Endocardial to Myocardial Notch-Wnt-Bmp Axis Regulates Early Heart Valve Development

Yidong Wang¹,², Bingruo Wu², Alyssa A. Chamberlain², Wendy Lui², Pratistha Koirala², Katalin Susztak³, Diana Klein⁴, Verdon Taylor⁵, Bin Zhou²,⁶,⁷*

¹State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan, China, ²Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University and Jiangsu Province Hospital, Nanjing, Jiangsu, China

Abstract

Endocardial to mesenchymal transformation (EMT) is a fundamental cellular process required for heart valve formation. Notch, Wnt and Bmp pathways are known to regulate this process. To further address how these pathways coordinate in the process, we specifically disrupted Notch1 or Jagged1 in the endocardium of mouse embryonic hearts and showed that Jagged1-Notch1 signaling in the endocardium is essential for EMT and early valvular cushion formation. qPCR and RNA in situ hybridization assays reveal that endocardial Jagged1-Notch1 signaling regulates Wnt4 expression in the atrioventricular canal (AVC) endocardium and Bmp2 in the AVC myocardium. Whole embryo cultures treated with Wnt4 or Wnt inhibitory factor 1 (Wif1) show that Bmp2 expression in the AVC myocardium is dependent on Wnt activity; Wnt4 also reinstates Bmp2 expression in the AVC myocardium of endocardial Notch1 null embryos. Furthermore, while both Wnt4 and Bmp2 rescue the defective EMT resulting from Notch inhibition, Wnt4 requires Bmp for its action. These results demonstrate that Jagged1-Notch1 signaling in endocardial cells induces the expression of Wnt4, which subsequently acts as a paracrine factor to upregulate Bmp2 expression in the adjacent AVC myocardium to signal EMT.

Citation: Wang Y, Wu B, Chamberlain AA, Lui W, Koirala P, et al. (2013) Endocardial to Myocardial Notch-Wnt-Bmp Axis Regulates Early Heart Valve Development. PLoS ONE 8(4): e60244. doi:10.1371/journal.pone.0060244

Editor: Masaru Katoh, National Cancer Center, Japan

Received January 8, 2013; Accepted February 24, 2013; Published April 1, 2013

Copyright: © 2013 Wang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Institute of Health (R01HL078881 and R01HL111770) to BZ and R01DK076077 to KS. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: bin.zhou@einstein.yu.edu

Introduction

Formation of heart valves is critical for heart function and is required for both embryogenesis and postnatal life. Defects in this process may cause congenital heart valve disease [1,2,3]. In mice, heart valve development begins with the mesenchymal transformation of endocardial cells (EMT) at embryonic day (E) 9.5 and E10.5 at endocardial cushions of atrioventricular canal (AVC) and outflow tract (OFT). EMT consists of a multiple cellular events including delamination of endocardial cells from the AVC endocardium, their invasion into the extracellular matrix, and the acquisition of mesenchymal phenotypes [4,5,6,7,8,9]. Even during EMT, endocardial cushions provide a valve-like function to prevent blood regurgitation in the developing heart [10].

Studies of EMT have identified Notch, Wnt, and Bmp pathways that act in the endocardium and/or myocardium of cushions to regulate this process. Global loss of Notch1 [11] or pan-endothelial loss of its ligand, Jagged1 [12] in the Notch pathway causes defective EMT, leading to hypocellular endocardial cushions. Similarly, ablation of endothelial Wnt [13] and myocardial Bmp [14,15] activities inhibit EMT and valve development. Furthermore, Ma and Martin et al. have reported that myocardial Bmp2 is required for endocardial Notch1 expression [15], and studies by Luna-Zurita and De la Pompa et al. have shown that Bmp2 drives EMT of ventricular endocardial cells which ectopically express active Notch1 [16]. How Notch, Wnt, and Bmp pathways coordinate in EMT process is still incompletely understood.

Global or pan-endothelial disruption of the Notch pathway also results in early vascular defects [17,18,19], in addition to the cardiac defects aforesaid. In this study, we seek to define specifically the role of endocardially produced Notch1 and Jagged1 in heart development by endocardial-specific deletion of Notch1 or Jagged1. We show that endocardial Jagged1-Notch1 signaling is required for EMT and regulates Wnt4 expression in the endocardium and Bmp2 expression in the myocardium. We also reveal that Wnt4 regulates Bmp2 expression. We further show that either Wnt4 or Bmp2 treatment rescues defective EMT resulting from Notch inhibition and Wnt4 rescuing requires Bmp activities. These results thus establish an endocardial to myocardial Notch-Wnt-Bmp signaling cascade essential for EMT during heart valve development.
Figure 1. Loss of Notch1 in the endocardium is embryonic lethal. A and B, wholemount X-gal staining of the E10.5 R26fslz;c1Cre embryo and yolk sac showing that the Cre recombinase-mediated LacZ expression (blue) was restricted to the heart (A) and not present in the yolk sac (B). C, sections of the X-gal stained embryos showing that LacZ expression was localized in the endocardium (arrowheads) and endocardial-derived cushion mesenchymal cells at the atrioventricular canal (AVC). D-G, Immunofluorescence showing that Notch1 protein is present in the AVC endocardium (D, arrowheads) and the pharyngeal vascular endothelium of N1ft/ft embryo (F, arrows), but not in the AVC endocardium of N1ft/c1Cre embryo (E, arrowheads). Note that Notch1 protein remains in the pharyngeal vascular endothelium of N1ft/c1Cre embryo (G, arrows). H and I, Wholemount views showing that E11.5 N1ft/c1Cre embryos were runted and had dilated pericardial sac (H) and E12.5, N1ft/c1Cre embryos were absorbed (I). J, summarizing the total number of embryos analyzed at different stages, indicating that N1ft/c1Cre embryos died between E11.5 and E12.5. The expected number of embryos at different stages is indicated in the parentheses.

