CYCLIN D1 IS A BONA FIDE TARGET GENE OF NFATc1 AND IS SUFFICIENT IN THE MEDIATION OF INJURY-INDUCED VASCULAR WALL REMODELING

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Running title: Cyclin D1 is a target gene for NFATc1 in HASMCs
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Platelet-derived growth factor BB (PDGF-BB) induced cyclin D1 expression in a time- and NFAT-dependent manner in human aortic smooth muscle cells (HASMCs) and blockade of NFATs prevented HASMC DNA synthesis and their cell cycle progression from G1 to S phase. Selective inhibition of NFATc1 by its siRNA also blocked HASMC proliferation and migration. Characterization of cyclin D1 promoter revealed the presence of several NFAT binding sites and the site at -1333 nt was found to be sufficient in mediating PDGF-BB-induced cyclin D1 promoter-luciferase reporter gene activity. In addition to its role in cell cycle progression, cyclin D1 mediated HASMC migration in NFATc1-dependent manner. Balloon injury-induced cyclin D1-CDK4 activity requires NFAT activation and adenovirus-mediated transduction of cyclin D1 was found to be sufficient to overcome the blockade effect of NFATs by VIVIT on balloon injury-induced vascular wall remodeling events, including SMC migration from medial to luminal region, their proliferation in the intimal region and neointima formation. Together, these results provide another mechanistic evidence for the role of NFATs, particularly NFATc1 in the regulation of HASMC proliferation and migration as well as vascular wall remodeling. NFATc1 could be a potential therapeutic target against the re-narrowing of artery after angioplasty.

Nuclear factor of activated T cells (NFATs) are a family of transcriptional factors that belong to the Rel/NFkB group (1). Among the five members of this family of transcriptional factors identified to date, NFATc1-c4 require Ca\(^{2+}\)/calcineurin for activation and play a pivotal role in transcriptional regulation of cytokine genes (2-4). On the other hand, NFAT5, which is also called as tonicity enhancer binding protein, although belongs to the same family of transcriptional factors, appears to be Ca\(^{2+}\)/calcineurin-independent and regulates genes associated with hypertonicity (5). The calcineurin-NFAT signaling has been shown to play a role in various developmental aspects, including cardiac valve formation (6), skeletal muscle growth (7), neuronal and osteoclast differentiation (8, 9) and vascular development (10). In addition, a large body of evidence indicates a role for calcineurin-NFAT signaling in the regulation of pathological cardiac hypertrophy (11, 12). In contrast, suppression of an endogenous calcineurin inhibitor, down syndrome candidate region-1 (DSCR1), in endothelial cells leads to hyper activation of calcineurin, which results in precocious endothelial cell apoptosis, thereby inhibiting tumorigenesis (13). Furthermore, in infantile hemangiomas (the tumors of endothelial cell origin), increased VEGFR2 signaling was found to be linked to suppression of NFAT-dependent expression of VEGFR1 (14). Thus, NFATs appear to be involved in the regulation of both positive and negative cell growth.

Dedifferentiation of vascular smooth muscle cells (VSMCs), followed by their migration from media to intima and proliferation in intima contribute to the pathophysiology of re-narrowing of the artery after angioplasty (15, 16). Previously, we have shown that NFATs play a role in the regulation of vascular
smooth muscle cell (VSMC) growth and migration (17, 18). Towards understanding the mechanisms of NFATs in the regulation of VSMC migration, we have shown that these transcriptional factors mediate interleukin-6 expression as one of the effector molecules in receptor tyrosine kinase (RTK) and G protein-coupled receptor (GPCR) agonist-induced VSMC motility (18). In addition, we have reported the involvement of NFATs in injury-induced neointima formation (19). Recently we have also demonstrated that activation of NFATc1 causes up-regulation of cell cycle dependent gene, cyclin A2, leading to increased CDK2 activity in VSMCs and the progression of these cells through the cell cycle (20). Since cell cycle progression is a complex multistep process orchestrated by different cyclins and their partner CDKs (21-23), it is quite possible that NFATc1 may also have a role in the regulation of other cell cycle dependent genes. In this manuscript, we identified cyclin D1 as a target gene for NFATc1 in human aortic smooth muscle cells (HASMCs) and it is sufficient to mediate the progression of these cells through the cell cycle as well as their motility downstream to NFATc1. In addition, adenovirus-mediated transduction of cyclin D1 was found to be sufficient in overcoming the inhibitory effect of NFAT blockade on balloon injury-induced SMC migration from medial to luminal surface and their proliferation in the intimal region forming neointima. These observations together with our previous findings provide a convincing evidence for the role of NFATc1 in vascular wall remodeling following injury.

MATERIALS AND METHODS
Reagents- Cyclosporin A (A-195) was bought from Biomol (Plymouth Meeting, PA). Recombinant human PDGF-BB (220-BB) was from R & D Systems Inc. (Minneapolis, MN). Anti-CDK4 antibodies (SC-260), anti-NFATc3 antibodies (SC-8321), anti-Rb antibodies (SC-50), anti-β-tubulin antibodies (SC-9104), truncated Rb protein (SC-4112) and mouse normal serum (SC-45051) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cyclin D1 antibodies (RB-010-P) were obtained from NeoMarkers (Fremont, CA). Anti-NFATc1 antibodies (MA3-024) were from Affinity BioReagents (Golden, CO). Anti-histone H1 antibodies (MAB 1276) were bought from Chemicon (Temecula, CA). Anti-pRb antibodies (Ser795) were obtained from Cell Signaling Technology (Beverly, MA). ABC kit, DAB kit and Vectashield mounting medium containing DAPI were purchased from Vector Laboratories Inc. (Burlingame, CA), respectively. Propidium Iodide, Lipofectamine LTX and plus reagent, and Pf50 Taq polymerase were from Invitrogen (Carlsbad, CA). pGEM-T vector was from Promega (Madison, WI). Stratagene Quickchange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). RNase A was purchased from Roche (Indianapolis, IN). T4 polynucleotide kinase was obtained from New England Biolabs (Ipswich, MA). [γ-32P]-ATP (s. a. 3000 Ci/mmol) was from MP Biomedicals (Irvine, CA) and protein-A Sepharose (CL-4B) was purchased from Amersham Biosciences (Piscataway, NJ). In situ cell death detection kit, TMR Red was purchased from Roche Diagnostics (Indianapolis, IN). Human NFATc1 siRNA (Human NFATc1, ON-TARGETplus SMART-pool L-003605-00-0020, NM_172390), human NFATc2 siRNA (Human NFATc2, ON-TARGETplus SMART-pool L-003606-00-0005, NM_173091), human cyclin D1 siRNA (ON-TARGETplus SMART-pool L-003606-00-0005, NM_173091), human cyclin A2 siRNA (ON-TARGETplus SMART-pool L-003205-00, NM_053056), control nontargeting siRNA (D-0012-06) and DharmaFECT 1 transfection reagent (T-2001-02) were bought from Dharmacon RNAi Technologies (Chicago, IL). All the primers and oligonucleotides were synthesized by IDT (Coralville, IA).

