Nuclear Factor of Activated T Cells 5 Regulates Vascular Smooth Muscle Cell Phenotypic Modulation

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Objective—The tonicity-responsive transcription factor, nuclear factor of activated T cells 5 (NFAT5/tonicity enhancer binding protein [TonEBP]), has been well characterized in numerous cell types; however, NFAT5 function in vascular smooth muscle cells (SMCs) is unknown. Our main objective was to determine the role of NFAT5 regulation in SMCs.

Methods and Results—We showed that NFAT5 is regulated by hypertonicity in SMCs and is upregulated in atherosclerosis and neointimal hyperplasia. RNAi knockdown of NFAT5 inhibited basal expression of several SMC differentiation marker genes, including smooth muscle α actin (SMαA). Bioinformatic analysis of SMαA revealed 7 putative NFAT5 binding sites in the first intron, and chromatin immunoprecipitation analysis showed NFAT5 enrichment of intronic DNA. Overexpression of NFAT5 increased SMαA promoter-intron activity, which requires an NFAT5 cis element at +1012, whereas dominant-negative NFAT5 decreased SMαA promoter-intron activity. Because it is unlikely that SMCs experience extreme changes in tonicity, we investigated other stimuli and uncovered 2 novel NFAT5-inducing factors: angiotensin II, a contractile agonist, and platelet-derived growth factor-BB (PDGF-BB), a potent mitogen in vascular injury. Angiotensin II stimulated NFAT5 translocation and activity, and NFAT5 knockdown inhibited an angiotensin II-mediated upregulation of SMαA mRNA. PDGF-BB increased NFAT5 protein, and loss of NFAT5 inhibited PDGF-BB-induced SMC migration.

Conclusion—We have identified NFAT5 as a novel regulator of SMC phenotypic modulation and have uncovered the role of NFAT5 in angiotensin II-induced SMαA expression and PDGF-BB-stimulated SMC migration. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiotensin II • growth factors • NFAT5 • phenotypic modulation • smooth muscle
rin dephosphorylation of the protein.\textsuperscript{13} NFAT5 acts independently of calcineurin signaling and is primarily stimulated by hypertonicity, leading to the phosphorylation and translocation of the protein.\textsuperscript{14,15} Although the nomenclature may suggest that NFAT5 and NFATc1–4 are regulated in a similar manner, the mechanisms of activation and downstream gene targets are indeed very different. Furthermore, NFATc1–4 transcription factors have been well characterized in SMCs, and our laboratory and others have shown that NFATc1–4 play a key role in the regulation of SMC phenotypic modulation and gene regulation in vascular injury.\textsuperscript{13,16–18} whereas the expression, regulation, and role of NFAT5 is unknown in vascular biology.

Herein, we show for the first time that NFAT5 protein is expressed in the SMCs of the vasculature, is upregulated in atherosclerosis and neointimal hyperplasia, and is sensitive to NaCl-induced hypertonicity. Although hypertonicity upregulates NFAT5 protein and activity in SMCs, it is unlikely that SMCs experience extreme changes in tonicity because blood osmolality remains relatively constant at 290 mOsm/L. Evidence for alternate methods of NFAT5 stimulation have been identified in other cell types, such as T-cell receptor activation in T cells,\textsuperscript{15} αβ integrin clustering in cancer cells,\textsuperscript{8} and interleukin-1β and tumor necrosis factor-α release in rheumatoid arthritis.\textsuperscript{19} These data support the idea that NFAT5 can respond to other stimuli in tissues that do not see large changes in tonicity, such as SMCs.

We have novel evidence demonstrating that both Ang II and PDGF-BB, 2 important stimuli in the context of vascular development and disease, positively regulate NFAT5 activity in SMCs. We have identified NFAT5 as a regulator of both the contractile and migratory phenotypes and show that NFAT5 is required for Ang II-induction of SMCa contractile gene expression, as well as PDGF-BB induced SMC migration. Our data highlight the critical role of NFAT5 in the regulation of SMC phenotypic modulation.

Methods
See Supplemental Materials and Methods, available online at http://atvb.ahajournals.org, for additional experimental protocols.

Plasmid Luciferase and Overexpression Experiments
Rat aortic SMCs (RASMCs) were transfected at 50% confluence with a promoter-driven (SMaA Int mut-luc [Dr Gary Owens])\textsuperscript{20} or reporter-driven (NFAT5-luc [Dr H.M. Kwon])\textsuperscript{21} NFATc1–4-luc (Clontech) luciferase constructs (500 ng/mL) using FuGENE 6 (Roche). Following a 48-hour growth arrest, cells were treated with NaCl (25, 50, or 100 mmol/L), Ang II (Sigma, 1 μmol/L), or PDGF-BB (Millipore, 30 ng/mL) for up to 24 hours and harvested with 10 ng of a SMaA WT-luc or SMaA Int mut-luc plasmid for 48 hours. Whole cell lysates were harvested with cold radioimmunoprecipitation assay + protease inhibitor (Roche), and nuclear/cytoplasmic protein was harvested according to the NE-PER protocol (Pierce). Protein concentrations were determined through bichinchoninic acid assay analysis (Pierce). Equal protein masses were run on 4% to 15% precast gels (Bio-Rad), and polyvinylidene difluoride membrane–transferred proteins were probed for accordingly (rabbit anti-NFAT5, Dr H.M. Kwon, 1:2000); mouse anti-SMaA, Sigma, 1:10,000; and rabbit anti-β-tubulin, Cell Signaling, 1:1000). Horse-radish peroxidase–conjugated secondary antibodies allowed for chemiluminescent protein detection. Band intensities were quantified with ImageJ and normalized to β-tubulin.

