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Nuclear factor of activated T-cells 5 regulates vascular smooth muscle cell phenotypic modulation

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Abstract

OBJECTIVE—The tonicity-responsive transcription factor, nuclear factor of activated T-cells 5 (NFAT5/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 show that NFAT5 is regulated by hypertonicity in SMCs and is upregulated in atherosclerosis and neointimal hyperplasia. RNAi knockdown of NFAT5 inhibits basal expression of several SMC differentiation marker genes, including smooth muscle alpha actin (SMαA). Bioinformatic analysis of SMαA reveals seven putative NFAT5 binding sites in the first intron, and ChIP analysis shows NFAT5 enrichment of intronic DNA. Overexpression of NFAT5 increases SMαA promoter-intron activity, which requires an NFAT5 *cis* element at +1012, while dominant-negative NFAT5 decreases SMαA promoter-intron activity. Since it is unlikely that SMCs experience extreme changes in tonicity, we investigated other stimuli and uncovered two 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 stimulates NFAT5 translocation and activity, and NFAT5 knockdown inhibits an angiotensin II-mediated upregulation of SMαA mRNA. PDGF-BB increases NFAT5 protein and loss of NFAT5 inhibits PDGF-BB-induced SMC migration.

CONCLUSIONS—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.

Keywords

smooth muscle; NFAT5; phenotypic modulation; angiotensin II; platelet-derived growth factor BB

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The primary function of a quiescent, mature vascular smooth muscle cell (SMC) is contraction. While contraction is necessary for the maintenance of overall vascular tone, SMCs also play an important role in vascular injury and disease by undergoing phenotypic modulation to a migratory or proliferative (i.e. synthetic) phenotype, moving into the lumen of the artery, and then differentiating back to the contractile phenotype in an effort to protect the artery and help regain contractile function.[1] The contractile SMC phenotype is characterized by high expression of SMC differentiation marker genes (i.e. smooth muscle alpha actin (SMαA), SM myosin heavy chain (SMMHC), SM 22 alpha (SM22α), calponin 1, smoothelin, and myocardin). These SMC-selective genes are regulated by serum response factor (SRF) dimerization and binding to CArG box ($CC(A/T)_6GG$) *cis* elements in the promoter and/or first intron, followed by myocardin recruitment to the transcriptional complex.[2–4] Since SMCs are not terminally differentiated, they can undergo rapid phenotypic modulation to the synthetic or contractile state in response to altered environmental cues. Various factors released both systemically and from the local vasculature stimulate phenotypic modulation. Platelet derived growth factor-BB (PDGF-BB) drives SMC proliferation and migration, and angiotensin II (Ang II) promotes SMC contraction and hypertrophy.[2, 5] This process of SMC phenotypic modulation requires precise epigenetic coordination and rapid transcription factor modulation to alter gene expression.[6]

The transcription factor nuclear factor of activated T-cells 5 (NFAT5/tonicity enhancer binding protein [TonEBP]) is sensitive to hypertonic stress and is directly involved in regulating gene expression to restore cellular homeostasis.[7] Additionally, NFAT5 has been shown to direct cellular migration in cancer cells[8] and skeletal muscle myoblasts[9] and regulate proliferation in lymphocytes and fibroblast-like synoviocytes.[10, 11] NFAT5 belongs to the Rel family of transcription factors and bears close homology to both NFATc1-4 and NFkB proteins through a highly conserved DNA binding domain.[7, 12] Importantly though, while NFATc1-4 transcription factors are activated by calcium-triggered calcineurin dephosphorylation of the protein,[13] NFAT5 acts independent of calcineurin signaling and is primarily stimulated by hypertonicity, leading to the phosphorylation and translocation of the protein.[14, 15] Thus, 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. Further, NFATc1-4 transcription factors have been well characterized in SMCs, and our lab and others have shown that NFATc1-4 play a key role in the regulation of SMC phenotypic modulation and gene regulation in vascular injury,[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 since blood osmolarity remains relatively constant at 290 mOsm/L. Evidence for alternate methods of NFAT5 stimulation have been identified in other cells types, such as T-cell[15] α_6/β_4 integrin clustering in cancer cells,[8] and IL-1β and TNF-α release in rheumatoid arthritis.[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, two 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 SMαA

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 online Supplemental Materials and Methods for additional experimental protocols.

