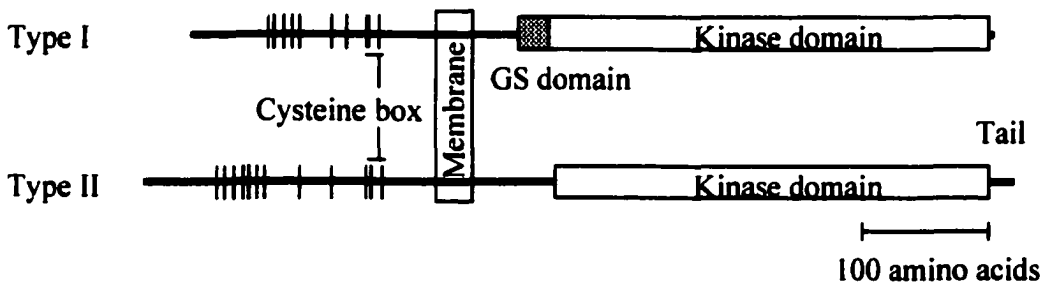

Figure 8. The structures of the type I and type II serine/threonine kinase receptors of the TGF-β superfamily. The vertical bars show the extracellular cysteines. All receptors for different members within the family are found to be around 550 amino acids in size. Both receptors contain a kinase domain, but the type I receptor lacks a tail on the C-terminus and contains a GS domain, a conserved 29-amino-acid region (based on Attisano et al. 1994).

brane protein (MW 200-400 kDa) that is heavily modified by glycosaminoglycan groups (Wang et al. 1991). The family of the type I and type II TGF-β receptors is summarized in Table 2.

Table 2. *Receptors of TGF-β family*[a]

Ligand	TGF-β	BMP	Dpp[c]	Activin
Type I receptor	TβR-I (ALK1, ALK5, ALK7[b])	BMPR-IA (ALK3) BMPR-IB (ALK6)	Punt	ActR-I (ALK2) ActR-IB (ALK4)
Type II receptor	TβR-II	BMPR-II	Tkv[c] Sax[c]	ActR-II

a. Data are from Massague et al. 1992; ten Dijke et al. 1994; Letsou et al. 1995; Wharton 1995; Tsuchida et al. 1996.
b. Orphan receptor, no ligand has been identified.
c. Tkv, thickveins, Sax, saxphone, Dpp, decapentaplegic.

BMP signaling--ligand binding and receptor activation. Binding of ligand to transmembrane receptors is the first step in signaling by the TGF-β superfamily. Wrana et al. (1994) proposed that TGF-β signaling begins with the ligand binding directly to the type II receptor; ligand binding leads to the assembly of a hetero-oligomeric receptor complex in which the type II receptor phosphorylates and activates the type I receptor. Further studies have established that this sequential binding mode (Massague 1998) is characteristic of TGF-β and activin receptors. In contrast to this mode, BMP binds to its receptors cooperatively, meaning that type I and type II receptors both have low affinity for the ligand and together achieve high affinity binding (Letsou et al. 1995; Rosenzweig et al. 1995).

Phosphorylation of the type I receptor is a central event in activation of signaling by ligand-receptor complexes. This process occurs in a cluster of five serine and threonine residues in the GS box (Massague and Weis-Garcia 1996) and is likely catalyzed by the type II receptor kinase whose activity is required for phosphorylation in the cell. Studies indicate that TβR-II (Wrana et al. 1994), ActR-II (Attisano et al. 1993), and ActR-IIB (Mathews and Vale 1993) are phosphorylated in the absence of ligand binding, and the mechanism of the phosphorylation remains mysterious. The serine and threonine in the cytoplasmic region of the type II receptors can be autophosphorylated when the receptor protein is expressed in the bacteria (Lin et al. 1992). Most data collected thus far suggest that the type II receptor alone can not signal but can only act as part of a signaling receptor complex (Massague 1998).

The intracellular events following the interactions between the type I and type II receptor had remained unknown until about four years ago when a candidate transducer mother against decapentaplegic (Mad) was identified in *Drosophila* by a genetic screen for mutations that modify the Dpp activity (Sekelsky et al. 1995).

BMP signaling mediators--Smad proteins. The search for Mad-related intracellular components of TGF-β signaling has led to the identification of three genes from C. elegans (Sma-2, -3, and –4; Savage et al. 1996) and nine vertebrate members (Smad1-9, Kretzschmar and Massague 1998; Fig. 9). One of the first reported human Smads is Smad4 (DPC4--deleted in pancreatic carcinoma locus 4), a gene frequently mutated in pancreatic cancer. Other human, mouse, *and Xenopus* Smads1-8 were cloned by screening expressed sequence tag (EST) database or cDNA libraries for Mad homologues

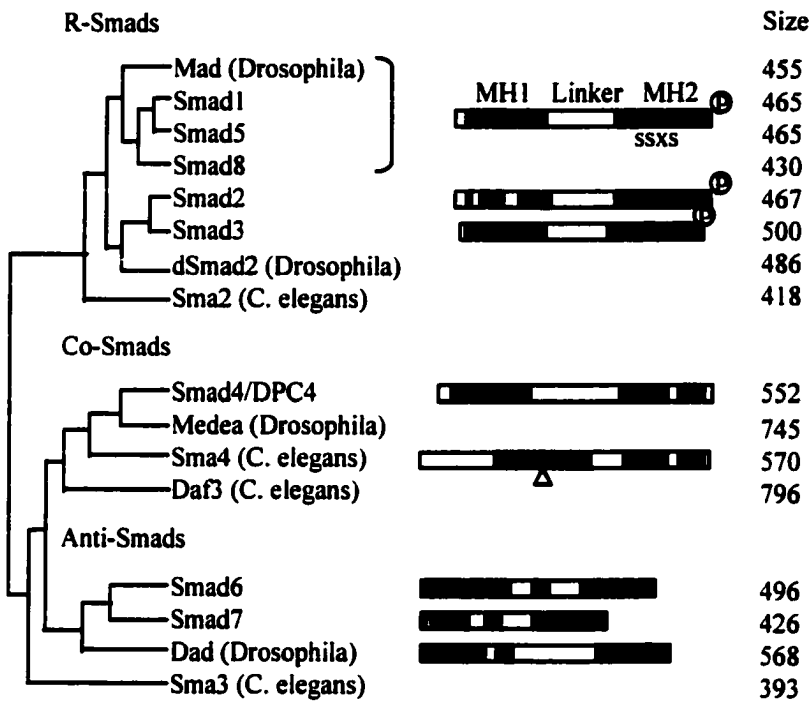

Figure 9. Phylogenetic relationships and domain structures of the Smad family members. Two domains are highly conserved within receptor-regulated (R-) Smads and common (Co-) Smads and are homologous to *Drosophila* Mad called MH1 and MH2. Sma4 and Smad2 contain small insertions in the MH1 domain; Sma4 has an unique long N-terminal extension. The linker region varies greatly among different Smads in both length (numbers of amino acids) and sequence of amino acids. R-Smads mediate the responses of a different set of TGF-β family ligands: Smad1, -5, and -8 are responsible for BMP signaling, whereas Smad2 and -3 transduce the TGF-β/activin signals into the cells. Co-Smads interact with both TGF-β and BMP pathway-restricted Smads. Antagonistic (Anta-) Smads lack most of the conserved MH1 domain, and their MH2 domain is also divergent from that of the other family members. A receptor phosphorylation motif (SSXS) at the end of MH2 domains is also indicated (based on Massague and Shi 1998; Raftery and Sutherland 1999).

(Graff et al. 1994; Chen et al. 1996; Eppert et al. 1996; Hoodless et al. 1996; Lechleider et al. 1996; Liu et al. 1996; Yingling et al. 1996; Zhang and Evans 1996; Imamura et al. 1997; Nakao et al. 1997a). *Xenopus* Smad2 was independently cloned in a cDNA screen for inducers of mesoderm formation in embryo (Liu et al. 1996). Two other Smads, Smad6 and –7 were identified as shear stress-induced genes in endothelial cells (Topper et al. 1997). The identification of Smads facilitated rapid progress in the TGF-β signaling field. Now it is clear that Smads play a central role in the transduction of receptor signals to target genes to the nucleus.

Smads are molecules of relative molecular mass 42-60 kDa. Vertebrate and *Drosophila* Smads fall into three classes based both on phylogenetic relationships and on functional assays. Two classes, the receptor-regulated Smads (R-Smads) and the common mediator Smads (Co-Smads), are directly involved in signaling transduction from membrane to the nucleus. A third class, the antagonistic Smads (anta-Smads), inhibit signaling.

R-Smads including five members, Smad1, -2, -3, -5, and -8, each of which can interact with and become phosphorylated by specific type I serine/threonine kinase receptors and thereby act in a pathway-restricted fashion. Smad1, -5, and -8 are phosphorylated and translocated to the nucleus after stimulation by BMP (Hoodless et al. 1996; Chen et al. 1997; Kretzschmar et al. 1997; Suzuki et al. 1997a), whereas Smad2 and Smad3 are phosphorylated and translocated to the nucleus in response to TGF-β or activin (Chen et al. 1996; Yingling et al. 1996). At the ends of their C-terminus, all R-Smads have a characteristic SS*X*S motif in which the last two of the serine residues are phosphorylated by their corresponding type I receptors (Abdollah et al. 1997; Kretzsch-

mar et al. 1997; Souchelnytskyi et al. 1997). The interaction of type I receptors and R-Smads is transient; after phosphorylation, the R-Smads are released from the receptors. Structurally, R-Smads encompass three distinct domains in R-smads: two highly conserved domains, referred to as Mad-homology domain1 (MH1 or N-domain) and MH2 (C-domain), respectively, and an intervening linker region, which is of variable length and sequences (Massague 1998). Each domain has different function(s) (Fig. 10). In their inactive state, the MH1 and MH2 domains associate with each other, forming a compact structure and inhibiting each domain's function. After activation by type I receptor, the compact structure opens up, allowing the R-Smads to form a heterocomplex with Co-Smads (see below). The complex then translocates into the nucleus where transcription of target genes is affected.

Co-Smads include Smad4 from vertebrates, medea from *Drosophila*, and Sma-4 from *C. elegans* (Hata et al. 1998). Smad4 has been shown to form a complex with receptor-phosphorylated R-Smads, which take it into the nucleus (Lagna et al. 1996; Onichtchouk et al. 1996). Formation of this complex is required for optimal binding and transcriptional activation of target genes in the response to TGF-β family members.

Anta-Smads consists of Smad6 (Hata et al. 1998) and Smad7 in vertebrates (Hayashi et al. 1997; Nakao et al. 1997b; Topper et al. 1997), Dad in *Drosophila* (Tsuneizumi et al. 1997), and Daf-3 in *C. elegans* (Patterson et al. 1997), which is distantly related to R- and Co-Smads and has a poorly conserved MH1 domain. When overexpressed at a high level, anta-Smads inhibit TGF-β, BMP, and activin signaling nonspecifically because of the stable binding of Smad6 and Smad7 to receptors, which blocks the receptors' ability to phosphorylate R-Smads. Studies showed that Smad6 preferen

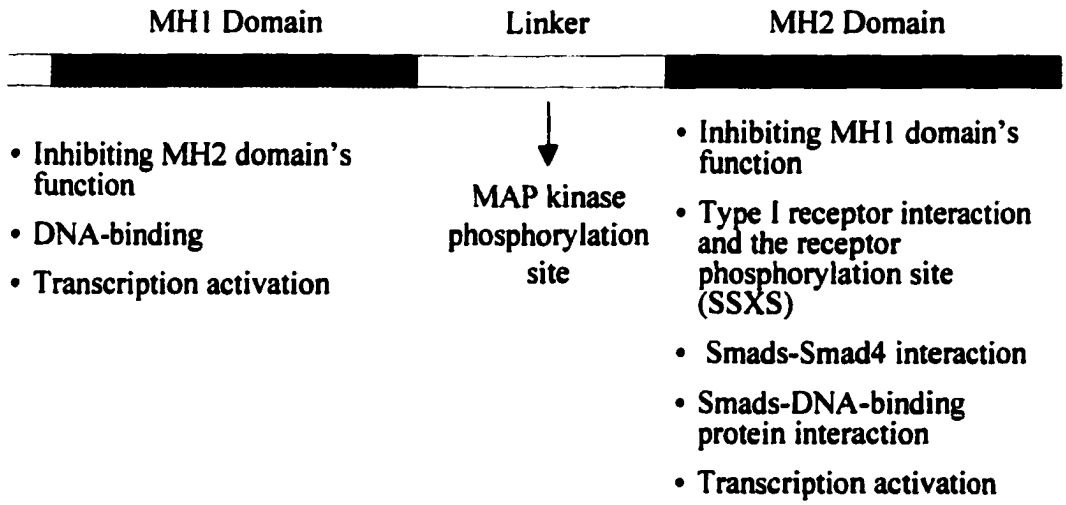

Figure 10. Smad domain functions. Smads contain two highly conserved N-terminal and C-terminal domains, namely MH1 and MH2, which share the homology with *Drosophila* Mad protein. The distinct functions of each domain are shown. The linker region is most variable and rich in proline, and it also contains several serine/threonine clusters. The serine/threonine residues within linker region can be phosphorylated by MAP kinase, which inhibits the nuclear translocation of the Smads (see text for details).

tially inhibits BMP signaling, whereas Smad7 may inhibit TGF-β and activin signaling. It appears that TGF-β and other stimuli induce the transcription of anta-Smads; thus, anta-Smads may act as an autoregulatory negative-feedback loop in the signal transduction of the TGF-β superfamily.

BMP signaling–Smad nuclear translocation and gene activation. Numerous reports have addressed the question of how TGF-β family members mediate their multifunctional effects by triggering transcriptional responses of target genes. It has become clear that upon phosphorylatoin by ligand-bound receptors, R-Smads dissociate from type I receptors and form a hetero-oligomer with Co-Smads. Subsequently, the complex translocates into the nucleus where the transcriptional control occurs (Lagna et al. 1996; Massague and Weis-Garcia 1996). Two modes have been proposed for Smad-mediated transcriptional activation of target genes (Whitman 1998).

In the first mode, the Smad complex forms a stable higher order protein-protein complex with other DNA-binding proteins. DNA recognition by this complex results from the combined DNA-binding affinities of the Smads and the additional transcription factor(s). For example, the Smad2/Smad4 complex interacts with the *Xenopus* winged-helix factor forkhead activating signal transducer-1 (FAST-1) to form the higher ordered transcriptional activation complex. This complex binds to an activin response element upstream of the homeobox gene *Mix.2* and activates gene transcription (Cavolina et al. 1997). A similar mechanism has been shown with the mammalian homolog FAST-2 in the activation of homeobox gene *goosecoid* (Farrington et al. 1997).

The second mode of Smad-mediated gene activation involves an intrinsic transactivation activity in which the Smads directly bind to their DNA element on the target genes. Many studies have suggested that the MH1 domains of both R-Smads and Co-Smads have an intrinsic DNA binding ability. The target DNA sequence has been identified (Kim et al. 1997; Dennler et al. 1998; Zawel et al. 1998), and the optimal core sequence GTCT (or its palindrome CAGA) box has been revealed by oligonucleotide selection (Johnson et al. 1999) and crystallographic studies (Shi et al. 1998). This Smad recognition core sequence has been found in many target genes, such as *collagenase*, *PAI-1* (Yingling et al. 1997), *c-Jun* (Wong et al. 1999), *Col* I (Chen et al. 1999), α2(I) *procollagen* (Chen et al. 1999), and human type VII *collagen* (Vindevoghel et al. 1998). The mechanisms of TGF-β family signaling and transcriptional activation are summarized in Fig. 11.

BMP signaling and osteoblastic differentiation. BMPs can induce bone cell formation in cell cultures; hence, such BMP-responsive cell lines have been used as ideal system for studies of the mechanisms of osteoblastic differentiation. Similar to in vivo process, in vitro, BMPs stimulate the expression of osteoblastic differentiation-related genes and induce mineralization, leading to the terminal differentiation of osteoblasts in a variety of masenchymal cell lines, such as the cell lines derived from osteosarcoma, including rat ROS 17/2.8 (Centrella et al. 1995) and UMR106 (Goto et al. 1996), human MG-63 (Cheng et al. 1998) and SaOS-2 (Laitinen et al. 1999), or from primary bone cell cultures such as MC3T3-E1 (Hino et al. 1999) and UMR201 (Zhou et al. 1993).

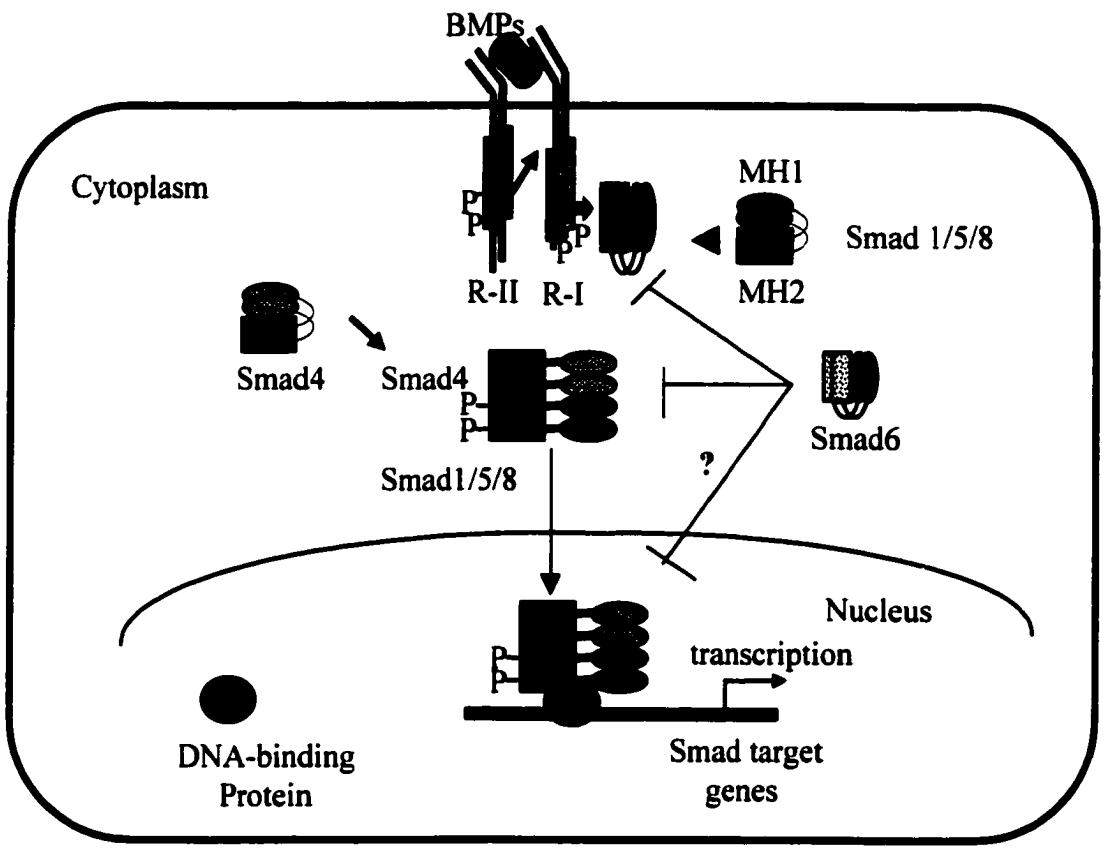

Figure 11. BMP signaling transduction through Smad family members. Smads form homo-oligomers through the interaction between MH1 and MH2 domains. Upon BMP ligand binding, BMPR-I receptors will phosphorylate Smad1, -5, or -8, the BMP pathway-restricted Smads, at the serine residues of the SSXS motif at the end of its C-terminus. When phosphorylated, the R-Smads (Smad1, -5, or -8) will form a complex with Smad4 and then translocate into the nucleus, where the complex can either directly bind to the DNA or recruit other DNA-binding proteins to initiate the target gene transcription. Smad6 is a specific antagonistic Smad which inhibits BMP signaling by preventing the phosphorylation of R-Smads by the BMPR-I receptor, by inhibiting phosphorylated R-Smads to interact with Smad4, or by inhibiting the R-Smads-Smad4 complex to interact with DNA-binding transcription factors.

Osteoblastic precursor cell lines also have been used as a model system for the study of cell differentiation in vitro. This system confers many advantages for such a study. It properly differentiates only as a result of correct signaling, and it responds to BMP signals. C3H10T1/2 is the most widely used multipotent MSC line. Depending upon the growth factor used, these cells can be induced to differentiate into multiple cell precursors, such as progenitors of chondrocytes, pre-osteoblasts, adipocytes, myotubes, or fibroblasts (Taylor and Jones 1979; Ahrens et al. 1993). In response to BMP2, cultured C3H10T1/2 cells express bone-specific marker genes, form bone mineral nodules, and undergo morphological changes necessary to become bone cells. Low concentrations of glucocorticoids can also stimulate these cells to differentiate into bone cells. Thus, C3H10T1/2 cells are used in this dissertation as a model system to study the mechanism of BMP signal transduction leading to bone cell differentiation.

Mechanisms of Osteoblastic Differentiatoin

Cells that participate in bone formation originate from pluripotenial MSCs. Stem cells seem to be under negative control by contact inhibition with other cells or suppression by negative regulators from their differential progeny (Abercrombie 1979). The molecular mechanisms underlying osteoblastic differentiation are largely unclear. Candidates or known factors controlling MSC properties range from growth factors and signal transduction molecules to transcription factors and determinants of chromosomal structure. Growth factors and signaling mediators enable communication with the external environment, whereas the transcription factors and structural determinants of chromosomes ultimately dictate the changing repertories of genes accessible and utilized by

MSCs in carrying out proliferation and differentiation decisions. The recent discovery of helix-loop-helix transcription factors encoded in so-called "master genes" sheds light on lineage differentiation patterns. The differentiation process is likely to be triggered by the induction of up-regulation of transcription factors in the osteogenitor cells, which in turn induce the osteoblast-specific genes that convert the osteoprogenitor cells to differentiated osteoblasts. However, such master genes controlling the initiation of the osteoblastic differentiation have not been reported.

Master genes, also called selector genes, act as stable binary switches that direct lineages of cells to adopt alternative developmental fates. Hox genes are master regulatory switches that function combinatorially and hierarchically to specify axial identity and to control the growth and differentiation of groups of cells related by position. (Botas 1993). Research in *Drosophila* has shown that Hox genes are continuously required during development for correct axial identity. Dominant gain and recessive loss-of-function mutations of Hox genes generate opposite segmental transformations (Gehring 1993). Selector genes start the cascade of sequential up-regulation of phenotype-specific genes that are responsible for lineage commitment and the specific phenotype of responsive cells.

It has been suggested that *CBFA*1 (*osf*-2), a member of the runt domain gene family, may act as a master gene responsible for osteoblastic lineage commitment (Ducy et al. 1997). Both TGF-β and BMP have been shown to up-regulate the expression of the *osf*-2 gene (Tsuji et al. 1998; Gori et al. 1999; Lee et al. 1999), indicating that *osf*-2 is one of the targets of BMP and participates in regulating the expression of genes related to osteoblastic phenotype. However, the cellular mechanisms responsible for the coordinate

expression and regulation of *osf*-2 and other specific transcription factors during osteoblastic differentiation and development are not clear. The role of *osf*-2 as a "master regulator" remains to be defined. Other transcription factors are also critical intrinsic components of multipotent stem cells that underlie processes of cell lineage commitment, proliferation, and survival. Of such factors, some of them show specificity for osteoblastic cells. For example, hXBP-1, a leucine zipper-containing protein, is only expressed in preosteoblasts and osteoblasts in the area of newly formed bones (Clauss et al. 1993), suggesting that hXBP-1 may play a role in regulating the expression of tissue-specific genes (*TIMP*, osteonectin, *opn*, and *oc*) expressed in osteoblasts. *Msx*-1/2 express restrictively in undifferentiated mouse calvarial osteoblasts (Hoffmann et al. 1994) and seem to act as transcriptional suppressors of the rat OC gene (Towler et al. 1994). Expression of the homeotic gene *Dlx*-5 is increased with progression of osteoblastic differentiation. BMP-4 has been shown to induce *Dlx*-5 expression, which leads to an increase in various osteogenetic markers, including increases in ALP activity, OC production, and appearance in mineralization of extracellular matrix (Miyama et al. 1999). These studies suggest an important role for Dlx-5 in promoting development of mineralized tissues, including bone, cartilage, and tooth (Ryoo et al. 1997). Clearly, the conversion of osteoprogenitor cells to mineralizing osteoblasts is a key event in bone cell formation. BMPs are molecular cues for osteoprogenitor cells to differentiate into osteoblasts (Reddi 1994b). BMPs also initiate bone formation events and act on each stage of the sequential cascade by binding specifically to their receptors and triggering intracellular responses that result in a mineralizing osteoblast (Yamashita et al. 1996b; Sakou 1998). Recent work has elucidated some of the intracellular signaling pathways for BMPs and subse-

quent gene activation leading to osteoblastic differentiation. This study was designed to elucidate the pathways by which BMPs activate specific osteoblastic genes that eventually lead to the final stage of osteoblastic differentiation. The advances in identification of Smad family proteins as downstream molecules of BMP signal transduction provide an excellent opportunity for us to reveal the mechanism underlying the conversion of osteoprogenitors to mature osteoblasts.

Central Hypothesis and Specific Aims

BMP-induced osteoblastic differentiation is mediated by the interaction of Smad1 with specific DNA-binding proteins. Smad1 specifically transduces BMP signals from the cytoplasm into the nucleus. Upon activation of Smads through phosphorylation by BMP type I serine/threonine kinase receptors, Smad1 will interact with Smad4 to form a complex, which will then be translocated into the nucleus to activate downstream genes. It is likely that Smad1 activates BMP target gene transcription through an interaction with other specific transcription factors. The goal of this research is to identify the protein(s) that interact with the specific BMP signal transducer, Smad1, and, further, to extend our current understanding by characterizing the molecular mechanism of BMP-induced osteoblastic differentiation. To achieve this goal, I will pursue the following specific aims.

Specific aim I: Identify and characterize the specificity of the interaction between Smad1 and the unknown DNA-binding protein(s) in the BMP signaling pathway. A mouse Smad1 cDNA will be cloned into a bait plasmid, which will be used to detect the Smad1-interactor in a yeast two-hybrid system. The specificity of the interaction between Smad1 and the DNA-binding protein will be determined by pull-down assays in

vitro and by immunoprecipitation assays in mammalian cells. The target for the DNA-binding protein(s) and the effect of the interaction between Smad1 and its interactor will also be identified by gel mobility shift assays.

Specific aim I: Map the domains of both Smad1 and its interactor to reveal the minimal sequences that are responsible for the interaction. A series of cDNA deletion constructs of the two proteins will be generated to examine the interaction of the two protein fragments. The effect of the interaction on DNA binding activity of the Smad1-interactor will be determined by gel shift assays. The effect of mapped Smad1 interaction domains on gene transcription will also be assessed in transient transfection assays. The significance of the interacting domains of Smad1 in the BMP-induced osteoblastic differentiation will be studied in permanent cell lines that express those fragments in a tetracycline-regulated mammalian expression system. Expression of the osteoblastic-specific genes that are induced by the Smad1 interaction domains will be determined by Northern hybridization, RNase protection assays, or reverse transcriptase-polymerase chain reaction. ALP activity assay and von Kossa staining to detect the mineralized particles will also be used to monitor the process of bone cell formation in those permanent cell lines.

Specific aim III: Identify and characterize Smad1 binding element(s) from osteoblastic marker genes. Apart from recruiting other DNA binding proteins to modulate gene transcription, Smad1 also binds directly to DNA and regulates gene transcription. The DNA elements and the core sequence GTCT for Smad3 and Smad4 have previously been reported (Zawel et al. 1998). By examining some of the osteoblastic marker genes, such as the osteopontin gene, we found several GTCT core sequences within the -300

base pair flanking region of the gene. The last part of this study will focus on identifying which of these core sequences is responsible for Smad1 binding and gene activation. The major techniques will include electrophoresis mobility shift assays to locate the Smad1 binding element(s) within the OPN gene and transfection to assay the transcriptional activity of the binding.

Significance of the Study

Throughout life, bone is constantly being broken down by osteoclasts and rebuilt by osteoblasts in a remodeling process. Pathology results from an imbalance between bone resorption and formation--too much bone resorption at the expense of formation leads to osteoporosis. In the United States, about 15 million individuals suffer from primary osteoporosis, and their annual medical care costs are close to $1 billion. Complications from overt fractures of the femoral neck, pelvis, or spine are frequent and result in 40,000 to 50,000 deaths per year, which is more than the combined mortality from carcinomas of the breast and endometrium (Rosenberg 1994). Effective treatment and prevention are imperative. Currently, most osteoporosis therapies are based on agents that restrict osteoclast activity. Drugs to stimulate bone formation to compensate bone loss are not available because the mechanism of osteoblastic differentiation is unclear. Understanding the factors regulating the continued remodeling of bone and regeneration of injured tissue is necessary if pharmaceuticals are to be truly effective in the treatment of osteoporosis.

This study will identify some important transcriptional factors that regulate osteoblastic differentiation. Our data will reveal the mechanism of the BMP-induced bone cell

formation through Smad1-mediated gene transcription activation. Further characterization of the interaction between Smad1 and its interactor will help us to understand the mechanism of BMP signaling and its role in bone cell differentiation and will provide us a scientific basis to develop rational clinical therapies. Fine mapping of the interacting domains of Smad1 and its interacting DNA-binding protein(s) will also facilitate pharmaceutical drug design.

SMAD1 INTERACTS WITH HOMEOBOX DNA-BINDING PROTEINS IN BONE MORPHOGENETIC PROTEIN SIGNALING

by

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The Journal of Biological Chemistry, 1999; 274:13711-13717

Abstract

Bone morphogenetic proteins (BMP) transduce their signals into the cell through a family of mediator proteins known as Smads. Upon phosphorylation by the BMP receptors, Smad1 interacts with Smad4 and translocates into the nucleus where the complex recruits DNA-binding protein(s) to activate specific gene transcription. However, the DNA binding protein(s) involved in BMP signaling has not been identified. Using a yeast two-hybrid approach, we found that Smad1 interacts with Hoxc-8, a homeodomain transcription factor. The interaction between Smad1 and Hoxc-8 was confirmed by a "pull-down" assay and a co-immunoprecipitation experiment in COS-1 cells. Interestingly, purified Smad1 inhibited Hoxc-8 binding to the osteopontin Hoxc-8 site in a concentration-dependent manner. Transient transfection studies showed that native osteopontin promoter activity was elevated upon BMP stimulation. Consistent with the gel shift assay, overexpression of Hoxc-8 abolished the BMP stimulation. When a wild-type or mutant Hoxc-8 binding element was linked to an SV40 promoter-driven reporter gene, the wild-type but not the mutant Hoxc-8 binding site responded to BMP stimulation. Again, overexpression of Hoxc-8 suppressed the BMP-induced activity of the wild-type reporter construct. Our findings suggest that Smad1 interaction with Hoxc-8 dislodges Hoxc-8 from its DNA binding element, resulting in the induction of gene expression.

Introduction

Transforming growth factor-β (TGF-β)–related molecules, or BMPs, regulate embryonic development, vertebral patterning, and mesenchymal cell differentiation (Wang et al. 1988; Francis et al. 1994). BMP-2 and -4 have been identified as bone inductive

growth factors and are important signaling molecules during the development of the skeleton in vertebrates (Wang et al. 1988; Ahrens et al. 1993; Mead et al. 1996). Signal transduction in the TGF-β superfamily requires the interaction of two types of serine/threonine transmembrane receptor kinases (Heldin et al. 1997). The signaling is mediated by direct phosphorylation of Smad proteins. Specifically, Smad2 and -3 are phosphorylated by TGF-β and activin receptors (Lagna et al. 1996; Heldin et al. 1997), whereas phosphorylation of Smad1 is induced by BMPs (Hoodless et al. 1996; Nishimura et al. 1998). Upon phosphorylation, these Smads interact with a common partner, Smad4, which then translocates to the nucleus where the complexes recruit DNA binding protein(s) to activate specific gene transcription (Hoodless et al. 1996; Zhang et al. 1996; Heldin et al.1997). The downstream DNA-binding proteins in the TGF-β signaling pathway, such as Fast-1, Fast-2, and TFE3, have been reported (Chen et al. 1997; Hua et al. 1998; Labbe et al.. 1998; . However, little is known about the downstream DNA-binding protein(s) beyond Smad1 in the BMP signal transduction machinery.

It has been suggested that homeobox genes play a role in downstream events in BMP signaling (Tang et al. 1998; Ladher et al. 1996). In vertebrates, there are 39 Hox transcription factor genes organized into four separated chromosome clusters, which play critical roles in the patterning of vertebrate embryonic development (Sharkey et al. 1997). These 39 genes are subdivided into 13 paralogous groups on the basis of duplication of an ancestral homeobox cluster during evolution, sequence similarity, and position within the cluster (Maconochie et al. 1996). Each paralog group has been demonstrated to be responsible for morphogenesis of a particular embryonic domain or structure (Sharkey et al. 1997).

