Mitogen-Activated Protein Kinase 14 Is a Novel Negative Regulatory Switch for the Vascular Smooth Muscle Cell Contractile Gene Program

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Objective—Several studies have shown through chemical inhibitors that p38 mitogen-activated protein kinase (MAPK) promotes vascular smooth muscle cell (VSMC) differentiation. Here, we evaluate the effects of knocking down a dominant p38MAPK isoform on VSMC differentiation.

Methods and Results—Knockdown of p38MAPKα (MAPK14) in human coronary artery SMCs unexpectedly increases VSMC differentiation genes, such as miR145, ACTA2, CNN1, LMOD1, and TAGLN, with little change in the expression of serum response factor (SRF) and 2 SRF cofactors, myocardin (MYOCD) and myocardin-related transcription factor A (MKL1). A variety of chemical and biological inhibitors demonstrate a critical role for a RhoA-MKL1-SRF–dependent pathway in mediating these effects. MAPK14 knockdown promotes MKL1 nuclear localization and VSMC marker expression, an effect partially reversed with Y27632; in contrast, MAP2K6 (MKK6) blocks MKL1 nuclear import and VSMC marker expression. Immunostaining and Western blotting of injured mouse carotid arteries reveal elevated MAPK14 (both total and phosphorylated) and reduced VSMC marker expression.

Conclusion—Reduced MAPK14 expression evokes unanticipated increases in VSMC contractile genes, suggesting an unrecognized negative regulatory role for MAPK14 signaling in VSMC differentiation. (Arterioscler Thromb Vasc Biol. 2013;33:378-386.)

Key Words: differentiation ■ myocardin ■ p38 mitogen-activated protein kinase ■ smooth muscle
from depleted globular actin and translocate into the nucleus where they associate with SRF to stimulate CArG-dependent target gene expression.22,33 This dynamic nucleocytoplasmic shuttling mechanism underscores a connection between actin cytoskeletal dynamics and nuclear changes in gene expression.33 Central to this mechanism of MKL1/2 shuttling is the activation of the small GTPase, RhoA.22 In VSMCs, RhoA-dependent actin polymerization14 coupled to MKL1 nuclear localization35 facilitates VSMC contractile gene expression. Several extracellular stimuli have been demonstrated to stimulate RhoA-MKL1, leading to VSMC-directed gene expression including transforming growth factor-β1 (TGFβ1)/BMP436 and sphingosine-1-phosphate.37,38 However, a full understanding of all signaling cascades that converge upon RhoA-MKL1 to initiate VSMC-restricted gene expression is incomplete.

Numerous reports document TGFβ1 and downstream signaling pathways in the activation of VSMC-restricted gene expression.39-50 p38 mitogen-activated protein kinase (p38MAPK) is among the signaling pathways shown to be pivotal for TGFβ1-induced VSMC differentiation.44,46,49,51 p38MAPK comprises 4 different isoforms encoded in 4 distinct gene loci. Among these isoforms, p38MAPKα (MAPK14) and p38MAPKβ (MAPK11) have received most attention in terms of expression and activity, based on their high sensitivity to the selective inhibitors, SB203580 and SB202190.52 Although there has been general consensus as to the inhibitory action of these SB compounds on dominant-negative mutants of p38MAPK on TGFβ1-directed VSMC differentiation, whether altered expression of MAPK14 per se has any effect on this program is unknown. Here, we report the unanticipated finding of an enhanced VSMC differentiated phenotype with knockdown of MAPK14 and an opposing downregulation of such VSMC contractile markers upon constitutive activation of MAPK14 with its upstream kinase, MKK6. Mechanistic studies support a role for the RhoA/ROCK–MKL1–SRF axis in mediating VSMC gene transcription with reduced p38MAPK. These results suggest a heretofore unrecognized role for p38MAPK in the negative control of the VSMC differentiation program.

Materials and Methods

An expanded Methods section is available in the online-only Data Supplement.

Cell Culture

Human coronary artery SMCs (HCASMCs), primary mouse aortic SMCs, human pulmonary artery SMCs, rat aortic SMCs, 10T1/2 cells, human lung fibroblasts, and Hek293 cells were used as indicated throughout.

siRNA Transfections

Growing cells were seeded in 6-well plates and cultured until 70% confluent and then transfected with the expression plasmids, scramble small interfering RNA (siRNA) controls, or siRNAs to MAPK14, SRF, MYOCD, or MKL1.

