VEGF and RANKL Regulation of NFATc1 in Heart Valve Development

Michelle D. Combs, Katherine E. Yutzey

Rationale: NFATc1 (nuclear factor of activated T-cells cytoplasmic 1) activity in endocardial cushion (ECC) endothelial cells is required for normal ECC growth and extracellular matrix (ECM) remodeling during heart valve development.

Objective: The mechanisms of NFATc1 activation and downstream effects on cell proliferation and ECM-remodeling enzyme gene expression were examined in NFATc1 mutant mice and chick ECC explants.

Methods and Results: NFATc1−/− mice display reduced proliferation of ECC endothelial and mesenchymal cells at embryonic day 10.5, whereas myocardial cells are unaffected. Vascular endothelial growth factor A (VEGF) activates NFATc1 and promotes ECC cell proliferation via the regulatory phosphatase, calcineurin, and mitogen-activated protein kinase–extracellular signal-regulated kinase 1–extracellular signal-regulated kinase 1/2 (MEK1-ERK1/2)–dependent signaling. As ECCs mature, RANKL (receptor activator of nuclear factor κB ligand) and the ECM-remodeling enzyme cathepsin K (CtsK) are expressed by ECC endothelial cells. RANKL inhibits VEGF-induced cell proliferation while causing increased expression of CtsK via calcineurin/NFATc1 and c-Jun N-terminal kinase (JNK)1/2-dependent signaling.

Conclusion: These data support a novel mechanism for the transition from ECC growth to remodeling in which NFATc1 promotes a sequential pattern of gene expression via cooperation with ligand-specific cofactors such as MEK1-ERK1/2 or JNK1/2. (Circ Res. 2009;105:565-574.)

Key Words: valve development ▪ NFATc1 ▪ VEGF ▪ RANKL ▪ JNK ▪ ERK

Congenital malformations of cardiac valves affect 1% to 2% of the population; however, the molecular mechanisms that govern valve development are still not completely understood.1 Cardiac valvulogenesis is a complex process that begins with the formation of endocardial cushions (ECCs) in the atrioventricular canal (AVC) and outflow tract regions of the looping heart. The ECCs are populated as overlying endothelial cells delaminate, undergo epithelial-to-mesenchymal transformation (EMT), and invade the extracellular matrix (ECM).2 During EMT, ECC endothelial cells are highly proliferative, and increased mitotic index is a feature of both endothelial and mesenchymal cells of the developing valves.2,3 After outgrowth, the ECM of the valves is remodeled into a highly organized, trilaminar architecture characteristic of mature cardiac valves. During ECM remodeling, valve interstitial cell proliferation is decreased and endothelial cells express ECM remodeling enzymes.4,5 It is clear that precise regulation of valve cell cycle, growth, and remodeling is required for normal valve development, as alterations in these processes are linked to valve defects.2,6,7 Although much is known about signaling mechanisms regulating ECC formation and EMT, mechanisms governing the transition from growth to remodeling in the developing valves are relatively uncharacterized.

NFATc1 (nuclear factor of activated T-cells cytoplasmic 1) (also known as NFAT, NFATc, and NFAT2), a transcription factor of the NFAT family, functions in development and homeostasis of the brain, skeleton, immune system, and heart.8 In both mouse and chick embryos, cardiac NFATc1 expression is specific to ECC endothelial cells and overlaps with expression of its regulatory phosphatase calcineurin (Cn).7,9,10 NFATc1-null mice, or those lacking NFATc1 expression specifically in endothelial cells, have normal ECC formation and EMT, however, these ECCs fail to grow and remodel, resulting in lethality at embryonic day (E)12.0 to E14.5.9,11 Mice lacking expression of Cnb1 specifically in endothelial cells or chick embryos treated with the Cn inhibitor cyclosporin A (CsA), just before cushion growth, phenocopy NFATc1−/− mouse models, thereby illustrating the importance of the Cn-NFATc1 interaction for valve growth and remodeling.7,10 A necessary spatiotemporal window for Cn-NFATc1 activation in ECC endothelial cells has been defined, however, upstream effectors and intersecting pathways of NFATc1 in ECC endothelial cells during this critical period were not previously identified.

Vascular endothelial growth factor A (VEGF) (also known as VEGFA and VEGF165) is critical for development and
Non-standard Abbreviations and Acronyms

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<tr>
<td>AVG</td>
<td>atrioventricular canal</td>
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<td>BrdU</td>
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<td>E</td>
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<td>ECC</td>
<td>endocardial cushion</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>EMT</td>
<td>epithelial to mesenchymal transformation</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>HH</td>
<td>Hamburger and Hamilton stage</td>
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<td>ICLSM</td>
<td>immunofluorescence and confocal laser scanning microscopy</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>MEK1</td>
<td>mitogen-activated protein kinase–extracellular signal-regulated kinase 1</td>
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<td>Mes</td>
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<td>NFATc1</td>
<td>nuclear factor of activated T-cells cytoplasmic 1</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>pH3H</td>
<td>phosphohistone H3</td>
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<td>RANKL</td>
<td>receptor activator of nuclear factor kB ligand</td>
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<td>sFlt1</td>
<td>soluble Flt receptor chimera</td>
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<td>VEGF</td>
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<td>vascular endothelial growth factor receptor</td>
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maintenance of heart, lung, and vascular tissues. VEGF levels must be tightly controlled during cardiac valve morphogenesis because VEGF signaling maintains the ECC endothelial cell layer during ECC formation and is also a potent inhibitor of EMT. VEGF is expressed by myocardium and ECC endothelial cells during ECC formation and growth; however, expression is extinguished during ECC remodeling. Although it is known that under or overexpression of VEGF in ECC endothelial cells disrupts ECC morphogenesis, the role of VEGF in post-EMT ECC growth is not known. VEGF/NFATc1 signaling has been implicated in homeostasis of valve endothelial tissue because VEGF treatment of adult human pulmonary valve endothelial cells (HPVECs) increases cell proliferation. However, VEGF regulation of NFATc1 in ECC cells during development has not been demonstrated.

