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

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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;109:00-00.)

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.1 Endocardial to mesenchymal transformation (EMT) gives rise to heart valve mesenchyme and plays a critical role in formation of heart valves.2–6 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,7–10 which is regulated at least in part by the extracellular matrix and soluble growth factors.11–15 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 atroventricular and semilunar valves,16,17 and similar morphogenetic pathways are shared in the atroventricular canal (AVC) and outflow tract (OFT),18–21 accumulating genetic data suggest that there are unique morphogenetic mechanisms that regulate semilunar valve formation.22–27 One morphogenetic process that differentiates AVC and OFT de-
development involves a contribution from the migration and subsequent differentiation of cardiac neural crest; aberrant cardiac neural crest function causes defects in semilunar valve remodeling/mutation.

In the mouse, valvelike function in the OFT is initially provided at embryonic day (E) 8.5 by apposition of regional swellings of the extracellular matrix, also known as endocardial cushions, which ensures unidirectional blood flow. Shortly after E9.5, cardiac neural crest cells migrate into the OFT from the aortic arch in mice. At E10.5, a subpopulation of endocardial cells of the proximal OFT (pOFT) undergo EMT and invade the endocardial cushions. By E11.5, the OFT endocardial cushions form 2 spiral OFT ridges, which can each be morphologically divided into the distal OFT (dOFT) and the pOFT according to an OFT curvature. Although it is still unknown when the EMT ceases, the cushion begins remodeling between E11.5 and E12.5. By E12.5, coincident with seption of the cardiac outlet by fusion of the pOFT ridges, the earliest analogues of semilunar valve leaflets emerge at the distal-proximal OFT boundary as 3 pairs of condensed mesenchymal swellings developing from these ridges.

Although extensive studies have shown that multiple signaling pathways between the endocardium and the myocardium at the AVC and OFT are involved in activating or promoting EMT, the mechanisms that regulate endocardial cell-fate decisions during EMT and elongation remain elusive. We and others have previously shown that Nfatc1 coordinates EMT and valve development by regulating valve endocardial cell 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. The embryos or hearts were collected between E9.5 and E11.5 for subsequent differentiation of cardiac neural crest; aberrant cardiac neural crest function causes defects in semilunar valve remodeling/mutation.

In the present study, we generated a novel valve endocardial cell–specific Cre mouse line for fate-mapping analyses of 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 allocating 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-enhancer Cre) transgenic lines were generated by microinjection of a construct that contained a nuclear localized Cre inserted into an HSP68 minimal promoter and a 4.1-kb Nfatc1 intron 1 fragment into the fertilized eggs. The neural crest–specific Wnt1-Cre transgenic line (Wnt1-Cre), the endothelium-specific Tie2-Cre transgenic line (Tie2-Cre), and R26reporter were purchased from The Jackson Laboratory (Bar Harbor, ME). The Nfatc1-null allele was maintained as a compound heterozygous for Tie2-Cre;Nfatc1+/− or Wnt1-Cre;Nfatc1+/−. 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. Noontime 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+ cell populations. To reveal the role of Nfatc1 in the fate development of endocardial cells during EMT and valve elongation, Tie2-Cre;Nfatc1+/− or Wnt1-Cre;Nfatc1+/− animals were crossed to R26reporter;Nfatc1+/− animals to generate wild-type Tie2-Cre;Nfatc1+/− or Wnt1-Cre;Nfatc1+/− and knockout Tie2-Cre;Nfatc1+/− or Wnt1-Cre;Nfatc1+/− embryos. Whole-mount X-gal staining of embryos or isolated hearts was performed as described previously. At least 5 age-matched litters 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, with modifications. E10.5 pOFT or E9.5 AVC explants were dissected from Nfatc1+/− or Nfatc1+/− 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

Homozygous Nfatc1+/− embryonic stem (ES) cells were injected into wild-type C57BL/6 blastocysts, which constitutively express...
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 Nfat1+/− or Nfat1−/− endocardial cells to the cushion mesenchyme was visualized and quantified in parallel in the same chimeric embryo as lacZ-expressing Nfat1+/− or lacZ-negative Nfat1−/− cells, respectively.

