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Control of sinus venous valve and sinoatrial node development by endocardial NOTCH1

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Aims Sinus venous valve (SVV) and sinoatrial node (SAN) develop together at the sinoatrial junction during embryogenesis. SVV ensures unidirectional cardiac input and SAN generates sinus rhythmic contraction, respectively; both functions are essential for embryonic survival. We aim to reveal the potential role of endocardial NOTCH signalling in SVV and SAN formation.

Methods and results We specifically deleted *Notch1* in the endocardium using an *Nfatc1*^{Cre} line. This deletion resulted in underdeveloped SVV and SAN, associated with reduced expression of T-box transcription factors, *Tbx5* and*Tbx18*, which are essential for the formation of SVV and SAN. The deletion also led to decreased expression of *Wnt2* in myocardium of SVV and SAN. WNT2 treatment was able to rescue the growth defect of SVV and SAN resulted from the *Notch1* deletion in whole embryo cultures. Furthermore, the *Notch1* deletion reduced the expression of *Nrg1* in the SVV myocardium and supplement of NRG1 restored the growth of SVV in cultured *Notch1* knockout embryos.

Conclusion Our findings support that endocardial NOTCH1 controls the development of SVV and SAN by coordinating myocardial WNT and NRG1 signalling functions.

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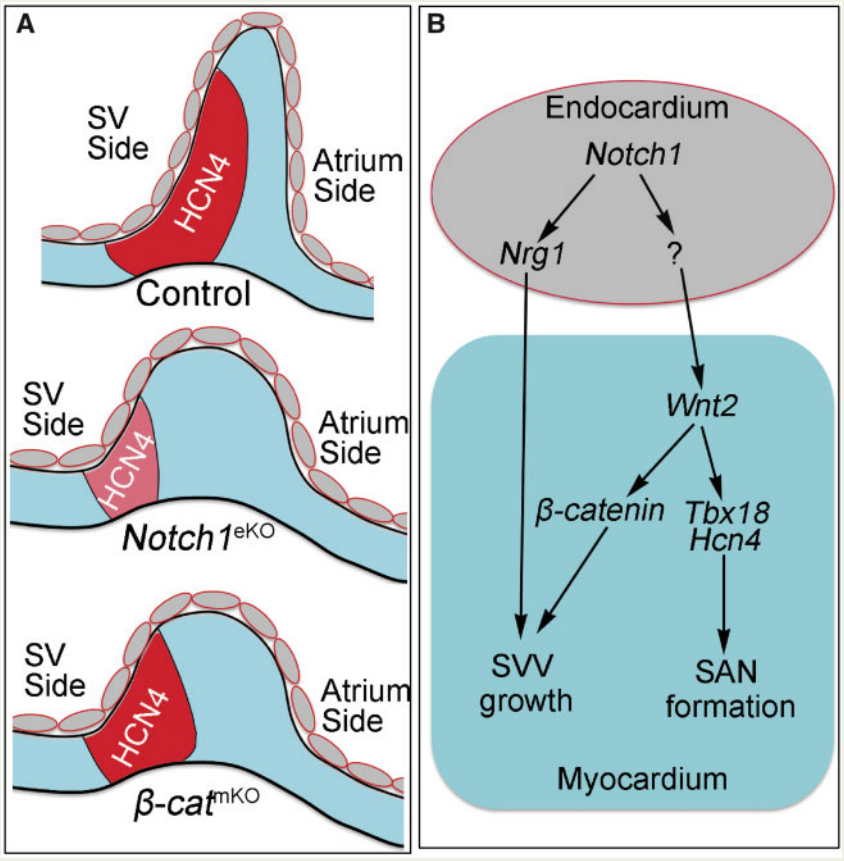
Graphical Abstract



Keywords Sinus venous valve • Sinoatrial node • NOTCH1 • WNT2 • NRG1

1. Introduction

Sinus venous valve (SVV) and sinoatrial node (SAN) are essential for maintaining unidirectional circulation and sinus rhythmic contraction, respectively.[1] SVV and SAN form together at the sinoatrial junction during development.[1] In mouse, SVV begins to form at embryonic day (E) 9.5 and is matured by E11.5.[2] It prevents the cardiac regurgitation in the early embryos before the formation of functional atrioventricular and semilunar valves.[3,4] At the same time of SVV development, a cluster of cardiomyocytes differentiates into specialized primary pacemaker cells that form the SAN at the base of the developing SVV.[5,6] SAN cardiomyocytes express HCN4 and generate the pacer action potential that controls the sinus heart rate and rhythm.[7]

Emerging data from recent studies have underpinned a unique gene regulatory network controlling the development of SVV and SAN, in particular, the differentiation of primary cardiac pacemaker cells.[8] Studies by Christoffels *et al.*[6] have shown that SAN progenitors arise from the cardiogenic mesoderm as a genetically distinct subpopulation.[9] The proliferation and differentiation of these progenitor cells into SVV/SAN myocardium are regulated by a transcriptional network consisting of the T-box transcription factors, *Tbx3*,[10] *Tbx5*,[11] and *Tbx18*,[12] as well as the short stature homeobox transcription factors, *Shox* and *Shox2*.[2,13,14] Furthermore, ectopic expression of *Tbx3* or *Tbx18* in the mature quiescent cardiomyocytes reprograms them into the pacemaker-like cells.[15,16] In contrast, ectopic expression of *Nkx2.5* suppresses the differentiation of SAN myocardium.[17] Despite of these findings, the signalling mechanisms that regulate the SVV and SAN transcriptional program remain understudied.

Molecular signalling from the endocardium to the myocardium are essential for myocardial development.[18] Among them, the NOTCH1 signalling in the endocardium is a well-studied paracrine signal that regulates myocardial gene expression to promote the proliferation and differentiation of working myocardium during ventricular chamber formation.[19] However, whether the NOTCH1 signalling in the endocardium has a role in the development of SVV and SAN myocardium are not addressed in the previous studies, as deletion of *Notch1* in the pan-endothelium (including vascular endothelium and endocardium) using the *Tie2*^{Cre} mouse results in severe vascular and cardiac defects, leading to early embryonic lethality around E9.5–E10.5 before the formation of mature SVV and SAN.[19,20]

In the present study, we addressed this question by using the *Nfatc1*^{Cre} mouse to specifically delete *Notch1* in the endocardium during heart development.[21,22] The endocardial *Notch1* knockout embryos died around E11.5 that allowed us to investigate the functions of endocardial NOTCH1 in the development of SVV and SAN between E9.5 and E11.5. Our results show that endocardial NOTCH1 controls the development of SVV and SAN by regulating Wnt2 and Nrg1.

2. Methods

Detailed methods were described in the Supplementary material online.

2.1 Ethics statement

All animal procedures conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institution of Health and was approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

2.2 Histology, LacZ staining, and immunofluorescence

The pregnant mice were sacrificed by an overdose of isoflurane in a sealed container. Embryos were fixed overnight at 4°C using 4% paraformaldehyde in phosphate-buffered saline, dehydrated through an ethanol gradient, treated with xylene, and embedded in paraffin wax. Embryos were oriented for sagittal sections and cut in 6 μm sections using a Leica microtome. Haematoxylin and eosin (HE), LacZ, and immunofluorescence staining were performed using standard protocols.

2.3 Cell proliferation assays

Cell proliferation was determined using an EdU assay.

2.4 RNA extraction and quantitative PCR

Total RNAs were isolated from pooled heart tissues from E9.5 and E10.5 control and endocardial *Notch1* knockout embryos using Trizol (Invitrogen, Thermo Fisher, USA). First strand cDNA was synthesized using the Superscript II Reverse Transcriptase Kit (Invitrogen, Thermo Fisher, USA). Quantitative PCR (qPCR) was performed using the Power SYBR Green PCR Master Mix (ABI, Thermo Fisher, USA). Gene specific primers were used for qPCR (Supplementary material online, *Table S1*). The expression of *Gapdh* is used as internal control.

2.5 RNA *in situ* hybridization

RNA *in situ* hybridization was performed according to a previously described protocol.[21]

2.6 Measurement of heartbeat frequency

The heartbeat was measured as described previously.[2]

2.7 Whole embryo culture

Whole embryo culture was carried out as described previously.[21] For rescue experiments, the culture media was supplemented with recombinant NRG1 proteins (1.25×10^{-8} M) (R&D system, USA) or WNT2 conditioned media.[21,23] The embryos were cultured in an incubator that contained specialized gas (60% oxygen, 5% CO_2, and 35% N_2) at 37°C for 24 h. The cultured embryos were subjected to gene expression analysis by whole mount *in situ* hybridization.

