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LIM Domain Only 2 Regulates Endothelial Proliferation, Angiogenesis, and Tissue Regeneration

Shu Meng, MD, PhD; Gianfranco Matrone, PhD; Jie Lv, PhD; Kaifu Chen, PhD; Wing Tak Wong, PhD; John P. Cooke, MD, PhD

Background—LIM domain only 2 (LMO2, human gene) is a key transcription factor that regulates hematopoiesis and vascular development. However, its role in adult endothelial function has been incompletely characterized.

Methods and Results—In vitro loss- and gain-of-function studies on LMO2 were performed in human umbilical vein endothelial cells with lentiviral overexpression or short hairpin RNA knockdown (KD) of LMO2, respectively. LMO2 KD significantly impaired endothelial proliferation. LMO2 controls endothelial G1/S transition through transcriptional regulation of cyclin-dependent kinase 2 and 4 as determined by reverse transcription polymerase chain reaction (PCR), western blot, and chromatin immunoprecipitation, and also influences the expression of Cyclin D1 and Cyclin A1. LMO2 KD also impaired angiogenesis by reducing transforming growth factor-β (TGF-β) expression, whereas supplementation of exogenous TGF-β restored defective network formation in LMO2 KD human umbilical vein endothelial cells. In a zebrafish model of caudal fin regeneration, RT-PCR revealed that the lmo2 (zebrafish gene) was upregulated at day 5 postresection. The KD of lmo2 by vivo-morpholino injections in adult zebrafish reduced 5-bromo-2'-deoxyuridine incorporation in endothelial cells, impaired neoangiogenesis in the resected caudal fin, and substantially delayed fin regeneration.

Conclusions—The transcriptional factor LMO2 regulates endothelial proliferation and angiogenesis in vitro. Furthermore, LMO2 is required for angiogenesis and tissue healing in vivo. Thus, LMO2 is a critical determinant of vascular and tissue regeneration. (J Am Heart Assoc. 2016;5: e004117 doi:10.1161/JAHA.116.004117)

Key Words: angiogenesis • endothelial cell • LIM domain only 2 • proliferation • regeneration • transcription factors
LMO2 is Required for Adult Endothelial Function  
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To assess the role of LMO2 in adult EC function, we performed in vitro loss- and gain-of-function experiments of LMO2 in human umbilical vein ECs (HUVECs) to assess major aspects of endothelial function such as proliferation, network formation, acetylated-low density lipoprotein (acLDL) uptake, NO production, and cell surface marker expression. We further screened and identified the key molecular targets of LMO2, cyclin-dependent kinase (CDK) 2 and CDK4, and related cyclins involved in endothelial proliferation and transforming growth factor-β (TGF-β1)–promoted angiogenesis. We examined the role of LMO2 in vivo in a caudal fin resection model in adult zebrafish. Our studies suggest that LMO2 is a critical determinant of angiogenesis and tissue regeneration.

Material and Methods
Chemicals and Antibodies
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. EGM-2 medium and bullet kit was from Lonza (Walkersville, MD). Angiogenesis RT Profiler PCR Array and chromatin immunoprecipitation (ChIP) polymerase chain reaction (PCR) primers were from Qiagen (Valencia, CA). TGF-β ELISA kits were from R&D Systems (Minneapolis, MN). Taqman primers, cell proliferation kit, AF594-conjugated acLDL, CellTracker Red, and Griess kit was from Invitrogen (Carlsbad, CA). PE-human CD31 antibody and APC-human CD144 antibody was from BD Biosciences (San Jose, CA). ChIP kit was from Cell Signaling Technology (Beverly, MA). Anti-human LMO2 antibody (clone SP51) was purchased from Spring Bioscience (Pleasanton, CA). Cyclin D1, p21, and horseradish peroxidase (HRP)–conjugated goat anti-mouse/rabbit antibodies were from Santa Cruz Biotechnology Inc. CT noncoding and control (CT) short hairpin RNA (shRNA) and LMO2 shRNA was from Abcam (Cambridge, UK). Lentiviral particles of LMO2 open reading frame (ORF) OE lentiviral particles were from Applied Biological Materials Inc (Richmond, BC, Canada).

