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13. ABSTRACT (Maximum 200 Words)

We identified a novel transcription factor LMO4 and found that LMO4 is highly expressed in breast epithelial cells and is related to cell proliferation and/or invasion *in vivo*. Because these cellular features are associated with breast carcinogenesis and because LMO4 is overexpressed in more than 50% of breast cancer cases, we hypothesize that LMO4 may play roles in oncogenesis of breast epithelial cells by regulating proliferation, invasion and/or other cellular features. As the initial step of the whole projects, we established several permanent cell line (including breast normal epithelial cells and cancer cells), which stably overexpress LMO4 protein or express LMO4 RNAi to delete LNO4 protein. Using these cell lines, We will analyze the biological effects of LMO4 on cell proliferation, invasion and other cellular features *in vitro*, and screen the target gene of LMO4 by gene microarray. We have obtained some exciting results and found that LMO4 modulates TGFβ signaling, which plays the crucial roles in both development and cancer. Our results show LMO4 associated with TGFβ signaling target gene promoter by interacting with Smad proteins, and enhanced the inhibitory effect of TGFβ on cell proliferation of mammary gland epithelial cells. These results strengthen the hypothesis that LMO4 may contribute to the oncogenesis of breast tissue and indicates that our whole work will play a role in solving the breast cancer problem on the support of the Army.

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Table of Contents

A. Figures 1-8

B. Abstracts:

Zhongxian Lu, Manuel Cortes, Bogi Andersen.2004. The LIM-only protein LMO4 activates TGFβ signaling by interacting with Smad proteins. The 2004 Conference of Chao Family Comprehensive Cancer Center, Palm Spring,California, Novermber

Zhongxian Lu, Keyes S. Lam, Manuel Cortes & Bogi Andersen. 2005. The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins. The 96th annual meeting of the American Association of Cancer Research, Anaheim,California, April.

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The 2005 Conference of DOD Breast Cancer Research Program, Philadelphia, Pennsylvania, June.

INTRODUCTION

My training program contains two distinct components: molecular laboratory research in breast cancer and computational biology and bioinformatics. The two components will be integrated because analyses of data obtained from my laboratory research will be one of my entry points into computational biology. In addition, results from the computational part of my project will spurn biological experiments. **A.** My training in computational biology will involve formal course work from the UCI Bioinformatics Training Program. These courses include *Basic Statistics (Math 7, 4u), Introduction to Computer Science (ICS 21, 6u), Representations and Algorithms for Molecular Biology (ICS 277A, 4u)* and *Probabilistic Modeling of Biological Data (ICS 277B, 4u).* **B.** My molecular laboratory research training focuses on the LIM-only factor (LMO) 4 gene. LMO4 belongs to a family of four mammalian LMO proteins, which are only composed of two LIM domains and little other sequence (1). LMOs are thought to act as adapter molecules in transcriptional complexes, tethering the co-activators CLIM (Nli/Ldb) to various DNA-binding proteins (2). LMOs family proteins show a crucial role not only during development, but also in tumorogenesis. LMO1 and LMO2 act as oncogenes in acute lymphblastic leukemia (3). LMO4 is also referred to as Human Breast Tumor Autoantigen based on that LMO4 was also first isolated from breast cancer tissue and overexpressed in more than 50% of breast cancer cases (4, 5). Furthermore, LMO4 interacts with BRCA1 and inhibits the activation of BRCA1 (6). In study comparing expression profiles in estrogen positive and negative breast cancer, LMO4 was found in a panel of genes that strongly predicted estrogen negative status of breast cancer. My hypothesis is that, analogous to the role of LMO2 in leukemia, LMO4 overexpression promotes oncogenesis of breast epithelial cells by deregulating one or more of the following cellular features: differentiation, proliferation, apoptosis or invasion. In addition, we hypothesize that LMO4 acts, at least in part, by interacting with BRCA1, thereby interfering with the regulation of BRCA1 target genes.

Our specific aims were: #1. To test the effects of LMO4 overexpression or LMO4 interference in breast cancer by conditional expression systems and an interfering RNA plasmid system to increase and decrease, respectively, LMO4 protein levels in breast cancer cell lines. #2. To use gene expression profiling in MCF-7 breast cancer cells to elucidate the mechanisms of action for LMO4 overexpression. To gain insights into how LMO4 acts at a molecular level, we will use Affymetrix microarrays to define the profile of genes altered by LMO4 in breast cancer cells. I will also use the same approach to compare the target genes of LMO4 and BRCA1.

BODY

Task 1. **To test the phenotypic effects of conditional LMO4 overexpression and LMO4 interference in human breast cancer cells.**

1. Create the gene transduction system by the retrovirus and overexpress LMO4 in breast cancer cell line.

To understanding completely the role of LMO4 in breast cancer, we will assay the biological effects of LMO4 overexpression or interference in several different cell lines, including normal mammary gland cells or breast cancer cells. For introducing efficiently LMO4 gene into the different target cell, a retroviral gene transfer and expression system (BD Retro-X^{TM}) was employed. Retrovirus can highly efficiently introducing gene into any dividing cell, and gene will be stably, heritably expressed in host cell. Using the retrovirus, LMO4 can be stably overexpressed in the target cell. In addition, LMO4 protein level can be controlled by adjusting the titer of virus, which allows the results from different cell types are compatible. Performing the retroviral gene transfer and expression system, I have obtained high titer virus containing LMO4 gene, and successful stably overexpressed LMO4 in several cell line: normal human mammary gland epithelial (HME) cells, murine mammary epithelial (NMuMG) cells, human breast cancer cell line MCF-7, MD-MBA-231 and T47D. Fig.1 shows the protein level of LMO4 or control protein in different cells.