2.8 Statistical analysis

All data were presented as means ± SEM. One-way ANOVA and Tukey's multiple comparisons test were used for calculating the statistical differences of means among multiple groups. Unpaired Student's *t*-test was used for statistical calculation between two groups. The Probability (*P*) values below 0.05 were considered as significant.

3. Results

3.1 Morphogenesis of SVV and SAN and expression of NOTCH1

We first examined the morphogenesis of SVV in normal mouse embryos between E9.5 and E10.5 by HE staining. The results showed that the myocardium at the sinoatrial junction started to bend towards the lumen of the heart tube at E9.5, this process continued at E9.75–E10.0, and by E10.5 the SVV structure was clearly formed, which demarks the boundary of sinus venous and atrium (*Figure 1A* and *D*). We next examined the SAN development by immunostaining for HCN4, which labels the primary pacemaker cells. The results showed that HCN4 was expressed by the cardiomyocytes at the sinoatrial junction of E9.5 embryos (*Figure 1B* and *D*). As the SVV elongated, the HCN4-expressing cardiomyocytes became to be clustered towards the sinus venous side at E9.5–E10.0, by E10.5 the HCN4-expressing cardiomyocytes was further condensed to form the SAN at the base of SVV at the sinus venous side (*Figure 1B*). We then performed immunostaining for NOTCH1 and found that NOTCH1 was highly expressed in the endocardium of developing SVV and SAN (*Figure 1C* and *D*). In addition, by *RNA in situ* hybridization and immunostaining, we found that the NOTCH ligand Jag1 but not Dll4 was expressed within SVV region (Supplementary material online, *Figure S1*). Together, these findings suggest that NOTCH1 may be involved in the developmental process of SVV and SAN.

3.2 Deletion of endocardial *Notch1* disrupts the development of SVV

To determine the possible roles of NOTCH1 in the formation of SVV and SAN, we crossed *Nfatc1*^{Cre} mice[22] with floxed *Notch1* mice[24] (*Notch1*$^{f/f}$) to generate endocardial *Notch1* knockout mice (*Notch1*$^{f/f}$:*Nfatc1*^{Cre}, *Notch1*^{eKO} thereafter). We first determined the Cre activities in the developing hearts by crossing the *Nfatc1*^{Cre} mouse with *R26*^{lacZ} reporter line.[25] X-gal staining showed that at E9.5 LacZ expression was present in the endocardial cells within the atrium, atrioventricular canal and ventricle, while it was absent in the endocardial cells of the SVV (*Figure 2A* and Supplementary material online, *Figure S2A and B*). At E10.5, the LacZ expression was expanded to the endocardial cells of SVV (*Figure 2A* and Supplementary material online, *Figure S2C–E*). These results indicated that the *Nfatc1*^{Cre} started to induce gene recombination in the endocardial cells of SVV around E9.5. Immunofluorescence confirmed that the expression of N1ICD in the endocardial cells of SVV was dramatically reduced in E10.5 *Notch1*^{eKO} embryos when compared to that in their littermate controls (*Figure 2B*). Quantitative RT–PCR confirmed that the E9.5 and E10.5 *Notch1*^{eKO} hearts had significantly reduced levels of *Notch1* transcripts (*Figure 2C*). Additionally, RNA *in situ* hybridization assays showed that NOTCH target gene *Hey1* but not *Hey2* was expressed within SVV region of E10.5 control embryos and such expression was reduced in *Notch1*^{eKO} embryos (Supplementary material online, *Figure S3*). We noted that the *Notch1*^{eKO} embryos were grossly normal at E10.5, but they appeared to be underdeveloped and smaller in size at E11.5 and died by E12.5 (Supplementary material online, *Figure S4*). We therefore analysed the heart histology by HE staining of serial sections crossing the whole heart and found that all the *Notch1*^{eKO} embryos had more primitive SVV when compared to that in controls (*Figure 2D and E* and Supplementary material online, *Figure S5*). In addition, we observed the cushion and trabecular defects that were reported by us previously.[21] These observations suggest that endocardial *Notch1* is required for the growth of SVV. This notion was confirmed by the

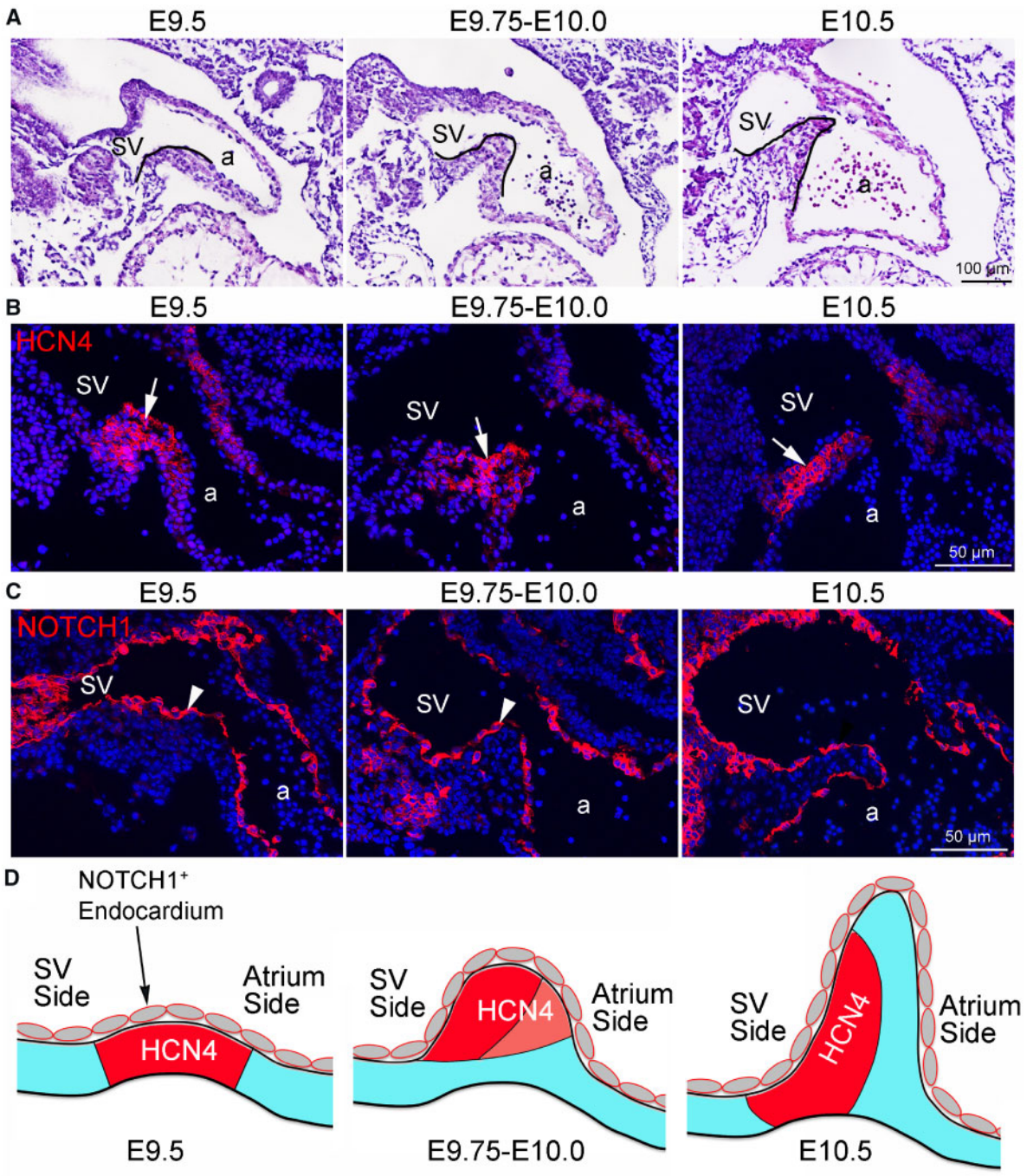


Figure 1 Morphogenesis of SVV and SAN and the expression of NOTCH1. (*A*) HE staining shows the morphology of developing SVV (outlined by black lines) in mouse embryos between E9.5 and E10.5. a, atrium; sv, sinus venous. (*B*) Immunofluorescence shows the expression of HCN4 (arrows) which marks the pacemaker cells of developing SAN. (*C*) Immunofluorescence shows the expression of NOTCH1 (arrowhead) in the endocardium of developing SVV. (*D*) Schematics show the morphogenic changes of developing SVV and SAN. At E9.5, the myocardium at the sinoatrial junction begins to bend towards the lumen of the heart tube and expresses HCN4. Between E9.75 and E10.0, the myocardium at the sinoatrial junction continues its protrusion towards the lumen. The HCN4-expressing cardiomyocytes are clustered at the sinus venous side of SVV. By E10.5, the SVV is clearly developed with an elongated tail (or leaflet) at the sinoatrial border. Meanwhile, the SAN head was formed by the HCN4-expressing cardiomyocytes at the base of SVV, with the HCN4 expression extended along the sinus side of the SVV, which is also called SAN tail.

