Inflammatory Cytokines Promote Mesenchymal Transformation in Embryonic and Adult Valve Endothelial Cells

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Objective—Inflammatory activation of valve endothelium is an early phase of aortic valve disease pathogenesis, but subsequent mechanisms are poorly understood. Adult valve endothelial cells retain the developmental ability to undergo endothelial-to-mesenchymal transformation (EndMT), but a biological role has not been established. Here, we test whether and how inflammatory cytokines (tumor necrosis factor-α and interleukin-6) regulate EndMT in embryonic and adult valve endothelium.

Methods and Results—Using in vitro 3-dimensional collagen gel culture assays with primary cells, we determined that interleukin-6 and tumor necrosis factor-α induce EndMT and cell invasion in dose-dependent manners. Inflammatory-EndMT occurred through an Akt/nuclear factor-κB–dependent pathway in both adult and embryonic stages. In embryonic valves, inflammatory-EndMT required canonical transforming growth factor-β signaling through activin receptor-like kinases 2 and 5 to drive EndMT. In adult valve endothelium, however, inflammatory-induced EndMT still occurred when activin receptor-like kinases 2 and 5 signaling was blocked. Inflammatory receptor gene expression was significantly upregulated in vivo during embryonic valve maturation. Endothelial-derived mesenchymal cells expressing activated nuclear factor-κB were found distal to calcific lesions in diseased human aortic valves.

Conclusion—Inflammatory cytokine–induced EndMT in valve endothelium is present in both embryonic and adult stages, acting through Akt/nuclear factor-κB, but differently using transforming growth factor-β signaling. Molecular signatures of valve EndMT may be important diagnostic and therapeutic targets in early valve disease. (Arterioscler Thromb Vasc Biol. 2013;32:121-130.)

Key Words: calcification ■ invasion ■ smooth muscle actin ■ transforming growth factor-β ■ tumor necrosis factor-α

Degenerative heart valve disease is a significant contributor to cardiovascular morbidity in the United States. Aortic valve dysfunction affects 2.5% of all Americans and 30% of the elderly.1 Heart valve disease directly claims 20 000 lives annually in the United States, and sufferers have twice the risk of heart attacks, strokes, and heart failure.1 In addition, congenital valve abnormalities affect 1% to 2% of live births and lead to early structural deterioration.2 Historically, aortic valve disease (AVD) was thought to be pathogenically similar to vascular atherosclerosis. Both diseases are characterized by loss of endothelial integrity, infiltration of inflammatory cells, accumulation of plasma lipoproteins, release of inflammatory cytokines, mesenchymal proliferation, extracellular matrix remodeling, and the growth of plaque lesions.3 Unlike atherosclerosis, however, AVD leads to large, obstructive lesions containing significant matrix mineralization.4 Randomized clinical trials using lipid-lowering agents to halt AVD severity or progression have been disappointing.5,6 These findings underscore the conclusion that mechanisms of AVD are distinctly different from vascular atherosclerosis. As a consequence, there are currently no established molecular biomarkers specific to AVD progression and no molecular targets for AVD therapy.7

We previously determined in mice that the degree of inflammation in aortic valves directly correlates with the degree of calcification.4 Diseased human valve cusps become progressively thickened, with increases in the presence of macrophages and cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).8-11 Both IL-6 and TNF-α can activate arterial endothelial cells, which is a phenotypic change characterized by upregulation of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), recruitment of leukocytes, and increased monolayer permeability.10,12,13 In atherosclerotic plaques, both IL-6 and TNF-α drive transcription through nuclear translocation.
of nuclear factor-κB (NFκB), but their role in valve endothelium is unknown.14,15

Fetal human aortic valve endothelial cells also express ICAM-1 and VCAM-1, which suggests that endothelial activation also occurs during valve development.16 TNF-α, IL-6, and NFκB expression levels are elevated in the myocardium of children with congenital heart defects affecting valve development.17,18 NFκB inhibition during avian heart development leads to valvuloseptal defects and impaired outlet formation.19

Leptin, a member of the IL-6 superfamily, was shown to induce endothelial-to-mesenchymal transformation (EndMT) in embryonic endocardium, in part through activating transforming growth factor-β (TGF-β).20 Akt has also been shown to drive embryonic and adult EndMT.21,22 Adult ovine aortic valve endothelium has been shown to undergo EndMT in vitro and in vivo.23–25 Collectively, these findings motivate the hypothesis that inflammatory signaling toward EndMT may be a developmentally conserved signaling pathway in valve endothelium, but a biological rationale is unclear.

In this study, we determine that both TNF-α and IL-6 induce EndMT in embryonic and adult valve endothelium via an Akt/NFκB-dependent pathway. In embryonic valve endocardium, NFκB acted upstream of TGF-β to induce EndMT through activin receptor-like kinases 2 and 5 (Alk2/5). We found increasing levels of TNF-α and IL-6 receptor gene expression in embryonic valve primordia during valve morphogenesis. Interestingly, although TGF-β could drive EndMT in adult valve endothelium through Alk2/5, TGFβ was not required for NFκB-induced EndMT. Finally, we identify evidence of EndMT in calcified human aortic valves. Taken together, these findings support a developmentally conserved mechanism of valve remodeling via NFκB-induced EndMT that may be a previously unrecognized molecular signature of early AVD.

Materials and Methods

We used 2 well-characterized valve endothelial cell culture systems to test inflammatory signaling on EndMT in vitro: porcine aortic valve endothelial cells (PAVEC) and embryonic quail endocardial explants (QEE).26–28 Additional details for each source are given in the online-only Data Supplement. PAVEC were isolated through collagenase digestion as previously described,29 with purity confirmed by nondetection of the mesenchymal ACTA2 gene expression (>37 cycles via real-time polymerase chain reaction). PAVEC (passage 5) were cultured as confluent surface monolayers on the surface of type 1 collagen hydrogels (1.5 mg/mL, 50,000 cells/cm²). Pretransformed (HH14 staged) QEE from the atrioventricular and outflow tract valvulogenic regions were cultured as monolayer patches (without myocardium) on 1.5 mg/mL collagen gels, as previously described.29

