Nfatc1 Coordinates Valve Endocardial Cell Lineage Development Required for Heart Valve Formation

Bingruo Wu, Yidong Wang, Wendy Lui, Melissa Langworthy, Kevin L. Tompkins, Antonis K. Hatzopoulos, H. Scott Baldwin, Bin Zhou

**Rationale:** Formation of heart valves requires early endocardial to mesenchymal transformation (EMT) to generate valve mesenchyme and subsequent endocardial cell proliferation to elongate valve leaflets. Nfatc1 (nuclear factor of activated T cells, cytoplasmic 1) is highly expressed in valve endocardial cells and is required for normal valve formation, but its role in the fate of valve endocardial cells during valve development is unknown.

**Objective:** Our aim was to investigate the function of Nfatc1 in cell-fate decision making by valve endocardial cells during EMT and early valve elongation.

**Methods and Results:** Nfatc1 transcription enhancer was used to generate a novel valve endocardial cell–specific Cre mouse line for fate-mapping analyses of valve endocardial cells. The results demonstrate that a subpopulation of valve endocardial cells marked by the Nfatc1 enhancer do not undergo EMT. Instead, these cells remain within the endocardium as a proliferative population to support valve leaflet extension. In contrast, loss of Nfatc1 function leads to enhanced EMT and decreased proliferation of valve endocardium and mesenchyme. The results of blastocyst complementation assays show that Nfatc1 inhibits EMT in a cell-autonomous manner. We further reveal by gene expression studies that Nfatc1 suppresses transcription of Snail1 and Snail2, the key transcriptional factors for initiation of EMT.

**Conclusions:** These results show that Nfatc1 regulates the cell-fate decision making of valve endocardial cells during valve development and coordinates EMT and valve elongation by allocating endocardial cells to the 2 morphological events essential for valve development. (Circ Res. 2011;109:183-192.)

**Key Words:** valves, heart defects, congenital, endocardium

Congenital heart valve defects occur in 2% to 3% of the population and are the leading cause of perinatal and neonatal mortality and morbidity. Endocardial to mesenchymal transformation (EMT) gives rise to heart valve mesenchyme and plays a critical role in formation of heart valves. A critical step of early valve formation is the cell-fate decision that determines whether an endocardial cell will undergo EMT, becoming a valve core mesenchymal component, or maintain an endothelial phenotype and participate in the generation of a valve leaflet during valve remodeling or elongation. A balance in allocation of endocardial lineages to these 2 morphogenic processes must be achieved to form functional heart valves. Therefore, studying the underlying molecular mechanisms of the cell-fate decision-making process of valve endocardial cells during valve formation may provide new insight into the pathogenesis of congenital valvular heart disease. Migration and invasion of some but not all endocardial cells into matrix-rich cushions is the hallmark of the EMT process, which is regulated at least in part by the extracellular matrix and soluble growth factors. However, the mechanisms that permit only some endocardial cells to undergo EMT have not been fully understood. Additionally, although EMT gives rise to most, if not all, of the valve mesenchyme of the atrioventricular and semilunar valves, and similar morphogenetic pathways are shared in the atrioventricular canal (AVC) and outflow tract (OFT), accumulating genetic data suggest that there are unique morphogenetic mechanisms that regulate semilunar valve formation. One morphogenetic process that differentiates AVC and OFT de-
the valve endocardial cells to test this hypothesis. We also performed in vivo loss-of-function and blastocyst complementation analyses, in vitro EMT and endocardial cell differentiation assays, and gene expression studies. We show that the valve endocardial cells marked by the Nfatc1 enhancer do not undergo EMT and remain within the endocardium as a proliferative population to support valve leaflet extension during valve elongation and that Nfatc1 inhibits EMT in a cell-autonomous manner and suppresses transcription of Snail1 and Snail2, the key transcriptional factors for initiation of EMT. Together, these results reveal a previously unknown function for Nfatc1 in endocardial cell-fate decision making and indicate that Nfatc1 coordinates EMT and valve elongation by activating the endocardial cells to the 2 morphological events essential for valve development.

Methods

Generation of Valve Endocardium-Specific Cre and LacZ Mouse Lines

Nfatc1-enhancer Cre (Nfatc1\textsuperscript{enCre}) transgenic lines were generated by microinjection of a construct that contained a nuclear localized Cre inserted between an HSP68 minimal promoter and a 4.1-kb Nfatc1 intron 1 fragment\textsuperscript{34} into the fertilized eggs. The nuclear–cytoplasmic Wnt1-Cre transgenic line (Wnt1\textsuperscript{Cre}),\textsuperscript{35} the endothelium-specific Tie2-Cre transgenic line (Tie2\textsuperscript{Cre}),\textsuperscript{36} and R26\textsuperscript{Zin40} reporter were purchased from The Jackson Laboratory (Bar Harbor, ME). The Nfatc1-null allele\textsuperscript{34} was maintained as a compound heterozygous for Tie2\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} or Wnt1\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−}. All genetically altered mouse lines were backcrossed to inbred C57BL/6 mice (Charles River Laboratories, Wilmington, MA) for at least 8 generations. Mice were housed on a 6:00 AM to 6:00 PM light-dark cycle. Nonotime on the day that vaginal plugs were detected was designated as E0.5. The maintenance of mice and animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine of Yeshiva University and Vanderbilt University School of Medicine.