Cell culture- HASMCs were purchased from Cascade Biologicals (Portland, OR), subcultured in Medium 231 containing smooth muscle cell growth supplements and used between 4 and 10 passages.

Western blot analysis- HASMCs with and without an appropriate treatment were
harvested and cell extracts were prepared. An equal amount of protein was analyzed by Western blotting for the protein of interest using its specific antibodies as described previously (20).

**CDK4 assay** - Protein extracts from cells or tissues were analyzed for CDK4 activity as described previously (24). The [32P]-labeled Rb protein was visualized by autoradiography and the band intensities were quantified using NIH Image J.

**RT-PCR** - Total cellular RNA was isolated from HASMCs with and without an appropriate treatment using Trizol reagent following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Reverse transcription was carried out with Superscript III First-Strand Synthesis System for RT-PCR based on supplier’s protocol (Invitrogen, Carlsbad, CA) and the cDNA was then used as template for PCR using primers specific for human cyclin D1 (NM_53056) (forward, 5'-AAG GCG GAG GAG ACC TGC GCG-3' and reverse, 5'-ATC GTG CGG CAT TGC GGC-3') and human β-actin (NM_001101) (forward, 5'-AGCCATGTACGTTGCTAT-3' and reverse, 5'-GATGTCCACGTCACACTTCA-3'). The PCR amplification was carried out up to 25 cycles on Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA), and the amplified RT-PCR products were separated on 8% native polyacrylamide gels, stained with ethidium bromide. Gel pictures were captured using AlphaEase Digital Imaging System (Alpha Innotech Corporation, SanLeandro, CA) and the band intensities were quantified using NIH Image J.

**DNA synthesis** - DNA synthesis in HASMCs with and without appropriate treatments was measured by pulse-labeling cells for the last 16 hrs of the 24-hrs treatment period with 2 µCi/ml [3H]-thymidine as described previously (20).

**FACS analysis** - Cell cycle progression of HASMCs with and without appropriate treatments was monitored using Propidium Iodide staining as described previously (20).

**Cell migration** - HASMC migration was measured by modified Boyden chamber method as described previously (25) and the migrated cells were stained with DAPI and counted under a Nikon diaphot fluorescence microscope with phometrics CH250 CCD camera (Nikon, Garden City, NY).

**Electrophoretic mobility shift assay** - Nuclear extracts of HASMCs with and without appropriate treatments were prepared and analyzed for DNA binding activity of NFATs as described previously (20). Double-stranded oligonucleotides that encompass the -1254 (GAC AGT AAA ATG TCC TTT TAT TTT TTA ATG), -1333 (TGA ACT ATA TTC AAA AGG AAG TAA ATG AAC), -2293 (GCT TTT CTT TTT GGA AAA GCG GGA GAA) and -2902 (GGG CTT AAC AAT GGA AAA CAT CGC GGA CGC T) NFAT regulatory elements from human cyclin D1 promoter were used as [32P]-labeled or unlabeled cold probes to measure NFAT DNA binding activity.

**Chromatin immunoprecipitation (ChIP) assay** - Binding of NFATc1 to different NFAT regulatory elements in cyclin D1 promoter was measured by using a ChIP assay kit following the supplier’s protocol (Upstate Biotechnology Inc. Lake Placid, NY). The immunoprecipitated and purified DNA was used as a template for PCR amplification using four sets of primers. The first set of primers (forward, 5'-CTG GCG AAG GGG AGA GGG CTT T-3' and reverse, 5'-GGT GTT CGT GGT TAC ATG AGA G-3') encompass the -1254 and -1333 NFAT binding elements that would give a PCR product of 276 bp. The second, third and fourth sets of primers (2nd set: forward, 5'-GCTTCGTGGTGGGTTTTAAG-3' and reverse, 5'-GACCAATACCTCTGGGCTT-3'; 3rd set: forward, 5'-GCTGAGCGCAAGTCCTTTCAAG-3' and reverse, 5'-GAGCGCTTCATTCAAGA-3'; 4th set: forward, 5'-
GGCTTTTCGAAGCGTTTT-3’ and reverse, 5’-GGACCAGTCCCGACCTTT-3’) encompass the -2293, -2801 and -2902 NFAT binding elements, respectively, that would give PCR products of 150 bp, 237 bp, 193 bp, respectively. Primers (forward, 5’-GCT AAG GGC CGC GCG GCT-3’ and reverse, 5’-CAC CGG ACC TTG CAG CTG C-3’) spanning the cyclin D1 gene region 2.5 kb away from the -1333 NFAT element were used as negative control. The resulting PCR products were resolved on 8% native polyacrylamide gels, stained with ethidium bromide, pictures were taken using AlphaEase Digital Imaging System (Alpha Innotech Corporation, SanLeandro, CA) and the band intensities quantified using NIH Image J.

**Cloning of human cyclin D1 promoter**- The human cyclin D1 promoter region -1430 to +50 bp relative to transcription start site was PCR amplified from genomic DNA using the following primers, forward, 5’-GCTGTGGGTACC CT GGGCGAAGGGGAGAGGGCT-3’ and reverse, 5’-AAGCTT GAGGCTCCAGGACTTTGC-3’. Reaction products were analyzed by agarose gel electrophoresis and cloned into the pGEM-T vector. The region -1430/+50 was released from pGEM-T vector by digestion with Kpn I and Hind III. The excised fragment was then subcloned into the pGL3 basic vector (Promega) at the same site yielding pGL3-CCND1p-(1.5 kb)-Luc. A series of 5’-deletion constructs, pGL3-CCND1p-(1.0 kb)-Luc, pGL3-CCND1p-(0.9 kb)-Luc, pGL3-CCND1p-(0.8 kb)-Luc and pGL3-pCCND1p-(0.5 kb)-Luc were generated via PCR amplification using following forward primers: 5’-GGTACC CGGTCTTGTCCCAGGCAGAG-3’, 5’-GGTACC GGATCACTGTTTCTCAGC-3’, 5’-GGTACC AGAGTCTCCAGGCTAGAAGG-3’, 5’-GGTACC GTGGCGTTCTTGGAAATGC-3’, and reverse primer, 5’-AAGCTTGAGGCTCCAGGACTTTGC-3’. The underlined sequences in the forward and reverse primers indicate Kpn I and Hind III sites, respectively. Site directed mutation within NFAT binding sites at -1333 to -1339 nt (second NFAT site) and -1254 to -1260 nt (first NFAT site), was introduced using the QuickChange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions using the following primers, forward, 5’-GAACATATTTCAAAAAATTCGTAATGGAACAGTT-3’, and reverse, 5’-AAGCTTCAATTACTGGAATTTTGAATATAGTT-3’; forward, 5’-GTGAACAGTAATAATGGGAATTATATT GTTTAATGCACTC-3’ and reverse, 5’-GGTGCAATTAAAAATAAAATCCATTACTGTTCAATAC-3’, respectively. Mutations at both the NFAT binding sites were introduced (pGL3-CCND1p-(1.5 kb DM)-Luc) by using the construct with single mutation at -1333 nt NFAT binding site as a template and the primers that were used to mutate the NFAT binding site at -1254 nt. The bold letters indicate the mutated bases. The nucleotide sequence of each construct was verified by DNA sequencing.