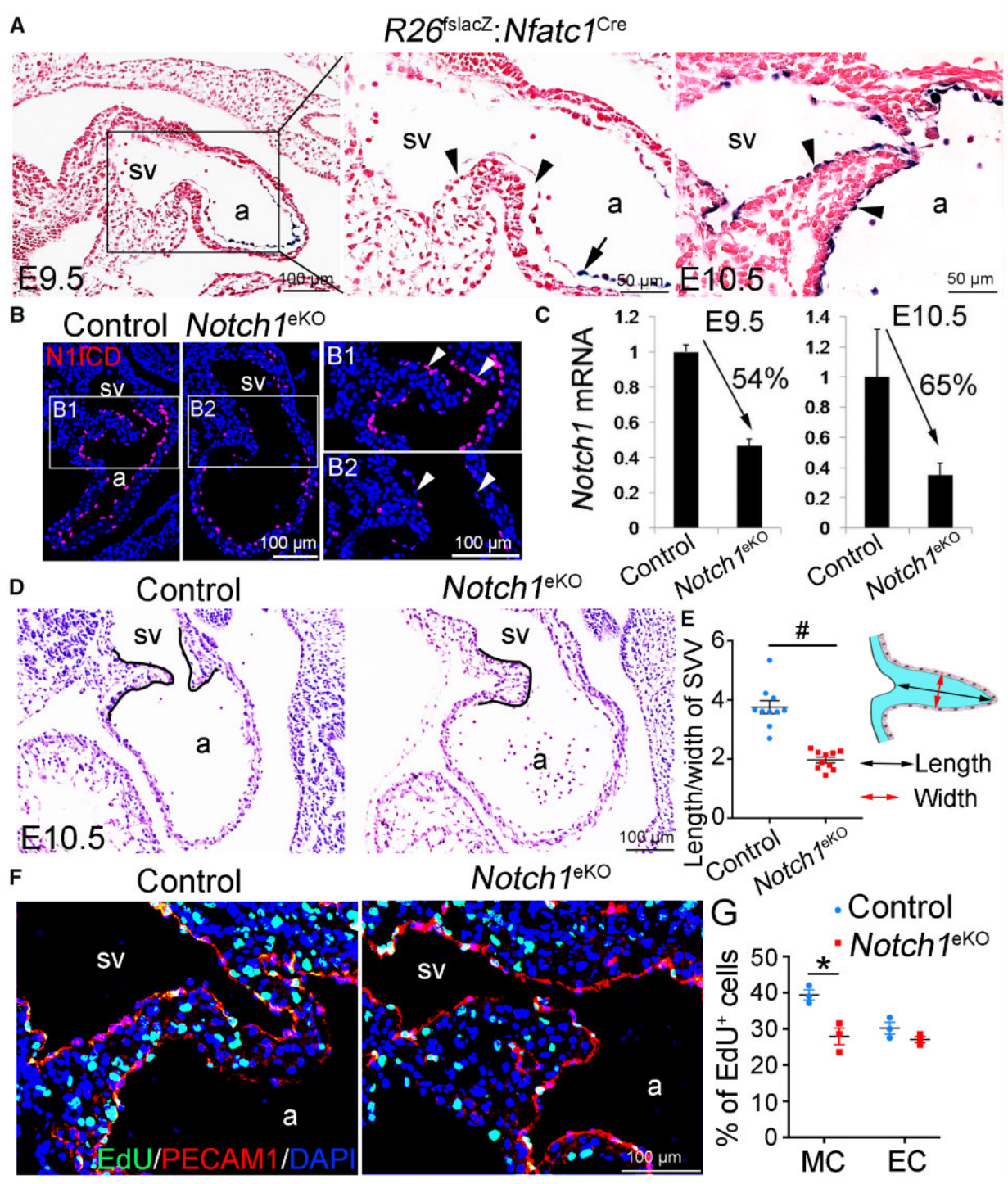


Figure 2 Deletion of endocardial *Notch1* results in defective growth of sinus venous valve. (*A*) X-gal staining shows the expression of LacZ reporter gene activated by the *Nfatc1*^{Cre} in the forming SVV of E9.5 and E10.5 embryos. Arrows and arrowheads indicate the endocardial cells in the atrium and SVV, respectively. (*B*) Immunofluorescence shows the cleaved NOTCH1 (N1ICD) expression in the endocardium (arrowhead) of SVV from the E10.5 control (*Notch1*$^{f/+}$) and endocardial *Notch1* knockout (*Notch1*$^{f/f}$: *Nfatc1*^{Cre}) embryos. (*C*) qPCR analysis shows the mRNA level of *Notch1* in E9.5 or E10.5 hearts of control and *Notch1*^{eKO} embryos. The expression of *Notch1* was normalized to that of *Gapdh*. Two to three hearts were pooled as one sample ($n = 3$/group). (*D*) HE staining shows histology of SVV (outlined by black lines). (*E*) Quantification of the ratio of the length/width of SVV of E10.5 control and *Notch1*^{eKO} embryos ($n = 10$/group). $^{\#}P < 0.001$. (*F*) EdU labelling shows the proliferating cells (green) in the SVV. The endocardial cells are marked by PECAM1immunostaining (red). (*G*) Quantification of proliferation rate in the cardiomyocytes (CM) and endocardial cells (EC) of SVV ($n = 3$/group). $^{*}P < 0.05$. Unpaired Student's *t*-test was used for statistical calculation. a, atrium; sv, sinus venous.

EdU labelling that revealed a significant reduction in the proliferation of SVV and atrial cardiomyocytes in the E10.5 *Notch1*^{eKO} embryos (*Figure 2F* and *G* and Supplementary material online, *Figure S6*). Together, these findings demonstrate that the endocardial *Notch1* is essential for the proliferation of SVV cardiomyocytes and the growth of SVV.

3.3 Deletion of endocardial *Notch1* impairs the formation of SAN

Since SVV and SAN develop concurrently in mouse between E9.5 and E10.5 (*Figure 1*), we next examined the expression of *Hcn4*. Whole mount RNA *in situ* hybridization showed that *Hcn4* transcripts marked the forming SAN at the sinoatrial boundary of control embryos at E10.5 (*Figure 3A*, top panel). In contrast, only a rudimentary SAN with markedly reduced *Hcn4* expression was present in the E10.5 *Notch1*^{eKO} embryos. Sectional analysis of the stained embryos clearly showed that the *Hcn4*-delineated SAN was present at the base of SVV in the control embryos, while the undersized SAN with much less *Hcn4* positive cells was observed in the same region of *Notch1*^{eKO} embryos (*Figure 3A*, bottom panel). Consistently, RT–qPCR and immunostaining confirmed a reduced level of HCN4 mRNA and protein respectively in the forming SAN of the E10.5 *Notch1*^{eKO} embryos (*Figure 3B* and *C*). In addition, EdU labelling revealed a reduction in the proliferation of the HCN4-expressing cardiomyocytes in the E10.0 *Notch1*^{eKO} embryos (*Figure 3D* and *E*). SAN generates the primary pacemaker pulse and enables the rhythmic heart contraction. To determine whether the defective SAN affects the heartbeat, we recorded the heartbeat frequencies and the results revealed a significantly reduced heartbeat rate by the E10.5 *Notch1*^{eKO} hearts when compared to controls (*Figure 3F* and Supplementary material online, *Movies S1 and S2*). These results indicate that endocardial *Notch1* is also required for the SAN formation and its function.

