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ORIGINAL ARTICLE

LMO4 can interact with Smad proteins and modulate transforming growth factor-β signaling in epithelial cells

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LIM-only protein 4 (LMO4) plays critical roles in mammalian development, and has been proposed to play roles in epithelial oncogenesis, including breast cancer. As LMO4 is highly expressed in the epithelial compartments at locations of active mesenchymal–epithelial interactions, we reasoned that LMO4 might act by modulating signaling pathways involved in mesenchymal–epithelial signaling. One such candidate signal is the transforming growth factor-β (TGFβ) cytokine pathway, which plays important roles both in development and cancer. We show here that the transcriptional response to TGFβ in epithelial cells is sensitive to LMO4 levels; both up- and downregulation of LMO4 can enhance TGFβ signaling as assessed by a TGFβ-responsive reporter gene. Furthermore, LMO4 can interact with the MH1 and linker domains of receptor-mediated Smad proteins, and associate with the endogenous TGFβ-responsive Plasminogen Activator Inhibitor-1 gene promoter in a TGFβ-dependent manner, suggesting that such interactions may mediate the effects of LMO4 on TGFβ signaling. When introduced into mammary epithelial cells, LMO4 potentiated the growth-inhibitory effects of TGFβ in those cells. These results define a new function for LMO4 as a coactivator in TGFβ signaling, and provide a potential novel mechanism for LMO4-mediated regulation in development and oncogenesis.
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Introduction

LIM-only factor (LMO) 4 belongs to a family of four mammalian LMO proteins (Grutz *et al*., 1998; Kenny *et al*., 1998; Sugihara *et al*., 1998; Racevskis *et al*., 1999); all family members are short transcriptional regulators composed almost entirely of two LIM domains (Bach, 2000). The four LMOs play roles in mammalian development (Yamada *et al*., 1998; Hahm *et al*., 2004; Tse *et al*., 2004; Lee *et al*., 2005). In addition, LMO1 and LMO2 act as oncogenes in acute lymphoblastic leukemia (Rabbitts, 1998), and recent studies have defined LMO3 as an oncogene in neuroblastoma (Aoyama *et al*., 2005) and LMO4 as a protumorigenic factor in breast cancer (Visvader *et al*., 2001; Sum *et al*., 2005b). LMOs interact strongly with transcriptional coregulators referred to as Co-factors of LIM domains (Clims)/LIM domain-binding proteins (Ldb)/nuclear LIM interactors (Nli) (Agulnick *et al*., 1996; Jurata *et al*., 1996; Bach *et al*., 1997, 1999; Visvader *et al*., 1997; Matthews and Visvader, 2003). The Clims also interact with the LIM domains of LIM homeodomain proteins as well as with some transcription factors that lack LIM domains (Torigoi *et al*., 2000; Matthews and Visvader, 2003). Clims, which interact with transcription factors via the C-terminus, are thought to coordinate the assembly of large multiprotein transcriptional complexes through their N-terminally located dimerization domains (Matthews and Visvader, 2003).

LMOs are thought to regulate transcription by several distinct mechanisms. First, by sequestering Clim coregulators participating in gene activation, upregulation of LMOs may repress transcription of genes that are activated by the association of Clims with LIM homeodomain factors (Milan *et al*., 1998; Zeng *et al*., 1998; Milan and Cohen, 2000). Second, LMOs interact with several DNA-binding proteins that lack LIM domains; the best characterized are certain Helix–Loop–Helix and GATA transcription factors (Wadman *et al*., 1994, 1997; de la Calle-Mustienes *et al*., 2003). LMOs are thought to recruit Clim cofactors to such complexes, thereby activating transcription of target genes. Third, because LMOs participate in multiprotein transcription complexes, the stoichiometry of these complexes is critical for transcriptional regulation (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). Coordinated upregulation of LMOs, Clims, and associated DNA-binding proteins may lead to activation, whereas both upregulation and downregulation of individual components may disrupt such complexes. While the levels of LMO4 and Clims are often coordinately regulated during development, in breast

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cancer cells, where LMO4 has been proposed to act in a pro-oncogenic fashion (Sum *et al*., 2005b), LMO4 is often upregulated disproportionately to Clims (Visvader *et al*., 2001; Wang *et al*., 2004).

In addition to neurons, LMO4 is highly expressed in epithelial cells, often at locations of active mesenchymal–epithelial interactions, such as in hair follicles, teeth, epidermis, mammary gland, kidney, and lungs (Sugihara *et al*., 1998; Hermanson *et al*., 1999; Wang *et al*., 2004; Sum *et al*., 2005a). We and others have found that LMO4 can interact with distinct DNA-binding proteins expressed at these locations (Sugihara *et al*., 1998; Sum *et al*., 2002; Kudryavtseva *et al*., 2003; Manetopoulos *et al*., 2003). As LMO4 is highly expressed at multiple sites of mesenchymal–epithelial interactions, it is attractive to propose that LMO4 interacts with and modulates the function of DNA-binding proteins in conserved signaling pathways involved in mesenchymal–epithelial signaling.

The Smad proteins, key mediators of the transforming growth factor-β (TGFβ)/bone morphogenic protein (BMP) superfamily of ligands, provide an example of DNA-binding proteins that play roles in mesenchymal–epithelial interactions in development and cancer (Massague and Wotton, 2000). Smads respond to phosphorylating signals by translocating into the nucleus and associating with target genes as a complex of receptor-activated Smads (R-Smads) and common mediator Smads (Co-Smad; Smad4). Previous work has shown that the Smad transcription complex interacts with several transcription factors, which can positively or negatively modulate TGFβ signal (Derynck and Zhang, 2003). By modulating the binding and activity of Smad proteins on target genes, these Smad-associating proteins are thought to play key roles in TGFβ/BMP signal transduction by affecting the specificity and magnitude of the TGFβ signal in response to environmental effects (Massague and Wotton, 2000).

In this paper, we demonstrate that LMO4 can modulate the proliferative response of epithelial cells to TGFβ signaling. Furthermore, we show that LMO4 interacts with R-Smads and is recruited to genomic Smad-binding sites, suggesting a mechanism for the ability of LMO4 to modulate TGFβ signaling. Our findings link LMO4 to a conserved signaling pathway that plays important roles in epithelial homeostasis.

Results

LMO4 enhances TGFβ-mediated transcriptional signal

LMO4 is upregulated in epithelial cells during the proliferative phase of mammary gland development and in about half of invasive breast cancer cases (Visvader *et al*., 2001; Wang *et al*., 2004). To determine whether LMO4 upregulation could modulate TGFβ signaling, we tested the ability of LMO4 to affect the expression of a well-characterized TGFβ-responsive reporter gene, 9xGAGA-Luciferase (Wieser *et al*., 1995; Dennler *et al*., 1998), which is derived from the regulatory region of the Plasminogen Activator Inhibitor 1 (PAI-1) gene. When the 9xGAGA-Luciferase plasmid was cotransfected with a constitutively active TGFβ receptor 1 (TβR1-AAD) into the kidney epithelial cell line HEK293T, luciferase expression was increased nine-fold (Figure 1a), consistent with previously published data (Dennler *et al*., 1998). Cotransfection of an expression plasmid encoding LMO4 resulted in a dose-dependent expression of LMO4 (Figure 1b) and markedly increased the TβR1-AAD-stimulated luciferase activity, also in a dose-dependent manner (Figure 1a). Moreover, we observed similar enhancing effects of LMO4 on TGFβ1-stimulated 9xGAGA-Luciferase expression in normal human mammary epithelial cells (HMEC) (Figure 1c), and the mouse mammary epithelial cell line NMuMG (Figure 1d). These results indicate that LMO4 can enhance TGFβ-mediated signaling as monitored by the PAI-1 promoter in HEK293T and mammary epithelial cells.

To test whether LMO4 could also modulate the expression of the endogenous PAI-1 gene, we used retroviral transduction to introduce the LMO4 protein into NMuMG cells, and measured PAI-1 mRNA levels with quantitative real-time PCR. Consistent with previous results (Dong-Le Bourhis *et al*., 1998), TGFβ1 increased PAI-1 mRNA expression several fold ($\Delta\Delta C_t = 3$). LMO4 increased PAI-1 mRNA several fold under both basal ($\Delta\Delta C_t = 2.3$) and TGFβ1-stimulated ($\Delta\Delta C_t = 5.7$) conditions (Figure 1e). Taken together, these results suggest that LMO4 upregulation is capable of enhancing TGFβ-stimulated transcription of the PAI-1 gene.