**Transient transfections and luciferase assays**- Equal number of HASMCs were transfected with pGL3-CCND1 promoter constructs using Lipofectamine LTX and Plus Reagent according to the manufacturer’s instructions in 231 Medium containing 0.1% serum (Invitrogen, CA). After transfection, cells were quiesced for 24 hrs in the above medium with 0.1% serum and treated with or without PDGF-BB (20 ng/ml) for 8 hrs. Cells were washed with cold PBS and lysed in 100 µl of lysis buffer (Promega). Cell extracts were assayed for luciferase activity using Luciferase Assay System (Promega) and a single tube luminometer (TD20/20, Turner Designs, Sunnyvale, CA).

**Tunnel assay**- HASMCs after appropriate treatments were fixed with paraformaldehyde and examined for cell death using in situ cell death detection kit essentially as per the instructions of the manufacturer.
Adenoviral vectors- Construction of Ad-GFP and Ad-GFPVIVIT were described previously (19). The human cyclin D1 was amplified by PCR using primers, forward, 5'-TTA ACC GTC GAC ATG GAC TAC AAA GAC CAT GAC-3' and reverse, 5'-ACA GGG ATG CCA CCC GGG ATC CTC TAG-3' from the p3XFLAG-cyclin D1 expression vector (26) and subcloned into Sal I and BamH I sites of pBlueScript. The underlined sequences in the forward and reverse primers represent Sal I and BamH I sites, respectively. The cyclin D1 insert was released from pBlueScript by digestion with Sal I and Not I and sub-cloned into the same sites of pENTR3C vector, which was then subjected to recombination with pAd/CMV/V5-DEST vector to generate pAd-cyclin D1. pAd-cyclin D1 was linearized with Pac I and used to transfect HEK293A cells to amplify the virus. The virus was purified with cesium chloride gradient centrifugation and titers were determined by plaque assay as described previously (27).

Carotid artery balloon injury- All animal protocols were performed in accordance with the relevant guidelines and regulations approved by the Institutional Animal Care & Use Committee of the University of Tennessee Health Science Center. BI was performed essentially as described previously (28). Briefly, rats weighing 250-300 g were anesthetized by injecting (ip) ketamine (60 mg/kg) and xylazine (5 mg/kg). Under a stereomicroscope, the right common, external and internal carotid arteries were exposed by a longitudinal midline cervical incision and blood flow was temporarily interrupted by ligation of the common and internal carotid arteries using vessel clips. External carotid artery was ligated permanently. A 2F Fogarty arterial embolectomy catheter was introduced through an arteriotomy in the external carotid artery just below the ligature and advanced to the common carotid artery. To produce carotid artery injury, the balloon was inflated with saline and passed 4 times with rotation from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After this, the balloon was deflated and the catheter was withdrawn. The external carotid artery was ligated with a 6-0 silk suture and the blood flow restored by removing the clips at the common and internal carotid arteries. After inspection to ascertain adequate pulsation of the common carotid artery, the surgical incision was closed and the rats were allowed to recover from anesthesia in a humidified and warmed chamber for 2 h to 4 h. At three days or two weeks after balloon injury, the animals were sacrificed with an overdose of pentobarbital (200 mg/kg) and the carotid arteries were collected and either protein was isolated or fixed to make cryosections.

Delivery of adenoviruses into injured arteries- After balloon injury, solutions of 100 µl Ad-GFP (10^10 pfu/ml), Ad-VIVIT (10^10 pfu/ml) or Ad-cyclin D1 (10^10 pfu/ml) were infused into the ligated segment of the common carotid artery for 30 min as described previously (28).

Double immunofluorescence staining- After dissecting out, arteries were embedded in OCT compound. Cryo-sections (5 µm) were made using Leica Kryostat machine (Model: CM3050S). After fixing in cold acetone and blocking in normal goat serum, the cryo-sections were incubated first with anti-SMCα-actin antibodies (1:500) followed by TRITC-conjugated secondary antibodies. After washing with PBS and blocking again in normal goat serum, these sections were incubated with anti-cyclin D1 (1:100) antibodies followed by FITC-conjugated secondary antibodies. Fluorescence was observed under Zeiss inverted fluorescence microscope (Model: AxioVision AX10).

In vivo SMC migration assay- The in vivo SMC migration was determined as described by Bendeck et al. (29). Briefly, three days after balloon injury, the middle 1 cm of the denuded (injured) and uninjured common carotid artery was cut and fixed in cold acetone for 15 minutes. The artery was then opened longitudinally and pinned down onto an agar plate with the luminal surface facing upward. The arteries were rinsed in PBS and then placed in 0.3% H₂O₂ for 30 min to block
endogenous peroxidase activity. Non-specific protein binding was blocked by incubating the arteries in 5% normal goat serum in PBS for 30 minutes. The arteries were incubated with anti-histone monoclonal antibodies (MAB 1276, Chemicon, Temecula, CA) diluted 1:300 in PBS for 1 h, followed by incubation in biotinylated goat anti-mouse IgG for 30 minutes. Peroxidase labeling was carried out by using ABC kit (Vector Laboratories) and the signals were visualized by using DAB kit (Vector Laboratories). After each step, the slides were rinsed 3 times for 5 min each in PBS. Finally, the opened arteries were placed intimal side up on glass slides with coverslips. As a negative control, samples of the same specimens without the primary antibody incubation were used. The intimal surface of the vessel was examined under a light microscope at X200 magnification and the total number of positively stained cells per 0.1 square millimeter of the luminal surface area was counted.

Histochemistry- The cryo-sections (5 µm) of balloon injured, Ad-GFP or Ad-VIVIT-transduced carotid arteries were immunostained for PCNA using its specific antibodies or stained with H & E. The stained sections were observed under light microscope (Model: Eclipse 50i, Garden City, NY). SMC proliferation was measured by counting PCNA-positive cells in the intimal and medial regions of each artery using NIH image J. The intimal (I)/medial (M) areas and lumen sizes in H & E stained sections were measured using NIH image J program and the I/M ratios were calculated.

Statistics- All the experiments were repeated several times with similar results. Data are presented as Mean ± S. D. The treatment effects were analyzed by Student’s t test. p values < 0.05 were considered to be statistically significant. In the case of ChIP analysis, EMSA, and Western blotting, one representative set of data is shown.

