Nuclear Factor of Activated T Cells c1 Mediates p21-activated Kinase 1 Activation in the Modulation of Chemokine-induced Human Aortic Smooth Muscle Cell F-actin Stress Fiber Formation, Migration, and Proliferation and Injury-induced Vascular Wall Remodeling*

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Background: We explore the mechanisms by which NFATc1 mediates vascular wall remodeling.

Results: MCP1 activates Pak1 in a Rac1-NFATc1-cyclin D1-CDK6-CDK4-dependent manner in the mediation of HASMC migration and proliferation.

Conclusion: Downstream of Rac1, NFATc1, via the cyclin D1-CDK6-CDK4 signaling axis, mediates Pak1 activation in the modulation of vascular wall remodeling.

Significance: Interference with NFATc1 activation could represent a novel therapeutic approach for the treatment of restenosis.

Recent literature suggests that cyclin-dependent kinases (CDKs) mediate cell migration. However, the mechanisms were not known. Therefore, the objective of this study is to test whether cyclin/CDKs activate Pak1, an effector of Rac1, whose involvement in the modulation of cell migration and proliferation is well established. Monocyte chemotactic protein 1 (MCP1) induced Pak1 phosphorylation/activation in human aortic smooth muscle cells (HASMCs) in a delayed time-dependent manner. MCP1 also stimulated F-actin stress fiber formation in a delayed manner in HASMCs, as well as the migration and proliferation of these cells. Inhibition of Pak1 suppressed MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation. MCP1 induced cyclin D1 expression as well as CDK6 and CDK4 activities, and these effects were dependent on activation of NFATc1. Depletion of NFATc1, cyclin D1, CDK6, or CDK4 levels attenuated MCP1-induced Pak1 phosphorylation/activation and resulted in decreased HASMC F-actin stress fiber formation, migration, and proliferation. CDK4, which appeared to be activated downstream of CDK6, formed a complex with Pak1 in response to MCP1. MCP1 also activated Rac1 in a time-dependent manner, and depletion/inhibition of its levels/activation abrogated MCP1-induced NFATc1-cyclin D1-CDK6-CDK4-Pak1 signaling and, thereby, decreased HASMC F-actin stress fiber formation, migration, and proliferation. In addition, smooth muscle-specific deletion of NFATc1 led to decreased cyclin D1 expression and CDK6, CDK4, and Pak1 activities, resulting in reduced neointima formation in response to injury. Thus, these observations reveal that Pak1 is a downstream effector of CDK4 and Rac1-dependent, NFATc1-mediated cyclin D1 expression and CDK6 activity mediate this effect. In addition, smooth muscle-specific deletion of NFATc1 prevented the capacity of vascular smooth muscle cells for MCP-1-induced activation of the cyclin D1-CDK6-CDK4-Pak1 signaling axis, affecting their migration and proliferation in vitro and injury-induced neointima formation in vivo.

Vascular smooth muscle cell (VSMC)3 migration to, and proliferation in, the intimal region are considered hallmarks of neointimal development following angioplasty and vein grafting (1, 2). In recent years, many reports showed that besides resident VSMCs, progenitor cell recruitment to and homing at the site of vascular injury also plays a role in neointima formation, at least in rodent animal models (3, 4). Among the many theories put forth in explaining the abnormal multiplication of these cells, their dedifferentiation gained momentum (5, 6). Although the exact cause for the switching of VSMCs from the contractile to the synthetic phenotype is currently unclear, a variety of molecules produced at the site of vascular injury appear to be involved in the modulation of their migration and proliferation (2, 7). One such molecule is monocyte chemotactic protein 1 (MCP1) (8). MCP1, also known as CCL2, belongs to the CC chemokine family, and it exhibits profound chemotactic activity toward monocytes, memory T cells, and dendritic cells and, thus, plays a major role in inflammation (9, 10). Previously, we and others have shown that MCP1 is produced at the site of vascular injury and is involved in vascular wall remodeling (11–13). Toward exploring the mechanisms under-

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3 The abbreviations used are: VSMC, vascular smooth muscle cell; NFAT, nuclear factor of activated T cells; HASMC, human aortic smooth muscle cell; SMC, smooth muscle cell; l, intimal; M, medial; SM, smooth muscle; MBP, myelin basic protein.
lying VSMC migration and proliferation, we have also reported previously that the transcriptional factor nuclear factor of activated T cells c1 (NFATc1) is activated by potent vascular mitogens such as platelet-derived growth factor and thrombin and that inhibition of NFATs blunts their effect on VSMC migration and proliferation and injury-induced neointima formation (14–16).