EndMT was quantified via real-time polymerase chain reaction (RT-PCR), immunohistochemistry, and Western blotting, whereas cell invasion was quantified at 60 μm depth with brightfield microscopy (Figure 1 in the online-only Data Supplement). The protein extraction and Western blot protocols are described in detail in the online-only Data Supplement. NFκB protein nuclear colocalization was quantified for 100 cells for each treatment using Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA). RNA extraction, purification, and RT-PCR were performed according to the manufacturer’s protocols. Primers used for RT-PCR are listed in Table 1 in the online-only Data Supplement. For NFκB transfection, PAVEC were trypsinized and electroporated with plasmid encoding the RelA subunit of NFκB (Addgene plasmid 23255) using Neon transfection system (Invitrogen, Carlsbad, CA) and further cultured in 5% serum, antibiotic-free DMEM for 24 hours.30 The cells were then trypsinized, resuspended in normal media (DMEM with 10% fetal bovine serum and 1% penicillin-streptomycin), and seeded onto gels. Invasion assay was performed as described above. Human diseased aortic valves were obtained from adults undergoing planned, nonelective valve replacement surgery by Dr Sanjay Samy at Robert Packer Hospital in Sayre, PA. All procedures were approved by Institutional Review Boards at Cornell and Robert Packer Hospital. Pediatric valves were obtained from Dr Jonathan Chen, Cornell-Weill Medical School and NY Presbyterian Hospital. Results are expressed as mean±SEM, n≥2 independent cultures per treatment condition, with 5 to 6 explants pooled for each QEE sample. Data were analyzed with GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA). Treatment effects were compared using ANOVA with Tukey post hoc paired tests, and data were transformed when necessary to obtain equal sample variances. Differences between means were considered significant at P≤0.05. Expanded methods are provided in the online-only Data Supplement.

Results

TNF-α and IL-6 Induce EndMT in Adult Valve Endothelial Cells

We first exposed 3-dimensional (3D), cultured, adult PAVEC monolayers to doses of TNF-α or IL-6 and quantified changes in EndMT-related gene expression, protein expression, and collagen matrix invasion. Both IL-6 and TNF-α in 3D culture induced loss of endothelial cell–cell contacts, including platelet endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial-cadherin (VE-cadherin), acquisition of mesenchymal α-smooth muscle actin (α-SMA), and invasion into collagen matrix in a dose-dependent manner (Figure 1A, 1D, 1E, and 1G; Figures I and II in the online-only Data Supplement). Genetic overexpression of NFκB alone was sufficient to cause 3D matrix invasion in PAVEC, supporting an NFκB-dependent EndMT mechanism (Figure 1A). Pro-EndMT–related gene expression (ACTA2, Snail, TGFβ1) was significantly upregulated in response to NFκB overexpression (238±20.6, 15.0±1.1, and 4.2±0.5 fold, respectively) and TNF-α (14.3±4.8, 3.8±0.7, and 3.8±0.6 fold, respectively; P<0.05), but not IL-6 (Figure 1B). EndMT-related protein expression (α-SMA, Snail increase, and VE-cadherin decrease) was shown in PAVEC, but not porcine aortic endothelial cells (PAEC), in response to TNF-α (Figure 1G). TGFβ, however, induced EndMT protein expression in both PAVEC and PAEC (Figure 1G). Nuclear translocation of NFκB was significantly upregulated in response to either IL-6 or TNF-α (1.7±0.1 and 2.0±0.1 fold increase, respectively; P<0.05; Figure 1C and 1F; Figure III in the online-only Data Supplement). To control for cell number, we quantified PAVEC proliferation (via BrdU incorporation) and apoptosis (via TUNEL) in response to doses of TNF-α. PAVEC proliferation increased with TNF-α dose (up to 2.7±0.1 at 100 ng/mL; P<0.05; Figure ID in the online-only Data Supplement), whereas apoptosis remained unchanged (Supplemental Figure IE). Collectively, these results suggest that IL-6 and TNF-α induce EndMT and matrix invasion in adult PAVEC in vitro, at least in part through NFκB, with TNF-α being a more potent EndMT inducer.

EndMT Is Present in Calcified Human Aortic Valves

In explanted human aortic valves with calcific lesions (Figure 1G–1I), we identified a population of subendothelial cells coexpressing α-SMA and PECAM-1, but not
Figure 1. Exposure to inflammatory cytokines induces mesenchymal transformation in adult endothelial cells. A, Porcine aortic valve endothelial cell (PAVEC) mesenchymal transformation and invasion of the collagen matrix after 48-hour exposure to 100 ng/mL interleukin-6 (IL-6), 100 ng/mL tumor necrosis factor-α (TNF-α), or genetic overexpression of nuclear factor-κB (NFκB). B, PAVEC endothelial-to-mesenchymal transformation (EndMT)-related gene expression after 48-hour exposure to 100 ng/mL IL-6 or TNF-α, or genetic overexpression of NFκB. C, PAVEC NFκB nuclear localization quantification. D, Confocal images of control, +100 ng/mL IL-6, and +100 ng/mL TNF-α PAVEC at a 48-hour time point stained for CD31 (red), α-smooth muscle actin (α-SMA; green), and DNA (blue). E, Confocal images of control, +100 ng/mL IL-6, and +100 ng/mL TNF-α PAVEC at a 48-hour time point stained for vascular endothelial-cadherin (VE-cadherin) (red), α-SMA (green), and DNA (blue). F, Confocal images of control, +100 ng/mL IL-6, and +100 ng/mL TNF-α PAVEC at a 48-hour time point stained for NFκB (green) and DNA (blue). G, Western blots for PAVEC and porcine aortic valve endothelial cells exposed to 100 ng/mL TNF-α or transforming growth factor-β1 (TGF-β1) for 48 hours. H, Diseased human aortic valve endothelial cells coexpressing α-SMA and CD31 (on the fibrosa or outflow side and invaded, calcified nodule shown in inset). I, Expanded frame of H. J, Nuclear translocation of NFκB in diseased human valve endothelial cells. Error bars show ±SEM, n ≥ 3 culture wells. Bars that do not share any letters are significantly different according to a 1-way ANOVA with Tukey posttest (P≤0.05). For EndMT gene expression (B), differences that are significantly different than the control according to an unpaired Student t test are indicated with an *. Differences were considered significant at P≤0.05. Scale bars, 25 μm (D and E) or 10 μm (F). PECAM indicates platelet endothelial cell adhesion molecule; PAEC, porcine aortic endothelial cells; VE-cadherin, vascular endothelial-cadherin; TGF-β1, transforming growth factor-β1.
CD45 (Figure IV in the online-only Data Supplement). This suggests the cells were EndMT-derived, but not from an immune origin.\textsuperscript{31} The number of invaded fibroblastic endothelial cells was 11.13±1.88 cells/×40 field (scanning confocal microscopy, n=3 calcified adult valves). Endothelial cells located near the calcified nodule and on the fibrosa side showed decreased VE-cadherin expression (Figure IVD in the online-only Data Supplement). EndMT and potential EndMT-derived cells were only found in the fibrosa layer, distal to sites of calcified lesions. Furthermore, we found that many of these transformed and invaded cells also coexpressed nuclear NFκB (Figure 1H). Healthy pediatric human valves did not coexpress α-SMA and VE-cadherin or nuclear NFκB (Figures V and VI in the online-only Data Supplement). These findings support that NFκB-mediated EndMT occurs in human calcific AVG but is likely not involved in the mineralization process.

