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***Abstract*—**In situ analysis of fetal semilunar valve leaflets has revealed cells coexpressing endothelial and mesenchymal markers along the endothelium, with diminished frequency seen in adult valves. To determine whether such cells are progenitor cells, we isolated clonal populations from human pulmonary valves. The clones expressed endothelial markers but showed potential to further differentiate into endothelium in response to vascular endothelial growth factor (VEGF)-A. When exposed to transforming growth factor (TGF)-β_2, individual clones adopted a mesenchymal phenotype to varying degrees and expressed markers of endothelial to mesenchymal transformation (EMT). Both VEGF- and TGFβ_2–induced phenotypic changes were partially reversible, indicating the plasticity of these cells. When challenged with VEGF or TGFβ_2, a hierarchy of endothelial/mesenchymal potential could be seen among the clonal populations: cells initially closer to an endothelial phenotype showed a strong response to TGFβ_2 that could be inhibited by VEGF, whereas cells closer to a mesenchymal phenotype responded to TGFβ_2 but were resistant to endothelial-inducing effects of VEGF. These findings suggest the presence of bipotential valve progenitor cells with ability to differentiate into either endothelial or interstitial cells of the valve leaflet. Understanding the differentiation potential and function of these cells may be important for understanding heart valve disease and may also be applied to current paradigms for creating tissue-engineered heart valves. **(*Circ Res*. 2006;99:861-869.)**

Key Words: cell culture ■ endothelial cell differentiation ■ endothelial cells ■ heart valves ■ progenitor cells ■ transdifferentiation ■ vascular endothelial growth factor ■ vascular endothelium

Valvular heart disease is a major cause of morbidity, often resulting in the need to replace the valve with a mechanical or bioprosthetic valve. There is a pressing need for improved approaches, especially for children because calcification and destruction of the replacement valve occurs more rapidly in children and young adults.[1] Possible strategies include stimulating endogenous repair pathways or using autologous progenitor cells to create tissue-engineered heart valves that will grow with the child. For both approaches, an increased understanding of mechanisms underlying the normal cellular turnover and repair throughout adult life will be essential.

During embryonic valve development, a subset of endocardial endothelial cells, driven by signals from the underlying myocardium, change their phenotype to mesenchymal cells and migrate into the cardiac jelly to form endocardial cushions, the primordial of valves and cardiac septa of the adult heart.[2] This transdifferentiation of endocardial cells to mesenchymal cells and migration away from the endothelial layer is termed endothelial-mesenchymal transformation (EMT). Studies from Markwald and coworkers show that during EMT, the activated endothelial cells lose cell-cell contacts, gain mesenchymal markers such as α-smooth muscle actin (α-SMA), reduce expression of endothelial markers, and migrate into the cardiac jelly.[3,4]

TGFβ superfamily members are well-studied signaling intermediates in endocardial cushion formation, with TGF$\beta_{1\ to\ 3}$ isoforms expressed at or near the onset of EMT during chick and mouse cardiogenesis.[4–6] Vascular endothelial growth factor (VEGF) is also expressed, by embryonic day 9.5, in the endothelial cells in the outflow tract and atrioventricular canal that undergo EMT.[7] Alterations in

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VEGF levels appear to have pronounced effects on the endocardial cushions depending on the timing, level, and location during heart development.[8–11] Following these embryonic events, Aikawa and colleagues demonstrated that human fetal valves possess a dynamic structure composed of proliferating cells, a nascent extracellular matrix (ECM) and α-SMA–positive cells.[12] Hence, dynamic changes in cell phenotype, cell proliferation and apoptosis, and ECM remodeling occur after the endocardial cushions have remodeled into leaflets and continue throughout human fetal and postnatal development.

Vascular progenitor cells derived from murine embryonic stem cells can differentiate into either endothelial or smooth muscle cells when treated with VEGF or platelet-derived growth factor (PDGF)-BB, respectively.[13] More recently, vascular progenitors have been isolated from arteries of adult mice.[14] That valve progenitors exist and contribute to cell regeneration or repair in adult valves has been speculated on[15]; however, only recently has experimental evidence for valve progenitors been reported.[16,17] Indeed, bone marrow transplantation experiments in mice have shown that clonal hematopoietic stem cells engrafted into the aortic, pulmonary, and atrioventricular valves can differentiate into fibroblast-like cells.[18] In the course of our studies on the endothelium of postnatal valves, we identified clonal populations from human pulmonary valve leaflets with progenitor-like properties, demonstrated by their ability to differentiate into endothelial cells in response to VEGF or into myofibroblasts/smooth muscle cells (SMCs), reminiscent of valve interstitial cells, in response to TGFβ_2.

Materials and Methods

Immunohistochemistry of Fetal and Adult Human Semilunar Valves

Fetal seminlunar valves at 14 to 19 weeks gestation, 20 to 39 weeks gestation, and from adults with a mean age 50.1±2.5 years were obtained at autopsy according to a protocol approved by the Human Research Committee at Brigham and Women's Hospital. No patient had documented cardiac disease or conditions known to predispose to heart valve disease. Valves were fixed in 10% buffered formalin, cut radially in the central cusp region through the adjacent arterial wall, and embedded in paraffin. Sections cut at 6-μm thickness were stained by the avidin/biotin/peroxidase method.[12,15] Images were analyzed using imaging software (IPLab version 3.9.3; Scanalytics Inc, Fairfax, Va).

Isolation and Culture of Clonal Population of Human Pulmonary Valve Endothelial Cells

Human pulmonary valve endothelial cells (HPVECs) were isolated from a pulmonary valve of a 9-month-old infant as described[19] and cultured on 1% gelatin-coated plates in EBM-2 supplemented with EGM-2 SingleQuots (Cambrex Bio Science, Walkersville, Md [except the hydrocortisone aliquot was omitted]), and 20% heat-inactivated FBS. This culture media will be referred to as EBM-2. Clonal populations were isolated from HPVECs at passage 2 by suspending in growth medium at 3.3 cells/mL and plating 100 μL/well of a 96-well plate. When the colonies covered two-thirds of the well, cells were passaged into 24-well dishes. Human dermal microvascular ECs (HDMECs) were isolated as reported[20] but cultured in the same medium, EBM-2, as the HPVECs and HPVEC-derived clones.

An expanded Materials and Methods section, available at http://circres.ahajournals.org, describes materials and methods for indirect immunofluorescence, RT-PCR, Western blot analysis, flow cytometry, cellular migration and invasion assays, and the HL-60 cell adhesion assay.

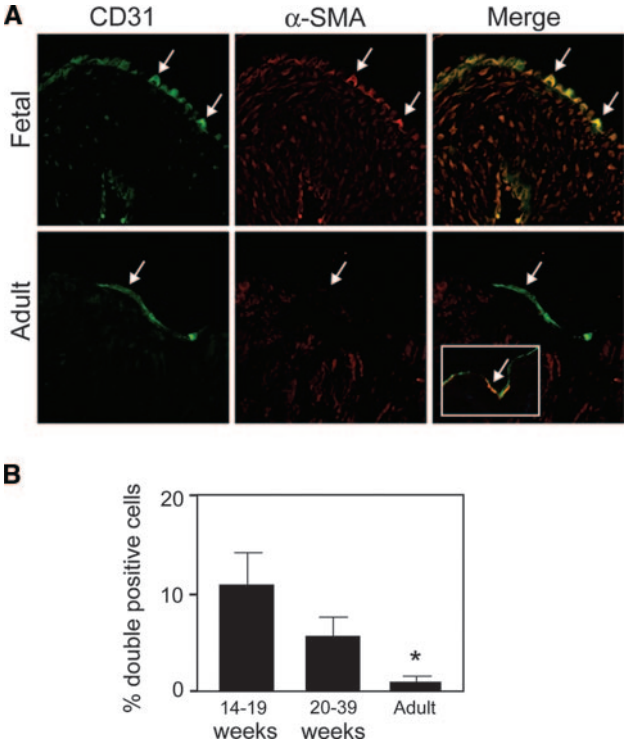

Figure 1. Cells coexpressing CD31 and α-SMA in fetal and adult semilunar valves. A, Sections were double-labeled with anti-CD31 (green) and anti–α-SMA (red). Arrows indicate double-positive cells along the arterial side of the leaflet. The inset in the lower right panel shows rare double-positive cells detected in adult valves. B, Cells coexpressing both markers were quantitated: fetal valves from 14 to 19 weeks of gestation, 10.8±3.3% (n=11); 20 to 39 weeks of gestation, 5.6±2.0%, (n=10); and adult valves, 1.0±0.5%, (n=10). Data are presented as mean±SEM. Probability values: 14 to 19 weeks vs 20 to 39 weeks, P=NS; 14 to 19 weeks vs adult, $P<0.05$; 20 to 39 weeks vs adult, $P<0.05$.

