A lineage study for cardiac conduction system in mice

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1. Summary

The heart is an essential organ for blood circulation to whole body via keeping heart beats with constant rhythm. The rhythm is produced by electric stimulation generated autonomously in the cardiac conduction system (CCS). The defects of CCS induce severe cardiac disease called cardiac arrhythmia. Popular therapies for the disease are the implantation of pacemakers and catheter ablation, but the quality of life of the patients is limited with these methods. For this reason, establishing a new therapy by transplantation of CCS cells is expected. However, the method is not established yet because the origin and developmental process of CCS cells have not been understood well.

The CCS is composed of special cardiomyocytes called pacemaker cells and Purkinje cells. Pacemaker cells in the sinoatrial node (SAN) and the atrioventricular node (AVN) produce electric stimulation and the stimulation is expanded to all cardiac chambers via Purkinje cells. Although CCS cells are a kind of cardiomyocyte, its developmental process is different from the myocardium based on the previous lineage tracing study using *Mesp1*-Cre mouse. Even though *Mesp1* lineage contributes to almost all cardiac components derived from the cardiac crescent, the CCS was not derived from the *Mesp1* lineage. On the other hand, cell tracking analyses by dye labeling using mouse and chick embryos implied that progenitor cells of CCS are localized in the caudal-lateral parts of the cardiac crescent. Based on these studies, I hypothesized that CCS progenitor cells can be identified by specific marker genes which are expressed on the cardiac crescent.

As a candidate gene possibly marking early CCS progenitor cells, I focused on the *Secreted-frizzled related protein 5* (*Sfrp5*) gene. *Sfrp5*-venusYFP (vYFP) knock-

in (KI) mice revealed that the expression of vYFP was observed in the cardiac crescent stage and gradually restricted to the CCS in the four-chambered heart stage. It suggests that *Sfrp5* is continuously expressed in CCS lineages during development. In order to trace the lineage of *Sfrp5*, I generated KI mice which Cre and CreERT2 is knocked-in into the *Sfrp5* locus. The lineage tracing analysis using *Sfrp5*-Cre mouse revealed that the descendants differentiated into many cardiac components including the CCS in all the chambers except for myocardium in the right ventricle. Furthermore, the lineage tracing analysis using *Sfrp5*-CreERT2 mouse indicated that the *Sfrp5* expressing cells at embryonic day (E) 7.5 contributed to the CCS. These results indicated that *Sfrp5* could mark CCS lineages much earlier than *Hcn4*, which is known as a CCS lineage marker gene and can mark CCS lineages only after E9.5. However, *Sfrp5* is not a specific marker for CCS progenitor cells. Therefore, next I focused on the difference of the contribution between *Mesp1* and *Sfrp5* lineages. In order to trace the cell lineage simultaneously in a single embryo, I established *Mesp1*-Cre/*Sfrp5*-Dre and *Mesp1*-Dre/*Sfrp5*-CreERT2 mice. Double lineage tracing analyses using *Mesp1*-Cre/*Sfrp5*-Dre mice showed that major parts of SAN head and Purkinje cells were derived from the cells once expressed *Sfrp5* but not *Mesp1* (*Mesp1*-negative/*Sfrp5*-positive cells), and the analyses using *Mesp1*-Dre/*Sfrp5*-CreERT2 mice confirmed that the *Mesp1*-negative/*Sfrp5*-expressing cells at E7.5 contributed to the SAN. To observe these cells at E7.5, I crossed *Mesp1*-Cre mouse with CAG-floxed-CAT-mCherry/*Sfrp5*$^{v/v}$ mice, and found that *Mesp1*-negative/*Sfrp5*-expressing cells were frequently observed on the posterior side of the cardiac crescent, implying that the area is the candidate that includes CCS specific progenitor cells. To further restrict the CCS progenitors, I conducted lineage tracing

analyses focusing on the *T*-Cre lineage that marks more posterior mesodermal cells than the *Mesp1*-Cre lineage. The lineage contribution was observed in the SAN and Purkinje cells, but not in the AVN. Furthermore, double lineage tracing analyses of *Mesp1*-Dre and *T*-Cre showed that a few *Mesp1*-negative/*T*-positive cells observed on the posterior cardiac crescent are strong candidates for SAN progenitors.

In conclusion, based on the lineage tracing analyses using different combinations of three markers, I found that SAN and Purkinje cells but not AVN were derived from a common origin. I expect that isolating the progenitor cells of each CCS component using these three markers should enable us to identify differentially expressed genes and help us to understand the developmental process of each CCS component. In addition, I showed that almost all differentiated cells except for the CCS lost the expression of *Sfrp5*, implying that SFRP5 has an important role for CCS cell development. Further analyses focusing on the function of SFRP5 would help in elucidating the molecular mechanism of CCS development.

2. Abbreviations

AVC	atrioventricular canal
AVN	atrioventricular node
CCS	cardiac conduction system
E	embryonic day
EMT	epithelial-mesenchymal transition
FHF	first heart field
GFP	Green Fluorescent Protein
IHC	immunohistochemistry
IVS	inter ventricular septum
KI	knock-in
KO	knock-out
LA	left atrium
LBB	left bundle branch
LV	left ventricle
OFT	outflow tract
PS	primitive streak
PSM	presomitic mesoderm
R26R	*Rosa26*-LacZ receptor mouse
RA	right atrium
RBB	right bundle blanch
RFP	Red Fluorescent Protein
RV	right ventricle
SAN	sinoatrial node
SHF	second heart field
SV	sinus venosus
SVC	superior vena cava
SVP	sinus venosus primordia

3. Gene Symbol

Cx40	*connexin40*
Fgf10	*fibroblast growth factor 10*
Foxa2	*forkhead box A2*
Hcn4	*Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4*
Islet1	*ISL1 transcription factor, LIM/homeodomain*
Mef2c	*Myocyte-specific enhancer factor 2C*
Mesp1	*mesoderm posterior 1*
Mlc2a	*myosin light chain 2*
Nkx2.5	*NK-2 transcription factor related, locus 5*
Sfrp	*Secreted frizzled related protein*
Shox2	*Short Stature Homeobox 2*
T	*T/brachyury*
Tbx18	*T-Box18*
Tbx2	*T-Box2*
Tbx3	*T-Box3*
Tbx5	*T-Box5*
Wt1	*Wilms tumor 1 homolog*

4. Introduction

The heart is an essential organ for blood circulation throughout the entire body and beats with a constant rhythm. The heart beating rhythm is initiated by the electric stimulation generated autonomously by pacemaker cells in the sinoatrial node (SAN) which exists on the posterior wall of the right atrium (RA) near the entrance of the superior vena cava (SVC). The stimulation spreads to the atrium to induce atrial contraction and converged atrioventricular node (AVN).The AVN is also composed of pacemaker cells that propagates the electric pulse to ventricles through Purkinje fibers for separating the contractions of atrial and ventricular chambers. This cellular network system for making the beating rhythm is called the cardiac conduction system (CCS). Defects in the CCS induce not only an irregular beating rhythm but also insufficient electric stimulation for contraction, which leads the cardiac dysfunction called cardiac arrhythmia. This disease occurs by both congenital and acquired reasons, and is known as one of the causes of sudden death. Implantation of pacemakers, defibrillators, catheter ablation, etc. are popular treatments for this disease, but these methods are burdensome to the patient and thus the quality of life of the patient is also limited. For this reason, the establishment of a new therapy by transplantation of CCS cells is expected. However, the method is not established yet because the origin of CCS cells and the process of CCS development have not been understood well. In my thesis study, I tried to identify the origin of the CCS for understanding the developmental process of the CCS in detail.