Fate-Mapping Analyses and X-Gal Staining

The embryos or hearts were collected between E9.5 and E11.5 for fate mapping of Nfatc1\textsuperscript{1/−} endocardial cells. To reveal the role of Nfatc1 in the fate development of endocardial cells during EMT and valve elongation, Tie2\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} or Wnt1\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} animals were crossed to R26\textsuperscript{Zin40};Nfatc1\textsuperscript{1/−} animals to generate wild-type Tie2\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} or Wnt1\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} and knockout Tie2\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} or Wnt1\textsuperscript{Cre+/−};Nfatc1\textsuperscript{1/−} embryos. Whole-mount X-gal staining of embryos or isolated hearts was performed as described previously.\textsuperscript{32} At least 5 age-matched littersmates were examined at each stage. The contribution of endocardial lineage to OFT morphogenesis was determined by measurement of the ratio of the length of dOFT and pOFT in E10.5 or E11.5 embryos.

In Vitro Collagen Gel Assays

Collagen gel assays for EMT were performed as described previously,\textsuperscript{33} with modifications. E10.5 pOFT or E9.5 AVC explants were dissected from Nfatc1\textsuperscript{1/−} or Nfatc1\textsuperscript{1/−} embryos and placed on collagen gels. An overnight adhesion was allowed, and the adhered explants were then cultured for 24 hours. Transforming endocardial cells were identified as those spindle-shaped cells that migrated away from the explants or invaded the gel, and these were counted manually.

Mouse Blastocyst Complementation Assay

Homzygous Nfatc1\textsuperscript{1/−} embryonic stem (ES) cells\textsuperscript{34} were injected into wild-type Zin40 blastocysts, which constitutively express

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β-galactosidase in nuclei46 (Figure 5A). Embryos of E9.5 to E10.5 were isolated, fixed, and X-gal–stained as above. Contribution of the Nfatc1+/− or Nfatc1−/− endocardial cells to the cushion mesenchyme was visualized and quantified in parallel in the same chimeric embryo as lacZ-expressing Nfatc1+/− or lacZ-negative Nfatc1−/− cells, respectively.

In Vitro Endocardial Cell Differentiation Assay
Mouse endothelial progenitor cells (EPCs) were transplanted with the Nfatc1 enhancer-lacZ construct. After transfection, the EPCs were induced to undergo endothelial/endocardial differentiation in vitro in the presence of cAMP as described previously,47 and the expression of endothelial/endocardial markers was determined by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR).

Additionally, some cells were fixed in 3% paraformaldehyde for 10 minutes before they were costained with Pecam1 (platelet endothelial cell adhesion molecule-1) and β-galactosidase to identify the Nfatc1h cells differentiated from EPCs.

Cell Proliferation and RT-PCR
Bromodeoxyuridine was used for pulse labeling of proliferating cells. Immunodetection of proliferating cells was performed with antibodies against bromodeoxyuridine and by the ABC method. For RT-PCR analysis, total RNAs were extracted from pooled E10.5 Nfatc1h or Nfatc1−/− hearts, cDNA templates were generated, and RT-PCR was performed with the gene-specific primers listed in Online Table I (available in the Online Data Supplement at http://circres.ahajournals.org).

Results
Nfatc1h Endocardial Cells Do Not Undergo EMT
Nfatc1 is required for heart valve formation,23,24 but its function in EMT remains unclear. We have previously identified a tissue-specific enhancer that autoamplifies Nfatc1 expression in Nfatc1h cells during EMT and subsequent valve elongation.42 To reveal the role of Nfatc1 in determining whether endocardial cells will undergo EMT or remain in the endocardium and proliferate for valve elongation, we first used the Nfatc1 enhancer to generate an Nfatc1enCre mouse line to trace the fate of Nfatc1h cells during EMT and early valve elongation from E10.5 to E12.5. When bred to R26fslz mice, Cre activated lacZ expression in a subpopulation of valve endocardial cells or Nfatc1h cells at E10.5 AVC (Figure 1B). The number of lacZ-expressing descendants of Nfatc1h cells in the AVC increased from E10.5 to E11.5 (Figure 1C) and extended to the OFT (Figure 1D). By E12.5, the X-gal–stained descendants of Nfatc1h cells were seen along the growing edge of the atrioventricular or OFT valves (Figures 1E and 1F). Surprisingly, no descendants of Nfatc1h cells were found in the cushion mesenchyme (Figures 1B through 1F). We thus used direct lacZ reporter lines driven by the same Nfatc1 enhancer (Nfatc1enlacZ) as an indicator of Cre expression during EMT (Online Figure I, A). The results confirmed that the Nfatc1h cells marked by the enhancer lacZ (or Cre expression) were a subpopulation of cushion endocardium in E10.5 Nfatc1+/− embryos during EMT (Online Figure I, B and C). After EMT, lacZ expression was continuously restricted to Nfatc1h cells during valve elongation from E11.5 to E12.5 (Online Figure I, D through G). Therefore, by comparing lacZ expression in Nfatc1enlacZ embryos (Online Figure I) to the Cre-mediated lacZ expression in the Nfatc1enCre embryos (Figure 1), we determined that Nfatc1h cells did not undergo EMT. Additionally, Nfatc1h lineages did not contribute to the core mesenchyme of the remodeling valves; instead, they remained on the endocardial edges of the growing valve leaflets at E14.5 (Figure 2). Taken together, the genetic fate-mapping analyses indicate that Nfatc1h cells do not undergo EMT and suggest that Nfatc1 regulates valvulogenesis by preventing a subset of endocardial cells from undergoing EMT, thereby allocating them to valve elongation.