doi:10.1371/journal.pone.0060244.g001

Methods

Mice

The endocardial Cre mouse line (Nfatc1Cre) has an IRES-Cre cassette inserted at the 3′ untranslated region of the mouse Nfatc1 [20][21]. The lacZ ROSA26 Cre reporter strain (R26fslz) [22] and Notch1ff (N1ff) [23] were obtained from the Jackson Laboratory (Bar Harbor, Maine). The Jagged1ff (J1ff) allele has been described previously [24]. The GFP Cre reporter line R26fg1flo [25,26] was a gift from Gordon Fishell (New York University Medical School). All mouse strains were maintained on C57B6 background and mouse experiments were performed according to the guideline of the National Institute of Health and the protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine (IACUC Approval Number: 20110303). Noontime on the day of detecting vaginal plugs was designated as E0.5. Mouse lines were PCR genotyped using tail or yolk sac DNA.

Histology and Immunohistochemistry

E9.5 and E10.5 embryos were dissected and fixed overnight at 4°C using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). They were then dehydrated through an ethanol gradient, treated with xylene, and embedded in paraffin wax. Embryos were oriented for sagittal sections (6 μm) using a Leica microtome. Hematoxylin and eosin (H&E) staining was performed for histology. H&E stained tissue sections were examined and photographed using a Zeiss Axios Observer Z1 inverted microscope. For quantification of the number of cushion mesenchymal cells, serial sections across the cushion region were used for cell
counting. The data were presented as the average number of cells per section from multiple embryos for each genotype. For immunohistochemistry, tissue sections were antigen retrieved by boiling for 10 minutes in sodium citrate (10 mM, pH 6.0) (Vector Laboratories) and blocked with 3% BSA in PBS before being incubated with primary antibodies and then secondary antibodies. Mouse anti-Notch1 antibodies (A6, Abcam) were used for detecting the membrane Notch1 as previously described [27]. The signal was amplified using the TSA Plus Cyanine 3 System (Perkin Elmer). Goat anti-jagged1 IgGs (C20, Santa Cruz) were used for detecting the membrane Jagged1. Immunostained tissue sections were photographed using a Zeica TCS SP5 confocal scope.

Cell Proliferation and Apoptosis Assays
Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation. A dose of 100 mg/kg BrdU (Invitrogen) was injected to the peritoneum of pregnant female mice two hours before isolation of embryos. E10.5 embryos were collected and fixed in 4% PFA at 4°C overnight. The fixed embryos were rinsed in PBS, dehydrated in a series of graded ethanol, paraffin embedded, and sectioned at 6 μm. BrdU-incorporated nuclei were detected using BrdU staining Kit (Invitrogen) following the manufacturer’s instructions. Serial sections of each embryo were examined and the results were presented as the mean percentage of BrdU-positive cells/total cells. The results from three age-matched control and endocardial Notch1 null littersmates were collected for statistical analysis. Cell apoptosis was analyzed by immunostaining using anti-cleaved-Caspase3 antibody (Cell Signaling Technology).

RNA Extraction and Quantitative PCR (qPCR)
Total RNAs were isolated from pooled AVC tissues from five E10.5 hearts using Trizol (Invitrogen). First strand cDNA was synthesized using the Superscript II Reverse Transcription Kit (Invitrogen). qPCR was performed using Power SYBR Green PCR Master Mix (ABI). Gene specific primers were used (Table S1). The relative level of gene expression was normalized to an internal control (level of Gapdh) and calculated using the 2^ΔΔCt method. Biological repeats were performed using three different samples for each genotype, and technical triplicates were carried out for each gene expression analysis. The mean relative expression of each gene between groups was used for statistical significant analysis.

Wholemount X-gal Staining
Wholemount X-gal staining was performed as previously described [28]. E10.5 embryos were dissected, fixed in 4% PFA for 30 minutes at 4°C, washed twice in PBS containing 2 mM MgCl2 and once in PBS (pH 7.5)/2 mM MgCl2/0.1% deoxycholic acid/0.2% NP-40. The X-gal staining/reaction was developed in the same buffer containing 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, and 0.6 mg/mL X-gal (Promega) at room temperature (RT) for six hours. The reaction was stopped by washing the embryos in PBS/0.5 mM EDTA. The stained embryos were photographed using a Zeiss Discovery V12 stereo scope. The stained embryos were then post-fixed, dehydrated, embedded in paraffin, sectioned and photographed using the Zeiss Axios Observer Z1 inverted microscope.

Wholemount Pecam1 Antibody Staining
E10.5 embryos were fixed in 4% PFA in PBS for 2 hours at 4°C, dehydrated through a methanol series, and bleached in 6% H2O2/methanol for 1 hour. Embryos were then rehydrated, post-fixed in 4% PFA/0.2% glutaraldehyde for 20 minutes at room temperature and washed twice in PBST containing 0.1% Tween-20. The embryos were then blocked in PBSST containing 1% BSA and 0.1% Tween-20 for two hours, and incubated with Rat anti-Pecam1 antibodies (MEC13.3, BD Bioscience) in PBST overnight at 4°C. The following day, the embryos were washed five times in PBST and incubated overnight at 4°C with an HRP-conjugated donkey anti-rat secondary antibody (Vector Laboratories). On the third day, the embryos were washed five times in PBST and incubated in A&B solution (Vector Laboratories) overnight at 4°C, before being washed four times in PBST; rinsed once in PBST (PBS containing 0.1% tween20), and developed with the DAB Kit (Vector Laboratories). The stained embryos were then washed in PBS, post-fixed in 4% PFA, dehydrated through a methanol series, and imaged using a Zeiss discovery microscope. Three age-matched embryos from control or null groups were examined simultaneously.