Immunofluorescence Microscopy

Both conventional immunofluorescence and confocal microscopy were performed to analyze expression of VSMC markers, actin cytoskeleton, and MKL1 nucleocytoplasmic shuttling.

Adenoviral-Mediated Gene Transfer

HCASMCs were transduced with the indicated adenoviral constructs at varying multiplicity of infection. Negative control cells were transduced with the same adenoviral backbone carrying the bacterial LacZ gene.

Luciferase Assay

10T1/2 cells were seeded in 24-well plates and grown to subconfluence, whereupon transfections were carried out with various expression plasmids and reporter genes, and luciferase assay was performed in standard fashion.

Mouse Carotid Artery Ligation

Twelve-week-old C57BL/6J mice were subjected to complete or partial common carotid artery ligation as described.53-54

Immunohistochemistry

Sections of carotid artery were prepared and quenched with 3% H2O2 and then subjected to antigen retrieval before applying primary antibody.

Results

Knockdown of Endogenous MAPK14 Induces the VSMC Contractile Phenotype

We recently reported that a commonly used p38MAPK inhibitor, SB203580, completely blocked TGFβ1-induced miR143/145 and other VSMC contractile genes.51 Each of the 4 p38MAPK isoforms is detectable in cultured HCASMCs as demonstrated by conventional and quantitative reverse transcriptase-polymerase chain reaction (Figure I in the online-only Data Supplement). Because the α isoform (MAPK14) is of higher expression than the other SB203580-sensitive isoform (MAPK11) and more is known about MAPK14 in VSMCs, we sought to design siRNA to MAPK14 to confirm whether its expression, per se, is important for TGFβ1-induced VSMC differentiation. Upon MAPK14 knockdown, miR145 levels increase after TGFβ1 stimulation (Figure I in the online-only Data Supplement). These results prompted us to examine whether VSMC contractile genes are similarly elevated upon knockdown of MAPK14. Indeed, CArG-dependent CNN1, ACTA2, LMOD1,55 and TAGLN increase with MAPK14 depletion in HCASMCs (Figure 1A). Such VSMC gene induction is specific because levels of KL5 and PDCD4 mRNA show little or no change (Figure 1A). Furthermore, other more widely expressed CArG-containing genes (e.g., SRF, VCL, and EGR1) exhibit minor alterations in expression with MAPK14 knockdown (Figure 1A and data not shown). We also note that reduced MAPK14 increases both baseline and TGFβ1-induced ACTA2 and CNN1 protein expression (Figure 1B). Comparable increases are seen with 3 independent siRNAs to MAPK14 (Figure II in the online-only Data Supplement). Primary mouse aortic SMCs treated with siRNA to Mapk14 also display strong induction of VSMC contractile markers at both the mRNA and protein levels.
An important characteristic of differentiated VSMCs is the increases filamentous actin in VSMCs upon knockdown of MAPK14. This novel and widely evident activation of the VSMC contractile program upon knockdown of MAPK14 strongly supports evidence to promote the formation of F-actin assembly in human VSMC.

### Knockdown of Endogenous MAPK14 Increases Filamentous Actin in VSMCs

An important characteristic of differentiated VSMCs is the existence of an array of myofilaments reflecting the polymerization of globular actin into filamentous actin (F-actin). We therefore analyzed the intracellular composition of F-actin by staining HCASMCs with phalloidin in the absence or presence of siRNA to MAPK14. Results show an increase in F-actin upon MAPK14 knockdown (Figure IV in the online-only Data Supplement). Together, these results provide strong evidence to support a novel and widely evident activation of the VSMC contractile program upon knockdown of MAPK14.