Zebrafish Husbandry
Tg(fli1:egfp)11 fish were raised according to standard procedures11 and kept at 28°C under a 14/10-hour light/dark cycle and fed with dry meal (Gemma Micro) twice per day. Animals were housed and all experiments were carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee. All surgery procedures were performed under anesthesia.

Stable Cell Generation
HUVEC were cultured in EBM-2 basal medium supplemented with EGM-2 bullet kits. HUVECs at passage 4 were grown to 60% confluence before infection with lentiviral particles containing shRNA targeting LMO2 or ORF of LMO2. Scramble shRNA containing virus or virus without LMO2 ORF were used as controls. Stable KD or OE of LMO2 was obtained by puromycin (1 μg/mL) selection for consecutive 14 days after viral infection. LMO2 KD or OE was confirmed by gene expression and protein level analysis.

Protein Extraction and Western Blot Analysis
Cultured cells or zebrafish tissue samples were collected and solubilized in RIPA buffer (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail. Protein concentration was measured using BCA assay. Samples (containing 50–100 μg protein) were subjected to polyacrylamide gel electrophoresis (4–12% gradient) and transferred onto nitrocellulose membranes. Blots were stained with 1% Ponceau S for loading controls. Blots were then blocked with 5% nonfat milk in PBST (PBS + 0.1% Tween) for 1 hour at room temperature and probed with primary antibody overnight at 4°C. They were then washed 4 times with PBST for 10 minutes. HRP-conjugated goat anti-mouse or rabbit antibodies were incubated for 1 hour at room temperature. Blots were washed 4 times with PBST for 10 minutes. Antigen-antibody complexes were then detected by exposure in FluorChem M system from ProteinSimple (San Jose, CA). β-tubulin was used as loading control.

RNA Extraction and RT-PCR
mRNA was extracted from cultured cells or homogenized zebrafish caudal fins using column purification.12 The mRNA was reverse transcribed into cDNA. Taqman primers targeting specific genes were then used for real-time PCR (RT-PCR; QuantStudio Real-Time PCR System, Life Technologies, Carlsbad, CA). 18S was used as an internal control for HUVEC studies and β-actin was used as an internal control for zebrafish studies. Angiogenesis RT Profiler PCR Array was performed according to the manufacturer’s manual.

Endothelial Proliferation Assay, Network Formation Assay, and Griess Assay
Cell proliferation was examined by proliferation assay. HUVECs were seeded at the same starting number 5 × 10⁴ cells in EGM-2 medium and counted and subcultured every other day for 4 days. Angiogenesis was examined by matrigel

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network formation assay. Briefly, 24-well plates were coated with Matrigel 300 μL (BD Biosciences), and incubated at 37°C for 30 minutes. In each well, 7 x 10⁴ cells in 500 μL EGM-2 medium was seeded. After 16 hours, cells were stained with CellTracker Red 5 μmol/L for 30 minutes and visualized under the fluorescence microscope. Griess assay was used for NO measurement based on the manufacturer’s manual.

**ELISA Assay**

TGF-β1 ELISA assay was performed following the manufacturer’s manual.

**ChIP-PCR Assay**

ChIP assay was performed following the manufacturer’s manual. Briefly, DNA and protein were crosslinked by 1% formaldehyde. Chromatin was isolated and digested with micrococcal nuclease. The DNA-protein complex was then precipitated with control IgG or antibodies against LMO2 overnight at 4°C and protein A/G–conjugated magnetic beads for 1 hour. Cross-links were reversed. The extracted DNA was used as a template for PCR amplification of the targeted promoter region. The putative LMO2 complex binding sites were predicted using the DECODE (Decipherment of DNA Elements) database.

**Vivo-Mo Injection in Zebrafish**

Suppression of lmo2 transcription factor was achieved by using antisense vivo-Mo 5′-GTAGAAGCCATTGCTATGATTC-3′ (Gene Tools LLC, Philomath, OR) targeting the LMO2 mRNA translation initiation site. Vivo-Mo is made by covalently binding a standard Mo to a synthetic scaffold containing guanidinium head groups as a delivery moiety. An antisense Vivo-Mo that targets human β-globin intron mutation 5′-CC TCTTACCTCATATGATTATA-3 was used as control. Vivo-Mo was injected into the retro-orbital vein, as previously described. Briefly, zebrafish were anesthetized in tricaine 0.05 mg/mL, and 2 μL of 0.1 mmol/L vivo-Mo solution was loaded in a glass capillary and injected with a standard microinjector (IM300 Microinjector; Narishige, Tokyo, Japan) on days 0, 2, 4, 6, 8, 10, 12, and 14.