RNA in situ Hybridization
RNA in situ hybridization (ISH) was performed according to a previously described protocol [29]. Digoxigenin-labeled complementary RNA probes for Wnt4 and Bmp2 mRNA were prepared from the linearized plasmids by reverse transcription. E10.5 embryos were isolated in RNase-free PBS, fixed overnight in 4%PFA in PBS at 4°C, dehydrated through a methanol series, rehydrated and treated for one hour at room temperature (RT) with 5% H2O2 to quench the endogenous peroxidases. The embryos were then proteinase K (10 μg/mL) digested for 15 minutes at RT, refixed with 4% PFA/0.2% glutaraldehyde, and hybridized overnight at 70°C with antisense DIG-labeled RNA probes. The following day, the embryos were washed, blocked, and incubated overnight with an alkaline-phosphatase (AP) conjugate anti-DIG antibody. AP activity was detected using BM Purple (Roche). Embryos were post-fixed in 4%PFA/0.1% glutaraldehyde prior to visualization and imaging in PBS using a Zeiss discovery microscope. The stained embryos were next post-fixed, dehydrated, embedded in paraffin, sectioned and photographed using the Zeiss Axios Observer Z1 inverted microscope. For each gene, four to five age-matched embryos from control or mutant groups were analyzed simultaneously.

Wnt2 Conditioned Medium
Wnt2-conditioned medium and control medium were prepared as described previously [30]. CHO cells were stably transduced with pcDNA3-mWnt2 or pcDNA3 vector. Cell lines were cultured in DMEM with 10% FBS until confluence. Medium was replaced with DMEM containing 0.5% FBS and the supernatant was collected after 24-hour culture and stored in −20°C.

In vitro EMT Assay
EMT assay was performed as described previously [5,9]. The AVC tissues were microdissected out from E9.5 hearts and cultured on rat-tail collagen gel in 4-well plates. Explants were cultured for 48 hours, and cells that migrated away from explants and invaded the gel were counted. For rescue experiments, DAPT (50 μM) (Tocris Bioscience), BMP2 (200 ng/ml) (R&D systems), Wnt2 conditioned medium, Wnt4 (250 μg/ml) (R&D systems), and Noggin (200 ng/ml) (R&D systems) were added to explant culture and changed after 24 hours.
Whole Embryo Culture

Whole embryo culture was performed as described previously [31]. E9.5 embryos were dissected out in pre-warmed Hank’s Balanced Salt Solution (HBSS) (Sigma) with intact yolk sac. The embryos were cultured with whole embryo culture media containing 75% Rat whole embryo culture serum (Harlan, Laboratories, BT4520), 25% HBSS, 1% Pen-strep and 2 mg/mL of glucose. The embryos were cultured in incubator with specialized gas (60% O2, 40% N2, 5% CO2).

Figure 2. Endocardial-specific deletion of Notch1 does not affect early vascular formation. A–C, wholemount view showing the yolk sac vessels in E9.5 N1ff (A) and N1ff;c1Cre (B) embryos; they are not present in the N1ff;Tie1Cre embryos (C). D–I, Pecam1 staining showing mature vessels in the yolk sac and head of N1ff (D and G) and N1ff;c1Cre embryos (E and H); they are not formed in the N1ff;Tie1Cre embryos (F and I). J–L, wholemount view showing mature yolk sac vessels in E10.5 N1ff (J) and N1ff;c1Cre (K), but not in N1ff;Tie1Cre embryos (L). M–R, Pecam1 staining showing mature vascular networks in the yolk sac and head of E10.5 N1ff (M and P) and N1ff;c1Cre (N and Q) but not N1ff;Tie1Cre (O and R) embryos. doi:10.1371/journal.pone.0060244.g002

Figure 3. Disruption of endocardial Jagged1-Notch1 signaling blocks EMT and formation of endocardial cushions. A–D, wholemount views showing that at E10.5 the gross morphology was comparable between N1ff (A) and N1ff;c1Cre (B) embryos. Similar results were observed between J1ff (C) and J1ff;c1Cre (D) embryos. E–L, H&E stained sections through the atrioventricular canal (avc) region of E10.5 embryos showing dense mesenchymal cells (arrows) in the cushion of N1ff (E and I) or J1ff (G and K) hearts, but fewer mesenchymal cells in the same region of N1ff;c1Cre (F and J) or J1ff;c1Cre (H and L) hearts. M and N, quantitative analysis of the number of mesenchymal cells in the cushion of N1ff;c1Cre (M) or J1ff;c1Cre (N) hearts. *p<0.001. O–Q, in vitro collagen gel EMT assay showing that while ~80 endocardial cells (arrows) migrated away from each N1ff explant (O and Q), fewer (25/explant) cells underwent this process in cultured N1ff;c1Cre explants (P and Q). *p<0.001. doi:10.1371/journal.pone.0060244.g003
Figure 4. Endocardial Jagged1-Notch1 signaling regulates expression of endocardial Wnt4 and myocardial Bmp2. A, qPCR analysis of EMT gene expression in the atrioventricular canal (avc) from E10.5 N1f/f or N1f/f;c1Cre hearts. Each cDNA sample was prepared from five avc tissues and three samples of each group were used for qPCR. Gene expression was normalized to Gapdh. *p<0.05; **p<0.01. B–E, RNA in situ hybridization showing endocardial Wnt4 (B, ec, arrowheads) and myocardial Bmp2 expression (D, myo, arrows) in E10.5 N1f/f hearts. Their expression is dramatically reduced in N1f/f;c1Cre hearts (C and E). F, qPCR analysis of EMT gene expression in the AVC cushions of E10.5 J1f/f or J1f/f;c1Cre hearts. G–J, RNA in situ hybridization showing that Wnt4 and Bmp2 expression is downregulated in J1f/f;c1Cre hearts. a, atrium and v, ventricle.