RANKL (receptor activator of nuclear factor kB ligand) (also known as TRANCE, TNFSF11, OPGL, and ODF) is a member of the tumor necrosis factor family of signaling molecules that is best known for its role in promoting osteoclast differentiation and production of ECM-remodeling enzymes, such as cathepsin K (CtsK). In myxomatous and diseased human mitral valves containing high levels of fragmented collagen and elastin, CtsK expression is upregulated by interstitial cells. Consistent with RANKL/NFATc1 pathway activity during valve remodeling, CtsK is normally expressed by murine valve endothelial cells at E13.5. However, CtsK is not expressed in NFATc1-null embryos. Conserved spatiotemporal expression of RANKL/NFATc1-related genes among vertebrates has not been previously demonstrated. Likewise, the ability of RANKL to promote CtsK transcription in a Cn-dependent manner in ECC cells has not been tested.

The present study examines the relationship of VEGF and RANKL signaling mechanisms in the regulation of NFATc1 in cardiac valve growth and remodeling. In isolated avian ECC cultures, VEGF signaling promotes cell proliferation via activation of Cn/NFATc1 together with MAPK-ERK Kinase1-Extracellular signal-Related Kinase1/2 (MEK1-ERK1/2) copathways. In vivo, NFATc1 is necessary for normal ECC growth, as mice lacking NFATc1 expression have decreased ECC endothelial cell proliferation at E10.5. In addition, RANKL/NFATc1 signaling is conserved among avian and mammalian embryos and RANKL acts during valve remodeling to promote CtsK expression via activation of Cn/NFATc1 and c-Jun N-terminal kinases (JNK)1/2 signaling. These data suggest NFATc1 plays a central role in the transition from ECC cell proliferation, in response to VEGF signaling, to ECM-remodeling enzyme production, in response to RANKL signaling. VEGF/NFATc1 signaling promotes cell proliferation and not CtsK expression via cooperation with MEK1-ERK1/2-dependent cofactors, whereas RANKL/NFAT signaling inhibits cell proliferation and increases CtsK expression via cooperation with JNK1/2-dependent cofactors.

**Methods**

E10.5 NFATc1 mouse embryos were collected and genotyped as previously described. Progeny of ECC cells were determined via quantification of bromodeoxyuridine (BrdU)-labeled nuclei versus total nuclei in paraffin embedded mouse heart sections. Explanted superior and inferior atrioventricular ECCs, isolated from Hamburger and Hamilton stage 25 (HH25; E4.5) chicken embryos, were maintained in culture for 2 to 7 days with one or more of the following treatments added to the culture media: VEGF (R&D Systems) 50 ng/mL, soluble Flt receptor chimera (sFlt1) (R&D Systems) 50 ng/mL, RANKL (R&D Systems) 800 ng/mL, osteoprotegerin (OPG) (R&D Systems) 50 ng/mL, CsA (Novartis) 1 μg/mL, BSA (Sigma) 100 to 800 ng/mL, U0126 (Promega) 10 μmol/L, SP600125 (Calbiochem) 2.5 μmol/L, and/or DMSO (Sigma) 0.005% to 0.01%. Real-time RT-PCR for CtsK expression and in situ hybridization for NFATc1, RANKL, and CtsK mRNA were performed as previously described.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**NFATc1+/− Mouse Embryos Have Decreased Proliferation of ECC Endothelial Cells In Vivo**

During ECC formation, endothelial cells populate the ECC mesenchyme by undergoing EMT and migrating into the cardiac jelly. ECCs then enter a growth period (mouse E10.5-E13.5) during which endothelial and mesenchymal cells of the ECC are highly proliferative. To determine whether NFATc1 is necessary for ECC endothelial cell proliferation, the proliferative index of ECC endothelial cells was examined in NFATc1+/+, NFATc1+/−, and NFATc1−/− E10.5 mouse embryos. Pregnant NFATc1+/− females were injected intraperitoneally with BrdU-labeling solution. The
ECC endothelial cells via EMT (Figure 1D). The proliferation also was observed in ECC mesenchymal cells that arise from BrdU-labeled nuclei (Figure 1D). Decreased proliferation in ECC cell proliferation cannot be decreased proliferation of ECC endothelial and myocardial cells in 6 embryos for each genotype (n = 6). \( *P \leq 0.01. \)

The proliferative index of endothelial cells overlying ECCs along with ECC mesenchymal cells and ventricular myocytes was assessed for at least 6 embryos of each genotype isolated from separate litters. Adjacent sections were labeled with MF20 antibody to visualize the myocardial boundaries of atrioventricular ECCs (Figure 1A through 1C). Wild-type (NFATc1+/+) and heterozygous (NFATc1+/−) mice have 32% and 30% of ECC endothelial cell nuclei labeled with BrdU, respectively, whereas NFATc1-null (NFATc1−/−) littersmates have a significantly lower (21%) percentage of BrdU-labeled nuclei (Figure 1D). Decreased proliferation also was observed in ECC mesenchymal cells that arise from ECC endothelial cells via EMT (Figure 1D). The proliferative defect observed is not attributable to general embryo failure, as no differences were detected in the mitotic index of ventricular myocytes among genotypes (Figure 1D). It is important to note that cardiac myocytes do not express NFATc1. This suggests that Cn/NFATc1 signaling is necessary during ECC formation and growth for normal ECC cell proliferation. No difference in the number of apoptotic cells were observed in ECCs of NFATc1+/+, NFATc1−/+ or NFATc1−/− E10.5 mice, as determined by anti–cleaved caspase3 labeling (data not shown). These results indicate that the hypoplastic ECC phenotype observed in NFATc1−/− embryos at E13.5 is likely attributable to decreased proliferation of ECC endothelial and endothelial-derived mesenchymal cells as early as E10.5, when these embryos are grossly indistinguishable from NFATc1+/+ and NFATc1−/− littersmates.