**BrdU Incorporation in Zebrafish**

To label proliferating cells, a dose of 5 μL of 5-bromo-2′-deoxyuridine (BrdU; 10 mmol/L in PBS) was injected into the retro-orbital vein at days 11 and 13 after the day 0 injection of vivo-MO. At day 15, fish were euthanized for enzymatic digestion in PBS containing trypsin 0.4%, 1 mmol/L EDTA, and collagenase P 0.05 mg/mL, and incubated for 45 minutes at 28.5°C with pipetting every 5 to 10 minutes to obtain single-cell suspension for fluorescence-activated cell sorter (FACS) analysis.

**Caudal Fin Resection and Regeneration**

Caudal fin resection in adult fishes was performed with sterile razor blades at day 4 after the day 0 injection of vivo-Mo and allowed to regenerate. Total regeneration was measured as previously described. Briefly, fin images were collected before amputation and time points after amputation. The new tissue area (in pixels) of the caudal fin from the new distal fin edge to the amputation plane was quantified in each fish up to 5 days postresection using Image J software. The percentage of regeneration for each fin at each time point was defined as percentage of regeneration = 100 x (regenerated tissue area/original fin area amputated).

**FACS Analysis**

HUVECs were washed with PBS and trypsinized to generate single-cell suspension. For cell surface marker detection, single-cell suspension cells were stained with indicated antibody. For cell cycle analysis, single-cell suspension was fixed with 70% EtOH at 4°C overnight. Cells were then stained using propidium iodide (PI)/RNase buffer for 30 minutes and immediately run on a BD LSR II machine (BD Biosciences). BrdU incorporation assay in zebrafish was performed as previously described. Data were analyzed by FlowJo software (FlowJo, LLC, Ashland, OR). Cell cycle distribution was analyzed using the cell cycle package of FlowJo.

**Data Analysis**

Statistical analysis was performed with Prism 7 software (GraphPad Software, Inc, La Jolla, CA). Results were expressed as mean±SEM. The Shapiro-Wilk test was used to confirm the null hypothesis that the data follow a normal distribution. Statistical comparisons between the two groups were then performed via Student t test, and one-way ANOVA test was used to analyze multiple groups. Bonferroni corrections test was applied for multiple comparisons. P<0.05 was considered significant.

**Results**

**Generation of LMO2 KD HUVECs and LMO2 OE HUVECs**

A stable line of LMO2 KD HUVECs was generated by infection with lentiviral particles containing shRNA targeting LMO2 followed by puromycin selection. A CT HUVEC line
was generated using lentiviral scramble shRNA. Compared with CT cells, the LMO2 KD cells exhibited a 70% decrease in LMO2 gene expression detected by RT-PCR (Figure 1A) and an 80% reduction in protein level by Western blot (Figure 1B). The morphology of LMO2 KD cells was similar to CT (Figure S1). A stable line of HUVECs overexpressing LMO2 was generated by lentiviral particles encoding the ORF of LMO2 followed by puromycin selection. The CT HUVEC line was generated using lentiviral particles without an LMO2 ORF. Compared with CT cells, the LMO2 OE stable cells had 8-fold increases in LMO2 gene expression detected by RT-PCR (Figure 1C) and 7-fold increases in protein level by Western blot (Figure 1D).