NFATs are a family of transcriptional factors belonging to the Rel group, and it consists of NFATc1-c4 and NFAT5 (17). The activation of NFATc1-c4 requires their dephosphorylation, which is mediated by a Ca2+/calmodulin-dependent phosphatase, calcineurin (18). Phosphorylation by kinases such as glycogen synthase kinase 3β in the nucleus invokes their nuclear export and inactivation. NFATs bind to the GGAAA core DNA element present in the promoter regions of the genes and influence their transcription (17). Toward elucidating the mechanisms by which NFATs are involved in vascular wall remodeling, we reported that NFATc1 enhances the expression of cyclins, particularly cyclin D1 and cyclin A2 in VSMCs mediating the migration and proliferation of these cells (19, 20). Although the role of cyclins in cell cycle progression has been established beyond doubt (21), their role in cell migration lacks mechanistic evidence. In this aspect, we have also reported that cyclin D1/CDK6 mediates the activation of the Rho GTPase effector PKN1 in mediating MCP1-induced HASMC migration and proliferation and injury-induced neointima formation in an NFATc1-dependent manner (22). In this study, we asked the question whether cyclin D1/CDK6 also influence the modulation of the Rac1 effector Pak1, whose role in the regulation of cell migration and proliferation is well established (23, 24), in mediating VSMC migration and proliferation downstream of NFATc1. Our findings reveal that MCP1 activates Pak1 in an NFATc1-, cyclin D1-, CDK6-, and CDK4-dependent manner downstream of Rac1 in mediating VSMC migration and proliferation. Furthermore, CDK4 was found to form a complex with and phosphorylate Pak1, and depletion of CDK4 levels or inhibition of its activity suppressed MCP1-induced Pak1 kinase activity. In addition, genetic deletion of NFATc1 in SMCs prevented the capacity of VSMCs in the activation of the cyclin D1-CDK6-CDK4-Pak1 signaling axis and to migrate and proliferate in response to MCP1 in vitro and attenuated injury-induced neointima formation in vivo.

MATERIALS AND METHODS

Reagents—EHT1864 (catalog no. 3872) and recombinant human MCP-1 (catalog no. 279-MC) were from R&D Systems Inc. (Minneapolis, MN). Anti-CDK4 (catalog no. SC-260), anti-CDK6 (catalog no. SC-56362), anti-β-tubulin (catalog no. SC-9104), anti-MEK1 (catalog no. SC-219), anti-p53 (catalog no. SC-6243), and anti-GFP (catalog no. SC-9996) antibodies and truncated Rb protein (catalog no. SC-4112) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cyclin D1 antibody (catalog no. RB-010-P) was bought from NeoMarkers (Fremont, CA). Anti-NFATc1 antibody (catalog no. MA3-024) was from Affinity BioReagents (Golden, CO). Anti-NFATc1 antibody (catalog no. 556602) was procured from BD Biosciences. Anti-pPak1 (catalog no. 2601) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Hoechst 33342 (catalog no. H3570), Lipofectamine 2000 reagent, and Prolong Gold antifade mounting medium (catalog no. P36930) were bought from Invitrogen. Rat anti-mouse MCP1 antibodies (catalog no. ab8101) and recombinant full-length Pak1 protein (catalog no. ab67946) were purchased from Abcam (Cambridge, MA). Fasiciplyn (catalog no. 341251) was procured from EMD Millipore (Billerica, MA). [γ-32P]ATP (specific activity 3000 Ci/mmol) was from MP Biomedicals (Irvine, CA). [3H]Thymidine (specific activity 20 Ci/mmol) was obtained from PerkinElmer Life Sciences (Waltham, MA). Protein A-Sepharose (catalog no. CL-4B) and protein G-Sepharose (catalog no. P36930) were bought from Biotium (Hayward, CA). Human NFATc1 siRNA (ON-TARGETplus SMARTpool L-003605-00, NM_172390), human cyclin D1 siRNA (ON-TARGETplus SMARTpool L-003210-00, NM_053056), human CDK4 siRNA (ON-TARGETplus SMARTpool L-003238-00, NM_000075), human CDK6 siRNA (ON-TARGETplus SMARTpool L-003240-00, NM_001259), human Pak1 siRNA (ON-TARGETplus SMARTpool L-003521-00, NM_002576), human cyclin D1 siRNA (siGENOME siRNA D-003210-07), human CDK6 siRNA (siGENOME siRNA D-003240-09), human Pak1 siRNA (siGENOME siRNA M-003521-04), and control non-targeting siRNA (D-001810-10) were purchased from Dharmacon RNAi Technologies (Chicago, IL). The ECL Western blotting detection reagents (catalog no. RPN2106) were obtained from GE Healthcare.

