NFATc1 Mediates Vascular Endothelial Growth Factor-induced Proliferation of Human Pulmonary Valve Endothelial Cells*

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Ehrin N. Johnson‡‡, You Mie Lee‡, Tara L. Sander‡, Elena Rabkin‡, Frederick J. Schoen‡, Sunjay Kaushal, and Joyce Bischoff**

From the ‡Department of Surgery, Children's Hospital and ¶Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Mice deficient for the transcription factor NFATc1 fail to form pulmonary and aortic valves, a defect reminiscent of some types of congenital human heart disease. We examined the mechanisms by which NFATc1 is activated and translocated to the nucleus in human pulmonary valve endothelial cells to gain a better understanding of its potential role(s) in post-natal valvular repair as well as valve development. Herein we demonstrate that activation of NFATc1 in human pulmonary valve endothelial cells is specific to vascular endothelial growth factor (VEGF) signaling through VEGF receptor 2. VEGF-induced NFATc1 nuclear translocation was inhibited by either cyclosporin A or a calcineurin-specific peptide inhibitor; these findings suggest that VEGF stimulates NFATc1 nuclear import in human pulmonary valve endothelial cells by a calcineurin-dependent mechanism. Importantly, both cyclosporin A and the calcineurin-specific peptide inhibitor reduced VEGF-induced human pulmonary valve endothelial cell proliferation, indicating a functional role for NFATc1 in endothelial growth. In contrast, VEGF-induced proliferation of human dermal microvascular and human umbilical vein endothelial cells was not sensitive to cyclosporin A. Finally, NFATc1 was detected in the endothelium of human pulmonary valve leaflets by immunohistochemistry. These results suggest VEGF-induced NFATc1 activation may be an important mechanism in cardiac valve maintenance and function by enhancing endothelial proliferation.

Defects in heart development are the most common congenital anomaly, occurring in 1% of all live births (1, 2). Some of the defects, especially those that involve the aortic and pulmonary valves, require surgical intervention, which can include replacement of a defective valve. In the past decade, researchers have elucidated some of the basic signaling interactions necessary for heart development. In addition to initiating heart development, many of these pathways are also required for optimal heart function in post-natal life (3). However, comparatively little is known about the interactions that control more specific events, for example, induction of cardiac valve formation (4). In the mouse, primordial heart valves known as cardiac cushions begin to form by embryonic day 9.5. These cushions appear as swellings in the atrioventricular junction and outflow tract. As development proceeds, the cushions contribute to chamber septation and ultimately result in development of four adult valves.

Initiation of endocardial cushion formation involves a distinct subset of endothelial cells in the cushion-forming area that undergo endothelial to mesenchymal transdifferentiation (EMT). It is believed that the underlying myocardium sends inductive signals to the endocardial cells, beginning the process of EMT (5). These newly formed mesenchymal cells migrate into the underlying extracellular matrix, where further remodeling transforms the cushions into fibrous leaflets. Studies have demonstrated that transforming growth factor-β (TGF-β) signaling (6) and the type III TGF-β receptor are required for EMT in avian models (7). Indeed, TGF-β-mediated EMT has been shown to occur in post-natal ovine aortic valve endothelial cells (8). More recently, the phenotypes of mice and zebrafish in which specific genes have been “knocked out” or mutated have provided new insight into the genes required for normal valve development.

For example, targeted gene deletion has demonstrated a specific requirement for NFATc1, also known as NFAT2, in valve development. Knockout of NFATc1 in the mouse leads to defective aortic and pulmonary valve development with subsequent death at embryonic days (E) 14–15 due to congestive heart failure (9, 10). The cardiac cushions in the outflow tract of these mice are hypoplastic, suggesting that lack of NFATc1 leads to dysregulation of an early step in cushion formation. Knockout of other NFAT proteins, such as NFATc2, c3, and c4, has no effect on valve development (11). Members of the NFAT family function as mediators of the CsA-sensitive calcineurin-NFAT signaling pathway. First discovered in the pathway of interleukin-2-mediated T-cell activation and proliferation (12),
NFAT (nuclear factor in activated T cells) signaling has since been shown to be crucial for neuronal guidance, skeletal and cardiac muscle hypertrophy, and, as cited above, cardiac valve development (13–15).