LMO4 regulates the transcriptional response to TGFβ in a biphasic manner

LMO4 regulates transcription by participating in multiprotein complexes that often involve both DNA-binding proteins and other transcriptional coregulators, such as Clims. The stoichiometry of these complexes is critical for their activity and LMO4 upregulation may therefore modulate transcription by disrupting such complexes (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). If this is true, then lowering of LMO4 levels might also lead to changes in gene expression that are similar to those found with LMO4 upregulation; both perturbations, up- and downregulation, would alter the stoichiometry of LMO4-containing transcription complexes. For example, both up- and downregulation of the *Drosophila* Clim homologue, Chip, lead to similar phenotypes in proneural (Ramain *et al*., 2000) and wing (Milan and Cohen, 1999; van Meyel *et al*., 1999) patterning.

To test this idea, we designed three siRNAs against human LMO4 and tested their ability to lower LMO4 levels in T47D breast cancer cells, which express LMO4 at a relatively high level, facilitating the monitoring of endogenous LMO4 protein levels. Of the three LMO4 siRNAs, LMO4 siRNA #1 and #3 effectively decreased endogenous LMO4 levels (Figure 2a; lanes 1 and 3) compared to a negative control siRNA. To test the

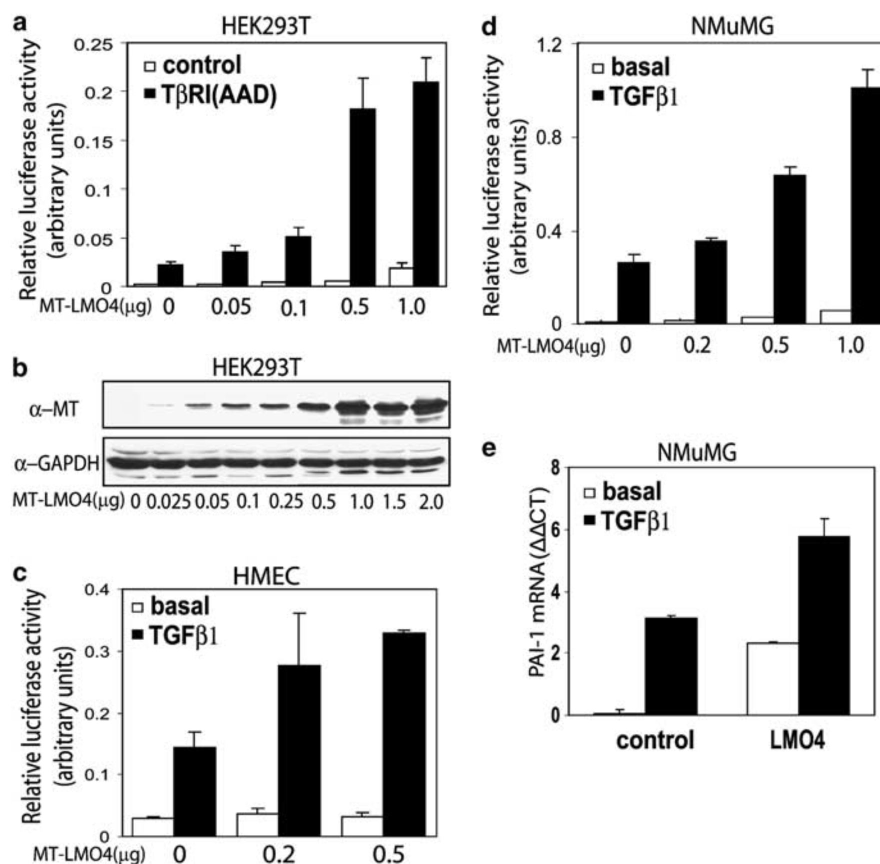

Figure 1 LMO4 potentiates TGFβ-mediated transcriptional activity in epithelial cells. (**a**) The 9xCAGA-Luciferase reporter plasmid (0.5 μg) was transiently cotransfected into HEK-293T cells with either an empty expression plasmid (control) or a plasmid encoding a constitutively activated receptor I of TGFβ (TβRI-AAD; 0.1 μg), which activates TGFβ signaling. An expression plasmid encoding MT-LMO4 was cotransfected in the indicated amounts, ranging from 0 to 1.0 μg; equal amount of DNA was included in all transfections by adjusting the amount of empty expression vector. We determined relative luciferase activity 40 h after the transfection. (**b**) The MT-LMO4 expression plasmid was transfected into HEK293T cells in the indicated concentrations. We isolated whole-cell lysates 40 h later and determined the expression of MT-LMO4 protein by Western blotting with an MT antibody (top panel). As a control for protein concentration and loading, the same blot was also bound to a GAPDH antibody (bottom panel). (**c**) Normal human mammary epithelial (HME) cells were cotransfected with the 9xCAGA-Luciferase reporter plasmid (0.5 μg) and an expression plasmid encoding MT-LMO4 in the indicated amounts. After 24 h, the cells were treated either with vehicle (basal) or TGFβ1 (1 ng/ml) for 20 h before relative luciferase activity was determined. (**d**) Mouse mammary gland (NMuMG) cells were cotransfected with the 9xCAGA-Luciferase reporter plasmid (0.5 μg) and an expression plasmid encoding MT-LMO4 in the indicated amounts. After 24 h, the cells were treated either with vehicle (basal) or TGFβ1 (1 ng/ml) for 20 h before relative luciferase activity was determined. (**e**) NMuMG cells were infected with a retroviruses expressing GFP (control) or LMO4-GFP fusion protein (LMO4). When approximately 80% of the cell monolayers were expressing the target proteins as judged by fluorescent microscopy, the cells were treated either with vehicle alone (basal) or TGFβ1 (1 ng/ml) for 6 h. Total RNA was extracted and endogenous PAI-1 mRNA relative to 18S mRNA levels were determined by real-time PCR. All experiments were carried out in triplicate, and luciferase activity and mRNA levels are expressed as the mean$\pm$s.d. Similar results were obtained in three different experiments, each one performed in triplicate.

effect of LMO4 siRNA on TGFβ signaling, we transfected into HEK293T cells an expression vector encoding LMO4 shRNA#1 with 9xGAGA-Luciferase reporter plasmid, with and without a TGFβ activator. While the control shRNA had little effect on TGFβ stimulation of reporter activity, the LMO4 shRNA markedly enhanced TGFβ stimulation (Figure 2b). The effect of the LMO4 shRNA was specific because the expression vector that encodes mouse LMO4, which is not targeted by the shRNA, could partially reverse the stimulatory effect of LMO4 shRNA (Figure 2c). As predicted from the experiments described previously (Figure 1), higher amounts of transfected LMO4 ultimately resulted in stimulation of gene expression, creating a U-shaped dose–response curve for the effect of LMO4 on TGFβ-stimulated gene expression (Figure 2c).

Together, these experiments show that in this system, TGFβ signaling is sensitive to LMO4 levels. Very high or low concentration of LMO4 can enhance TGFβ-dependent transcription of the PAI-1 gene reporter. These findings are consistent with results from other systems, showing that the stoichiometry of the components of transcription complexes involving LIM domain transcription factors is critical for regulation of gene activation (Milan and Cohen, 1999; van Meyel *et al.*,

1999; Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003; Matthews and Visvader, 2003).