VEGF Treatment Increases ECC Cell Proliferation via Cn/NFATc1 and MEK1-ERK1/2 Signaling

VEGF regulates endothelial cell adhesion, cell cycle, and inflammatory cell recruitment during development and throughout postnatal life.13 The ability of VEGF to increase NFATc1 nuclear localization and cell proliferation was examined in ECC cells isolated from HH25 (E4.5) chicken embryos and placed in cell culture. The avian ECC culture system was used because HH25 chick embryos have much larger ECCs than murine embryos at the same stage in development, large numbers of synchronously staged ECCs can be collected at one time, and cultured ECC explants can be treated with cytokines or inhibitors to manipulate developmental pathways without the confounding effects of myocardial or systemic interactions.3,12,21,23 These explants contain valve progenitor cells as well as precursors of septum intermedium.2 NFATc1-positive ECC cells are endothelial as indicated by coexpression with endothelial markers such as Sox17 and VEGF receptor (VEGFR2) (Online Figure A, A through A’; data not shown).12,24 NFATc1-positive cells from nondissociated ECC explants exist as clusters of Sox17 or VEGFR2-positive endothelial cells surrounded by smooth muscle α-actin–positive, MF20-negative, and Sox17-negative mesenchymal cells. Myocyte contamination of cultures was not detected by immunofluorescence and confocal laser scanning microscopy (ICLSM) or real-time RT-PCR. Isolated atrioventricular ECCs were treated with VEGF, soluble Flt1 receptor chimera (sFlt1, a VEGF inhibitor), cyclosporin A (CsA) (a Cn inhibitor), VEGF+sFlt1, VEGF+CsA, or BSA (as a vehicle control). Nuclear localization of NFATc1 of was evaluated by ICLSM.

Cultures treated with VEGF contained significantly more cells with nuclear NFATc1 labeling than control cultures (76% versus 16%), indicating that VEGF promotes NFATc1 nuclear localization (Figure 2A, 2B, and 2G). In contrast, cultures treated with VEGF+sFlt1 or sFlt1 alone had a comparable number of cells containing nuclear NFATc1 as control cultures, indicating that increased NFATc1 nuclear localization was a specific effect of VEGF treatment (Figure 2C, 2E, and 2G). ECC cells treated with VEGF+CsA or CsA alone also showed low level NFATc1 nuclear localization comparable to controls, demonstrating that NFATc1 nuclear accumulation following VEGF treatment is Cn-dependent (Figure 2D, 2F, and 2G). It is important to note that nuclear size and morphology in all cultures of this study were comparable and apparently normal with no evidence of toxicity. Because NFATc1 is predominantly expressed by endothelial cells of the ECC in vivo and in culture, these results demonstrate that VEGF treatment promotes NFATc1 nuclear localization in ECC endothelial cells.

To determine whether VEGF induces proliferation of ECC cells, HH25 chick ECCs were cultured and treated with VEGF, VEGF+sFlt1, VEGF+CsA, sFlt1, CsA, or BSA. The proliferative index was calculated as the percent of total nuclei labeled with the M-phase marker anti–phosphohistone H3 (pH3) antibody. Cultures treated with VEGF had a significantly higher percentage of pH3 positive cells (4.4%) than BSA-treated controls (2.3%) (Figure 3A, 3B, and 3G).
Proliferation indices from cultures treated with a combination of VEGF/sFlt, VEGF/CsA, or sFlt1 alone were comparable to BSA-treated controls, indicating that the increased proliferation is a specific effect of VEGF treatment and is Cn-dependent (Figure 3C through 3E and 3G). Cultures treated with CsA alone had a significantly lower percentage of pHH3 positive nuclei, which could be attributable to the ability of CsA treatment to block endogenous Cn/NFAT signaling and affect cell proliferation in the absence of added VEGF (Figure 3F and 3G). Endogenous expression of VEGFR2, the main receptor for VEGF signaling, is specific to ECC endothelial cells and it was determined that VEGFR2 is localized to clusters of endothelial cells in culture (data not shown).14,25 Similarly, the majority of pHH3-positive cells coexpress endothelial markers such as Sox17 and NFATc1 in VEGF-treated cultures (Online Figure I, B and C). Together, these data indicate that VEGF increases proliferation of ECC endothelial cells in a Cn-dependent manner.

**Figure 2.** VEGF treatment of ECC cells induces NFATc1 nuclear localization. A through F, ICLSM of avian ECC cultures treated with BSA (A), VEGF (B), VEGF+sFlt (C), VEGF+CsA (D), sFlt (E), or CsA (F) and labeled with anti-NFATc1 antibody (green) and ToPro3 iodide nuclear stain (blue) shows cells with nuclear NFATc1 labeling (arrows) and cells lacking nuclear NFATc1 labeling (arrowheads). G, Quantification of percentage of cells with nuclear NFATc1 labeling in 3 independent experiments for each treatment (n=3). *P<0.01.

**Figure 3.** VEGF-induced proliferation of ECC cells is dependent on Cn signaling. A through F, ICLSM of ECC cultures treated with BSA (A), VEGF (B), VEGF+sFlt (C), VEGF+CsA (D), sFlt (E), or CsA (F) and labeled with anti-pHH3 antibody (pink) and ToPro3 (blue) shows M-phase ECC cells (arrows). G, Quantification of percentage of mitotic cells in 3 independent experiments for each treatment (n=3). *P<0.01.

**RANKL Increases CtsK Gene Expression via Cn/NFATc1**

In osteoclasts, RANKL/NFATc1 signaling promotes bone ECM remodeling by inducing expression of NFATc1 target genes, including the ECM-remodeling enzyme CtsK.17 RANKL/NFATc1 pathway components are expressed by murine ECC endothelial cells during physiological valve remodeling and by human ECC cells during pathological remodeling, suggesting that this pathway has a role in normal valve development and human disease mechanisms.5,19,26 Because species-specific differences between mouse and chicken in transforming growth factor β signaling molecules have been noted during ECC formation, we sought to determine that the spatiotemporal expression patterns of NFATc1,
RANKL, and CtsK during valve growth and remodeling are conserved in chicken embryos. In situ hybridization revealed that in chicken embryos, NFATc1 is expressed by ECC endothelial cells throughout growth and remodeling (chicken E4.5-E14) (Figure 4A and 4B; data not shown). In contrast, RANKL and CtsK transcripts are absent in E5 ECCs (C-E); however, RANKL and CtsK mRNAs are expressed by E7 mitral valve (MV) endothelial cells (arrows) (D, D’, F, F’).

