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The secondary heart field is a new site of Calcineurin/Nfatc1 signaling for semilunar valve development

Chieh-Yu Lin^{a,*}, Chien-Jung Lin^{a,*}, Chen-Hao Chen^a, Richard M. Chen^a, Bin Zhou^b, and Ching-Pin Chang^{a,+}

^aDivision of Cardiovascular Medicine, Department of Medicine, Stanford Cardiovascular Institute, Stanford University, Stanford, California 94305, USA

^bDepartments of Genetics, Medicine, and Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461, USA

Abstract

Semilunar valve malformations are common human congenital heart defects. Bicuspid aortic valves occur in 2-3 percent of the population, and pulmonic valve stenosis constitutes 10% of all congenital heart disease in adults (Brickner et al., 2000)[1]. Semilunar valve defects cause valve regurgitation, stenosis, or calcification, leading to endocarditis or congestive heart failure. These complications often require prolonged medical treatment or surgical intervention. Despite the medical importance of valve disease, the regulatory pathways governing semilunar valve development are not entirely clear. In this report we investigated the spatiotemporal role of calcineurin/Nfatc1 signaling in semilunar valve development. We generated conditional knockout mice with calcineurin gene disrupted in various tissues during semilunar valve development. Our studies showed that calcineurin/Nfatc1 pathway signals in the secondary heart field (SHF) but not in the outflow tract myocardium or neural crest cells to regulate semilunar valve morphogenesis. Without SHF calcineurin/Nfatc1 signaling, the conal endocardial cushion-- the site of prospective semilunar valve formation-- first develop and then regress due to apoptosis, resulting in a striking phenotype with complete absence of the aortic and pulmonic valves, severe valve regurgitation, and perinatal lethality. This role of calcineurin/Nfatc1 signaling in the SHF is different from the requirement of calcineurin/Nfatc1 in the endocardium for semilunar valve formation (Chang et al., 2004)[2], indicating that calcineurin/Nfatc1 signals in multiple tissues to organize semilunar valve development. Also, our studies suggest distinct mechanisms of calcineurin/Nfat signaling for semilunar and atrioventricular valve morphogenesis. Therefore, we demonstrate a novel developmental mechanism in which calcineurin signals through Nfatc1 in the secondary heart field to promote semilunar valve morphogenesis, revealing a new supportive role of the secondary heart field for semilunar valve formation.

Keywords

calcineurin; Nfatc1; secondary heart field; semilunar valve

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*Author for correspondence: Ching-Pin Chang, M.D., Ph.D., CCSR Building, Room 3115-C, 269 Campus Drive, Stanford, CA 94305-5169, Office: +1 650-736-8539, Fax: +1 650-723-6903, chingpin@stanford.edu.

*Equal contribution

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1. Introduction

Development of semilunar valves (aortic and pulmonary valves) requires the formation of endocardial cushions in the cardiac outflow tract (OFT) and remodeling of these cushions into elongated valve leaflets. These OFT cushions have truncal and conal parts, each of which has distinct developmental origins. The truncal cushions, located in the distal OFT, are populated by mesenchymal cells originating from the neural crest, whereas conal cushions residing in the proximal OFT are occupied by mesenchymal cells derived from the endocardium through the endocardial-mesenchymal transition (EMT)[3-6]. Subsequent to EMT, the conal cushions undergo tissue remodeling and elongate to form mature valve leaflets. Mechanisms of the valve elongation process remain poorly understood but likely involve differential proliferation and apoptosis of cushion mesenchymal cells [7], as well as tissue-tissue interactions among mesenchymal cells, endocardial cells, and myocardial cells [8], the latter originating from the secondary heart field (SHF) [9]. Recent studies using SHF-targeting Cre lines [9, 10] show that both Fgf8/BMP [10-12] and Notch signaling pathways [13-15] in the SHF are inhibitory for semilunar valve development, indicating that SHF provides signals to prevent semilunar valve hyperplasia.