Small Interfering RNA Transfections
RASMCs and A404 mouse SMC precursor cells were transfected at 30% confluence with 60 mmol/L rat or mouse NFAT5 dicer-substrate small interfering RNA (siRNA) duplex (rat sequence: CCAGTTCTACAATGTAATAACACTGA) or a scrambled negative control (Integrated DNA Technologies). Following a 3-day knockdown, cells were treated with Ang II (Sigma, 1 μmol/L), PDGF-BB (Millipore, 30 ng/mL), or retinoic acid (RA) (Sigma, 1 μmol/L, A404 experiments) for 48 hours.

Chromatin Immunoprecipitation
RASMCs were grown arrested at 70% confluence for 24 hours. Cells were fixed with 1% formaldehyde, DNA was sonicated into 500- to 1000-bp fragments, and immunoprecipitation was performed using the following antibodies: rabbit anti-NFAT5 (Dr H.M. Kwon),\textsuperscript{21} rabbit nonimmune IgG (Millipore), or rabbit anti-RNA polymerase II (Santa Cruz Biotechnology). DNA immunoprecipitation concentrations were measured through Picogreen (Invitrogen) fluorescence, followed by polymerase chain reaction amplification of 2 ng of DNA with primers specific for the SMaA first intron containing the overlapping CArG/NFAT5 sequence. NFAT5 enrichment values were normalized to total input DNA (starting quantitySpecific / starting quantityInput). The nonimmune IgG immunoprecipitation group and the RNA polymerase immunoprecipitation group served as a negative and positive controls, respectively.

Electric Cell Substrate Impedance Sensing Migration Assay
Electric cell substrate impedance sensing (ECIS) measures cellular migration through detecting changes in electric impedance produced by cells moving over ten 250-μm-wide electrode sensors in each well. siRNA-transfected RASMCs were replated onto 10-well ECIS chamber slides (Applied Biophysics) in insulin-free serum-free medium at 7 × 10^4 cells/cm\textsuperscript{2} and subsequently growth arrested for 18 hours. Baseline electrode impedance readings were recorded for each well using the ECIS devise (Applied Biophysics), followed by a 60-second, 64,000 Hz electrode pulse to kill the cells seeded on the electrodes. Wells were immediately treated with PDGF-BB (Millipore, 30 ng/mL) or serum (10% FBS) for 24 hours. Three independent experimental replicates were performed, and a representative graph is shown.

Boyden Chamber Chemotaxis Migration Assay
siRNA-transfected RASMCs and mouse embryonic fibroblasts were plated in the top chamber of a Transwell (Costar) at 7 × 10^5 cells/cm\textsuperscript{2} in medium containing 10% FBS. PDGF-BB (Millipore, 30 ng/mL) suspended in insulin-free serum-free medium or serum (10% FBS) was pipetted into the bottom chamber. Following a 24-hour incubation, cells were fixed with 4% paraformaldehyde, stained with 0.02% crystal violet, and imaged using a dissecting microscope. ImageJ was used to quantify pixel intensity of migrated cells.

Proliferation Assays
siRNA-transfected RASMCs were treated with PDGF-BB (Sigma, 30 ng/mL) or serum (10% FBS) for 24 hours. Each assay proceeded as follows. For the 5-bromo-2′-deoxyuridine (BrdU) assay, BrdU (Roche, 10 μmol/L) was added to each well with treatment. Cells were fixed with Fixdenat (Roche) and incubated with an anti-BrdU-
POD (Roche) antibody plus TMB substrate (Pierce). A spectrophotometer was used to measure absorbance at 370 nm, and values were normalized to background absorbance. For the Picogreen assay, deionized water, and tris EDTA were added to each well to lyse the cells. A 1:1 ratio of sample to Picogreen (Invitrogen) was combined in a 96-well plate and read on fluorescence plate reader. For 5-ethynyl-2'-deoxyuridine flow cytometry, cells were handled according to the Click-It 5-ethynyl-2'-deoxyuridine (Invitrogen) protocol. 5-ethynyl-2'-deoxyuridine (Invitrogen, 10 μmol/L) was added to each well with treatment. Cells were fixed with Click-It fixative (Invitrogen), incubated with a fluorescent dye azide (Invitrogen), and analyzed by flow cytometry.

