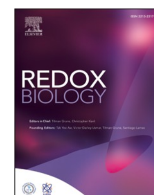
Redox Biology 59 (2023) 102586

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox



MKL1 fuels ROS-induced proliferation of vascular smooth muscle cells by modulating FOXM1 transcription

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A R T I C L E I N F O

Keywords:
Transcriptional regulation
Post-translational modification
Vascular injury
Vascular smooth muscle cell
Proliferation
Phenotypic modulation

A B S T R A C T

Reactive oxygen species (ROS) promotes vascular injury and neointima formation in part by stimulating proliferation of vascular smooth muscle cells (VSMC). The underlying transcriptional mechanism, however, is not completely understood. Here we report that VSMC-specific deletion of MKL1 in mice suppressed neointima formation in a classic model of vascular injury. Likewise, pharmaceutical inhibition of MKL1 activity by CCG-1423 similarly mollified neointima formation in mice. Over-expression of a constitutively active MKL1 in vascular smooth muscle cells enhanced proliferation in a ROS-dependent manner. On the contrary, MKL1 depletion or inhibition attenuated VSMC proliferation. PCR array based screening identified forkhead box protein M1 (FOXM1) as a direct target for MKL1. MKL1 interacted with E2F1 to activate FOXM1 expression. Concordantly, FOXM1 depletion ameliorated MKL1-dependent VSMC proliferation. Of interest, ROS-induced MKL1 phosphorylation through MK2 was essential for its interaction with E2F1 and consequently FOXM1 *trans*-activation. Importantly, a positive correlation between FOXM1 expression and VSMC proliferation was identified in arterial specimens from patients with restenosis. Taken together, our data suggest that a redox-sensitive phosphorylation-switch of MKL1 activates FOXM1 transcription and mediates ROS fueled vascular smooth muscle proliferation. Targeting the MK-2/MKL1/FOXM1 axis may be considered as a reasonable approach for treatment of restenosis.

1. Introduction

Vascular smooth muscle cells (VSMCs) display remarkable flexibility that enable them to adapt (or misadjust) to a host of different pathophysiological conditions. Normally, fully differentiated VSMCs adopt a contractile phenotype by exiting the cell cycle and producing muscle-related molecules to execute rhythmic contraction/dilation of blood vessels in response to vasoactive substances. However, VSMCs can switch between several morphologically and functionally distinct states in a process commonly known as phenotypic modulation (PM). PM is considered the linchpin of vascular injury contributing to the pathogenesis of a wide range of human diseases including atherosclerosis [1], aortic aneurysm [2], and pulmonary hypertension [3]. Narrowly put, PM designates the process in which VSMCs transition from the contractile phenotype to the synthetic phenotype characterized by augmented ability to proliferate, migrate, and produce excessive extracellular matrix (ECM) proteins. Undoubtedly this narrow definition does not encompass the complexity of the process wherein VSMC tailors its phenotypic flexibility to vascular injury because multiple distinct types of VSMCs have been identified in different vascular disorders [4].

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https://doi.org/10.1016/j.redox.2022.102586
Received 17 December 2022; Accepted 22 December 2022
Available online 29 December 2022
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Enhanced proliferation of VSMCs is considered a hallmark event during vascular injury that contributes to the pathogenesis of restenosis, defined as 50% decline in vessel diameter [5]. Studies performed in model animals and humans have shown that the process of restenosis bears striking resemblance to wound healing and can be roughly divided into three stages: it begins with an acute pro-inflammatory phase, followed by a migroproliferative phase, and concludes with an ECM remodeling phase [6]. Typically, VSMCs may expand as much as 5 times the original volume and account for 90% of the increased luminal area (neointima) [7]. Overall, this process is subjected to the regulation by a sea of growth factors and cytokines, of which platelet derived growth factor (PDGF) is the most extensively studied [8]. From a cell biological perspective, restenosis is characterized by VSMCs exiting the G0 phase and re-entering the G1 phase of cell cycling [9]. PDGF relays a pro-proliferative signal through the MAPK pathway, which in turn activates a host of transcription factors in the nucleus to reprogram VSMC behaviors [10]. Notably, PDGF-mediated VSMC proliferation and, by extension, pathogenesis of restenosis, are sensitive to intracellular redox status. Increased ROS production is observed in VSMCs exposed to PDGF treatment [11] and in the vessels of patients with restenosis [12]. ROS accumulation promotes VSMC proliferation likely through differential activation of MAPK signaling [13]. In accordance, ROS cleansing by antioxidants inhibits PDGF-induced VSMC proliferation and attenuates restenosis [14,15].

Megakaryocytic leukemia 1 (MKL1), also known as myocardin-related transcription factor A (MRTF-A), is a transcriptional regulator involved in a wide range of pathophysiological processes in the cardiovascular system. MKL1, along with myocardin and MKL2, was initially identified as a co-factor that interacts with serum response factor (SRF) to activate the transcription of contractile genes in muscle cells [16]. Forced expression of MKL1 in non-muscle cells leads to robust up-regulation of contractile genes [17]. Germline deletion of either myocardin or MKL2 in mice results in embryonic lethality owing to failed vasculogenesis with defective VSMC differentiation whereas mice with MKL1 deletion are developmentally normal suggesting that MKL1 may play a redundant role in maintaining the contractile phenotype of VSMCs [18]. Although MKL1-null mice are phenotypically indistinguishable from wild type littermates under physiological conditions, recent investigations have portrayed MKL1 as a stress-responsive regulator of cardiovascular diseases in a lineage-specific manner. For instance, vascular endothelial cell-specific deletion of MKL1 attenuates hypoxia-induced pulmonary hypertension and pressure overload-induced cardiac hypertrophy [19,20]. In the present study, we report that VSMC-specific MKL1 deletion attenuates neointima formation in mice. Mechanistically, redox-sensitive phosphorylation of MKL1 by MK2 promotes FOXM1 transcription to drive VSMC proliferation.

2. Methods

2.1. Animals

All animal protocols were reviewed and approved the intramural Ethics Committee on Humane Treatment of Laboratory Animals of Nanjing Medical University. The mice were maintained in an SPF environment with 12 h light/dark cycles and libitum access to food and water. *Mkl1*$^{f/f}$ mice [21] were crossed to *Myh11*-Cre^{ERT2} mice [22] to generate SMC-specific MKL1 knockout mice. Vascular injury was induced in mice by carotid artery ligation as previously described [23].

2.2. Cell culture and treatment

Primary vascular smooth muscle cells were isolated from 3 to 5wk C57/BL mice and maintained in DMEM supplemented with 15% FBS as previously described [1]. Typically, cells with passages between 3 and 6 were used. FOXM1 expression constructs [24], FOXM1 promoter-luciferase constructs [25,26], FLAG-tagged MKL1 expression constructs [27], and GFP-tagged E2F1 expression constructs [28] have been described previously. Mutagenesis was performed a QuikChange kit (Thermo Fisher Scientific, Waltham, MA, United States) as previously described [29]. All DNA constructs were verified by direct sequencing. Small interfering RNAs were purchased from GenePharma. Cells were harvested 24–48h after the transfection. Transient transfections were performed with Lipofectamine 2000. Luciferase activities were assayed using a luciferase reporter assay system (Promega) as previously described [30].

2.3. Chromatin immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assays were performed essentially as described before [31–33]. In brief, chromatin in control and treated cells were 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 and PMSF. DNA was fragmented into ~200 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-FLAG (Sigma, F1804), anti-MKL1 (Proteintech, 21166-1), anti-E2F1 (Cell Signaling Tech, 3742), or pre-immune IgG.

2.4. RNA isolation and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen) as previously described [34]. Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen) as previously described [35]. Real-time PCR reactions were performed on an ABI Prism 7500 system with the following primers: Mouse *Foxm1*, 5′-CTGATTCTCAAAAGACGGAGGC-3′ and 5′-TTGATAATCTTGATTC CGGCTGG-3'. Ct values of target genes were normalized to the Ct values of housekeeping control gene (18s, 5′-CGCGGTTCTATTTTGTTGGT-3′ and 5′-TCGTCTTCGAAACTCCGACT-3′ for both human and mouse genes) using the ΔΔCt method and expressed as relative mRNA expression levels compared to the control group which is arbitrarily set as 1 as previously described [36].

2.5. PCR array

A customized PCR array (Qiagen) in a 96-well format was performed to screen for MRTF-A target genes. 1 μg total RNA extracted from VSMCs was reverse-transcribed using the RT^2 First Strand kit supplied by the vendor. Then, the cDNA was mixed with 2x RT^2 SYBR Green Mastermix and 25 μl of the mix was dispensed into the customized 96-well plate that contained 45 pre-selected genes in duplicate plus 3 housekeeping genes for normalization. Quantitative PCR was performed on an Applied Biosystems StepOne Plus system. Cycle threshold (CT) values were calculated using StepOne software v2.1. The fold-change for each gene was calculated using the ΔΔCT method and normalized by the housekeeping genes.

2.6. Protein extraction, immunoprecipitation, and Western blotting

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche) as previously described [37]. 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 blotting was performed with anti-FLAG (Sigma,

F3165), anti-GFP (Proteintech, 50430-1), anti-MKL1 (Proteintech, 21166-1), anti-E2F1 (Cell Signaling Tech, 3742), anti-FOXM1 (Abcam, ab207298), anti-phosphorylated serine/threonine (Cell Signaling Tech, 9631), anti-MK2 (Cell Signaling Tech, 3042), anti-phosphorylated MK2 (Cell Signaling Tech, 3316), and anti-β-actin (Sigma, A2228).

2.7. Histology

Histological analyses were performed essentially as described before [38,39]. Paraffin sections were stained with were blocked with 10% normal goat serum for 1 h at room temperature and then incubated anti-PCNA (Proteintech, 10205-1, 1:250) or anti-FOXM1 (Abcam, ab207298, 1:200) antibodies. Staining was visualized by incubation with anti-rabbit secondary antibody and developed with a streptavidin-horseradish peroxidase kit (Pierce) for 20min. Pictures were taken using an Olympus IX-70 microscope. Quantifications were performed with Image J.

2.8. Immunofluorescence staining

Paraffin sections or formaldehyde-fixed cells were incubated with anti-Ki67 (Abcam, ab15580, 1:200) or anti-FOXM1 (Abcam, ab207298, 1:200) followed by incubation with donkey secondary antibodies (Jackson ImmunoResearch). The nuclei were counterstained with DAPI (Sigma). Quantifications were performed with Image J.

2.9. Human restenosis specimens

Vascular biopsies were collected from patients with restenosis referring to Nanjing Drum Tower Hospital. Written informed consent was obtained from subjects or families of liver donors. All procedures that involved human samples were approved by the Ethics Committee of the Nanjing Drum Tower Hospital and adhered to the principles outlined in the Declaration of Helsinki. Paraffin sections were stained with indicated antibodies. Slides were observed under a light microscope at high power (X40) by two pathologists independently in a double-blind fashion.

