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NOTCH maintains developmental cardiac gene network through WNT5A

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Abstract

NOTCH and WNT signaling pathways play critical roles in cardiac chamber formation. Here we explored the potential interactions between the two pathways in this developmental process by using genetically modified mouse models and whole embryo culture systems. By deletion of *Notch1* to inactivate NOTCH1 signaling in the endocardium in vivo and ex vivo rescue experiments, we showed that myocardial WNT5A mediated endocardial NOTCH1 signaling to maintain the gene regulatory network essential for cardiac chamber formation. Furthermore, genetic deletion of β-catenin in the myocardium and inhibition of the WNT/Ca^{2+} signaling by FK506 resulted in a similar disruption of the gene regulatory network as inactivation of endocardial NOTCH1 signaling. Together, these findings identify WNT5A as a key myocardial factor that mediates the endocardial NOTCH signaling to maintain the gene regulatory network essential for cardiac chamber formation through WNT/β-catenin and WNT/Ca^{2+} signaling pathways.

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The authors report no commercial or proprietary interest in any product or concept discussed in this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2018.10.014.

Keywords

NOTCH; WNT; Cardiac gene network; Cardiac chamber formation

1. Introduction

Normal formation of cardiac chamber is critical for maintaining heart function and indispensable for fetal survival and adult life [1–3]. Heart development starts with the formation of the first and secondary heart field by cardiac progenitors from the mesoderm around the embryonic day (E) 7.0 in mice. These progenitors cells coalesce at the midline of the embryos to form a linear heart tube that consists of the endocardium and the myocardium and soon begins to beat around E8.0 [4]. The heart tube then loops and separates to form a four-chamber heart, with formation of trabecular and compact myocardium [4,5] and development of conduction myocardium [6–9]. In these developmental processes, cell proliferation and differentiation are precisely controlled by a complex gene regulatory network consisting of key cardiac transcriptional factors including *Nkx2.5, Tbx5,* and *Gata4.* Human mutations in those genes are associated with congenital heart disease [10–16].

Cardiac gene regulatory network is modulated by molecular signals, between endocardium and myocardium. Previous studies have documented that NOTCH and WNT signaling pathways play critical roles in heart looping, chamber morphogenesis, and conduction system development [17,18]. In particular, NOTCH signaling in the endocardium regulates trabeculation and its dysregulation is associated with left ventricular non-compaction cardiomyopathy or hypoplastic left ventricular syndrome [19,20]. Mechanistically, *Nrg1* is downstream of NOTCH signaling in the endocardium and upstream of ErBB receptors in the myocardium [19]. Endocardial NOTCH signaling has been shown to interact with WNT signaling in early cardiac progenitor cell formation and heart valve development [21,22]. Whether endocardial NOTCH signaling interacts with myocardial WNT signaling during cardiac chamber formation remains largely unknown.

In this study, we addressed this question by genetic inactivation of *Notch1* in mice and ex vivo rescue experiments. The results of these experiments identified that WNT5A in the myocardium acts downstream of endocardial NOTCH1 to maintain the developmental cardiac gene network and support cardiac chamber formation.

2. Results

2.1. Endocardial NOTCH1 maintains developmental gene program in myocardium

To precisely reveal myocardial genes regulated by endocardial *Notch1,* we deleted *Notch1* by crossing floxed *Notch1* mice with $Nfatc1^{Cre}$ mice [22,23] to generate the $Notch1^{f/f}$:$Nfatc1^{Cre}$ mouse model *(*$Notch1^{eKO}$ hereafter). We confirmed the tissue-specific deletion by immunostaining for NOTCH1. The results showed dramatically reduced expression of NOTCH1 in the endocardium of E10.5 $Notch1^{eKO}$ embryos (Fig. S1A). In addition, qPCR analysis showed significantly reduced expression of *Hey1* and *Hey2*,

known targets of NOTCH, in E10.5 *Notch1*^{eKO} hearts (Fig. S1B). We then investigated the heart morphology by HE staining and found that E10.5 *Notch1*^{eKO} embryos exhibited underdeveloped ventricular chambers evident by underdeveloped trabeculae and thinner myocardial wall compared to controls (Fig. S2A). In line with these phenotypes, EdU labeling showed that E10.5 *Notch1*^{eKO} embryos had significantly reduced cell proliferation in both trabecular and compact myocardium, without changes in proliferation in the endocardium (Fig. S2B and C). These findings are consistent with previous observations made with the pan-endothelial deletion of *Notch1* [19], and our data precisely define the essential role of endocardial *Notch1* in cardiac chamber development.

We next investigated the expression of key genes involved in cardiac chamber formation in endocardial *Notch1* knockout hearts by using RNA in situ hybridization (ISH) and qPCR. The results showed that E10.5 *Notch1*^{eKO} embryos had significantly reduced expression of cardiac transcription factors, *Tbx5, Nkx2.5, Gata4, Hand1,* and *Irx4,* when compared to the littermate controls (Fig. 1A-C). During ventricular chamber development, a subset of cardiomyocytes is differentiated into conduction cardiomyocytes and expresses genes encoding gap junction proteins, Cx40, Cx43 and Cx45. We found that E10.5 *Notch1*^{eKO} embryos had significantly reduced expression of *Cx40* and *Cx45,* while they maintained a normal expression level of *Cx43* (Fig. 2A and B). *Bmp10* is a growth factor predominately expressed in the trabecular myocardium and involved in the growth of trabeculae downstream of endocardial NOTCH at E9.5 [24]. Unexpectedly, our results showed that *Bmp10* expression in the trabecular myocardium of E10.5 *Notch1*^{eKO} embryos was not affected, but it was ectopically expressed in the atrial myocardium (Fig. S3A and B). Together, the gene expression analysis reveals that endocardial *Notch1* is indeed required for maintaining the developmental gene regulatory network for cardiac chamber formation after heart looping at E9.5.

2.2. Endocardial NOTCH1 sustain myocardial gene regulatory network through NRG1

We then examined the expression of genes likely involved in the endocardial NOTCH1 signaling in the endocardium. qPCR showed *Nfatc1,* a specific marker for the endocardium [25,26], was dramatically downregulated in *Notch1*^{eKO} hearts, while the expression level of pan-endothelial marker *Pecam1* was not changed (Fig. 3A). Consistently, immmnuostaining revealed a reduced level of NFATC1 protein in the endocardium of *Notch1*^{eKO} hearts (Fig. 3B). The down-regulation of NFATC1 in *Notch1*^{eKO} hearts suggests that endocardial NOTCH1 might be required for maintaining the endocardial cell fate. *Nrg1* is a known downstream target of NOTCH signaling in the endocardium to support the cardiac gene regulatory network during chamber development at E9.5 [27]. We found that *Nrg1* expression was also significantly reduced in E10.5 *Notch1*^{eKO} hearts (Fig. 3A and C). In addition, E10.5 *Notch1*^{eKO} hearts showed decreased expression of *EphrinB2* and *Hand2* (Fig. 3A and D), two known upstream regulators of *Nrg1* at E9.5 [19,28]. To determine whether the reduced expression of *Nrg1* contributed to the disrupted gene regulatory network in E10.5 *Notch1*^{eKO} hearts, we cultured E9.5 *Notch1*^{eKO} embryos and treated the embryos with or without recombinant NRG1 protein for 24 h RNA ISH and qPCR analysis of cultured embryos showed that the exogenous NRG1 was able to restore the expression of *Tbx5, Nkx2.5, Hand1, Irx4, Cx40* and *Cx45* in *Notch1*^{eKO} hearts (Fig. 4A and B).