The cellular origin and developmental process of the CCS may be different from those of other cardiac components, since even though pacemaker cells and Purkinje cells express general cardiomyocyte markers α-actinin and desmin, those also express CCS specific markers, Hcn4 or Cx40 and can produce the electric stimulation[1,2]. It was

reported that almost all cardiac components are derived from cells transiently expressing the transcription factor Mesp1, which is the master regulator of cardiovascular development at embryonic day 6.5 (E6.5) in mice[3-5]. However, some cell clusters that were not derived from the *Mesp1* lineage (*Mesp1*-negative lineage) were observed in the four-chambered heart at E13.5. The clusters were identified as components of the CCS by the comparison with the expression pattern of CCS-LacZ reporter mouse, which is expected to stain all CCS components[6,7]. However, it was not clear whether the CCS-LacZ reporter mouse really marks all CCS components because marker genes for CCS were not identified previously.

The origin of the CCS was suggested by several researchers. One is cells in the sinus venosus primordia (SVP)[8] marked by *Hcn4*, which encodes a potassium/sodium channel protein, at E9.5[9]. The SVP is known as a temporal structure formed above the primitive atrium from E8.5, and the SVP contains progenitor cells contributing to the SAN and the sinus venosus (SV) in four-chambered heart. In addition to *Hcn4*, *Tbx18* and *Shox2,* which encode transcription factors, are expressed in the SVP from E8.5, and the expression become restricted to the SAN in later stages[10-12]. These transcription factors are known as regulators that induce differentiation of pacemaker cells *in vivo* and in a culture system[11-16]. However, it is not known whether cells expressing *Tbx18* or *Shox2* at E8.5 contain the population that contributes to the SAN because no time-dependent lineage tracing analysis is reported.

Another report indicates that CCS progenitors can be identified in the cardiac primordium called the cardiac crescent at E7.5. The cardiac crescent is mainly derived from the *Mesp1* lineage. The cardiac mesoderm cells derived from the *Mesp1* lineage ingress through the primitive streak and migrate with the cranial mesoderm, and form a

cardiac primordium exhibiting a crescent shape. It can be divided into the first heart field (FHF) and second heart field (SHF) based on the difference in lineage contribution in the four-chambered heart[17]. The FHF is known as classical primordia and expresses several cardiac specific genes, *Nkx2.5, Mlc2a* and *Tbx5,* at E7.5. The cells differentiate into myocardium, in all chambers[18-21]. The SHF is another cardiac progenitor field located medial and posterior to the FHF and contains cells expressing *Islet1, Mef2c* and *Fgf10*. The lineage tracing analysis of SHF using *Islet1*-Cre mice revealed that the lineage contributes to not only the myocardium in right ventricle (RV), outflow tract (OFT), and left and right atrium (LA and RA) but also the pacemaker cell in the SAN[22-24]. However, it is not revealed that whether the *Islet1* expressing cells on the cardiac crescent contributes to the SAN or not because no time-dependent *Islet1*-lineage tracing analysis is reported. In chick, cell tracking analysis by dye-labeling implied that CCS-specific progenitor cells exist on the lateral-caudal cardiac crescent, and differentiated into pacemaker cells[25]. Furthermore, cell tracking analysis in mice also showed that the lateral-caudal side of the cardiac crescent contains *Nkx2.5*-negative mesodermal cells that contribute to the SVP marked by *Tbx18*, which are thought to be SAN progenitors by embryonic culture system[26]. These reports suggest that CCS specific progenitor cells are located in a similar position on the cardiac crescent in both chick and mouse. Taken together, although it is likely that CCS progenitor cells at E9.5 are derived from the *Mesp1*-negative cell lineage, the origin and developmental process is not clarified yet because a CCS specific marker gene for the early stages has yet to be identified.

To know the developmental process of the CCS in detail, identifying appropriate marker genes in the early stage is necessary. Recently, our laboratory identified the *Secreted frizzled related protein 5* (*Sfrp5*)-expressing progenitor

population in the cardiac crescent at E7.5[8]. *Sfrp5* is a family gene of SFRP family proteins 1, 2 and 5 and an inhibitor of Wnt signaling, which is known as an important signal for cardiac mesoderm[1,27-32]. The expression of *Sfrp5* was observed from E7.5 in the lateral-caudal side of the cardiac crescent as a distinct population from the FHF and SHF. The reporter analysis indicated that the *Sfrp5* expression was gradually confined to the SVP cells that contained CCS progenitors. Furthermore, its expression pattern at the later stage (E14.5) is quite similar to that of the CCS. Therefore, I speculated that *Sfrp5* could be a good marker for the identification of the CCS population. Although venus YFP knock-in mouse (*Sfrp5*-venus) line was established previously, no detailed lineage-tracing analysis or any correlation study with other lineages has been conducted. Therefore, I started my thesis study to identify CCS progenitor cells using *Sfrp5* and other early progenitor marker genes. The identification of the CCS progenitors and the markers useful to isolate such progenitors should contribute to the development of therapeutic methods in the near future.

5. Results

5-1. *Sfrp5* is a useful marker for CCS progenitors and differentiated CCS components.

In order to clarify the relationship between *Sfrp5* expression and CCS lineage, I took advantage of the *Sfrp5*$^{v/+}$ mouse line, in which a venus YFP (vYFP) reporter is knocked into the endogenous *Sfrp5* locus[33]. Using these mice, I could monitor *Sfrp5* expression without immunostaining. The vYFP expression was observed from E7.5 in the cardiac crescent (Fig. 1A) and the expression was continuously observed in the whole heart tube at E8.5-E9.5. However, stronger expression was observed in the posterior heart tube, including the SVP (Fig. 1B, C). At E10.5, the vYFP signal was clearly concentrated between the RA and SVP. Interestingly, additional signals became stronger in the trabecular zone of the LV and RV (Fig. 1D-H). The distribution pattern of vYFP signals at E14.5 was very similar with the left and right bundle branches (LBB and RBB) of the CCS, which is known to function in spreading the electric power from the AVN to the ventricles (Fig. 1I)[7]. Based on these observations, I hypothesized that the *Sfrp5* is expressed in the CCS during heart development.

To confirm that *Sfrp5*$^{v/+}$ expressing cells represent the CCS and the progenitors, I conducted double immunohistochemistry (IHC) using anti-HCN4 and anti-GFP antibodies. HCN4 is known as a marker for both CCS progenitor cells at E9.5 and differentiated SAN[9,34] at E14.5-16.5. I found that most of the HCN4-positive cells were also marked by strong *Sfrp5*$^{v/+}$ signals, although vYFP signals were more widely observed at E9.5 (Fig. 2A, B). At E16.5, the vYFP signals were well overlapped with HCN4 signals in cells located between the RA and SV, suggesting that pacemaker

cells in the SAN also express *Sfrp5* (Fig. 2C, D). I also observed vYFP signals in the trabecular region of the left ventricle. I speculated that these signals represented the ventricular Purkinje cells. Connexin40 (Cx40) is a gap junction protein expressed in Purkinje cells in the trabecular zone that spread the electric stimulation[35]. The vYFP signals were widely observed in the trabecular zone of the LV and RV, and these signals were overlapped with Cx40 suggesting that the *Sfrp5* expression was kept in the differentiated Purkinje cells (Fig. 2E, F).

These observations indicate that the expression of *Sfrp5* is kept in all CCS components and the progenitor cells; therefore, *Sfrp5* may represent a useful marker for understanding CCS development.

5-2. *Sfrp5* expressing cells on the cardiac crescent differentiate into the CCS.

Differentiated CCS and the progenitor cells kept expression of *Sfrp5*. However, *Sfrp5* is expressed from E7.5 and its lineage contributes to not only the SVP but also the heart tube at E10.5[8]. Therefore it has not been clear whether *Sfrp5*-expressing cells on the cardiac crescent contribute to CCS. . In order to investigate the *Sfrp5* expressing cell lineage, I generated *Sfrp5*-Cre KI mice and crossed them with the CAG-CAT-eGFP (GFP reporter) mice[8,36]. GFP positive cells were broadly observed in the upper side of the RA, RA, LA and LV, except for the RV at E16.5. I conducted double fluorescent immunohistochemistry with the CCS markers (Fig. 3A). As expected, GFP-positive cells also expressed the CCS marker HCN4 in the E16.5 heart suggesting that *Sfrp5* derived cells differentiated into the SAN (Fig. 3B, B'). The lineage contribution to the compact layer of RV was lower than those of other cardiac chambers, but several GFP-positive cells were observed in the trabecular

zone of the RV (Fig. 3B, C). The GFP-positive cells were also positive for Cx40, similar with those in the atrium and trabecular zone of the LV, demonstrating that cells expressing *Sfrp5* differentiated into Purkinje cells in all the chambers (Fig. 3C, C'). These results were consistent with the *Sfrp5* expression observed in *Sfrp5*$^{v/+}$ embryos (Fig. 2).