Results

EMT in Developing Fetal Heart Valves

We showed previously that endothelial cells coexpressing α-SMA exist in aortic valve leaflets in juvenile sheep.[15] To gain insights on the nature of these cells, we quantitated CD31/α-SMA–positive cells in paraffin-embedded sections from human fetal and adult semilunar valve leaflets. Three subsets of cells were observed along the leaflet endothelium: cells expressing only CD31 (green), only α-SMA (red), and those coexpressing both CD31 and α-SMA (yellow) (Figure 1A). In fetal valves, some CD31-positive cells were seen in the interstitium of the leaflet. The CD31 expression might correspond to residual endothelial characteristics of the interstitial cells, similar to endothelial-specific green fluorescence protein (GFP) expression seen in embryonic valve leaflets.[21] α-SMA–positive cells were observed predominantly along the subendothelial lining. In fetal valves, 5% to 10% of the CD31-positive cells were also α-SMA positive, whereas only 1% double-positive cells were detected in adult valves (Figure 1B). The presence of dual CD31/α-SMA–positive cells suggests that a subset of valve endothelial cells have the

potential to differentiate toward a mesenchymal phenotype and perhaps migrate into the interstitial regions of the leaflet.

Isolation and Characterization of HPVEC Clones

To gain insight into the EMT potential of the CD31-positive and CD31/α-SMA–positive cells in human cardiac valves, we used a limiting dilution technique to isolate clonal populations. We isolated 5 clones, of which 4 exhibited features of a mesenchymal phenotype and yet expressed endothelial markers. These clones showed slower growth kinetics and exhibited spindle-shaped morphology instead of the cobblestone morphology seen in parental HPVECs or HDMECs (Figure 2A). Of the clones obtained, clone 5 and clone 8 were chosen for further characterization of markers specific to endothelial and mesenchymal lineages by 3 different techniques: RT-PCR (Figure 2B), indirect immunofluorescence (Figure 2C), and flow cytometry (Figure 2D). Both clones expressed the endothelial markers tested: CD31, VE-cadherin, von Willebrand factor (vWF), VEGF-R2, endothelial NO synthase (eNOS), CD34, CD146, and CD105 as determined by PCR and by flow cytometry (Figure 2D). HDMECs served as a positive control, whereas parental HPVECs and human saphenous vein smooth muscle cells (HSVSMCs) were analyzed for comparison. Clones 5 and 8 also expressed TGFβ receptors I, II, and III (Figure 2B). Immunofluorescence staining of clone 8 and clone 5 showed cell/cell border staining for CD31 and VE-cadherin, a punctate cytoplasmic staining for vWF, consistent with its localization in Weibel–Palade bodies, but little α-SMA (Figure 2C). The flow cytometry analysis showed a similar pattern among HPVECs, clone 8, clone 5, and HDMECs and a clear distinction from HSVSMCs. The clones were negative for mesenchymal marker CD90 (Figure 2D). Based on the expression patterns, clone 8 and clone 5 appeared to be endothelial cells despite lacking a typical cobblestone appearance.

TGFβ2 Induces Expression Smooth Muscle Markers in HPVEC Clones

TGFβ isoforms are well documented in the differentiation of endothelial to mesenchymal cells in mouse and chick endocardial cushion formation.[4,6,15,19,22,23] However, whether TGFβ isoforms induce EMT in human valve endothelial cells is unknown. We tested whether the clonal populations from HPVECs can be induced to undergo EMT in response to TGFβ_1, -β_2, or -β_3. Clonal cells are essential for this experiment to ensure that effects are not attributable to preferential growth of mesenchymal cells that could contaminate a nonclonal endothelial cell culture. We treated the cells with TGF$\beta_{1,2,\text{ or }3}$ for 10 days and examined the changes in the expression of smooth muscle cell/mesenchymal markers α-SMA and calponin (Figure 3A). TGFβ_2, but not TGFβ_1 or TGFβ_3 isoforms, induced robust expression of α-SMA and calponin in clone 8. Induction was also evident in clone 5 and in the parental HPVECs, but to a lesser extent because the cells expressed α-SMA in the absence of TGFβ_2. Increased expression of α-SMA and calponin was confirmed by RT-PCR (data not shown). HDMECs did not express α-SMA or calponin in either the absence or presence of TGFβ_2. Consistently, TGFβ_2 treatment attenuated CD31 expression in clone 8 and clone 5. In Figure 3B, clone 8 and clone 5 treated without or with TGFβ_2 for 10 days were analyzed for coexpression of CD31 and α-SMA by double-label immunofluorescence. The presence of both CD31 and α-SMA in individual cells supports the finding that the cells are undergoing EMT. The increase in α-SMA expressing (red) CD31-positive cells in response to TGFβ_2, quantitated by counting fluorescent cells, was significant.