3.4 Deletion of endocardial *Notch1* impairs the expression of *Tbx5*, *Tbx18*, and *Wnt2*

Previous studies have shown that the progenitors arise from the cardiogenic mesoderm to form the SVV and SAN.[9] The proliferation and differentiation of these progenitor cells are regulated by T-box transcription factors including *Tbx3*,[10] *Tbx5*,[11] and *Tbx18*.[12] We therefore examined the expression of these genes in the E10.5 *Notch1*^{eKO} embryos by RNA *in situ* hybridization and qPCR. The results showed that the expression of *Tbx3* in SAN was comparable between the control and *Notch1*^{eKO} embryos (*Figure 4A* and *G*). In contrast, the expression of *Tbx5* and *Tbx18* in the SVV and SAN region were dramatically reduced in E10.5 *Notch1*^{eKO} embryos (*Figure 4B, C,* and *G*). We also examined the expression of *Shox2*, a key regulator of SVV and SAN development,[2,13,14] and found that its expression was not affected in the *Notch1*^{eKO} embryos (*Figure 4D* and *G*). Since *Wnt2* and *Bmp4* have been implicated in the development of SVV and SAN,[26–28] we next examined their expression in the *Notch1*^{eKO} embryos by RNA *in situ* hybridization. We found that *Wnt2* was predominantly expressed in the SVV and SAN region in control embryos, while such expression was greatly reduced in the *Notch1*^{eKO} embryos (*Figure 4E* and *G*). On the contrary, *Bmp4* was expressed at a comparable level in the SVV and SAN region between the control and *Notch1*^{eKO} embryos (*Figure 4F* and *G*). These results suggest that decreased expression of *Wnt2* might cause reduced WNT/β-catenin signalling, leading to the SVV and SAN defects in the *Notch1*^{eKO} embryos.

3.5 Endocardial NOTCH1 regulates SVV and SAN formation through myocardial WNT2

To determine whether myocardial WNT2 acts downstream of the endocardial NOTCH1 signalling to promote the development of SVV and SAN, we performed the rescue experiments by treating the cultured E9.5 *Notch1*^{eKO} embryos with the recombinant WNT2 protein. The cultured embryos were then subjected to gene expression analysis of *Hcn4*, *Tbx5,* and *Tbx18* using RNA *in situ* hybridization and qPCR. The results showed that addition of WNT2 was able to partially restore the expression of *Hcn4* and *Tbx18* in the *Notch1*^{eKO} embryos, while it failed to do so for *Tbx5* (*Figure 5A* and *B*). Of note, the WNT2 treatment also rescued the growth defect of SVV in the *Notch1*^{eKO} embryos (*Figure 5C*). In addition, we found that WNT2 treatment restored the heartbeat rate in *Notch1*^{eKO} embryos (*Figure 5D* and Supplementary material online, *Movies S3–S5*). Together, these findings indicate that endocardial NOTCH1 regulates the formation of SVV and SAN, at least partially, through WNT2.

3.6 WNT/β-catenin signalling is essential for SVV growth

Since WNT2 is a ligand for activating canonical WNT (WNT/β-catenin) signaling,[29] we then sought to determine whether the WNT/β-catenin signalling is required for the formation of SVV and SAN. We first traced WNT/β-catenin activities in the developing SVV by using a canonical WNT signalling reporter mouse model in which the expression of GFP was directed by of six tandem TCF/LEF binding sites.[30] The results showed that the WNT/β-catenin activities were exclusively present in the SVV myocardium and rare in the SVV endocardium (*Figure 6A*). This finding suggests that the myocardial WNT/β-catenin activities might be involved in the development of SVV and SAN. To test this idea, we generated myocardial *β-catenin* knockout mice (*β-catenin*$^{f/f}$: *Tnt*^{Cre}, *β-cat*^{mKO} thereafter) by crossing the floxed *β-catenin* mice[31] with the *Tnt*^{Cre} mice.[32] Histological analysis by HE staining showed that *β-cat*^{mKO} embryos had primitive SVV (*Figure 6B* and *C*, and Supplementary material online, *Figure S7*) that was similar to the phenotypes observed in the *Notch1*^{eKO} embryos (*Figure 2D* and *E*). Consistent with the reduced size of SVV, EdU labelling revealed a significantly decreased cell proliferation in the SVV cardiomyocytes of *β-cat*^{mKO} embryos (*Figure 6D and E*). RNA *in situ* hybridization and qPCR were then performed to determine the expression of *Hcn4* and the results showed that the deletion of *β-catenin* in the myocardium had no effect on the expression of *Hcn4* (Supplementary material online, *Figure S8A* and *B*). Consistently, *β-cat*^{mKO} embryos had a normal heartbeat rate (Supplementary material online, *Figure S8C*). Together, these results indicate that the WNT/β-catenin signalling in the myocardium is required for the growth of SVV, but not for the formation of SAN.

3.7 Endocardial NOTCH1 regulates the elongation of SVV through myocardial NRG1

Nrg1 is specifically expressed in the endocardium and known to mediate the endocardial NOTCH signalling to regulate myocardial gene expression during cardiac chamber formation.[11,19,33] To determine whether *Nrg1* plays a similar role in the development of SVV and SAN, we examined its expression in the *Notch1*^{eKO} embryos by RNA *in situ* hybridization and qPCR. We found that the expression of *Nrg1* in the ventricular