As shown in Fig. 1, *Sfrp5* is expressed from the cardiac crescent stage to the differentiated CCS stage. Next, I wanted to define the earliest time point of *Sfrp5* expression, by which we can mark progenitors for CCS. For this purpose, I generated *Sfrp5* CreERT2-KI mice and crossed them with *Rosa26-LacZ* reporter mice (R26R)[8,37]. I treated tamoxifen (TM) once by oral administration to pregnant R26R mice at E7.5, E8.5, E9.5 or E10.5 and obtained the fetal heart at E16.5 for LacZ staining (Fig. 4A). The distribution pattern of β-gal positive cells in the heart that was given TM at E7.5 (E7.5TM heart) was similar with the results of the *Sfrp5* Cre lineage tracing analysis (Fig. 3); the staining was observed in the upper side of the RA, SV, LA, RA and LV, except for the RV (Fig. 4B-B''), but the population of LacZ positive cells was significantly lower in the E7.5 TM heart compared with the results of the *Sfrp5*-Cre lineage tracing study[8].In addition, LacZ positive cells in the LV were decreased in the E8.5 TM and E9.5 TM hearts (Fig. 4C-D''), and the staining was detected only in the SV when TM was administrated at E10.5 (Fig. 4E-E''), suggesting that the *Sfrp5* expressing cells becomes restricted along with heart development. The β-gal-positive cells were observed in the upper side of the RA and LV of the E7.5 TM heart. As the SAN is located inside of the RA aperture and it is hard to observe by macroscopically, therefore, I prepared frozen sections for IHC from *Sfrp5*CreERT2 mice crossed with GFP reporter mice and administrated

TM at E7.5 (Fig. 5A). In the fetal heart at E16.5, GFP-positive cells were observed in the RA aperture and these cells also expressed HCN4 (Fig. 5B, C), confirming that the lineage of *Sfrp5* from E7.5 differentiated into the SAN. GFP-positive cells were also observed in the trabecular zone of the ventricle and those were positive for Cx40 (Fig. 5D), confirming that the lineage of *Sfrp5* from E7.5 also differentiated into Purkinje cells. In addition, GFP-positive cells were observed in the compact layer of the ventricle and were positive for the myocardium marker actinin (Sup. Fig. 1A), indicating that the lineage of *Sfrp5* from E7.5 differentiated into not only CCS but also myocardium. These observations indicate that *Sfrp5* expressing cells at the cardiac crescent stage contain CCS progenitor cells, although *Sfrp5* is not a specific marker for CCS progenitor cells.

5-3. *Sfrp5* expressing cells derived from the *Mesp1*-negative lineage differentiate into pacemaker cells in the SAN head and chamber Purkinje cells.

The lineage analyses indicated that *Sfrp5* expressing cells at E7.5 contain CCS progenitor cells. In addition, previous reports suggested that the CCS is derived from the *Mesp1*-negative lineage (the cells that never expressed *Mesp1* are defined as *Mesp1*-negative lineage)[6]. Thus, I expected that combinatory usage of the *Mesp1* and *Sfrp5* markers may define CCS progenitors in the early embryo. However, the detailed cell fate contribution of the *Mesp1*-negative lineage has not been examined using differentiation markers. In order to re-examine the CCS contribution of the *Mesp1*-negative lineage, I crossed *Mesp1*-Cre mice with GFP reporter mice and examined with CCS differentiation markers at E16.5 (Fig. 6A). The SAN can be separated into three parts based on differential gene expression (Fig. 6E). The most cranial part is

called the sinus horn, which expresses *Tbx18*, *Hcn4* and *Cx43*. The SAN head is around the superior vena cava (SVC) and expresses *Tbx3*, *Tbx18* and *Hcn4,* and the SAN tail is along the venous valve and expresses *Hcn4* and *Nkx2-5* [11]. I used HCN4 as a marker for the SAN, because HCN4 is commonly expressed in all SAN compartments. GFP-positive cells (*Mesp1*-lineage) were mainly observed in the sinus horn and the SAN tail, but were significantly reduced in the SAN head (Fig. 6B-D). To know the contribution of the *Mesp1*-negative lineage to the SAN head, I counted the GFP-negative/HCN4-positive cells among HCN4 positive cells. Approximately 72.8% of the HCN4 positive cells in the SAN head were negative for GFP, suggesting that the SAN head is mainly composed of *Mesp1*-negative lineages. Furthermore, GFP-positive cells in the cardiac chamber were negative for the Purkinje cell marker Cx40, suggesting that Purkinje cells are not derived from the *Mesp1* lineage (Fig. 6F). These observations together with the results of *Sfrp5*-CreERT2 lineage tracing indicate that pacemaker cells in the SAN head and chamber CCS are derived from *Mesp1*-negative/*Sfrp5*-positive cells from E7.5.

In order to trace the cell lineage of *Mesp1* and *Sfrp5* simultaneously in one embryo, I employed Cre-LoxP and Dre-Rox systems[38,39]. The Dre-Rox system is like the Cre-LoxP homologous recombination system, but Dre protein does not recognize the sequence of LoxP. Thus, using Cre-LoxP and Dre-Rox systems makes it possible to trace the two cell lineages in one embryo. For this purpose, I generated *Sfrp5*-Dre KI mice (Sup Fig. 2) and CAG-Roxed Neo-lynmRFP reporter mice, and established *Mesp1*-Cre/*Sfrp5*-Dre (double driver) and CAG-Loxed CAT-eGFP/CAG-Roxed Neo-lynmRFP (double reporter) mice. I crossed *Mesp1*-Cre/ *Sfrp5*-Dre with the double reporter, and examined the fetal heart at E16.5 for IHC with CCS markers (Fig.

7A). In the SAN head, many HCN4-positive cells were positive for lynmRFP but not for GFP, suggesting that the pacemaker cells in the SAN head consist of the cells that once expressed *Sfrp5*; however, the cells are not derived from the *Mesp1* lineage (Fig. 7B). In the ventricles, Cx40-positive cells were positive for lynmRFP and the half of them were positive for GFP (Fig. 7C). This suggests that ventricular CCS components consist of not only the *Mesp1*-negative lineage but also *Mesp1*-positive lineage, and these different lineages commonly expressed *Sfrp5*. In addition, GFP-negative cells were observed in the atrium and these cells were positive for the myocardium marker actinin even though the atrial myocardium has been thought to be derived from *Mesp1*-positive cells (Fig. 7D). This suggests that *Mesp1*-negative/*Sfrp5*-positive cells also differentiate into atrial myocardium.

