Myocardin-Related Transcription Factor A Mediates OxLDL-Induced Endothelial Injury

Fei Fang, Yuyu Yang, Zhibin Yuan, Yuqi Gao, Jiliang Zhou, Qi Chen, Yong Xu

**Rationale:** Atherosclerosis proceeds through a multistep reaction that begins with endothelial injury caused by a host of stress signals, among which oxidized low-density lipoprotein (oxLDL) plays a critical role. OxLDL disrupts normal functionality of the endothelium by upregulating adhesion molecules (eg, ICAM-1) and concomitantly downregulating endothelial nitric oxide synthase (eNOS) expression. The transcriptional modulator that mediates the cellular response to oxLDL remains largely obscure.

**Objective:** Our goal was to determine whether myocardin-related transcription factor (MRTF)-A, a key protein involved in the transcriptional regulation of smooth muscle cell phenotype, is responsible for the endothelial injury by oxLDL, and, if so, how MRTF-A promotes the proatherogenic agenda initiated by oxLDL.

**Methods and Results:** OxLDL stimulated the expression of MRTF-A in endothelial cells as evidenced by Western blotting and immunofluorescence. Overexpression of MRTF-A synergistically enhanced the induction of ICAM-1 and suppression of eNOS by oxLDL. In contrast, disruption of MRTF-A, either by small interfering RNA or dominant negative mutation, abrogated the pathogenic program triggered by oxLDL. Finally, chromatin immunoprecipitation assays indicate that oxLDL preferentially augmented MRTF-A binding to ICAM-1 and eNOS promoters and that MRTF-A drove differential epigenetic alterations taking place on these promoters in response to oxLDL.

**Conclusions:** Therefore, our data provide the first demonstration that MRTF-A is critically linked to pivotal pathophysiological events in the vascular endothelium. (Circ Res. 2011;108:797-807.)

**Key Words:** MRTF-A ■ oxLDL ■ ICAM-1 ■ transcription ■ endothelial cell

Atherosclerosis is a polygenic pathology progressing through a series of interdependent processes that eventually result in the formation in the aortic artery of an atheromatous plaque. A host of extrinsic and intrinsic insults, which include oxidative stress, inflammatory stress, shear stress, and ischemic hypoxic stress, coordinately act on the vasculature to promote the atherogenic agenda. Upregulation of oxidized low-density lipoprotein (oxLDL) levels is a hallmark feature of atherosclerosis. Accumulation of oxLDL, acting as a stress signal, alters the transcription and hence the function of the vasculature, contributing to the development and progression of atherosclerotic injury.

It is generally accepted that chronic inflammation plays a major role in atherosclerosis whereas injury to the endothelial layer of vessels is considered a pivotal step in initiating the inflammatory response. For proinflammatory leukocytes to gain access to the vasculature, the expression of adhesion molecules (ICAM, VCAM) is markedly induced by oxLDL to facilitate the infiltration of leukocytes, which in turn perpetuates the chronic inflammation and aggravates atherosclerotic injury. Meanwhile, expression of endothelial nitric oxide synthase (eNOS), which, through producing NO, affords the endothelium much of its physiological function, is severely disrupted by various pathogenic insults including elevated oxLDL levels paralleling the loss of vascular tone during atherogenesis. Thus, oxLDL can impair endothelial function by stimulating ICAM and VCAM transcription while suppressing eNOS transcription. The specific transcriptional modulator(s) that drives the differential expression profiles in the endothelium during atherogenesis as well as the underlying mechanism, however, is not fully appreciated.

Myocardin-related transcription factor (MRTF)-A, also known as MKL1 (megakaryoblastic leukemia 1), belongs to a group of transcriptional regulators that also include myocardin and MRTF-B and is involved in the organogenesis of the cardiovascular system with a prominent role in steering the development and function of smooth muscle cells (SMCs). Recent investigations have implicated MRTF-A in a number
MRTF-A Potentiates the Activation of Adhesion Molecules by OxLDL

Because adhesion molecules are upregulated by oxLDL and contribute to the initiation of endothelial injury during atherogenesis, we set to examine the role of MRTF-A in the transcriptional activation of these genes. MRTF-A, but not MRTF-B, activated the promoter activities of ICAM-1, ICAM-2, and VCAM-1 genes in a dose response manner (Figure 2A; Online Figure II, A and B). In addition, suboptimal doses of MRTF-A and oxLDL synergistically stimulated the transcription of adhesion molecules, confirming a functional interplay between oxLDL and MRTF-A (Figure 2B; Online Figure II, C). Similar observations were made for endogenous ICAM-1 expression when MRTF-A was overexpressed by viral infection (Figure 2C). On the contrary, disruption of MRTF-A function by siRNA mediated knockdown (Figure 2D for siRNA validation) or overexpression of a dominant negative MRTF-A (DN-MRTF-A) abrogated the induction of ICAM-1 by oxLDL and significantly blocked the interaction between endothelial cells and leukocytes (Figure 2E and 2F; Online Figure II, D through F). Combined, our data suggest that MRTF-A is instrumental for oxLDL-induced adhesion of leukocytes to the endothelium by activating the transcription of adhesion molecules.