RESULTS

Previously, we have demonstrated that NFATc1 mediates PDGF-BB-induced expression of cyclin A2 in VSMCs (20). Since sequential activation of cyclin D/CDK4, cyclin E/CDK2, cyclin A/CDK2 and cyclin B/CDK1 is required for orderly cell cycle progression (21-23), we asked the question whether NFATc1 besides cyclin A2 targets other cyclins in the regulation of PDGF-BB-induced VSMC proliferation. To address this question, we have studied the role of NFATs in PDGF-BB-induced expression of cyclin D1, as it plays a determinant role in cell cycle progression from G0 to G1 (21). PDGF-BB (20 ng/ml) induced cyclin D1 expression both at mRNA and protein levels as measured by semi-quantitative RT-PCR and Western blot analysis, respectively, in a time-dependent manner with maximum increases at 8 h and 16 h in HASMCs (Figures 1A & 2A). To examine the role of NFATs in PDGF-BB-induced cyclin D1 expression, we tested the effect of CsA, a potent inhibitor of calcineurin (1) and VIVIT, a pentapeptide that competes with NFATs for binding to calcineurin and inhibits NFATs (30). Both CsA (10 µM) and VIVIT (80 MOI) blocked PDGF-BB-induced cyclin D1 expression at mRNA and protein levels (Figures 1B & 2B; Supplemental Figure 1A & B). Since cyclin D1 forms a complex with and activates CDK4, we next studied the effect of PDGF-BB on CDK4 activity. Consistent with its effect on cyclin D1 levels, PDGF-BB (20 ng/ml) induced CDK4 activity in a time-dependent manner with maximum 2- to 3-fold increases at 8 h and 16 h (Figure 2A). In addition, CsA and VIVIT inhibited PDGF-BB-induced CDK4 activity (Figure 2B; Supplemental Figure 1B). To find whether PDGF-BB-induced cyclin D1/CDK4 activity correlates with phosphorylation of endogenous Rb protein, we also studied the time course effect of PDGF-BB on Rb phosphorylation levels. Consistent with its effect on cyclin D1/CDK4 activity, PDGF-BB-induced Rb (Ser795) phosphorylation in a time-dependent manner with maximum effects at 8 h and 16 h and VIVIT significantly blunted this effect (Figure 2A & B). As cyclin D1/CDK4 plays an important role in cell cycle progression, we next examined the effects of CsA and VIVIT on PDGF-BB-induced DNA synthesis and cell cycle progression. PDGF-BB induced DNA
synthesis by 4-fold as measured by [3H]-thymidine incorporation and this effect was blocked by both CsA and VIVIT (Figure 3A; Supplemental Figure 2A). FACS analysis showed that treatment with PDGF-BB induced about 45% of quiescent HASMCs to enter S phase from G1 phase of the cell cycle as compared to untreated cells (Figure 3B; Supplemental Figure 2B). Consistent with their effect on DNA synthesis, both CsA and VIVIT abrogated PDGF-BB-induced HASMC progression from G1 to S phase (Figure 3B; Supplemental Figure 2B). Our previous studies have demonstrated a role for NFATc1 in the regulation of VSMC growth by both GPCR and RTK agonists (17, 20). Therefore, we asked the question whether NFATc1 also plays a role in PDGF-BB-induced cyclin D1 expression in HASMCs. We used NFATc1 siRNA molecules to down-regulate NFATc1 levels and determined its effects on PDGF-BB-induced cyclin D1 expression. NFATc1 siRNA while having no effect on NFATc3 levels depleted NFATc1 levels by more than 90% (Figure 4A). NFATc1 siRNA also abolished PDGF-BB-induced cyclin D1 expression at both mRNA and protein levels in HASMCs (Figure 4B & C) and their DNA synthesis and migration (Figure 4D & E). To find whether other members of the NFAT family of transcription factors have any role in PDGF-BB-induced cyclin D1 expression, we also studied the role of NFATc2. Depletion of the steady-state levels of NFATc2 by its siRNA had no noticeable effect on PDGF-BB-induced cyclin D1 expression at mRNA or protein levels (Figure 5A-C).

To understand the mechanisms of NFATc1 involvement in the regulation of cyclin D1 expression, we analyzed about 3.0 kb of human cyclin D1 promoter for transcriptional factor binding sites by TFsearch. Five NFAT regulatory elements were found in the 3.0 kb cyclin D1 promoter, one each at -1254, -1333, -2293, -2801 and -2902 nt position relative to transcription start site (31). In order to understand which of these NFAT-binding elements are involved in PDGF-BB-induced cyclin D1 expression, we cloned a 1.5 kb promoter region that contains the first two NFAT-binding sites from the transcription start site with serial 5'-deletions in pGL3 basic vector and determined their effects on luciferase reporter gene activity (Figure 6A & B). PDGF-BB induced luciferase reporter gene activity only with 1.5 kb cyclin D1 promoter that contains the first two NFAT-binding sites. Deletion of these two NFAT-binding sites in the promoter failed to respond to PDGF-BB in the induction of luciferase activity (Figure 6B). Furthermore, mutation in the NFAT-binding site at -1333 nt but not at -1254 nt abolished PDGF-BB-induced luciferase activity, suggesting that the second NFAT-binding site from the transcription start site is essential for cyclin D1 expression by PDGF-BB (Figure 6B). Serial deletions of AP-1, NFKb and E2F transcriptional factor binding sites had no effect on PDGF-BB-induced promoter activity. To obtain further evidence for the role of NFAT-binding site at -1333 nt in PDGF-BB-induced cyclin D1 expression, we next studied the time course effect of PDGF-BB on NFAT DNA binding activity using the -1333 NFAT element as a [32P]-labeled oligonucleotide probe. PDGF-BB induced protein-DNA binding activity in a time-dependent manner with about 2-fold increase at 2 h using this element as a [32P]-labeled probe (Figure 7A). The specificity of NFAT binding activity to this site was further confirmed by lack of binding of a [32P]-labeled mutant -1333 NFAT oligonucleotide probe (Figure 7B). To check if NFATs bind preferentially to -1333 NFAT-binding site compared to those at -1254, -2293, -2801 and -2902 nt positions in response to PDGF-BB, we used 10-fold molar excess of the cold oligonucleotide probes representing these NFAT sites in a competition binding assay with the [32P]-labeled -1333 NFAT oligonucleotide probe. Only the cold -1333 NFAT-binding oligonucleotides but not the other NFAT-binding oligonucleotides competed with the [32P]-labeled -1333 NFAT oligonucleotide probe for binding to nuclear extracts of PDGF-BB-induced HASMCs (Figure 7C).
regulation of cyclin D1, we also measured NFATc1 binding to cyclin D1 promoter using chromatin immunoprecipitation (ChIP) assay. ChIP analysis showed that NFATc1 binds to cyclin D1 promoter region containing NFAT sites at -1254/-1333 nt positions in a time-dependent manner in response to PDGF-BB (Figure 7F). In contrast, although NFATc2 binds to NFAT elements at -1254/-1333 nt at basal level, its binding was not influenced by PDGF-BB, suggesting that NFATc1-binding to these sites is specific (Figure 7G). To test whether NFATc1 binds to other NFAT-binding sites, we also performed ChIP assay using primers specific for these sites. As shown in Figure 7H, NFATc1 preferentially binds to NFAT sites at -1254 and -1333 nt but not to NFAT sites at -2293, -2801 and -2902 nt positions. Furthermore, PDGF-BB-induced NFATc1 binding to -1254/-1333 NFAT-sites was inhibited by VIVIT (Figure 7I). As a negative control, we designed primers for the cyclin D1 gene 2.5 kb downstream to the -1333 NFAT binding site, and these primers did not give any amplification product with the anti-NFATc1 immunoprecipitated and purified DNA as a template. These results clearly indicate that NFATc1 binds preferentially to the NFAT sites at -1254/-1333 nt in response to PDGF-BB. We should also point out that although it is technically difficult to differentiate between these two sites to which one NFATc1 binds, the mutagenesis, and EMSA data clearly point out that NFATc1 binds to -1333 NFAT-element. Besides its involvement in cell cycle regulation, cyclin D1 has been reported to play a role in cell migration (32). In this aspect, we recently reported that STAT-5B-dependent expression of cyclin D1 plays a role in PDGF-BB-induced HASMC migration (24, 33). Since NFATc1 plays a role in PDGF-BB-induced HASMC DNA synthesis and migration and it mediates PDGF-BB-induced cyclin D1 expression, we were intrigued to find whether cyclin D1 has any role in HASMC migration downstream to NFATc1. Depletion of cyclin D1 levels by the use of its siRNA inhibited PDGF-BB-induced HASMC DNA synthesis and migration (Figure 8A, B & C). In contrast, depletion of cyclin A2 while inhibiting DNA synthesis had no effect on PDGF-BB-induced HASMC migration (Figure 8B & C). To find whether depletion of cyclin D1 levels triggers cellular apoptosis and thereby accounts for decreased DNA synthesis and migration, we tested cyclin D1 siRNA effect on apoptosis. Inhibition of cyclin D1 levels had no effect on HASMC apoptosis suggesting that the decreased HASMC DNA synthesis and migration by cyclin D1 depletion were not due to increased apoptosis (Figure 8D). To determine whether forced expression of cyclin D1 alone is sufficient to rescue PDGF-BB-induced DNA synthesis and migration from inhibition by NFATc1-depletion, HASMCs that were transfected with NFATc1 siRNA and transduced with various titers of adenovirus expressing cyclin D1 and quiesced were tested for PDGF-BB-induced DNA synthesis and migration. Adenovirus-mediated expression of cyclin D1 while alone inducing HASMC DNA synthesis and migration ablated the inhibitory effect of NFATc1 siRNA on PDGF-BB-induced DNA synthesis and migration in a dose-dependent manner with maximum effect at 40 MOI (Figure 9A & B). However, at higher doses of adenovirus (80 MOI), cyclin D1 expression alone is causing maximal DNA synthesis and under these circumstances the cells appear to be less responsive to a subsequent challenge by PDGF-BB. To validate these findings in vivo, we used a rat aortic balloon injury model. BI induced cyclin D1 expression and CDK4 activity 3 days post injury and adenovirus-mediated transduction of VIVIT inhibited these effects (Figure 10A). In addition, double immunofluorescence staining of the cross sections of the artery at 3 days post BI for cyclin D1 and SMCα-actin revealed that the induction of cyclin D1 expression occurs in SMC and it is mediated by NFAT activation (Figure 10B). BI caused migration of SMC from medial to luminal region 3 days post injury as determined by staining of longitudinally opened BI arteries with anti-histone H1 antibodies and counting the positively-stained cells (Figure 11A). Similarly, BI induced SMC proliferation in the intimal region at one-week post injury as measured by immunostaining of the artery.
cross-sections for PCNA expression (Figure 11B). In addition, H & E staining and morphometry analysis of the cross-sections of the arteries at 2-weeks post BI showed substantial neointimal growth (Figure 12). Adenovirus-mediated expression of VIVIT significantly blocked BI-induced SMC migration from medial to luminal surface and their proliferation in the intimal region resulting in reduced neointima (Figures 11A, B & 12). It is interesting to find that adenovirus-mediated transduction of cyclin D1 soon after BI while alone enhancing the effects of BI on SMC migration from media to intima and their proliferation in intima leading to neointima formation, rescued these responses from inhibition by VIVIT (Figures 11A, B & 12).