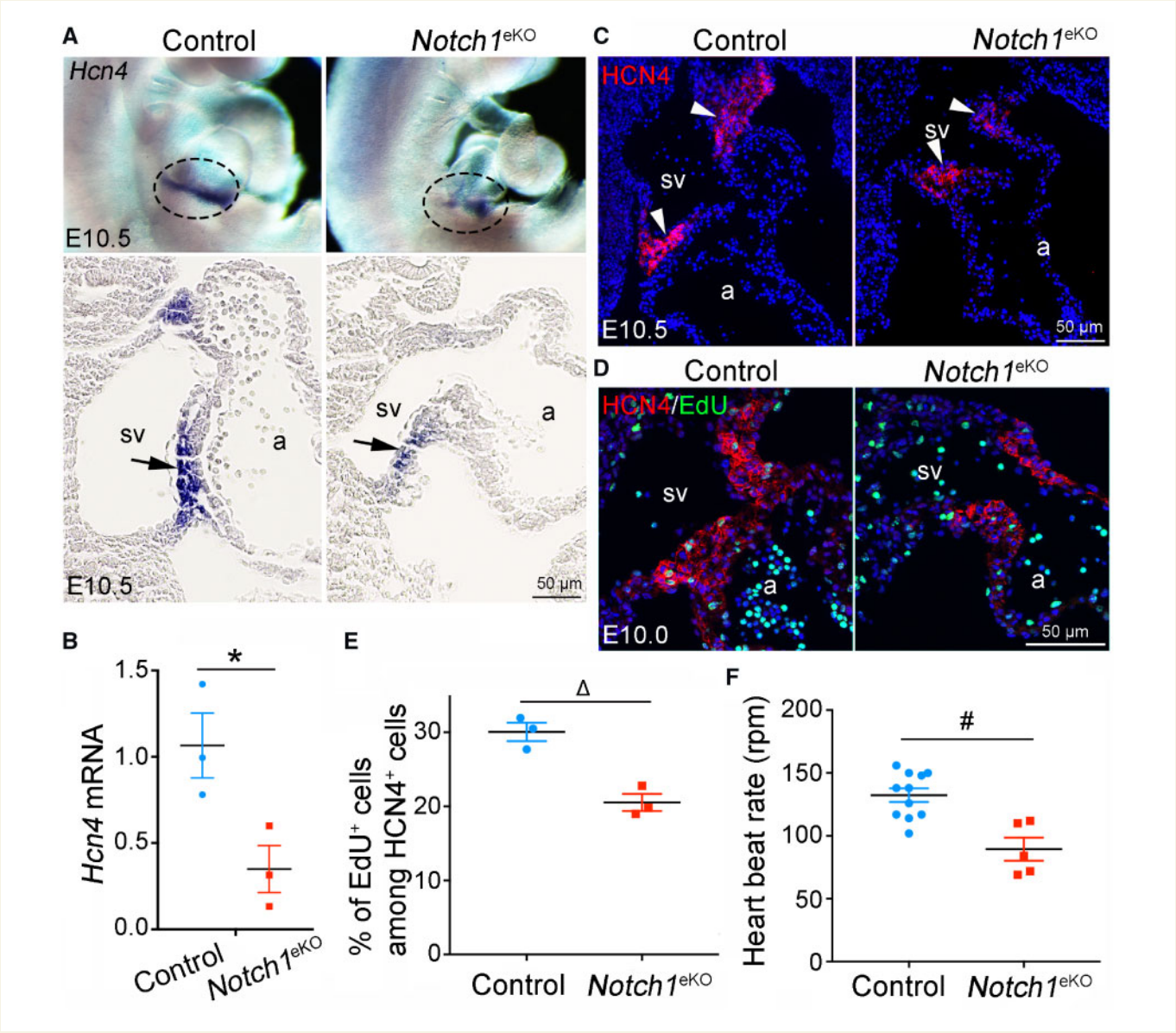


Figure 3 Deletion of endocardial *Notch1* results in defective development of sinoatrial node. (*A*) Whole mount RNA *in situ* hybridization shows that the mRNA level of *Hcn4* is markedly reduced in the SAN of E10.5 *Notch1*^{eKO} embryos when compared to that in control embryos. Note that 3–5 embryos from each genotype were analysed simultaneously. Representative images of whole mount (top panel) or sectional views (bottom panel) show the expression of *Hcn4* in the SAN (indicated by circle or arrow) in E10.5 control and *Notch1*^{eKO} embryos. a, atrium; sv, sinus venous. (*B*) RT–qPCR analysis of the *Hcn4* mRNA expression in E10.5 control and *Notch1*^{eKO} hearts. The expression of *Hcn4* is normalized to that of *Gapdh* ($n = 3$/group). $^{*}P < 0.05$. (*C*) Immunostaining shows a reduced level of HCN4 protein (arrowhead) in the forming SAN of E10.5 *Notch1*^{eKO} embryos. (*D* and *E*) EdU labelling shows decreased proliferation of the HCN4-expressing cardiomyocytes in the E10.0 *Notch1*^{eKO} embryos ($n = 3$/group). $^{\Delta}P < 0.01$. (*F*) Live recording heart rate indicates a reduced beating rate by the E10.5 *Notch1*^{eKO} hearts ($n = 5$) compared with the control hearts ($n = 11$). $^{\#}P < 0.001$. Unpaired Student's *t*-test was used for statistical calculation.

endocardium was dramatically reduced in the *Notch1*^{eKO} embryos compared to that of controls (*Figure 7A–C*). Unexpectedly, we noted that *Nrg1* was expressed in both endocardium and myocardium of SVV tail in the control embryos and such expression was markedly reduced in the *Notch1*^{eKO} embryos (*Figure 7A–C*). Consistent with its expression pattern, addition of NRG1 in the cultured E9.5 *Notch1*^{eKO} embryos for 24 h was able to rescue the SVV growth defect (*Figure 7D* and *F*), but not the expression of *Hcn4* and *Wnt2* in the SVV head (*Figure 7D* and *E* and Supplementary material online, *Figure S9*). These findings suggest that NRG1 acts downstream of endocardial NOTCH1 to promote the SVV growth.

4. Discussion

NOTCH signalling mediates signal communications between neighbouring cells and play multiple roles during heart development.[34] NOTCH signalling critically regulates early cardiomyocyte differentiation,[35,36] trabeculation,[19,37] heart valve development,[21,38] atrioventricular node

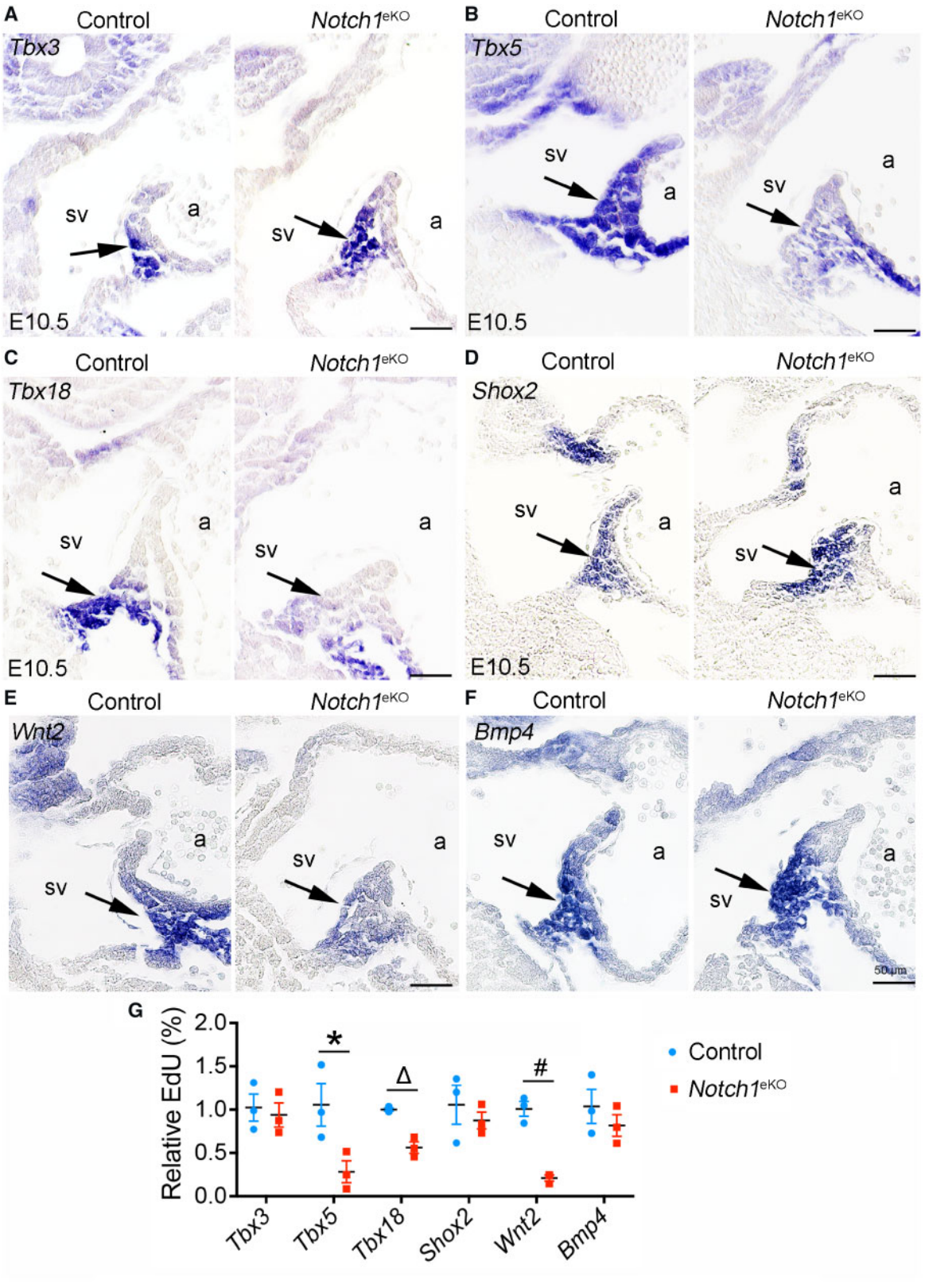


Figure 4 Deletion of endocardial *Notch1* reduces the expression of *Tbx5*, *Tbx18*, and *Wnt2*. (*A–F*) RNA *in situ* hybridization shows the expression of *Tbx3* (*A*), *Tbx5* (*B*), *Tbx18* (*C*), *Shox2* (*D*), *Wnt2* (*E*), and *Bmp4* (*F*) in the E10.5 control and *Notch1*^{eKO} embryos. The results indicate a reduced expression of *Tbx5* and *Wnt2* in the forming SAN and SVV, as well as *Tbx18* in the forming SAN, of the *Notch1*^{eKO} embryos. In each experiment, at least three embryos from each genotype were analysed simultaneously for one gene. Representative images are present to show the expression of these genes in SAN/SVV (arrow). a, atrium; sv, sinus venous. (*G*) RT–qPCR analysis of gene expression in the hearts from E10.5 control and *Notch1*^{eKO} embryos. The expression of *Gapdh* is used as internal control (n = 3/group). Unpaired Student's t-test was used for statistical calculation (n = 3/group). *P < 0.05; ΔP < 0.01; $^{\#}P$ < 0.001.