Here we focused on the role of calcineurin/Nfatc1 signaling in SHF for semilunar valve morphogenesis. In contrast to previous work showing that SHF signals are inhibitory for semilunar valve formation [10-15], we show that calcineurin/Nfatc1 signaling in SHF is essential for semilunar valve development. Mouse embryos lacking calcineurin or Nfatc1 in the SHF develop conal cushions that regress and fail to develop into semilunar valve leaflets. The absence of SHF calcineurin causes increased apoptosis of conal cushion mesenchyme, leading to the regression of these cushions and failure of semilunar valve formation. This function of calcineurin/Nfatc1 in SHF is distinct from its roles in other tissues for heart valve development [2, 16-18].

2. Materials and Methods

2.1. Mouse Husbandry

All mouse strains were maintained in outbred backgrounds. *Cnb1^{ff}* [19], *Nfatc1^{ff}* [20], *Mef2cCre* [9], *Isl1Cre* [10], *Wnt1Cre* [21], and *Sm22aCre* [22, 23] mice were described previously. The date of observation of a vaginal plug was set as embryonic day E0.5. The gestational dates of embryos were confirmed by ultrasonography [24] and by embryo morphology. All animal studies were approved by Stanford University School of Medicine.

2.2. In utero Echocardiography

Pregnant female mice were scanned using an Acuson Sequoia C256 (Siemens) ultrasonography system with a linear 15L8 transducer. The dams were held steadily in the hand of the operator without the use of anesthetics. Sonographic gel was applied to eliminate trapped air in the abdominal fur to facilitate abdominal ultrasonography of the pregnant mice. Color Doppler analysis was used to assess the aortic and pulmonary valve insufficiency of embryos *in utero*. After imaging, embryos were harvested and examined to confirm gene mutation and semilunar insufficiency by genotyping and histopathology.

2.3. Tissue Processing and Histopathology

Harvested embryos were fixed overnight at 4°C in a 4% paraformaldehyde in phosphate-buffered saline (PBS) solution, dehydrated with serial ethanol treatment, cleared with xylenes, and stored overnight at 62°C in melted paraffin wax. The paraffinized embryos were then embedded in transverse orientation, sectioned at 7 µm consecutively using a Leica

microtome, and mounted on Superfrost Plus Microscope Slides. After rehydration, tissue sections were stained using hematoxylin and then counterstained with eosin, dehydrated, and mounted using Permount (Fisher). Alcian blue staining was performed on rehydrated slides using a 1% solution of Alcian blue (pH 2.5) in acetic acid and counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA).

2.4. Immunostaining

Paraffin tissue sections were rehydrated, immersed in a dilution of Antigen Unmasking Solution (Vector Laboratories), and then cooked for 10 minutes in a high pressure cooker. While cooling down to the room temperature, the tissue slides were first blocked with 0.3% hydrogen peroxide and the M.O.M. blocking reagent (Vector Laboratories), followed by incubation with the primary antibody overnight at 4°C. The next day, visualization was accomplished by incubating the slide in a biotinylated goat anti-rabbit secondary antibody (Jackson Laboratories, West Grove, PA) followed by labeling with a streptavidin-based ABC Kit (Vector Laboratories). The slides were then developed using a DAB chromogen kit (Dako, Carpinteria, CA). For immunofluorescence, tissue sections were incubated with Alexa 488-conjugated goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA). Primary antibodies used in this study include rabbit anti-calcineurin (Upstate, Charlottesville, VA), rabbit anti-phosphorylated Smad1/5/8 (9511, Cell Signaling Technology, Danvers, MA), rabbit anti-Sox9 (sc-20095, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Nfatc1 (7A6, Santa Cruz Biotechnology) and rabbit anti-versican (PA3-11, Pierce Thermo Scientific, Rockford, IL). A BrdU staining kit (Invitrogen) was used following the manufacturer's instructions.

2.5. TUNEL staining

Paraffin tissue sections were rehydrated, blocked with 100 mM glycine, and antigen-retrieved with 10 µg/ml protease K in PBS at 37°C. TUNEL reaction was performed using fluorescent in situ Cell Death Detection Kit (Roche) according to manufacturer's instructions. The slides were counterstained with Hoechst 33342 and mounted with VectaShield Hard Set mounting media (Vector Laboratories).