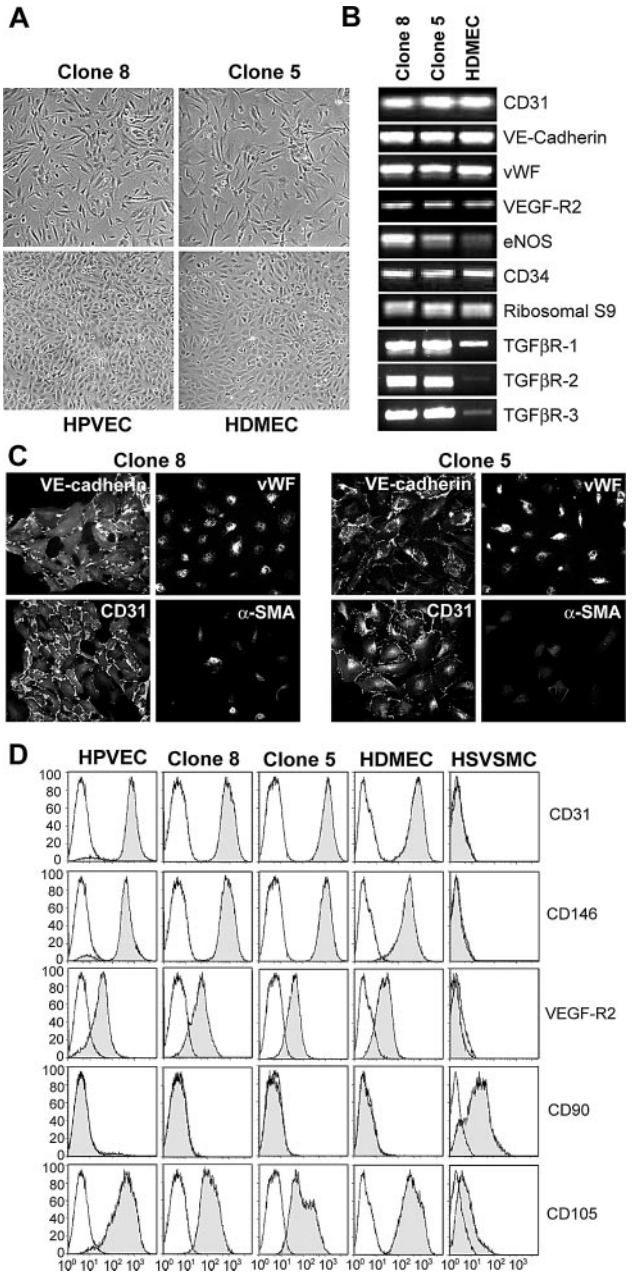

Figure 2. Clonal cell populations from human pulmonary valve express endothelial-specific markers. A, Phase-contrast micrographs of clone 8 and clone 5 (top panels) parental HPVECs and HDMECs (bottom panels) grown in EBM-2. B, RT-PCR analysis of clones 8 (lane 1), 5 (lane 2), and HDMECs (lane 3) for endothelial-specific transcripts and TGFβ receptor transcripts. Ribosomal S9 served as a control. C, Immunostaining of clone 8 (left panels) and clone 5 (right panels) with anti–VE-cadherin, anti-vWF, anti-CD31, and anti–α-SMA antibodies. D, Flow cytometric analysis of parental HPVECs, clone 8, clone 5, HDMECs, and HSVSMCs stained with fluorescein isothiocyanate (FITC)-conjugated CD31, CD146, VEFG-R2, CD90, and CD105 Abs.

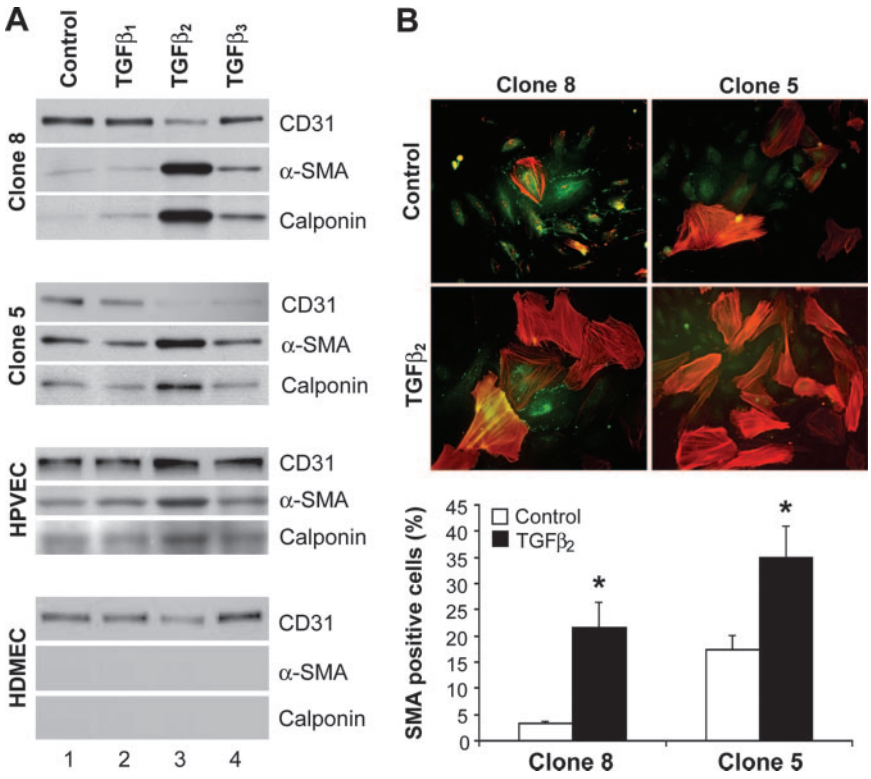

Figure 3. TGFβ_2-induced expression of α-SMA and calponin. A, Clones 8 and 5, the parental HPVECs and HDMECs, were grown for 10 days in absence (control, lane 1) or presence of 2 ng/mL of TGFβ_1 (lane 2), TGFβ_2 (lane 3), or TGFβ_3 (lane 4). Cell lysates were analyzed by western blot for the expression of CD31, α-SMA, and calponin. B, Clone 8 (left panels) and clone 5 (right panels) were grown for 10 days in absence (control, top panels) or presence of 2 ng/mL of TGFβ_2 (bottom panels). Cells were double-labeled with goat anti-human CD31/FITC-conjugated secondary antibody and mouse anti–α-SMA/Texas Red–conjugated secondary antibody. α-SMA–positive cells, which also expressed CD31, were counted to determine number of positive cells in each clone. *$P<0.05$.

TGFβ_2 Treatment Results in Upregulation of EMT-Related Transcripts

Slug and its homolog Snail are zinc finger transcription factors that mediate both epithelial and endothelial transdifferentiation.[24] Slug expression in developing valves is TGFβ_2 dependent,[25] and downregulating Slug with antisense oligonucleotides inhibited EMT in chick endocardial cushion explants.[26] Therefore, we investigated if these markers were upregulated when clone 5 and clone 8 cells were induced to undergo EMT (Figure 4A). TGFβ_2 induced expression of Slug and caused a modest enhancement of Snail. The expression of other known EMT-related proteins, hHex, bone morphogenetic protein (BMP)-2, or Jagged-1, was unaltered by TGFβ_2 (data not shown). Enhanced migration is another hallmark of EMT and crucial players involved in this process belong to the family of matrix metalloproteases (MMPs).[6] TGFβ_2 significantly enhanced the expression of MMP-1 and MMP-2 in HPVEC clones as assessed by RT-PCR (Figure 4A). To measure cellular migration directly, different factors were tested for ability to stimulate migration of clone 8 (Figure 4B). Increased migration in response to serum, PDGF, and basic fibroblast growth factor (bFGF) was observed in cells treated with TGFβ_2 compared with untreated control cells. We also measured the ability of clone 8 to

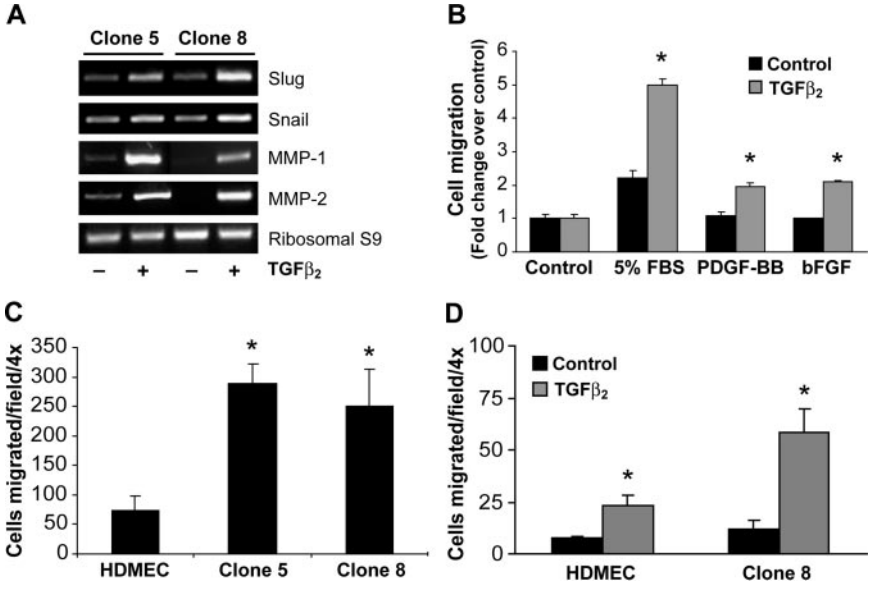

Figure 4. TGFβ_2-induced expression of EMT markers, migration, and invasion. A, Clone 5 (lanes 1 and 2) and clone 8 (lanes 3 and 4) were grown for 10 days in absence (lanes 1 and 3) or presence of 2 ng/mL TGFβ_2 (lanes 2 and 4). RNA was extracted and RT-PCR was performed with Slug, Snail, MMP-1, and MMP-2 primers. Ribosomal S9 served as a control. B, Clone 8 cells cultured in absence (black bars) or presence (gray bars) of TGFβ_2 for 10 days were tested for ability to migrate toward EBM (control), EBM with 5% FBS, 10 ng/mL PDGF-BB, and 10 ng/mL bFGF. C, HDMECs and clone 5 and clone 8 cells were cultured in EBM-2 medium, and basal migration toward control medium was tested. D, HDMECs and clone 8 cells were cultured in absence (black bars) or presence (gray bars) of TGFβ_2 in EBM-2 medium for 10 days and were tested for invasion into collagen gels for 72 hours. Data from the migration and invasion assays are mean±SD of 2 independent experiments performed in triplicates. In B through D, *$P<0.05$ compared control.