doi:10.1371/journal.pone.0060244.g004
oxygen, 5% CO2 and 35% N2) at 37 C for 24 hours. In some experiments, Wnt inhibitor Wif1 (2.5 mg/ml) (R&D systems) or Wnt4 (250 ng/ml) was added into the culture medium. The cultured embryos were used for gene expression assays using qPCR and/or RNA in situ Hybridization as described above. Statistical Analysis

Statistical analysis was performed using Microsoft Excel and all data were presented as mean ± SD. Student’s t-Test was used for comparison between groups and Probability (p) values <0.05 were considered as significant.

Results

Loss of Notch1 or Jagged1 in the Endocardium is Embryonic Lethal

To further characterize the role of Notch signaling in the endocardium for heart development, we deleted Notch1 or Jagged1 in the endocardium during early mouse embryogenesis using the Nfat1-Cre mouse (c1Cre) [21], a knockin Cre at the Nfat1 locus which codes an endocardial-specific gene [28,32,33,34,35]. As shown in Figure 1A and 1C, c1Cre activated lacZ expression specifically in the endocardium and its mesenchymal derivatives of E10.5 Rosa26 floxed-stop-lacZ (R26fslz) embryos. However, the lacZ expression was not found in the yolk sac (Figure 1B). We crossed c1Cre with floxed Notch1 mice (N1f/f) to generate endocardial Notch1 knockout embryos (N1f/f;c1Cre, hereafter) and confirmed by immunofluorescent staining that Notch1 protein in endocardium (but not vascular endothelium) was abolished at E10.5 (Figure 1D to 1G). All N1f/f;c1Cre embryos died between E11.5 and E12.5 with severe pericardial effusion (Figure 1H to 1J), suggesting insufficient heart function. Of note, the timing of embryonic death in our study is different from that in previous studies of germline or pan-endothelial deletion of Notch1, which cause embryonic death before E10.5 [17,18].

Figure 5. Bmp2 expression is regulated by Wnt signaling. A, qPCR analysis showing that Bmp2 expression was inhibited by Wif1 and induced by Wnt4. E9.5 embryos were cultured in the control media or media with Wnt inhibitor Wif1 or recombinant mouse Wnt4. After 24-hour culture, each RNA sample was prepared from AVC tissues of 5 hearts for each treatment. The data from three independent samples for each group were used for statistical calculation. *p<0.05 and **p<0.01. B–D, RNA in situ hybridization analysis showing Bmp2 expression (indicated by arrows) in cultured wild type embryos under indicated condition; cushion myocardial Bmp2 expression was inhibited by Wif1 (C) and induced by Wnt4 (D). E–G, RNA in situ hybridization showing Bmp2 expression in cultured N1f/f (E), N1f/f;c1Cre embryos (F), and N1f/f;c1Cre embryo treated with Wnt4 (G). The data indicated that Bmp2 expression was reduced in the N1f/f;c1Cre embryo when compared to the N1f/f embryo. However, Wnt4 treatment restored its expression in the N1f/f;c1Cre embryo (G). a, atrium and v, ventricle.

doi:10.1371/journal.pone.0060244.g005
We then compared control N1f/f, N1f/f;c1Cre, and pan-endothelial Notch1 null embryos (N1f/f;Tie1Cre) (Figure 2). N1f/f;c1Cre embryos developed to E9.5–E10.5 with global appearance comparable to control N1f/f embryos (Figure 2A, 2J vs. 2B and 2K). However, N1f/f;Tie1Cre embryos were runted (Figure 2C and 2L). Pecam1 immunostaining showed normal vessels in the yolk sac (Figure 2D and 2E) and head (Figure 2G and 2H) of E9.5 N1f/f and N1f/f;c1Cre embryos, but not in N1f/f;Tie1Cre embryos (Figure 2F and 2I). By E10.5 we noticed small differences in gross morphology of yolk sac and head vessels between N1f/f embryos (Figure 2M and 2P) and N1f/f;c1Cre embryos (Figure 2N and 2Q), but the patterning of vasculature appeared normal, suggesting a potential defect in vascular development secondary to insufficient cardiac function. In contrast, early vascular development in N1f/f;Tie1Cre embryos at this stage was completely arrested (Figure 2O and 2R), as described before [18]. In addition, we generated endocardial Jagged1 knockout (J1f/f;c1Cre) mice using the c1Cre and floxed Jagged1 mice (J1f/f) and confirmed its endocardial deletion by immunofluorescence (Figure S1). This deletion resulted in similar defects in vascular development (data not shown). Our results demonstrate that the Jagged1-Notch1 signaling in the endocardium is essential for embryonic survival, likely through its primary functions in the regulation of heart development [11,12,16,36,37,38].