2.6. BrdU and TUNEL Quantification

All histological and staining sections were imaged using a Nikon Eclipse 90i microscope and the Nikon NIS-Elements Advanced Research software. Mesenchymal cells were identified by morphology; the total number of mesenchymal cells or the area occupied by mesenchymal cells was determined by the count or area function of the NIS-Elements software. The number of BrdU- or TUNEL-positive cells was determined relative to the total number of mesenchymal cells or the total area occupied by mesenchymal cells.

3. Results

3.1. Calcineurin deletion in the SHF leads to embryonic lethality

To understand the role of calcineurin/Nfat signaling in the secondary heart field (SHF), we used *Mef2c-Cre* [9] and loxP-flanked alleles of calcineurin B1 (*Cnb1^{fl}*) [2, 19] to ablate *Cnb1* in the SHF of *Mef2cCre;Cnb1^{fl/fl}* mice. In normal embryos, *Cnb1* was widely expressed in the developing heart tissues, including the endocardium, myocardium, and cushion mesenchyme (Fig. 1A-B and supplementary Fig. 1A, C). However, in *Mef2cCre;Cnb1^{fl/fl}* embryos, *Cnb1* was absent in the right ventricular and interventricular septal myocardium (Fig. 1C-D and supplementary Fig. 1B, D), consistent with the deletion of *Cnb1* by *Mef2cCre* in the SHF [9] that provides myocardial precursors for the right ventricle and outflow tract. Although *Mef2cCre* was reported to be active in the endocardium of right heart [9], we found that *Cnb1* in *Mef2cCre;Cnb1^{fl/fl}* embryos was still expressed in

endocardial cells and the endocardially-derived mesenchymal cells (Fig. 1C-D and supplementary Fig. 1B, D). This expression pattern indicates that *Cnb1* is deleted in the SHF myocardial progenitors, but not endocardial progenitors of *Mef2cCre;Cnb1^{ff}* mice. At E17-E18, the *Mef2cCre;Cnb1^{ff}* embryos were grossly indistinguishable from the control littermate (*Mef2cCre;Cnb1^{ff/+}*, *Cnb1^{ff}*, and *Cnb1^{ff/+}*) (Fig. 1E-F and data not shown). Despite the normal gross morphology at the end of gestation, very few *Mef2cCre;Cnb1^{ff}* embryos survived to or beyond postnatal day 0 (P0) (Supplementary Fig. 2). All mutant embryos that survived postnatally were offspring of the same male founder; the other mutants died before birth or perinatally. These findings suggest that calcineurin in the SHF myocardial progenitors is essential for perinatal viability.

3.2. SHF calcineurin is essential for semilunar valve development

To determine the cause of perinatal lethality, we examined the littermate control and *Mef2cCre;Cnb1^{ff}* embryos for cardiac abnormalities. At E17.5, control embryos had elongated aortic and pulmonic valve leaflets (Fig. 1G-H), whereas the *Mef2cCre;Cnb1^{ff}* embryos displayed a complete absence of aortic or pulmonic valve leaflets, leaving the cardiac outflow tract wide open (Fig. 1I-J). In the outflow tract, only remnants of mesenchymal tissue were present at the junction between the right ventricle and great arteries as shown by Alcian blue staining (Fig. 1K-L). A total of 41 *Mef2cCre;Cnb1^{ff}* embryos older than E13.5 was examined histologically for semilunar valves, and all these mutant embryos lacked aortic and pulmonic valves. The few embryos that survived neonatally and originated from the same male founder (Supplementary Fig. 2) had aortic valves but no pulmonic valves (data not shown). This founder line was considered as a partially escaper line and discarded for subsequent analysis. In contrast to semilunar valve defects, the mitral and tricuspid atrioventricular valves were normal in *Mef2cCre;Cnb1^{ff}* embryos (Supplementary Fig. 3). These results indicate that calcineurin in the SHF is required for semilunar valve formation.