### Heterologous MAPK14 Rescues Synergistic Activation of ACTA2 With Combined MKL1 and MAPK14 Knockdown

MKL1 can potently stimulate SRF-dependent VSMC contractile gene expression. We asked whether MAPK14 knockdown can synergize with MKL1 to potentiate ACTA2 expression in Hek293 cells. Indeed, we observe clear synergy with combined MKL1 and MAPK14 knockdown and ectopic MKL1 expression (Figure IID and IIE in the online-only Data Supplement). Importantly, this synergy is lost when we introduce an expression plasmid carrying mouse MAPK14 that is not targeted for knockdown with the siRNA to human MAPK14 (Figure IIF in the online-only Data Supplement). Collectively, these data indicate that MAPK14 knockdown is not a result of an off-target effect and that MKL1 may be an important mediator for siMAPK14-induced VSMC gene expression (see below).

### siMAPK14-Mediated VSMC Gene Induction Is RhoA/SRF-Dependent

RhoA activity plays a critical role in mediating VSMC gene expression through F-actin assembly, MKL1 nuclear translocation, and interaction with SRF. To test whether RhoA signaling underlies the increase in VSMC contractile proteins with MAPK14 knockdown, we used the Rho-associated protein kinase (ROCK) inhibitor, Y27632. Results show that Y27632 attenuates the induction of CNN1 and TAGLN after MAPK14 knockdown (Figure 2A); expression of a dominant-negative RhoA also abolishes the increase in VSMC marker expression with siMAPK14 (Figure 2B). The RhoA inhibitor, CCG-1423, blocks siMAPK14-induced VSMC contractile proteins in both HCASMCs (Figure 2C) and pulmonary artery SMCs (Figure V in the online-only Data Supplement). Furthermore, we observe a consistent increase in phosphorylated cofilin (CFL1), a downstream target of activated RhoA, with MAPK14 knockdown (Figure 2D). Latrunculin B, a compound that blocks MKL1 nuclear shuttling through depolymerization...
siMAPK14 expression of MYOCD mRNA (Figure 3A) and protein (data not shown) with apparent low-level expression of MKL1 mRNA (Figure 3A). siMAPK14 knockdown, suggesting that activation of the downstream VSMC contractile program is independent of the expression level of this cofactor (Figure 1C and 1E). Because MKL1, rather than MYOCD, is the principal cofactor for MKL1-mediated vascular smooth muscle cell (VSMC) contractile protein expression, following mitogen-activated protein kinase (MAPK) 14 knockdown.

siMAPK14-Mediated VSMC Gene Induction Requires MKL1, But Not MYOCD

We next sought to ascertain the relative contribution of MYOCD versus MKL1 in the activation of the VSMC contractile program with MAPK14 knockdown. We first examined the effects of siMAPK14 in human lung fibroblast cells because these cells express abundant MKL1 mRNA (Figure 3A) and protein (data not shown) with apparent low-level expression of MYOCD mRNA (Figure 3A). siMAPK14 treatment of human lung fibroblasts induces protein expression of CNN1 and ACTA2 (Figure 3B). Although knockdown of MYOCD in HACSMCs results in an expected decrease in VSMC contractile markers, induction of such markers with MAPK14 knockdown persists even with reduced MYOCD expression (Figure 3C and D). In contrast, MKL1 knockdown abrogates siMAPK14-induced VSMC contractile proteins in HACSMCs (Figure 3E). Similar results are found in human lung fibroblast cells (data not shown). These findings suggest that MKL1, rather than MYOCD, is the principal cofactor for siMAPK14-induced VSMC contractile marker expression.

Reduced MAPK14 Promotes Nuclear Translocation of MKL1 in a ROCK-Dependent Manner

MKL1 mRNA and protein are not induced with MAPK14 knockdown, suggesting that activation of the downstream VSMC contractile program is independent of the expression level of this cofactor (Figure 1C and 1E). Because MKL1...
exhibits nucleocytoplasmic shuttling, we asked whether reduced MAPK14 has any influence on the cellular distribution of MKL1. We initially treated HCASMCs with siRNA to MAPK14 and then transduced cells with an adenovirus carrying HA-MKL1 to examine its cellular distribution profile (repeated attempts to interrogate endogenous MKL1 shuttling were not successful). Most ectopic MKL1 staining is confined to the cytosol of serum-starved HCASMCs with normal levels of MAPK14. However, MKL1 redistributes to the nuclear compartment upon siMAPK14 knockdown (Figure 4A). Quantitative analysis reveals that >80% of control cells display MKL1 in the cytosol, with a nearly equivalent percentage of cells residing in the nucleus upon MAPK14 knockdown (Figure 4B). Importantly, the effects of MAPK14 knockdown could be partially reversed upon cotreatment with the ROCK inhibitor, Y27632 (Figure 4A and 4B). Collectively, these observations suggest that depletion of endogenous MAPK14 enables MKL1 to translocate into the nucleus in a Rho-dependent manner, thereby effecting SRF-dependent VSMC contractile gene expression.