Together, these findings demonstrate that endocardial NOTCH1/NRG1 signaling supports the developmental cardiac gene regulatory network during cardiac chamber formation after heart looping at E9.5.

2.3. WNT5A in myocardium mediates the roles of endocardial NOTCH1/ NRG1 signaling

WNT signaling is known to regulate cardiomyocyte differentiation and proliferation during early cardiogenesis [17]. To determine potential interactions between NOTCH and WNT signaling pathways in regulation of cardiac chamber development, we compared the expression of 12 WNT pathway genes between the control and the *Notch1*^{eKO} hearts at E10.5 by qPCR analysis. The results showed while most of the genes were expressed at a similar level between control and *Notch1*^{eKO} hearts, the expression of *Wnt2, Wnt4* and *Wnt5a* was significantly downregulated in *Notch1*^{eKO} hearts (Fig. 5A). *Wnt2* is mainly expressed in inflow tract and essential for inflow tract development during early embryogenesis [29], whereas *Wnt4* is expressed in the cushion endocardium and regulates EMT through modulating myocardial BMP signaling [22]. In contrast, *Wnt5a* is broadly expressed in the early developing heart and works redundantly with *Wnt11* to regulate secondary heart field development [30]. We found by RNA ISH that *Wnt5a* was predominately expressed in the chamber myocardium of control hearts, and such expression was dramatically reduced in *Notch1*^{eKO} hearts (Fig. 5B). Moreover, the reduced expression of *Wnt5a* in cultured *Notch1*^{eKO} embryos was partially but significantly restored by supplementation of NRG1 recombinant proteins (Fig. 5C and D). These observations suggest that endocardial NOTCH1 regulates the expression of myocardial *Wnt5a* via NRG1. To test whether WNT5a acted as a myocardial mediator of endocardial NOTCH-NRG1 signaling to maintain the myocardial gene regulatory network, we treated E9.5 *Notch1*^{eKO} embryo cultures with WNT5A for 24 h and examined the expression of *Tbx5, Hand1, Nkx2.5, Irx4, Gata4, Cx40* and *Cx45* by RNA ISH and qPCR. The results showed that addition of WNT5A restored the expression of *Tbx5, Hand1, Nkx2.5, Irx4, Cx40* and *Cx45* in cultured *Notch1*^{eKO} embryos, except for that of *Gata4* (Fig. 6A and B). Together, these findings indicate that myocardial WNT5A mediates endocardial NOTCH1/NRG1 signaling to maintain the developmental gene regulatory network essential for cardiac chamber development.

2.4. Myocardial WNT/β-catenin signaling promotes ventricular chamber growth

Since WNT5A can induce canonical and non-canonical WNT signaling in a cell context and receptor dependent manner [30–32]. Therefore, we asked whether WNT/β-catenin signaling is required for sustaining the myocardial gene program. We first examined the WNT/β-catenin activities in developing chambers using a new WNT reporter mouse, in which the reporter GFP expression is under the control of 6 tandem TCF/LEF binding sites [33]. The results showed that WNT/β-catenin activities were strong in the ventricular myocardium but weak in the endocardium from E9.5 to E11.5 (Fig. S4), suggesting that the myocardial WNT/β-catenin signaling may be involved in the regulation of myocardial gene expression. To test this, we generated a myocardial specific β-catenin (also known as *Ctnnb1*) knockout mice (β-catenin$^{f/f}$:*Tnt*^{Cre}, referred as β-cat^{mKO} hereafter) by crossing the floxed β-catenin mice with myocardial specific Cre mice, Tnt^{Cre34} and confirmed the specific-deletion of β-catenin in the myocardium by immunostaining (Fig. S5A). Histological analysis showed that

β-cat^{mKO} embryos had thinner compact myocardium at E10.5 and E11.5 when compared to the controls (Fig. S5B). In contrast, the trabecular myocardium was comparable between β-cat^{mKO} and control embryos from E9.5 to E11.5. Consistently, EdU labeling showed that *Ctnnb1*^{mKO} embryos had significantly reduced proliferation in the compact myocardium, while they maintained a normal proliferative rate in the trabecular myocardium (Fig. S5C and D). Together, these findings demonstrate that the WNT/β-catenin signaling is essential for the growth of the compact myocardium.

2.5. WNT/β-catenin and WNT/Ca^{2+} regulate two distinct sets of cardiac genes

We then characterized the molecular changes caused by the inactivation of myocardial β-catenin using qPCR and RNA ISH. The results showed that deletion of myocardial β-catenin had no effects on the expression of gap junction genes (*Cx40*, *Cx43* and *Cx45*), cardiomyocyte genes (*Mlc2a*, *Mlc2v*, *cTnt*, and *Nppa*), and cell cycle related genes *(Ccnd1, p21* and *p53),* and cardiac transcriptional factors *(Tbx5* and *Gata4*). In contrast, the deletion resulted in significantly reduced expression of key cardiac transcriptional factors including *Nkx2.5, Hand1* and *Irx4* (Fig. 7A and B). Given that the supplement of WNT5A was able to restore the expression of broader cardiac genes including *Tbx5, Cx40, Cx45, Nkx2.5, Hand1,* and *Irx4* in cultured *Notch1*^{eKO} embryos (Fig. 6), we speculated that WNT5A might also regulate the myocardial gene expression through the WNT/Ca^{2+} signaling pathway [35]. To test this hypothesis, we cultured E9.5 wild type embryos with the media containing FK506, an inhibitor for the WNT/Ca^{2+} signaling, and found that the FK506 treatment specifically downregulated the expression of *Tbx5, Cx40,* and *Cx45,* but had no impact on the expression of *Nkx2.5, Hand1,* and *Irx4* (Fig. 7C and D). These findings support that WNT/β-catenin and WNT/Ca^{2+} signaling regulates two distinct sets of myocardial genes during cardiac chamber development (Fig. 7E).

3. Discussion

Previous global or pan-endothelial *Notch1 /Rbpj* knockout studies in mice have reported that the NOTCH1 signaling in the endocardium is essential for early ventricular chamber development by regulating cardiomyocyte proliferation and differentiation at E9.5 [19]. Different from these studies, our study applied an endocardial-specific *Notch1* knockout strategy to isolate the endocardial-specific NOTCH1/NRG1 signaling in cardiac chamber morphogenesis at the later stage E10.5. We were able to achieve this goal by avoiding the early lethality of the global or pan-endothelial *Notch1/Rbpj* knockout embryos due to severe cardiac and vascular defects [19,22,36], as well as potential secondary cardiac phenotypes due to a dysfunctional vasculature in the yolk sac and the collapse of embryonic circulation [37,38]. We also provide genetic evidence to support that endocardial NOTCH1 signaling acts upstream of myocardial WNT signaling to maintain the myocardial gene regulatory network essential for cardiac chamber morphogenesis.