Results

NFAT5 Is Expressed in Vascular Smooth Muscle and Is Upregulated in Vascular Injury

To determine whether NFAT5 protein was expressed in vascular SMCs and regulated by hypertonicity as it is in other cell types, we stimulated rat and human SMCs with increasing concentrations of NaCl and observed a maximal 3-fold increase in rat NFAT5 protein and an 8-fold increase in human NFAT5 protein (Figure 1A and 1B). Cell death was not observed. To test for NFAT5 activity, we transfected RASMCs with a synthetic NFAT5 reporter plasmid construct (NFAT5-luc) expressing the luciferase gene downstream of 3 NFAT5 consensus binding sites. NFAT5 activity was positively correlated with increasing NaCl concentrations, and even the smallest dose of 25 mmol/L NaCl produced a 7-fold increase in NFAT5 activity (Figure 1C). As a control experiment, we showed that hypertonicity did not stimulate NFATc1–4 activity in SMCs (Supplemental Figure I). We next performed immunohistochemistry to identify NFAT5 protein expression patterns in vascular disease. Apolipoprotein E−/− mice placed on a high-fat diet for 20 weeks generated atherosclerotic lesions in the aorta that displayed differential expression patterns of NFAT5 protein within the SMCs of the artery (Figure 1D). Although NFAT5 expression was low in medial SMCs where there was no overlying lesion (Figure 1E), NFAT5 expression was high in both the cells of the lesion and surrounding medial SMCs. G, Midgrade human atherosclerotic lesions showed high NFAT5 protein expression in the medial (M) SMCs surrounding the plaque (P). H, NFAT5 protein was upregulated at 7 days and 21 days in the SMCs of the rat carotid artery following balloon injury. M indicates media; NI, neointima; L, lumen. I and J, Western blot analysis of rat balloon-injured artery protein showed that NFAT5 protein was upregulated at 21 days (J) and that SMαA protein was increasing in parallel with NFAT5 back to basal levels following injury (J). Equal protein masses were loaded into each well (Supplemental Figure II) (n=5 mice/group, **P<0.005). SMMHC indicates smooth muscle myosin heavy chain.
NFAT5 Positively Regulates SMαA

Following our discovery that NFAT5 positively regulates several SMC-selective genes, we chose to focus our studies solely on the contractile protein SMαA. Bioinformatic analysis of the SMαA nucleotide sequence surrounding the transcriptional start site (−2.5 to +2.8 kb) predicted a total of 7 NFAT5 binding sites (T/A)GGAAA, all located within the first intron of the gene (Figure 3A). (Bioinformatic analysis of the other 5 SMC-selective genes is shown in Supplemental Figure IV.) Of significance, 1 of the predicted NFAT5 sites overlaps the intronic CArG box (containing SRF binding sites) that is critical for SMαA expression. To further investigate NFAT5 regulation of SMαA, we cotransfected RASMCs with a SMαA WT-luciferase plasmid containing the SMαA promoter and first intron (Figure 3A) with increasing concentrations of either an overexpressing (active) or dominant-negative (inactive) NFAT5 plasmid. At the lowest concentration of 100 ng, overexpressing NFAT5 increased SMαA promoter-intron activity 2.7-fold, whereas dominant-negative NFAT5 significantly decreased SMαA promoter-intron activity by 50% (Figure 3B). To determine whether SMαA promoter-intron activity required NFAT5

regulation of the sequence overlapping the first intronic CArG box, a second luciferase plasmid with a mutated SMαA intronic CArG and overlapping NFAT5 consensus sequence was utilized (SMαA Int mut-luc, Figure 3A). Our data showed that mutation of the intronic CArG/NFAT5 binding site inhibited an NFAT5 overexpression-induced upregulation in SMαA promoter-intron activity (Figure 3B). Although the SRF binding site is also mutated in this construct, the literature indicates that this same mutation does not inhibit a myocardin overexpression-driven increase in SMαA promoter-intron activity, whereas our data show that this mutation completely ablated an NFAT5 overexpression-driven increase SMαA promoter-intron activity. We also show, as an addendum in Supplemental Figure V, that NaCl-induced hypertonicity increased SMαA WT promoter-intron activity, and mutation of the intronic CArG/NFAT5 site attenuated this induction. To investigate NFAT5-mediated regulation of SMαA in a model of SMC differentiation, we used the RA-induced A404 cell line. RA stimulation of A404 smooth muscle precursor cells results in increased expression of virtually all SMC-selective genes; therefore, this cell line is a helpful tool for studying SMC differentiation. Day 0 (D0) A404 cells did not receive RA treatment and therefore represented NFAT5 and SMαA gene
expression in an undifferentiated smooth muscle precursor cell. Addition of RA to cell culture medium for 7 days (D1 to D7) resulted in increased NFAT5 mRNA preceding increased SMαA expression (Figure 3C). A404 cells were transfected with NFAT5 siRNA and treated with RA, and reverse transcription–polymerase chain reaction data showed that the percentage of NFAT5 knocked down in each biological replicate directly correlated with the percentage of reduced SMαA mRNA in each replicate ($R^2 = 0.975$, Figure 3D).

Additionally, we showed that NFAT5-null mouse embryonic fibroblasts had a 98% reduction in SMαA mRNA compared with WT mouse embryonic fibroblasts (Supplemental Figure VI). To determine whether NFAT5 bound to the region spanning the SMαA intronic CArG/NFAT5 sequence, primers for chromatin immunoprecipitation were made to encompass the intronic CArG/NFAT5 site (around +1.0 kb). We showed NFAT5 to enrich SMαA intronic DNA (Figure 3E).