2.10. Statistical analysis

One-way ANOVA with post-hoc Scheff'e analyses were performed by SPSS software (IBM SPSS v18.0, Chicago, IL, USA). Unless otherwise specified, values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. MKL1 deletion or inhibition ameliorates neointima formation in mice

In order to investigate the role of MKL1 in VSMC phenotypic modulation, the *Mkl1*$^{f/f}$ strain was crossed to the *Myh11*-Cre^{ERT2} strain to generate an SMC-specific MKL1 knockout strain (*Mkl1*$^{\Delta SMC}$, Fig. 1A). When both the *Mkl1*$^{f/f}$ mice and the *Mkl1*$^{\Delta SMC}$ mice were subjected to the carotid artery ligation procedure for 4 weeks, H&E staining showed that neointima formation, as evidenced by narrowing of the vascular lumen, was more severe in the *Mkl1*$^{f/f}$ mice than in the *Mkl1*$^{\Delta SMC}$ mice (Fig. 1B). Quantification of both relative medial areas (Fig. 1C) and neointima/media ratios (Fig. 1D) confirmed that Mkl1 deletion in VSMCs attenuated neointima formation. Further, immunofluorescence staining with an anti-Ki67 antibody detected more proliferating VSMCs in the neointima in the *Mkl1*$^{f/f}$ mice than in the *Mkl1*$^{\Delta SMC}$ mice (Fig. 1E).

Next, C57/B6 mice were subjected to the carotid artery ligation procedure followed by daily administration of an MKL1 inhibitor CCG-1423 [40] to test the hypothesis that MKL1 inhibition might equally mitigate vascular injury (Fig. 1F). Indeed, H&E staining (Fig. 1G), quantification of relative medial areas (Fig. 1H), neointima/media ratios (Fig. 1I), and Ki67 immunofluorescence staining (Fig. 1J) all provided support for the conclusion that MKL1 activity might be essential for VSMC expansion and neointima formation during vascular injury.

3.2. MKL1 promotes VSMC proliferation in a ROS-dependent manner

Next, we sought to establish a relationship between MKL1 and VSMC proliferation by over-expressing a constitutively active (CA) MKL1 [41] in primary murine VSMCs. MKL1 over-expression significantly boosted VSMC proliferation as evidenced by Ki67 immunofluorescence staining (Fig. 2A) and MTT assay (Fig. 2B); treatment with N-acetyl cysteine (NAC), a ROS scavenger, suppressed cell proliferation induced by MKL1 over-expression. Next, primary VSMCs were isolated from *Mkl1*$^{f/f}$ mice and tranduced with adenovirus carrying a Cre vector to delete MKL1. MKL1 deletion in VSMCs attenuated cell proliferation induced by PDGF treatment but did not alter basal-level cell proliferation (Fig. 2C and D). Of interest, NAC treatment suppressed proliferation of wild type cells (Ad-GFP) but not MKL1-null cells (Ad-Cre), indicating that NAC might target MKL1 to regulate VSMC proliferation. Likewise, CCG-1423 treatment down-regulated VSMC proliferation in the presence of PDGF but rendered the cells insensitive to NAC treatment (Fig. 2E and F). Combined, these data suggest that MKL1 might regulate VSMC proliferation in a redox-sensitive manner.

3.3. MKL1 regulates FOXM1 transcription

In order to determine the downstream target of MKL1 that might mediate its redox-sensitive effect on VSMC proliferation, we performed a micro-array based screening of redox-related genes. Using 2Xfold change as a cutoff, 16 genes met this criterion with 11 being up-regulated and 5 being down-regulated in the MKL1 CA over-expressing VSMCs compared to the control VSMCs (Fig. 3A). Because MKL1 is generally considered as a transcriptional activator, we focused on the top 3 genes that were up-regulated by MKL1 CA over-expression: NCF1, SOD3, and FOXM1. Whereas NCF1 (p47^{phox}) and SOD3 have been well characterized for their roles in neointima formation [42,43], relatively little is known with regard to FOXM1. FOXM1 expression was up-regulated by PDGF treatment whereas Cre-mediated MKL1 deletion significantly attenuated FOXM1 expression at both mRNA (Fig. 3B) and protein levels (Fig. 3C). Consistent with a previous report by Park et al. [44], FOXM1 expression was also induced by H_2O_2 treatment (Fig. 3D and E); it was also observed that FOXM1 expression was more strongly augmented in the wild type VSMCs (Ad-GFP) than in the MKL1-null VSMC (Ad-Cre). Similarly, MKL1 inhibition by CCG-1423 greatly repressed PDGF-induced (Fig. 3F and G) and H_2O_2-induced (Fig. 3H and I) FOXM1 expression in VSMCs. Additionally, immunofluorescence staining confirmed that VSMC-specific MKL1 deletion (Fig. 3K) or CCG-1423 administration repressed FOXM1 expression in the vessels following carotid artery ligation in mice. Of note, FOXM1 expression was detected in endothelial cells and comparable between the *Mkl1*$^{f/f}$ mice and the *Mkl1*$^{\Delta SMC}$ mice but the pathophysiological relevance of this observation is not clear (Fig. S1).

Ki67 staining (Fig. 4A) and MTT assay (Fig. 4B) showed that FOXM1 depletion by siRNAs attenuated VSMC proliferation in the presence of a constitutively active MKL1, suggesting that FOXM1 can be placed downstream of MKL1 in regulating VSMC proliferation. Next, reporter assay was performed to determine the region of the FOXM1 promoter to which MKL1 might bind. A series of FOXM1 promoter-luciferase fusion constructs were transfected into HEK293 cells with or without MKL1. As shown in Fig. 4C, MKL1 over-expression could stimulate the reporter activity even when the deletion was extended as far as −110 relative to the transcription start site indicating that MKL1 might be associated with the −110/+75 region of the FOXM1 promoter. ChIP assay confirmed that when VSMCs were treated with PDGF, binding of MKL1 to the proximal region (−132/+25) of the FOXM1 promoter, where a conserved E2F motif is located, was appreciably strengthened (Fig. 4D). Mutation of the E2F motif rendered the FOXM1 promoter unresponsive

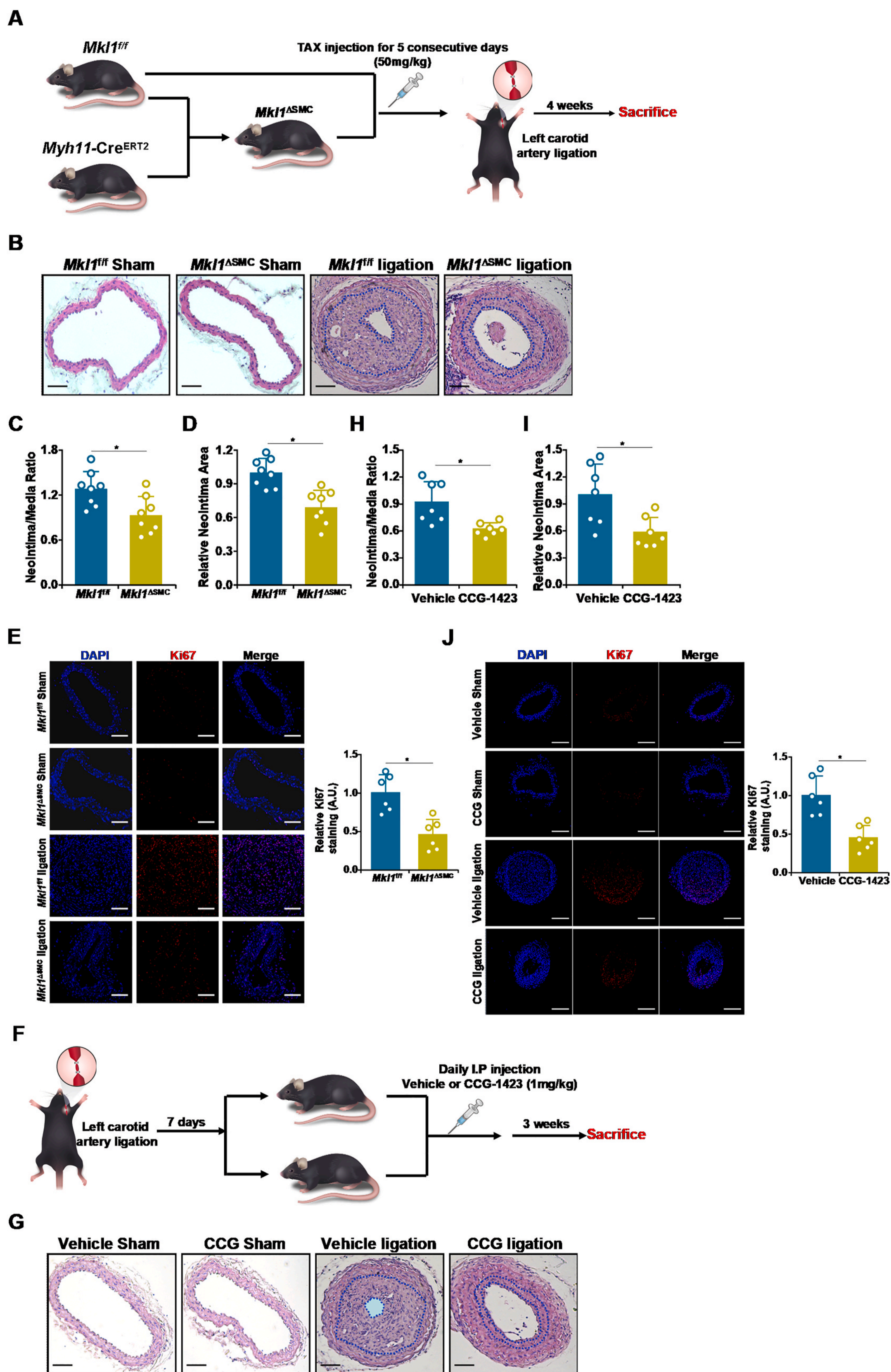

Fig. 1. ***MKL1 deletion or inhibition ameliorates neointima formation in mice.*** (**A-E**) *Mkl1*$^{f/f}$ mice and *Mkl1*$^{\Delta SMC}$ mice were subjected to carotid artery ligation or the sham procedure for 4wk (A). (B) Representative HE staining images of vessels. (C) Relative medial areas. (D) Neointima/media ratios. (E) Ki67 staining. (**F-J**) C57/B6 mice were subjected to carotid artery ligation or the sham procedure for 4wk; peritoneal injection of CCG-1423 (1 mg/kg) was administered daily starting day 7 post-surgery (F). (G) Representative HE staining images of vessels. (H) Relative medial areas. (I) Neointima/media ratios. (J) Ki67 staining.