Plasmid luciferase and overexpression experiments

Rat aortic SMCs (RASMCs) were transfected at 50% confluency with a promoter-driven (SMαA WT-luc, SMαA Int mut-luc [Dr. Gary Owens][20]) or reporter-driven (NFAT5-luc [Dr. H.M. Kwon],[21] NFATc1-4-luc [Clontech]) luciferase constructs (500 ng/mL) using FuGENE 6 (Roche). Following a 48 hr growth arrest, cells were treated with NaCl (25, 50, or 100 mM), Ang II (Sigma, 1 μM), or PDGF-BB (Millipore, 30 ng/mL) for up to 24 hrs and harvested with 1× passive lysis buffer (Promega). Luminescence values were measured on a FLUOstar Omega (BMG Labtech) plate reader and normalized to total protein. For overexpression experiments, RASMCs were co-transfected with increasing concentrations of either an overexpressing (OE) or dominant-negative (DN) NFAT5 plasmid, an empty pCMV-Tag2 vector to ensure equal concentrations transfected per well, and 100 ng of a SMαA WT-luc or SMαA Int mut-luc plasmid for 48 hrs.

Western blot analysis

Whole cell lysates were harvested with cold RIPA + protease inhibitor (Roche) and nuclear/cytoplasmic protein was harvested according to the NE-PER protocol (Pierce). Protein concentrations were determined through BCA analysis (Pierce). Equal protein masses were run on 4–15% pre-cast gels (Bio-Rad) and PVDF membrane-transferred proteins were probed for accordingly (rabbit anti-NFAT5, Dr. H.M. Kwon, 1:2000;[21] mouse anti-SMαA, Sigma, 1:10,000; and rabbit anti-β-tubulin, Cell Signaling, 1:1000). HRP-conjugated secondary antibodies allowed for chemiluminescent protein detection. Band intensities were quantified with ImageJ and normalized to β-tubulin.

siRNA transfections

RASMCs and A404 mouse SMC precursor cells were transfected at 30% confluency with 60 nM of a rat or mouse NFAT5 dicer-substrate siRNA duplex (rat sequence: CCAGTTCCTACAATG ATAACACTGA) or a scrambled negative control (IDT). Following a 3-day knockdown, cells were treated with Ang II (Sigma, 1 μM), PDGF-BB (Millipore, 30 ng/mL), or retinoic acid (Sigma, 1 μM, A404 experiments) for 48 hrs.

Chromatin Immunoprecipitation

RASMCs were growth arrested at 70% confluency for 24 hrs. Cells were fixed with 1% formaldehyde, DNA sonicated into 500–1000 bp fragments, and IP performed using the following antibodies: rabbit anit-NFAT5 (Dr. H.M. Kwon),[21] rabbit non-immune IgG (Millipore), or rabbit anti-RNA polymerase II (Santa Cruz). DNA IP concentrations were measured through Picogreen (Invitrogen) fluorescence, followed by PCR amplification of 2 ng of DNA with primers specific for the SMαA first intron containing the overlapping CArG/NFAT5 sequence. NFAT5 enrichment values were normalized to total input DNA ($SQ_{Specific}/SQ_{Input}$). The non-immune IgG IP group and the RNA polymerase IP group served as a negative and positive control, respectively.

Electric cell substrate impedance sensing (ECIS) migration assay

ECIS measures cellular migration through detecting changes in electrical impedance produced by cells moving over ten, 250 micrometer-wide electrode sensors in each well.

siRNA-transfected RASMCs were re-plated onto 10-well ECIS chamber slides (Applied Biophysics) in insulin-free serum-free media at 7×10^4 cells/cm^2 and subsequently growth arrested for 18 hrs. 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). Impedance measurements for each well were recorded every 20 seconds for 48 hrs. Three independent experimental replicates were preformed, and a representative graph is shown.