2. Establish breast cancer cell lines that express LMO4-RNAi

For deleting the LMO4 protein, Three LMO4-specific siRNAs, which were designed based on the human LMO4 cDNA sequence (accession number, NM_006769), were obtained from Ambion. The siRNAs were introduced into breast cancer cell T47D, which express LMO4 at a relatively high level. Of the three LMO4 siRNAs, LMO4 siRNA #1 and #3 effectively decreased endogenous LMO4 levels (Fig. 2A; lanes 1 and 3) compared to a negative control siRNA. Then LMO4 siRNA #1 and #3 and the control siRNA were cloned into siRNA vector. Using the construct of LMO4 siRNA, I have established the permanent cell line of MD-MBA-231 and T47D which stably expressed LMO4-RNAi (Fig 2B).

3. Test the phenotypic effects of altering LMO4 levels in breast cancer cell lines.

A. Check the effects of LMO4 in normal mammary epithelial cells. LMO4 is highly expressed in epithelial cell, and relate to mammary gland development. We therefore explored the biological effects of LMO4 on normal mammary gland epithelial cell. We first overexpressed GFP-tagged LMO4 in Primary human mammary gland epithelial (HME) cells, and observed the cell grow. Compare the control cell expressing GFP, HME-LMO4 cells had no significant different on the growth (Fig 3). Using overexpressing TAPc tagged LMO4 HME cell, we evaluated the effects of LMO4 on cell proliferation and apoptosis. LMO4 overexpression also didn't show significant effects (Fig. 4A and 4B). However, based on the fact that LMOs protein are relate to TGFβ/BMP signaling pathway in development (7, 8), and that LMO4 is highly expressed in locations of epithelial-mesenchymal interactions where TGFβ cytokine signaling plays important roles both in development and in cancer (5, 9), we treated HME cell overexpressed LMO4 with TGFβ and found exciting results: LMO4 significantly potentiated the cytostatic effect of TGFβ. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay show HME-LMO4GFP cell is more strong response to TGFβ stimulation and grow slow than the control cell(Fig 3). 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 (CSF) assay. The introduction of LMO4 by retroviral transduction inhibited proliferation of HMEC (Fig. 4A; lower panels). To test whether cell death was modulated by LMO4, we monitored apoptosis after introduction of LMO4 in the presence and absence of TGFβ in HMEC, using Annexin V staining in combination with FACS analysis. TGFβ treatment increased the fraction of apoptotic HMEC from 9.8% to 16.4% and this effect was not significantly affected by LMO4 (Fig. 4B), suggesting that LMO4 does not affect 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.

To determine whether LMO4 upregulation could modulate TGFβ signaling, we tested the ability of LMO4 to affect the expression of a well-known TGFβ-responsive reporter gene, 9xGAGA-Luciferase, which is derived from the regulatory region of the Plasminogen Activator Inhibitor 1 (PAI-1) gene. Co-transfection of an expression plasmid encoding LMO4 resulted in a dose-dependent expression of LMO4 (Fig. 5B) and markedly increased a constitutively active TGFβ receptor 1 (TβR1-ADD)-stimulated luciferase activity, also in a dose-dependent manner (Fig. 5A). We observed similar enhancing effects of LMO4 on TGFβ1-stimulated 9xGAGA-Luciferase expression in normal human mammary epithelial cells (HMEC) (Fig. 5C), and the mouse mammary epithelial cell line, NMuMG (Fig. 5D). These results indicate that LMO4 can enhance TGFβ-mediated signaling as monitored by the PAI-1 promoter in HEK293T and mammary epithelial cells. Using quantitative PCR to measured PAI-1 mRNA, we found LMO4 increased PAI-1 mRNA several fold under both basal ($\Delta\Delta CT=2.3$) and TGFβ-stimulated ($\Delta\Delta CT=5.7$) conditions (Fig. 5E). Taken together, these results suggest that LMO4 up-regulation is capable of enhancing TGFβ-stimulated transcription of the PAI-1 gene.

LMO4 regulates transcription by participating in multiprotein complexes that are likely to involve both DNA-binding proteins and other transcriptional co-regulators, such as Clims. The stoichiometry of these complexes is thought to be critical for their activity and LMO4 upregulation may therefore modulate transcription by disrupting such complexes. 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 up-regulation; both perturbations, up- and down-regulation, would alter the stoichiometry of LMO4-containing transcription complexes. To test this idea, we transfected into HEK293T cells an expression vector encoding LMO4 siRNA#1 with 9xGAGA-Luciferase reporter plasmid, with and without a TGFβ activator. While the control siRNA had little effect on TGFβ stimulation of reporter activity, the LMO4 siRNA markedly enhanced TGFβ stimulation (Fig. 6A). The effect of the LMO4 siRNA was specific because the expression vector that encodes mouse LMO4 (not targeted by the siRNA) could partially reverse the stimulatory effect of LMO4 siRNA (Fig. 6B). As predicted from the experiments described previously (Fig. 5), 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 (Fig. 6B). Together,

these experiments show that either decreasing or increasing the concentration of LMO4 can enhance TGFβ-dependent transcription of the PAI-1 gene reporter.

TGFβ regulates transcription of the PAI-1 gene by facilitating the nuclear translocation and DNA-binding of a complex composed of an R-Smad (Smad2 and/or Smad3) and the co-Smad, Smad4 (10). To investigate the mechanisms of action for the effect of LMO4 on TGFβ-mediated transcription, we therefore tested whether LMO4 could interact with the key mediators of TGFβ-regulated transcription, the Smad proteins. 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 a myc tag antibody followed by SDS gel electrophoresis and immunoblotting with an HA antibody. Smad1, Smad2, Smad4 and Smad5 were all co-immunoprecipitated with LMO4 (Fig. 7A; top panel), suggesting that LMO4 is capable of interacting with several Smad 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 interacts with Smad2, Smad3, Smad4, Smad5, and Smad8, with weakest and strongest LMO4-interactions detected with Smad4 and Smad8, respectively (Fig. 7B). To map the interaction domains on Smads, which are responsible for interactions with LMO4, we tested the interactions between LMO4 and domains of the Smad3 and Smad4 proteins. Smad proteins are composed of an N-terminal Mad Homology (MH) domain 1, which is responsible for DNA-binding, attached with a C-terminally located MH2 domain; a less conserved linker domain joins the two MH domains. All three domains have been shown to interact with several transcription factors. In these experiments, LMO4 interacted with the MH1 and linker domains in both Smad3 and Smad4; no interaction was found with the MH2 domain (Fig. 7C). These data suggest that LMO4 may modulate the transcriptional response to TGFβ by interacting with Smad proteins.