OxLDL Promotes the Recruitment of MRTF-A to the ICAM-1 Promoter

To demarcate the region on ICAM-1 promoter where MRTF-A exerts its effect, ICAM-1 promoter constructs harboring serial deletions were used. MRTF-A activated ICAM-1 transcription only when a small region (~393/~176) is preserved (Figure 3A). Indeed, ChIP assays using primers spanning this region showed the binding of MRTF-A, which was further augmented by oxLDL; no amplification was detected using primers surrounding the first intron of the ICAM-1 gene, confirming the specificity of MRTF-A binding (Figure 3B).

Because RhoA, the small G protein that has been shown to induce the nuclear accumulation of MRTF-A, can be acti-
we also examined whether MRTF-A could be placed down-stream of RhoA in the transcriptional regulation of adhesion molecules. Constitutively active RhoA (RhoA CA) potently augmented the promoter activity of ICAM-1 with response element being mapped to the same region as MRTF-A (Online Figure III, A). Activation of adhesion molecules by RhoA, however, was completely blunted in the presence of MRTF-A siRNA (Figure 3C; Online Figure III, B and C). Moreover, overexpression of a dominant negative RhoA (RhoA DN) abolished the recruitment of MRTF-A to the ICAM-1 promoter induced by oxLDL (Figure 3D). Thus, it is likely that an oxLDL-RhoA-MRTF-A axis regulates the induction of adhesion molecules during endothelial injury.

Figure 1. MRTF-A is activated by oxLDL in endothelial cells. A and B, HUVECs (EAhy926) (left) or aortic endothelial cells (right) were treated with oxLDL (20 to 100 μg/mL) and probed for protein (A) and mRNA (B) levels of MRTF-A, ICAM-1, and eNOS. MRTF-A protein expression from 3 independent experiments was corrected by β-actin levels for normalization and expressed as relative MRTF-A protein levels. Unfilled indicates control; light gray, oxLDL (20 μg/mL); dark gray, oxLDL (50 μg/mL); filled, oxLDL (100 μg/mL). C, Luciferase construct driven by the human MRTF-A promoter (−1585/+114) was transfected into HUVECs followed by treatment with oxLDL (20 to 100 μg/mL). Luciferase activities were normalized by both protein concentration and GFP fluorescence. Data are presented as normalized relative luciferase unit (NRLU). *P<0.05. D, HAECs were treated with oxLDL (50 μg/mL) for 24 hours. Cellular MRTF-A was visualized by immunofluorescence staining. The nuclei were counterstained with DAPI. The number of cells located in the nucleus in each group was counted using Image-Pro, and data were expressed as percentages compared with the overall cell count. Scale bar, 50 μm.
MRTF-A Is Recruited to the ICAM-1 Promoter by Nuclear Factor κB

As a transcriptional cofactor without the ability to bind directly to DNA, MRTF-A relies on sequence-specific transcriptional factor(s) to be recruited to its targets. Therefore, we examined how MRTF-A is recruited to the ICAM-1 promoter. Bioinformatic analysis of the ICAM-1 promoter region (−393/−176) revealed a conserved binding element for the sequence-specific transcription factor nuclear factor (NF)-κB (Online Figure IV, A), indicating that NF-κB might play a role in bringing MRTF-A to the ICAM-1 promoter. Indeed, MRTF-A formed a complex with p65 (Figure 4A; Online Figure IV, B). Further analysis on the association of MRTF-A with NF-κB/p65 indicated that MRTF-A interacted with p65 via its N-terminal
glutamine-rich (Q) domain whereas p65 interacted with MRTF-A via its Rel-homology domain (Online Figure IV, C and D). Moreover, MRTF-A interacted with p65 on the ICAM-1 promoter region as demonstrated by gel shift assay (Figure 4B). Most importantly, oxLDL enhanced the interaction between MRTF-A and p65 on the ICAM-1 promoter as demonstrated by Re-ChIP (Figure 4C).

Cotransfection of p65 and MRTF-A activated the ICAM-1 promoter synergistically (Online Figure IV, E). On the other hand, mutation of the NF-κB site or shRNA-mediated silencing of p65 disrupted the binding of MRTF-A to the ICAM-1 promoter region and blocked the activation of ICAM-1 transcription by MRTF-A (Figure 4D through 4F), pointing to a functional interplay between MRTF-A and p65. In further support of this notion, MRTF-A augmented the luciferase activity driven by 2 tandem repeats of the NF-κB response element (Online Figure IV, F). In aggregate, these data suggest that p65 recruits MRTF-A to the ICAM-1 promoter in response to increased oxLDL levels.