The upstream signaling events regulating NFATc1 activity in the valve endothelium are unknown, although all known NFAT proteins are dependent upon cytosolic Ca²⁺ flux for nuclear translocation. Increased cytosolic Ca²⁺ leads to activation of calmodulin and ultimately calcineurin, a serine/threonine phosphatase. When activated, calcineurin dephosphorylates residues in the conserved N-terminal region of various NFAT isoforms (16, 17). This dephosphorylation prompts a conformational change in NFAT that exposes a previously inaccessible nuclear localization sequence. NFAT is then shuttled into the nucleus, where it interacts with other transcription factors, including AP-1 and NF-κB, to alter gene transcription (18). Although Ca²⁺ flux across the membrane is a ubiquitous signaling mechanism, specificity can be accomplished by differential expression of NFAT isoforms or use of specific ligand-receptor complexes that alter the pattern of calcium flux. Inhibitors of NFAT activation include the pharmacological agents CsA, FK506, and the synthetic peptide VIVIT. CsA and FK506 inhibit its enzymatic activity (17). VIVIT is a hydrophobic 16-amino acid peptide that mimics the calcineurin-docking motif of NFAT proteins and thereby inhibits calcineurin-mediated activation of NFATc1 (20).

Recent studies have demonstrated that VEGF, an endothelial mitogen known to induce calcium mobilization upon receptor activation, may regulate NFATp activity in endothelial cells (21). VEGF is a potent stimulator of angiogenesis and vascular permeability with the ability to induce endothelial proliferation, migration, and survival in vitro (22). In human umbilical vein endothelial cells (HUVECs), VEGF has been shown to induce nuclear localization of NFATc2, also known as NFATp, by a CsA-sensitive mechanism, which resulted in increased tissue factor expression (23). In a separate study, administration of CsA prohibited VEGF from activating expression of cyclooxygenase-2 and inhibited angiogenesis in a corneal neovascularization model, again suggesting an important role for NFAT in vascular endothelial cells (24).

Studies of VEGF expression in transgenic mice provide evidence for its role in cardiac valve development. LacZ-tagged VEGF knock-in mice were found to express VEGF at E 9.0 in endocardial cells along the entire heart tube (25). By E 9.5, VEGF expression was restricted to endothelial cells of the outflow tract and atrioventricular valve area. This expression pattern coincides precisely with the timing and location of NFATc1 expression in the embryonic valve (9, 10). In a different study, transgenic mice that overexpress VEGF in the embryonic myocardium at E 10.5 demonstrated decreased EMT in the endocardial cushions (26, 27). Although these studies suggest a role for VEGF in heart valve development, little is known regarding the signaling cascade downstream of VEGF in valvular endothelial cells. To study the role of VEGF in NFATc1 signaling in endothelial cells, we performed experiments on human pulmonary valve endothelial cells (HPVECs). Our results demonstrate a unique pathway for VEGF-mediated proliferation of these cells involving an NFATc1-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—HPVEC were isolated from human pulmonary valve leaflets obtained from patients undergoing cardiovascular surgical procedures at Children’s Hospital, Boston under an institutional review board-approved protocol. Removal of the pulmonary valve was a planned part of each procedure. Patients’ ages ranged from 5 months to 20 years. The time interval from surgical excision to cell isolation was less than 1 h. Endothelial cells in primary culture of human pulmonary valve leaflets were isolated using *Ulex europaeus* I-coated Dynabeads (28) as previously described (8). HPVECs were cultured on 1% gelatin-coated tissue culture plates in endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with endothelial cell growth media-2 SingleQuots (human VEGF, EGF, bFGF, insulin-like growth factor-1, ascorbit acid, gentamycin, and heparin; Clonetics), 20% heat-inactivated FBS, and glutamine/penicillin/streptomycin (GPS, Inverogen). This culture media will be referred to as EBM-2. Cells were maintained in 5% CO₂ at 37 °C. Human umbilical vein endothelial cells (HUVECs) were generously provided by Dr. F. W. Luscinskas at Brigham and Women’s Hospital, Boston. Human dermal microvascular endothelial cells (HDMEC) were isolated and cultured as described previously (29).