Hoxc-8, as one of the three members of paralog VIII, is predominantly expressed at a high level in limbs, backbone, and spinal cord in early mouse embryos (Simeone et al. 1987; Le Mouellic et al. 1988). Null mutant mice showed that Hoxc-8 is expressed in neuron, chondrocyte, fetal liver, and adult bone marrow (Le Mouellic et al. 1992; Shimamoto et al. 1999). Bending and fusion of the ribs, anterior transformation of the vertebrae, and abnormal pattern of ossification in the sternum were observed in adult *hoxc*-8 null mice (Le Mouellic et al. 1992). Studies published recently demonstrated that tissue-specific overexpression of a Hoxc-8 transgene inhibits chondrocyte maturation and stimulates chondrocyte proliferation (Yueh et al. 1998). The other two members in the Hox VIII group are Hoxb-8 and Hoxd-8. Hoxb-8 has been shown to activate the Sonic hedgehog gene, an essential mediator in forelimb development (Charite et al. 1994; Lu et al. 1997), whereas generalized expression of Hoxd-8 modifies *Drosophila* anterior head segments (Bachiller et al. 1994).

Despite the fact that homeobox genes are DNA-binding proteins, little has been learned about their natural DNA response elements and the role in transcription (Maconochie et al. 1996). In the current study, we report that Smad1 interacts with Hoxc-8, and this interaction specifically activates the osteopontin gene transcription in response to BMP stimulation. Our data suggest that Hoxc-8 functions as a transcription repressor and that the interaction of Smad1 with Hoxc-8 dislodges Hoxc-8 binding from its element resulting in initiation of gene transcription.

Experimental Procedures

Two-hybrid Library Screening

A full-length Smad1 coding sequence from pBluescript-Smad1 (Zhang et al. 1996) was cloned into *SalI/PstI* sites of pGBT9 (Clontech, CA) to generate the pGBT9/Smad1 bait plasmid. The human U2 OS osteoblast-like pACT2 cDNA library was screened according to the manufacturer's instruction (Clontech). To confirm the interaction between Hoxc-8 and Smad1, a full-length mouse Hoxc-8 cDNA (Le Mouellic et al. 1988) was subcloned into pACT2 vector (Clontech) between *XhoI* and *EcoRI* sites. The pACT/Hoxc-8 was co-transformed with pBGT9/Smad1 into Y190 and the colonies were assayed for the production of β-galactosidase using both filter lift and liquid assays.

Expression and Purification of Glutathione S-transferase (GST) Fusion Proteins

GST fusion constructs of GST-Smad1 and -Smad3 were generated by restriction digest of pGBT-Smad1 (*SalI/Hind*III) and pCMV5-Smad3 (Zhang et al. 1996) (*BamHI/SalI*) and subsequently inserted into the *SalI/Hind*III and *BamHI/SalI* sites of the pGEX-KG vector, respectively. GST-Smad2 and -Smad4 were digested with *EcoRI/SalI* from pCMV5-Smad2 and pCMV5-Smad4 (Zhang et al. 1996) and inserted into *EcoRI/SalI* sites of the pGEX-5X-2 and pGEX-5X-1 vector (Amershem Pharmacia Biotech), respectively. The GST-Hoxc-8 and GST-Hoxa-9 (Catron et al. 1996) were amplified by polymerase chain reaction, using high fidelity *Pfu*-Turbo DNA polymerase (Stratagene) and cloned in the *BamHI/EcoRI* and *BamHI/XbaI* sites of the pGEX-KG vector, respectively. The GST-Msx-1 and –Msx-2 expression plasmids (Izon et al. 1998) were provided by Dr. C Abate-Shen (Center for Advanced Biotechnology and Medicine,

Piscataway, NJ). The GST constructs described above were transformed into BL21. The expression and purification of the fusion proteins were performed as described (Sterner et al. 1995)

GST Pull-down Assay

[^{35}S]-methionine-labeled Hoxc-8 protein was synthesized using the TNT-coupled transcription and translation system (Promega) with linearized pBluescript-Hoxc-8 plasmid according to manufacturer's instruction. The production of labeled protein was confirmed by SDS-polyacrylamide gel electrophoresis. An equivalent amount (1 μg) of purified GST or GST-Smad1 fusion protein was pre-incubated with [^{35}S]-labeled Hoxc-8 protein (5 μl) for 30 min on ice. Following the addition of GST-agarose, the samples were incubated for another 30 min at 4°C. The agarose beads were washed four times in 0.1% Triton X-100 in phosphate-buffered saline solution, and bound proteins were eluted by boiling in 2X SDS buffer for 5 min before loading onto 10% SDS polyacrylamide gel.

Immunoprecipitation and Western Blot

HA-tagged Hoxc-8 was subcloned from pACT2/Hoxc-8 into a mammalian expression vector pcDNA3 (Invitrogen) at *Bgl*II/*BamH*I and *Xho*I. Expression vectors for Flag-tagged Smad1 and Smad4 were provided by Dr. Rik Derynck (University of California, San Francisco, CA). Expression plasmids for constitutively active BMP type IA (ALK3) and IB receptor (ALK6; Macias-Silva et al. 1998) were provided by Dr. Jeffrey L. Wrana (Hospital for Sick Children, Toronto, Canada). COS-1 cells were transfected with expression constructs as indicated in Fig. 2B using Tfx-50 according to the manufacturer's description (Promega). Cells were lysed 48 hr post-transfection, and lysates

were immunoprecipitated with anti-HA antiserum (Babco) and immunoblotted with anti-Flag M2 (Eastman Kodak) as described (Hoodless et al. 1996).

Gel Shift Assay

Gel shift assays were performed as previously described (Cao et al. 1996). In brief, DNA fragments OPN1, OPN2, and OPN3 were generated by polymerase chain reaction using primers specific for the osteopontin promoter. The double-stranded oligomers were created by annealing the pairs of synthetic oliogonucleotides (only top strands are shown): 5'-CATGACCCCAATTAGTCCTGGCAGCA-3' (Probe-M); 5'-CC TTTCCTT ATGGATCCCTG-3' (OPN-4); 5'-GGTAGTTAATGACATCGTTCATCAG -3'(OPN-5); 5'-GGTAGT**GCCG**GACATCGTTCATCAG-3'(mOPN-5); 5'-GACATCG TTCATCAGTAATGCTTTG-3' (OPN-6). Mutated nucleotides in mOPN-5 are bolded. These DNA fragments were radio-labeled by T_4 polynucleotide kinase and [γ-^{32}P]ATP.

Transfection

The osteopontin promoter from region -266 to -1 relative to the transcription start site was amplified by polymerase chain reaction from CH10T1/2 cell genomic DNA and cloned into *Sma*I and *Xho*I sites of pGL3-basic vector (Promega) to generate luciferase reporter construct (OPN-266). Hox-pGL3 reporter bearing the Hoxc-8 binding site (-290 to -166) was constructed using the same strategy but was put into the pGL3-control vector (Promega). The Hox recognition core TAAT was replaced with GCCG in Hox-pGL3 by polymerase chain reaction to create mutant Hox-pGL3 (mHox-pGL3). C3H10T1/2 cells (2.5 x 10^5 cells/60-mm dish) were transfected using Tfx-50 with 0.5 μg of luciferase

reporter plasmid (OPN-266, Hox-pGL3, or mHox-pGL3) and different expression plasmids as indicated. Total DNA was kept constant by addition of pSV-β-galactosidase plasmid. Luciferase activities were assayed 48 hr post-transfection using the dual luciferase assay kit (Promega) according to the manufacturer's direction. Values were normalized with the *renilla* luciferase activity expressed from pRL-SV40 reporter plasmid. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.

Results and Discussion

Yeast Two-hybrid Library Screening

To investigate the transcription mechanism in BMP-induced gene activation, we have used a yeast two-hybrid system to identify transcription factors that interact with Smad1. An intact Smad1 cDNA fused with the Gal4 DNA-binding domain was used as a bait plasmid to screen a human U-2 OS osteoblast-like cell cDNA library constructed in the pACT2 plasmid. After two rounds of screening, we obtained 25 positive clones. DNA sequence analysis identified one clone as Hoxc-8 and two clones as Smad4. Because our objective is to identify downstream transcription factors in the BMP signaling pathway and Hoxc-8 is a homeodomain DNA-binding protein, we chose the Hoxc-8 cDNA clone for further study. Cloning of Smad4 provided a positive control for the two-hybrid library screening because the interaction between Smad1 and Smad4 is known. The other 22 clones were not characterized.

The initial Hoxc-8 cDNA clone (Fig. 1B, clone 19) encodes amino acids 68 to 237 of a 242-amino-acid Hoxc-8 protein. Fig.1A shows the growth properties of the two-

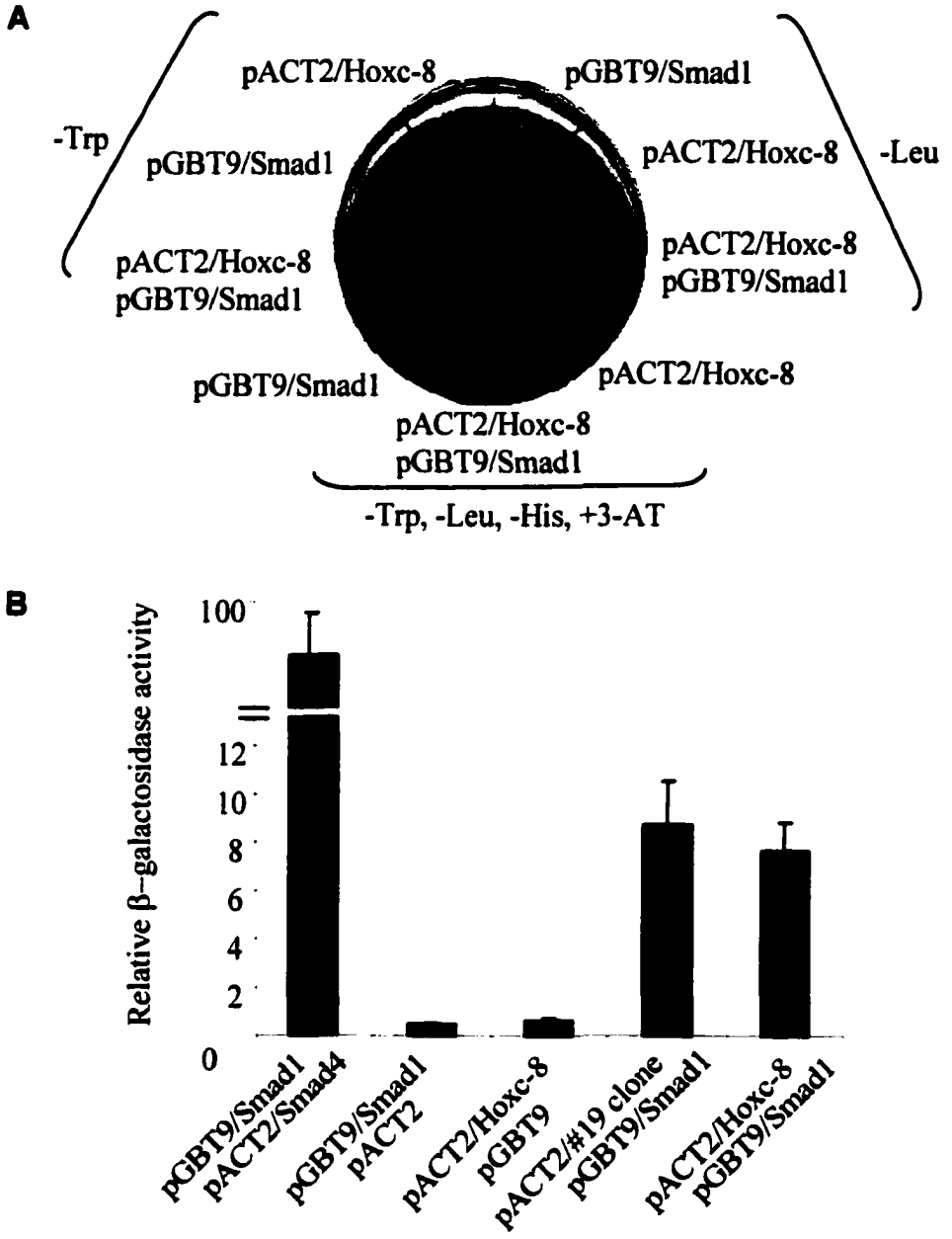

Figure 1. Specific interaction of Smad1 with Hoxc-8 in a yeast two-hybrid system. (*A*) Growth properties of two-hybrid clones. The interaction was assayed in a yeast strain, which requires His, Leu, and Trp to grow. pGBT9-Smad1 and pACT2-Hoxc-8 plasmids carry Trp and Leu as their selective markers, respectively. The interaction between Smad1 and Hoxc-8 enables the yeast to synthesize His. Only clones bearing both pGBT9-Smad1 and pACT2-Hoxc-8 plasmids grew in medium lacking His, Leu, and Trp. All assays were done in medium containing 45 mM 3-aminotriazole (3-AT), which inhibits growth because of nonspecific interaction. (*B*) β-galactosidase liquid assay for two-hybrid interaction. β-Gal activities for yeast bearing plasmids as indicated were plotted.

hybrid clones, suggesting that there is an interaction between Smad1 and Hoxc-8 in vivo. The yeast bearing both Smad1 and Hoxc-8 plasmids grew on medium deficient in Trp, Leu, and His. The interaction between Hoxc-8 and Smad1 was further confirmed with a β-galactosidase filter lift assay (data not shown) and quantified by a liquid β-galactosidase assay (Fig. 1B). When the full length of Hoxc-8 fused with the GAL4 transcriptional activation domain was tested in the two-hybrid system, it showed an interaction similar to clone 19. The assays of both empty prey vector (pACT2) with Smad1 in bait plasmid and empty bait vector (pGBT9) with full-length Hoxc-8 in the prey vector showed very little activity. Compared with the interaction between Smad1 and Smad4, the interaction of Smad1 with Hoxc-8 is weaker in the yeast two-hybrid β-galactosidase assay (Fig. 1B).

Smad1 Interacts With Hoxc-8 In Vitro and in COS-1 Cells

The interaction between Smad1 and Hoxc-8 was examined in an in vitro pull-down experiment using [^{35}S]methionine-labeled Hoxc-8 and purified GST-Smad1 or GST alone. As shown in Fig. 2A, Hoxc-8 was precipitated with the purified GST-Smad1 fusion protein (lane 3) but not with the GST alone (lane 2), demonstrating a direct interaction between the two proteins in vitro.

BMP-2 stimulates phosphorylation of Smad1, and phosphorylated Smad1 in turn binds to Smad4 and takes the complex into the nucleus. It is of interest whether Smad1, Smsd4, or the complex of Smad1 and Smad4 also interacts with Hoxc-8 in cells. COS-1 cells were transiently co-transfected with expression plasmids for Flag-Smad1, Flag-Smad4, HA-Hoxc-8, and/or constitutively active BMP type IA receptor ALK3 (Q233D). The cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted

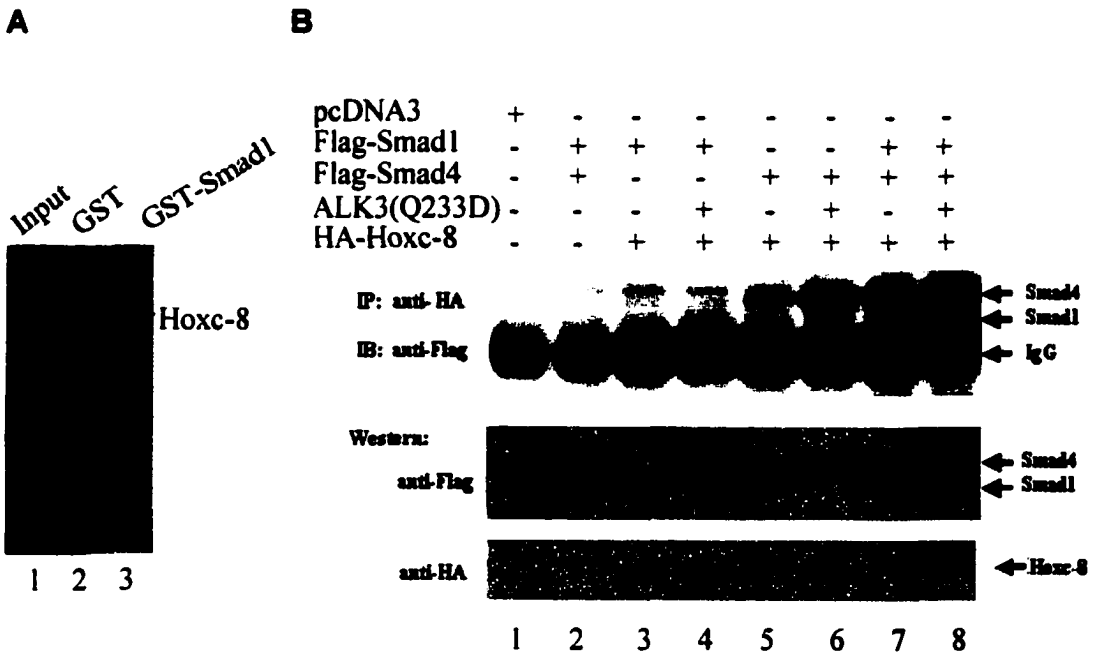

Figure 2. Interaction of Smad1 with Hoxc-8 in vitro and in vivo. (*A*) Specific interaction of Smad1 with Hoxc-8 in vitro. Hoxc-8 protein was labeled with [^{35}S] methionine by in vitro translation and incubated with purified GST-Smad1 or GST-protein. Samples were subsequently incubated with GST-Sepharose, washed, eluted in SDS buffer, and separated on 10% SDS-polyacrylamide gel electrophoresis. (*B*) The interaction of both Smad1 and 4 with Hoxc-8 in vivo. Flag-tagged Smad1 and -4 and HA-tagged Hoxc-8 were co-transfected with or without ALK3 (Q233D). Cell lysates were immunoprecipitated by anti-HA antibody, and the resulting complexes were analyzed by Western blotting with anti-Flag antibody. The expression levels of Smad1 and -4 were shown by Western blot with anti-Flag antibody (*middle panel*) and of Hoxc-8 with anti-HA antibody (*bottom panel*).

with anti-Flag antibody. Fig. 2B demonstrates that Smad1 (lane 3), Smad4 (lane 5) or both (lane 7) were co-immunoprecipitated with HA-Hoxc-8 in cells. Co-transfection of ALK3 (Q233D) enhanced the interaction of Smad1 (lane 4) or Smad4 (lane 6) with Hoxc-8. However, ALK3 (Q233D) did not significantly enhance the interaction of Smad1 and Smad4 complex with Hoxc-8 (lane 8). These results show both Smad1 and Smad4 interact with Hoxc-8 in COS-1 cells with or without BMP stimulation, indicating that the phosphorylation of Smad1 is not required for its interaction with Hoxc-8. If this is the case, the BMP-dependent regulation of the interaction is inherent in the intracellular localization of the proteins. Hox proteins are homeodomain transcription factors localized in the nucleus (Gendron-Maguire et al. 1993), whereas both Smad1 and Smad4 are cytoplasmic (Mead et al. 1996). It is likely that the interaction occurs only when Smad1 or the complex translocates to nucleus upon its phosphorylation induced by BMP receptors.

Osteopontin Promoter Contains a Hoxc-8 Binding Element

To examine the effect of the interaction between Hoxc-8 and Smad1 on Hoxc-8 DNA binding activity, we turned our attention to BMP-2 inducible genes. Putative Hox binding sites were found in four BMP-2 inducible bone matrix protein genes including bone sialoprotein, osteopontin, osteonectin and osteocalcin (Ahrens et al. 1993; Barone et al. 1993). These genes have served as markers for osteogenic differentiation. The osteopontin promoter was examined for this purpose because its mRNA expression is rapidly induced in response to BMP-2 treatment in C3H10T1/2 mesenchymal cell (Barone et al. 1993). Five putative Hox binding sites with a core sequence of Tt/aAT (Craig and Den-

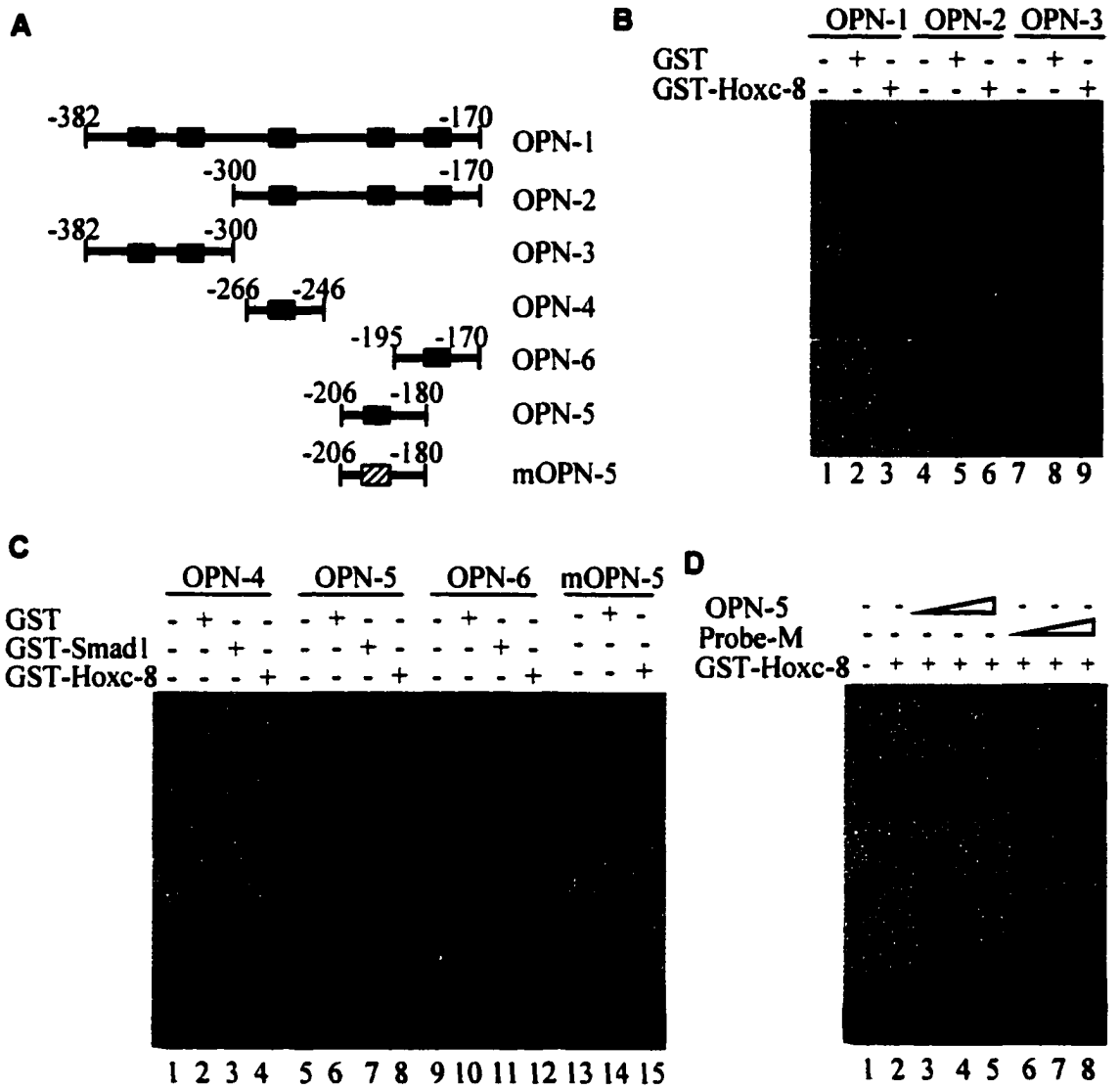

Figure 3. Characterization of a Hoxc-8 DNA-binding site from the osteopontin promoter. (*A*) DNA fragments of osteopontin promoter used for gel shift assays in panels B, C, and D. Nucleotides are numbered relative to the transcription start site. Filled boxes indicate putative Hox binding sites. The striped box represents a putative Hox binding site containing the mutated core sequence. (*B*) OPN-2 DNA fragment contains a Hoxc-8 binding site. A gel shift assay was performed using different ^{32}P-labeled DNA fragments as indicated. (*C*) OPN-5 is the Hoxc-8 binding element. A gel shift assay was performed using shorter DNA fragments as marked. The Hoxc-8 binding element is located from -206 to -180. (*D*) Hoxc-8 specifically binds to OPN-5. A gel shift assay was performed using OPN-5 alone (lane 1) or with GST-Hoxc-8 (lanes 2-8). Lanes 3-5 and 6-8 contained 5-, 25-, and 100-fold molar excess of unlabeled OPN-5 and MSX-2 DNA binding element (Probe-M), respectively.

hardt 1991) were identified within the first 382 base pairs of the 5'-flanking region in the osteopontin gene (Fig. 3A). When a 212-base pair DNA fragment from -382 to -170 (OPN-1 in Fig. 3A) containing all five putative Hox sites was used for a gel shift assay with purified GST-Hoxc-8 protein, one shifted band (Fig 3B, lane 3) was observed. This band was not present in lane 1, containing probe only, or in lane 2, containing probe with GST (Fig. 3B). This result indicates that there is only one Hoxc-8 binding site in this osteopontin promoter fragment. Further gel shift assays with shorter probes (OPN-2, and OPN-3 in Fig. 3A) indicated that OPN-2 contains this Hoxc-8 binding element (Fig. 3B, lane 6). When three single putative Hox binding probes (OPN-4, -5, and –6; Fig. 3A) were used, Hoxc-8 only bound to OPN-5, located at –206 to –180 (Fig. 3C, lane 8). Neither GST alone nor GST-Smad1 fusion protein could bind to any of the probes used in this series of gel shift assays (Fig. 3 B, lanes 2, 5, and 8; Fig. 3C, lanes 2, 3, 6, 7, 10, and 11). When the TAAT core sequence of Hoxc-8 binding site in OPN-5 was mutated to GCCG (mOPN5), Hoxc-8 binding was abolished (Fig. 3C, lane 15).

The specificity of the Hoxc-8 binding to the DNA was determined by a gel shift competition assay. Unlabeled Hoxc-8 DNA binding element inhibited the shifted band in a concentration-dependent manner (Fig. 3D, lanes 3-5) in which a 100-fold excess of the specific cold probe eliminated the Hoxc-8 binding, whereas a 100-fold excess of the Msx-2 DNA binding element (Towler et al. 1994) did not (Fig. 3D). Msx-2 is a homeo-domain-containing protein, but it does not belong to the Hox family. The Msx-2 DNA-binding element was identified from osteocalcin promoter, and its flanking regions of the core sequence is different from Hoxc-8 binding site.

There are three TAAT and two TTAT putative Hox sites identified from the osteopontin promoter. Hoxc-8 binds to only one of the TAAT core sequences (–206 to -180), suggesting that the flanking regions are also important for Hoxc-8 binding. The Hoxc-8 binding site, including its flanking regions, is highly conserved in chicken, mouse, pig and human. The other four putative Hox sites may be involved in other homeodomain protein binding or may not be authentic Hox binding sites.

Smad1 Inhibits Binding of Hox Proteins to DNA

Purified GST-Smad1 was examined for the effect of its interaction with Hoxc-8 on Hoxc-8 DNA binding activity. When GST-Hoxc-8 protein and its DNA binding element (OPN-5) were incubated with increasing amounts of GST-Smad1 protein, the binding of Hoxc-8 to the DNA probe was inhibited in a concentration-dependent manner (Fig. 4A, lanes 5-7). The same amount of GST protein did not have an effect on Hoxc-8 binding activity (Fig. 4B, lane 4). These results suggest that the interaction of Smad1 with Hoxc-8 dislodge Hoxc-8 from its response element.

Because the signaling networks of the TGF-β superfamily are very complex, it is important to understand the specificity of the interaction between Hox and Smad proteins. Hoxa-9 was chosen as a well-characterized homeobox DNA-binding protein (Fromental-Ramain et al. 1996; Cohn et al. 1997) to examine its interaction with different Smad proteins. Two other homeodomain proteins, Msx-1 and Msx-2, were also used for gel shift assays for the same purpose. *Msx*-1 and *Msx*-2, found at different loci from the Hox gene clusters, are involved in development of teeth. The expression of both

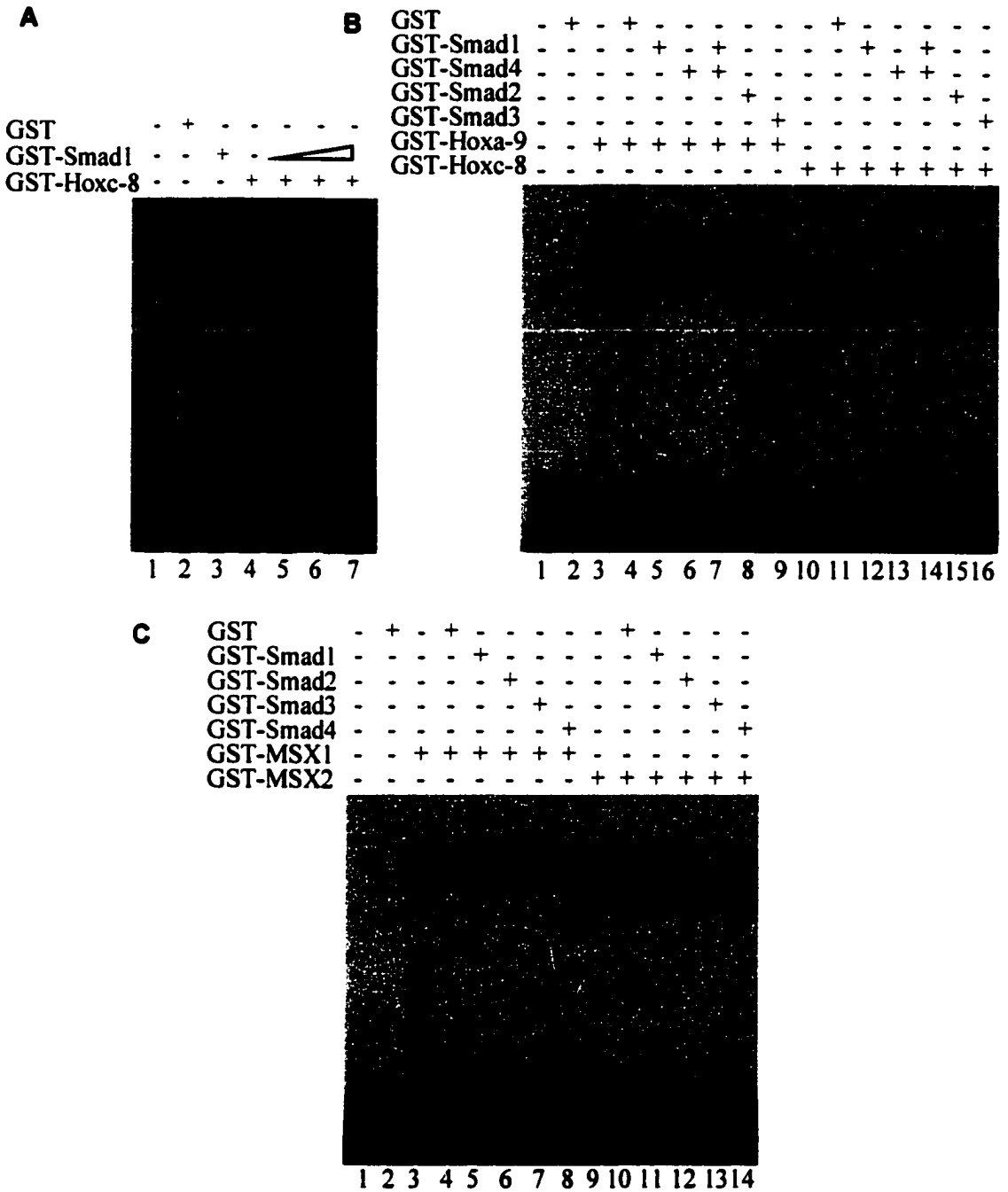

Figure 4. Smad1 inhibits binding of Hox proteins to DNA. (*A*) Smad1 inhibits binding of Hoxc-8 to OPN-5 in a concentration-dependent manner. Gel-shift assays were performed using OPN-5 alone (lane 1), with 1.5 μg GST (lane 2), 1.5 μg GST-Smad1 (lane 3) or 0.2 μg GST-Hoxc-8 protein (lanes 4-7) and different amounts of GST-Smad1 (1.5, 3 and 4.5 μg for lanes 5-7, respectively). (*B*) Hox proteins interact with Smad1 and 4 but not Smad2 and 3. Hoxa-9 and Hoxc-8 GST fusion proteins (0.2 μg) were tested for their ability to interact with Smad1, 2, 3, 4 or GST (3 μg) in a gel-shift assay. (*C*) Smads do not inhibit binding of Msx-1 and Msx-2 homeodomain containing proteins to their cognate DNA element. Purified GST-Msx-1 or Msx-2 (0.5 μg) was incubated together with probe-M and different Smads (3 μg).

genes is coordinately regulated by BMP-2 and BMP-4 (Jowett et al. 1993; Catron et al. 1996; Phippard et al. 1996).