Disruption of Endocardial Jagged1-Notch1 Signaling Blocks EMT and Endocardial Cushion Formation

We then examined E10.5 N1f/f;c1Cre or J1f/f;c1Cre embryos and found that they appeared normal comparing to their N1f/f or J1f/f littermates (Figure 3A to 3D). However, on cross sections, we
Figure 7. Working model shows Notch-Wnt-Bmp signaling axis that regulates EMT and early valve formation. A, Schematic showing the cardiac phenotypes found in the endocardial Jagged1 or Notch1 knockout embryos. During E9.5 to E10.5, cushion endocardial cells undergo EMT and form endocardial cushions at the atrioventricular canal and outflow tract of the wild-type (WT) embryos. This process is disrupted in the endocardial Jagged1 (N1f/f;C1Cre) or Notch1 (N1f/f;C1Cre) knockout (KO) embryos, which results in hypocellular endocardial cushions. B, Endocardial Jagged1-mediated Notch1 activation induces expression of Wnt4, which subsequently upregulates expression of Bmp2 in the adjacent myocardium. Myocardial Bmp2 then acts on endocardial cells to promote EMT. This Notch-Wnt-Bmp signaling axis promotes EMT during heart valve development.

doi:10.1371/journal.pone.0060244.g007

noted that while mesenchymal cells derived from cushion endocardial cells via EMT had populated endocardial cushions at the AVC of N1f/f (Figure 3E and 3I) and J1f/f embryos (Figure 3G and 3K), much fewer mesenchymal cells were present in the cushions of N1f/f;C1Cre (Figure 3F and 3J) and J1f/f;C1Cre embryos (Figure 3H and 3L). Quantitative analysis showed significant reduction in the number of mesenchymal cells in AVC cushions of N1f/f;C1Cre and J1f/f;C1Cre embryos (Figure 3M and 3N). We also observed the same phenotype in the outflow tract (OFT) cushions of E10.5 N1f/f;C1Cre and J1f/f;C1Cre embryos (data not shown). We then studied EMT process by fate mapping cushion endocardial cells in E10.5 control (N1f/f;R26flx;floxed), and endocardial Notch1 knockout (N1f/f;R26flx;floxed) embryos. X-gal staining showed that LacZ-positive mesenchymal descendants of endocardial cells populated the AVC cushions of N1f/f;R26flx;floxed embryos (Figure S4A; A); in contrast, the number of LacZ-positive mesenchymal cells was dramatically reduced in the AVC cushions of N1f/f;R26flx;floxed embryos (Figure S4B, A). The same result was found at OFT cushions (Figure S4, A and D). We subsequently examined EMT by culturing AVC of E9.5 hearts on collagen gels. We found that while endocardial cells migrated away from N1f/f explants and became elongated mesenchymal cells that invaded the gel (Figure 3O and 3Q), fewer endocardial cells underwent EMT in N1f/f;C1Cre AVC explants (Figure 3P and 3Q). Our findings are consistent with previous observations made in the standard knockout [11] or pan-endothelium knockout [12] and confirm that endocardial Jagged1-Notch1 signaling is required for EMT.

Endocardial Jagged1-Notch1 Signaling Regulates Expression of Endocardial Wnt4 and Myocardial Bmp2

To identify the factors that mediate endocardial Jagged1-Notch1 signaling for EMT, we examined expression of Notch targets and genes known to be involved in EMT by qPCR. The results showed that expression of Hey1, a major nuclear factor mediating canonical Notch signaling [39], was significantly reduced in AVC cushions of E10.5 N1f/f;C1Cre hearts, whereas expression of p21, a non-canonical Notch effector [39], was not affected (Figure 4A). Expression of Snail1, Snail2, Msx1, and Msx2, transcription factors involved in EMT [11, 40, 41] was also downregulated in N1f/f;C1Cre AVC cushions. However, expression of Vcad was unchanged in AVC cushions of E10.5 N1f/f;C1Cre hearts. Interestingly, we found that expression of Wnt4, Wnt4b, Bmp2, Bmp4, Bmp5, Bmp6, and Bmp7 was significantly decreased in the N1f/f;C1Cre AVC cushions (Figure 4A). In addition, expression of Tgfb2 and Nog1 was decreased in the AVC cushions of E10.5 N1f/f;C1Cre hearts. Using RNA in situ hybridization, we further examined expression of Wnt4 and Bmp2, which are specific for AVC endocardium and myocardium, respectively. The results showed that their expression was abolished in the E10.5 N1f/f;C1Cre hearts (Figure 4B to 4E). Similar changes in gene expression were observed in E10.5 J1f/f;C1Cre hearts using qPCR (Figure 4F)
RNA in situ hybridization (Figure 4G to 4J). These results indicate that endocardial Jagged1-Notch1 signaling regulates endocardial Wnt and myocardial Bmp activities to ensure proper EMT at the valve ontogenic site. They also suggest that endocardially produced Wnt ligands may be responsible for Bmp2 expression in the adjacent myocardium.

Endocardial Wnt Ligands Regulate Myocardial Bmp2 Expression

To determine whether expression of Bmp2 and other genes involved in the EMT was regulated by Wnt ligands, we cultured E9.5 embryos for one day with Wnt antagonist Wi1 [42] or recombinant mouse Wnt4 protein and examined their effect on the expression of genes listed in Figure 4A using qPCR. The results showed that among the genes examined, expression of Bmp2 and Mesp1 was significantly decreased after Wi1 treatment, and conversely, significantly increased after Wnt4 treatment (Figure 5A). We further confirmed the Wnt-dependent expression of Bmp2 in the AVC myocardium using RNA in situ hybridization (Figure 5B to 5D). Moreover, Wnt4 treatment recovered Bmp2 expression in the AVC myocardium of cultured E9.5 N/Dp10f/f c1CreVE-cad embryos (Figure 5E to 5G). These results demonstrate that cushion myocardial Bmp2 expression is regulated by Wnt signals from the adjacent AVC endocardium. This most likely occurs through the cushion endocardial-specific Wnt ligand Wnt4, whose expression is dependent upon endocardial Jagged1-Notch1 signaling.