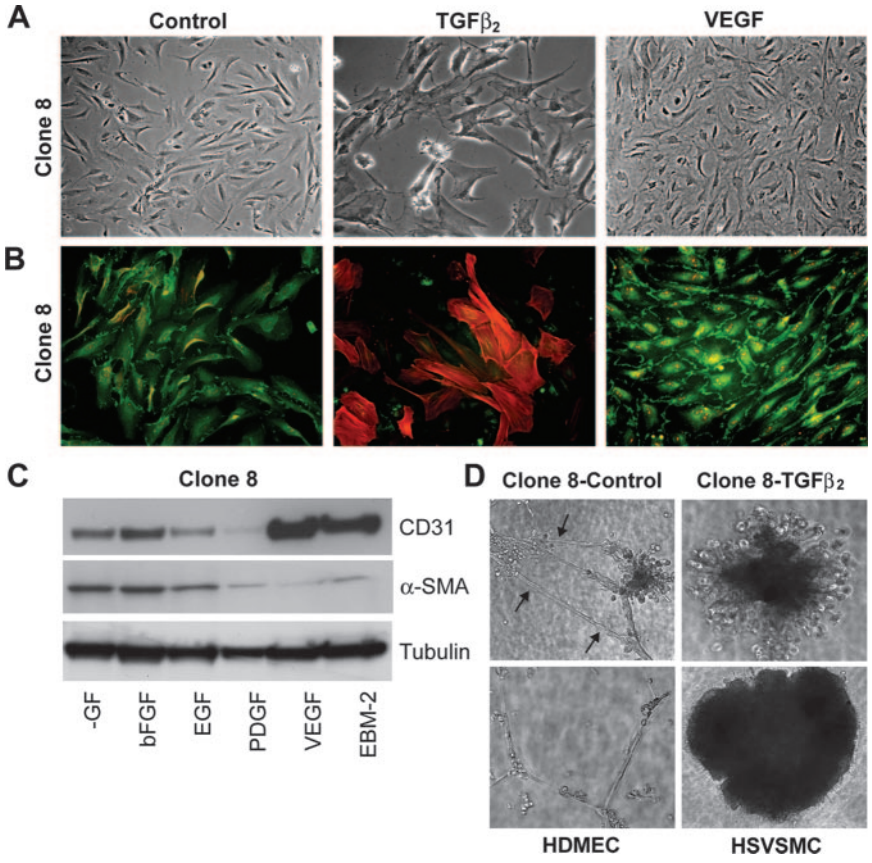

Figure 5. Phenotypic modulation of HPVEC clones. Clone 8 cells were cultured in absence (control) or presence of 2 ng/mL TGFβ_2 or 10 ng/mL VEGF in EBM-2 medium for 10 days. A, Phase-contrast micrographs show cell morphology. B, Double-label immunostaining of same cells with anti-CD31 (FITC) and anti–α-SMA (Texas Red). C, Clone 8 cells were cultured in growth factor–depleted medium (-GFl) or supplemented with bFGF (10 ng/mL), EGF (10 ng/mL), PDGF (10 ng/mL), VEGF (10 ng/mL), and EBM-2 medium for 10 days. Cell lysates were analyzed for the expression of CD31 and α-SMA, and tubulin to confirm equal loading. D, Tube formation on Matrigel. Clone 8 cells were cultured in absence (control) or presence of TGFβ_2 in EBM-2 medium for 10 days and then plated on Matrigel. Tube formation was assessed after 24 hours in comparison with HDMECs and HSVSMCs that had not been treated with TGFβ_2.

invade a collagen gel. TGFβ_2-treated or untreated clone 8 cells were compared with HDMECs treated with or without TGFβ_2. In this assay, TGFβ_2-treated cells were more invasive compared with the control cells, with TGFβ_2-treated clone 8 cells showing the most robust invasion (Figure 4D). In addition, clone 5 and clone 8 exhibited 4- to 5-fold higher levels of basal migration compared with the HDMECs (Figure 4C). As enhanced basal migration is a hallmark of endothelial progenitor cells,[27] the increased migratory activity of clone 5 and clone 8 is consistent with a progenitor phenotype.

HPVEC Clones Exhibit Endothelial/Mesenchymal Plasticity

We also examined the effects of adding additional VEGF-A (10 ng/mL) on differentiation of these clones. (EBM-2 supplemented with EGM-2 SingleQuots contains 2 to 5 ng/mL VEGF-A.) The additional VEGF caused the cell clusters to form a confluent cobblestone-like monolayer (Figure 5A), with CD31 localized at cell/cell borders (Figure 5B). To determined if this effect was specific to VEGF or a general response to growth factors, cells were treated with EBM-2 media without the added growth factors from SingleQuots (-GF), with -GF media to which bFGF, EGF, PDGF, or VEGF had been added, and with full EBM-2 growth medium with SingleQuots. In media without growth factors, both CD31 and α-SMA were expressed; the levels were not significantly altered by addition of bFGF or EGF. PDGF treatment led to reduction in CD31 and α-SMA expression. In contrast, cells treated with 10 ng/mL VEGF or with complete EBM-2 medium showed enhanced CD31 expression and reduced α-SMA expression (Figure 5C), suggesting that VEGF drives these cells to stronger endothelial phenotype. Taken together, the results demonstrate that these cells have a dual potential to differentiate to an endothelial phenotype with VEGF or a mesenchymal phenotype with TGFβ_2 (Figures 4 and 5A). The fact that the clones were expanded from a single cell, coupled with the above findings, suggests that these clones are bipotential progenitors that reside in the pulmonary valve leaflets.

TGFβ_2 Abrogates the Functional Endothelial Properties of the HPVEC Clones

We then asked whether these clones have the potential to function as normal endothelial, and, if so, what effect does TGFβ_2 have. Tube formation in Matrigel is a characteristic feature of endothelial cells. Therefore, we investigated the tube-forming ability of these cells in presence or absence of TGFβ_2 (Figure 5D). Untreated clone 8 cells formed tube-like structures with translucent slits along the length of the cords, indicating lumen formation. However, prior treatment with TGFβ_2 inhibited tube formation of clone 8 and, instead, caused the cells to contract into a large clump that appeared similar to untreated HSVSMCs. HDMECs treated with TGFβ_2 were able to form tubes (data not shown), as were the nontreated HDMECs (Figure 5D).

A second functional property of endothelial cells is expression of adhesion molecules E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 in response to tumor necrosis factor (TNF)-α.