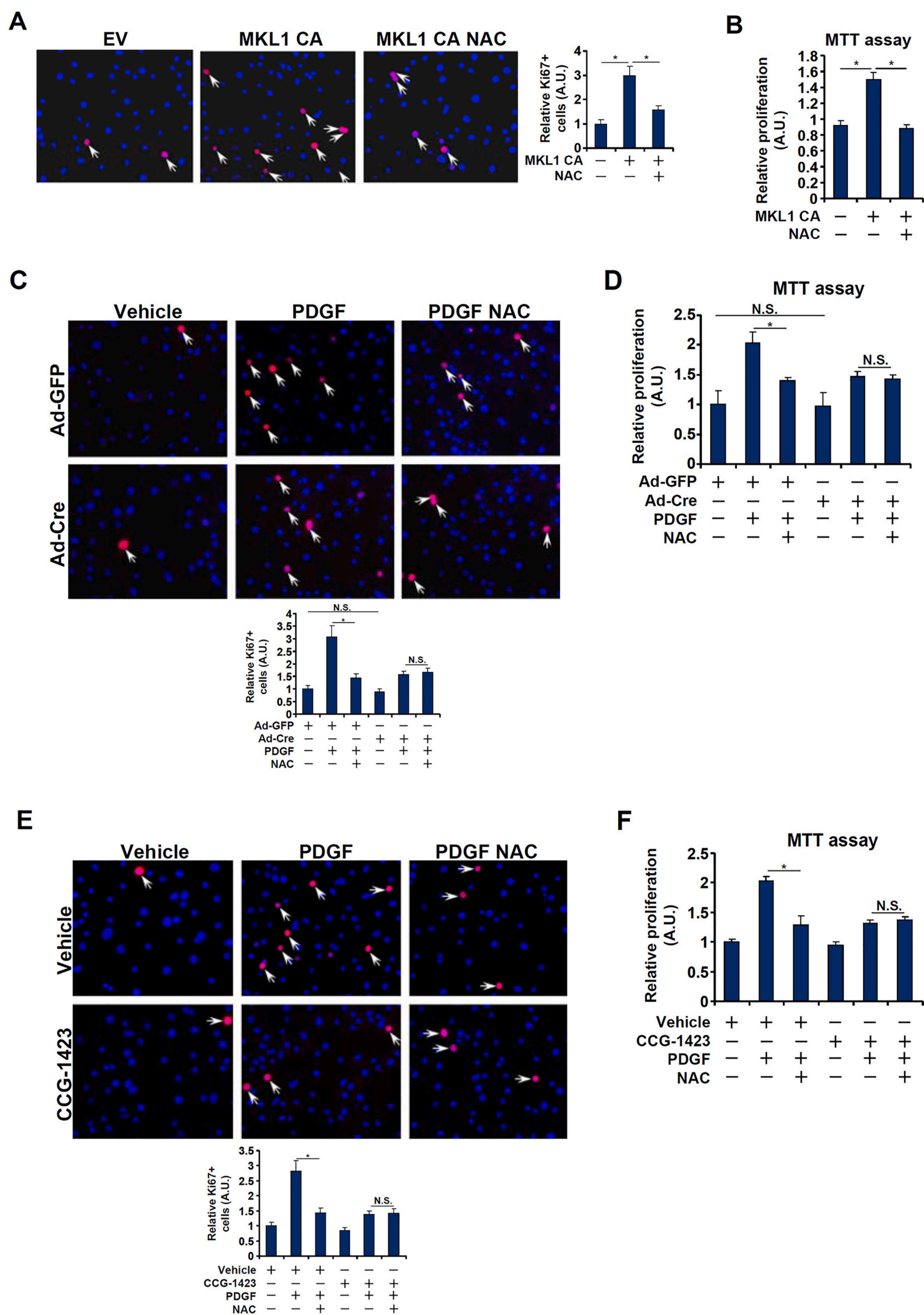

Fig. 2. ***MKL1 promotes VSMC proliferation in a ROS-dependent manner*.** (**A, B**) Primary VSMCs were infected with lentivirus carrying constitutively active MKL1 or an empty vector (EV) in the presence or absence of NAC (5 mM) for 24h. Cell proliferation was evaluated by MTT assay and immunofluorescence staining with an anti-Ki67 antibody. (**C, D**) Primary VSMCs were isolated from *Mkl1*$^{f/f}$ and transduced with Cre-carrying adenovirus (Ad-Cre) or the control adenovirus (Ad-Cre) followed by treatment with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) for 24h. Cell proliferation was evaluated by MTT assay and immunofluorescence staining with an anti-Ki67 antibody. (**E, F**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) or CCG-1423 (5 μM) for 24h. Cell proliferation was evaluated by MTT assay and immunofluorescence staining with an anti-Ki67 antibody.

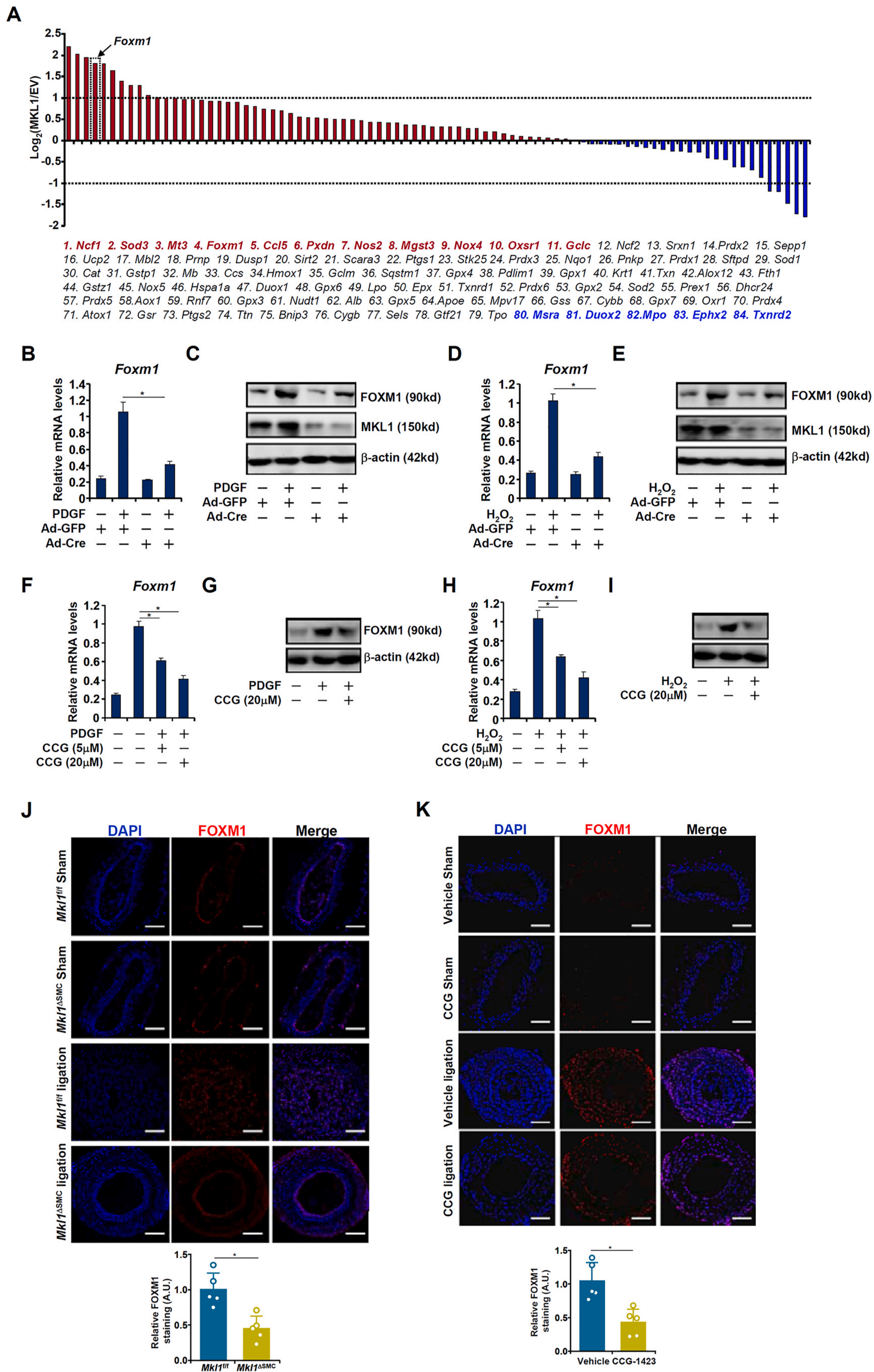

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Fig. 3. ***MKL1 regulates FOXM1 expression.*** (**A**) Primary VSMCs were infected with lentivirus carrying constitutively active MKL1 or an empty vector (EV) for 48h. PCR array was performed as described in Methods. (**B, C**) Primary VSMCs were isolated from *Mkl1*$^{f/f}$ and transduced with Cre-carrying adenovirus (Ad-Cre) or the control adenovirus (Ad-Cre) followed by treatment with PDGF for 24h. FOXM1 expression was examined by qPCR and Western. (**D, E**) Primary VSMCs were isolated from *Mkl1*$^{f/f}$ and transduced with Cre-carrying adenovirus (Ad-Cre) or the control adenovirus (Ad-Cre) followed by treatment with H_2O_2 for 24h. FOXM1 expression was examined by qPCR and Western. (**F, G**) Primary VSMCs were treated with PDGF in the presence or absence of CCG (5 μM or 20 μM) for 24h. FOXM1 expression was examined by qPCR and Western. (**H, I**) Primary VSMCs were treated with H_2O_2 in the presence or absence of CCG (5 μM or 20 μM) for 24h. FOXM1 expression was examined by qPCR and Western. (**J**) *Mkl1*$^{f/f}$ mice and *Mkl1*$^{\Delta SMC}$ mice were subjected to carotid artery ligation or the sham procedure for 4wk. FOXM1 expression in the vessels was examined by immunofluorescence staining. (**K**) *Mkl1*$^{f/f}$ mice and *Mkl1*$^{\Delta SMC}$ mice were subjected to carotid artery ligation or the sham procedure for 4wk. C57/B6 mice were subjected to carotid artery ligation or the sham procedure for 4wk; peritoneal injection of CCG-1423 (1 mg/kg) was administered daily starting day 7 post-surgery. FOXM1 expression in the vessels was examined by immunofluorescence staining.

to MKL1 over-expression (Fig. 4E), suggesting that MKL1 might be recruited by E2F(s) to the FOXM1 promoter.

In order to determine which E2F(s) might be responsible for FOXM1 *trans*-activation, siRNAs were employed to knock down individual E2F proteins (Fig. S2A). Depletion of E2F1, but not other E2F proteins, completely abrogated the induction of FOXM1 expression by either PDGF treatment or H_2O_2 treatment in VSMCs (Fig. S2B). Indeed, PDGF treatment promoted E2F1 binding to the FOXM1 promoter whereas E2F1 knockdown prevented MKL1 from binding to the FOXM1 promoter (Fig. 4F). Moreover, Re-ChIP assay detected an E2F1-MKL1 complex on the FOXM1 promoter in VSMCs upon PDGF stimulation (Fig. 4G). Together, these data demonstrate that MKL1 interacts with E2F1 to regulate FOXM1 transcription in VSMCs.