LMO4 interacts with several R-Smads

TGFβ regulates transcription of the PAI-1 gene by facilitating the nuclear translocation and DNA binding of a complex composed of R-Smads (Smad2 and/or Smad3) and the co-Smad, Smad4 (Massague and Wotton, 2000; Derynck and Zhang, 2003). To investigate the mechanisms of action for the effect of LMO4 on TGFβ-mediated transcription, we tested whether LMO4 could interact with these key mediators of TGFβ-regulated transcription. An expression vector encoding myc-tagged LMO4 was transfected into HEK293T cells with or without HA-tagged Smad1, Smad2, Smad4, and Smad5. Whole-cell extracts were isolated and immunoprecipitated with an myc-tagged antibody followed by SDS gel electrophoresis and immunoblotting with an HA antibody. Smad1, Smad2 and Smad5 were all clearly co-immunoprecipitated with LMO4 (Figure 3a; top panel), suggesting that LMO4 is capable of interacting with several Smad proteins. A weak interaction was also detected between LMO4 and the co-Smad, Smad4 (Figure 3a; lane 5). LMO4 was also co-immunoprecipitated with a Smad2 antibody in non-transfected HEK293T cells (Figure 3b), indicating interaction of endogenous LMO4 and Smad2 proteins.

To validate the co-immunoprecipitation results, and to test whether the LMO4–Smad interactions are direct, we performed GST pull-down assays. We found that LMO4 clearly interacts with Smad2, Smad3, Smad5, and Smad8, with the strongest LMO4 interactions detected with Smad8 (Figure 4a). Consistent with the co-immunoprecipitation results, a weak LMO4 interaction was also detected with Smad4. To map the Smad domains that are responsible for interactions with LMO4, we tested the interactions of LMO4 with subregions of the Smad3 protein. Smad proteins are composed of an N-terminal Mad homology (MH) domain 1, which is responsible for nuclear import and DNA binding, except in the case of the major splice form of Smad2, which contains an insertion in these regions and does not directly bind DNA. A C-terminal MH2 domain, which mediates Smad oligomerization, is linked to the MH1 domain with a less-conserved linker domain (Massague and Wotton, 2000; Derynck and Zhang, 2003). All three domains have been shown to interact with several transcription factors as well as cytoplasmic adaptors (Massague and Wotton, 2000; Derynck and Zhang, 2003). In these experiments, LMO4 interacted with the MH1 and linker domains of Smad3; no interaction was found with the MH2 domain (Figure 4b).

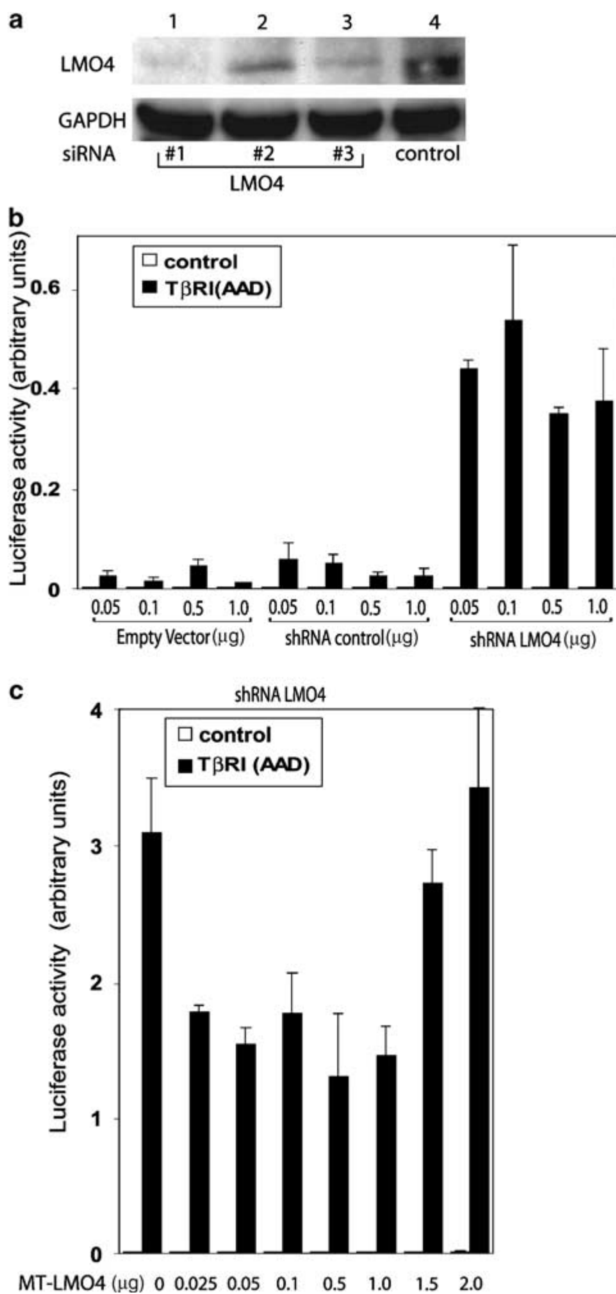

Figure 2 Biphasic regulation of PAI-1 reporter activity by LMO4. (**a**) Three distinct siRNAs targeting human LMO4 and a control siRNA were transfected into T47D breast cancer cells, using RNAiFect transfection reagent (Qiagen). After 40 h, LMO4 protein levels were determined by Western blotting of whole-cell lysates with LMO4 antibody (top panel). As a control, the same blot was bound to GAPDH antibody (bottom panel). (**b**) HEK293T cells were cotransfected with the 9xCAGA-Luciferase construct (0.5 μg) and either an empty expression plasmid (control) or a plasmid encoding a TGFβ activator (TβRI-AAD; 0.1 μg). To test the effect of lowering LMO4, we also transfected the indicated amounts of empty shRNA expression vector, control shRNA expression vector, and LMO4 shRNA expression vector. (**c**) HEK293T cells were cotransfected with the 9xCAGA-Luciferase construct (0.5 μg) and either an empty expression plasmid (control) or a plasmid encoding a TGFβ activator (TβRI-AAD; 0.1 μg). In addition, the vector expressing human LMO4 shRNA#1 (0.5 μg) was included under all conditions. An expression vector that encodes mouse MT-LMO4 in the indicated concentrations was cotransfected. At 40 h after transfection, luciferase activity was determined; relative luciferase activity is expressed as the mean $\pm$ s.d. from triplicate transfection. Similar results were obtained in three independent experiments.

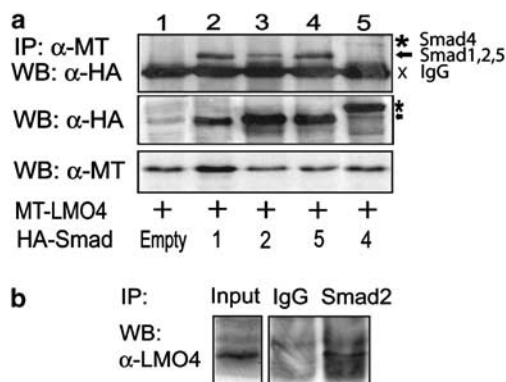

Figure 3 LMO4 interacts with several Smad proteins. (**a**) MT-tagged LMO4 and HA-tagged Smad1 (lane 2), Smad2 (lane 3), Smad4 (lane 5), and Smad5 (lane 4) were cotransfected into HEK293T cell. At 2 days after transfection, whole-cell lysates were isolated. Cell lysates were immunoprecipitated with anti-MT and the Smad proteins in the complex identified with immunoblotting with anti-HA (top panel). Smad and LMO4 protein expression was demonstrated with direct immunoblotting of cell lysates with HA antibody (middle panel) and MT antibody (bottom panel), respectively. The asterisk indicates the location of Smad4, the arrow the location of Smad2, -3 and -5, and the X the location of IgG. (**b**) Lysates from HEK293T cells were immunoprecipitated with either IgG or Smad2 antibody, and immunoblotted with an LMO4 antibody.

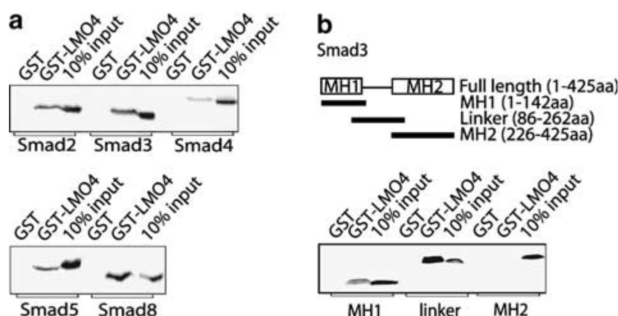

Figure 4 LMO4 interacts with the MH1 and linker regions of Smad proteins. (**a**) Full-length, ^{35}S-labeled Smad2, Smad3, Smad4, Smad5, and Smad8 were incubated with either GST alone or GST-LMO4. LMO4–Smad interactions were determined with GST pull-down assays and compared to 10% of the Smad protein input as visualized by SDS–PAGE and autoradiography. (**b**) GST pull-down assays were used to determine interactions between GST-LMO4 and the indicated ^{35}S-labeled subdomains of Smad3.