Figure 4. NFATc1, RANKL, and CtsK mRNA expression in developing chick AVC. In situ hybridization of E5 and E7 chick heart sections. A through B’, NFATc1 mRNA is expressed by endothelial cells of ECC and mitral valves (arrows). C through F’, RANKL and CtsK transcripts are absent in E5 ECCs (C-E); however, RANKL and CtsK mRNAs are expressed by E7 mitral valve (MV) endothelial cells (arrows) (D, D’, F, F’).

To examine the ability of RANKL to induce NFATc1 nuclear localization in ECC cells, avian ECCs were cultured and treated with RANKL, osteoprotegerin (OPG) (a soluble RANKL inhibitor), CsA, RANKL+OPG, RANKL+CsA, or BSA. The percentage of cells with nuclear NFATc1 was determined by ICLSM. Cells treated with RANKL had a significantly higher percentage of cells containing nuclear NFATc1 (37%) than BSA-treated controls (16%), indicating that RANKL promotes NFATc1 nuclear localization in ECC cells (Figure 5A, 5B, and 5G). Cells treated with RANKL+OPG or OPG alone had NFATc1 nuclear localization comparable to controls, indicating that increased nuclear NFATc1 is a specific effect of RANKL treatment (Figure 5C, 5E, and 5G). ECC cells treated with RANKL+CsA or CsA alone also showed NFATc1 nuclear localization comparable to controls, demonstrating that NFATc1 nuclear accumulation induced by RANKL is Cs-dependent (Figure 5D, 5F, and 5G). Together, these results indicate that RANKL treatment promotes NFATc1 nuclear localization in cultured ECC cells.

To determine whether RANKL induces expression of the ECM-remodeling enzyme CtsK in ECC cells, avian ECCs were explanted and cultured for 7 days with the aforementioned treatments added to media. In these experiments, addition of RANKL to cultured ECCs resulted in 7.1-fold higher CtsK mRNA expression than BSA-treated controls, as detected by real-time RT-PCR (Figure 5H). Treatment with RANKL+OPG or OPG alone yielded CtsK expression levels...
comparable to controls, indicating that increased CtsK expression is a specific effect of RANKL treatment (Figure 5H). Treatment with RANKL+/CsA significantly inhibited the induction of CtsK expression, whereas CsA treatment alone had expression comparable to BSA-treated cells (Figure 5H). For all real-time RT-PCR experiments, GAPDH and β-actin mRNA levels before normalization were comparable among culture groups, indicating that cultures contained a similar number of live cells at collection. Taken together, these results indicate that RANKL promotes expression of CtsK via Cn signaling in ECC cells.

The results obtained for 7-day cultures are comparable to those obtained when cells were maintained in culture for 4 days. However, no induction of CtsK mRNA expression was observed at 48 hours, even though RANKL induced NFATc1 nuclear localization at this time point (Figure 5B and 5G; data not shown). Expression of markers associated with ECC maturation were assessed for ECC explants maintained in culture for 2 days versus those cultured for 7 or 10 days. Although NFATc1 expression remained comparable among all cultures, CtsK expression increased over the culture period. This increase in CtsK mimics gene expression of ECCs and valves in vivo (Online Figure II, A and B). Similarly, expression of periostin and versican in ECC cultures increased over time, whereas scleraxis mRNA levels remain relatively unchanged. This pattern of gene expression closely resembles gene transcription in vivo (Online Figure II, A and B). Together, these data suggest that cultured ECC cells have a pattern of gene expression consistent with maturing valves in vivo and that RANKL-induced CtsK expression in ECC cells is not only ligand-dependent but also time-dependent.

Cn/NFATc1 Activation Is a Nodal Point in RANKL and VEGF Signaling

The specificity of VEGF and RANKL induction of cell proliferation and ECM-remodeling enzyme expression was examined. To determine whether VEGF/NFATc1 signaling can induce CtsK transcription, HH25 avian ECC cells were cultured in the presence of VEGF, VEGF+sFlt1, VEGF+CsA, sFlt1, or CsA. None of these treatment groups expressed increased Ctsk mRNA compared to BSA-treated controls (Figure 6A). To determine whether RANKL/NFATc1 signaling promotes ECC cell proliferation, ECCs were cultured in the presence of RANKL, RANKL+OPG, RANKL+CsA, OPG, CsA, or BSA. In these experiments, none of the treatment groups exhibited increased proliferation compared to BSA-treated controls (Figure 6C). These results show that downstream effects of NFATc1 activation in ECC cells are ligand-dependent.

To examine the signaling hierarchy and crosstalk at the level of VEGF and RANKL receptors upstream of NFATc1, ECC explants were cultured and treated with VEGF+sFlt1 (RANKL inhibitor) or RANKL+sFlt (VEGF inhibitor). These data demonstrated that VEGF-induced ECC cell proliferation does not require RANK receptor function (Online Figure III, A) and, likewise, that RANKL-induced CtsK mRNA expression does not require VEGF receptor function (Online Figure III, B). Therefore, VEGF and RANKL signaling act independently with separable downstream effects in ECCs, however, both VEGF-induced cell proliferation and RANKL-induced CtsK expression are Cn/NFATc1-dependent.

