Cyclosporine Up-Regulates Krüppel-Like Factor-4 (KLF4) in Vascular Smooth Muscle Cells and Drives Phenotypic Modulation In Vivo^S

Sean M. Garvey, Daniel S. Sinden, Pamela D. Schoppee Bortz, and Brian R. Wamhoff

Department of Medicine, Division of Cardiovascular Medicine (S.M.G., D.S.S., P.D.S.B., B.R.W.), Robert M. Berne Cardiovascular Research Center (S.M.G., B.R.W.), University of Virginia, Charlottesville, Virginia

Received November 16, 2009; accepted January 19, 2010

ABSTRACT

Cyclosporine A (CSA, calcineurin inhibitor) has been shown to block both vascular smooth muscle cell (VSMC) proliferation in cell culture and vessel neointimal formation following injury in vivo. The purpose of this study was to determine molecular and pathological effects of CSA on VSMCs. Using real-time reverse transcription-polymerase chain reaction, Western blot analysis, and immunofluorescence microscopy, we show that CSA up-regulated the expression of Krüppel-like factor-4 (*KLF4*) in VSMCs. *KLF4* plays a key role in regulating VSMC phenotypic modulation. *KLF4* antagonizes proliferation, facilitates migration, and down-regulates VSMC differentiation marker gene expression. We show that the VSMC differentiation marker genes smooth muscle α -actin (*ACTA2*), transgelin (*TAGLN*), smoothelin (*SMTN*), and myocardin (*MYOCD*) are all down-regulated by CSA in VSMC monoculture, whereas cyclin-

dependent kinase inhibitor-1A (*CDKN1A*) and matrix metalloproteinase-3 (*MMP3*) are up-regulated. CSA did not affect the abundance of the VSMC microRNA (MIR) markers MIR143 and MIR145. Administration of CSA to rat carotid artery in vivo resulted in acute and transient suppression of *ACTA2*, *TAGLN*, *SMTN*, *MYOCD*, and smooth muscle myosin heavy chain (*MYH11*) mRNA levels. The tumor suppressor genes *KLF4*, *p53*, and *CDKN1A*, however, were up-regulated, as well as *MMP3*, *MMP9*, and collagen-VIII. CSA-treated arteries showed remarkable remodeling, including breakdown of the internal elastic lamina and reorientation of VSMCs, as well as increased *KLF4* immunostaining in VSMCs and endothelial cells. Altogether, these data show that cyclosporin up-regulates *KLF4* expression and promotes phenotypic modulation of VSMCs.

Cyclosporine A (CSA) is a fungus-derived immunosuppressant used clinically to combat organ graft rejection in transplant subjects. CSA suppresses the immune system by inhib-

iting proliferation of lymphocytes. Lymphocyte proliferation is facilitated by the cytokine interleukin-2 (IL-2), whose secretion is blocked by CSA. The transcriptional activation of IL-2 is dependent on the nuclear factor of activated T cells (NFAT) family of transcription factors (Shaw et al., 1988). NFAT nuclear translocation from the cytoplasm and subsequent transcriptional activity is facilitated through its dephosphorylation by the calcineurin (Cn) phosphatase (Flanagan et al., 1991). Cn activity can be inhibited by interaction with a complex of CSA and the endogenous cellular protein cyclophilin A (CYPA). In a similar fashion, the bacterium-derived immunosuppressant FK506 (tacrolimus, Prograf) binds to the cellular protein FK506-binding protein 1A, 12 kDa (FKBP12) to inhibit Cn activity. CSA and FK506 are thus powerful immunosuppressants used to combat organ

This study was supported by the American Heart Association Scientist Development Grant (to B.R.W.); the National Institutes of Health [Grant R01-HL081682] (to BRW); and the American Physiological Society (Postdoctoral Fellowship in Physiological Genomics to S.M.G. and Undergraduate Summer Research Fellowship to D.S.S.).

The following abstract was presented as posters: Garvey SM, Sinden DS, Schoppee Bortz PD, and Wamhoff BR (2009) Cyclosporin A-dependent regulation of genes associated with vascular smooth muscle phenotypic modulation in *Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference*; 2009 Apr 29-May 1; Washington, DC. American Heart Association, Dallas, TX.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.109.163949.

^S The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: CSA, cyclosporine A; CAD, coronary artery disease; Cn, calcineurin; CYPA, cyclophilin A; ECM, extracellular matrix; IEL, internal elastic lamina; *KLF4*, Krüppel-like factor 4; MMP, matrix metalloproteinase; NFAT, nuclear factor of activated T cells; PDGF-BB, platelet-derived growth factor-BB; siRNA, short interfering RNA; SMC, smooth muscle cell; VSMC, vascular smooth muscle cell; A-285222, 3,5-bis(trifluoromethyl) pyrazole; FK506, tacrolimus; FBS, fetal bovine serum; RT-PCR, reverse transcriptase-polymerase chain reaction; ELK1, ETS gene like-1; oxPL, oxidized phospholipid; ROS, reactive oxygen species; DMSO, dimethyl sulfoxide; COL8, collagen-VIII; *CDKN1A*, cyclin-dependent kinase inhibitor-1A; *ACTA2*, smooth muscle α -actin; *TAGLN*, transgelin; *SMTN*, smoothelin; *MYOCD*, myocardin; *MYH11*, smooth muscle myosin heavy chain; MIR, microRNA.

rejection in transplant subjects. Sustained CSA and FK506 immunosuppression is associated with higher risk for post-transplant arteriopathy, coronary artery disease (CAD), hypertension, and hyperlipidemia (Miller, 2002; Lindenfeld et al., 2004). There is also evidence that CSA treatment is associated with greater risk for CAD than FK506 treatment in a study of liver transplant subjects (Bianchi et al., 2008). It remains to be understood how calcineurin inhibitors affect vascular smooth muscle cells (VSMCs) in health and in vascular disease.

