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A role of TGFß/BMP in leukemogenesis through interaction between Smad4 and Hoxa9

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A ROLE OF TGFβ/BMP IN LEUKEMOGENESIS THROUGH INTERACTION BETWEEN SMAD4 AND HOXA9

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Hematopoiesis is tightly regulated by the continuous proliferation and differentiation of hematopoietic cells. Understanding the molecular basis for the regulation of hematopoiesis may help to elucidate the pathogenesis of hematological disorders such as leukemia and to identify new therapeutic targets.

Transforming growth factor β (TGFβ) is a crucial regulator of hematopoiesis. However, the molecular mechanism of how TGFβ modulates hematopoiesis is not well characterized. In this dissertation, the studies demonstrate that TGFβ/bone morphogenetic protein (BMP) regulates the bone marrow transformation capability of Hoxa9 and Nup98-Hoxa9 through Smad4 and show how Smad4 mutations identified in human acute myeloid leukemia patients may affect its normal function.

Smad4 directly interacts with the homeodomain of Hoxa9, which is conserved in all 39 homeobox (Hox) proteins and functions as their DNA-binding domain. This interaction blocks the DNA-binding ability of Nup98-Hoxa9 and, thus, suppresses its downstream gene transcription. Mapping data revealed that the amino-terminus of Smad4 is responsible for this interaction. Overexpression of this Hoxa9 interaction domain of Smad4 was sufficient to inhibit the leukemic transformation capability of Nup98-Hoxa9. Thus, our studies establish a novel mechanism by which TGFβ/BMP regulates hemato-

poiesis and raise the possibility that Hox DNA-binding activity may serve as a novel therapeutic intervention for those leukemias that involve Hox deregulations.

In addition, Smad4 mutants exhibited significantly decreased protein stability. Importantly, we found that F-box protein β-transducin-repeat-containing protein 1 (β-TrCP1) in Skp1-cullin-F box protein (SCF) E3 ligase interacts with Smad4, and exhibits stronger interaction affinity with the acute myelogenous leukemia-derived Smad4 mutants. Consequently, E3 ligase complex $SCF^{\beta\text{-}TrCP1}$ mediates ubiquitination of Smad4 mutants. When small interference RNA (siRNA)-induced F-box protein β-TrCP1 gene silencing was used, the protein steady-state level of Smad4 was elevated in acute myelogenous leukemia cells.

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LIST OF ABBREVIATIONS

A	adenine
AML	acute myeloid leukemia
BEX	blue-excited green fluorescence protein variant
BME	β-mercaptoethanol
BMP	bone morphogenetic protein
C	cytosine
CBF	core-binding factor
CBP	cyclic-adenosine monophosphate-responsive element-binding protein binding protein
CDK	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CHIP	carboxyl-terminus of Hsc70-interacting protein
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
Co-Smad	common partner Smad
CREB	cyclic-adenosine monophosphate-responsive element binding protein
DMEM	Dulbecco's modification of Eagle medium
DNA	deoxyribonucleic acid

Ecto	ectodermin
EMSA	electrophoretic mobility shift assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
G	guanine
GFP	green fluorescent protein
GST	glutathione S transferase
Hox	homeobox
HSC	hematopoietic stem cell
I-Smad	inhibitory Smad
IL	interleukin
IRES	internal ribosome entry site
MAP	mitogen-activated protein
MCR	mutation cluster region
MPD	myeloproliferative disease
mRNA	messenger ribonucleic acid
MSCV	murine stem cell virus
NK	natural killer
NLS	nuclear localization signal
OPG	osteoprotegerin
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PKC	protein kinase C
PML	promyelocytic leukemia
R-Smad	receptor-regulated Smad
RT-PCR	reverse transcription-polymerase chain reaction
SARA	Smad anchor for receptor activation
SCF	Skp1-cullin-F box protein
SDS	sodium dodecyl sulphate
Smurf	Smad-ubiquitin-regulatory factor
siRNA	small interference RNA
siSmad4	glutathione S transferase
T	thymine
TALE	3-amino acid loop extension
TGFβ	transforming growth factor β
VEX	violet-excited green fluorescence protein variant
WT	wild-type

INTRODUCTION

Hematopoiesis

Hematopoiesis, the process by which mature blood cells of distinct lineages are produced from pluripotent hematopoietic stem cells (HSCs) that reside in the bone marrow, is a highly orchestrated process (Kondo *et al*, 2003; Szilvassy, 2003) (Figure 1). These cells insure the production of blood cells that function in gas exchange, cellular and humoral immunity and homeostasis throughout one's life. Two fundamental characteristics define these cells (Kondo *et al*, 2003; Reya, 2003). First, they can generate more HSCs, a process called self-renewal. Second, they have the potential to differentiate into various progenitor cells that eventually commit to further maturation along specific pathways. The end result of these events is the continuous production of sufficient but not excessive numbers of cells of all lineages.

HSCs differentiate along two major pathways: the lymphoid pathway and the myeloid pathway (Kondo *et al*, 2003). The differentiation process of each pathway originates from a common progenitor cell, the common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). CLPs terminally give rise to lymphocytes, including B cells, T cells and natural killer (NK) cells. CMPs produce granulocytes, monocytes, megakaryocytes, and erythrocytes. Granulocytes include neutrophils, eosinophils and basophils. Monocytes are precursors of tissue macrophages and osteoclasts. Megakaryocytes secrete platelets. Erythrocytes remain within the blood vessels and transport O_2 and

CO_2 bound to hemoglobin. Granulocytes, monocytes and lymphocytes, together called white blood cells, combat infection, phagocytose and digest debris.

The study of hematopoiesis is greatly facilitated by the characterization of cell-surface markers presented on hematopoietic cells. A certain combination of surface markers is used to identify the lineage or stage of maturation of certain hematopoietic cells. For example, c-Kit, a tyrosine kinase receptor for stem cell factor (SCF), is highly expressed by early progenitors such as HSCs (Ikuta and Weissman, 1992). CD34, a glycoprotein that is expressed on endothelial and some hematopoietic cells, functions in cell adhesion and is often used as a marker of HSCs (Sutherland *et al*, 1989; Krause *et al*, 1994; Morel *et al*, 1996). Gr-1 and Mac-1 are expressed by the myeloid lineage in a developmentally regulated manner in the bone marrow and are used to monitor myeloid cells (Springer *et al*, 1979; Fleming *et al*, 1993).

Regulation of Hematopoiesis

The regulation of hematopoiesis is a complex process that is an orchestrated process incorporating effects from both external cytokines and internal transcription factors. The fine tuning between the proliferation and differentiation of hematopoietic stem/progenitor cells is critical for normal blood hematopoiesis.

Transcription Factors

Recent studies of regulation of normal genes, as well as the study of leukemias, have suggested that transcription factors play a major role in both myeloid differentiation and leukemogenesis.

PU.1. PU.1 is a member of the Ets transcription family (Klemsz *et al*, 1990). Ets factors all contain a characteristic DNA-binding domain of approximately 80 amino acids (Karim *et al*, 1990; Wasylyk *et al*, 1993). The amino-terminus of PU.1 protein contains an activation domain that has been implicated in interactions with other regulatory proteins (Klemsz *et al*, 1990; Hagemeier *et al*, 1993; Klemsz and Maki, 1996). The expression of PU.1 is restricted to myeloid and B cells (Klemsz *et al*, 1990; Hromas *et al*, 1993). During hematopoietic development, PU.1 mRNA is expressed at low levels in human CD34+ stem cells and is specifically upregulated during myeloid development (Cheng *et al*, 1996). Thus, these expression studies suggest that regulation of PU.1 mRNA may play a significant role in the commitment of early multipotent progenitors to the myeloid lineages, as well as in the further differentiation and maturation of these cells. PU.1 is positively autoregulated (Oka *et al*, 1991). Such autoregulation of PU.1 may play a major role in irreversible commitment and differentiation of hematopoietic multipotent progenitors. Mice with targeted disruption of PU.1 demonstrated different levels of hematopoietic defects, especially in myeloid lineages (Scott *et al*, 1994; McKercher *et al*, 1996).

GATA-1. GATA-1 is a zinc-finger protein that interacts with PU.1 (Rekhtman *et al*, 1999; Nerlov *et al*, 2000). After high levels of PU.1 commit hematopoietic progenitors to a myeloid fate, GATA-1 is thought to modify the DNA-binding properties of PU.1, allowing the cells that express it to become erythrocytes or megakaryocytes (McDevitt *et al*, 1997). These observations are confirmed by the phenomenon that overexpression of GATA-1 will cause hematopoietic progenitor cells to reprogram to three lineages: erythrocytes, eosinophils and megakaryocytes (Kulessa *et al*, 1995).

C/EBPα. C/EBPα is a member of one of several leucine zipper transcription factor families, including the fos/Jun and ATF/CREB family (Johnson *et al*, 1987; Landschulz *et al*, 1988). Although C/EBPα has been detected in some other cells such as adipocytes, expression of C/EBPα in the hematopoietic system may be limited to myeloid cells (Scott *et al*, 1992). Heterozygous knockout mice of the C/BEPα gene are normal, but homozygous mice die within the first few hours after birth (Wang *et al*, 1995; Flodby *et al*, 1996). Analysis of the hematopoietic system in embryonic and newborn mice has demonstrated a significant defect in production of granulocytic cells, suggesting that C/EBPα is a crucial regulator of granulocytic development.

Arabidopsis Mei2-like 1 (*AML1*). AML1 is a member of the core binding factor (CBF) family of transcription factors (Levanon *et al*, 1994; Tanaka *et al*, 1995; Takahashi *et al*, 1995). AML1, also known as Runx1 and CBFA2, is expressed in hematopoietic tissues and during myeloid development (Miyoshi *et al*, 1995; Cheng *et al*, 1996). AML1 knockout mice show no hematopoietic defect during embryonic development, but severe impairment of definitive hematopoiesis was observed in knockout animals (Okuda *et al*, 1996; Wang *et al*, 1996). AML1 was identified by studying one of the most frequent chromosomal translocations found in human AML, t (8; 21), which produces the fusion oncogene, AML1-ETO (Downing, 1999).

Homeobox (Hox) proteins. Hox proteins are crucial regulators of hematopoiesis and will be discussed in detail later.

Cytokines

Hematopoiesis takes place primarily in bone marrow, where hematopoietic stem/progenitor cells are exposed to different cytokines and growth factors (Zhu and Emerson, 2002). Stromal cells, including specialized fibroblasts, endothelial cells, osteoblasts and perhaps adipocytes, secrete a variety of cytokines. Stem cell factors (SCF), TPO, Flt3L and GM-CSF are all produced from within this stromal compartment, primarily from a small, highly proliferative subset of stromal fibroblasts that display cell surface markers of smooth muscle cells, as well. These cytokines regulate the proliferation and lineage commitment of hematopoietic stem/progenitor cells.

Among the various cytokines identified, members of the transforming growth factor β (TGFβ) superfamily, including TGFβs, bone morphogenetic proteins (BMPs) and activins, emerged as key cytokines in recent studies.

Acute Myeloid Leukemia (AML)

Pathology

There are two major types of leukemia: lymphocytic leukemia, which involves lymphoid-committed cells that form and mature in the lymphatic system, and myelogenous leukemia, which affects myeloid-committed cells that form and mature in the bone marrow. Each of these types can occur in either the acute or chronic form. While the chronic form involves more mature cells that have stopped dividing or do so at a relatively slow rate, the acute form affects young cells still involved in the growth process that divide quickly and hasten the progress of the disease (Gilliland and Tallman, 2002). Myeloid leukemia develops from a chronic phase to an acute phase after latency or a relapse from treatment for

chronic myeloid leukemia (CML). Eighty percent of adult patients with acute leukemia have an AML form (Stone *et al*, 2004). AML is chiefly an adult disease with a median age at presentation of 50 years. The rate of incidence increases with age. AML represents a group of clonal HSC disorders that result in accumulation of nonfunctional immature cells termed myeloblasts. Infiltration of myeloblasts to spleen, liver and brain will cause other pathological disorders that originate from AML. Only about one-third of those between ages 18–60 who are diagnosed with AML can be cured; disease-free survival is rare, and current therapy is devastating in older adults.

Molecular Genetics of AML

In contrast to CML, which is caused by constitutively activated tyrosine kinases such as BCR/ABL that confer a proliferative and survival advantage to normal hematopoietic progenitors but do not affect differentiation, AML is frequently associated with balanced reciprocal translocations that involve transcription factors as one or both of the fusion partners (Dash and Gilliland, 2001). Cloning of these translocation break points has provided important insights into the pathogenesis of disease, as well as novel therapeutic approaches. Chromosomal translocations in AML most often result in gain-of-function mutations where transcription factors that are required for normal hematopoietic development become constitutively active. For example, t (8; 11) translocation gives rise to the AML1-ETO fusion protein. However, there is also expression of the normal AML1 gene from the unaffected chromosome 21. There is a large body of evidence that supports the hypothesis that the AML-ETO fusion protein is a dominant negative inhibitor of the AML1 gene (Frank *et al*, 1995; Meyers *et al*, 1995; Takahashi *et al*, 1995).

These mutations in transcription factors that impair the differentiation program of hematopoietic stem/progenitor cells, however, are not sufficient to cause AML (Dash and Gilliland, 2001). Activating mutations in the hematopoietic tyrosine kinases FLT3 and c-KIT and in N-RAS and K-RAS confer proliferative advantage to hematopoietic progenitors and cooperate with other mutations in hematopoietic transcription factors to cause an acute leukemia phenotype characterized by proliferation and impaired differentiation. This concept is suggested by the studies where transplanted animals that receive oncogene-transduced bone marrow cells usually need a long latency for the overt onset of AML, during which a second genetic change may occur. Co-expression of these two types of oncogenes can greatly shorten the latency and accelerate the onset of AML (Dash *et al*, 2002; Schessl *et al*, 2005). Disease progression from CML to AML provides indirect support for this hypothesis, as both types of mutations are detected simultaneously in clinical patients at the blast crisis phase of CML (Look, 1997; Rowley, 1999).

TGFβs and BMPs in the TGFβ Superfamily

The TGFβ superfamily is comprised of TGFβs, BMPs, activins and related proteins (Massague, 1990; Derynck and Zhang, 2003). These secreted proteins are considered pleiotropic factors because they have been shown to play a regulatory role in most processes linked to the control of somatic tissue development and renewal (Massague, 1990). Evidence suggests that TGFβ/BMP play a key role in hematopoietic stem/progenitor cell quiescence, proliferation and differentiation (Fortunel *et al*, 2000; Kim and Letterio, 2003). In this dissertation, I focus on the regulation of hematopoiesis by TGFβ/BMP.

TGFβ/BMP Signaling Pathway

Three similar isoforms of TGFβ, called TGFβ1, -β2, and -β3, were identified and cloned from mammals (Derynck *et al*, 1985; de Martin *et al*, 1987; Ten Dijke *et al*, 1988). All three isoforms of TGFβ are involved in the regulation of hematopoiesis. There are more subtypes of BMP have been identified recently. Each subtype of BMP may have distinct physiological functions that are different from those of other subtypes.

The functional complex of TGFβ family receptors at the cell surface consists of two type II and two type I transmembrane serine/threonine kinase receptors (Derynck and Feng, 1997; Itoh *et al*, 2000; Massague, 2000) (Figure 2). Type I receptors have a characteristic Gly-Ser (GS) sequence upstream from the kinase domain. In the absence of ligand, type II and type I receptors exist as homodimers at the cell surface. Upon ligand binding, type II and type I receptors form a heterodimeric complex, inside of which constitutively activated type II receptor activates the type I receptor through phosphorylation at its GS domain. In mammals, five type II receptors and seven type I receptors have been identified. Their functional interactions with various ligands in the TGFβ superfamily control the specificity of downstream effects in a variety of cell types.

The intracellular TGFβ signaling pathway involves the Smad protein family as substrates for the signaling receptors (Derynck and Feng, 1997; Itoh *et al*, 2000; Massague, 2000; Moustakas *et al*, 2001). There are three subclasses of Smad proteins that are distinguished by distinct functions in TGFβ signal transduction. The first class of Smads is called receptor-activated Smads (R-Smads). R-Smads are directly phosphorylated by activated TβR-I. While Smad1, -5 and -8 are specific for the BMP signaling pathway, Smad2 and -3 are TGFβ-specific R-Smads. Upon phosphorylation, R-Smads interact with com-

mon partner Smad (Co-Smad), Smad4, with which they form a heterodimer and translocate into the nucleus, where they regulate target gene expression either by directly binding to a specific promoter sequence or by further interacting with other cofactors. Smad6 and Smad7 belong to the third subclass of Smads, which is called inhibitory Smads (I-Smads) (Hata *et al*, 1998; Hanyu *et al*, 2001). Smad7 inhibits signaling by both TGFβ and BMPs, whereas Smad6 inhibits TGFβ signals less effectively (Itoh *et al*, 2000; Massague, 2000; Moustakas *et al*, 2001). The expressions of I-Smads are inducible, representing an auto-inhibitory feedback mechanism for the signaling pathway. Smad6 and Smad7 inhibit TGFβ family signaling by forming stable associations with their activated type I receptors, blocking phosphorylation of receptor-regulated Smads in the cytoplasm. Smad6 also interferes with the heterodimerization of BMP-activated Smads with Smad4, preventing the formation of an effector Smad complex. In addition, recruitment of a complex of Smad7 with Smad-ubiquitin-regulatory factor 1 (Smurf1) or Smurf2 to the type I TGFβ receptor results in the ubiquitylation and degradation of the receptor (Kavsak *et al*, 2000; Ebisawa *et al*, 2001).

Ubiquitin-Proteosome Degradation of Smads

Proper TGFβ signaling requires precise control of Smad functions. Smurf1 and Smurf 2 antagonize TGFβ signaling by interacting with R-Smads and targeting them for degradation (Arora and Warrior, 2001). Smurf1 is more specific for BMP signaling, as evidenced by the fact that Smurf preferentially interacts with Smad1 and Smad5 (Zhu *et al*, 1999). Smurf2 interacts broadly with R-Smads, enabling it to suppress signaling from nearly all TGFβ superfamily members (Zhang *et al*, 2001). Smad4, the central mediator of

all TGFβ superfamily signaling, is also subject to ubiquitin-proteasome degradation through a different mechanism. Smad4 was shown to interact with Jab1, which was initially identified as a co-activator of c-Jun, and it also induces degradation of cell cycle inhibitor p27 and tumor suppressor p53 (Wan *et al*, 2002). This interaction induces ubiquitylation and degradation of Smad4 through the 26S ubiquitin-proteosome pathway. Ectodermin (Ecto) was also identified as a RING-type ubiquitin ligase for Smad4 (Dupont *et al*, 2005). Depletion of Ecto in human cells enforces TGFβ-induced cytostasis and, moreover, plays a causal role in limiting the antimitogenic effects of Smad4 in tumor cells. CHIP (carboxyl-terminus of Hsc70-interacting protein), which was previously shown as a cochaperon protein and a U-box-dependent E3 ligase, interacts with Smad1 and Smad4, and expression of the CHIP protein suppresses the activation of the Smad-mediated signaling pathways through degradation of Smad4 (Li *et al*, 2004). Suppression of CHIP expression by siRNA significantly enhanced Smad1/Smad4- or BMP-induced gene transcription *in vitro*.

The Role of TGFβ/BMP in Hematopoiesis

TGFβ is one of the most potent endogenous negative regulators of hematopoiesis (Kim and Letterio, 2003). TGFβ plays an important role in regulating the balance between proliferation and differentiation in hematopoietic cells. TGFβ activities are orchestrated by a mixture of soluble factors and matrix-associated proteins, and the effects of those activities are often context and stage dependent (Keller *et al*, 1991; Fortunel *et al*, 2000). TGFβ1 is known to influence both the proliferation and differentiation of the uncommitted stem cell precursors and of myeloid progenitors by regulating the response of progenitors

to cytokines or modulating cytokine receptors at the progenitor cell surface (Jacobsen *et al,* 1991; Keller *et al*, 1991). Similar to its role in epithelial cells, TGFβ is widely considered the most potent inhibitor of cell cycle progression in committed progenitors when compared with other growth factors that regulate hematopoiesis (Hatzfeld *et al*, 1991). This is demonstrated by its ability to suppress *in vitro* colony formation in cytokine-supplemented suspension cultures.

Autocrine production of TGFβ by hematopoietic stem cells acts to maintain their quiescence and protect hematopoietic stem cells from agents that selectively kill cycling cells (Grzegorzewski *et al*, 1994). This effect may be mediated by several events that are activated downstream of the TGFβ receptor from both Smad-dependent and Smad-independent pathways such as the MAP kinase pathway (Derynck and Zhang, 2003). These events include the transcriptional repression of the proto-oncogene c-myc, downmodulation of the expression and activities of G1 and G2 cyclin-dependent kinase (CDK) and cyclins (Ewen *et al*, 1993; Geng and Weinberg, 1993; Iavarone and Massague, 1997) and activation of the genes encoding $p15^{INK4b}$ (Hannon and Beach, 1994; Reynisdottir *et al*, 1995; Sandhu *et al*, 1997; Warner *et al*, 1999; Seoane *et al*, 2001), $p21^{Cip1}$ (Datto *et al*, 1995; Landesman *et al*, 1997; Miyazaki *et al*, 1998; Claassen and Hann, 2000; Ducos *et al*, 2000), or $p27^{Kip1}$ that encode CDK inhibitors (Polyak *et al*, 1994; Depoortere *et al*, 2000; Pierelli *et al*, 2000). However, there are also data supporting a model that TGFβ can exert growth arrest independently of this class of cell cycle regulators (Cheng *et al*, 1998).

Besides controlling cell cycle regulators, which are also the well characterized targets of TGFβ in many other tissues, TGFβ can directly control the expression of the stem

cell antigen CD34 through both the classical Smad pathway and MAP kinase pathways (Batard *et al*, 2000).

Thus, previous observations point out the involvement of divergent downstream intracellular intermediates in the TGFβ pathway and illustrate how the balance of these signals is what may ultimately determine the differentiation of progenitors in response to TGFβ as an extracellular signal. Meanwhile, these observations show that our understanding of the mechanism through which TGFβ regulates hematopoiesis is still at the beginning stage.