During TGFβ signaling pathway, R-Smad is phosporylated by activated receptor and form a complex with co-Smad (Smad4). Then, the complex of R-Smad and Smad4 enter into into the nucleus and associating with target genes (10). LMO4 interacts with Smads protein and possible affects these steps. HEK293T cells were transfected with LMO4, and then treated with TGFβ. The phosporylation of Smad2 was assessed with phosporylating Smad2 antibody by western blotting. Overexpression of LMO4 didn't affects TGFβ-induced Smad2 phosporylation (Fig. 8A). In addition, Flag tagged Smad3 and HA tagged Smad4 were co-transfected into HEK293T cell with or without MT-LMO4. After TGFβ treatment, the interaction between Flag-Smad3 and HA-Smad4 was analyzed with immunoprecipitation and western blotting. LMO4 overexpression has also no influences on the complex formation of Smad3 and Smad4 (Fig. 8B). These results suggested LMO4 don't involve in R-Smad activation and interaction with Co-Smad, and indicated LMO4 may modulates target gene transcription as a part of Smads-DNA complex. To confirm this hypothesis, we performed Chromatin Immunoprecipitation (ChIP) assays to test whether LMO4 associate with the PAI-1 promoter. HEK293T cells, untreated or treated with TGFβ, were transfected with an empty vector or expression vectors encoding myc tagged Smad4 or myc tagged LMO4. ChIP assays were performed as previously described, using myc antibodies and IgG as a control, with binding to the

PAI-1 promoter detected with PCR using specific oligonucleotides. As expected, Smad4 associates with the PAI-1 promoter with binding greatly increased after TGFβ treatment (Fig. 8C; lanes 1 and 2). Interestingly, LMO4 also associates with the PAI-1 promoter in a TGFβ-dependent manner (Fig. 8C; 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 (Fig. 8C; lanes 3), and non-specific IgG did not precipitate the PAI-1 promoter (Fig. 5B; lanes 1-4) in an experiment where LMO4 associated with the promoter in a TGFβ-dependent manner (Fig. 8D; lanes 5 and 6). The association of LMO4 to the PAI-1 is also promoter specific because no binding was detected to the GAPDH promoter (Fig. 8E), 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 associate with Smad proteins on the TGFβ-responsive PAI-1 promoter in a TGFβ-dependent fashion, resulting in modulation of its promoter activity.

B. Assess the effects of LMO4 on breast cancer cell (in working).

Task 2. **To use gene expression profiling in MCF-7 breast cancer cells to elucidate the mechanisms of action for LMO4 overexpression. (in working):**

Task 3. **Formal training in bioinformatics.**

a. I have studied the basic statistic course. This course completely introduces the basic statistic method, and strenthens my ability to design experiments, harvest the original data, analyze data and make conclusion from the results.
b. I have finished the Representations and Algorithms for Molecular Biology course, which introduces mechanisms of computer processing for biological data, such as how to compare different sequences of gene or protein, how to analyze the structure of protein and genomic material, and especially how to harvest and analyze microarray data. This course greatly improves my ability in working on molecular experiments (such as designing primer, analyzing the sequence of novel promoter, defining the new gene sequence, etc.) and to do gene expression profiling, which is the major experiment in specific aim #2.

KEY RESEARCH ACCOMPLISHMENT

1. Defining the regulation mechanisms of LMO4 on cell growth and finding LMO4 regulated cell growth by modulating TGFβ signal.
2. Demonstrating that LMO4 modulates TGFβ signaling pathway by interaction with Smad proteins.

REPORTABLE OUTCOMES TO DATE

1. Permanent breast cancer cell lines of LMO4 overexpression.

2. Permanent breast cancer cell lines of LMO4 siRNA

3. Manuscrip: **Zhongxian Lu**, Kaye Star Lam, Manuel Cortes, Bogi Andersen, 2005.The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins. To be submitted to J Biol Chem.

4.Abstract: **Zhongxian Lu**, Manuel Cortes, Bogi Andersen.2004. The LIM-only protein LMO4 activates TGFβ signaling by interacting with Smad proteins. The 2004 Conference of Chao Family Comprehensive Cancer Center, Palm Spring,California, Novermber

5. Abstract:**Zhongxian Lu**, Kaye Star Lam, Manuel Cortes & Bogi Andersen. 2005. The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins. The 96th annual meeting of the American Association of Cancer Research, Anaheim,California, April.

6. Abstract: **Zhongxian Lu**, Kaye Star Lam, Manuel Cortes, Bogi Andersen, 2005.The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins. The 2005 Conference of DOD Breast Cancer Research Program, Philadelphia, Pennsylvania, June.

CONCLUSION

In summary, with the support of the Army Fellowship Award, I am able to obtain excellent training on both molecular lab research in breast cancer and computational biology and bioinformatics and acquire expertise in working on the breast cancer problem at a high level. During the first year of the funding period, I made significant progress on specific aims, and my training in breast cancer has been greatly enhanced. I have published three first-author abstract and am preparing a manuscript for submission that describes my recent finding with LMO4 in breast cancer. My major achievements are described as followes: (1) I have developed a retroviral gene transfer and expression system, and overexpressed LMO4 in several breast cancer and normal cell lines. (2) I have established breast cancer cell lines that stably express LMO4 siRNA and have a constitutively low LMO4 protein expression. As the initial steps of the whole projects, both types of cell lines serve solid foundation for next experiments. (3) I found that LMO4 modulates TGFβ signaling as a part of Smads-DNA complex and promotes TGFβ inhibition of cell proliferation of mammary gland epithelial cell.(4) I have finished two computational science courses, which enhance greatly my ability to analyze data, especially gene microarray data. Based on the crucial role of TGFβ in both development and cancer, our findings strengthen the hypothesis that LMO4 may contribute to the oncogenesis of breast tissue. Together, these results show a good start to the whole project and provide a strong support for the completion of the whole project. These results also indicate that this whole work will play a role in solving the breast cancer problem on the support of the Army.