Adenoviral Vectors—The construction of Ad-GFP and Ad-dnRac1 was described previously (16, 25).

Cell Culture—HASMCs were purchased from Invitrogen, subcultured in medium 231 containing smooth muscle cell growth supplements, and used between four and ten passages. Aortic smooth muscle cells from WT and SM22Cre: NFATc1fl/fl mice were isolated by collagenase II/elastase digestion, grown in DMEM/F12 containing 10% FBS, and used between three to ten passages.

Transfections and Transductions—HASMCs were transfected with non-targeted control or ON-TARGETplus SMARTpool or siGENOME SMARTpool siRNA molecules at a final concentration of 100 nM using Lipofectamine 2000 transfection reagent according to the instructions of the manufacturer. When adenoviral vectors were used to block the function of a specific molecule, cells were transduced with adenovirus harboring either GFP (control) or a dominant negative mutant of the target molecule at 40 multiplicity of infection overnight in complete medium. After transfections or transductions, cells were growth-arrested for 36 h and used as required.

CDK4/6 and Pak1 Assays—Cell extracts were analyzed for CDK4/6 and/or Pak1 activities as described previously (26, 27). The [32P]-labeled Rb protein, Pak1, and/or myelin basic protein (MBP) were visualized by autoradiography, and the band intensities were quantified using National Institutes of Health Image. In the case of the coupled kinase assay, first Pak1 (1 μg) was added to the CDK4 immunoprecipitate kinase reaction mix and incubated for 30 min at 37 °C. Then, MBP (10 μg) was added, and incubation continued for another 30 min at 37 °C. After the end of the incubation period, the reaction was stopped by adding an equal volume of 2× sample buffer and...
boiling for 5 min. The reaction mix was separated by SDS-PAGE and subjected to autoradiography.

DNA Synthesis—DNA synthesis was measured by [3H]thymidine incorporation as described previously and expressed as counts/min/dish (26).

Cell Migration—Cell migration was measured using a modified Boyden chamber method as described previously (28). Cell motility is presented as number of migrated cells/field.

Western Blot Analysis—Cell or tissue extracts containing an equal amount of protein from the control and each treatment/condition were analyzed by Western blotting for the indicated molecule using its specific antibody as described previously (26). The band intensities were quantified using National Institutes of Health ImageJ.

Common Carotid Artery Wire Injury—C57BL/6 mice and SM22Cre mice (Tg(Tagln-Cre)1Her/J, stock no. 004746) were purchased from The Jackson Laboratory, Bar Harbor, ME. NFATc1<sup>d/d</sup> mice were a gift from Dr. Laurie H. Glomcher, Harvard Medical School, Boston, MA (29). Mice were bred and maintained according to the Institutional Animal Care and Use facility guidelines. All experiments involving the use of animals were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, TN. SM-specific NFATc1-deficient mice (SM-NFATc1<sup>d/d</sup> or SM22Cre: NFATc1<sup>d/d</sup>) were generated by cross-breeding NFATc1<sup>d/d</sup> mice with SM22Cre mice. NFATc1<sup>d/d</sup> and SM22Cre:NFATc1<sup>d/d</sup> were routinely genotyped using DNA from a tail biopsy and a set of the following three primers, which can differentiate the WT, NFATc1<sup>fl/fl</sup>, and NFATc1<sup>d/d</sup> from a tail biopsy and a set of the following three primers, as described previously (26). The band intensities were quantified using National Institutes of Health ImageJ.