The primary function of the adult VSMC is contraction. VSMCs express a distinct class of contractile and regulatory proteins to maintain the differentiated, contractile phenotype (Owens, 1995). Classic smooth muscle contractile genes include smooth muscle α -actin (*ACTA2*), smooth muscle myosin heavy chain (*MYH11*), transgelin (*TAGLN*), and smoothelin (*SMTN*) (Owens et al., 2004). VSMC differentiation requires the transcription factor myocardin (MYOCD), which complexes with serum response factor to activate transcription at the promoters of several contractile genes, including *ACTA2*, *MYH11*, and *TAGLN* (Chen et al., 2002). In response to various pathological stimuli, contractile VSMCs can remodel toward a spectrum of proliferative, migratory, and inflammatory phenotypes (Owens et al., 2004; Gerthoffer, 2007). This process is called phenotypic modulation. Phenotypic modulation is coupled to both down-regulation of VSMC differentiation marker genes and up-regulation of proliferative, inflammatory, and/or extracellular matrix (ECM) genes (Wamhoff et al., 2006; Orr et al., 2010). The phenotypically modulated VSMC is functionally primed to proliferate, migrate toward the vessel lumen, and promote blood vessel repair after injury. After migration in atherosclerosis, VSMCs can further remodel to form a fibrous, plaque-stabilizing cap. However, VSMC phenotypic modulation also contributes to vessel wall inflammation and plaque destabilization (Lusis, 2000).

Cn inhibitors have an inhibitory effect on VSMC phenotypic modulation. An established model for phenotypic modulation in vitro involves treatment of VSMCs with platelet-derived growth factor-BB (PDGF-BB) to induce proliferation (Owens et al., 2004; Wamhoff et al., 2004b). VSMCs and platelets produce PDGF-BB in response to acute vascular injury. In cell culture, CSA decreases PDGF-BB-induced VSMC proliferation (Liu et al., 2005b; Lee et al., 2010). CSA inhibits Cn activity and subsequent NFAT nuclear translocation in VSMCs (Boss et al., 1998; Stevenson et al., 2001; Gomez et al., 2002; Jabr et al., 2007). Specific inhibition of NFAT activity with A-285222 (Djuric et al., 2000; Trevillyan et al., 2001) also decreases PDGF-BB-induced proliferation (Nilsson et al., 2007). Another NFAT-specific inhibitor, the peptide MAGPHPVIVITGPHEE, and CSA both reduce balloon injury-induced neointima formation by approximately 40% in the rat carotid model (Liu et al., 2005b).

Although CSA clearly prevents VSMC proliferation, very little is known about the direct effects of CSA on VSMC molecular phenotype. We hypothesized that CSA inhibition of VSMC proliferation would parallel a MYOCD-dependent pathway to promote VSMC differentiation. Surprisingly, we show here that CSA suppressed the expression of MYOCD and VSMC markers, concomitant with up-regulation of the transcription factor Krüppel-like factor-4 (KLF4). KLF4 is involved with many cellular processes, including proinflam-

matory endothelial activation (Hamik et al., 2007), tumor development (Rowland et al., 2005), and stem cell biology (Takahashi and Yamanaka, 2006). In VSMCs, KLF4 both promotes phenotypic modulation and inhibits proliferation. PDGF-BB treatment caused acute up-regulation of KLF4 and down-regulation of VSMC marker genes that was prevented by siRNA knockdown of KLF4 (Liu et al., 2005a). Despite down-regulating VSMC marker genes, KLF4 activates the tumor suppressor gene *CDKN1A* (p21) in a p53-dependent manner, resulting in reduced VSMC proliferation (Wassmann et al., 2007). In vivo, conditional deletion of murine *KLF4* enhanced neointima formation and delayed down-regulation of VSMC marker genes following vascular injury (Yoshida et al., 2008b). Consistent with antiproliferative effects of CSA, we show that CSA increased VSMC expression of KLF4 in both cell culture and in vivo, with down-regulation of VSMC differentiation marker genes.

Materials and Methods

Cell Culture. Rat aortic SMCs were plated and allowed to attach for 24 h in Dulbecco's modified Eagle's medium/F12 growth media supplemented with 10% FBS, L-glutamine (1.6 mM), penicillin G (100 U/ml), and streptomycin sulfate (100 μ g/ml). For subconfluent protocol, cells were growth-arrested at 50 to 75% confluence for 48 to 72 h in insulin-free serum-free media supplemented with L-ascorbic acid (3.52 mg/ml), apotransferrin (5 μ g/ml), and selenium selenite (6.25 ng/ml) in addition to L-glutamine, penicillin, and streptomycin. For postconfluent protocol, cells were grown to confluence and then growth-arrested for 3 days in serum-free media. Before PDGF-BB stimulation, cells were pretreated with inhibitor for 30 min. Reagents used were PDGF-BB (30 ng/ml; Millipore, Billerica, MA); cyclosporine A (1–10 μ M; Sigma-Aldrich, St. Louis, MO); FK506 (10 μ M; Sigma-Aldrich); and A-285222 (10 μ M, a gift from Abbott Laboratories, Abbott Park, IL) (compound **19** in Djuric et al., 2000).

Quantitative Real-Time RT-PCR. At time of harvest, VSMCs were washed once in phosphate-buffered saline and lysed in 350 μ l of RNeasy lysis buffer (QIAGEN, Valencia, CA). Total RNA was prepared according to manufacturer's instructions (RNeasy Kit; QIAGEN). cDNA was synthesized from 0.2 μ g of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). SYBR Green dye-based quantitative real-time polymerase chain reaction (RT-PCR) was used to measure DNA amplification (iCycler; Bio-Rad). Results were normalized to expression of the 18S rRNA gene and expressed as starting quantity. Oligonucleotide primer sequences are listed in Supplemental Table 1. For microRNA (MIR) expression studies, the QIAGEN miRNeasy preparation kit, miScript cDNA synthesis kit, and SYBR Green dye-based real-time assays (rat MIR143, MIR145, MIR221, MIR222, and RNU6B; www.geneglobe.com) were used. All target MIR expression values were normalized to the MIR control RNU6B. SigmaStat 3.1 software was used to perform *t* test and determine statistical significance in all datasets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Error bars show 1 S.E.M.