To estimate the relative strength of the interactions between Smads and homeodomain proteins, the same amounts of Hoxc-8 and Hoxa-9 or Msx-1 and Msx-2 proteins were used in each of the gel shift assays with a fixed amount of different Smad proteins (Fig. 4B and 4C). Smad1 and Smad4 inhibited both Hoxc-8 and Hoxa-9 binding, and the inhibition was enhanced when both Smad proteins were added together (Fig. 4B, lanes 7, 14). In contrast, neither Smad2 nor Smad3 interacted with these two Hox proteins. Fig. 4C shows that neither of the Msx proteins interacted with any of the four Smad proteins. GST did not affect Hox or Msx protein binding (Fig. 4B, lane 4 and 11; Fig. 4C, lane 4 and 10). The homeodomain, a well-conserved DNA binding motif, is the region highly conserved between Hoxc-8 and Hoxa-9, suggesting that Smad1 interacts with other Hox proteins involved in BMP signaling.

Hox Binding Site Mediates BMP-induced Transcription

To examine whether the Hoxc-8 binding site functions as a BMP response element, we cloned a 266-base pair osteopontin promoter fragment containing the Hoxc-8 binding site into the pGL3-basic luciferase reporter vector to generate an OPN-266 reporter plasmid (Fig. 5A). Transfection of the OPN-266 construct in C3H10T1/2 mesenchymal cells showed that the reporter activity was stimulated moderately when Smad1 or Smad4 expression plasmids were co-transfected. The luciferase activity was significantly enhanced when the OPN-266 reporter construct was co-transfected with ALK3 (Q233D),

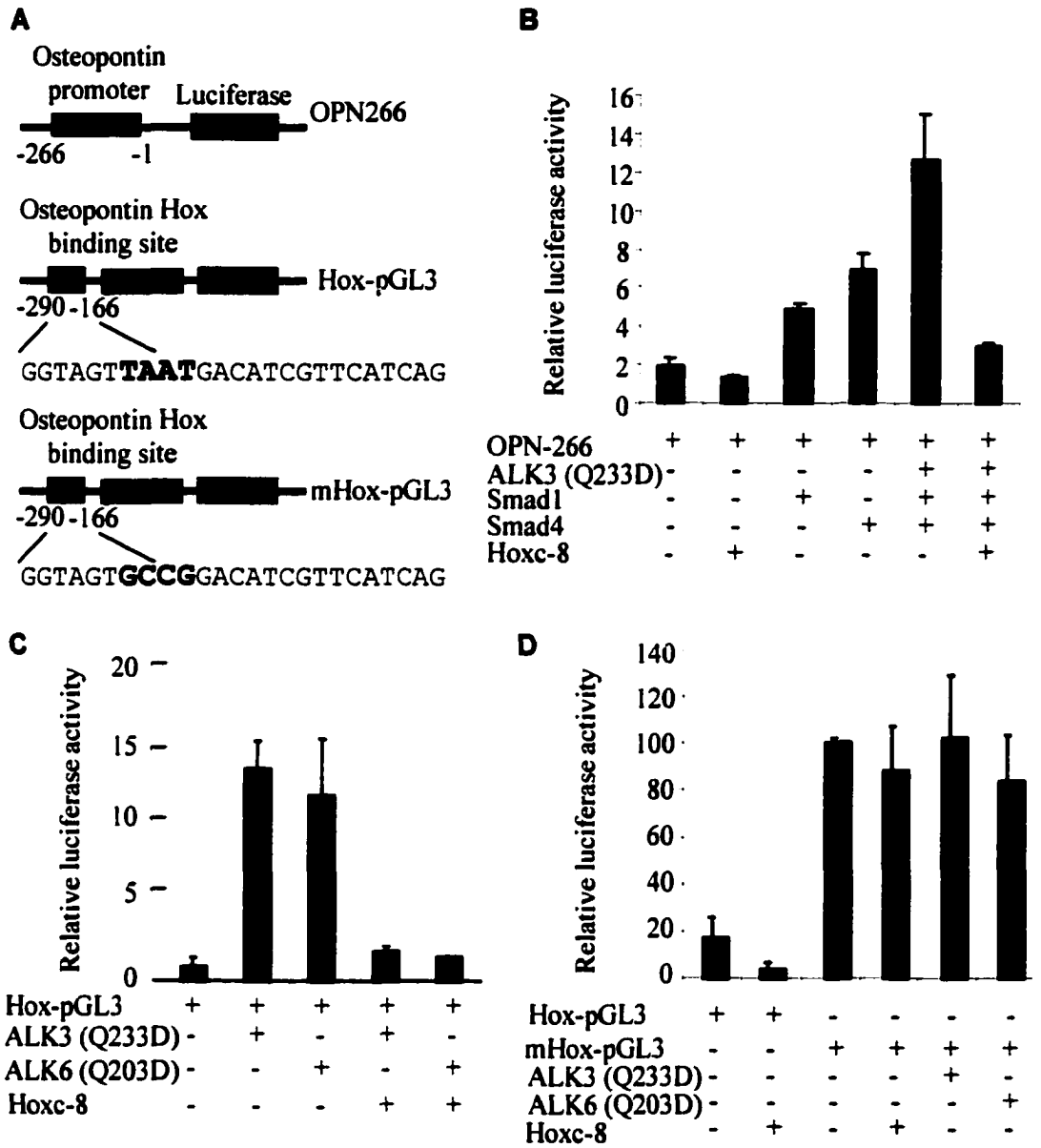

Figure 5. BMP-induced osteopontin gene transcription is mediated by Hoxc-8 binding site. (*A*) Schematic description of the constructs used in the transfection assays: OPN266 is the native osteopontin construct; Hox-pGL3 contains the osteopontin Hox binding site linked to SV40 promoter; mHox-pGL3 contains the mutated osteopontin Hox binding site. (*B*) BMP activates the osteopontin promoter. The OPN-266 plasmid was co-transfected in C3H10T1/2 mesenchymal cells with Hoxc-8, Smad1, or Smad4 plasmids alone or in combination of all three in the presence or absence of ALK3 plasmid. (*C*) The osteopontin Hox binding site mediates BMP-induced transcription. Hox-pGL3 construct was co-transfected with ALK6 or ALK3 in C3H10T1/2 mesenchymal cells. (*D*) Mutation of Hox binding site abolishes BMP stimulation. Hox-pGL3 construct or mHox-pGL3 pGL3 control plasmid was co-transfected with ALK6, ALK3, or Hoxc-8 plasmids in C3H10T1/2 mesenchymal cells. Cell lysates in *B*, *C*, and *D* were assayed for luciferase activity normalized to *Renilla* luciferase levels 48 hr after transfection. Experiments were repeated twice in triplicates.

Smad1, and Smad4 expression plasmids. Furthermore, the ALK3 (Q233D)-induced transcriptional activity was completely abolished when Hoxc-8 was overexpressed (Fig. 5B).

To further define the transcription activity of the Hoxc-8 binding site, we linked a shorter osteopontin promoter fragment containing the Hoxc-8 binding site to a luciferase reporter vector under the control of the SV40 promoter (Hox-pGL3, Fig. 5A). When the Hox-pGL3 construct was co-transfected in C3H10T1/2 cells with ALK3 (Q233D) or ALK6 (Q203D), luciferase reporter activity was induced more than 13- and 11-fold, respectively. Most importantly, overexpression of Hoxc-8 suppressed the ALK3 (Q233D)-induced or ALK6 (Q203D)-induced reporter activity (Fig. 5C). These results suggest that the Hox binding site mediate BMP signaling and that Hoxc-8 functions as a transcription repressor. In comparison with osteopontin native promoter, the Hox-pGL3 construct does not require overexpression of Smad1 and 4 in responding to BMP stimulation. This is an SV40 promoter-driven construct with a much shorter osteopontin promoter fragment, which does not contain many other transcription elements like the native osteopontin promoter construct.

To validate whether the Hoxc-8 site mediates BMP signaling, we mutated the core nucleotides of the Hoxc-8 binding site from TAAT to GCCG to create mHox-pGL3 (Fig. 5A). Transfection of the mutant construct, mHox-pGL3, completely abolished the ALK3 (Q233D)-induced or ALK6 (Q203D)-induced reporter activity and eliminated Hoxc-8 inhibition in C3H10T1/2 cells (Fig. 5D). These results confirm that the osteopontin Hox binding site is a BMP response element.

Several Smad downstream transcription factors have been characterized in the TGF-β pathway. Here, we first show that Hoxc-8 interacts with Smad1 as a downstream

DNA-binding protein in the BMP pathway. Our data demonstrate that Hoxc-8 binds to the osteopontin promoter and represses the gene transcription. BMP stimulation activates gene transcription by derepressing the Hoxc-8 protein, through the interaction of Smad1 with the Hoxc-8 protein. The direct interaction between Smad1 and Hox protein(s) suggests their functional relationship and the mechanisms in BMP-induced skeleton development.

Acknowledgments

We are grateful to R. Wuthier, V. Darley-Usmar, and H. Jo for discussions and comments on the manuscript. We thank J. Wrana for kindly providing the constitutively active type IB (ALK6) and IA (ALK3) BMP receptor expression vectors, R. Derynck for human Smad1, -2, -3 and -4 cDNA clones, H. Le Mouellic for Hoxc-8 cDNA, C. Largman for Hoxa-9 cDNA, and C. Abate-Shen for GST-MSX1 and -MSX2 expression vectors.

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SMAD1 DOMAINS INTERACTING WITH HOXC-8 INDUCE OSTEOBLAST DIFFERENTIATION

by

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The Journal of Biological Chemistry, 2000; 275:1065-1072

Abstract

Bone morphogenetic proteins are potent osteotropic agents that induce osteoblast differentiation and bone formation. The signal transduction of bone morphogenetic proteins has recently been discovered to involve Smad proteins. Smad1 is an essential intracellular component that is specifically phosphorylated by bone morphogenetic protein receptors and translocated into the nucleus upon ligand stimulation. Previously, we have reported that Smad1 activates osteopontin gene expression in response to bone morphogenetic protein simulation through an interaction with a homeodomain transcription factor, Hoxc-8. In the present study, the interaction domains between the two proteins were characterized by deletional analysis in both yeast two-hybrid and gel shift assays. Two regions within the amino-terminal 87 amino acid residues of Smad1 were mapped to interact with Hoxc-8, one of which binds to the homeodomain. Overexpression of recombinant cDNAs encoding the Hoxc-8 interaction domains of Smad1 effectively activated osteopontin gene transcription in transient transfection assays. Furthermore, stable expression of these Smad1 fragments in 2T3 osteoblast precursor cells stimulated osteoblast differentiation-related gene expression and led to mineralized bone matrix formation. Our data suggest that the interaction of amino-terminal Smad1 with Hoxc-8 mimics bone morphogenetic protein signaling and is sufficient to induce osteoblast differentiation and bone cell formation.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF-β) superfamily, which plays a vital role in regulating cell proliferation, dif-

ferentiation, and apoptosis, and which supports the formation, patterning, and repair of particular morphological features (Wozney et al. 1989; Kingsley et al. 1994). BMPs induce de novo bone formation in post-fetal life through the process of intramembranous and endochondrial ossification. BMP-2, -4, and -7 are the most potent osteotropic factors that promote new cartilage and bone formation both in vitro and in vivo (Katagiri et al. 1990; Wang et al. 1990; Ahrens et al. 1993).

Smad1 is the downstream effector of BMP signaling and is phosphorylated by BMP type I receptors (Hoodless et al. 1996; Kretzschmar et al. 1997; Macias-Silva et al. 1998). The phosphorylation of Smad1 induces its accumulation in the nucleus where it regulates gene transcription by associating with a nuclear transcription factor (Shi et al. 1999; Verschueren et al. 1999) or by binding directly to DNA (Kim et al. 1997). Smad1 consists of three distinct domains: two highly conserved amino- (NH_2-) and carboxyl- (COOH-) terminal domains, referred to as MH1 (mad homology 1) and MH2, respectively, and a more divergent intervening linker region. MH1 has DNA binding activity when MH2 is removed. MH2 contains a conserved receptor phosphorylation motif, SS*X*S, and has transactivation activity. Cross-talk between Smad and mitogen-activated protein kinase signaling pathways is conferred by the linker region in which the serine residues can be phosphorylated by mitogen-activated protein kinases, leading to an inhibition of Smad1 translocation into the nucleus (Kretzschmar et al. 1997). In the inactive state, MH1 and MH2 bind to one another, mutually inhibiting the function of each domain. In the active state, the phosphorylation of Smad1 opens up this structure to allow association with Smad4 or with other DNA-binding proteins via the MH2 domain (Massague et al. 1998; Tsukazaki et al. 1998; Whitman et al. 1998).

Studies on the mechanism by which Smads mediate TGF-β/activin-regulated gene transcription have led to the discovery of several Smad-interacting nuclear transcription factors and their *cis*-acting DNA elements. In particular, the *Xenopus* FAST-1 (forkhead activin signal transducer-1) binds to an activin response element upstream of the homeobox gene *Mix*.2. The transcription activation requires the presence of activin and assembly of a FAST-1-Smad2-Smad4 complex (Chen et al. 1997). The mammalian homolog FAST-2 activates the Hox gene *goosecoid* where formation of a higher order complex of FAST-2-Smad2-Smad4 is also essential for transactivation (Labbe et al. 1998). Transcription factor μF3 binds to the E-box of plasminogen activator inhibitor-1 promoter, whereas Smad3 and Smad4 bind to a sequence adjacent to the transcription factor μF3 binding site to cooperatively activate plasminogen activtator inhibitor-1 gene transcription (Hua et al. 1998).

In contrast to the TGF-β/activin pathway, little progress has been made in the identification of factors involved in Smad1-mediated transcriptional regulation in response to BMP signaling. We have reported that Smad1 interacts with homeodomain transcription factor Hoxc-8 (Shi et al. 1999). Hoxc-8 belongs to a highly conserved Hox gene family and is expressed in limbs, backbone rudiments, neural tube of mouse mid-gestation embryos, and in the cartilage and skeleton of newborns (Simeone et al. 1987; Le Mouellic et al. 1988; Yueh et al. 1998). Its expression is also found in the mouse hematopoietic organs, fetal liver, and adult bone marrow (Shimamoto et al. 1999). *Hoxc*-8 knockout mice displayed skeletal abnormalities in ribs, sternum and lumbar vertebra, and neuronal tissues (Le Mouellic et al. 1992; Tiret et al. 1998). Similar alterations of axial skeletal structures also occurred in Hoxc-8 transgenic mice (Pollock et al. 1992,

1995). Overexpression of the Hoxc-8 transgene in mice demonstrated that Hoxc-8 could regulate chondrocyte differentiation at the level of the proliferating chondrocyte or its immediate precursor (Yueh et al. 1998).

The present study is aimed at investigating the mechanism by which the Smad1-Hoxc-8 interaction mediates induction of osteoblast differentiation. We show that two domains, one within MH1 and the other at the MH1-linker boundary of Smad1, interact with the Hoxc-8 and are functionally sufficient to activate bone marker gene transcription. More importantly, permanent expression of these Hoxc-8 interaction domains in 2T3 osteoblast precursor cells is able to mimic BMP signaling and induce osteoblast terminal differentiation.

Experimental Procedures

Yeast Two-hybrid Interactions

cDNAs encoding full-length forms of Smad1 and Hoxc-8 were fused in-frame to the GAL4 DNA binding domain of pGBT9 vector and to the GAL4 activation domain of pACT2 vector, respectively, to obtain the bait and prey plasmids as described previously (Shi et al. 1999). All of the Smad1 or Hoxc-8 deletion constructs as indicated in figures were generated by *Pfu* (Stratagene) polymerase chain reaction-based strategy and inserted into their respective bait (pGBT9) or prey (pACT2) vectors. Resultant constructs were sequenced and subjected to yeast two-hybrid assays. To facilitate the interaction test, we first transformed a yeast reporter strain Y190 with the bait or prey plasmids and selected on SD/-Trp plate for pGBT9/Smad1 and SD/-Leu plate for the pACT2/Hoxc-8. Various deletions of Hoxc-8 were transformed individually into Y190 bearing pGBT9/Smad1,

whereas the different truncated constructs of Smad1 were transformed into Y190 containing pACT2/Hoxc-8.

In Vitro Binding Assays

The methods of constructing the bacterial expression plasmids for GST-Smad1 and GST-Hoxc-8 have been described previously (Shi et al. 1999). GST fusion constructs of all the Smad1 and Hoxc-8 deletion mutants were made by the polymerase chain reaction-based strategy with the pGEX-KG vector. Bacterially expressed GST fusion proteins were purified with glutathione substrate-affinity agarose beads (Sigma) as described (Guan and Dixon 1991) and analyzed for their purity by 10% SDS-polyacrylamide gel electrophoresis. In vitro binding was studied using a gel shift assay (Cao et al. 1996) with constant amounts (1.5 μg for all forms of GST-Smad, 0.2 μg for GST-Hoxc-8, and 10 ng for deletions of GST-HDC and GST-HD) of purified GST-fusion proteins. A double-stranded oligomer corresponding to the osteopontin promoter, -206 to -180 relative to the transcription start site (OPN-5) labeled using T_4 polynucleotide kinase and [γ–^{32}P]ATP, was used as probe.

Co-transfection Analysis

The single Hoxc-8 binding site and its flanking region derived from osteopontin promoter -290 to -166 were inserted in the pGL3-control vector (Promega) that uses luciferase as reporter (Hox-pGL3; Shi et al. 1999). The plasmids encoding various forms of Smad1 fused with a nuclear localization signal were constructed by inserting the polymerase chain reaction-amplified fragments into the cytomegalovirus (CMV) pro-

moter-based mammalian expression vector pCMV5. Each construct contained one of the following regions: Smad1-NL (amino acids (aa) 3-278), Smad1-L (aa 145-278), and Smad1-M (aa 101-191). The expression plasmid for Hoxc-8 was made by subcloning the cDNA from pACT2/Hoxc-8 into pcDNA3 (Invitrogen). C3H10T1/2 cells (5×10^4 cells/well in a 12-well culture dish) were transfected with 0.5 μg of Hox-pGL3 plasmid together with 200 ng of the indicated constructs using Tfx-50 as instructed (Promega). Luciferase activity was determined 16 hr after the start of transfection, and values were normalized with protein content. The luciferase activity shown in the figures is representative of transfections performed in triplicate in at least three independent experiments.

Establishment of Permanent Cell Lines

The tetracycline-regulated expression system (Life Technologies, Inc.) was used for inducible expression of Smad1-Hoxc-8 interaction domains of Smad1 in 2T3 osteoblast precursor cells (Ghosh-Choudhury et al. 1996). Smad1-NL, -L, or -M linked to a nuclear localization signal was subcloned from pCMV5 into pTet-Splice vector (Life Technologies, Inc.). 2T3 cells (10^5cells/60-mm dish) were transfected with a mixture of 2 μg of either empty pTet-Splice vector (control), recombinant pTet-Splice/Smad1-NL, -L, or -M along with 2 μg of pTet-tTAk, and 12 μl of Tfx-50 (Promega) in α–minimal essential medium containing 0.5 μg/ml tetracycline. pcDNA3 (40 ng) was also included in the DNA mixture to provide a selective marker plasmid that expresses resistance to G418. 2 days after transfection, cells were replated and α–minimal essential medium containing 10% fetal bovine serum, 0.5 μg/ml tetracycline, and 400 μg/ml G418 was

added to exclude nontransfected cells. 2-3 weeks later, drug-resistant colonies developed from single cells were isolated and maintained in α–minimal essential medium containing 10% fetal bovine serum, 0.2 mg/ml G418, and 0.5 μg/ml tetracycline. To induce the expression of Smad1 fragments, tetracycline was omitted from the growth medium. The expression was analyzed with Slot-Blot (Bio-Rad) by Northern hybridization using 5 μg of total RNA and 5 x 10^5 cpm/ml [α-^{32}P] dCTP-labeled appropriate cDNA probes. Total RNA was prepared with STAT-60 (Tel-Test) from G418-resistant clones grown in a medium with or without tetracycline. 10-20 clones of each indicated construct were analyzed. The tetracycline-regulated Smad1 fragment-expressing clones were kept for further characterization of the osteoblastic phenotype as described below.

Bone Marker Gene Expression

2T3 and its derivative cell lines containing empty pTet-Splice (vector control) and those displaying regulated expression of indicated Smad1 fragments were grown in the absence of tetracycline to reach confluence. Then the medium was replaced with a medium consisting of 5% fetal bovine serum, 0.1 mg/ml ascorbic acid, and 5 mM β-glycerol phosphate with or without 50 ng/ml recombinant human (rh) BMP-2 in α–minimal essential medium. mRNA was isolated at day 4 with MicroPoly(A)Pure (Ambion) and 3 μg of poly-A$^+$ RNA was used for Northern blot using Rapid-Hyb buffer (Amersham Phamacia Biotech) according to the manufacturer's direction. Probes for osteopontin and *osf*-2/*cbfa*1 were polymerase chain reaction amplified using cDNA prepared from rhBMP-2-treated C3H10T1/2 cells as template. Type I procollagen α1(1) cDNA was kindly provided by D. Chen (University of Texas).

Alkaline Phosphatase and Mineralized Bone Matrix Formation Assays

Bone cell differentiation was monitored by alkaline phosphatase (ALP) assay (Begley et al. 1993) and von Kossa stain of mineralized bone matrix (Begley et al. 1993). In brief, cells were prepared in a fashion similar to that described in the Northern analysis except that the cell density was 5 x 10^4cells/well in 12-well culture plates for ALP assay and 2 x 10^4cells/well in 24-well plates for mineralization staining. Cells were lysed with 0.05% Triton 2 days after reaching confluency. ALP activity was assayed using *p*-nitrophenol phosphate (Sigma) as the substrate. The A_{405} was normalized to protein content, and the data shown are representative of positive clones. For von Kossa staining, cells were washed with phosphate-buffered saline, fixed with formalin, and stained with 2% silver nitrate and with 1% acid fuchsin as described (Bellows et al. 1986) with the exception of the dehydration and rehydration steps, which were omitted.

Results

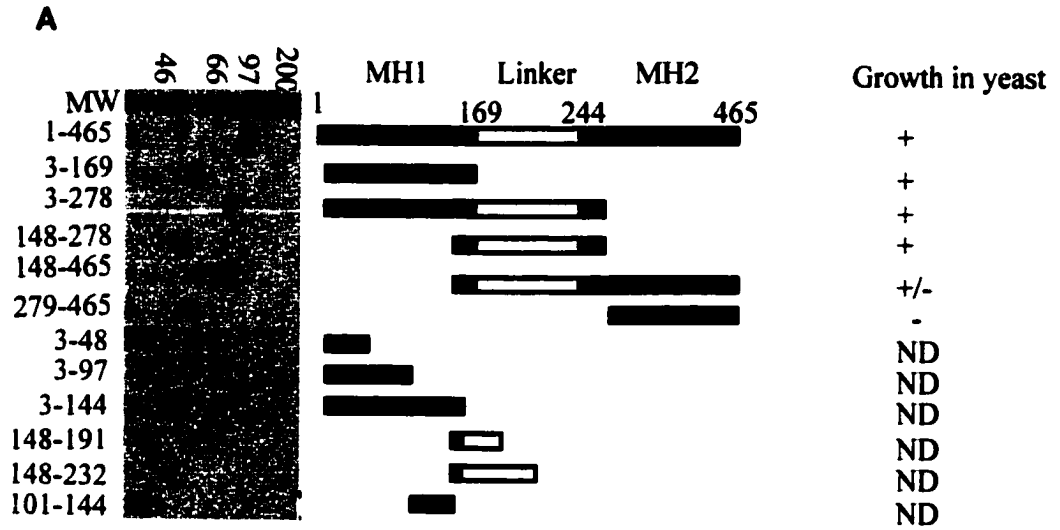
MH1 and Linker of Smad1 Contribute to the Interaction with Hoxc-8

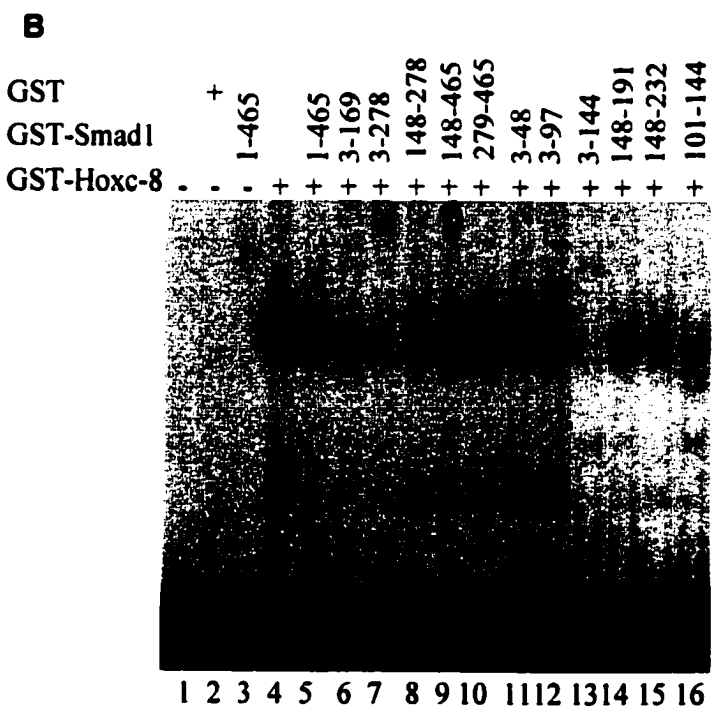
The interaction of Smad1 and Hoxc-8 in yeast by two-hybrid assay, in mammalian cells by co-immunoprecipitation, and in vitro by pull-down analysis has been demonstrated previously (Shi et al. 1999). To resolve region(s) mediating the protein-protein interaction, we first constructed five Smad1 deletions by removing various amino acids from either the NH_2 terminus, COOH terminus, or both. All of these deletion forms of Smad1, pGBT9/Smad1 (positive control), and empty bait plasmid pGBT9 (negative control) were transformed individually into yeast reporter strain Y190 carrying a prey plasmid pACT2/Hoxc-8 to test their associations with full-length Hoxc-8. Transformants

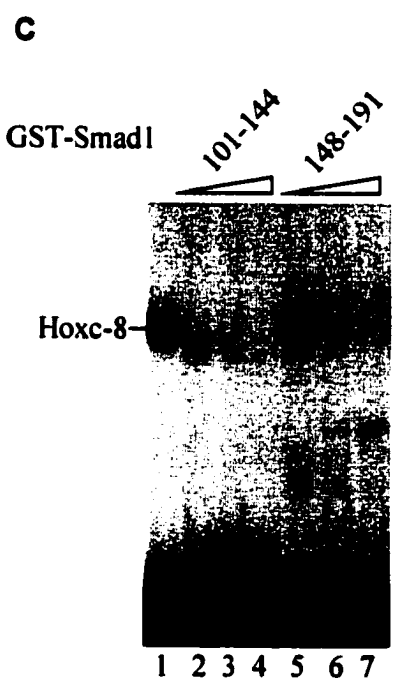
were plated on a medium deficient in His, Trp, and Leu. Fig. 1A shows that all forms of Smad1 containing MH1 (3-169) and/or linker domains (148-278) were able to grow. In contrast, yeast containing empty pGBT9 or pGBT9/MH2 (279-465) failed to grow. These results suggest that the Hoxc-8 interaction domains of Smad1 may locate within the MH1 and linker domains.

To localize further the domains involved in the protein-protein interaction, we took advantage of our previous observation from gel shift assays in which GST-Smad1 fusion protein inhibited Hoxc-8 binding to OPN-5, a 27-base pair element derived from the osteopontin promoter from -206 to -180 (Shi et al. 1999). A set of Smad1 fragments fused with GST was expressed in bacteria and purified to homogeneity as shown in Fig. 1A. An equal amount (1.5 μg) of each purified GST-Smad1 or its deletion mutants was incubated with 0.2 μg of GST-Hoxc-8 and OPN-5 probe for gel shift assays. Fig. 1B shows that the binding of Hoxc-8 (lane 4) was reduced by the addition of wild-type Smad1 (lane 5) or mutant Smad1 containing either MH1 (3-169, lane 6) or linker (148-278, lane 8) to the binding reaction. A strong inhibition was observed in the Smad1 retaining both MH1 and linker domain (3-278, lane 7). In contrast, the binding of Hoxc-8 remained unchanged when GST-MH2 was added (lane 10). The Hoxc-8 binding was only slightly reduced by Smad1 containing both linker and MH2 (148-465, lane 9). It is possible that the MH2 domain prevents the linker from interacting with Hoxc-8 through MH2-linker interaction. These data are consistent with earlier observations in the yeast two hybrid analysis (Fig. 1A). To define the inhibitory regions within each domain, MH1 and linker domains were truncated further into smaller segments (Fig. 1A). Similar gel shift assays using these smaller Smad1 derivatives resolved two regions of aa 101-

Figure 1. NH2-terminal domains of Smad1 interact with Hoxc-8. (*A*) *left* panel, SDS-polyacrylamide gel electrophoresis profile of purified GST-Smad1 fragments used in the gel shift assays. Bacterially expressed GST recombinant Smad1 proteins were purified on glutathione-agarose. Glutathione elutions were subjected to 10% SDS-polyacrylamide gel electrophoresis and proteins were visualized by Coomassie Blue staining. Each deletion is marked with the end point amino acid residues. Molecular mass markers in kDa are shown on the top lane of the gel. *Middle* panel, schematic presentation of Smad1 deletion constructs. *Right* panel, the growth property of Yeast clones. Yeast strain Y190 containing the plasmid pACT2/Hoxc-8 was transformed with pGBT9 (control, not shown) or pGBT9 harboring various Smad1 deletions as indicated. Transformants were plated on SD plates with 30 mM 3-amino-1, 2, 4-Triazole and without His, Trp, and Leu. ND, not determined. (*B*) Two regions of Smad1 confer an inhibitory effect on Hoxc-8 binding to OPN-5. Gel shift assay was performed using purified GST fusion proteins and ^{32}P-labeled probe derived from osteopontin promoter (nt –206 to –180). Lanes contained probe alone (lane 1), with GST (lane 2), with GST-Smad1 (lane 3), or with GST-Hoxc-8 (lanes 4-16) in the absence (lane 4) or presence of various sized Smad1 proteins (lanes 5-16). (*C*) Inhibition of Hoxc-8 binding to DNA by Smad1 fragments is dose-dependent. Hoxc-8 was incubated with the same probe in the absence (lane 1) and the presence of Smad1 fragments 101-144 (lanes 2-4) or 148-191 (lanes 5-7) with a 2-fold increase in Smad1 concentration between successive lanes (1.5, 3, and 6 μg respectively).







144 within the Smad1 MH1 domain and aa 148-191 at the MH1-linker junction which were sufficient for the interaction (Fig. 1B, lanes 13-16). Both fragments were shown to inhibit Hoxc-8 binding to OPN-5 in a dose-dependent manner (Fig. 1C, lanes 2-7) where 6 μg of each diminished the binding (Fig. 1C, lanes 4 and 7). These results indicate that two regions within the NH_2-terminus of Smad1 can account for the inhibition of Hoxc-8 binding to its cognate DNA element.

Homeodomain is Responsible for Hoxc-8 Association with Smad1

Hox proteins have in common a similar homeodomain (HD) consisting of a highly conserved DNA binding motif of 60 amino acid residues (Sharkey et al. 1997). In addition to the HD that lies from aa 149 to 209, Hoxc-8 contains two other conserved regions (CR), CR1 (aa 1-8) and a hexapeptide (aa 137-142) located upstream from the homeodomain (Le Mouellic et al. 1988; Fig. 2). The hexapeptide of Leu-Met-Phe-Pro-Trp-Met is presumably involved in the interaction with Hox-assisting cofactors such as the Pbx family (Phelan et al. 1995). The regions outside of the HD may determine the functional specificity of Hox proteins (Sharkey et al. 1997; Sreenath et al. 1996).