**TNFα and IL-6 Induce EndMT in Embryonic Valve Endocardium**

Similarly, we quantified the EndMT response characteristics of 3D-cultured HH14–pretransformed QEE monolayers from outflow tract or atrioventricular regions to doses of TNF-α and IL-6. As in adult valve endothelium, both IL-6 and TNF-α induced loss of QH1, acquisition of α-SMA, and collagen matrix invasion (Figure 2A and 2D). Embryonic endocardial EndMT-related genes (ACTA2, Slug, and TGFβ3) were significantly upregulated in valvulogenic endocardium by both IL-6 and TNF-α (Figure 2B). PECAM1 gene expression was significantly increased in response to inflammatory cytokine (Figure 2A). PECAM1 has been shown to be an early marker of cardiovascular development, and the increase in PECAM1 in culture is likely related to embryonic endocardial differentiation.\textsuperscript{32} Downstream NFκB protein nuclear translocation also occurred in response to either IL-6 or TNF-α (Figure 2C and 2E; Figure VII in the online-only Data Supplement). There was no difference in explant area (a control for cell number) with IL-6 or TNF-α stimulation compared with control (Figure VIIIC and VIIID in the online-only Data Supplement). Collectively, these results suggest that both TNF-α and IL-6 induce EndMT in embryonic valve endocardial cells in vitro with similar potency. We further quantified the expression of inflammatory signaling–related genes in the atrioventricular and outflow tract valve, forming regions of embryonic hearts across the period of valve morphogenesis (day 2 to day 10). We determined that ICAM-1, NFκB-1, IL-6RA (coding for...
IL-6Rα), and TNFRSF1A (coding for TNFR1) gene expression were significantly upregulated in both valve regions, as EndMT and valve remodeling progressed (Figure IX in the online-only Data Supplement). These results suggest that inflammatory signaling is active in valves during morphogenesis in vivo and directly causes EndMT in valve endocardium in vitro.

**Inflammatory-EndMT Acts Through an Akt/NFκB Pathway Present in Both Adult and Embryonic Valve Endothelium**

We next sought to identify the signaling pathway by which inflammatory cytokines induced EndMT through NFκB. Previous studies have shown that TNF-α activates NFκB-mediated transcription through Akt or mitogen-activated protein kinase kinase 1 (MEK1).33–37 IL-6 activates signal transducer and activator of transcription 3 (STAT3) most potently, but there is also evidence of IL-6–induced NFκB activation through phosphoinositide 3-kinase (PI3K) and Akt.38–41 Using our 3D culture system, molecular inhibition of either Akt (via 5 μM Akt inhibitor XI) or MEK1 (via 25 μM PD98059) significantly reduced TNF-α–induced NFκB expression and nuclear translocation in PAVEC, but only Akt inhibition resulted in complete knockdown (Figure 3C and 3D). Akt inhibition also completely blocked IL-6–induced NFκB gene expression and protein nuclear translocation, whereas STAT3 inhibition (via 5 μM PpYLKTk STAT3 inhibitor) had no effect (Figure XC and XD in the online-only Data Supplement). Inhibition of Akt completely blocked TNF-α–induced inflammatory receptor activation (VCAM-1, ICAM-1, PAVEC), and subsequent matrix invasion by PAVEC (Figure 3A–3C). MEK1 blockade also inhibited TNF-α–induced inflammatory receptor activation, EndMT gene expression, and subsequent invasion, but to a lesser degree than Akt inhibition. In a similar fashion, Akt inhibition completely blocked IL-6–induced upregulation of inflammatory receptors, EndMT gene expression, and subsequent matrix invasion (Figure XA–XC in the online-only Data Supplement). STAT3 inhibition, however, had no effect on any of these responses from IL-6.

Identical experiments were conducted on avian pre-EndMT valvulogenic endocardial monolayers. As in adult valve endothelium, molecular inhibition of Akt or MEK1 both blocked TNF-α–induced NFκB gene expression and nuclear translocation. Akt inhibition completely blocked NFκB, whereas MEK1 inhibition only partially blocked NFκB (Figure 4C and 4D). IL-6–induced NFκB gene expression and protein nuclear translocation were also blocked with Akt inhibition but not STAT3 inhibition (Figure XIC and XID in the online-only Data Supplement). Akt or MEK1 inhibition also blocked inflammatory receptor (VCAM-1, ICAM-1) upregulation, EndMT-related gene expression (ACTA2, Slug, TGFβ3), and collagen invasion by QEE cells (Figure XIA–XIC in the online-only Data Supplement). As a positive control, AKT1 gene expression was blocked by Akt inhibition in both PAVEC and QEE subjected to TNF-α (Figures XII and XIII in the online-only Data Supplement). Collectively, these results suggest that inflammatory cytokines initiate EndMT at the gene, protein, and function levels through an AKT/NFκB pathway that is conserved in both adult valve endothelial and embryonic valvulogenic endocardium. TNF-α was a more potent inducer of EndMT and resulted in more consistent pathway responses in both cell types compared with IL-6. IL-6 may also induce EndMT genes more strongly at an earlier or later time point.