RANKL Inhibits VEGF-Induced Proliferation of ECC Cells

The above results are consistent with a mechanism whereby VEGF activation of NFATc1 promotes ECC proliferation, followed by RANKL activation of NFATc1 to induce ECM-remodeling enzyme expression in maturing valves. Therefore, experiments were performed to examine the response of ECC cells in the presence of both VEGF and RANKL signals concurrently. Cells treated with VEGF+RANKL together
VEGF and RANKL Require MEK1-ERK1/2 and JNK1/2 Signaling, Respectively, to Induce Proliferation and CtsK Expression

RANKL stimulates ECM-remodeling enzyme production via coactivation of Cn/NFATc1 and JNK1/2 pathways in osteoclasts. JNK1/2 activation in cardiac valves was examined in vivo via immunohistochemistry on E12.5 mouse heart sections with anti–phosphorylated JNK(Thr183/Tyr185) antibody. JNK1/2 activation was detected in mitral and tricuspid valve endothelial cells, consistent with RANKL and JNK1/2 activity during valve ECM remodeling in vivo (Figure 7). To determine whether RANKL-induced CtsK expression requires JNK1/2 signaling in ECC cells, avian ECC explants were cultured for 7 days in the presence of RANKL+DMSO, RANKL+SP600125 (a JNK1/2 inhibitor), SP600125, or DMSO (vehicle control). ICLSM was used to determine that SP600125 treatment of cultured ECC cells significantly decreases phosphorylated JNK(Thr183/Tyr185)-expressing ECC cells, whereas RANKL-induced NFATc1 nuclear localization is not significantly altered (Online Figure IV, E through H; data not shown). Real-time RT-PCR demonstrated that SP600125 treatment significantly decreases RANKL-induced CtsK expression (Figure 8C). Therefore, RANKL-induced CtsK expression in ECC cells is JNK1/2-dependent.

In vascular endothelial cells, VEGF stimulation of VEGFR2 activates Cn/NFAT and ERK1/2 copathways to promote gene transcription. To determine whether ERK activation is specifically required for VEGF-mediated effects downstream of NFATc1 in ECC cells, ECCs were cultured in the presence of SP600125 (a MEK1-ERK1/2 inhibitor), U0126, or DMSO (vehicle control). ICLSM was used to determine that U0126 treatment of cultured ECC cells significantly decreased diphosphorylated ERK1/2-expressing ECC cells, whereas VEGF-induced NFATc1 nuclear localization was not significantly altered (Online Figure IV, A through C; data not shown). VEGF and DMSO control treated cells had mitotic indices of 4.0% and 2.6%, respectively, as determined by pH3 immunoactivity (Figure 8A). Addition of U0126 to cultures either alone or in combination with VEGF blocked the effects of VEGF treatment, and presumably endogenous VEGF signaling on cell proliferation, resulting in a significantly decreased mitotic index of 1.3% for both, compared to control cultures (Figure 7A). In contrast, MEK1-ERK1/2 inhibition has no effect on RANKL-induced CtsK expression in cultured ECC cells. As a specificity control, treatment with the phosphoinositide 3-kinase inhibitor LY294002 has no effect on either VEGF-induced proliferation or RANKL-induced CtsK expression in ECC cells (Figure 7D; data not shown). These data demonstrate that VEGF-induced ECC cell proliferation is MEK1-ERK1/2-dependent; however, RANKL-induced CtsK expression does not require MEK1-ERK1/2 activity.

Conversely, JNK1/2 inhibition with SP600125 does not significantly alter VEGF-induced ECC cell proliferation, demonstrating that VEGF-induced ECC cell proliferation is JNK1/2-independent (Figure 8B). To determine whether RANKL inhibition of VEGF-induced ECC cell proliferation requires JNK1/2 activation, ECC cells were treated with VEGF, RANKL, and SP600125. These experiments showed that RANKL-mediated inhibition of VEGF-induced ECC cell proliferation is JNK1/2-dependent (Figure 8E). Interestingly, JNK1/2-dependent signaling has not previously been associated with maturation of ECC cells. Overall, these results show that, in conjunction with Cn/NFATc1, MEK1-ERK1/2 activation is necessary to achieve VEGF-induced ECC cell proliferation.
proliferation, whereas JNK1/2 activation is necessary for RANKL-induced CtsK expression and for RANKL-mediated inhibition of ECC cell proliferation.

Discussion
During heart valve morphogenesis, ECCs transition from growth, characterized by high cell proliferation, to remodeling, during which the ECM is stratified and mature valve leaflets become apparent.3 Investigation into the role of NFATc1 in valve maturation supports a model whereby VEGF/NFATc1/ERK1/2 signaling promotes ECC cell proliferation during ECC growth, and RANKL/NFATc1/JNK1/2 signaling inhibits VEGF-induced cell proliferation, while promoting CtsK expression, during valve remodeling. These data also support a novel mechanism for the transition from ECC growth to remodeling in which NFATc1 promotes a sequential pattern of gene expression via cooperation with ligand-specific cofactors MEK1-ERK1/2 and JNK1/2 (Online Figure V).

NFATc1−/− mouse embryos exhibit decreased proliferation of ECC endothelial and mesenchymal cells at E10.5. Before this time, ECCs are apparently normal, demonstrating that NFATc1 is not required for ECC formation and EMT.7,9,11 VEGF is an upstream activator of Cn/NFATc1 and requires MEK1-ERK1/2 activation in promoting proliferation of cultured ECC cells. In vivo ECC growth is characterized by nuclear localization of NFATc1 in endothelial cells in addition to expression of VEGF and VEGFR2.7,15,27 VEGF signaling must be tightly regulated for normal valvulogenesis to occur, because VEGF is necessary for endothelial proliferation and maintenance, as well as being a potent inhibitor of EMT during initial formation of the ECCs.15,27 In the AVC myocardium, VEGF expression is negatively regulated by NFATc3/c4; however, NFATc1 has not been shown to regulate VEGF transcription in the ECC. By E14.5 in mouse, valve remodeling has begun and expression of VEGF and VEGFR2 are lost in valve endothelial cells, supporting a model whereby loss of VEGF signaling in the ECC endothelial cells...
is associated with the transition from growth to remodeling during valvulogenesis.\textsuperscript{14,15}