Endocardial to Myocardial Notch-Wnt-Bmp Signaling Axis Regulates EMT

To further determine whether Wnt-dependent Bmp2 expression functions directly downstream of Notch signaling to mediate EMT, we performed rescue experiments using the collagen gel EMT assay. We cultured AVC explants isolated from E9.5 R26fsGFP;c1CreVE-cad embryos, in which the cushion endocardial cells and their mesenchymal descendants were marked by GFP expression, allowing direct visualization of migration and invasion by endocardial cells (Figure 6A). While explants treated with DAPT to block Notch activities abolished EMT by the endocardial cells (Figure 6B and 6G), Wnt2, Wnt4, or BMP2 treatment was able to rescue EMT defect caused by inhibition of Notch signaling (Figure 6C, 6D or 6E, respectively). Of particular note, blocking Wnt2, Wnt4, or BMP2 expression in the AVC myocardium using RNA in situ hybridization (Figure 6F) was sufficient to induce myocardial Bmp2 expression (Figure 4). Moreover, the rescue experiments demonstrate that Wnt2 or Wnt4 can restore Bmp2 expression in the AVC myocardium of endocardial Notch1 knockout embryos (Figure 5). Together these results suggest that endocardial Notch-Wnt signaling regulates myocardial Bmp2 expression.

We do not know how endocardial Notch signaling regulates expression of Wnt ligands in the endocardium. Further studies are needed to determine whether canonical or non-canonical Notch signaling or both regulate expression of Wnt. Similarly, how endocardial Wnt regulates myocardial Bmp2 expression needs to be investigated in the future. In addition, previous studies have shown that the levels of cushion myocardial Bmp2 expression are not changed in the Rbpj germline knockout embryos [16]. However, our study indicates that endocardial Notch signaling is required for Bmp2 expression in the AVC myocardium. This discrepancy might be due to the difference in the embryonic stages analyzed in the previous and our studies. Alternatively, Rbpj mutant affects mainly the canonical Notch signaling, whereas endocardial Notch1 deletion impairs both canonical and non-canonical signaling.

It is well known that signals from the AVC myocardium induce EMT by the endocardial cells [4,6,7,8]. For instance, myocardial Bmp2 regulates endocardial Notch1 expression and is required for EMT [15,16]. Our findings indicate that endocardial Notch signaling regulates Bmp2 expression in the AVC myocardium. We also show that multiple Wnt ligands are downregulated in the endocardial Notch1 or Jagged1 knockout hearts (Figure 4) and either Wnt2 or Wnt4 can rescue the impaired EMT resulting from Notch inhibition (Figure 6). It is worth to mention that Wnt4 knockout mice survive to birth [37,48], whereas Wnt2-null AVC explants exhibit poor EMT [49]. Loss of the common nuclear

Discussion

Previous studies have shown that Notch, Wnt, and Bmp signals in the endocardium and myocardium of the valve ontogenic site regulate EMT and early heart valve formation [1,11,12,16,36,37,38,43,44]. In addition, interactions between Notch and BMP signaling have been found in the developing endocardial cushions [15,16]. This study further addresses how endocardial and myocardial signals coordinate to direct EMT. We used genetic knockouts, EMT assays, and rescue experiments to uncover a signaling cascade from endocardial Notch-Wnt to myocardial Bmp that directs EMT to form the primitive heart valves.

We show that endocardial-specific Notch1 or Jagged1 knockout embryos are embryonic lethal and they die around E11.5 with pericardial effusion (Figure 1). This finding is of significance as endocardial Notch1 or Jagged1 null embryos develop normal vascular networks in the yolk sac and embryo at E9.5–10.5, whereas embryos with pan-endothelial loss of Notch1 or Jagged1 die around E9.5–10.5 with severely defective vascular angiogenesis (Figure 2, data not shown) as shown previously [18,19]. Thus, our mouse models allowed us localize the primary role of endocardial Jagged1-Notch1 signaling in EMT and early heart valve development (Figure 3), which has been described in previous studies [11,12,16,38].
effect of Wnt signaling, beta-catenin, in the endocardium, results in severely defective EMT and hypocellular endocardial cushions [13]. These observations suggest that the redundant expression of Wnt ligands in the AVC endocardium [50], also found in our study (Figure 4), compensates each other’s function.

Together with previous studies [15,16], our study suggests a reciprocal Notch-Bmp signaling between the endocardium and myocardium at the valve ontogenic site. This endocardial-myocardial signaling begins with endocardial Jagged1-Notch1 signaling; it regulates expression of endocardial Wnt4 or other Wnt ligands, which induces myocardial Bmp2 expression; myocardial Bmp2 then promotes endocardial cells to undergo EMT [15,51] (Figure 7). This study thus improves the current understanding of EMT in early heart valve development and may provide new insights into the genesis of congenital valve defects.