These data suggest that LMO4 may modulate the transcriptional response to TGFβ by interacting with Smad proteins, and that both the MH1 and linker domains of Smad3 participate in the interaction.

LMO4 can associate with the PAI-1 endogenous promoter in vivo *in response to TGF*β

During TGFβ signaling, R-Smads are phosphorylated by the activated receptor and form complexes with the co-Smad Smad4, after which the R-Smad/Smad4 complex enters the nucleus and associates with target genes (Massague and Wotton, 2000). To test whether LMO4 affects the phosphorylation of R-Smads, HEK293T cells were transfected with a control vector or LMO4, followed by treatment with vehicle or TGFβ1. We assessed the phosphorylation of endogenous Smad2 by Western blotting with an antibody recognizing phosphorylated Smad2. LMO4 had no effect on TGFβ1-induced Smad2 phosphorylation (Supplemental Figure 1A). To test whether LMO4 affects the R-Smad–Smad4 interaction, a Flag-tagged Smad3 and an HA-tagged Smad4 were cotransfected into HEK293T cell with or without MT-LMO4. After TGFβ1 treatment, the interaction between Flag-Smad3 and HA-Smad4 was analysed with immunoprecipitation and Western blotting. While TGFβ1 markedly enhanced Smad3/Smad4 complex formation, LMO4 had no effect on the complex formation (Supplemental Figure 1B). Together, these results suggest that LMO4 affects TGFβ signaling downstream of R-Smad phosphorylation and R-Smad/Smad4 complex formation. Based on these experiments and the protein–protein interaction results (Figures 3 and 4), we hypothesized that LMO4 might associate with Smad complexes on target genes.

To test whether LMO4 can associate with the PAI-1 promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays. HEK293T cells, untreated or treated with TGFβ1, were transfected with an empty vector or expression vectors encoding MT-Smad4 or MT-LMO4. ChIP assays were performed as previously described using myc(MT) antibodies with binding to the endogenous PAI-1 promoter detected with PCR using specific oligonucleotides (Kurisaki *et al*., 2003). As expected, Smad4 associates with the PAI-1 promoter, with binding greatly increased after TGFβ1 treatment (Figure 5a; lanes 1 and 2). Interestingly, LMO4 also associates with the PAI-1 endogenous promoter in a TGFβ1-dependent manner (Figure 5a; lanes 4 and 5), consistent with its ability to interact with Smad proteins and regulate the PAI-1 promoter. The MT antibody is specific in this assay because the PAI-1 promoter was not precipitated in cells transfected with an empty vector (Figure 5a; lane 3), and nonspecific IgG did not precipitate the PAI-1 promoter (Figure 5b; lanes 1–4) in an experiment where LMO4 associated with the promoter in a TGFβ1-dependent manner (Figure 5b; lanes 5 and 6). The association of LMO4 to the PAI-1 regulator, region is also promoter specific because no binding was detected to the GAPDH promoter (Figure 5c), which is regulated neither by TGFβ nor LMO4. Taken together with the results from transient transfection assays and protein–protein interaction studies, these data suggest that LMO4 can bind the PAI-1 promoter in a TGFβ-dependent fashion. This may occur via direct association with Smad proteins, resulting in modulation of promoter activity.

*LMO4 potentiates TGF*β*-mediated inhibition of cell proliferation*

Among the many different effects of TGFβ, inhibition of epithelial cell growth, either by suppression of cell proliferation or enhanced apoptosis, is one of the best-characterized (Derynck *et al*., 2001). Therefore, to test

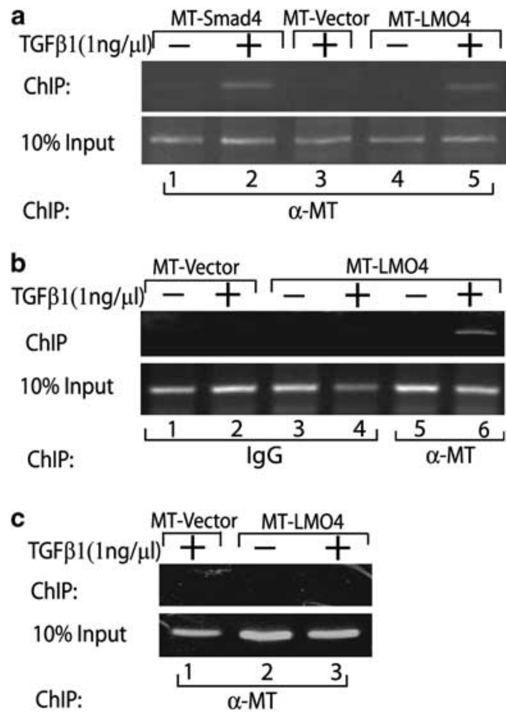

Figure 5 LMO4 associates with the endogenous PAI-1 promoter in a TGFβ-dependent fashion. (**a–c**) HEK293T cells grown in 100 mm dishes were transfected with 2 μg of empty expression vector or the same amount of expression vectors encoding MT-LMO4 or MT-Smad4, using Lipofectamine 2000. On the third day after transfection, cells were treated with vehicle or TGFβ1 (1 ng/ml) for 2 h. LMO4-associated DNA was isolated by ChIP with anti-MT or normal mouse IgG as a negative control, followed by PCR with primers specific for the PAI-1 promoter (**a** and **b**) or the GAPDH promoter (**c**). As a control, 10% of the input DNA was also PCR-amplified (lower panels in **a**, **b**, and **c**).

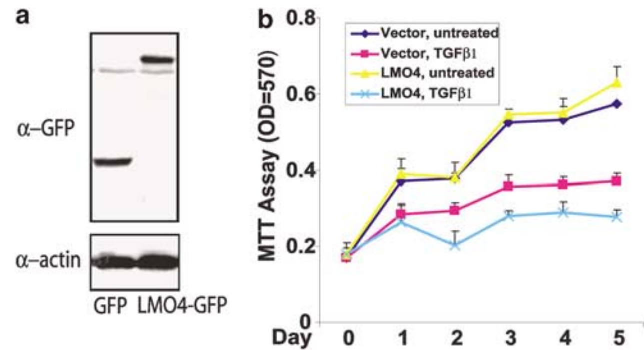

Figure 6 LMO4 enhances the inhibitory effect of TGFβ on human mammary epithelial cell growth. (**a**) HME cells were infected with equivalent pfu of retroviruses encoding GFP alone or LMO4-GFP. After 2–3 rounds of infections, about 80% of HME cells were expressing the target proteins as assessed by immunomicroscopy (not shown). At that time, whole-cell lysates were isolated and analysed by Western blotting with GFP antibody (top panel). As a control, the same blot was also bound to actin antibody (bottom panel). (**b**) HME cells expressing either LMO4-GFP or the control protein GFP were plated onto 96-well plates (5000 cells/well). After treatment with TGFβ1 (1 ng/ml) for 24 h, cells were grown in fresh grow medium for another 4 days; cell growth was monitored, using the MTT assay. MTT assays were performed in 10-replicate determination and results are expressed as the mean$\pm$s.d. at OD = 570 nm. Three independent experiments were performed; the data from a representative experiment are shown.

whether LMO4 can modulate the *in vivo* function of TGFβ signaling, we introduced viral vectors expressing either green fluorescent protein (GFP) or LMO4-GFP fusion proteins into normal HMEC. Expression from the GFP and LMO4-GFP vectors was equivalent in these experiments (Figure 6a) and for both vectors about 80% of cells expressed the proteins as determined by the GFP signal (data not shown). Cells were treated either with vehicle or TGFβ1 for 24 h and their growth was monitored over the course of 5 days, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. As expected, TGFβ1 inhibited the growth of HMEC in a time-dependent manner (Figure 6b). Interestingly, LMO4 significantly potentiated the cytostatic effect of TGFβ1 (Figure 6b). In contrast, LMO4 had no significant effect on the growth of untreated HMEC (Figure 6b).