Double lineage tracing using *Mesp1*-Cre/*Sfrp5*-Dre revealed that many SAN head cells and half of the ventricular Purkinje cells were derived from the *Mesp1*-negative/*Sfrp5*-positive lineage. *Mesp1* is transiently expressed at E6.5, but *Sfrp5* is continuously expressed from the cardiac crescent to the differentiated CCS. Therefore, I next wanted to ask whether *Sfrp5* expressing cells at E7.5 contributing to the CCS are derived from the *Mesp1*-negative cell lineage. For this purpose, I generated *Mesp1*-Dre KI mice (Sup Fig. 3) and established *Mesp1*-Dre/*Sfrp5*-CreERT2 mice. I crossed *Mesp1*-Dre/*Sfrp5*-CreERT2 mice with double reporter mice and administered TM at E7.5. I obtained embryos at E10.5 and the fetal heart at E16.5 for IHC with the CCS marker HCN4 because *Hcn4*-expressing cells at E10.5 are known as the specific progenitor cells for pacemaker cells[9] (Fig. 8A). At E10.5, a few GFP-positive cells were observed in the SVP and heart tube (Fig. 8B). GFP-positive cells in the SVP were negative for lynmRFP, but positive for HCN4 (Fig. 8C-D). On the other hand,

GFP positive cells in the heart tube were positive for lynmRFP, but negative for HCN4 (Fig. 8E). These observations suggest that *Sfrp5* expressing cells at E7.5 consist of the *Mesp1* negative lineage and the positive lineage, and *Mesp1*-negative/*Sfrp5*-positive cells provide CCS progenitor cells, whereas *Mesp1*-positive/*Sfrp5*-positive cells provide the progenitor cells for myocardium. In the later stage, GFP-positive cells were observed in the upper side of the RA, atrium and LV of the E16.5 heart (Fig. 8F). GFP-positive cells were positive for HCN4, but negative for lynmRFP in the SAN, suggesting that some *Sfrp5* expressing cardiac crescent cells are derived from the *Mesp1*-negative lineage and these cells differentiate into pacemaker cells in the SAN (Fig. 8G, H). Taken together, my observations suggest that *Sfrp5* expressing cells, which are derived from the *Mesp1*-negative lineage, exist in the cardiac crescent as CCS progenitor cells.

5-4. *Mesp1*-negative/*Sfrp5*-expressing cells were concentrated on the posterior side of the cardiac crescent.

In order to observe *Mesp1*-negative/*Sfrp5*-expressing cells on the cardiac crescent, I generated CAG-CAT-mCherry mouse (Sup. Fig. 4) and established CAG-CAT-mCherry/*Sfrp5*$^{v/v}$ mouse line. I crossed *Mesp1*-Cre mice with CAG-CAT-mCherry/*Sfrp5*$^{v/v}$ mice and obtained embryos at E7.5. vYFP-positive cells were observed like a crescent and mCherry-positive cells were observed broader than vYFP-positive region in 0-3 somite stage embryos(Fig. 9A-D). Interestingly, the mCherry-negative vYFP-positive cells i.e., *Sfrp5* expressing *Mesp1*-negative lineage cells, were observed at E7.5 (Fig. 9E), and the cells were concentrated on the posterior side of the cardiac crescent (Fig. 9F). I quantified the mCherry-negative vYFP-

positive cells and found that the number of the cells located on the posterior side of the cardiac crescent was 2.2-times higher than that on the anterior side (Fig. 9g). These results showed that the *Sfrp5* expressing cells derived from *Mesp1*-negative lineage were concentrated on the posterior side of the cardiac crescent.

5-5. *T*-expressing mesoderm cells at E6.5 contribute to SAN and Purkinje cells in all chambers but not to the AVN.

My observations suggested that *Mesp1*-negative *Sfrp5* expressing cells on the posterior side of the cardiac crescent are candidates for CCS progenitor cells. In a previous study, the cell tracking analysis showed that *Nkx2-5*-negative mesodermal cells provided CCS progenitors[25,26]. Based on this observation, I hypothesized that CCS components, which are derived from the *Mesp1*-negative/*Sfrp5*-positive lineage, are also descendants of mesoderm. In order to examine the contribution of different mesodermal lineages, I focused on *T*. *T,* which encodes a transcription factor also called *Brachyury*, is expressed from the onset of gastrulation. The expression is detected from E6.5 in the primitive streak (PS) where mesoderm progenitor cells exist, and is kept in the PS, presomitic mesoderm (PSM) and notochord with embryonic growth[40]. The contribution of the *T* lineage into the heart area has been shown using *T*-Cre/R26R mice and the descendants were observed in most mesodermal lineages[41,42]. It was reported that the atrium and endocardium of the heart tube is derived from the *T* lineage; however, it has not been examined if the *T* lineage contributes to the CCS. Therefore, I first examined the contribution of the *T* lineage to the CCS by LacZ staining. I crossed *T*-Cre mice with R26R mice and obtained the fetal heart at E16.5 (Fig. 10A). LacZ-positive cells were observed in the SVC, atrium,

SV and a part of the ventricles in the heart (Fig. 10B, C). To observe the distribution of LacZ-positive cells in detail, I made frozen sections of E16.5 heart and stained with LacZ. LacZ-positive cells were mainly observed in the upper side of the RA and atrium, and some LacZ-positive cells were observed in the trabecular zone of ventricular chambers but not observed in the AVN, implying that many CCS components, except for the AVN, are derived from the *T* lineage (Fig. 10D- L, Q, R). Furthermore, LacZ-positive cells were observed in the AV valves, aortic valve, pulmonary valve and epicardium, suggesting that these cardiac components were also derived from the *T* lineage (Fig. 10M-P, S, T). To examine whether the *T*-lineage includes the CCS cell population, I crossed *T*-Cre with GFP reporter mice. The pattern of GFP distribution was mainly observed on the caudal side of the embryo (Fig. 11B). In the heart, the SV and atrium showed GFP signal, but the signal was significantly weaker in the future ventricle and in a part of the AVC of the heart tube (Fig. 11B'). I conducted IHC using the CCS progenitor marker HCN4. GFP-positive cells were positive for HCN4 in the SVP (Fig. 11E), suggesting that SAN and Purkinje cell progenitors are derived from the *T* lineage. However, even though *T* expression starts from E6.5, and is maintained in the PS and PSM, *T* expression was not observed in the cardiac primordium or heart[42]. Therefore, I next examined the important timing of *T*-expression that can contribute to the heart. For this purpose, I traced the cell lineage using *T*-CreERT2 mice[42] (Fig. 11A). I crossed *T*-CreERT2/*Rosa26*$^{mTmG/mTmG}$ mice with MCH (wild type) mice, and administrated TM at E6.5, E7.5 or E8.5 (Fig. 11A) and obtained E10.5 embryos. I found that GFP-positive cells in the heart tube were only observed in E6.5TM embryos but not in E7.5TM (Fig. 11D) or E8.5TM (Sup. Fig. 5) embryos. The GFP signals were observed in the atrium and the SVP but

not in the ventricle (Fig. 11C, C'), and the GFP-positive cells were positive for HCN4 in the SVP of E6.5TM embryos (Fig. 11F), suggesting that the *T* lineage only from E6.5 can contribute to CCS progenitors. The distribution pattern of GFP-positive cells using *T*-Cre was very similar to that of *T*-CreERT2 (E6.5-TM) at both E10.5 (Fig. 11B-C) and E16.5 (Fig. 11G-J), indicating that T-lineage cells, which invaginated through the primitive streak at E6.5, but not after E7.5 contribute to the cardiac region.

5-6. *Sfrp5* expressing cells derived from the *T* lineage are candidates for SAN and Purkinje cell progenitors.