**TGF-β Signaling Through Alk2/5 Is Required for Inflammation-Induced Embryonic EndMT, But Not for Adult EndMT**

Previous studies have identified a canonical TGF-β signaling pathway that induces EndMT and is isoform-specific: the
TGF-β1 isomorf in adult valve endothelial cells, whereas EndMT invasion is mediated by TGF-β3 in avian embryonic valve endocardium.23,42,43 We therefore tested whether TGF-β signaling was involved in inflammatory-induced EndMT. As a positive control, exogenous TGF-β3 (100 ng/mL) induced EndMT matrix invasion in embryonic valve endocardium (Figure 5A), whereas exogenous TGFβ1 induced EndMT in PAVEC (Figure 6A; Figure XIV in the online-only Data Supplement). TNF-α induced TGF-β1 gene expression in PAVEC and TGFβ3 in QEE (3.79±0.57 and 12.03±1.64; Figures 1B and 2B). IL-6 induced TGFβ3 only in QEE (7.61±0.53; Figure 2B). Previous studies demonstrate that TGF-β type I Alk2/5 mediate TGF-β–induced EndMT.44,45 Molecular inhibition of Alk2/5 signaling (via 10 μM SB 431542) in QEE reduced EndMT-related gene expression (Figure 5B; Figure XV in the online-only Data Supplement), cell invasion induced by either TNF-α or IL-6 (Figure 5A; Figure XVA in the online-only Data Supplement), and Smad2/3 phosphorylation and nuclear localization (Figure 5E), but did not affect inflammatory activation–related gene expression (Figure 5C; Figure XVC in the online-only Data Supplement) or NFκB protein nuclear localization (Figure 5D; Figure XD in the online-only Data Supplement). Together, these results support that inflammatory cytokine–induced EndMT in embryonic valves acts at least in part by canonical TGF-β–Alk–Smad signaling that is downstream of NFκB.

In contrast to embryonic valve endocardium, Alk2/5 inhibition in adult valve endothelial cells (PAVEC) treated with TNF-α significantly decreased Smad2/3 phosphorylation and nuclear translocation (Figure 6E), but did not result in decreased cell invasion (Figure 6A), did not effect EndMT-related or inflammatory activation–related gene expression (Figure 6B and 6C), and did not inhibit NFκB nuclear localization (Figure 6D). Interestingly, endothelial cells from the porcine aorta show increased pSmad2 in response to TGF-β1, but not to TNF-α treatment, suggesting a unique signaling behavior of PAVEC in response to TNF-α (Figure 6F). These results suggest that Alk2/5-dependent TGF-β signaling is not downstream of TNF-α–induced EndMT in adult valve endothelium. Therefore, although both embryonic and adult valve endothelium activate an EndMT program in response to TNF-α through Akt/NFκB, TGF-β signaling is required downstream in embryonic but not in adult valve endothelium. Adult cells may bypass TGF-β signaling because activated NFκB has been shown to stabilize Snail, an EndMT-regulating transcription factor, in adult cells.46 In mouse cardiac endothelial cells, treatment with SB431542, but not PD98059, completely blocked TGF-β2–induced EndMT.47

Discussion

The molecular and cellular events that initiate and propagate AVD are poorly understood and are understudied, particularly with respect to endothelial dysfunction. Although the occurrence of EndMT has been previously noted in adult valve endothelium, a biological role remains unclear. EndMT studies in embryonic valves have identified >100 different regulatory genes, including members of the TGF-β, bone morphogenetic protein, and vascular endothelial growth factor signaling pathways and matrix proteins, such as periostin and versican.48-51 Misexpression and mutation of many of these genes have been identified in diseased aortic valves.1 Our results identify a novel, inflammatory cytokine–induced EndMT pathway in aortic valve endothelium through Akt/NFκB and further support that this pathway is conserved during embryonic valve development. These findings collectively suggest that an inflammatory reactivation of embryonic-like EndMT may be a mechanism of early aortic valve dysfunction.

Identifying mechanisms conserved between embryonic and adult valve endothelium remains challenging because there is currently no species for which both embryonic and adult valve endothelial cell cultures have been obtained. Porcine aortic valve endothelial cells are the well-characterized and

Figure 4. Inflammatory cytokines induce endotheelial-to-mesenchymal transformation (EndMT) through Akt/mitogen-activated protein kinase/nuclear factor-κB (NFκB) in quail endocardial explants (QEE). A, Cell invasion after a 48-hour exposure to 100 ng/mL tumor necrosis factor-α (TNF-α) or 100 ng/mL TNF-α with 5 μM Akt inhibitor XI or 25 μM PD98059 MEK1 inhibitor. B, EndMT-related gene expression after a 48-hour exposure to 100 ng/mL TNF-α with inhibitors. C, Inflammatory activation–related genes after a 48-hour exposure to 100 ng/mL TNF-α with inhibitors. D, QEE NFκB nuclear localization quantification after a 48-hour exposure to 100 ng/mL TNF-α with inhibitors. Error bars show ±SEM, n≥3 culture wells with pooled explants. Bars that do not share any letters are significantly different according to a 1-way ANOVA with Tukey posttest (P≤0.05). TGF-β1 indicates transforming growth factor-β1; VCAM-1, vascular cell adhesion protein 1; and ICAM-1, intercellular adhesion molecule 1.
well-studied adult valve endothelial population and can be obtained from animals without disease. Human aortic valve endothelial cells have been obtained from recipient hearts from cardiac transplantation surgeries or rejected valve donors. Although no gross calcification may be present, the valve endothelium exhibits non-negligible inflammatory activation. Paruchuri et al. identified clonally isolated subpopulations of human pulmonary valve endothelial cells that were capable of EndMT in response to TGF-β, which supports our findings in pure, whole valve endothelial cells populations. Holliday et al. recently showed that in vitro cultured human aortic valve endothelial cells isolated from heart transplant surgeries express endothelial phenotype markers and elevated α-SMA, which is in contrast to in vivo expression patterns in nondiseased human and porcine aortic valves. These coexpression findings suggest that human aortic valve endothelium from these patients may have an increased propensity or capacity for EndMT. Furthermore, several studies demonstrate that inflammatory endothelial activation and calcific degeneration occur preferentially on the fibrosa side of the valve. Our analysis of calcified human aortic valves found invaded cells coexpressing α-SMA and CD31 on the fibrosa surface, suggesting that EndMT correlates with side-specific propagation of AVD. In situ mRNA profiling of valve endothelial cells determined hundreds of genes that are differentially expressed between sides, with many further modified in experimental hypercholesteremia. The inflow or ventricular surface is exposed to pulsatile unidirectional shear stress, whereas the outflow or fibrosa surface experiences oscillatory shear stress. When either endothelial surface is exposed to opposite-sided flow profiles, they similarly upregulate inflammatory receptors. Recently, Wu et al. demonstrated that both sides of valve endothelium have the same genetic origin. Our results support and extend these findings to suggest that fibrosa-sided environmental conditions are more conducive to inflammatory activation and EndMT, but additional studies on side-specific endothelial populations are warranted.