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Fig 1

A   MCF-7

GFP

GFP-
LMO4

B   MB-MDA-231

C   HME cell
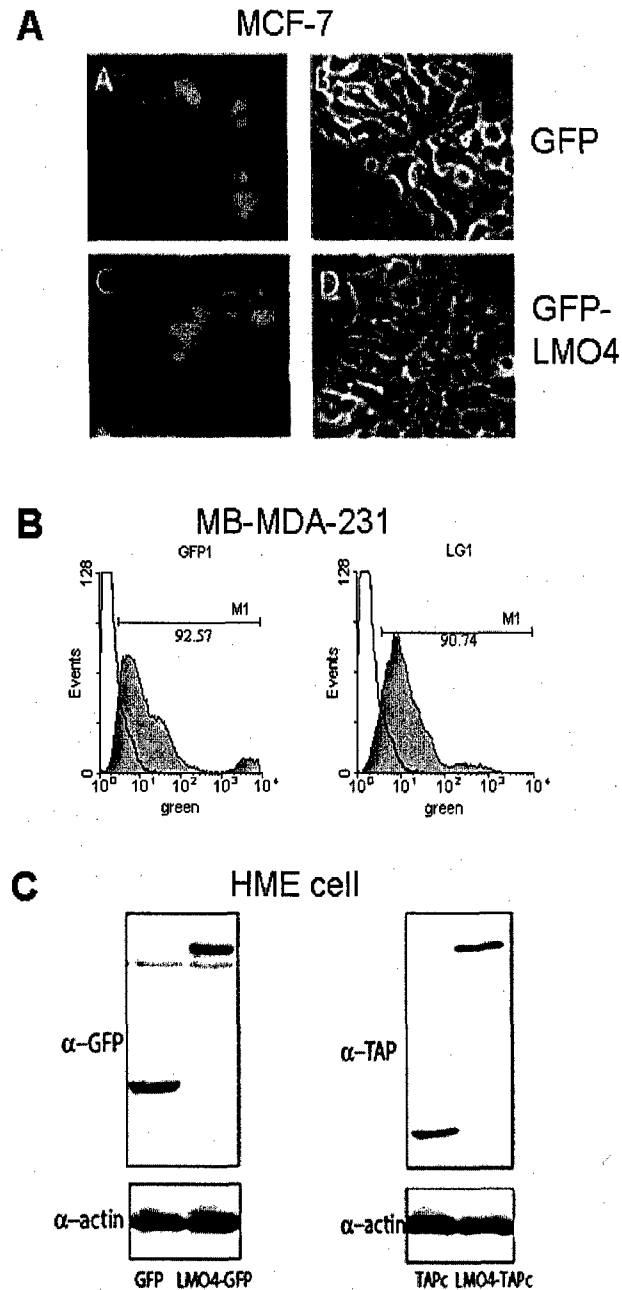
Fig. 1. LMO4 was stably expressed in several cell line, including normal mammary gland epithelial cells or breastcancer cell lines. After virus infection, we evaluate infection efficiency using microscope (example as **A** in MCF-7 cell), flow cytometry (example as **B** in MB-MDA-231 cell) and western bloting (example as **C** in HME cell).

Fig 2

A.

B.
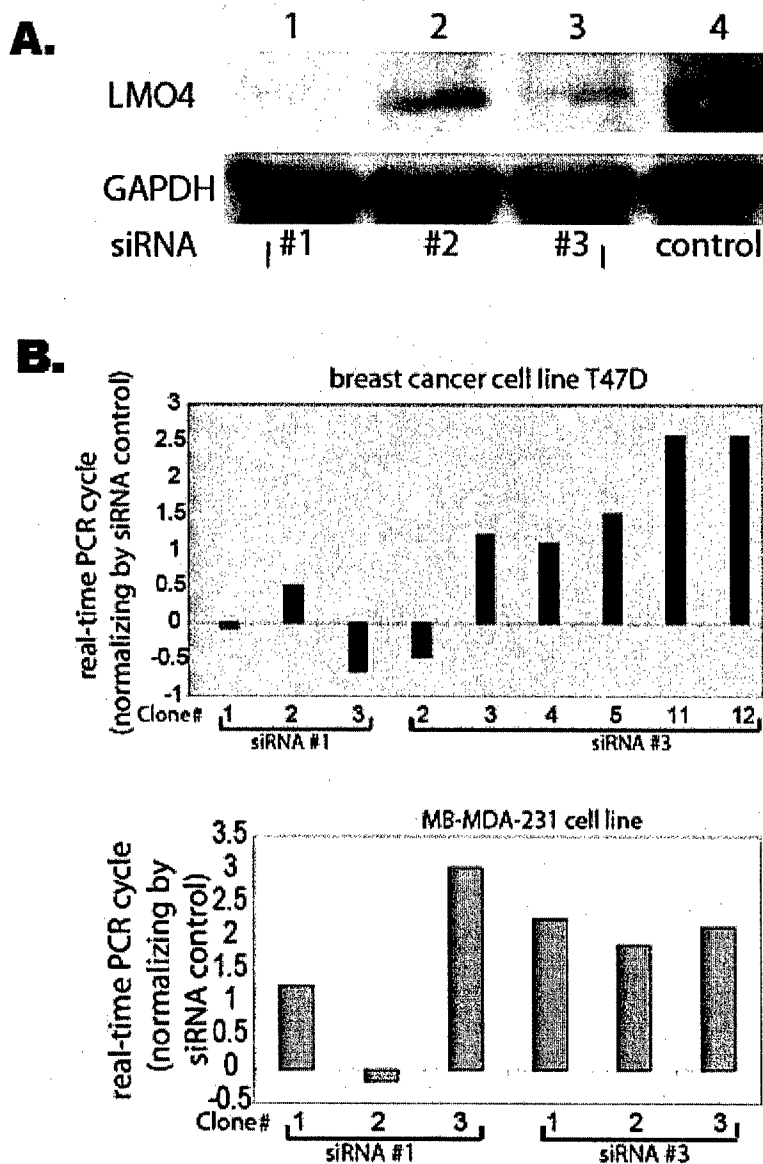
Fig. 2 **LMO4 protein was deleted by siRNA in breast cancer cell T47D and MB-MDA-231. A.** Three specific siRNAs of human LMO4 were introduced into breast cancer cell T47D.Western blotting showed LMO4 siRNA #1 and #3 effectively decreased endogenous LMO4 levels (lanes 1 and 3) compared to a negative control siRNA. **B.** Real-time PCR show siRNA vector expressed LMO4 siRNA #1 and #3 were stably expressed and deleted the LMO4 protein expression in breast cancer cell line of MD-MBA-231 and T47D.
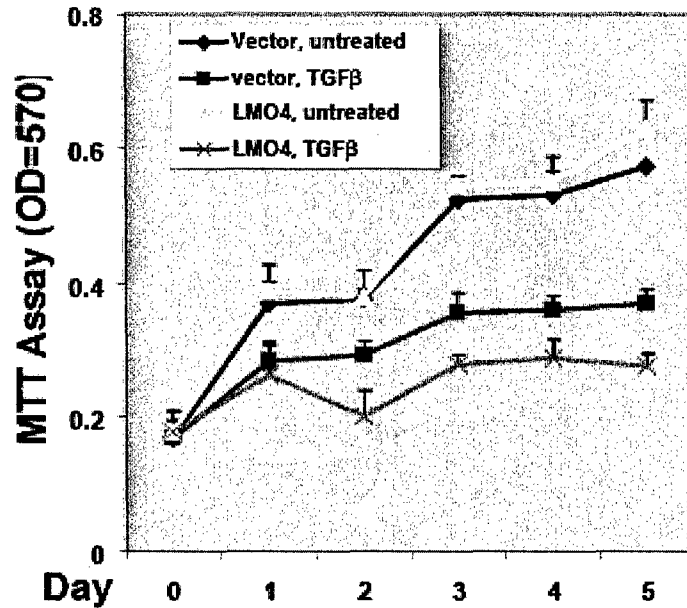
Figure 3