Because of the existence of 6 other NFAT5 consensus sequences in close proximity to the CArG/NFAT5 site, we could not use the chromatin immunoprecipitation assay to resolve whether NFAT5 was binding specifically to the CArG/NFAT5 site at +1012; however, we show in Figure 3B that mutation of this CArG/NFAT5 cis element blocked NFAT5 induction of SMαA promoter-intron activity. Thus, we tested the hypothesis that NFAT5 physically interacts with the CArG-binding transcription factor, SRF. A coimmunoprecipitation assay was performed whereby NFAT5 protein was overexpressed in human embryonic kidney cells, SRF/protein complexes were pulled down, and NFAT5 protein was probed for. We did not detect NFAT5 interaction with SRF (Figure 3F).

Ang II Increases NFAT5 Translocation and Transcription Factor Activity, and NFAT5 Is Required for An Ang II-Mediated Upregulation in SMαA Promoter-Intron Activity and mRNA Expression

In Figure 1A to 1C, we showed that NFAT5 was regulated by high NaCl-induced hypertonicity in SMCs; however, it is unknown whether SMCs of the artery experience hypertonic conditions such as these. Therefore, we surveyed a panel of candidate physiological/pathophysiological regulators of...
NFAT5 in vascular SMCs (Supplemental Figure VII). A screen of cytokines (interleukin-1, interleukin-8, interleukin-10, tumor necrosis factor-α, interferon-γ), mitogens (PDGF-BB, sphingoside-1-phosphate, oxidized phospholipid), and contractile agonists (endothelin-1, Ang II, thrombin, transforming growth factor-β) led to the discovery that Ang II, a potent contractile agonist and known positive regulator of SMαA activity and expression (Figure 4A and 4F), stimulated NFAT5 activity in SMCs. Of note, Ang II upregulated NFAT5 reporter activity maximally at 24 hours (63-fold, Figure 4A) and did so in a calcineurin-independent manner (Supplemental Figure VIIIB). Ang II stimulation also increased NFAT5 translocation to the nucleus, as shown by immunofluorescence and nuclear/cytosolic protein preparation (Figure 4B). However, Ang II treatment did not alter NFAT5 mRNA (Figure 4C) or protein expression (Figure 4D). To determine whether NFAT5 was required for Ang II-mediated upregulation in SMαA promoter-intron activity and mRNA expression, RASMCs were transfected with NFAT5 siRNA or scrambled control and treated with Ang II. Results showed that NFAT5 knockdown inhibited an Ang II-induced increase in both SMαA promoter-intron activity (Figure 4E) and SMαA mRNA (Figure 4F), indicating that NFAT5 is necessary for an Ang II-mediated upregulation in SMαA expression.

PDGF-BB Upregulates NFAT5 Protein and NFAT5 Reporter Activity and Stimulates A More Delayed NFAT5 Activation Compared With NFATc1–4 Proteins

In addition to our discovery of Ang II as an effective regulator of NFAT5 translocation and activity, we also identified PDGF-BB as another novel regulator of NFAT5 in SMCs (Supplemental Figure VII). This finding is noteworthy because it suggests that NFAT5 regulates gene expression not only in the Ang II-driven contractile SMC phenotype, but also in the PDGF-BB-mediated migratory or proliferative SMC phenotype. We showed PDGF-BB to significantly increase NFAT5 protein expression in a calcineurin-independent manner (Figure 5A and Supplemental Figure VIIIIB) and increase NFAT5 reporter activity maximally following 24 hours of PDGF-BB treatment (Figure 5B). It is known that PDGF-BB is also a stimulator of NFATc1–4 activity,13 and we herein showed that PDGF-BB stimulated NFATc1–4 and NFAT5 activity along different time courses, with NFATc1–4 activity peaking early at 3 hours (7-fold) and NFAT5 activity peaking at a more delayed 24 hours (12-fold, Figure 5B). Thus, PDGF-BB differentially regulates NFATc1–4 and NFAT5 transcription factor activity in SMCs.

NFAT5 Positively Regulates PDGF-BB and Serum-Induced SMC Migration But Not Proliferation

To determine the function of PDGF-BB-simulated NFAT5 expression and activity in SMCs, we knocked down NFAT5 mRNA and measured PDGF-BB and serum (10% FBS)-induced SMC migration and proliferation. RASMCs transfected with NFAT5 siRNA appeared to be smaller and were less elongated compared with scramble control SMCs (Figure 6A). To test for altered SMC migration, we used 2 different

Figure 4. Angiotensin II (Ang II) increases nuclear factor of activated T cells 5 (NFAT5) translocation and transcription factor activity, and NFAT5 is required for an Ang II-mediated upregulation in SMαA promoter-intron activity and mRNA expression. A, Ang II upregulated SMαA promoter-intron activity and NFAT5 reporter activity in rat aortic smooth muscle cells (RASMCs) ($n=3$, *$P<0.05$, **$P<0.005$). B, Immunofluorescence images show NFAT5 translocation to the nucleus following Ang II treatment, verified by Western blot analysis ($n=3$). C and D, RASMCs treated with Ang II showed no change in NFAT5 mRNA ($n=3$) (C) or NFAT5 protein ($n=12$) (D). E and F, Small interfering RNA (siRNA) knockdown of NFAT5 inhibited a 24-hour Ang II-mediated upregulation in SMαA promoter-intron activity (E) and mRNA expression (F) ($n=3$, *$P<0.05$).
NFAT5 Regulates SMC Phenotypic Modulation