Different from the previous studies that have shown normal expression of myocardial differentiation markers *(MLC2v, C-actin, SM- actin* and *Irx4)* [19], we found that the deletion of *Notch1* in the endocardium disrupted a broader cardiac gene regulatory network, including the dramatically decreased expression of *Nkx2.5*, *Gata4*, *Tbx5, Hand1, Irx4,*

Cx40 and *Cx45* at E10.5. This difference likely reflects a temporal function of the endocardial NOTCH1 signaling in the developing cardiac chamber and/or is due to the stage difference between previous and our studies. Nonetheless, our findings provide new evidence to support that the endocardial NOTCH1 signaling is essential for maintaining the developmental gene program for cardiac chamber morphogenesis after early events in cardiogenesis and heart looping.

In addition to the paracrine regulation of myocardial development, we showed that NOTCH in endocardium is required for maintaining the endocardial cell fate. Gene expression analysis showed that the expression of *Nfatc1,* a specific marker for the endocardium in the developing heart [26], was dramatically downregulated, while the expression of *Pecam1* , the general endothelial cell marker, was not affected by the deletion of *Notch1* . Consistent with the previous reports, $Notch1^{eKO}$ embryos showed significantly decreased expression of *Nrg1* as well as *EphrinB2* and *Hand2* [19,28], two known upstream regulators of *Nrg1* . These findings suggest that NOTCH1 signaling is also essential for maintaining endocardial fate in addition to its roles in the myocardial proliferation and differentiation.

Perhaps the most significant new finding is that our study identified and characterized WNT5A as a myocardial mediator between endocardial NOTCH and myocardial WNT signaling in regulating the developmental cardiac gene regulatory network. By screen 12 WNT pathway genes, we found significantly decreased expression of *Wnt5a* in the ventricular myocardium of the $Notch1^{eKO}$ embryos at E10.5 and this reduction was restored by the NRG1 treatment. NRG1 in endocardium is required for sustaining the myocardial gene regulatory networks during cardiac chamber development [27]. These findings strongly suggest WNT5A as a functional linker between the endocardial NOTCH1-NRG1 and the myocardial WNT signaling in regulating myocardial gene expression. This idea was further supported by the rescue experiments which showed that exogenous WNA5A could restore the defective cardiac gene program in the cultured $Notch1^{eKO}$ embryos. Of note, although WNT5A is generally considered as a non-canonical WNT family member, accumulating reports have shown that WNT5A can also induce the canonical WNT signaling in different cells and tissues [30,31]. Consistent with this notion, inactivation of β-catenin resulted in the decreased expression of *Nkx2.5, Hand1* and *Irx4* but had no impact on the expression of *Tbx5, Cx40 andCx45.* In contrast, inhibition of WNT/Ca^{2+} signaling by FK506 repressed the expression of *Tbx5, Cx40* and *Cx45* but did not affect the expression of *Nkx2.5, Hand1* and *Irx4.* Taking together, these findings suggest that WNT5A maintains the cardiac gene program possibly through regulating two distinct sets of cardiac genes via WNT/β-catenin and WNT/Ca^{2+} signaling, respectively.

Our data showed that WNT5A expression is restricted to the compact myocardium where strong WNT/β-catenin activities are present (Fig. 5 and Fig. S4) and WNT/β-catenin signaling regulates the growth of compact myocardium but not the trabecular myocardium (Fig. S5B-D). These data indicate that WNT5A/β-catenin signaling mediates endocardial NOTCH-NRG1 signaling primarily in the compact myocardium but not in the trabecular myocardium. Several signaling pathways and factors have been shown to be required for trabecular myocardium development. *Bmp10* is a well-known growth factor specific for trabecular myocardium development by inhibiting p57kip2 which is a negative regulator

of cell cycle [24]. However, our data showed that *Bmp10* expression in the ventricular chamber was not affected in E10.5 *Notch1* mutant embryos (Fig. S3), indicating that *Bmp10* is not the downstream effector of endocardial NOTCH-NRG1 signaling at this stage. In addition, *FKBP12* is essential for trabecular development and the *FKBP12*-deficient embryos exhibit ventricular hyper-trabeculation and noncompaction [39]. Further studies are needed to determine whether *FKBP12* is regulated by the endocardial NOTCH- NRG1 signaling during cardiac chamber development. The work by Lai et al. showed that NRG1 regulates gene regulatory network in both trabecular and non-trabecular myocardium through activating ErbB-ERK½ signaling [27]. How NOTCH-NRG1 and ErbB-ERK½ signaling differentially regulate the compact versus trabecular myocardial gene expression needs further investigations.

In conclusion, by using genetically modified mice models and whole embryo cultures, we have identified WNT5A in the myocardium as a downstream effector of the endocardial NOTCH1/NRG1 signaling to maintain the developmental gene regulatory network essential for cardiac chamber morphogenesis.

4. Materials and Methods

4.1. Experimental mouse models

Nfatc1^{Cre} mice [23] were crossed with the floxed *Notch1* mice [40] to generate endocardial-specific *Notch1* knockout mice. *Tnt*^{Cre} mice [34] were crossed with the floxed *β-catenin* mice [41] to delete *β-catenin* in myocardium. TCF/Lef:H2B-GFP transgenic mice [33], which express an H2B-EGFP fusion protein under the control of six copies of a T cell specific transcription factor/lymphoid enhancer-binding factor 1 response element and a heat shock protein 1B minimal promoter, were used for visualizing canonical WNT/β-catenin signaling activity in the developing heart. The GFP Cre reporter line R26^{fsGFP} was a gift from Gordon Fishell (New York University Medical School). All mouse strains were maintained on the 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. Noontime on the day of detecting vaginal plugs was designated as E0.5. Adult mice and mouse embryos were PCR genotyped using tail and yolk sac DNA, respectively.

4.1.1. Histology—Embryos were dissected between E9.5 and E11.5, 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 or front sections and cut at 6 μm using a Leica microtome. Hematoxylin and eosin (H&E) staining was performed for histology using standard protocol. H&E stained tissue sections were examined and photographed using a Zeiss Axio Observer Z1 inverted microscope.