Nfatc1 Is Required to Establish OFT Mesenchymal Boundary
To determine whether Nfatc1 regulates endocardial cell fate during EMT, we next performed endocardial cell lineage tracing in Nfatc1-null embryos using Tie2Cre. We did not use Nfatc1enCre because the enhancer was autoregulated by Nfatc1 and was inactivated in the Nfatc1-null embryos.42 The combination of the Tie2Cre and R26fslz reporter allowed us to trace endocardial progenies that populated the cushion mesenchyme through EMT.16 We bred Tie2Cre;Nfatc1+/− to R26fslz;Nfatc1−/− mice to trace endocardial cells in Tie2Cre;R26fslz;Nfatc1+/− or Tie2Cre;R26fslz;Nfatc1−/− embryos (Figure 3A). We found a segmental contribution of endocardium-derived mesenchyme to the pOFT and non-endocardium-derived mesenchyme to the dOFT that generated a tissue boundary in E10.5 Nfatc1+/− embryos (Figure
3B). The boundary formed at the OFT bend, the anatomic site for future semilunar valves. However, in E10.5 *Nfatc1*<sup>−/−</sup> embryos, the boundary was disrupted by an extended endocardium-derived mesenchyme to the dOFT cushion (Figure 3C). At E11.5, although mixed mesenchymal cells invested the pOFT of *Nfatc1*<sup>+/+</sup> embryos (Figure 3D), only endocardium-derived mesenchyme occupied the region in *Nfatc1*<sup>−/−</sup> embryos (Figure 3E). Further measurement of the lengths of dOFT and pOFT showed a relative shorting of the pOFT from E10.5 to E11.5 in *Nfatc1*<sup>+/+</sup> embryos (Figures 3F and 3G). In contrast, shortening did not take place in *Nfatc1*<sup>−/−</sup> embryos, which led to a significantly longer pOFT in these embryos at E11.5 (Figure 3F). Additionally, the AVC of *Nfatc1*<sup>−/−</sup> embryos appeared elongated and rigid and packed with endocardium-derived mesenchymal cells (Figure 3E).
Cardiac neural crest cells populate the dOFT cushion.17,31,34,48 A potential defect in migration of cardiac neural crest in Nfatc1−/− mice might result in their reduced contribution to the OFT tissue boundary. To determine whether this might have occurred, we used Wnt1Cre to trace migration of cardiac neural crest cells.33 We did not detect a difference in migration between Nfatc1+/+ and Nfatc1−/− embryos at E9.0 or E10.0 (Online Figure II, A through D); however, there appeared to be an attenuation of neural crest–derived mesenchyme in the dOFT of E12.5 Nfatc1−/− embryos (Online Figure II, E and F), which affected the base of the forming aortic valve (Online Figure II, G and H). Together, the reciprocal cell tracings revealed that Nfatc1 was essential for establishment of the OFT mesenchymal boundary and suggest that Nfatc1 regulates OFT morphogenesis through EMT at pOFT and cardiac neural crest cell extension at dOFT.

**Nfatc1 Regulates EMT Through Matrix Adhesiveness and Cell-Cell Contact**

We then applied in vitro collagen gel assays to quantify Nfatc1 regulation of EMT. The pOFT explants from E10.5 hearts were dissected according to the anatomic bend at OFT to avoid contamination of the endocardium-derived mesenchyme by migratory cardiac neural crest–derived mesenchymal cells. Using Wnt1Cre;R26SloES embryos, we confirmed that the bend was a reliable landmark that separated the distal migratory neural crest cells from the proximal transforming endocardial cells in the OFT (Figure 4A). We first noticed poor adhesion of the Nfatc1−/− OFT explants to the collagen gels. In 4 different experiments, 20 (87%) of 23 Nfatc1+/+ OFT explants adhered to the collagen gel after a 24-hour incubation, whereas only 11 (52%) of 21 Nfatc1−/− OFT explants attached to the gel at the end of the incubation. A similar observation was made with E9.5 AVC explants. Among the attached explants, the average number of transformed cells from Nfatc1+/+ pOFT or AVC explants was 30 or 68, respectively. In contrast, in Nfatc1−/− pOFT or AVC explants, the number was increased to 54 (P=0.003) or 93 (P=0.005), respectively (Figures 4B through 4F). These results indicate that Nfatc1 regulates EMT by maintaining adhesion of endocardial cells to extracellular matrix or stabilizing endocardial cell-cell contacts, thus inhibiting EMT in the Nfatc1−/− population of the endocardium.