**SRF Is Essential for ICAM-1 Transactivation by OxLDL**

It is well documented that MRTF-A interacts with SRF to promote gene transcription specific for the smooth muscle cell lineage and that SRF physically and functionally interacts with p65. Because it was observed that SRF expression was induced by oxLDL in endothelial cells, we examined the role of SRF in MRTF-A mediated ICAM-1 transactivation. Disruption of endogenous SRF by RNAi prevented activation of ICAM-1 promoter by MRTF-A and oxLDL (Figure 5A and 5B; Online Figure V, A and B). Moreover, SRF depletion resulted in amelioration of endogenous ICAM-1 mRNA and protein levels as well as leukocyte adhesion induced by oxLDL (Figure 5C; Online Figure V, C).

There is no conserved SRF binding element (CARG) on the proximal ICAM-1 promoter (−393/−176), indicating that SRF likely impacts ICAM-1 transcription through other mechanisms than direct DNA binding. Sequential immunoprecipitation assay and gel shift assay showed that MRTF-A, p65, and SRF formed a tertiary complex (Figure 5D; Online Figure V, D). In addition, oxLDL promoted the binding of SRF to the same region that MRTF-A and p65 occupy on the ICAM-1 promoter (Figure 5E). Similar to what was observed for MRTF-A, oxLDL also augmented the interaction between SRF and p65 on the ICAM-1 promoter as demonstrated by Re-ChIP (Online Figure V, E), whereas knockdown of p65 markedly disrupted the recruitment of SRF to the ICAM-1 promoter (Online Figure V, F). Combined, these data clearly illustrated that oxLDL induced the formation of a p65/MRTF-A/SRF complex on the ICAM-1 promoter to induce its transcription.

**MRTF-A Binds to eNOS Promoter and Suppresses Its Transcription in Response to OxLDL**

eNOS dependent NO production plays a significant role in endothelial physiology and is impaired by oxLDL during atherogenesis. We then sought to determine the effect of MRTF-A on
Figure 4. MRTF-A is recruited to the ICAM-1 promoter by NF-κB. A, Immunoprecipitation assays were performed with anti-p65, anti-MRTF-A, or preimmune IgG (P.I.I.) using HUVEC lysate. Asterisk symbol represents nonspecific band; H.C., heavy chain. B, Electrophoresis mobility shift assay (EMSA) was performed with a DNA probe harboring the p65 site on the ICAM-1 promoter as described in Methods. C, HAECs were treated with oxLDL (50 μg/mL). Re-ChIP assays were performed with indicated antibodies. Precipitated DNA was amplified by qPCR. *P<0.05. D, ICAM-1 promoter constructs (WT and NF-κB site mutant) were transfected into HEK293 cells and HUVECs with or without MRTF-A. Luciferase activities are expressed as NRLU. *P<0.05. E, HAECs were infected with lentivirus carrying shRNA plasmid targeting p65 (shp65), or a control shRNA plasmid (shC) followed by treatment with oxLDL (50 μg/mL). ChIP assays were performed with anti-MRTF-A. Precipitated DNA was amplified by qPCR. *P<0.05 (bottom) Lysates from HAECs infected with shp65 or shC were probed for p65 protein by Western blot. F, ICAM-1 promoter construct was transfected into HUVECs and HAECs with MRTF-A, shp65 or shC as indicated followed by treatment with oxLDL (20 μg/mL). Luciferase activities are expressed as NRLU. *P<0.05.
the expression and activity of eNOS. Under basal conditions, MRTF-A modestly suppressed the activity of eNOS promoter whereas its activity was markedly enhanced by oxLDL (Figure 6A). oxLDL also repressed endogenous eNOS mRNA better when cells were infected with retrovirus carrying MRTF-A (Figure 6B). In contrast, both MRTF-A siRNA and DN-MRTF-A alleviated the repression of eNOS by oxLDL (Figure 6C; Online Figure VI, A through C). Moreover, depletion of MRTF-A in endothelial cells restored NO bioavailability in the presence of oxLDL (Figure 6D; Online Figure VI, D).