**In Vitro Endocardial Cell Differentiation Assay**

Mouse endothelial progenitor cells (EPCs) were transfected with the Nfat1 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 Nfat1h 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 Nfat1+/− or Nfat1−/− hearts. cDNA templates were generated, and RT-PCR was performed with the gene-specific primers listed in Online Table 1 (available in the Online Data Supplement at http://circres.ahajournals.org).

**Results**

**Nfat1h Endocardial Cells Do Not Undergo EMT**

Nfat1 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 Nfat1 expression in Nfat1h cells during EMT and subsequent valve elongation.42 To reveal the role of Nfat1 in determining whether endocardial cells will undergo EMT or remain in the endocardium and proliferate for valve elongation, we first used the Nfat1 enhancer to generate an Nfat1enCre mouse line to trace the fate of Nfat1h 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 Nfat1h cells at E10.5 AVC (Figure 1B). The number of lacZ-expressing descendants of Nfat1h 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 Nfat1h cells were seen along the growing edge of the atrioventricular or OFT valves (Figures 1E and 1F). Surprisingly, no descendants of Nfat1h cells were found in the cushion mesenchyme (Figures 1B through 1F). We thus used direct lacZ reporter lines driven by the same Nfat1 enhancer (Nfat1enCre) as an indicator of Cre expression during EMT (Online Figure I, A). The results confirmed that the Nfat1h cells marked by the enhancer lacZ (or Cre expression) were a subpopulation of cushion endocardium in E10.5 Nfat1enCre embryos during EMT (Online Figure I, B and C). After EMT, lacZ expression was continuously restricted to Nfat1h cells during valve elongation from E11.5 to E12.5 (Online Figure I, D through G). Therefore, by comparing lacZ expression in Nfat1h embryos (Online Figure I) to the Cre-mediated lacZ expression in the Nfat1enCre embryos (Figure 1), we determined that Nfat1h cells did not undergo EMT. Additionally, Nfat1h 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 Nfat1h cells do not undergo EMT and suggest that Nfat1 regulates valvulogenesis by preventing a subset of endocardial cells from undergoing EMT, thereby allocating them to valve elongation.

**Nfat1 Is Required to Establish OFT Mesenchymal Boundary**

To determine whether Nfat1 regulates endocardial cell fate during EMT, we next performed endocardial cell lineage tracing in Nfat1-null embryos using Tie2Cre. We did not use Nfat1enCre because the enhancer was autoregulated by Nfat1 and was inactivated in the Nfat1-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;Nfat1+/− to R26fslz;Nfat1−/− mice to trace endocardial cells in Tie2Cre;R26fslz;Nfat1−/− (or Tie2Cre;R26fslz;Nfat1+/−) 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 Nfat1+/− embryos (Figure
3B). The boundary formed at the OFT bend, the anatomic site for future semilunar valves. However, in E10.5 \( \text{Nfatc1}^{+/+} \) 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 \( \text{Nfatc1}^{+/+} \) embryos (Figure 3D), only endocardium-derived mesenchyme occupied the region in \( \text{Nfatc1}^{+/-} \) 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 \( \text{Nfatc1}^{+/-} \) embryos (Figures 3F and 3G). In contrast, shortening did not take place in \( \text{Nfatc1}^{-/-} \) embryos, which led to a significantly longer pOFT in these embryos at E11.5 (Figure 3F). Additionally, the AVC of \( \text{Nfatc1}^{-/-} \) embryos appeared elongated and rigid and packed with endocardium-derived mesenchymal cells (Figure 3E).

Figure 2. Fate mapping of endocardial cells in mature valves shows that they do not contribute to valve mesenchyme. A and B, Whole-mount X-gal staining of E14.5 \( \text{Nfatc1}^{+/+};\text{R26fslz} \) heart shows \( \text{Nfatc1}^{+/-} \) cell lineage restricted to 4 heart valves. C-F, Sectional views indicate that cells of the \( \text{Nfatc1}^{+/-} \) lineage only contribute to the endocardium of mature valves; they do not become valve mesenchymal cells. Ao indicates aorta; Pa, pulmonary artery; AV, aortic valve; PV, pulmonary valve; MV, mitral valve; TV, tricuspid valve; LV, left ventricle; RV, right ventricle; and IVS, interventricular septum.