Boyden chamber chemotaxis migration assay

siRNA-transfected RASMCs and mouse embryonic fibroblasts (MEFs) were plated in the top chamber of a transwell (Costar) at 7×10^4 cells/cm^2 in media containing 10% FBS. PDGF-BB (Millipore, 30 ng/mL) suspended in IFSF media or serum (10% FBS) was pipetted into the bottom chamber. Following a 24 hr 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 hrs. Each assay proceeded as follows: ***BrdU assay***: BrdU (Roche, 10 μM) 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. ***Picogreen assay***: diH_2O and TE 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. ***EdU flow cytometry***: Cells were handled according to the Click-It EdU (Invitrogen) protocol. EdU (Invitrogen, 10 μM) 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 if 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 (Figures 1A & B). 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 three NFAT5 consensus binding sites. NFAT5 activity was positively correlated with increasing NaCl concentrations, and even the smallest dose of 25 mM NaCl produced a 7-fold increase in NFAT5 activity (Figure 1C). As a control experiment, we show that hypertonicity does not stimulate NFATc1-4 activity in SMCs (Supplemental Figure I). We next performed immunohistochemistry to identify NFAT5 protein expression patterns in vascular disease. ApoE$^{-/-}$ mice placed on a high fat diet (HFD) 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). While NFAT5 expression was low in medial SMCs where there was no overlaying lesion (Figure 1E), NFAT5 expression was high in both the cells of the lesion and the surrounding SMCs (Figure 1F). Similarly, mid-grade human atherosclerotic lesions showed high expression of NFAT5 in the medial SMCs surrounding the lesion (Figure 1G). Following our initial characterization of NFAT5 protein expression in atherosclerosis, we analyzed rat carotid balloon-injured arteries (Figure 1H) and found a

significant 3.3-fold increase in NFAT5 protein 21 days following balloon injury (Figure 1I). Additionally, we made the observation that increased NFAT5 protein expression at 21 days corresponds with increased SMαA expression back to basal levels following injury (Figure 1J), which is known to correlate with lesion resolution and SMC phenotypic switching back to the contractile state.[22] Coomassie-stained gels show equal masses of protein loaded in each well (Supplemental Figure II).

siRNA knockdown of NFAT5 significantly inhibits basal expression levels of four SMC-selective genes

In Figure 1J we showed that SMαA protein levels increased in parallel with NFAT5 protein expression in the rat balloon injury model. In our RNAi loss-of-function studies, we were able to determine whether loss of NFAT5 altered SMC-selective gene expression in SMCs. Utilizing our highly efficient siRNA to NFAT5 (Figure 2B, Supplemental Figure III), we knocked down NFAT5 mRNA in RASMCs and analyzed expression of six SMC-selective genes. Loss of NFAT5 inhibited basal mRNA expression levels of SMαA, Calponin1, Smoothelin, and SM22a, but not SMMHC or Myocardin (Figure 2A). We validated the specificity of our siRNA by showing a 93% knockdown of NFAT5 mRNA, compared to no significant change in expression of transcription factors that share the same Rel homology domain, NFKB and NFATc1-4 (Figure 2B).

NFAT5 positively regulates SMαA

Following our discovery that NFAT5 positively regulates several SMC-selective genes, we chose 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 seven 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, one of the predicted NFAT5 sites overlaps the intronic CArG box (containing SRF binding sites) that is critical for SMαA expression.[23] To further investigate NFAT5 regulation of SMαA, we co-transfected RASMCs with a SMαA WT-luciferase plasmid containing the SMαA promoter and first intron (Figure 3A) with increasing concentrations of either an overexpressing (OE, active) or dominant-negative (DN, inactive) NFAT5 plasmid. At the lowest concentration of 100 ng, OE NFAT5 increased SMαA promoter-intron activity 2.7-fold, while DN NFAT5 significantly decreased SMαA promoter-intron activity by 50% (Figure 3B). To determine if 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, literature indicates that this same mutation does not inhibit a myocardin overexpression-driven increase in SMαA promoter-intron activity,[24] while our data show that this mutation completely ablates an NFAT5 overexpression-driven increase SMαA promoter-intron activity. We also show as an addendum in Supplemental Figure V that NaCl-induced hypertonicity increases SMαA WT promoter-intron activity, and mutation of the intronic CArG/NFAT5 site attenuates this induction. To investigate NFAT5-mediated regulation of SMαA in a model of SMC differentiation, we utilized the retinoic acid (RA)-induced A404 cell line. RA stimulation of A404 smooth muscle precursor cells results in increased expression of virtually all SMC-selective genes;[25] 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 media for 7 days (D1–D7) resulted in increased NFAT5 mRNA preceding