To test whether the effect of LMO4 on the growth of HMEC was due to inhibition of proliferation or increased apoptosis, we first examined the effect of LMO4 on proliferation of HMEC, using the 5-(and 6-) carboxy fluoroscein diacetate succimidyl ester (CFSE) assay. As expected, TGFβ1 inhibited the proliferation of HMEC in a time-dependent fashion (Figure 7a; top panels). The introduction of LMO4 by retroviral transduction inhibited proliferation of HMEC (Figure 7a; middle panels). Expression from the control vector (TAP) and the vector expressing LMO4-TAP was similar (Figure 7b). To test whether cell death was modulated by LMO4, we monitored apoptosis after introduction of LMO4 in the presence and absence of TGFβ1 in HMEC, using Annexin V staining in combination with FACS analysis. TGFβ1 treatment increased the fraction of apoptotic HMEC from 6.43 to 11.21% and this effect was not significantly modulated by LMO4 (Figure 7c), suggesting that LMO4 does not alter the growth of HMEC by affecting apoptosis. Together, these experiments suggest that LMO4 affects cell growth by potentiating the inhibitory effect of TGFβ on cell proliferation.

In summary, our results suggest a novel function for LMO4 in TGFβ signaling. Based on our findings, we propose a model in which LMO4 interacts with Smad proteins on target genes, thereby modulating the cytostatic response of TGFβ.

Discussion

In this manuscript, we provide new information that the transcriptional coactivator LMO4 can modulate the cytostatic effects of TGFβ in epithelial cells. Using ChIP and transient transfection transcription assays, we demonstrate that LMO4 can associate with and regulate a prototype Smad target promoter.

One of the striking features of TGFβ signaling is the pleiotropic nature of its biological effect (Massague and

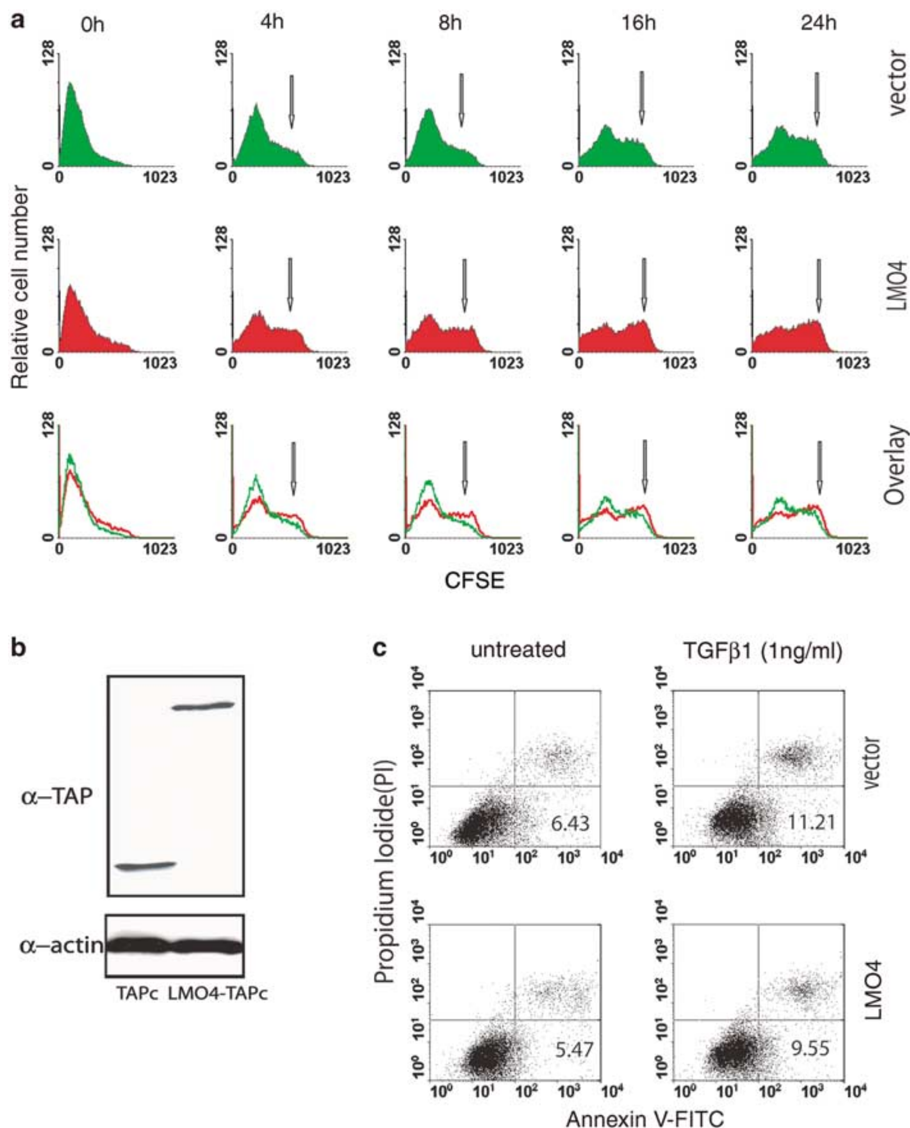

Figure 7 LMO4 enhances the inhibitory effect of TGFβ on HME cell proliferation, but has no effect on TGFβ-induced apoptosis. (**a**) HME cells were infected with retroviruses encoding LMO4-TAPc fusion protein or TAP alone as described for the experiment in Figure 6. HME cells expressing either control protein TAP (top panel) or LMO4-TAPc (middle panel) were stained with CFSE and then plated onto six-well plates (10 000 cells/well). On the second day, cells were treated with TGFβ1 (1 ng/ml) for the indicated time, and then grown in fresh medium for another 3 days. Cell proliferation was assessed with a FACS based on CFSE quantity. The CFSE amount in a single cell will decrease by 50% with each cell division. The arrows point to cells that contain large amount of CSFE, indicating slow proliferation. The third panel contains overlay of the TAPc (vector) and LMO4-TAPc (LMO4) panels and shows the relative abundance of slow-growing cells in the LMO4-infected panel. The data from a single representative experiment (out of three) are shown. (**b**) Expression of TAP and LMO4-TAPc in HME cell lysates was assessed by immunoblotting with TAP antibody (top panel). As a control, the same blot was also analysed by an Actin antibody (bottom panel). (**c**) HME cells expressing either TAP control protein or LMO4-TAPc were seeded onto 60-mm dishes (1×10^5 cells/dish). The next day, cells were treated with either vehicle (untreated) or TGFβ1 (1 ng/ml) for 24 h. Cell apoptosis was analysed with combined propidium iodide/annexin-V-FITC staining. The number in right-bottom half in each panel indicates the percentage of apoptotic cells. Similar results were obtained from three different experiments.

Wotton, 2000; Derynck and Zhang, 2003). Depending on context, TGFβ can selectively regulate proliferation, apoptosis, migration, epithelial–mesenchymal transition, as well as other cellular features. In addition, the effects of TGFβ are highly dependent on the responding cell type. Our data add to the growing literature suggesting that interactions of Smad proteins with other transcription factors may, at least in part, underlie the specificity of the multitude of TGFβ actions. Thus, our data suggest that LMO4 has selective effects on TGFβ actions because it modulates cell proliferation (Figures 6 and 7), but has no effect on apoptosis (Figure 7) and epithelial–mesenchymal transition (data not shown). Also, since LMO4 expression is restricted to epithelial cells, our findings suggest one mechanism whereby TGFβ effects are selectively modulated in distinct cell types.

Interestingly, our data predict that within the same cell type, changes in LMO4 levels may either increase or decrease TGFβ signaling, depending on the levels of LMO4 under the basal condition and the magnitude of LMO4 change (Figure 2c). For example, under conditions of very low LMO4 levels, moderate increases in LMO4 may lead to decreased TGFβ effect. However, under conditions of higher basal levels of LMO4, a further increase may enhance TGFβ effect. Smad proteins participate in multiprotein complexes that include transcriptional coactivators and corepressors, as well as DNA-binding proteins (Massague and Wotton, 2000; Derynck and Zhang, 2003). Since both upregulation and downregulation of LMO4 can lead to potentiation of TGFβ activation of the PAI-1 promoter, it is tempting to speculate that LMO4 helps to coordinate complexes on the PAI-1 gene, and that the stoichiometry of the components of these complexes is important. In such a case, both removal and excess of LMO4 is predicted to disrupt multiprotein complexes (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). Our findings are consistent with data in *Drosophila* showing that either upregulation or downregulation of the Clim homologue Chip leads to similar developmental phenotypes (Ramain *et al*., 2000).