4.1.2. Immunohistochemistry and Immunofluorescence staining—Tissue sections were prepared as described above for histology and then antigen retrieved by boiling for 10 min in sodium citrate (10 mM, pH 6.0) (Vector Laboratories). The tissues were incubated in 1% H2O2 for 30 min to quench the endogenous hydroperoxide

activities. M.O.M kit (Vector Laboratories) was used for immunostaining for NFATC1 antibody (BD Pharmingen, 556,602). The brown color was developed using DAB kit (Vector Laboratories). The tissues were then blocked with 5% normal horse serum in PBS before being incubated with PECAM1 antibodies (Santa Cruz Biotechnology, sc-1506) and secondary anti-bodies. The red color was developed using Vector Red Kit (Vector Laboratories). Immunofluorescence staining of NOTCH1 was performed on frozen sections. The embryos were collected at E10.5 and fixed in 4%PFA at 4 °C for one hour, soak in 15% and 30% sucrose sequentially and embedded in OCT compounds with orientation for front sections at 8 μm on the positive charged slides. Tissue sections were then post-fixed in the cold ethanol and acetone (1:1) solution for 5 min and stored at − 80 °C. Tissue sections were air dried for 45 min before staining. The tissues were then blocked with 5% normal horse serum in PBS before being incubated with NOTCH1 antibodies (R&D, AF5267) and secondary antibodies. Stained tissue sections were photographed using a Zeiss Imager A2 microscope.

4.1.3. EdU staining—Cell proliferation was determined using an EdU assay. Pregnant female mice were injected with EdU (Life Technology) through intraperitoneal injection at a concentration of 100 mg/kg. After a 2-h pulse, embryos were collected and processed for frozen sections. Briefly, E10.5 or E11.5 embryos were fixed in 4%PFA at 4 °C for one hour, soak in 15% and 30% sucrose sequentially and embedded in OCT compounds with orientation for front sections at 8 μm on the positive charged slides. Tissue sections were then post-fixed in the cold ethanol and acetone (1:1) solution for 5 min and stored at −80 °C. Tissue sections were air dried for 45 min before staining. Serial sections crossing the entire cushion region were first stained with PECAM1 antibody followed by EdU staining with EdU imaging Kit (Life Technology) and counterstained with DAPI (Vector Laboratories). The stained sections were photographed using a Zeiss Imager M2 microscope. The positive EdU labeling was quantified for the endocardium, trabeculae, or compaction myocardium using Image J software. Three embryos were analyzed for each genotype.

4.1.4. RNA extraction and quantitative PCR (qPCR)—Total RNAs were isolated from three pooled E10.5 hearts using Trizol (Invitrogen). First strand cDNA was synthesized using the Superscript II Reverse Transcriptase Kit (Invitrogen). qPCR was performed using the 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 $2^{-\Delta\Delta CT}$method. Biological repeats were performed using three different samples of three pooled hearts for each genotype, and technical triplicates were carried out for each run. The mean relative expression of each gene between groups was used for statistical significant analysis.

4.1.5. RNA in situ hybridization—RNA in situ hybridization (ISH) was performed as previously described [22]. Digoxigenin-labeled complementary RNA probes for *Tbx5, Nkx2.5, Hand1, Hand2, Gata4, Irx4, Bmp10, Cx40, Cx43* and *Cx45* were prepared from the linearized plasmids or PCR products by reverse transcription. E10.5 embryos were isolated in RNase-free PBS, fixed overnight in 4%PFA at 4 °C, dehydrated through a methanol series, rehydrated and treated for one hour at room temperature (RT) with 3%

H_2O_2 to quench the endogenous peroxidases. Embryos were then digested by proteinase K (10 μg/ml) for 15 min at RT, fixed 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 then post-fixed, dehydrated, embedded in paraffin and sectioned. The images were collected using a Zeiss Axio Imager M2 microscope. For each gene, three to five age-matched embryos from control or mutant groups were analyzed simultaneously.

4.1.6. Whole embryo culture—Whole embryo culture was performed as described previously [22]. 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% oxygen, 5% CO2 and 35% N2) at 37C for 24h. For rescue experiments, NRG1 (1.25×10^{-8}M) or WNT5a (200 ng/ml) (R&D systems) was added into the culture medium. For inhibition of WNT/Ca^{2+} pathway, 1 μg/ml of FK506 was added into the culture media. The cultured embryos were used for gene expression assays using qPCR and/or RNA ISH as described above.

4.2. Statistical analysis

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

Supplementary Material

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Acknowledgments

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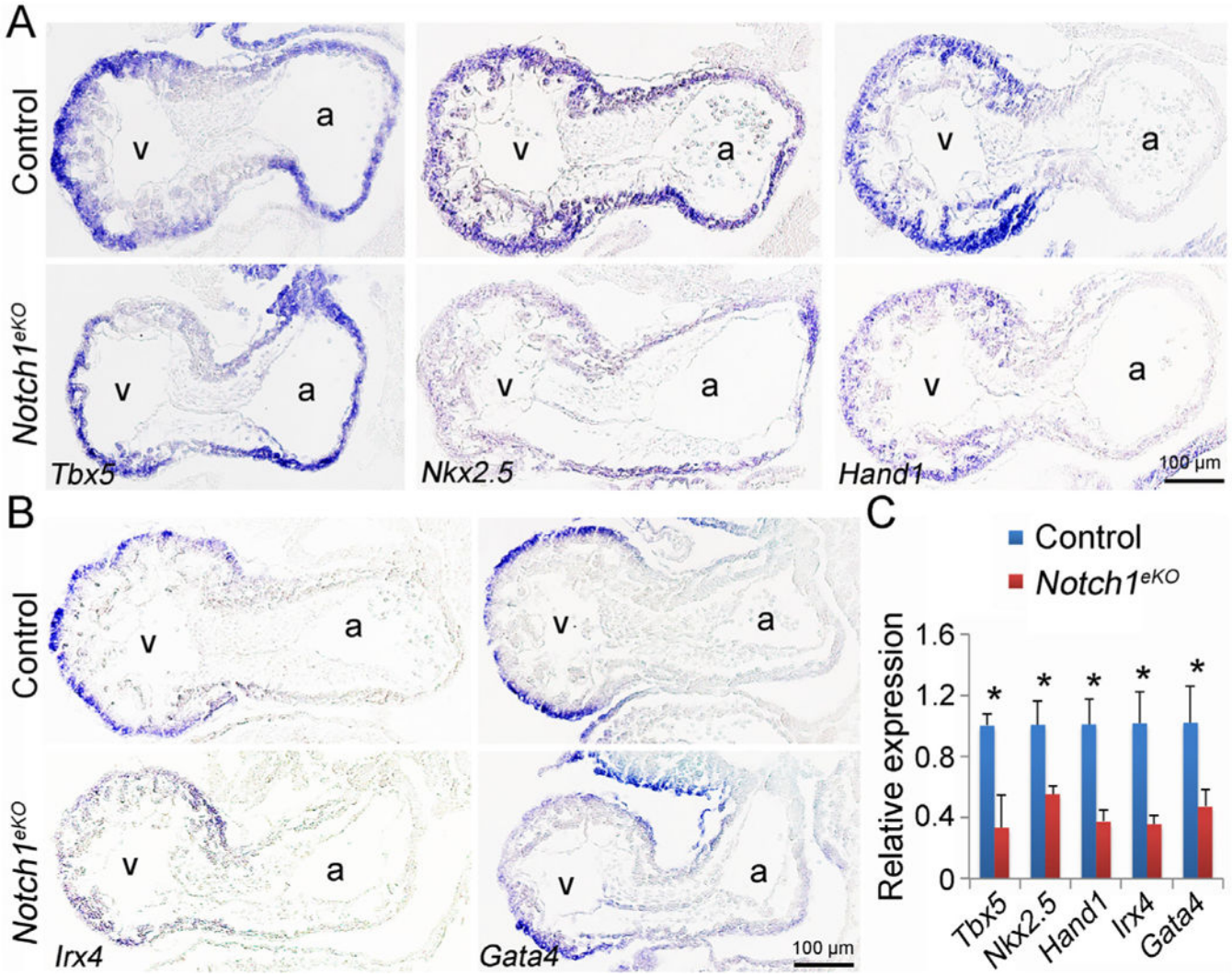

Fig. 1.