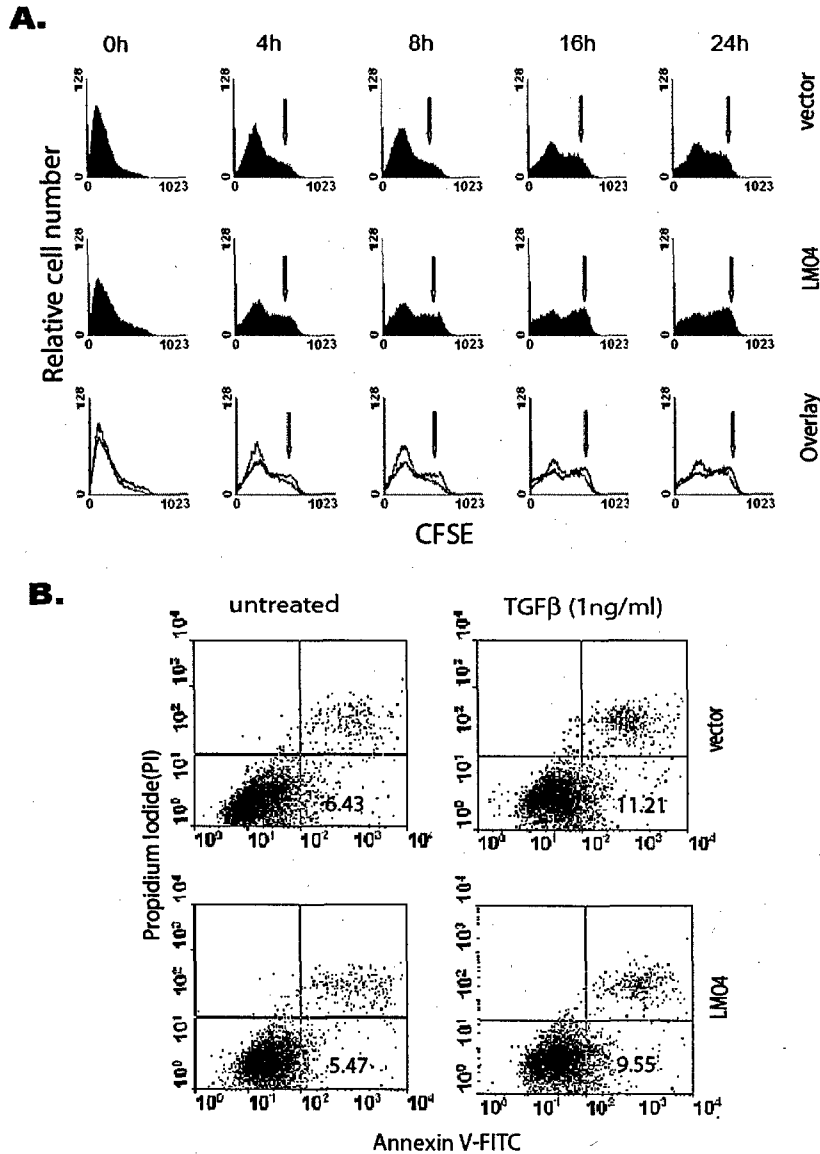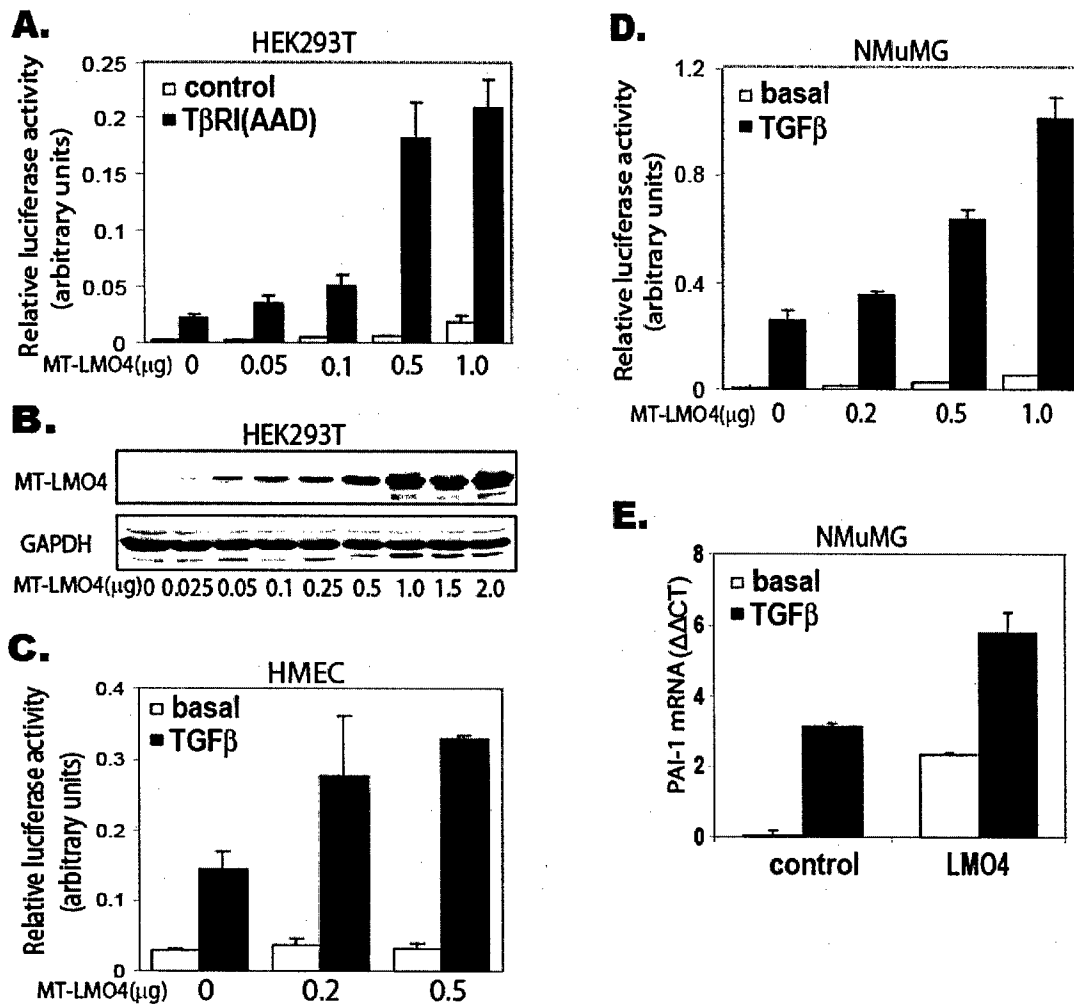**Figure 3. LMO4 enhances the inhibitory effect of TGFβ on human mammary epithelial cell growth.** HME cells expressing either LMO4-GFP or the control protein GFP were plated onto 96-well plates. After treatment with TGFβ (1ng/ml) for 24h, cells were grow in fresh grow medium for another 4 days. After the beginning of TGFβ treatment (day 0), cell growth was monitored, using the MTT assay.