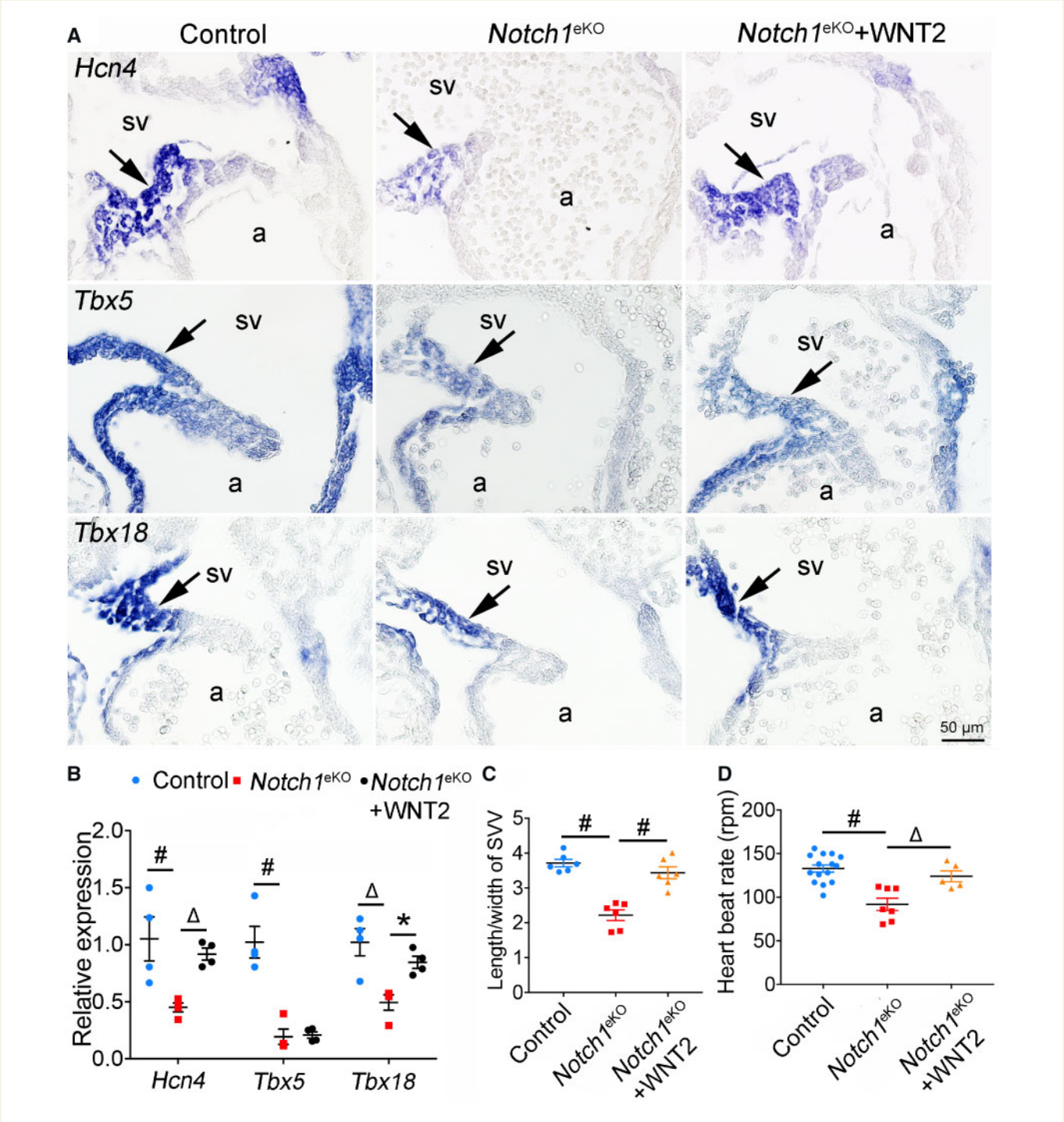


Figure 5 Endocardial NOTCH1 regulates the formation of SVV and SAN through WNT2. (*A*) E9.5 control and *Notch1*^{eKO} embryos were cultured with or without the WNT2 treatment for 24 h. RNA *in situ* hybridization shows the expression of *Hcn4*, *Tbx5*, and *Tbx18* in SAN (arrow) of cultured embryos. In each experiment, 3–5 embryos from each group were analysed simultaneously. (*B*) RT–qPCR analysis of the expression of *Hcn4*, *Tbx5*, and *Tbx18* in the hearts from cultured embryos. The expression of *Gapdh* is used as internal control ($n = 4$/group). (*C*) Quantification of the length/width ratio of SVV in cultured embryos indicates the rescued SVV growth in the *Notch1*^{eKO} embryos by WNT2 ($n = 6$/group). $^{\#}P < 0.001$. (*D*) Live recording heart rate of cultured embryos. Control ($n = 15$); *Notch1*^{eKO} ($n = 7$); *Notch1*^{eKO} + WNT2 ($n = 5$). Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparisons test. a, atrium; sv, sinus venous. $^{*}P < 0.05$; $\Delta P < 0.01$; $^{\#}P < 0.001$.

formation,[39] and coronary artery development.[40,41] Here we present the first genetic evidence that demonstrates a previous unknown role of endocardial NOTCH1 in regulation of SVV and SAN development (*Figure 8A*). Collectively, our data support that endocardial *Notch1* controls the formation of SVV and SAN through regulating Wnt2 and Nrg1 (*Figure 8B*).