Supporting Information

Figure S1 Endocardial deletion of Jagged1 by CItcre. Immunofluorescence showing Jagged1 protein in the cushion endocardial cells (arrows) in E10.5 J1f/f hearts (A). In contrast, the level of Jagged1 protein in the endocardium was greatly reduced in the J1f/f;CItcre (B) hearts. (TIF) Figure S2 Hypocellular cushions at the outflow tract region in endocardial Notch1 or Jagged1 null embryos. Histological analysis of E10.5 embryo sections through the outflow tract (OFT) region showing that endocardial Notch1 knockout (J1f/f;CItcre) (G and D) or Jagged1 knockout (J1f/f;CItcre) (G and H) hearts have hypocellular OFT cushions compared to control, J1f/f (A and B) or J1f/f (E and F). (TIF) Figure S3 Endocardial specific disruption of Notch1 does not affect cell proliferation in the endocardial cushions. A–C, BrdU incorporation and immunostaining showing that the percentage of proliferating cells in the endocardium (arrowheads) or cushion mesenchyme (arrows) is comparable at the atrioventricular canal (avc) and endocardial Notch1 knockout (J1f/f;CItcre) embryos. Serial sections throughout the avc of each embryo were used for cell counting and data from three embryos from each group were analyzed for statistical significance. (TIF)

Figure S4 Lineage tracing of endocardial cells in endocardial Notch1 knockout hearts. A–D, photos of X-gal stained sections through the endocardial cushions of E10.5 hearts showing that the descendants of endocardial cells contribute a dense cushion mesenchyme to the atrioventricular canal (vc), and outflow tract (ot), cushions of the J1f/f;R26flslz;c1Cre embryos. Such contribution is reduced in the J1f/f;R26flslz;c1Cre;BZ embryos (B and D). (TIF)

Table S1 List of primers used in qPCR. (DOCX)

Acknowledgments

We thank Bernice Morrow for Wnt4 RNA probe and Gordon Fishell for R26flslz mice.

Author Contributions

Designed and generated the Nfatc1-Cre mouse line: BW BZ. Obtained permission for use of floxed Jagged1 conditional mouse line: VT. Obtained permission for use of Wnt2 cell line: DK. Conceived and designed the experiments: YW BW BZ. Analyzed the data: YW BW AAC WL PK BZ. Contributed reagents/materials/analysis tools: YW BW KS DK VT BZ. wrote the paper: YW BZ.

References

1. Armstrong EJ, Bischoff J (2004) Heart valve development: endothelial cell signaling and differentiation. Circ Res 95: 459–470.
2. Combs MD, Yutzey KE (2009) Heart valve development: regulatory networks in development and disease. Circ Res 105: 408–421.
3. Lin CJ, Lin CY, Chen CH, Zhou B, Chang CF (2012) Partitioning the heart: mechanisms of cardiac seption and valve development. Development 139: 3277–3289.
4. Eisenberg LM, Markwald RR (1995) Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circulation Research 77: 1–6.
5. Camenisch TD, Molin DG, Person A, Runyan RB, Gittenberger-de Groot AC, et al. (2002) Temporal and distinct TGFbeta ligand requirements during mouse cardiac valve development. Circulation Research 77: 1–6.
6. Schroeder JA, Jackson LF, Lee DC, Camenisch TD (2003) Endocardial-epicardial reciprocal Notch-Bmp signaling between the endocardium and myocardium in the developing heart valves: coordination by extracellular matrix and growth factor signaling. Circulation Research 103: 392–403.
7. Barnett JV, Desgrosellier JS (2003) Early events in valvulogenesis: a signaling perspective. Birth Defects Research Part C, Embryo Today: Reviews 69: 58–72.
8. Lencinas A, Tavares AL, Barnett JV, Runyan RB (2011) Gelatin gel analysis of epithelial-mesenchymal transition in the embryo heart: an in vitro model system for the analysis of tissue interaction, signal transduction, and environmental effects. Birth defects research Part C, Embryo today: reviews 93: 298–311.
9. Wu B, Wang Y, Liu Y, Langworthy M, Tompkins KL, et al. (2011) Nfatc1 coordinates valve endocardial cell lineage development required for heart valve formation. Circulation Research 109: 183–192.
10. Baldwin HS (1999) Advances in understanding the molecular regulation of cardiac development.Curr Opin Pediatr 11 Suppl 1: i1–10.
11. Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, et al. (2004) Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev 18: 99–115.
12. Hofmann JJ, Briot A, Enciso J, Zovein AC, Ren S, et al. (2012) Endothelial deletion of murine Jag1 leads to valve calcification and congenital heart defects associated with Alagille syndrome. Development 139: 4449–4460.
13. Liebner S, Cattelino A, Gallini R, Rudini N, Iurlaro M, et al. (2004) Beta-catenin is required for endothelial-mesenchymal transformation during heart development in the mouse. J Cell Biol 166: 359–367.
14. Jiao K, Kulessa H, Tompkins K, Zhou Y, Batt L, et al. (2003) An essential role of Bmp2 in the atrioventricular seption of the mouse heart. Genes Dev 17: 2959–2967.
15. Ma I, Lu MF, Schwarz RJ, Marin JF (2005) Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. Development 132: 3601–3611.
16. Luna-Zurita I, Prados B, Grego-Bessa J, Luxan G, del Monte G, et al. (2010) Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation. The Journal of clinical investigation 120: 3495-3507.
17. Krohs LT, Xue Y, Norton CR, Shutter JR, Maguire M, et al. (2000) Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 14: 1343–1352.
18. Limbourg FP, Takeshita K, Radtke F, Bronson RT, Chin MT, et al. (2005) Essential role of endothelial Notch1 in angiogenesis. Circulation 111: 1832–1832.
19. High FA, Lu MM, Pear WS, Loesmes KM, Kaestner KH, et al. (2008) Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc Natl Acad Sci U S A 105: 1953–1959.
20. Zhou B, Cron RJ, Wu B, Gerini A, Wang Z, et al. (2004) Regulation of the murine Nfatc1 gene by NFATc2. J Biol Chem 279: 10704–10711.
21. Wu B, Zhang Z, Liu W, Chen X, Wang Y, et al. (2012) Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. Cell 151: 1083–1096.
22. Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21: 70–71.
23. Yang X, Klein R, Tian X, Cheng HT, Kopan R, et al. (2004) Notch activation induces epithelial-mesenchymal transition in neural progenitor cells through a p35-dependent pathway. Dev Biol 266: 81–94.
24. Nyfeler Y, Kirch RD, Mantei N, Leone DP, Radtke F, et al. (2005) Jagged1 signaling is essential for vascular morphogenesis in mice. Genes Dev 19: 1832.
25. Sousa VH, Miyoshi G, Hjerling-Leffler J, Karayannis T, Fishell G (2009) Characterization of Nkx6-2-derived neocortical interneuron lineages. Cerebral cortex 19 Suppl 1:i1–i10.