Discussion

We have identified NFAT5 as a novel transcriptional regulator of vascular smooth muscle gene expression and phenotypic modulation. We show for the first time that NFAT5 protein is expressed in vascular SMCs and is sensitive to NaCl-induced hypertony in cell culture, and we report novel evidence demonstrating that both Ang II and PDGF-BB positively regulate NFAT5 activity in SMCs. Our results highlight the important role of NFAT5 in the regulation of SMC phenotypic modulation, in that NFAT5 positively regulates Ang II-induced SMOA contractile gene expression, yet NFAT5 is also required for PDGF-BB induced SMC migration. Additional in vivo data show that NFAT5 is upregulated in the artery following acute vascular injury and in chronic atherosclerosis.

NFAT5 sensitivity to changes in tonicity to maintain cellular homeostasis is well described in other cell types. SMCs of the vasculature, however, do not experience extreme changes in tonicity. The kidney precisely regulates solute levels in the blood, and therefore blood osmolarity is strictly maintained at 290 mOsm/L. Although it would be difficult to test, we and others speculate that hypertonic experimental techniques: (1) ECIS to measure changes in electric impedance produced by migrating cells, and (2) the Boyden chamber assay to quantify chemotaxis-induced SMC migration. ECIS analysis indicated that RASMCs deficient in NFAT5 showed significantly inhibited PDGF-BB and serum-induced migration compared with scrambled controls (Figure 6B). A similar trend was seen in Boyden chamber assay experiments (Figure 6C). Additionally, NFAT5 knockout mouse embryonic fibroblasts did not migrate at basal levels (vehicle) or in response to PDGF-BB or serum treatment (Figure 6D). We also tested the effect of NFAT5 knockdown on PDGF-BB and serum-induced proliferation. Several proliferation assays, including BrdU incorporation, Picogreen quantification, and 5-ethyl-2′-deoxyuridine-based flow cytometry, confirmed that NFAT5 does not regulate SMC proliferation (Figure 6E).

Figure 5. Platelet-derived growth factor-BB (PDGF-BB) (BB) upregulates nuclear factor of activated T cells 5 (NFAT5) protein and NFAT5 reporter activity and stimulates a more delayed NFAT5 activation compared with NFATc1–4 proteins. A, Western blot analysis of rat aortic smooth muscle cells (RASMCs) treated with PDGF-BB for 24 hours showed increased NFAT5 protein expression (n = 6, *P < 0.05). B, RASMCs treated with PDGF-BB showed NFATc1–4 reporter activity to peak early at 3 hours, unlike the more delayed response in NFAT5 activity, which peaked at 24 hours (n = 3, *P < 0.05). VEH indicates vehicle.

NFAT5 contributes to both Ang II and PDGF-BB-induced SMC phenotypic modulation. Of note, NFAT5 not only regulates gene expression in the Ang II–driven contractile SMC phenotype, but it also regulates the PDGF-BB-mediated migratory SMC phenotype. Transcription factors classically regulate a single cellular process or phenotypic change, such as nuclear factor-κB, which promotes inflammation, or p53, which induces growth arrest and apoptosis. However, some transcription factors, such as SRF and Krüppel-like factor-4, regulate gene expression in both the contractile and synthetic SMC phenotypes. For example, SRF activation of several SMC-selective genes is critical for SMC differentiation and maintenance of the contractile phenotype. In the event of an acute vascular injury such as a balloon angioplasty, environmental cues and targeted histone modifications lead to the compaction of SMC-selective DNA and the coordinate epigenetic relaxation of chromatin that directs SMC migration or proliferation. SRF responds to these changes in chromatin dynamics by abandoning SMC-selective regulatory regions and concomitantly binding to the promoter of the growth-promoting gene c-fos to drive SMC proliferation. This SRF-mediated, rapid shift in gene transcription and SMC phenotype enables the SMC to quickly respond to its environment. We hypothesize that NFAT5 in SMCs regulates gene expression in a manner similar to SRF, whereby NFAT5 is sensitive to alterations in environmental signals and can relocate from regulatory regions of SMC-selective genes to promigratory genes. Preliminary data from our laboratory show that NFAT5 positively regulates basal expression levels of the promigratory genes Cyr61 and vascular endothelial growth factor-C (data not shown). In addition, we see that NFAT5 is differentially expressed in the SMCs of murine atherosclerotic lesions (Figure 1D to 1F). These data are important in that they fit the hypothesis that not all SMCs in an artery and lesion are one and the same, but that SMC phenotypes can vary depending on location and stage of the disease.