**Sustained Activation of MAPK14 Reduces VSMC Markers and MKL1 Nuclear Localization**

Previous work has shown that a transient increase in MAPK14 activity mediates TGFβ1-induced VSMC contractile phenotype, an effect blocked by SB203580. MKK6 is an upstream, dual-specificity kinase that selectively phosphorylates MAPK14. Overexpressing constitutively active MKK6 in HCASMCs results in a dose-dependent increase in phospho-MAPK14 (Figure 5A). Importantly, similarly treated cells show a concurrent decrease in the expression of VSMC markers (Figure 5A; Figure VIA in the online-only Data Supplement). The MKK6-mediated decrease in VSMC markers seems to be specific for phosphor-MAPK14 because simultaneous knockdown of MAPK14 abolishes

**Figure 4.** Reduced mitogen-activated protein kinase (MAPK) 14 promotes MKL1 nuclear translocation in human coronary artery smooth muscle cells (HCASMCs). Growing HCASMCs were transfected with siControl or siMAPK14 for 7 h and then refed fresh growth medium overnight. Cells were then transduced with adenovirus carrying HA-MKL1 for an additional 24 h, whereupon the medium was switched to basal (no serum) medium for an additional 24 h. Cells were then visualized by confocal immunofluorescence microscopy (A). Quantitative analysis of MKL1 cellular localization from 100 consecutive cells categorized as having MKL1 in the nuclear (N), cytosolic (C), or both nuclear and cytosolic (N+C) compartments (B). Data are representative of 3 independent experiments performed by >1 person.

**Figure 5.** Constitutively active MKK6 suppresses vascular smooth muscle cell (VSMC) differentiation. Human coronary artery SMCs (HCASMCs) were transduced with varying multiplicity of infection of adenovirus carrying Lacz or constitutively active MKK6 for 48 h before Western blotting (A). HCASMCs were treated with siControl or siMAPK14 for 24 h and then transduced with adenovirus-MKK6 for 48 h before Western blotting (B). 10T1/2 cells were transfected with Tagln promoter±MKK6 in the presence of MAPK14 or dominant-negative mitogen activated protein kinase (MAPK) 14 (dnMAPK14) for 24 h before luciferase assay. Luciferase activity was normalized to Tagln+MAPK14-treated group (set to 1). Representative results from 3 independent experiments are shown (C). HCASMCs were transduced with adenovirus carrying HA-MKL1±MKK6 for 24 h and then refed fresh growth medium for an additional 24 h to promote nuclear importation of MKL1. Cells were then visualized by confocal immunofluorescence microscopy, and quantitative analysis of MKL1 cellular localization was carried out (D, E).
such repression (Figure 5B). MKK6 abrogates basal Tagln promoter activity in the presence of ectopic MAPK14, but such inhibition is rescued upon coexpression of a dominant-negative (kinase-deficient) MAPK14 (Figure 5C). In contrast to the elevation in MKL1 nuclear localization with MAPK14 knockdown (Figure 4), ectopic MKK6 expression completely suppresses serum-induced MKL1 nuclear localization (Figure 5D and 5E). Furthermore, both SB203580 (Figure VIB in the online-only Data Supplement) and a kinase-deficient MAPK14 (Figure VIC in the online-only Data Supplement) partially block the suppressive effect of MKK6 on VSMC contractile marker expression, suggesting that the inhibitory effect of MKK6 is through activation of MAPK14.

**Reciprocal Expression of MYH11 and MAPK14 in Neointima After Arterial Injury**

To determine relative expression of MAPK14 versus VSMC markers in vivo, we turned to a well-defined model of neointimal formation. In the uninjured vessel wall, there is abundant expression of MYH11, the gold standard marker for VSMC lineages. Upon ligation injury, levels of MYH11 are lower in the neointima versus underlying medial tissue. However, MAPK14 expression is notably elevated in the neointima versus media (Figure 6A). Similar changes are observed on Western blot analysis (Figure 6B). Importantly, most neointimal cells exhibit clear phosphorylated MAPK14 expression (Figure 6A, panel e), a finding consistent with Western blotting studies (Figure 6B).