Western Blot Analysis. VSMCs were washed twice with ice-cold 1 $\times$ phosphate-buffered saline and lysed in either a modified radioimmunoprecipitation buffer or 2 $\times$ Laemmli SDS sample buffer (Bio-Rad). Lysates were snap-frozen, thawed, vortexed for 30 s, and then centrifuged for 60 s at 12,000g. Supernatant was heated for 60 s at 95°C, loaded in denaturing 4 to 15% polyacrylamide Tris-HCl gels (Bio-Rad), and separated at 20 mA. Gels were transferred to polyvinylidene difluoride membranes using semi-dry blot apparatus (Bio-Rad). Membranes were blocked with 5% nonfat dry milk, 1% donkey serum, and 0.1% Tween 20 in Tris-buffered saline and stained with primary antibody for 60 min at room temperature in blocking buffer. Membranes were then stained with horseradish peroxidase-conju-

gated goat anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 45 min at room temperature in blocking buffer and washed 1×15 min and then 3×5 min each in 0.1% Tween 20 in Tris-buffered saline. Immunodetection was carried out with the enhanced chemiluminescence (ECL) Plus Western blotting detection system (GE Healthcare Life Sciences, Piscataway, NJ). Primary antibodies for Western blot included KLF4 peptide antibody (1:1000, raised against mouse KLF4 from amino acid 15 to 29, ASGPAGREKTLRPAG; a gift from Dr. G. K. Owens) (Yoshida et al., 2008a) and β -tubulin antibody (1:1000; Cell Signaling Technology Inc., Danvers, MA). Optical scanning densitometry was performed using ImageJ software.

KLF4 Promoter-Reporter Assay. VSMCs were growth-arrested at 50% confluence and transfected with a 520-base pair KLF4 promoter-luciferase reporter construct (Deaton et al., 2009) in FuGENE 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN). Three days later, cells were harvested in $1 \times$ passive lysis buffer (Promega, Madison, WI) at designated times. Luciferase activity was determined with a FLUOstar plate reader (OMEGA; BMG Labtech, Cary, NC), and fluorescence units were normalized to total protein.

siKLF4 Transfection. Three short interfering double-stranded RNAs to KLF4 (siKLF4-I: 5'-GGUACAGAACGUGUUCUAAUAGCCT-3'; siKLF4-II: 5'-GCAAGUCAGUUGUGAAUGGAUAAT-3'; siKLF4-III: 5'-CCAUAUCAAGAGCUCAUGCCACCG-3') and the Universal scrambled control were purchased from DNA Technologies, Inc. (Corallville, IA). At 50% confluence, cells were growth-arrested and simultaneously transfected with 10 nM siKLF4 in Oligofectamine reagent (Invitrogen, Carlsbad, CA) for 3 days before treatment.

Administration of Pluronic Gel to Rat Carotid Arteries. The animal protocol was approved by the Animal Care and Use Committee at the University of Virginia. This procedure has been described previously (Pidkovka et al., 2007). In brief, six Sprague-Dawley rats (150–180 g) were used per group per experiment. Rats were anesthetized with ketamine/xylazine. Ice-cold Pluronic F127 gel (150 μ l; 50% in sterile water), containing 100 μ M CSA or A-285222, was pipetted around the adventitial surface of the right carotid artery. Treated and untreated arteries were harvested 12 and 24 h later and prepared for quantitative real-time RT-PCR analysis and paraffin embedding.

Immunohistochemistry. Paraffin sections were assessed for histopathology using the Modified Russell-Movat Pentachrome Method and hematoxylin and eosin. KLF4 immunohistochemistry has been described previously (Yoshida et al., 2008b). In brief, 5- μ m paraffin sections underwent microwave antigen retrieval (Vector Laboratories, Burlingame, CA) and incubation with KLF4 antibody. Primary antibody was detected with the VECTASTAIN Elite Kit (Vector Laboratories) and visualized with 3,3'-diaminobenzidine (Dako North America, Inc., Carpinteria, CA). Counterstaining was done using Harris hematoxylin (Thermo Scientific, Waltham, MA).

Results

CSA Up-Regulates KLF4 in Vascular SMCs. PDGF-BB treatment of subconfluent VSMCs induced KLF4 mRNA levels acutely with peak levels at 1 h (>30-fold induction;

Fig. 1. CSA up-regulates KLF4 expression in subconfluent VSMCs. **A**, quantitative real-time RT-PCR analysis of KLF4 mRNA levels in rat aortic SMCs treated with 30 ng/ml PDGF-BB alone (■), and PDGF-BB with 30-min pretreatments with 3 μ M CSA (▲) or 10 μ M A-285222 (■). Samples were harvested at 0.5, 1, 2, 3, 4, 6, 8, 12, 18, and 24 h following addition of PDGF-BB. KLF4 starting quantity mRNA values were normalized to 18S rRNA and expressed as fold change compared with vehicle-treated controls. **B**, real-time RT-PCR analysis of KLF4 mRNA levels treated with either CSA (■) or A-285222 (▲) alone. **C**, dose-response of KLF4 mRNA levels to CSA in VSMCs. **D**, Western blot analysis of VSMCs treated with vehicle, PDGF-BB, and CSA for 24 h. Arrow points to the anti-KLF4 immunoreactive band that corresponds to nuclear protein. **E**, optical scanning densitometry of anti-KLF4 immunoreactivity normalized to β -tubulin. **F**, time course of KLF4 protein levels following CSA treatment. **G**, effect of 12-h DMSO vehicle treatment on KLF4 indirect immunofluorescence. **H**, effect of 12-h CSA treatment on KLF4 indirect immunofluorescence in VSMCs. Arrows point to enhanced nuclear staining. VEH, vehicle; CSA, cyclosporine A. *, $p < 0.05$, CSA versus vehicle control.