increased SMαA expression (Figure 3C). A404 cells were transfected with NFAT5 siRNA and treated with RA, and RT-PCR 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 show that NFAT5 null mouse embryonic fibroblasts (MEFs) have a 98% reduction in SMαA mRNA compared to WT MEFs (Supplemental Figure VI). To determine whether NFAT5 bound to the region spanning the SMαA intronic CArG/NFAT5 sequence, primers for chromatin immunoprecipitation (ChIP) were made to encompass the intronic CArG/NFAT5 site (around +1.0 kb). We show NFAT5 to enrich SMαA intronic DNA (Figure 3E). Due to the existence of six other NFAT5 consensus sequences in close proximity to the CArG/NFAT5 site, we could not use the ChIP 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 blocks NFAT5 induction of SMαA promoter-intron activity. Thus, we tested the hypothesis that NFAT5 physically interacts with the CArG-binding transcription factor, SRF. A co-immunoprecipitation (Co-IP) assay was performed whereby NFAT5 protein was overexpressed in human embryonic kidney (HEK) 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–C, we showed that NFAT5 was regulated by high NaCl-induced hypertonicity in SMCs; however, it is unknown if 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 (IL-1, IL-8, IL-10, TNFα TNFγ), mitogens (PDGF-BB, S1P, oxPAPC), and contractile agonists (ET-1, Ang II, Thrombin, TGFβ) led to the discovery that angiotensin II (Ang II), a potent contractile agonist and known positive regulator of SMαA activity and expression (Figures 4A & 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 if NFAT5 was required for Ang II-induced 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), therefore 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 to NFATc1-4 proteins

In addition to our discovery of Ang II as an effective regulator of NFAT5 translocation and activity, we also identified platelet-derived growth factor BB (PDGF-BB) as another novel regulator of NFAT5 in SMCs (Supplementary Figure 7). 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 show PDGF-BB to significantly increase NFAT5 protein expression in a calcineurin-independent manner (Figure 5A, Supplemental Figure VIIIA) 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 show that PDGF-BB stimulates NFATc1-4 and NFAT5 activity along different timecourses, 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 in size and were less elongated compared to scramble control SMCs (Figure 6A). To test for altered SMC migration, we utilized two different experimental techniques: 1) Electric cell substrate impedance sensing (ECIS) to measure changes in electrical 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 to scrambled controls (Figure 6B). A similar trend was seen in Boyden chamber assay experiments (Figure 6C). Additionally, NFAT5 knockout (KO) mouse embryonic fibroblasts (MEFs) did not migrate at basal levels (VEH) 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 EdU-based flow cytometry, confirmed that NFAT5 does not regulate SMC proliferation (Figure 6E).

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 hypertonicity 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 SMαA 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 microenvironments could develop within the vessel wall during atherosclerotic lesion progression, possibly due to foam cell necrosis and subsequent release of intracellular contents into the extracellular space, or in response to swelling caused by acute injury. These hypotheses are difficult to test because of both the lack of tools to assess changes in arterial osmolarity *in vivo* and the problematic issues involved in measuring the osmolarity of tissues *ex vivo*. Therefore, we sought to determine if other known SMC phenotype-modulating factors stimulated NFAT5 activity in SMCs.

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 NFkB which promotes inflammation[26] or p53 which induces growth arrest and apoptosis.[27] However, some transcription factors, such as SRF and kruppel-like factor-4 (KLF4), 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.[28] However, 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.[6] 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.[6, 29] 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 pro-migratory genes. Preliminary data from our lab shows that NFAT5 positively regulates basal expression levels of the pro-migratory genes Cyr61 and VEGF-C (data not shown). In addition, we see that NFAT5 is differentially expressed in the SMCs of murine atherosclerotic lesions (Figure 1D–F). These data are important in that they fit the hypothesis that not all SMCs in an artery and lesion are one in the same, but that SMC phenotypes can vary depending on location and stage of the disease.[30]

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, i.e. 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 ApoE null mouse model of atherosclerosis (unpublished data). Although preliminary, these data and this current study herein lay the 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 two stimuli drive the SMC into two very different phenotypes: Ang II to the contractile phenotype and PDGF-BB to the proliferative and/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, upon developmental formation of the mature vessel or resolution of the vascular injury, Ang II signaling may require pre-existing NFAT5 protein to enable rapid activation of SMαA 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 unknown. Several kinases have been implicated in the phosphorylation and subsequent translocation of NFAT5 to the nucleus,[31–34] and we hypothesize that PDGF-BB and Ang II stimulation of downstream kinases such as ERK, p38, and PKC may play a role in signal propagation and NFAT5 activation. Although our lab has begun to investigate these possible mechanisms, the signaling pathways required for NFAT5 phosphorylation in SMCs remain undetermined.