**Nfatc1 Regulates EMT in a Cell-Autonomous Manner**

We then used mouse blastocyst complement assay to determine whether the excessive EMT of Nfatc1−/− endocardial cells was the result of an intrinsic defect in these cells. Nfatc1−/− ES cells were microinjected into Zin40-lacZ–labeled Nfatc1+/+ blastocysts26,46 (Figure 5A). Chimeric embryos were harvested at E9.5 or E10.5 and analyzed by cross-section analysis after X-gal staining. LacZ-negative cells, derived from Nfatc1−/− ES cells, were found in both the endocardium and early mesenchyme of E9.5 AVC or E10.5 pOFT (Figures 5B through 5D), which indicates that Nfatc1 is not required for endocardial cell specification or the initiation of EMT. However, further assessment of the ratio of
mesenchymal cells to endocardial cells revealed an enhanced EMT by the Nfatc1−/− cells (Figure 5E). In contrast, control blastocyst complementation experiments with wild-type ES cells showed no difference in EMT between endocardial cells derived from ES cells and blastocysts (data not shown). This observation demonstrates a cell-autonomous role for Nfatc1 in limiting EMT.

**Nfatc1 Promotes Proliferation and Survival of Valve Endocardial Lineages**

The hallmark of the cardiac phenotypes of Nfatc1−/− embryos is the absence of semilunar valve leaflets, which indicates that the extended endocardium-derived mesenchyme in the dOFT of E10.5 and E11.5 Nfatc1−/− embryos results in a defect in remodeling of the mesenchyme, poor outgrowth of Nfatc1+/h cells, or both. We thus examined cell proliferation and programmed cell death to determine whether they were affected in Nfatc1−/− embryos. By bromodeoxyuridine staining, we found a significant decrease in the proliferation of both endocardial cells and cushion mesenchyme at E11.5 in pOFT or AVC of Nfatc1−/− embryos (Figure 6). Between E12.5 and E13.5, the decreased proliferation of Nfatc1+/h endocardial cells in Nfatc1−/− embryos was more pronounced at the leading edge of the primitive semilunar valves, whereas in Nfatc1+/− embryos, the outgrowth of Nfatc1+/h cells began to form primitive leaflets (Online Figure III, A and B). Programmed cell death was also determined by cleaved caspase 3 staining. We observed somewhat increased activated caspase 3 staining in the primitive semilunar valves of Nfatc1+/− embryos after E12.5 (Online Figure III, C and D), although activated caspase 3 was not detected in either Nfatc1+/+ or Nfatc1−/− embryos at E10.5 or E11.5 (data not shown). Together, these data indicate that in addition to its inhibitory role in the EMT, Nfatc1 positively regulates valve elongation by promoting the proliferation of Nfatc1+/h cells and survival of valve mesenchymal cells.

**Nfatc1 Regulates Expression of Genes Involved in EMT and Cell-Cell Contact**

To further understand how Nfatc1 regulates endocardial cell fate, we developed an in vitro endocardial cell differentiation assay using an EPC line. In this assay, EPCs were able to differentiate into endothelial cells, which mainly consisted of Nfatc1−/− cells and survival of valve mesenchymal cells.

*Figure 5: Blastocyst complementation analysis shows Nfatc1 inhibits EMT in a cell-autonomous manner. A. Diagram shows generation of chimeric embryos by wild-type blastocyst (LacZ-labeled) injection with Nfatc1+/− ES cells. B, X-gal–stained E9.5 heart section shows Nfatc1+/− endocardial cells (negative for LacZ) were integrated into the endocardium at AVC and OFT (arrowheads) and invaded the cushions (arrows). C and D, X-gal–stained E10.5 heart sections show transformed Nfatc1+/+ (positive for LacZ) Nfatc1−/− (negative for LacZ) endocardial cells at AVC and OFT cushions. More Nfatc1+/− transformed cells appear in both cushions (+). E, Quantitative analyses demonstrate significant increases in the transformation of Nfatc1−/− endocardial cells compared with Nfatc1+/− endocardial cells (n=8 or 6 chimeric embryos examined at E9.5 or E10.5; Student’s t-test; bar=SD).*
and especially Snail2, the transcriptional repressors of VE-Cad and EMT, and downregulation of their target, VE-Cad, in E10.5 Nfatc1−/− hearts (Figure 7D). Furthermore, immunostaining of Snail 2 revealed that its expression was upregulated in cushion endocardial and mesenchymal cells of E10.5 Nfatc1−/− embryos (Figures 7E and 7F). Together, the expression results indicate that Nfatc1 maintains endocardial phenotype by suppressing Snail2 expression.