Recent studies in lower organisms and the mouse have suggested that BMPs, important members of the TGFβ superfamily, may play a critical role in the specification of hematopoietic tissue from the mesodermal germ layer. Similar to TGFβs, BMPs also inhibit proliferation and induce differentiation of highly purified human hematopoietic cells (Bhatia *et al*, 1999). Constitutive activation of BMPs causes an increase in commitment of hematopoietic progenitors to myeloid differentiation (Walters *et al*, 2002). Inhibition of Smad5 in human hematopoietic progenitors blocks erythroid differentiation induced by BMP-4 (Fuchs *et al*, 2002). In addition, loss of the Smad5 gene leads to enhanced proliferation of highly proliferative potential precursors during embryonic hematopoiesis (Liu *et al*, 2003).

Abnormality of TGFβ in Leukemia

Identification of inactivation of genes involved in the TGFβ signal transduction pathway may represent a possible mechanism by which some hematopoietic progenitors escape from quiescent and cell-cycling inhibition. Abnormalities in the expression of

TGFβ receptors have been described in proliferative syndrome of both myeloid and lymphoid leukemias (DeCoteau *et al*, 1997; Lagneaux *et al*, 1997; Rooke *et al*, 1999). In these situations, more TGFβ cytokines are produced by those cells that have already lost responsiveness to TGFβ due to receptor defects. Thus, proliferative advantage is given to these cells, while growth of normal cells is inhibited by the overproduced TGFβ (Knaus *et al*, 1996; Schiemann *et al*, 1999).

Besides receptors, a missense mutation of the Smad4 gene in the MH1 domain (P102L) and a frame shift mutation resulting in termination in the MH2 domain (Δ (483-552)) have been identified in AML (Imai *et al*, 2001). In some cases, Smad4 loss is also observed at the protein level with normal transcription of Smad4 mRNA in AML (Wierenga *et al*, 2002). Both of the mutated Smad4 proteins are subject to faster degradation through the ubiquitin-proteosome pathway. Loss of Smad3 is also reported to be responsible for the pathogenesis of lymphoid leukemia.

In addition to the direct loss of TGFβ signal components, aberrant expressions of oncoproteins that abrogate TGFβ responses are also implicated in myeloid leukemia. Evi-1, a zinc-finger oncoprotein, was reported to induce hematopoietic cells insensitive to TGFβ regulation through interaction with Smad3 and suppression of its transcriptional activity (Kurokawa *et al*, 1998). Cytoplasmic promyelocytic leukemia (PML) physically interacts with Smad2/3 and Smad anchor for receptor activation (SARA) and is required for association of Smad2/3 with SARA and for the accumulation of the SARA and TGFβ receptor in the early endosome (Lin *et al*, 2004). The PML-RARα oncoprotein formed by chromosome translocation is identified in human acute promyelocytic leukemia (APL) patients.

The resulting fusion protein, PML-RARα, can antagonize cytoplasmic PML function and make APL cells unresponsive to TGFβ signaling (Lin *et al*, 2004).

Hox Genes

Hox genes are a group of proteins defined by a specific helix-turn-helix DNA-binding motif, the 61-amino-acid homeodomain, which is encoded by a highly conserved 183-bp DNA sequence (Boncinelli *et al*, 1989). The chromosomal organization of these genes in mammals, including humans, is arranged in four genomic clusters that are localized in four different chromosomes (Boncinelli *et al*, 1989). Each cluster is made up of 9-11 genes arranged in a homologous sequence organization called a paralog group. Interestingly, Hox genes are expressed in a temporal fashion corresponding to their physical position in the cluster. Studies demonstrated that, except for D clusters of Hox genes, whose expressions are confined to primitive subpopulations, all of the A, B and C clusters are transcribed during normal definitive hematopoiesis (Sauvageau *et al*, 1994; Giampaolo *et al*, 1995; Pineault *et al*, 2002). Differential expression of Hox genes is closely associated with specific lineages and stages of hematopoietic differentiation, indicating the crucial roles of Hox genes during both primitive and definitive hematopoiesis.

Among all of the Hox genes studied, Hoxa9 emerged as an oncogene for leukemogenesis (Kroon *et al*, 1998; Calvo *et al*, 2000). Overexpression of Hoxa9 may serve as a prognosis marker of leukemia patients, with its overexpression suggesting treatment failure (Golub *et al*, 1999). Overexpression of Hoxa9 in primary bone marrow cells causes myeloid leukemia in recipient mice after a latency period, the development of which is greatly accelerated by the co-expression of Meis1 (Kroon *et al*, 1998). Hoxa9 and Meis1

are frequent targets of endogenous retroviral insertional activation in the leukemic cells of the BXH-2 mice, suggesting leukemogenic collaboration between Meix1 and Hoxa9 (Nakamura *et al*, 1996b). Studies of the mechanism of the process indicate that Meis1 interacts with and directs the nuclear localization of PBX Homeodomain proteins (Chang *et al*, 1997; Rieckhof *et al*, 1997; Berthelsen *et al*, 1999). Both PBX and Meis1 are co-factors of Hoxa9. Although Hoxa9 may immortalize myeloid progenitors in the absence of PBX and although PBX can not enhance the leukemogenic potential of Hoxa9 as Meis1 *in vivo* (Kroon *et al*, 1998; Calvo *et al*, 2000), *in vitro* assays indicate that the interaction with PBX proteins is required for Hoxa9 to transform fibroblasts (Kasper *et al*, 1999). A trimeric DNA-binding complex composed of Hoxa9, PBX and Meis1 has been detected in leukemic cells (Shen *et al*, 1999). Defects of Hoxa9 will cause myeloid developmental defects in Hoxa9 knockout mice (Lawrence *et al*, 1997).

Another Hox gene that has received intense attention is Hoxb4. Hoxb4 was shown to expand HSCs and induce HSC differentiation from embryonic stem cells (Antonchuk *et al*, 2002; Kyba *et al*, 2002; Krosl *et al*, 2003). These two properties have tremendous clinical value and need to be further characterized.

Nup98-Hoxa9 and Other Fusion Proteins

The identification of Nup98-Hoxa9 resulting from t (7; 11) chromosomal translocation was the first direct evidence of Hoxa9 involvement in AML (Borrow *et al*, 1996; Nakamura *et al*, 1996a). The fusion protein contains the amino-terminus of Nup98 fused with the carboxyl-terminus of Hoxa9, which contains the PBX1a interaction motif, the DNA-binding Homeodomain and the carboxyl-end. Experiments suggest that

Nup98-Hoxa9 works as an aberrant transcription factor that dysregulates the target gene of Hoxa9 (Ghannam *et al*, 2004). A preliminary study using gene array confirmed this hypothesis. Of the 102 Nup98-Hoxa9 target genes identified, 92 were upregulated, and only 10 were downregulated, suggesting a transcriptional activation function. A similar analysis of wild-type Hoxa9 revealed 13 target genes, 12 of which were upregulated and 1 of which was downregulated. Nup98-Hoxa9 is primarily localized inside the nucleus (Kasper *et al*, 1999). Mice transplanted with bone marrow cells expressing Nup98-Hoxa9 through retroviral transduction acquire a myeloproliferative disease (MPD) and eventually succumb to AML (Kroon *et al*, 2001). The Nup98 portion of the fusion protein was shown to be responsible for transforming a clinically silent preleukemic phase observed for Hoxa9 into a chronic, stem cell-derived MPD (Kroon *et al*, 2001). The co-expression of NUP98-HOXA9 and Meis1 accelerated the transformation of MPD to AML, identifying a genetic interaction previously observed for Hoxa9 and Meis1 (Kroon *et al*, 2001). This effect was further confirmed in a Nup98-Hoxa9 transgenic mice model in an effort to search for cooperative genes (Iwasaki *et al*, 2005). Nup98-Hoxa9 chimeras transformed NIH/3T3 fibroblasts, and this transformation required the HOXA9 domains for DNA-binding and PBX interaction (Kasper *et al*, 1999). The FG repeats in Nup98 acted as very potent transactivators of gene transcription. Transactivation by FG repeat-rich segments of NUP98 correlates with their ability to interact functionally and physically with the transcriptional co-activators cAMP regulatory element-binding protein (CREB)-binding protein (CBP) and p300 (Kasper *et al*, 1999).

In addition to Hoxa9, Abdominal-B Hox genes in leukemia, including Hoxa11, Hoxa13, Hoxc11, Hoxc13, Hoxd11 and Hoxd13, have been identified as being fusion

partners with Nup98 (Moore, 2005). In all cases, the Nup98 amino-terminus is fused with the carboxyl-terminus of the Hox protein, which contains the complete DNA-binding homeodomain. Besides these homeobox genes, nonhomeobox genes are also on the list of NUP98 fusion partners. A central characteristic for them is that they contain regions capable of forming coiled-coil domains. Such domains have been shown to be critical for the leukemogenic function of PML-RARα and AML1-ETO, as well (Hussey and Dobrovic, 2002).

Central Hypothesis and Specific Aims

We have shown that Hox proteins are TGFβ/BMP downstream transcription factors (Shi *et al*, 1999, 2001). Smad4 and BMP-specific R-Smad, Smad1, can interact with Hox proteins, but TGFβ-specific Smad2 and Smad3 cannot do so. During mesenchymal development and osteoblast differentiation, Hoxa9 or Hoxc8 functions as a transcription repressor in mesenchymal stem cells (Lei *et al*, 2005). Upon TGFβ/BMP stimulation, Smad1/4 can directly interact with Hox proteins and block their DNA-binding activity. Ectopic expression of the interaction domain of Smad1 with Hoxc8 sufficiently stimulates BMP downstream gene expression and results in osteoblast differentiation and bone formation both *in intro* and *in vivo* (Yang *et al*, 2000; Liu *et al*, 2004). As a negative regulation mechanism, Smad6, the BMP-specific I-Smad, forms a heterodimer with Hox on DNA elements and prevents Smads from binding to Hox proteins, therefore repressing BMP-induced gene transcription (Bai *et al*, 2000; Bai and Cao, 2002). Besides our investigations of Smad/Hox interaction, other research has shown that Smad4 interacts with DLX1 at its homeodomain and blocks activin signaling in hematopoietic cells (Chiba *et al*,

2003). In summary, study suggests a broad picture of the crucial regulatory functions of Smads/Hox interactions during hematopoietic development.

On the basis of previous findings, we hypothesize that TGFβ/BMPs induce hematopoietic cell differentiation and inhibit hematopoietic cell proliferation mediated though Smad4 interaction with Hoxa9. The proposed studies are designed to extend our findings and characterize the molecular mechanism of the role of TGFβ/BMP in hematopoiesis. To achieve this goal, I will pursue the following specific aims:

Specific Aim I: Characterize the domains of the interaction between Smad4 and Hoxa9 proteins in TGFβ/BMP signaling and the effect on downstream gene transcription. We will examine the specificity of the interaction between Smad4 and Hoxa9 in a hematopoietic progenitor cell line by using immunoprecipitation assays. A series of cDNA deletion constructs of Smad4 will be generated, and the interaction of these two protein fragments will be examined by electrophoretic mobility shift assay (EMSA). We will also examine the effect of Smad4 on HOXA9 transcriptional activities by luciferase assay and monitor HOXA9 downstream gene expression by RT-PCR and Western blot.

Specific Aim II: Characterize the effects of the interaction between Smad4 and Hoxa9 on hematopoiesis. We will use retrovirus-mediated gene transfer to overexpress Smad4 together with HoxA9 in primary bone marrow cells isolated from femurs and tibias of mice.

Specific Aim III: Characterize the effects of Smad4 on Nup98-Hoxa9-induced AML.

1. We will test the effect of interaction of Smad4 with its domain(s) with Nup98-Hoxa9 on DNA-binding ability and downstream gene transcription and expression.
2. We will use retrovirus-mediated gene transfer to overexpress Smad4 together with Nup98-Hoxa9 in primary bone marrow cells isolated from femurs and tibias of mice and *in vitro*.

The studies presented in this work demonstrate that TGFβ/BMP regulates bone marrow transformation capability of Hoxa9 and Nup98-Hoxa9 through Smad4 and indicate how Smad4 mutations identified in human AML patients may affect its normal function. Smad4 directly interacts with the homeodomain of Hoxa9, which is conserved in all 39 members of Hox proteins and functions as their DNA-binding domain. This interaction blocks the DNA-binding ability of Nup98-Hoxa9 and, thus, suppresses its downstream gene transcription. Mapping data revealed that the amino-terminus of Smad4 is responsible for this interaction. Overexpressioin of this Hoxa9 interaction domain of Smad4 was sufficient to inhibite the leukemic transformation capability of Nup98-Hoxa9. Thus, our studies establish a novel mechanism by which TGFβ/BMP regulates hematopoiesis and raise the possibility that modifying Hox DNA-binding activity may serve as a novel therapeutic intervention for those leukemias that involve *Hox* deregulations. In addition, I also examined the protein stability of Smad4 mutants found in AML. We found that the Smad4 mutants exhibited significantly decreased protein stability. Importantly, we found that the F-box protein β-TrCP1 in SCF E3 ligase interacts with Smad4 and exhibits stronger interaction affinity with the acute myelogenous leukemia-derived Smad4 mutants.

Consequently, E3 ligase complex $SCF^{\beta\text{-}TrCP1}$ mediates ubiquitylation of Smad4 mutants. When we used small interference RNA (siRNA)-induced F-box protein β-TrCP1 gene silencing, the protein steady-state level of Smad4 was elevated in acute myelogenous leukemia cells.

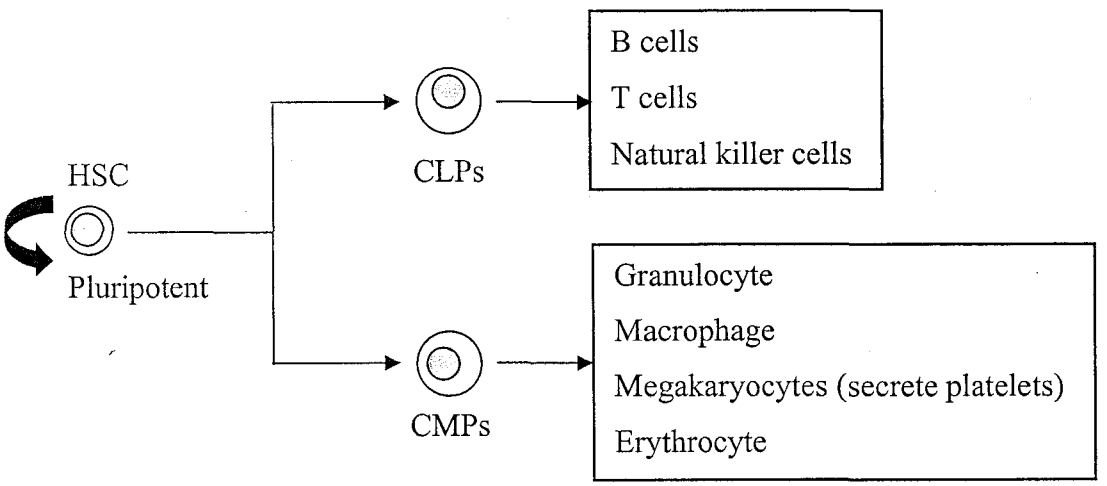

Figure 1 Schematic view of adult hematopoiesis. The pluripotent HSC normally divides to generate either more pluripotent HSCs (self-renewal) or destined progenitor cells, such as CMPs or CLPs, which are irreversibly determined to produce only one type or a few types of blood cells in the lymphoid or myeloid lineage. The progenitor cells are stimulated to proliferate by specific growth factors but progressively lose their capacity for division and develop into terminally differentiated blood cells, which usually live for only a few days or weeks in the peripheral blood.

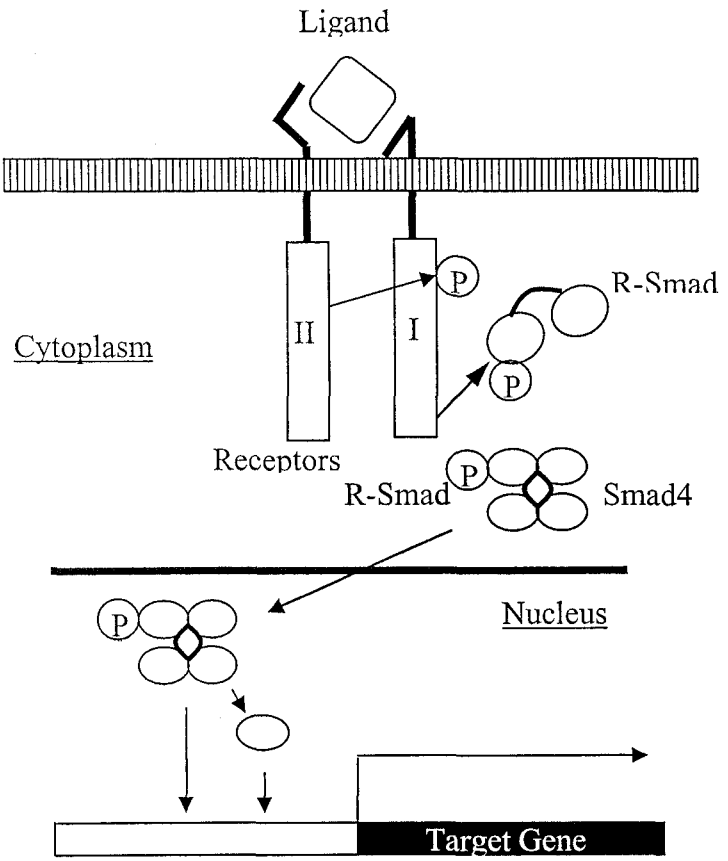

Figure 2 TGFβ signaling pathway. Ligand binds cooperatively to the type I and II receptors and results in the phosphorylation (P) of the type I receptor in the GS domain by the constitutively activated type II receptor. Phosphorylated and activated type I receptor in turn interacts with R-Smads, leading to the phosphorylation of Smad proteins, their association with Smad4 and movement into the nucleus. In the nucleus, this complex binds to the DNA sequence in the transcriptional regulatory region of target genes. The I-Smads inhibit both binding of R-Smads to the activated type I receptor and the nuclear translocation of Smad4.

TGFB/BMP INHIBITS LEUKEMOGENESIS BY REPRESSING HOX DNA-BINDING ACTIVITY

by

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Abstract

Homeobox (*Hox*) gene mutations and altered expressions are frequently linked to human leukemia; however, how Hox is regulated during hematopoiesis is not well characterized. Here, we report that transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) inhibits the bone marrow transformation capability of Hoxa9 and Nup98-Hoxa9, the chimeric fusion form of Hoxa9 identified in human acute myeloid leukemia (AML), through Smad4, the common Smad (Co-Smad) in the TGFβ/BMP signaling pathway. Smad4 directly interacts with the homeodomain of Hoxa9, which is conserved in all 39 members of Hox proteins and functions as their DNA-binding domain. This interaction blocks the DNA-binding ability of Nup98-Hoxa9 and, thus, suppresses its downstream gene transcription. Mapping data revealed that the amino-terminus of Smad4 is responsible for this interaction. Overexpression of this Hoxa9 interaction domain of Smad4 was sufficient to inhibit the leukemic transformation capability of Nup98-Hoxa9. Thus, our studies establish a novel mechanism by which TGFβ/BMP regulates hematopoiesis and raise the possibility that modifying Hox DNA-binding activity may serve as a novel therapeutic intervention for those leukemias that involve *Hox* deregulations.

Introduction

The transforming growth factor β (TGFβ) superfamily consists of TGFβs, bone morphogenetic proteins (BMPs), activins and related proteins. These secreted proteins regulate a broad range of cellular responses during hematopoiesis, including cell proliferation, differentiation and apoptosis (Bhatia *et al*, 1999; Fortunel *et al*, 2000). Smad proteins are intracellular signal transducers in the TGFβ/BMP signaling pathway. Upon

ligand binding cooperatively to the type I and II transmembrane receptors, receptor-regulated Smads (R-Smads) are activated through phosphorylation (Derynck and Zhang, 2003). Specifically, Smad2 and Smad3 are phosphorylated by TGFβs, whereas phosphorylation of Smad1 is induced by BMPs. Consequently, phosphorylated R-Smads form heteromeric complexes with Smad4, the common Smad (Co-Smad) shared in both TGFβ and BMP signaling pathways, and translocate to the nucleus (Attisano and Wrana, 2002), where they control target gene expression either by directly binding to DNA or by interacting with other cofactors (Wotton *et al*, 1999; Hata, 2001).

Although TGFβ/BMP's function in hematopoiesis is increasingly recognized, the mechanism of how TGFβ/BMP regulates hematopoiesis is not well characterized. TGFβ is considered to be one of the most potent autocrine negative regulators of hematopoiesis, and accumulated evidence indicates its role as tumor suppressor in hematological malignancy (Tessier and Hoang, 1988; Sing *et al*, 1988). Loss of responsiveness to TGFβ has been identified in leukemia. Abnormalities in the expression of TGFβ receptors have been described in proliferative syndromes, including both early myeloid (Bousse-Kerdiles *et al*, 1996; Rooke *et al*, 1999) and lymphoid leukemia (Lagneaux *et al*, 1997; DeCoteau *et al*, 1997). A missense mutation of the *Smad4* gene in the MH1 domain (P102L) and a frame shift mutation resulting in termination in the MH2 domain (Δ(483-552)) have been identified in acute myelogenous leukemia (Imai *et al*, 2001). Both of the mutated Smad4 proteins are subject to faster degradation through the ubiquitin-proteosome pathway. Besides disruptions of the components in the TGFβ signaling pathway, aberrant expressions of oncoproteins that abrogate TGFβ responses are also implicated in myeloid leukemia (Kurokawa *et al*, 1998; Lin *et al*, 2004). Similar to TGFβ,

BMPs also inhibit proliferation and induce differentiation of highly purified human hematopoietic cells (Bhatia *et al*, 1999). Constitutive activation of BMPs causes an increase in commitment of hematopoietic progenitors to myeloid differentiation (Walters *et al*, 2002). Inhibition of Smad5 in human hematopoietic progenitors blocks erythroid differentiation induced by BMP-4 (Fuchs *et al*, 2002). In addition, loss of the *Smad5* gene leads to enhanced proliferation of highly proliferative potential precursors during embryonic hematopoiesis (Liu *et al*, 2003).