Figure 3. Fate mapping of endocardial cells shows that \( \text{Nfatc1} \) deletion results in abnormal OFT morphogenesis. A, Schematic of endocardial cell fate mapping in \( \text{Tie2}^{\text{Cre}};\text{R26fslz};\text{Nfatc1}^{+/-} \) or \( \text{Tie2}^{\text{Cre}};\text{R26fslz};\text{Nfatc1}^{-/-} \) embryos. B and D, E10.5 and E11.5 \( \text{Nfatc1}^{-/-} \) heart sections stained with X-gal show that \( \text{Tie2}^{\text{Cre}} \)-marked endocardium-derived mesenchymal cells locate at the pOFT, and LacZ-negative neural crest-derived mesenchyme locate at the dOFT. The 2 populations form a tissue boundary at the OFT curvature (B; line). C and E, E10.5 and E11.5 \( \text{Nfatc1}^{-/-} \) heart sections show increased endocardium-derived mesenchyme that extends into the dOFT (line). F and G, Graphs show the lengths of dOFT and pOFT and indicate a relative shorting of the pOFT from E10.5 to E11.5 in \( \text{Nfatc1}^{-/-} \) but not in \( \text{Nfatc1}^{+/-} \) embryos. n=5 paired embryos analyzed for each time point. A indicates atrium; AS, aortic sac; and V, ventricle.
Cardiac neural crest cells populate the dOFT cushion. 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. 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::R26lox/lox 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+/+ blastocysts (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,24 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+/+ 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+/+ 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+/+ 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+/+ 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.47 In this assay, EPCs were able to differentiate into endothelial cells, which mainly consisted of Nfatc1-possitive endocardial cells. We used Nfatc1+/+ or Nfatc1−/− endocardial cells (n=8 or 6 chimeric embryos examined at E9.5 or E10.5; Student 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, immuno-staining of Snail 2 revealed that its expression was upregulated in cushion endocardial and mesenchymal cells of E10.5 \( Nfatc1^{1/2} \) embryos (Figures 7E and 7F). Together, the expression results indicate that \( Nfatc1 \) maintains endocardial phenotype by suppressing Snail2 expression.

**Discussion**

Two waves of \( Nfatc1 \) 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.\(^{41}\) \( Nfatc1 \) is expressed by the endocardium from the stage of the primary heart tube to the looping heart between E8.5 and E10.5.\(^{21,24}\) Subsequently, its expression is downregulated in the chamber endocardium but maintained at a high level in valve endocardial cells or \( Nfatc1^{ipher} \) cells during EMT and valve elongation. We have previously shown that a tissue-specific enhancer autoamplifies \( Nfatc1 \) expression in \( Nfatc1^{ipher} \) cells during EMT and subsequent growth of primitive valve leaflets.\(^{52}\) This enhancer activity corresponds to the second wave of calcineurin/\( Nfatc1 \) activity required for valve elongation.\(^{41}\)

In the present study, we aimed to understand the role of \( Nfatc1^{ipher} \) cells in EMT and valve elongation using a combination of genetic fate-mapping, loss-of-function, and blastocyst complementation approaches. We generated \( Nfatc1^{Icre} \) and control \( Nfatc1^{Ilz} \) mice as an indicator of Cre expression (Online Figure II). Comparisons of these new transgenic lines revealed that \( Nfatc1^{ipher} \) 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
During early OFT morphogenesis is essential for subsequent derived mesenchyme and neural crest–derived mesenchyme, suggesting that the proper spatiotemporal contact of endocardium-derived mesenchyme and neural crest–derived mesenchyme is required for post-EMT valve elongation and maturation.30,31 Thus, NFATC1 may also regulate OFT morphogenesis and semilunar valve formation through an additional non–cell-autonomous effect on neural crest–derived mesenchymal function. The disruption of normal tissue boundary where semilunar valves develop in Nfatc1−/− embryos suggests that the proper spatiotemporal contact of endocardium-derived mesenchyme and neural crest–derived mesenchyme during early OFT morphogenesis is essential for subsequent semilunar valve remodeling. However, how this tissue interaction regulates development of semilunar valves is currently unclear.

We propose such an interaction is determined, at least in part, by Nfatc1-enhancer–regulated valve endocardial cells (Figure 8A). During normal semilunar valve formation, a mixed endocardium- and neural crest–derived mesenchyme forms at approximately E11.5 in the OFT, immediately before valve elongation or remodeling begins. Nfatc1 regulates proper spatiotemporal contact between the 2 mesenchymal populations, which may be a prerequisite for valve elongation. Nfatc1 suppresses transcription of Snail1 and Snail2, thereby inhibiting EMT initiated by Snail1- and Snail2-dependent downregulation of 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.