**Endothelial Cell Characterization**—Immunofluorescence was performed as previously described (8). Briefly, cells plated onto gelatin-coated glass coverslips were fixed with 2 °C methanol and incubated with primary antibody diluted 1:1000 followed by FITC- or Texas Red-conjugated secondary antibody at 5 μg/ml. von Willebrand factor (vWF) was detected using rabbit anti-human vWF (Dako) and Texas Red anti-rabbit IgG. CD31 (also known as platelet endothelial cell adhesion molecule, PECAM-1) was detected using goat anti-human CD31 (Santa Cruz Biotechnology) and Texas Red anti-goat IgG. To detect lipopolysaccharide (LPS)-induced E-selectin, cells were treated with 1 μg/ml LPS for 15 h prior to staining with mouse anti-human E-selectin (29) and FITC-anti-mouse IgG.

**Growth Factor Stimulation**—HPVECs were plated onto gelatin-coated glass cover slips for 1–3 days and then switched from EBM-2 medium to endothelial cell basal medium (EBM) containing 10% FBS (without the additive growth factors described above) for at least 24 h. Cells were then stimulated for 20 min with either 50 ng/ml VEGF, bFGF (R&D Systems), or 1 μM calcium ionophore A23187 (Sigma). 5 μM CsA or 1 μM PK506 (Sigma) were added 2 h before VEGF stimulation.

Cells were also stimulated with receptor-selective variants of VEGF: KDR-select was shown to have wild-type affinity for KDR/VEGFR2 but 2000-fold reduced binding to Flt-1, whereas Flt-1-select was shown to have wild-type affinity for Flt-1 but 470-fold reduced binding to KDR/VEGFR2 (30). Drs. Bing Li and Abraham M. de Vos (Genentech, Inc., South San Francisco, CA) kindly provided these receptor-selective variants, as well as comparably produced wild-type VEGF (VEGFwt, amino acids 1–109). NFATc1 expression was analyzed by fixing cells in 4% paraformaldehyde, permeabilizing with 0.5% Triton X-100, and incubating with mouse anti-human NFATc1 mAb (7A6 from Santa Cruz Biotechnology) diluted 1:500 followed by FITC-conjugated anti-mouse IgG. An aliquot of the anti-human NFATc1 mAb for preliminary experiments was kindly provided by Dr. Gerald Crabtree (Stanford University). Cellular localization of NFATc2, NFATc3, and NFATc4 were analyzed as described above using monoclonal antibodies (mAbs) purchased from Santa Cruz Biotechnology.

**Western Blotting**—Cell lysates were prepared as described previously (8) and subjected to SDS-PAGE using commercially available gradient gels. Gels were transferred to nitrocellulose membrane (Millipore). The membrane was rinsed in TBS-T solution (0.1% Tween 20 in TBS, pH 7.5) and incubated in blocking buffer (5% skim milk in TBS-T) at room temperature. The membranes were incubated with the mouse anti-human NFATc1 (7A6 from Santa Cruz Biotechnology), mouse anti-human NFATc2 (G1-D10 from Santa Cruz Biotechnology), mouse anti-human NFATc3 (F-1 from Santa Cruz Biotechnology), or goat anti-human NFATc4 (C-20 from Santa Cruz Biotechnology) antibodies diluted 1:500 for overnight binding at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 for 1 h at room temperature. The immunoreactive bands were visualized using chemiluminescent reagent (LumiGLO, KPL).