We have discovered that NFAT5 positively regulates basal levels of CArG box-driven SMC-selective genes, namely SMαA, Calponin 1, Smoothelin, and SM22α. To elucidate the mechanism of NFAT5 regulation of SMαA, we identified the location of seven putative NFAT5 binding sites in the SMαA first intron, and ChIP analysis indicated that NFAT5 enriched SMαA intronic DNA (Figure 3E). One of the NFAT5 sites we identified overlaps the intronic CArG that is critical for SMαA expression. Gonzalez Bosc *et al.* previously recognized this overlapping intronic CArG site as an NFATc1-4 consensus sequence.[35] 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%.[35] These findings thereby suggest that a non-calcineurin activated GGAAA-binding protein (i.e. NFAT5) could also modulate SMαA at that site. We demonstrate that NFAT5 positively regulates SMαA by showing that overexpression of NFAT5 increases SMαA promoter-intron activity, while dominant-negative NFAT5 decreases SMαA promoter-intron activity. To determine if SMαA promoter-intron activity required NFAT5 regulation of the sequence overlapping the first intronic CArG, we utilized a pre-existing luciferase construct in which two mutations were made: one at the start of the intronic CArG box and one in the overlapping CArG/NFAT5 site.[20] 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 SMαA promoter-intron activity[24]. It would then be hypothesized that this mutation would not inhibit NFAT5 overexpression-induced SMαA promoter-intron activity, yet we see that this mutation completely ablates an NFAT5 overexpression-induced increase in SMαA promoter-intron activity. This data therefore supports our hypothesis that NFAT5 is regulating SMαA through the overlapping CArG/NFAT5 binding site in the SMαA first intron. We subsequently tested the hypothesis that NFAT5 physically interacts with SRF, but 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 is too weak to detect with a Co-IP assay, or 3) the SRF/NFAT5 interaction blocks the NFAT5 antibody-binding epitope. Finally, we show that RNAi-mediated NFAT5 knockdown inhibits an Ang II-induced increase in both SMαA promoter-intron activity and SMαA mRNA, therefore demonstrating that NFAT5 is necessary for an Ang II-mediated upregulation in SMαA 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 pervading thought that a SMC could modulate to one of two phenotypes: the contractile phenotype or synthetic (i.e. migrating and proliferating) phenotype.[2] Our current understanding of SMC phenotypic modulation is now much more complex. The contractile SMC can modulate towards an array of phenotypes and can become proliferative, migratory, or inflammatory.[36] Therefore, NFAT5 could specifically direct SMC migration and not proliferation, as these processes are mutually exclusive. Literature shows that NFAT5 is required for migration of carcinomas, skeletal myoblasts, and endothelial cells during angiogenesis,8, 9, 19 while NFAT5 has been documented as regulating proliferation in lymphocytes and fibroblast-like synoviocytes.10, 19, 37 These data indicate that NFAT5 can direct different cellular functions in diverse tissues. In an effort to validate the pro-migratory 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 due to the inconsistency of neointimal hyperplasia in the $NFAT5^{+/-}$ mice. Further 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 two newly-identified NFAT5 stimuli, Ang II and PDGF-BB. NFAT5 is required for both Ang II stimulation of SMαA 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 atherosclerosic 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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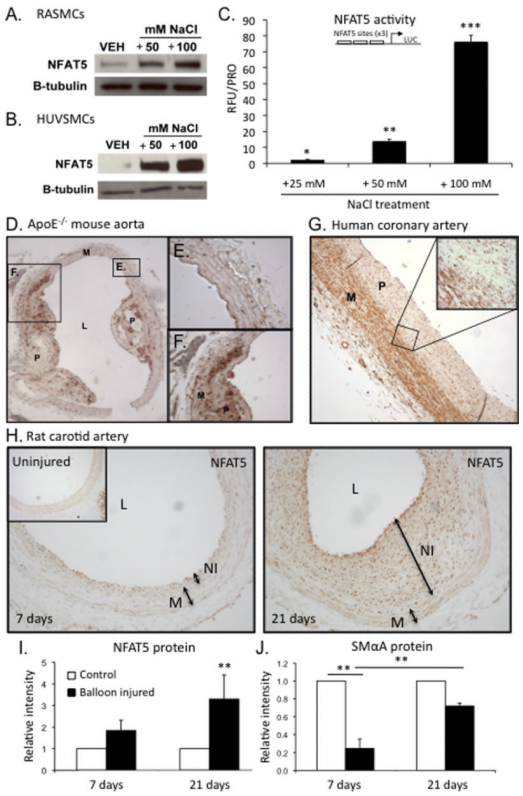