It is well known that Ang II and PDGF-BB are released systemically and from the local vasculature during periods of acute injury and chronic disease, ie, atherosclerosis, to regulate SMC phenotypic modulation. We speculate that NFAT5 could be regulating gene expression (and therefore phenotypic modulation) in response to signaling in these localized vessel wall microenvironments. Recently, our group has shown that NFAT5 haploinsufficiency inhibits atherosclerotic lesion formation in the apolipoprotein E–null mouse model of atherosclerosis (unpublished data). Although preliminary, these data and this current study herein lay the...
Figure 6. Nuclear factor of activated T cells 5 (NFAT5) positively regulates platelet-derived growth factor-BB (PDGF-BB) and serum-induced smooth muscle cell (SMC) migration, but not proliferation. A, Rat aortic smooth muscle cells (RASMCs) transfected with NFAT5 small interfering RNA (siRNA) were less elongated and appeared to be smaller. B, NFAT5 siRNA-transfected SMCs treated with PDGF-BB and serum displayed decreased migration compared with scrambled control SMCs, as quantified by electric cell substrate impedance sensing (ECIS) (n=10053, *P<0.05). C, The Boyden chamber assay displayed a trend of impaired chemotaxis-induced migration of siRNA-transfected RASMCs (n=3). D, NFAT5 knockout (KO) mouse embryonic fibroblasts (MEFs) showed an ablated migratory response to basal, PDGF-BB, and serum-induced migration compared with wild-type (WT) MEF controls (n=3, *P<0.05). E, Exhaustive SMC proliferation analyses revealed that NFAT5 is not critical for SMC proliferation (5-bromo-2’-deoxyuridine [BrdU] assay, n=5; Picogreen assay, n=5; flow cytometry 5-ethynyl-2’-deoxyuridine assay, n=3).
foundation for establishing a role for NFAT5 in atherosclerosis, and further studies will unmask any potential therapeutic or translational implications for NFAT5 as a target in atherosclerosis. We show that Ang II and PDGF-BB both positively regulate NFAT5 activity in SMCs. Specifically, Ang II increases NFAT5 reporter activity and nucleo-cytoplasmic translocation of NFAT5 protein, but Ang II does not affect levels of NFAT5 mRNA or protein. PDGF-BB stimulation increases both NFAT5 protein expression and NFAT5 reporter activity. These 2 stimuli drive the SMC into 2 very different phenotypes: Ang II to the contractile phenotype and PDGF-BB to the proliferative or migratory phenotype. NFAT5 may serve as a transcriptional hub for processing these different yet complementary phenotypes. For example, in both vascular development and vascular injury, we speculate that PDGF-BB orchestrates SMC migration partly through upregulation of NFAT5 protein and downstream target genes. Subsequently, on developmental formation of the mature vessel or resolution of the vascular injury, Ang II signaling may require preexisting NFAT5 protein to enable rapid activation of SmoA contractile gene expression and SMC phenotypic switching to the quiescent, contractile state. The exact mechanism of Ang II and PDGF-BB-mediated activation of NFAT5 in SMCs is still undefined. Several kinases have been implicated in the phosphorylation and subsequent translocation of NFAT5 to the nucleus, and we hypothesize that PDGF-BB and Ang II stimulation of downstream kinases, such as extracellular signal regulated kinase, p38, and protein kinase C, may play a role in signal propagation and NFAT5 activation. Although our laboratory has begun to investigate these possible mechanisms, the signaling pathways required for NFAT5 phosphorylation in SMCs remain undefined.

We have discovered that NFAT5 positively regulates basal levels of CArG box-driven SMC-selective genes, namely SmoA, calponin 1, smoothelin, and SM22α. To elucidate the mechanism of NFAT5 regulation of SmoA, we identified the location of 7 putative NFAT5 binding sites in the SmoA first intron, and chromatin immunoprecipitation analysis indicated that NFAT5 enriched SmoA intronic DNA (Figure 3E). One of the NFAT5 sites we identified overlaps the intronic CArG that is critical for SmoA expression. Gonzalez Bosc et al previously recognized this overlapping intronic CArG site as an NFATc1–4 consensus sequence. The authors showed that treatment of SMCs with the calcineurin inhibitors FK506 and CsA decreased reporter activity of this intronic region by roughly 60%, suggesting that this region is partially regulated by NFATc1–4 proteins. However, direct mutation of the NFAT site further decreased reporter activity by approximately 27%. These findings thereby suggest that a noncalcineurin activated GGAAA-binding protein (ie, NFAT5) could also modulate SmoA at that site. We demonstrated that NFAT5 positively regulates SmoA by showing that overexpression of NFAT5 increases SmoA promoter-intron activity, whereas dominant-negative NFAT5 decreases SmoA promoter-intron activity. To determine whether SmoA promoter-intron activity required NFAT5 regulation of the sequence overlapping the first intronic CArG, we used a preexisting luciferase construct in which 2 mutations were made: 1 at the start of the intronic CArG box and 1 in the overlapping CArG/NFAT5 site. Although there is a mutation in both the SRF and NFAT5 binding sites, Yoshida et al showed that this mutation does not inhibit myocardin overexpression-induced SmoA promoter-intron activity. It would then be hypothesized that this mutation would not inhibit NFAT5 overexpression-induced SmoA promoter-intron activity, yet we saw that this mutation completely ablated an NFAT5 overexpression-induced increase in SmoA promoter-intron activity. These data therefore support our hypothesis that NFAT5 is regulating SmoA through the overlapping CArG/NFAT5 binding site in the SmoA first intron. We subsequently tested the hypothesis that NFAT5 physically interacts with SRF, but we could not detect an interaction. This could be due to (1) the fact that SRF and NFAT5 do not interact, (2) the SRF/NFAT5 interaction being too weak to detect with a coimmunoprecipitation assay, or (3) the SRF/NFAT5 interaction blocking the NFAT5 antibody-binding epitope. Finally, we showed that RNAi-mediated NFAT5 knockdown inhibits an Ang II-induced increase in both SmoA promoter-intron activity and SmoA mRNA, thereby demonstrating that NFAT5 is necessary for an Ang II-mediated upregulation in SmoA expression.