Fig. 1A). Pretreatment with either the Cn inhibitor CSA or the NFAT inhibitor A-285222 did not significantly affect PDGF-BB-mediated early accumulation of KLF4 mRNA (Fig. 1A). KLF4 mRNA levels returned to baseline levels following 24 h of treatment with PDGF-BB or with A-285222 in addition to PDGF-BB. However, pretreatment with CSA, not A-285222, resulted in a >15-fold accumulation of KLF4 mRNA at 24 h (Fig. 1A; Supplemental Fig. 1, A and C). We then treated subconfluent VSMCs with CSA or A-285222 alone. CSA increased the abundance of both KLF4 mRNA and protein. The increase in KLF4 mRNA was time-dependent, increasing gradually to 13-fold greater abundance than vehicle-treated cells at 24 h after treatment (Fig. 1B; Supplemental Fig. 1, B and D). The effect of CSA was also dose-dependent (Fig. 1C). The increase in KLF4 mRNA translated to a 2.5-fold increase in KLF4 protein by 24 h compared with both vehicle and PDGF-BB treatments (Fig. 1, D and E). A sustained increase in KLF4 protein was observed 4.5 h after treatment (Fig. 1F). Twelve hours after treatment, accumulation of the KLF4 transcription factor was observed within nuclei of CSA-treated VSMCs compared with vehicle-treated controls (Fig. 1, G and H). KLF4 minimal promoter-luciferase plasmid construct activity was not affected by CSA treatment (Supplemental Fig. 2).

CSA Down-Regulates VSMC Differentiation Marker Genes. Induction of KLF4 has been shown to down-regulate myocardin and other classic markers of VSMC differentiation (Liu et al., 2005a). Given the induction of KLF4 with CSA treatment, we hypothesized that CSA would down-regulate the transcription factor MYOCD and VSMC contractile genes. To test this hypothesis, we assayed target gene expression by quantitative real-time RT-PCR in VSMCs grown 3 days beyond confluence (postconfluent protocol), where up-regulation of contractile genes and differentiation of VSMCs are remarkably

enhanced (Wamhoff et al., 2004a). In the postconfluent model, 10 μ M CSA treatment increased KLF4 mRNA levels 33-fold compared with vehicle-treated controls (Fig. 2). The Cn inhibitor FK506 increased KLF4 mRNA levels 7-fold. A-285222 increased KLF4 by 1.5-fold. CSA (10 μ M) and FK506 treatment decreased myocardin mRNA levels by 86 and 64%, respectively, compared with vehicle-treated controls (Fig. 2). CSA also decreased ACTA2, TAGLN, and SMTN mRNA levels by 91, 79, and 76%, respectively. FK506 decreased ACTA2, TAGLN, and SMTN mRNA levels by 63, 46, and 48%, respectively (Fig. 2). A-285222 had no effect.

Consistent with the antiproliferative effects of CSA, tumor suppressor CDKN1A (p21) mRNA levels were increased 2.2-fold. CSA treatment also caused cell detachment (data not shown), so we investigated the expression of lamina-degrading matrix metalloproteinases (MMPs). CSA and FK506 increased MMP3 mRNA levels 4.2- and 3.7-fold, respectively, compared with vehicle-treated controls (Fig. 2). Neither CSA nor FK506 increased MMP2 or MMP9 mRNA levels in cultured VSMCs (Fig. 2). CSA and FK506 did, however, increase collagen-VIII (COL8) mRNA levels 1.7- and 7.5-fold, respectively (Fig. 2). COL8 is an ECM protein associated with VSMC pathology and phenotypic modulation and regulated by KLF4 (Cherepanova et al., 2009).

The KLF4 dependence of CSA-induced VSMC differentiation marker gene down-regulation was tested in the subconfluent protocol with short interfering RNA oligonucleotides designed against KLF4 (siKLF4). In particular, siKLF4-III caused the largest reduction (69%) in basal vehicle-treated KLF4 mRNA compared with scrambled siSCR control (Fig. 3A). siKLF4-III also reduced CSA induction of KLF4 by 91% compared with siSCR. Consistent with the role of KLF4 in regulating ACTA2, transfection with siKLF4 increased basal ACTA2 mRNA levels by 68% (Fig. 3C). Despite KLF4

Fig. 2. Real-time RT-PCR analysis of the effects of CSA, FK506, and A-285222 on VSMC differentiation marker gene expression and other genes associated with phenotypic modulation. Starting quantity mRNA values were normalized to 18S and expressed as fold change compared with DMSO vehicle-treated cells. Cells were cultured according to the postconfluent protocol (see *Materials and Methods*), which enhanced VSMC differentiation marker gene expression. VEH, vehicle; CSA3, 3 μ M cyclosporine-A; CSA10, 10 μ M cyclosporine A; FK506 at 10 μ M. *, $p < 0.05$; **, $p < 0.01$, treatment versus vehicle control.

Fig. 3. CSA-induced down-regulation of MYOCD and ACTA2 is not dependent on KLF4. A, validation of siKLF4 knockdown by quantitative real-time RT-PCR analysis. Three separate siRNA oligonucleotides targeting KLF4 were tested in VSMCs treated with CSA (3 μ M) and vehicle control. B, effect of siKLF4-III on MYOCD mRNA levels in VSMCs treated with CSA. C, effect of siKLF4-III on ACTA2 mRNA levels. D, effect of 10% FBS or CSA on the expression of MIR143, MIR145, MIR221, and MIR222. MIR starting level values were normalized to control MIR RNU6B. *, $p < 0.05$; **, $p < 0.01$, treatment versus vehicle. †, $p < 0.05$, ††, $p < 0.01$, siKLF4/VEH versus siSCR/VEH control. ns, not significant.

knockdown, ACTA2 and MYOCD mRNA levels still decreased significantly with CSA treatment (Fig. 3, B and C).

We also tested the effects of CSA on the expression of several MIRs recently implicated in VSMC phenotypic modulation. Overexpression of MIR145 up-regulates the expression of VSMC marker genes in culture and inhibits neointima formation after injury in vivo (Cheng et al., 2009). Conversely, MIR221 down-regulates VSMC marker genes and promotes proliferation. Knockdown of MIR221 and MIR222 inhibits neointima formation after injury in vivo (Liu et al., 2009). As predicted, 10% FBS decreased MIR143/MIR145 expression and increased MIR221/222 expression (Fig. 3D). CSA had no significant effect on the expression of these MIRs (Fig. 3D). Differential regulation of these MIRs probably does not play a role in CSA-induced VSMC phenotypic modulation.