Figure 1. NFAT5 is expressed in vascular smooth muscle and is upregulated in vascular injury

Increasing concentrations of NaCl induces: **A.** NFAT5 protein expression in RASMCs (n=5), **B.** NFAT5 protein expression in human umbilical vein SMCs (n=3), and **C.** NFAT5 reporter expression (i.e. NFAT5 activity) in RASMCs. (n=3, *p<0.05, **p<0.005, ***p<0.0001) **D.** NFAT5 is differentially expressed in the ApoE$^{-/-}$ atherosclerotic plaque at 20 wks. (M=media, P=plaque, L=lumen) **E.** NFAT5 expression is low in medial SMCs with no overlaying lesion. **F.** NFAT5 expression is high in both the cells of the lesion and surrounding medial SMCs. **G.** Mid-grade human atherosclerotic lesions show high NFAT5 protein expression in the medial (M) SMCs surrounding the plaque (P). **H.** NFAT5 protein is upregulated at 7 days and 21 days in the SMCs of the rat carotid artery following balloon injury. (M=media, NI=neoinitma, L=lumen) **I–J.** Western blot analysis of rat balloon-injured artery protein shows that NFAT5 protein is upregulated at 21 days (**I**) and that SMαA protein is 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)

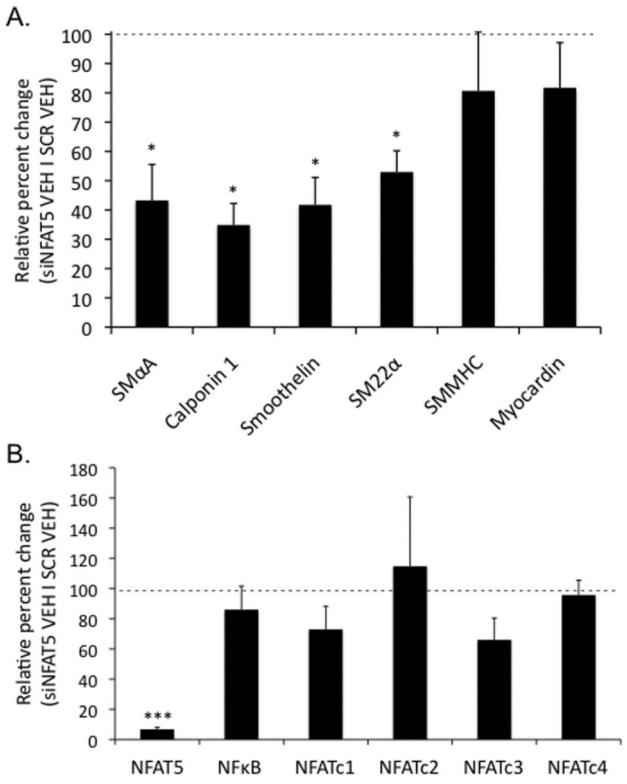


Figure 2. siRNA knockdown of NFAT5 significantly inhibits basal expression levels of four SMC-selective genes

A. RT-PCR analysis of SMC-selective genes shows inhibited basal mRNA expression of SMαA, Calponin1, Smoothelin, and SM22α in RASMCs transfected with NFAT5 siRNA. **B.** RT-PCR analysis validates siRNA specificity (93% knockdown of NFAT5) in relation to other transcription factors that share the same Rel homology domain with NFAT5. (n=4, *p<0.05 ***p<0.0001)