To determine region(s) that are involved in the association with Smad1, we constructed five deletion mutants of Hoxc-8 in addition to the originally identified truncated form of Hoxc-8 clone 19 (Fig. 2). pACT2 prey plasmid containing non-related cDNA (randomly chosen from cDNA library (Shi et al. 1999) as a control), the full-length, and the deletion mutants of Hoxc-8 were transformed separately into Y190 harboring pGBT9/Smad1 bait plasmid, and β-galactosidase activity was assayed. Fig. 2 shows that the full-length Hoxc-8 (1-242) and all four HD-containing deletions (67-237, 137-242,

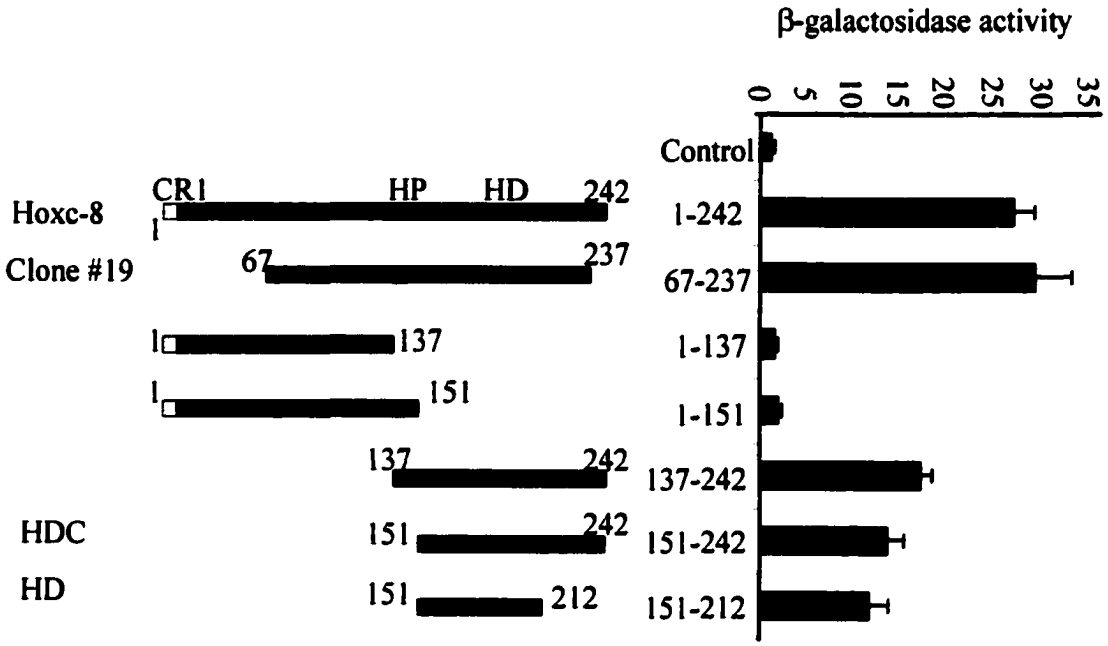


Figure 2. Interaction between Smad1 and Hoxc-8 deletions in yeast. *Left* panel, schematic illustrations of various deletion mutants of Hoxc-8 used for interaction studies. The size of each is labeled by the amino acid residues. CR1, conserved region 1; HP, hexapeptide; clone 19, the originally identified clone by two-hybrid technology (9); HDC, homeodomain and its COOH-terminal extension. *Right* panel, interaction between Smad1 and Hoxc-8 in yeast two-hybrid system. Yeast strain Y190 containing the plasmid pGBT9/Smad1 was transformed with pACT2 containing non-related cDNA (see Experimental Procedures) as a control or pACT2 containing various sized Hoxc-8 cDNA as indicated. Transformants (colonies) were assayed for β-galactosidase activity, and values (OD_{420}) were normalized with cell densities (OD_{600}). Each bar represents the mean ± SD from three independent determinations.

151-242, and 151-212) interacted with Smad1 as indicated by higher β-galactosidase activity compared with the negative control. The association was stronger with full-length Hoxc-8 and clone 19 compared with HD alone (151-212), indicating that the NH_2-terminal region of Hoxc-8 contributes to the interaction. However, two constructs containing only the NH2-terminal region (1-137 and 1-151) failed to bind Smad1, showing negligible β-galactosidase activity (Fig. 2). This suggests that the NH_2 terminus does not participate directly in the protein-protein interaction between Hoxc-8 and Smad1. The NH2-terminal region may help to stabilize the proper configuration of the homeodomain increasing its interaction with Smad1. Elimination of conserved region-1 (compare 1-242 with clone 19) and the hexapeptide (compare 137-242 with 151-242) appeared to produce no significant reduction in the association (Fig. 2). In fact, HD alone is sufficient to support the interaction (Fig. 2, 151-212), suggesting that HD is involved directly in Hoxc-8-Smad1 interaction.

To delineate whether the COOH-terminal extension of HD contributes to the protein-protein interaction by gel shift assay, cDNAs encoding HD (151-212) or HDC (151-242) were cloned into pGEX-KG vector to make truncated forms of GST-Hoxc-8 fusion proteins. Purified GST-HDC and GST-HD (10 ng) bound to the OPN-5 probe. Reaction with full-length GST-Hoxc-8 (0.2 μg) was run alongside for comparison (Fig. 3A). The affinity of GST-HDC and GST-HD to the DNA probe is at least 20 times higher, suggesting that the NH_2-terminus not only assists the Hoxc-8 to interact with Smad1 (Fig. 2) but also modulates the protein binding to its cognate element. Both deletions of Hoxc-8 were also tested for their interaction with either wild-type or mutant Smad1. As shown in Fig. 3, B and C, the binding pattern of HDC and HD to the OPN-5

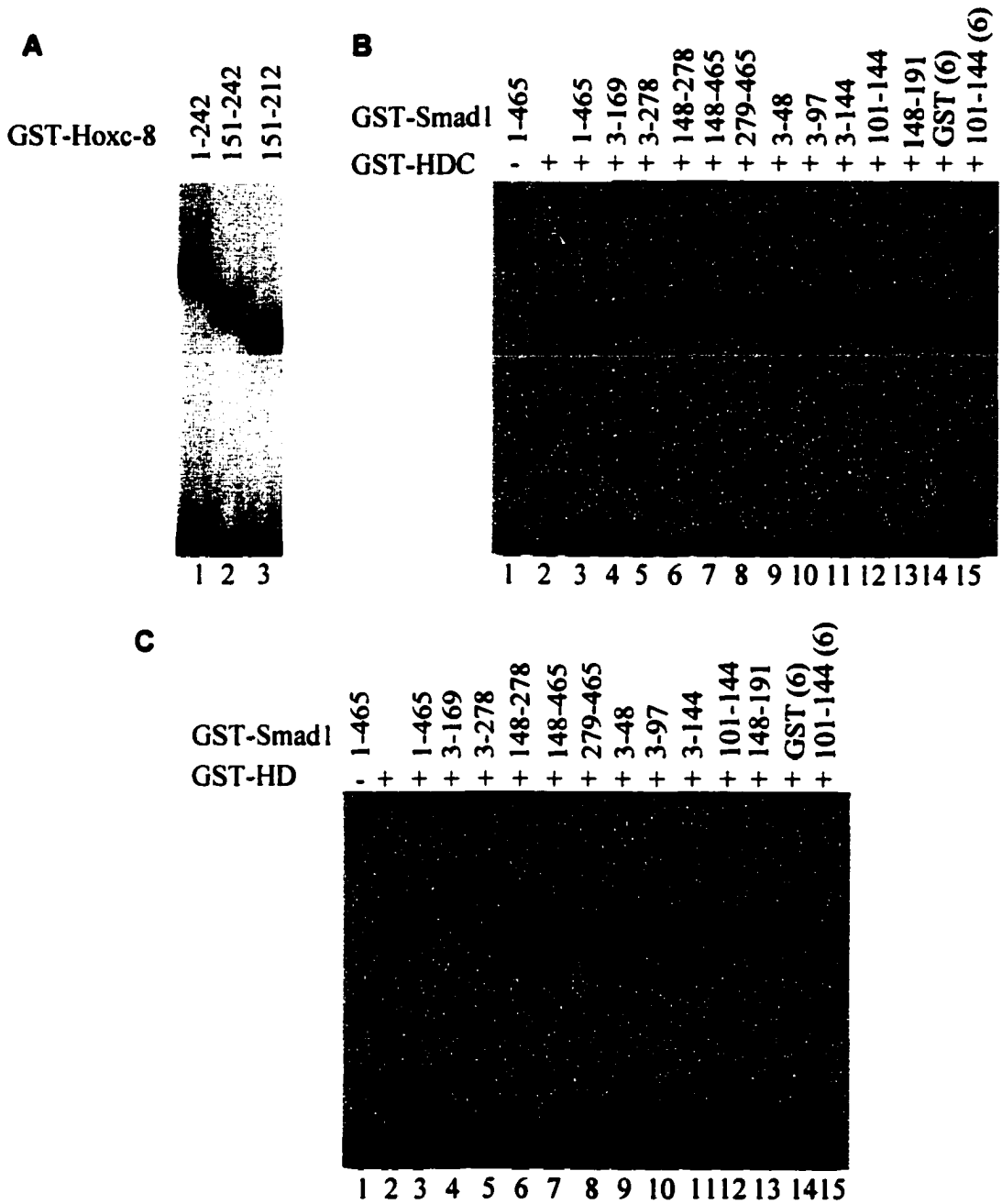


Figure 3. HD of Hoxc-8 interacts with Smad1. (*A*) Binding of Hoxc-8 and its derivatives to OPN-5 probe. 10 ng of both GST-HDC (151-242) and GST-HD (151-212) were incubated with probe OPN-5 (5 x 10^4 cpm) and subjected to 5% native polyacrylamide gel. The reaction of GST-Hoxc-8 (0.2 μg) was run alongside for comparison. (*B*) Interaction between HDC of Hoxc-8 and various forms of Smad1. GST-HDC was incubated with probe OPN-5 in the absence (lane 2) or presence (lanes 3-15) of various deletions of Smad1 as indicated on the top. Negative controls include probe with Smad1 (lane 1) and probe with 6 μg of GST (lane 14). (*C*) HD of Hoxc-8 interacts with various Smad1 derivatives. GST-HD was assayed for binding activity to the OPN-5 in presence or absence of various Smad1 fragments as indicated for each lane.

is nearly identical and is also comparable to that of full-length Hoxc-8 (Fig. 1B). Similarly, Smad1 and all other MH1 or linker-containing mutants inhibited the binding of both HDC and HD to the probe. Interestingly, the MH1 domain showed the strongest inhibition of HDC and HD domains, which is different from the pattern seen earlier with full-length Hoxc-8 binding (compare Fig. 3, B and C, 3-169 and 3-144 with Fig. 1B, 3-278). Note that the linker region alone (148-278) and its smaller deletion (148-191) had no effect on the HDC and HD binding (Fig. 3, B and C). The binding of both HDC and HD to DNA was reduced by the smaller domain (aa 101-144), but it required a higher amount (6 μg; Fig. 3, B and C). As a control, the same amount of GST (6 μg) had no effect on the binding (lane 14 in Fig. 3, B and C). Collectively, these data suggest that the HD of Hoxc-8 is sufficient for the interaction with Smad1 possibly by making direct contact with amino acid residues within MH1 domain.

Hoxc-8 Interaction Domains of Smad1 Are Sufficient to Induce Osteopontin Promoter Activation

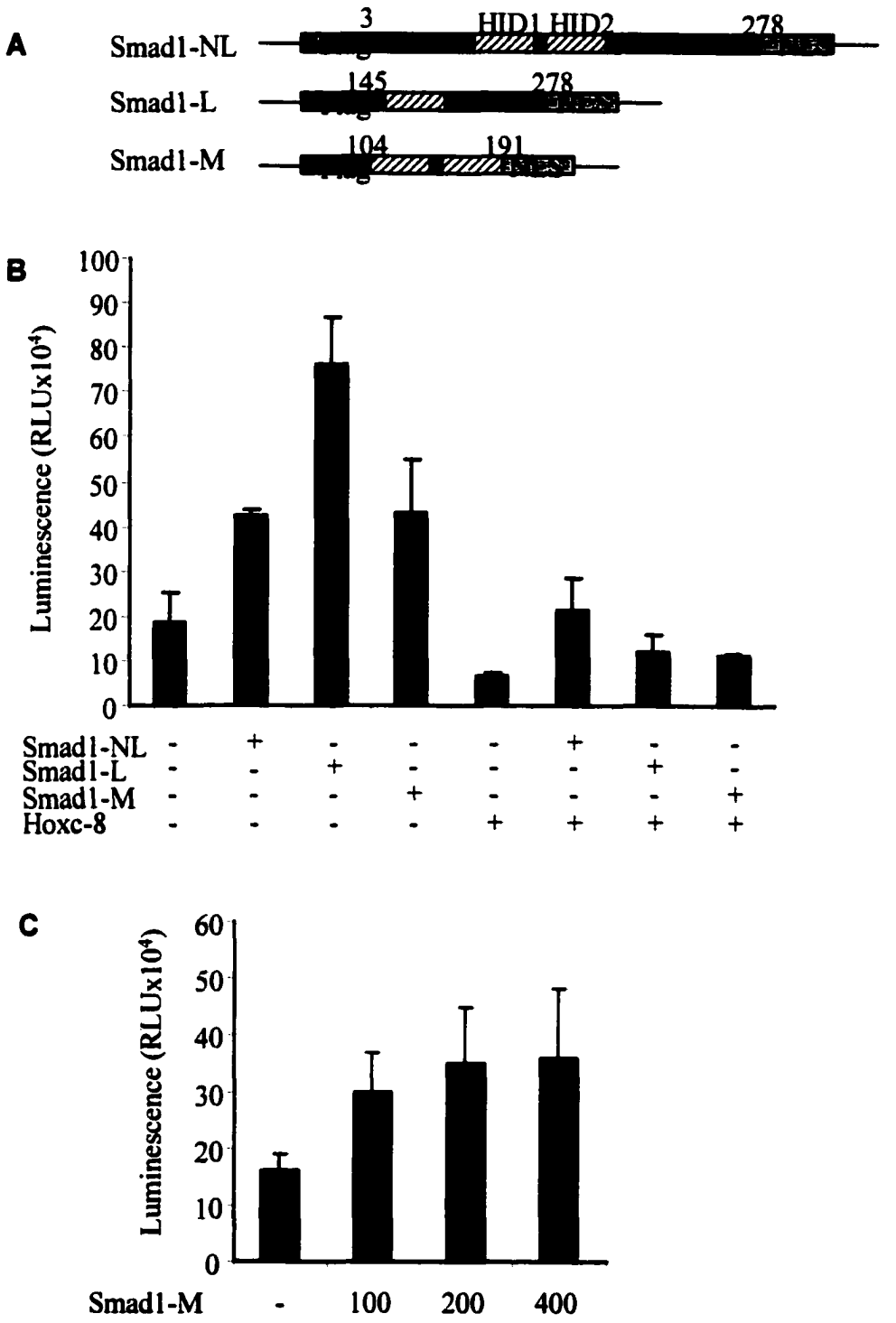
The C3H10T1/2 (C3H) mesenchymal cell line provides an ideal model system for exploring the mechanism of Smad1-mediated BMP signaling. In response to BMP stimulation, C3H expresses bone markers, including osteopontin, ALP, type I collagen, and sialoprotein genes, leading to mineral deposition, the terminal stage of osteoblast differentiation (Taylor and Jones 1979; Ahrens et al. 1993). Previously, we have reported that Hoxc-8 binds to a 266-base pair osteopontin promoter fragment and represses reporter gene transcription. Co-transfection of Smad1, Smad4, and a constitutively active form of the BMP type I receptor ALK3 (Q233D) in C3H cells induces reporter gene transcription (Shi et al. 1999). Two regions of Smad1, Hoxc-8 interaction domains (HIDs),

aa 101-144 (HID1) and aa 148-191 (HID2), are sufficient to inhibit Hoxc-8 binding to DNA (Fig. 1). To investigate the transcription activity of the HIDs of Smad1, we constructed three cDNA fragments. One fragment, Smad1-L (linker region), contained one Hoxc-8 interaction domain (HID2). Two others, Smad1-NL (N-domain and linker) and Smad1-M (minimal region of HIDs), held both HIDs. These constructs were fused with a nuclear localization signal into a CMV5 mammalian expression vector (Fig. 4A). The same reporter Hox-pGL3 containing a Hoxc-8 binding site in front of the luciferase gene and SV-40 promoter was used for the transfections (Shi et al. 1999). Co-transfection was performed with Hox-pGL3 or its mutant (mHox-pGL3) and plasmids for various Smad1 fragments separately. Results showed that Smad1-NL (3-276), -L (145-276), and -M (101-191) containing either one or both HIDs stimulated osteopontin promoter activity as monitored by the luciferase assay (2-4-fold). The reporter's activity was suppressed when Hoxc-8 was co-expressed (Fig. 4B). The minimal region containing both HIDs increased Hox-pGL3 reporter activity in a dose-dependent manner (Fig. 4C). In contrast, none of these Smad1 fragments affected the luciferase activity of the mHox-pGL3 reporter in which the Hox binding site was mutated (data not shown). These data indicate that the HIDs of Smad1 are sufficient to activate osteopontin gene transcription by inhibiting Hox-8 binding to its cognate site.

HIDs of Smad1 Induce Bone Cell Formation

To examine the function of the Hoxc-8 interaction domains of Smad1 in osteoblast differentiation, Smad1-NL, Smad1-L, and Smad1-M were cloned into the tetracycline-regulated expression vector pTet-Splice. These plasmids and a control vector were

Figure 4. Smad1-Hoxc-8 interaction domains (HID) activate osteopontin gene transcription. (*A*) Constructs of various deletion forms of Smad1. Smad1-NL (3-278), Smad1-L (148-278), and Smad1-M (101-191) were cloned into pCMV5 mammalian expression vector. HID1 and HID2 represent aa 101-144 and aa 148-191, respectively, of Smad1 (see Fig. 1). A nuclear localization signal (NLS) was fused to each construct to allow the expressed truncated proteins to enter the nucleus. (*B*) Overexpression of Smad1-Hoxc-8 interaction domains stimulates osteopontin gene transcription. C3H10T1/2 cells plated at a density of 5×10^4 cells/well in 12-well plates were transiently transfected with 0.5 μg of luciferase reporter plasmid containing a Hoxc-8 binding site (Hox-pGL3; Shi et al. 1999) and indicated plasmids (200 ng each). (*C*) Overexpresion of Smad1-M enhances osteopontin gene transcription dose-dependently. Indicated amounts of Smad1-M DNA together with Hox-pGL3 reporter (0.5 μg/well) were introduced into C3H10T1/2 cells as described in B. Luciferase activity was measured 24 hours post-transfection.

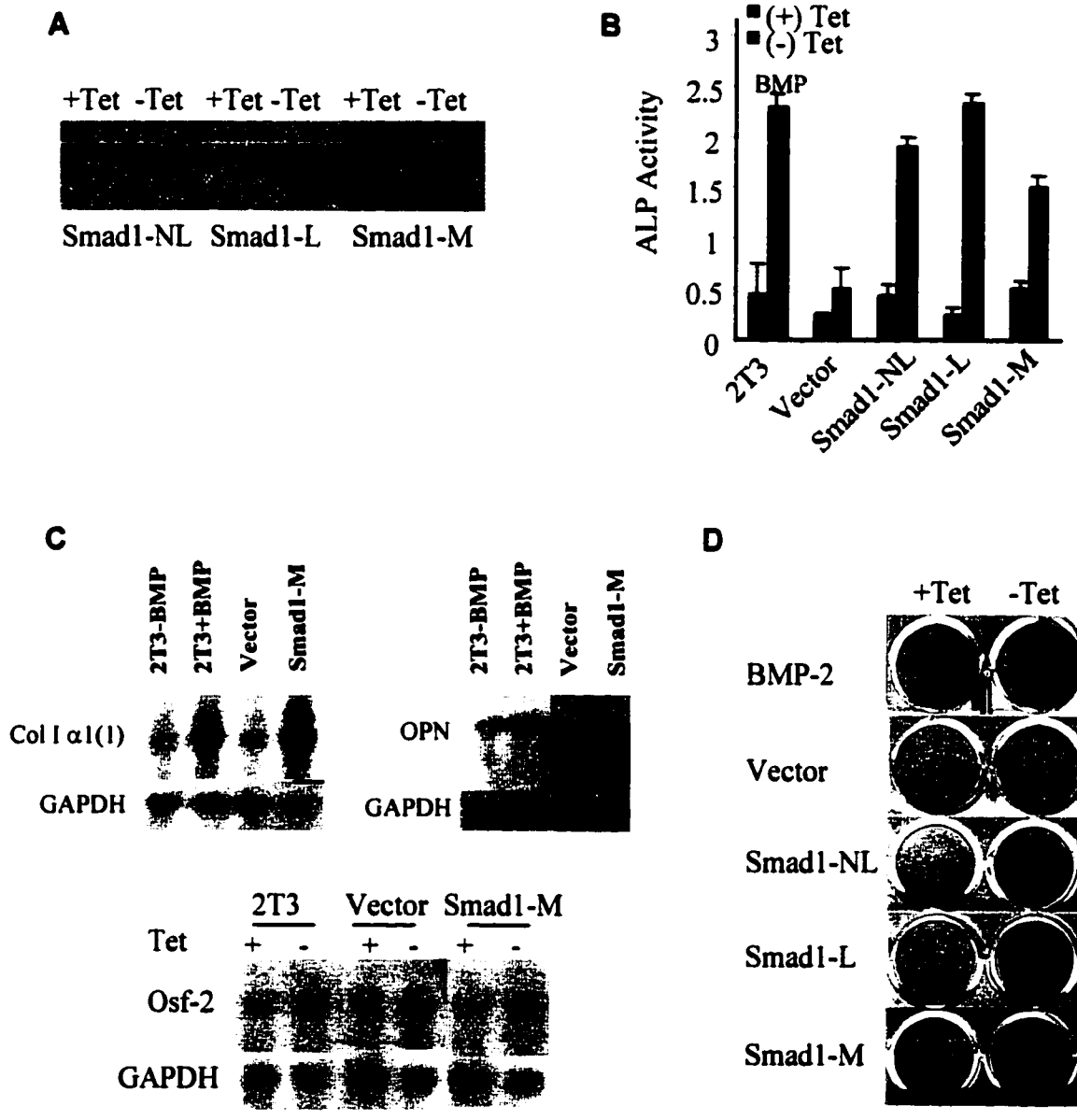


permanently transfected into 2T3 cells. 5-10 tetracycline-regulated positive clones of each were selected by slot-blot and Northern hybridization using corresponding cDNA probes. Fig. 5A demonstrates tetracycline-regulated expression of the three different Smad1 fragments of such clones.

ALP activity is a hallmark of bone formation, and induction of its activity in progenitor cells marks the entry of a cell into the osteoblastic lineage. BMP and its constitutively active receptors have been shown to induce effectively 2T3 cells to express high level of ALP activity (Begley et al. 1993; Chen et al. 1998). The effect of the HID-containing fragments of Smad1 on ALP activity was examined in these stable cell lines. The expression of Smad1 fragments was induced by withdrawal of tetracycline from the growth medium. ALP activity was determined in 5-10 stable clones (for details, see "Experimental Procedures"), all of which showed increased ALP activity after tetracycline withdrawal. Fig. 5B shows the results for one of each of the Smad1-NL, -L, or –M clones. ALP activity remained at a basal level in all cells that were kept in tetracycline-containing medium. 2T3 cells that were permanently transfected with pTet-Splice vector showed little or no increased ALP activity upon tetracycline removal. These data indicate that the ALP activity in the stable clones is induced by the expression of HID-containing fragments of Smad1.

The progression of osteoblastic differentiation can also be monitored by the temporal expression of other bone cell phenotypic genes, such as osf-2, type I collagen, osteopontin, osteocalcin, and bone sialoprotein genes. To investigate the effect HIDs of Smad1 on the expression of such genes, Northern analysis was performed using mRNAs from 2T3 cells or HID-expressing clones after tetracycline withdrawal for 4 days. An

Figure 5. Smad1 domains induce bone cell differentiation. (*A*) Inducible expression of Smad1 fragments. Constructs shown in Fig. 4A were subcloned into pTet-Splice vector to make tetracycline (Tet)-regulated expression plasmids for Smad1-NL, -L, and -M. These were transfected permanently into 2T3 osteoblast precursor cells, and the total RNA was extracted from 2-day cultures grown in the absence or presence of tetracycline. Slot-blot hybridization assay (Bio-Rad) was carried out using 5 μg of total RNA from indicated clones and ^{32}P-labeled corresponding cDNA probes. (*B*) ALP activity is induced by the HIDs of Smad1. Stable 2T3 cell lines bearing pTet-Splice vector (vector), or pTet-Splice recombinant constructs containing each of the Smad1-HIDs of Smad1 were cultured in an osteoblastic promotion medium with or without tetracycline. 2T3 cells were cultured in the absence or presence of rhBMP-2 (50 ng/ml) as controls. ALP activity was determined as described under "Experimental Procedures," and the A_{405} was normalized to protein content. Data shown are representative of positive clones. (*C*) NH2-terminal domains of Smad1 induce osteoblast differentiation-related gene expression. mRNA was extracted from indicated cell clones grown in the presence or absence of tetracycline for 4 days, and 3 μg of each was used for Northern analysis with indicated probes. Bone marker gene expression in 2T3 cells that were grown in the presence or absence of rhBMP-2 shown in the figure serves as a positive control. (*D*) HIDs of Smad1 induce mineralized bone matrix formation. 2T3 cells and the indicated stable clones were cultured for 12 days in osteoblastic promotion medium with/without tetracycline or with/without 50 ng/ml rhBMP-2. Cells were then fixed and stained by silver nitrate and acid fuchsin as described under "Experimental Procedures" to visualize mineralized bone matrix (black spots).



increased level of *osf*-2 expression is not detectable. However, consistent with ALP activity, the expression of type I procollagen α1(1) and osteopontin genes is elevated upon tetracycline withdrawal. The levels of the marker gene expression are compatible with positive controls (BMP-treated, Fig. 5C). A much higher expression of osteopontin gene was observed in both 2T3 cells that were treated with rhBMP-2 and clones that express HIDs at 12 days (data not shown). These data indicate that the Hoxc-8 interaction domains of Smad1 induce bone marker gene transcription in 2T3 cells and thus mimic BMP signaling.

The terminal differentiation of bone cells is characterized by the onset of extracellular matrix mineralization, which can be visualized by van Kossa staining. To confirm the role of Smad1 HIDs in induction of the final stage of osteoblast differentiation, we determined the formation of mineralized bone matrix in prolonged cultures of 2T3 and its derivative cell lines in the presence of rhBMP-2 or under indicated conditions. Stable cell lines were cultured in parallel in the presence or absence of tetracycline to modulate the expression of the Smad1 fragments. As expected, rhBMP-2 treated 2T3 cells and permanent 2T3 cell lines that expressed Smad1-NL, -L, or -M (-Tet) underwent mineralization at day 12 showing black stained spots. However, no mineralized bone matrix was observed in cells that were stably transfected with the empty vector or in cells that did not express Smad1-NL, -L, -M (+Tet; Fig. 5D). Together, these data indicate that the interaction between Smad1 and Hoxc-8 is mediated through HID1 and HID2 of Smad1 and HD of Hoxc-8. The HID of Smad1 mimics BMP signaling by inducing ALP activity, osteoblast marker gene transcription, and mineralization in 2T3 cells.

Discussion

This study focused on mapping functional domains that are involved in the interaction between Smad1 and Hoxc-8 and subsequently on investigating the role of these domains in the induction of osteoblast differentiation and bone cell formation. From both yeast two-hybrid and gel shift assays with a series of deletion forms of the two proteins, we identified two regions of Smad1, namely HID1 and HID2, which interact with Hoxc-8, one of which (HID1) interacts specifically with Hoxc-8 at the homeodomain.

HID1 lies within the MH1 domain between aa 101 and 144 of Smad1, and it inhibits both the full-length and the HD of Hoxc-8 binding to DNA (Figs. 1 and 3). The MH1 domain of *Drosophila* Mad (a homolog of mammalian Smad1), Smad3, and Smad4 binds to the DNA (Kim et al. 1997; Dennler et al. 1998; Jonk et al. 1998). Knowing that MH1 is highly conserved among Smads, one would wonder whether the inhibition of Hoxc-8 binding by Smad1 to osteopontin promoter proximal sequence was caused by a competition for a cognate site. To address this issue, we tested all forms of GST-Smad1, none of which bound to the OPN-5 probe except for HID1, which showed a very weakly shifted band when 2 μg of the protein was used. Increasing the amount of HID1 had no effect on binding (data not shown). Thus, the HID1 is unlikely to have an intrinsic DNA binding activity on the OPN-5 of osteopontin promoter. This conclusion is in agreement with the observation from the crystallographic study (Shi et al. 1998). NH_2-terminal sequence alignment of five pathway-restricted Smads that contain MH1 and MH1-linker junction domains is shown in Fig. 6, with the two HIDs, the 7-aa insertion, the β-hairpin, and the double loops indicated. Interestingly, the double loops of Smad3 are exposed to the surface of the DNA-MH1 complex and are readily available for interactions with

other factors. Furthermore, the DNA-binding aa in the β-hairpin are mostly conserved among pathway-restricted Smads (Fig. 6). Because the HID1 domain does not include this DNA binding motif, we believe that the inhibition of Hoxc-8 binding to DNA by Smad1 depends upon a protein-protein interaction instead of a protein-DNA competition.

The HID2, mapped to the junction of the MH1-linker, binds outside of the Hoxc-8 homeodomain since it only inhibits full-length Hoxc-8 but not HD binding (Figs. 1 and 3). These results are in agreement with the yeast two-hybrid assay data in which clone 19 and full-length Hoxc-8 interacted with Smad1 more strongly than did the HD alone or any other HD-containing deletions (Fig. 2). Binding of HID2 to Hoxc-8 may mask the accessibility of HD to DNA, thereby inhibiting the protein-DNA interaction.

Our previous data showed that Smad2 and Smad3 have no effect on the Hoxc-8 binding to OPN-5 probe (Shi et al. 1999). Sequence alignment analysis revealed that several residues within the two HIDs are highly conserved, the actual aa differ between BMP and TGF-β pathway-restricted Smads (Smad1, -5, and -8 versus Smad2 and -3 in Fig. 6, boxed amino acids). Interestingly, a 7-aa insertion in BMP pathway-restricted Smad1, -5, and -8 is absent in both TGF-β pathway-restricted Smad2 and -3. It is also noteworthy that high homology exists between Smad2 and Smad3 in HID2 but is relatively less conserved among Smad1, -5, and -8 (Fig. 6). This is appealing because the subtle differences of HID2 among the three BMP pathway-restricted Smads may be implicated in the specificity of each.

The well-conserved homeodomain consists of three α helices and a flexible NH_2-terminal arm, which makes contact with the DNA (Sharkey et al. 1997). Residues that contact the DNA directly are usually conserved among multiple Hox paralog groups and

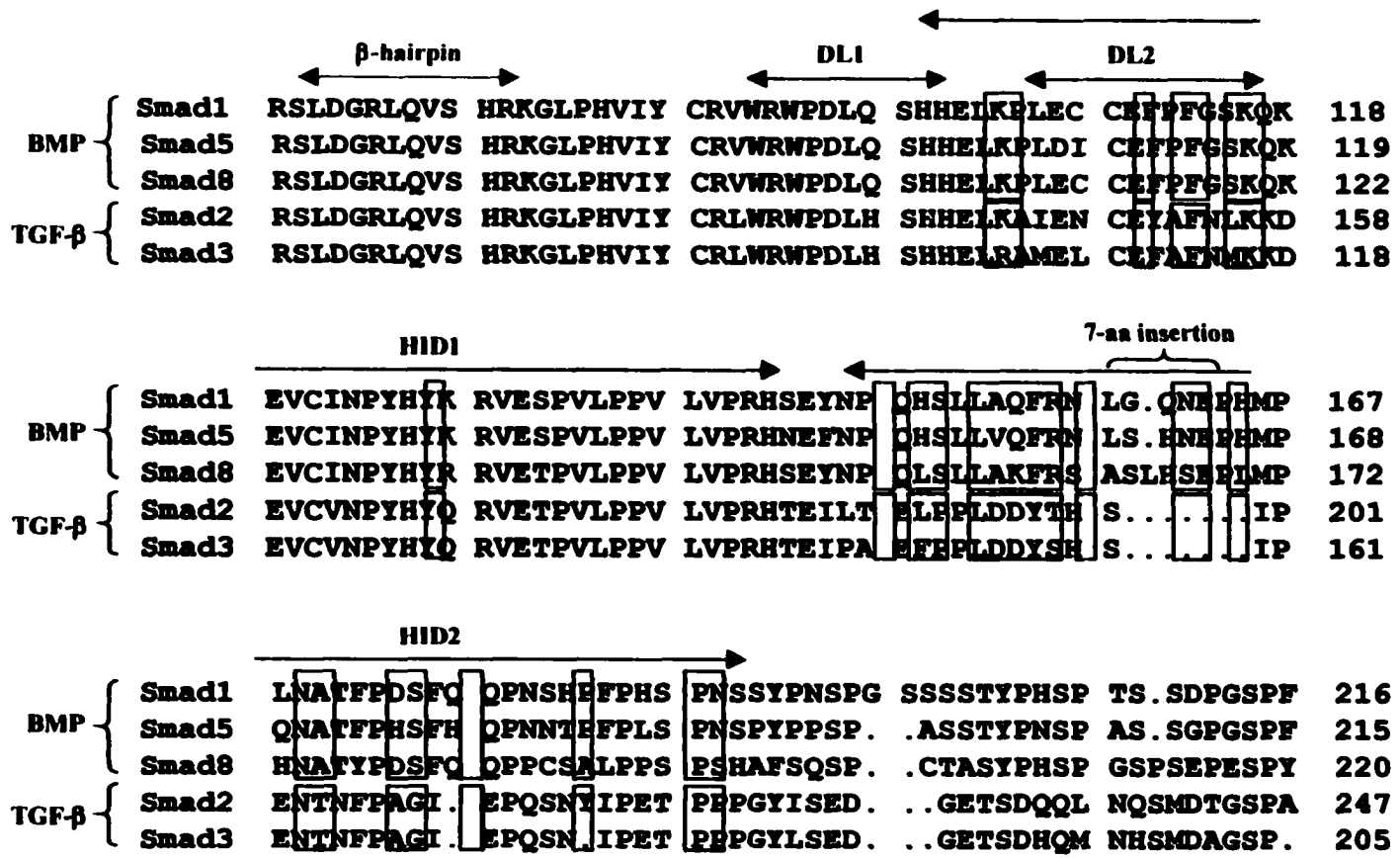


Figure 6. Partial sequence alignment of five human Smad proteins. Boxed amino acid residues are conserved within each group, but differ between BMP and TGF-β pathway-restricted Smads (labeled as BMP or TGF-β). Regions that mapped to interact with Hoxc-8 are indicated as HID1 (aa 101-144) and HID2 (aa 148-191). The β-hairpin stucture that binds to DNA and the double loops (DL) identified in MH1 domain of Smad3 (Shi et al. 1998), are also labeled.

appear to provide a general means of binding. The functional specificity of Hox proteins may be determined by "characteristic residues" within or outside of the HD which are likely to contact other partners, such as Hox, Pbx, Extradenticle, or Engrailed homeoproteins (Krumlauf 1992; Mak and Johnson 1993; van Dijk and Murre 1994; Phelan et al. 1995). A recent crystallographic study has revealed that a hexapeptide of Hoxb-1 binds to its DNA-binding partner at a pocket formed partly by a three-amino acid insertion in the Pbx1 homeodomain (Piper et al. 1999). HD helix-1 of Hoxd-8 also mediates a direct contact with Hoxc-9 and inhibits the latter's binding to DNA (Vigano et al. 1998). We found previously that two Hox proteins, Hoxc-8 and Hoxa-9, are able to interact with Smad1, yet the only homology between the two is the HD. Here, we show that the binding of HD to OPN-5 is reduced by the HID1 of Smad1 (Fig. 3), indicating that the HD is also responsible for the protein-protein interaction.