Cellular and molecular analysis of embryonic valve formation is almost exclusively conducted with avian and mouse models. Although there are some differences in cardiac anatomy between avians and mammals, the morphogenesis, remodeling, and final anatomy of the clinically important left side of the heart are more similar between chicks and humans than in mice. Biological assays with avian and mouse valve progenitors demonstrate identical molecular mechanisms and participants, although the isoforms used may differ. Importantly, chick embryos are inexpensive, can be obtained without additional animal euthanization, and are very tolerant of experimentation. Our findings of progressive upregulation of inflammatory receptors in valve formation in embryonic chicks are supported by observations in fetal human aortic valves. Our result that EndMT and matrix invasion occurs...
at least through TGF-β1 in adult porcine cells and TGF-β3 in embryonic quail is supported by several studies. Therefore, we have confidence that the general molecular regulation we identify is likely conserved across age, but we cannot definitively rule out species differences in the isoforms used.

The downstream fate of EndMT-derived mesenchyme in adult valves is of great interest, but was not a focus of this study. Bischoff and Aikawa recently proposed that EndMT creates a type of progenitor-like cell that renews the valve interstitial cell population and maintains tissue homeostasis. Both embryonic and adult valvular mesenchymal cells have the potential for osteogenic differentiation, but whether these cells are directly EndMT-derived or from subsequent differentiation processes is not yet known. TNF-α and NFκB have been linked to the mesenchymal cell molecular and cellular mechanisms that mediate valvar calcification. TNF-α expression and nuclear localization of NFκB are present in stenotic human aortic valves, and TNF-α treatment of valve interstitial cells in 2-dimensional culture induces calcium nodule formation and expression of runx2, independent of osteogenic media. We found EndMT-derived subendothelial cells in calcified human aortic valves but were always located distal to calcific lesions.

This was not unexpected, because valve calcifications are associated with endothelial loss. Taken together, these results support that inflammatory EndMT is a component of early stage AVD, but how these progeny contribute to and regulate downstream calcific progression is still unclear.

In conclusion, our results establish an inflammatory mechanism of early aortic valve pathogenesis that is, in part, developmentally conserved. Age-dependent use of canonical TGF-β signaling may be an important mode of inflammatory disease pathogenesis distinct from potentially healthy tissue formation and remodeling. Selective molecular inhibition of the AKT/NFκB pathway in valve endothelium may therefore be a potential strategy for impacting early stage AVD.

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Supplemental Material

Supplemental Methods

Porcine aortic valve endothelial cell justification and culture

Porcine aortic valve endothelial cells (VEC) are by far the best-characterized and most studied valve endothelial population\textsuperscript{1-4}. Non-calcified adult endothelial cells can be obtained from acute traumatic non-cardiac injuries, but are more often acquired from cardiac transplantation surgeries or rejected donor valves. VEC from these sources have a sub-clinical, but likely non-negligible, amount of inflammation and pathology\textsuperscript{5-7}. Holliday et al. have shown that human VEC isolated from heart transplant surgeries have elevated $\alpha$-SMA expression, suggesting some pathobiology may be present, including potentially EndMT\textsuperscript{8}. Healthy human VEC have no $\alpha$--SMA expression \textit{in situ}. Like humans, pigs develop atherosclerotic vascular and valvular lesions without intervention\textsuperscript{9,10}. In addition, pigs fed a high fat and cholesterol diet for up to 6 months develop LDL lipid profiles similar to those in humans and form arthelerosclerotic lesions, preferentially on the valve fibrosa, with lipid disposition, calcification, cholesterol clefts, signs of continuous inflammation, and prothrombotic tendency\textsuperscript{11-15}. No study to date has demonstrated a pure population of cultured valve endothelial cells from small animals such as mice. Collectively, these findings support the use of porcine valve endothelial cells for the adult valve studies.

Porcine aortic valve endothelial cells were isolated using the method shown by Butcher et al. and Gould and Butcher\textsuperscript{1,16}. Porcine heart valves were kindly donated by Shirk Meats of Dundee, NY. Porcine aortic valve endothelial cells (PAVEC) were grown in flasks coated with 50 µg/mL rat tail collagen I (BD Biosciences, San Jose, CA). Cells were cultured at 37°C and 5% CO$_2$ in DMEM supplemented with 10% FBS (Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen), and 50 U/mL heparin (Sigma-Aldrich, St. Louis, MO). Culture medium was changed every 48 hours and cells were passaged with 0.05% Trypsin-EDTA (Invitrogen) 1:3 at confluence. Endothelial culture purity was confirmed at the most conservative level via real-time PCR for alpha-smooth muscle actin (not expressed in VEC but expressed in VIC and in cells undergoing EndMT). Cultures with undetectable expression (cycle threshold > 37 cycles) were used in subsequent experiments.

For experiments, 95,000 cells were seeded onto collagen gels at passage 5. After 2 hours 1, 10, or 100 ng/mL of human IL-6 (Sigma-Aldrich) or human TNF-$\alpha$ (Sigma-Aldrich) was added to the culture medium for treated cells. For IL-6 inhibitor experiments seeded cells were allowed to attach for 2 hours before the addition of 100 ng/mL IL-6 and 5 µm Akt Inhibitor XI (EMD Chemicals, Gibbstown, New Jersey) or 25 µm MEK1 inhibitor PD 98059 (EMD Chemicals) , or 10 µm SB 431542 ALK5 inhibitor (Sigma-Aldrich) was added to cultures. After 48 hours cell invasion was quantified manually at 60 µm depth into the gel (see Supplementary Figure I). Immediately after quantifying cell invasion cells were fixed for immunofluorescence in 4% paraformaldehyde or processed for RNA isolation.