Harnessing a similar strategy, we identified a short stretch of DNA (−430/168) on the eNOS promoter as the response element for MRTF-A (Online Figure VII, A) and ChIP assays in both HUVECs and HAECs confirmed that oxLDL preferentially stimulated the binding of MRTF-A to this region but not to the first intron of the eNOS gene (Figure 6E). Likewise, MRTF-A requires functional RhoA signaling to be recruited to the eNOS promoter as forced expression of RhoA DN prevented the binding of MRTF-A (Online Figure VII, B). Conversely, RhoA seemed to be dependent on MRTF-A to suppress eNOS expression (Online Figure VII, C and D). Taken together, our data suggest that MRTF-A contributes to oxLDL-dependent impairment of vascular tone by repressing eNOS transcription in endothelial cells in a RhoA-dependent manner.

**MRTF-A–Mediated Transcription Involves Differential Epigenetic Alterations**

At the chromatin level, altered epigenetic modifications are responsible for differential gene expression. We next probed how MRTF-A might affect the epigenetic alterations taking place on the ICAM-1 and eNOS promoters. In keeping with the expression pattern of ICAM-1, active histone modifications (acetylated histones H3 and H4, dimethylated histone H3 lysine 4) were preferentially induced by oxLDL on its promoter whereas levels of trimethylated H3 lysine 9, usually associated with transcriptional repression, were suppressed (Figure 7A; Online Figure VIII, A). Intriguingly, this set of epigenetic alterations was completely reversed when MRTF-A levels were knocked down by siRNA. Knockdown of endogenous SRF with siRNA had a similar impact (Online Figure VIII, B).
Figure VIII, B and C). In sharp contrast, basal levels of active histone marks were higher on the eNOS promoter in the absence of oxLDL (Figure 6B). Treatment with oxLDL erased the active marks and put in place repressive histone modifications only in the presence of endogenous MRTF-A (Figure 7B; Online Figure VIII, D). SRF depletion, however, did not alter the histone modifications on the eNOS promoter (data not shown). Thus, our data indicate that the MRTF-A is central to oxLDL-orchestrated epigenetic regulation of gene expression in endothelial cells.

Discussion

OxLDL is present in the circulation as well as in the atherosclerotic plaque.\(^3,14\) It is believed to be a critical risk...
factor/biomarker that links several concurrent pathophysiological processes including chronic inflammation, metabolic disorder, and oxidative stress during atherogenesis rendering the vasculature dysfunctional. Among many of its deleterious effects, oxLDL-induced endothelial injury is often considered a predisposing trigger leading up to further progression and deterioration of atherosclerosis. Here we present evidence that MRTF-A, a transcriptional modulator, is responsible for oxLDL induced endothelial injury by mediating key transcriptional events in the endothelium. We demonstrate here that regulation of MRTF-A by oxLDL is at least 4-fold. First, oxLDL increased MRTF-A expression at the transcriptional level. Second, oxLDL prompted nuclear accumulation of MRTF-A. Third, oxLDL increased the occupancy of MRTF-A on target promoters. Finally, oxLDL promoted the interaction between MRTF-A and p65 on the ICAM-1 promoter.

Accumulating evidence has linked MRTF-A to cellular adaptation to stress cues. In the cardiovascular system, upregulation of MRTF-A levels has been linked to increased incidence of coronary artery disease. Moreover, 2 independent groups have demonstrated that MRTF-A deficiency attenuates tissue impairment and protects against myocardial infarction and cardiac hypertrophy. Our data presented here allude to the notion that activation of MRTF-A underscores the maladaptation of endothelial cells in response to stress exerted by oxLDL, indicating endothelial-specific loss-of-function of MRTF-A would alleviate atherosclerotic injury. Future studies using tissue-specific knockout model of MRTF-A and high-output expression profiling may shed more light on the vascular pathophysiology of MRTF-A.