**MTT Assay**—1 × 10⁶ cells/well were plated on gelatin-coated 48-well plates. One day later, cells were washed with PBS and incubated with either 5 μM CsA at 1, 5, 10, and 20 μM in low serum (2% FBS) or high serum (20% FBS) containing EBM-2 media for 48 h. Five hours before the end of the incubation, 50 μl of MTT solution (5 mg/ml) was added into each well. Media was removed, and 500 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. Residual MTT crystals were visible as dark purple crystals after 4 h incubation at 37 °C. Absorbance was measured at 550 nm. Dissolved crystals were transferred into 96-well plates, and absorbance at 550 nm was measured.

**Cell Proliferation Assay**—Cell proliferation was assayed by [³H]thymidine incorporation (31). Cells were plated onto gelatin-coated 48-well plates at a density of 1 × 10⁴ cells/well. The next day, cells were washed with PBS and incubated in thymidine-free endothelial basal labeling medium (Clonetics) with 2% FBS and GPS (starvation medium) for 24 h. The quiescent cells were stimulated with fresh starvation medium.
containing 10 ng/ml VEGF, KDR-sel2, or Flt-sel, with or without CsA. Approximately 18–24 h after growth factor stimulation, 0.7 μCi of [3H]thymidine (6.7 Ci/mmol, PerkinElmer Life Sciences) was added to each well, and incubations were continued for another 4 h. [3H]Thymidine DNA incorporation in the cell lysates was quantitated in a scintillation counter. Each condition was tested in triplicate, and the mean ± S.D. of [3H]thymidine DNA incorporation was calculated. Statistical significance was determined by Student’s t test, with a p value of <0.05 considered statistically significant. The fold induction compared with cells with no VEGF stimulation was also determined. Proliferation assays on retrovirally transduced HPVEC were performed as above after the GFP-positive cells were sorted by FACS.

**Northern Blot Analysis**—Total RNA was isolated from human endothelial cells using an RNeasy kit (Qiagen). Blots were hybridized with 32P-labeled cDNA probes for KDR/VEGFR2, Flt-1/VEGFR-1, and neuropilin-1.

**Results**

**Isolation and Characterization of HPVEC**—The function of NFATc1 in valvular endothelial cell proliferation was studied using HPVEC isolated from human pulmonary valve leaflets. The cells uniformly expressed the endothelial-specific markers von Willebrand factor (vWF), CD31/PECAM-1, and LPS-inducible E-selectin, demonstrating that HPVEC retain expression of endothelial-specific markers in culture (Fig. 1).

**VEGF Stimulates NFATc1 Nuclear Translocation by a KDR/VEGFR2-specific and Calcium-dependent Mechanism**—To identify extracellular ligands that stimulate NFATc1 activation in valvular endothelial cells, growth factors were tested for their ability to induce NFATc1 nuclear import in HPVEC. HPVEC were stimulated with 50 ng/ml VEGF, KDR-sel2, Flt-sel, or bFGF and then immunostained with an antibody specific for NFATc1. VEGF (50 ng/ml) was shown previously to be sufficient for stimulating NFATc2 (also known as NFATp) nuclear localization (23). VEGF stimulated the nuclear translocation of NFATc1 (Fig. 2A, panel c) compared with untreated HPVECs (Fig. 2A, panel b), at concentrations of VEGF as low as 10 ng/ml (data not shown). The pharmacological agents CsA (Fig. 2A, panel d) and FK506 (data not shown) inhibited the VEGF-induced nuclear translocation, suggesting a calcineurin-dependent mechanism. As expected, the calcium ionophore A23187 also stimulated NFATc1 nuclear import (data not shown). KDR-sel2 stimulated NFATc1 nuclear translocation (Fig. 2A, panel g), and, as observed with VEGF, the translocation was inhibited when CsA was included (Fig. 2A, panel h).