The RANKL/NFATc1 pathway is conserved among vertebrates and is active in endothelial cells of remodeling valves. This study is the first to report that RANKL induces CtsK expression via costimulation of Cn/NFATc1 and JNK1/2 pathways in ECC cells. In the skeletal system, NFATc1 is a key regulator of osteoclast differentiation and function in response to RANKL signaling.\textsuperscript{17} On RANKL binding in osteoclasts, the RANK receptor recruits adaptor molecules that costimulate NFAT and JNK1/2 pathways, ultimately leading to NFATc1/activator protein 1 (AP1)–mediated expression of ECM-remodeling enzymes such as Ctsk and matrix metalloproteinase 9.\textsuperscript{19} Expression of the RANKL/NFATc1 pathway components RANKL, CtsK, and matrix metalloproteinase 9 is associated with increased pathogenic ECM remodeling and calcification of human valves suggesting this pathway may play a role in valve maturation and disease.\textsuperscript{19,26}

In contrast, VEGF stimulation of NFATc1 in human pulmonary valve endothelial cells induces endothelial cell proliferation, which implicates NFATc1 in normal homeostasis of the valve endothelium.\textsuperscript{16}

Work presented here and elsewhere demonstrates that NFATc1 participates in complex regulatory interactions during valve development. In developing osteoclasts and endothelial cells, NFATc1 forms complexes with other NFATs, as well as unrelated transcription factors such as Elks, GATAs, and AP1, to bind DNA.\textsuperscript{8,29,30} Ligand-specific responses to Cn/NFATc1 activation occur through selective costimulation of NFATc1 partners in T cells, where genes associated with increased immune response are targeted by NFATc1/AP1 complexes, while genes associated with dampened immune response are activated by NFATc1 in the absence of AP1.\textsuperscript{31} JNK1/2 signaling is important for outflow tract development, but its role in valve development has not been previously reported.\textsuperscript{32} MEK1-ERK1/2–activated transcription factors are necessary for EMT and ECC cell proliferation.\textsuperscript{33,34} Together these data suggest NFATc1 plays a role in regulating the transition from ECC growth to valve remodeling via partnership with ligand-specific cofactors to elicit gene expression. Further interrogation of NFATc1 and its costimulatory pathway functions in valve maturation and homeostasis may reveal new therapeutic targets for prevention and treatment of congenital valve defects and disease.

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Disclosures

None.

References


VEGF and RANKL Regulation of NFATc1 in Heart Valve Development
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Supplementary Methods

Chicken and mouse embryo collection
Fertilized white leghorn chicken eggs (CBT Farms, MD and Charles River Laboratories, CT) were incubated at 38°C under high humidity. Embryos were collected at Hamburger and Hamilton (HH) stages 25, 26, 30 and 36 corresponding to E4.5, 5, 7 and 10 days, respectively. NFATc1 heterozygous mutant mice were obtained from Dr. Laurie Glimcher. Mouse embryos were generated via timed matings with observation of a copulation plug designated as E0.5. Embryos were collected at E10.5 and genotyping for NFATc1 mutation was performed by PCR using primers designed for the wild type and targeted alleles as described in Ranger et al. All animal procedures were approved and performed in accordance with institutional guidelines.

Immunohistochemical analysis and quantification of cell proliferation
Pregnant female mice were injected on E10.5 intraperitoneally with 10 µL/g body weight Bromodeoxyuridine (BrdU) Labeling Solution (Zymed). Females were sacrificed two hours post injection and embryos were collected, fixed, and processed for immunohistochemistry as previously described. Proliferation of endocardial cushion (ECC) endothelial and mesenchymal cells was determined as described in Bushdid et al., except that the proliferative index for ECC endothelial cells was determined by the number of BrdU positive ECC endothelial cell nuclei divided by the total number of endothelial cell nuclei within a 5 µm section. The proliferative index for ECC mesenchymal cells was determined as the number of BrdU positive ECC mesenchymal cell nuclei divided by the total number of ECC mesenchymal cell nuclei per section. The proliferative index for ventricular myocytes was determined as the number of BrdU positive myocyte nuclei divided by the total number of myocyte nuclei per section. At least five comparable heart sections were analyzed per embryo for six embryos of each genotype. For MF20 antibody labeled sections, MF20 antibody (Iowa Hybridoma Bank) was diluted 1:200 in 0.5% blocking solution (Pierce) and incubated on sections overnight at 4°C. For anti-phosphorylated JNK(Thr183/Tyr185) labeled sections, E11.5 and E12.5 mouse embryos were collected into ice cold 1x phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were dehydrated and paraffin embedded as previously described. 5 µm mouse sections were deparaffinized, rehydrated, and primary antibody applied per manufacturer’s protocol (Abcam). This procedure included antigen retrieval with Tris–EDTA pH9.0 buffer. Washing steps were performed using 1x Tris-buffered saline with 0.01% Tween 20 (TBST). Anti-phosphorylated JNK(Thr183/ Tyr185) antibody (Abcam) was diluted in 1:400 in 0.5% blocking solution (Pierce). For MF20 and pJNK labeling, secondary antibody application and HRP detection was performed according to manufacturer’s instructions (using TBS) with the Pierce Ultra-sensitive ABC Kit and DAB Enhanced Metal Substrate Kit (Pierce).