To assess the hemodynamic impact of absent semilunar valves, we scanned pregnant mice carrying E17.5 embryos with *in utero* cardiac ultrasonography (Supplementary movie). In control embryos, cardiac systolic flow was detected by two forward flow jets from the ventricles into the great arteries (aorta and main pulmonary artery) (Fig. 2A, upper panels). In diastole there was no flow going back from arteries to the ventricles because of semilunar valve closure (Fig. 2B, upper panels). However, in mutant embryos, the systolic forward flows collided with the regurgitant flows, causing turbulence in the ventricles (Fig. 2A, lower panels). During diastole, the mutant embryos showed large regurgitant jets returning from arteries to the ventricles (Fig. 2B, lower panels). These regurgitant diastolic jets were comparable in magnitude but reverse in direction when compared to the normal systolic jets (Fig. 2A upper panels and 2B lower panels), indicating a wide-open cardiac outflow tract with severe blood regurgitation, consistent with the absence of semilunar valves in embryos lacking SHF calcineurin. Severe regurgitation increases the cardiac mechanical load, thereby leading to heart failure and perinatal lethality.

3.3. Loss of SHF calcineurin leads to endocardial cushion regression

Because the conal endocardial cushions in the outflow tract are essential for semilunar valve formation, we examined the development of conal cushions in the control and *Mef2cCre;Cnb1^{ff}* embryos harvested between E12.5 and E16.5. At E12.5, *Mef2cCre;Cnb1^{ff}* embryos developed two prongs of endocardial cushion along the outflow tract comparable to those of control embryos (Fig. 2C-D). However, the cushion development in *Mef2cCre;Cnb1^{ff}* embryos began to diverge from the controls at E14.5 (Fig. 2E-F). Conal cushions regressed progressively from E14.5 to E17.5 until they were nearly absent (Fig. 2D, F, H; Fig. 1I-J). In contrast to the conal cushion defects, the development of

truncal cushions and neural crest cells in the outflow tract remained intact in *Mef2cCre;Cnb1^{fl/fl}* embryos, as evidenced by the normal migration of neural crest cells into truncal cushions (neural crest cells marked by Plexin A2 [25])(Supplementary Fig. 4A-B) as well as the normal septation of truncus arteriosus into aorta and main pulmonary artery (which depends on truncal cushions and neural crest cells [3]) (Supplementary Fig. 4C-D). Therefore, neural crest cells are not involved in semilunar valve defects of *Mef2cCre;Cnb1^{fl/fl}* embryos. Collectively, these studies indicate that *Cnb1* in the SHF specifically regulates conal but not truncal cushion development. The regression of conal cushions provides an explanation for the absence of semilunar valves.

To further investigate the cushion mesenchyme in *Mef2cCre;Cnb1^{fl/fl}* embryos, we assayed the activity of BMP signaling by immunostaining phosphorylated Smad1/5/8. Analysis of E13.5 embryos revealed normal expression of phosphorylated Smad1/5/8 in cushion mesenchymal cells of *Mef2cCre;Cnb1^{fl/fl}* embryos (Supplementary Fig. 5A-B). The mesenchymal expression of Sox9 and versican, downstream effectors of the BMP pathway, was also similar in control and *Mef2cCre;Cnb1^{fl/fl}* embryos (Supplementary Fig. 5C-F). These molecular studies, together with morphological examination (Fig. 2C-D), suggest that the development of conal cushion mesenchyme is initially normal. It is the later regression of conal cushions that causes the absent semilunar valve phenotype.

3.4. Loss of SHF calcineurin increases conal cushion apoptosis

The regression of conal cushions in *Mef2cCre;Cnb1^{fl/fl}* embryos may result from decreased proliferation of cushion mesenchymal cells or increased cell death. To distinguish these two possibilities, we first pulse-labeled the embryos with bromodeoxyuridine (BrdU) to measure cell proliferation. BrdU immunostaining showed that cushion mesenchyme of control and *Mef2cCre;Cnb1^{fl/fl}* embryos incorporated BrdU at similar rates at E12.5 and E13.5 (Fig. 3A-F). Mesenchymal cell density within the conal cushions was also comparable between control and mutant embryos at E13.5 (Fig. 3C, F). We next tested whether apoptosis contributed to cushion regression in mutant embryos. TUNEL staining of E14.5 embryos revealed that the percentage of cushion mesenchymal cells undergoing apoptosis increased by 2.6 folds in *Mef2cCre;Cnb1^{fl/fl}* embryos (Fig. 3G-I). Together, these results demonstrate that loss of calcineurin in the SHF enhances mesenchymal apoptosis in conal cushions, contributing to cushion regression and absent semilunar valves.