The lineage tracing analysis using *T*-Cre and *T*-CreERT2 revealed that CCS progenitor cells could be marked using *T*-Cre from the gastrulation stage. Together with the result of the lineage tracing analysis using *Sfrp5*-CreERT2, I hypothesized that a subset of *T*-expressing cells at E6.5 would express *Sfrp5* at E7.5, and *T* lineage-derived *Sfrp5* expressing cells differentiate into the SAN. In order to trace the lineage of *T* and *Sfrp5*, I crossed *T*-Cre/*Sfrp5*-Dre mice with double reporter mice and examined E10.5 embryos and E16.5 heart (Fig. 12A). As expected, the GFP signal, which was derived from the *T* lineage, was observed in the CCS progenitor region, atrium and caudal part of the embryo at E10.5. In addition, RFP signal derived from the *Sfrp5* lineage was observed in the CCS progenitor region and the heart tube except for the primitive RV (Fig. 12B-C'). GFP and RFP were overlapped in the SVP and atrium. In addition, the double positive cells also expressed HCN4 in the SVP suggesting that CCS progenitor cells were derived from the *T*/*Sfrp5*-double positive lineage (Fig. 12D, D'). In later stages, GFP and RFP signals were overlapped in the atrium and SV at E16.5 (Fig. 12E), and the double positive cells co-expressed HCN4

in the SAN (Fig. 12F). These observations imply that the *T*/*Sfrp5*-double positive lineage provides CCS progenitor cells and the progenitor cells differentiate into pacemaker cells in the SAN. Moreover, GFP/RFP-double positive cells were observed not only in the CCS but also in the atrium suggesting that the *T*/*Sfrp5*-double positive lineage also provide progenitor cells contributing to the atrium. Different from the SAN, the AVN was composed of GFP-negative/RFP-positive cells, suggesting that the AVN is derived from the *T*-negative/*Sfrp5*-positive lineage (Fig. 12G), as observed with the LacZ reporter of the *T*-lineage (Fig. 10J). These data suggested that the SAN and AVN are derived from different origins. Interestingly, Purkinje cells that projected from the AVN were GFP/RFP-double positive (Fig. 10J), suggesting that Purkinje cells are of the *T*/*Sfrp5*-double positive lineage, same as the SAN.

5-7. *Sfrp5* expressing cells on the posterior cardiac crescent derived from the *T*-lineage are candidates for SAN and Purkinje cell progenitors.

Based on the double lineage tracing study of *T/Sfrp5* and each single lineage tracing studies of *T*-CreERT2 and *Sfrp5*-CreERT2, I hypothesized that some of the cells expressing *T* at E6.5 would express *Sfrp5* as CCS and atrium progenitors on the posterior cardiac crescent. In order to verify whether *T* lineage-positive *Sfrp5* expressing cells exist on the cardiac crescent, I crossed *T*-Cre with CAG-CAT-mCherry/*Sfrp5*$^{v/v}$ mice and obtained embryos at E7.5 (Fig. 13A). mCherry-positive cells i.e., *T* lineage, slightly overlapped with vYFP-positive cells in the posterior side of the cardiac crescent in 0 somite-stage embryos (Fig. 13B). Double positive cells were increased in the posterior part in 1 and 2 somite-stage embryos (Fig. 13C, D),

and some double positive cells existed in the anterior part of the cardiac crescent. Therefore, *T*–positive/*Sfrp5*-expressing cells were concentrated on the posterior part of the cardiac crescent and the position was similar with the position of the *Mesp1*-negative/*Sfrp5*-expressing cells (Fig. 9E-G),). These results indicate the possibility that the posterior part of the cardiac crescent includes *Sfrp5*-expressing CCS progenitors derived from *Mesp1*-negative and/or *T*-positive lineages.

5-8. The cells derived from the *Mesp1*-negative/*T*-positive lineage provide SAN and Purkinje cell progenitors.

My double lineage tracing experiments of *Mesp1/Sfrp5* and *T/Sfrp5* demonstrated that the cells on the posterior side of the cardiac crescent must be SAN and Purkinje cell progenitors. However, it is still unclear whether these progenitors are derived from the *Mesp1*-negative/*T*-positive lineage. To compare the lineages directly, I crossed *Mesp1*-Dre/*T*-Cre mice with double reporter mice, and examined at E10.5 and E16.5. RFP signal representing the *Mesp1*-lineage was observed broadly in E10.5 embryos and E16.5 heart, whereas GFP signal representing the *T*-lineage was observed in the posterior part of the embryo and SVP and atrium of the heart tube at E10.5, and in the atrium, SV and SVC in the E16.5 heart (Fig. 14A, B). To examine CCS contribution of these cell lineages, I conducted IHC with anti-HCN4 using E10.5 embryos and observed both RFP-negative/GFP-positive cells expressing HCN4 and triple-positive cells in the SVP (Fig. 14C, C'). These two population were always segregated, and RFP-negative/GFP-positive/HCN4-positive cells were found at a distance from the heart tube, while triple-positive cells were found near the heart tube (Fig. 14C, C') As the *Mesp1*-negative lineage mainly contributed to the SAN head (Fig. 6C) and a part

of the Purkinje cells (Fig. 6F), it is likely that RFP-negative/GFP-positive cells expressing HCN4 at E10.5 give rise to the SAN head, triple-positive cells give rise the rest of the SAN, and both populations give rise to Purkinje cells.

5-9. The cells derived from the *Mesp1*-negative/*T*-positive lineage exist on the posterior cardiac crescent.

Double lineage tracing of *Mesp1*/*T* suggested that at least the SAN head and a subset of Purkinje cells are derived from the *Mesp1*-negative/*T*-positive lineage. When I examined the *Mesp1*-negative/*T*-positive lineage on the cardiac crescent, I was able to identify the SAN head and Purkinje progenitors on the crescent. In order to observe the distribution of *Mesp1* and *T* lineages, I crossed *Mesp1*-Dre/*T*-Cre mice with double reporter mice and examined at E7.5 (Fig. 15A). lynmRFP-positive cells were observed in the entire cardiac crescent and GFP-positive cells overlapped on the posterior side of the cardiac crescent but not on the anterior side (Fig. 15B). These observations indicate that the cells derived from the *Mesp1*-negative/*T*-positive lineage existing on the posterior cardiac crescent are strong candidates for SAN and Purkinje cell progenitors. Together with these results, I conclude that a subset of CCS progenitor cells exists on the posterior side of the cardiac crescent, and that the *T*-Cre/*Mesp1*-Dre double lineage tracing system provides a useful method to isolate the CCS progenitor cells.

6. Discussion

In a previous study, it was suggested that the CCS is derived from the *Mesp1*-negative lineage[6]. However, the origin of CCS cells has not been addressed because of the lack of specific markers required for identification of CCS cells from early mouse embryos. On the other hand, cell tracking analysis by dye labeling using chick and mouse embryos suggested that CCS-specific progenitor cells exist on the caudal-lateral cardiac crescent[25,26]. In my thesis, by the extensive lineage tracing studies using various combinations among *Sfrp5, Mesp1* and *T*, I was able to identify SAN head and Purkinje progenitor cells on the posterior side of the cardiac crescent as *Sfrp5* expressing cells derived from the *Mesp1*-negative but *T*-positive mesodermal lineage. The comparison between cell lineages derived from *Mesp1* and *T* was particularly interesting because it indicated that the lineage restriction occurred before forming the cardiac crescent at a much earlier stage than I thought. *Mesp1*-expressing mesoderm represents the earliest mesoderm invaginating through the PS and contributes to the most heart components[3,5]. Although the T-lineage (it is noted that this study used *T*-Cre TG mouse in which *Cre* expression is different from that of the endogenous *T* gene[41]) represents the mesodermal group that invaginates later than the *Mesp1*-population, it mainly contributes to the posterior part of the mouse embryo, which includes the posterior part of the cardiac crescent fated to become the SAN and atrium. In addition, it was recently reported that the *Foxa2*-expressing cells at E6.5 contribute to the anterior part of the cardiac crescent and differentiate into some cardiac components in ventricles but not into atrium or the SAN[43]. Taken together, the distribution pattern of the four-chambered heart may be determined with the timing of mesoderm cell ingression from the PS at E6.5, and the relative distribution is maintained from the cardiac crescent to the four-chambered heart

(Fig. 16). In particular, CCS development strictly retains this distribution pattern. At E10.5, the cells derived from the *Mesp1*-negative/*T*-positive lineage were specifically observed in the area distant from the heart tube among the HCN4-positive SAN progenitor population in the SVP, while the *Mesp1*-positive/*T*-positive lineage was observed near the heart tube (Fig. 14C, C'). Each population gives rise to the SAN head and tail, respectively, at E16.5 (Fig. 6). Previously, the SAN has been divided into three parts: the sinus horn, SAN head and SAN tail by gene expression[6,9,44]. In this study, I revealed that the origin of the SAN head and tail could be clearly divided also by *Mesp1* expression.