MRTF-A forges extensive protein-protein interactions inside the cell, which in turn affect its subcellular localization, and/or target specificity. To date, most studies have focused on the interaction between MRTF-A and SRF, which recruits MRTF-A to the conserved CArG sequence found in many SMC-specific genes. There is no conserved CArG element in the proximal ICAM-1 promoter. Instead, we demonstrate here that MRTF-A is recruited to the ICAM-1 promoter by interacting with NF-kB/p65. Because MRTF-A, unlike myocardin, is universally expressed, this interaction is likely to have broader physiological and/or pathological implications. Intriguingly, by chromatin immunoprecipitation coupled with microarray (ChIP-chip), several p65 binding sites have been located on the proximal promoter and within the first intron of the MKL1/MRTF-A gene. In light of our new data, we intend to propose a scenario wherein MRTF-A, as a downstream target of p65, reciprocally fine-tunes p65 activity. A well-established example in support of this hypothesis is the nuclear receptor peroxisome proliferator activated receptor (PPAR). PPAR upregulates the expression of CAAT/enhancer binding protein (C/EBP) and cooperate with the latter to dictate adipogenesis in preadipocyte whereas it activates and facilitates CIITA to regulate matrix production and chronic inflammation in smooth muscle cells. Interestingly, SRF forms a tertiary complex with MRTF-A and p65 and is brought to the ICAM-1 promoter by p65 (Figure 5; Online Figure V). Previously, it has been shown that p65 interacts with SRF and enhances SRF-driven transcription without binding to DNA directly. Our data suggest that the functional interaction between SRF and p65 could be reciprocal such that they act as coactivators for each other. It remains to be determined at a genome-wide scale how the p65/MRTF-A duet (and possibly SRF) regulates human...
pathophysiology. On the other hand, we demonstrate that MRTF-A directly binds to the eNOS promoter and represses its transcription in response to oxLDL likely involving epigenetic maneuvers. It remains enigmatic what sequence-specific transcription factor (more precisely, repressor) recruits MRTF-A to the eNOS promoter. Although several transcriptional repressors for the eNOS gene, including STAT3,23 AP-1,26 and ATF2,27 have been identified, their binding sites do not overlap with the putative MRTF-A-response element (~430/~168). Of note, there is no conserved SRF element in the proximal eNOS promoter and depletion of SRF had no impact on eNOS expression and epigenetic alterations on the eNOS promoter (data not shown). Ongoing investigation in our laboratory harnessing DNA affinity chromatography coupled with mass spectrometry will hopefully resolve this issue.

Epigenetic alterations are associated with differential gene expression patterns in the vasculature.28 Little is known, however, about the molecular switch that dictates the epigenetic alterations leading up to atherogenesis. Our data suggest that MRTF-A is a master coordinator in the epigenetic regulation of ICAM-1 and eNOS expression in response to oxLDL (Figure 7). MRTF-A is known to interact with members of the epigenetic machinery, including the ATP-dependent chromatin remodeling proteins (Brg1 and Brm) and the histone demethylase Jmjd1a.30,31 Our preliminary data suggest that Brg1 and, to a lesser extent, Brm are required for MRTF-A-dependent epigenetic alterations and transcriptional outcomes in endothelial cells (Fang F. and Xu Y., unpublished data). Several outstanding questions regarding the precise maneuvering that underlies the epigenetic regulation of gene expression by MRTF-A in response to stress await answers. For instance, it remains enigmatic how MRTF-A provokes 2 opposite sets of epigenetic alterations that herald transcriptional activation and repression simultaneously. Our data favor a model (Figure 8) where MRTF-A is recruited to multiple spatially separated transcriptional foci (zones) that differ in cofactor and modification enzyme. Recent reports indicate that MRTF-A is both SUMOylated and phosphorylated in vivo.32 Because protein post-translational modifications (PTM) including SUMOylation and phosphorylation are actively involved in the organization of nuclear compartments,34 it is likely that the PTM machinery modifies and hence assigns MRTF-A to different loci to mediate either transcriptional activation or repression depending on the local enrichment of cofactors. Of note, several proteins that are important regulators of cardiovascular pathophysiology are subject to signal-induced modifications, which in turn affect their activities. PPARγ, for instance, can promote lipid uptake of macrophages by activating the scavenger receptor gene CD36 while, at the same time, reining in the inflammatory response by downregulating iNOS transcription; only the latter requires ligand-dependent SUMOylation on lysine 365 of PPARγ.35 We have previously reported that CIITA, in response to the proinflammatory cytokine interferon-γ, modulates an array of key transcriptional events involved in atherogenesis including activation of MHC II genes and repression of collagen type I genes.36 Spatiotemporal control of CIITA activity is attributed to differential modifications including phosphorylation, ubiquitination, and acetylation.39 Proteomic studies combined with animal models deficient in the PTM machinery may help unveil the precise mechanism whereby MRTF-A differentially regulates transcription in the vasculature.

In summary, we have delineated an oxLDL–MRTF-A axis that contributes to endothelial injury during atherogenesis. Because MRTF-A plays a major role in this process, therapeutic strategies targeting MRTF-A could serve as a potential treatment option to correct endothelial dysfunction.

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Disclosures
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MRTF-A downregulates eNOS expression and NO bioavailability in response to oxLDL.

Endothelial dysfunction and oxidation of LDL are believed to be early and critical events in atherogenesis. OxLDL significantly alters the endothelial transcriptome in vascular endothelial cells. Here we report that oxLDL activates MRTF-A in endothelial cells by inducing its transcription, prompting its nuclear enrichment, and increasing its binding to target promoters. MRTF-A, in turn, upregulates ICAM-1 transcription and downregulates eNOS expression in response to oxLDL by engaging the epigenetic machinery. Depletion of MRTF-A in endothelial cells alleviates leukocyte adhesion and restores NO bioavailability. Our findings assign a previously unidentified role for MRTF-A in atherogenesis and suggest novel therapeutic strategies for treating atherosclerosis.
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SUPPLEMENTAL MATERIAL

Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC/EAhy926, ATCC), human monocyte/macrophage cells (THP-1, ATCC), and human embryonic kidney cells (293FT, Invitrogen) were maintained in RPMI-1640 and DMEM (Invitrogen), respectively, supplemented with 10% fetal bovine serum (FBS, Hyclone). Human primary aortic endothelial cells (HAEC, Cambrex/Lonza) were maintained in EGM-2 media with supplements supplied by the vendor; experiments were performed in HAECs between 3rd and 6th passages.