3.5. Calcineurin functions in SHF myocardial progenitors

To determine the spatiotemporal requirements of *Cnb1* for semilunar valve formation, we generated conditional knockouts of *Cnb1* in different cell populations of the cardiac outflow tract. First, we ablated *Cnb1* using *Isl1Cre*, another widely used SHF-targeting Cre line that causes gene deletion in SHF and derivatives [10]. *Isl1Cre;Cnb1^{fl/fl}* embryos, like *Mef2cCre;Cnb1^{fl/fl}* embryos, had *Cnb1* deleted in the right ventricular and cushion myocardium but not endocardium (Supplementary Fig. 1F, H, J). Interestingly, we found that *Cnb1* was present in the interventricular septal myocardium of *Isl1Cre;Cnb1^{fl/fl}* embryos but not *Mef2cCre;Cnb1^{fl/fl}* embryos (Supplementary Fig. 1B, D, F, H), revealing a different expression domain of these Cre lines in the interventricular septum. *Isl1Cre;Cnb1^{fl/fl}* embryos phenocopied the cushion regression and absent semilunar valves of *Mef2cCre;Cnb1^{fl/fl}* embryos (Fig. 4A-B). The semilunar valve defects of *Isl1Cre;Cnb1^{fl/fl}* and *Mef2cCre;Cnb1^{fl/fl}* embryos suggest that *Cnb1* functions either in the SHF myocardial progenitors or mature cushion myocardium to regulate semilunar valve formation. To test the role of *Cnb1* in mature cushion myocardium, we used *Sm22aCre* to delete *Cnb1* in the myocardium [22, 23, 26]. Surprisingly, *Cnb1* deletion in the myocardium did not affect semilunar valve development (Fig. 4C-D, Supplementary Fig. 6A-B). These results suggest that *Cnb1* functions in the SHF myocardial progenitors but not the mature cushion myocardium for

semilunar valve development. This is in contrast to the essential role of myocardial *Cnb1* for atrioventricular valve development [2], indicating a specific function of *Cnb1* in the SHF for semilunar valve development.

We next tested whether *Cnb1* acted in neural crest cells for heart valve development by using *Wnt1Cre* to delete *Cnb1* in the neural crest cells and neural crest cell-derived truncal cushion mesenchyme [21, 27]. *Wnt1Cre*-mediated deletion of *Cnb1* had no influence on aortopulmonary septation or semilunar valve formation (Fig. 4E-F), suggesting that *Cnb1* is not required in neural crest cells for outflow tract septation or semilunar valve development. This is consistent with the conventional thought that neural crest cells have no direct roles in semilunar valve formation [28-30]. Collectively, these studies indicate that *Cnb1* programs SHF myocardial progenitors before they differentiate into cushion myocardium that interacts with cushion mesenchyme and endocardium to regulate semilunar valve development.

3.6. Calcineurin signals through *Nfatc1* in SHF

We next tested whether *Cnb1* signaled through *Nfatc1* to regulate semilunar valve development. Although previous studies show that *Nfatc1* functions in the endocardium to regulate heart valve development [2, 17, 18], the role of *Nfatc1* in SHF is unknown. We generated SHF-specific *Nfatc1* conditional knockout embryos (*Mef2cCre;Nfatc1^{ff}*). Strikingly, without deleting *Nfatc1* in the endocardium (Supplementary Fig. 6C-D), *Mef2cCre;Nfatc1^{ff}* embryos phenocopied the absent semilunar valves of *Mef2cCre;Cnb1^{ff}* mice (Fig. 5A-B), indicating that *Cnb1* in the SHF signals through *Nfatc1* to control semilunar valve development. This is in contrast to the essential roles of *Cnb1* and *Nfatc1* in the endocardium for heart valve development [2]. These findings thus uncover SHF as a previously unknown site of *Cnb1/Nfatc1* signaling for semilunar valve development [2, 17, 18].

4. Discussion

Our studies reveal a new developmental mechanism of semilunar valve morphogenesis. We demonstrate that calcineurin functions through *Nfatc1* in SHF myocardial progenitors to regulate semilunar valve development. In the absence of calcineurin/*Nfatc1* signaling, the conal cushions first develop and then regress at least partly due to increased mesenchymal apoptosis, resulting in a complete absence of aortic and pulmonic valves. Although further investigations are needed to elucidate the detailed mechanisms of how calcineurin/*Nfatc1* suppresses mesenchymal cell apoptosis and maintains the conal cushion, our current studies provide several insights into heart valve development.