Figure 4

A.


B.

Figure 4. LMO4 énhances the inhibitory effect of TGFβ on HME cell proliferation, but has no effect on TGFβ-induced apoptosis. A, HME cells expressing either control protein TAPc (top panel) or LMO4-TAPc (middle panel) were stained with CFSE and then plated onto 6-well plates (10000 cells/well). On the second day, cells were treated with TGFβ (1ng/ml) for indicated time, and then grown in fresh medium for another 3 days. Cell proliferation was assessed with a FACS based on CFSE quantity. B, HME cells expressing either TAPc control protein or LMO4-TAPc or were seeded onto 60-mm dishes (1 x 10^5 cells/dish). The next day, cells were treated with either vehicle (untreated) or TGFβ (1ng/ml) for 24h. Cell apoptosis was analyzed with combined propidium iodide/annexin-V-FITC staining.

Figure 5

A.
HEK293T

□ control
■ TβRI(AAD)

Relative luciferase activity (arbitrary units)

0.25, 0.2, 0.15, 0.1, 0.05, 0

MT-LMO4(µg) 0   0.05   0.1   0.5   1.0

B.
HEK293T

MT-LMO4

GAPDH

MT-LMO4(µg) 0 0.025 0.05 0.1 0.25 0.5 1.0 1.5 2.0

C.
HMEC

□ basal
■ TGFβ

Relative luciferase activity (arbitrary units)

0.4, 0.3, 0.2, 0.1, 0

MT-LMO4(µg)   0   0.2   0.5

D.
NMuMG

□ basal
■ TGFβ

Relative luciferase activity (arbitrary units)

1.2, 0.8, 0.4, 0

MT-LMO4(µg) 0   0.2   0.5   1.0

E.
NMuMG

□ basal
■ TGFβ

PAI-1 mRNA ($\Delta\Delta$CT)