Avian endocardial explant culture

The embryonic avian (chick and quail) is a standard animal model for understanding heart and valve development and is ideal for these studies. The avian (quail, chick) embryo does not require a placental circulation and can be cultured outside of the egg for almost all of its gestation\textsuperscript{17,18} therefore not requiring death of the mother. Though there are some differences in cardiac anatomy between avians and mammals, the morphogenesis, remodeling, and final anatomy of the clinically important left side of the heart is remarkably similar between chicks and humans\textsuperscript{19-22}. Late fetal and postnatal avian aortic and mitral valves exhibit well developed
trilaminar matrix striation and fibroblastic cell phenotype similar to humans, whereas matrix stratification is barely detectable in mice\textsuperscript{19}. Biological assays with avian and mouse valve progenitors demonstrate identical molecular mechanisms and participants (though specific isoforms may differ)\textsuperscript{23,24}. As each assay we perform pools multiple explants per sample, conducting similar experiments with mice would require an impractical number of identically timed pregnant females. The avian is the only embryo model that enables isolation of sufficient numbers of valve explants for 3D \textit{in vitro} experimentation, and therefore suitable for the embryonic experiments. We utilize the chick for its sequenced genome (enabling PCR design), and quail for its QH1 antigen identifying endocardial cells.

Fertile quail eggs were acquired from Lake Cumberland Game Bird Farm (Monticello, KY). Eggs were incubated at 37°C and 60% humidity to stage HH14. The embryos were placed into sterile Earl’s Basic Salt Solution (EBSS, Invitrogen) and staged according to the criteria of Hamburger and Hamilton\textsuperscript{25}. Quail endocardial explant isolation has been previously described\textsuperscript{26}. Following endocardial cell isolation, M199 was added to the wells containing 1, 10, or 100 ng/mL of human IL-6 or human TNF-\(\alpha\). For inhibitor experiments 100 ng/mL IL-6 was added with 5 \(\mu\)m Akt Inhibitor XI, 25 \(\mu\)m STAT3 Inhibitor Peptide, or 10 \(\mu\)m SB 431542 ALK5 inhibitor or 100 ng/mL TNF-\(\alpha\) was added with 5 \(\mu\)m Akt Inhibitor XI, 25 \(\mu\)m MEKK1 inhibitor PD 98059, or 10 \(\mu\)m SB 431542 ALK5 inhibitor. Cell invasion was quantified manually at a 60 \(\mu\)m depth into the gel after 48 hours (see Supplementary Figure I). The cultures were then fixed in 4% paraformaldehyde for immunofluorescence or processed for RNA isolation.

### Three dimensional collagen constructs

Three dimensional collagen gels at a concentration of 1.5 mg/mL collagen were made by combining ice-cold 3X Dulbecco’s Modified Eagle’s Medium (DMEM, PAVEC; Invitrogen, Carlsbad, CA) or 3X M199 (QEE, Invitrogen), 10% Fetal Bovine Serum (FBS, PAVEC; Invitrogen) or 1% Chick Serum (CS, QEE; Invitrogen), sterile 18 M\(\Omega\) water, 0.1 M NaOH, and rat tail collagen I (BD Biosciences). A 0.3 mL aliquot of the collagen solution was pipetted into 4 well tissue culture plates (1.9 cm\(^2\) growth area; Nunc, Rochester, NY) and allowed to gel for at least 1 hour at 37\(^{\circ}\)C and 5% CO\(_2\).

### Collagen gel immunofluorescence

Fixed samples on collagen gels were washed for 15 minutes on a rocker 3 times with PBS, permeabilized with 0.2% Triton-X 100 (VWR International, West Chester, PA) for 10 minutes, and washed another 3 times with PBS. Samples were incubated overnight at 4\(^{\circ}\)C in a 1% BSA (Rockland Immunochemicals, Inc., Gilbertsville, PA) blocking solution followed by another 4\(^{\circ}\)C overnight incubation with mouse anti-porcine PECAM 1:100 (AbD Serotech, Raleigh, NC), rat anti-porcine VE-cadherin 1:100 (AbD Serotech), or mouse anti-quail QH1 (DSHB, Iowa City, Iowa) and rabbit anti-human \(\alpha\)-SMA 1:100 (Spring Bioscience, Pleasanton, CA). For NF\(\kappa\)B staining, samples were incubated with rabbit anti-human NF\(\kappa\)B p105 / p50 1:100 (Abcam, Cambridge, MA). For pSMAD staining, samples were incubated with pSMAD2 rabbit anti-human 1:50 (Cell Signaling Technology, Beverly, MA). After 3 washes for 15 minutes with PBS, samples were exposed to Alexa Fluor\textsuperscript{880} 488 or 568 conjugated (Invitrogen), species specific secondary antibodies at 1:100 in 1% BSA for 2 hours at room temperature. Three more washes with PBS for 15 minutes were followed by incubation with DRAQ\textsuperscript{5} far red nuclear stain (Enzo Life Sciences AG, Lausen, Switzerland) at 1:1000. Samples were washed once more with 18 M\(\Omega\) water and stored in 18 M\(\Omega\) water at 4\(^{\circ}\)C. Images were taken with a Leica TCS SP2 (Exton, PA) or Zeiss 710 (Thornwood, NY) laser scanning confocal microscope. NF\(\kappa\)B and pSMAD2/3 nuclear co-localization was measured with Metamorph 7.1 software (Molecular Devices). Green (NF\(\kappa\)B) and far red (nuclei) channels were each thresholded. Regions were
created around each nuclei. The regions were transferred onto the green channel and the thresholded stained area was quantified within the regions.

**Human valve section immunofluorescence**

Diseased human aortic valves were obtained from adults undergoing planned, non-elective valve replacement surgery by Dr. Sanjay Samy at Robert Packer Hospital in Sayre, PA. Pediatric valves were obtained from Dr. Jonathan Chen, Cornell-Weill Medical School and NY Presbyterian Hospital. Fixed human valves were paraffin embedded and sectioned at 6 µm. Sections were placed on slides, de-waxed, hydrated, and antigens were retrieved by placing the slides in 10 mM TRIS base buffer at pH 10.0 at 90°C for 40 minutes. Following antigen retrieval sections were washed with PBS, permeabilized with 0.1% Tween-20, washed again with PBS, and blocked for 1 hour with 5% BSA in PBS. Primary antibodies were added at a concentration of 1:100 and incubated overnight at 4°C. The following primary antibodies were used: human anti-mouse P2B1 (DSHB), rat anti-porcine VE-cadherin (AbD Serotech), rabbit anti-human α-SMA (Spring Bioscience), rabbit anti-human NFκB p105 / p50 (Abcam), and anti-human Cy5.5-labelled CD45 (generously provided by Dr. Michael King, Department of Biomedical Engineering, Cornell University, Ithaca, NY). Sections were incubated with species-specific secondary antibodies at 1:100 for 1 hour, washed with PBS, incubated with 1:1000 DRAQ5 (Enzo Life Sciences AG) or 2:10,000 Hoechst 33342 (Invitrogen) for 30 minutes, washed again with PBS, mounted with Prolong Gold antifade reagent (Invitrogen), and imaged with a confocal microscope.