The lineage tracing analysis of *T* gave us further novel insights about the origin of each cardiac component, especially in the AVN and AV valves. Although the AVN is a component of the CCS derived from *Mesp1*-negative lineage, its origin is different from the SAN. The progenitor cells of the SAN were identified in the SVP by the expression of *Hcn4* at E9.5, whereas the progenitor cells of the AVN were observed in the atrioventricular canal (AVC). The AVC is the region, which is located between primitive atrium and ventricle of the heart tube, and it is reported that the AVN and AV valves are derived from the cells located in the AVC[9,31,32,35,45,46]. In addition, AV valves are derived from the *Mesp1*-positive endocardial cells located in the AVC[47-50]. These studies suggest that the AVC is comprised of several cell lineages including *Mesp1*-positive and negative cells. My experiments of the lineage tracing study of *T* suggested that AV valves are derived from the *T*-positive cells located in the atrial side of the AVC, whereas the AVN is derived from the *T*-negative cells located in the ventricular side of the AVC. This study is the first report that clarified the difference in their origin. The congenital arrhythmia caused by AVN defects is known as a major congenital complication of AV valve

dysplasia and is thought to be caused during the heart tube looping stage. However, the mechanism has not been elucidated because the arrhythmia results from an abnormal AVN and the AV valve dysplasia arises in a close region that is interlinked. Detailed analysis of AVC development using *T* lineage as a marker will contribute to the understanding of the mechanism of the formation of AVN and AV valves, and it will help to understand the morphogenesis of the AVN and the malformation of AV valves.

By the lineage tracing analysis using *Mesp1* and *T*, I found that the SAN and AVN were derived from distinct origins, and the relative distribution of each component are determined at E6.5 and kept until the four-chambered heart stage. However, distribution of Purkinje cells, the other component of the CCS, markedly changes. Purkinje cells connect between the SAN and AVN, and then further extend into the ventricles. There are contradictory reports about the formation of the Purkinje network. One states that Purkinje cells originate from the AVN and extend toward the SAN and ventricles[46]. The other is that Purkinje cells originate from the SVP, which contains SAN progenitors but not AVN progenitors[9]. My observations clearly support the latter case, because Purkinje progenitors are derived from *Sfrp5*-positive and *T*-positive but *Mesp1*-negative mesodermal lineages, same as the SAN head. Even though SAN and Purkinje cells are derived from the same origin, the marker gene expression is quite different as Purkinje cells do not express the SAN marker HCN4, but instead express Cx40. Purkinje cells may acquire migratory ability after E10.5 and the marker gene expression may also change along with the character change.

Although the *T* lineage in general did not contribute to the ventricle, a few cells derived from the *T* lineage were observed in the compact layer of the ventricles and the cells were present near the epicardium derived from the *T* lineage (Fig. 10 Q, S, T). The

previous report of *Wt1*-lineage tracing showed that a subset of cells in the SVP differentiated into epicardium, and the epicardium further differentiated into myocardium via the EMT[51]. As *T*-positive lineage cells were observed in the SVP[42]. The *T* lineage cells might provide *Wt1*-expressing proepicardium, which give rise to myocardium of the ventricle via the EMT of epicardium.

Previous studies demonstrated that *Hcn4* is a common marker for the SAN and AVN[32,52], and *Cx40* is a marker for Purkinje cells[1,35,53]. However, there was no report showing a common gene marking the entire CCS. I revealed that *Sfrp5* is expressed in the cardiac crescent at E7.5, and the expression is restricted to the CCS with heart development (Fig. 1A-H, Fig. 2C-F). Furthermore, even though the *Sfrp5* expressing cells at E7.5 contributed to both the CCS and myocardium, the expression is kept only in the CCS (Fig. 4B, Fig. 5B-D). These results suggest that *Sfrp5* may be a progenitor marker and may function to prevent CCS lineages from differentiating into other cardiac lineages. However, *Sfrp5* KO mice do not exhibit any phenotype in the heart[54], implying that the function of *Sfrp5* is compensated by other genes. *Sfrp1* and *Sfrp2* are family genes of *Sfrp5,* and are upregulated in the infarcted area[55,56]. Although *Sfrp1* and *Sfrp2* are expressed in relation with cardiac repair, these genes are not detected in the embryonic heart[33,57,58]. All KO and double KO mice showed no phenotype in the heart, but *Sfrp1/2/5* triple KO (*Sfrp* TKO) mice exhibit abnormal heart tubes at E9.5 and die thereafter[8,33]. We reported that the cell proliferation rate was increased in the SVP of *Sfrp* TKO mice[8], suggesting that *Sfrp1/2/5* suppresses the proliferation in cardiac progenitor cells. However, HCN4 was expressed in *Sfrp* TKO mice (Sup. Fig. 6), suggesting that CCS progenitor cells exist until E9.5. In order to understand the function of the *Sfrp* family for CCS development, I have to examine the embryo after E10.5 because maturation of the CCS

occurs from E10.5 to E16.5. To avoid embryonic lethality, I am trying to knock out *Sfrp1/2* and *5* after E9.5 using *Hcn4*-CreERT2. It has been shown that the HCN4-expressing lineage at E10.5 contributes to the CCS and SAN. I hope this will help to clarify the function of *Sfrp1/2/5* in CCS development.

In addition, the expression of *Sfrp5* was retained in the entire CCS but was lost in the mature myocardium, indicating that the expression was regulated by some CCS related genes. TBX3 is a transcription factor known as a suppressor for myocardial differentiation, and is expressed in CCS progenitors and differentiated pacemaker cells. Its expression also overlaps with that of *Sfrp5*. It is reported that *Tbx3* hypomorphic mice exhibit lethal arrhythmia because of SAN hypoplasia until E15.5[59], and TBX3 directly binds to the site existing 4.5 kbp downstream of the *Sfrp5* gene in the adult heart[60]. Taken together, it is possible that the expression of *Sfrp5* is controlled by TBX3. Further analysis is necessary to clarify this possibility.

Detailed analysis about the *Mesp1*-negative/*T*-positive/*Sfrp5*-expressing cells (candidates of CCS progenitors) in the developmental process can identify the essential gene that determines the cell fate. After identifying the gene, I would like to verify its function *in vitro*. If the forced expression of *Sfrp5* or new factor induces pacemaker-like cells, it will help to establish CCS cell transplantation therapy.

7. Experimental Procedures

7-1. Mice

Sfrp5 venusYFP knock-in mice were kindly gifted from Dr. Akihiko Shimono[33]. *T*-Cre, *T*-CreERT2 transgenic mice were kindly provided from Dr. Mark Lewandoski[41,42]. *Sfrp5*-Cre, *Sfrp5*-CreERT2, *Mesp1*-Cre, *R26*R, *CAG-floxed CAT-eGFP* mouse lines have been previously described[5,8,36,37].

To generate the *Sfrp5* Dre knock-in mouse line, the targeting vector was constructed using pBluescript (SK+) with a cassette containing the *Dre* gene with frt-PGK-*neomycin*^{r} (neo) -bpA-frt, which was ligated with KpnI-SmaI and SpeI-XbaI fragments for long and short homologous arms, respectively. A MC1-DTA-negative selection marker cassette was also added to the outside of the short homologous arm. A pair of oligo DNAs corresponding to *Sfrp5* sgRNA (5'-GGCCAGGAGTACGACTACTA-3') sequence was inserted into the pX330 plasmid (addgene). For recombination, the targeting vector and pX330/Sfrp5 were then co-transfected into TT2 ES cell lines with Lipofectamine 2000 (Invitrogen). Homologous recombination was confirmed by PCR using the following primer set: Neo6: 5'-CGACCACCAAGCGAAACATC-3' and SAcontRR9: 5'-CCCAGGGAAGGGAATTGTTCTAG-3'. Deletion of Neo by crossing with the ROSA-Flp mouse line was confirmed by PCR using the following primer set: *Sfrp5* Dre deltaNeo F1: 5'-GGCGCCGGTCCTAAGAAGAAG-3' and *Sfrp5* DeltaNeo R2: 5'-CCTAGCGACCCCAAGCATCC-3' (Sup. Fig. 2).