Our data, which suggest that TGFβ regulation of at least some genes may be sensitive to LMO4 levels, are likely to have implications for understanding LMO4-mediated gene regulation because LMO4 is highly regulated under a variety of conditions that include normal and cancer development, as well as in response to physiological stimuli (Hinks *et al*., 1997; Wang *et al*., 2004). Owing to the cell- and developmental-specific regulation of LMO4, our findings may provide a mechanistic basis for aspects of cell-type- and context-specific gene regulation by TGFβ. Our results, showing that LMO4 overexpression enhances TGFβ-mediated cytostasis, may seem to contradict recent studies, which indicate that LMO4 overexpression promotes tumorigenic properties of mammary epithelial cells (Visvader *et al*., 2001; Sum *et al*., 2005b). However, there are at least two potential explanations for this apparent contradiction. First, because of the U-shaped TGFβ response curve to LMO4 (Figure 2c), the starting point will determine whether LMO4 potentiates or decreases TGFβ signaling; LMO4 overexpression in tumors may inhibit TGFβ signaling. Second, in addition to a direct cytostatic effect, TGFβ has direct and indirect protumorigenic effects; it is possible that LMO4 potentiates the protumorigenic effects of TGFβ *in vivo*.

A striking feature of LMO4 gene expression is its prominent expression in epithelial cells at locations of active reciprocal mesenchymal–epithelial interactions (Sugihara *et al*., 1998; Hermanson *et al*., 1999; Wang *et al*., 2004; Sum *et al*., 2005a). In such organs, including the developing hair follicles, teeth, mammary gland, lungs, and kidneys, BMP signaling has been shown to be very important (Arias, 2001; Waite and Eng, 2003). While our study has focused on the role of TGFβ signaling, it is quite possible that LMO4 could also modulate BMP signaling because we found that it interacts with Smad1, Smad5, and Smad8, which are primarily responsible for mediating BMP signals (Derynck and Zhang, 2003). In this respect, a recent study that used the yeast two-hybrid assay to screen for Smad8-interacting proteins identified LMO4 as a Smad8 partner (Colland *et al*., 2004). This is consistent with our findings that of all Smads tested, the strongest interaction was found between LMO4 and Smad8. This study also showed that LMO4 siRNA could inhibit BMP-7-stimulated transcription of a BMP-responsive reporter gene and the alkaline phosphatase gene in HepG2 cells (Colland *et al*., 2004). Yet, another potential link between LMO4 and BMP signaling comes from studies in *Xenopus* where it was shown that xLMO4 transcripts in ventral mesoderm and the neural plate are upregulated by BMP-4 (de la Calle-Mustienes *et al*., 2003). Functional studies indicate that xLMO4 plays roles in ventral mesoderm identity and neural plate regionalization. Thus, depending on the context, LMO4 may be both induced by BMP signaling and a modulator of the transcriptional effects of BMPs.

Many of the experiments in our study, including the ChIP experiments, were performed with exogenously expressed LMO4. However, it is important to note that we provide strong support for the potential role of endogenous LMO4 in TGFβ signaling. First, we demonstrated an interaction between endogenous LMO4 and Smad2 proteins, suggesting that LMO4 and Smad2 can interact *in vivo* at normal cellular concentrations (Figure 3b). Second, we showed that RNAi-mediated knockdown of LMO4 affected TGFβ signaling, supporting an *in vivo* role for endogenous LMO4 in TGFβ signaling (Figure 2).

For unknown reasons, LMO4 knockout mice die during later stages of embryogenesis or perinatally (Hahm *et al*., 2004; Tse *et al*., 2004; Lee *et al*., 2005). While a significant portion of these mice show exencephaly, even mice without this abnormality die perinatally. In addition, LMO4 knockout mice have skeletal patterning defects involving the basal skull, vertebrae, and ribs. Other homeotic transformations such as fusions of cranial nerves IX and X and defects in cranial nerve V were also observed (Hahm *et al*., 2004). No mice deleted for genes encoding TGFβ superfamily ligands phenocopy all aspects of the LMO4 knockout mice. However, strikingly, mice deleted for the TGFβ2 gene show defects in the sphenoid bone highly similar to those found in LMO4 mutant mice, including a missing presphenoid body; TGFβ2 knockout mice also exhibit rib cage abnormalities similar to the LMO4 knockout mice (Sanford *et al*., 1997). As in the LMO4 knockout mice, skeletal defects of the basal skull, vertebrae, and ribs are prevalent in BMP7 gene-deleted mice (Luo *et al*., 1995). These skeletal abnormalities include rib cage abnormalities that are common to the two, such as misalignment of the ribs on the sternum. Deletion of the BMP antagonist Noggin leads to altered patterning of somites and the neural tube in the mouse, including neural tube closure defects in the cranial region, similar to those found in the LMO4 knockout mice (McMahon *et al*., 1998). Similarly, Smad5 knockout mice exhibit

failure of cranial neural tube closure and exencephaly (Chang *et al*., 1999). Furthermore, mice deleted for the c-ski gene, which encodes a transcriptional repressor involved in TGFβ/BMP signaling, show both exencephaly and defects in the basal skull bones similar to those found in LMO4 knockout mice (Berk *et al*., 1997). Thus, it is possible that altered signaling by TGFβ superfamily ligands plays roles in some of the abnormalities in LMO4 knockout mice.

In addition to a developmental role, there are several lines of evidence suggesting that LMO4, like other members of this gene family, may play roles in oncogenesis. LMO4 was originally identified as an autoantigen in human breast cancer (Racevskis *et al*., 1999) and subsequently shown to be upregulated in over 50% of breast cancer cases (Visvader *et al*., 2001). Additionally, it was found that LMO4 could interact with the BRCA1 tumor suppressor gene (Sum *et al*., 2002). Consistent with a role in mammary epithelial cells, we have shown that overexpression of a dominant-negative LMO4 inhibits ductular and lobuloalveolar development in the mammary glands of transgenic mice (Wang *et al*., 2004), and others have demonstrated that mammary gland-specific deletion of the LMO4 gene leads to impaired lobuloalveolar development during pregnancy (Sum *et al*., 2005c). LMO4 has also been shown to be upregulated at the invasive fronts of oral cancers, suggesting a role in cancer cell invasion (Mizunuma *et al*., 2003). In the prostate, LMO4 was downregulated during tumor progression and lowered in hormone refractory tumors (Mousses *et al*., 2002). In breast cancers and in breast cancer cell lines, LMO4 levels appear to be disproportionately upregulated as compared to the levels of Clim factors (Visvader *et al*., 1997; Wang *et al*., 2004). Therefore, the effects we have observed may have particular relevance for such situations where LMO4 and Clim levels are not coordinately regulated.

Materials and methods

Cell culture, retroviruses, and transfection assays

Normal HMEC were purchased and cultured according to protocols from Cambrex. The murine mammary epithelial (NMuMG) cells, human embryonic kidney (HEK293T) cells, and human breast cancer cell line T47D were cultured according to the ATCC protocol.

Retroviruses expressing LMO4 gene and control protein were based on the Retro-X™ System from BD Biosciences. Construction of the LMO4 retroviruses and the infection of virus into cells were performed according to the manufacturer's protocol. LMO4 was fused in frame at the C-terminus to the tandem affinity purification (TAPc) tag, which contains two IgG-binding domains of *Staphylococcus aureus* protein A and a calmodulin-binding peptide separated by a TEV protease cleavage site (Puig *et al*., 2001). Another vector was created in which LMO4 was fused in frame at the C-terminal site to GFP. Retrovirus was harvested from the stably transfected packaging cell line GP2-293, and the titer of virus was determined using NIH3T3 cell. In experiments, cells were infected with equivalent virus titer for each construct and for the same length of time. Protein expression was determined by Western blotting to ensure similar expression from the control and experimental viruses.