Homeobox (Hox) genes are also key regulators of hematopoiesis (Lawrence and Largman, 1992). In vertebrates, Hox genes are grouped into four clusters (Hox-A to Hox-D) on separate chromosomes (Sharkey *et al*, 1997). The expressions of *Hox* genes during hematopoietic development are stage dependent and tightly controlled. *Hoxa9*, a member of the abdominal-B subclass of *Hox* genes, is one of the most studied *Hox* genes in hematopoiesis. *Hoxa9* is abundantly expressed in early self-renewing CD34+ cells and gradually shuts down when cells undertake maturation and terminal differentiation (Sauvageau *et al*, 1994; Lawrence *et al*, 1997). *Hoxa9*-deficient mice show defects in blood formation, while enforced expression of *Hoxa9* immortalizes and blocks the differentiation of myeloid progenitors, eventually leading to acute myeloid leukemia (AML) in mice (Kroon *et al*, 1998). *Hoxa9* is also upregulated in human AML (Golub *et al*, 1999b). The detection of *Nup98-Hoxa9* fusion genes in cases of AML, with the amino-terminal portion of Nup98 fused to the Hoxa9 carboxyl-terminal DNA-binding homeodomain as a result of the t (7; 11) chromosomal translocation, further confirms the direct oncogenic effect of Hoxa9 in leukemia (Nakamura *et al*, 1996; Borrow *et al*, 1996). Overexpression of Nup98-Hoxa9 in murine bone marrow cells causes immortalization *in vitro* and induces

chronic and acute myeloid leukemia *in vivo* (Kroon *et al*, 2001; Calvo *et al*, 2002). It is widely accepted that Nup98-Hoxa9 works as an aberrant DNA-binding transcription factor by upregulating a broader range of genes than Hoxa9 alone does (Kasper *et al*, 1999; Ghannam *et al*, 2004). Meis1 and PBX1a, members of the 3-amino-acid loop extension (TALE) homeodomain family, are cofactors of Hox (Moskow *et al*, 1995). Meis1 can collaborate with Nup98-Hoxa9 to accelerate the onset of leukemia. PBX1a is shown to enhance the DNA-binding affinity of Nup98-Hoxa9 through heterodimerizing with Nup98-Hoxa9 on the consensus sequence TGATTTA (C/T) (Kasper *et al*, 1999b). However, the mechanism of Hox in hematopoiesis is not well characterized.

We have shown that Hox proteins are TGFβ/BMP downstream transcription factors (Shi e*t al*, 1999, 2001). Upon TGFβ/BMP stimulation, Smad4 and BMP-specific R-Smad, Smad1, can directly interact with Hox proteins and block their DNA-binding activity. Besides our investigations of Smad/Hox interaction, other research has shown that Smad4 interacts with DLX1 at its homeodomain and blocks activin signaling in hematopoietic cells (Chiba *et al*, 2003). Here we report that TGFβ/BMP inhibits the bone marrow transformation capability of Hoxa9 and Nup98-Hoxa9 through Smad4. Biochemical and cellular data demonstrate that the interaction between the amino-terminus of Smad4 and Nup98-Hoxa9 mediates this effect by inhibiting Hox DNA-binding ability. This study reveals a novel regulatory mechanism through which TGFβ/BMP regulates hematopoiesis and raises the possibility that Hox DNA-binding activity may serve as a potential therapeutic target.

Results

TGFß/BMP Inhibits Bone Marrow Transformation Capability of Hoxa9 and Nup98-Hoxa9

Previously, we have shown that Smad1 and Smad4 directly interact with Hox proteins such as Hoxc8 or Hoxa9 at their conserved homeodomains and inhibit their DNA-binding activity. This suggests that TGFβ/BMP may have an inhibitory effect on the bone marrow transformation capability of Hoxa9 or Hoxa9 fusion proteins by modulating their DNA-binding activity through Smads. To test this possibility, we first used myeloid colony formation assay to analyze the effects of TGFβ/BMP on bone marrow cells expressing *Hoxa9* or *Nup98-Hoxa9*. To achieve this goal, cDNAs encoding *Hoxa9* or *Nup98-Hoxa9* were individually cloned into the upstream of an internal ribosome entry site (IRES) linked with a blue-excited green fluorescent protein (GFP) variant (BEX) within murine stem cell virus (MSCV) (Figure 1A) (Anderson *et al*, 1996). Western blotting of extracts from transiently transfected BOCS23 retrovirus packaging cells confirmed that both *Hoxa9* and *Nup98-Hoxa9* constructs were efficiently expressed (Figure 1C). Bone marrow cells were infected with retrovirus bearing *BEX*, *Hoxa9* or *Nup98-Hoxa9*. Transduced cells (BEX positive) were then isolated by fluorescence-activated cell sorting (FACS) (Figure 1D) and cultured in methylcellulose for 7-10 days. Transduction efficiencies ranged from 5 to 20% for Hoxa9 and Nup98-Hoxa9 and from 35 to 45% for BEX (Figure 1C and data not shown).

Cells transduced with *Hoxa9* or *Nup98-Hoxa9* gave rise to large compact colonies with an average of 50 and 100 myeloid colonies per 2600 plated cells, respectively (Figures 1E and 2A and J). TGFβ1 (2 ng/mL) reduced the number of colonies formed from *Hoxa9* and *Nup98-Hoxa9*-transduced cells by 3.1 fold and 4 fold, respectively (Figure

1D). BMP-2 (300 ng/mL) exhibited similar effects and reduced the number of colonies formed from *Hoxa9*- and *Nup98-Hoxa9*-transduced cells by 2.5 fold and 3.2 fold, respectively (Figure 1D). TGFβ/BMP showed inhibitory effects on BEX-transduced cells due to the expression of endogenous Hox genes in bone marrow progenitor cells (Figure 1D).

To test the replating ability of cells within the primary cultures, primary colonies were harvested and replated without further treatment (Figure 1B). While BEX-transduced cells showed lowered replating efficiency due to exhaustion of their proliferation capacity (data not shown), *Nup98-Hoxa9*-transduced cells, consistent with a previous report (Kroon *et al*, 2001), exhibited enhanced replating efficiency (Figure 1E). However, cells harvested from cultures previously treated with TGFβ1 or BMP-2 showed significantly lowered replating efficiency than nontreated cells did (Figure 1F), indicating that TGFβ/BMP reduces the frequency of colony-forming cells in the first round of plating.

Further examination of the morphology of cells within each colony showed that myeloid differentiation was blocked in *Hoxa9*- and *Nup98-Hoxa9*-expressing colonies (Figure 2G and M). Strikingly, both TGFβ1 and BMP-2 were able to induce myeloid differentiation of *Hoxa9* and *Nup98-Hoxa9* colonies into cells that exhibited macrophage and granulocytic cell morphology (Figure 2H, I, N and O). Control BEX-expressing cells exhibited an average of 20 myeloid colonies of heterogeneous size and morphology similar to those of colonies obtained from normal or mock-infected cells (Figure 1E and data not shown). All colonies were florescence positive, indicating that retroviral gene transductions were stable (Figure 2D, E and F). In summary, these data allow us to conclude that TGFβ/BMP inhibits the bone marrow transformation capability of Hoxa9 and Nup98-Hoxa9 and induces myeloid differentiation.

Smad4 Inhibits Binding of Nup98-Hoxa9 to DNA

Nup98-Hoxa9 is a human AML-associated *Hox* gene that is constitutively over-expressed in patients. To understand the mechanism through which TGFβ/BMP inhibits the function of Nup98-Hoxa9, we examined the effect of TGFβ/BMP on Nup98-Hoxa9 downstream gene transcription. Since the downstream transcriptional targets of Nup98-Hoxa9 are not clear, we selected *Hoxa9* as a potential target gene because (a) *Hoxa9* expression is induced in primary bone marrow cells transduced with *Nup98-Hoxa9* (Calvo *et al*, 2002), (b) there are multiple Hox-binding sites (TTA(C/T)) in Hoxa9 promoter (Patel *et al*, 1999), (c) it has been determined that *Hox* genes are positively autoregulated by their own products or cross-regulated by the products of other *Hox* genes (Gould *et al*, 1997). These findings suggest that Hoxa9 could be a direct transcription target of Nup98-Hoxa9. *Hoxa9* promoter luciferase reporter (*Hoxa9*-luc) was transfected in NIH/3T3 cells together with *Nup98-Hoxa9* in combination with *PBX1a*, *Smad1*, *Smad4* or Activin-receptor-like kinase (ALK3), which is a constitutively active BMP type I receptor (Q233D) (Shi *et al*, 2001). Nup98-Hoxa9 alone significantly stimulated the transcription activity, which was further enhanced by co-expression of its heterodimer partner, PBX1a. Importantly, these transactivations were inhibited by Smad1, Smad4 or ALK3 (Figure 3A). In Ba/F3 hematopoietic progenitor cells, TGFβ1 or Smad4 inhibited Nup98-Hoxa9-induced transcription, but Smad2 had no such effect (Figure 3B). This result was consistent with our prior finding that TGFβ-specific R-Smads do not interact with Hox proteins (Shi *et al*, 1999d). There were no significant effects of Smad2, Smad4, TGFβ or ALK3 on the basal activity of *Hoxa9* promoter, indicating that the inhibitory effects were specific to Nup98-Hoxa9-induced transcriptional activation. To verify the critical role of

Smad4 in TGFβ/BMP-mediated inhibition, we eliminated endogenous Smad4 expression with a small interference RNA (siRNA) against Smad4 (si-Smad4) (Wan *et al*, 2004) and repeated the transfection assay in NIH/3T3 cells. As Figure 3C shows, si-Smad4 completely abolished TGFβ/BMP-induced inhibition of Nup98-Hoxa9 transactivation and elevated Nup98-Hoxa9-induced transcription on Hoxa9 promoter, while control si-GFP lacked such an effect. In summary, Nup98-Hoxa9 activates transcription on *Hoxa9* promoter, and this transcription activation is suppressed by TGFβ/BMP through Smad4.

To further characterize the mechanism of Smad4-mediated inhibition, we examined whether Smad4 disrupts Nup98-Hoxa9 DNA-binding in an electrophoretic mobility shift assay (EMSA). Purified glutathione S transferase (GST)-Nup98-Hoxa9, GST-PBX1a or both were incubated with 32^{P}-labeled sequence fragment from Hoxa9 promoter containing the Hox/PBX consensus DNA-binding site (TGATTTAC) (Shen *et al*, 1999). GST-Nup98-Hoxa9 formed a retarded band in a dose-dependent manner (Figure 3D, lane 2-4), and the band shifted when PBX1a protein was added (Figure 3D, lanes 5-7), indicating that Nup98-Hoxa9 directly binds to *Hoxa9* promoter and that Nup98-Hoxa9 can heterodimerize with PBX1a. The specificity of the binding was demonstrated by a dose-dependent competition of Nup98-Hoxa9 binding with an excess of unlabeled Hoxa9 DNA probe (Figure 3D, lanes 8-10), but not with an excess of unlabeled random oligos (Figure 3D, lanes 11-13). Most importantly, Smad4 effectively inhibited the binding of Nup98-Hoxa9 to the DNA element in a concentration-dependent manner (Figure 3E), whether in the presence of Pbx1a or not. These results suggest that TGFβ/BMP inhibits Nup98-Hoxa9-induced transcription activity by disrupting the binding of Nup98-Hoxa9 with DNA through Smad4.

Smad4 MH1 Domain Contributes to the Interaction With Nup98-Hoxa9

To map the domain(s) of Smad4 that interacts with the homeodomain of Hoxa9 protein, we generated a series of GST-Smad4 truncated fusion proteins as shown in Figure 4A. In the absence of Hoxa9, none of the GST-Smad4 or truncated GST-Smad4 fusion proteins bound to the probe (Figure 4B, lanes 3-8). Smad4 full-length, MH1 domain and Smad4 MH1 domain with linker region inhibited Hoxa9 DNA-binding activity. Moreover, neither Smad4 MH2 domain nor Smad4 MH2 domain plus linker region had such an effect (Figure 4B). The results clearly indicate that the amino-terminus of Smad4 mediates the interaction of Smad4 with Hoxa9. To localize the interaction region within the amino-terminus, we generated four smaller fragments as shown in Figure 4C. Both fragment containing amino acids 52-148 and that containing amino acids 101-148 of the MH1 domain inhibited the binding of Hoxa9 to DNA (Figure 4D), but neither the fragment containing amino acids 1-51 nor that containing amino acids 52-101 showed any inhibition (Figure 4D). This result suggests that the residue 101-148 is crucial for the interaction with the Hoxa9 homeodomain. Then we tested whether the mapped domains also inhibited DNA-binding activity of Hoxa9 and Nup98-Hoxa9 with PBX1a. As expected, both Smad4D (MH1 domain plus linker region) (Figure 4E, lanes 4 and 10) and Smad4.4 (fragment containing amino acids 101-148) (Figure 4E, lanes 5 and 11) inhibited Hoxa9/PBX1a or Nup98-Hoxa9/PBX1a binding to DNA, while Smad4E (MH2 domain plus linker) had no such effect (Figure 4E, lanes 6 and 12).

Smad4 Inhibits Endogenous Hoxa9 Expression Induced by Nup98-Hoxa9

Having shown that Smad4 inhibits Nup98-Hoxa9 transactivation by blocking its DNA-binding activity, we then attempted to examine whether Smad4 inhibits *Hoxa9* endogenous gene transcription induced by Nup98-Hoxa9. Cells were infected with retrovirus bearing HA-tagged Nup98-Hoxa9 or Nup98b-Hoxa9, which is an alternatively spliced form of Nup98-Hoxa9 (Kasper *et al*, 1999). Their expressions were confirmed by RT-PCR and Western blot (Figure 5B and C). Consistent with an earlier report (Calvo *et al*, 2002), both Nup98-Hoxa9 and Nup98b-Hoxa9 enhanced endogenous Hoxa9 mRNA (Figure 5A) and protein expression (Figure 5B), and enforced expression of Smad4 inhibited the induction of Hoxa9 expression (Figure 5A and B), suggesting that Smad4 suppresses Nup98-Hoxa9-induced gene transcription in cells.

Smad4 Inhibits Binding of Nup98-Hoxa9 to DNA in Cells

To determine whether TGFβ/BMP regulates Nup98-Hoxa9 downstream gene transcription in cells by blocking Nup98-Hoxa9 DNA-binding, we investigated the binding of Nup98-Hoxa9 to chromatin-associated Hoxa9 promoter and analyzed the effect of TGFβ/BMP on this binding by chromatin immunoprecipitation (ChIP) assay. Retrovirally transduced NIH/3T3 cells were treated with TGFβ or BMP for 2-4 h and then with formaldehyde to cross-link DNA-protein complexes. After sonication, chromatin fragments were immunoprecipitated with HA antibody specific for HA-Nup98-Hoxa9, and the immunoprecipitated fragment DNA was subjected to PCR to amplify Hoxa9 promoter DNA (between -284 and -91) containing Hox-PBX consensus-binding element TGATTTAC.

As illustrated in Figure 6A, the Hoxa9 promoter element co-immunoprecipitated with HA-Nup98-Hoxa9 from NIH/3T3 cells. Furthermore, both TGFβ1 and BMP-2 inhibited co-immunoprecipitation of Nup98-Hoxa9 with Hox-binding DNA element. These results indicate specific association of Nup98-Hoxa9 with the proximal Hoxa9 gene promoter, and TGFβ and BMP-2 suppress the association. We have previously shown that Smad4 mediates TGFβ/BMP inhibition of Hoxa9 DNA-binding and that Smad2 does not interact with Hoxa9 (Shi *et al*, 1999). Consequently, we examined whether Smad4 or Smad2 affects the association of Nup98-Hoxa9 with the proximal Hoxa9 gene promoter. In a similar ChIP assay, NIH/3T3 cells infected with retrovirus expressing HA-Nup98-Hoxa9 or GFP were transiently transfected with Smad4 or Smad2 or with empty vector as a control. Consistent with the formerly described result in EMSA, Smad4 specifically inhibited binding of both Nup98-Hoxa9 and Nup98b-Hoxa9 to DNA (Figure 6B).

Smad4 Inhibits Leukemic Transformation Induced by Nup98-Hoxa9

Our results suggest that TGFβ/BMP inhibits the function of Nup98-Hoxa9 through Smad4. To examine whether Smad4 alone can inhibit Nup98-Hoxa9-induced leukemic transformation of primary bone marrow cells, cDNAs of *Smad4* and the Smad4/Hoxa9 interaction domain (amino acids 52-148) (*Smad4FG*) were fused with a nuclear localization signal (NLS) and cloned into the MSCV retroviral vector, which carries a spectrally distinct GFP variant, violet-excited GFP (VEX) (Figure 7A). Expression of BEX and VEX constructs can be detected in a single cell due to their differential excitation properties (Anderson *et al*, 1996) (Figure 7B). We also generated a chimera construct, *Smad2/4*, in which the Smad4/Hox interaction MH1 domain of Smad4 was re-

placed by the MH1 domain of Smad2. Equivalent numbers of double-transduced cells were then isolated by FACS and cultured in methylcellulose for 7-10 days. Expression of *Smad4*, *Smad4FG* and *Smad2/4* alone produced a decreased number of colonies in series platings; these results are similar to those found for control (data not shown). *Nup98-Hoxa9/VEX* cells gave rise to sharp-edged large colonies with continuously enhanced re-plating ability (Figure 7D), which suggests the leukemic transformation capability of Nup98-Hoxa9. Co-expression of *Smad4* or *Smad4FG* together with *Nup98-Hoxa9* not only significantly inhibited the colony numbers in the first round of plating but also impaired replating ability enhanced by Nup98-Hoxa9. Examination of colony morphology showed smaller sizes in *Smad4*- or *Smad4FG*-expressing cultures than in *Nup98-Hoxa9* alone (Figure 7E). Giemsa-Wright staining confirmed that *Smad4* or *Smad4FG* expression sufficiently induced myeloid differentiation into macrophages and granulocytic cells (Figure 7E). Co-expression of *Smad2/4* with *Nup98-Hoxa9* did not have a significant suppression effect on colony formation in comparison with *Nup98-Hoxa9/VEX* cultures and was not able to induce myeloid differentiation (Figure 7D and E).

On the basis of our previous finding that Smad1 also interacts with homeodomain protein at their HD and blocks its DNA-binding activity, we tested Smad1 in the inhibition of Hoxa9-induced leukemic transformation on murine primary bone marrow cells in order to confirm that Hoxa9 DNA-binding activity is crucial for its oncogenicity. Toward this end, cDNAs of *Smad1* and its Hox interaction domain, *Smad1C*, fused with an NLS (Yang *et al*, 2000), were cloned into MSCV-IRES-VEX retrovirus vector and coexpressed with *Hoxa9* in primary bone marrow cells (Figure 7A). Both *Smad1* and *Smad1C* expressions were shown to inhibit the sustained replating ability of Hoxa9-transformed

bone marrow cells (Figure 7C). As expected, myeloid differentiations into macrophages and granulocytic cells were induced in *Smad1*- and *Smad1C*-expressing colonies, while *Hoxa9/VEX* expression appeared to be immature myeloblasts (Figure 7E). Taken together, these data indicate that the interaction of Smad4 with Hoxa9 inhibits leukemic transformation.

Discussion

In the present study, we characterized a novel mechanism that mediates the inhibitory effect of TGFβ/BMP on the leukemic transformation of murine primary bone marrow cells by Hoxa9 or Nup98-Hoxa9, the chimeric fusion form of Hoxa9 identified in AML. Biochemical studies demonstrate that Smad4 inhibits the DNA-binding activities of Nup98-Hoxa9 through its amino-terminus and suppresses Hox downstream gene transcription via a TGFβ/BMP-mediated mechanism. Furthermore, both Smad4 and Smad4FG, the 98-amino-acid interaction domain of Smad4 with Hoxa9, are sufficient to inhibit the leukemic transformation of primary bone marrow cells by Nup98-Hoxa9. These results reveal a critical antagonistic role of TGFβ/BMP in controlling Hox activity and provide a model of regulatory orchestration between external cytokines and intrinsic transcription factors during hematopoiesis (Figure 7F). Hoxa9 maintains HSCs and progenitor cells in an undifferentiated stage. Under physiological conditions and upon stimulation of TGFβ/BMP, Smads interact with Hoxa9 and remove it from its DNA target, resulting in myeloid differentiation. However, in pathological situations, mutations causing overexpression of Hoxa9 break the balance between Smads and Hoxa9, which results in constitutive activation of Hox function and leads to leukemia.

TGFβ is the most potent negative regulator of cell cycle progression in general, and a line of evidence indicates that TGFβ maintains quiescence of hematopoietic stem cells through autocrine secretion of this cytokine (Ploemacher *et al*, 1993). In addition, a number of related mechanisms can mediate the effect of cell cycle inhibition. These mechanisms are primarily involved in transcriptional regulation of cell cycle regulators including downregulation of cyclin-dependent kinases (CDKs) and cyclins in G1 and G2 phases or upregulation of the cell cycle inhibitors such as p27, p21, and p15 (Kim and Letterio, 2003). However, it has also been shown that TGFβ inhibition of cell cycle progression in hematopoietic cells is independent of cell cycle regulators (Cheng *et al*, 1998). Here we have shown that TGFβ/BMP-induced interaction of Smads with Hoxa9 negatively regulates Hoxa9 activity by inhibiting Hoxa9 DNA-binding activity in murine primary bone marrow cells, which may represent a major mechanism through which TGFβ/BMP controls hematopoietic cells since most of the 39 Hox genes are expressed in the hematopoietic system and, in general, maintain hematopoietic cells in an undifferentiated and self-renewal stage. Overexpression of *Smad4FG* or *Smad1C* is sufficient to inhibit the leukemic transformation of murine primary bone marrow cells by Nup98-Hoxa9 and Hoxa9, respectively. The fact that neither Smad4FG nor Smad1C is able to elicit the broad range of responses of TGFβ/BMP suggests that Smads/Hox interaction plays a key role in this inhibition. To further demonstrate this point, we substituted the MH1 domain of Smad4 with the corresponding sequence of Smad2 to generate Smad2/4. As expected, the resulting chimeric protein lost the inhibitory effect. Taken together, these data suggest that the Smad4/Hox interaction pathway plays a major role in how TGFβ/BMP modulates myeloid development.