**Discussion**

We previously reported that SB203580, a widely used inhibitor of p38MAPK, blocks TGFβ1-induced miR143/145 and other VSMC contractile genes, which is consistent with the results reported by other groups. These studies suggest that under conditions of TGFβ1 stimulation, p38MAPK signaling is important for VSMC differentiation.

**Figure 6.** Increased mitogen-activated protein kinase (MAPK) 14 in neointima after arterial injury. Immunohistochemistry (A) for MYH11 (panels a, c), MAPK14 (b, d), and phospho-MAPK14 (pMAPK14; panel e) in the unligated (a, b) and complete ligated (c, d, e) mouse carotid artery. Magnifications are ×600 for each panel. Western blotting (B) of the indicated proteins in the absence (−) or presence (+) of carotid artery partial ligation. All data are representative of at least 3 independent experiments.

**Figure 7.** Hypothetical model for the dual regulation of vascular smooth muscle cell (VSMC) differentiation through mitogen-activated protein kinase (MAPK) 14. Stimulation of VSMC through, for example, growth or inflammatory proteins induced after vascular injury can lead to chronic MAPK14 activation (schematized as larger font of pMAPK14) and subsequent suppression of the RHOA–MKL1–serum response factor (SRF) axis, thus attenuating VSMC contractile gene expression. In contrast, certain agonists, such as transforming growth factor-β1 (TGFβ1), may stimulate transient SB203580-sensitive activation of MAPK14 and promote VSMC differentiation via serum response factor (SRF)/myocardin (MYOCD). TNFα indicates tumor necrosis factor-α; and PDGF, platelet-derived growth factor.
through its stimulation of SRF/myocardin expression \(^\text{51,59-61}\) (Figure 7). The current study predicted that knockdown of p38MAPK would replicate results obtained with SB203580. However, we found the opposite result; knockdown of the dominant p38MAPK isoform (MAPK14) evokes a consistent elevation in VSMC contractile markers. Mechanistic studies support the involvement of a RhoA–MKL1–SRF axis in the activation of VSMC contractile genes upon knockdown of MAPK14. Consistent with these findings, we observe that overexpression of a constitutively active upstream kinase for p38MAPK, MKK6, inhibits VSMC differentiation, and this inhibitory effect is reversed upon treatment with either SB203580 or a kinase-deficient MAPK14. Importantly, injury to the vessel wall is associated with p38MAPK expression and activity in the evolving neointima concomitant with reduction in VSMC differentiation. Thus, we surmise that p38MAPK has opposing effects on VSMC differentiation depending on the nature of the stimulus: some factors (TGF\(\beta\)) cued upon addition of exogenous mouse MAPK14. Third, we tested whether reducing p38MAPK expression phenocopies the inhibition of VSMC differentiation via SB203580. Here, we have clearly demonstrated the unexpected augmentation of VSMC differentiation marker expression upon knockdown of MAPK14 and further demonstrate that constitutively active MKK6 overexpression decreases VSMC genes. A previous study revealed that siRNA knockdown of p38MAPK blocked platelet-derived growth factor–induced VSMC proliferation, although there was no investigation into VSMC marker expression\(^7\); however, MKK6 has been demonstrated to suppress VSMC markers.\(^6\) Importantly, we show that the MKK6-mediated suppression of VSMC markers can be partially rescued with either a dominant-negative MAPK14 or SB203580, suggesting that the inhibition of VSMC markers is, at least in part, dependent on the kinase activity of MAPK14. It will be of interest to define the downstream substrates of MAPK14 that are mediating the suppressive effects on VSMC phenotype. Computational prediction of Ser-Thr phosphorylation sites using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) reveals RhoA, G-actin, SRF, and MKL1 as potential substrates for MAPK14. Studies are underway to define substrates of MAPK14 that explain the negative regulatory role of this signaling protein in VSMC contractile gene expression, as well as the in vivo function of MAPK14 through VSMC-specific Mapk14 knockout mice. There is increasing appreciation for an active role of the actin cytoskeleton in mediating transcriptional programs of gene expression.\(^23\) Here, we provide several findings that support a role for actin dynamics involving RhoA–SRF–MKL1 in the control of VSMC differentiation gene expression through MAPK14 knockdown. First, Y27632 (a ROCK inhibitor) and a dominant-negative RhoA decrease siMAPK14-induced VSMC contractile gene expression. Second, CCG-1423, a small-molecule inhibitor of RhoA transcriptional signaling,\(^57\) and latrunculin B, an inhibitor of actin polymerization, attenuate the effect of siMAPK14 treatment on VSMC marker expression. Third, knockdown of SRF abolishes the activation of VSMC differentiation markers upon siMAPK14 knockdown. Finally, siMAPK14 knockdown enhances F-actin assembly and MKL1.
nuclear localization, an effect partially reversed with Y27632 treatment, and activation of VSMC markers is attenuated upon concurrent knockdown of MKL1. It will be informative to investigate how MAPK14 affects the RhoA–MKL1–SRF axis to attenuate VSMC marker expression in, for example, a disease setting, such as acute vascular occlusion.