Previous work showed that calcineurin/*Nfatc1* signaling in the endocardium is essential for valve elongation [2, 16-18, 31]. Our current studies demonstrate an additional site at the SHF myocardial progenitors where *Cnb1/Nfatc1* signals to regulate semilunar valve development (Fig. 5C). Endocardial and SHF *Cnb1/Nfatc1* signaling contribute to valve formation through different mechanisms. Endocardial *Cnb1/Nfatc1* is required for cushion remodeling and valve elongation, whereas SHF *Cnb1/Nfatc1* is essential for maintaining cushion mesenchyme and preventing its regression. Besides the distinct contributions of *Cnb1/Nfatc1* in the endocardium and SHF to semilunar valve formation, *Cnb1/Nfatc1* signaling in the myocardium plays different roles for atrioventricular and conal cushion development. In atrioventricular cushions, *Cnb1/Nfatc2/3/4* in the myocardium is essential for initiating EMT [2], whereas in conal cushions, EMT initiation does not require *Cnb1* in the SHF myocardial progenitors or conal myocardium. Furthermore, *Cnb1/Nfatc1* functions in a previously unknown time window to regulate valve development. The phenotypic difference of SHF-*Cre* and *Sm22aCre* mediated *Cnb1* deletion suggests a critical developmental window of *Cnb1* action for semilunar valve formation. SHF-*Cre* deletes

target genes in the SHF at E7.5 [9], while *Sm22aCre* deletes genes in the myocardium at E8.5-E9.0 [22, 23, 26], thus indicating that *Cnb1/Nfatc1* acts at E7.5-E8.5 in the SHF myocardial progenitors, before these progenitors become mature cardiomyocytes, to control conal cushion development (Fig. 5C). These studies, therefore, define a new spatiotemporal action of *Cnb1/Nfatc1* essential for the formation of aortic and pulmonic valves.

The SHF calcineurin/Nfatc1 signaling is unique in semilunar valve development. It is the only SHF signaling currently known to promote semilunar valve formation. Previous tissue-specific gene deletions in SHF suggest that SHF is inhibitory to semilunar valve formation since disruption of signaling pathways in SHF such as Bmp and Notch pathways all lead to semilunar valve hyperplasia/stenosis [10-15]. Our studies provide an example that SHF myocardial progenitors, before they differentiate into more mature cardiomyocytes, are capable of supporting semilunar valve development, since the disruption of *Cnb1/Nfatc1* signaling in those progenitor cells results in absent semilunar valves. In the SHF, *Cnb1/Nfatc1* signaling may counteract Bmp and Notch signaling and programs myocardial progenitors for semilunar valve formation. Over- and under- signaling from SHF may cause hyper- or hypo- plastic semilunar valves, leading to obstruction or leakage of blood flow. Together with the previous work on the inhibitory SHF signaling [10-15], the stimulatory role of *Cnb1/Nfatc1* signaling in the SHF suggest that a balance of signaling within the SHF determines the extent of semilunar valve morphogenesis, thus preventing valvular stenosis or leakage.

Besides interacting with other SHF signaling pathways, *Cnb1/Nfatc1* may interact with other pathways within the conal cushion to regulate valve leaflet development. Several pathways have been identified in the cushion to regulate valve remodeling. HB-EGF, for example, is an EGF family growth factor expressed in the endocardium and essential for cushion remodeling. In the absence of HB-EGF, conal cushions first develop normally but later enlarge excessively due to increased proliferation of cushion mesenchymal cells [32]. Such cushion hyperplasia is also observed in mice bearing homozygous D61G allele of *Ptpn11*, an EGF signaling gene [33]. The phenotypes resulting from HB-EGF disruption in the endocardium are opposite to that of *Mef2cCre;Cnb1^{ff}* embryos, in which *Cnb1* is deleted in the progenitors of conal myocardium. These studies suggest that myocardial calcineurin/Nfatc1 signaling may counteract endocardial EGF signaling in determining the extent of mesenchymal expansion in the conal cushion. Further understanding of how calcineurin/Nfat interacts with other pathways within the SHF and within the conal cushion will be essential to elucidate how SHF signaling sculpts semilunar valves in the developing embryo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research highlights

Calcineurin deletion in the secondary heart field leads to absence of semilunar valve.