**Western blots**

P4 PAVEC and PAEC were grown to 70% confluency in a 6-well plate and treated with culture medium alone, 100 ng/mL TNF-α, or 100 ng/mL TGFβ-1 for 48 hours at 37°C. Cells were lysed directly on the plate using RIPA buffer supplemented with 25mM NaF, 1mM NaVO4, and 0.5% protease inhibitor cocktail (Sigma-Aldrich), incubated for 15 min at RT, scraped and homogenized by pipetting up and down, and centrifuged at 15,000 rpm for 10 min. 10ug of protein was loaded into a 10% gel with Laemmli buffer at 1:1 ratio and run for 1 hour at 120V in 25mM Tris, 0.2M Glycine, 1% SDS running buffer. Western blot transfer to nitrocellulose membrane (Thermo Scientific, Rockford, IL) was performed at 400mA for 1 hour in 25mM Tris, 20% methanol transfer buffer. After rinsing in PBS with 0.1% Tween-20, the membrane was blocked for 1 hour in Odyssey Blocking Buffer at RT. Mouse anti-pig CD31 (Springer Bioscience, 1:1000), mouse anti-human VE-cadherin (Abcam, 1:500), rabbit anti-human alpha-smooth muscle actin (Abcam, 1:1000), rabbit anti-human Smad2 and pSmad2 (Cell Signaling, 1:2000), rabbit anti-Sna1 (SCBT, 1:200), and mouse anti-human GAPDH (Ambion, 1:2000) were used in Odyssey blocking buffer+0.1% Tween-20 to detect protein expression. The membrane was washed 4x in PBS-tween, 1X in PBS, and incubated overnight at 4°C with gentle agitation. The same washes were then performed and the membrane was incubated in Odyssey blocking buffer +0.1% Tween-20, +0.2% SDS with 1:20,000 anti-mouse and anti-goat secondary antibodies (Li-Cor IRDye). Blots were imaged using the Odyssey Infrared system (Li-Cor).

**RT-PCR**

Total RNA was extracted using a Norgen total RNA purification kit (Norgen Biotek Corp., Thorold, ON) and RNA was reverse transcribed to cDNA using the SuperScript III RT-PCR kit with oligo(dT) primer (Invitrogen). RT-PCR was performed on all samples using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and a MiniOpticon Real-Time PCR Detection System (Biorad, Hercules, CA). Primer sequences are listed in Supplementary Table S1.
Apoptosis Assay

Fixed samples on collagen gels were examined for apoptosis using the APO-BrdU TUNEL assay (Invitrogen) according to the instructions provided by the manufacturer. Briefly, the samples were washed 3 times in the wash buffer and then incubated in DNA labeling solution for 2 hours at 37°C. Samples were washed for 5 minutes on a rocker with rinse buffer, then incubated in Anti-BrdU mouse monoclonal antibody PRB-1, Alexa Fluor 488 conjugate 1:50 (Invitrogen) and Draq5 1:1000 (Enzo Life Sciences AG) for 1 hour at room temperature. 200uL of propidium iodide stain was added to each sample and then incubated for 30 minutes at room temperature. Samples were stored in PBS at 4°C and imaged less than 24 hours later. Images were taken with a Zeiss 710 laser scanning spectral confocal microscope. Custom Matlab code was used to threshold the red (propidium iodide), green (BrdU labeling) and far red (nuclei) channels according to the automated global thresholding level given in analysis of the far red channel. Cell boundaries, nuclei, and BrdU labeling were confirmed visually for each sample. Numbers of apoptotic cells per sample were counted via Matlab using BrdU labeling within a cell boundary as the criteria for identifying an apoptotic cell.

Proliferation Assay

BrdU labeling reagent (Invitrogen) was added to PAVEC culture medium 6 hours prior to experiment completion. At 48 hours cells were fixed as described above and processed in the same manner as the apoptosis assay. BrdU labeling within a cell nucleus was the criteria for a proliferating cell.

Statistics

Results are expressed as mean ± standard error, n ≥ 3. Data was analyzed with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). An unpaired student's t-test or a one-way ANOVA with Tukey's post test was used to compare differences between means and data was transformed when necessary to obtain equal sample variances. Differences between means were considered significant at p < 0.05.
**Supplemental Table**

**Table I. Primer sequences**

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Supplemental Figures and Figure Legends