RESULTS

Pak1 Mediates MCP1-induced HASMC F-actin Stress Fiber Formation, Migration, and Proliferation—We have reported previously that vascular injury produces MCP1 and that it mediates VSMC migration and proliferation (12, 13). In addition, we have demonstrated that MCP1-induced VSMC migration and proliferation require PKN1 (22), a Rho GTPase effecter (30). However, many studies have reported that Pak1, a Cdc42/Rac1 effector, plays an essential role in the regulation of cell migration and proliferation (31, 32). Therefore, to understand the mechanisms by which MCP1 modulates VSMC migration and proliferation, we have studied the role of Pak1. MCP1 induced Pak1 phosphorylation and its activity in a delayed time-dependent manner, with maximum effects at 4–8 h (Fig. 1A). MCP1 also induced F-actin stress fiber formation in a delayed time-dependent manner, with the maximum effect at 4–8 h (Fig. 1B). In addition, down-regulation of Pak1 levels by its siRNA (SMARTpool) substantially inhibited MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation (Fig. 1, C–E). Depletion of Pak1 levels using another set of its siRNA molecules (siGENOME siRNA) also attenuated MCP1-induced HASMC Pak1 activity, migration, and proliferation (Fig. 1, F–H).
CDK6 and CDK4 Mediate MCP1-induced Pak1 Activation

We have shown previously that CDKs, particularly CDK6, mediate MCP1-induced PKN1 activation and that this event requires NFATc1-dependent cyclin D1 expression (22). To find out whether there is any link between CDKs and Pak1 in the regulation of MCP1-induced VSMC migration and proliferation, we examined the role of CDK6 and CDK4. In line with our previous observations (22), MCP1 induced CDK6 and CDK4 activities in a time-dependent manner, with maximum effects at 4–8 h (Fig. 2A). Surprisingly, depletion of CDK6 or CDK4 levels inhibited MCP1-induced Pak1 phosphorylation and its activity (Fig. 2B). Down-regulation of CDK6 or CDK4 levels also inhibited MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation (Fig. 2, C–E). Because both

FIGURE 1. Pak1 mediates MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation. A, equal amounts of protein from quiescent and MCP1-treated (50 ng/ml) HASMCs were analyzed by Western blotting for pPak1 levels using its phosphospecific antibodies, and the blot was reprobed for total Pak1 levels for normalization. To measure Pak1 activity, equal amounts of protein from the control and each treatment were immunoprecipitated with anti-Pak1 antibodies, and the immunocomplexes were assayed for its kinase activity using MBP and [γ-32P]ATP as substrates. B, quiescent and MCP1-treated (50 ng/ml) HASMCs were stained with phalloidin for F-actin stress fiber formation. C, upper panel, HASMCs were transfected with non-targeted control or SMARTpool Pak1 siRNA (100 nM). Two days after transfection, cell extracts were prepared, equal amounts of protein from each condition were analyzed by Western blotting for Pak1 levels using its specific antibodies, and the blot was reprobed for β-tubulin levels to show the effect of the siRNA on its target and off-target molecule levels. Lower panel, HASMCs that were transfected with non-targeted control or SMARTpool Pak1 siRNA (100 nM) and quiesced were treated with and without MCP1 (50 ng/ml) for 24 h and 8 h, and DNA synthesis (D) and cell migration (E) were measured, respectively. F, HASMCs that were transfected with non-targeted control or siGENOME Pak1 siRNA (100 nM) and quiesced were treated with and without MCP1 (50 ng/ml) for 8 h. Cell extracts were prepared, and equal amounts of protein from control and each treatment were analyzed for Pak1 activity as described in A. Equal amounts of protein from the same cell extracts were also analyzed by Western blotting for Pak1 and β-tubulin levels to show the effect of the siRNA on its target and off-target molecule levels. SiGsiRNA, siGENOME siRNA. G and H, all the conditions were the same as in F, except that after quiescence, cells were treated with and without MCP1 (50 ng/ml) for 24 h and 8 h, and DNA synthesis (G) and migration (H) were measured, respectively. DNA synthesis was measured by [3H]thymidine incorporation, and cell migration was measured by the Boyden chamber method. *, p < 0.01 versus control siRNA; **, p < 0.01 versus control siRNA + MCP1.
CDK4 Mediates Pak1 Phosphorylation and Activation

CDK6 and CDK4 activities are induced by MCP1, we wanted to find out whether there is any cross-talk between their activation. Down-regulation of CDK6 levels completely blocked MCP1-induced CDK4 activity (Fig. 2A). On the other hand, depletion of CDK4 levels inhibited MCP1-induced CDK6 activity to a modest level (Fig. 2F). These findings suggest a cooperative dependence between CDK6 and CDK4 activation, with CDK6 priming the effect. These findings also infer that CDK4 is likely the immediate upstream modulator of Pak1 phosphorylation/activation. Therefore, to confirm the role of CDK4 in MCP-1-induced Pak1 activation and F-actin stress fiber formation, we used a pharmacological approach. Fascaplysin, a potent and specific inhibitor of CDK4 (33), completely blocked MCP1-induced CDK4 and Pak1 activities and F-actin stress fiber formation (Fig. 2G and H).