SHF-calcineurin deletion results in normal formation of endocardial cushions which subsequently regress.

Calcineurin signals through *Nfatc1* in the SHF to control endocardial cushion remodeling.

Fig. 1. Calcineurin is essential in SHF for semilunar valve development

(A-D) Immunostaining of Cnb1 (brown) in *control* (Ctrl) and *Mef2cCre;Cnb1^{ff}* embryos at E11.5-12.0. The dashed lines in (B) and (D) mark the junctions between myocardium and cushion mesenchyme. myo: myocardium. RV: right ventricle. epi: epicardium.

(E, F) Gross morphology of E17.5 *control* and *Mef2cCre;Cnb1^{ff}* embryos.

(G-J) Hematoxylin-eosin staining of semilunar valves in *control* and *Mef2cCre;Cnb1^{ff}* mice at E17.5. PA: pulmonary artery. Ao: Aorta. RV: right ventricle.

(K, L) Alcian blue staining (blue) of the pulmonary valve in *control* and *Mef2cCre;Cnb1^{ff}* mice at E17.5. Red: nuclear red counterstain.

Fig. 2. Cushion regression and severe regurgitation in mice lacking SHF *Cnb1*

(A, B) Doppler *in utero* echocardiography of control (upper panels) and *Mef2cCre;Cnb1^{fl/fl}* (lower panels) mice at E17.5. Orange color indicates blood flow towards the probe on the abdominal surface, and blue color shows blood flow moving away from the probe. Arrows: turbulent flows. Arrowheads: regurgitant flows.

(C-H) Hematoxylin-eosin staining of the pulmonary valve in control (C, E, G) and *Mef2cCre;Cnb1^{fl/fl}* (D, F, H) at different gestational ages. The dashed lines mark the junctions between myocardium and cushion mesenchyme. Ao: aorta. RV: right ventricle. PA: pulmonary artery.

Fig. 3. Loss of SHF calcineurin increases OFT cushion apoptosis

(A, B, D, E) 5-bromo-2'-deoxyuridine (BrdU) staining of *control* and *Mef2cCre;Cnb1^{fl/fl}* pulmonary valve at E12.5 (A, B) and E13.5 (D, E) after 6-hour BrdU labeling. The dashed lines marked the junctions between myocardium and cushion mesenchyme. RV: right ventricle.

(C, F) Quantitation of BrdU-positive cell percentage (left panels) and total cell density (right panels) in mesenchymal regions in *control* and *Mef2cCre;Cnb1^{fl/fl}* mice.

(G, H) TUNEL staining of *control* and *Mef2cCre;Cnb1^{fl/fl}* pulmonary valve at E14.5. Dashed line marked the junction between mesenchymal cells and myocardium.

(I) Quantitation of TUNEL-positive cell percentage in mesenchymal regions in *control* and *Mef2cCre;Cnb1^{fl/fl}* mice.

Fig. 4. Calcineurin functions in SHF myocardial progenitors

(A, B) Hematoxylin-eosin staining of *control* and *Isl1Cre;Cnb1^{ff}* pulmonary valves at E14.5. Ao: aorta. RV: right ventricle. PA: pulmonary artery.

(C, D) Hematoxylin-eosin staining of *control* and *Sm22aCre;Cnb1^{ff}* pulmonary valves at E15.5.

(E, F) Hematoxylin-eosin staining of *control* and *Wnt1Cre;Cnb1^{ff}* pulmonary valves at E16.5.

Fig. 5. Calcineurin signals through Nfatc1 in SHF

(A, B) Hematoxylin-eosin staining of *control* and *Mef2cCre;Nfatc1^{ff}* pulmonary valves at E16.5. Ao: aorta. RV: right ventricle. PA: pulmonary artery.

(C) Spatiotemporal windows of calcineurin/Nfat signaling during heart valve development. SHF: secondary heart field.