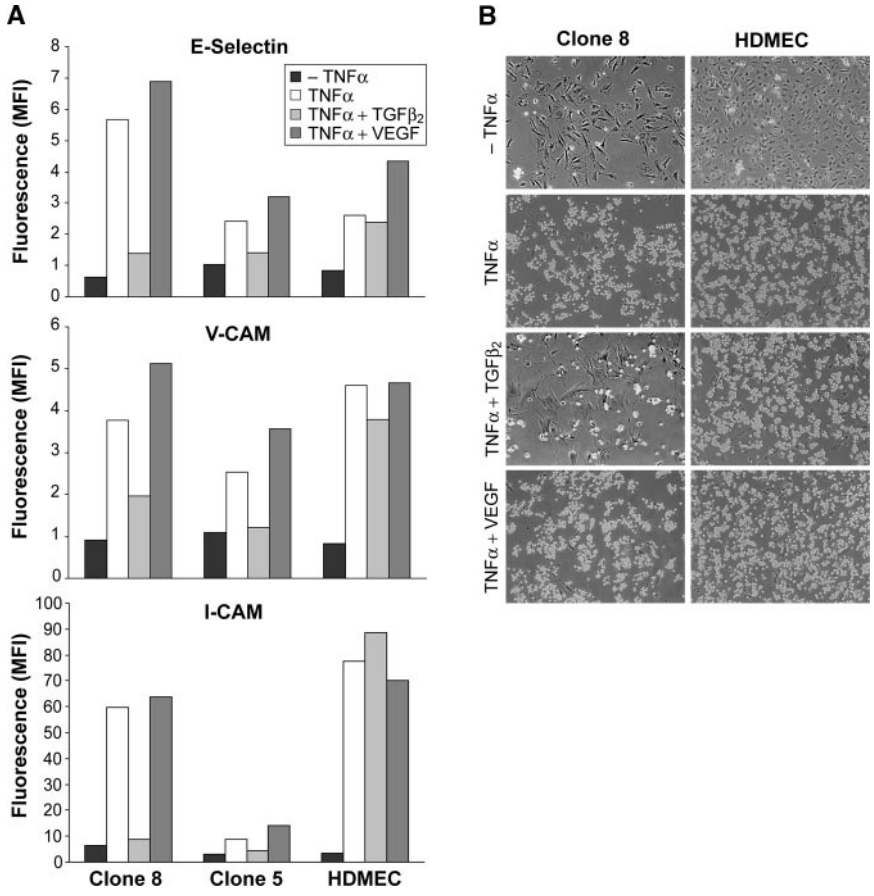

Figure 6. Cytokine-induced expression of leukocyte adhesion molecules and adherence of leukocytes. Clone 8 cells were cultured in absence or presence of 2 ng/mL TGFβ_2 or 10 ng/mL VEGF in EBM-2 medium for 10 days. Cells were further treated without (-TNFα) or with TNF-α (TNF-α +) for 5 hour and analyzed for the expression of leukocyte adhesion molecules by flow cytometry. B, HL-60 cell adhesion to clone 8 and HDMECs with similar treatments as in A was determined by light microscope.

Therefore, HPVEC clones cultured in either VEGF or TGFβ_2 were exposed to TNF-α for 5 hours. Both clones as well as HDMECs showed TNF-α–induced expression of E-selectin, VCAM-1, and ICAM-1. Prior treatment with TGFβ_2 greatly attenuated the TNF-α–induced expression of the adhesion molecules in clone 8 and clone 5 but had no effect on the induction level in HDMECs (Figure 6A). Complementing these findings, a leukocyte adhesion assay revealed strong adhesion of HL-60 cells, a human promyelocytic leukemia line, to both clone 8 and clone 5 when stimulated with TNF-α for 5 hours. However, prior treatment with TGFβ_2 inhibited binding of HL-60 cells to clone 8 but not to HDMECs (Figure 6B). Treatment with VEGF before TNF-α induction led to modest increase in this adhesion response consistent with results in Figure 6A. These data demonstrate that TGFβ_2 treatment abrogates endothelial functional properties specifically in HPVEC-derived clones but not in HDMECs.

Reversibility of Endothelial and Mesenchymal Phenotypes

The above results prompted us to investigate if the changes we observed with TGFβ_2 and VEGF are transient or whether differentiation status is maintained after removal of the factors. We treated clonal cells with TGFβ_2 or VEGF for 10 days, trypsinized and reseeded the cells in presence of TGFβ_2 and VEGF according to the scheme in Figure 7A. Lanes 1 to 3 show that the cells differentiated as expected based on our previous results. When cells were first induced to a mesenchymal phenotype with TGFβ_2, we found that removing TGFβ_2 reversed the phenotype (compare lanes 4 and 5). The presence of additional VEGF did not result in any further decrease in α-SMA (compare lane 4 and lane 6). After TGFβ_2 treatment, a 10 day treatment with VEGF did not fully restore the endothelial phenotype (compare lanes 3 and 6). When cells were first cultured in VEGF for 10 days, subsequent exposure to TGFβ_2 resulted in a full reversal (compare lane 8 to lanes 2 or 5). These results suggest that (1) VEGF is required for the cells to maintain an endothelial phenotype; (2) TGFβ_2-induced α-SMA can be partially reversed; and (3) prior treatment with TGFβ_2 or VEGF does not diminish the plasticity of the cells (Figure 7B). Similar results were obtained with both clone 5 and clone 8. The reversibility may be influenced by the ECM such that removal of the cells from the ECM produced in response to TGFβ_2 might exert control over the cellular phenotype. To address the role of ECM, we examined the effect of plating the cells on different ECM to determine whether exogenous ECM might alter EMT. As seen in Figure 7C, cells plated on fibronectin showed markedly enhanced TGFβ_2 induction of α-SMA, consistent with the potential role for fibronectin in EMT in endocardial cushions.[6]

A Hierarchy of Endothelial/Mesenchymal Plasticity Among HPVEC Clonal Populations

To further understand the role of VEGF in the endothelial to mesenchymal differentiation, we analyzed 3 different clones, clones 1, 8, and 5, that varied in their potential to undergo EMT (Figure 8A). Clone 1 cells did not express α-SMA or

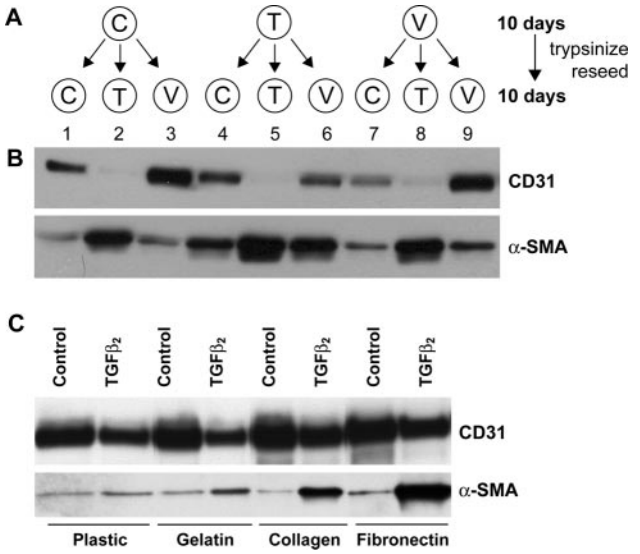

Figure 7. Changes induced by TGFβ_2 and VEGF were reversible and effects of ECM on TGFβ_2-induced EMT. A, Schematic of culture conditions: clone 8 cells were cultured in EBM-2 medium (C), EBM-2 medium with 2 ng/mL TGFβ_2 (T), or EMB-2 medium with 10 ng/mL VEGF (V) for 10 days. Cells were then replated and cultured for 10 days in (C), (T), or (V). B, Cell lysates were analyzed for the expression of CD31 and α-SMA. It is important to note that the EBM-2 medium (C) is supplemented with the commercially available SingleQuots, which contains 2 to 5 ng/mL VEGF-A. (V) medium is (C) supplemented with an additional 10 ng/mL VEGF-A. C, Clone 8 cells were plated on different ECM substratum in the absence or presence of TGFβ_2 to assess the effects on induction of α-SMA.

calponin in response to TGFβ_2, suggesting that this clone is unable to undergo EMT. However, addition of VEGF resulted in increased CD31 and VE-cadherin protein levels (Figure 8A); coincubation with TGFβ_2 had no effect on VEGF-enhanced expression of these endothelial markers. Clone 8 responded to both TGFβ_2 and VEGF; TGFβ_2 induced expression of α-SMA and calponin and a decrease in CD31, whereas VEGF increased CD31 and VE-cadherin. Strikingly, VEGF reduced the TGFβ_2 induction of α-SMA and calponin in clone 8. Clone 5, on the other hand, showed spontaneous transdifferentiation as seen by the coexpression of CD31 and α-SMA under control conditions (Figure 8A, and 3B). TGFβ_2 caused increased α-SMA and calponin and decreased CD31 as expected. However, VEGF was unable to block the effects of TGFβ_2 on α-SMA and calponin. This suggests that clone 5 is further differentiated toward a mesenchymal phenotype than clone 8. The band intensities for CD31 and α-SMA for each clone in each condition from 2 separate experiments were quantitated by densitometry to substantiate the results (Figure 8B). In summary, these results support the concept that a hierarchy exists among these clonal cell populations in their propensity to transition from an endothelial to mesenchymal phenotype in response to VEGF or TGFβ_2 (Figure 8C).