Transient transfections and luciferase reporter assays were performed as previously described, using calcium precipitation for HEK293 cells, and Lipofectamine™ 2000 (Invitrogen) for HME and NMuMg cells (Sugihara *et al*., 2001). Luciferase activity was normalized for differences in transfection efficiency, using the Renilla luciferase vector (Promega). The plasmids used in these studies have been previously described: 9xGAGA-Luciferase (Dennler *et al*., 1998), pCS2-MT-LMO4 (Sugihara *et al*., 1998), and pCMV5-TβR1-AAD (Chen *et al*., 1997).

The LMO4-specific siRNAs, which were designed based on the human LMO4 mRNA sequence (accession number, NM_006769), were obtained from Ambion. The target sequences of the LMO4 duplex siRNAs are: GGCAATGTGT ATCATCTTA (LMO4#1), GGTCTGCTAAAAGGTCAGA (LMO4#2), and GGAAACGTGTTTCAATCAA (LMO4#3). The control siRNA was unrelated to the LMO4 sequence and not known to affect any endogenous genes (Ambion). The siRNAs were introduced into T47D cells using RNAiFect transfection reagent (Qiagen). For transcriptional assays, LMO4 shRNAs and the control shRNA were synthesized and cloned into RNAi-Ready PSIREN-RetroQ-ZsGreen vector (BD Biosciences). The duplex sequences of the LMO4 shRNAs are: 5′-gatccggcaatgtgtatcatcttattcaagagataagatgatac acattgccttttg-3′ (shRNA #1), 5′-gatccggaaacgtgtttcaatcaattcaa gagattgattgaaacacgtttcctttttg-3′ (shRNA #3), and 5′-gatccgtgcg ttgctagtaccaacttcaagagatttttttacgcgtg-3′ (shRNA control).

Recombinant mature human TGFβ1 (R&D Systems) was used according to the manufacturer's recommendations. Unless otherwise indicated, all other chemicals were from Fisher/ICN.

Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and complementary DNA was synthesized using 5 μg of total RNA with the High-Capacity cDNA archive kit (Applied Biosystems) (Lin *et al*., 2004). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7900HT platform (384-well plates; Applied Biosystems), following standard protocols from the supplier to detect threshold cycle (C_t). ΔC_t values were calculated by comparing the C_t measurements of experimental wells to the untreated (basal) wells that were infected with the control virus. All values were then normalized to 18S rRNA to obtain $\Delta\Delta C_t$ values.

Co-immunoprecipitations, Western blots, and GST pull-down assays

Co-immunoprecipitations of extracts from transfected HEK293T cells were performed as previously described (Sugihara *et al*., 2001), using MT (myc) antibody (Invitrogen; R950-25) recognizing tagged LMO4, and HA antibody (Covance; MMS-101R) detecting tagged Smads. The following vectors, pCMV5/Smad1-HA, pCMV5/Smad2-HA, pCMV5/Smad4-HA, and pGCN/HA-Smad5, were described previously (Chen *et al*., 1997; Hata *et al*., 1997). For co-immunoprecipitation of endogenous LMO4 and Smad2 proteins in HEK293T cells, we used antibodies directed against LMO4 (Santa Cruz; SC-11122) and Smad2 (Zymed; 51-1300). For GST pull-down assays, Smad mutant genes were generated by PCR-based deletion, followed by cloning into vectors allowing *in vitro* transcription/translation; the sequences were confirmed by DNA sequencing. Western blot analysis was performed as described previously (Wang *et al*., 2004), using antibodies to

phosphor-Smad2 (Cell Signaling; 3101), LMO4 (Sum *et al*., 2002), MT (Invitrogen; R950-25), HA (Covance; MMS-101R), GFP (Upstate Cell Signaling Solution; 06–896), TAPc (Peroxidase-anti-peroxidase (PAP) antibody, Sigma-Aldrich; P-2026), GAPDH (Ambion; 4300), and β-actin (Santa Cruz; SC-8432).

The GST pull-down assays were performed as previously described (de la Calle-Mustienes *et al*., 2003). Briefly, GST protein or GST-LMO4 fusion protein were incubated with ^{35}S-labeled *in vitro* translated Smad proteins at room temperature for 30 min. After washing three times, the glutathione–agarose beads were resuspended in SDS sample buffer, boiled, and analysed on 10% SDS–polyacrylamide gels.

ChIP

ChIP assays were performed according to the protocol from Upstate Cell Solution. Chromatinized DNA was crosslinked in 1% formaldehyde for 10 min at 37°C. Cells were then washed twice using ice-cold phosphate-buffered saline containing protease inhibitors (Roche Applied Science; 10752800) and then harvested in PBS with protease inhibitors. Thereafter, cells (1×10^6) were resuspended in 0.2-ml SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated on ice for 10 min, and sonicated to reduce the chromatin DNA length to 1 kb. The lysates were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and precleared with sperm DNA-protein A-agarose beads (Upstate Cell Signaling Solutions) at 4°C for 1 h. Following overnight incubation with 2 μg of anti-MT or IgG, immune complexes were immobilized by salmon sperm DNA protein A agarose beads. After extensive washing and elution with 1% SDS and 0.1 M $NaHCO_3$, crosslinks were reversed by incubation at 65°C for 4 h in the presence of 0.2 M NaCl. The released DNA was phenol–chloroform-purified, and the PAI-1 and GAPDH promoter sequences were detected by PCR followed by agarose gel visualization. The ChIP primers for PAI-1 are 5′-CCT CCAACCTCAGCCAGACAAG-3′ (forward) and 5′-CCCAG CCCAACAGCCACAG-3′ (reverse) (Kurisaki *et al*., 2003). The primers for GAPDH are 5′-CGGCTACTAGCGGTTTT ACG-3′ (forward) and 5′-AAGAAGATGCGGCTGACTGT-3′ (reverse).

Cell growth assays

Cells were incubated overnight at a density of 5000 cells/well in 96-well plates, and treated with TGFβ1 (1 ng/ml) for 24 h. Then, cells were grown in a fresh growth medium for up to 5 days. Cell growth was assessed daily using the conversion of MTT to formazan production (Matsuda *et al*., 2002). Briefly, cells from 10 wells were incubated with MTT (62.5 μg/well) for 4 h. Cellular MTT was solubilized with acidic isopropanol, and absorbance was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). Results were plotted as the mean $\pm$ s.d. of 10 determinations for each time point. Four independent experiments were performed; the data from a representative experiment are shown.

Cell proliferation assays

To determine cell proliferation, cells were labeled with 5-(and 6-) carboxy fluoroscein diacetate succimidyl ester (CFSE; Molecular probes, Eugene, USA) to quantify cell division (Lee *et al*., 2004). Briefly, cells were resuspended in PBS at 2×10^7 cells/ml and labeled by incubation in 5 μM CFSE for 8 min at RT. Cells were then quenched with Fetal Bovine Serum, washed three times with PBS and plated onto six-well plates (10 000 cells/well). On the second day, cells were treated with TGFβ1 (1 ng/ml) for 4, 8, 16, 24 h, and then grown in fresh medium for another 3 days. Cells were detached by 0.05% trypsin (Invitrogen), suspended in 1 ml PBS, and analysed by a FACSCaliber flow meter (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

Apoptosis assays

For annexin V staining, cells were seeded at a density of 1×10^5 cells/60-mm dish on day 0. On day 1, cells were treated with TGFβ1 (1 ng/ml) for 24 h. Then, cells and supernatant were collected and stained with annexin-V-FITC and propidium iodide (PI), using the Annexin V-FLUOS Staining Kit (Roche Applied Science; 1858777). Duplicate samples were analysed on a FACSCaliber flow meter (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

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References

Agulnick AD, Taira M, Breen JJ, Tanaka T, Dawid IB, Westphal H. (1996). *Nature* **384**: 270–272.

Aoyama M, Ozaki T, Inuzuka H, Tomotsune D, Hirato J, Okamoto Y *et al*. (2005). *Cancer Res* **65**: 4587–4597.

Arias AM. (2001). *Cell* **105**: 425–431.