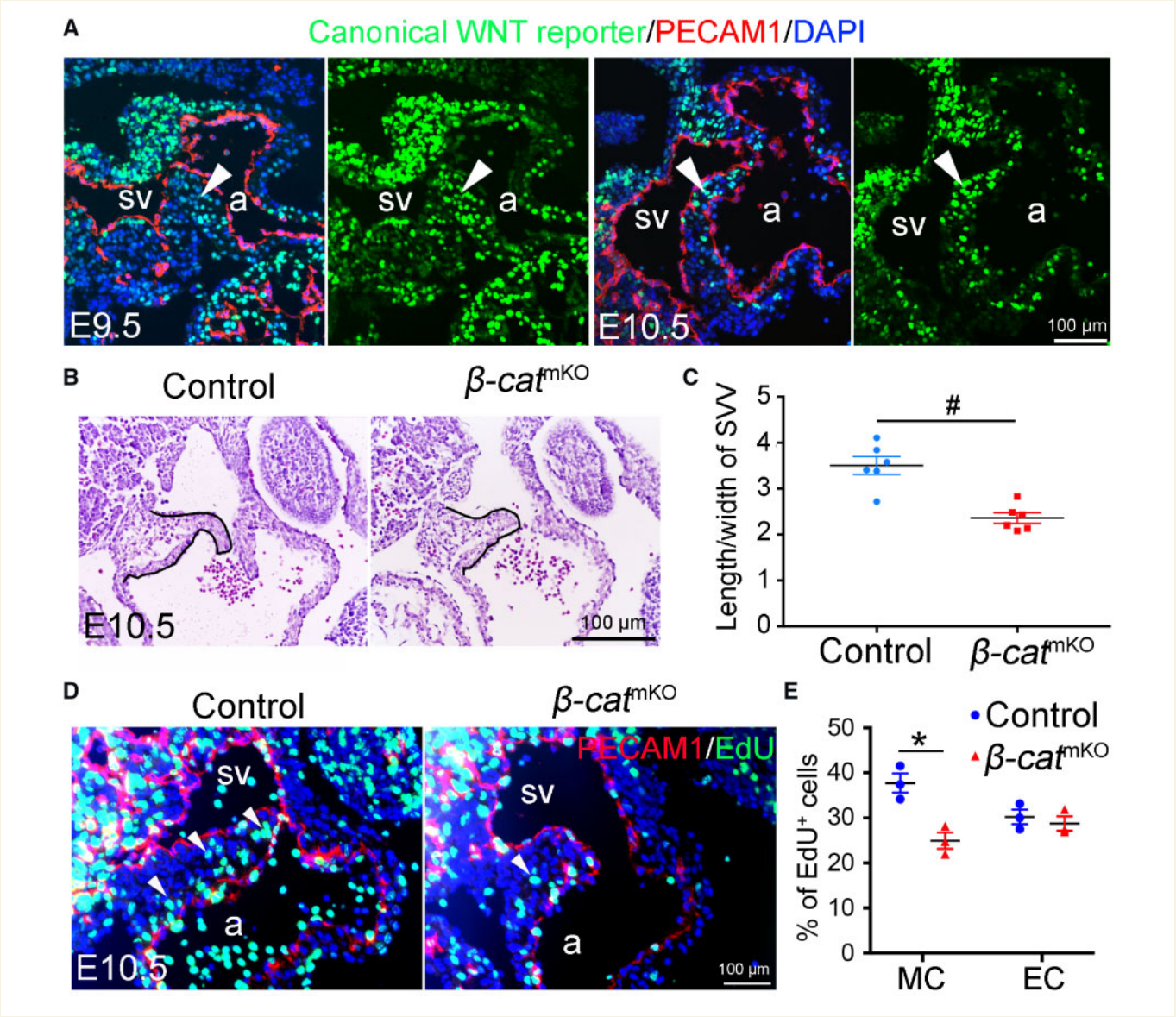


Figure 6 Deletion of myocardial *β-catenin* impairs the growth of SVV. (*A*) A canonical WNT signalling reporter line shows the canonical WNT activities (arrowhead) in the SVV/SAN region of E9.5 or E10.5 hearts. Endocardial cells were marked by immunostaining for PECAM1 (red). (*B*) HE staining shows the histology of SVV (outlined by black lines) in E10.5 control and myocardial *β-catenin* knockout (*β-cat*^{mKO}) embryos. (*C*) Quantification of the ratio of the length/width of SVV in the E10.5 control and *β-cat*^{mKO} embryos ($n = 6$/group). $^{\#}P < 0.001$. (*D*) EdU labelling shows the proliferating cells (green) in SVV, while endocardial cells are marked by PECAM1immunostaining (red). (*E*) Quantification of cell proliferation rate in the cardiomyocytes (CM) and endocardial cells (EC) within the SVV of E10.5 control and *β-cat*^{mKO} embryos ($n = 3$/group). $^{*}P < 0.05$. Unpaired Student's *t*-test was used for statistical calculation. a, atrium; sv, sinus venous.

We specifically deleted *Notch1* in the endocardium, without affecting the vascular endothelium.[21] The endocardial *Notch1* knockout embryos died around E11.5, thereby allowing us to study the functions of *Notch1* in heart development between E9.5 and E11.5. We found that loss of *Notch1* results in the primitive SVV that may cause blood regurgitation from the atrium to the sinus venous. In addition, loss of *Notch1* leads to markedly reduced expression of *Hcn4*, a functional marker specific for SAN that generate the primary pacemaker pulse.[7,42] The reduced heartbeat rate in the endocardial *Notch1* knockout embryos further suggests a defective SAN function. Together, the primitive SVV and defective SAN function may contribute to the embryonic lethality of the endocardial *Notch1* knockout embryos, in addition to the chamber and endocardial cushion defects.[21]

Previous studies have uncovered that the development of SAN is achieved through highly localized suppression of the working cardiomyocyte differentiation by a transcriptional network consisting of *Tbx3*, *Tbx5*, *Tbx18*, and *Shox2*.[6,9,14,43] *Tbx18*, initially expressed in a distinct precursor cell population within the septum transversum for the sinus venous horn myocardium, is required for the specification, differentiation, and localization of these cells into the myocardium to form the SAN head.[9,12,44] In addition, *Tbx3* and *Shox2* are essential for the establishment of SAN gene program and the differentiation of SAN cardiomyocytes.[2,10,12–14,45] Our

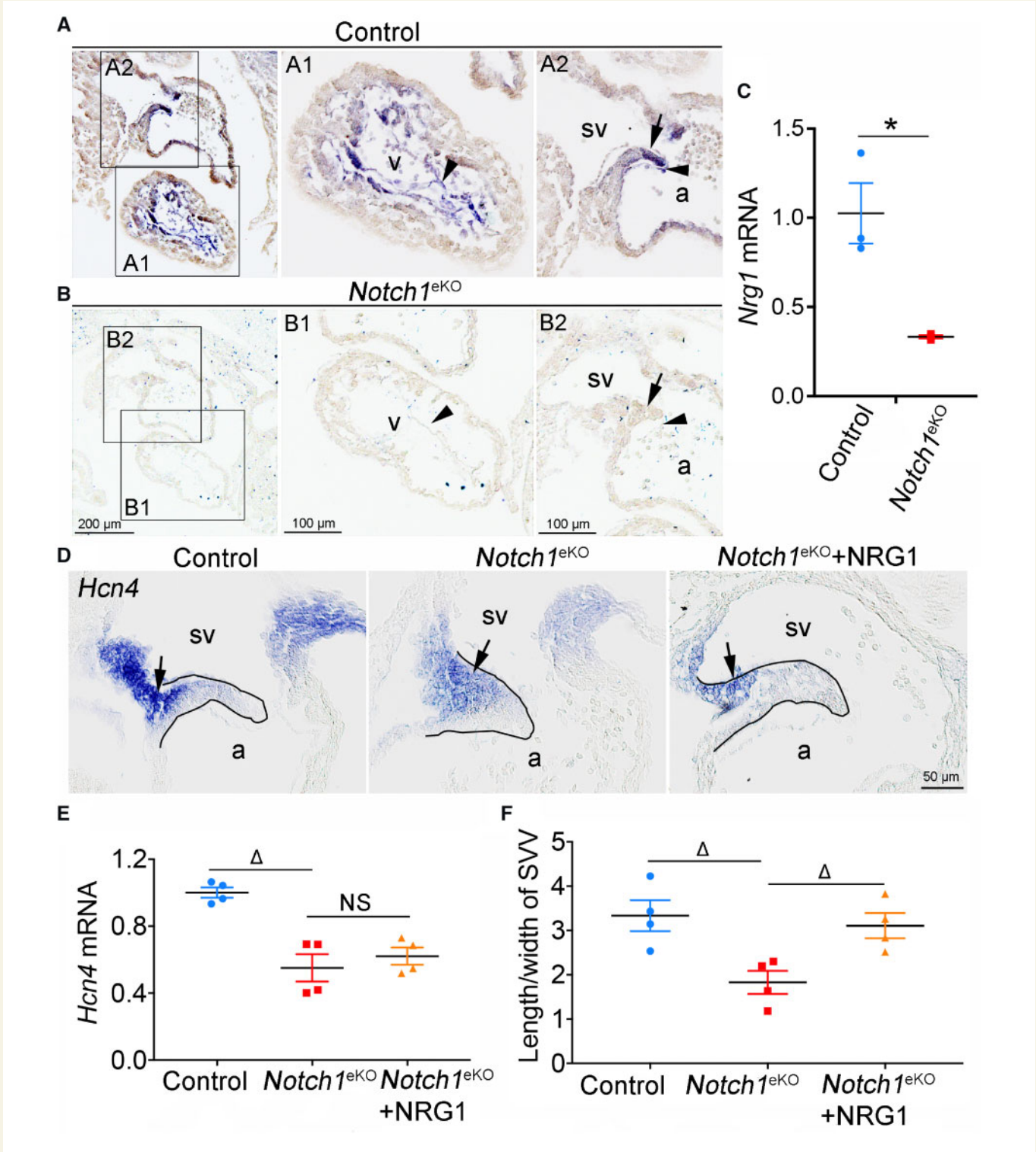


Figure 7 Endocardial NOTCH1 regulates the SVV formation through myocardial NRG1. (*A* and *B*), RNA *in situ* hybridization shows the expression of *Nrg1* in E10.5 control and *Notch1*^{eKO} embryos. The high magnification of ventricle (A1 and B1) and SVV (A2 and B2) regions is showed on the right. The arrowhead and arrow indicate the endocardium and myocardium respectively ($n = 3$/group). (*C*) RT–qPCR analysis of the *Nrg1* mRNA expression in E10.5 control and *Notch1*^{eKO} hearts. The expression of *Nrg1* was normalized to that of *Gapdh* ($n = 3$/group). (*D*) RNA *in situ* hybridization shows the expression of *Hcn4* (arrow) in the cultured E9.5 control and *Notch1*^{eKO} embryos with or without the NRG1 treatment for 24 h. a, atrium; sv, sinus venous. (*E*) RT–qPCR analysis of the *Hcn4* mRNA expression. The expression of *Hcn4* is normalized to that of *Gapdh* ($n = 4$/group). (*F*) Quantification of the ratio of the length/width of SVV in cultured embryos indicates the rescued SVV growth in the *Notch1*^{eKO} embryos by NRG1 ($n = 4$/group). Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparisons test. $^{*}P < 0.05$, $\Delta P < 0.01$.