Discussion

In the present study, we report for the first time that clonal endothelial-like cells isolated from human pulmonary valves transition to a mesenchymal phenotype, analogous to EMT, specifically in response to TGFβ_2. TGFβ_2 induced expression of EMT markers and increased cellular migration and invasion capacity. Endothelial functions such as ability to form tube-like structures and to adhere leukocytes in response to inflammatory signals were lost in TGFβ_2-treated cells. The cells were not responsive to TGFβ_1, consistent with the functional role for TGFβ_2 in EMT in avian and murine endocardial cushions.[28] In contrast, VEGF promoted formation of cell/cell contacts, a cobblestone morphology, and increased levels of CD31 and VE-cadherin. Our finding that VEGF can inhibit TGFβ_2-induced EMT provides a potential mechanism to explain the negative role reported for VEGF in endocardial cushion formation.[8,11] We postulate that the clonal populations are bipotential valve progenitor cells based on their plasticity and their ability to grow in culture from a single cell. We further propose that these cells represent the CD31/α-SMA–positive cells seen by in situ immunostaining of human pulmonary valve leaflets. Whether the reversibility of the endothelial and mesenchymal phenotypes seen in vitro occurs in vivo will require further investigation. Finally, the differentiation potential seems to be restricted as clones failed to differentiate into adipocytes, chondrocytes osteocytes, or hematopoietic progenitors in in vitro assays (data not shown).

We isolated valvular progenitor cells from discarded, surgically resected pulmonary valve tissue from 3 different patients. Two were infants, ages 5 months and 9 months, and the third was a 15-year old. The number of single cells that grew into clonal populations was approximately 30%, but the number of bipotential clones ranged from 1% from the 15-year old to 3% to 4% from the infant specimens. This corresponds well to our in vivo data showing that cells expressing both CD31 and α-SMA are found in higher numbers in fetal specimens compared with the adult valves (Figure 1). Although the valve progenitor cells are different from normal human microvascular ECs, such as HDMECs, they express endothelial markers and behave as functional endothelial cells in 2 in vitro assays. The endothelial properties we detected in the clones could be explained by that fact that they were expanded in a medium designed for growth of human endothelial cells, EBM-2, which contains 2 to 5 ng/mL VEGF. This level of VEGF was not sufficient to block the action of TGFβ_2 in clones 5 and 8 but may have been in clone 1.

HDMECs were not responsive to TGFb$_{1,2,\text{ or }3}$, suggesting that postnatal EMT is specific to valve endothelium and perhaps to a subset of valve EC with progenitor properties. However, TGFβ-induced differentiation of large vessel-derived EC into SMC-like cells has been reported, suggesting that EMT may not be unique to the valve endothelium and that vascular progenitors may reside in the vessel wall. In 1992, bovine aortic ECs were shown to express α-SMA, with a concomitant decrease in EC markers, in response to TGFβ_1.[29] DeRuiter et al traced EC in the embryonic dorsal aorta that migrated into the subendothelial space and expressed α-SMA, indicating EMT in embryonic endothelium.[30] A small percentage of cells in EC preparations from bovine arteries were also shown to undergo EMT.[31] More recently, clonal populations of human umbilical vein ECs were shown to differentiate into SMC-like cells when FGF-1 was re-

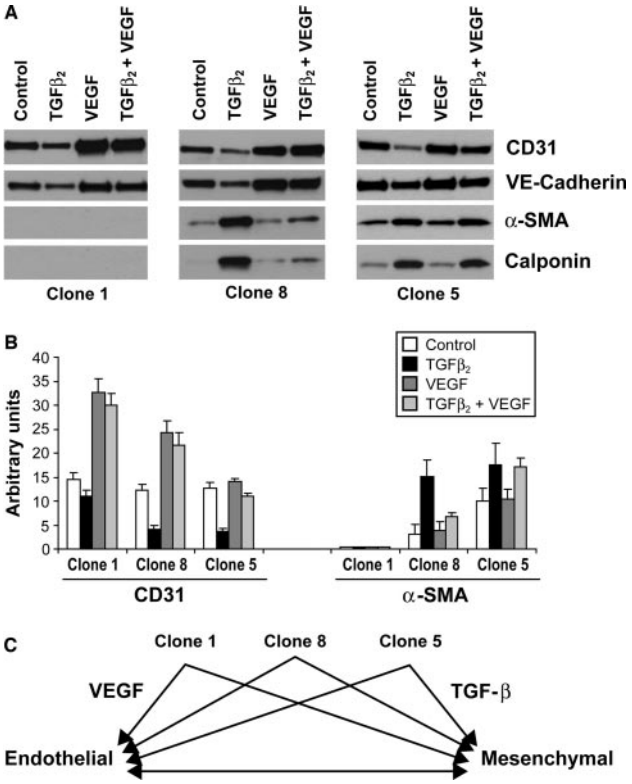

Figure 8. Endothelial/mesenchymal hierarchy in HPVEC clones. Clone 1 (left), clone 8 (middle), and clone 5 (right) were grown for 10 days in absence (control), 2 ng/mL of TGFβ_2, 10 ng/mL VEGF, or TGFβ_2 and VEGF (TGFβ_2+VEGF). Cell lysates were analyzed by western blot for the expression of CD31, VE-cadherin, α-SMA, and calponin. B, Quantitation of bands seen in A by densitometry. C, Schematic representation of hierarchy in plasticity of pulmonary valve progenitor cells.

moved.[32] Hence, although the valve endothelium appears to harbor endothelial cells that can be easily prompted to undergo EMT, endothelial cells with capacity to undergo EMT may reside throughout the vasculature and perhaps through embryonic, fetal, childhood, and adult life.

How closely the EMT we observe in valve EC progenitors in vitro reflects the EMT that occurs in vivo, either during valve development or during postnatal life, is a critical question. Many of the hallmarks of EMT are seen in our in vitro system: loss of cell/cell contacts in the EC monolayer; increased expression of α-SMA, MMP-1, MMP-2, and Slug; increased cellular migration and invasion; and induction of Sox9 (J.H.Y. and J.B., 2006 unpublished results). The specificity of TGFβ_2 for inducing these events is also consistent with in vivo studies of EMT in mouse endocardial cushions.[28] α-SMA is expressed transiently during EMT in developing valves and during steps of endothelial delamination and migration[4] but is found only in focal regions along the endothelium in healthy postnatal valves.[12,15] Therefore, we postulate that the α-SMA–positive valve progenitors are recapitulating early events in EMT. Identification of specific markers of the interstitial cells of heart valves would provide important tools for studying the entire differentiation process.