LMO2 KD Hindered G1/S Transition and Reduced Proliferation

Cell growth was attenuated in the LMO2 KD as shown by total cell numbers counted every other day for 4 days (Figure 2A). This effect was likely due to an impairment of cell proliferation, as cell cycle analysis by PI staining and FACS revealed that the fraction of cells in S phase was decreased (4.5% versus 9% in CT) and that in G1 phase was slightly increased (Figure 2B and 2C), indicating an impairment of G1/S transition. Further examination of G1/S transition-related cell cycle genes by Western blot showed decreased levels of CDK4, CDK2, Cyclin D1, Cyclin A1, while Cyclin E and p21 expression were not significantly changed (Figure 2D). ENCODE database prediction suggests that both the CCGATAACGG region starting at +232 of CDK4 (Gene ID: 1019) and the CCCTCAGGTGGTG region starting at +2700 of CDK2 (Gene ID: 1017) could be potential LMO2 complex binding sites. ChIP assays confirmed that LMO2 complex binds to both regions (Figure 2E and 2F). In LMO2 KD HUVECs, LMO2 complex binding is reduced in the CDK2 gene (by 50%; Figure 2E) and in the CDK4 gene (by 75%; Figure 2F) consistent with a partial KD of LMO2 and downregulated gene expression of CDK2 and CDK4, respectively.

LMO2 OE Increased G1/S Transition but Not Proliferation

Cell cycle analysis showed that the fraction of cells in S phase were increased (14.7% versus 7.9% CT) and that in G1 phase decreased in LMO2 OE condition (Figure 3A and 3B). However, cell proliferation was not changed by LMO2 OE (Figure S2). Following LMO2 OE, Cyclin D1 mRNA level was increased, whereas CDK2 and CDK4 mRNA levels remained stable (Figure 3C). Interestingly, LMO2 OE increased only Cyclin D1 and Cyclin A1 protein levels but not that of CDK2, CDK4, Cyclin E, or p21 (Figure 3D).

LMO2 KD Impaired Endothelial Network Formation: Role of TGF-β1

The LMO2 KD impaired endothelial network formation in matrigel (Figure 4A). Further analysis of 84 angiogenesis-related genes using the Qiagen Angiogenesis RT Profiler PCR Array revealed that there was a substantial reduction in the expression of core angiogenesis genes (33 of 84; Figure 4B) in the LMO2 KD cells. Among them, TGF-β1 promoter region is predicted to have LMO2 complex binding sites, indicating that it could be a direct transcriptional target of LMO2. We confirmed by RT-PCR (Figure 4C) and ELISA (Figure 4D) that TGF-β1 was downregulated in LMO2 KD cells. To determine the contribution of TGF-β downregulation to impaired angiogenic processes in LMO2 KD cells, we determined whether network formation could be rescued by TGF-β. Indeed, supplementation of the HUVEC LMO2 KD with exogenous TGF-β restored defective network formation (Figure 4F). ENCODE database prediction suggests that two regions on TGF-β1 (Gene ID: 7040) promoter

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could potentially have LMO2 complex binding. One region, CCGCAGCTGCTGC, starts at the proximal –901 position and the other region, CAGATAGGGG, starts at the distal –4030 position. The ChIP assay revealed that LMO2 complex binds to both regions (Figure 4G), confirming that TGF-β1 is a direct transcriptional target of LMO2. Interestingly, in LMO2 KO cells, the decreased binding of LMO2 complex was only observed in the proximal –901 binding site, suggesting that this binding site is more sensitive to LMO2 downregulation. Notably, OE of LMO2 did not further increase TGF-β1 level, probably because LMO2 binding to DNA involves the participation of other components of the binding complex (Figure 4E). This explanation is consistent with the observation that LMO2 OE did not further promote endothelial network formation (Figure S3).

Figure 2. LMO2 knockdown (KD) reduced cell proliferation and hindered G1/S transition. A, Cell growth curve. B, Cell cycle analysis by propidium iodide (PI) staining. C, Quantification of cell fractions in G1, S, and G2/M phases. D, Western blot examination of G1/S transition-related genes. E and F, chromatin immunoprecipitation polymerase chain reaction analysis of LMO2 complex binding to CDK2 gene (+2700) and CDK4 gene (+232) in both control (CT) and LMO2 KD cells. Data are presented as mean±SEM (n=3). *P<0.05 vs CT.
LMO2 KD Did Not Cause Global Loss of Endothelial Function

Because the LMO2 KD caused a severe impairment in angiogenic processes, we assessed other basic functions of ECs after LMO2 KD. We assessed the expression of endothelial surface markers, the uptake of acLDL, and the elaboration of NO. Compared with CT cells, LMO2 KD did not significantly alter acLDL uptake, the expression of CD31 and CD144 as assessed by FACS analysis, or the synthesis of NO as assessed by Griess assay (Figure S4).