**DISCUSSION**

The important observations of the present study are as follows: 1. PDGF-BB induced cyclin D1 expression and CDK4 activity in HASMCs in NFATc1-dependent manner. 2. Depletion of NFATc1 blocked PDGF-BB-induced HASMC DNA synthesis and their cell cycle progression from G1 to S phase. 3. Downregulation of NFATc1 also inhibited PDGF-BB-induced HASMC migration. 4. TransFac analysis of human cyclin D1 promoter revealed the presence of five NFAT binding elements spanning in about 3.0 kb region starting from the transcription start site and the site at -1333 nt was found to be sufficient to mediate PDGF-BB-induced promoter activity. 5. NFATc1-mediated expression of cyclin D1 was found to be required for PDGF-BB-induced HASMC DNA synthesis and migration. 6. Adenovirus-mediated transduction of cyclin D1 into artery while enhancing the BI-induced SMC migration from media to luminal surface and their proliferation in the intimal region forming neointima rescued these vascular wall remodeling responses from inhibition due to blockade of NFATs by VIVIT. Together, these observations provide a mechanistic evidence for the role of NFATc1 in PDGF-BB-induced HASMC proliferation and migration in vitro and BI-induced vascular wall remodeling in vivo.

In addition to their well-characterized function in the regulation of cytokine gene expression in immune cells, NFATs have been shown to play a role in skeletal muscle gene expression (7), neuronal and osteoclast differentiation (8, 9), cardiac valve formation (6), vascular development (10) as well as pathological cardiac hypertrophy (11, 12). It was demonstrated that constitutively active forms of NFATc1 and NFATc2 induce two distinct phenotypes in NIH 3T3 cells; while NFATc1 increases their proliferation capacity and transformation (34, 35), NFATc2 causes their cell cycle arrest and apoptosis (35). NFATc1 has also been shown to mediate VEGF-induced endothelial cell proliferation (36). It was reported that conditional expression of NFATc1 enhances pancreatic β-cell proliferation (37). In addition to these observations, work from our laboratory showed that NFATs mediate VSMC growth and migration in response to both RTK and GPCR agonists (17, 18). A role for NFATs in the regulation of VSMC growth has also been supported by the findings that forced expression of constitutively active sarco/endoplasmic reticulum calcium ATPase inhibits VSMC proliferation through inactivation of calcineurin and its target transcription factor NFAT (38). In contrast to these reports, many studies have also provided evidence for a negative role of NFATs in the regulation of cell proliferation. Specifically, it was shown that NFATc1 via repressing CDK4 maintains hair follicle stem cells in quiescence (39). In infantile hemangiommas, suppression of NFAT-dependent VEGFR1 expression was reported to be associated with altered VEGFR2 signaling (14). In addition, depletion of calcineurin inhibitor, DSCR1, has been shown to prevent tumor growth (13). Based on these findings, it appears that NFAT isoforms carry out differential roles in the regulation of cell proliferation and/or differentiation in different cell types. In this aspect, it may be pointed out that NFATc2-/- lymphocytes grow faster than wild type cells and express higher levels of cyclins (40). NFATc2 has also been shown to repress cyclin A2 and CDK4 expression in T cells (41, 42). In contrast, work from our laboratory showed a positive
role for NFATs, particularly NFATc1 in the regulation of cyclins in VSMCs (20). It was well established that cyclin D1/CDK4 plays a role in cell cycle progression from G1 to S phase (43, 44). Similarly, cyclin A2/CDK2 is involved in the progression of a cell through the S phase of the cell cycle (45). The role of cyclins in the regulation of cell proliferation can be appreciated by the capacity of cyclin D1/CDK4 to trigger a proliferative response even in a terminally differentiated cell type such as cardiomyocytes (46). In the present study, we identified cyclin D1, a signature marker for cell proliferation, as a downstream effector molecule of NFATc1 in mediating HASMC DNA synthesis in response to PDGF-BB. The differential effects of NFATc1 and NFATc2 on the regulation of cyclin D1 levels as observed in the present study also correlate well with their differential effects on cell proliferation as reported by other studies (34, 35, 40-42). The characterization of cyclin D1 promoter revealed a role for NFAT-binding site at -1333 nt in PDGF-BB-induced cyclin D1 expression in HASMCs.