HPVEC clones also exhibit a hierarchy in which some clones are more endothelial like, whereas others are more mesenchymal-like in terms of response to exogenous factors. We hypothesize that these cells are valve progenitors that participate in cellular renewal of the valve endothelium and the interstitial cells of the leaflet. Differentiation toward one cell type or the other would depend on exposure to biochemical and mechanical signals and possibly even to pathological signals arising from disease or structural malformations. Alterations in valve progenitor cell proliferation or differentiation may occur in any number of deleterious conditions leading to calcification, leaflet thickening, or even loss of integrity. Understanding the functional characteristics of these cells may lead to greater understanding of heart valve disease and eventually to strategies to manipulate these cells for therapeutic benefit.

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Disclosures

None.

References

1. Rabkin-Aikawa E, Mayer JE Jr, Schoen FJ. Heart valve regeneration. *Adv Biochem Eng Biotechnol*. 2005;94:141–179.
2. Markwald RR, Fitzharris TP, Manasek FJ. Structural development of endocardial cushions. *Am J Anat*. 1977;148:85–119.
3. Bernanke DH, Markwald RR. Migratory behavior of cardiac cushion tissue cells in a collagen-lattice culture system. *Dev Biol*. 1982;91:235–245.
4. Nakajima Y, Mironov V, Yamagishi T, Nakamura H, Markwald RR. Expression of smooth muscle alpha-actin in mesenchymal cells during formation of avian endocardial cushion tissue: a role for transforming growth factor beta3. *Dev Dyn*. 1997;209:296–309.
5. Nakajima Y, Yamagishi T, Hokari S, Nakamura H. Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat Rec*. 2000;258:119–127.
6. Person AD, Klewer SE, Runyan RB. Cell biology of cardiac cushion development. *Int Rev Cytol*. 2005;243:287–335.
7. Miquerol L, Gertsenstein M, Harpal K, Rossant J, Nagy A. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. *Dev Biol*. 1999;212:307–322.
8. Dor Y, Camenisch TD, Itin A, Fishman GI, McDonald JA, Carmeliet P, Keshet E. A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. *Development*. 2001;128:1531–1538.
9. Hallaq H, Pinter E, Enciso J, McGrath J, Zeiss C, Brueckner M, Madri J, Jacobs HC, Wilson CM, Vasavada H, Jiang X, Bogue CW. A null mutation of Hhex results in abnormal cardiac development, defective vasculogenesis and elevated Vegfa levels. *Development*. 2004;131:5197–5209.
10. Miquerol L, Langille BL, Nagy A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development*. 2000;127:3941–3946.
11. Chang CP, Neilson JR, Bayle JH, Gestwicki JE, Kuo A, Stankunas K, Graef IA, Crabtree GR. A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis. *Cell*. 2004;118:649–663.
12. Aikawa E, Whittaker P, Farber M, Mendelson K, Padera RF, Aikawa M, Schoen FJ. Human semilunar cardiac valve remodeling by activated cells from fetus to adult: implications for postnatal adaptation, pathology, and tissue engineering. *Circulation*. 2006;113:1344–1352.
13. Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T, Naito M, Nakao K, Nishikawa S. Flk1-positive cells derived from

embryonic stem cells serve as vascular progenitors. *Nature*. 2000;408: 92–96.
14. Sainz J, Al Haj Zen A, Caligiuri G, Demerens C, Urbain D, Lemitre M, Lafont A. Isolation of "side population" progenitor cells from healthy arteries of adult mice. *Arterioscler Thromb Vasc Biol*. 2006;26:281–286.
15. Paranya G, Vineberg S, Dvorin E, Kaushal S, Roth SJ, Rabkin E, Schoen FJ, Bischoff J. Aortic valve endothelial cells undergo transforming growth factor-beta-mediated and non-transforming growth factor-beta-mediated transdifferentiation in vitro. *Am J Pathol*. 2001;159:1335–1343.
16. Veinot JP, Prichett-Pejic W, Song J, Waghray G, Parks W, Mesana TG, Ruel M. CD117-positive cells and mast cells in adult human cardiac valves—observations and implications for the creation of bioengineered grafts. *Cardiovasc Pathol*. 2006;15:36–40.
17. Deb A, Wang SH, Skelding K, Miller D, Simper D, Caplice N. Bone marrow-derived myofibroblasts are present in adult human heart valves. *J Heart Valve Dis*. 2005;14:674–678.
18. Visconti RP, Ebihara Y, Larue AC, Fleming PA, McQuinn TC, Masuya M, Minamiguchi H, Markwald RR, Ogawa M, Drake CJ. An in vivo analysis of hematopoietic stem cell potential. Hematopoietic origin of cardiac valve interstitial cells. *Circ Res*. 2006;98:690–696.
19. Johnson EN, Lee YM, Sander TL, Rabkin E, Schoen FJ, Kaushal S, Bischoff J. NFATc1 mediates vascular endothelial growth factor-induced proliferation of human pulmonary valve endothelial cells. *J Biol Chem*. 2003;278:1686–1692.
20. Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. *J Clin Invest*. 2001;107:745–752.
21. Gitler AD, Zhu Y, Ismat FA, Lu MM, Yamauchi Y, Parada LF, Epstein JA. Nf1 has an essential role in endothelial cells. *Nat Genet*. 2003;33: 75–79.
22. Nakajima Y, Miyazono K, Kato M, Takase M, Yamagishi T, Nakamura H. Extracellular fibrillar structure of latent TGF beta binding protein-1: role in TGF beta-dependent endothelial-mesenchymal transformation during endocardial cushion tissue formation in mouse embryonic heart. *J Cell Biol*. 1997;136:193–204.
23. Nakajima Y, Yamagishi T, Nakamura H, Markwald RR, Krug EL. An autocrine function for transforming growth factor beta 3 in the atrioventricular endocardial cushion tissue formation during chick heart development. *Ann N Y Acad Sci*. 1998;857:272–275.
24. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol*. 2000;2:76–83.
25. Romano LA, Runyan RB. Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. *Dev Biol*. 2000;223:91–102.
26. Romano LA, Runyan RB. Slug is a mediator of epithelial-mesenchymal cell transformation in the developing chicken heart. *Dev Biol*. 1999;212: 243–254.
27. Khan ZA, Melero-Martin JM, Wu X, Paruchuri S, Boscolo E, Mulliken JB, Bischoff J. Endothelial progenitor cells from infantile hemangioma and umbilical cord blood display unique cellular responses to endostatin. *Blood*. 2006;108:915–921.
28. Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, McDonald JA, Klewer SE. Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev Biol*. 2002;248:170–181.
29. Arciniegas E, Sutton AB, Allen TD, Schor AM. Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro. *J Cell Sci*. 1992;103(pt 2):521–529.
30. DeRuiter MC, Poelmann RE, VanMunsteren JC, Mironov V, Markwald RR, Gittenberger-de Groot AC. Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ Res*. 1997;80:444–451.
31. Frid MG, Kale VA, Stenmark KR. Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ Res*. 2002;90:1189–1196.
32. Ishisaki A, Hayashi H, Li AJ, Imamura T. Human umbilical vein endothelium-derived cells retain potential to differentiate into smooth muscle-like cells. *J Biol Chem*. 2003;278:1303–1309.

Supplemental Materials and Methods.