To generate the *Mesp1*-Dre knock-in mouse line, targeting vectors were constructed using pBluescript (SK+) with a cassette containing the *Dre* gene with a bpA signal that was inserted with a 1 kb fragment from the 5' sequence upstream of the *Mesp1* translation initiation site, and 1 kb *Xba*I fragment starting from exon 2, upstream and

downstream of the *Dre*-pA cassette, respectively. A pair of oligo DNAs corresponding to *Mesp1-5'* sgRNA (5'-GCTACAGCGGACCCAATGGTC-3') sequence or *Mesp1-3'* sgRNA (5'-GCCGATTGTGCTAGTGTCATT-3') sequence was inserted into the pX330 plasmid (addgene). The *Mesp1*-Dre targeting vector, pX330-Mesp1-5', and pX330-Mesp1-3' were injected into pronuclei of the fertilized embryos from the B6C3F1 cross. The injected zygotes were transferred into oviducts of pseudopregnant MCH females. Homologous recombination was confirmed by PCR using the following primer sets: Mesp1-5'arm L1:5'- GCTCTTGAAGAACCCAAGGTTCTG-3' and Dre-R2:5'-CCTCAGGAATCCTCCAGAGGAGCC-3', NeoAL2:5'-GGGGATGCGGTGGGCTCTATGGCTT-3' and Mesp1-GR1:5'-ATATGCCAAGTCATTGAGGTGAGCTTTC-3' (Sup. Fig. 3).

To generate transgenic reporter mice for Dre and Cre, the CAG-rox Neo-lynmRFP vector was made by inserting the lyn-mRFP cassette into the CAG-rox-Neo vector. The CAG-floxed CAT-mCherry vector was made by replacing the lyn-eGFP cassette from the CAG-CAT-lyn-eGFP vector with the mCherry cassette. These vectors were linearized and injected into fertilized embryos as described above (Sup. Fig. 4). The mice are maintained as the mixed genetic background composed of C57BL/6, CBA and MCH.

All of the mice were maintained according to the institutional guidelines for the animal facilities of NIG.

7-2. Tamoxifen administration

Tamoxifen (SIGMA) was dissolved in sesame oil (Nacalai Tesque, 25620-65) at 55°C and administered orally to 6-8-week pregnant mice 0.1-0.4 g/kg (body weight).

7-3. Immunohistochemistry (IHC)

The embryos and fetal hearts were obtained at E9.5-16.5, fixed in 4% paraformaldehyde (PFA) for 60-90 min. at 4°C, and then embedded in OCT to make 6-μm frozen sections. After drying at room temperature, sections were blocked with 5% BSA in PBS and reacted with antibodies. The antibodies and conditions used for each analysis are described in Table1.

7-4. Whole-mount *in situ* hybridization (WISH)

Embryos were obtained at E7.5-10.5 and fixed with 4% PFA in PBS overnight. After dehydration using a methanol series, embryos were rehydrated with 0.1% Tween 20 in PBS (PBST), bleached with 6% H_2O_2 in PBS, treated with Protease K and then hybridized with digoxygenin (DIG)-labelled probe for *Sfrp5* at 68 °C overnight. The embryos were then washed with 5 × and 2 × SSC/50% formamide, PBST and blocking reagent for 1 h. For detection of the DIG-labelled probe, embryos were incubated with anti-DIG-AP antibody (Roche) and detected using BM Purple (Roche) according to the manufacturer's instructions.

7-5. Lac-Z staining

Whole Lac-Z staining

The fetal hearts were obtained at E16.5 and fixed in 4% PFA for 60 min. at 4°C, and stained for 2 hours to overnight at 37°C in the dark with 1 mg/ml X-gal, 5 mM $K_4Fe(CN)_63H_2O$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS to detect beta-galactosidase activity.

Section Lac-Z staining

The embryos and fetal hearts were obtained at E9.5-16.5, fixed in 4% PFA for 60-90 min. at 4°C, and then embedded in OCT to make 15-μm frozen sections. After drying at room temperature for less than 10 min., the sections were stained for 2 hours to overnight at 37°C in the dark with 1 mg/ml X-gal, 5 mM $K_4Fe(CN)_63H_2O$, 5 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS to detect beta-galactosidase activity.

7-6. Imaging

Images of whole embryos or whole hearts with fluorescent reporters, except for the double reporter samples at E7.5 stage, WISH samples and whole Lac-Z staining samples, were taken with a Leica MZ16F fluorescent stereomicroscope equipped with a Pixera Penguin 600CL camera. Section Lac-Z staining sample images were taken with an Olympus BX61 fluorescent microscope with a DP80 camera. Images of IHC samples and the double reporter samples at E7.5 stage were taken with an Olympus FV1200 confocal microscope. The double reporter samples at E7.5 stage were dissected and transferred to PBS in a glass-bottom dish, Z-stack images were taken at 5-μm intervals, and the images were projected for the figures.

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9. Figure Legends

Fig. 1. *Sfrp5* is expressed from the cardiac crescent to four-chambered heart.

(A-H) vYFP pattern of *Sfrp5*$^{v/+}$ at E7.5 (A), E8.5 (B), E9.5 (C), E10.5 (D), E11.5 (E), E12.5 (F), E13.5 (G) and E14.5 (H). arrows; SVP and SV, arrow head; apex of vYFP signal in ventricle (n≥5). Dotty circle in (A) indicates the cardiac crescent.

(I) The cartoon showing the CCS networks.

AVN; atrioventricular node, IVS; interventricular septum, LA; left atrium, LV; left ventricle, LBB; Left bundle branch, RA; right atrium, RBB; right bundle branch, RV; right ventricle, SAN; sinoatrial node, SV: sinus venosus, SVP: sinus venosus primordia.

Fig. 2. *Sfrp5* is expressed in CCS progenitor cells and differentiated CCS of four-chambered heart.

(A, B) Double immunohistochemistry of E9.5 *Sfrp5*$^{v/+}$ embryo using anti-HCN4 (red) and anti-GFP (green). Boxed area of (A) is magnified in (B). Asterisks indicate the co-expressing GFP and HCN4 cells.

(C-F) Double immunohistochemistry of E16.5 *Sfrp5* $^{v/+}$ heart using anti-HCN4, anti-Cx40 (red) and anti-GFP (green). Boxed area of (C, E) is magnified in (D, F).

R; right-hand side, L; left-hand side.

Fig. 3. *Sfrp5* lineage contribute to SAN and Purkinje cells in all cardiac chambers.

(A) The scheme of lineage tracing analysis using *Sfrp5*-Cre/CAG-floxed CAT-eGFP.

(B-C') Double immunohistochemistry of E16.5 *Sfrp5* Cre/CAG-floxed CAT-eGFP heart using anti-HCN4, anti-Cx40 (red) and anti-GFP (green). Boxed area of (B, C) is magnified in (B', C').

L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle.

Fig. 4. *Sfrp5* lineage contribute to all chambers except for RV.

(A) The scheme of lineage tracing analysis using *Sfrp5*-CreERT2/R26R mouse.

(B-E) Whole-mount *in situ* hybridization using *Sfrp5* probe. The expression pattern correspond to the timing of TM administration (n≥4).

(B'-E') Ventral view of LacZ stained *Sfrp5* CreERT2/ Rosa26-LacZ E16.5 hearts.

(B''-E'') Dorsal view of LacZ stained *Sfrp5* CreERT2/ Rosa26-LacZ E16.5 hearts.

HT; Heart tube, L; left-hand side, LA; right atrium, LV; left ventricle. R; right-hand side, RA; right atrium, RV; right ventricle, SVP; sinus venosus primordia

Fig. 5. *Sfrp5* lineage from E7.5 differentiated into SAN and chamber CCS cell.

(A) The scheme of lineage tracing analysis using *Sfrp5*-CreERT2/CAG-floxed CAT -eGFP.