Deletion of *Notch1* in endocardium impairs the expression of key cardiac transcriptional factors. (A and B) *RNA* in situ hybridization was used to determine gene expression in control (*Notch1*$^{f/f}$ or *Notch1*$^{f/+}$) and *Notch1*^{eKO} (*Notch1*$^{f/f}$*: Nfatc1*^{Cre}) embryos at E10.5. Three to five embryos were analyzed for each genotype and each gene simultaneously. The representative images show the expression of indicated genes. a, atrium; v, ventricle. (C) Quantitative PCR (qPCR) was used to determine gene expression in the hearts of E10.5 control and *Notch1*^{eKO} embryos. The expression of target genes was normalized to the expression level of *Gapdh*. Two to three hearts were pooled as one sample. $n = 3$/group. *$p < 0.05$.

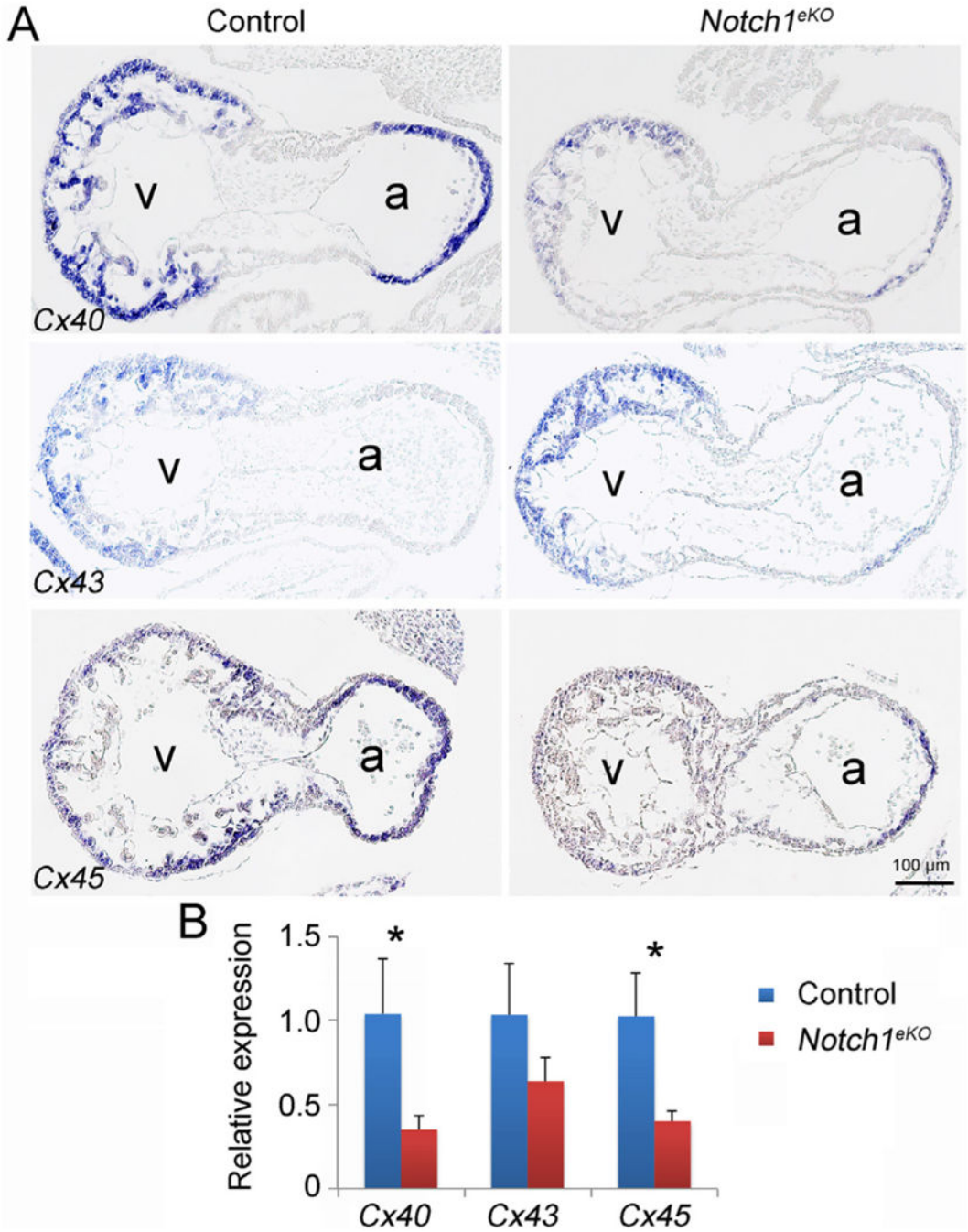

Fig. 2.

Deletion of *Notch1* in endocardium impairs the expression of gap junction genes. (A) *RNA* in situ hybridization was used to determine gene expression in control and *Notch1*^{eKO} embryos at E10.5. Three to five embryos were analyzed for each genotype and each gene simultaneously. The representative images show the expression of *Cx40*, *Cx43* and *Cx45.* a, atrium; v, ventricle. (B) qPCR was used to determine gene expression in the hearts of E10.5 control and *Notch1*^{eKO} embryos. The expression of target genes was normalized to

the expression level of *Gapdh.* Two to three hearts were pooled as one sample. n = 3/ group. *p < 0.05.