Upregulation of lmo2 Gene Expression during Regeneration of the Caudal Fin

To test whether lmo2 is required for tissue regeneration, we assessed the gene expression of lmo2 and another early endothelial marker gene tie2 in caudal fin tissue at 0, 1, 2, and 5 days after caudal fin resection (Figure 5A). We observed that tie2 gene expression remained stable (Figure 5C) from day 0 to day 5 (at which time about 50% of the caudal fin was regenerated). Conversely, lmo2 gene expression level significantly increased by 50% at day 5 when about 50% of the caudal fin was regenerated (Figure 5B).

Adult Endothelial Proliferation Was Impaired with lmo2 KD

To test the hypothesis that LMO2 plays a key role in the proliferation of adult ECs in vivo, we injected vivo-Mo targeting lmo2 translation start site in adult Tg(fli1:egfp)y1 zebrafish (2.5 months old) and quantified cell proliferation by BrdU incorporation (Figure 6A). The lmo2 vivo-Mo significantly downregulated lmo2 expression compared with the CT group injected with a vivo-Mo targeting human β-globin, as shown by Western blot (Figure 6E). The BrdU incorporation rate of GFP+ ECs was 13.7% in the CT vivo-Mo group, whereas it decreased to 7.2% in the lmo2 vivo-Mo–injected group (Figure 6B and 6D). In contrast, the BrdU incorporation rate in GFP– cells was similar between the CT vivo-Mo (1.77%) and lmo2 vivo-Mo injection groups (1.85%) (Figure 6B and 6C).

Regeneration of the Vasculature and Parenchyma Was Impaired with LMO2 KD

The caudal fin was resected at 4 days after the first vivo-Mo injection and its regeneration was followed up to day 15 (Figure 7A). At day 1 and 2 postresection, the percentage of fin regeneration was similar in LMO2 KD and CT fish (Figure 7A and 7B). However, at 5 days postresection, the percentage of

Figure 3. LMO2 overexpression promoted endothelial G1/S transition. A, Cell cycle analysis by propidium iodide (PI) staining. B, Quantification of cell fractions in G1, S, and G2/M phases. C, mRNA level of G1/S-related genes. D, Western blot examination of G1/S-related genes. Data are presented as mean±SEM (n=3). *P<0.05 vs control (CT).
caudal fin regeneration was only 28% in LMO2 KD fish compared with 44% in CT fish (Figure 7A and 7B). Furthermore, neoangiogenesis was impaired as the regenerated vasculature area was significantly smaller (0.47 mm²) in lmo2 KD fish compared with CTs (0.74 mm²) at day 5 postresection (Figure 7C and 7D). Interestingly, the morphology of the neovessels in the lmo2 KD fish were aberrant with tangled vessel complexes (Figure 7C), in comparison to the standard vascular branching observed in the CT fish.

**Discussion**

**Transcriptional Factor LMO2**

LMO2 is a key transcription factor that regulates hematopoiesis\(^1\) and embryonic vascular development.\(^4\) LMO2 is considered as an oncogene in T cells as aberrant activation of LMO2 by chromosomal translocations contributes to T-cell acute lymphoblastic leukemia in humans.\(^2,3\) Lmo2 transgenic mice develop T-cell leukemia as Lmo2 OE impedes T-cell differentiation.\(^17,19\) LMO2 expression in adult murine and human ECs has been reported.\(^4\) However, the role of LMO2 in adult angiogenesis has not been systematically studied. Our studies indicate that LMO2 is a critical factor in adult angiogenesis and endothelial proliferation, and in the response to injury, as it mediates the vascular regeneration required for tissue repair.