PLOS ONE | www.plosone.org 11 April 2013 | Volume 8 | Issue 4 | e60244
26. Miyoshi G, Hjerling-Leffler J, Karayannis T, Sousa VH, Butt SJ, et al. (2010) Genetic fate mapping reveals that the caudal ganglionic eminence produces a large and diverse population of superficial cortical interneurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 30: 1582–1594.

27. Del Monte G, Grego-Bessa J, Gonzalez-Rajal A, Bolos V, De La Pompa JL (2007) Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. Dev Dyn 236: 2594–2614.

28. Zhou B, Wu B, Tompkins KL, Boyer KL, Grindley JC, et al. (2005) Characterization of Nfatc1 regulation identifies an enhancer required for gene expression that is specific to pro-valve endocardial cells in the developing heart. Development 132: 1137–1146.

29. Stankunas K, Hang CT, Tsun ZY, Chen H, Lee NV, et al. (2008) Endocardial Brg1 represses ADAMTS1 to maintain the microenvironment for myocardial morphogenesis. Dev Cell 14: 298–311.

30. Klein D, Demory A, Peyre F, Kroll J, Augustin HG, et al. (2008) Wnt2 acts as a cell type-specific, autocrine growth factor in rat hepatic sinusoidal endothelial cells cross-stimulating the VEGF pathway. Hepatology 47: 1018–1031.

31. Hang CT, Chang CP (2012) Use of whole embryo culture for studying heart development. Methods in Molecular Biology 843: 3–9.

32. Misfeldt AM, Boyle SC, Tompkins KL, Bautch VL, Labosky PA, et al. (2009) Endocardial cells are a distinct endothelial lineage derived from Flk1+ multipotent cardiovascular progenitors. Dev Biol 333: 78–89.

33. de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, et al. (1998) Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum.[see comment]. Nature 392: 182–189.

34. Ranger AM, Grusby MJ, Hodge MR, Gravallese EM, de la Brousse FC, et al. (1998) The transcription factor NF-ATc is essential for cardiac valve formation.[see comment] Nature 392: 182–189.

35. Chang CP, Neilson JR, Bayle JH, Gestwicki JE, Kuo A, et al. (2004) A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis.[see comment] Cell 118: 679–693.

36. Noseda M, McLean G, Niessen K, Chang L, Pollet I, et al. (2004) Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. Circ Res 94: 910–917.

37. Grego-Bessa J, Luna-Zurita L, del Monte G, Bolos V, Melgar P, et al. (2007) Notch signaling is essential for ventricular chamber development. Dev Cell 12: 415–429.

38. Leong KG, Niessen K, Kulir I, Raouf A, Eaves C, et al. (2007) Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. The Journal of experimental medicine 204: 2933–2948.

39. Niesen K, Karsan A (2008) Notch signaling in cardiac development. Circ Res 102: 1189–1181.

40. Chen YH, Ishii M, Sucov HM, Masson RE, Jr. (2008) Mx1 and Mx2 are required for endothelial-mesenchymal transformation of the atrioventricular cushions and patterning of the atrioventricular myocardium. BMC Dev Biol 8: 75.

41. Niesen K, Fu Y, Chang I, Hoodless PA, McFadden D, et al. (2008) Slug is a direct Notch target required for initiation of cardiac cushion cellularization. J Cell Biol 182: 323–325.

42. Hu J, Dong A, Fernandez-Ruiz V, Shan J, Kawa M, et al. (2009) Blockade of Wnt signaling inhibits angiogenesis and tumor growth in hepatocellular carcinoma. Cancer Res 69: 6951–6959.

43. de la Pompa JL (2009) Notch signaling in cardiac development and disease. Pediatric Cardiol 30: 645–650.

44. High FA, Epstein JA (2008) The multifaceted role of Notch in cardiac development and disease. Nat Rev Genet 9: 49–61.

45. Romano LA, Runyan RB (1999) Slug is a mediator of epithelial-mesenchymal cell transformation in the developing chicken heart. Dev Biol 212: 243–254.

46. Romano LA, Runyan RB (2000) Slug is an essential target of TGFbeta2 signaling in the developing chicken heart. Developmental Biology 223: 91–102.

47. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. Nature 397: 405–409.

48. Stark K, Vainio S, Vasileva G, McMahon AP (1994) Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372: 679–683.

49. Tian Y, Yuan L, Goss AM, Wang T, Yang J, et al. (2010) Characterization and in vivo pharmacological rescue of a Wnt2-Gata6 pathway required for cardiac inflow tract development. Developmental cell 18: 275–287.

50. Aliferi CM, Cheek J, Chakraborty S, Yutzey KE Wnt signaling in heart valve development and osteogenic gene induction. Dev Biol 338: 127–135.

51. Gaussin V, Van de Patte T, Mishina Y, Hanks MC, Zwijsen A, et al. (2002) Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. Proc Natl Acad Sci U S A 99: 2878–2883.