Plasmids, Transient Transfection, Viral Infection, and Luciferase Assay

FLAG-tagged MRTF-A (WT, CA, DN) and MRTF-B, constitutively active RhoA (Q63L), dominant negative RhoA (N19), GFP-tagged p65, FLAG-tagged p65, and V5-tagged p65 were gifts from Dr. Prywes, Dr. Olson, Dr. Fluriot, Dr. Parmacek, Dr. Faessler, Dr. Akeda, Dr. Colburn, Dr. Matsuoka, and Dr. Sasaki. Promoter luciferase fusion constructs for eNOS, ICAM-1, ICAM-2, and VCAM-1 were provided by Dr. Liang, Dr. Stratowa, Dr. Redondo, Dr. Tiruppathi, and Dr. Pearse. Retroviral constructs for RhoA and MRTF-A, Myc-tagged MRTF-A, and short hairpin RNA for p65 have been described previously. Small interfering RNA (siRNA) sequences for MRTF-A were as follows: #1, 5'-GUGUCUUGGUAGUGUA-3', #2, 5'-CUGCGUGCAAUACAGAACAA-3', #3, 5'-CAGTTGAUUAAUCCGAAAUG-3', siRNA sequences for SRF were as follows: #1, 5'-GCAGAGGGCAACUGCAUACAUUG-3', #2, 5'-AUGUUUGCCAGAUGUAUUA-3', #3, 5'-GAAUGAGUGCCACUGCUU-3'. MRTF-A promoter luciferase construct was made by amplifying genomic DNA spanning the proximal promoter and the first exon of MKL1 gene (-1585/+114) and ligating into a pGL3-basic vector (Promega). Transient transfections were performed with Lipofectamine 2000 (Invitrogen). Retroviruses were generated in 293FT cells as previously described. Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells and repeated three times.

Preparation of OxLDL

LDL was isolated from the serum of healthy Chinese donors by sequential ultracentrifugation. Oxidation reaction was performed by incubating native LDL for 4 h at 37°C in phosphate-buffered saline (PBS) containing 5mM CuSO₄ and then extensively dialyzed against PBS and sterilized by filtration. The oxidative extent of each lot was monitored by agarose gel electrophoresis and colorimetrically by the TBARS assay as described before.

Protein Extraction, Immunoprecipitation, and Western Blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor tablet (Roche). Nuclear proteins were prepared with the NE-PER Kit (Pierce) following manufacturer’s recommendation. Specific antibodies or pre-immune IgGs (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1X SDS electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with lysates overnight. Precipitated immune complex was eluted with 3X FLAG peptide (Sigma). Western blot analyses were performed with anti-FLAG, anti-GFP, anti-β-actin (Sigma), anti-ICAM-1 (Abgent), anti-eNOS, anti-SRF, anti-p65, and anti-MRTF-A (Santa Cruz) antibodies.

Electrophoresis mobility shift assay (EMSA)

EMSA was performed with biotin-labeled DNA probe spanning the NF-κB/p65 binding site of the ICAM-1 gene (wild type: 5'-TAGCTTTAAAAATCCGAGC-3' and mutant 5'-TAGCTTTTTAAAATCCGAGC-3') and nuclear protein extracted from HUVECs using a LightShift
chemiluminescent EMSA Kit (Pierce) following the vendor’s recommendations. The CArG box found in the c-fos promoter was used as a competitor for SRF binding: 5’-GATGTCCATATTAGGACATC-3’.

Immunofluorescence microscopy
Endothelial cells were fixed with 4% formaldehyde, permeabilized with TBST (.25% Triton X-100, 150mM NaCl, 50mM Tris pH7.4), blocked with 5% BSA, and incubated with indicated primary antibodies overnight. After several washes with PBS, cells were incubated with FITC-labeled secondary antibodies (Jackson) for 30 minutes. DAPI (Sigma) was added and incubated with cells for 5 minutes prior to observation. Immunofluorescence was visualized on a co-focal microscope (LSM 710, Zeiss).

RNA Isolation and Real-time PCR
RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on an ABI Prism 7500 system. Primers and Taqman probes used for real-time reactions to detect MRTF-A, ICAM-1, and eNOS were purchased from Applied Biosystems.