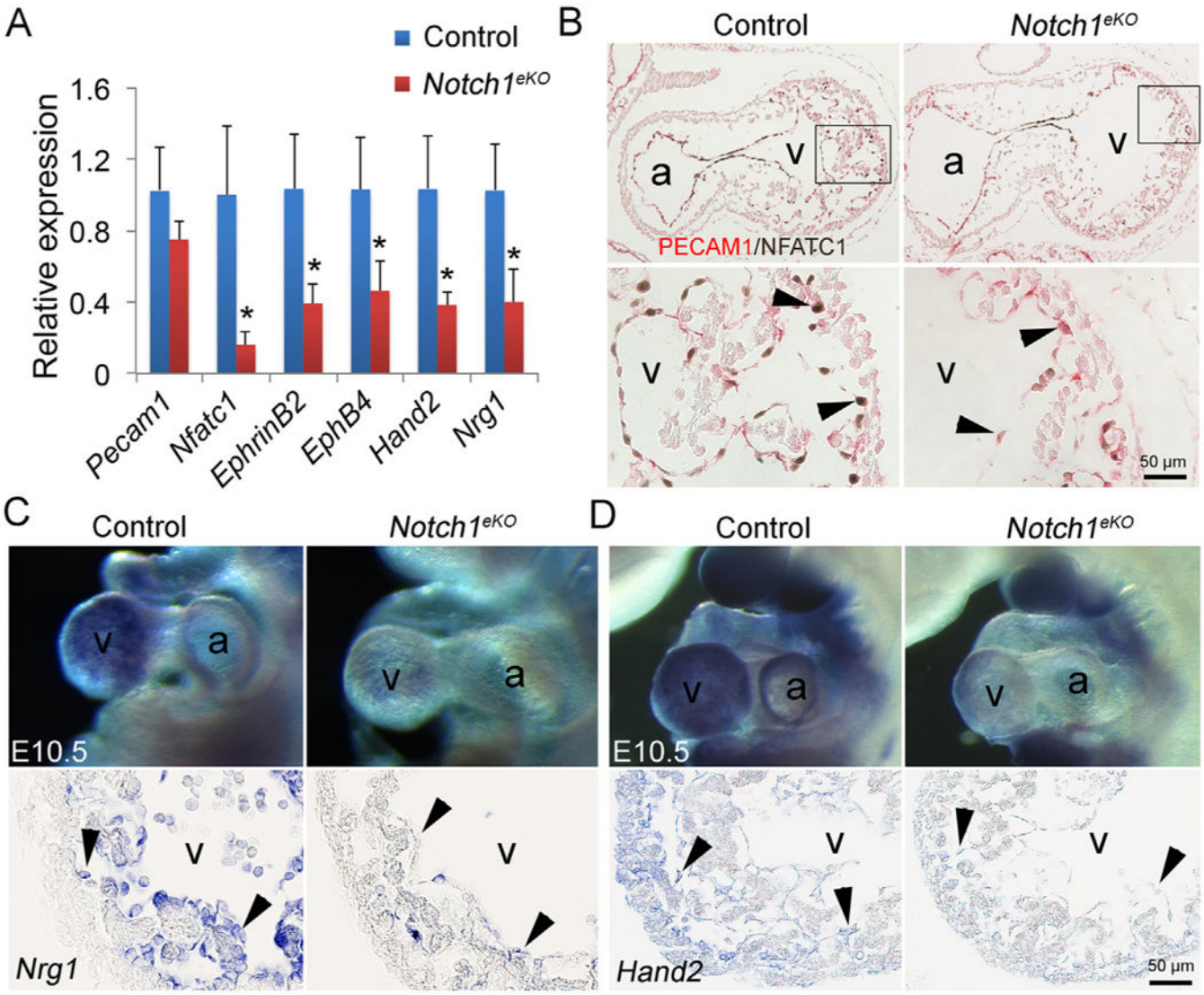

Fig. 3.

Deletion of *Notch1* impairs the expression of genes in endocardium. (A) qPCR was used to determine the expression of endocardial genes in the hearts of E10.5 control and *Notch1*^{eKO} embryos. The expression of target genes was normalized to the expression level of *Gapdh.* Two to three hearts were pooled as one sample. n = 3/group. *p < 0.05. (B) Co-Immunostaining of NFATC1 (brown) and PECAM1 (red) show NFATC1 expression in the hearts of E10.5 control and Notch1^{eKO} embryos. The arrowheads indicated endocardium. The bottom panel shows the high magnification of boxed regions in upper panel. a, atrium; v, ventricle. (C and D) *RNA* in situ hybridization was used to determine the expression of *Nrg1* and *Hand2* in control and *Notch1*^{eKO} embryos at E10.5. Three to five embryos were analyzed for each genotype and each gene simultaneously and the representative images were shown. a, atrium; v, ventricle. The arrowheads indicated endocardium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

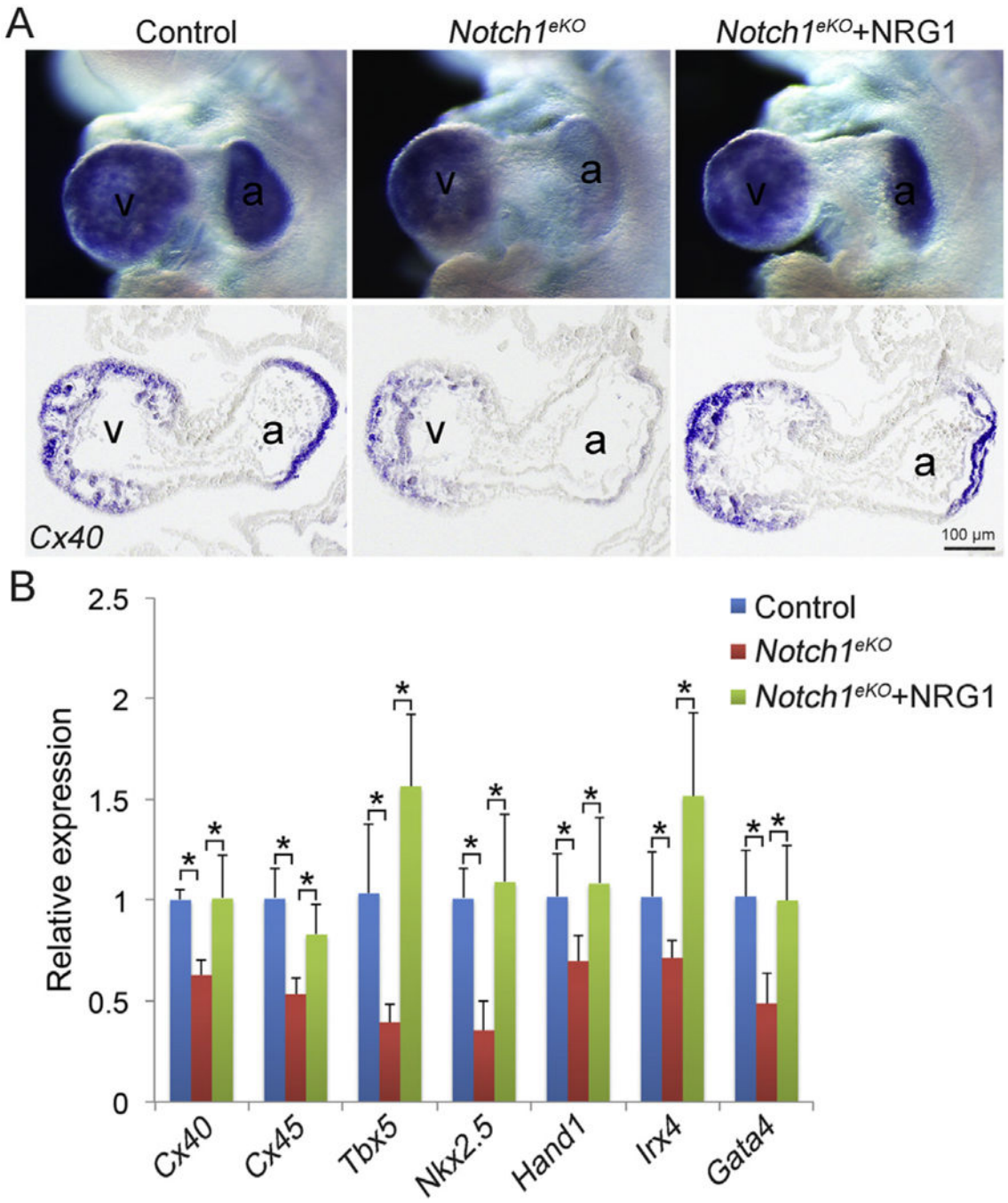

Fig. 4.