8, 6, 4, 2, 0

control   LMO4

Figure 5. **LMO4 potentiates TGFβ-mediated transcriptional activity in epithelial cells.** A dose-dependent expression of LMO4 (**B**) was co-transfected with a Plasminogen Activator Inhibitor-1(PAI-1) gene reporter plasmid into HEK293T(**A**), normal human mammary epithelial (HMEC) (**C**) and mouse mammary epithelial NMuMG (**D**) cells, and markedly increased the effects of TGFβ1(1ng/ml)-stimulated Luciferase activity. By using retroviral transduction to introduce the LMO4 protein into NMuMG cells, LMO4 increased PAI-1 mRNA several folds under both basal ($\Delta\Delta$CT=2.3) and TGFβ-stimulated ($\Delta\Delta$CT=5.7) conditions (**E**), based on quantitative PCR measurements.
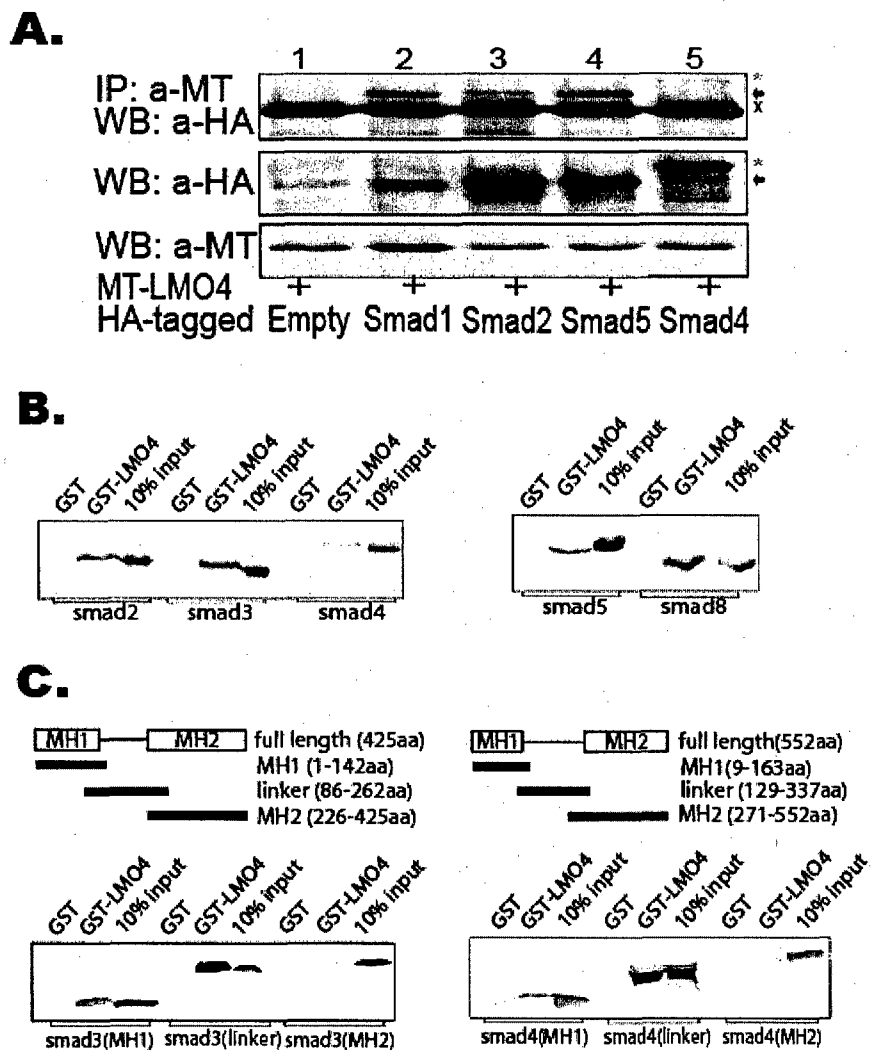
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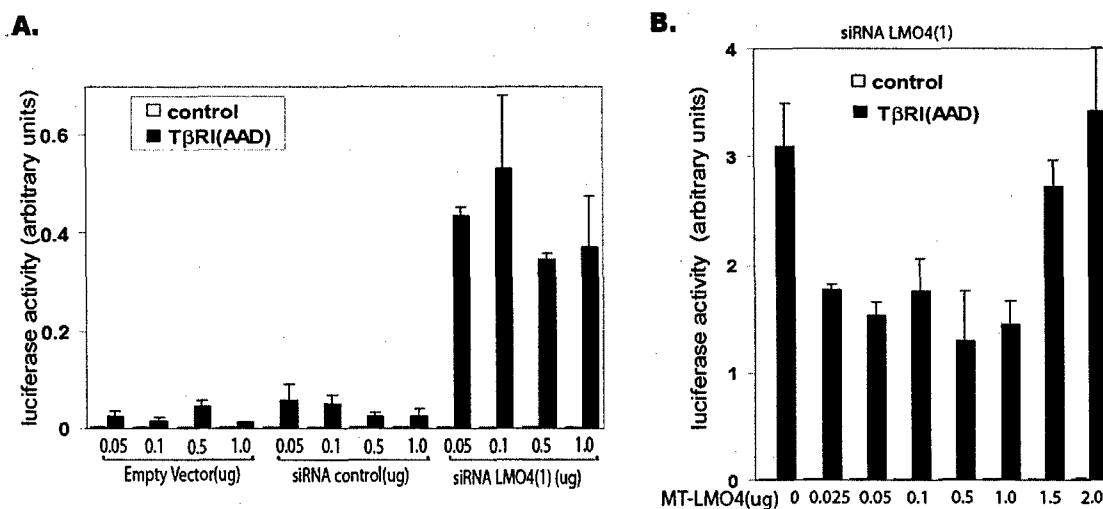
Figure 6

Figure 6. Biphasic regulation of PAI-1 reporter activity by LMO4. A, a vector expressing LMO4 siRNA#1 markedly enhanced TGFβ stimulation on luciferase expression in HEK293T cell. **B.** The expression vector that encodes mouse LMO4 (not targeted by the siRNA) partially reverse the stimulatory effect of LMO4 siRNA in HEK293T cell cell. Adding higher amounts of transfected mouse 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 .

16

Figure 7

A.