Both Smad1 and Smad5 have been shown to induce ALP activity (Yamamoto et al. 1997) and osteocalcin production (Nishimura et al. 1998) in a pluripotent mesenchymal cell line C2C12. Our data demonstrate that the HIDs interact with Hoxc-8, preventing binding of Hoxc-8 to osteopontin promoter (Figs. 1 and 3). Thus, HIDs release the repression of Hoxc-8 leading to activation of the gene transcription and mimic the Smad1 activity. HIDs appear to be sufficient to activate bone cell phenotypic gene transcription and, subsequently, to cause mineralized bone matrix formation (Figs. 4 and 5).

Three Hox proteins, namely Hoxa-7, Hoxc-8, and Hoxb-4, are all found to repress gene transcription (Schnabel and Abate-Shen 1996), and previous work from our laboratory showed that Hoxc-8 functions as a transcriptional repressor of osteopontin gene (Fig. 5; Shi et al. 1999). It has been suggested that repression may be a general mode of action

for Hox proteins, which may be required for maintaining cells in an undifferentiated state during development to prevent premature differentiation of precursor cells (Violette et al. 1992; Catron et al. 1995; Schnabel and Abate-Shen 1996). Overexpression of Hoxc-8 in skeletal tissue results in an accumulation of progenitors in the hypertrophic area (Yeuh et al. 1998). It is likely that the involvement of Hoxc-8 in both osteo- and chondrogenic processes prevents a switch from proliferation to differentiation.

BMPs are important growth factors that participate in many processes during embryonic development. In addition to the well-known fact that BMPs induce bone and cartilage formation in ectopic sites in vivo, BMP-2 induces the undifferentiated mesenchymal progenitors to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro (Katagiri et al. 1990; Ahren et al. 1993). BMP-2 also inhibits myogenic cells from differentiating into myotubes (Yamaguchi 1995). Given the fact that both BMPs and Hox genes play a fundamental role in directing cell fate, Smad-mediated BMP signaling through the interaction with Hox proteins might be also involved in some of the above processes. Various functional domains of both Smad and Hox proteins may be utilized selectively for mediating protein-protein or protein-DNA interaction and for repression or activation of gene transcription, depending upon developmental stage, cell type, and promoter context. Clearly, a detailed study of the structural and functional properties of Smad and Hox proteins will provide important insights into deciphering the complexity of their roles in embryogenesis and cell differentiation.

Acknowledgments

We thank R. Derynck for the human Smad1 and H. Le Mouellic for Hoxc-8 cDNAs. We are also grateful to S. E. Harris for 2T3 cells. We also thank N. G. Abney for critical reading of this manuscript.

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SMAD1 BINDS TO A SEQUENCE ADJACENT TO THE HOXC-8 BINDING SITE OF THE OSTEOPONTIN PROMOTER AND STIMULATES TRANSCRIPTION

by

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In preparation for submision to Journal of Biological Chemistry

Abstract

Smad1 is an intracellular component that transduces bone morphogenetic protein signaling from the plasma membrane to the nucleus where it mediates transcriptional activation of downstream target genes. Previously, we identified a conserved Hoxc-8 binding element in the osteopontin promoter (nucleotides -206 to -180) which confers a strong repression to the expression of a fused luciferase reporter gene. Smad1 stimulates the transcription of the osteopontin gene in response to bone morphogenetic protein signaling by inhibiting Hoxc-8 from binding to the Hoxc-8 binding element. In this study, the 5'-flanking region of the osteopontin gene was scanned for Smad1 binding element(s) by gel shift assays. Smad1 specifically bound to a DNA fragment -229 to -205 in the promoter, a DNA sequence immediately adjacent to the Hoxc-8 binding element. Deletional analysis showed that NH2-terminal amino acid residues 101-145 of the Smad1 MH1 domain were sufficient for binding to Smad binding element. Furthermore, overexpression of Smad1 alone or in combination with Smad4 and the bone morphogenetic protein constitutively active receptor ALK3 (Q233D) enhanced transcription of an osteopontin-luciferase reporter gene. Our observations suggest that, in addition to relieving the depression caused by Hoxc-8 binding to its element, Smad1 also directly binds to a DNA element immediately adjacent to Hoxc-8 binding element in the osteopontin promoter and activates gene transcription in the bone morphogenetic protein signaling.

Introduction

Smad family proteins are mediators for the signaling of the multifunctional transforming growth factor-β (TGF-β) superfamily that also includes bone morphogenetic

proteins (BMPs), activin, and other morphogens. The pathway-restricted Smads (R-Smads; Whitman 1998) are selectively phosphorylated by their respective type I and type II serine/threonine kinase receptor complexes upon appropriate ligand stimulation. Phosphorylation of R-Smads induces the association of individual R-Smads with Smad4 to form a heteromeric complex, which moves to the nucleus to activate target gene transcription (Kretzschmar and Massague 1998). There are three mechanisms by which Smads activate downstream gene activation.

First, Smads regulate gene activation by interacting with specific DNA binding transcription factors, such as the *Xenopus* winged-helix factor forkhead activin signal transducer-1 (FAST-1; Chen et al. 1997) and its mammalian homolog FAST-2 (Labbe et al. 1998), Smad-interacting protein, and TGIF (Chen et al. 1998). In search of the Smad1 interactors, we have identified an interaction between Smad1 and Hoxc-8, and a conserved Hoxc-8 binding element in the osteopontin (OPN) promoter (Shi et al. 1999). The Hoxc-8 binding site confers transcriptional repression to a reporter gene. The interaction of Smad1 with Hoxc-8 inhibits the latter from binding its DNA cognate, resulting in release of repression by Hoxc-8 and gene activation. This derepression mechanism has been further studied by fine mapping of the interaction domains between Smad1 and Hoxc-8 (Yang et al. 2000). We have shown that the N-terminal domains of Smad1 responsible for inhibiting Hoxc-8 from binding to DNA are also sufficient to activate gene transcription.

In addition to interacting with other transcription factors, Smads also directly bind to DNA to regulate gene transcription. Smads contain three distinct domains. The highly conserved amino-terminal and carboxyl-terminal regions are termed MH1 and MH2, re-

spectively, and the poorly conserved intervening region is called linker. The DNA binding activity is mediated by the MH1 domain of R-Smads, such as *Drosophila* Mad (Kim et al. 1997), Smad3 (Yingling et al. 1997; Shi et al. 1998), and Smad1 (Shi et al. 1998; Johnson et al. 1999). The optimal recognition site, an inverted repeat GTCTAGAC, for Smad3 and Smad4 MH1has been identified by binding site selection approach (Zawel et al. 1998), and a half site, with a GTCT motif, for binding of a single MH1 domain has been revealed by crystallographic studies (Shi et al. 1998). Several native response elements of Smads have been identified from a spectrum of genes, such as *Drosophila vestigial* (Kim et al. 1997), *collagenase* (Candia et al. 1997), *plasminogen activator inhibitor*-1 (*PAI;* Stroschein et al. 1999), *JunB* (Dennler et al. 1998), *Col*7A1 (Jonk et al. 1998), *Mix.*2 (Song et al. 1998), and *goosecoid* (Labbe et al. 1998).

The third mechanism is that Smads bind to a sequence adjacent to the binding sites of other DNA binding proteins to synergistically activate promoters. This does not necessarily involve a physical association between Smads and those DNA-binding proteins. The synergy may be mediated by other coactivators (Derynck 1998). The complex of Smad3 and Smad4 has been shown to bind to a sequence adjacent to TFE3 binding site within the E-box of the PAI-1 promoter, and the cooperation of Smads and the TFE3 proteins is required for TGF-β–inducible transcription (Hua et al. 1998). Similarly, *Drosophila* Mad- and Tinman-binding sites are both essential for decapentaplegic (Dpp)-induced homeobox gene *Tinman* transcription (Xu et al. 1998). Dpp-induced *Ubx* gene transcription also requires both Mad- and cAMP-response elements (Szuts et al. 1998), suggesting a cooperative mechanism.

BMP stimulation rapidly elevates OPN mRNA in osteoblast-like cells (Noda et al. 1988) and pluripotent mesenchymal cells (Ahrens et al. 1993; Shi et al. 1999). OPN is a major noncollagenous protein in the bone matrix, and it promotes bone formation by facilitating the cell adhesion and attachment of osteoblasts to the extracellular matrix (Butler 1989; Neame and Butler 1996). OPN also promotes migration of human smooth muscle cells in a time- and gradient-dependent manner (Zhao et al. 1996; Yue et al. 1994). Studies demonstrate that OPN expression is elevated in the neoplastic transformation of certain cells (Kubota et al. 1989), suggesting there are broader functions of this protein. OPN is synthesized by osteoblastic cells at all stages of their differentiation (Mark et al. 1988), and its gene transcription is regulated by a number of hormones and growth factors, such as 1,25-dihydroxyvitamin D3, retinoic acid, BMP, and TGF-β. Although considerable divergence exists, an approximately 285-bp sequence of immediately upstream of the OPN transcription start site, which directs high-level gene expression (Craig and Denhardt 1991), is largely conserved across species, from human to chicken (Butler et al. 1996). Several regulatory elements within this region have been identified, such as SP1 and AP1 (Zhang et al. 1992), CCAAT box-binding factor (Tezuka et al. 1996), CBFA1, and Ets-1 (Sato et al. 1998).

Previously, we have shown that BMP-induced *OPN* gene transcription can be mediated by the interaction between Smad1 and Hoxc-8. Our current study addresses the role of Smad1 in the enhancement of *OPN* gene expression in response to BMP. The *OPN* gene was scanned for Smad1 binding by gel shift assays using a series of oligo probes derived from the OPN promoter region at nucleotide (nt) –266 to -170. Smad1 specifically bound to a DNA fragment nt -229 to -205 from the transcription start site, a

DNA sequence immediately adjacent to Hoxc-8 binding element, nt –206 to –180, and up-regulated the gene expression.

Experimental Procedures

Cell Lines

C3H10T1/2 mouse mesenchymal cells were purchased from American Type Culture Collection and maintained in Dulbecca's modified essential medium (DMEM), supplemented with 10% bovine albumin, and penicillin-streptomycin. Permanent cell lines that overexpress Smad1-NL and Smad1-L were established by methods previously described (Yang et al. 2000), but using C3H10T1/2 cells instead of 2T3 cells. The cells were maintained in the same medium containing 400 μg/ml G418.

GST-Smad1 Expression and Purification

Constructs encoding Smad1 were made by inserting cDNAs from the mammalian expression vectors pCMV5B/Smad1 into bacterial expression vectors pGEX-KG between the *Sal*I and *Hind*III sites. Deletions of Smad1 were generated by polymerase chain reactions and inserted at the same restriction sites. pGEX-KG/Smad1 or its deletions was then transformed and expressed in bacterial strain BL21. Expression of the fusion proteins was induced by addition of isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM to the cell culture at A_{600} of 0.4 ~0.6. After 5 hr induction, cells were harvested and GST fusion proteins were purified with glutathione-argrose (Sigma) by established methods (Guan and Dixon 1991). Briefly, pellets of BL21 cells bearing expressed fusion proteins were lysed with NETN buffer (20 mM Tris, pH 8.0, 100 mM

NaCl, 1 mM EDTA, 0.5% Nonidet P-40) in the presence of lysozyme and proteinase inhibitors. Following sonication and centrifugation at 10,000 rpm for 1 hr at 4°C, the supernatant was collected and incubated with glutathione-agarose beads at 4°C that had been previously equilibrated with NETN. After three washes with NETN, the fusion proteins were eluted and visualized by staining of SDS-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining.

Oligonucleotide Probes

The probes were derived from OPN promoter region at nt –264 to –170. Sequences of upper strands are as follows: OPN-11 (nt -264 to –170) is gagctcTTCC TTTCCTTATG GATCCCTGAT GGATCCCTGA TGCTCTTCCG GGATTCTAAA TGCAGTCTAT AAATGAAAAA GGGTAGTTAA TGACATCGTT CATCAGTAAT GCTTTGTG; OPN-133 (nt -247 to –206) is tataggtacc TGATGGATCC CTGATGCTCT TCCGGGATTC TAAATGCA**GT CT**ATAAATGA AAAAGCCATc tcgagtata. OPN-5 (nt -206 to –180) is GGGTAG**TTAA** TGACATCGTT CATCAGT. OPN-153 (nt -229 to -206) is CTAAATGCA**G TCT**ATAAATG AAAAAG. mOPN-153 is CTAAATGC**ca tgg**ATAAATG AAAAAG. Lowercase letters are nucleotides added to OPN promoter sequence, boldface letters are the core binding site for Smad1 (GTCT) or Hoxc-8 (TAAT), and lower and boldface letters are nucleotides substituted from wild-type sequences. Nucleotide substitutions were done by polymerase chain reaction using mutated primers.

In Vitro Binding Assays

In vitro binding was studied using a gel shift assay with 1.5 μg GST-Smad1 or its deletion forms. Double-stranded oligomers corresponding to the OPN promoter sequences shown above were labeled using T_4 polynucleotide kinase and [γ-^{32}P]ATP. Binding reactions were preincubated for 20 min at 22°C with indicated proteins in 75 mM NaCl, 1mM EDTA, 1 mM DTT, 10 mM Tris-HCl (pH 7.5), 1 mg/ml of bovine serum albumin, and 25 ng polydIdC in a volume of 19 μl. One μl of DNA probe (0.5 ng, 5 x 10^4 cpm) was added to each of the reactions. The retardation of the probes by indicated proteins was resolved by 4% polyacrylamide nondenaturing gel. Nuclear extracts were isolated using the standard procedures, and 20 μg of total nuclear proteins were used in each binding reaction. For the supershift assays, 6 μg of Smad1 monoclonal antibody (Santa Cruz) was added to each reaction.

Co-transfection Analysis

The sequence derived from OPN promoter (nt-290 to –166) containing a single Smad1 and Hoxc-8 binding site was inserted in pGL3-control vector (Promega) and used as a luciferase reporter (Hox-pGL3). Two other reporter constructs containing either mutated Smad1 or Hoxc-8 binding sites were made by substituting the wild-type with the mutant sequences in Hox-pGL3. The plasmids encoding various forms of Smad1 fused with a nuclear localization signal were constructed by inserting the PCR-amplified fragments into the cytomegalovirus promoter-based mammalian expression vector pCMV5. Each construct contained one of the following regions: Smad1-NL (amino acids (aa) 3-276) or Smad1-L (aa 145-276). C3H10T1/2 cells (5 x 10^4 cells/well in a 12-well culture

dish) were transfected with 0.5 μg of Hox-pGL3 plasmid together with 200 ng of indicated constructs using Tfx-50 as instructed (Promega). Luciferase activity was determined 16 hr after the start of transfection, and values were normalized to protein content. Luciferase activity shown in the figures is representative of transfections performed in triplicate in at least three independent experiments.

Results

Overexpression of Smad1/4 Complex Activates OPN Native Promoter

We previously showed that BMP stimulation induces activation of a native OPN promoter reporter construct (Yang et al. 2000). In this study, we examined the OPN promoter in more detail. The OPN native promoter sequence (nt -266 ~ -1) was inserted upstream of the promoter-less luciferase gene to generate the reporter construct (Fig. 1A). The ability of this promoter sequence to mediate BMP-induced activation of luciferase gene expression was examined by transient tranfection in C3H10T1/2 cells. Expression plasmids for Smad1, Smad4, or ALK3 (Q233D) were expressed either alone or in a combination with others as indicated in Fig. 1B. Overexpression of Smad1 or Smad4 alone induced transcriptional activation 1- or 3-fold, respectively. Co-transfection of the constitutively active BMP type I receptor, ALK3 (Q233D) with either Smad1 alone or Smad4 alone only slightly increased the activation. However, co-expression of ALK3 (Q233D) with both Smad1 and Smad4 induced 6-7 times higher reporter gene activity. Together, these data indicate that the *OPN* gene is transcriptionally regulated by the Smad-mediated signaling pathway.

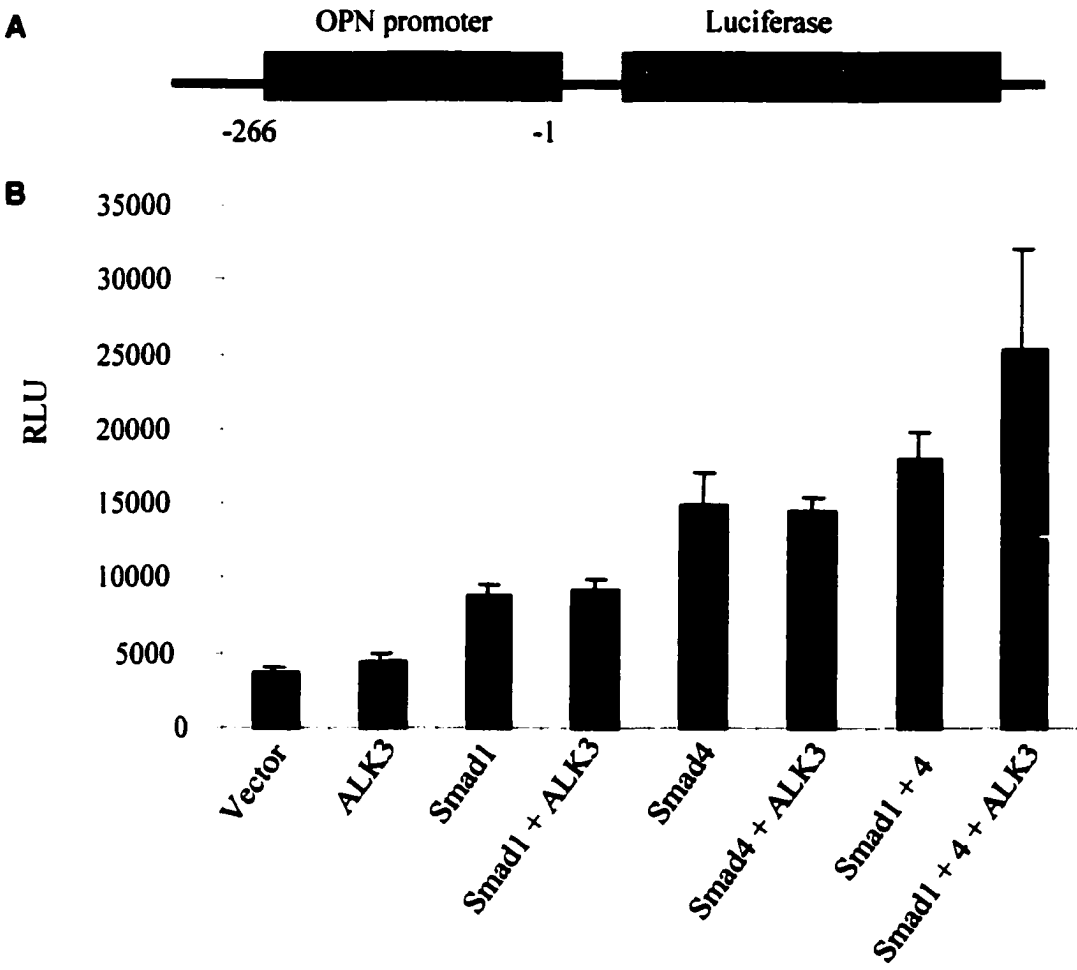

Figure 1. Overexpression of Smad1/4 complex stimulates osteopontin gene transcription. (*A*) Schematic representation of the reporter gene construct. The 5'-flanking region of the OPN gene (nt -266 ~ -1) was inserted in the pGL3-basic luciferase reporter vector. (*B*) Overexpression of Smads enhances reporter's activity. Smad1, Smad4, and a constitutively active form of BMP type I receptor were transfected into C3H10T1/2 cells either alone or in combination as indicated. Luciferase activity was determined 24 hr post-transfection.

Smad1 Binds To A Sequence Adjacent To Hoxc-8 Binding Element In The OPN Promoter

Smad3 and Smad4 have been shown to activate specific genes by directly binding to the DNA. In many cases, the Smads recognition sites are near the elements of their interacting DNA binding proteins, which supports a synergistic cooperation of the two different classes of transcription factors (Hua et al. 1998; Shen et al. 1998; Stroschein et al. 1999). We previously showed that Smad1 stimulates a native promoter's (OPN-266) activity in C3H10T1/2 cells and Mvlu cells (Shi et al. 1999 and Fig. 1). To determine whether Smad1 binds to the BMP-responsive region between nt –266 and –170 upstream of the initiation site in the OPN promoter, we performed gel shift assays with a series of synthesized oligonucleotide probes (Fig. 2A). GST-Smad1 was purified from *Escherichia coli* and 1.5 μg of purified protein was incubated with the [^{32}P]-labeled 95-base pair (bp) DNA fragment (OPN-11). Fig. 2B shows that GST-Smad1 bound to the probe with one shifted band (lane 3), but GST did not bind to this 94-bp probe (lane 2). This result suggests that at least one Smad1 recognition element is present in OPN promoter nt –266 to –180. To further pinpoint the region that mediates Smad1 binding, three additional overlapping oligos were tested for their association with the GST-Smad1. As shown in Fig. 2B, GST-Smad1 bound to the OPN-133 (nt -247 ~ -205, lane 9), but not to the other two downstream probes (OPN-5, lanes 4-6; OPN-6, lanes 10-12). Again, GST did not bind any of these three probes (lanes 5, 8, and 11). Together, the results demonstrate that Smad1 binds to the OPN promoter between nt –247 and –205.

We have previously identified a 27-bp Hoxc-8 binding element (HBE, –180 ~ -206) in the OPN promoter (Yang et al. 2000). Hoxc-8 binds to this element and suppresses transcription. Smad1 or HIDs of Smad1 inhibits Hoxc-8 from binding to its

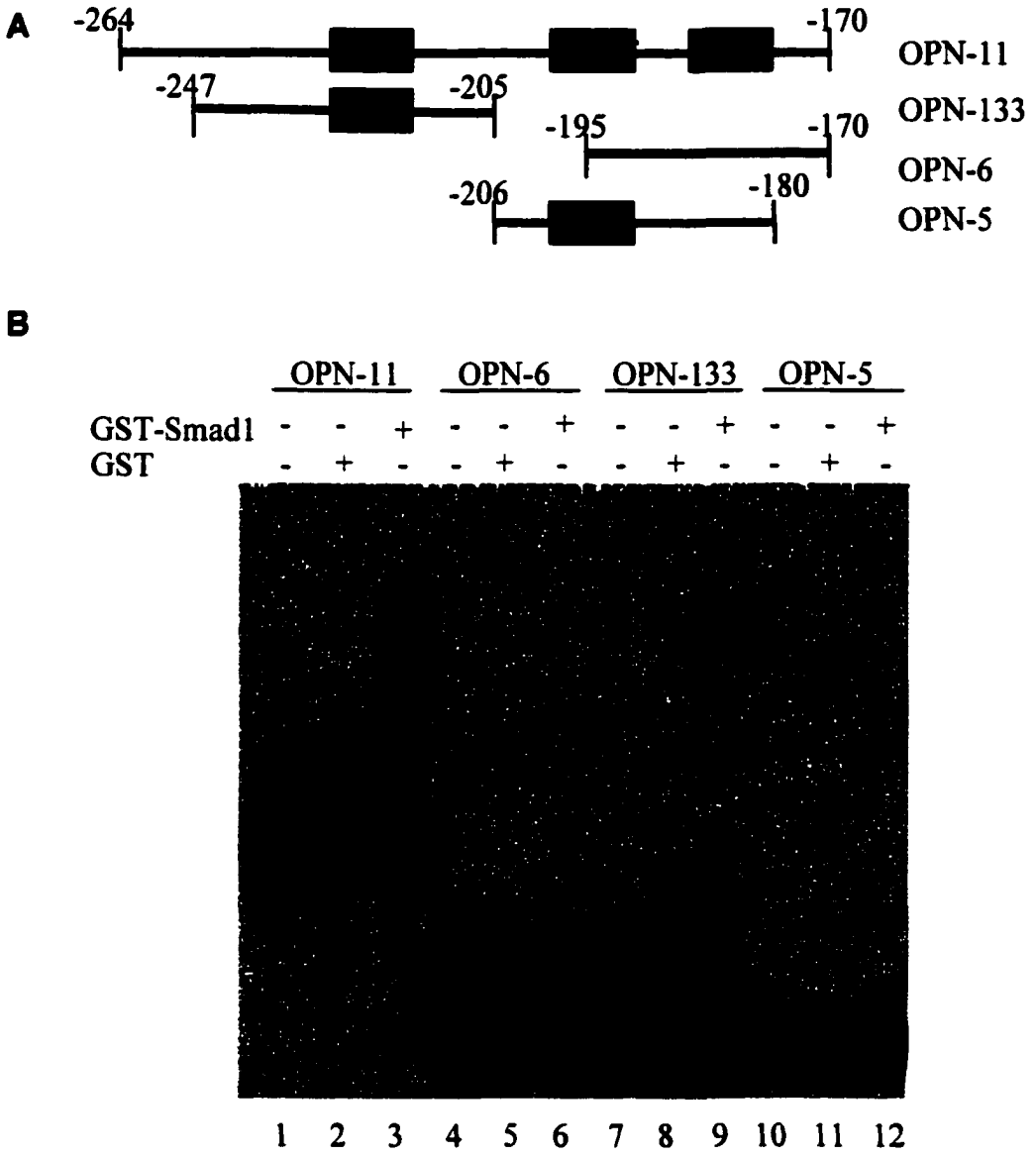

Figure 2. Mapping of the Smad1 binding element in the OPN promoter. (A) Schematic representation of OPN promoter regions used as probes in gel shift assays. Solid boxes represent the core sequences of the Smad1 binding element (SBE, GTCT) and Hoxc-8 binding element (HBE, TAAT). The sizes of probes are indicated by the nucleotide numbers rlative to the transcription start site. (B). GST-Smad1 binds to OPN-133. Double-strained oligonucleotide probes were generated by polymerase chain reaction and labeled by a kinase reaction using ^{32}P-ATP. GST-Smad1 was expressed in *E. coli*, and purified GST-fusion proteins (1.5 μg) were incubated with various probes as indicated. GST was used as negative control in the binding reactions. Protein-DNA complexes were resolved on a nondenaturing 5% polyacrylamide gel.

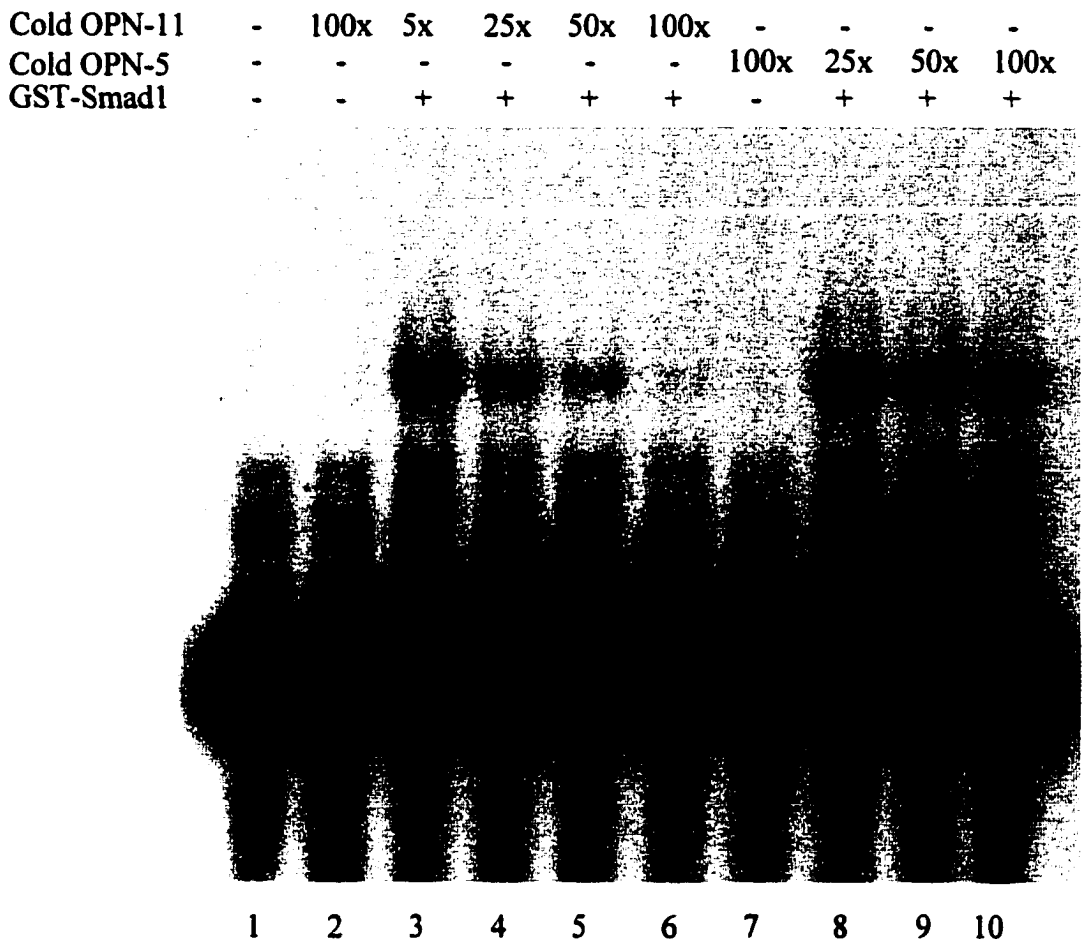

Figure 3. Smad1 specifically binds to a sequence adjacent to the HBE. Purified GST-Smad1 was incubated with the labeled probe OPN-11 (5 x 10^4 cpm), and complexes were resolved on a nondenaturing 5% polyacrylamide gel. Specific competitor probe OPN-11 containing SBE or non-specific probe OPN-5 containing HBE (see Fig. 2A) were added to some of the reactions (lanes 2-11). The numbers on the top represent molar excess of unlabeled probes used in the competition analysis.

OPN-11 inhibited the shifted band by the GST-Smad1 in a concentration-dependent manner (lanes 3-7), in which a 100X excess of the specific cold probe eliminated the Hoxc-8 binding. The presence of the OPN-5 containing HBE failed to compete with the binding (Fig. 3, lanes 8-10). From this result, we inferred that the SBE resided between nt -229 and -205, a DNA sequence immediately adjacent to HBE. To confirm these results, GST-Smad1 was tested for its binding to two smaller fragments derived from OPN-11, OPN-153 (nt -228 ~ -205) and OPN-5. GST-Smad1 binds only to OPN-153 (data not shown), but not to OPN-5 (Fig. 2B, lanes 10-12), indicating that the SBE is located at nt -229 ~ -206, which is immediately upstream of the HBE.

MH1 Domain of Smad1 Is Responsible For The DNA Binding

Drosophila Mad protein is able to bind to the *Mix.2* gene promoter region when the MH2 domain is removed (Kim et al. 1997). To localize the DNA binding domain of Smad1, we constructed five deletion forms of Smad1 and fused them with GST. Purified deletion forms of GST-Smad1 were examined for their DNA binding activity to OPN-153. Fig. 4 shows both the full-length Smad1 and the MH1 of Smad1 interacted with the probe (lanes 2 and 3, respectively). It is likely that the MH1 domains associated with the OPN-153 with a higher affinity than the GST-Smad1 full-length protein, showing an intense shifted band (lane 2). However, the linker region and the MH2 domain alone failed to bind to the probe (lanes 4 and 5, respectively). Further mapping of the MH1 domain showed that a small form of the Smad1 containing only aa 101-144 was sufficient for the DNA binding (Fig. 4, lane 7). Consistent with previous studies, the MH1 domain of Smad1 has an intrinsic DNA binding activity (Kim et al. 1997; Zawel et al. 1998).