**Fig. 1.** HPVECs express endothelial-specific markers. HPVEC were stained by indirect immunofluorescence using rabbit control IgG (A), rabbit anti-human von Willebrand Factor (vWF) (B), goat anti-human CD31/PECAM-1 (C), or mouse anti-human E-selectin (D). In panel D, HPVECs were stimulated with 1 μg/ml LP8 for 3 h to induce E-selectin expression. Photographs were taken at 630× magnification.

In contrast, Flt-1-sel did not induce translocation (Fig. 2A, panel f), indicating that VEGF-induced NFATc1 translocation is mediated by KDR/VEGFR-R2 and not Flt-1/VEGFR-R1. When cells were treated with other endothelial mitogens such as bFGF, NFATc1 remained in the cytosol, as evident by a diffuse pattern of immunofluorescence (Fig. 2A, panel e). Other growth factors, such as insulin-like growth factor-1, EGF, and heparin-binding EGF, and the endothelial factors angiopoietin-1 and -2, had no effect on NFATc1 localization (data not shown). These results demonstrate that the mechanism of NFATc1 activation and nuclear translocation in HPVEC is specific to VEGF signaling events mediated by KDR/VEGFR-R2.

NFATc1 activation was further examined by Western blot (Fig. 2B). Diphosphorylation of NFATc1 results in increased mobility on SDS-PAGE (20). As seen in Fig. 2B, three variants of NFATc1, with apparent molecular masses between 90 and 140 kDa, were detected with anti-human NFATc1 mAb (Fig. 2B, lane 1). Removal of growth factors from the culture medium for 24 h had no effect on migration of the NFATc1 isoforms (lane 2). Treatment with VEGF for 15 min (lane 3) or for 24 h (lane 5) resulted in an increase in electrophoretic mobility of all three isoforms. This apparent diphosphorylation at 15 min and 24 h was inhibited in the presence of 5 μM CsA (lanes 4 and 6). Tubulin expression in each cell lysate is shown as a control (Fig. 2B, lower panel).
family members are activated by VEGF. NFAT family members c1, c2, and c3, but not c4, were detected in the cytoplasm of HPVEC in non-stimulated cells by indirect immunofluorescence using isomform-specific mAbs (Fig. 2C, panels a–d). However, only NFATc1 was translocated into the nucleus when HPVEC were treated with 50 ng/ml VEGF for 30 min (Fig. 2C, panels e–h). In Fig. 2D, protein expression of NFATc1, c2, and c3 was analyzed by Western blot of HPVEC (lane 3), HUVEC (lane 4), and HDMEC (lane 5) cell lysates. Human lymphoma cell lines served as positive controls (lanes 1 and 2). As seen in Fig. 2D, NFATc1, c2, and c3 were detected in all three human endothelial cultures. NFATc4 was not detected by Western blot (data not shown). NFATc2 expression was highest in HUVEC, compared with NFATc1 and NFATc3, consistent with previous studies showing NFATc2 (i.e. NFATp) in HUVECs. Also consistent with Armesilla et al. (23), NFATc2 was translocated into the nucleus of HUVECs in response to VEGF (data not shown). These results suggest that VEGF specifically induces translocation of NFATc1, but not NFATc2 or NFATc3, in vascular endothelial cells.