**LMO2 and Adult Angiogenesis**

To examine the role of LMO2 in endothelial function, we generated stable LMO2 KD cells using lentiviral shRNA in HUVECs. The LMO2 KD was associated with severely impaired angiogenesis as assessed by endothelial network

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**Figure 4.** LMO2 knockdown (KD) impaired endothelial network formation, which can be rescued by exogenous transforming growth factor-β1 (TGF-β1). A, Network formation of control (CT) and LMO2 KD human umbilical vein endothelial cells (HUVECs). B, List of downregulated genes identified in LMO2 KD in comparison to CT HUVECs using angiogenesis polymerase chain reaction (PCR) array. C, Reverse transcription PCR (RT-PCR) of TGF-β1. D, ELISA for TGF-β levels in culture medium of CT and LMO2 KD cells. E, RT-PCR of TGF-β1 in CT and LMO2 overexpression HUVECs. F, Network formation of CT and LMO2 KD HUVECs with or without supplementation of TGF-β 10 ng/mL. G, Chromatin immunoprecipitation PCR analysis of LMO2 complex binding to TGF-β1 promoter (−901 and −4030 region) in both CT and LMO2 KD cells. Data are presented as mean±SEM (n=3). *P<0.05 vs CT.
formation. We hypothesized that LMO2 may modulate the expression of genes involved in angiogenesis. Accordingly, we used an angiogenesis PCR array for the network of core angiogenic genes and found that almost half of these genes (33 of 84), including TGF-β1, were downregulated more than 2-fold. TGF-β1 is the most downregulated angiogenic cytokine detected in our experimental system. Further, ENCODE database prediction suggests one proximal (starting at −901) and one distal (starting at −4030) consensus sequence in the TGF-β1 promoter, which was confirmed by our ChIP assays. TGF-β is an angiogenic cytokine that regulates physiological and tumor angiogenesis.\textsuperscript{20,21} We found that supplementation of TGF-β could rescue the impaired network formation in the LMO2 KD, indicating that TGF-β is a critical mediator of the angiogenic effects of LMO2. Subsequently, we used ChIP analysis to confirm that LMO2 binds to both the proximal and distal sites in the TGF-β1 promoter. Notably, the proximal binding site seems to be more sensitive to a reduced level of LMO2. We further confirmed that KD of LMO2 reduces TGF-β1 gene expression.

Additional analysis (Data S1) using the Gene Expression Omnibus (GEO) database ChIP-seq data indicates that the LMO2 transcriptional complex may bind to more than 100 gene promoters in murine hemogenic endothelium\textsuperscript{22} and mouse hematopoietic progenitor cells (Figure S5A). Among these genes, only 627 Lmo2 downstream genes are identified in both hemogenic endothelium and hematopoietic progenitor cells, suggesting Lmo2 complex binding targets vary between cell types. This observation might be explained by the existence of differences between cell types in the prevalence of LMO2 binding partners. Interestingly, five genes from the GEO database that contain consensus sequences for the LMO2 binding complex (Figure S5B), specifically COL18A1, EPHB4, IGF1, PF4, and SPHK1, were also detected in our angiogenesis PCR array. These genes could potentially be direct transcriptional targets of LMO2 in adult ECs. Besides direct transcriptional regulation, the mechanism of LMO2-mediated indirect regulation requires future elucidation.

**Figure 5.** Lmo2 gene expression increased following caudal fin resection and the regeneration process. A, To analyze changes in gene expression in the regenerating caudal fin, the amputated tissues from 15 fish were collected at day 0 (left panel) and regenerating distal tissues were resected and collected at 1, 2, or 5 days after the original resection (n=5 for each time point) (right panel, a fin at 2 days postresection). B, Gene expression of Lmo2 in the regenerating fin. Data are presented as mean±SEM (n=5). *P<0.05 vs control.

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**EC Proliferation is Regulated by LMO2**

Our data further suggest that in ECs, LMO2 is a key regulator of G1/S transition and cell proliferation. A recent bioinformatics analysis\textsuperscript{23} predicted binding of the LMO2 complex to promoter regions of cell cycle genes such as CDK2 and CDK4, suggesting a potential regulation by LMO2 of cell cycle progression. This prediction has not been experimentally verified until now. We found that LMO2 KD in HUVECs decreased cell proliferation (Figure 2A) and hindered the G1/S transition (Figure 2B). Master genes that regulate G1/S transition\textsuperscript{24} such as CDK2, CDK4, Cyclin D1, and Cyclin A1 were downregulated in LMO2 KD, while Cyclin E and p21 did not change. Consistent with a previous bioinformatics prediction report,\textsuperscript{23} our ChIP assay (Figure 2E and 2F) confirms the binding of LMO2 complex to CDK2 (CCCTAGGTGGTG site at +2700) and CDK4 (CCGATAACGG at +232). We also confirmed that CDK4 is a direct transcriptional target of LMO2 (Figure S5B).