**Discussion**

Two waves of Nfatc activities are required for valvulogenesis in mice, one in E9.5 myocardium for initiation of EMT and the other in E11.5 endocardium for valve elongation. Nfatc1 is expressed by the endocardium from the stage of the primary heart tube to the looping heart between E8.5 and E10.5. Subsequently, its expression is downregulated in the chamber endocardium but maintained at a high level in valve endocardial cells or endocardium-derived mesenchymal cells of E10.5 Nfatc1−/− embryos (Figures 7E and 7F). Together, the expression results indicate that Nfatc1 maintains endocardial phenotype by suppressing Snail2 expression.

Leaflets. This enhancer activity corresponds to the second wave of calcineurin/Nfatc activity required for valve elongation.

In the present study, we aimed to understand the role of Nfatc1b cells in EMT and valve elongation using a combination of genetic fate-mapping, loss-of-function, and blastocyst complementation approaches. We generated Nfatc1bCre mice to map the fate of Nfatc1b cells in the developing cushions and valves (Figure 1; Online Figure I) and control Nfatc1bfl/wt mice as an indicator of Cre expression (Online Figure II). Comparisons of these new transgenic lines revealed that Nfatc1b cells do not undergo EMT; instead, they remain in the endocardium during EMT and valve elongation (Figures 1 and 2). Further fate-mapping analyses showed that OFT is not a continuous structure of a uniform mesenchymal cell population in mice; rather, OFT mesenchymal cell populations form a segmented structure, with cardiac neural crest mesenchyme occupying the dOFT and the endocardium-derived mesenchyme populating the pOFT (Figure 3). Their interface establishes the dOFT/pOFT mesenchymal border, which
corresponds to the site for developing semilunar valves in humans.37,49

However, in Nfatc1−/− embryos that did not form semilunar valves, this tissue boundary was disrupted by an increased endocardium-derived mesenchyme and a decreased cardiac neural crest–derived mesenchyme. The observation indicates that the 2 mesenchymal populations must interact coordinately to give rise to the semilunar valves. In vitro collagen gel assays revealed a premature loss of cellular adhesiveness and excessive EMT by Nfatc1−/− endocardial cells (Figure 4), and blastocyst complementation confirmed enhanced EMT by Nfatc1−/− endocardial cells (Figure 5). In addition, Nfatc1 promotes endocardial and OFT mesenchymal proliferation during valve elongation (Figure 6). Augmented expression of Nfatc1 and other endothelial markers, including VE-Cad, correlated with endocardial cell differentiation from EPCs in vitro, and Nfatc1 suppressed expression of Snail1 and Snail2, thereby maintaining the VE-Cad expression necessary for a tight cell-cell contact of the valve endocardial cells that prevents them from undergoing EMT. Nfatc1 also positively regulates the proliferation of valve endocardial cells necessary for the growth of valve leaflets mediated by unknown factors.

Figure 8. Working model for the role of Nfatc1 in semilunar valve development. A, Diagram showing the role of Nfatc1 in defining valve mesenchymal interaction during semilunar valve morphogenesis. In E11.5 Nfatc1−/− embryos, apposition of endocardium-derived mesenchyme from EMT at pOFT (blue cells) and cardiac neural crest–derived mesenchyme from migration at dOFT (green cells) establishes a mesenchymal tissue boundary for semilunar valve formation. From E11.5 to E12.5, the valve elongates from the boundary and becomes primitive valve leaflets. In Nfatc1−/− embryos, this tissue boundary is shifted into the dOFT because of an increased endocardium-derived mesenchyme from augmented EMT in the pOFT and subsequently decreased neural crest–derived mesenchyme in the dOFT. The alteration of heterogeneous mesenchymal tissue populations (and reduced valve endocardial proliferation not shown in the model) disrupts post-EMT valve elongation. B, A simple diagram shows that Nfatc1 transcriptionally regulates EMT and valve endocardial cell proliferation. Nfat1 maintains an endocardial cell phenotype through suppression of expression of Snail1 and Snail2, thereby maintaining the VE-Cad expression necessary for a tight cell-cell contact of the valve endocardial cells that prevents them from undergoing EMT. Nfatc1 also positively regulates the proliferation of valve endocardial cells necessary for the growth of valve leaflets mediated by unknown factors.
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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- During development, the semilunar valves are generated by cells of endocardial and cardiac neural crest lineages.
- Endocardial to mesenchymal transformation (EMT) and cardiac neural crest cell migration are involved in semilunar valve formation.
- Nfatc1 (nuclear factors of activated T cells, cytoplasmic 1) is required for semilunar valve formation.
- Nfatc1 expression is restricted to endocardial cells.
- A transcription enhancer regulates high Nfatc1 expression in valve endocardial cells.

**What New Information Does This Article Contribute?**

- Proper contact between endocardium- and cardiac neural crest–derived mesenchymal cells precedes normal elongation of semilunar valves.
- A previously unidentified population of valve endocardial cell does not undergo EMT during valve formation.
- Nfatc1 plays an important role in maintaining endocardial cell fate during EMT.
- **NFATC1** is a candidate gene for human congenital heart valve disease.