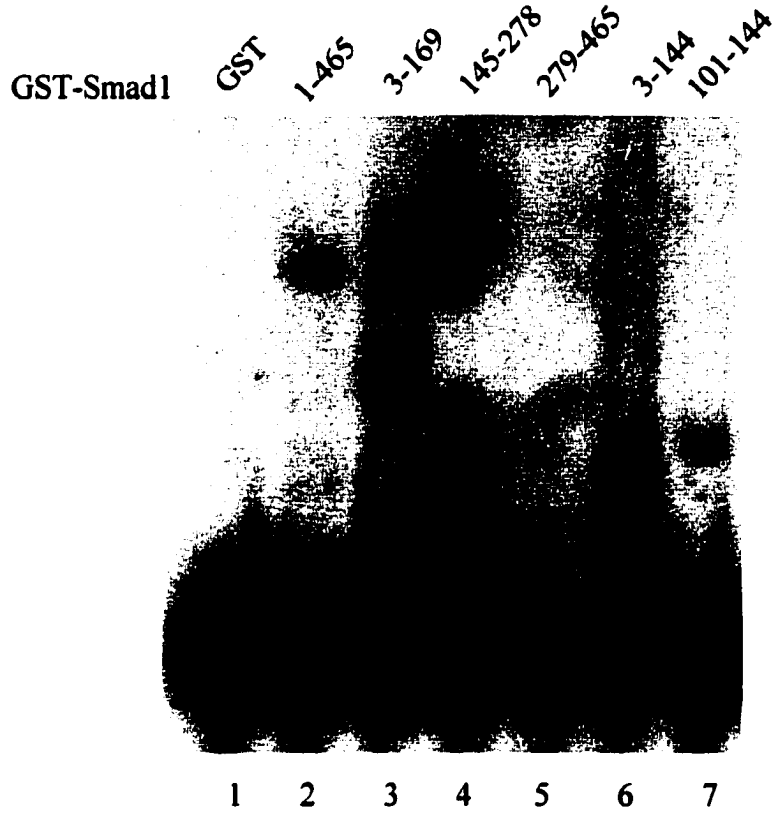

Figure 4. MH1 domain of Smad1 binds to the OPN promoter. Smad1 was expressed as full-length or truncated forms of GST-fusion proteins in *E. coli*. The sizes of each fragment in amino acid numbers are shown on the top. Purified proteins were incubated with the [^{32}P]-labeled probe (5 x 10^4 cpm), and complexes were resolved on a 5% nondenaturing polyacrylamide gel.

GTCT in the OPN-153 Is Essential for Binding of Smad1-MH1 Domain

The SBE (nt -229 to –206) identified above contains a consensus core GTCT for Smad3 and Smad4 binding (Shi et al. 1998; Dennler et al. 1998). A careful examination of the DNA sequences between nt –229 and –205 revealed one potential motif similar to the optimal Smad-binding core sequence, GTCTAGAC. However, we found that OPN-153 contains only a half site (GTCT) of the optimal SBE. To investigate whether this core sequence is important for the association with Smad1, we mutated the SBE by substituting the AGTCT with CATGG (mOPN153) and tested its binding activity to GST-Smad1 and the MH1 of Smad1. We used only the MH1 domain for further gel shift assays because it has a stronger DNA binding activity. The result shows that both GST-Smad1 (data not shown) and GST-Smad1/MH1 bind to OPN-153 (Fig. 5A, lane 2). However, the binding was decreased when mOPN153 was used in the reaction (Fig. 5A, lane 4), demonstrating that the core sequence of GTCT in the OPN-153 is essential for binding of the MH1 domain of Smad1.

MH1 Domain of Smad1 Isolated From Mammalian Cells Binds SBE

Having shown that the bacterially produced GST-Smad1 and GST-MH1 of Smad1 bind to the OPN-153 region, we further examined the DNA binding of Smad1 isolated from mammalian cells. We first constructed expression vectors for Smad1-NL (aa 3-276) and Smad1-L (aa 148-276) that were linked to a nuclear localization signal, which facilitates the entrance of the expressed protein into the nucleus. Equal amounts of the nuclear extract (20 μg) from C3H10T1/2 cells that permanently express these Smad1 fragments were incubated with probe OPN-153. As shown in Fig. 5B, the nuclear extract

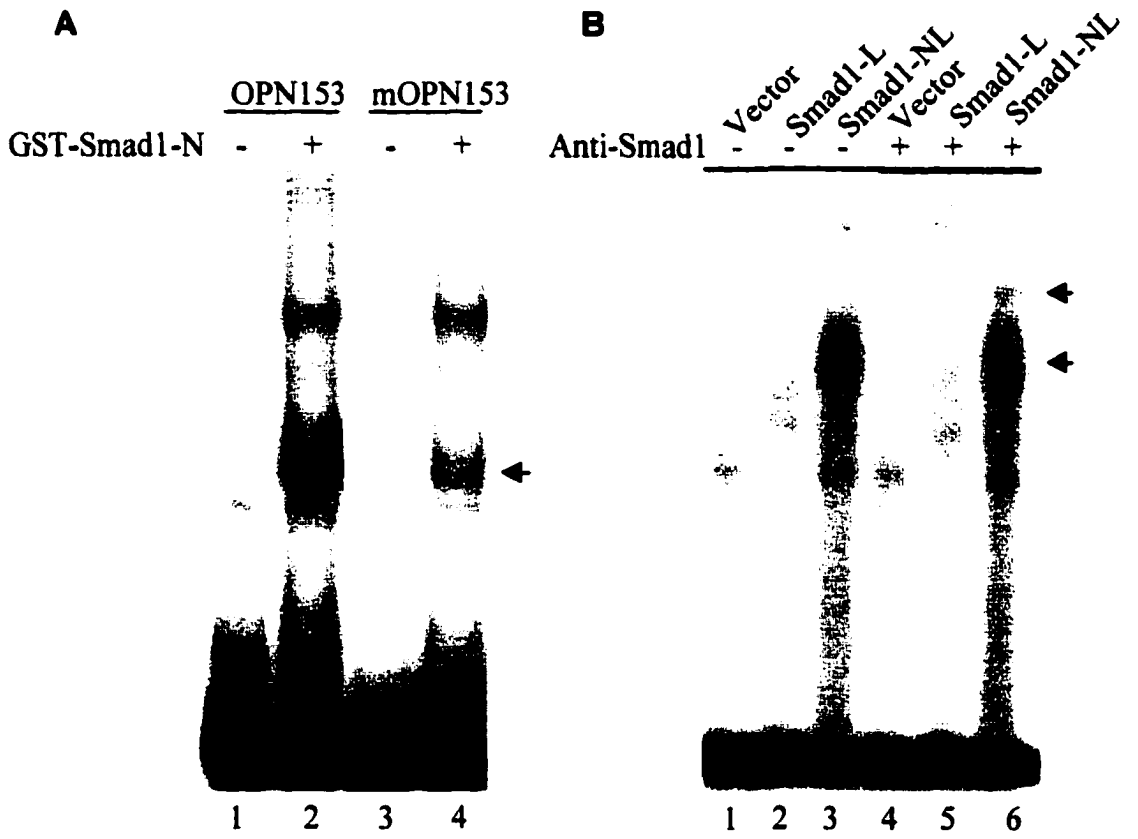

Figure 5. The core sequence of GTCT from the OPN-153 is essential for binding of MH1 domain of Smad1. (*A*) Bacterially produced GST-Smad1-N (MH1 domain) was incubated with end-labeled wild-type (lanes 1 and 2) or mutated (lanes 3 and 4) probe OPN-153, and the complexes were resolved by electrophoresis as in Figs. 1. (*B*) Removal of the MH2 domain of Smad1 allows its binding to DNA. Nuclear extracts (20 μg) of total protein) of cell lines that permanently express Smad1-NL or Smad1-L (negative control) were incubated with OPN-153, and complexes were resolved as in Fig. 2. Monoclonal antibody of Smad1 (Santa Cruz) was used for supershift assays (lanes 4-6).

of Smad1-NL (lane 3) contained the OPN-153 binding protein, whereas the nuclear extract of control vector (lane 1) and Smad1-L expressing cells had no OPN-153 binding (lanes 1 and 2). These results suggest the Smad1-MH1 isolated from mammalian cells is able to bind the OPN promoter. The protein-DNA complex was supershifted by the Smad1 specific antibody, although weakly, verifying the identity of the shifted band containing the Smad1 protein (Fig. 5B, lane 6).

SBE Mediates the Activation of OPN Gene Transcription

Previously, we have shown that a reporter construct of a 266-bp OPN promoter fragment (OPN-266), containing both the HBE and the SBE, is able to mediate BMP-induced transcription of a luciferase reporter gene (Fig. 1 and (Shi et al. 1999)). To examine whether the SBE functions as a BMP response element, we cloned an OPN promoter fragment containing only nt –290 ~ -170 into a TK-pGL3 luciferase reporter vector to generate a reporter plasmid (S/HBE-pGL3, Fig. 6A). To dissect transcriptional activity of SBE and HBE, we made three additional reporter constructs containing mutated HBE (SBE-pGL3, mutation of TAAT to GCCG), SBE (HBE-pGL3, mutation of AGTCT to CATGG), or both (mH/SBE-pGL3) (Fig. 6A). The Hox-pGL3 reporter construct responded to both constitutively active forms of the BMP type I receptor, ALK3 (Q233D) and ALK6 (Q203D) (Shi et al. 1999). S/HBE-pGL3 contains the same OPN promoter region, but it is linked to a TK promoter-driven luciferase reporter because TK is a weaker promoter than SV-40 in the Hox-pGL3. When the SBE-pGL3 construct was co-transfected in C3H10T1/2 cells with Smad1-NL, luciferase reporter activity was induced about 5-fold. However, in the presence of HBE (S/HBE-pGL3), the reporter's activity

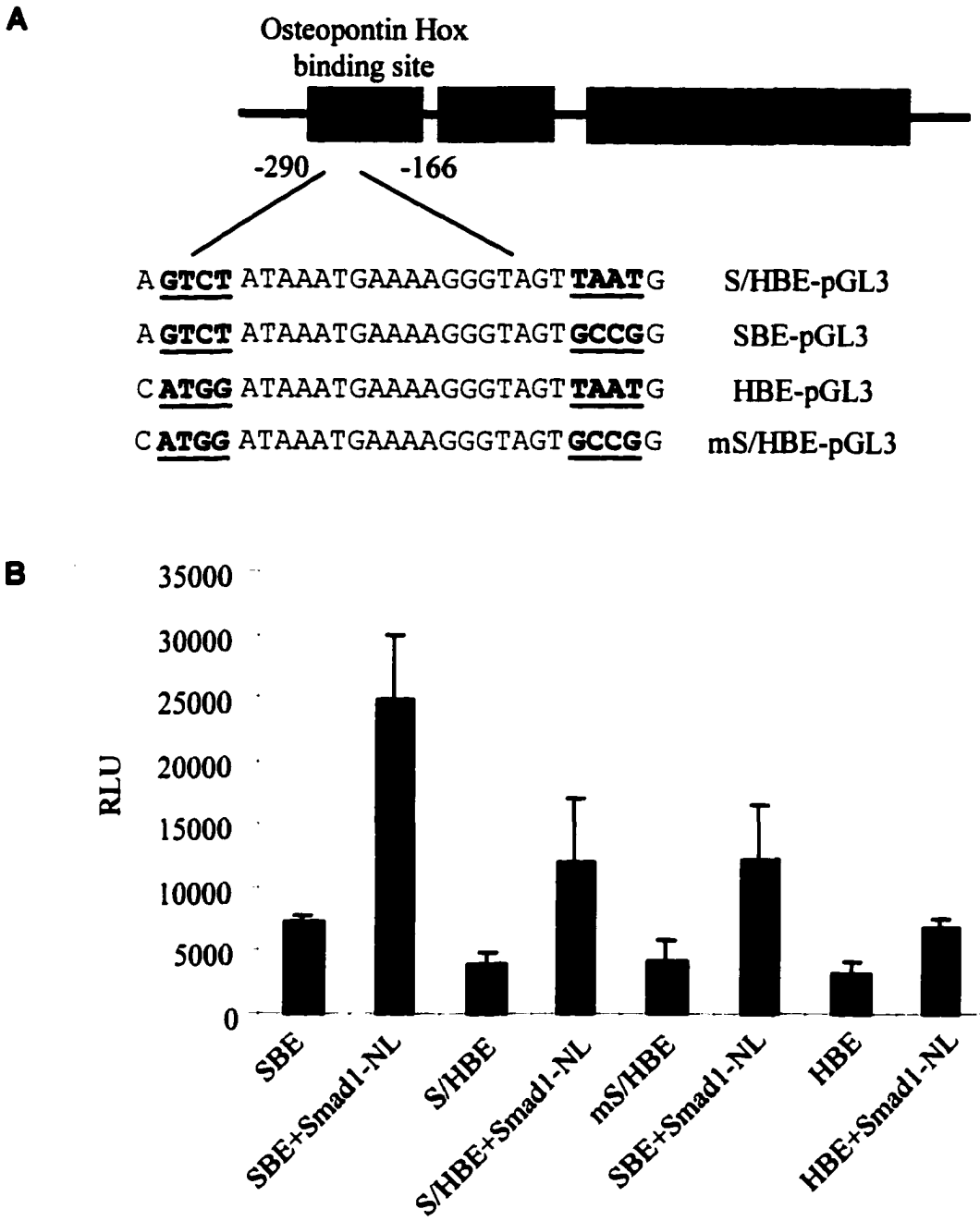

Figure 6. HBE and SBE both contribute to the activation of OPN gene transcription. (*A*) Schematic representation of the nucleotide sequences of wild-type and mutant HBE and SBE in the luciferase reporter constructs used in the transactivation experiments. (*B*) C3H10T1/2 cells were transfected with 0.25 μg of indicated reporter constructs listed in (A), with or without 0.1 μg of Smad1-NL. Luciferase activity was determined as described under "Experimental Procedures." The experiment was repeated three times with duplicates of each treatment. Data shown in the figure are from one set of results and are representative of the others.

and its response to Smad1-NL were reduced by half. Similarly, mutation of both SBE and HBE (mS/HBE-pGL3) inhibited the reporter gene transcription and the response to the stimulation by Smad1-NL. Furthermore, the HBE-pGL3 reduced the response of the reporter gene to Smad1-NL by half (Fig. 6B). Collectively, our results suggest that both HBE and SBE contribute partially to the activation of the OPN gene transcription, in which HBE inhibits reporter gene transcription, whereas the SBE enhance the reporter's activity and its response to the Smad1-NL stimulation.

Discussion

OPN gene transcription is regulated by a number of hormones and cytokines (Butler et al. 1996). Early studies have shown that TGF-β1 and TGF-β2 stimulate OPN mRNA expression in ROS 17/2.8 rat sarcoma cells and MC 3T3-E1 mouse preosteoblastic cells (Kubota et al. 1989). Noda and Denhardt (1995) have also reported that BMPs up-regulate OPN mRNA expression in osteoblast-like cells. Our previous studies provide direct evidence that BMP-induced activation of OPN gene transcription is mediated by the interaction between Smad1 and Hoxc-8 (Shi et al. 1999). We have identified a HBE in the OPN promoter region at nt –206 ~ -180. In addition, our study indicates that this element confers a strong inhibition to gene transcription. The inhibition of HBE can be released by either co-expression of the Smad1/Smad4 complex or co-expression of constitutively active BMP type I receptor. Also, Smad1, the specific mediator of the BMP signaling pathway, interacts with Hoxc-8 and inhibits Hoxc-8 from binding to HBE. On the basis of these data, we propose that Smad1 mediates the activation of OPN gene transcription by inhibiting Hoxc-8 from binding to its DNA element.

Here, we have identified a SBE from the OPN promoter at nt –229 ~ -205, which is immediately next to the HBE (nt -206 ~ -180). Bacterially expressed GST-Smad1 directly binds to the SBE. The Smad1 binding activity has also been confirmed by gel shift assay using a nuclear extract from the cells that permanently express Smad-NL. Our mapping data suggest that MH1 domain is responsible for the DNA binding. Finally, the SBE containing reporter gene shows responsiveness to BMP stimulation. Our study suggests that in addition to inhibiting the Hoxc-8 repressor, Smad1 also directly interacts with the DNA to regulate OPN gene transcription. Our studies reveal a dual function of Smad1 that provides additional information to explain the mechanism of Smad1-mediated control of OPN gene expression in vivo. On the one hand, Smad1 interacts with Hoxc-8 and inhibits the repressor Hoxc-8 binding to HBE, leading to a partial activation of the gene transcription. On the other hand, Smad1 binds to the SBE and may interact with other transcription factors to induce a full activation of the OPN gene expression.

The SBE from the 5'-flanking region in the OPN promoter has been identified by gel shift assays using a series of oligo probes (Fig. 1A). Fig. 1 clearly shows that one band is shifted when Smad1 was incubated with OPN-11, which covers the sequence of nt -264 to –170 relative to the transcription start site of the *OPN* gene. GST-Smad1 also binds to smaller probes, OPN-133 (nt -247 ~ -205) and OPN-153 (nt -229 ~ -205). Further characterization of the binding of Smad1 to SBE, by competition assay and substitution of nucleotides within the SBE (Figs. 1-3) has revealed that Smad1 specifically binds to the fragment at nt -229 ~ -205, a DNA sequence immediately adjacent to HBE. Importantly, there is a GTCT Smad recognition core sequence present in this element

(Dennler et al. 1998; Shi et al. 1998). It has been reported that Smad3/4 complex binds to three abutting GTCT sequences and that arrays of such sites elevate reporter expression relative to arrays of binding sites containing only two GTCTs, that is GTCTAGAC (Johnson et al. 1999). Compared with this, our SBE lacks half of the optimal core. However, the binding affinity of Smad1 to this GTCT-containing element is strong. And functionally, the SBE-pGL3 shows a response to the overexpression of Smad1-NL. Thus, we believe the SBE in the OPN 5'-flanking region is accountable for the Smad1 binding.

Accumulating data indicate that some of the receptor-regulated Smads and common Smads (Smad4) contain a DNA binding activity (Whitman, 1998). It is anticipated that Smad1 has an intrinsic DNA binding activity. However, most studies show Smads binding to DNA only when the MH2 domain is deleted (Kim et al. 1997). Here, for the first time, a full-length Smad1 is shown to interact with its cognate DNA element (Figs. 1-3). It might be possible that some bacterial kinases phosphorylate Smad1 non-specifically, which opens up the Smad1 structure and allows the MH1 to interact with DNA. Some studies have shown that full-length Smad3 binds to the DNA (Dennler et al. 1998). Supershift data from our study with antibody also detected that the intact Smad3 binds to DNA (manuscript in preparation). It seems that the phosphorylation of R-Smad is required only for translocation but not for DNA binding. In fact, Smad4, a common Smad for both the TGF-β and the BMP signaling pathways, binds to DNA strongly (Dennler et al. 1998), yet lacks the conserved SS*X*S phosphorylation site. However, it is unclear whether Smad1's binding to DNA is independent of phosphorylation by type I receptor serine/threonine kinases.

Consistent with published studies, we have also shown that the MH1 domain of Smad1 is responsible for the DNA-binding activity. Smad1-NL isolated from mammalian cells recognized the same probe, OPN-153 (Fig. 5). Importantly, overexpression of Smad1-NL induced the activation of the reporter gene containing SBE (Fig. 6B). Mutated SBE inhibits the activation induced by the Smad1-NL by 50%, directly indicating that SBE contributes to the regulation of OPN gene transcription. Our results also suggest that mutated HBE partially abolished the reporter's activation by overexpressed Smad1-NL, although it confers a higher level of the reporter gene expression as a result of derepression of Hoxc-8 (Shi et al. 1999).

The action of endogenous Smad1 on the regulation of OPN gene transcription is not known. Our previous study demonstrated that overexpression of Hoxc-8 interaction domains of Smad1 alone is sufficient to induce endogenous OPN gene activation in the absence of BMP stimulation. Whether the gene activation is induced solely by derepression of Hoxc-8 remains to be answered. In this study, we have shown that the small fragment of MH1 domain, aa 101-145, has DNA binding activity (Fig. 3), and this same domain is also sufficient to inhibit Hoxc-8 from binding HBE (Yang et al. 2000). Both depression by association with the repressor Hoxc-8 and activation by interacting with SBE contribute to the full activation of endogernous OPN gene. Further studies are certainly needed to verify this speculation.

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SMAD INTERACTORS IN BMP SIGNALING PATHWAY

by

XIANGLI YANG AND XU CAO

In press in *The Two-Hybrid Systems: Methods and Protocols*

Introduction

The yeast genetic-based two-hybrid system has been widely used in identifying a pair of interacting proteins. The basis for this technique is that eukaryotic transcription activators are modular with respect to their DNA-binding domain and transcription activation domain (Keegan et al. 1986). The two domains are required for activating gene expression, but they need not be covalently linked. Indeed, they may be located on entirely separately proteins. It is sufficient that the interaction between two (or more) proteins brings these domains into close proximity (Sadowski et al. 1988). Therefore, the transcriptional activity of the target gene can be used as a measure of the protein-protein interaction.

A variety of versions of the two-hybrid system are commercially available. The most commonly used are the yeast Gal4 (Fields and Song 1989) and the *Escherichia coli* LexA (Brent and Ptashne 1985)-derived systems. Three components are essential for all the two-hybrid systems. The first is a bait plasmid that directs the synthesis of the protein being studied, whose cDNA has been fused to a DNA-binding domain of Gal4 or LexA proteins. The second is a prey plasmid that directs the synthesis of a second protein fused to an activation domain like those of Gal4 or Herpes virus VP16 (Sadowski, et al. 1988). This second protein can either be one chosen specifically for study or can be an unknown protein derived from a cDNA library. Finally, a yeast reporter strain that contains one or more reporter genes, like yeast *Leu2* and *His3* genes and *E. coli LacZ*, with upstream binding sites for the bait is also required. Productive interaction between the bait and prey proteins leads to expression of the reporter gene(s) that can be determined by the

ability of yeast strains to grow on a selective medium and/or to turn blue with the right substrate.

Smads are a family of newly discovered downstream mediators of transforming growth factor-β (TGF-β) superfamily signaling. In mammalian systems, eight members of Smads identified thus far can be categorized into three subgroups. The receptor-regulated Smads (R-Smads) can be phosphorylated by TGF-β/activin receptors (Smad2 and Smad3; Eppert et al. 1996; Lagna et al. 1996; Yingling et al. 1996) or by bone morphogenetic protein (BMP) receptors (Smad1, Smad5, and Smad8; Hoodless et al. 1996; Kretzschmar et al. 1997; Macias-Silva et al. 1998). Hence, they determine the specificity of the signaling pathways by interacting with different receptor molecules. The common Smads (Co-Smads) shared by both TGF-β and BMP signaling pathways is composed solely of Smad4 (Lagna et al. 1996), which interacts with the R-Smads and translocates into the nucleus where the complex activates gene transcription. The antagonist Smads (Anta-Smads), including Smad6 and Smad7 (Topper et al. 1997), inhibit the signaling by either inhibiting the phosphorylation of R-Smads by their receptors (Imamura et al. 1997; Nakao et al. 1997) by competing with R-Smads for the Co-Smad4 (Hata et al. 1998), or by preventing the R-Smads from interacting with other DNA-binding molecules (our unpublished data).

Similar to other signaling molecules, Smads transduce their signal by interacting with other Smads or with other cellular proteins. In addition, Smad complexes also function as transcription activators, which bind directly to other nuclear DNA-binding proteins or to DNA (Massague 1998). The two-hybrid system, as a tool for studying protein-protein interactions, is exceedingly helpful in hunting for Smad interactors in the TGF-β

superfamily signaling pathway, because it allows us to build individual binary links to more complex patterns of connections (Bartel et al. 1993).

Another feature of Smads that makes the yeast two-hybrid system so useful is that Smads contain three distinct domains, termed MH1, linker, and MH2, for amino-terminal, intervening region, and carboxyl-terminal domains, respectively. Each domain possesses characteristic structures that support the interaction with specific proteins. In particular, MH2 of all the R-Smads has a SS*X*S motif that is recognized and phosphorylated by TGF-β or BMP type I receptors. The MH2 domain is also the transactivator, which interacts with other transcription factors. The linker of R-Smads can be phosphorylated by MAP-kinases, which leads to an inhibition of nuclear translocation of Smad complexes. The MH1 domain associates with MH2 of the same R-Smads when the protein is in the inactivate state (Whitman 1998). The MH1 domain of Smad1 and Smad4 is also found to bind other proteins, such as Hoxc-8 and Hoxa-9 (manuscript in revision). Thus, different domains of Smads can be separately fused with DNA-binding domains and used as baits to pull out their specific interactors.

The yeast two-hybrid assay has advantages over cross-linking, co-precipitation (pull-down), co-immunoprecipitation, co-chromatography, or other approaches for the study of protein-protein interaction. It has a higher sensitivity, which supports identification of weak and transient interactions in vivo. Further, it detects the interactions in yeast host cells, a physiologically relevant environment. Many groups have successfully identified Smad interactors by using either intact Smads (Shi et al. 1999) or truncated Smads (Verschueren et al. 1999) as baits in two-hybrid assays. The two-hybrid system is also used to study the interaction of individual domains of Smads (Hata et al. 1997; Shioda et

al. 1998) or of Smad and its interactor (Topper et al. 1997; Verschueren et al. 1999) Here, we describe detailed yeast two-hybrid protocols that have been sucessfully used to identify the Smad1 interactor, Hoxc-8, and to map the interaction domains between the two proteins.

Materials

Vectors, Yeast and Bacterial Strains, Equipment

1. pGBT9 cloning vector (5.4 kb, Clontech) for generating the fusion of the Smad1 bait protein with the GAL4 DNA-binding domain. pGBT9 carries the *trp1* gene that confers a Trp+ phenotype to yeast transformants.
2. Human osteosarcoma MATCHMAKER cDNA library (Clontech, HL4026AH). The cDNA library was fused with the GAL4 activation domain in pACT2 cloning vector (8.1 kb). pACT2 contains the *leu2* gene which gives a Leu+ phenotype to its host yeast strains.
3. Y190 *Sccharomyces cerevisiae* yeast reporter strain that requires histidine (His), leucine (Leu), and tryptophan (Trp) in the medium to grow. Y190 also contains *E. coli lacZ* and yeast *HIS3* reporter genes.
4. Two primers for sequencing the fusion genes. GAL4 DNA-binding domain primer TCATCGGAAGAGAGTAG; and GAL4 activation domain primer TACCACTACAATGGATG.
5. *E. coli* competent cells, such as DH5α, or HB101.
6. Spectrophotometer, 30°C and 37°C incubators, 42°C water-bath, electroporator.

Media and Plates

1. YDP medium: 2% Difco peptone (w/v), 1% yeast extracts (w/v), and 2% glucose (use separately autoclaved 50% stock).
2. SD synthetic medium: 0.67% (w/v) Difco yeast nitrogen base without amino acids (Difco 0919-15), 2% glucose (autoclave and add separately), and 1x dropout solution (see 2.3; autoclave and add separately). Store at 4°C.
3. LB broth: 10 g bacto-tryptone, 5 g bacto-yeast extract, and 5 g NaCl in 1 L of water. Autoclave and store at room temperature.
4. YPD/SD/LB plates: 15% agar in appropriate media and autoclave. Pour the 100x 15-mm or 150 x 15-mm plates and store at 4°C.

Stock Solutions and Chemicals

All chemicals were obtained from Sigma unless stated otherwise.

1. 10x dropout solutions: dissolve following in water and autoclave. Store at 4°C up to 1 year.

Table 1. *Amino acid components in 10x dropout solution*

Compound	g/L	Sigma	Compound	g/L	Sigma
L-Isoleucine	0.3	I-7383	L-Valine	1.5	V-0500
L-Adenine hemisulfate salt	0.2	A-9126	L-Arginine HCl	0.2	A-5131
L-Lysine HCl	0.3	L-1262	L-Methionine	0.2	M-9625
L-Phenylalanine	0.5	P-5030	L-Threonine	2.0	T-8625
L-Tyrosine	0.3	T-3754	L-Uracil	0.2	U-0750

2. 200x L-tryptophan (Sigma T-0254): 0.4 g/100 ml water, autoclave. Store at 4°C.
3. 100x L-leucine (Sigma L-1512): 1 g/100 ml water, autoclave and store at 4°C.
4. 1000x L-histidine (Sigma H-8511): 2 g/100 ml water, autoclave and store at 4°C.

5. 1 M 3-amino-1, 2, 4-triazole, a competitive inhibitor of the His3 protein.
6. 50% PEG 4000 (polyethylene glycol, avg. MW 3350, Sigma P-3640). Autoclave and store at room temperature (RT).
7. 100% DMSO (dimethyl sulfoxide, Sigma D-8779).
8. 100% glycerol
9. 10x TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5. Store at RT after autoclaving.
10. 10x LiAc: 1 M lithium acetate (Sigma L-6883). Autoclave and store at RT.
11. Salmon sperm single-stranded DNA: 10 mg/ml.
12. Z buffer: 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, and 0.246 g $MgSO_4 \cdot 7H_2O$. Add water to 1 L and autoclave. Store at RT.
13. ONPG: o-nitrophenylgalactoside (Sigma N-1127).
14. 1 M Na_2CO_3.
15. Yeast lysis solution: 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% Triton x-100, and 1% SDS.
16. Phenol/chloroform/isoamyl alcohol (25:24:1).
17. Acid-washed glass beads (425-600 μm; Sigma G-8772).
18. 70% and 95% EtOH
19. 1000x ampicillin: 100 mg/ml in H_2O, filter sterile and store at –20°C.

Methods

All yeast manipulations are based on the MATCHMAKER GAL4 two-hybrid system user manual (Clontech, PT3061-1) with modifications. Unless stated otherwise, any

bacterial or yeast culture should start from a single colony grown to fresh overnight cultures. All the materials and solutions should be sterilized by autoclaving or filtering.

Construction of Bait Plasmid

We used an intact human Smad1 cDNA inserted between the *Sal*I and *Pst*I sites in pGBT9 vector as a bait to screen a human osteoblast-like cell cDNA library constructed in pACT2 vector. The first step was to construct a bait plasmid that directs the expression of GAL4 DNA-BD-Smad1 fusion. A multi-step cloning strategy was used to obtain the bait plasmid because the *Sal*I/*Pst*I sites were too close to be used together.

1. Digest the pGBT9 vector with *Pst*I and dephosphorylate with alkaline phosphatase.
2. Insert any DNA fragment in the *Pst*I site for the purpose of providing some distance between the two intended cloning sites.
3. Sequentially digest the pGBT9/insert with *Sal*I and then *Pst*I to release the pGBT9 vector.
4. Digest pCMV5B/Samd1 (9) with *Sal*I and *Pst*I to release Smad1 cDNA.
5. Ligate pGBT9 vector and Smad1 cDNA insert and transform bacteria with the ligation mix.
6. Verify the hybrid construct by restriction digestion with *Sal*I/*Pst*I and sequencing using the GAL4 DNA-BD primer to confirm the in-frame fusion of Smad1 cDNA and the GAL4-DB.
7. (Optional) Check expression of fusion protein of GAL4 DNA-BD-Smad1 by Western blot with Smad1 antibody.

Generation of Bait Plasmid-Carrying Strain and Testing of the Transactivation Activity of the Bait Protein

It is not uncommon that the target protein activates transcription when linked to a DNA-binding domain. In this case, the yeast two-hybrid system is not suitable for a library search for the unknown target-interacting proteins. The pGBT9/Smad1 bait plasmid was transformed into Y190 reporter strain and plated onto SD/-Trp and SD/-Trp-His plates. The phenotype of the transformants should be Trp+His- if the bait alone does act as a transactivator.

Transformation of Y190 with the Bait Plasmid (pGBT9/Smad1)

1. Inoculate Y190 cells from a single colony (2-3 mm in diameter) into 3 ml of YPD medium and incubate at 30°C with shaking at 200 rpm until A_{600} = 1.5 (1-2 days).
2. Transfer enough (1-2 ml) of the culture to 40 ml YPD medium in a 250-ml flask to get an A_{600} = 0.2. Grow this culture at 30°C with shaking at 200 rpm for 3 hr.
3. Pour culture into a 50-ml Falcon tube and pellet cells by centrifugation at 1,000x *g* for 5 min at RT.
4. Discard the medium and wash cells by resuspension of the cells in water and harvesting the cells as above.
5. Discard the supernatant and resuspend the cells in 0.2 ml of 1x TE/LiAc (made freshly by mixing 0.1 ml of each 10x TE and 10x LiAc and 0.8ml of sterile water).
6. Transfer the cell suspension to a 1.5-ml microcentrifuge tube.
7. Boil 0.1 ml of single-stranded DNA for 5 min and quickly chill in ice water.
8. To the 0.1 ml cell suspension add the following components onto the top of the cells in the order shown. Mix well by vortexing: 0.48 ml PEG (50%); 60 µl of 10x TE

buffer; 60 µl of 10 x LiAc; 10 µl of ssDNA (10 mg/ml); and 0.1 µg of pGBT9/Smad1.