Besides its involvement in cell cycle regulation, cyclin D1 has been reported to play a role in cell migration (32). Since NFATs were found to be involved in the regulation of migration and cyclin D1 expression in VSMCs, it was intriguing to ask the question whether this cell cycle regulator has any role in cell migration downstream to NFATc1. The finding that a reduction in cyclin D1 but not cyclin A2 levels blocked PDGF-BB-induced migration and adenovirus-mediated expression of cyclin D1 rescued PDGF-BB-induced HASMC migration from inhibition by NFATc1-depletion clearly point out a selective involvement of cyclin D1 in the regulation of cell migration. Previously, we have demonstrated that NFATs mediate both RTK and GPCR agonist-induced VSMC migration via induction of expression of IL-6 (18). Based on these observations, it may be suggested that NFATs target several genes including IL-6 and cyclin D1 in the mediation of cell migration, perhaps different aspects associated with cell motility. We have also reported that cyclin D1 mediates both RTK and GPCR agonist-induced VSMC migration downstream to STAT-5B (24, 33). Based on these observations, one can postulate that several mechanisms may be involved in the regulation of cyclin D1 by RTK and GPCR agonists and such concerted actions of various transcriptional factors in the regulation of expression of a gene such as cyclin D1 may be needed in tilting the balance between anti- and pro-migratory state to pro-motility state of the cell.

It is also exciting to find that adenovirus-mediated expression of cyclin D1 while enhancing BI-induced SMC migration from media to luminal surface, their proliferation in the intimal region leading to neointima formation, rescued these injury-induced vascular wall remodeling responses from inhibition by VIVIT. These findings reinforce the role of cyclin D1 in the regulation of SMC migration and proliferation downstream to NFAT, most likely NFATc1, in vivo as well. In recent years, it was reported that the knockdown of any single cyclin or CDK gene does not affect the development variably (47, 48), but deletion of a combination of these molecules is lethal (49). Since NFATs regulate more than one cyclin in VSMCs that seem to be needed for the mediation of proliferation, migration or both, these transcriptional factors could be better targets for the development of therapeutic agents for the control of vascular diseases such as re-narrowing of coronary artery after angioplasty.

ACKNOWLEDGEMENTS
This work supported in part by a grant from the National Institutes of Health (HL069908) to GNR.

REFERENCES
FIGURE LEGENDS

Figure 1. PDGF-BB induces cyclin D1 mRNA expression in NFAT-dependent manner in HASMCs. A. Quiescent HASMCs were treated with and without PDGF-BB (20 ng/ml) for the indicated times and total cellular RNA was isolated. An equal amount of RNA was subjected to RT-PCR analysis of cyclin D1 and β-actin mRNA levels using their respective primers. B. HASMCs that were transduced with either Ad-GFP or Ad-VIVIT at 80 MOI, and quiesced were treated with and without PDGF-BB (20 ng/ml) for the indicated times and total cellular RNA was isolated and analyzed for cyclins D1 and β-actin mRNA levels as described in panel A. The bar graph in each panel represents the quantitative analysis of three independent experiments. * p<0.01 vs control or Ad-GFP; † p<0.01 vs Ad-GFP + PDGF-BB.

Figure 2. PDGF-BB induces cyclin D1 levels and CDK4 activity in NFAT-dependent manner in HASMCs. Quiescent HASMCs were treated with and without PDGF-BB (20 ng/ml) for the indicated times and cell extracts were prepared. A. An equal amount of protein from control and each treatment was analyzed by Western blotting for cyclin D1, CDK4, pRb (S795) and Rb levels using their specific antibodies. To measure CDK4 activity, an equal amount of protein from control and each treatment was immunoprecipitated with anti-CDK4 antibodies and the kinase activity was measured. B. The bar graph in each panel represents the quantitative analysis of three independent experiments. * p<0.01 vs control or Ad-GFP; † p<0.01 vs Ad-GFP + PDGF-BB.
activity in the immunocomplexes was measured using recombinant retinoblastoma protein, Rb, and \([\gamma^{32}P]\)-ATP as substrates. B. HASMCs that were transduced with either Ad-GFP or Ad-VIVIT at 80 MOI and quiesced were treated with and without PDGF-BB (20 ng/ml) for the indicated times, cell extracts were prepared and analyzed for cyclin D1, CDK4, pRb (S795) and Rb levels as well as CDK4 activity as described above in panel A. The bar graph in each panel represents the quantitative analysis of three independent experiments. * p<0.01 vs control or Ad-GFP; † p<0.01 vs Ad-GFP + PDGF-BB.

Figure 3. Blockade of NFAT activation inhibits PDGF-BB-induced DNA synthesis and cell cycle progression of HASMCs. A. HASMCs that were transduced with either Ad-GFP or Ad-VIVIT at 80 MOI, and quiesced were treated with and without PDGF-BB (20 ng/ml) for 24 h and DNA synthesis was measured by \([^{3}H]\)-thymidine incorporation. B. All the conditions were the same as in panel A except that after 16 h of treatment with PDGF-BB, cells were subjected to FACS analysis. * p<0.01 vs Ad-GFP; † p<0.01 vs Ad-GFP + PDGF-BB.

Figure 4. NFATc1 mediates PDGF-BB-induced cyclin D1 expression, DNA synthesis and migration of HASMCs. A. HASMCs were transfected with scrambled or NFATc1 siRNA and 36 h later cell extracts were prepared and analyzed by Western blotting for NFATc1 and NFATc3 levels using their specific antibodies. B & C. All the conditions were the same as in panel A except that after transfection, HASMCs were quiesced, treated with or without PDGF-BB (20 ng/ml) for 16 h and analyzed for cyclin D1 mRNA and protein levels as described in Figure 1, panel A and Figure 2, panel A legends, respectively. D & E. All the conditions were the same as in panel B except that after transfection with scrambled or NFATc1 siRNA and quiescence, HASMCs were subjected to PDGF-BB-induced DNA synthesis or migration. The bar graph in each panel represents the quantitative analysis of three independent experiments. * p<0.01 vs TR alone or Scr siRNA; † p<0.01 vs TR + PDGF-BB or Scr siRNA + PDGF-BB.