The OE of LMO2 increased the expression of Cyclin D1 and Cyclin A1 and was associated with an increased G1/S transition. Since the LMO2 complex does not have a putative
binding site for the promoter regions of Cyclin D1 and Cyclin A1, its indirect regulation of these genes may be mediated by an effect of LMO2 on other transcription factors. Although OE of LMO2 in HUVECs promoted G1/S transition and increased S phase (Figure 3A), it did not further promote proliferation (Figure S2). Thus, while LMO2 is necessary for normal proliferation of mature ECs, it is not sufficient to accelerate their proliferation. This may be due to the fact that LMO2 binds to DNA with the help of other protein components of its DNA binding complex. As LMO2 forms protein-protein interactions with a variety of known binding partners, it may also regulate gene expression through nontranscriptional mechanisms. These other complex components may become rate limiting in the setting of LMO2 OE. This hypothesis would be consistent with the observation that the expression of CDK2 and CDK4 was not increased after LMO2 OE. In T cells where LMO2 is an oncogene, LMO2 OE does not cause increased cycling but does cause quiescence, suggesting that LMO2 functions differently in different cell types.

Notably, LMO2 appears to have specificity for regulation of angiogenic processes. We found that LMO2 KD did not cause a global impairment of endothelial function as it did not alter acLDL uptake, NO secretion, or the expression of specific surface markers. To summarize, our in vitro studies indicate that LMO2 is a critical and fairly specific regulator of endothelial proliferation and angiogenesis. Accordingly, we further investigated whether LMO2 is involved in the response to injury as endothelial proliferation and angiogenesis are required for tissue regeneration.

**LMO2 Mediates Vascular and Tissue Regeneration After Injury**

As a model for vascular and tissue regeneration, we chose the caudal fin resection model in zebrafish. Following amputation, LMO2 is Required for Adult Endothelial Function  

![Figure 6](http://ahajournals.org)  

**Figure 6.** lmo2 knockdown (KD) by vivo-morpholino (Mo) in zebrafish reduced proliferating GFP+ cells. A, Adult Tg(fl1:egfp)y1 zebrafish fish (2.5 months old) received a retroorbital injection of PBS solution containing lmo2 or control (CT) vivo-Mo, then the caudal fin was resected and injected with 5-bromo-2′-deoxyuridine (BrdU), as shown in the diagram. B, At day 15, whole fish were enzymatically digested and cell suspension was analyzed by flow cytometry. Negative control was Wik (non-GFP) fish line not injected with BrdU. BrdU+ and GFP+ events are shown in CT and lmo2 vivo-Mo–injected fish. C and D, Diagrams showing the percentage of BrdU+GFP+/GFP− cells and BrdU+GFP+/GFP+ cells. E, Western blot of lmo2 expression at day 10 in zebrafish without vivo-Mo injection or injected with CT or lmo2 vivo-Mo injection. Data are presented as mean±SEM (n=10). *P<0.05 vs CT.
lineage-restricted mesenchymal progenitor cells form at the wound edge, presumably via dedifferentiation of mature somatic cells of the stump. ECs sprout into the avascular area. Angiogenic cytokines such as vascular endothelial growth factor have been shown to play a crucial role in this process. Other major signaling pathways that regulate fin regeneration include fibroblast growth factor, Hedgehog, bone morphogenetic protein, retinoic acid, Wnt/β-catenin, insulin-like growth factor, activin, Notch, mechanistic target of rapamycin complex 1, and calcineurin.

We first examined lmo2 gene expression during the regeneration of the resected caudal fin in zebrafish. We discovered that lmo2 gene expression is increased in regenerating caudal fin at day 5 postresection (Figure 5B), whereas the expression of tie2, an early endothelial marker known to promote vascular stability, was unchanged. This
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Our studies provide the first evidence that LMO2 is a critical transcription factor that regulates adult EC proliferation and angiogenesis. In addition, LMO2 appears to be critically involved in the response to injury. By its regulation of vascular regeneration, LMO2 is required for normal tissue repair. These results indicate that LMO2 may be a good target for therapeutic modulation in tissue regeneration.