CSA Induces Remodeling of Rat Carotid Artery Medial Layer. CSA treatment of intact, uninjured rat carotid arteries in vivo caused rapid structural remodeling of the carotid arteries. The carotid artery was exposed to a chilled solution of Pluronic gel containing CSA. Upon application,

the Pluronic gel hardens around the vessel and delivers CSA to the artery wall. At 12 h after surgery, both CSA-treated right carotid arteries and untreated left carotid arteries were harvested and embedded in paraffin for light microscopy. Cross-sections of the CSA-treated arteries show reorientation of the elongated medial SMC nuclei (Fig. 4, B–D), which normally align perpendicular to the radial axis of the vessel (Fig. 4A). CSA treatment also stimulated breakdown of the internal elastic lamina (IEL) (Fig. 4, F–H). Endothelial and adventitial layers displayed no abnormal morphology.

CSA and A-285222 Down-Regulate VSMC Differentiation Marker Genes In Vivo. Several markers of VSMC differentiation and phenotypic modulation showed evidence of differential expression in both CSA- and A-285222 Pluronic gel-treated carotid arteries. After 12 h of CSA treatment of right carotid arteries, KLF4 mRNA levels were increased 1.9-fold compared with untreated left carotid arteries (Fig. 5). By 24 h, KLF4 mRNA levels had returned to baseline levels. At 12 h, A-285222 increased KLF4 mRNA levels by 40%. In a separate control experiment, 12-h DMSO vehicle/Pluronic gel-treated right arteries showed no significant change in KLF4, MYOCD, CDKN1A, MMP3, and MMP9 mRNA levels compared with untreated left carotid arteries

Fig. 4. Acute exposure to CSA promotes vascular wall remodeling. CSA/Pluronic gel was administered to rat carotid arteries for 12 h. A to D, hematoxylin and eosin staining of CSA-treated arteries. E to H, modified Russell-Movat pentachrome staining of CSA-treated arteries. A and E, untreated control; B to D and F to H, 100 μ M CSA/50% Pluronic gel. pg, Pluronic gel; ad, adventitia; m, media; lu, lumen.

Fig. 5. Acute exposure of rat carotid arteries to A-285222 and CSA differentially affects expression of genes involved with VSMC phenotypic modulation. 100 μ M CSA/Pluronic gel was administered to rat carotid arteries for 12 and 24 h. A-285222/Pluronic gel (100 μ M) was administered for 12 h only. Starting quantity mRNA values were normalized to 18S and expressed as percentage change between treated and untreated control arteries (six arteries per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, CSA versus untreated control.

(Supplemental Fig. 3). CSA-induced down-regulation of VSMC differentiation marker genes was again observed here in vivo. MYOCD, ACTA2, MYH11, and SMTN mRNA levels were decreased by 32, 38, 57, and 41% respectively, compared with untreated left carotid arteries (Fig. 5). TAGLN expression showed no statistically significant change. In response to A-285222, MYOCD, ACTA2, MYH11, TAGLN, and SMTN mRNA levels were decreased by 47, 40, 44, 35, and 50%, respectively, compared with untreated left carotid arteries. By 24 h after CSA treatment, ACTA2 and MYH11 levels were still decreased by 29 and 32%. mRNA levels of the tumor suppressor genes p53 and CDKN1A were increased 2.4- and 2.0-fold, respectively, and mRNA levels of the matrix metalloproteinases MMP3 and MMP9 were increased 2.3- and 1.9-fold, respectively. MMP2 levels were decreased by 20%. CSA increased COL8 mRNA levels 2.5-fold. A-285222 treatment also increased mRNA levels of TP53, CDKN1A, MMP3, MMP9, and COL8. Most CSA-induced changes in gene expression returned to baseline levels after 24 h of treatment. Relative starting quantity mRNA values with standard deviations are shown in Supplemental Fig. 4. Altogether, these data show that the effects of CSA/Pluronic gel administration on rat carotid arteries are both rapid and transient.

**CSA Increases KLF4 Immunostaining of Rat Vascu-
lature.** Immunohistochemistry was used to test the abundance and localization of KLF4 protein after 12-h administration of CSA/Pluronic gel to rat carotid arteries in vivo. Under normal conditions, KLF4 was observed in some cells of

the adventitial layer, with minimal staining of the medial and endothelial layers (Fig. 6A). CSA treatment caused a dramatic increase in KLF4 immunostaining within the medial wall (Fig. 6, B–F). KLF4 localized to both cytoplasmic and nuclear compartments of SMCs clearly undergoing phenotypic modulation (Fig. 6B, dotted line). The internal elastic lamina was disorganized and often absent (Fig. 6, asterisks). KLF4 immunostaining also increased in the medial wall of vessels that showed normal structural morphology 12 h after treatment (Fig. 6E). Intriguingly, CSA increased KLF4 levels within the endothelial layer as well (Fig. 6, arrowheads). In rat endothelial cell monoculture, 24-h CSA treatment increased KLF4 and MMP3 mRNA levels 5.4- and 11.1-fold, respectively (Supplemental Fig. 5).

Discussion

CSA immunosuppression is associated with post-transplant arteriopathy and higher risk for CAD, hypertension, and hyperlipidemia (Miller, 2002; Lindenfeld et al., 2004; Bianchi et al., 2008), but little is known about how CSA affects cellular phenotypes in the vasculature. We show here that CSA treatment of rat VSMCs increased both KLF4 mRNA and protein. We also show that CSA treatment of rat carotid arteries in vivo up-regulated KLF4. KLF4 is a pivotal transcription factor involved with VSMC phenotypic modulation from the contractile phenotype toward proliferative, migratory, and/or inflammatory phenotypes. In both VSMC

Fig. 6. CSA treatment of rat carotid artery increases KLF4 immunostaining. CSA/Pluronic gel was administered to rat carotid arteries for 12 h. Arteries were subsequently prepared for paraffin embedding and immunohistochemistry. A, untreated control. B to F, 100 μ M CSA/Pluronic gel. KLF4 immunoreactivity, brown; nuclei, blue; arrow, nuclear staining; arrowhead, endothelial cell staining; asterisk, internal elastic lamina breakdown; dotted line, zone of differential VSMC morphology; pg, Pluronic gel; ad, adventitia; m, media; lu, lumen.

culture and in vivo, CSA treatment was associated with down-regulation of VSMC differentiation marker genes and up-regulation of CDKN1A, MMP3, and COL8. CSA treatment in vivo also caused remarkable changes in VSMC morphology. Altogether, we conclude that CSA is a protagonist of VSMC phenotypic modulation.