Our studies have revealed that NFAT5 is required for PDGF-BB and serum-driven SMC migration but is not required for SMC proliferation. It was once the prevailing thought that an SMC could modulate to 1 of 2 phenotypes: the contractile phenotype or the synthetic (ie, migrating and proliferating) phenotype. Our current understanding of SMC phenotypic modulation is now much more complex. The contractile SMC can modulate toward an array of phenotypes and can become proliferative, migratory, or inflammatory. Therefore, NFAT5 could specifically direct SMC migration and not proliferation, as these processes are mutually exclusive. The literature shows that NFAT5 is required for migration of carcinoma, skeletal myoblasts, and endothelial cells during angiogenesis, whereas NFAT5 has been documented as regulating proliferation in lymphocytes and fibroblast-like synoviocytes. These data indicate that NFAT5 can direct different cellular functions in diverse tissues. In an effort to validate the promigratory effect of NFAT5 in vivo, we studied the effect of vascular injury on NFAT5+/− mice. These mice underwent carotid ligation or carotid wire injury to test the hypothesis that mice haploinsufficient in NFAT5 would exhibit decreased neointimal hyperplasia. Unfortunately, results from these studies were inconclusive because of the inconsistency of neointimal hyperplasia in the NFAT5+/− mice. Additional acute injury studies could provide valuable insight into this mechanism.

In conclusion, these combined data demonstrate the importance of NFAT5 transcription factor activity in vascular SMCs and further elucidate the role of NFAT5 in the differential regulation of SMC phenotypic modulation. We show that NFAT5 is not only sensitive to hypertonicity in SMCs but is also regulated by 2 newly identified NFAT5 stimuli, Ang II and PDGF-BB. NFAT5 is required for both Ang II stimulation of SmoA expression and PDGF-BB-mediated SMC migration. Our in vivo data show that NFAT5 expression in the artery is upregulated following acute vascular injury and that NFAT5 is differentially expressed the
cells of the atherosclerotic lesion. We have therefore identified NFAT5 as an important player in SMC phenotypic modulation, and further studies may establish that NFAT5 is a viable therapeutic target to potentially block SMC migration in occlusive vascular disease.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Materials and Methods

Cell culture
Rat aortic SMC (RASMCs) were isolated and cultured (passages 11-15) in DMEM/F12 media containing 10% FBS as previously described.\textsuperscript{1} For RNA and protein analysis, cells were growth arrested in insulin-free serum-free (IFSF) media at 70% confluency for 48 hours, followed by treatment with NaCl (25, 50, or 100 mM), Ang II (Sigma, 1 μM), or PDGF-BB (Millipore, 30 ng/mL) for up to 24 hrs.

Immunohistochemistry
5 um paraffin sections and a polyclonal rabbit anti-NFAT5 (H.M. Kwon) antibody were used for immunohistochemistry (IHC) analysis. In brief, Antigen Retrieval (Vector Laboratories) was performed using a microwave followed by primary antibody detection with a Vectastain Elite Kit (Vector Laboratories) and DAB (Dako Corp) for visualization. Negative controls were run with the omission of the primary antibody. A light microscope was used to take 4x, 10x, and 20x images of the arteries.

Human atherosclerotic lesions
Coronary arteries were collected from patients at the University of Virginia Hospital ranging from ages 4 months to 65 years who expired due to complications other than cardiovascular disease. 24 hrs post-mortem, tissues were extracted, fixed in 10% neutral buffered formalin and paraffin-embedded, and IHC was performed. A representative mid-grade lesion is shown in Figure 1G.

ApoE\textsuperscript{-/-} mouse atherosclerotic lesions
8-week-old ApoE\textsuperscript{-/-} mice obtained from the Jackson Laboratory were placed on a high-fat diet (HFD) (by weight 21% fat, 0.15% cholesterol, 19.5% casein) for 20 wks. Upon euthanasia, aortas were removed, fixed, and paraffin-embedded, and IHC was performed. A representative ApoE\textsuperscript{-/-} lesion is shown in Figure 1D.