ECC cell cultures and treatments
Prefused superior and inferior atrioventricular canal (AVC) ECCs were harvested from embryonic chicken hearts at HH25, as previously reported by Lincoln et al., with the exception that the ECC cells were not dissociated before being placed into one well of a two-well collagen coated chamber slide. Six hearts were used for each experimental condition in duplicate. For RNA isolation in all experiments except where noted, ECC cells were incubated for 7 days. Recombinant human (rh) VEGF-165 (R&D Systems) 50ng/mL, rhVEGFR1/Flt1/Fc Chimera (sFlt1)(R&D Systems) 50ng/mL, Cyclosporin A (CsA) (Novartis) 1µg/mL, rhRANKL (R&D Systems) 800ng/mL, rhOPG (R&D Systems) 1µg/mL, BSA (Sigma) 100-800ng/mL (as a vehicle control), U0126 (Promega) 10µM, SP600125 (Calbiochem) 2.5µM, LY294002 (Calbiochem) 25µM, or Dimethyl sulfoxide (DMSO) (Sigma) 0.005-0.01% (as a vehicle control) was added to
culture media at the time of dissection and replenished after 3 days. For immunofluorescence, ECCs were incubated 1-7 days in M199 media (Cellgro) containing 10% Fetal Bovine Serum (Hyclone), 1% Penicillin-Streptomycin (Gibco), and 0.1% Chick Embryo Extract (Sera Labs International). For nuclear localization and proliferation studies, media was replaced after 24 hours with EGM-2MV-Microvascular Endothelial Cell Medium-2 (Cambrex) with all SingleQuot additives except VEGF and incubated another 24h. For examination of VEGF and RANKL regulation of NFATc1 nuclear localization, ECCs were treated with rhVEGF-165 50ng/mL, sFlt1 50ng/mL, CsA 1µg/mL, BSA 100ng/mL for 20 minutes or rhRANKL 800ng/mL, rhOPG 1µg/mL, CsA 1µg/mL, BSA 800ng/mL for 30 minutes. Cultures with inhibitors (sFlt1, CsA, or OPG) were pre-treated for 2 hours prior to addition of rhVEGF or rhRANKL. To validate U0126 inhibition of VEGF-induced ERK1/2 activation and SP600125 inhibition of RANKL-induced JNK1/2 activation, ECCs were treated with rhVEGF-165 50ng/mL, U0126 (Promega) 10µM, DMSO 0.01% for 20 minutes or rhRANKL 800ng/mL, SP600125 (Calbiochem) 2.5µM, DMSO 0.005% for 30 minutes. Cultures with inhibitors (U0126 or SP600125) were pre-treated for 2 hours prior to addition of rhVEGF or rhRANKL. Cultures used to determine proliferation were treated for 24 hours with rhVEGF, rhRANKL, sFlt1, rhOPG, CsA or BSA. For each experiment, samples were collected in biological duplicate and data were collected from 3-6 independent experiments for each condition.

Immunofluorescence and scanning laser confocal microscopy
ECC cultures were fixed and prepared for confocal microscopy as detailed in Evans-Anderson et al. 5, with the following exception; fixed cultures were incubated for 10 minutes in 0.3% Triton-X in PBS and then washed 3x5 minutes with PBS before being incubated for 1 hour in blocking solution (1% BSA, 0.1% cold water fish skin gelatin, 0.1% Tween-20, 0.05% NaN3/PBS). Mouse monoclonal anti-NFATc1 (Santa Cruz) (1:100), rabbit polyclonal anti-phosphohistone H3 (Ser10) (pHH3) (Upstate) (1:100), guinea pig anti-Sox 17 (generous gift from Dr. Jeffrey Whitsett) (1:1000), mouse monoclonal anti-Cathepsin K (Santa Cruz) (1:100), rabbit polyclonal anti-Flk1 (Santa Cruz) (1:200), mouse monoclonal anti-MAP kinase, activated (diphosphorylated ERK1/2) (Sigma clone MAPK-YT) (1:10,000), or rabbit polyclonal anti-phosphorylated JNK(Thr183/Tyr185) (Abcam) (1:400) primary antibodies were diluted in 1:1 blocking solution/PBS and incubated on slides overnight at 4ºC. Corresponding Alexa-goat anti-mouse-488, Alexa-goat anti-guinea pig-488, Alexa-donkey anti-rabbit-488, Alexa-goat anti-guinea pig-568 and/ or Alexa-goat anti-rabbit-568 (Molecular Probes) secondary antibodies were applied at a concentration of 1:200 in PBS for 1-3 hours. ToPro3 iodide nuclear stain (Molecular Probes) (1:1000 in PBS for 10 minutes) was used to label cell nuclei. Immunofluorescence was detected using a Zeiss LSM 510 confocal microscope and images were obtained using Zeiss LSM version 3.2 SP2 software. Ten random microscopic fields were imaged for each experimental condition and all images were captured in parallel using identical confocal laser settings, constant PMT filters and integration levels. Percent cells with nuclear NFATc1 fluorescence was determined by dividing the number of cells with nuclear NFATc1 labeling by the total number of NFATc1 positive cells per microscopic field. The percent pHH3 positive cells was determined by dividing the number of pHH3 positive nuclei by total nuclei in a microscopic field. Data were collected from 3-6 independent experiments with biological duplicates for each condition.

RNA isolation and Real Time RT-PCR
ECC cell cultures were collected in 200µL Trizol reagent (Invitrogen), total RNA isolated, and cDNA generated using SuperScript II (Invitrogen) per manufacturer’s protocol. 600ng of cDNA in Power SyberGreen Master Mix (ABI) was subjected to Quantitative Real Time PCR (MJ Research, Opticon 2) analysis using the following 20pmol primers: CtsK- 5’-

- 2 -
AAAGCAGTACAACGGCAAGG-3' and 5'-GAGCTCACATCTTGGGGAAG-3'; *NFATc1*- 5'-CTCTGGAGAGCCCTAGAATTGA-3' and 5'-CGCAGAAGTTTCTTCTTG-3'. The identity of the *CtsK* and *NFATc1* PCR products were confirmed by sequencing. Amplification reactions were performed as 95º 5 minutes, (94º 30 seconds, 64º 30 seconds, 72º 30 seconds, plate read) x35 cycles, 72º 10 minutes, melting curve from 65º-95º read every 1º and hold 10 seconds, then 10º 5 minutes. Primers and reaction conditions used for *GAPDH*, *β-actin*, *Periostin*, *Versican*, and *Scleraxis* were previously reported. 4,6,7 Samples were run in triplicate and gene expression levels were determined as previously described by Lincoln et al. 4, with the standard curve for each primer set generated with HH34 whole heart cDNA. All reported values were normalized to corresponding *GAPDH* levels. 4 Gene expression levels were also confirmed by normalization to *β-actin*. For each experiment, samples were collected in biological duplicates and data were collected from three independent experiments for each condition.