Chromatin Immunoprecipitation (ChIP)
Chromatin was cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-MRTF-A, anti-SRF (Santa Cruz), anti-acetyl histone H3, anti-acetyl histone H4, anti-trimethyl H3K9, anti-dimethyl H3K4 (Millipore), or pre-immune IgG. For re-ChIP, immune complexes were eluted with the elution buffer (1% SDS, 100mM NaCO₃), diluted with the re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris pH 8.1), and subject to immunoprecipitation with a second antibody of interest. Precipitated genomic DNA was amplified by real-time PCR with primers listed in supplemental Table I.

Leukocyte Adhesion Assay
THP-1 cells were stained with a fluorescent die (2′,7′-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis (acetoxymethyl) ester) (Sigma) for 30 min at 37°C. After several washes with PBS, THP-1 cells were co-incubated for 30 min with endothelial cells. Unbound leukocytes were removed by washing and the number of adhered cells was visualized by fluorescence microscopy and analyzed with Image-Pro Plus (Media Cybernetics).

NO measurement
Prior to each assay, endothelial cells were switched to and maintained in the Kreb's solution (118 mM NaCl, 4.6 mM KCl, 27.2 mM NaHCO₃, 1.2mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 11.1 mM glucose) for 1 hour at 37°C. Afterwards, 100 μl supernatant from each well was collected and the nitrate content was measured with a Griess reagent system (Promega).

Statistical Analysis
One-way ANOVA with post-hoc Scheffe analyses were performed using an SPSS package. P values smaller than .05 were considered statistically significant.