9. To the other 0.1 ml cell suspension, add above mix but leave the bait plasmid out (control).
10. Incubate the tubes at 30°C for 30 min with shaking or with occasional inversion.
11. Add 70 µl of DMSO to each of the tubes and mix by vortexing gently.
12. Heat-shock for 15 min at 42°C and then chill on ice briefly.
13. Pellet cells by centrifugation at 14,000 rpm in a microcentrifuge for 5 sec.
14. Carefully aspirate the supernatant and resuspend the cells with 0.5 ml 1x TE buffer.
15. Plate 0.1 ml and 0.4 ml of each transformation mixture onto 100-mm SD/-Trp and SD/-Trp-His+3-AT (25 mM) plates, respectively.
16. Incubate the plates at 30°C for 2-4 days.

Score the Phenotype of the Transformants

1. Y190 straining bearing pGBT9/Smad1 should grow on SD/-Trp plate but not on the SD/-Trp-His+3-AT plate. Y190 control cells should not grow on any of above selective media.
2. If any visible colonies formed on the SD/-Trp-His+3-AT plates before 3 days, the bait cannot be used for the interactor hunt. In this case, the activation domain of the bait needs to be removed. If no visible transformants are His-, continue following steps.
3. Pick up 1-5 transformants from SD/-Trp plate and inoculate 3 ml of SD/-Trp liquid medium and grow at 30°C for 1-2 days until the cell density is OD_{600} = 1.5.

4. Make bait-bearing yeast (Y190/pGBT9/Smad1) stocks by mixing 0.75 ml of the liquid culture and 0.25 ml of glycerol. Store at –80°C.
5. (Optional) Perform β-galactosidase (β-gal) liquid assay on the bait-carrying clones to verify the negative for transactivation activity (see later sections for β-gal assay methods).

The above steps establish whether the bait can be used in a Smad1 interactor hunt. The bait-bearing strain can also be used in the two-step transformation for the library screening (see *Library Screening*). In our practice, transformants of Y190/pGBT9/Smad1 failed to grow on SD/–His+3AT plates, and they were also negative in β-gal activity, so the bait fusion is suitable for the library screening. It is advisable to include a control plamid encoding a GAL4-DB fused with an intrinsic transactivator, such as pVA3 (Clontech), which can grow on SD/–His+3-AT plates.

Amplification of cDNA Library

One of the main uses of the two-hybrid system is to screen a relevant cDNA library for genes encoding the bait interactors. Smad1 as a BMP downstream intracellular transducer may play a role in osteoinduction. To this end, we used the human osteoblast-like cDNA library in pACT2 (Clontech). The cDNA library plasmids, supplied as a population of bacterial-carrying clones, were amplified by the method described below. Before the amplification, one should aliquot the library bacterial culture after the first thawing to avoid multiple freeze/thaw cycles and titer the library to estimate the colony-forming units (cfu) present per ml.

Titer the cDNA library

1. Thaw one tube containing 1 ml of the library bacterial culture and chill nine 0.5-ml Eppendorf tubes on ice. Gently vortex the culture and then transfer 0.1 ml aliquots into each of the 0.5 Eppendorf tubes. Leave one tube on ice and store all the rest at -80°C.
2. Prepare three 1.5–ml Eppendorf tubes, each containing 990 µl of LB/Amp medium.
3. Transfer 10 µl of library culture to first tube to obtain a 10^{-2} dilution. Mix well by vortexing.
4. Transfer 10 µl of the 10^{-2} dilution to the second tube and mix well to obtain 10^{-4} dilution. Do the same from the second to the third tube, which is the 10^{-6} dilution.
5. Plate 0.1-ml aliquots from each of the second and the third dilutions onto LB/Amp plates.
6. Incubate the plates at 30°C until colonies appear.
7. Count the colonies formed on each plate and calculate the cfu as following:

   2^{nd} dilution = # colonies x 10^5 = cfu/ml.

   3^{rd} dilution = # colonies x 10^7 = cfu/ml.

   We titered our library culture and obtained the same titer as Clontech expected (>10^8cfu/ml). Several factors reduce the titer of a library culture, such as repeated freeze-thaw cycles, prolonged storage, and storage of diluted culture.

Amplify the Human Osteosarcoma cDNA Library

1. Prepare 250 LB/Amp plates (150 x 15-mm) and let them dry at room temperature for 3 days.

2. Dilute the library with LB/Amp (100 μg/ml) to a concentration of 1.5 x 10^5 cfu/ml to make a total of 50 ml cell suspension.
3. Spread 0.2 ml of the cell suspension onto each LB/Amp plate with a sterile glass or metal spreading rod over agar surface until all visible liquid has been absorbed.
4. Incubate the plates at 30°C until confluent (36-48 hr).
5. Add 5 ml of LB containing 25% glycerol to each plate to resuspend colonies.
6. Pool all the suspension into one flask and mix well.
7. Remove 1/3 of the library culture for plasmid DNA preparation using any standard Maxi-prep method.
8. Store the remainder library culture in 50-ml aliquots at –70°C for later use.
9. Expected yield of plasmid DNA per 1 x 10^6 cfu (cfu as referred in step 2) for pACT2 library is >0.25 mg.

This protocol was used to amplify the human osteoblast-like library, and it produced enough cDNA library plasmids for at least seven rounds of screening.

Library Screening

Both bait and prey plamids can be co-transformed into a single yeast cell; however, it is often more efficient to perform two-step (sequential) transformation. We describe here a sequential transformation procedure in which the cDNA library plasmids are transformed into a yeast strain already carrying the bait plasmid. The PEG/LiAc method described below usually gives us a transformation efficiency of 10^5 transformants/μg DNA. In theory, to screen 2-3 x 10^6 independent clones in a library, 20-30 μg cDNA and 1-2

mg carrier ssDNA are required. We used 500 μg cDNA library plasmid to ensure that proteins encoded by low abundance transcripts are represented.

Prepare competent Y190 carrying pGBT9/Smad1 bait plasmid

1. Grow a 3-ml overnight culture of the pGBT9/Smad1 bait-carrying Y190 from a single colony (2-3 mm in diameter) at 30°C.
2. Inoculate 100 ml of SD/-Trp with the 3-ml culture and incubate at 30°C for overnight.
3. Remove 60-100 ml of the overnight cell culture to inoculate 1 L YPD in a 2800-ml flask to obtain an OD_{600} = 0.4. Incubate at 30°C for 4 hr with shaking at 200 rpm.
4. Pellet the cells by centrifugation at 2500 rpm for 5 min at RT.
5. Decant the supernatant, wash the cells in 500 ml water, and pellet the cells as above.
6. Discard the supernatant and resuspend cells in 20 ml 1x TE/LiAc solution.
7. Leave the cell suspension at RT for 10 min.

Transform the bait carrying yeast strain with cDNA library plasmids

1. To an autoclaved 2-L beaker, add following components in the order shown and mix well by vigorous vortexing: 112 ml PEG (50%); 14 ml TE (10x); 14 ml LiAc (10x); 2 ml of single-stranded DNA (10 mg/ml); 500 μg of cDNA library plasmid.
2. Add the 20 ml competent cells from step 7 in *Prepare competent Y190 carrying pGBT9/Smad1 bait plasmid* to the mix.
3. Mix well by swirling and incubate at room temperature for 30 min.
4. Add 17.6 ml of DMSO and mix well by swirling the beaker gently and heat shock in a 42°C water-bath for 6 min with constant swirling to equilibrate the temperature.

5. Add 50 ml sterile water to cool the cells to room temperature and transfer the transformation mix to five 50-ml conical tubes.
6. Pellet the cells by centrifugation at 2500 rpm for 5 min at room temperature.
7. Aspirate the supernatant and wash the cells by resuspension of the cells with 25 ml of TE buffer to each tube and collect the cells by centrifugation as above.
8. Discard the supernatant and resuspend cells in 1 L YPD in a 2.8-L flask.
9. Incubate the cells in 30°C for 1 hr with shaking at 200 rpm.
10. Harvest the cells as above and resuspend cells in 10 ml TE buffer.
11. Plate 10 μl (dilute in 0.2 ml of TE) of the same transformation mix and its dilution onto separate SD/-Trp-Leu plates for estimation of the transformation efficiency.
12. Plate 0.2 ml of the cell suspension onto each 150 x 15-mm agar plates containing SD/-Trp-Leu-His+AT (45 mM) medium (50 plates).
13. Incubate all the plates at 30°C for 3-5 days until His+ colonies are visible. To search for weak interactions, the incubation time can be prolonged to 8 days.
14. On the next day, count the colonies formed on the SD/-Trp-Leu plates and calculate the transformation efficiency (# of the transformants/μg DNA).

Preparation of the master plate

Colonies formed within 1 week are collected onto one or more master plate by streaking all the His+ colonies in a grid pattern to facilitate future identification of the colonies.

1. Mark the back of 100-mm SD/-Trp-Lue-His+3-AT plates with grid pattern.
2. Streak single His+ colonies to each grid.

3. Incubate the master plates for 1-2 days until the colony size reach 2 mm in diameter.
4. Seal the plates with Parafilm and store at 4°C if not used immediately. These are your master plates.
5. Restreak fresh plates every 3-4 weeks until all colonies are examined (see *Elimination of false positives*).

Elimination of False Positives

One of the disadvantages of the two-hybrid assays is that the His+ transformants sometimes are false positives; that is, the His+ clones contain no plasmids encoding hybrid proteins that directly interact with Smad1 target proteins. The true positives should be examined for the expression of the second reporter gene by β-gal activity assays described below. Filter β-gal assays allow one to select positive clones quickly by blue/white screening. If one wishes to obtain quantitative β-gal activity data, the liquid assay can be performed to measure the production of a yellow compound, o-nitrophenol, from the substrate ONPG.

Filter β-gal assay

1. Prepare Z-buffer/X-gal solution: 10 ml Z-buffer, 27 μl β-mercaptoethanol, and 167 μl X-gal (20 mg/ml).
2. Prepare a 1-L liquid nitrogen bucket.
3. Place a sterile Whatman #1 filter paper (75 mm in diameter) over the surface of the master plate and poke holes at the edge through the filter into the agar to orient the filter paper.

4. Lift the filter off the plate with forceps, drop the filter in the liquid nitrogen, and leave it in for 5 sec to freeze the colonies.
5. Put the plate back in the incubator to allow yeast colonies to grow at 30°C for 1-2 days.
6. Take out the filter and place it in the 100-mm dish (colony side up) to allow it to thaw at room temperature for 2-5 min to permeabilize the cells.
7. Add 0.5 ml of the Z-buffer/X-gal solution to the dish by touching the pipet tip to the edge of the dish. Allow the solution to migrate over the filter paper.
8. Incubate the filter at 37°C and check periodically for the appearance of blue colonies.
9. Identify the blue colonies by aligning the filter paper with the master plate using the orienting marks.
10. Grow liquid cultures by inoculating the corresponding positive colonies from the master plate to 3 ml of SD/-Trp-Leu-His medium to make stocks for further analysis.

Liquid β-gal assay

1. Inoculate individual yeast positive colonies from the master plate to a 3 ml SD/-Trp-Leu-His and incubate at 30°C overnight.
2. Inoculate 2 ml of each overnight culture from step 1 to a 8 ml the YPD medium and incubate at 30°C for 3-5 hr until the culture reach A_{600} = 0.5–0.8 (mid-log phase).
3. Record the A_{600} value for each transformant culture.
4. Prepare ONPG solution by dissolving 40 mg of ONPG in 10 ml of Z-buffer.
5. Transfer 1.5 ml culture into a 1.5-ml microcentrifuge tube and spin at 14,000 rpm for 30 sec to pellet cells.

6. Carefully remove the supernatant and wash the cells once with 1.5 ml of Z-buffer.
7. Resuspend each pellet in 0.3 ml of Z-buffer, which concentrates the cell culture 5-fold.
8. Transfer 0.1 ml of the cell suspension to a fresh microcentrifuge tube.
9. Freeze (1 min in liquid nitrogen) and thaw (1 min at 37°C) the cells three times to permeablize the cells.
10. Prepare Z-buffer/β-mercaptoethanol by mixing 10 ml Z-buffer with 27 μl of β-mercaptoethanol and set up a blank tube with 0.1 ml of Z-buffer.
11. Add 0.7 ml of the Z-buffer/β-mercaptoethanol to each reaction and the blank tubes.
12. Add 0.16 ml of ONPG solution to each tube and record the start time for the reaction.
13. Incubate reactions at 37°C until the yellow color develops. Add 0.4 ml of 1 M Na_2CO_3 to stop the reaction and record the elapsed time in minutes for each reaction.
14. Centrifuge reaction tubes for 10 min at 14,000 rpm to pellet cell debris.
15. Calibrate the spectrophotometer with the blank tube at A_{420} and read A_{420} for all the reactions. The linear range of A value is 0.02–1.0. Make dilutions and re-read the A_{420} if needed.
16. Calculate the β-gal activity by Miller's equation:

$$\beta\text{-gal units} = 1000 \times (A_{420}/T \times V \times A_{600})$$

where T = minutes of the reaction duration; V = 0.1 ml x concentration factor (5, in this case).

The time it takes colonies or reactions to develop color varies from minutes to hours. Prolonged incubation (>24 hr) may give false positives. We found fresh colonies take less time to turn blue or yellow. Thus, when one tries to make comparisons of the β-gal

activity on candidate clones, the growth condition, the age and the size of the colonies, and the efficiency with which the cells are permeablized should be similar or identical. The liquid assay is less sensitive than the filter lift assay, and the result varies from time to time. We recommend analyzing all the clones at once if one wishes to compare β-gal activities.

Isolation of Leu+Trp- Clones

His/LacZ double-positive transformants need to be isolated and the prey plasmids need to be sequenced for further characterization. The genetic manipulation described below was used to isolate transformants that contain only prey plasmids (putative Smad1 interactors).

1. Restreak β-gal-positive clones on selection medium containing SD/+Trp+Leu to segregate the cDNA fusion plasmids.
2. Re-assay for LacZ+ (β-gal active) to verify the positive clones.
3. Grow each transformant (Trp+Leu+LacZ+) in 3 ml of liquid SD/Leu- medium, which maintains only the prey plasmid (Leu+), until the culture is saturated (8-10 days) to allow random loss of the pGBT9/Smad1 plasmid.
4. Dilute the liquid culture and plate onto SD/Leu- plates. Incubate plates at 30°C for 2-3 days.
5. Patch 30-50 colonies on the SD/-Leu and SD/-Leu-Trp agar plates in parallel.
6. Pick up the colonies that grow on SD/-Leu but not on SD/-Trp-Leu (Trp auxotrophs, presumably, have lost their bait plasmids but maintain the prey plasmid).

7. Assay for LacZ phenotype and save the ones that is β-gal negative for isolation of pACT2/prey cDNA.

Theoretically, 10–20% of the yeast cells will lose their plasmid spontaneously when the selective pressure is absent. Patching 30-50 colonies should give at least a few Trp-Leu+ clones. However, we sometimes have to patch more than 100 colonies to isolate one Trp-Leu+ clone. An alternative is to isolate prey plasmids from yeast and transform into bacteria to isolate Leu+ clones. In this case, the HB101 bacterial strain can be used as the host because the yeast *leu2* gene in pACT2 can complement the *leuB* mutation of HB101.

Isolation of Plamids from Y190/prey cDNA

1. Inoculate single colonies into 2 ml of YPD medium, and grow overnight at 30°C for 16-14 hr.
2. Transfer 1.5 ml of the culture into microcentrifuge tube and spin at 14,000 rpm to pellet cells.
3. Add 0.2 ml of yeast lysis solution mix well and then 0.2 ml of phenol/chloroform/ isoamyl alcohol.
4. Add 0.3 g of acid-washed glass beads and vortex for 2 min or longer to break the cell wall.
5. Centrifuge at 14,000 rpm for 5 min at room temperature.
6. Transfer the supernatant to a clean Eppendorf tube and add 1/10 V of 3 M NaAc and 2.5 V of 95% ethanol to precipitate DNA.
7. Wash the DNA once with 75% EtOH and dry the DNA at RT for 5 min.

8. Resuspend the plasmid DNA in 20 μl of TE buffer.

Amplification of Prey Plasmid in Bacteria and cDNA Analysis

Because of the low yield of yeast plasmid DNA, the isolated pACT2/prey cDNA needs to be transformed and amplified in bacteria for further characterization.

1. Transform competent bacteria with 1-5 μl of isolated prey cDNA plasmids by either electroporation or chemical method.
2. Grow overnight and isolate plasmid DNA by mini-prep from bacterial transformants.
3. Restriction enzyme digest to verify the cDNA insert.
4. Sequence the insert cDNA using the GAL4-AD primer.

Other Methods to Verify Positive Interactions

Once an interaction has been identified by yeast two-hybrid, several methods are available to further confirm the interaction in vitro and in vivo.

1. Pull-down assay to verify the interaction in vitro.
2. Co-immunoprecipitation to verify the interaction in mammalian cells.
3. Functional studies, such as transfection, to verify the biological relevance of the interaction.

Mapping Interaction Domains of Two Known Proteins

Another main use of yeast two-hybrid system is to identify interacting domains of two known proteins. We also examined the interaction domains of Smad1 and Hoxc-8 by the yeast two-hybrid technology described below.

1. Analyze sequence characteristics of two proteins to locate important structural or functional domains.
2. Make deleted forms (cDNA fragments) of each by polymerase chain reaction.
3. Clone each cDNA fragment into respective bait or prey vector.
4. Transform Y190/pGBT9/Smad1 with deletions of pACT2/Hoxc-8 or Y190/pACT2/ Hoxc-8 with deleted forms of pGBT9/Smad1.
5. Plate on SD/-Trp-Leu-His+3-AT (25 mM) plates and incubate at 30°C for 3-5 days.
6. Filter lift and liquid β-gal assay to access the strength of each pair of interacting domains.

Notes

1. After two rounds of screening for Smad1 interactor, we have obtained 25 positive clones. DNA sequence analysis found one clone as Hoxc-8 and two clones as Smad4. Smad4 is known to interact with Smad1, which provided a positive control for the system. The interaction between Smad1 and Hoxc-8 was confirmed by a pull-down assay in vitro and a co-immunoprecipitation in COS-1 cells (Shi et al. 1999) The interaction domains of the two proteins were mapped at the N-terminal domains of Smad1 and Homeodomain of Hoxc-8 (Yang et al. 2000).

2. Smads are signaling mediators and transcription factors, located in both cytoplasm and nucleus, which is convenient for the use of the two-hybrid assay in the yeast. However, some Smads might activate reporter gene transcription when fused to GAL4 DNA-binding domain; thus, the transactivation activity of Smads must be tested before using them as baits in the interactor hunt. If they activate reporter gene transcription, modification is required to remove the activation domains.
3. Transformation efficiency is critical to ensure all the possible target interactors are trapped. Generally, an efficiency of 10^4 transformants/μg DNA or greater is acceptable. In our practice, we used a two-step transformation by first generating a bait-carrying strain, then transforming it with the cDNA library plasmids. We usually get an efficiency of 10^5 or greater when performing a large-scale transformation. Two-step transformation sometimes can be a problem if the bait protein is toxic or introduces some growth advantage to the yeast host cells. If this is a concern, one can break the large-scale transformation into 5-10 small-scale ones to transform the bait and prey plasmids simultaneously. Small-scale transformation can sometimes give a drastically higher efficiency.
4. Y190 strain is very leaky for His3 expression, so 3-AT must be included when growing Y190 transformants on plates with appropriate SD medium. We found 45 mM 3-AT is required to eliminate the background growth. However, 3-AT is omitted when we grow liquid culture and lowered to 25 mM when we grow clones on plates for the β-gal assays. If desired, optimize the 3-AT concentration by growing Y190/pGBT9-bait transformants on SD/-Trp-His plates containing a range of 25-60

mM. Use the lowest concentration that allows only small (<1 mm in diameter) colonies to form after 1 week.

5. Y190 carries the *ade*2-101 mutation that confers a pink color to colonies and its colonies can grow to >2 mm in diameter. Spontaneous white mutants and smaller colonies may form at a rate of 1-2% (we found the rate is higher). Select pink colonies when inoculating cultures.
6. The production of β-gal can be used to assess the binding affinity of the bait and the prey proteins. However, the β-gal activity may not necessarily correlate with the in vivo strength of interaction. We have experienced difficulties when we tried to test the interaction of Hoxc-8 with Smad1 deletions. Positive interactions (confirmed by gel shift assays) activated only *His*3 gene transcription, but β-gal activity was only barely detectable by filter lift assays. The fusion proteins appear to be expressed at levels ranging from 50 nM to 1 μM (Phizicky and Fields 1995). At this concentration, it should be possible to detect very low affinity interactions, but when compared with other methods, the two-hybrid sometimes seems to be more sensitive and sometimes less. It is speculated that the folding of fusion bait and/or prey proteins can be somewhat distorted, causing steric hindrance to interfere with the interaction (Phizicky and Fields 1995).
7. Lastly, because the yeast two-hybrid uses the transcriptional activity of reporter genes as a readout, the interaction must take place in the nucleus. For membrane-bound proteins and proteins that normally do not enter the nucleus easily, the yeast two-hybrid system, as presented, may not be suitable. In some cases, a nuclear localiza-

tion signal can be fused in front of the bait gene, and/or the transmembrane domain can be removed to assist the target proteins in entering the nucleus.

References

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SUMMARY AND IMPLICATIONS

How growth factor-mediated signaling leads to changes in gene expression and ultimately to changes in cell fate or function is a fundamental question in biology. BMPs are potent early inducers of bone formation and cartilage development, as well as of bone reconstitution after injury. Recent genetic and molecular studies started unraveling the molecular basis for BMP-induced pattern formation, which strongly affects skeletal development. Regional signals and gradients of factors all provide the cues for proliferation and/or differentiation. However, even as specialized tissues emerge during development, undifferentiated precursors and stem cells must be preserved because stem cells continuously replenish normal tissues and allow repair of damage in the adults. The biological mechanisms by which cells are kept in an undifferentiated state stimulate intense intellectual curiosity.

The primary objective of this study was to further define the regulatory mechanisms that control the differentiation of MSCs into osteoblastic cells through Smad1-mediated BMP signaling. In this dissertation, my colleagues and I provide evidence for the mechanisms by which BMPs induce bone formation through transcriptional control. We show that Smad1, the BMP pathway-specific signaling transducer, activates OPN bone marker gene transcription through two major events. First, Smad1 associates with Hoxc-8, a repressor for OPN gene transcription, and dislodges the Hoxc-8 from its DNA element in the OPN promoter. Second, Smad1 directly binds to its cognate element adjacent to the Hoxc-8 binding site of the OPN gene. Furthermore, we

demonstrate that the NH2-terminal domains responsible for mediating the interaction between Smad1 and Hoxc-8, and DNA binding are sufficient to induce osteoblastic differentiation in cell culture.

In the first article, my colleagues and I have characterized the direct interaction between the nuclear transcription factor Hoxc-8 and the BMP signaling pathway-specific mediator Smad1 (Shi et al. 1999). We have identified one Hoxc-8 binding site (OPN-5), which spans nucleotides -206 to -180 in the OPN promoter. OPN is an important early marker gene for osteoblastic differentiation, and its mRNA is rapidly induced by BMPs (Ahrens et al. 1993; Cheifetz et al. 1996; Li et al. 1996). In addition, we show that Smad1 inhibits Hoxc-8 binding to the OPN Hox recognition site in a concentration-dependent manner. The Hoxc-8 binding element (HBE) confers a strong inhibition to the reporter gene trancription. Smad1 interacts with Hoxc-8 and inhibits the binding of Hoxc-8 to HBE. In addition, overexpression of Hoxc-8 abolishes the constitutively active BMP type I receptor-induced reporter gene transcription. Collectively, these data suggest that the Hoxc-8 act as a transcription repressor in the OPN gene. Here, we propose a model that Smad1, after BMP-induced translocation into the nucleus, interacts with Hoxc-8 and dislodges Hoxc-8 from its DNA binding element, resulting in the induction of gene expression (Fig. 1).

The second article of this study describes the mapping of the domains that are involved in the interaction of Smad1 and Hoxc-8. By deletional analysis in both yeast two-hybrid and gel shift assays, within the MH1 and linker regions of Smad1, my colleagues and I have identified 87 amino acid residues that interact with Hoxc-8, some of which bind to the homeodomain. Overexpression of these interaction domains of Smad1 effec-

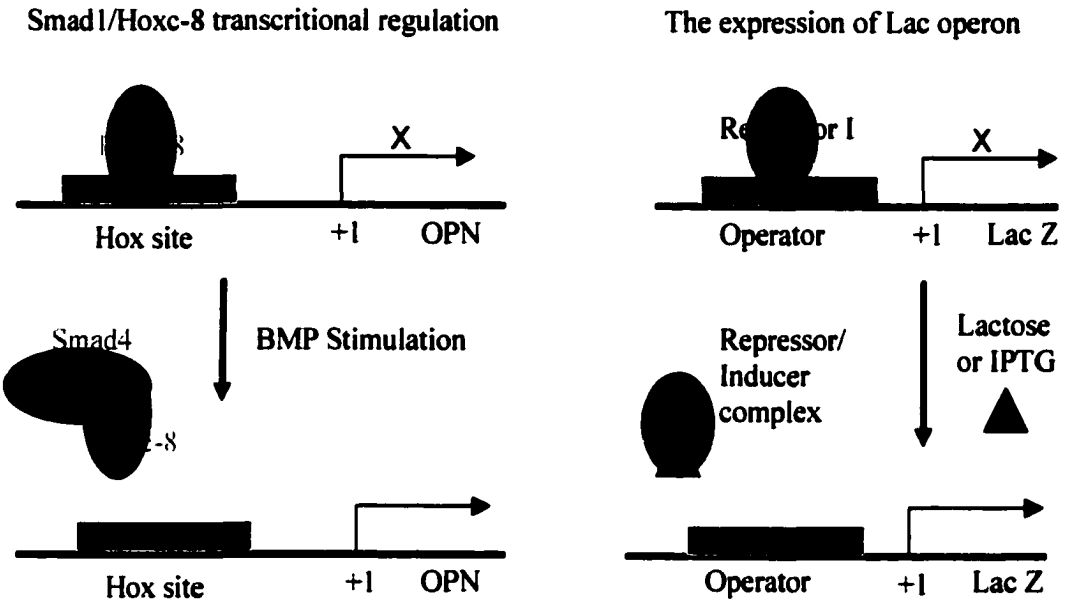

Figure 1. A model of BMP-induced activtion of OPN gene transcription. The left panel represents BMP type I receptors phosphorylating Smad1 after BMPs bind. The phosphorylated Smad1 forms a complex with Smad4, which then translocates into the nucleus, where it associates with Hoxc-8 and inhibits the latter from binding to the DNA. Because Hoxc-8 is a transcriptional repressor, the interaction between the Smad1/4 complex and Hoxc-8 releases the repression by Hoxc-8, resulting in activation of gene transcription. The right panel shows an analogy of the model with the classical Lac operon. Equivalent to Hoxc-8, the repressor I binds to DNA and represses LacZ gene transcription. Upon induction by lactose or IPTG, the repressor I binds to inducers and releases the repression of gene transcription, achieving activation of LacZ expression.

tively activates OPN gene transcription in C3H10T1/2 cells. We have also established cell lines that express the Hoxc-8 interacting domains (HIDs) permanently under tetracycline control. Our study demonstrates that HIDs of Smad1 in 2T3 osteoblastic precursor cells stimulate the expression of endogenous osteoblastic differentiation-related genes, including *Col* I and *opn*. Furthermore, the HIDs also stimulate ALP activity and mineralized bone matrix formation. Collectively, these data suggest that the interaction of HIDs of Smad1 with Hoxc-8 mimics BMP signaling and is sufficient to induce osteoblastic differentiation and bone cell formation. As for these findings, we have amended the first model as Fig. 2.

In addition to preventing the Hoxc-8 binding, I have also provided evidence that Smad1 stimulates OPN gene transcription by directly binding to the promoter. The third article in this dissertation describes a analysis of the 5'-flanking region of the OPN gene by gel shift assays to identify a Smad1-binding element (SBE). Smad1 specifically binds to a DNA fragment (nt -229 to –205) in the promoter, a DNA sequence immediately adjacent to the HBE. The MH1 domain of Smad1 directly participates in the binding to the SBE. Furthermore, overexpression of MH1-linker of Smad1 enhanced transcription of a SBE containing a luciferase reporter gene. The luciferase activity was increased further when the constitutive active BMP type I receptor ALK3Q233D and the common partner, Smad4, were co-transfected. These observations suggest that Smad1 plays a dual role in activating OPN gene transcription. By binding to Hoxc-8, Smad1 relieves the repression from Hoxc-8 caused by binding to its element, and by directly binding to SBE, it stimulates the gene leading to full activation of gene transcription.

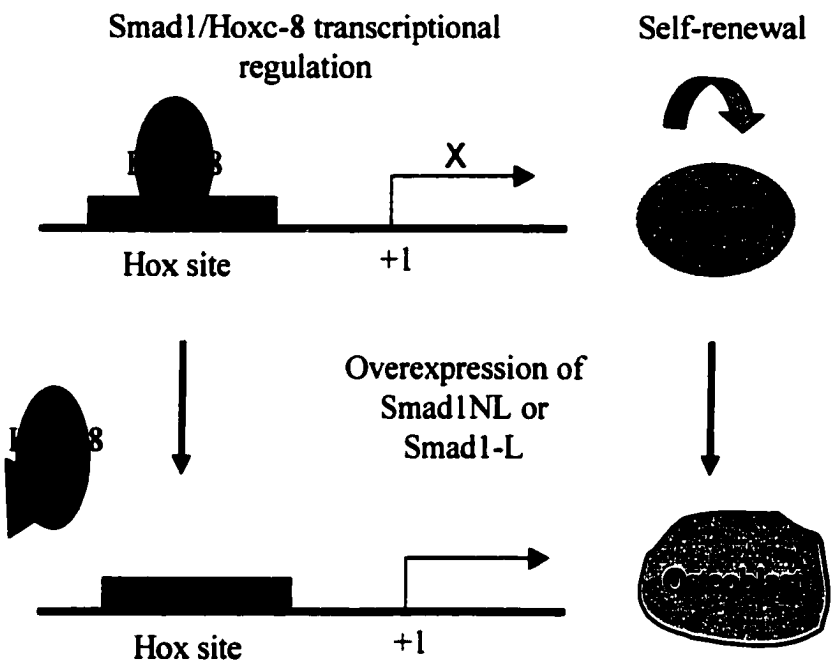

Figure 2. Overexpression of Smad1-NL or Smad1-L induces osteoblastic phenotype-related gene transcription and osteoblastic differentiation. Smad1-NL and Smad1-L mimic full-length Smad1's function, which associate with Hoxc-8 and release transcription repression by Hoxc-8. Smad1-NL contains both Hoxc-8 interaction domains (HID1 and HID2), and the Smad1-L contains only HID2 (see text for details).

Hox Genes are Indispensable in Skeletal Development

The discovery that Smad proteins act as a central link between membrane receptors and nucleus target genes has allowed us to trace an entire signaling pathway. This, in turn, increases our understanding of the regulatory mechanisms underlying target gene activation. Employing yeast two-hybrid technology, we first reported that Hoxc-8 and Hoxa-9 are the downstream interactors of Smad1 and Smad4 (Shi et al. 1999). Later studies of other groups have also shown that Hox proteins are the interacting molecules of Smad2 (Verschueren et al. 1999; Wotton et al. 1999).

Hox genes appeared early in evolution and increased in number through gene duplication (Krumlauf 1994). Homeobox genes are defined by the presence of a characteristic 183-base-pair DNA sequence (the homeobox) coding for 61 relatively conserved amino acids, termed the homeodomain (HD), which binds DNA containing ATTA cores with high affinity, thus acting as transcription regulators. In mammals, there are about 40 genes organized in four clusters, *Hoxa* to *Hoxd*, with up to 13 genes per cluster, named paralogs 1-13. The four Hox gene clusters are a highly conserved group of genes evolutionarily related to the *Drosophila* Antennapedia- and Bithorax-complexes. Each paralog has three to four members that are responsible for the morphogenesis of a particular embryonic domain or structure (Sharkey et al. 1997). Extensive analyses of the patterns of gene expression for members from all four of the hox clusters show that domains of gene expression are spatially restricted in different embryonic sites and axes. An important feature of these Hox complexes is the linear correlation between the position of a gene in a hox cluster and its relative antero-posterior or axial domain of expression in many embryonic tissues. This relationship, named "co-linearity", is believed to be one of the

mechanisms for the control of the antero-posterior axis of the embryo (Fig. 3; Von Allmen et al. 1996; Cohn et al. 1997).