Endocardial *Notch1* regulate myocardial gene expression via *Nrg1.* (A and B) E9.5 control and *Notch1*^{eKO} embryos were cultured in media with or without recombinant NRG1 proteins at 1.25×10^{-8} M. The embryos were cultured for 24h and subjected to gene expression analysis using *RNA* in situ hybridization (A) and qPCR (B). For *RNA* in situ hybridization assay, three to five embryos were analyzed for each genotype simultaneously. For qPCR analysis, the expression of target genes was normalized to the expression level of

Gapdh. Two to three hearts were pooled as one sample. n = 3/group. *p < 0.05. a, atrium; v, ventricle.

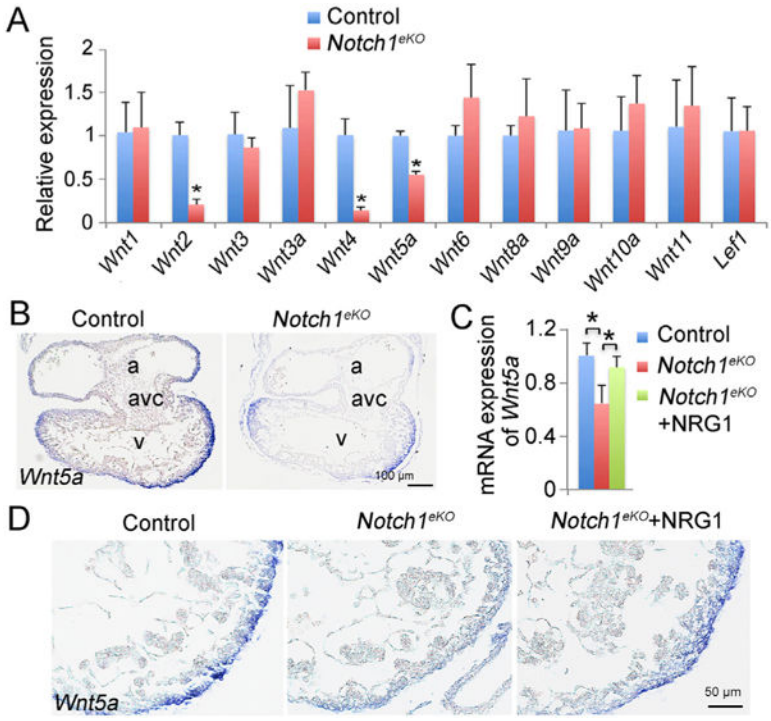

Fig. 5.

Endocardial *Notch1* regulates myocardial *Wnt5a* via *Nrg1.* (A and B) qPCR was used to determine the expression of 12 genes in WNT pathway in the hearts of E10.5 control and *Notch1*^{eKO} embryos. (B) *RNA* in situ hybridization was used to determine the expression of *Wnt5a* in control and *Notch1*^{eKO} embryos at E10.5. Three to five embryos were analyzed for each genotype simultaneously and the representative images were shown. a, atrium; v, ventricle; avc, atrioventricular canal. (C and D) E9.5 control and *Notch1*^{eKO} embryos were cultured in media with or without recombinant NRG1 proteins at 1.25 $\times 10^{-8}$ M. The embryos were cultured for 24 h and subjected to gene expression analysis using qPCR (C) and *RNA* in situ hybridization (D). For RNA in situ assay, three to five embryos were analyzed for each genotype simultaneously. For qPCR analysis, the expression of *Wnt5a* was normalized to the expression level of *Gapdh*. Two to three hearts were pooled as one sample. n = 3/group. *$p < 0.05$.