References
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**Online Figure I**: (A) HUVECs and HAECs were treated with oxLDL (50μg/ml). Cells were harvested at various time points post-treatment and MRTF-A mRNA levels were measured by qPCR. (B) HUVECs and HAECs were treated with oxLDL (50-100μg/ml) for 24 hours. Cells were harvested and nuclear proteins were probed for MRTF-A. (C) HUVECs were treated with oxLDL (50μg/ml) for 24 hours. Cellular MRTF-A was visualized by immunofluorescence staining. The nuclei were counter-stained with DAPI. Scale bar: 50μM. (D) HUVECs and HAECs were treated with oxLDL (50μg/ml) for 24 hours. mRNA levels for MRTF-A, myocardin, MRTF-B, and SRF were measured by qPCR. *, p<0.05; ND, not detectable. (E) A representative full gel image of MRTF-A blot (same as shown in Figure 1A).
Online Figure II: (A) ICAM-1 promoter construct was transfected with increasing concentrations of MRTF-A or MRTF-B into HEK293 cells. Luciferase activities are expressed as RLU. Cell lysates from the same experiments were probed for the expression levels of MRTF-A and MRTF-B by Western. (B) ICAM-2 or VCAM-1 promoter construct was transfected with increasing concentrations of MRTF-A into HEK293 cells. Luciferase activities are expressed as RLU. (C) ICAM-2 promoter construct was transfected with MRTF-A into HUVECs followed by oxLDL (20 μg/ml) treatment. Luciferase activities are expressed as RLU. *, p < 0.05. (D) An ICAM-1 promoter construct (-1393) was transfected into HUVECs with dominant negative MRTF-A (DN-MRTF-A) followed by oxLDL (50 μg/ml) treatment. Luciferase activities are expressed as RLU. *, p < 0.05. (E) HUVECs and HAECs were transfected with MRTF-A siRNA (#1, #2, #3) or scrambled siRNA followed by oxLDL (50 μg/ml) treatment. ICAM-1 mRNA levels were measured by qPCR. *, p < 0.05 (F) HUVECs were transfected with MRTF-A siRNA (#1, #2, #3) or scrambled siRNA followed by oxLDL (50 μg/ml) treatment. Leukocyte adhesion assay was performed as described under Methods. Relative adhesion was quantified by measuring fluorescent intensities in each group and comparing them to the control group which is set arbitrarily as 1. *, p < 0.05
**Online Figure III:** (A) ICAM-1 promoter constructs of various lengths were transfected into HEK293 cells or HUVECs along with a constitutively active RhoA (RhoA CA). Luciferase activities are expressed as RLU. *, p < 0.05 (B) ICAM-2 or VCAM-1 promoter construct was transfected with constitutively active RhoA (RhoA CA) into HUVECs in the presence of either MRTF-A siRNA (#1) or scrambled siRNA. Luciferase activities are expressed as NRLU. *, p < 0.05 (C) HAECs were infected with retrovirus carrying a constitutively active form of RhoA (RhoA CA) followed by transfection with either MRTF-A siRNA (#1) or scrambled siRNA. ICAM-1 mRNA and protein (bottom) levels were determined by qPCR and Western. *, p < 0.05
**Online Figure IV:** (A) A diagram of the ICAM-1 proximal promoter. The NF-κB response site (boxed) is conserved among human, mouse and rat. X indicates the nucleotides that are mutated. (B) HEK293 cells were transfected with GFP-tagged p65 and FLAG-tagged MRTF-A. Immunoprecipitation assays were performed with anti-GFP, anti-FLAG, or pre-immune IgG (P.I.I.) as indicated. Immunoblottings were performed with either anti-GFP or anti-FLAG. ※: Non-specific band. (C, D) HEK293 cells were transfected indicated expression plasmids. Immunoprecipitations were performed with anti-FLAG. Eluates were probed for the interaction between MRTF-A and p65 with Western. (E) ICAM-1 promoter construct was transfected into HUVECs or HAECs with p65 and MRTF-A followed by treatment with oxLDL (20μg/ml). Luciferase activities are expressed as NRLU. *, p < 0.05 (F) 2XκB-LUC was transfected into HUVECs with MRTF-A and/or p65 followed by treatment with oxLDL (20μg/ml). Luciferase activities are expressed as NRLU. *, p<0.05
**Online Figure V**: (A) Validation of SRF siRNA. (B) An *ICAM-1* promoter construct was transfected into HUVECs with MRTF-A, SRF siRNA (#1, #2, #3), or scrambled siRNA (SCR). Luciferase activities expressed as NRLU. (C) SiSRF or SCR was transfected into HUVECs followed by treatment with oxLDL. Leukocyte adhesion was measured as described earlier. (D) FLAG-MRTF-A and V5-p65 were transfected into HEK293 cells with or without GFP-SRF. Sequential immunoprecipitations were performed with anti-FLAG and anti-GFP as indicated. (E) HAECs were treated with oxLDL (50 μg/ml). Re-ChIP assays were performed with indicated antibodies. Precipitated DNA was amplified by qPCR. *, p<0.05 (F) HAECs were infected with lentivirus carrying shRNA plasmid targeting p65 (shp65), or a control shRNA plasmid (shC) followed by treatment with oxLDL (50 μg/ml). ChIP assays were performed with anti-SRF. Precipitated DNA was amplified by qPCR. *, p<0.05
Online Figure VI: (A) An eNOS promoter construct (-2091/+22) was transfected into HUVECs with either MRTF-A siRNA or scrambled siRNA followed by oxLDL (50 μg/ml) treatment. Luciferase activities are expressed as RLU. *, p<0.05 (B) An eNOS promoter construct (-2091/+22) was transfected into HUVECs with dominant negative MRTF-A (DN-MRTF-A) followed by oxLDL (50 μg/ml) treatment. Luciferase activities are expressed as RLU. *, p<0.05 (C) HUVECs and HAECs were transfected with MRTF-A siRNA (#1, #2) or scrambled siRNA followed by oxLDL (50 μg/ml) treatment. ICAM-1 mRNA levels were measured by qPCR. *, p<0.05 (D) HUVECs were transfected with MRTF-A siRNA (#1, #2, #3) or scrambled siRNA followed by oxLDL (50 μg/ml) treatment. eNOS activity was measured by NO assay as described in Methods. *, p<0.05
Online Figure VII: (A) eNOS promoter constructs of various lengths were transfected into HEK293 cells along with MRTF-A WT, MRTF-A CA, or Rho A CA as indicated. Luciferase activities are expressed as RLU. *, p<0.05; N.S., not significant. (B) HAECs were infected with retrovirus carrying a dominant negative form of RhoA (N19) followed by treatment with oxLDL (100 μg/ml). ChIP assays were performed with an anti-MRTF-A antibody as described under Methods. Precipitated DNA was amplified by qPCR with primers spanning the eNOS promoter. *, p<0.05. (C) eNOS promoter construct was transfected with constitutively active RhoA (RhoA CA) and/or dominant negative MRTF-A (MRTF-A DN) into HUVECs. Luciferase activities are expressed as RLU. *, p<0.05. (D) HAECs were infected with retrovirus carrying a constitutively active form of RhoA (RhoA CA) followed by transient transfection with either MRTF-A siRNA (#1) or scrambled siRNA. eNOS mRNA and protein levels were determined by qPCR and Western. *, p<0.05
**Online Figure VIII:** (A, B, C, D) HAECs were transfected with MRTF-A siRNA (#1), SRF siRNA (#1), or scrambled siRNA followed by treatment with oxLDL (50 μg/ml). ChIP assays were performed with indicated antibodies. Precipitated DNA was amplified with qPCR primer/probe sets for ICAM-1 (A, B, C) and eNOS (D) promoters as described under Methods.
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