3.4. Activation of FOXM1 transcription by MKL1 is ROS-sensitive

Consistent with the micro-array data, over-expression of MKL1 CA markedly up-regulated FOXM1 expression in VSMCs; NAC treatment, however, blunted FOXM1 up-regulation suggesting that activation of FOXM1 by MKL1 is ROS-sensitive (Fig. 5A and B). ChIP assay confirmed that NAC treatment weakened the binding of MKL1 to the FOXM1 promoter (Fig. 5C). Of note, NAC treatment did not influence the binding of E2F1 on the FOXM1 promoter (Fig. 5D). These observations pointed to the possibility that interaction between MKL1 and E2F1 might be subjected to the regulation of redox status in VSMCs. Re-ChIP data supported this hypothesis by showing that NAC treatment disrupted the formation of an E2F1-MKL1 complex on the FOXM1 promoter (Fig. 5E). In addition, co-immunoprecipitation assay provided corroborating evidence that NAC treatment dampened the association of MKL1 with E2F1 (Fig. 5F).

3.5. ROS-induced MKL1 phosphorylation mediates its interaction with E2F1

In order to explore the mechanism whereby ROS regulates the interaction between MKL1 and E2F1, we first determine the region within MKL1 that mediates its association with E2F1. It has been well-documented the N-terminal region right behind the RPEL domain, including the basic (B) domain, the glutamine-rich (Q) domain, and the SAP domain, is responsible for mediating the interaction of MKL1 with several different sequence-specific transcription factors including SRF, SMAD, NF-κB and ERG-1. Indeed, deletion of this region (ΔQ/B/SAP) completely abrogated the interaction of MKL1 with E2F1 (Fig. 6A). Proteomic studies have identified at least 6 serine/threonine (S/T) residues within this region that potentially can be phosphorylated (Fig. 6B). To test the possibility that ROS-sensitive phosphorylation of MKL1 mediates its interaction with E2F1, the six S/T residues were simultaneously mutated to either alanine (6A) or aspartic acid (6D). Exposure of VSMCs to PDGF treatment prompted an increase in phosphorylation of WT MKL1, which was dampened by NAC treatment. The 6A MKL1 mutant, on the other hand, completely lost the responsiveness to ROS-induced phosphorylation (Fig. 6C). Congruently, the 6A mutant displayed much weaker whereas the 6D mutant displayed stronger affinity for E2F1 than WT MKL1 as evidenced by immunoprecipitation assay (Fig. 6D). Consequently, the 6A mutant activated the FOXM1 promoter less potently whereas the 6D mutant activated the FOXM1 promoter more potently than WT MKL1 in reporter assay (Fig. 6E). ChIP assay confirmed that there was diminished recruitment of the 6A mutant and enhanced recruitment of the 6D mutant to the FOXM1 promoter compared to WT MKL1 (Fig. 6F).

3.6. MK-2 mediated MKL1 phosphorylation contributes to ROS-induced FOXM1 transcription

Ronkina et al. have reported that MAPK activated kinase 2 (MK-2) can phosphorylate two serine residues within the B/Q/SAP domains of MKL1 [45]. Because MK-2 has a documented role in VSMC proliferation [46], we hypothesized that phosphorylation of MKL1 by MK-2 could potentially impact its interaction with E2F1 and consequently recruitment to the FOXM1 promoter. Exposure of VSMCs to PDGF treatment robustly activated MK-2, as evidenced by Thr^{222} phosphorylation, which was dampened by NAC treatment (Fig. 7A). In accordance, treatment of VSMCs with a specific MK-2 inhibitor (PF-3644022) blunted ROS-induced phosphorylation of MKL1 (Fig. 7B). More important, MK-2 inhibition blocked the interaction between MKL1 and E2F1 (Fig. 7C). ChIP assay showed that MKL1 recruitment, but not E2F1 recruitment, to the FOXM1 promoter was weakened by MK-2 inhibition (Fig. 7D). Indeed, the formation of an E2F1-MKL1 complex on the FOXM1 promoter was disrupted as a result of MK-2 inhibition (Fig. 7E). Consistent with these observations, PF-3644022 treatment attenuated FOXM1 induction by MKL1 CA over-expression (Fig. 7F and G) and suppressed proliferation of VSMCs (Fig. 7H and I). Collectively, these data suggest that MK-2 may contribute to VSMC proliferation by mediate ROS-induced MKL1 phosphorylation.

3.7. Correlation between FOXM1 and VSMC proliferation in human biopsy specimens

We finally made an attempt to correlate FOXM1 expression and VSMC proliferation in human biopsy samples taken from patients with restenosis. Immunohistochemial staining showed that in patients whose vessels displayed strong VSMC proliferation (PCNA staining) FOXM1 expression appeared to be high whereas in patients whose vessels display weak VSMC proliferation FOXM1 expression appeared to be relatively low (Fig. 8A). Linear regression confirmed a positive correlation between FOXM1 expression and VSMC proliferation (Fig. 8A).

4. Discussion

Dysregulated VSMC proliferation leads to expansion of the vascular medial layer and neointima formation, which constitutes the pathophysiological basis of restenosis and limits the success rate of interventional approaches to restore blood flow in the clinics [5]. Herein we describe a novel pathway whereby post-translational modification modulated MKL1 activates FOXM1 to promote VSMC proliferation and neointima formation (Fig. 8B). Our data show that VSMC-specific MKL1 deletion or pharmaceutical inhibition of MKL1 attenuated medial expansion in mice possibly by intercepting the migroproliferative transition of VSMCs. This observation echoes previous findings that link MKL1 to VSMC phenotypic modulation. Yang et al. have previously

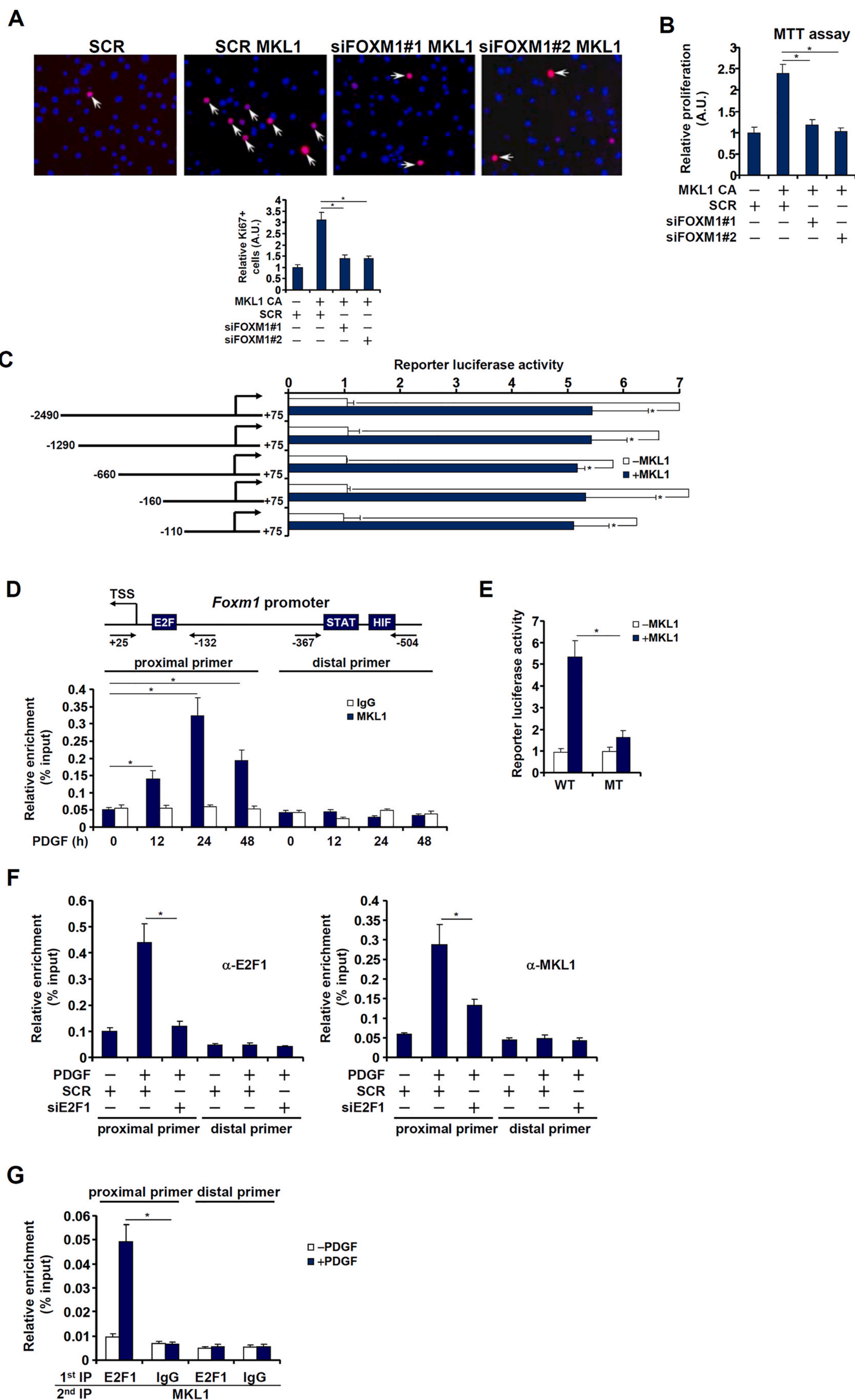

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Fig. 4. ***FOXM1 is a direct transcriptional target of MKL1.*** (**A, B**) Primary VSMCs were infected with lentivirus carrying constitutively active MKL1 or an empty vector (EV) in the presence or absence of indicated siRNAs for 24h. Cell proliferation was evaluated by MTT assay and immunofluorescence staining with an anti-Ki67 antibody. (**C**) FOXM1 promoter-luciferase constructs were transfected into HEK293 cells with or without MKL1. Luciferase activities were normalized by protein concentration and GFP fluorescence. (**D**) Primary VSMCs were treated with PDGF and harvested at indicated time points. ChIP assays were performed with anti-MKL1 or IgG. (**E**) Wild type and mutant FOXM1 promoter-luciferase constructs were transfected into HEK293 cells with or without MKL1. Luciferase activities were normalized by protein concentration and GFP fluorescence. (**F**) Primary VSMCs were transfected with siRNAs targeting E2F1 or scrambled siRNA (SCR) followed by treatment with PDGF for 24h. ChIP assays were performed with anti-MKL1 and anti-E2F1. (**G**) Primary VSMCs were treated with or without PDGF for 24h. Re-ChIP assay was performed with indicated antibodies.