Figure I. (A) Cell invasion assay. Embryonic valve endocardial or adult valve endothelial cells are plated onto a 3D collagen gel. After 48 hours, invaded, mesenchymally transformed cells are quantified using a 10X objective and an inverted microscope. First the microscope is focused on the endocardial or endothelial layer on the surface of the gel. Next the microscope is focused 60 μm down into the gel, and the number of in-focus invaded cells is quantified. This process is repeated for all endocardial explants present on the gel, or for four fields in the center of the gel for endothelial monolayers. (B-E) Inflammatory cytokine dose response, proliferation and apoptosis in porcine aortic valve endothelial cells (PAVEC). (B) 100 ng/mL IL-6 treatment for 48 hours significantly increases cell invasion when compared with controls. (C) A 48 hour 100 ng/mL TNF-α treatment significantly increases cell invasion when compared with control, +1 ng/mL, and +10 ng/mL treatments. PAVEC showed a significant increase in proliferation (D), but not apoptosis (E), in response to increasing doses of TNF-α for 48 hours. Error bars show ±SEM, n ≥ 3 independent monolayers. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
**Figure II.** Exposure to inflammatory cytokines induces mesenchymal transformation and invasion in adult endothelial cells. Confocal images of control porcine aortic valve endothelial cells (PAVEC) 48 hours after plating at the surface of the 3D collagen gel (A, B). PAVEC 48 hours following exposure to 100 ng/mL TNF-α (D, E). PAVEC 48 hours following exposure to 100 ng/mL TNF-α at the surface of the gel (C) and -60 µm into the gel (F), cells co-express CD31 and α-SMA. Arrows show collagen fiber arrangement, invaded cells exposed to TNF-α remodel collagen fibers.
Figure III. Separate green and blue channels for confocal images of control (A, B), +100 ng/mL IL-6 (C, D), and +100 ng/mL TNF-α (E, F) PAVEC at a 48 hour time point stained for NFκB (green) and DNA (blue).
Figure IV. Diseased human aortic valve immunohistochemistry. A, B) Human aortic valve endothelial cells co-expressing CD31 and α-SMA do not also express CD45, an immune cell marker. Arrows show the same cell in consecutive sections co-expressing CD31 and α-SMA, but not expressing CD45. C) CD45-positive immune cells are present in the diseased human valve. D) The endothelium near the nodule (*) is degraded, and VE-cadherin expression is decreased. Scale bar = 10 μm.
Figure V. Healthy human pediatric valve immunohistochemistry. Healthy human aortic valve endothelial cells do not co-express VE-cadherin and α-SMA and do not express nuclear NFκB. A-C) fibrosa, D-F) ventricularis. A,D) α-SMA. B,E) VE-cadherin. C,F) NFκB, arrows show lack of nuclear staining in fibrosa and ventricularis endothelial cells. Scale bar = 10µm.
Figure VI. Healthy human pediatric valve. A) fibrosa, B) ventricularis. Green: α–SMA, red: CD31, blue: DNA. Scale bar = 10µm.
Figure VII. Separate green and blue channels for confocal images of control (A, B), +100 ng/mL IL-6 (C, D), and +100 ng/mL TNF-α (E, F) QEE at a 48 hour time point stained for NFκB (green) and DNA (blue).
Figure VIII. Inflammatory cytokine dose response migration and proliferation in quail endocardial explants (QEE). A, B) A 48 hour exposure to 100 ng/mL IL-6 or TNF-α significantly increases cell invasion when compared with control, +1 ng/mL, and +10 ng/mL treatments. C,D) QEE size increased by ~250% after 48 hours, cytokine exposure had no significant effect of QEE proliferation. Error bars show ±SEM, n ≥ 3 batches of pooled explants (QEE). Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure IX. Inflammatory gene expression in embryonic valve forming regions. VCAM1, ICAM1, TNFRSF1A (the gene that codes for TNFR1), IL6RA (the gene that codes for IL-6Rα), and NFKB1 in chick day 4, day 7, and day 10 atrioventricular (AV) cushions or valves and outflow tracts (OFT) normalized to day 2 chick AV or OFT. Error bars show ±SEM, n ≥ 3. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure X. Inflammatory cytokines induce EndMT through Akt/NFκB in PAVEC. A) Cell invasion after to exposure to 100 ng/mL IL-6 or 100 ng/mL IL-6 with 5 µm Akt inhibitor XI or 5 µm PpYLKTK STAT3 inhibitor. B) ENDMT-related gene expression after exposure to 100 ng/mL IL-6 with inhibitors. C) Inflammatory activation-related genes after exposure to 100 ng/mL IL-6 with inhibitors. D) PAVEC nuclear factor kappa b (NFκB) nuclear localization quantification after exposure to 100 ng/mL IL-6 with inhibitors. Error bars show ±SEM, n ≥ 3 independent monolayers. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure XI. Inflammatory cytokines induce EndMT through Akt/NFκB in QEE. A) Cell invasion after to exposure to 100 ng/mL IL-6 or 100 ng/mL IL-6 with 5 µm Akt inhibitor XI or 5 µm PpYLKTK STAT3 inhibitor. B) EndMT-related gene expression after exposure to 100 ng/mL IL-6 with inhibitors. C) Inflammatory activation-related genes after exposure to 100 ng/mL IL-6 with inhibitors. D) QEE nuclear factor kappa b (NFκB) nuclear localization quantification after exposure to 100 ng/mL IL-6 with inhibitors. Error bars show ±SEM, n ≥ 3 batches of pooled explants. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure XII. AKT1 gene expression was blocked by Akt inhibition and MEK1 gene expression was blocked by MEK1 inhibition in PAVEC. A) AKT1 expression was blocked in PAVEC following exposure to 100 ng/mL TNF-α and 5 µm Akt inhibitor XI. B) AKT1 expression was blocked in PAVEC following exposure to 100 ng/mL IL-6 and 5 µm Akt inhibitor XI. C) MEK1 expression was blocked in PAVEC following exposure to 100 ng/mL TNF-α and 25 µm PD98059 MEK1 inhibitor. Error bars show ±SEM, n ≥ 3 independent monolayers. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure XIII. AKT1 gene expression was blocked by Akt inhibition and MEK1 gene expression was blocked by MEK1 inhibition in QEE. A) AKT1 expression was blocked in QEE following exposure to 100 ng/mL TNF-α and 5 µm Akt inhibitor XI. B) AKT1 expression was blocked in QEE following exposure to 100 ng/mL IL-6 and 5 µm Akt inhibitor XI. C) MEK1 expression was blocked in QEE following exposure to 100 ng/mL TNF-α and 25 µm PD98059 MEK1 inhibitor. Error bars show ±SEM, n ≥ 3 batches of pooled explants. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure XIV. EndMT related (A) and inflammatory activation-related (B) gene expression in porcine aortic valve endothelial cells (PAVEC) after exposure to 1 ng/mL or 100 ng/mL TGFβ-1. Error bars show ±SEM, n ≥ 3 independent monolayers. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
Figure XV. Embryonic endocardial monolayers co-opt TGFβ in inflammatory-EndMT signaling. A) Cell invasion after to exposure to 100 ng/mL TGFβ-3, 100 ng/mL IL-6, or 100 ng/mL IL-6 with 10 µm SB 431542 ALK5 inhibitor. B) EndMT-related gene expression after exposure to 100 ng/mL IL-6 with inhibitors. C) Inflammatory activation-related genes after exposure to 100 ng/mL IL-6 with inhibitors. D) QEE nuclear factor kappa b (NFκB) nuclear localization quantification after exposure to 100 ng/mL IL-6 with inhibitors. Error bars show ±SEM, n ≥ 3 batches of pooled explants. Bars that do not share any letters are significantly different according to a one-way ANOVA with Tukey’s post test (p ≤ 0.05).
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