**VEGF Induces Proliferation by a CsA-sensitive Mechanism in HPVEC but Not in HDMEC or HUVEC**—We next sought to investigate the cellular effects of preventing VEGF-induced NFATc1 nuclear import. Because VEGF is a mitogen of endothelial cells (21) and NFAT signaling can increase proliferation in other cell types (16), we used CsA to test whether VEGF induced valvular endothelial proliferation by an NFATc1-dependent mechanism. First, the potential cytotoxicity of CsA (34) was addressed. MTT assays were performed on HPVEC, HDMEC, and HUVEC at 1, 5, 10, and 20 μM CsA in the presence of 2 and 20% FBS (2% FBS was used in the growth factor-induced proliferation assays). Cytotoxicity was not detected at 1, 5, or 10 μM CsA in these three human endothelial cell cultures in either 2% or 20% FBS (data not shown). Quiescent HPVEC were stimulated with VEGF, VEGFwt, KDR-sel2, or Flt-sel2 (30), in the absence or presence of 5 μM CsA (Fig. 3A). Cell proliferation was assayed by [3H]thymidine incorporation. We found that VEGF, VEGFwt, and KDR-sel 2 induced HPVEC proliferation by 4– to 6-fold in the absence of CsA (Fig. 3A, open bars), but the proliferation was attenuated significantly by CsA (Fig. 3A, black bars). CsA inhibited VEGF-induced proliferation by 30% ($p = 0.032$), VEGF-wt-induced proliferation by 64% ($p = 0.031$), and KDR-sel2-induced proliferation by 37% ($p = 0.004$). A similar inhibitory effect was observed with FK506 (data not shown). Flt-sel did not induce proliferation compared with control cells. Thus, VEGF stimulates HPVEC proliferation, via KDR/VEGF-R2, by a calcineurin-dependent mechanism that may be dependent upon the dephosphorylation and nuclear translocation of NFATc1. This result was observed in cultures of HPVEC isolated from the pulmonary valve leaflets of three different patients. The CsA-mediated inhibition of VEGF-induced proliferation appears to be specific to the valve endothelium, because proliferation of HDMEC and HUVEC was not inhibited by CsA-mediated NFATc1 inactivation (Fig. 3B). 5 μM CsA was sufficient to inhibit VEGF-induced NFATc1 nuclear translocation in HDMEC and HUVEC (data not shown). It is unlikely that this cell-specific CsA effect is due to differences in VEGF receptor expression, because mRNA expression levels for KDR/VEGF-R2, neuropilin-1, and Flt-1/VEGF-R1 did not vary more than 2-fold among the three types of human endothelial cells tested (data not shown). It is possible that the interaction of NFATc1 with valve-specific nuclear factors accounts for the CsA-sensitive proliferation response to VEGF observed in HPVECs.

**The NFAT-specific Inhibitor, VIVIT, Inhibits VEGF-induced Proliferation of HPVEC**—Because CsA and FK506 can potentially disrupt calcineurin-dependent pathways other than the activation of NFAT, we used the synthetic peptide VIVIT to
inhibit NFAT activation selectively without disrupting other calcineurin-dependent pathways (20). We demonstrated that VIVIT inhibits VEGF-induced NFATc1 nuclear translocation and that this results in inhibition of VEGF-mediated HPVEC proliferation (Fig. 4). HPVEC retrovirally transduced with GFP-VIVIT (an oligonucleotide coding for VIVIT-containing peptide fused to the N terminus of the green fluorescent protein) did not import NFATc1 to the nucleus upon VEGF stimulation (Fig. 4, C and D). HPVEC expressing GFP alone did show VEGF-induced NFATc1 nuclear localization (Fig. 4, A and B), indicating that the inhibition of NFATc1 nuclear import is specific to the VIVIT peptide and that NFATc1 nuclear shuttling was not disrupted by retroviral transduction. VEGF-induced proliferation was inhibited by 35% (p = 0.022), whereas KDR-sel-2-induced proliferation was inhibited 62% (p = 0.001) (Fig. 4E). Although VIVIT peptide could potentially inhibit NFATc2 and c3, our data show that these two family members are not translocated to the nucleus in response to VEGF (Fig. 2C) and therefore would not be functionally disrupted in this experiment. These data provide strong evidence that nuclear translocation of NFATc1 is required for maximal VEGF-induced proliferation of HPVEC. The smaller -fold induction observed in this experiment compared with Fig. 3 was likely due to the sequential retroviral transduction, FACS sorting, and 24 h serum starvation prior to measuring VEGF-induced proliferation.