In summary, we found a heretofore unrecognized role for p38MAPK (MAPK14) in the negative regulation of VSMC differentiation. The fact that higher levels of MAPK14 and phospho-MAPK14 are observed in the neointima where less VSMC contractile protein expression exists suggests that p38MAPK may be an important therapeutic target for combating vascular diseases.

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Disclosures

None.

References


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Supplemental Material

Expanded Methods Section

**Cell Culture** - Human coronary artery SMC (HCASMC) isolates were purchased from Invitrogen and maintained in medium 231 with growth supplements as described previously 1. Mouse primary aortic SMC were isolated enzymatically and maintained in DMEM containing 10%FBS, 100 U/ml penicillin and streptomycin. Human lung fibroblasts (HLF) were a generous gift from Dr. Nickolai Dulin (University of Chicago) and were cultured as described 2. Human primary pulmonary artery smooth muscle cells (PASMCs) were generously provided by Dr. Akiko Hata (University of California, San Francisco) and maintained in Sm-GM2 medium (Lonza). Hek293 cells and rat aortic SMC (RASMC) were purchased from ATCC and maintained in DMEM containing 10% FBS. All primary cell cultures were within 7 passages. For TGF-β1 treatment, HCASMC were plated in 6-well dishes and grown until 70% confluent. Following overnight serum starvation, cells were treated with TGF-β1 (2 ng/ml) for 24 h. For the inhibitor studies, HCASMC were pretreated with each indicated inhibitor for 30 min followed by TGF-β1 stimulation as above. The inhibitors used in this study include: SB203580 (Calbiochem), Y27632 (Calbiochem), CCG1423 (Cayman), and LatrunculinB (Enzo Life Sciences).

**RNA extraction and RT-PCR** – Total RNA was extracted using the miRNeasy kit according to the manufacture’s instruction (Qiagen). Total RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Both semi-quantitative PCR and SYBR green-based quantitative PCR (MyIQ, Bio-Rad) were used to measure steady-state mRNA levels. The housekeeping gene GAPD was utilized as a loading control. Taqman quantitative PCR (Applied Biosystems) was used to measure microRNA levels and SnoRNA48 was used as a loading control. All qPCR raw data were normalized to the loading control using 2^{−ΔΔCt} method as described 1. The primers used for different target mRNA and microRNAs are listed in the Supplemental Table.