CSA is a potent repressor of VSMC differentiation marker gene expression (Figs. 2 and 5). The prevailing model of *ACTA2* down-regulation involves direct binding of the KLF4 repressor to the proximal promoter. *ACTA2* down-regulation in response to PDGF-BB or serum is dependent on KLF4 binding to the *ACTA2* promoter (Liu et al., 2005a). Oxidized phospholipid (oxPL)-mediated down-regulation of *ACTA2* is also dependent on KLF4 (Yoshida et al., 2008a). In vivo, *ACTA2* down-regulation was delayed following vascular injury in *KLF4*-conditional knockout mice (Yoshida et al., 2008b). This in vivo response, albeit delayed, suggests that KLF4-independent mechanisms for *ACTA2* down-regulation do exist. Intriguingly, we show that *ACTA2* down-regulation was not dependent on KLF4 in response to CSA treatment (Fig. 3). There are several possible explanations. First, transcriptional activators may be more strongly blocked by CSA treatment. Indeed, the dramatic depletion (86%) of MYOCD mRNA in response to CSA (Fig. 2), and perhaps even the MYOCD-related transcription factors, may be sufficient to deactivate *ACTA2* transcription. Second, functionally redundant transcriptional repressors may be activated by CSA. One such repressor could be the transcription factor ETS gene like-1 (ELK1), which has already been shown to cooperate with KLF4 in down-regulation of *ACTA2* transcription (Wang et al., 2004; Yoshida et al., 2008a). Intriguingly, ELK1 is a validated target of the Cn phosphatase in several non-SMC cell types (Sugimoto et al., 1997). Cn activation and subsequent ELK1 dephosphorylation inactivates the transcriptional activity of ELK1 (Tian and Karin, 1999). Conversely, Cn inhibition restores ELK1 activity and downstream target gene regulation (Tian and Karin, 1999). Induction and/or activation of ELK1 could mediate down-regulation of *ACTA2* in response to CSA.

The mechanism of CSA-induced VSMC phenotypic modulation does not exclusively involve inhibition of the NFAT family of transcription factors in cell culture. It has previously been shown that NFATc1 and NFATc3 proteins positively regulate *MYH11* and *ACTA2* expression, respectively, and that a combination of CSA and FK506 decreases *ACTA2* protein levels in rat A7r5 aortic SMCs (Wada et al., 2002; Gonzalez Bosc et al., 2005). The A-285222 compound used in this study specifically inhibits NFAT dephosphorylation. Compared with CSA and FK506, A-285222 treatment of VSMCs caused little increase in KLF4 mRNA levels and did not significantly affect expression of VSMC marker genes in cell culture (Fig. 2). Whereas VSMC marker gene induction may require NFATc1 or NFATc3, it does not appear that NFAT proteins affect basal levels of high VSMC marker gene expression in postconfluent VSMCs. It is intriguing that A-285222 down-regulated VSMC marker gene expression in vivo in intact carotid arteries (Fig. 5). These data suggest that either NFAT expression in VSMCs enhances contractile gene expression in vivo or that A-285222 has NFAT-independent effects that suppress VSMC marker gene expression. Regardless, A-285222 had little effect on VSMC marker gene in cell culture. Thus, CSA and FK506 treatment probably

leads to phenotypic modulation through either 1) alteration of the activity of a non-NFAT calcineurin target (such as ELK1), or 2) Cn-independent effects. To address the first hypothesis, we attempted to reduce Cn activity by siRNA-mediated knockdown of calcineurin-B1 (CNB1). Although CNB1 mRNA was sufficiently reduced, KLF4 mRNA levels did not change (data not shown). However, we were unable to successfully assay loss of Cn activity.

Cn-independent effects of CSA have been well characterized in VSMCs. Perhaps the most evident Cn-independent effect results from the interaction of CSA and the chaperone protein CYPA, needed for Cn inhibition. The binding of CSA to CYPA inhibits its peptidylprolyl *cis-trans*-isomerase activity. CYPA activity is critical in vascular remodeling. Targeted deletion of *CYPA* in mice reduced medial and neointimal hyperplasia and VSMC proliferation following carotid ligation (Satoh et al., 2008). Conversely, SMC-specific transgenic *CYPA* overexpression increased medial and neointimal hyperplasia and VSMC proliferation (Satoh et al., 2008). In addition, murine aortic SMCs derived from the *CYPA* knockout and transgenic mice show decreased and increased migratory/proliferative responses to serum, respectively. Reduced CYPA activity could explain the acute vascular remodeling observed after Pluronic gel delivery of CSA to rat carotid arteries (Fig. 4). Another Cn-independent effect of CSA is inhibition of the mitochondrial permeability transition pore complex, which can disrupt calcium handling and respiration (Bernardi, 1999). CSA also stimulates VSMC production of reactive oxygen species (ROS), including superoxide (Krauskopf et al., 2005). ROS are key signaling molecules involved with VSMC gene regulation, proliferation, migration, and contraction (Lyle and Griendling, 2006). It is intriguing that ROS up-regulates the expression of KLF4 in VSMCs (Nickenig et al., 2002). ROS generation may partly explain *KLF4* up-regulation in response to CSA; however, pretreatment with the antioxidant *N*-acetyl-cysteine or ebselen did not curb KLF4 induction (data not shown).

Unlike the early and transient response to PDGF-BB and serum, CSA caused a gradual and sustained increase in KLF4 in VSMC culture (Fig. 1). Although PDGF-BB increased *KLF4* minimal promoter (520 bp) activity, CSA had no effect (Supplemental Fig. 2). These data suggest that downstream effectors of CSA may require *cis*-acting regulatory elements outside the 520-bp region sufficient for PDGF-BB activation. Very little is known about positive regulation of the *KLF4* promoter in VSMCs. The SP1 transcription factor appears to play the role of a coactivator in response to PDGF-BB (Deaton et al., 2009), but it is unclear which factors truly initiate activation of *KLF4*.