Congenital heart valve defects are a major cause of perinatal and neonatal mortality and morbidity. EMT generates heart valve mesenchyme and thus plays a critical role in the formation of heart valves. Expression of Nfatc1 is restricted to the valve endocardial endocardium during valve development through a tissue-specific enhancer. To address its potential role in endocardial cell-fate decision making during EMT, we generated a novel valve endocardial cell–specific Cre mouse line for fate-mapping analyses of valve endocardial cells. We also performed in vivo loss-of-function and blastocyst complementation, in vitro EMT, and endocardial cell differentiation assays, as well as gene expression studies. The results from these experiments showed that valve endocardial cells marked by the Nfatc1 enhancer did not undergo EMT and remained within the endocardium as a proliferative population to support post-EMT valve elongation. Nfatc1 inhibits EMT in a cell-autonomous manner by suppressing transcription of Snail1 and Snail2, the key transcriptional factors for the initiation of EMT. These studies reveal a previously unknown function of Nfatc1 in endocardial cell-fate decision making in allocating the endocardial cells to EMT and post-EMT valve elongation, and they identify **NFATC1** as a candidate gene for human congenital heart valve disease.
Nfatc1 Coordinates Valve Endocardial Cell Lineage Development Required for Heart Valve Formation
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Detailed Methods

Generation of endocardial Cre or lacZ mouse lines Nfatc1 Cre transgenic lines (Nfatc1<sup>enCre</sup>) were generated by microinjection of a construct containing a nuclear localized Cre inserted between a HSP68 minimal promoter and a 4.1-kb Nfatc1 intron 1 fragment into the fertilized eggs from C57BL/6 strain (Fig. 1A). We also made an Nfatc1-enhancer lacZ line (Nfatc1<sup>enhiz</sup>) by replacement of the Cre with a nuclear localized lacZ as a control (Online Fig. I). Multiple lines were made for each construct so that the consistency of expression was verified between the lines. The Cre lines were evaluated for the specific Cre-mediated recombination in the Nfatc1<sup>h</sup> cells by breeding to the Rosa26-flox-stop-lacZ reporter (R26<sup>floxiz</sup>)<sup>2</sup> and subsequent X-gal staining of the whole embryos or the hearts of the Nfatc1<sup>enCre</sup>;R26<sup>floxiz</sup> (Fig. 1B-F). The staining of the Nfatc1<sup>enhiz</sup> embryos or hearts of the same development stages was simultaneously carried out as an indicator of Cre expression in the Nfatc1<sup>h</sup> cells thereby determining the window between Cre expression and Cre-mediated lacZ expression.

The neural crest-specific Wnt1-Cre transgenic line (Wnt1<sup>Cre</sup>)<sup>3</sup>, the endothelial-specific Tie2-Cre transgenic line (Tie2<sup>Cre</sup>)<sup>4</sup>, and the R26<sup>floxiz</sup> reporter were purchased from the Jackson Laboratory (Bar Harbor, Maine). The Nfatc1 null allele<sup>5</sup> was maintained as a compound heterozygous for Tie2<sup>Cre+</sup>;Nfatc1<sup>+/−</sup> or Wnt1<sup>Cre+</sup>;Nfatc1<sup>+/−</sup>. Mice were housed on a 6:00 am to 6:00 pm light-dark cycle. Noontime on the day of detecting vaginal plugs was designated as embryonic day 0.5 (E0.5). The maintenance of mice and animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine of Yeshiva University and Vanderbilt University School of Medicine.

Cell lineage analysis and X-gal staining The embryos or hearts were collected between E9.5 to E11.5 for the lineage tracing of Nfatc1<sup>h</sup> endocardial cells. To reveal Nfatc1 role in the fate development of endocardial cells during EMT and valve elongation, Tie2<sup>Cre+</sup>;Nfatc1<sup>+/−</sup> or Wnt1<sup>Cre+</sup>;Nfatc1<sup>+/−</sup> animals were crossed to R26<sup>floxiz</sup>;Nfatc1<sup>+/−</sup> animals to generate wild-type Tie2<sup>Cre+</sup>;Nfatc1<sup>+/−</sup> or Wnt1<sup>Cre+</sup>;Nfatc1<sup>+/−</sup> or knockout Tie2<sup>Cre+</sup>;Nfatc1<sup>−/−</sup> or Wnt1<sup>Cre+</sup>;Nfatc1<sup>−/−</sup> (Fig. 2 and Online Fig. II). Wholemount X-gal staining of embryos or isolated hearts were carried out as described before<sup>1</sup>. After staining and post-fixation, the embryos or hearts were paraffin embedded and sectioned at 6-µm thickness. Serial sections were counterstained with eosin, inspected under an inverted microscope (Zeiss Axio Observer Z1). At least 5 age-matched littermates at each stage were examined. For E10.5 or E11.5 embryos, the lengths of the proximal OFT (pOFT) populated by the EMT-derived cells and the distal OFT (dOFT) invested by the invasion of cardiac neural crest cells was measured using Axiovision Rel.4.7 software equipped with the scope. The pOFT/dOFT ratio was determined. The difference between Nfatc1<sup>+/+</sup> and Nfatc1<sup>−/−</sup> embryos was analyzed using the Student’s t-test, and p < 0.05 was considered as statistically significant (Fig. 3F,G).