Although BMP exhibited an effect comparable with that of TGFβ, it may have more regulatory influence on Hox activity. In fact, we have shown that both Smad4 and BMP-specific R-Smad, Smad1, inhibit Hox proteins binding to DNA but that none of the TGFβ-specific R-Smads (Smad2 and Smad3) have such an inhibitory effect on Hox DNA-binding activity (Shi *et al*, 1999). There are two inhibitory Smads (I-Smads): Smad6, which preferentially inhibits BMP signaling (Hata *et al*, 1998), and Smad7, which has a broader inhibition profile (Itoh *et al*, 2000). Interestingly, Smad6 forms heterodimers with Hox transcription factors when binding to DNA as a negative feedback loop in the nucleus, but Smad7 does not interact with Hox proteins (Bai *et al*, 2000). Once the Smad6/Hox heterodimer is formed, neither Smad1 nor Smad4 is able to regulate its DNA-binding and transcription activity. Smad4 and the BMP-specific Smad1 and Smad6 have been observed to interact with Hox transcription factors from each of 13 paralogs in vertebrate animals. It is likely that Smad1, Smad4 and Smad6 interact with all 39 Hox proteins depending on promoter context and cell type. Apparently, BMP employs the interaction of Smads with Hox in regulation of hematopoiesis. The understanding of BMP function in hematopoiesis is still at the early stage, but it is known that BMPs are involved in hematopoietic development and that Hox also plays a critical role in this process. If the numerous BMP ligands, multiple BMP receptors and R-Smads and a complex pattern of Hox gene expression during hematopoiesis are considered, it seems highly likely that interactions between Hox and BMP-regulated Smads generate intricate signals to negatively modulate Hox transcription activity in hematopoietic cell lineage commitment and maturation. Both TGFβ and BMP regulate this composite transcription network in hematopoiesis, and much of the detailed mechanism remains unexplored.

Elevated expression of *Hox* genes is frequently found in leukemia, particularly in AML (Golub *et al*, 1999). Animal models, as well as retrovirus-mediated overexpression of individual Hox genes, indicate that many if not all *Hox* genes can cause leukemia. In addition to *Hoxa9*, *Nup98* has been identified as a fusion partner with other *Hox* genes in leukemia, including *Hoxa11*, *HoxC13* and *HoxD13* (Moore, 2005). In all cases, the Nup98 amino-terminus is fused with the carboxyl-terminus of the Hox protein, which contains the complete DNA-binding homeodomain. However, how the function of Hox protein is regulated remains poorly described. Hox proteins work as transcription factors and mediate a wide variety of gene families (Dorsam *et al*, 2004; Ghannam *et al*, 2004). The DNA-binding activity is required for its function. For example, protein kinase C (PKC) was reported to phosphorylate Hoxa9 at S204 and to impair the DNA-binding activity of Hoxa9, thus inducing differentiation of hematopoietic cell lines as well as Hoxa9-immortalized murine bone marrow cells (Vijapurkar *et al*, 2004). Mutations causing loss of the DNA-binding activity of Nup98-Hoxa9 abolish their transformation ability in NIH/3T3 cells (Kasper *et al*, 1999). Here we report the negative regulation of Hox DNA-binding activity by TGFβ/BMP through Smads. This observation points toward the functional importance of the Smads/Hox interaction in hematopoiesis.

Although mutations and abnormal expression of Hox genes are frequently observed in leukemia, particularly in AML, mutations of TGFβ signaling components are not common in leukemia. Two distinct *Smad4* mutations, as well as proteolytic degradation of Smad4 protein, have been described in human myeloid leukemia (Imai *et al*, 2001; Wierenga *et al*, 2002). Smad4 is a potent inhibitor of Hox DNA-binding activity; therefore, mutations or loss of Smad4 may elevate Hoxa9 activity that contributes to myeloid

leukemia. Thus, inhibition of Hox binding to DNA by Smads provides a potential therapeutic intervention for those leukemias that involve dysregulation of Hox expression.

Materials and Methods

Bone Marrow Harvesting and Culture

C57B/6-Ly-5.2 mice were injected intraperitoneally with 150 mg/kg body weight 5-FU (Sigma) dissolved in sterile phosphate-buffered saline (PBS). Four days later, mice were killed, and bone marrow was harvested from the femurs and tibias by using a 25-gauge needle and sterile PBS. After a single cell suspension was obtained, red cells were lysed for 7 min on ice in 1.0 mL ACK (8.3 g ammonium chloride and 1.0 g potassium bicarbonate in 1 L distilled deionized water) per mouse equivalent of bone marrow. Cells were then washed in PBS, filtered, and then resuspended in Dulbecco modified eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), penicillin 100 IU/mL, streptomycin 100 IU/mL (Gibco), 1× nonessential amino acids, 1× sodium pyruvate (1 mM), 1×L-glutamine (2 mM; Gibco) and 50 μM β-mercaptoethanol (BME; Sigma). To induce stem cell cycling, cytokines were added to the culture medium as follows: 5 ng/mL IL-6 (R&D Systems), 50 ng/mL stem cell factor (R&D Systems) and 1x leukemia inhibitory factor (ESGRO). Cells were prestimulated for 24 h in a sterile incubator at 37°C in 5% CO_2.

Retrovirus Production and Transduction

Retrovirus-packaging BOSC23 cells were transfected by standard calcium-phosphate transfection. After 24 h of transfection, the cells were irradiated (3000 rads)

with a Cobalt irradiator. The prestimulated bone marrow cells were then plated on top of the irradiated, retroviral producer cells along with 8 μg/mL polybrene (hexadimethrine bromide; Sigma). Transduced cells were then sorted in the core facility after 48 h.

Methylcellulose Colony-Forming Assay

Cells were sorted into Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS and then were plated into methylcellulose by using MethCult 3434 medium (Stem Cell Technologies), which contains the recombinant cytokines, stem cell factor, IL-3, IL-6 and erythropoietin (EPO). Seven days later, colonies of more than 50 cells were counted and examined for BEX expression by using an inverted fluorescent microscope. Representative colonies were picked, and cells were cytospun onto microscopic glass slides. Wright-Giemsa staining was performed on the slides to examine cell morphology.

GST Fusion Proteins and EMSA

GST fusion constructs were generated by using pGEX5-N1 vector and were transformed into the BL21 strain of *Escherichi coli*. GST proteins were purified as previously described (Shi *et al*, 1999). DNA probes (50 000 cpm/binding reaction) consisted of PCR-generated, gel-purified, end-labeled oligonucleotides. EMSA was performed as described (Chang *et al*, 1995). Briefly, DNA-binding reactions were performed at 4°C for 30 min and contained purified GST fusion proteins in a total volume of 20 μl containing 2 μg poly [d(I-C)], 75 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM Tris-HCl (pH 7.5), 6% glycerol, 2 μg BSA and 50 000 cpm of DNA probe. Binding reactions were subject to

nondenaturing electrophoresis on a 6% polyacrylamide gel (Acrylamide:Bis-Acrylamide, 29:1) in 0.25X TBE buffer at 10 mA per gel. Dried gels were autoradiographed at -80°C overnight.

Plasmid and Constructs

Nup98-Hoxa9 cDNA was a generous gift from Nakamura. *Nup98b-Hoxa9* was generated by PCR mutagenesis. Both *Nup98-Hoxa9* chimeras were cloned into pcDNA3 vector tagged with HA. Different cDNAs as indicated in the figures were also cloned into parental vectors MSCV-IRES-BEX and MSCV-IRES-VEX. All genes were cloned into the *Eco*RI and *Xho*I sites upstream of the IRES sequence. si-RNA-GFP and si-RNA-Smad4 have been described elsewhere (Wan *et al*, 2004).

Cell Culture and Transfection

Ba/F3 cells were cultured in RPMI-1640 containing 10% FBS and 1 ng/mL recombinant murine IL-3 (Sigma, I4144). NIH/3T3 cells were cultured in DMEM containing 10% FCS and 4 mM L-glutamine. Ba/F3 cells were transfected by electroporation. Briefly, 107 cells in RPMI-1640 were mixed with 20-30 μg of DNA (typically 5 μg reporter, 2 μg prl-0 to normalize, expression constructs and pcDNA3 empty vectors to an equivalent total) and electroporated in a 4-mM gap cuvette (Bio-Rad) at 350 V, 950 μf. NIH/3T3 cells were transfected by Lipofectamine according to the manufacturer's suggestions.

Western-blotting analysis and RT-PCR

Western-blotting analysis of cell lysates was performed as described previously (Wan *et al*, 2004). All blots were developed by the enhanced chemiluminescence technique (Amersham, Little Chalfont, UK). In RT-PCR analysis, total RNA was isolated from cultured cells by using RNA STAT-60 (Tel-test, INC). One microgram of total RNA was used for the synthesis of first-strand cDNA by using the Superscript preamplification system (Life Technologies). Primers used were as follows: Smad4: forward, 5'-TGGCC-TGATCTTCACAAAAA-3', and reverse, 5'-CACAGTGTTAATCCTGAG-AGAT-3'; GAPDH: forward, 5'-TAAAGGGCATCCTGGGCTACACT-3', and reverse, 5'-TTA-CTCCTTGGAGGCCATGTAGG-3'; Nup98-Hoxa9: forward, 5'-AGCTCCTC-CACCACTAATTCAGGCTTT-3', and reverse, 5'-AGAGAAGGCGCCTTCGCTGGG-TTG-3'; endogenous Hoxa9: forward, 5'-CTACGTGCACCCCCAGGCGC-3', and reverse, 5'-CTTCTGGCCGACAGCGGTTCAGGTTTA-3'.

Luciferase Assays and Statistical Analysis

Luciferase activities were assayed with the Dual-Luciferase assay kit (Promega, Madison, WI) according to the manufacturer's directions. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.

ChIP Assay

NIH/3T3 cells were seeded in 100-mm plates at 40% confluency and were transfected with pcDNA3, pRK5-Smad2-flag, pRK5-Smad4-flag and Hoxa9-luc promoter.

After transfection, cells were infected with virus-containing supernatant for 18-24 h with 8 μg/mL polybrene. Fresh medium was used. After 24 h, cells were cross-linked with 1% formaldehyde for 25 min at room temperature, and glycine (125 mM) was added for 10 min at room temperature to quench the formaldehyde. The cells were washed twice with ice-cold PBS and lysed in 500 mL of lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP-40, protease inhibitors) for 30 min on ice in 0.4 mL nuclear lysis buffer (50mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors). The samples were sonicated four times for 10 sec (60% duty cycle). The average DNA fragments ranged from 300 to 1000 bp. The lysates were then clarified by centrifugation at max speed for 10 min at 4°C. 2% of the samples were saved as input control and the rest were diluted 10 times in ChIP dilution buffer (15 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, and protease inhibitors). Samples were then incubated with antibodies overnight at 4°C. Immunoprecipitation was carried out by using Protein G-sepharose beads for 1 h at 4°C. Immune complexes were washed consecutively for 10 min with each of the following solutions: low-salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.10% SDS and 2 mM EDTA), high-salt wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.10% SDS and 2 mM EDTA), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 1% SDS) and twice with TE. Complexes were then eluted twice at 65°C for 10 min in elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS). Immunoprecipitated DNA was reversed cross-linked at 65°C overnight and purified by using a PCR purification kit (Qiagen). Two microliters of purified DNA were subjected to PCR for 30 cycles. Primers used for amplication of Hoxa9 promoter were: forward, 5'-

ACCCCATCGTAGAGCGGCACGA-3', and reverse, 5'-GGAAGTACAGTCACCTA-ATAAGTTGCCG-3'. PCR products were dissolved in 2% agarose gel containing EB.

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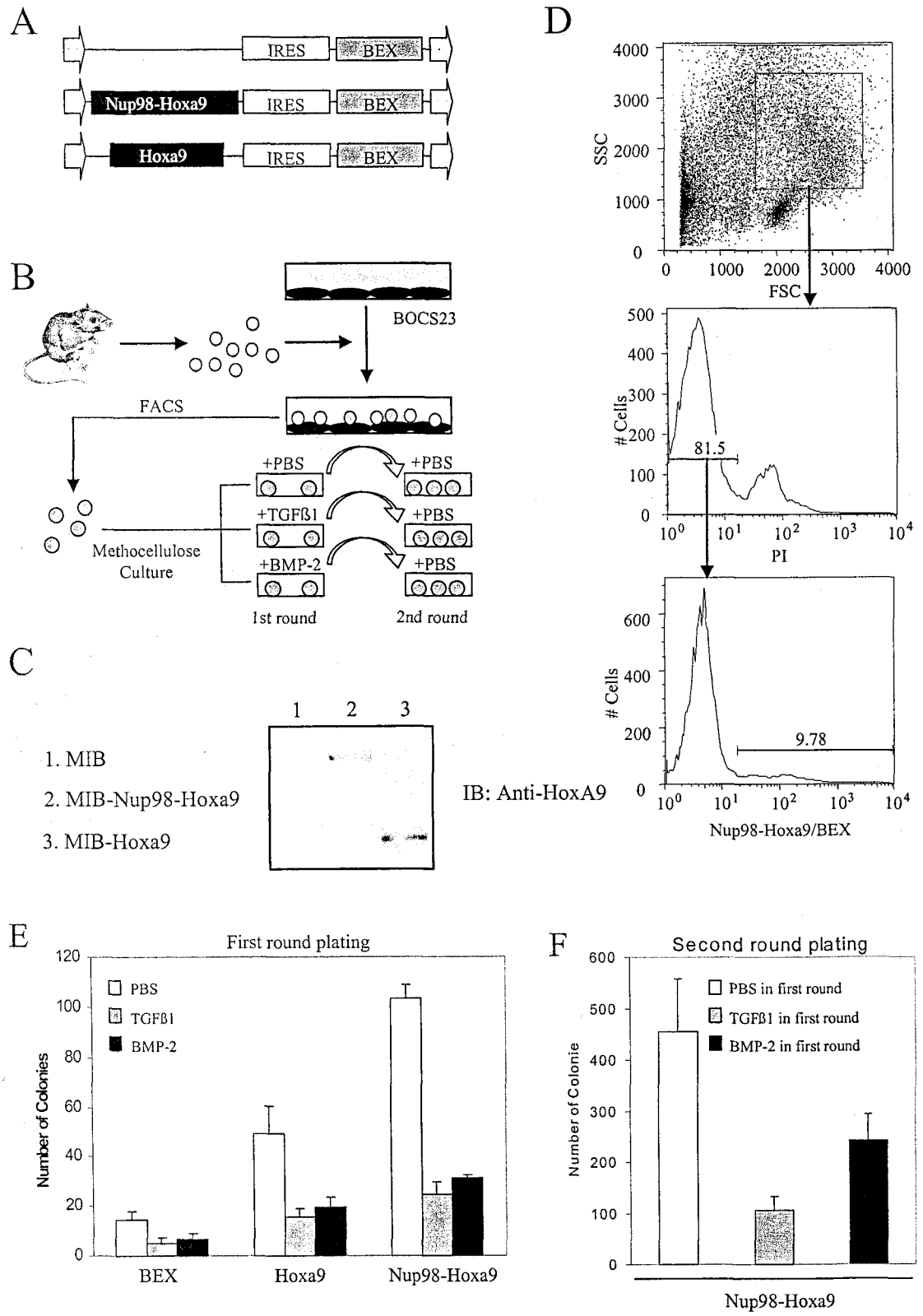
Figure 1 TGFβ/BMP inhibits bone marrow transformation capability of Hoxa9 or Nup98-Hoxa9. (**A**) Diagram of retroviral constructs expressing Hoxa9 and Nup98-Hoxa9 generated in MSCV. MSCV consists of long terminal repeat, IRES, and BEX. (**B**) Schematic presentation of retroviral transduction procedures. Bone marrow cells were purified from 5-fluorouracil-injected C57BL/6-Ly5.2 mice and infected through cocultivation with transfected BOSC 23 retrovirus packaging cells for 24-48 h. BEX-positive cells were isolated by FACS and grew in methylcellulose culture with various treatments as indicated. (**C**) Western blot analysis of BOSC 23 cells transfected with MIB-Hoxa9 or MIB-Nup98-Hoxa9 as detected with an anti-Hoxa9 polyclonal antibody. (**D**) Bone marrows were gated on myeloid cells by forward scatter (FSC) and side scatter (SSC) and on propidium iodide (PI)-negative cells. Histograms indicate the percentage of BEX positive cells, which were isolated by FACS. (**E**) Colony numbers generated in the first plating of 2600 transduced bone marrow cells are shown. TGFβ1 (2 ng/mL) and BMP-2 (300 ng/mL) were used for treatment as indicated. Data presented are an average of at least three independent experiments with error bars. (**F**) Replating of 2600 transduced bone marrow cells harvested from first-round plating. Data presented are an average of at least three independent experiments with error bars.



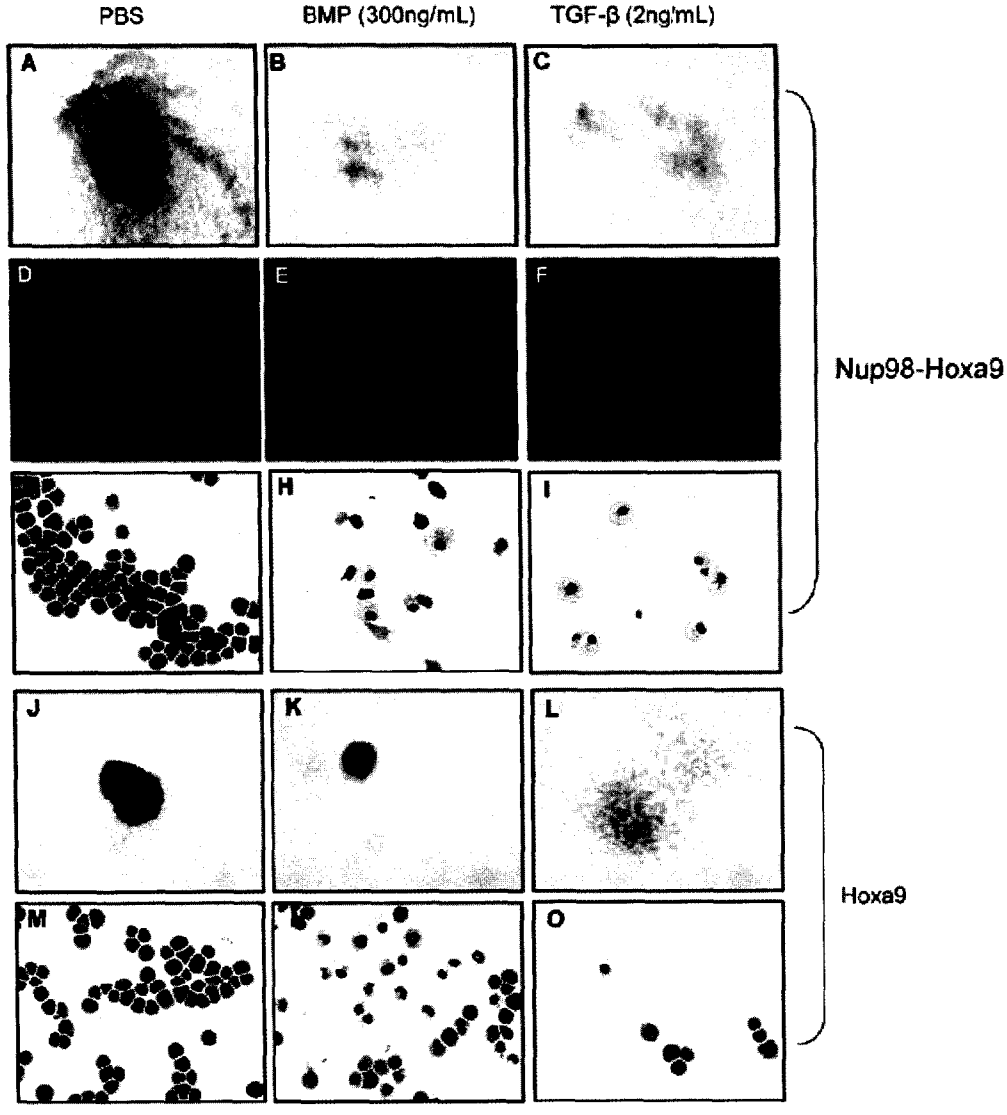


Figure 2 TGFβ/BMP-induced myeloid differentiation of bone marrow cells immortalized by Hoxa9 or Nup98-Hoxa9. (**A-C, J-L**) Morphology of colonies formed in methylcellulose assays. Original magnification ×5. (**D-F**) Fluorescence photomicrographs of colonies. (**G-I, M-O**) Wright-Giemsa-stained cytospin preparation of cells derived from the second round of the colonies. Orginal magnification ×40.

Figure 3 TGFβ/BMP represses Nup98-Hoxa9 transcription through Smad4-mediated inhibition of Nup98-Hoxa9 binding to DNA. (**A**) The luciferase activities under the control of Hoxa9-luc were measured in the NIH/3T3 cells expressing Nup98-Hoxa9, in the absence or presence of PBX1a, in combination with Smad1, Smad4 or ALK3 as indicated. Each bar represents the mean and the standard deviations of at least three independent experiments. (**B**) Effect of TGFβ on Nup98-Hoxa9-induced transcription, in the absence or presence of PBX1a, on *Hoxa9* promoter measured by *Hoxa9*-luc transcription reporter assay in Ba/F3 cells. Each bar represents the mean and the standard deviations of at least three independent experiments. (**C**) Effect of loss of Smad4 by si-RNA on Nup98-Hoxa9 transcription on Hoxa9 promoter. Western blot (bottom) confirms the absence of Smad4 in si-RNA Smad4 transfected cells. (**D**) EMSA. Nup98-Hoxa9 alone dose-dependently binds to *Hoxa9* promoter (lane 2-4). PBX1a supershifts this protein-DNA complex (lanes 5-7). Increasing amount of specific (lanes 8-10) but not of nonspecific cold competitors (lanes 11-13) at 0, 50× and 100× excess of radiolabeled probe inhibits binding of Nup98-Hoxa9 to *Hoxa9* promoter probe. (**E**) EMSA. Smad4 dose-dependently inhibits the DNA-binding ability of Nup98-Hoxa9 to *Hoxa9* promoter probe, whether in the absence or in the presence of PBX1a.