**In situ hybridization**
The chicken *CtsK/JTAP1* (GenBank accession #NM_204971) and *RANKL* (GenBank accession #NM_001083361) sequences were amplified from HH34 whole heart cDNA using the following primers: *CtsK*- 5'-AAAGCAGTACACGGCAAGG-3' and 5'-GAGCTCACATCTTGGGGAAG-3' and *RANKL*- 5'-ACACGCCCTTTGAAAATCAG-3' and 5'-AATGCCCCAAAGTAAGTTGC-3'. Sequences of *CtsK* (891bp) or *RANKL* (630bp) were ligated into pGEM-T vector (Promega) and confirmed by DNA sequencing. Vectors containing *CtsK* and *RANKL* sequence inserts were linearized with *NcoI* and DIG-labeled riboprobes were prepared using SP6 polymerase as previously described. 6,8 *NFATc1* plasmid was a generous gift from Dr. D.W. Benson. Chicken *NFATc1* sequence was generated using primers: 5'-CTCTGGAGAGCCCTAGAATTGA-3' and 5'-CAATGAACAGCTGTAGCGTGAG-3' and ligated into pBluescript SK+ vector. Vector containing the *NFATc1* (1216bp) sequence was linearized with *BamHI* and DIG-labeled riboprobe was prepared as described above using T7 polymerase. In situ hybridization of chick tissue sections was performed as described in Shelton and Yutzey et al. 6 Proteinase K (20 µg/mL) was applied to E5 sections for 5 minutes and E7 for 8 minutes. 4-Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3- indolyl-phosphate solution (Roche) was applied to all sections in parallel with developing times of 30 minutes for *CtsK*, 45 minutes for *RANKL* and 60 minutes for *NFATc1* chick heart sections.

**Statistical Analysis**
Statistical significance was determined by Student’s *t*-test with *p*≤0.01 or *p*≤0.05 as indicated. Data are reported as a mean with standard error of the mean (SEM).

**References**


**Online Figures**

**Online Figure I. NFATc1 positive cells co-express endothelial markers, and VEGF induces proliferation of endothelial cells in cultured ECCs.** Immunofluorescence with confocal laser scanning microscopy (ICLSM) of cultured ECC cells. A'-A'') Coexpression of the endothelial marker Sox17 (pink) and NFATc1 (green) in cultured ECC cells (arrows). B-C) Coexpression of M-phase marker pH3 (pink) and endothelial markers Sox17 (green) (B) and NFATc1 (green) (C) induced by VEGF treatment (arrows). D) Coexpression of Sox17 (pink) and CtsK (green) in ECC cells (arrows). ToPro3 iodide nuclear stain was used to visualize cell nuclei (blue).

**Online Figure II. Expression of endothelial and mesenchymal markers by cultured ECC cells over time mimics gene expression observed in maturing ECCs/ mitral valves in vivo.** A) Expression of endothelial (*NFATc1, CtsK*) and mesenchymal (*Periostin, Versican, Scleraxis*) marker mRNAs in 2, 7, and 10 day cultured ECC cells. B) Expression of endothelial (*NFATc1, CtsK*) and mesenchymal (*Periostin, Versican, Scleraxis*) marker genes in avian ECCs/ mitral valves isolated at E4.5, E7, and E10. Fold change in mRNA expression was determined by Real Time RT-PCR and normalized to *GAPDH* levels. Data presented is representative of 3 independent experiments performed in biological duplicate with PCR performed in triplicate.

**Online Figure III. OPG does not inhibit VEGF-induced cell proliferation and sFlt1 does not inhibit RANKL-induced CtsK expression.** A) Quantification of percent pH3 positive nuclei in treated chick ECC cultures for three independent experiments per treatment (n=3). B) Quantification of fold difference in CtsK mRNA expression for treated avian ECC cultures was determined by real time RT-PCR. Samples were run in triplicate and normalized to *GAPDH* mRNA expression for three independent experiments per treatment (n=3). *p≤0.01

**Online Figure IV. Percent diphosphorylated ERK1/2 positive cells is significantly reduced in cultures treated with U0126 compared to controls.** Percent phosphorylated JNK positive cells is significantly reduced in cultures treated with SP600125 compared to controls. A) Quantification of percent anti-diphosphorylated ERK1/2 (dpERK) positive cells per total cell nuclei for three independent experiments per treatment (n=3). *p≤0.01 B-D) Representative images showing anti-dpERK positive cells (arrows) in VEGF+DMSO (B), VEGF+U0126 (C), and DMSO (D) treated ECC cultures. Green= dpERK and blue= ToPro3
iodide nuclear stain. E) Quantification of percent anti-phosphorylated JNK(Thr183/Tyr185) (pJNK) positive cells per total cell nuclei for three independent experiments per treatment (n=3). *p≤0.01  
F-H) Representative images showing anti-pJNK positive cells (arrows) in RANKL+DMSO (F), RANKL+SP600125 (G), and DMSO (H) treated ECC cultures. Green=pJNK and blue= ToPro3 iodide nuclear stain. 

**Online Figure V. Model of NFATc1 function in the transition from ECC growth to valve remodeling.** During ECC growth, VEGF/ NFATc1/ MEK1-ERK1/2 signaling promotes cell proliferation. As remodeling begins, RANKL/ NFATc1/ JNK1/2 signaling increases CtsK mRNA expression while inhibiting cell proliferation. These data support a novel mechanism for the transition from ECC growth to remodeling in which NFATc1 promotes a sequential pattern of gene expression via cooperation with ligand-specific cofactors MEK1-ERK1/2 and JNK1/2.
Online Figure I
Online Figure II
Online Figure III
Online Figure IV
Online Figure V

VEGF

RANKL

(+JNK1/2)

Calcineurin

MEK1

ERK1/2

JNK1/2

NFATc1

Proliferation

CtsK

Cushion growth

Cushion ECM remodeling

Early Cushion

Mature Valve