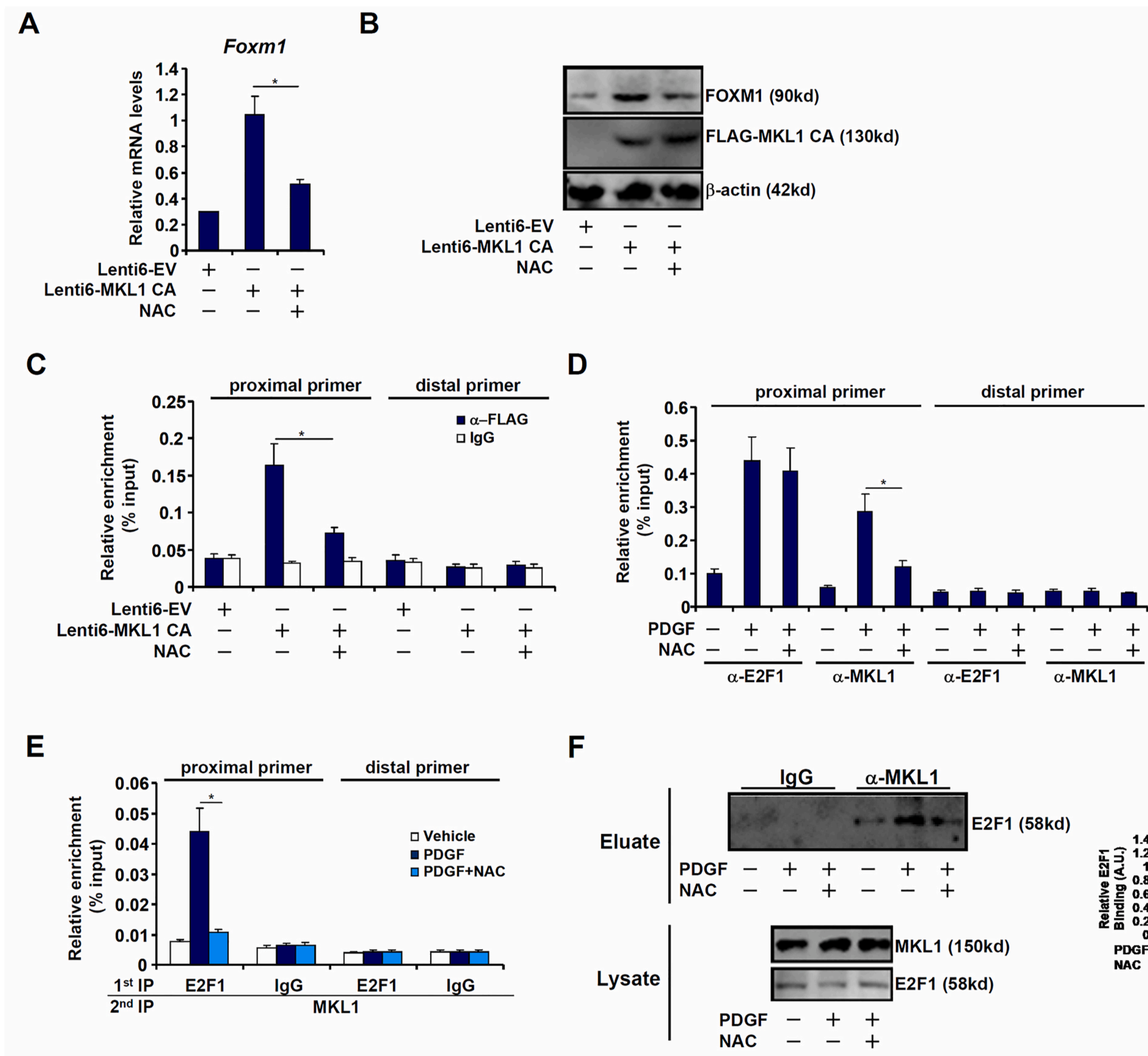

Fig. 5. ***Activation of FOXM1 by MKL1 is ROS-sensitive.*** (**A-C**) Primary VSMCs were infected with lentivirus carrying constitutively active MKL1 or an empty vector (EV) in the presence or absence of NAC (5 mM). FOXM1 expression was examined by qPCR and Western. ChIP assay was performed with anti-FLAG. (**D**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) for 24h. ChIP assay was performed with anti-E2F1 and anti-MKL1. (**E**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) for 24h. Re-ChIP assay was performed with indicated antibodies. (**F**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) for 24h. Immunoprecipitation was performed with anti-MKL1 or IgG.

demonstrated that MKL1, through interacting with and recruiting a multi-protein epigenetic complex, activates the transcription of a slew of cytokines and chemokines in human aortic smooth muscle cells to pivot VSMCs to a pro-inflammatory phenotype [47]. More recently, Long and colleagues have reported that global MKL1 deletion in mice attenuates the incidence and severity of aortic dissection, a vascular disorder acutely influenced by VSMC phenotypic modulation [48]. Accordingly to Gao et al., this observed phenotype could be attributed to, at least in part, reduced senescence and inflammation in VSMCs. In this regard, our findings provide heretofore the first *in vivo* evidence that modulation of VSMC phenotype by MKL1 might be cell-autonomous. It is noteworthy that our data do not foreclose the possibility that MKL1 might contribute to the pathogenesis of restenosis in a VSMC non-autonomous manner. For instance, Zhang et al. have shown that MKL1 in macrophages stimulates the synthesis and release of IL-6, which in turn acts on the vasculature to inflict injuries [49]. Notwithstanding the lineage-specific roles, MKL1 certainly appears to be an attractive interventional target for amelioration of vascular disorders.

Our data indicate that MKL1 regulates VSMC proliferation via redox-sensitive FOXM1 *trans*-activation reaffirming a previous finding by Park

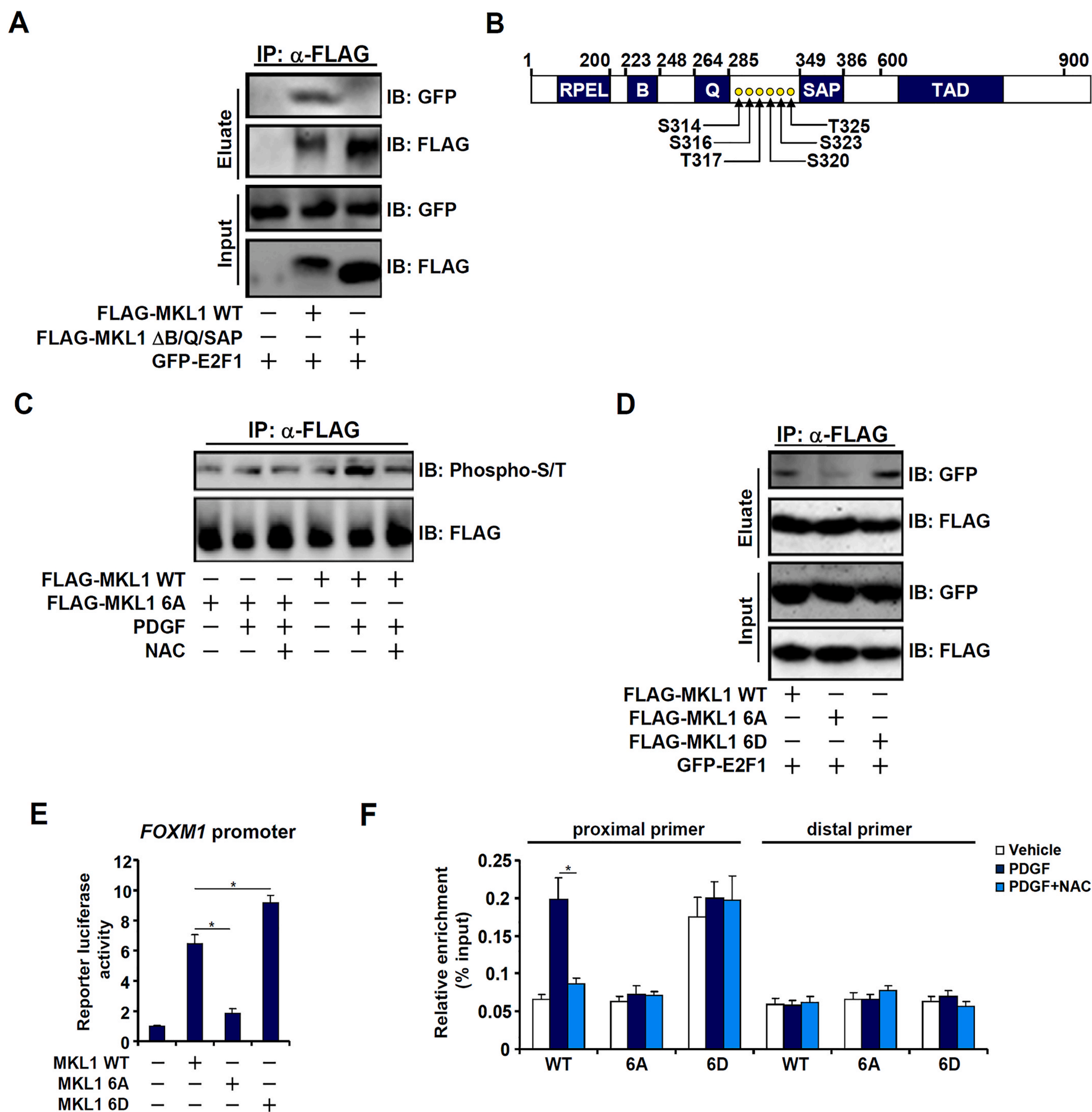

Fig. 6. ***ROS-induced MKL1 phosphorylation mediates its interaction with E2F1*****.** (**A**) HEK293 cells were transfected with FLAG-tagged MKL1 WT expression construct, FLAG-tagged MKL1 ΔB/Q/SAP expression, and/or GFP-tagged E2F1 expression construct as indicated. Immunoprecipitation was performed with anti-FLAG. (**B**) A schematic domain arrangement of MKL1 protein. The six identified S/T residues are highlighted. (**C**) VSMCs were infected with FLAG-tagged WT MKL1 or 6A MKL1 followed by treatment with PDGF and/or NAC. Immunoprecipitation was performed with anti-FLAG. MKL1 phosphorylation was detected by an anti-phospho-S/T antibody. (**D**) HEK293 cells were transfected with indicated expression constructs. Immunoprecipitation was performed with anti-FLAG. (**E**) A FOXM1 promoter-luciferase construct was transfected into HEK293 cells with indicated MKL1 expression constructs. Luciferase activities were normalized by protein concentration and GFP fluorescence. (**F**) VSMCs were infected with FLAG-tagged WT MKL1 or 6A MKL1 followed by treatment with PDGF and/or NAC. ChIP assay was performed with anti-FLAG.