NFATc1, via Enhancing Cyclin D1-CDK6/4 Signaling, Mediates MCP1-induced Pak1 Activation—Activation of CDKs requires type D cyclins (21). In addition, our previous work showed that NFATc1 mediates cyclin D1 expression in response to various agonists, including MCP1 (20, 22, 34). Therefore, we next tested the role of NFATc1 and cyclin D1 in MCP1-induced Pak1 phosphorylation/activation. Consistent

FIGURE 2. CDK4/6 mediate MCP1-induced Pak1 activation in HASMCs. A, quiescent HASMCs were treated with and without MCP1 (50 ng/ml) for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from the control and each treatment were analyzed by immunocomplex kinase assay for CDK4/6 activities using truncated recombinant retinoblastoma protein and [γ-32P]ATP as substrates. B, after transfection with non-targeted control or SMARTpool CDK6 or CDK4 siRNA (100 nM) and quiescence, HASMCs were treated with and without MCP1 (50 ng/ml) for 8 h, and cell extracts were prepared. Equal amounts of proteins from the control and each treatment were also assayed for Pak1 activity by immunocomplex kinase assay using MBP and [γ-32P]ATP as substrates. C, conditions were the same as in B, except that after treatment with and without MCP1, cells were stained with phalloidin for F-actin stress fiber formation. D and E, after transfections with non-targeted control or SMARTpool CDK6 or CDK4 siRNA and quiescence, cells were treated with and without MCP1 (50 ng/ml) for 24 h and 8 h, and DNA synthesis (D) and cell migration (E) were measured, respectively. F, HASMCs that were transfected with non-targeted control or SMARTpool CDK6 or CDK4 siRNA and quiesced were treated with and without MCP1 (50 ng/ml) for 8 h. Cell extracts were prepared, and equal amounts of protein from the control and each treatment were analyzed by immunocomplex kinase assay for CDK6 and CDK4 activities as described in A. Cell extracts containing an equal amount of protein from the control and each treatment were also analyzed by Western blotting for CDK6 and CDK4 levels to show the effect of their siRNAs on their total levels. G and H, quiescent cells were treated with and without MCP1 (50 ng/ml) in the presence and absence of fascaplysin (5 μM) for 8 h, and either cell extracts were prepared and analyzed for CDK4 and Pak1 activities as described in A and B, respectively, or stained with phalloidin for F-actin stress fiber formation as described in C, *, p < 0.01 versus control siRNA; **, p < 0.01 versus control siRNA + MCP1.
with our previous findings (22), MCP1 induced cyclin D1 expression in a time-dependent manner, with the maximum effect at 4–8 h (Fig. 3A). In addition, down-regulation of NFATc1 levels using its siRNA suppressed MCP1-induced cyclin D1 expression (Fig. 3B). Depletion of NFATc1 or cyclin D1 levels also inhibited CDK6 and CDK4 activities as well as...
CDK4 Mediates Pak1 Phosphorylation and Activation

Pak1 phosphorylation and its activity (Fig. 3B). In accordance with these observations, depletion in NFATc1 or cyclin D1 levels attenuated MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation (Fig. 3, C–E). To strengthen the role of cyclin D1/CDK6 in MCP1-induced CDK4 (and its apparent downstream target, Pak1) activities, we used a second set of their siRNA molecules that are distinct from SMARTpool. Depletion of cyclin D1 and CDK6 levels using their siGENOME siRNA molecules also blocked MCP1-induced CDK4 and Pak1 activities in HASMCs as well as the migration and proliferation of these cells (Fig. 3, F–H). Because CDK6 was found to be upstream to CDK4 (Fig. 2F), we next wanted to find out whether Pak1 is a substrate for CDK4. To test this, we performed a time course effect of MCP1 on CDK4 activity using recombinant Pak1 (rPak1) as a substrate. CDK4 activity, as measured by immunocomplex kinase assay using rPak1 as a substrate, was increased by MCP1 in a time-dependent manner (Fig. 4A). Pak1 activity using MBP as a substrate