The Hox genes are believed to function in the specification and interpretation of positional information in the embryo through the particular combination of genes that are expressed at any one regional level. This idea is supported by phenotypes arising from experimental perturbation of their expression in vertebrate embryos. Classical genetics and mutagenesis studies have shown that loss-of-function mutations of certain hox genes in mice cause anterior transformations in the axial skeleton, recognized by changes in vertebral morphology. Examples of such genes are listed in Table 1. Specifically, homozygous *hoxa*-2 mutant mice die at birth, and defects have been found in the branchial region of the head and homeotic transformation of second to first arch skeletal elements (Rijli et al. 1993). The *hoxb*-2 knockout mice also die within 24 hr of birth. A majority of these mice have severe sternal defects that compromised their ability to breathe. The sternal defects are similar to those previously reported for *hoxb*-4 mutant mice (Ramirez-Solis et al. 1993). All of the mutants show marked facial paralysis similar to, but more severe than, that of *hoxb*-1 mutant homozygotes (Goddard et al. 1996). Target disruption of the *hoxc*-9 gene reveals that anterior homeotic transformation occurred from the tenth thoracic vertebrae to the first lumbar vertebra with bending and fusion of the ribs (Suemori et al. 1995). The sternum shows an abnormal pattern of ossification. Phenotypes resembled those of the *hoxc*-8 mutant mice (Le Mouellic et al. 1992) and *hoxc*-6 mutant mice (Jegalian and De Robertis 1992).

Compound mutants in the group 4 hox genes, *hoxa*-4, *hoxb*-4, and *hoxd*-4, display clear alterations in regional identity, including a nearly complete transformation of the

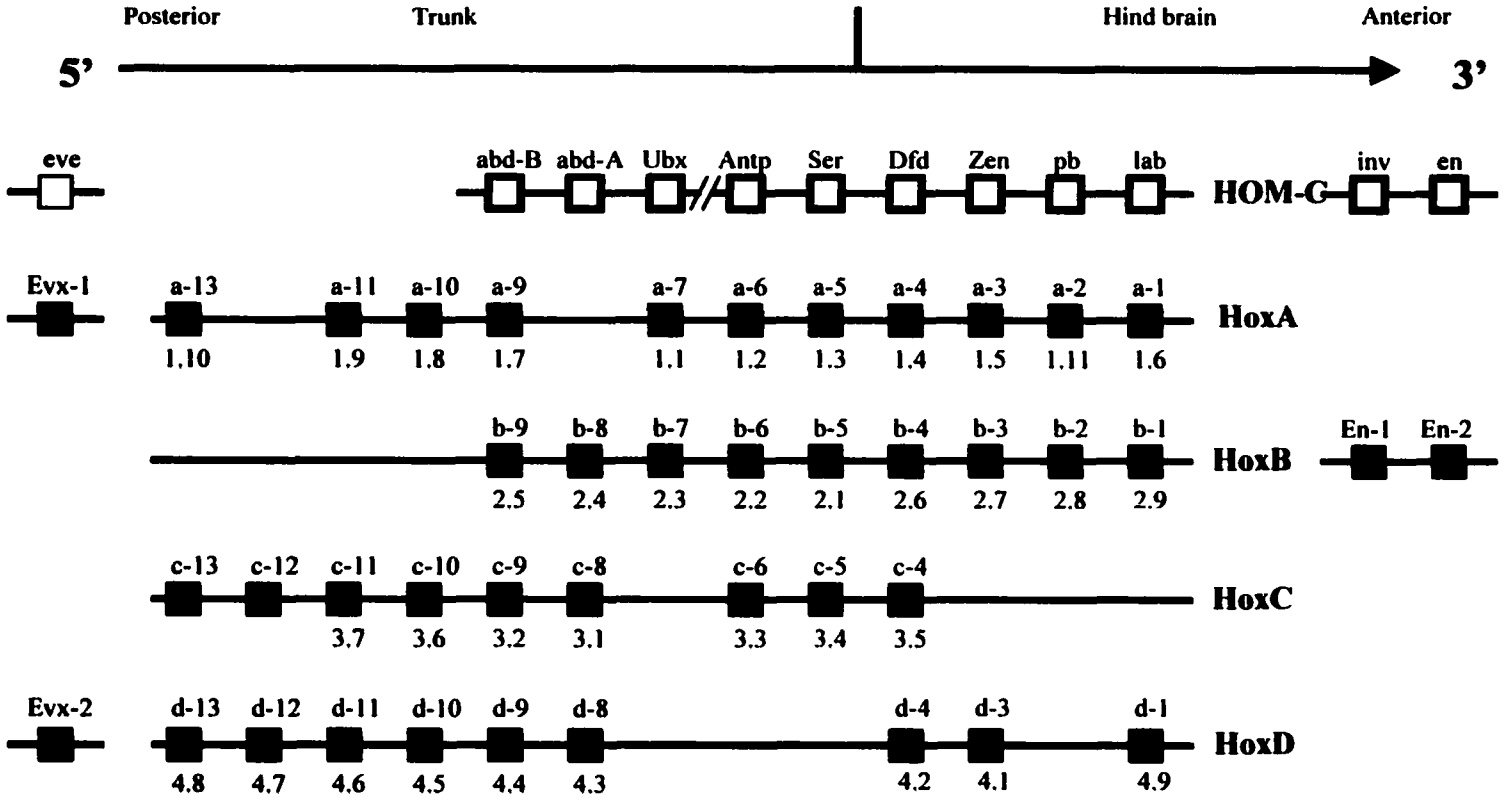

Figure 3. Homology between *Drosophila HOM-C* and mouse Hox gene clusters. The 38 mouse Hox genes are organinzed into four separate chromosomal clusters, named HoxA-HoxD, which are located in chromosome 6, 11, 15, and 2, respectively. Shaded boxes represent the 38 mouse Hox genes in the HoxA-D, clusters. They can be subdivided into 13 paralogs, and each paralogous group has 2-4 members (vertically aligned). Mammalian Hox genes are closely related to the *Drosophila* HOM-C genes (empty boxes). The expression of each gene in a particular cluster is colinear with respect to timing and position (from posterior to anterior showing as an arrow). The numbers at the bottom of each cluster are the old nomenclature (modified from Maconochie, 1996).

second to the first cervical vertebrae (Horan et al. 1995). Loss-of-function mutations in *hoxa*-1 and *hoxa*-3 produce effects on cranial and mesenchymal neural crests and cause changes in hind-brain segmentation (McKay et al. 1994). Mutations in *hoxa*-5 perturbed the specification of axial identity and reduced viability (Aubin et al. 1998), whereas mutations in *hoxd*-13 cause fusion of digits (Muragaki et al. 1996). Overexpression of *hoxd*-6 produces an extra digit in the chick wing (Morgan et al. 1992). Mice lacking *hoxa*-11 and *hoxd*-11 completely lack the radius and ulna (Davis et al. 1995). The available data strongly suggest that a functional interaction between Hox proteins is involved in segmental determination.

Functions of Hoxc-8

Hoxc-8 is one of the three members in paralog VIII and is predominantly expressed at a high level in limbs and backbone rudiments as shown by Northern hybridization studies (Simeone et al. 1987). The null mutation of *hoxc-8* has been generated in mice by substituting part of the coding region with the *lacZ* reporter gene in embryonic stem cells (Le Mouellic et al. 1992). The *hoxc*-8 null mice showed severe skeletal abnormalities between the seventh thoracic vertebra and the first lumbar vertebra. The most notable transformation was the attachment of the eighth pair of ribs to the sternum and the appearance of a pair of fourteenth ribs on the first lumbar vertebra, showing anterior transformation. The frequency of transformation correlated with both the level of expression of *hoxc*-8 in cells and the density of *hoxc*-8-expressing cells in each segment (Tiret et al. 1993). The expression of *hoxc-8* in chondrocytes has been shown by in situ hybridization (Yueh et al. 1998). Overexpression of a *hoxc*-8 transgene specifically in

skeletal tissue causes an accumulation of proliferating chondrocytes in the hypertrophy area, where mature chondrocytes should situate, and a reduction of maturation. The severity depends on the expression level of the transgene. These results suggest that Hoxc-8 continue to regulate skeletal development well beyond patterning in a tissue-specific manner, presumably by controlling the progression of cells along the chondrocyte differentiation pathway.

Other than its substantial function in normal cartilage and bone differentiation in the axial skeleton, Hoxc-8 may also play a crucial role in hematopoiesis. Hoxc-8 is expressed in the mouse hematopoietic organs, fetal liver, and adult bone marrow. The *hoxc*-8-null mice showed a significant reduction in the number of erythroid burst-forming units and in granulocyte/macrophage colony-forming units (Shimamoto et al. 1999). However, the hematopoietic cells from the homozygous animals exhibited normal expansion capability in a liquid culture system. These data suggest that the decreased number of progenitor cells may be due to a defect extrinsic to the hematopoietic cells. Additional reports also demonstrated that *hoxc*-8 is highly expressed in motoneurons within spinal cord segments C7 to T1 during embryogenesis. Mice deficient for the *hoxc*-8 gene showed a numerical deficit of motoneurons and an irreversible disorganization of motor pools caused by a specifically enhanced apoptosis in C7-T1 motoneurons (Tiret et al. 1998).

It has been hypothesized that hox genes within one paralog group would be responsible for the positional identity and morphogenesis of a particular embryonic domain or structure (Maconochie et al. 1996). Studies have shown that mutations in the same hox paralog genes exhibit a considerable degree of overlap in phenotype and may have a

common role. For example, the *hoxa*-11 and *hoxd*-11 products are functionally equivalent, and extra doses of *hoxd*-11 can rescue *hoxa*-11 loss of function (Zakany et al. 1996). Similarly, another member of the hox VIII group, *hoxb*-8, has been shown to activate the Sonic hedgehog gene, a key regulator of chondrocyte differentiation in forelimb development (Charite et al. 1994; Lu et al. 1997). Likewise, misexpression of *hoxd*-8, the third member of this hox VIII paralog, modifies *Drosophila* anterior head segment(s) (Bachiller et al. 1994). Taken together, these findings indicate that *hoxc*-8 is directly implicated in proliferation and differentiation of chondrocytes and osteoblasts.

BMPs Regulate Hox Gene Expression

Given the fact that both BMPs and Hox genes play a vital role in skeletal development, it is plausible that BMPs are regulators for hox gene expression. Available evidence has shown that hox genes also regulate BMP expression at the transcriptional level (Kim et al. 1998), suggesting an interplay between these two important regulators. BMPs act as inductive signals between germ layers in the embryo, and they regulate the expression of several transcription factors, including hox genes. *Bmp*-2 and *bmp*-4 are expressed in many regions of the developing embryo (Francis-West et al. 1995; Wall and Hogan 1995) and have been shown to be involved in many developmental processes including epithelial-mesenchymal interactions during tooth and limb development (Niswander and Martin 1993; Vainio et al. 1993). *Msx*-1 and *msx*-2, homologs of the *Drosophila* muscle segment homeobox gene, *msh* (Sassoon et al. 1989), are the HD-containing proteins encoded by different loci from the Hox gene clusters. Three different *msx* genes have been identified in mice (Shimeld et al. 1996), and two of them, *msx*-1 and

msx-2, have been found also in human (Jabs et al. 1993) and *Xenopus* (Su et al. 1991). In the mouse embryo, *bmp*-4 is expressed in the dental ectoderm during the early stages of tooth formation and induces *msx*-1 and *msx*-2 expression (Vainio et al. 1993). Overlapping with that of *bmp*-2 and *bmp*-4, the expression pattern of *msx*-1 and *msx*-2 is found in the developing dental mesenchyme, limb, neural tube, and craniofacial complex, especially in regions of epithelial-mesenchymal organogenesis. Later studies indicated that BMP-induced *msx*-1 and *msx*-2 gene expression is required for the dorsal parts of vertebratal cartilage differentiation (Monsoro-Burq et al. 1996). The timing of expression, embryonic distribution, and the function of *Xenopus* msx genes also parallels that of *bmp*-4 (Suzuki et al. 1997b), where Msx-1 induces ventralization and inhibits neural differentiation. A recent study demonstrated that msx genes are the direct targets of BMP signaling (Hollnagel et al. 1999).

By in situ hybridization, the co-expression and co-localization of BMP and Hox genes were also observed in many other instances. A recent study suggests that the expression of *tlx*, a homeobox gene expressed in the primitive streak of mouse embryos, is rapidly induced by exogenous BMP-2. Similar to the mutants of BMP-2, BMP-4, and ALK3 (BMP type I receptor), disruption of *tlx*-2 function leads to early embryonic lethality. The mutant embryos display severe defects in the primitive streak and in mesoderm formation (Tang et al. 1998). *Bmp*-11 is expressed in the primitive streak and tail bud regions, sites at which new mesodermal cells are generated. Homozygous mutant mice carrying a targeted deletion of *bmp*-11 exhibit posterior displacement of the hind limbs and anteriorly directed homeotic transformations throughout the axial skeleton. Mutant embryos show alterations in expression patterns of hox genes, including *hoxc*-6,

hoxc-8, *hoxc*-10, and *hoxc*-11, suggesting that *bmp*-11 acts upstream of the hox genes (McPherron et al. 1999). BMP-2 has been shown to induce ectopic *hoxa*-13 expression in the anterior region of the limb bud (Hashimoto et al. 1999). BMP-7 is able to induce the expression of *hoxa*-1 in pluripotent human embryonic carcinoma cells (Andrews et al. 1994). BMP-2 and BMP-4 have been shown to induce *Xhox*3 gene expression during early ventral-posterior mesoderm formation in *Xenopus* embryos (Hemmati-Brivanlou and Thomsen 1995).

The temporal and spatial correlation of the expression patterns of many BMP and Hox genes suggests that Hox genes act as downstream executors of BMP signaling in vivo. Genetic and in situ hybridization techniques have demonstrated that many Hox genes functionally overlap with BMP. BMP-4 is a ventralizing factor and an epidermal inducer of early *Xenopus* development. Inhibition of BMP-4 function in isolated ecto-dermal cells causes the formation of neural tissue. Local inhibition of BMP-4 function in whole embryos causes the formation of an additional dorsal axis. *Xom* is a homeobox gene whose expression pattern is similar to that of *bmp*-4 and whose expression requires BMP-4 signaling. Overexpression of *xom* causes a phenotype similar to that caused by overexpression of *bmp*-4, suggesting that this gene act downstream of BMP-4 to mediate its effects (Ladher et al. 1996). The functional overlapping of BMP-4 is also seen in some other *hox* genes. During gastrulation, *mix*.1, a paired class homeobox gene of *Xenopus*, is induced by and co-expressed with *bmp*-4 in the ventral region of the embryo. Genetic studies demonstrate that *mix*.1 is positioned downstream of the BMP-4 signaling cascade that participates in ventral patterning (Mead et al. 1996). The *hox*7.1 (*msx*-1)

gene is capable of rescuing neutralization imposed by dominant negative BMP receptors (Suzuki et al. 1997a).

Smads Interact with Hox Proteins

Smads were found to be universally required for TGF-β-like signal transduction by phenotypic complementary studies in *Drosophila* and *C. elegans* (Sekelsky et al. 1995; Savage et al. 1996) and by molecular identification of homologous components in distantly related organisms. The discovery of Smads as the TGF-β signaling transducers has made it possible to understand the events between signal initiation by ligand binding and downstream gene activation. In the past 5 years, intensive studies on the mechanism by which Smads transduce TGF-β and BMP-regulated gene transcription have led to the identification of many Smad-interacting nuclear transcription factors and their *cis*-acting DNA elements. Some of the Smad-interactors are Hox family members, including Hoxc-8 and Hoxa-9 (Shi et al. 1999), Smad1- and Smad4-binding proteins, and TGIF, a ubiquitously expressed homeodomain protein, a Smad2- and Smad3-binding protein.

Interaction Between Smad1 and Hoxc-8

The data presented in this study suggest both Smad1 and Smad4 interact with Hoxc-8 in mammalian cells with or without BMP stimulation. The constitutively active BMP type I receptor ALK3 Q233D does not enhance Smad1 and Smad4's interaction with Hoxc-8, indicating the interaction is not dependent upon phosphorylation of the Smads. In addition, data from gel shift assays demonstrate that both Smad1 and Smad4 inhibit Hoxc-8 binding to DNA. Because Smad4 is shared by both BMP and TGF-β

pathways, we questioned whether the interaction between Smad1 and Hoxc-8 is restricted to the BMP signaling pathway. In similar immunoprecipitation experiments, Smad2 and Smad3, the TGF-β pathway-restricted Smad proteins, were not seen to interact with Hoxc-8. Additionally, neither Smad2 nor Smad3 was able to inhibit Hoxc-8 binding to the DNA (Shi et al. 1999). These lines of evidence suggest that TGF-β/activin pathway-restricted Smads do not interact with Hoxc-8. Furthermore, evidence has shown Smad4 can only be translocated into the nucleus after the pathway-restricted Smads (R-Smads) are activated by ligand-induced phosphorylation. Given the fact that Hoxc-8 is located in the nucleus under physiological conditions, the interaction between Smads1 and 4 with Hoxc-8 presumably occurs only after the complex of Smad1/Smad4 is translocated into the nucleus upon BMP stimulation.

Based on our gel shift and transfection assays, we have also reported that Hoxc-8 acts as a transcription repressor to the OPN gene. Hoxc-8 binds to -206 to -180 of its promoter. When this element is linked to the SV40 promoter, the reporter expression is dramatically inhibited, whereas the mutated Hoxc-8 binding site does not have this effect. These data suggest that Hoxc-8 binding repress OPN gene transcription (Shi et al. 1999). Importantly, the Hoxc-8 binding sequence is conserved in pig, mouse, chicken, and human (Butler 1995), suggesting its biological significance. In addition, we show that the native OPN promoter is activated by co-overexpression of Smad1 and Smad4, and this activation is further enhanced in the presence of a constitutively active BMP receptor. Overexpression of Hoxc-8 suppressed OPN native promoter and SV40 promoter transcription, but this repression was absent when the Hoxc-8 binding site was mutated. Collectively, the first article of this dissertation demonstrates that through the OPN pro-

moter, Hoxc-8 represses gene transcription. BMP-activated Smad1 dissociates Hoxc-8 from its DNA element, which releases the repression leading to gene activation.

Smad1 Iinteracts with Other Hox Proteins

Hoxa-9, a proto-oncogene related to leukemia in human (Nakamura et al. 1996; Lawrence et al. 1997), also binds to the OPN promoter at the same Hoxc-8 recognition site (Article 1, Fig. 4). Hoxa-9 is expressed in limb buds and along the vertebral axis. *Hoxa*-9-deficient mutants show homeotic transformations corresponding to anteriorizations of vertebrae L1 to L5 in the lumbar region (Fromental-Ramain et al. 1996). Interestingly, both Smad1 and Smad4 inhibit Hoxa-9's binding to the DNA, with the complex of these two Smads inhibiting more profoundly (Article 1, Fig. 4). In agreement with this, transfection data also show that Hoxa-9 inhibits the transcription of reporter genes containing the Hoxc-8/Hoxa-9 binding site of the OPN promoter. Furthermore, overexpression of Hoxa-9 inhibits the TGF-β-induced OPN promoter activity (our unpublished data). All these data indicate that Hoxa-9 acts in a way similar to Hoxc-8 on the OPN gene as a transcriptional repressor.

Smad1 interacts with these two Hox proteins at this domain, possibly by binding the HD. Our mapping data indicate that Smad1 binds to the HD of Hoxc-8 (Article 2, Fig. 3), and the HD is the only region highly conserved between Hoxc-8 and Hoxa-9. It is also possible that Smad1 interacts with other Hox proteins involved in BMP signaling because that the HD is the region conserved among Hox proteins. Given this conservation, we attempted to determine the specificity of the interaction between Smad1 and other HD-containing proteins. Msx-1 and Msx-2 are shown to be transcription repressors

(Zhang et al. 1996; Newberry et al. 1998), and their expression is also coordinately regulated by BMP-2 and BMP-4. Interestingly, these two Msx proteins also bind to HBE identified from OPN gene, although with a lower affinity (unpublished data). Yet, in our gel shift analysis, none of them interacts with any of the tested Smads, including Smad1, -2, -3, and -4 (Article 1, Fig. 3). Consistently, the probe-M, the Msx-1/2 binding site, is unable to strip the OPN-5 probe off Hoxc-8 (Article 1, Fig. 2), whereas the Hoxc-8 also fails to bind the probe-M (data not shown). Apparently, Hoxc-8 binds to only one TAAT of the five TAAT/TTAT core sequences. The other four putative Hox sites in the OPN promoter may be involved in the binding of other homeobox proteins. These data suggest that the flanking nucleotides of the consensus core also contribute to the binding specificity of Hox proteins (Catron et al. 1993).

Specificity of Hox Gene Function

Each of the many known Hox proteins is expressed in a complex pattern during embryogenesis. As a result of overlap of these patterns, binding sites are exposed to a mixture of Hox proteins that varies in composition in a complex spatial-temporal fashion. A variety of experiments suggested that the minor differences in DNA binding specificity that distinguish Hox monomers in vitro have little influence on their ability to target different downstream genes (Hayashi and Scott 1990; Mann 1995). A paradox in our understanding of homeoprotein's function is that these proteins act with a high degree of specificity in development that is not easily explained by differences in DNA binding specificity.

All of the tested HD-containing proteins in our study, including Hoxc-8, Hoxa-9, Msx-1, and Msx-2, share the same recognition site. A critical question, therefore, concerns how the regulatory specificity of Hox proteins is determined. One possible mechanism invokes interactions of Hox proteins with other regulatory proteins (Pomerantz et al. 1992). The protein-protein contacts within these complexes are believed to include crucial interactions that allow Hox proteins to discriminate among target regulatory elements. The dimerization of homeoproteins generally results in a change in transcriptional activity, often mediated by changes in sequence specificity or degree of activation or repression (Tomei et al 1992; Wilson et al. 1993; Phelan and Featherstone 1997). For instance, the Dlx (distal-less *hox*) binds to Msx by forming a heterodimeric complex through their HDs, which mutually exclude each other's DNA binding activities. More interestingly, the transcriptional properties of Msx and Dlx proteins display reciprocal inhibition. Specifically, Msx-1 and -2 proteins act as transcriptional repressors (Zhang et al. 1996), and Dlx-2 and -5 proteins act as activators, whereas in combination, Msx and Dlx proteins counteract each other's transcriptional activities (Zhang et al. 1997). Hoxa-9 protein has been shown to physically interact with Meis1 proteins by forming heterodimeric binding complexes on a DNA target containing a Meis1 site, thus stabilizing the Meis1 binding to its DNA element (Shen et al. 1997). Although no Hoxc-8 partners that cooperatively bind to a DNA element have been reported thus far, we anticipate Hoxc-8 might use proteins other than Meis1, such as the Pax (paired hoxgene) family (Wilson et al. 1993).

Smad1/4 complex's interaction with Hox, but not with Msx, may reflect another level of control on the transcriptional activity and specificity of the HD-containing pro-

teins. Our studies suggests that the Smad1/4 complex interacts with Hox proteins and inhibits their binding to DNA. The formation of the complex of Smad1/4/Hox can occur in the absence DNA, as seen in the immunoprecipitation assays (Article 1, Fig. 2 and unpublished data). Nonetheless, Smad2 and Smad3 associate with none of the four Hox proteins despite their being closely related to Smad1 and Smad4 (Article 1, Fig. 3 and unpublished data). These data support the view that Hox proteins specifically interact with BMP pathway-restricted Smads. Similar actions of Smad1/4 also exist with other Hox proteins. As an example, Lhx2, a LIM-type homeoprotein, specifically interacts with Msx-1 in the absence of DNA. The interaction between Msx-1 and Lhx2 is mediated through the HDs of both proteins. Intriguingly, the Msx-Lhx complex is unable to bind DNA, suggesting that the functional specificity of Hox proteins in vivo can be determined by a balance between their association with DNA and their protein partners (Bendall et al. 1998).

Homeobox Proteins as Transcriptional Repressors

Repressors maintain genes in a transcriptionally inactive state, or specifically down-regulate a gene response in vital biological processes, such as development and the regulation of cell growth (Gray and Levine 1996). Some Hox proteins possess intrinsic repressor activity; they repress transcription without their partner proteins. It has been suggested that repression may be a general mode of action for Hox proteins, which may be required for maintaining cells in an undifferentiated state during development to prevent premature differentiation of precursor cells (Violette et al 1992; Catron et al. 1995; Schnabel and Abate-Shen 1996).

Here, we provide evidence that Hoxc-8 functions as a transcriptional repressor of the OPN gene. Transient transfections suggest that overexpression of Hoxc-8 suppresses the gene transcription that is induced by constitutively active BMP type I receptor. Endogenous Hox proteins that bind to the Hoxc-8 recognition site also suppress reporter gene expression (Article 1, Fig. 5). Other groups have shown that overexpression of Hoxc-8 in skeletal tissue results in an accumulation of progenitors in the hypertrophic area (Yueh et al. 1998). Thus, it is likely that the involvement of Hoxc-8 in both osteo- and chondrogenic processes is to prevent the switch from proliferation to differentiation. Several other mammalian Hox proteins, namely Hoxa-7, Hoxb-4, Hoxc-6, Msx-1, and Msx-2, are all found to repress gene transcription (Violette et al. 1992; Chariot et al. 1996; Schnabel and Abate-Shen 1996; Zhang et al. 1996). Msx-1 is a potent repressor of transcription and can function through both TATA-containing and TATA-less promoters.

It is likely that there are Hox co-repressors, which may contribute to the selection of Hox targets that are subject to repression (Pinsonneault et al. 1997; Li et al. 1999). One study suggests a dual function of human Hoxd-8. By interacting with Hoxd-9, it inhibits transcription of human Hoxd-9 gene promoter in cultured cells. When alone, however, Hoxd-8 or Hoxd-9 activates different target genes (Zappavigna et al. 1994). It seems that repression dominates over activation in the regulation of gene transcription when different Hox genes are co-expressed (Capovilla and Botas 1998). The dominance of repression over activation also exists in other Hox-interacting proteins. Specifically, Smad2 is recognized as a co-activator when interacting with p300/CREB-binding protein or AP-1 (Pouponnot et al. 1998; Shen et al. 1998) for the expression of most target genes identified thus far. But it acts as a co-repressor when it binds TGIF, a ubiquitously ex-

pressed HD-containing protein that binds to the RXR response element (Bertolino et al. 1995; Wotton et al. 1999).

The mechanism by which Hox proteins suppress gene transcription is not well defined. Some studies in a purified reconstituted assay system revealed that Msx-1 interacts with protein complexes composed of TBP and TFIIA and of TBP, TFIIA, and TFIIB, suggesting that the mechanism of repression is mediated through interaction(s) with a component(s) of the core transcription complex (Catron et al. 1995; Zhang et al. 1996). Another well-accepted explanation of repression is that the repressors interact with histone deacetylase complexes (HDACs), which stabilize and maintain transcriptionally repressed states (Kuo and Allis 1998). The Smad2/4 complex has been shown to interact with HDAC1 and also to interact with TGIF, conferring gene transcription repression (Wotton et al. 1999). We also have indications that Smad6 may act as a corepressor of Hoxc-8 for OPN gene transcription (unpublished data). Whether the respression of the Hoxc-8/Smad6 complex is mediated by the HDAC is under active investigation.

Interaction of Smad1/Hoxc-8 Induces Osteoblastic Differentation

The unique capacity of bone for complete structural and functional renewal depends on the presence of the MSCs that reside predominantly in the bone marrow. The expression of Hoxc-8 in adult animals becomes restricted to limited areas including the bone marrow, which contains both mesenchymal and hematopoietic stem cells (Shimamoto et al. 1999). Using reverse transcriptase-polymerase chain reaction, we detected the expression of Hoxc-8 in the multipotent mesenchymal C3H10T1/2 cells and in the human bone marrow stromal cells (data not shown). Both C3H10T1/2 and bone mar-

row stromal cells have been shown to proliferate rapidly and differentiate into osteoblasts in response to BMP stimulation. The pattern of Hoxc-8 expression at the different stages osteoblastic lineage differentiation remains to be determined.

The exact pathway of BMP-induced patterns and molecular regulation of mesenchymal cell lineage differentiation, especially in its early stage, is still obscure. Our study suggests that Hoxc-8 play a role in maintaining the undifferentiated state of the MSCs. The findings in this study provide evidence that the interaction between Smad1 and Hoxc-8 is an important mechanism for BMP signaling leading to the induction of osteoblastic differentiation. Unlike other negative regulators that shut down BMP signaling, Hoxc-8 seems to be submissive to BMP stimulation. That is, when the HIDs of Smad1 are overexpressed, the inhibition of Hoxc-8 on OPN gene transcription is relieved (Article 2, Fig. 4). This derepression can include an entire program of osteoblastic differentiation. This conclusion is based on several lines of evidence. First, the Hoxc-8 interaction domains of Smad1, namely HID1 (amino acid (aa) 101to145) and HID2 (aa 148 to 191), imitate full-length Smad1 and are sufficient to inhibit the full-length Hoxc-8 DNA binding activity. HID1 is also able to inhibit the HD of Hoxc-8 binding to the DNA. Secondly, transfection assays show that Smad1-M, the minimal region (85 aa in length) of Smad1 containing only these two HIDs, stimulates OPN reporter gene transcription in a dose-dependent manner. Since most of Smad1 is absent in this construct, we infer that gene transcription activation results from the derepression of Hoxc-8 by the HIDs of Smad1. Thirdly, Smad1-M also effectively enhances endogenous OPN and Col I gene transcription, as seen in Northern blots (Article 2, Fig. 5). In addition, overexpression of the Smad1-M is sufficient to drive the 2T3 osteoblastic precursor cells to differ-

entiate into mature osteoblasts, as indicated by increased ALP activity and formation of positive mineralized matrix in the Smad1-M-expressing cell lines (Article 2, Fig. 5). Taken together, these data suggest that the HIDs are sufficient to bind endogenous Hoxc-8 that otherwise would bind to the DNA element, resulting in derepression, which eventually leads to the terminal differentiation of osteoblasts.

Moreover, Smad1 binds to an element adjacent to the Hoxc-8 binding site in the OPN gene, and this binding site confers a transcriptional activation of the reporter gene (Article 3, Fig. 4). Thus, it is likely that a full activation of OPN gene transcription in vivo results from a dual action of endogenous Smad1. By interacting with Hoxc-8, Smad1 removes Hoxc-8 from its binding site, leading to a partial activation of the gene transcription. Meanwhile, by binding to the SBE, Smad1 may interact with other transcription factors to induce a full activation of the OPN gene expression. Although we have not been able to shown the action of endogenous Smad1 on OPN gene transcription, our study demonstrates that overexprssion of Smad1-M alone is sufficient to induce bone cell differentiation in the absence of BMP stimulation. Presumably, the induction of bone cell differentiation does not involve the Smad1's DNA binding activity because the inactive (unphosphorylated) form of Smad1 is not able to move into the nucleus. However, it is possible that 2T3 cells synthesize a certain level of BMP under the culture conditions, which may activate the endogenous Smad1 and allow a certain degree of its nuclear translocation. Therefore, the binding of Smad1 to the DNA may also contribute to the induction of osteoblastic differentiation. Further studies are certainly needed to verify this speculation.

Future Work

Besides inducing bone and cartilage formation in vivo, BMP-2 plays an important role in the induction of the undifferentiated mesenchymal progenitors to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro (Katagiri et al. 1990; Ahrens et al. 1993). BMP-2 also inhibits myogenic cells from differentiating into myotubes (Yamaguchi 1995). Given the fact that both BMPs and Hox genes play a fundamental role in directing cell fate, Smad-mediated BMP signaling through an interaction with Hox proteins might also be involved in some of the above processes. Progress over the past several years provides a framework for new questions and opportunities to analyze what specific combination of BMP receptors, Smads, and associated proteins leads to specific responses. Our findings revealed that Hoxc-8 acts as a downstream transcription factor that may play an important role in the control of undifferentiated MSCs. Upon BMP signaling, activated Smad1 moves into the nucleus where it binds to Hoxc-8 and removes it from its DNA element. This removal initiates a program of cascade events, including activation of many osteoblastic phenotype-related genes that are responsible for lineage commitment. Smad1 also directly binds to DNA and induces the transcription of osteoblastic marker genes. It is anticipated that Smad1's DNA binding activity is also involved in the induction of osteoblastic differentiation.

By defining roles that BMPs play in developmental biology and bone regeneration, significant progress has been made in identifying cell-signaling molecules and their regulators. Exploiting BMPs and Smads may generate new therapeutic options for bone repair. Our future work will focus on identifying a carrier/delivery system, a recognized challenge in the field. By collaborating with outside pharmaceutical companies, we are

actively investigating the potential of a retroviral system to deliver Hoxc-8 and the Hoxc-8 interaction domains of Smad1 for their expression in primary cells to examine the effects on human osteoblastic differentiation. We also plan to generate transgenic mice with these constructs to further define the function of Smad1 fragments that interact with Hoxc-8 in vivo.

BMPs induce bone formation postnatally, and they have therapeutic potential in reparative osteogenesis and odontogenesis. Implicit in our search for a better understanding of MSCs in mammals, particularly in humans, is the potential for application within clinical medicine. If MSCs can be grown to infinite numbers in culture, allowing for ex vivo genetic manipulation and lasting gene repair through homologous recombination, and if MSCs can be reimplanted in vivo to undergo normal differentiation, then some genetic diseases could be permanently alleviated. This will be the long-term practical goal of research into MSCs.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

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Graduate Program Pathology

Title of Dissertation Bone morphogenetic proteins induce gene transcription and osteoblastic differentiation through the interaction between Smad1 and Hoxc-8

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that _he may be recommended for the degree of Doctor of Philosophy.

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