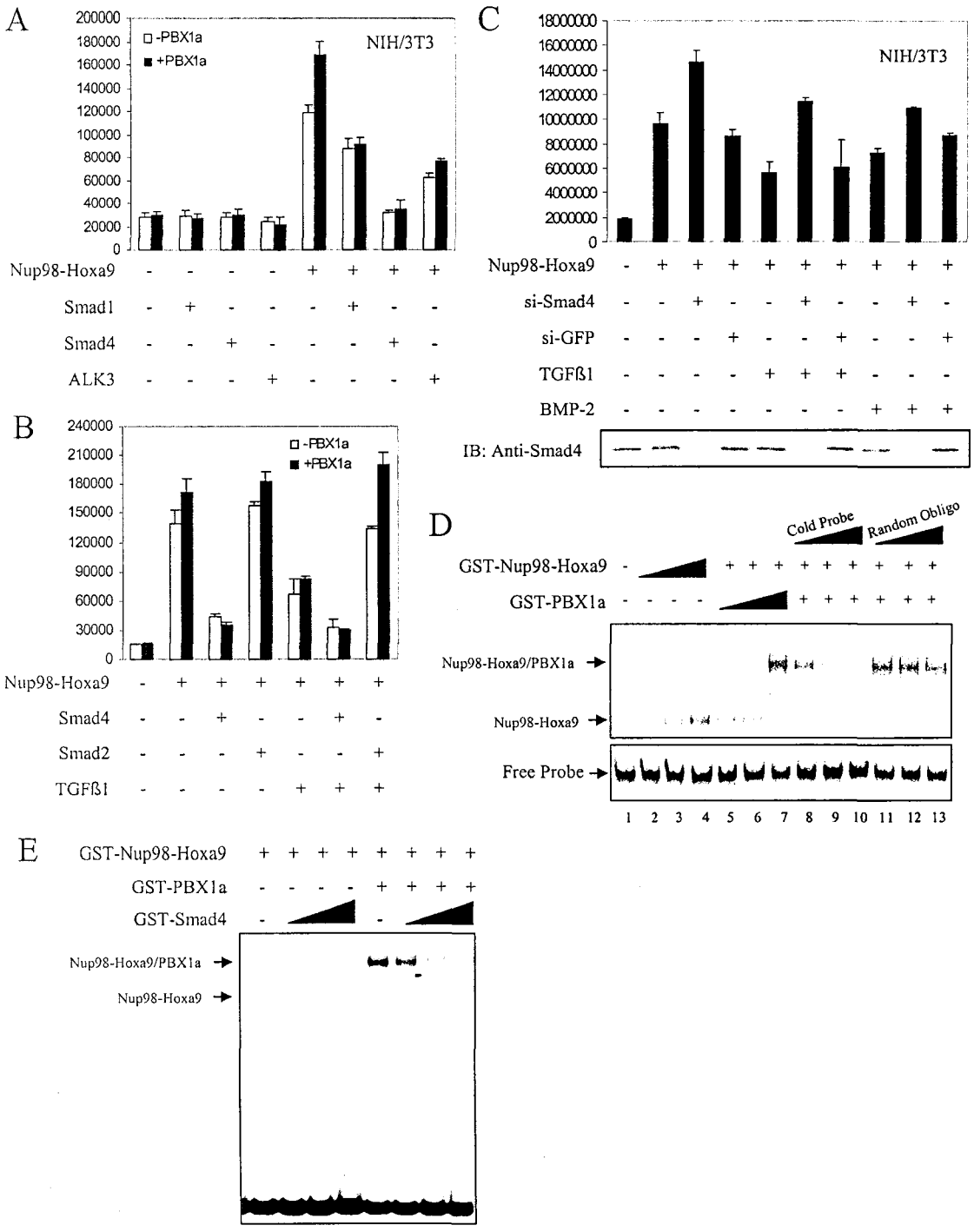
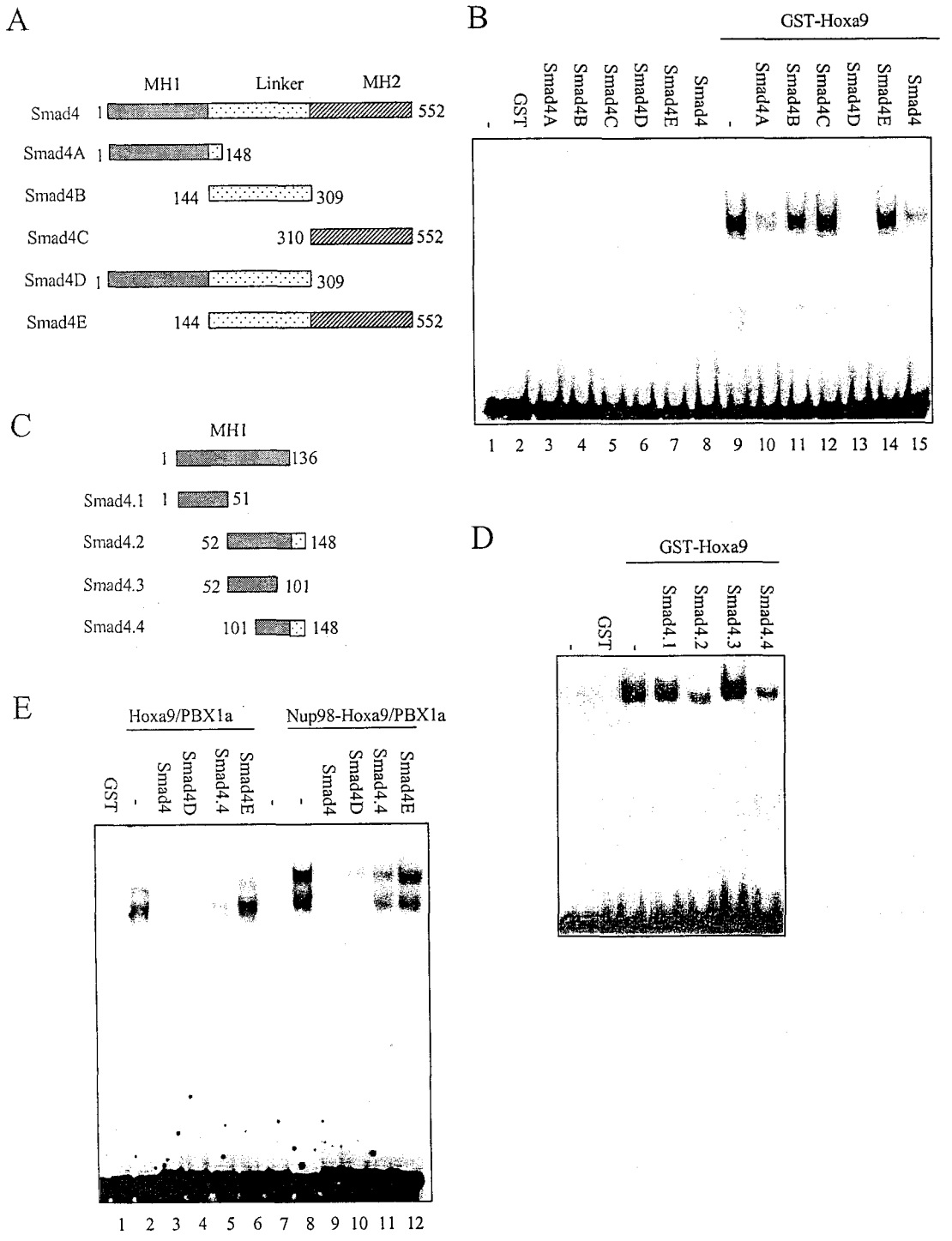


Figure 4 Amino-terminal domain of Smad4 interacts with Hoxa9. (**A**) Schematic representation of Smad4 deletion constructs. (**B**) EMSA was performed by using purified GST fusion proteins illustrated in panel A and 32^{P}-labeled probe. (**C**) Schematic representation of Smad4 MH1 plus partial linker region deletion constructs. (**D**) EMSA was performed by using purified GST fusion proteins illustrated in panel B and 32^{P}-labeled probe. (**E**) EMSA was performed by using purified GST fusion proteins as indicated and 32^{P}-labeled probe.



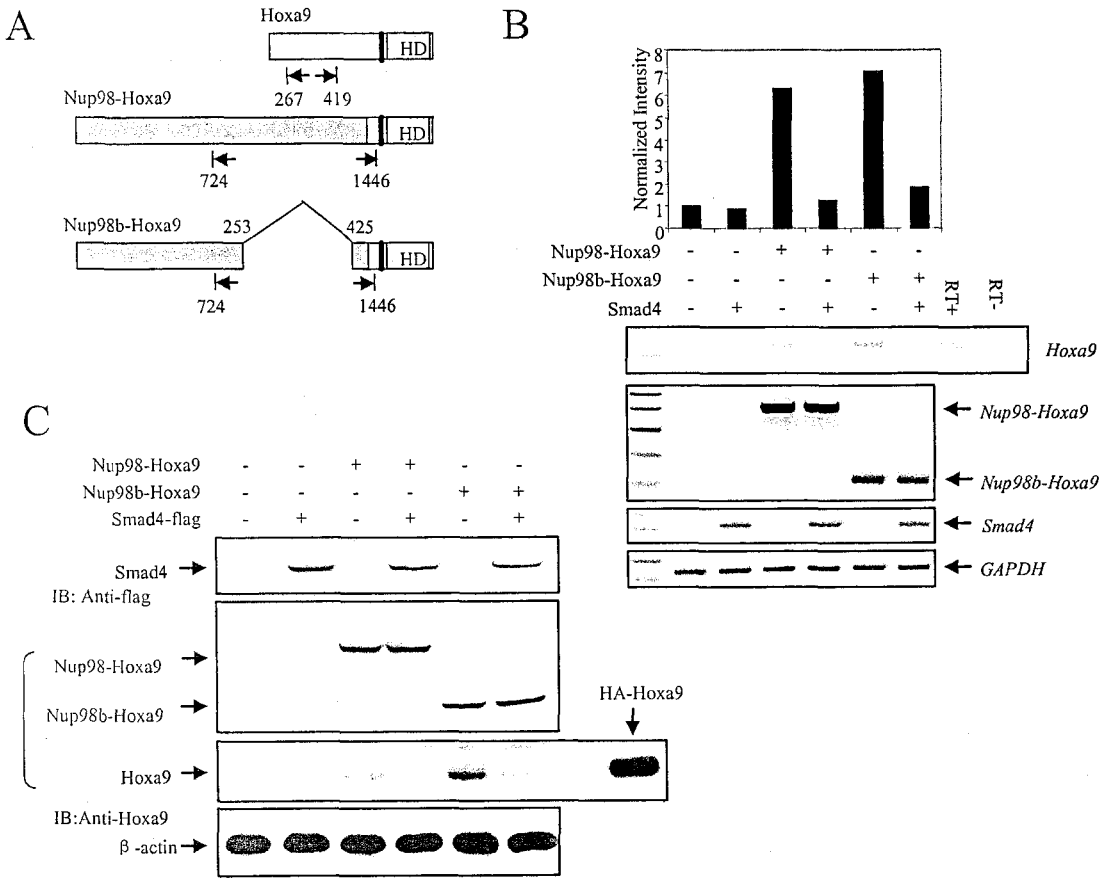
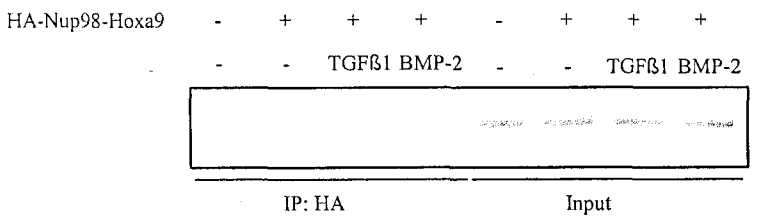


Figure 5 Smad4 inhibits endogenous Hoxa9 expression induced by Nup98-Hoxa9. (**A**) Schematic representation of primers used for RT-PCR analysis. (**B**) RT-PCR analysis of the *Hoxa9* gene expression in NIH/3T3 cells. NIH/3T3 cells were infected with retrovirus vectors encoding GFP, Nup98-Hoxa9 or Nup98b-Hoxa9 and transfected with vectors encoding Smad4-flag or empty vector (EV) as control. Total cellular RNA was isolated from parent cells. After reverse transcription, PCR was performed with specific primers as indicated. A 1-kb DNA ladder was used for size markers (Fisher Scientific). The density of the bands was quantitated with Amersham Pharmacia Biotech Storm System and image analysis software. (**C**) Western blot analysis of endogenous Hoxa9 expression. Overexpressed HA-tagged Hoxa9 was used as positive control to indicate the size of Hoxa9.

A



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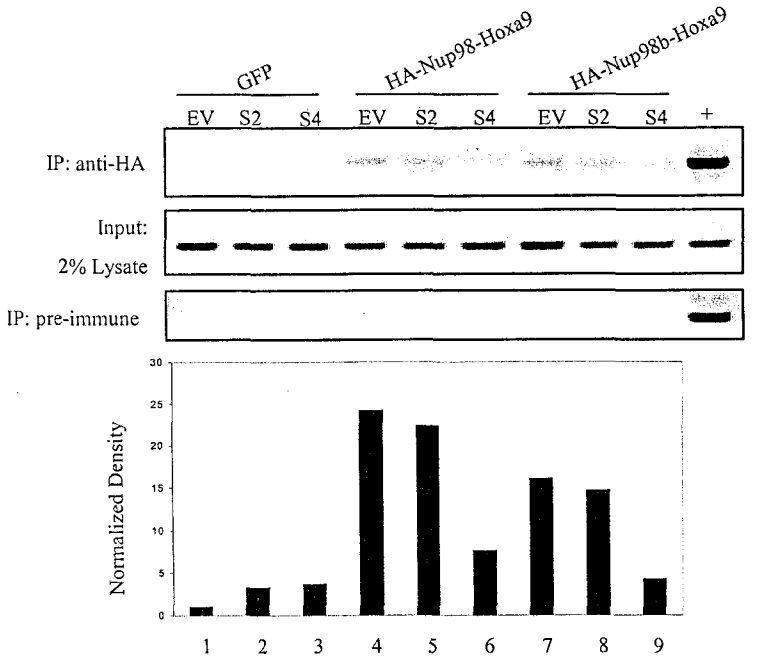
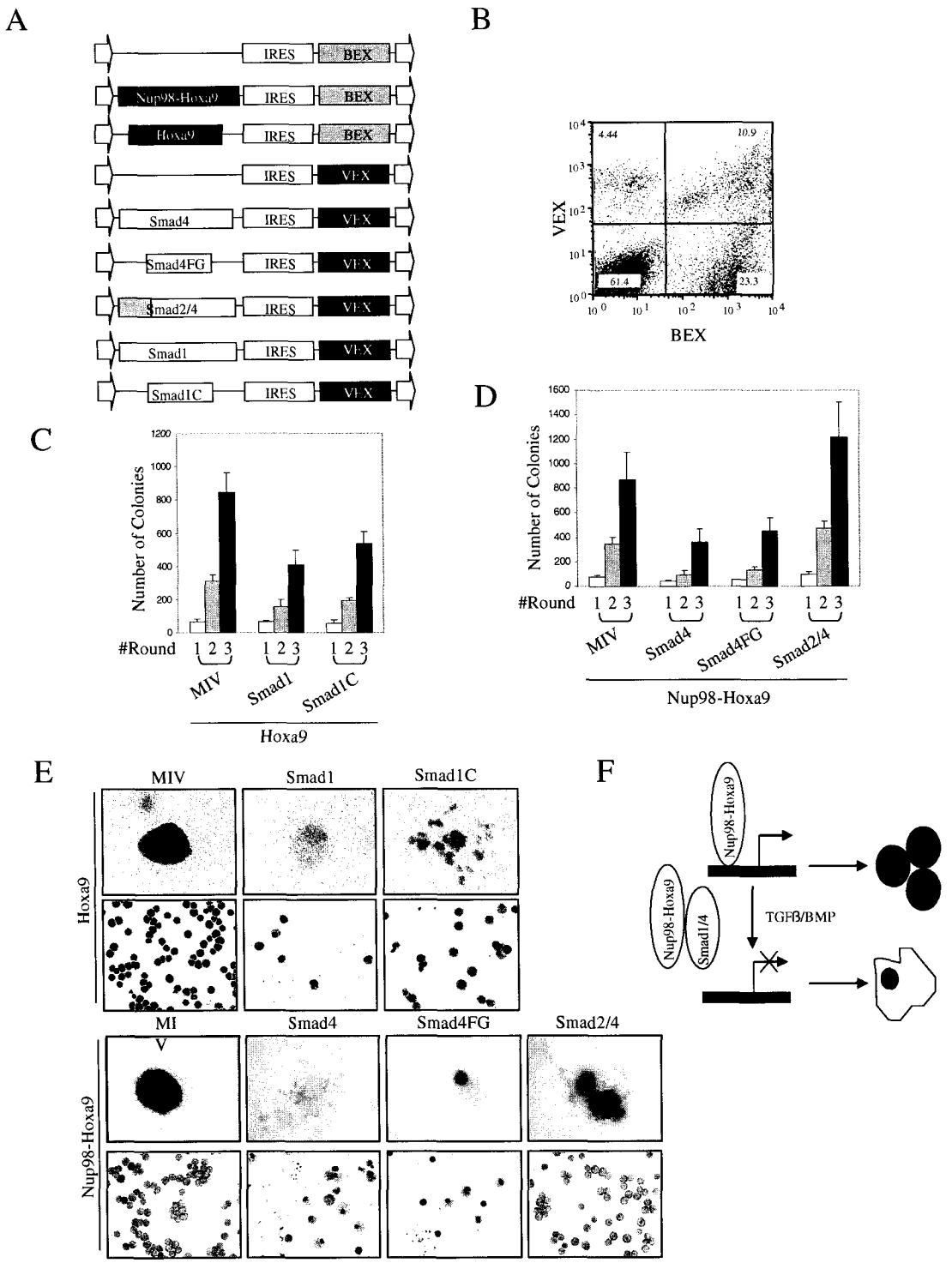


Figure 6 TGF-β/BMP inhibits binding of Nup98-Hoxa9 to DNA *in vivo*. (**A**) NIH/3T3 cells were treated with or without TGFβ1 or BMP-2 for 2 h, and ChIP assays were performed with the indicated antibody and PCR primers. (**B**) ChIP assays. Overexpression of Smad4 but not Smad2 inhibited the binding of Nup98-Hoxa9 to *Hoxa9* promoter. The density of the bands was quantitated by using Amersham Pharmacia Biotech Storm System and image analysis software.

Figure 7 Smads inhibit bone marrow transformation capability of Hoxa9 or Nup98-Hoxa9. (**A**) Schematic representation of retrovirus constructs generated in MSCV. (**B**) Representative FACS plot indicates the BEX and VEX double positive cell population. (**C**) Smad1 inhibits the replating ability of bone marrow cells by Hoxa9. Colony numbers generated in the first, second and third round of plating of 3000 double-transduced bone marrow cells isolated from FACS are shown. Data presented are an average of at least two independent experiments with error bars. (**D**) Smad4 inhibits transformation capability of bone marrow cells by Nup98-Hoxa9. Colony numbers generated in the first, second and third round of plating of 3000 double-transduced bone marrow cells isolated from FACS are shown. Data presented are an average of at least two independent experiments with error bars. (**E**) Morphology of colonies formed in methylcellulose assays (upper panel). Wright-Giemsa-stained cytospin preparation of cells from methylcellulose colonies. (lower panel). (**F**) Model illustrates the regulation of Hoxa9 activity of TGFβ/BMP. During undifferentiated stage of myeloid cells, Hoxa9 occupies target gene promoter and regulates its transcription. Upon TGFβ/BMP stimulation, Smad1/4 translocate into nucleus, where they interact with Hoxa9 or its fusion proteins and inhibit their DNA-binding activity, resulting in myeloid differentiation.



ACUTE MYELOGENOUS LEUKEMIA-DERIVED SMAD4 MUTATIONS TARGET THE PROTEIN TO UBIQUITIN-PROTEOSOME DEGRADATION

by

LEI YANG, NING WANG, XU CAO AND MEI WAN

Submitted to *Human Mutation*

Format adapted for dissertation

Abstract

Disruption of transforming growth factor β (TGFβ) signaling contributes to the formation of human hematological malignancies. Smad4, a tumor suppressor, functions as an essential intracellular signal transducer of the TGFβ signaling pathway. Recent studies demonstrated that some tumor-derived mutations of Smad4 are associated with protein instability, but the precise mechanism by which mutated Smad4 protein undergoes rapid degradation remains to be elucidated. A missense mutation of the Smad4 gene in the MAD homology 1 (MH1) domain (P102L) and one frame shift mutation resulting in termination in the MAD homology 2 (MH2) domain (Δ483-552) have been identified in acute myelogenous leukemia. Whether the protein instability of these Smad4 mutants is one of the major contributors to TGFβ signaling disruption in acute myelogenous leukemia is not known. Here we report that these two acute myelogenous leukemia-derived Smad4 mutants are degraded rapidly in comparison with their wild-type counterpart. We demonstrated that both mutated proteins exhibit enhanced polyubiquitination and proteosomal degradation. Importantly, we found that β-transducin-repeat-containing protein 1 (β-TrCP1), an F-box protein in the Skp1-cullin-F box protein (SCF) E3 ligase complex, directly interacts with and acts as a critical determinant for the protein degradation of both mutated Smad4 proteins. In addition, small interference RNA (siRNA)-triggered endogenous β-TrCP1 suppression increased the protein expression level of both overexpressed Smad4 mutants and endogenous mutated Smad4 protein in acute myelogenous leukemia cells. These data suggested that mutated Smad4 protein undergoes rapid degradation in acute myelogenous leukemia cells via $SCF^{\beta\text{-}TrCP1}$ E3 ligase-mediated protein ubiquitination and subsequent proteosomal degradation pathway.

Introduction

Transforming growth factor β (TGFβ) signaling plays a central role in regulation of a broad range of cellular responses in a variety of organisms, including cell proliferation, recognition, differentiation, apoptosis and specification of developmental fate (Blobe *et al*, 2000; Derynck and Zhang, 2003). Loss of TGFβ signaling has been implicated in malignant transformation of various tissues (Massague, 1998; Yue and Mulder, 2001). Recent studies have demonstrated that TGFβ plays important roles in hematopoiesis by regulating the proliferation and differentiation of hematopoietic cells (Fortunel *et al*, 2000; Kim and Letterio, 2003). Inhibition of TGFβ signaling by an oncogene is involved in formation of human hematological malignancies (Kurokawa *et al*, 1998), suggesting that the TGFβ signaling pathway has a tumor suppression effect in hematopoietic cells.

The Smad family proteins mediate TGFβ signaling from the cell membrane to the nucleus and are therefore critical components of the TGFβ signaling pathway. Smad4, characterized as a key downstream determinant in TGFβ signaling (Massague, 1998), is a transcriptional comodulator capable of integrating cellular responses to multiple signalling cascades. Smad4, also known as DPC4 (deleted in pancreatic carcinoma locus 4), was originally isolated from human chromosome 18q21.1 as a tumor suppressor gene for pancreatic cancer (Hahn *et al*, 1996). Mutations in Smad4/DPC4 are frequently found in tumors such as pancreatic adenocarcinomas and colorectal cancer (Hahn *et al*, 1996; Schutte *et al*, 1996), suggesting a possible pivotal role of Smad4 in TGFβ functional loss in tumorigenesis. In fact, defects in Smad4 play a significant role in the malignant progression of tumors. Tumors lacking functional Smad4 tend to be more invasive and an-

giogenic and consequently are more likely to form metastatic lesions (Sunamura *et al*, 2002).