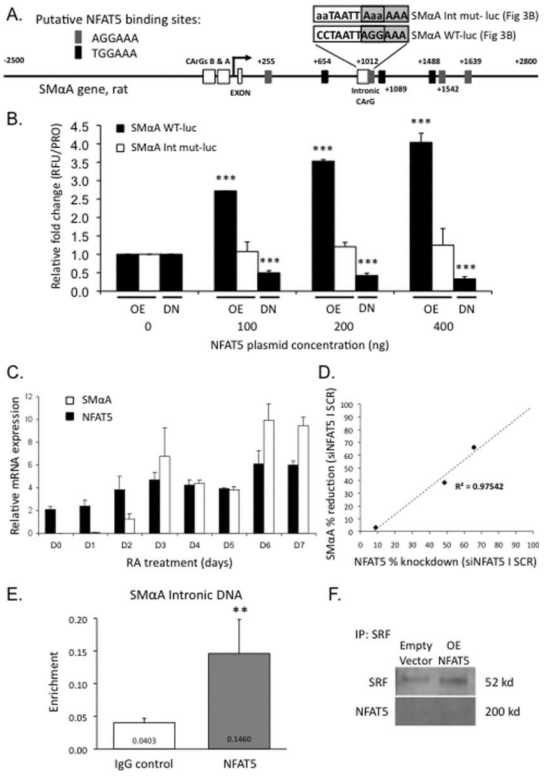


Figure 3. NFAT5 positively regulates SMαA

A. Bioinformatic analysis revealed seven putative NFAT5 binding sites ((T/A)GGAAA) in the SMαA first intron. **B.** the intronic NFAT5 binding site ablates an OE NFAT5 induction in SMαA promoter-intron activity, and mutation of the intronic NFAT5 binding site ablates an OE NFAT5 induction in SMαA promoter-intron activity. (n=3, ***p<.0001) **C.** NFAT5 mRNA expression precedes increased SMαA expression over 7 days in RA-treated differentiating A404 cells. (n=3) **D.** The percentage of NFAT5 knocked down in A404 cells in each biological replicate directly correlates with the percentage of reduced SMαA mRNA. (n=3, R^2=0.975) **E.** Chromatin immunoprecipitation performed in untreated RASMCs shows NFAT5 enrichment of tne SMαA intronic CArG region. (n=10, **p<0.005) **F.** Co-immunoprecipitation performed in HEK cells indicates that NFAT5 and SRF do not interact.

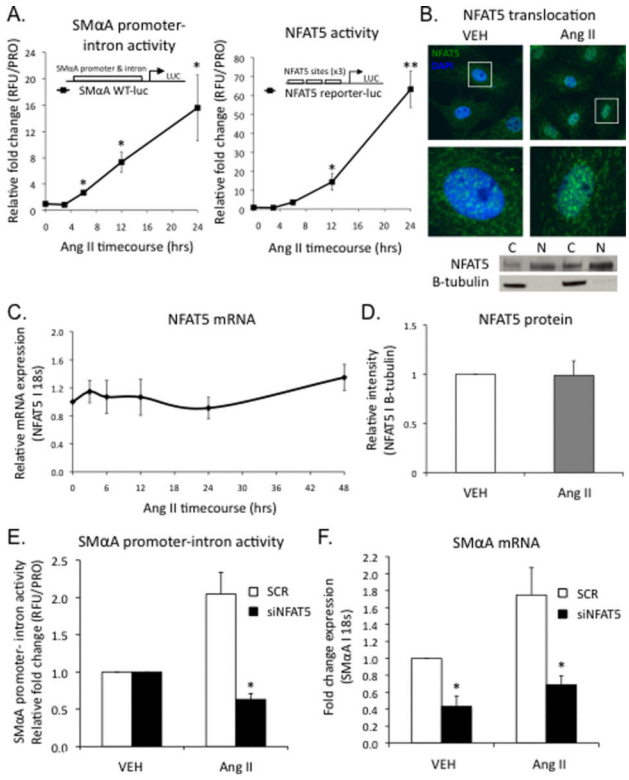


Figure 4. Ang II increases NFAT5 translocation and transcription factor activity, and NFAT5 is required for an Ang II-mediated upregulation in SMαA promoter-intron activity and mRNA expression

A. Ang II upregulates SMαA promoter-intron activity and NFAT5 reporter activity in RASMCs. (n=3, *p<0.05 **p<.005) **B.** Immunofluorescence images show NFAT5 translocation to the nucleus following Ang II treatment, verified by western blot analysis. (n=3) **C-D**. RASMCs treated with Ang II show no change in NFAT5 mRNA (n=3) (**C**) nor NFAT5 protein (n=12) (**D**). **E-F.** siRNA knockdown of NFAT5 inhibits a 24 hr Ang II-mediated upregulation in SMαA promoter-intron activity (**E**) and mRNA expression (**F**). (n=3, *p<0.05)