IP: a-MT
WB: a-HA

WB: a-HA

WB: a-MT

MT-LMO4   +   +   +   +   +

HA-tagged Empty Smad1 Smad2 Smad5 Smad4

B.

smad2   smad3   smad4

smad5   smad8

C.

full length (425aa)
MH1 (1-142aa)
linker (86-262aa)
MH2 (226-425aa)

full length(552aa)
MH1(9-163aa)
linker (129-337aa)
MH2 (271-552aa)

smad3(MH1) smad3(linker) smad3(MH2)

smad4(MH1) smad4(linker) smad4(MH2)

Figure 7. LMO4 interacts with the MH1 and linker regions of several Smad proteins. A. MT-tagged LMO4 and HA-tagged Smads plasmids were co-transfected into HEK293T cell. Smad1, Smad2, Smad4 and Smad5 were all co-immunoprecipitated with LMO4 (Fig. 3; top panel).**B.** Full length, ^{35}S-labelled Smad2, Smad3, Smad4, Smad5, and Smad8 were incubated with either GST alone or GST-LMO4. LMO4-Smad interactions were determined with GST-pulldown assays and compared to 10% of the Smad protein input as visualized by SDS-PAGE and autoradiography. **C,** GST-pulldown assays were used to determine interactions between GST-LMO4 and ^{35}S-labelled subdomains of Smads.

17
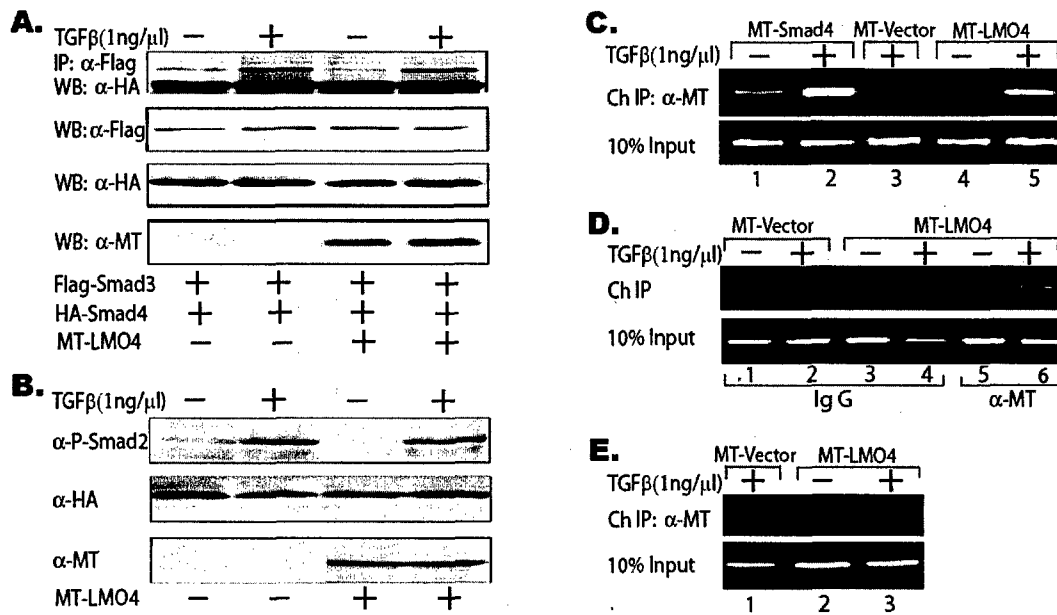
Figure 8

Figure 8. LMO4 associates with the PAI-1 promoter in a TGFβ-dependent fashion.
A. Overexpression of LMO4 didn't affects TGFβ-induced Smad2 phosporylation in HEK 293T cell based on the western blotting phosporylating Smad2 antibody. **B.** LMO4 overexpression has also no influences on the interaction between Flag-Smad3 and HA-Smad4 HEK 293T cell under TGFβtreatment. **C-D.** LMO4 was found to associates with the PAI-1 promoter in a TGFβ-dependent fashion performing ChIP assays in HEK293T cell transiently-expressed myc-LMO4 (C). As a negative control, non-specific IgG did not precipitate the PAI-1 promoter (D), and no binding was detected to the GAPDH promoter (E).

18

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ABSTRACT FORM

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LIM-only protein 4 (LMO4) has been established to have crucial functions in development. In addition, it is often overexpressed in breast cancer cases and is proposed to play roles in oncogenesis. LMO4 is highly expressed in locations of epithelial-mesenchymal interactions where TGFβ cytokine signaling plays important roles, both in development and in cancer. We therefore explored the possibility that LMO4 might modulate TGFβ signaling in breast cancer cells. Primary human mammary gland epithelial (HME) cells were infected with retroviruses expressing LMO4-GFP or GFP as a control. We found that LMO4 potentiated the TGFβ-mediated inhibition of cell proliferation in HME cells, indicating that under these conditions LMO4 has positive effects on TGFβ function. Consistent with this finding, in transient transfection assays, LMO4 potentiated the TGFβ effect on the well known TGFβ target gene PAI-1. In contrast, a dominant negative LMO4 strongly blocked the regulation by TGFβ in 293T, HMEC, and mouse mammary gland epithelial cells (NMuMg). To test the mechanisms of action for LMO4, we co-transfected MT-LMO4 and HA-tagged Smads proteins 1, 2, 4, 5 into 293T cells, and found that LMO4 interacts with these Smads. GST protein-protein interaction assays showed that LMO4 binds to the MH1 domain of Smad1, Smad3 and Smad4. Together, these experiments suggest that LMO4 modulates TGFβ signaling by binding directly to Smad proteins. In support of this idea, we found in chromatin immunoprecipitation assays that LMO4 is part of the Smad-containing transcriptional protein complex on the PAI-1 gene after TGFβ treatment. In summary, we have found that the transcriptional adapter LMO4 may regulate the binding of transcription factors on TGFβ target genes. These results define a new function for LMO4 as a coactivator of TGFβ signaling, and provide a potential novel mechanism for LMO4 regulation in development and oncogenesis.

Abstract Title: **The LIM-only Protein LMO4 Activates TGFβ Signaling by Interacting with Smad Proteins**

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4485 The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins

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THE LIM-ONLY PROTEIN LMO4 MODULATES TGFB SIGNALING BY INTERACTING WITH SMAD PROTEINS

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