Figure 5. The lack of effect of NFATc2 on PDGF-BB-induced cyclin D1 expression in HASMCs. A. HASMCs were transfected with scrambled or NFATc2 siRNA and 36 h later cell extracts were prepared and analyzed by Western blotting for NFATc2 levels using its specific antibodies. B & C. All the conditions were the same as in panel A except that after transfection, HASMCs were quiesced, treated with or without PDGF-BB (20 ng/ml) for 16 h and analyzed for cyclin D1 mRNA and protein levels as described in Figure 1, panel A and Figure 2, panel A legends, respectively. The bar graph in each panel represents the quantitative analysis of three independent experiments. * p<0.01 vs TR alone.

Figure 6. PDGF-BB-induced cyclin D1 promoter-luciferase reporter gene activity requires -1333 NFAT-binding element. A. Sequence of the cloned 1.5 kb human cyclin D1 promoter showing the -1254 and -1333 NFAT-binding sites. B. HASMCs were transfected with empty vector or cyclin D1 promoter-luciferase constructs with serial 5’-deletions or mutagenesis, quiesced, treated with and without PDGF-BB for 8 h and cell extracts were prepared and analyzed for luciferase activity. M1 and M2 indicate mutations in NFAT binding sites located at -1254 and -1333, respectively, whereas DM stands for mutations in both the NFAT binding sites. * p < 0.01 vs vehicle control; † p<0.01 vs pGL3-CCND1p-(1.5 kb)-Luc + PDGF-BB. Dotted vertical lines indicate mutated NFAT sites.

Figure 7. PDGF-BB induces NFAT binding to human cyclin D1 promoter. A. Quiescent HASMCs were treated with and without PDGF-BB (20 ng/ml) for the indicated times and nuclear extracts were prepared. An equal amount of nuclear protein from control and each treatment was analyzed for DNA binding activity using \([^{32}P]\)-labeled oligonucleotide probe representing -1333
NFAT element in the cyclin D1 promoter. B. Nuclear extracts from control and 2-h PDGF-BB-treated HASMCs were analyzed for NFAT DNA binding activity using the mutated [32P]-labeled -1333 NFAT oligonucleotide probe. C. The NFAT DNA binding activity using [32P]-labeled -1333 NFAT oligonucleotide probe was subjected to competition with 10-fold molar excess of cold oligonucleotides that encompass the NFAT binding elements at -1254, -1333, -2293, -2801 and -2902 regions of cyclin D1 promoter. D. All the conditions were the same as in panel A except that cells were treated with or without PDGF-BB (20 ng/ml) in the presence or absence of CsA (10 µM) for 1 h and nuclear extracts were prepared and analyzed for NFAT DNA binding activity using [32P]-labeled -1333 NFAT oligonucleotide as a probe. E. All the conditions were the same as in panel D, except that cells were transduced with either Ad-GFP or Ad-VIVIT at a MOI of 80 and quiesced before subjecting to treatment with and without PDGF-BB and analyzing for NFAT DNA binding activity. F & G. All the conditions were the same as in panel A except that after treatment with and without PDGF-BB, cells were subjected to ChIP assay using either NFATc1 (F) or NFATc2 (G) antibodies and primers specific for -1254/-1333 NFAT-binding sites. H. All the conditions were the same as in panel F & G except that cells were treated with and without PDGF-BB for 2 h and subjected to ChIP assay using NFATc1 antibodies and primers specific for -1254/-1333, -2293, -2801 and -2902 NFAT-binding sites. I. The HASMCs were transduced with either Ad-GFP or Ad-VIVIT at 80 MOI, quiesced, treated with and without PDGF-BB (20 ng/ml) for 2 h and subjected to ChIP assay using NFATc1 antibodies and primers specific for -1254/-1333 NFAT-binding sites.

Figure 8. Cyclin D1 expression is required for both PDGF-BB-induced HASMC DNA synthesis and migration. A. HASMCs were transfected with either scrambled, cyclin D1 or cyclin A2 siRNA and 36 h later cell extracts were prepared and analyzed by Western blotting for cyclin D1 and cyclin A2 levels using their respective antibodies. B & C. After transfection with the indicated siRNAs, cells were quiesced and treated with and without PDGF-BB (20 ng/ml) for either 24 h to determine DNA synthesis (B) or 8 h to measure migration (C). D. HASMCs were transfected with cyclin D1 siRNA and 36 h later cells were examined for apoptosis using TUNEL assay. E & F. HASMCs that were transfected with NFATc1 siRNA were transduced with Ad-GFP or various doses of Ad-cyclin D1, quiesced for 24 h and subjected to PDGF-BB-induced DNA synthesis and migration. * p < 0.01 vs TR alone or Scr siRNA; † p < 0.01 vs TR + PDGF-BB or Scr siRNA + PDGF-BB.

Figure 9. Forced expression of cyclin D1 rescues PDGF-BB-induced HASMC DNA synthesis and migration from inhibition by depletion of NFATc1. A & B. HASMCs that were transfected with NFATc1 siRNA were transduced with Ad-GFP or various doses of Ad-cyclin D1, quiesced for 24 h and subjected to PDGF-BB-induced DNA synthesis (A) and migration (B). * p < 0.01 vs Scr siRNA; † p < 0.01 vs Scr siRNA + PDGF-BB; †† p < 0.01 vs NFATc1 siRNA + PDGF-BB.

Figure 10. Blockade of NFAT activation suppresses balloon injury-induced cyclin D1 expression and CDK4 activity in the arteries. A. Soon after balloon injury the rats received adenovirus expressing either Ad-GFP or Ad-VIVIT by infusion into the injured arteries. Three days after balloon injury, rats were sacrificed and the injured right common carotid arteries and uninjured left common carotid arteries were dissected out and tissue extracts prepared. An equal amount of protein from uninjured and balloon-injured Ad-GFP or Ad-VIVIT-transduced arteries were analyzed for cyclin D1 and β-tubulin expression and CDK4 activity by Western blotting and immunocomplex kinase assay, respectively, as described in Figure 2, panel A legend. The bar graph represents mean ± SD values of six animals in each group. B. Double immunofluorescence staining of balloon injured Ad-GFP or Ad-VIVIT-transduced carotid artery sections for SMCα-actin and cyclin D1. * p < 0.01 vs UI; † p < 0.01 vs Ad-GFP-BI. UI, uninjured.
Figure 11. Transduction of cyclin D1 in rat carotid arteries rescues injury-induced SMC migration from medial to luminal surface and their proliferation in intimal region from inhibition by Ad-VIVIT. A. Three days after balloon injury and transduction with Ad-GFP or Ad-VIVIT in combination with and without Ad-cyclin D1, rats were sacrificed, the injured and uninjured common carotid arteries were isolated, fixed, opened longitudinally, stained with anti-histone H1 antibodies and the positively stained cells were counted. B. All the conditions were the same as in panel A except that arteries were isolated one week after balloon injury, fixed, cross-sections were made, stained with anti-PCNA antibodies and PCNA-positive cells were counted. The representative pictures were shown in the upper panels and quantitative analysis was presented as bar graphs in the bottom panels. † p < 0.05 vs Ad-GFP-BI (n = 6); †† p < 0.05 vs Ad-VIVIT-BI (n = 6).