Recently, it has been demonstrated that one missense mutation in the MAD homology 1 (MH1) domain (P102L) and one frameshift mutation resulting in termination of the MAD homology 2 (MH2) domain (Δ483-552) in the Smad4 gene are associated with the pathogenesis of acute myelogenous leukemia, indicating that disruption of the TGFβ signaling pathway could lead to acute myelogenous leukemia (Imai *et al*, 2001). The mechanisms underlying the Smad4 inactivation caused by mutations in cancers are not fully understood. Recently, some mutations in Smad4 have been shown to target the proteins for rapid degradation via the ubiquitin-proteosome pathway (Xu and Attisano, 2000; Maurice *et al*, 2001; Moren *et al*, 2003), indicating that protein instability of Smad4 may contribute to the loss in cellular responsiveness to TGFβin tumors. Although it has been shown that the mutations identified in acute myelogenous leukemia lead to functional inactivation (Imai *et al*, 2001), it is possible that these two mutations cause Smad4 protein instability. Since cellular responses are highly sensitive to the level of Smad protein (Shimizu *et al*, 1999), studies of the protein degradation of mutated Smad4 should be instructive for further understanding of the role of Smad4 in human acute myelogenous leukemia.

Ubiquitin-dependent degradation by the 26S proteosome has emerged as a central mechanism to control protein turnover. A polyubiquitin chain is built onto one or multiple lysine residues of a substrate to target it for capture and degradation by the 26S proteosome (DeSalle *et al*, 2001; Pickart *et al*, 2001). The polyubiquitination reaction requires the coordination of three classes of different enzymes: E1, E2 and E3. As a key

component of ubiquitination pathways, the ubiquitin ligases (E3 ligases) control both the specificity and the timing of substrate ubiquitination. Several classes of E3 ligases have been identified, among which are Cullin-based E3 ligases. The Skp1–Cul1–F-box protein (SCF) complex is so far the best-characterized group of Cullin-based ligases (Deshaies *et al*, 1999; Jackson *et al*, 2002). The variable F-box protein serves as the substrate recognition subunit. The β-transducin repeat-containing protein (β-TrCP), members of the Fbw subfamily of F-box proteins, are known to recognize the phosphorylated $DSG(X)_{2+n}S$ motif within their substrates, which include many important cellular regulatory proteins (Yaron *et al*, 1998; Fuchs *et al*, 1999; Hart *et al*, 1999; Hatakeyama *et al*, 1999; Kitagawa *et al* 1999; Latres *et al*, 1999; Spencer *et al*, 1999; Suzuki *et al*, 1999; Winston *et al*, 1999; Lassot *et al*, 2001; Fong *et al*, 2002; Busino *et al*, 2003; Guardavaccaro *et al*, 2003; Jin *et al*, 2003; Kumar *et al*, 2003; Lang *et al*, 2003; Mantovani *et al*, 2003; Margottin-Goguet *et al*, 2003; Li *et al*, 2004; Watanabe *et al*, 2004; Zhou *et al*, 2004). Therefore, $SCF^{\beta\text{-}TrCP}$ E3 ligases ubiquitinate and regulate the stability of specific protein substrates and play a pivotal role in the regulation of cell division and various signal transduction pathways that are essential for many aspects of tumorigenesis.

Previously, we have identified $SCF^{\beta\text{-}TrCP1}$, a ubiquitin (E3) ligase, as a critical determinant for the protein degradation of Smad4 protein (Wan *et al*, 2004). We also demonstrated that $SCF^{\beta\text{-}TrCP1}$ is one of the contributors to protein rapid degradation of pancreatic tumor-derived Smad4 mutants (Wan *et al*, 2005). In this study, we examined the protein stability of Smad4 mutants found in acute myelogenous leukemia. We found that the Smad4 mutants exhibited significantly decreased protein stability. Importantly, we found that the F-box protein β-TrCP1 in SCF E3 ligase interacts with Smad4 and exhibits

stronger interaction affinity with the acute myelogenous leukemia-derived Smad4 mutants. Consequently, E3 ligase complex $SCF^{\beta\text{-}TrCP1}$ mediates ubiquitination of Smad4 mutants. When small interference RNA (siRNA)-induced F-box protein β-TrCP1 gene silencing was used, the protein steady-state level of Smad4 was elevated in acute myelogenous leukemia cells.

Results

Low Protein Expression Level of Mutated Smad4 Derived From Acute Myelogenous Leukemia

The stability of two mutated Smad4 proteins derived from acute myelogenous leukemia was examined. Flag-tagged Smad4 expression constructs harboring Smad4 mutations identified from acute myelogenous leukemia were generated, among which one is a point mutation in the MH1 domain (P102L) and the second is a deletion in the MH2 domain (Δ483-552) in Smad4 (Figure 1A). We first examined the expression levels of mutated Smad4 protein in 293T cells by transient transfection of the same amount of Smad4 wild-type (WT), P102L or Δ(483-552) plasmid, respectively. Figure 1B shows that P102L exhibits a much lower level of Smad4 protein expression compared with WT Smad4 protein (lane 2) and Δ(483-552), which almost lacks Smad4 protein expression (lane 3).

Instability of Mutated Smad4 Proteins Derived From Acute Myelogenous Leukemia

The observed low expression level of the mutated Smad4 suggested that the two mutations might lead to an increased turnover of the proteins. To investigate this possibil-

ity, COS-1 cells were transfected with WT Smad4 and the two mutated Smad4s. After 40 h, cycloheximide (80 μg/mL) was added to prevent further synthesis. Whole cell extracts were prepared from the cells at different times after cycloheximide addition, and the Smad4 protein was visualized by Western blotting. WT Smad4 was very stable and almost remained the same after 12 h of addition of cycloheximide (Figure 2A, lanes 1-5). The level of Smad4 P102L decreased much faster than that of WT Smad4 did (Figure 2A, lanes 6-10). The level of Smad4 Δ(483-552) rapidly decreased after the addition of cycloheximide, and the protein disappeared after 6 h (Figure 2A, lanes 11-15). The half-life of WT Smad4 was >12 h, that of P102L was ~5 h, and that of Δ(483-552) was ~3 h (Figure 1B). The results indicate that rapid protein degradation is the main contributor to protein loss in acute myelogenous leukemia-derived Smad4 mutations.

Smad4 Mutants Undergo Protein Degradation Through the Ubiquitin-Proteosome Pathway

To determine whether the two mutated Smad4 proteins are targeted for degradation via the ubiquitin-proteosome pathway, we examined the expression of Smad4 mutants by using MG-132, a highly specific proteosome inhibitor (Lee *et al*, 1998). 293T cells were transfected with different amount of plasmids (Flag-tagged WT Smad4 800 ng/dish, P102L 800 ng/dish and Δ(483-552) 6 μg /dish), and MG-132 was added. MG132 significantly elevated the Smad4 protein expression level in both point-mutated and deleted Smad4 transfected cells (Figure 3A, lanes 4 and 6) but not in WT Smad4 transfected cells (lane 2), indicating that the mutated Smad4 undergoes rapid degradation via the 26S proteosome.

We also examined whether the enhanced turnover observed in Smad4 mutants is mediated through ubiquitination. Individual mutation or WT Smad4 expression plasmids were cotransfected with or without ubiquitin expression plasmid in 293T cells. As shown in Figure 3B, WT Smad4 exhibits very little ubiquitination with ubiquitin overexpression (lane 2 vs. lane 1). Stronger ladders of high molecular weight, ubiquitin-conjugated Smad4 products were observed in both point-mutated and deleted Smad4 transfected cells (lanes 4 and 6). These results suggest that Smad4 harboring acute myelogenous leukemia-derived mutations is degraded more rapidly through the ubiquitin-proteosome pathway than their WT counterparts are.

Instability of Smad4 Mutants Mediated by $SCF^{\beta\text{-}TrCP1}$

Previously, we found that an E3 ligase complex $SCF^{\beta\text{-}TrCP1}$ is responsible for Smad4 degradation. F-box protein β-TrCP1 in this complex associates with Smad4, and $SCF^{\beta\text{-}TrCP1}$-overexpressing cells display increased ubiquitination and degradation of Smad4 (Wan *et al*, 2004). The low protein stability of acute myelogenous leukemia-derived Smad4 mutants raises the question of whether the mutated Smad4 protein interacts with β-TrCP1. To address this question, 293T cells were transfected with empty vector, WT Smad4 or mutated Smad4 plasmids individually, and immunoprecipitation assays were performed. Both WT and mutated Smad4 form an interaction complex with β-TrCP1 (Figure 4A). The results indicate that β-TrCP1 is the F-box protein that recognizes Smad4 P102L and Δ(483-552) protein.

Since we demonstrated that the use of vector-based siRNA was successful in inhibiting the expression of β-TrCP1 and enhancing the expression of endogenous Smad4

(Wan *et al*, 2004), we asked whether this RNAi functioned to increase the stability of acute myelogenous leukemia-derived Smad4 mutants. WT Smad4 and mutated Smad4 were cotransfected with either BS/U6/β-TrCP1 (si-β-TrCP1) or BS/U6 (empty vector control) plasmids in 293T cells. Si/β-TrCP1 increased the expression level of mutated Smad4 (Figure 4B, lanes 4 and 6), suggesting that β-TrCP1 is a key factor in mediating the instability of acute myelogenous leukemia-derived Smad4 mutants.

β-TrCP1 Gene Silencing Elevated Smad4 Protein Level in Acute Myelogenous Leukemia Cells

CTV-1 acute myelogenous leukemia cells, which harbor an MH2 deletion (Δ483-552), provide a useful tool with which to investigate the effect of β-TrCP1 on Smad4 mutant degradation. A 1:10 amount of pCDNA3-GFP was cotransfected with BS/U6/β-TrCP1 (si-β-TrCP1) or BS/U6 (empty vector control), and immunofluorescence assays were performed. Since GFP and si-β-TrCP1 were cotransfected at a ratio of 1:10, the cells showing green light (GFP transfected) should also express si-β-TrCP1. Figure 5A demonstrated that cells expressing GFP exhibited much less expression of β-TrCP1 in si-β-TrCP1-transfected cells (lower panel showing in red), whereas GFP had no significant effect in empty vector-transfected cells (upper panel). The result indicates that si-β-TrCP1 successfully suppressed β-TrCP1 expression. Importantly, cells expressing GFP exhibited a level of Smad4 protein expression that was significantly elevated (Figure 5B, lower panel showing in red) in comparison with that in non-GFP-expressed cells or in cells expressing siRNA empty vector (upper panel). This result indicated that β-TrCP1 suppression in cells increased Smad4 expression.

The efficiency of transient transfection of siRNA into CTV-1 cells is relatively low, and a Western blot could hardly show the differences. We therefore generated retrovirus constructs that contain ΔU/U6/GFP (si-GFP, irrelevant siRNA control) and ΔU/U6/β-TrCP (si-β-TrCP1). High infection efficiencies of the GFP in CTV-1 cells were obtained (Figure 5C). Consistent with the findings illustrated in Figure 5A and B, si-β-TrCP1 significantly increased Smad4 expression level in CTV-1 cells as determined by both immunohistochemistry and Western blot as demonstrated in Figure 5D and E. Taken together, these results demonstrate that β-TrCP1 is a critical determinant regulating mutated Smad4 protein stability in acute myelogenous leukemia cells.

Discussion

Loss of Smad4 is associated with poor prognosis of human cancers (Sunamura *et al*, 2002). Recently, several lines of evidence have shown that Smad4 mutations identified in human cancer patients are rapidly degraded via the ubiquitin-proteosome pathway (Xu *et al*, 2000; Maurice *et al*, 2001; Moren *et al*, 2003), indicating that protein instability of Smad4/DPC4 contributes significantly to a loss in cellular responsiveness to TGFβ in tumorigenesis. In the present work, we provide evidence that acute myelogenous leukemia-derived Smad4 mutations target the protein to the ubiquitin-proteosome pathway, thereby possibly playing an important role in acute myelogenous leukemia progression. Importantly, our data give us new insight into the mechanism through which Smad4 point mutations undergo rapid degradation in acute myelogenous leukemia cells. We found a strong association between $SCF^{\beta\text{-}TrCP1}$ and the point-mutated Smad4 protein. Conse-

quently, the ubiquitination of the point-mutated Smad4 is significantly higher than that of WT Smad4 is.

The detailed mechanisms by which the β-TrCP1 more easily degrades these mutated Smad4 remain to be determined. It has been demonstrated that an SCF complex containing β-TrCP as the F-box protein (SCF $^{\beta\text{-TrCP}}$) recognizes phosphorylated substrates and mediates their protein degradation. One possibility is that some point-mutated Smad4 might be phosphorylated easily by certain protein-associated kinases. If this is the case, the mutated protein would be much easier for β-TrCP1 to recognize. Smad4 is a multi-functional protein that contains a DNA-binding MH1 domain, a protein-interaction MH2 domain and a linker region (Massague *et al*, 1998). A mutational hot spot within the MH2 domain corresponding to codons 330 to 370 was termed the mutation cluster region (MCR) in a recent work (Iacobuzio-Donahue *et al*, 2004). The immunohistochemical studies indicated that the majority of missense mutations inactivate Smad4 by protein degradation, whereas carcinomas with missense mutations within the MCR retain Smad4 protein stability. It would be interesting to examine whether other mutations within MCR would affect their recognition by the F-box protein β-TrCP1.

Functional inactivation caused by these Smad4 mutations, especially deletions in the MH2 domain, cannot be excluded as a possibility. Some Smad4 mutations lost the ability to form homo- or heterotrimeric Smad complexes (Shi *et al*, 1997) or increased auto-inhibition of the Smads by stabilizing intramolecular interactions between the MH1 and MH2 domains (Hata *et al*, 1997). In contrast, Xu *et al* (2000) reported that some Smad mutations do not interfere with most of the functions of these tumor suppressor proteins but instead inactivate the proteins by inducing targeting for the ubiquitin-

proteosome system. The two Smad4 mutations identified in acute myelogenous leukemia have been reported to disrupt TGFβ signaling (Imai *et al*, 2001). However, cellular responses are highly sensitive to the level of Smad protein (Shimizu *et al*, 1999). Therefore, we suggest that the reduction in the steady-state levels of these Smad proteins, which is insufficient to mediate TGFβ signaling, may be the primary defect in acute myelogenous leukemia. Smad4 is a key signaling molecule of TGFβ signaling and therefore is an attractive therapeutic target in oncology because of its strong cancer suppression activity. Our study and other publications indicate that it is becoming apparent that the instability of Smad4 proteins is a general phenomenon in cancer cells harboring point mutations in Smad4. Therefore, further characterization of the structure and function of Smad4/$SCF^{\beta\text{-}TrCP1}$ interaction might lead to the discovery of new targets for anticancer drug discovery.

Materials and Methods

Constructs

The wild-type Smad4 expression plasmid was amplified by PCR by using pRK5-Smad4 as the template and was subcloned into the *Eco*RI and *Sal*I sites of the pCMV5B vector with a Flag tag at the amino-terminus. Flag-tagged point mutated (P102L) and deleted (Δ483-552) Smad4 expression plasmid was constructed by using site-directed mutagenesis with the Quick Change kit (Stratagene) according to the manufacturer's protocol.

GFP and β-TrCP1 siRNA plasmids were generated by using BS/U6 vector (Sui *et al*, 2002). Briefly, a 22-nt oligo (oligo 1) corresponding to nucleotides 106-127 of GFP or nucleotides 453-474 of the human β-TrCP1 coding region was first inserted into the

BS/U6 vector digested with *Apa*I (blunted) and *Hind*III. The inverted motif that contains the 6-nt spacer and five Ts (oligo 2) was then subcloned into the *Hind*III and *Eco*RI sites of the intermediate plasmid to generate BS/U6/GFP and BS/U6/β-TrCP1. For cloning into retrovectors, the U6 promoter region plus the siRNA cassette were digested with *Xba*I and cloned into the *Xba*I site of a retrovirus vector, ΔU3.

Antibodies

Anti-human Smad4 monoclonal antibody, anti-human β-TrCP polyclonal antibody, anti-ubiquitin polyclonal antibody, anti-human p21 monoclonal antibody, anti-human p15 monoclonal antibody and anti-human caspase 3 monoclonal antibody were purchased from Santa Cruz Biotechnology. Anti-Flag and anti-HA monoclonal antibodies were purchased from CPR Inc. Anti-Myc and anti-β-actin monoclonal antibodies were purchased from Sigma-Aldrich.

Cell Culture and Transfection

293T, COS-1 cells were obtained and cultured according to protocols from the American Type Culture Collection (ATCC). CTV-1 cells were maintained in RPMI-1640 with 10% fetal calf serum (FCS). 293T cells were transfected with Tfx^{TM}-20 Reagent (Promega, WI) according to the manufacturer's instructions. For transient transfection, CTV-1 cells were transfected by the electroporation method (Schakowski *et al*, 2004) by using an electroporation apparatus (GENE PULSER^{R} II, Bio-Rad). In brief, 5×10^6 cells were suspended in 500 μL complete medium, mixed with 30 μg of plasmid in a 4-mm electroporation cuvette and incubated on ice for 10 min. After electroporation with a sin-

gle pulse, the cells were transferred into complete medium at a density of 1×10^6cells per milliliter.

Immunoprecipitation and Western blotting Analysis

Cells were lysed in buffer A (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 50mM Tris buffer, pH7.5, 1mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/ml leupeptin). For immunoprecipitation, lysed cells were incubated with different antibodies as indicated in the figures and were incubated at 4°C for 3h. Protein G plus agarose beads (Amersham) were added to the antigen-antibody mixture, which was then further incubated overnight. Immunocomplexes were washed thoroughly with buffer A containing 0.1% SDS and were separated on a 12% SDS-polyacrylamide gel and blotted to nitrocellulose. All blots were developed by the enhanced chemiluminescence (ECL) technique (Amersham).

In Vivo Ubiquitination Assays

Forty hours after transfection, cell lysates were immunoprecipitated by using antibody against Flag, boiled in SDS, and then reprecipitated prior to immunoblotting. To detect ubiquitination of precipitated Smad4, Western blot analysis was performed with anti-HA antibody.

Immunofluorescence

CTV-1 cells were cotransfected with GFP plasmid (2 μg) and β-TrCP1 RNAi plasmid (20 μg) or GFP plasmid (2 μg) with BS/U6 vector (20 μg). After 48h, cells were

harvested and reseeded on the poly-L-lysine (Sigma) pretreated glass coverslips. Cells were fixed in 3.7% formaldehyde, and permeabilized with 0.1% Triton X-100. After pre-blocking, the cells were treated with the anti-Smad4 or anti-β-TrCP1 antibodies and then incubated with Texas-red-conjugated donkey anti-mouse IgG or Texas-red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) as secondary antibodies. Images were observed with a fluorescence microscope (Olympus IX70; Tokyo, Japan), and photos were taken with the same optical parameters to ensure comparable luminosity.

Immunohistochemistry

CTV-1 cells were treated and fixed as described above. To eliminate nonspecific staining, the glass coverslides were incubated with the appropriate pre-immune serum for 20 min at room temperature, followed by incubation with anti-Smad4 antibody (1:100; Santa Cruz Biotechnology Inc.) at 4°C overnight. Slides were washed three times in 0.01 M TBS and then incubated with a 1:200 dilution of biotinylated secondary antibodies at 37°C for 30 min, after which they were washed three times with TBS. After incubation with the avidin-biotin-horseradish peroxidase complex (Vectastain ABC Peroxidase kit, Standard Elite Series; Vector Laboratories, Inc.) for 30 min at room temperature, slides were washed three times in TBS, rinsed in 0.5% Triton X-100/TBS for 30 s and developed by immersion in 0.1% of a freshly prepared solution of 3, 3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) for 6-8 min.

Acknowledgments

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A.

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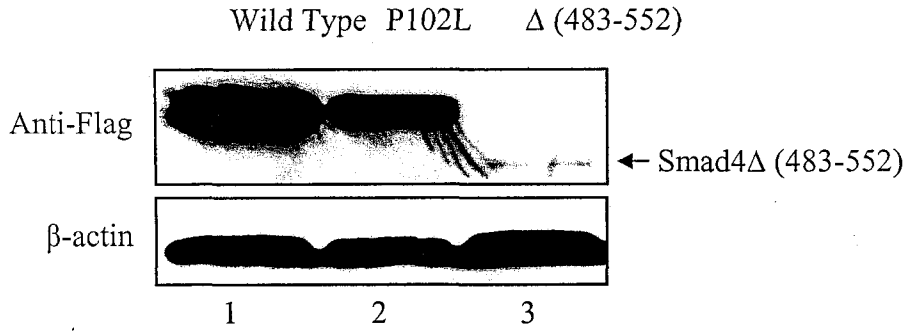

Figure 1 Low protein expression level of mutated Smad4 derived from acute myelogenous leukemia. (**A**) Partial gene sequences of the two acute myelogenous leukemia-derived Smad4 mutants. The arrows indicate the mutated sites of the Smad4 sequences. (**B**) The expression levels of acute myelogenous leukemia-derived Smad4 mutants. 293T cells were transfected with same amount of Flag-tagged wild-type and mutated Smad4 expression plasmids as indicated. Extracts were assayed by Western Blot with antibodies specific for Flag and β-actin.