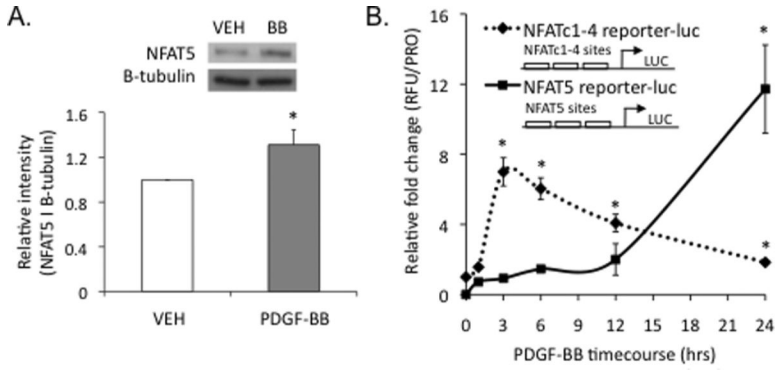
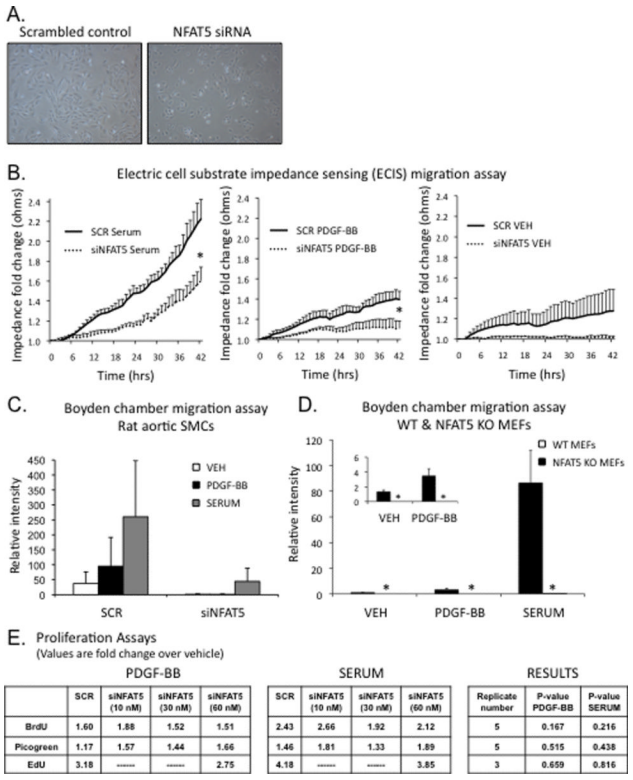


Figure 5. PDGF-BB upregulates NFAT5 protein and NFAT5 reporter activity and stimulates a more delayed NFAT5 activation compared to NFATc1-4 proteins

A. Western blot analysis of RASMCs treated with PDGF-BB for 24 hrs shows increased NFAT5 protein expression. (n=6, *p<0.05) **B.** RASMCs treated with PDGF-BB show NFATc1-4 reporter activity to peak early at 3 hrs, unlike the more delayed response in NFAT5 activity, which peaks at 24 hours. (n=3, *p<0.05)



E. Proliferation Assays
(Values are fold change over vehicle)

	PDGF-BB				SERUM				RESULTS		
	SCR	siNFAT5 (10 nM)	siNFAT5 (30 nM)	siNFAT5 (60 nM)	SCR	siNFAT5 (10 nM)	siNFAT5 (30 nM)	siNFAT5 (60 nM)	Replicate number	P-value PDGF-BB	P-value SERUM
BrdU	1.60	1.88	1.52	1.51	2.43	2.66	1.92	2.12	5	0.167	0.216
Picogreen	1.17	1.57	1.44	1.66	1.46	1.81	1.33	1.89	5	0.515	0.438
EdU	3.18	------	------	2.75	4.18	------	------	3.85	3	0.659	0.816

Figure 6. NFAT5 positively regulates PDGF-BB and serum-induced SMC migration, but not proliferation

A. RASMCs transfected with NFAT5 siRNA are less elongated and appear to be smaller in size.**B.** NFAT5 siRNA-transfected SMCs treated with PDGF-BB and serum display decreased migration compared to scrambled control SMCs, as quantified by ECIS. (n=3, *p<0.05) **C.** The Boyden chamber assay displays a trend of impaired chemotaxis-induced migration of siRNA-transfected RASMCs. (n=3) **D.** NFAT5 KO mouse embryonic fibroblasts (MEFs) show an ablated migratory response to basal, PDGF-BB, and serum-induced migration compared to WT MEF controls. (n=3, *p<0.05) Exhaustive SMC proliferation analyses reveal that NFAT5 is not critical for SMC proliferation. (BrdU assay [n=5], Picogreen assay [n=5], flow cytometry EdU assay [n=3])