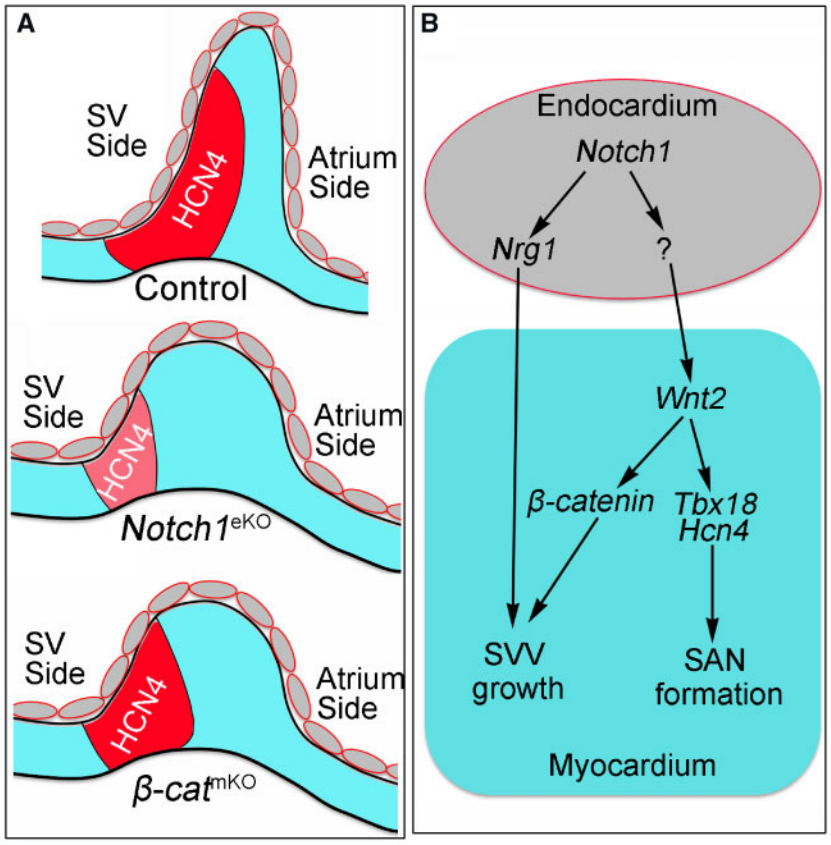


Figure 8 Schematic summary of the roles of endocardial *Notch1* in SVV and SAN formation. (*A*) A carton shows the normal development of SVV and SAN in the E10.5 control *Notch1*^{eKO} or *β-cat*^{mKO} embryos. Red area marks the HCN4-expressing SAN (head and tail). The expression of HCN4 is reduced in the *Notch1*^{eKO} embryos, but not affected in the *β-cat*^{mKO} embryos when compared to that in controls, whereas the elongation of SVV is affected in both mutant embryos. (*B*) A schematic working model shows the possible signalling mechanisms through which endocardial *Notch1* regulates the SVV growth and SAN formation. Endocardial *Notch1* promotes the SVV growth through regulating *Nrg1* expression in the endocardium. Endocardial *Notch1* also regulates myocardial *Wnt2 to* promote the SVV growth through *β-catenin*, as well as supports the SAN formation and maturation through regulating *Tbx18*.[16] The downstream mediators of *Notch1* in the endocardium that regulate *Wnt2* expression in myocardium are unknown and future studies are needed to identify them.

data showed that deletion of *Notch1* significantly reduced the expression of *Tbx18* but had no effect on the expression of *Tbx3* and *Shox2*. These findings suggest that endocardial NOTCH1 might recruit the TBX18-expressing cardiomyocytes and/or promote their proliferation during the formation of SAN. Thus, the reduced expression of *Hcn4* in the endocardial *Notch1* knockout embryos represents the underdeveloped SAN structure, not an early differentiation defect. It is worth to discuss that, while previous genetic studies have revealed a link between *Tbx5*, *Shox2*, and *Bmp4* in the developing SAN where *Tbx5* regulates *Bmp4* expression through *Shox2*,[11] our data show that the endocardial *Notch1* knockout embryos had reduced expression of *Tbx5* in the developing SVV and SAN, whereas the expression levels of *Shox2* and *Bmp4* are not changed. One explanation for this discrepancy between our and the previous study is that the reduced *Tbx5* expression in the endocardial *Notch1* knockout embryos is less dramatic and thus not sufficient to have an impact on the *Shox2* expression.

To explore the molecular mechanisms by which the endocardial NOTCH1 promotes myocardial cell proliferation and SVV growth, we examined the expression of candidate genes involved in the development of SVV and SAN. *Wnt2* has been reported to induce the WNT/β-catenin signalling that promotes cell proliferation and cardiac inflow tract development.[29] Interestingly, we found that the expression of *Wnt2* in the SVV and SAN myocardium was significantly reduced in the *Notch1* knockout embryos. In addition, we observed a high level of WNT/β-catenin signalling in the SVV and SAN myocardium of the transgenic mice that specifically report the canonical WNT signalling. These observations suggest that *Wnt2* might be involved in the formation of SVV and SAN by activating the WNT/β-catenin signalling. Indeed, our rescue experiments showed that recombinant WNT2 could rescue the SVV growth defect and partially restore the expression of *Hcn4* in the cultured *Notch1*^{eKO} embryos. Furthermore, we showed that disruption of *β-catenin* in the myocardium recapitulated the SVV growth defect resulted from the deletion of *Notch1* in the endocardium. However, loss of *β-catenin* had no effect on the expression of *Hcn4*. Consistent with these observations, Norden *et al.*[27] have shown that the WNT/β-catenin signalling is required to maintain the proliferation of the *Tbx18*-expressing mesenchymal progenitors, but not for the later formation of SAN. Taken together, these observations support that WNT2 regulates the

development of SVV through a β-catenin dependent manner, while it modulates the SAN formation independent of β-catenin.

One limitation of our study is that we do not know the downstream factors that mediate the endocardial NOTCH1 signalling to induce the expression of *Wnt2* in the myocardium. One such candidate would be *Nrg1*, since previous studies have shown that *Nrg1* works downstream of NOTCH1 signalling in the endocardium to regulate myocardial development including the central cardiac conduction system.[19,33,46,47] We found *Nrg1* expression in both endocardium and myocardium of SVV in control embryos and this expression was markedly reduced in *Notch1*^{eKO} embryos. Furthermore, addition of NRG1 was able to rescue the SVV growth defect resulted from the endocardial *Notch1* deletion, suggesting that NRG1 acts downstream of endocardial NOTCH1 signalling to promote SVV growth. This finding is consistent with the requirement of NOTCH1-NRG1 signalling in the ventricular endocardium for trabeculation,[19,33] suggesting a common NOTCH1-NRG1 mechanism regulating SVV elongation and trabecular protrusion. In contrast, exogenous NRG1 failed to restore the expression of *Hcn4* and *Wnt2*, indicating that the formation of SAN and the expression of *Wnt2* expression are not dependent on the endocardial NOTCH-NRG1 signalling. Future studies are required to identify other endocardial factors that relay the NOTCH signalling to the SVV and SAN myocardium.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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Translational perspective

Sinus venous valve and sinoatrial node control unidirectional cardiac flow and heart rhythm, respectively, during foetal development. By revealing that NOTCH1 regulates development of sinus venous valve and sinoatrial node through coordinating WNT2 and NRG1 signalling in genetic modified mouse models and whole embryo cultures, this study helps understanding of the potential disease mechanisms in congenital malformation and dysfunction of sinus venous valve and sinoatrial node.