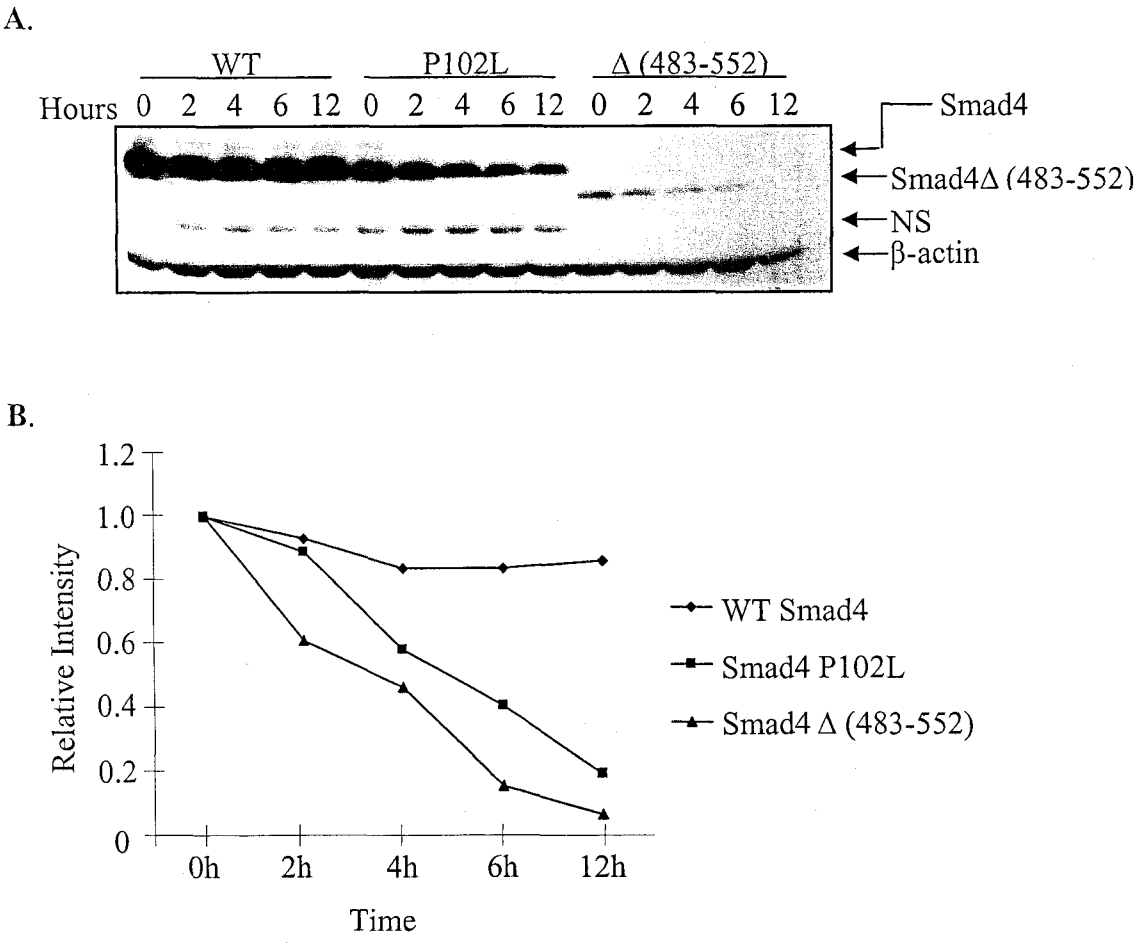

Figure 2 Instability of mutated Smad4 proteins derived from acute myelogenous leukemia. (**A**) The steady-state levels of acute myelogenous leukemia-derived Smad4 mutants. 293T cells were transfected with Flag-tagged wild type and mutated Smad4 plasmids as indicated. After 40 h, cycloheximide (40 μg/mL) was added to the culture to prevent any further Smad4 synthesis. Whole cell extracts were prepared at different time points as indicated and assayed by Western blot with an anti-Flag antibody recognizing both the full-length and truncated Smad4 proteins. (**B**) The intensity of the bands was quantitated by phosphorimaging and plotted relative to the amount present at time 0.

Figure 3 Smad4 mutants undergo protein degradation through the ubiquitin-proteosome pathway. (**A**) Proteosome inhibitor elevated the protein level of mutated Smad4. 293T cells were transfected with different amounts of wild-type and mutated Smad4 plasmids, respectively (wild type 800 ng/dish, P102L 800 ng/dish and Δ (483-552) 6 μg/dish). MG-132 (20 μM), an inhibitor of the 26S proteosome was added 4 h before cell lysis. Total cell extracts were analyzed by immunoblot with antibodies against Flag and β-actin. (**B**) Higher ubiquitination of mutated Smad4 than of wild-type Smad4 protein. 293T cells were transfected with indicated plasmids, and MG132 (20 μM) was added 4 h before cell lysis. Cell lysates were immunoprecipitated with Flag antiserum and then reprecipitated prior to immunoblot with anti-ubiquitin.

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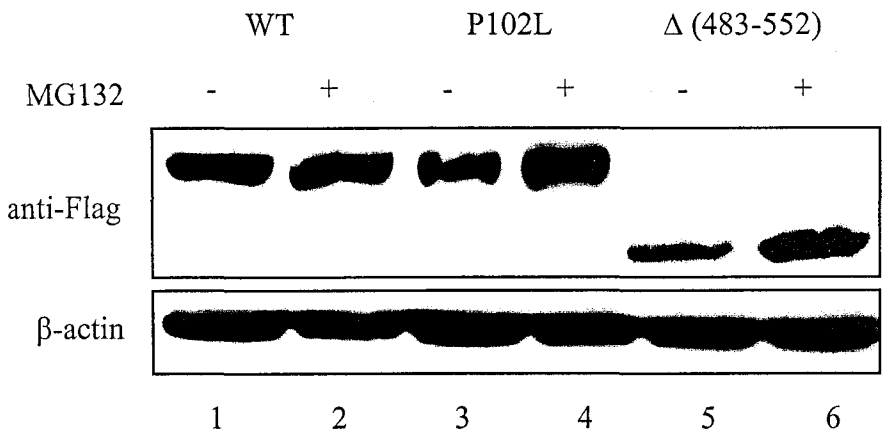

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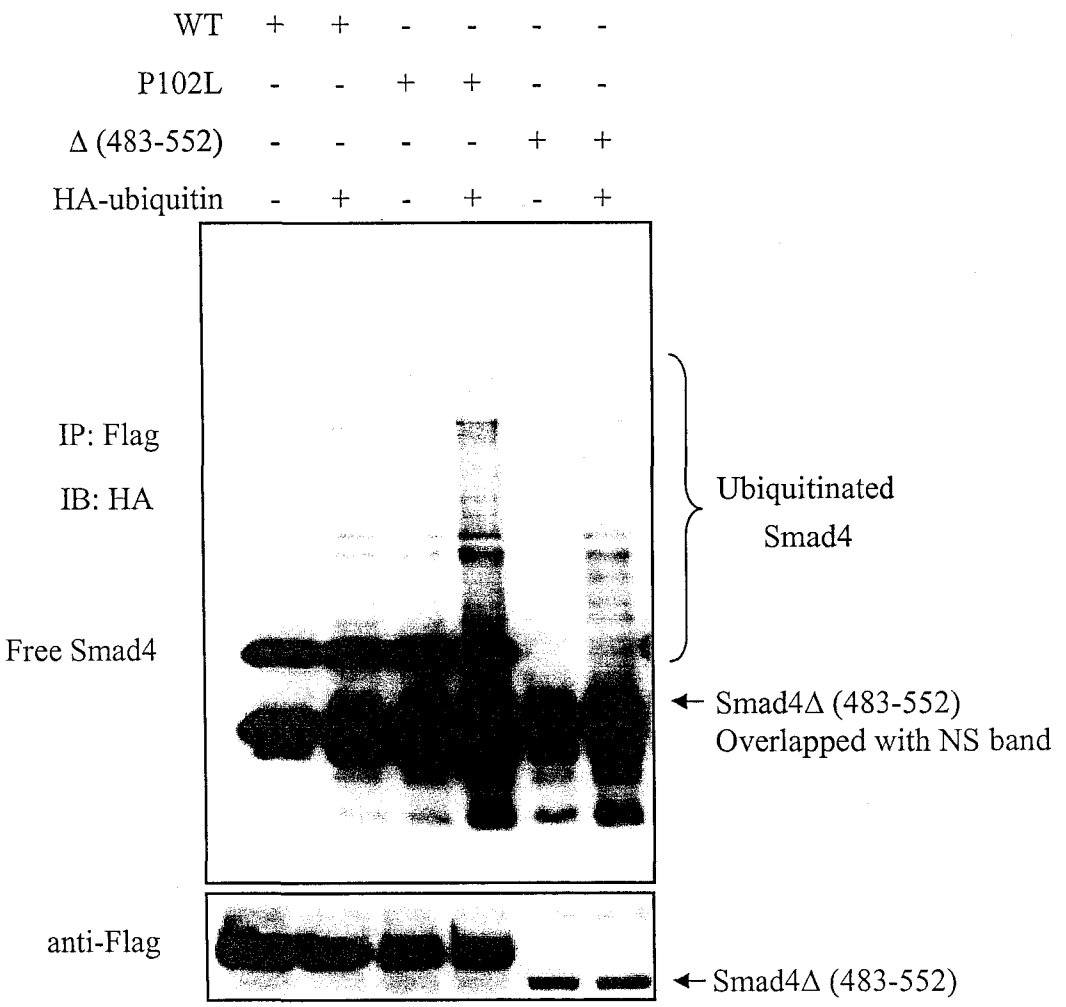

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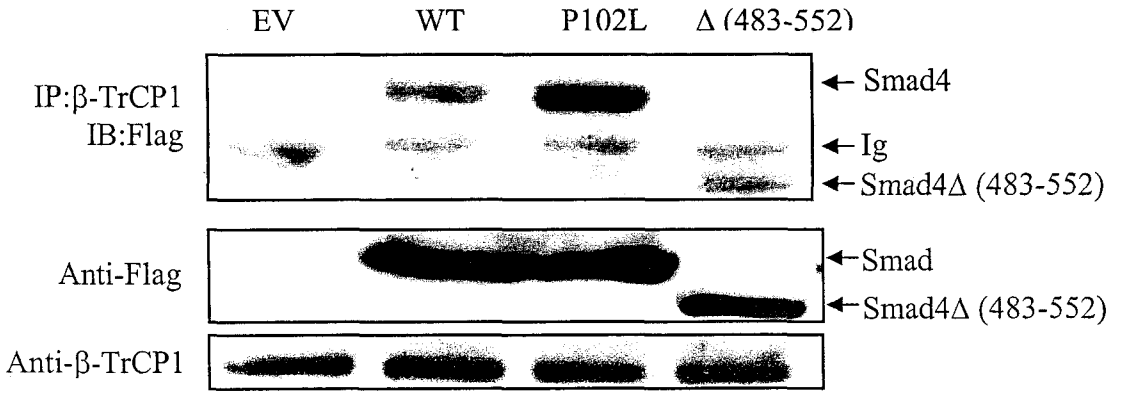

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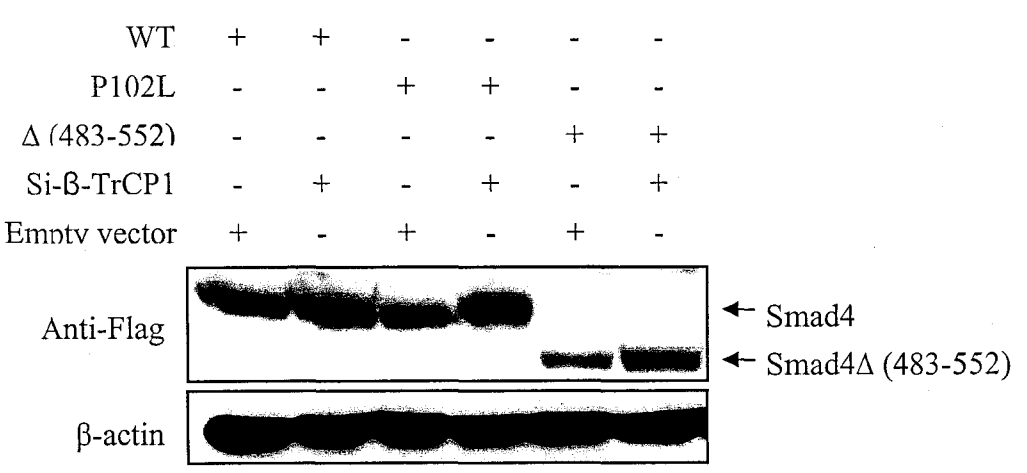

Figure 4 Instability of Smad4 mutants mediated by β-TrCP1. (**A**) Interaction of Smad4 mutants with β-TrCP1. 293T cells were transfected with the indicated plasmids. Immunoprecipitation assays were performed by using anti-β-TrCP1 antibody, and the immunocomplex was detected by Western-Blot with anti-Flag antibody. The expression levels of Flag proteins in cells were also detected, as indicated in the lower panel. (**B**) si-β-TrCP1 elevated protein level of acute myelogenous leukemia-derived Smad4 mutants. BS/U6 (empty vector control) or BS/U6/β-TrCP1 (si-β-TrCP1) plasmid as indicated were cotransfected into 293T cells with wild-type or individual Smad4 mutants. Cells were harvested 3 days later, and the extracts were assayed by Western blot with antibodies specific for Flag (upper panel) or β-actin (lower panel).

Figure 5 Suppression of endogenous β-TrCP1 inhibits the degradation of Smad4 in acute myelogenous leukemia cells. (**A**) Lowered β-TrCP1 expression in CTV-1 cells by si-β-TrCP1 transient overexpression. Empty BS/U6 (empty vector control) or BS/U6/β-TrCP1 (si-β-TrCP1) plasmids were cotransfected with 1:10 amount of pCDNA3-GFP plasmid into CTV-1 cells. Three days later, cells were fixed on the poly-L-lysine pretreated glass coverslips with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. After preblocking, the cells were treated with the anti-β-TrCP1 and then incubated with Texas-red-conjugated donkey anti-mouse IgG. Nuclei were counter-stained with To-Pro-3 iodide (blue). Images were observed with a fluorescence microscope (Olympus IX70; Tokyo, Japan). (**B**) si-β-TrCP1 transient overexpression in CTV-1 cells elevated Smad4 protein expression. Empty BS/U6 vector or BS/U6/β-TrCP1 plasmids were cotransfected with 1:10 amount of pCDNA3-GFP plasmid into CTV-1 cells. Cells were fixed, incubated with anti-Smad4 antibodies and developed by using the same methods described above. (**C**) High infection efficiency of GFP in CTV-1 cells by retrovirus delivery. CTV-1 cells were infected with virus containing GFP. Green light representing GFP expression in cells was detected under fluorescence microscope (left panel). Western-Blot assay was performed by using anti-β-TrCP1 and anti-β-actin antibody (right panel). (**D**) si-β-TrCP1 enhances Smad4 protein expression in CTV-1 cells. CTV-1 cells were infected with virus containing ΔU3/U6-GFP (si-GFP) or ΔU3/U6-β-TrCP1 (si-β-TrCP1). Three days later, cells were fixed on the poly-L-lysine pretreated glass coverslips with 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. After preblocking, the cells were treated with the anti-Smad4 and incubated with biotinylated secondary antibodies. After incubation with the avidin-biotin-horseradish peroxidase complex, slides were washed and developed by immersion in 3, 3'-diaminobenzidine tetrahydrochloride (left panel). (**E**) CTV-1 cells were infected with virus containing ΔU3/U6-GFP (si-GFP) or ΔU3/U6-β-TrCP1 (si-β-TrCP1). Three days later, cells were harvested, and a Western blot assay assay was performed with anti-Smad4 and anti-β-actin antibody (right panel).

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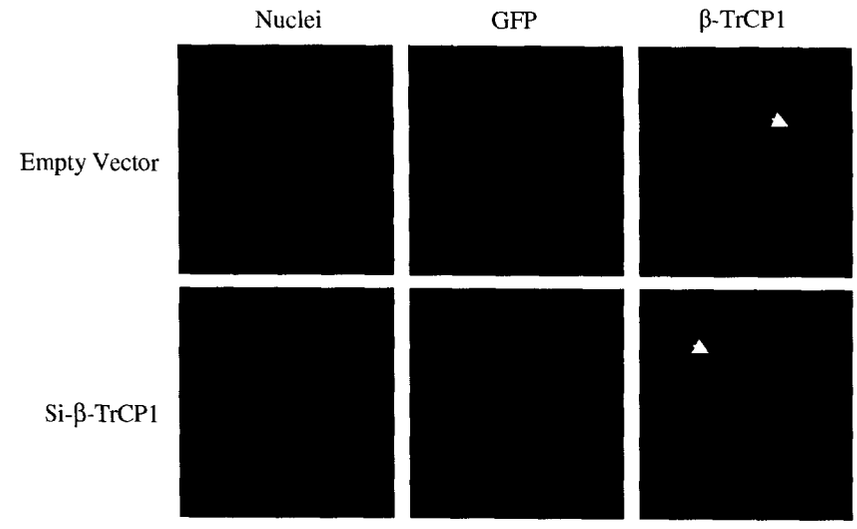

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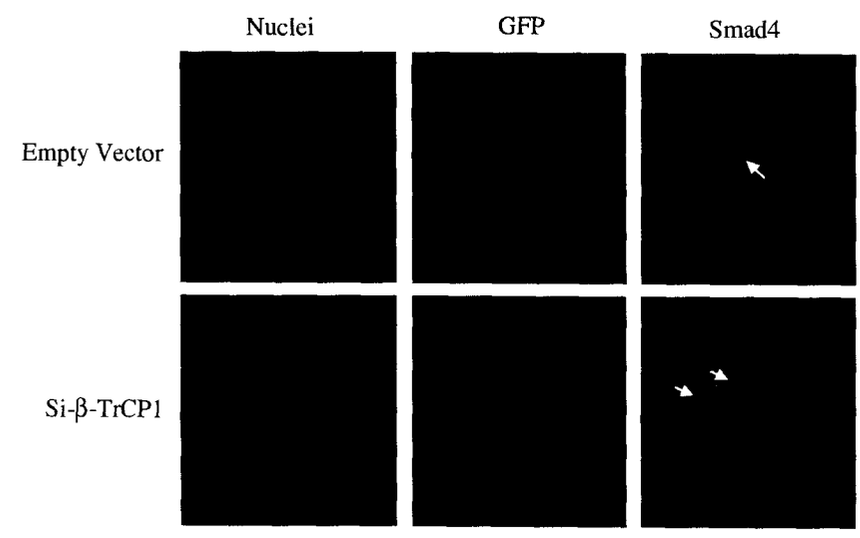

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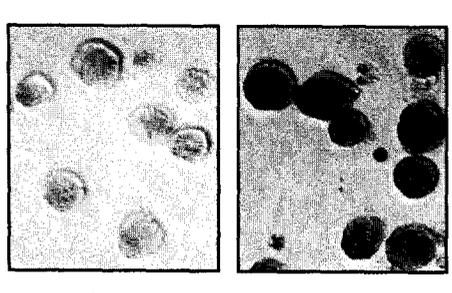

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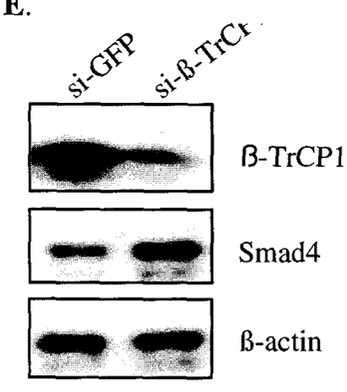


DISCUSSION

Hematopoiesis is a tightly regulated process that is characterized by the modulation of the hematopoietic stem/progenitor cells by external cytokines and internal transcription factors. Thus, how cytokine-mediated signaling leads to changes in gene expression and ultimately to alterations in the hematopoietic cell fate is a crucial question in the study of the biology of blood. Working as a transcription factor, Hoxa9 is a key regulator during hematopoiesis and a proto-oncogene in human leukemia. The identification of the Nup98-Hoxa9 fusion oncoprotein in AML patients pointed to a direct involvement of Hoxa9 in malignant hematopoiesis. However, the regulation of Hoxa9 is poorly characterized. For example, during progenitor cell maturation, besides physical removal of Hoxa9, what is the mechanism responsible for turning off the function of Hoxa9? Although recent reports demonstrate the negative functional regulation of Hoxa9, few studies link the activity of Hoxa9 to the environments of hematopoietic stem/progenitor cells exposed in the bone marrow. Protein kinase C (PKC) was shown to phosphoralyte Hoxa9 (Vijapurkar *et al*, 2004). The phosphorylation impairs the DNA-binding activity. PKC inhibition significantly reduced the phorbolester induced differentiation of the PLB985 hematopoietic cell line, as well as HOXA9-immortalized murine bone marrow cells. Another report demonstrated that Cul-4 directly interacts with Hoxa9 and leads to the degradation of Hoxa9 (Zhang *et al*, 2003). Interfering with Cul-4A biosynthesis by ectopic expression or by RNA-mediated interference resulted in alterations of the steady-state levels of HOXA9, as manifested by impairment of the ability of 32D myeloid progenitor

cells to undergo proper terminal differentiation into granulocytes. However, what trigger these interactions were not well understood and questions about the mechanism of the process remained unanswered.

On the basis of our previous findings (Shi *et al*, 1999, 2001), my colleagues and I investigated the potential regulatory role of TGFβ/BMP in hematopoiesis through Smads; the results of this research are reported in the first article in this dissertation. We used Nup98-Hoxa9 as a disease model. Since the target gene of Nup98-Hoxa9 is not well characterized, we first characterized Hoxa9 as a direct downstream gene of Nup98-Hoxa9. We found that Nup98-Hoxa9 induced Hoxa9 promoter activity in a dose-dependent manner (article 1, Figure 3). Overexpression of PBX1a, which is the DNA-binding partner of Hoxa9, further enhanced the transcription activity. Nup98-Hoxa9 also binds to the Hoxa9 promoter, both *in vitro* and *in vivo,* to a region containing a TGATTTAC PBX-HOX consensus sequence. Then we found that TGFβ/BMP inhibits the bone marrow transformation capability of Nup98-Hoxa9. Biochemical and cellular data demonstrate that Smad4 can interact with Nup98-Hoxa9 at its conserved HD and block their DNA-binding ability *in vitro* and *in vivo*, thus resulting in the suppression of their downstream gene transcription. Mapping data revealed that the amino-terminus of Smad1/4 mediates this effect. Most importantly, the interaction domains of Smads with Hoxa9 are sufficient to inhibit the leukemic transformation of primary bone marrow cells induced by Nup98-Hoxa9. This study provides a comprehensive analysis of a novel regulatory mechanism through which TGFβ/BMP regulates hematopoiesis (Figure 1): HOXA9 is expressed in primitive hematopoietic cells and maintains the undifferentiated stage of these cells. When cells are stimulated by TGFβ/BMP, the DNA-binding of Hoxa9 is suppressed

through interaction with Smads, resulting in myelocytic maturation (Figure 1A). When t (7; 11) chromosomal translocation results in overexpression of Nup98-Hoxa9, the regulatory effect of TGFβ/BMP through Smad4 becomes insufficient, and this leads to AML. Overexpression of Smads can ameliorate this oncogenetic transformation (Figure 1B).