et al. that oncogenic induction of FOXM1 in mouse embryonic fibroblasts (MEFs) is ROS-dependent [44]. This is also consistent with a previous report by Franco et al. in which increased FOXM1 expression was detected in the carotid arteries of rats subjected to balloon injury [50]. Additional corroborating evidence can also be drawn from a study wherein Dai et al. harnessed both VSMC-specific gain-of-function and loss-of-function FOXM1 transgenic mouse models to demonstrate a positive correlation between FOXM1 and VSMC proliferation in the context of pulmonary hypertension [51]. All these observations combined appear to suggest that FOXM1 may function as an essential regulator of cell proliferation by sensing and responding to cellular redox status. There are, however, several caveats regarding our model that deserve further attention. First, MKL1 target genes other FOXM1 as shown in Fig. 3A may play an equally important role mediating

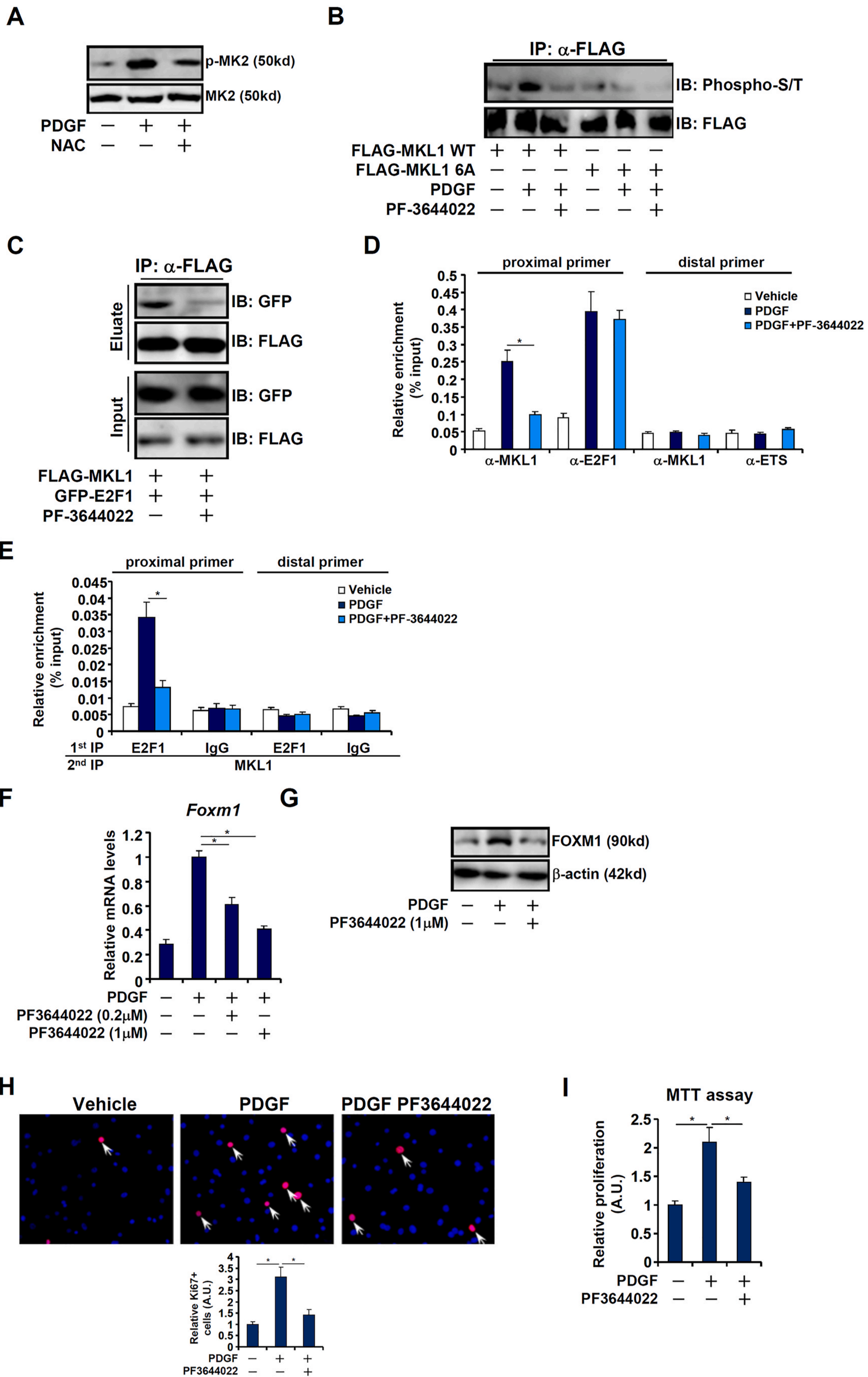

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Fig. 7. ***MK-2 mediated MKL1 phosphorylation contributes to ROS-induced FOXM1 transcription.*** (**A**) Primary VSMCs were isolated from wild type and MKL1 KO mice followed by treatment with PDGF (20 ng/ml) in the presence or absence of NAC (5 mM) for 30min. MK-2 phosphorylation was examined by Western. (**B**) VSMCs were infected with FLAG-tagged WT MKL1 or 6A MKL1 followed by treatment with PDGF and/or PF-3644022. Immunoprecipitation was performed with anti-FLAG. MKL1 phosphorylation was detected by an anti-phospho-S/T antibody. (**C**) HEK293 cells were transfected with indicated expression constructs followed by treatment with PF-3644022. Immunoprecipitation was performed with anti-FLAG. (**D**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of PF-3644022 for 24h. ChIP assay was performed with indicated antibodies. (**E**) Primary VSMCs were treated with PDGF (20 ng/ml) in the presence or absence of PF-3644022 for 24h. Re-ChIP assay was performed with indicated antibodies. (**F–I**) Primary VSMCs were infected with lentivirus carrying constitutively active MKL1 or an empty vector (EV) in the presence or absence of PF-3644022. FOXM1 expression was examined by qPCR and Western. Cell proliferation was evaluated by MTT assay and immunohistochemical staining with an anti-Ki67 antibody.

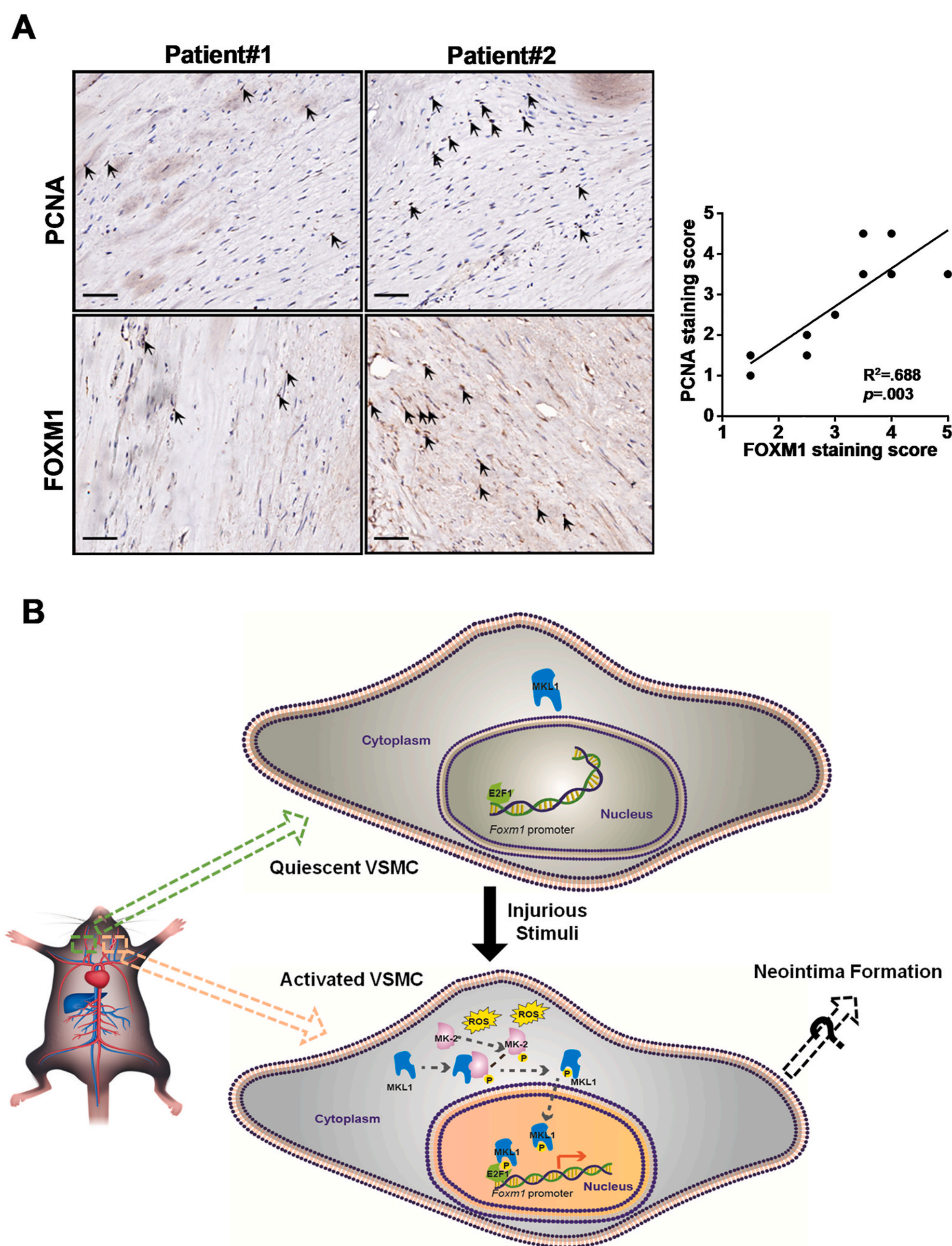

Fig. 8. ***Correlation between FOXM1 and VSMC proliferation in human restenosis biopsy specimens***. (**A**) Representative pictures showing IHC staining of vessel paraffin sections with an anti-FOXM1 antibody and an anti-PCNA antibody. Linear regression was performed with Graphpad. (**B**) A schematic model.

MKL1-dependent VSMC proliferation. For instance, Ncf1/p47^{phox} was the most robustly up-regulated target gene by MKL1 over-expression in the micro-array screening. Several independent investigations have strongly implicated Ncf1 as a key regulator of VSMC proliferation by promoting ROS production [52–54]. Of note, Ncf1 has previously been identified as direct transcriptional target for SRF [55]. Thus, it is plausible that MKL1 may drive ROS-induced VSMC proliferation by interacting with SRF to activate Ncf1 transcription. Second, Su et al. have recently shown that FOXM1 is induced in cancer cells through NOX4-dependent ROS production [56]. Because NOX4 is a known

transcriptional target for MKL1 [40] and appears to be a positive hit in the micro-array screening (Fig. 3A), we propose that MKL1 could contribute to FOXM1 up-regulation via an alternative, indirect mechanism in which MKL1 activates NOX4 transcription to elevate intracellular ROS levels and consequently stimulate FOXM1 expression.