Materials

Materials used were Endothelial Basal Medium (EBM)-2 and EGM-2 Single Quots (CC-3156 and CC-4176 respectively, Cambrex Bio Science, Walkersville, MD), fetal bovine serum (FBS) (Hyclone, Logan, UT), GPS, PSF (Invitrogen, Carlsbad, California), Immobilon-P membrane (Millipore, Bedford, MA), Hyperfilm ECL, FITC-streptavidin, and Texas Red-streptavidin (Amersham Life Sciences, Arlington Heights, IL), Lumiglo (KPL, Gaithersburg, MD), TGFβ_{1-3}, PDGF-BB, VEGF, bFGF, FITC-conjugated anti-human KDR (R&D Systems, Minneapolis, MN), FITC-anti-goat IgG, Texas Red anti-mouse IgG, peroxidase conjugated anti-mouse IgG, anti-goat IgG (Vector Laboratories, Burlingame, CA), FITC-conjugated anti-human CD31 (Ancell, Bayport, MN), FITC-conjugated mouse anti-human CD146, human fibronectin (Chemicon Int., Temecula, CA), mouse anti-human a-SMA (Sigma Aldrich Co., St. Louis, MO), goat anti-human CD31, goat anti-human VE-Cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human von Willebrand Factor (vWF) and anti-human calponin (DAKO, Carpinteria, CA), RNeasy kit and RNase-free DNase (Qiagen, Valencia, CA), Collagenase A (Roche Diagnostics, Indianapolis, IN), 6.5mm Transwells with 8.0 μm pore polycarbonate membrane inserts (Corning Life Sciences, Acton, MA), FITC-mouse anti-human CD90-PE (BD Pharmingen, San Diego, CA) CD105 (Serotec, Raleigh, NC), TNF-ά (R&D Systems, Minneapolis, MN), Type I collagen (Cohesion Technologies Inc., Palto Alto, CA and BD Biosciences, Bedford MA), phenol red-free Matrigel (BD Biosciences, Bedford, MA).

Indirect immunofluorescence. Immunofluorescence was performed as described[1]. Briefly, cells plated onto gelatin-coated glass coverslips were fixed with ice-cold methanol, rinsed and incubated with respective primary Abs followed by species-specific FITC- and Texas Red-conjugated secondary Abs, and then analyzed using a fluorescence microscope.

RNA extraction and PCR. Total RNA was isolated from using the RNeasy kit and RNase-free DNase. cDNA was synthesized from RNA with Superscript II RNase H^- RT (Invitrogen , Carlsbad**,** CA). DNAse I digestion of RNA samples (0.5ug) was performed prior to reverse transcription. PCR reactions were run for 25 cycles. Primer sequences are shown in Table 1, On-line Supplemental Data. Primers for human TGFβ-receptors I, II and III were purchased from Superarray Bioscience (Frederick, MD).

Western blotting. Cells were lysed with 4 mol/L urea, 0.5% SDS, 0.5% NP-40, 100 mmol/L Tris, and 5 mmol/L EDTA, pH 7.4, containing 100 μmol/L leupeptin, 10 mmol/L benzamidine, 1 mmol/L PMSF, and 12.5 μg/ml aprotinin. Lysates were subjected to 10% SDS-PAGE (10 μg of protein per lane) and transferred to Immobilon-P membrane. Membranes were incubated with respective primary Abs, goat anti-human CD31, goat anti-human VE-cadherin, mouse anti-human α-SMA, and mouse anti-human calponin diluted in 1x PBS, 5% dry milk, 0.1% Tween-20, and then with secondary Ab (peroxidase-conjugated anti-mouse or anti-goat). Antigen-Ab complexes were visualized using Lumiglo and chemiluminescent sensitive film.

Flow cytometry. HPVECs clones were cultured in EBM-2 with or without the indicated treatment for the specified time periods. At the end of the treatment, cells were trypsinized, washed and incubated either with primary Ab (20 min) followed by secondary Ab (20 min) or PE/ FITC-conjugated Abs on ice for 20 min. Cells were then washed twice and resuspended in 1% paraformaldehyde in PBS. Flow cytometric analyses were performed using a Becton Dickinson FACScan flow cytometer.

Migration Assay. Cellular migration was assayed as described [2]. One hundred μg/mL rat tail collagen type I (0.02N acetic acid) was used to coat 6.5mm Transwells with 8.0 μm pore polycarbonate membrane inserts for 24 hours at 37°C followed by one wash with PBS. Cells, not treated or treated as specified, were trypsinized and seeded in the upper chamber of the transwell plate at a density of 10,000 cells/well (100 μL volume). Six hundred μL of control media (EBM-2/serum- and growth factor-free) or control media with PDGF-BB or bFGF (10 ng/mL) was added to the lower chamber. Cells were then

allowed to migrate for 4 hours at 37°C. The cells in the upper chamber were gently removed using a cotton swab and the lower surface was fixed with ice-cold methanol. Cells were visualized by DAPI fluorescence (Vector Laboratories) and counted using a fluorescent microscope.

Invasion in collagen gels. 3-Dimensional collagen gels were made by mixing 400μL rat tail collagen type I (3mg/mL), 500μL water, 100 μL 10X medium, 50 μL 0.8M $NaHCO_3$ on a vortex mixture and the resulting mixture was immediately poured into 24 well plate. Cells treated with or without $TGF\beta_2$ (2ng/mL) for 10 days were plated on the top of the collagen gel and cultured for an additional 72 hours in EBM-2 medium in the presence or absence of $TGF\beta_2$. Migration of the cells to the bottom of the gel was counted using inverted light microscope.

HL-60 adhesion assay and analysis of expression of cell surface markers. HPVECs and HDMEC plated on 35-mm dishes were assayed at 80% confluency as described [3]. Briefly, cells were treated with or without TNF-α (10 ng/mL) for 5 h, after which the medium was removed, cells were washed once with RPMI and were incubated with 2 x 10^6 HL-60 cells in a volume of 0.6 mL at 4°C on a rocking platform for 45 min. Plates were washed five times, fixed with 2.5% glutaraldehyde in PBS and photographed. To analyze cell surface expression of leukocyte adhesion molecules, cells were removed from the cell culture plate following TNF-α treatment by brief trypsinization and stained with mAbs directed against human E-selectin, human VCAM-1, or human ICAM-1, and then analyzed by flow cytometry as described [3] using a Becton-Dickinson FACScan flow cytometer.

Tube formation in Matrigel. Matrigel (200μL) was added to 48-well plates and allowed to polymerize for 30 minutes at 37°C. Cells treated with or without $TGF\beta_2$ were suspended in EBM-2 medium and added to the Matrigel plates at a concentration of 10,000 cells/ well and incubated at 37°C for 12 hours after which tube formation was monitored.

References

1. Johnson EN, Lee YM, Sander TL, Rabkin E, Schoen FJ, Kaushal S, Bischoff J. NFATc1 mediates vascular endothelial growth factor-induced proliferation of human pulmonary valve endothelial cells. *J Biol Chem*. 2003;278:1686-92.
2. Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. *J Clin Invest*. 2001;107:745-52.
3. Dvorin EL, Jacobson J, Roth SJ, Bischoff J. Human pulmonary valve endothelial cells express functional adhesion molecules for leukocytes. *J Heart Valve Dis*. 2003;12:617-24.

Online Table 1: Primer Sequences used for RT-PCR

CD31	F- TCCCACGCCAAAATGTTAAGTGAG R- GATCAAGAGAGCAATGATCACTCC
VE-Cadherin	F- CCTTGGGTCCTGAAGTGACCT R- CAGGGCCTTCCTTCTGCAA
vWF	F- CACCGTTTGCCCACCCTTCG R- GCCCACTGGGAGCCGACACT
VEGF-R2	F- TCAAAGGAGAAGCAGAGCCATGTG R- GCACTCTTCCTCCAAC TGCCAATA
eNOS	F- AGGACATTTTCGGGCTCACGCTGCGACCCC R- TGG GGTAGGCACTTTAGTAGTTCTCCTACC
CD34	F- GCGCTTTGCTTGCTGAGTTTGC R- GCCTCATTGCCATGTTGAGACAC
RS9	F- GATGAGAAGGACCCACG GCGTCTGTTCG R- GAGACAATCCAGCAGCCCAG GAGGGACA
Slug	F- CGCCTCCA AAAAGCCAAAC R- CGGTAGTCCACACAGTGATG
Snail	F-ACCCCACATCCT TCTCACTG R- GAGGACACAGAACCAGAAAATG)
MMP-1	F- ATTCTACTG ATATCGGGGCTTTGA R- ATGTCCTTGGGGTATCCGTGTAG
MMP-2	F- AATGCCATCCCCGATAACC R- AACTTCACGCTCTTCAGAC

F = forward; R = reverse