NFATc1 Expression in Adult Human Pulmonary Valve Leaflets—In the mouse, expression patterns of NFATc1 in the developing heart have been examined thoroughly to gain insights into its role in valvulogenesis. NFATc1 can be detected in developing murine hearts beginning at embryonic day E7.5, becomes increasingly restricted to nascent valvular structures by E11.5, but is then undetectable by either reverse transcription-PCR or immunohistochemistry, after E13.5 (9, 10). NFATc1 has been reported undetectable in newborn or adult murine valves as well (9). Despite the lack of reported evidence for NFATc1 expression in post-natal murine valves, we examined NFATc1 protein expression in adult pulmonary valve leaflets, because our experiments in cultured HPVEC show that NFATc1 is expressed in adult HPVECs and suggest that NFATc1 may play an important physiological role in post-natal valve leaflets. Paraffin sections from human pulmonary and aortic valve leaflets were stained with anti-human NFATc1 mAb, followed by a Texas Red-conjugated anti-mouse IgG (B and D). Photographs were taken at 630× magnification. For proliferation assays (E), quiescent HPVEC were stimulated with 10 ng/ml VEGF or KDR-sel-2 for 24 h and assayed for [3H]thymidine incorporation. Data represent mean ± S.D. of a representative experiment (n = 3), each performed in triplicate. Asterisks denote a statistically significant (p < 0.05) difference.

**Fig. 3.** VEGF induces proliferation by a cyclosporin-sensitive mechanism in HPVEC but not in HDMEC or HUVEC. A, quiescent HPVEC were stimulated with 10 ng/ml VEGF or with 10 ng/ml of the VEGF receptor-selective variants, VEGFwt, KDR-sel-2, or Flt-sel, in the absence (open bars) or presence (black bars) of 5 μM CsA. Cell proliferation was assayed by [3H]thymidine incorporation. Data represent mean ± S.D. of a representative experiment (n = 3), each performed in triplicate. Asterisks denote a statistically significant (p < 0.05) difference. B, HDMEC and HUVEC were stimulated with 10 ng/ml VEGF in the absence (open bars) or presence (black bars) of 5 μM CsA. Data are plotted as -fold induction. The change in VEGF-mediated proliferation of these cell types after addition of CsA was not statistically significant.

**Fig. 4.** VIVIT inhibits VEGF-induced proliferation of HPVEC. HPVEC were retrovirally transduced with GFP or GFP-VIVIT. The brightest GFP-positive cells (fluorescence signal > 106) were sorted for analysis of NFATc1 localization in GFP-positive (A and B) and GFP-VIVIT-positive (C and D) cells and for proliferation assays (E). GFP-positive and GFP-VIVIT-positive sorted cells are shown in A and C, respectively. For indirect immunofluorescence, the same cells were stimulated with 10 ng/ml VEGF for 30 min followed by staining with anti-human NFATc1 mAb, followed by a Texas Red-conjugated anti-mouse IgG (B and D). Photographs were taken at 630× magnification. For proliferation assays (E), quiescent HPVEC were stimulated with 10 ng/ml VEGF or KDR-sel-2 for 24 h and assayed for [3H]thymidine incorporation. Data represent mean ± S.D. of a representative experiment (n = 3), each performed in triplicate. Asterisks denote a statistically significant (p < 0.05) difference.
NFATc1 is expressed in the lining of the pulmonic, aortic, and atrioventricular valves (9, 10). Similarly, VEGF is expressed in the endocardial cells lining the endocardial cushions at day E8.5 as well as in the myocardium from E9.5 to E13.5 (25). Interestingly, endocardial cells that have undergone transdifferentiation to mesenchymal cells are negative for both NFATc1 and VEGF expression (9, 10, 25), suggesting that NFATc1 and VEGF play pivotal roles both spatially and temporally in development of the endocardial cushion.