![CDK4 Mediates Pak1 Phosphorylation and Activation](image_url)
was also induced by MCP1, and it correlated with the time course of CDK4 activity. Both CDK4 and Pak1 activities were also correlated with cyclin D1 expression in response to MCP1. Down-regulation of NFATc1, cyclin D1, CDK6, or CDK4 levels blocked MCP1-induced CDK4-mediated rPak1 phosphorylation (Fig. 4B). To test whether CDK4-mediated phosphorylation of rPak1 is sufficient for its activity, we performed a coupled immunocomplex kinase assay. The CDK4 immunoprecipitates from MCP1-treated HASMCs phosphorylated rPak1 (Fig. 4C). Next, we incubated CDK4 immunoprecipitates first with rPak1 for 30 min at 37 °C. Then, the Pak1 substrate, MBP, was added, and incubation continued for another 30 min at 37 °C. We found that, upon phosphorylation by CDK4, rPak1 exhibited kinase activity on its substrate, MBP. To rule out the possibility of CDK4 acting directly on MBP as a substrate, CDK4 immunoprecipitates were incubated with MBP in a kinase reaction. Surprisingly, CDK4 immunoprecipitates phosphorylated MBP. To find whether the phosphorylation of MBP was due to CDK4-associated Pak1, we immunoprecipitated Pak1, and its kinase activity was measured using MBP as a substrate. MBP was phosphorylated by immunoprecipitated Pak1 with more effect in MCP1-treated HASMCs as compared with the control. Coinmunoprecipitation experiments revealed that, in response to MCP1, CDK4 was found to

FIGURE 5. Rac1 acts upstream of MCP1-induced activation of NFATc1-cyclin D1-CDK6-CDK4-Pak1 signaling in the modulation of HASMC F-actin stress fiber formation, migration, and proliferation. A, quiescent HASMCs were treated with and without MCP1 (50 ng/ml) for the indicated time periods, and cell extracts were prepared. Equal amounts of protein from the control and each treatment were analyzed by pull-down assays for Rac1 and RhoA activation using GST-Pak1 and GST-Rhotekin beads, respectively. Equal amounts of protein from the same cell extracts were also analyzed by Western blotting for total Rac1 and RhoA levels using their specific antibodies. B–D, HASMCs that were transduced with Ad-GFP or Ad-dnRac1 (10^9 plaque-forming units) and quiesced were subjected to MCP1 (50 ng/ml)-induced F-actin stress fiber formation (B), DNA synthesis (C), or migration (D). E, all conditions were the same as in B, except that after treatment with and without MCP1, cytoplasmic and nuclear extracts were prepared, and equal amounts of protein from each cell fraction were analyzed by Western blotting for NFATc1 levels using its specific antibodies. The blots were reprobed sequentially for MEK1, p53, and β-tubulin levels to show the purity of the cytoplasmic (CF) and nuclear (NF) extracts. F, all conditions were the same as in B, except that after treatment with and without MCP1, cell extracts were prepared, and equal amounts of protein from the control and each treatment were analyzed by either Western blotting for cyclin D1, pPak1, Pak1, Rac1, GFP, and β-tubulin levels using their specific antibodies or immunocomplex kinase assays for CDK6/4 and Pak1 activities using truncated recombinant retinoblastoma protein and MBP along with [γ-32P]ATP as substrates, respectively. *, p < 0.01 versus Ad-GFP; **, p < 0.01 versus Ad-GFP + MCP1.
be associated with Pak1 in a sustained manner (Fig. 4D). Down-regulation of Pak1 levels attenuated total Pak1 and CDK4-associated Pak1 activity (Fig. 4E). To confirm that the phosphorylation of Pak1 by CDK4 is sufficient for its activity, endogenous Pak1 levels were depleted by its siRNA, CDK4 was immunoprecipitated, and its activity was determined using rPak1 as a substrate. CDK4 phosphorylated rPak1, and this effect was more in MCP1-treated cells compared with the control (Fig. 4F). On the other hand, CDK4 that was immunoprecipitated from Pak1-depleted and MCP1-treated HASMCs did not phosphorylate MBP. When rPak1 and MBP were used as substrates in a coupled kinase assay, both were phosphorylated by CDK4. Without endogenous Pak1 depletion, the phosphorylation of MBP by CDK4 immunoprecipitates was found to be more than that with endogenous Pak1 depletion. These results indicate that Pak1 is associated with, and is phosphorylated and activated by, CDK4 or its associated kinase(s) in response to MCP1.