Figure 12. Transduction of cyclin D1 in rat carotid arteries rescues injury-induced neoitima from inhibition by Ad-VIVIT. Two weeks after balloon injury and transduction with Ad-GFP or Ad-VIVIT in combination with and without Ad-cyclin D1, rats were sacrificed, the injured and uninjured common carotid arteries were isolated, fixed, and cross-sections were made and stained with H & E. After morphometry analysis, lumen sizes were measured and the I/M ratios were calculated. The upper panel shows the representative cross-sections of the arteries that were stained with H & E. The bar graphs in the bottom panel show the quantitative analysis of the I/M ratios and lumen sizes of the balloon-injured arteries. † p < 0.05 vs Ad-GFP-BI (n = 6); †† p < 0.05 vs Ad-VIVIT-BI (n = 6).
Figure 1

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Cyclin D1 (381 bp)

β-Actin (478 bp)

B

PDGF-BB (h) PDGF-BB (h)

| 8 | 16 |
| 8 | 16 |

Cyclin D1 (381 bp)

β-Actin (478 bp)

Ad-GFP Ad-VIVIT

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Ad-GFP Ad-VIVIT

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Arbitrary densitometric units

- Control
- 2
- 4
- 8
- 16

* p < 0.05
† p < 0.01
‡ p < 0.001
Figure 3

A

![Graph showing [\(^3\)H]-Thymidine incorporation](image)

- **PDGF-BB**
- Ad-GFP
- Ad-VIVIT

B

![Cell number (%)](image)

- Ad-GFP
- Ad-GFP + PDGF-BB
- Ad-VIVIT
- Ad-VIVIT + PDGF-BB

![Cell number (%) by phase](image)

- G1
- S
- G2

* and † indicate statistical significance.
Figure 4

A

NFATc1
NFATc3

TR  +  +  +
Scr siRNA  –  +  –
NFATc1 siRNA  –  –  +

B

Cyclin D1
(381 bp)

β-actin
(478 bp)

C

Cyclin D1
CDK4

D

Arbitrary densitometric units

E

[3H] Thymidine incorporation

(1 X 10^3 cpm)
Figure 5

A

NFATc2
NFATc3
TR + + + +
Scr siRNA – + – –
NFATc2 siRNA – – + +

B

Cyclin D1
(381 bp)
β-actin
(478 bp)

Arbitrary densitometric units

C

Cyclin D1
CDK4

Arbitrary densitometric units

TR + + + + + +
Scr siRNA – – + + – –
NFATc2 siRNA – – – – + +
PDGF-BB – + – + – +

* *
Figure 7

A

Free Probe
Control
PDGF-BB, 1 h
PDGF-BB, 2 h

B

Free probe
Control
PDGF-BB
Free probe
Control
PDGF-BB

C

Free Probe
Control
NFAT –1333
NFAT –1254
NFAT –1333
NFAT –2293
NFAT –2801
NFAT –2902
Excess Cold

D

Free probe
Control
PDGF-BB
CsA + PDGF-BB
CsA

E

Free probe
Ad-GFP
Ad-GFP + PDGF-BB
Ad-VIVIT + PDGF-BB
Ad-VIVIT
Figure 7

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<td>+ - + - + - + -</td>
<td>- - + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Pre-immune serum</td>
<td>+ - + - + - + -</td>
<td>- - + + + + + +</td>
<td></td>
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<tr>
<td>PDGF-BB</td>
<td>- - + + + + + +</td>
<td>+ + - - + - + +</td>
<td></td>
</tr>
<tr>
<td>Anti-NFATc1 ab</td>
<td>+ + - - + + - -</td>
<td>- - + + + + + +</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 8**

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclin D1</th>
<th>β-Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scr siRNA</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cyclin D1 siRNA</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclin A2</th>
<th>β-Tubulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Scr siRNA</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cyclin A2 siRNA</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

B

[Graph showing thymidine incorporation (1 x 10^4 cpm) with various treatments.]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[^H]-Thymidine Incorporation (1 x 10^4 cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR</td>
<td>+</td>
</tr>
<tr>
<td>Scr siRNA</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin A2 siRNA</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin D1 siRNA</td>
<td>–</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>–</td>
</tr>
</tbody>
</table>
Figure 8

C

Number of migrated cells/field

<table>
<thead>
<tr>
<th>TR</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scr siRNA</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin A2 siRNA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin D1 siRNA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

D

Tunnel (red)  DAPI (blue)  Merged (pink)

scr RNA

Cyclin D1 siRNA

Positive control

Negative control

Downloaded from http://www.jbc.org/ by guest on October 5, 2017
Figure 9

A

[^3H] Thymidine Incorporation (1 x 10^3 cpm)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>PDGF-BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scr siRNA</td>
<td>+ +</td>
<td>- -</td>
</tr>
<tr>
<td>NFATc1 siRNA</td>
<td>- -</td>
<td>+ +</td>
</tr>
<tr>
<td>Ad-cyclin D1</td>
<td>- -</td>
<td>+ +</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>PDGF-BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scr siRNA</td>
<td>+ +</td>
<td>- -</td>
</tr>
<tr>
<td>NFATc1 siRNA</td>
<td>- -</td>
<td>+ +</td>
</tr>
<tr>
<td>Ad-cyclin D1</td>
<td>- -</td>
<td>+ +</td>
</tr>
</tbody>
</table>
Figure 10

A

Cyclin D1
β-tubulin

CDK4 activity

<table>
<thead>
<tr>
<th></th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>UI</td>
<td></td>
</tr>
<tr>
<td>Ad-GFP</td>
<td></td>
</tr>
<tr>
<td>Ad-VIVIT</td>
<td></td>
</tr>
</tbody>
</table>

Cyclin D1
CDK4 activity
β-tubulin

Arbitrary densitometric units

- Cyclin D1
- CDK4 activity
- β-tubulin

B

SMCα-actin (red)  | Cyclin D1 (green)  | Merge (yellow)

Ad-GFP-BI

Ad-VIVIT-BI
Figure 11

A

Ad-GFP-BI  Ad-VIVIT-BI

Ad-cyclin D1-BI  Ad-VIVIT + Ad-cyclin D1-BI

Number of migrated SMC onto luminal surface (cells/0.1 mm²)

<table>
<thead>
<tr>
<th></th>
<th>Ad-GFP</th>
<th>Ad-VIVIT</th>
<th>Ad-cyclin D1-BI</th>
<th>Ad-VIVIT + Ad-cyclin D1-BI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 11

B

Ad-GFP-BI

Ad-VIVIT-BI

Ad-cyclin D1-BI

Ad-VIVIT + Ad-cyclin D1-BI

Ratio of PCNA-positive cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>+</th>
<th>-</th>
<th>-</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-GFP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ad-VIVIT</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ad-cyclin D1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BI</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Cyclin D1 is a bona fide target gene of NFATc1 and is sufficient in the mediation of injury-induced vascular wall remodeling
Manjula Karpurapu, Dong Wang, Dong Van Quyen, Tae-Kang Kim, Venkatesh Kundumani-Sridharan, Srinidhi Pulusani and Gadiparthi N. Rao

J. Biol. Chem. published online November 22, 2009

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