Bioinformatics analysis
The NCBI GENE database was utilized to examine the -2.5 kb upstream and +2.0 kb downstream nucleotide sequence of the SMaA (Acta2) gene to identify putative NFAT5 binding sites (GGAAA).
**Immunofluorescence**

RASMCs seeded on chamber slides were growth arrested at 70% confluency and treated with Ang II (Sigma, 1 μm) for 24 hrs. Cells were fixed with 4% paraformaldehyde and incubated with primary antibody (rabbit anti-NFAT5, H.M. Kwon, 1:1000) and secondary antibody conjugated to Alexa Fluor 488 (Invitrogen). DAPI was used to for nuclear visualization. FITC and DAPI images were captured on a confocal microscope and merged. All images were equally brightened (75%) using Adobe Photoshop; originals images were saved.

**Mouse embryonic fibroblasts (MEFs)**

WT and NFAT5 null MEFs were prepared as previously described\(^2\) and given as a gift by Dr. H.M. Kwon.

**Statistical analyses**

Statistical significance was confirmed through a 1-way ANOVA or t-test (p<0.05).

**SUPPLEMENTAL REFERENCES**


2. NFAT5 TonEBP mutant mice define osmotic stress as a critical feature of the lymphoid microenvironment. William Y. Go, Xuebin Liu, Michelle A. Roti, Forrest Liu, and Steffan N. Ho
Supplemental Figure I. Hypertonicity does not stimulate NFATc1-4 activity in SMCs. RASMCs were transfected with the NFATc1-4-luc reporter and stimulated with +50 mM or +100 mM NaCl. Luciferase values were measured at 24 hrs. (n=3)
Supplemental Figure II. **A.** Western blot film from rat balloon-injured carotid artery protein (quantified in Figure 1I-J). **B.** Coomassie-stained gels show equal masses of protein loaded in each well.
Supplemental Figure III. Western blot analysis verifies siRNA knockdown of NaCl, Ang II, and PDGF-BB-induced NFAT5 protein expression. A-C. RASMCs were transfected with scrambled (SCR) or NFAT5 siRNA (siNFAT5) and treated with 100 mM NaCl (A, n=3), Ang II (B), or PDGF-BB (C). Protein samples were collected at 24 hrs and analyzed by western blot. Coomassie-stained gels show equal masses of protein loaded in each well (B-C).
Number of putative NFAT5 binding sites

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<tr>
<th>Gene, Rat</th>
<th>(-2500 to +1)</th>
<th>(+1 to +2000)</th>
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<tbody>
<tr>
<td>SMαA*</td>
<td>0</td>
<td>7</td>
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<tr>
<td>Calponin 1*</td>
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<td>2</td>
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<tr>
<td>Smoothelin*</td>
<td>4</td>
<td>3</td>
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<tr>
<td>SM22α*</td>
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<td>5</td>
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<tr>
<td>SMMHC</td>
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<td>4</td>
</tr>
<tr>
<td>Myocardin</td>
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</table>

Supplemental Figure IV. Putative NFAT5 binding sites in 6 SMC-selective genes. Bioinformatic analysis identified NFAT5 cis elements (sequence: GGAAA). *Regulated by NFAT5 at basal levels (Figure 2A).
Supplemental Figure V. NaCl-induced hypertonicity increases SMαA WT promoter-intron activity and mutation of the intronic CArG/NFAT5 site attenuates this induction. RASMCs were transfected with either a SMαA WT-luc plasmid or a SMαA intronic CArG/NFAT5 site mutant (Int mut-luc) plasmid and stimulated with +50 mM or +100 mM NaCl. Luciferase values were measured at 24 hrs. (n=3, **p<0.005 ***p<0.0001)
Supplemental Figure VI. SMαA mRNA expression is reduced in NFAT5 KO MEFs compared to WT. RT-PCR analysis of untreated WT and NFAT5 KO MEFs shows a 98% decrease in SMαA mRNA expression in NFAT5 KO MEFs.
Supplemental Figure VII. Preliminary experiments identified PDGF-BB as a novel NFAT5-stimulating factor in SMCs. RASMCs transfected with an NFAT5 reporter-luciferase construct were growth arrested for 2 days and treated with various stimuli for 24 hrs. (n=2) (Note: Ang II stimulation of RASMCs was not included in this preliminary study, but was identified at a later date.)
Supplemental Figure VIII. PDGF-BB and Ang II stimulate NFAT5 in a calcineurin-independent manner. A. RASMCs were pre-treated with the competitive NFAT peptide mimic A-285222 (ABT, 10 μM, Abbott Labs) or the calcineurin inhibitor FK506 (100 nM, Sigma) and stimulated with PDGF-BB for 24 hrs. (n=2) B. RASMCs were transfected with the NFAT5-luc reporter, pre-treated with ABT or FK506 and stimulated with Ang II. Luciferase values were measured at 24 hrs. (n=3)
A. *Rattus norvegicus* RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence (5'-3')</th>
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<tbody>
<tr>
<td>SMαA</td>
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<td>(F)ATCCGATAGAACACGGGACGTC (R)AGGCATAGAGGGACAGCACAGCA</td>
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<td>NC_005107.2</td>
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<td>(F)GCTTAAGAGGGAGGTTACACAGACAC (R)GCCTTCTCTTCTAATGATGAC</td>
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<td>SMMHHC</td>
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B. *Rattus norvegicus* ChIP primer sequences

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**Supplemental Figure IX.** Primer sequences for RT-PCR (A) and ChIP (B).