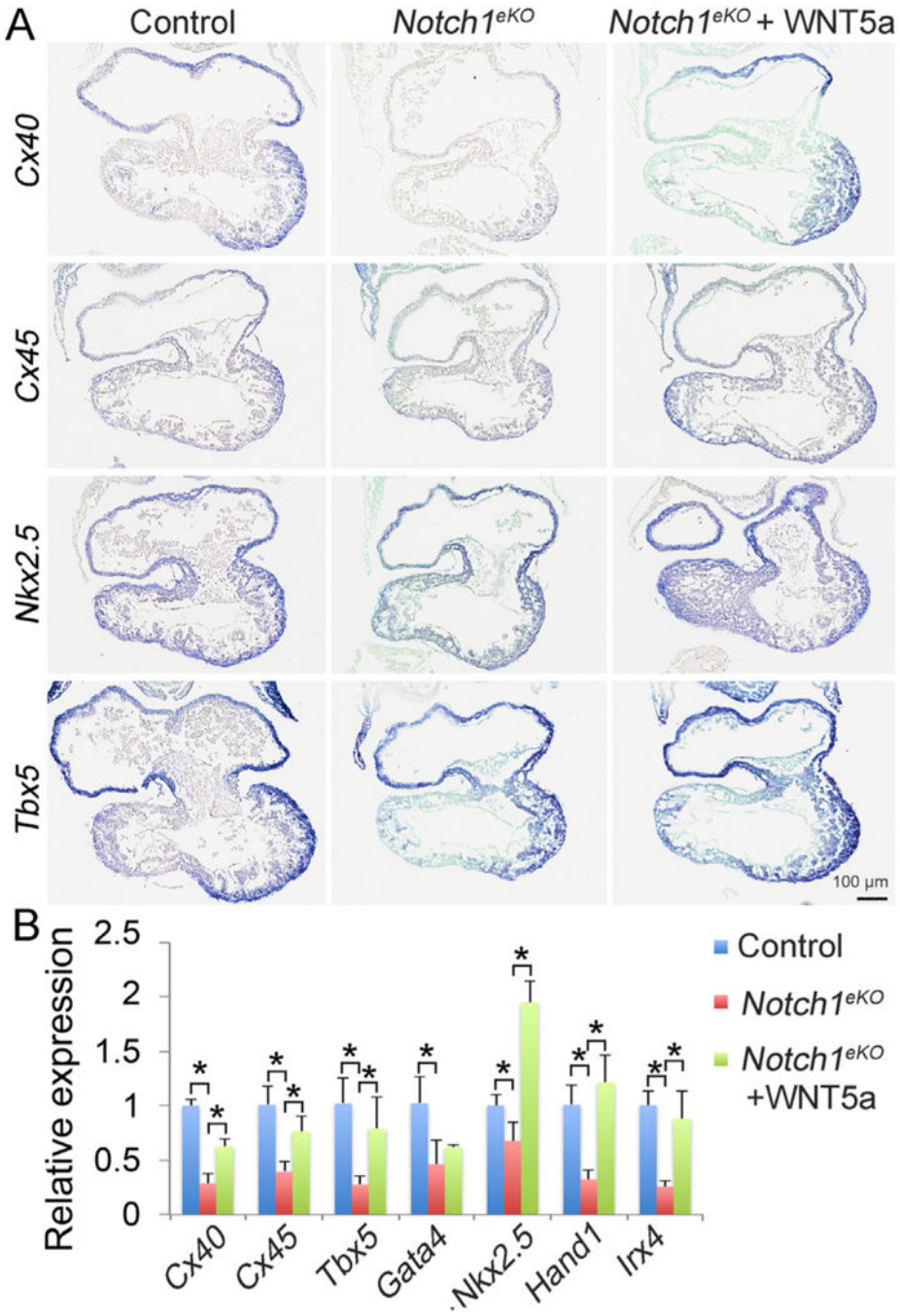

Fig. 6.

Endocardial *Notch1* regulates myocardial gene expression partially through WNT5A. (A and B) E9.5 control and *Notch1*^{eKO} embryos were cultured in media with or without recombinant WNT5A proteins at 200ng/ml. The embryos were cultured for 24 h and subjected to gene expression analysis using *RNA* in situ hybridization (A) and qPCR (B). For *RNA* in situ hybridization assay, three to five embryos were analyzed for each genotype and each gene simultaneously. For qPCR analysis, the expression of target genes

was normalized to the expression level of *Gapdh*. Two to three hearts were pooled as one sample. n = 3/group. *p < 0.05.

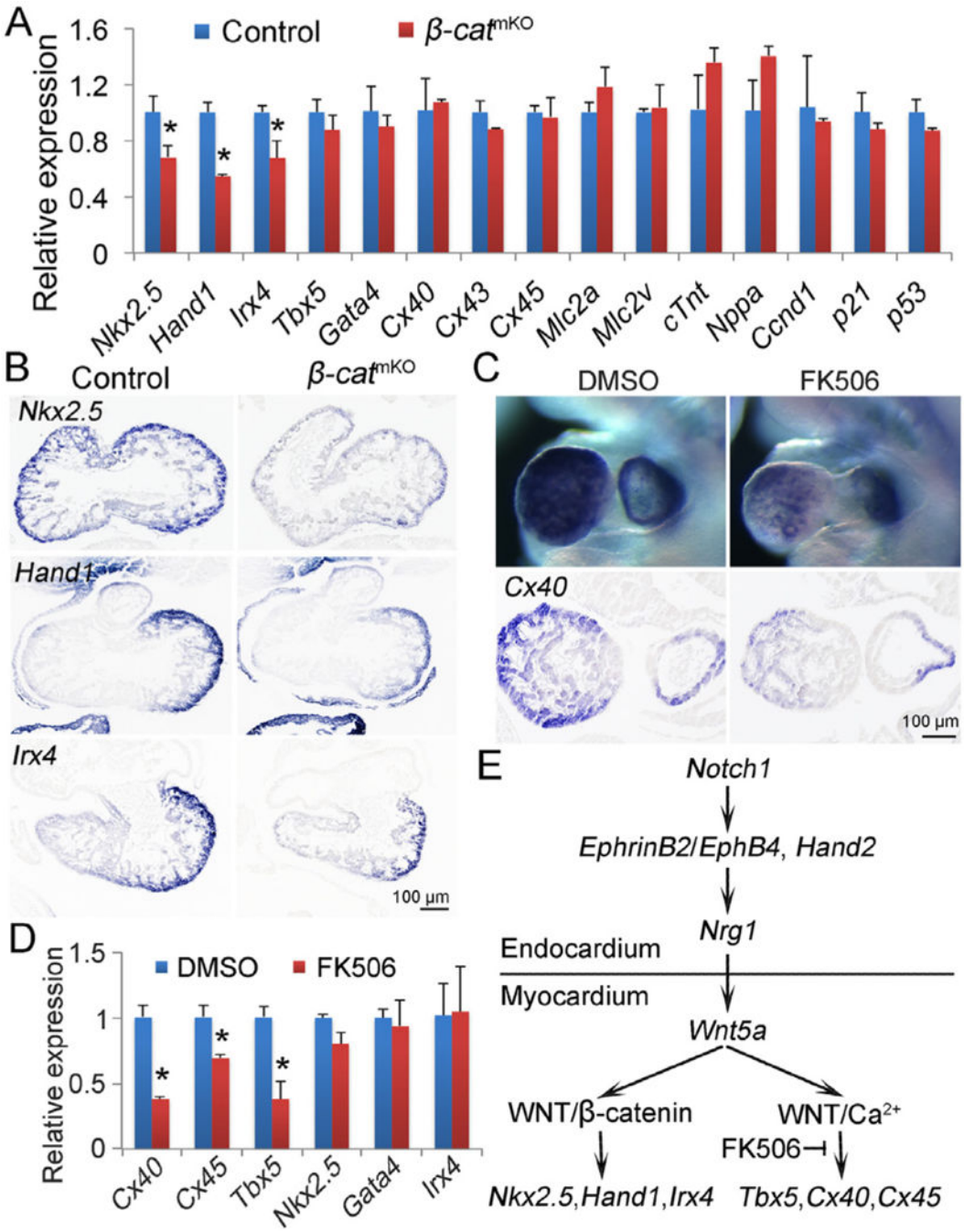

Fig. 7.

WNT/β-Catenin and WNT/Ca^{2+} signaling regulate distinct sets of genes. (A) qPCR was used to determine gene expression in the hearts of E10.5 control (β-catenin$^{f/f}$ or β-catenin$^{f/+}$) and β-cat^{mKO} (β-catenin$^{f/f}$:*Tnt*^{Cre}) embryos. (B) *RNA* in situ hybridization was used to determine the expression of *Nkx2.5, Hand1* and *Irx4* in E10.5 control and β-cat^{mKO} hearts. (C and D) E9.5 WT embryos were cultured in media with or without FK506 (100 ng/ml). The embryos were cultured for 24 h and subjected to gene expression analysis using *RNA* in situ hybridization (C) and qPCR (D). For RNA in situ hybridization assay, three

to five embryos were analyzed for each condition simultaneously. For qPCR analysis, the expression of target genes was normalized to the expression level of *Gapdh*. Two to three hearts were pooled as one sample. n = 3/group. *$p < 0.05$. (E) A schematic model shows that endocardial *Notch1* promotes the expression of *Nrg1* through *ephrinB2/Hand2* in endocardium. The *Nrg1* then induces the expression of *Wnt5a* in myocardium. WNT5A regulates two distinct sets of genes through WNT/β-Catenin and WNT/Ca^{2+} pathway respectively.