In the investigation reported in the second manuscript included in this disserstation, my colleagues and I studied AML-associated Smad4 mutations: a missense mutation of the Smad4 gene in the MH1 domain (P102L) and one frame shift mutation resulting in termination in the MH2 domain (Δ483-552). We showed that mutations in AML lead to protein instability caused by increased interaction affinity with β-TrCP in SCF E3 ligase compared with the wild-type Smad4. These two AML-derived Smad4 mutants are degraded rapidly in comparison with their wild-type counterpart. We also demonstrated that both mutated proteins exhibit enhanced polyubiquitylation and proteasome degradation. In addition, siRNA-triggered endogenous β-TrCP1 suppression increased the protein expression level of both overexpressed Smad4 mutants and endogenous mutated Smad4 protein in acute myelogeneous leukemia cells. These data suggested that mutated Smad4 protein undergoes rapid degradation in acute myelogeneous leukemia cells via $SCF^{\beta\text{-TrCP1}}$ E3 ligase-mediated protein ubiquitylation and subsequently in the proteasome degradation pathway.

Although the functional change of mutated Smad4 remains to be investigated, loss of Smad4 through the ubiquitylation proteasome pathway will enhance Hox activity (Figure 1C). In this way, Hox proteins will become constitutively active by resisting the negative regulation coming from TGFβ/BMP signaling in bone marrow where hematopoietic stem/progenitor cells reside, and will immortalize these cells in which their ex-

pressions are restricted, finally leading to leukemogenetic transformation. Thus, the study of the functional balance between Smad4 and Hox will be instructive in the understanding of both normal and malignant hematopoiesis.

Interaction Between Hox and Smads

Hoxa9 Interaction With Smad1 and Smad4

Previously, in an effort to characterize the mechanism of BMP-induced osteoblast formation, Shi and his colleagues (1999, 2001) showed that Smad1 and Smad4 interact with the Hox protein and displace it from its cognate DNA-binding site in response to BMP stimulation. Hoxc8 and Hoxa9 are strong transcriptional repressors in mesenchymal stem cells. Displacement of Hoxc8 and Hoxa9 activates osteopontin (OPN) and osteoprotegrin (OPG) transcription and results in osteoblast differentiation. Further studies confirmed the function of these protein interactions in bone formation. Ectopic expression of the interaction domain of Smad1 with Hoxc8 sufficiently stimulates BMP downstream gene expression and results in osteoblast differentiation and bone formation, both *in vitro* and *in vivo* (Yang *et al*, 2000; Liu *et al*, 2004).

Smad4 With Other Hox

Mapping data indicate that Smad4 and Smad1 interact with the DNA-binding homeodomain of Hoxc8 and Hoxa9. Since the homeodomain is conserved in all 39 members of Hox, it is likely that Smad4 is able interact with other Hox. Smad4 is shown to be able to interact with all Hox proteins from all 13 paralogs (unpublished observation) and is a more potent interactor with Hox than Smad1 is.

In agreement with this, Smad4 was shown to interact with the homeobox protein DLX-1, also at its homeodomain, and to block a signal from Activin A (Chiba *et al*, 2003). DLX1 is the product of a member of the distal-less homeobox gene family and is known to have important roles in embryogenesis. In hematopoietic cells, DLX1 is expressed in a lineage-dependent manner. This study broadens the view of Smad interaction with Hox during hematopoietic development (Chiba *et al*, 2003).

Other Smads With Hox

Beside R-Smads and Smad4, there are two I-Smads: Smad6, which preferably inhibits BMP signaling (Hata *et al*, 1998), and Smad7, which has a broader inhibition profile (Itoh *et al*, 2000). Interestingly, Smad6 forms heterodimers with Hox transcription factors when binding to DNA as a negative feedback loop in the nucleus, but Smad7 does not interact with Hox proteins (Bai *et al*, 2000). Once the Smad6/Hox heterodimer is formed, neither Smad1 nor Smad4 is able to regulate its DNA-binding and transcription activity.

It is likely that Smad1, Smad4 and Smad6 interact with all 39 Hox proteins depending on promoter context and cell type. It is known that TGFβ/BMPs are involved in hematopoietic development and that Hox also plays a critical role in this process. The understanding of the mechanism of TGFβ/BMP in hematopoiesis is still at the early stage. A number of related mechanisms can mediate the effect of cell cycle inhibition by TGFβ/BMP. These mechanisms are primarily involved in transcriptional regulation of cell cycle regulators, including downregulation of CDKs in G1 and G2 and activation of the expression of the cell cycle inhibitors of p27, p21 and p15 (Kim *et al*, 2003). However, it has also been shown that TGFβ inhibition of cell cycle progression in hematopoietic cells

is independent of cell cycle regulators (Cheng *et al*, 1998). Thus, BMP employs the interaction of Smads with Hox in the regulation of hematopoiesis. When the numerous TGFβ/BMP ligands, multiple TGFβ/BMP receptors and R-Smads and complex pattern of Hox gene expression during hematopoiesis are considered, it seems highly likely that interactions between Hox and TGFβ/BMP-regulated Smads generate intricate signals to negatively modulate Hox transcription activity in hematopoietic cell lineage commitment and maturation. Both TGFβ and BMP regulate this composite transcription network in hematopoiesis, and much of the detailed mechanism remains to be explored.

Potential Therapeutic Intervention by Targeting Hox

Targeting Hoxa9 DNA-Binding Activity

Targeted cancer therapy refers to treatment strategies designed to inhibit the product of an oncogene involved in the process of neoplastic transformation. Our study raises the possibility that Hox DNA-binding activity may serve as a potential target for therapeutic intervention. For example, a compound that can specifically recognize Nup98-Hoxa9, the chimeric fusion form of Hoxa9, may be used to treat t (7; 11)-positive AML patients (Figure 2). In a broader view, after the identification of the *Nup98-Hoxa9* fusion gene in AML, many chromosome translocations involving *Hox* have been reported, reflecting increasing interest in the role of *Hox* in leukemia (Moore, 2005). In addition to elevated expression of *Hox* due to chromosome translocation, mutations in other critical upstream effectors of *Hox* genes have been identified. For example, transformation of myeloid progenitors by MLL oncoproteins is dependent on *Hoxa9* (Ayton and Cleary, 2003). Although the downstream targets of Hox have not yet been elucidated, it is widely

accepted that the function of Hox is dependent on its DNA-binding activity. Thus, targeting the DNA-binding activity of leukemia-associated Hox may serve as a novel therapeutic intervention to treat those leukemias that involve Hox deregulation. In fact, by using a high-throughput screening assay based on mimicking the displacement of Hoxc8 binding to DNA by the Smad1 interaction domain with Hoxc8, we identified chemical entities that exhibit bone anabolic activity in cell and bone organ cultures (Liu *et al*, 2004). This raises the possibility that these compounds may be used as chemotherapeutic drugs for certain leukemias.

Besides being involved in leukemia, Hox genes have been implicated in many other types of cancers (Cillo *et al*, 2001). In many solid tumors and derivative cell lines, Hox deregulation in cancer is often associated with gain of expression in primary tumors and cell lines from brain, breast, colon, lung and kidney (Abate-Shen, 2002). Insights into the function of Hox genes indicate that they are overexpressed and promote cellular transformation in culture. Thus, functional inhibition of Hox will serve as a potential target in other tumors, as well.

Potential Pitfall of Targeting Hoxa9

However, induction of myeloid leukemia by Nup98-Hoxa9 requires a long latency period of 6 to 9 months, which suggests that formation of overt leukemia requires the cooperation of several genes that fulfill complementary functions (Figure 3) (Kroon *et al*, 2001). In this case, the effectiveness of targeting Nup98-Hoxa9 may be stage dependent since subsequent oncogenetic shock(s) may be responsible for the formation of leukemic

stem cells, and the recent cancer stem cell theory suggests that such therapy might not prove effective (Reya *et al*, 2001).

Smad Mutations, Leukemia and Other Cancers

Loss of Smad4 is associated with poor prognosis of human cancers. Most significantly, mutations in Smad4/DPC4 have been identified in approximately 50% of pancreatic adenocarcinomas. Recently, several lines of evidence have shown that Smad4 mutations identified in human cancer patients are rapidly degraded via the ubiquitin-proteasome pathway (Xu and Attisano, 2000; Maurice *et al*, 2001; Moren *et al*, 2003), indicating that protein instability of Smad4/DPC4 contributes significantly to a loss in cellular responsiveness to TGFβ in tumorigenesis. Loss of TGFβ in turn will stimulate the secretion of the TGFβ ligand, which in turn inhibits the growth of normal cells. This inhibition may give tumor cells a growth advantage over normal cells.

Functional inactivation caused by these Smad4 mutations, especially deletions in the MH2 domain, cannot be excluded as a possibility. Some Smad4 mutations lost the ability to form homo- or heterotrimeric Smad complexes (Shi *et al*, 1997) or increased auto-inhibition of the Smads by stabilizing intramolecular interactions between the MH1 and MH2 domains (Hata *et al*, 1997). In contrast, it has been reported that some Smad mutations do not interfere with most of the functions of these tumor suppressor proteins but instead inactivate the proteins by inducing targeting for the ubiquitin-proteasome system (Xu and Attisano, 2000; Maurice *et al*, 2001; Moren *et al*, 2003).

The two Smad4 mutations identified in acute myelogeneous leukemia have been reported to disrupt TGFβ signaling (Imai et al, 2001). However, cellular responses are

highly sensitive to the level of Smad protein. Therefore, we suggest that the reduction in the steady-state levels of these Smad proteins, which is insufficient to mediate TGFβ signaling, may be the primary defect in acute myelogeneous leukemia. Smad4 is a key signaling molecule of TGFβ signaling and therefore is an attractive therapeutic target in oncology because of its strong cancer suppression activity.

Besides loss of Smad4 in myeloid leukemia, loss of Smad3 protein is a specific feature of pediatric T-cell acute lymphoid leukemia. A reduction in Smad3 expression and the loss of $p27^{Kip1}$ work synergistically to promote T-cell leukemogenesis in mice (Wolfraim *et al*, 2004). However, a mutational analysis of the gene coding for the TGFβ signal transducer Smad2 has been performed on 50 primary lymphoid and myeloid leukemia cells, but no genetic defects were found in this gene (Wieser *et al*, 1998). Nonetheless, a larger panel of hematological disorders should be analyzed before the possible existence of various mutations in Smad genes in some of these pathologies is excluded.

Future Studies

In Vivo Model of Smad and Hox Protein Interaction

We attempted to generate an *in vivo* model of Hox/Smads protein interaction by transplanting double-infected primary bone marrow cells. However, due to the clonal nature of Nup98-Hoxa9-induced AML in mice, only a few transformed primary bone marrow cells can result in overt leukemia in recipient mice (Kroon *et al*, 2001). Nup98-Hoxa9 single expression in the bone marrow cells may dominate the outcome in the transplanted animals, which may make double infection resulting in cells that co-express Nup98-Hoxa9 and Smad4 insignificant. Thus, transplantation of whole infected bone

marrow cells that are subject to two different retrovirus infections may give results of no consequence.

The double-infection efficiency of the primary bone marrow cells is low. Thus, the technical difficulty in acquiring enough such cells through fluorescent activated cell sorting (FACS) may make it difficult to ensure the survival of the recipient mice, which need enough donor cells to reconstitute the whole blood system. It is plausible to design a retrovirus that links Nup98-Hoxa9 to Smad4 with an internal ribosome entry site (IRES) site in the middle. This will ensure co-expression of two interesting proteins in the same cell. Prolonged latency of Nup98-Hoxa9-mediated disease *in vivo* is expected on basis of the preliminary data that Smad4 inhibits the primary bone marrow cell transformation *in vitro* in methylcellulose culture.

Function of Smad4 Mutation in Leukemia

Is the loss of Smad4 the only reason for oncogenetic transformation? Is it possible that the mutated Smad4 acts as an oncogene with dominant gain of function in myeloid leukemia? While this dissertation was in preparation, a study showed that the R100T mutation of Smad4 identified in pancreatic cancer can activate the Wnt signaling pathway in Drosophila (Takaesu *et al*, 2005). The Wnt signaling pathway can stimulate the self-renewal of hematopoietic stem cells. There is a high possibility that this can contribute to leukemogenesis (Reya *et al*, 2003). Furthermore, mutated Smad4 may also act as an oncogene with dominant loss of function that interferes with wild-type Smad4 at heterodimer formation with R-Smad, translocation into the nucleus, DNA-binding activity or other tumor-suppressing activities that remained to be identified.

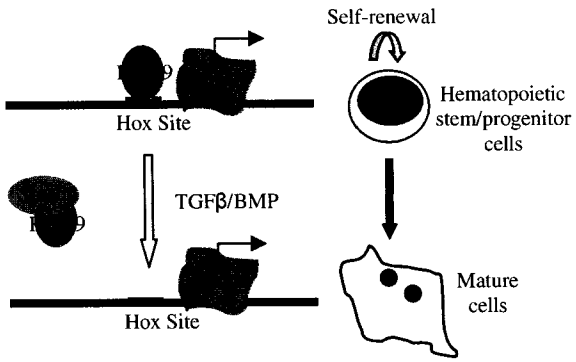
The Leukemogenesis Mechanism of Nup98-Hoxa9

Since the identification of Nup98-Hoxa9 in 1996, most research has been focused on illuminating its mechanism of leukemogenesis (Kasper *et al*, 1999; Kroon *et al*, 2001; Calvo *et al*, 2002; Ghannam *et al*, 2004). However, it is still not clear what target cells Nup98-Hoxa9 transforms, the HSCs or the progenitor cells downstream of the hierarchy of hematopoiesis. Investigation of this will help to improve the understanding of how leukemia arises, as well as to design better treatment through targeted therapy.

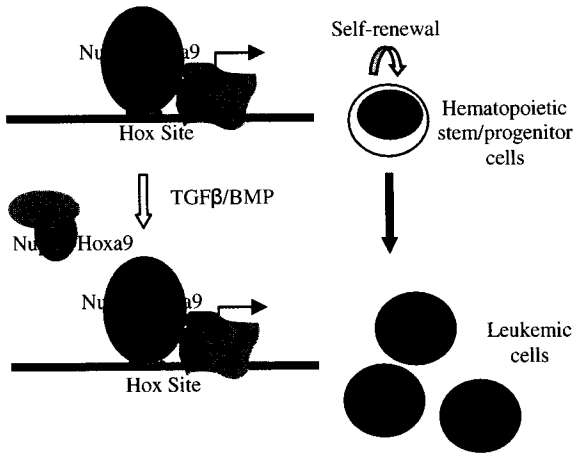
Although Nup98-Hoxa9 has been directly implicated as a dominant-acting transcription factor-type oncoprotein, the target genes that mediate its oncogenic properties are largely unknown. Gene expression profiling of transformed cells has suggested a number of provisional candidate target genes (Ghannam *et al*, 2004). However, genetic analyses in combination with biologically relevant functional assays for tissue-specific oncogenesis are essential for the correct assignment of the true downstream genes that make crucial contributions to the malignant transformation of bone marrow cells that cause overt hematological malignancy. Thus, studies that aim at illuminating the vital downstream genes of Hox will provide valuable information about the leukemic transformation mechanism, as well as molecular insight into the regulation of hematopoiesis by Hox.

Figure 1. Models of Hoxa9 and Smad4 interaction in hematopoietic development. (A) Activated R-Smads downstream of TGFβ/BMP signal interacting with Hoxa9 and inhibiting the latter from binding to the DNA. This results in the suppression of Hoxa9 target gene transcription and myeloid differentiation. (B) In the situation of t (7; 11) chromosomal translocation, transcription of Nup98-Hoxa9 is controlled by Nup98 promoter. This causes the constitutive expression of Nup98-Hoxa9 throughout all cell stages independently of regular cytokines and transcription factor regulation of Hoxa9 expression. Smad4 would appear insufficient in this situation. Dysregulation of Hoxa9 target genes by Nup98-Hoxa9 leads to immortalization of myeloid progenitors and AML. (C) In case of Smad4 mutation, faster Smad4 degradation will cause deficiency of antagonistic effect of Hoxa9 activity. This will also lead to enhanced Hoxa9 activity, resulting in immortalization of myeloid progenitors and AML.

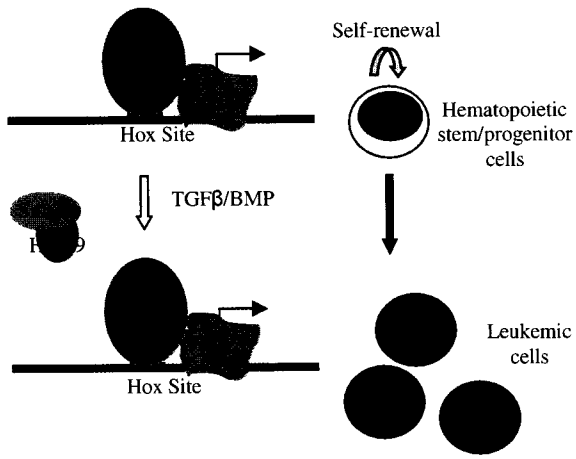
A.



B.



C.



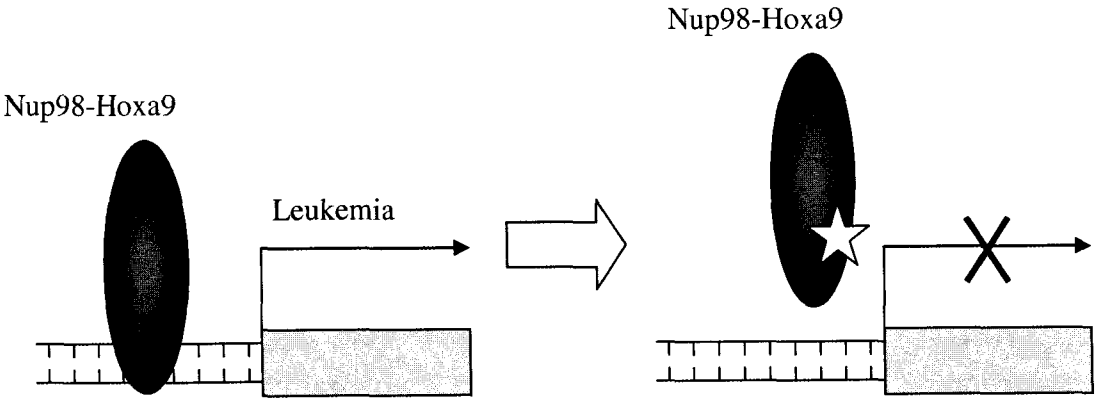


Figure 2. Rationale of targeted therapy against Nup98-Hoxa9. A chemical entity specifically designed to recognize the DNA-binding site of Nup98-Hoxa9 will bind to Nup98-Hoxa9, inhibit its DNA-binding activity, and suppress of its downstream gene transcription. Because Nup98-Hoxa9 is a transcription factor, abolishing its DNA-binding activity will deplete its function as an oncogene.

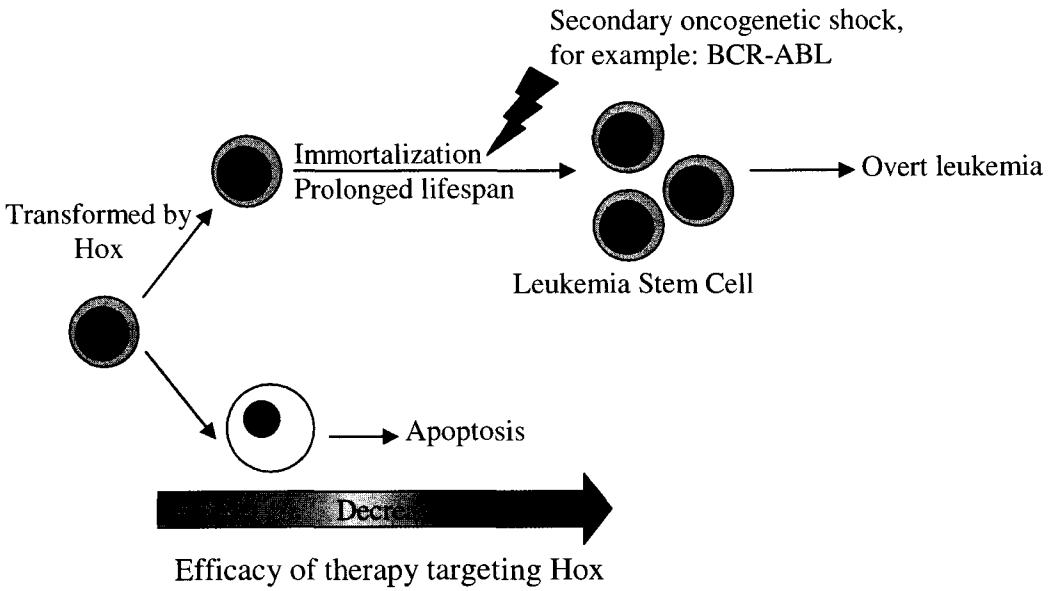


Figure 3. Potential inefficacy of leukemia therapy by targeting Hox. Constitutive activation of Hox gene expression causes immortalization of hematopoietic stem/progenitor cell. Unexpected prolonged lifespan of these cells will suffer from genomic instability and a possible secondary oncogenetic mutation, which may collaborate with Hox gene overexpression and transform these immortalized cells into leukemic stem cells with further enhanced proliferation potential and insensitivity to apoptotic signals. At this time, therapies targeting Hox may prove to be inefficient since the other oncogenes may contribute to the malignant phenotypes of these leukemic stem cells.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE FORM

NOTICE OF APPROVAL

DATE: November 2, 2004

TO: Xu Cao, Ph.D.
VH-G003 0019
FAX: 934-1775

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Smad6/7 as Sex Steroid Hormone Receptor Co-Repressors
Sponsor: NIH
Animal Project Number: 040906015

On September 14, 2004, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	144

Animal use is scheduled for review one year from September 2004. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 040906015 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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

Name of Candidate Ning Wang

Graduate Program Pharmacology and Toxicology

Title of Dissertation A Role of TGFβ/BMP in Leukemogenesis Through Interaction Between SMAD4 and HOXA9

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.

Dissertation Committee:

Name		Signature
Xu Cao	, **Chair**	Xu Cao
Christopher A. Klug		Christopher A. Klug
Jeffrey E. Kudlow		J E Kudlow
Elias Meezan		Elias Meezan
Kevin A. Roth		Kevin A. Roth

Director of Graduate Program C.A. Lambert

Dean, UAB Graduate School Bryce D. Roe

Date 12/22/05