**Rac1 Acts Upstream of NFATc1-Cyclin D1-CDK6/4-Signaling in the Modulation of Pak1 Activation**—Because Pak1 is an effector molecule of Rho GTPases, particularly Cdc42/Rac1 (23, 24, 35, 36), we wanted to find out whether there is any link between Rac1 and the NFATc1-cyclin D1-CDK6/4-Pak1 signaling axis. To address this point, we have tested the effect of MCP1 on Rac1 activation. Interestingly, MCP1, without having any effect on RhoA, time-dependently activated Rac1 as measured by pull-down assays (Fig. 5A). In addition, adenovirus-
mediated expression of dnRac1 blocked MCP1-induced HASMC F-actin stress fiber formation, migration, and proliferation (Fig. 5, B–D). Adenovirus-mediated expression of dnRac1 also inhibited MCP1-induced NFATc1 translocation from the cytoplasm to the nucleus (Fig. 5E). Next, we tested the effect of dnRac1 on MCP1-induced cyclin D1 expression and CDK6, CDK4, and Pak1 activities. Adenovirus-mediated expression of dnRac1 completely blocked MCP1-induced cyclin D1 expression and CDK6, CDK4, and Pak1 activities (Fig. 5F). It is well established that RhoA, but not Rac1, mediates stress fiber formation (37, 38). Therefore, to confirm the role of Rac1 in MCP1-induced F-actin stress fiber formation, we also used a pharmacological approach. Consistent with the effects of dnRac1, EHT1864, a highly specific inhibitor of Rac1 (39), blocked MCP1-induced NFATc1 nuclear translocation, cyclin D1 expression, CDK6 and CDK4 activities, and Pak1 phosphorylation/activation in HASMCs and prevented the F-actin stress fiber formation, migration, and proliferation of these cells (Fig. 6, A–E).

**SM-specific Deletion of NFATc1 Blunts MCP1-induced Cyclin D1-CDK6-CDK4-Pak1 Stimulation in Vitro and Injury-induced Neointima Formation in Vivo**—To find the importance of SM-specific NFATc1 in the modulation of the cyclin D1-CDK6-CDK4-Pak1 signaling axis in vascular wall remodeling, we generated transgenic mice for SM-specific deletion of NFATc1 by cross-breeding NFATc1fl/fl mice with SM22Cre mice. SM-specific deletion of NFATc1 in VSMCs of SM22Cre: NFATc1fl/fl mice was confirmed by lack of its expression (Fig. 7A). Next, we tested the role of SM-specific NFATc1 in VSMC-induced vascular wall remodeling. MCP1 induced Rac1 activity in VSMCs of both WT and SM22Cre: NFATc1fl/fl mice (Fig. 7B). However, compared with VSMCs from WT mice, VSMCs from SM22Cre: NFATc1fl/fl mice failed to respond to MCP1 in the induction of cyclin D1 expression, CDK6 and CDK4 activities, and Pak1 activation (Fig. 7B). VSMCs from SM22Cre: NFATc1fl/fl mice also did not respond to MCP1-induced proliferation or migration compared with VSMCs from WT mice (Fig. 7, C and D). Furthermore, guide wire injury induced MCP1

![Figure 7](image-url)
expression and Pak1 phosphorylation in WT mice, mostly in SMCs (Fig. 7E). On the other hand, SM-specific knockdown of NFATc1, although having no effect on injury-induced MCP1 expression, attenuated Pak1 phosphorylation in SMCs of SM22Cre:NFATc1fl/fl mice (Fig. 7E). Consistent with these observations, guide wire injury induced neointima formation in WT mice, whereas in SM22Cre:NFATc1fl/fl mice, this effect was attenuated substantially (Fig. 7F).