In vitro collagen gel assays Collagen gel assays for EMT were performed as described before with modifications<sup>6</sup>. E10.5 pOFT or E9.5AVC explants were dissected out from Nfatc1<sup>+/+</sup> or Nfatc1<sup>−/−</sup> embryos and placed on the collagen gels. An overnight adhesion was allowed and the adhered explants were then cultured for 24 hours. The EMT activity of endocardial cells was examined under the Zeiss Axiovert 40C microscope. Transforming endocardial cells were identified as those spindle-shaped cells migrating away from the explants or invading the gel and counted manually. The results from 4 independent experiments were analyzed and compared by the Student’s t-Test (Fig. 4).
**Mouse blastocyst complementation Assay** Homozygous Nfatc1\(^{+/+}\) embryonic stem (ES) cells were injected into wild-type Zin40 blastocysts, which constitutively express beta-galactosidase in nuclei \(^8\) (Fig. 5A). Embryos of E9.5 to E10.5 were isolated, fixed, and X-gal stained as above. Contribution of the Nfatc1\(^{+/+}\) or Nfatc1\(^{-/-}\) endocardial cells to the cushion mesenchyme was visualized in parallel in the same chimeric embryo as lacZ-expressing Nfatc1\(^{+/+}\) or lacZ-negative Nfatc1\(^{-/-}\) cells, respectively (Fig. 5A-D). Nfatc1\(^{+/+}\) or Nfatc1\(^{-/-}\) endocardial cells and their mesenchymal progenies were counted throughout the pOFT and AVC in serial sections. The effect of Nfatc1 on EMT was determined by comparing the ratio of the transformed Nfatc1\(^{+/+}\) cells in the cushion to the Nfatc1\(^{+/+}\) endocardial cells with the same ratio obtained in the Nfatc1\(^{-/-}\) cells. The results from 4 age-matched chimeric littermates were analyzed using the Student’s t-test (Fig. 5E).

**Cell proliferation, apoptosis, and RT-PCR** BrdU was used for pulse-labeling proliferating cells. One ml of the BrdU solution (Invitrogen Corp., Carlsbad, CA) per 100 g was injected into timed pregnant female mice via the intraperitoneal route 2 hours prior to embryo harvest. Embryos were fixed in 4% paraformaldehyde for 2 hours in the cold room, paraffin embedded, and sectioned. Immunodetection of proliferating cells were performed using antibodies against BrdU (Invitrogen) or Ki67 (Fig. 6, Online Fig. III). Apoptotic cells were identified by their expression of activated caspase-3 by using the antibody against cleaved caspase-3 (Cell Signaling Technology Inc., Davers, MA). The immunohistochemistry was performed using the ABC method (Vector Laboratories, Inc., Burlingame, CA). The results were examined and photographed under the Zeiss Axio Observer Z1 microscope. For RT-PCR analysis, total RNAs were extracted from pooled E10.5 Nfatc1\(^{+/+}\) or Nfatc1\(^{-/-}\) hearts using the Trizol solution (Invitrogen). The cDNA templates were generated by using the SuperScript® III reverse transcriptase kit (Invitrogen). Semi-quantitative RT-PCR was performed with gene-specific primers listed in Online Table I.

**In vitro endocardial cell differentiation assay** Mouse endothelial progenitor cells (EPCs) were transfected with the Nfatc1 enhancer-lacZ construct (Fig. 7). After transfection, the EPCs were induced to undergo endothelial/endocardial differentiation in vitro in the presence of cAMP as described before \(^9\). After a 72-hour culture, the expression of endothelial markers and Nfatc1 and their relationship were determined by semi-quantitative RT-PCR using extracted total RNAs. Additionally, some cells were fixed in 3% paraformaldehyde for 10 minutes prior to co-staining of Pecam1 using the Mec13.1 rat monoclonal antibody (BD Bioscience, San Jose, CA) and beta-galactosidase with X-gal solution to identify the Nfatc1\(^{h}\) endocardial cells differentiated from EPCs (Fig. 7).
References cited:


Online Figures and Figure Legends

Online Figure I. Mouse transgenic reporter gene assays shows that Nfatc1-enhancer activities mark Nfatc1<sup>h</sup> valve endocardial cells. A. A diagram showing a reporter construct generated by using an Nfatc1-enhancer and the heat shock protein minimal promoter (HSP68) to direct the lacZ. B,C. X-gal stained E10.5 heart sections showing the enhancer activities in the valve endocardial cells (Nfatc1<sup>h</sup> cells, arrows) of the atrioventricular canal (AVC) and outflow tract (OFT) during endocardial to mesenchymal transformation (EMT). D-G. E11.5 and E12.5 heart sections showing that the enhancer activities mark the Nfatc1<sup>h</sup> cells (arrows) of AVC and OFT during valve elongation.
Online Figure II. Wnt1-Cre fate mapping of cardiac neural crest shows that early migration of cardiac neural crest cells is not affected in Nfatc1<sup>−/-</sup> embryos. **A,B.** X-gal stained E9.0 embryo sections showing no differences in Wnt1-Cre marked neural crest (arrowheads) migrating into the distal OFT (dOFT) between Nfatc1<sup>+/−</sup> (A) and Nfatc1<sup>−/-</sup> embryos (B). **C,D.** X-gal stained E10.0 embryo sections showing neural crest-derived mesenchyme populating the dOFT and reaching the boundary of distal and proximal OFT (arrowhead) in both Nfatc1<sup>+/−</sup> (C) and Nfatc1<sup>−/-</sup> embryos (D). **E,F.** Whole mount X-gal stained E12.5 hearts showing attenuated cardiac neural crest-derived mesenchyme at dOFT (arrowhead) of Nfatc1<sup>−/-</sup> (F). **G,H.** Transverse sections of E12.5 hearts at the level of semilunar valve primordia showing a diminished population of cardiac neural crest-derived mesenchyme at the base of the primitive aortic valve of Nfatc1<sup>−/-</sup> embryos (H, arrowhead). Note that contribution of cardiac neural crest to aortopulmonary septum is comparable between Nfatc1<sup>+/−</sup> and Nfatc1<sup>−/-</sup> embryos (G,H, arrows). A, atrium; AS, aortic sac; dOFT/pOFT, distal/proximal OFT; V, ventricle.
Online Figure III. Nfatc1 promotes the proliferation and survival of the valve endocardial cells (Nfatc1<sup>h</sup> cells) during valve elongation. A,B. Ki67 immunostained E13.5 heart sections showing the Ki67-positive Nfatc1<sup>h</sup> cells at the growing edge of the primitive pulmonary valve leaflets in Nfatc1<sup>+/+</sup> embryos (A, indicated by arrowheads), but a decreased proliferation of Nfatc1<sup>h</sup> cells in the same area in Nfatc1<sup>-/-</sup> embryos (B, indicated by arrowheads). C,D. Caspase 3 stained E13.5 heart sections showing isolated apoptotic Nfatc1<sup>h</sup> cells at the growing edge of the primitive pulmonary valve leaflets in Nfatc1<sup>+/+</sup> embryos (C, indicated by arrow), but extended apoptosis of Nfatc1<sup>h</sup> cells in the same area in Nfatc1<sup>-/-</sup> embryos (D, indicated by arrows). The primitive valve cups are hypoplastic in Nfatc1<sup>-/-</sup> embryos (D, indicated by asterisk).
Online Table I. List of Primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence</th>
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| Id1       | Forward: 5’-GCCACGCAACTCCCAAGTGA-3’  
            | Reverse: 5’-TTAACCCTCCCAAGTCTCTG-3’ |
| Id2       | Forward: 5’-GCCGCTGACCCAAGCCTGAAC-3’  
            | Reverse: 5’-GGCCGGAAGAACGACACTGTG-3’ |
| Id3       | Forward: 5’-AACCAGCCCTCTCTACTACC-3’  
            | Reverse: 5’-CAGGGGAGCCACTCGCATCC-3’ |
| Tgf2      | Forward: 5’-CCCAGGCTTCCACCCCCACTCTC-3’  
            | Reverse: 5’-CCCACGGCTTCAACGAC-3’ |
| Bmp2      | Forward: 5’-TCAGGAATGGTGGAGGTGTTG-3’  
            | Reverse: 5’-AACAGGTTGCGAGGAAACAC-3’ |
| a-SMA     | Forward: 5’-ATTGATGGCTGGGTGTGGTAATAGTG-3’  
            | Reverse: 5’-TGATGTCAGGACCTCGAATCTGC-3’ |
| Snail1    | Forward: 5’-GGCCCTCTCGGACCTGCTCGCT-3’  
            | Reverse: 5’-CTTTCTCCCGGGCCACCTTG-3’ |
| Snail2    | Forward: 5’-CGTCGAGCCACTCCACCTCTCTC-3’  
            | Reverse: 5’-TCAGGCTGTTCCACAGCAG-3’ |
| VE-Cad    | Forward: 5’-CACGCAGGGGTGCAAAGC-3’  
            | Reverse: 5’-GCCCTCGTAGGGCTAGATAG-3’ |
| Nfatc1    | Forward: 5’-CTGACAGGATAGCAGAGAC-3’  
            | Reverse: 5’-GCTGTGATGGACAGAATGT-3’ |
| Gapdh     | Forward: 5’-ACGGGCAATTCACGAGCAGTCA-3’  
            | Reverse: 5’-TGGGGCAATTCACGAG-3’ |