One of most interesting findings in the present study is that MK2 licenses VSMC proliferation by modifying MKL1 to broker its interaction with E2F1 in a redox-sensitive manner. This observation adds to a wealth of data that support a pivotal role for MK2 in ROS-dependent regulation of VSMC phenotypes. The Griendling laboratory was among the first to suggest that MK2 activation by pro-proliferative stimuli in VSMCs is achieved through NOX2-mediated ROS production [57]. Further, Kapopara et al. have found that MK2 deletion in the *Ldlr*$^{-/-}$ background attenuates neointima formation with a concomitant down-regulation of pro-proliferative genes in the vessels [46] although the observed phenotype may not be solely attributed to VSMC-intrinsic MK2 since MK2 deletion was generated indiscriminately in all cell lineages. The most intriguing discovery regarding MK2-dependent regulation of vascular injury was recently reported by Tierney et al. in which a nanopolyplexed MK2 inhibitory peptide (MK2i-NP) displayed potent anti-restenosis effect in rabbits [58]. RNA-seq experiments performed in human VSMCs indicated that MK2 inhibition by MK2i-NP led to down-regulation of pro-inflammatory genes (e.g., VCAM1), pro-proliferative genes (e.g., MYC) and ECM molecules (e.g., COL1A1), many of which are well-documented MKL1 targets, further alluding to an intimate functional relationship between MK2 and MKL1. Despite all the corroborating evidence that points to an MK2-MKL1 axis, one needs to interpret our data with caution. First, MK2 substrates other than MKL1 could mediate its pro-proliferative effects to drive neointima formation. It has been shown that phosphorylation of SRF by MK2 at serine residue 103 significantly potentiates its transcriptional activity [59,60]. Because SRF is a known to interact with MKL1 to regulate VSMC phenotypic modulation it is plausible to speculate that an MK2-SRF axis may play an equally important role in neointima formation during vascular injury. Second, a pan-phospho-serine/threonine antibody was used in the present study such that ROS-sensitive but non-MK2 phosphorylation sites in MKL1 may have evaded detection. Indeed, MKL1 can be phosphorylated and consequently activated by both MAPK14 (p38α) and Rho kinase (ROCK) [49,61]. Since p38α [62] and ROCK [63] have been shown to play essential roles in VSMC proliferation and neointima formation, it is tempting to speculate that a phosphorylation barcode of MKL1 imprinted by multiple different kinases determines the stress-dependent MKL1 activity and, by extension, VSMC phenotype in disease settings. Further studies aided by proteomic tools will be needed to test this hypothesis.

In summary, our data unveil a previously unrecognized MK2-MKL1-FOXM1 axis that functions as a redox-sensitive switch to regulate VSMC proliferation and neointima formation. In light of the findings that both MK2 inhibitor [58] and FOXM1 inhibitor [50] show high efficacy in blocking neointima formation in model animals, our data provide renewed rationale that targeting this MK2-MKL1-FOXM1 axis is a feasible therapeutic approach in preventing restenosis.

Data availability statement

The data that support the findings of this study are available upon reasonable request.

Author contributions

Y Xu, YY Yang, and LL Zhuo conceived the project; all authors designed experiments; T Wu, N Li, QM Zhang, RQ Liu, HW Zhao, ZW Fan, and LL Zhuo performed experiments, collected data, and analyzed data; all authors wrote and edited the manuscript; Y Xu, YY Yang, N Li, and T Wu secured funding.

Declaration of competing interest

None.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (82121001, 82201718, 81800426, 81725001, and 81870326) and Nanjing Medical University (NMUB20210007).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102586.

References

[1] L.S. Shankman, D. Gomez, O.A. Cherepanova, M. Salmon, G.F. Alencar, R. M. Haskins, et al., KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis, Nat. Med. 21 (6) (2015) 628–637. Epub 2015/05/20.
[2] G. Ailawadi, C.W. Moehle, H. Pei, S.P. Walton, Z. Yang, I.L. Kron, et al., Smooth muscle phenotypic modulation is an early event in aortic aneurysms, J. Thorac. Cardiovasc. Surg. 138 (6) (2009) 1392–1399. Epub 2009/11/26.
[3] B. Lechartier, N. Berrebeh, A. Huertas, M. Humbert, C. Guignabert, L. Tu, Phenotypic diversity of vascular smooth muscle cells in pulmonary arterial hypertension: implications for therapy, Chest 161 (1) (2022) 219–231. Epub 2021/08/16.
[4] M.R. Alexander, G.K. Owens, Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease, Annu. Rev. Physiol. 74 (2012) 13–40. Epub 2011/10/25.
[5] S.O. Marx, H. Totary-Jain, A.R. Marks, Vascular smooth muscle cell proliferation in restenosis, Circul. Cardiovac. Interven. 4 (1) (2011) 104–111. Epub 2011/02/18.
[6] J.S. Forrester, M. Fishbein, R. Helfant, J. Fagin, A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies, J. Am. Coll. Cardiol. 17 (3) (1991) 758–769. Epub 1991/03/01.
[7] F.G. Welt, C. Rogers, Inflammation and restenosis in the stent era, Arterioscler. Thromb. Vasc. Biol. 22 (11) (2002) 1769–1776. Epub 2002/11/12.
[8] A. Levitzki, PDGF receptor kinase inhibitors for the treatment of restenosis, Cardiovasc. Res. 65 (3) (2005) 581–586. Epub 2005/01/25.
[9] J.C. Tsai, M.A. Perrella, M. Yoshizumi, C.M. Hsieh, E. Haber, R. Schlegel, et al., Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis, Proc. Natl. Acad. Sci. U. S. A. 91 (14) (1994) 6369–6373. Epub 1994/07/05.
[10] C. Li, Q. Xu, Mechanical stress-initiated signal transductions in vascular smooth muscle cells, Cell. Signal. 12 (7) (2000) 435–445. Epub 2000/09/16.
[11] Y. Zhou, M.J. Zhang, B.H. Li, L. Chen, Y. Pi, Y.W. Yin, et al., PPARgamma inhibits VSMC proliferation and migration via attenuating oxidative stress through upregulating UCP2, PLoS One 11 (5) (2016), e0154720. Epub 2016/05/06.
[12] R.P. Juni, H.J. Duckers, P.M. Vanhoutte, R. Virmani, A.L. Moens, Oxidative stress and pathological changes after coronary artery interventions, J. Am. Coll. Cardiol. 61 (14) (2013) 1471–1481. Epub 2013/03/19.
[13] A.S. Baas, B.C. Berk, Differential activation of mitogen-activated protein kinases by H2O2 and O2- in vascular smooth muscle cells, Circ. Res. 77 (1) (1995) 29–36. Epub 1995/07/01.
[14] T.C. Wu, Y.H. Chen, H.B. Leu, Y.L. Chen, F.Y. Lin, S.J. Lin, et al., Carvedilol, a pharmacological antioxidant, inhibits neointimal matrix metalloproteinase-2 and -9 in experimental atherosclerosis, Free Radic. Biol. Med. 43 (11) (2007) 1508–1522. Epub 2007/10/30.
[15] T. Kohno, N. Urao, T. Ashino, V. Sudhahar, R.D. McKinney, T. Hamakubo, et al., Novel role of copper transport protein antioxidant-1 in neointimal formation after vascular injury, Arterioscler. Thromb. Vasc. Biol. 33 (4) (2013) 805–813. Epub 2013/01/26.
[16] D.Z. Wang, S. Li, D. Hockemeyer, L. Sutherland, Z. Wang, G. Schratt, et al., Potentiation of serum response factor activity by a family of myocardin-related transcription factors, Proc. Natl. Acad. Sci. U. S. A 99 (23) (2002) 14855–14860. Epub 2002/10/25.
[17] J. Zhou, M. Zhang, H. Fang, O. El-Mounayri, J.M. Rodenberg, A.N. Imbalzano, et al., The SWI/SNF chromatin remodeling complex regulates myocardin-induced smooth muscle-specific gene expression, Arterioscler. Thromb. Vasc. Biol. 29 (6) (2009) 921–928. Epub 2009/04/04.
[18] Y. Sun, K. Boyd, W. Xu, J. Ma, C.W. Jackson, A. Fu, et al., Acute myeloid leukemia-associated Mkl1 (Mrtf-a) is a key regulator of mammary gland function, Mol. Cell Biol. 26 (15) (2006) 5809–5826. Epub 2006/07/19.
[19] D. Chen, Y. Yang, X. Cheng, F. Fang, G. Xu, Z. Yuan, et al., Megakaryocytic leukemia 1 directs a histone H3 lysine 4 methyltransferase complex to regulate hypoxic pulmonary hypertension, Hypertension 65 (4) (2015) 821–833. Epub 2015/02/04.
[20] X. Weng, L. Yu, P. Liang, D. Chen, X. Cheng, Y. Yang, et al., Endothelial MRTF-A mediates angiotensin II induced cardiac hypertrophy, J. Mol. Cell. Cardiol. 80 (1) (2015) 23–33. Epub 2014/12/03.