References


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 Sna1 and Sna2, 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.
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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<sup>1</sup> 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>flox</sup>)<sup>2</sup> and subsequent X-gal staining of the whole embryos or the hearts of the Nfatc1<sup>enCre</sup>;R26<sup>flox</sup> (Fig. 1B-F). The staining of the Nfatc1<sup>eniz</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>flox</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−/− or Wnt1<sup>Cre</sup>+/−;Nfatc1−/−. 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−/− or Wnt1<sup>Cre</sup>+/−;Nfatc1−/− animals were crossed to R26<sup>flox</sup>+/−;Nfatc1+/− animals to generate wild-type Tie2<sup>Cre</sup>+/−;Nfatc1+/− or Wnt1<sup>Cre</sup>+/−;Nfatc1+/− or knockout Tie2<sup>Cre</sup>−/−;Nfatc1−/− or Wnt1<sup>Cre</sup>−/−;Nfatc1−/− (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 Axiovission Rel.4.7 software equipped with the scope. The pOFT/dOFT ratio was determined. The difference between Nfatc1−/− and Nfatc1+/− 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+/− or Nfatc1−/− 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<sup>+/+</sup> embryonic stem (ES) cells<sup>7</sup> were injected into wild-type Zin40 blastocysts, which constitutively express beta-galactosidase in nuclei<sup>8</sup> (Fig. 5A). Embryos of E9.5 to E10.5 were isolated, fixed, and X-gal stained as above. Contribution of the Nfatc1<sup>+/+</sup> or Nfatc1<sup>-/-</sup> endocardial cells to the cushion mesenchyme was visualized in parallel in the same chimeric embryo as lacZ-expressing Nfatc1<sup>+/+</sup> or lacZ-negative Nfatc1<sup>-/-</sup> cells, respectively (Fig. 5A-D). Nfatc1<sup>+/+</sup> or Nfatc1<sup>-/-</sup> 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<sup>+/+</sup> cells in the cushion to the Nfatc1<sup>-/-</sup> endocardial cells with the same ratio obtained in the Nfatc1<sup>-/-</sup> 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., Daves, 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<sup>+/+</sup> or Nfatc1<sup>-/-</sup> 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<sup>9</sup>. 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<sup>h</sup> 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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<tbody>
<tr>
<td>Id1</td>
<td>Forward: 5'-GCCCATGGACAAAAAAATGGA-3’&lt;br&gt;Reverse: 5'-CAGGCCACCTCCACTCTCTG-3’</td>
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<tr>
<td>Id2</td>
<td>Forward: 5'-GCCGCTGACCAACCCTGAAC-3’&lt;br&gt;Reverse: 5'-GGCCGAGAAAGACACCTG-3’</td>
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<tr>
<td>Id3</td>
<td>Forward: 5'-AAGCCAGCCCTCTCTCACTTACC-3’&lt;br&gt;Reverse: 5'-CAGGGCAGCCACTCGCATCC-3’</td>
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<tr>
<td>Tgf2</td>
<td>Forward: 5'-CCCGCATTTACCCCACGCTC-3’&lt;br&gt;Reverse: 5'-CCCGCCCTCCACCCACCAT-3’</td>
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<tr>
<td>Bmp2</td>
<td>Forward: 5'-TCAAGGATTGCGAGTGGTGGG-3’&lt;br&gt;Reverse: 5'-AACAGGGTGAGGAGAAACATA-3’</td>
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<td>a-SMA</td>
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<td>Snail1</td>
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<tr>
<td>Snail2</td>
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<td>VE-Cad</td>
<td>Forward: 5'-CAGGCAGGTGCAGAAC-3’&lt;br&gt;Reverse: 5'-GCCCTCGTAGCCATGAGAT-3’</td>
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<tr>
<td>Nfatc1</td>
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<tr>
<td>Gapdh</td>
<td>Forward: 5'-ACGGCATACTCAGGGCAGAGG-3’&lt;br&gt;Reverse: 5'-TGGGGCATCGGCAGAAG-3’</td>
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