KLF4 activation has been previously implicated in VSMC migration. The promigratory effect of oxPLs was inhibited by both knockdown of KLF4 in rat aortic SMCs and targeted deletion of *KLF4* in mouse aortic SMCs (Cherepanova et al., 2009). The authors also showed that oxPL-induced SMC migration was dependent on COL8. OxPL treatment increased KLF4 occupancy at the *COL8* promoter, suggesting that KLF4 activates transcription of this promigratory collagen. We observed a similar up-regulation of COL8 in VSMCs and carotid arteries in response to CSA (Figs. 2 and 5). Treatment of VSMCs with CSA may trigger KLF4 to both 1) up-regulate MMPs to clear the extracellular matrix and 2) activate a subset of collagens to redefine the functional ECM landscape.

We observed up-regulation of MMP3 and MMP9 mRNA levels in rat carotid arteries following CSA treatment (Fig. 5), concomitant with reorientation of medial SMCs and IEL breakdown (Fig. 4). MMP3 and MMP9 have previously been implicated in the breakdown of the IEL, VSMC migration, and vascular pathology. For example, *MMP9* knockout mice show increased levels of collagen in the vascular wall and decreased neointimal hyperplasia in the carotid flow cessation model (Galis et al., 2002). In cell culture, *MMP9*-deficient aortic SMCs showed that both decreased migratory behavior in a cell culture wound assay (Galis et al., 2002). We hypothesize that KLF4 activity promotes *MMP3* and *MMP9* expression in response to CSA, similar to the requirement for KLF4 activation of *COL8* in response to oxPL.

To potentiate a promigratory signaling cascade, a VSMC may need to block subthreshold proliferative cues. CSA treatment of rat carotid arteries was associated with up-regulation of the tumor suppressors *CDKN1A* and *p53* (Fig. 5). In cell culture, it has been shown that KLF4 directly activates *CDKN1A* and inhibits VSMC proliferation (Wassmann et al., 2007). KLF4 appears to block VSMC proliferation while simultaneously promoting VSMC de-differentiation. Thus, KLF4 may be critical for creating a window of "stemness" or phenotypic opportunity, during which time the VSMC stalls commitment to a particular proliferative, migratory, or inflammatory phenotype. After activation of KLF4 in response to CSA, for example, up-regulation of *MMPs* and *COL8* may drive a migratory phenotype.

Altogether, we show that CSA has direct molecular consequences on VSMCs and whole arteries, consistent with phenotypic modulation. CSA up-regulated KLF4, down-regulated classic VSMC differentiation marker genes, and up-regulated genes associated with VSMC migration. Although our findings do not completely explain the higher incidence of vascular complications in immunosuppressed post-transplant subjects, they do show that CSA has direct effects on the vascular wall.

Acknowledgments

We thank Dominique Rose, Monica Lee, Oana Nicoara, and Melissa Bevard for technical assistance and Themis Karaoli and Jeffrey Wilson for comment on the manuscript.