Bach I. (2000). *Mech Dev* **91**: 5–17.

Bach I, Carriere C, Ostendorff HP, Andersen B, Rosenfeld MG. (1997). *Genes Dev* **11**: 1370–1380.

Bach I, Rodriguez-Esteban C, Carriere C, Bhushan A, Krones A, Rose DW *et al*. (1999). *Nat Genet* **22**: 394–399.

Berk M, Desai SY, Heyman HC, Colmenares C. (1997). *Genes Dev* **11**: 2029–2039.

Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A. (1999). *Development* **126**: 1631–1642.

Chen YG, Liu F, Massague J. (1997). *EMBO J* **16**: 3866–3876.

Colland F, Jacq X, Trouplin V, Mougin C, Groizeleau C, Hamburger A *et al*. (2004). *Genome Res* **14**: 1324–1332.

de la Calle-Mustienes E, Lu Z, Cortes M, Andersen B, Modolell J, Gomez-Skarmeta JL. (2003). *Dev Biol* **264**: 564–581.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. (1998). *EMBO J* **17**: 3091–3100.

Derynck R, Akhurst RJ, Balmain A. (2001). *Nat Genet* **29**: 117–129.

Derynck R, Zhang YE. (2003). *Nature* **425**: 577–584.

Dong-Le Bourhis X, Lambrecht V, Boilly B. (1998). *Br J Cancer* **77**: 396–403.

Grutz G, Forster A, Rabbitts TH. (1998). *Oncogene* **17**: 2799–2803.

Hahm K, Sum EY, Fujiwara Y, Lindeman GJ, Visvader JE, Orkin SH. (2004). *Mol Cell Biol* **24**: 2074–2082.

Hata A, Lo RS, Wotton D, Lagna G, Massague J. (1997). *Nature* **388**: 82–87.
Hermanson O, Sugihara TM, Andersen B. (1999). *Cell Mol Biol (Noisy-le-grand)* **45**: 677–686.
Hinks GL, Shah B, French SJ, Campos LS, Staley K, Hughes J *et al*. (1997). *J Neurosci* **17**: 5549–5559.
Jurata LW, Kenny DA, Gill GN. (1996). *Proc Natl Acad Sci USA* **93**: 11693–11698.
Kenny DA, Jurata LW, Saga Y, Gill GN. (1998). *Proc Natl Acad Sci USA* **95**: 11257–11262.
Kudryavtseva EI, Sugihara TM, Wang N, Lasso RJ, Gudnason JF, Lipkin SM *et al*. (2003). *Dev Dyn* **226**: 604–617.
Kurisaki K, Kurisaki A, Valcourt U, Terentiev AA, Pardali K, Ten Dijke P *et al*. (2003). *Mol Cell Biol* **23**: 4494–4510.
Lee MKt, Moore DJ, Jarrett BP, Lian MM, Deng S, Huang X *et al*. (2004). *J Immunol* **172**: 6539–6544.
Lee SK, Jurata LW, Nowak R, Lettieri K, Kenny DA, Pfaff SL *et al*. (2005). *Mol Cell Neurosci* **28**: 205–214.
Lee SK, Pfaff SL. (2003). *Neuron* **38**: 731–745.
Lin KK, Chudova D, Hatfield GW, Smyth P, Andersen B. (2004). *Proc Natl Acad Sci USA* **101**: 15955–15960.
Luo G, Hofmann C, Bronckers AL, Sohocki M, Bradley A, Karsenty G. (1995). *Genes Dev* **9**: 2808–2820.
Manetopoulos C, Hansson A, Karlsson J, Jonsson JI, Axelson H. (2003). *Biochem Biophys Res Commun* **307**: 891–899.
Massague J, Wotton D. (2000). *EMBO J* **19**: 1745–1754.
Matsuda K, Idezawa T, You XJ, Kothari NH, Fan H, Korc M. (2002). *Cancer Res* **62**: 5611–5617.
Matthews JM, Visvader JE. (2003). *EMBO Rep* **4**: 1132–1137.
McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. (1998). *Genes Dev* **12**: 1438–1452.
Milan M, Cohen SM. (1999). *Mol Cell* **4**: 267–273.
Milan M, Cohen SM. (2000). *Development* **127**: 3069–3078.
Milan M, Diaz-Benjumea FJ, Cohen SM. (1998). *Genes Dev* **12**: 2912–2920.
Mizunuma H, Miyazawa J, Sanada K, Imai K. (2003). *Br J Cancer* **88**: 1543–1548. (2: van Meyel DJ, *et al*. Ssdp proteins bind to LIM-int.[PMID:12642495]Related Articles, Links).
Mousses S, Bubendorf L, Wagner U, Hostetter G, Kononen J, Cornelison R *et al*. (2002). *Cancer Res* **62**: 1256–1260.
Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E *et al*. (2001). *Methods* **24**: 218–229.
Rabbitts TH. (1998). *Genes Dev* **12**: 2651–2657.
Racevskis J, Dill A, Sparano JA, Ruan H. (1999). *Biochim Biophys Acta* **1445**: 148–153.
Ramain P, Khechumian R, Khechumian K, Arbogast N, Ackermann C, Heitzler P. (2000). *Mol Cell* **6**: 781–790.
Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP *et al*. (1997). *Development* **124**: 2659–2670.
Sugihara TM, Bach I, Kioussi C, Rosenfeld MG, Andersen B. (1998). *Proc Natl Acad Sci USA* **95**: 15418–15423.
Sugihara TM, Kudryavtseva EI, Kumar V, Horridge JJ, Andersen B. (2001). *J Biol Chem* **276**: 33036–33044.
Sum EY, O'Reilly LA, Jonas N, Lindeman GJ, Visvader JE. (2005a). *J Histochem Cytochem* **53**: 475–486.
Sum EY, Peng B, Yu X, Chen J, Byrne J, Lindeman GJ *et al*. (2002). *J Biol Chem* **277**: 7849–7856.
Sum EY, Segara D, Duscio B, Bath ML, Field AS, Sutherland RL *et al*. (2005b). *Proc Natl Acad Sci USA* **102**: 7659–7664.
Sum EY, Shackleton M, Hahm K, Thomas RM, O'Reilly LA, Wagner KU *et al*. (2005c). *Oncogene* **24**: 4820–4828.
Thaler JP, Lee SK, Jurata LW, Gill GN, Pfaff SL. (2002). *Cell* **110**: 237–249.
Torigoi E, Bennani-Baiti IM, Rosen C, Gonzalez K, Morcillo P, Ptashne M *et al*. (2000). *Proc Natl Acad Sci USA* **97**: 2686–2691.
Tse E, Smith AJ, Hunt S, Lavenir I, Forster A, Warren AJ *et al*. (2004). *Mol Cell Biol* **24**: 2063–2073.
van Meyel DJ, O'Keefe DD, Jurata LW, Thor S, Gill GN, Thomas JB. (1999). *Mol Cell* **4**: 259–265.
Visvader JE, Mao X, Fujiwara Y, Hahm K, Orkin SH. (1997). *Proc Natl Acad Sci USA* **94**: 13707–13712.
Visvader JE, Venter D, Hahm K, Santamaria M, Sum EY, O'Reilly L *et al*. (2001). *Proc Natl Acad Sci USA* **98**: 14452–14457.
Wadman I, Li J, Bash RO, Forster A, Osada H, Rabbitts TH *et al*. (1994). *EMBO J* **13**: 4831–4839.
Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A *et al*. (1997). *EMBO J* **16**: 3145–3157.
Waite KA, Eng C. (2003). *Nat Rev Genet* **4**: 763–773.
Wang N, Kudryavtseva E, Ch'en IL, McCormick J, Sugihara TM, Ruiz R *et al*. (2004). *Oncogene* **23**: 1507–1513.
Wieser R, Wrana JL, Massague J. (1995). *EMBO J* **14**: 2199–2208.
Yamada Y, Warren AJ, Dobson C, Forster A, Pannell R, Rabbitts TH. (1998). *Proc Natl Acad Sci USA* **95**: 3890–3895.
Zeng C, Justice NJ, Abdelilah S, Chan YM, Jan LY, Jan YN. (1998). *Proc Natl Acad Sci USA* **95**: 10637–10642.

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