Conclusions

Our studies provide the first evidence that LMO2 is a critical transcription factor that regulates adult EC proliferation and angiogenesis. In addition, LMO2 appears to be critically involved in the response to injury. By its regulation of vascular regeneration, LMO2 is required for normal tissue repair. These results indicate that LMO2 may be a good target for therapeutic modulation in tissue regeneration.

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Disclosures

None.

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Data S1

ChIP-seq data analysis

ChIP-seq data from Gene Expression Omnibus (GEO) database were analyzed to identify potential LMO2 direct transcriptional target. LMO2 ChIP-seq data of murine hemogenic endothelium (HE) (GEO accession no: GSM1692846) and of murine hematopoietic progenitor cells (HPC) (GSE69101), and input data of HE (GSM1692808) and of HPCs (GSM1692809) were used.

For ChIP-seq analysis, Bowtie was used to map ChIP-Seq reads to the mouse reference genome version mm9, requiring single best match for each read across the genome. We used the function dpeak in DANPOS2 to calculate reads density from the mapped reads, normalized the total reads number of each ChIP-Seq sample to 25 million and defined enriched peaks with cutoff being Poisson test P value 1e-50. The extending length was set to 200bp and bin size to 10bp in the calculation of reads density. We set the smooth width to 0bp to not use any smoothing step in the calculation. Input effect was subtracted from the ChIP-Seq data by DANPOS2. For reference gene set, we used the UCSC KnownGene provided at the Table Browser page of UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTables), with the last updated date being 2011-03-02. To define LMO2 binding genes, we used the sub-command intersect of the bedtools suite to retrieve the subset of UCSC knownGenes transcripts with LMO2 binding regions overlapping with either their transcript bodies or the 5kb upstream regions of their transcription start sites (TSS).
Figure S1

CT  LMO2 KD
Figure S2

Cell growth

Number of cells ($1 \times 10^4$)

CT

LMO2 OE

0 1 2 3 4

0 10 20 30 40

0d
Figure S3

CT | LMO2 OE
Figure S4

A

CT

LMO2 KD

Cell number

ac-LDL

B

ac-LDL uptake

MFI of ac LDL

CT

LMO2 KD

C

NO production

OD548nm

CT

LMO2 KD

D

CT

LMO2 KD

CD144

CD31

E

CD31 expression

MFI of CD31

CT

LMO2 KD

F

CD144 expression

MFI of CD144

CT

LMO2 KD
Figure S5

A  
Hemogenic Endothelium  
1223 genes  

Hematopoietic progenitor cells  
1633 genes  

596  627  1006  

Direct transcriptional targets of LMO2

| Hemogenic endothelium | Hematopoietic progenitor cells |
|-----------------------|--------------------------------|
| Ephb4                 | Ephb4                         |
| Col18a1               | Pf4                            |
| lgf1                  | Sphk1                          |
|                       | Cdk4                           |
Figure S1-5 Legends

Figure S1. Representative bright field image of CT and LMO2 KD HUVECs.

Figure S2. LMO2 OE did not promote HUVEC proliferation.

Figure S3. LMO2 OE did not promote network formation on matrigel.

Figure S4. LMO2 knockdown in ECs did not interfere with ac-LDL uptake, NO secretion and cell surface marker levels. A. FACS analysis of AF594-acLDL uptake in both CT and LMO2 KD HUVEC. B. Quantification of mean fluorescence intensity (MFI) of AF594-acLDL. C. NO production measurement using Griess assay. D. FACS analysis of PE-CD31 and APC-CD144. E&F. Quantification of MFI of CD31 and CD144. All data represented as mean ± S.E.M. (n=3).

Figure S5. ChIP analysis of the LMO2 transcriptional targets in hemogenic endothelium and hematopoietic progenitor cells. A. LMO2 transcriptional targets. B. LMO2 transcriptional targets that are also identified in adult ECs.