References

- Bernardi P (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol Rev* **79**:1127–1155.
- Bianchi G, Marchesini G, Marzocchi R, Pinna AD, and Zoli M (2008) Metabolic syndrome in liver transplantation: relation to etiology and immunosuppression. *Liver Transpl* **14**:1648–1654.
- Boss V, Abbott KL, Wang XF, Pavlath GK, and Murphy TJ (1998) The cyclosporin A-sensitive nuclear factor of activated T cells (NFAT) proteins are expressed in vascular smooth muscle cells. Differential localization of NFAT isoforms and induction of NFAT-mediated transcription by phospholipase C-coupled cell surface receptors. *J Biol Chem* **273**:19664–19671.
- Chen J, Kitchen CM, Streb JW, and Miano JM (2002) Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol* **34**:1345–1356.
- Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES, and Zhang C (2009) MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ Res* **105**:158–166.
- Cherepanova OA, Pidkova NA, Sarmento OF, Yoshida T, Gan Q, Adiguzel E, Bendeck MP, Berliner J, Leitinger N, and Owens GK (2009) Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ Res* **104**:609–618.
- Deaton RA, Gan Q, and Owens GK (2009) Sp1-dependent activation of KLF4 is required for PDGF-BB-induced phenotypic modulation of smooth muscle. *Am J Physiol Heart Circ Physiol* **296**:H1027–H1037.
- Djuric SW, BaMaung NY, Basha A, Liu H, Luly JR, Madar DJ, Sciotti RJ, Tu NP, Wagenaar FL, Wiedeman PE, et al. (2000) 3,5-Bis(trifluoromethyl)pyrazoles: a novel class of NFAT transcription factor regulator. *J Med Chem* **43**:2975–2981.
- Flanagan WM, Cortés B, Bram RJ, and Crabtree GR (1991) Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature* **352**:803–807.
- Galis ZS, Johnson C, Godin D, Magid R, Shipley JM, Senior RM, and Ivan E (2002) Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ Res* **91**:852–859.
- Gerthoffer WT (2007) Mechanisms of vascular smooth muscle cell migration. *Circ Res* **100**:607–621.
- Gomez MF, Stevenson AS, Bonev AD, Hill-Eubanks DC, and Nelson MT (2002) Opposing actions of inositol 1,4,5-trisphosphate and ryanodine receptors on nuclear factor of activated T-cells regulation in smooth muscle. *J Biol Chem* **277**:37756–37764.
- Gonzalez Bosc LV, Layne JJ, Nelson MT, and Hill-Eubanks DC (2005) Nuclear factor of activated T cells and serum response factor cooperatively regulate the activity of an alpha-actin intronic enhancer. *J Biol Chem* **280**:26113–26120.
- Hamik A, Lin Z, Kumar A, Balcells M, Sinha S, Katz J, Feinberg MW, Gerzsten RE, Edelman ER, and Jain MK (2007) Kruppel-like factor 4 regulates endothelial inflammation. *J Biol Chem* **282**:13769–13779.
- Jabr RI, Wilson AJ, Riddervold MH, Jenkins AH, Perrino BA, and Clapp LH (2007) Nuclear translocation of calcineurin A-beta but not calcineurin A-alpha by platelet-derived growth factor in rat aortic smooth muscle. *Am J Physiol Cell Physiol* **292**:C2213–C2225.
- Krauskopf A, Lhote P, Petermann O, Ruegg UT, and Buetler TM (2005) Cyclosporin A generates superoxide in smooth muscle cells. *Free Radic Res* **39**:913–919.
- Lee MY, Garvey SM, Baras AS, Lemmon JA, Gomez MF, Schoppe Bortz PD, Daum G, LeBoeuf RC, and Wamhoff BR (2010) Integrative genomics identifies DSCR1 (RCAN1) as a novel NFAT-dependent mediator of phenotypic modulation in vascular smooth muscle cells. *Hum Mol Genet* **19**:468–479.
- Lindenfeld J, Miller GG, Shakar SF, Zolty R, Lowes BD, Wolfel EE, Mestroni L, Page RL 2nd, and Kobashigawa J (2004) Drug therapy in the heart transplant recipient: part II: immunosuppressive drugs. *Circulation* **110**:3858–3865.
- Liu X, Cheng Y, Zhang S, Lin Y, Yang J, and Zhang C (2009) A necessary role of miR-221 and miR-222 in vascular smooth muscle cell proliferation and neointimal hyperplasia. *Circ Res* **104**:476–487.
- Liu Y, Sinha S, McDonald OG, Shang Y, Hoofnagle MH, and Owens GK (2005a) Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. *J Biol Chem* **280**:9719–9727.
- Liu Z, Zhang C, Dronadula N, Li Q, and Rao GN (2005b) Blockade of nuclear factor of activated T cells activation signaling suppresses balloon injury-induced neointima formation in a rat carotid artery model. *J Biol Chem* **280**:14700–14708.
- Lusis AJ (2000) Atherosclerosis. *Nature* **407**:233–241.
- Lyle AN, and Griending KK (2006) Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda)* **21**:269–280.
- Miller LW (2002) Cardiovascular toxicities of immunosuppressive agents. *Am J Transplant* **2**:807–818.
- Nickenig G, Baudier S, Müller C, Werner C, Werner N, Welzel H, Strehlow K, and Böhm M (2002) Redox-sensitive vascular smooth muscle cell proliferation is mediated by GKLF and Id3 in vitro and in vivo. *FASEB J* **16**:1077–1086.
- Nilsson LM, Sun ZW, Nilsson J, Nordström I, Chen YW, Molkentin JD, Wide-Swensson D, Hellstrand P, Lydrup ML, and Gomez MF (2007) Novel blocker of NFAT activation inhibits IL-6 production in human myometrial arteries and reduces vascular smooth muscle cell proliferation. *Am J Physiol Cell Physiol* **292**:C1167–C1178.
- Orr AW, Hastings NE, Blackman BR, and Wamhoff BR (2010) Complex regulation and function of the inflammatory smooth muscle cell phenotype in atherosclerosis. *J Vasc Res* **47**:168–180.
- Owens GK (1995) Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* **75**:487–517.
- Owens GK, Kumar MS, and Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* **84**:767–801.
- Pidkova NA, Cherepanova OA, Yoshida T, Alexander MR, Deaton RA, Thomas JA, Leitinger N, and Owens GK (2007) Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. *Circ Res* **101**:792–801.
- Rowland BD, Bernards R, and Peeper DS (2005) The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat Cell Biol* **7**:1074–1082.
- Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, et al. (2008) Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation* **117**:3088–3098.
- Shaw JP, Utz PJ, Durand DB, Toole JJ, Emmel EA, and Crabtree GR (1988) Identification of a putative regulator of early T cell activation genes. *Science* **241**:202–205.
- Stevenson AS, Gomez MF, Hill-Eubanks DC, and Nelson MT (2001) NFAT4 movement in native smooth muscle. A role for differential Ca(2+) signaling. *J Biol Chem* **276**:15018–15024.
- Sugimoto T, Stewart S, and Guan KL (1997) The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. *J Biol Chem* **272**:29415–29418.
- Takahashi K and Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**:663–676.
- Tian J and Karin M (1999) Stimulation of Elk1 transcriptional activity by mitogen-activated protein kinases is negatively regulated by protein phosphatase 2B (calcineurin). *J Biol Chem* **274**:15173–15180.
- Trevillyan JM, Chiou XG, Chen YW, Ballaron SJ, Sheets MP, Smith ML, Wiedeman PE, Warrior U, Wilkins J, Gubbins EJ, et al. (2001) Potent inhibition of NFAT activation and T cell cytokine production by novel low molecular weight pyrazole compounds. *J Biol Chem* **276**:48118–48126.
- Wada H, Hasegawa K, Morimoto T, Kakita T, Yanazume T, Abe M, and Sasayama S (2002) Calcineurin-GATA-6 pathway is involved in smooth muscle-specific transcription. *J Cell Biol* **156**:983–991.
- Wamhoff BR, Bowles DK, McDonald OG, Sinha S, Somlyo AP, Somlyo AV, and Owens GK (2004a) L-type voltage-gated Ca2+ channels modulate expression of

- smooth muscle differentiation marker genes via a rho kinase/myocardin/SRF-dependent mechanism. *Circ Res* **95**:406–414.
- Wamhoff BR, Bowles DK, and Owens GK (2006) Excitation-transcription coupling in arterial smooth muscle. *Circ Res* **98**:868–878.
- Wamhoff BR, Hoofnagle MH, Burns A, Sinha S, McDonald OG, and Owens GK (2004b) A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis. *Circ Res* **95**:981–988.
- Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A, and Olson EN (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428**:185–189.
- Wassmann S, Wassmann K, Jung A, Velten M, Knuefermann P, Petoumenos V, Becher U, Werner C, Mueller C, and Nickenig G (2007) Induction of p53 by GKL is essential for inhibition of proliferation of vascular smooth muscle cells. *J Mol Cell Cardiol* **43**:301–307.
- Yoshida T, Gan Q, and Owens GK (2008a) Kruppel-like factor 4, Elk-1, and histone deacetylases cooperatively suppress smooth muscle cell differentiation markers in response to oxidized phospholipids. *Am J Physiol Cell Physiol* **295**:C1175–C1182.
- Yoshida T, Kaestner KH, and Owens GK (2008b) Conditional deletion of Kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. *Circ Res* **102**:1548–1557.

Address correspondence to: Dr. Brian R. Wamhoff, Department of Medicine, Division of Cardiovascular Medicine, University of Virginia, P.O. Box 801395, Charlottesville, VA 22908-1395. E-mail: wamhoff@virginia.edu