Given our results that VEGF stimulation leads to strong HPVEC proliferation in vitro, one might hypothesize that overexpression of VEGF would result in hyperplastic cardiac valves. However, the opposite result is observed in mice. Premature induction of VEGF expression in developing mouse embryos results in decreased endocardial cushion formation (27). This is phenotypically the same result observed with the NFATc1 murine knockout (9, 10). To explain these findings, we propose that VEGF-mediated NFAT signaling influences a cascade of events, including proliferation, migration, and differentiation, that is critical for valve development and post-natal valvular endothelial function (Fig. 6). In this model, VEGF induces proliferation of valvular endothelial cells and at the same time may influence the TGF-β-induced differentiation to a mesenchymal phenotype. Inhibition of VEGF signaling, either by genetic ablation of NFATc1 or by addition of CsA, would reduce the number of endothelial cells available to undergo EMT.

We postulate that this model may apply to heart valve regeneration and repair. Because our experiments were carried out on postnatal cells, we hypothesize that the signaling pathways used during development are re-established in mature valve leaflets to replenish endothelial and interstitial cells as needed throughout adult life. The focal expression of NFATc1 in mature valve leaflets (Fig. 5) is consistent with this hypothesis. The source of VEGF that would activate NFATc1 expressed in the leaflet endothelium is unknown. Possible sources of VEGF include the endothelial cells, the mesenchymal interstitial cells, or release of VEGF from circulating cells in the blood. Also consistent with the repair concept, Paranya et al. (8) demonstrated that clonal populations of valve endothelial cells isolated from post-natal valves can undergo EMT in a manner similar to what occurs in fetal valve development. Therefore, TGF-β-mediated EMT could be a common mechanism in both developing and adult heart valves, whereas VEGF-mediated proliferation may be a mechanism for repopulation of the valvular endothelium. VEGF may also elicit other endothelial responses in the valve. Further studies will be required to determine the full spectrum of VEGF-induced events in cardiac valve endothelium.

Our results demonstrate a unique signaling pathway for endothelial cell proliferation. Previous studies have demonstrated that VEGF signal transduction in endothelial cells is dominated by inositol 3,4,5-phosphate/diacylglycerol and ex-
tracellular-related kinase (ERK/mitogen-activated proliferation kinase (MAPK) pathways (35), the latter being dependent on Ras activation (36). Activation of inositol 3,4,5-phosphate leads to mobilization of cytosolic Ca\textsuperscript{2+}, which may ultimately stimulate calcineurin to dephosphorylate NFATc1. Because \textsuperscript{3}H/thymidine incorporation in HPVECs was reduced \textemdash\textup{30\textendash}60\% after administration of CsA (Fig. 3), calcineurin appears to be an important mediator for VEGF stimulation of HPVEC proliferation. We used the VIVIT peptide to dissect this signaling pathway further and to circumvent potential endothelial toxicity that has been observed with CsA (34). This approach revealed that expression of VIVIT in valvular endothelial cells reduced HPVEC proliferation by \textup{30–60\%}. A role for ERK and MAPK signaling in HPVECs remains to be defined but may represent the remaining proliferative activity of HPVECs after addition of CsA or VIVIT peptide.

In conclusion, our data provide evidence that VEGF and the receptor KDR/VEGF-R2 are upstream mediators of NFATc1 activation and nuclear translocation in HPVEC, which may lead to the expression of endothelial-specific genes required for valvular endothelial cell proliferation. To elucidate fully the function of NFATc1 in heart valve formation and function, it will be essential to identify additional upstream regulators of NFATc1 activity and downstream valve-specific target genes whose expression is mediated by NFATc1. We speculate that expression of NFATc1 in subsets of endothelium along the native valve leaflets from human adult pulmonary and aortic valve specimens may be a repair mechanism for replacing damaged endothelial cells. Although we have demonstrated an important role for NFATc1 in mediating valvular endothelial proliferation in cultured HPVEC, its functional role in mature heart valves in vivo must be investigated. Understanding the signaling mechanisms controlling valve endothelium proliferation and differentiation will provide insights on heart valve disease and for creating tissue-engineered valves that mimic normal valve function.

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