[21] L. Liu, Q. Zhao, M. Kong, L. Mao, Y. Yang, Y. Xu, Myocardin-related transcription factor A regulates integrin beta 2 transcription to promote macrophage infiltration and cardiac hypertrophy in mice, Cardiovasc. Res. 118 (3) (2022) 844–858. Epub 2021/03/23.
[22] A. Wirth, Z. Benyo, M. Lukasova, B. Leutgeb, N. Wettschureck, S. Gorbey, et al., G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension, Nat. Med. 14 (1) (2008) 64–68. Epub 2007/12/18.
[23] B. Zhou, S. Zeng, N. Li, L. Yu, G. Yang, Y. Yang, et al., Angiogenic factor with G patch and FHA domains 1 is a novel regulator of vascular injury, Arterioscler. Thromb. Vasc. Biol. 37 (4) (2017) 675–684. Epub 2017/02/06.
[24] L. Anders, N. Ke, P. Hydbring, Y.J. Choi, H.R. Widlund, J.M. Chick, et al., A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells, Cancer Cell 20 (5) (2011) 620–634. Epub 2011/11/19.
[25] J. Millour, N. de Olano, Y. Horimoto, L.J. Monteiro, J.K. Langer, R. Aligue, et al., ATM and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance, Mol. Cancer Therapeut. 10 (6) (2011) 1046–1058. Epub 2011/04/27.
[26] X. Kong, L. Li, Z. Li, X. Le, C. Huang, Z. Jia, et al., Dysregulated expression of FOXM1 isoforms drives progression of pancreatic cancer, Cancer Res. 73 (13) (2013) 3987–3996. Epub 2013/04/20.
[27] W. Dong, M. Kong, H. Liu, Y. Xue, Z. Li, Y. Wang, et al., Myocardin-related transcription factor A drives ROS-fueled expansion of hepatic stellate cells by regulating p38-MAPK signalling, Clin. Transl. Med. 12 (2) (2022) e688. Epub 2022/02/21.
[28] W. Zhang, W. Ji, X. Liu, G. Ouyang, W. Xiao, ELL inhibits E2F1 transcriptional activity by enhancing E2F1 deacetylation via recruitment of histone deacetylase 1, Mol. Cell Biol. 34 (4) (2014) 765–775. Epub 2013/12/18.
[29] X. Wu, W. Dong, M. Kong, H. Ren, J. Wang, L. Shang, et al., Down-regulation of CXXC5 de-represses MYCL1 to promote hepatic stellate cell activation, Front. Cell Dev. Biol. 9 (2021), 680344.
[30] Z. Zhang, B. Chen, Y. Zhu, T. Zhang, X. Zhang, Y. Yuan, et al., The Jumonji domain-containing histone demethylase homolog 1D/lysine demethylase 7A (JHDM1D/KDM7A) is an epigenetic activator of RHOJ transcription in breast cancer cells, Front. Cell Dev. Biol. 9 (2021), 664375.
[31] Y. Yang, G. Yang, L. Yu, L. Lin, L. Liu, M. Fang, et al., An interplay between MRTF-A and the histone acetyltransferase TIP60 mediates hypoxia-reoxygenation induced iNOS transcription in macrophages, Front. Cell Dev. Biol. 8 (2020) 484. Epub 2020/07/07.
[32] Y. Yang, Z. Li, J. Guo, Y. Xu, Deacetylation of MRTF-A by SIRT1 defies senescence induced down-regulation of collagen type I in fibroblast cells, Biochim. Biophys. Acta, Mol. Basis Dis. 1866 (5) (2020), 165723. Epub 2020/02/18.
[33] B. Chen, Z. Fan, L. Sun, J. Chen, Y. Feng, X. Fan, et al., Epigenetic activation of the small GTPase TCL contributes to colorectal cancer cell migration and invasion, Oncogenesis 9 (9) (2020) 86. Epub 2020/10/02.
[34] L. Liu, Q. Zhao, M. Kong, L. Mao, Y. Yang, Y. Xu, Myocardin-related transcription factor A (MRTF-A) regulates integrin beta 2 transcription to promote macrophage infiltration and cardiac hypertrophy in mice, Cardiovasc. Res. 118 (3): 844-858 (2022) Epub 2021/03/23.
[35] B. Chen, Y. Zhu, J. Chen, Y. Feng, Y. Xu, Activation of TCL transcription by lysine demethylase KDM4B in colorectal cancer cells, Front. Cell Dev. Biol. 9 (2021), 617549.
[36] Y. Zhang, H. Wang, M. Song, T. Xu, X. Chen, T. Li, et al., Brahma-related gene 1 deficiency in endothelial cells ameliorates vascular inflammatory responses in mice, Front. Cell Dev. Biol. 8 (2020), 578790. Epub 2020/12/18.
[37] Y. Yang, H. Wang, H. Zhao, X. Miao, Y. Guo, L. Zhuo, et al., A GSK3-SRF Axis mediates angiotensin II induced endothelin transcription in vascular endothelial cells, Front. Cell Dev. Biol. 9 (2021), 698254. Epub 2021/08/13.
[38] X. Wu, W. Dong, T. Zhang, H. Ren, J. Wang, L. Shang, et al., Epiregulin (EREG) and myocardin related transcription factor A (MRTF-A) form a feedforward loop to drive hepatic stellate cell activation, Front. Cell Dev. Biol. 8 (2020), 591246. Epub 2021/02/02.
[39] T. Wu, H. Wang, X. Xin, J. Yang, Y. Hou, M. Fang, et al., An MRTF-A-Sp1-PDE5 Axis mediates angiotensin-II-induced cardiomyocyte hypertrophy, Front. Cell Dev. Biol. 8 (2020) 839. Epub 2020/10/06.
[40] L. Yu, G. Yang, X. Zhang, P. Wang, X. Weng, Y. Yang, et al., Megakaryocytic leukemia 1 (MKL1) bridges epigenetic activation of NADPH oxidase in macrophages to cardiac ischemia-reperfusion injury, Circulation 138 (24) (2018) 2820–2836. Epub 2018/07/19.
[41] B. Cen, A. Selvaraj, R.C. Burgess, J.K. Hitzler, Z. Ma, S.W. Morris, et al., Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes, Mol. Cell Biol. 23 (18) (2003) 6597–6608. Epub 2003/08/29.
[42] J.N. Wang, N. Shi, S.Y. Chen, Manganese superoxide dismutase inhibits neointima formation through attenuation of migration and proliferation of vascular smooth muscle cells, Free Radic. Biol. Med. 52 (1) (2012) 173–181. Epub 2011/11/09.
[43] J.X. Chen, A. Stinnett, Critical role of the NADPH oxidase subunit p47phox on vascular TLR expression and neointimal lesion formation in high-fat diet-induced obesity, Lab. Invest. 88 (12) (2008) 1316–1328. Epub 2008/09/10.
[44] H.J. Park, J.R. Carr, Z. Wang, V. Nogueira, N. Hay, A.L. Tyner, et al., FoxM1, a critical regulator of oxidative stress during oncogenesis, EMBO J. 28 (19) (2009) 2908–2918. Epub 2009/08/22.
[45] N. Ronkina, J. Lafera, A. Kotlyarov, M. Gaestel, Stress-dependent phosphorylation of myocardin-related transcription factor A (MRTF-A) by the p38(MAPK)/MK2 axis, Sci. Rep. 6 (2016), 31219. Epub 2016/08/06.
[46] P.R. Kapopara, J. von Felden, O. Soehnlein, Y. Wang, L.C. Napp, K. Sonnenschein, et al., Deficiency of MAPK-activated protein kinase 2 (MK2) prevents adverse remodelling and promotes endothelial healing after arterial injury, Thromb. Haemostasis 112 (6) (2014) 1264–1276. Epub 2014/08/15.
[47] Y. Yang, X. Cheng, W. Tian, B. Zhou, X. Wu, H. Xu, et al., MRTF-A steers an epigenetic complex to activate endothelin-induced pro-inflammatory transcription in vascular smooth muscle cells, Nucleic Acids Res. 42 (16) (2014) 10460–10472. Epub 2014/08/28.
[48] P. Gao, J. Zhao, S. Shan, W. Luo, O.J. Slivano, W. Zhang, et al., MKL1 cooperates with p38MAPK to promote vascular senescence, inflammation, and abdominal aortic aneurysm, Redox Biol. 41 (2021), 101903. Epub 2021/03/06.
[49] M. Zhang, J. Gao, X. Zhao, M. Zhao, D. Ma, X. Zhang, et al., p38alpha in macrophages aggravates arterial endothelium injury by releasing IL-6 through phosphorylating megakaryocytic leukemia 1, Redox Biol. 38 (2021), 101775. Epub 2020/11/11.
[50] S. Franco, A. Stranz, F. Ljumani, G. Urabe, M. Chaudhary, D. Stewart, et al., Role of FOXM1 in vascular smooth muscle cell survival and neointima formation following vascular injury, Heliyon 6 (6) (2020), e04028. Epub 2020/06/25.
[51] J. Dai, Q. Zhou, H. Tang, T. Chen, J. Li, P. Raychaudhuri, et al., Smooth muscle cell-specific FoxM1 controls hypoxia-induced pulmonary hypertension, Cell. Signal. 51 (2018) 119–129. Epub 2018/08/10.
[52] J.A. Moraes, A.C. Frony, A.M. Dias, M. Renovato-Martins, G. Rodrigues, C. Marcinkiewicz, et al., Alpha1beta1 and integrin-linked kinase interact and modulate angiotensin II effects in vascular smooth muscle cells, Atherosclerosis 243 (2) (2015) 477–485. Epub 2015/11/02.
[53] I.H. Park, H.M. Hwang, B.H. Jeon, H.J. Kwon, K.L. Hoe, Y.M. Kim, et al., NADPH oxidase activation contributes to native low-density lipoprotein-induced proliferation of human aortic smooth muscle cells, Exp. Mol. Med. 47 (2015) e168. Epub 2015/06/13.
[54] T. Zou, W. Yang, Z. Hou, J. Yang, Homocysteine enhances cell proliferation in vascular smooth muscle cells: role of p38 MAPK and p47phox, Acta Biochim. Biophys. Sin. 42 (12) (2010) 908–915. Epub 2010/11/12.
[55] M. Kong, X. Chen, F. Lv, H. Ren, Z. Fan, H. Qin, et al., Serum response factor (SRF) promotes ROS generation and hepatic stellate cell activation by epigenetically stimulating NCF1/2 transcription, Redox Biol. 26 (2019), 101302. Epub 2019/08/24.
[56] X. Su, Y. Yang, Q. Yang, B. Pang, S. Sun, Y. Wang, et al., NOX4-derived ROS-induced overexpression of FOXM1 regulates aerobic glycolysis in glioblastoma, BMC Cancer 21 (1) (2021) 1181. Epub 2021/11/07.
[57] Y. Taniyama, M. Ushio-Fukai, H. Hitomi, P. Rocic, M.J. Kingsley, C. Pfahnl, et al., Role of p38 MAPK and MAPKAPK-2 in angiotensin II-induced Akt activation in vascular smooth muscle cells, Am. J. Physiol. Cell Physiol. 287 (2) (2004) C494–C499. Epub 2004/04/16.
[58] J.W. Tierney, B.C. Evans, J. Cheung-Flynn, B. Wang, J.M. Colazo, M.E. Polcz, et al., Therapeutic MK2 inhibition blocks pathological vascular smooth muscle cell phenotype switch, JCI insight 6 (19) (2021). Epub 2021/10/09.
[59] O. Heidenreich, A. Neininger, G. Schratt, R. Zinck, M.A. Cahill, K. Engel, et al., MAPKAP kinase 2 phosphorylates serum response factor in vitro and in vivo, J. Biol. Chem. 274 (20) (1999) 14434–14443. Epub 1999/05/13.
[60] N. Ronkina, M.B. Menon, J. Schwermann, J.S. Arthur, H. Legault, J.B. Telliez, et al., Stress induced gene expression: a direct role for MAPKAP kinases in transcriptional activation of immediate early genes, Nucleic Acids Res. 39 (7) (2011) 2503–2518. Epub 2010/11/27.
[61] M. Lee, A. San Martin, A. Valdivia, A. Martin-Garrido, K.K. Griendling, Redox-sensitive regulation of myocardin-related transcription factor (MRTF-A) phosphorylation via palladin in vascular smooth muscle cell differentiation marker gene expression, PLoS One 11 (4) (2016), e0153199. Epub 2016/04/19.
[62] W. Wu, W. Zhang, M. Choi, J. Zhao, P. Gao, M. Xue, et al., Vascular smooth muscle-MAPK14 is required for neointimal hyperplasia by suppressing VSMC differentiation and inducing proliferation and inflammation, Redox Biol. 22 (2019), 101137. Epub 2019/02/17.
[63] K. Noma, Y. Rikitake, N. Oyama, G. Yan, P. Alcaide, P.Y. Liu, et al., ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury, J. Clin. Invest. 118 (5) (2008) 1632–1644. Epub 2008/04/17.