**Transfection and adenoviral transduction experiments** – Cells were transfected with one of three different siRNA molecules to MAPK14 (ABI s3583 or s3585 or Invitrogen, 21430970) or MAPK11 (ABI s11155) in absence or presence of Myc-MKL1 or mouse flag-MAPK14 for 7 h using Lipofectamine 2000. Cells were then refed growth medium for 24 h after which total protein or RNA was collected (48 h post-transfection). Cells were then refed fresh growth medium and cultured an additional 24 h post-transfection. For double knock-down experiments, cells were transfected initially with either siRNA to SRF (ABI, s13427), MYOCD (Dharmacon, NM_153604) or MKL1 (Invitrogen, s33393) followed 24 h later with siMAPK14. 24 h following
siRNA delivery, chemical inhibitor, adenovirus, or expression plasmid was introduced into the cells as indicated in the figure legends. RNA or protein was extracted 48–72 h after initial transfection and qPCR or Western blotting was done for further analysis. For luciferase assays, Lipofectamine 2000 was used to transflect indicated plasmids into cells with a Renilla reporter gene to normalize transfection efficiency. Cell lysates were prepared 24 h after transfection and subject to the luciferase assay as described by the manufacturer (Promega). Transfections were performed in quadruplicate and repeated in at least three independent experiments. Data were analyzed with GraphPad Prism Software (version 4.0, GraphPad Software Inc.) and expressed as the normalized -fold increase over controls ± S.D. Indicated cells were transduced with adenovirus carrying either HA-MKL1 (kindly provided by Dr. Paul Herring), a constitutively active MKK6 3, a dominant negative MAPK14 4, or a dominant negative RhoA in the growth medium overnight as described previously 5.

**Protein Extraction and Western Blotting** – Cells were rinsed in cold phosphate-buffered saline (PBS) and total protein was isolated with cold lysis buffer (Cell Signaling) containing 1% protease inhibitor cocktail (Sigma). A detergent-compatible protein assay kit (Bio-Rad) was utilized to assess the concentration of total protein. Equal amounts of protein were resolved in a 10% SDS-PAGE gel and then transferred onto nitrocellulose membranes for 2 h. Following two brief rinses in PBS, membranes were blocked with 5% nonfat milk for 1 h at RT and then incubated with the indicated primary antibody overnight at 4°C. Membranes were incubated with HP-conjugated secondary antibody for 1 h at RT and signals were revealed either with enhanced chemiluminescence reagent (Pierce) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The primary antibodies used in this report were as follows: MAPK14 (Santa Cruz Biotechnology, sc-535); ACTA2 (Sigma, A2547); CNN1 (DAKO, M3556); Myc (Santa Cruz Biotechnology, sc-789), HA (Cell Signaling, 3724S); SRF (Santa Cruz Biotechnology, sc-335); TUBA (Sigma, T-5168), TAGLN (Abcam, ab10135); MKL1 (kind gift from Guido Posern, Max Planck Institute); cofilin and phospho-cofilin (Cell Signaling) and Flag M2 (Sigma, F3165).

**Immunofluorescence Microscopy** – HCASMC were seeded in 35 mm plates mounted with coverslips and grown until 70% confluence. Cells were then transfected with siRNA to MAPK14 overnight and refed the next day with growth medium for 24 h. After 24 h serum starvation, cells were stimulated with TGF-β1 (2 ng/ml) for 24 h and immunofluorescence microscopy was carried out. Briefly, cells were washed twice with PBS and then
fixed in freshly prepared 4% paraformaldehyde for 10 min at RT. After rinsing three times with PBS-Tween20, cells were permeabilized with 0.1% Triton X-100 for 5 min. A 1:200 dilution of mouse anti-human CNN1 and a 1:200 diluted goat anti-mouse IgG Texas Red conjugate (Abcam) were used to detect CNN1. For filamentous actin staining, 4% paraformaldehyde fixed cells were incubated with a 1:100 dilution of phalloidin (Molecular Probes) for 20 min. For detecting HA-tagged MKL1, cells were fixed with ice cold methanol for 10 min, rinsed with PBST, and incubated sequentially with a 1:200 dilution of HA antibody and a 1:200 dilution of goat anti-rabbit IgG FITC. Nuclear profiles were revealed with a 5 min incubation in a 1:10,000 dilution of DAPI (Molecular Probes) prior to microscopic observation. Fluorescence was visualized with an inverted Olympus IX70 fluorescence microscope and photographed for direct importation into Adobe Photoshop. All images were processed in an equivalent manner to faithfully capture the real time images of each sample. Quantitation of the nuclear versus cytosolic distribution of HA-tagged MKL1 was done by two independent observers who were blinded to the condition. For immunohistochemistry, 5 μm sections were blocked with 5% normal goat serum in PBS for 30 min and then incubated with rabbit antiserum to MAPK14 (1:1000), pMAPK14 (1:200) or MYH11 (1:200) overnight at 4°C. After rinsing in PBST, sections were incubated with biotinylated goat anti-rabbit IgG (1:400) for 30 min and the HP standard PK6100 complex (Vector, Burlinghame, CA) for an additional 30 min. Finally, sections were stained by either DAB kit (Thermal fisher, TA060HDX) for MAPK14/pMAPK14 or vector red alkaline phosphatase substrate kit (Vector Labs, BA-2000) for MYH11.
Supplemental Figures