**DISCUSSION**

The important findings of this study are as follows. 1) MCP1 activated Pak1 in a sustained manner; 2) Pak1 activation is required for MCP1-induced HASMC migration and proliferation; 3) MCP1 induced CDK6 and CDK4 activities in a time-dependent manner; 4) both CDK6 and CDK4 activities are required for MCP1-induced migration and proliferation; 5) MCP1 induced cyclin D1 expression in a time- and NFATc1-dependent manner; 6) MCP1-induced CDK6 and CDK4 activities required NFATc1-dependent cyclin D1 expression; 7) MCP1-induced Pak1 activation also required NFATc1 activation, cyclin D1 expression, and CDK6 and CDK4 activities; and 8) CDK4 phosphorylated rPak1 in vitro, and this phosphorylation is sufficient for rPak1 activity. Many studies have shown that cyclin D1 mediates agonist-induced cell migration (40, 41). Similarly, a role for CDKs in cell migration has also been reported (42). We have demonstrated previously that cyclin D1 mediates cell migration and proliferation (20). Many studies have shown that cyclin D1 mediates agonist-induced cell migration (40, 41). Similarly, a role for CDKs in cell migration has also been reported (42). We have demonstrated previously that cyclin D1 mediates cell migration and proliferation (20). In this study, we show that NFATc1, by enhancing cyclin D1 expression and, thereby, CDK6 and CDK4 activities, mediates Pak1 phosphorylation/activation in MCP1-induced HASMC migration and proliferation. Although the role of cyclin D1 in cell proliferation is well established (43), the mechanisms by which it mediates cell migration are not clear. In this aspect, studies from other laboratories have shown that cyclin D1 and CDKs mediate filamin A phosphorylation (42). More excitingly, in this study, we discovered that Pak1, whose role in cell migration is well established (23, 24), is a downstream effector of CDK4. A large body of evidence shows that Pak1 is an effector of Cdc42/Rac1 in mediating cell migration (35, 36). In this aspect, we found that Rac1 acts upstream to NFATc1 in mediating cyclin D1 expression and CDK6 and CDK4 activities. Activation of NFATc1 requires its dephosphorylation by calcineurin (18). In addition, the earlier studies have demonstrated a negative role for JNKs in the stimulation of NFATs (44), the later studies have reported that JNKs mediate NFAT activation (45, 46). Similarly, the role of Rac1 in the regulation of JNKs has also been reported (47, 48). In view of these observations, it is possible that Rac1 via JNKs may mediate the activation of NFATc1 and its downstream effectors.

In regard to the role of small Rho GTPases, an established dogma is that although RhoA mediates focal adhesions and stress fiber formation, Rac1 facilitates lamellipodia formation and membrane ruffling (37, 38). In this context, the intriguing finding of this study is that CCL2, which is a potent chemokine, although activating Rac1 in a sustained manner, does not stimulate RhoA. Furthermore, because interrupting Rac1 activation prevented CCL2-induced F-actin stress fiber formation, it appears that Rac1 hijacks RhoA function in F-actin cytoskeletal remodeling. A similar observation was made in regard to the role of Rac1 in F-actin stress fiber formation in mouse embryonic fibroblasts in response to platelet-derived growth factor.
NFATc1fl/fl mice carotid artery by injury was not affected, suggesting that injury induces MCP1 expression in a rat carotid artery injury model (16, 19, 20). In this study, we have reported previously that NFATs mediate injury-induced vascular wall remodeling (16, 19, 20). In addition, SM-specific knockdown of NFATc1 reduced MCP1 expression in the neointimal region of SM22Cre:NFATc1fl/fl mice. The lack of MCP1 expression in the carotid artery, mostly in SMCs, of both WT and SM22Cre:NFATc1fl/fl mice. This suggests that injury-induced MCP1 expression in a rat carotid artery injury model (12). In agreement with this observation, guide wire injury induced MCP1 expression in the carotid artery, mostly in SMCs, of both WT and SM22Cre:NFATc1fl/fl mice. The lack of MCP1 expression in the neointimal region of SM22Cre:NFATc1fl/fl mice may be explained by the reduction in the neointima formation because of SM-specific NFATc1 deletion. However, its expression in the medial SMCs of SM22Cre:NFATc1fl/fl mice carotid artery by injury was not affected, suggesting that injury-induced MCP1 expression was independent of NFATc1 activation. On the other hand, SM-specific NFATc1 deletion decreased injury-induced Pak1 phosphorylation. In addition, SM-specific knockdown of NFATc1 reduced neointima formation in response to injury. These results infer that MCP1 expression in the medial SMC stimulates their migration to, and proliferation in, the intimal region, resulting in the development of neointima and that these effects require NFATc1 activation. On the basis of these findings, as depicted in Fig. 8, we conclude that Pak1 is an effector of CDK4 and that its activation is dependent on NFATc1-mediated enhancement of cyclin D1 expression and CDK6 activity downstream to Rac1. In addition, SM-specific NFATc1 plays a crucial role in mediating injury-induced cyclin D1 expression and, thereby, CDK6 and CDK4 activities, leading to Pak1 activation and vascular wall remodeling.

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CDK4 Mediates Pak1 Phosphorylation and Activation

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