Supplemental Figure I. siMAPK14-induced miR145 expression in HCASMC.
Conventional PCR (A) and qPCR (B) of different isoforms of p38MAPK expression in growing HCASMC.
HCASMC pretreated with siMAPK14 for 24 h ± 2 ng/ml TGFβ1 for an additional 24 h and RT-PCR was performed for miR145 expression (C). Shown are representative data from 3 independent experiments. Similar findings were seen in four independent isolates of HCASMC (data not shown).

Supplemental Figure II. Specificity and rescue of siMAPK14 knockdown effects.
Three independent siRNAs targeting different coding regions of MAPK14 yielded similar induction of VSMC contractile proteins in HCASMC (A). HCASMC treated with two independent control siRNA or siMAPK14 (B) or siEGLN (C) and indicated markers analyzed 72 h by Western blotting. Growing Hek293 cells were transfected with negative control siRNA (-) or siMAPK14 ± Myc-MKL1 for 48 h and ACTA2 mRNA levels were assessed by qPCR (D). Hek293 cells were transfected with siMAPK14 and the indicated plasmids for 48 h, followed by Western blotting (E, F).

Supplemental Figure II. Inhibitory effect of MAPK14 on Tagln promoter activity. 10T1/2 cells were co-transfected with increasing amounts of a MAPK14 expression plasmid and a fixed level of a -505 Tagln promoter-linked to luciferase. The activity of the Tagln promoter was assessed by normalizing raw luciferase to the renilla control reporter. Data reflect quadruplicate samples (± standard deviation).

Supplemental Figure IV. siMAPK14 effects on the actin cytoskeleton. (A) HCASMC (a-f) were treated with sicontrol (a, c, e) or siMAPK14 (b, d, f) for 24 h, followed by overnight serum starvation and then TGF β1 stimulation for 24 h. Immunofluorescence microscopy for phalloidin staining of F-actin (a-d) and CNN1 (e, f) are shown. Magnifications are 10x (a, b) and 60x (c-f). (B) Western blot of G-actin and F-actin levels from HCASMC treated with either sicontrol or siMAPK14 for 72 h.

Supplemental Figure V. CCG-1423 abolishes siMAPK14-induced VSMC contractile protein expression.
PASMC were transfected with siMAPK14 for 8 h and then refed basal medium ± CCG-1423 (3.3uM) overnight before stimulation with TGFβ1 (2 ng/ml) for an additional 24 h. Western blotting for indicated proteins was done in two independent experiments with similar results.
Supplemental Figure VI. MKK6 suppresses VSMC contractile proteins. HCASMC transduced with adenovirus-LacZ or adenovirus-MKK6 overnight were switched to differentiating medium for 48 h for immunofluorescent staining of ACTA2 (A). Similarly transduced HCASMC were pretreated with SB203580 (10 uM) for 30 min and then switched to differentiating medium for 48 h before Western blotting (B). HCASMC were transduced with the indicated adenoviruses overnight and then treated with TGFβ1 for 40 h before Western blotting (C).

Supplemental Figure VII. Dose-dependent effects of p38MAPK inhibitors on VSMC contractile protein expression. Growing HCASMC were serum starved overnight and then pre-treated with indicated concentration of SB203580 (A, B) or BIRB (B) for 30 min followed by TGFβ1 treatment for 24 h and Western blotting for the indicated proteins.

References


Relative Tag/luc Activity

MAPK14
Long et al, Supplemental Figure V

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- MAPK14
- TUBA
- ACTA2
- CNN1
- TAGLN
Long et al, Supplemental Figure VI

A
Ad-lacZ

Ad-MKK6

B

MKK6  -  +  -  +
SB203580 - - + +

pMAPK14
ACTA2
TAGLN
TUBA

C

MKK6  -  +  +
Flag-dnMAKP14 - - +

TUBA
ACTA2
CNN1
Flag
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