(B-D) Double immunohistochemistry of E16.5 *Sfrp5* CreERT2/CAG-floxed CAT -eGFP heart using anti-GFP (green), anti-HCN4 (red) or anti-Cx40 (red). Asterisks; cells co-expressing GFP and HCN4/Cx40 cells.

L; left-hand side, R; right-hand side.

Fig. 6. Contribution of *Mesp1*-negative lineage to the CCS.

(A) The scheme of lineage tracing analysis using *Mesp1*-Cre/CAG-floxed CAT-eGFP mouse.

(B-D) Double immunohistochemistry of E16.5 *Mesp1*-Cre/CAG-floxed CAT-eGFP heart

using anti-GFP (green) and anti-HCN4 (red). (B) A section of dorsal part SAN tail and sinus horn. (C) A section of middle part of SAN head. (D) A section of ventral part of SAN head.

(E) Cartoon indicate the contribution of *Mesp1* lineage in the SAN.

(F) Double immunohistochemistry of E16.5 *Mesp1*-Cre/CAG-floxed CAT-eGFP heart using anti-GFP (green) and anti-Cx40 (red).

Fig.7. Contribution of *Sfrp5*-positive/*Mesp1*-negative lineage to the SAN.

(A) The scheme of double lineage tracing analysis using *Mesp1*-Cre/*Sfrp5*-Dre mouse and double reporter mouse.

(B-D) The immunohistochemistry of E16.5 *Mesp1*-Cre/*Sfrp5*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP heart using anti-HCN4 (B, SAN), anti-Cx40 (C, Purkinje cell) or anti-actinin (D, myocardium). GFP (*Mesp1* lineage) and lynmRFP (*Sfrp5* lineage) were directly observed without antibody. Asterisk indicate GFP-negative/RFP-positive/Cx40-positive cell.

(E) The cartoon showing the lineage contribution of *Mesp1* and *Sfrp5* lineages.

L; left-hand side, R; right-hand side, RA; right atrium, SVC; superior vena cava.

Fig. 8. Cells derived from *Sfrp5*-positive/*Mesp1*-negative lineage provide CCS progenitor cells and differentiate into SAN.

(A) The scheme of double lineage tracing analysis using *Mesp1*-Dre/*Sfrp5*-CreERT2 and the reporter CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mice.

(B) eGFP and lynmRFP pattern of E10.5 *Sfrp5*-CreERT2/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mouse (E7.5 TM, n=8). Upper panel: A bright field

image of the embryo. Lower panel: A magnified confocal image of the heart tube. Arrows indicate GFP-positive *Sfrp-5* lineage.

(C-E) Immunohistochemistry of (B) using anti-HCN4 (white, CCS progenitor cells). Boxed areas of heart tube (C) are magnified in panels (D and E).

(F) The eGFP and lynmRFP pattern of E16.5 *Sfrp5*-CreERT2/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP heart (E7.5 TM, n=13).

(G) Immunohistochemistry of (F) using anti-HCN4 (white, SAN) merged with GFP and mRFP signals. Nuclei were stained with DAPI.

(H) The magnified pictures of the boxed area in (G). Asterisks indicate eGFP-positive /lynmRFP-negative/HCN4-positive (blue) cells.

L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle, SVP; sinus venosus primordia.

Fig.9. *Sfrp5* expressing cells derived from *Mesp1* negative lineage on the cardiac crescent.

(A-D) vYFP and mCherry pattern of *Mesp1*Cre/CAG-floxed CAT-mCherry/*Sfrp5*$^{v/+}$ at 0 somite (A), 1 somite (B), 2 somite (C) and 3 somite (D) stage embryos (n=5).

(E) The magnified picture of (A) focused on the mCherry-negative/vYFP-positive cells in the anterior and posterior cardiac crescent. Arrows indicate *Sfrp5* expressing cells derived from *Mesp1*-negative lineage.

(F) The mapping of mCherry-negative/vYFP-positive cells (indicated by white dots) observed in (E).

(G) The quantification of (F). Anterior: 6.16 ± 0.74, Posterior: 13.3 ± 2.82 (P=0.057)

R; right-hand side, L; left-hand side.

Fig. 10. *T*-lineage contributes to SAN, Purkinje cells and atrium.

(A) Scheme of the lineage tracing analysis using *T*-Cre/R26R mouse.

(B-C) Ventral view (B) and dorsal view (C) of LacZ stained *T*-Cre/R26R heart at E16.5 (n=4).

(D-H) Frontal section of LacZ stained *T*-Cre/R26R heart at E16.5.

(I-T) Magnified picture of (D)-(H). SAN (I) and AVN (J) is shown in dotted circles. LacZ-positive cells were observed in right (K) and left (L) atria, pulmonary valve (M), aortic valve (N), AV valve (O, P), myocardial layer of right (Q) and left (R) ventricle and epicardium (S and T). Black arrow indicate β-gal positive cell.

L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand, RA; right atrium, RV; right ventricleside, SV; sinus venosus, SVC; superior vena cava.

Fig. 11. *T* lineage from E6.5 contribute to the CCS and atrium.

(A) Scheme of the lineage tracing analysis using *T*-Cre/CAG-floxed CAT-eGFP or *T*-CreERT2/*Rosa26*$^{mTmG/+}$. When *T*-CreERT2 mouse was crossed with *Rosa26*$^{mTmG/+}$, TM was administrated at E6.5 or E7.5.

(B, B') eGFP pattern of E10.5 *T*-Cre/CAG-floxed CAT-eGFP embryo (n=11). (B') is magnified pictures of heart tube of (B).

(C, C') mGFP pattern of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryo (E6.5 TM, n=18). (C') is magnified pictures of heart tube of (C). Dotted lines in (B) and (C) indicate the anterior limit of GFP signal.

(D) mGFP pattern of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryo (E7.5 TM, n=3).

(E) Immunohistochemistry of (B) using anti-HCN4 (red, CCS progenitor cell) and anti-GFP (green, *T* lineage reporter).

(F) Immunohistochemistry of (C) using anti-HCN4 (red, CCS progenitor cell) and anti-GFP (green, *T* lineage reporter).

(G, H) eGFP pattern of E16.5 *T*-Cre/CAG-CAT-eGFP heart (n=21). (G) is the ventral view and (H) is the dorsal view.

(I, J) mGFP pattern of E16.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ heart (E6.5 TM, n=18). (I) is the ventral view and (J) is the dorsal view.

A; atrium, AVC; atrio-ventricular canal, L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle, SV; sinus venosus, SVC; superior vena cava, V; ventricle.

Fig. 12. *T*-positive/*Sfrp5*-positive lineage provide the CCS progenitor cells.

(A) Scheme of the double lineage tracing analysis using *T*-Cre/*Sfrp5*-Dre and CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mouse.

(B-C') eGFP and lynmRFP pattern of double reporter at E10.5 (n= 6). (B) is left side view and (B') is right view of the embryo and (C) and (C') are magnified picture of boxed area in (B) and (B').

(D, D') Immunohistochemistry of the E10.5 embryonic heart using anti-HCN4 (white, CCS progenitor cell). Boxed area of (D) is magnified in (D'). White arrow indicate GFP/RFP/HCN4 triple positive cell.

(E) eGFP and lynmRFP pattern of *T*-Cre/*Sfrp5*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP at E16.5 (n = 12). Upper panel is the ventral view, middle panel is the dorsal view and lower panel is magnified images of SV (boxed area of the middle panels).

(F, G) Immunohistochemistry of section confining the SAN (F) and AVN (G) prepared from E16.5 embryo using anti-HCN4 (white, SAN and AVN marker) merged with

confocal images of GFP and lynmRFP. White arrowhead indicate GFP/RFP/HCN4 triple positive cell, Dotty circle indicates the AVN.

AVN; atrio-ventricular node, L; left-hand side, LA; left atrium, LV: left ventricle, OFT: outflow tract, R; right-hand side, RA; right atrium, RV: right ventricle, SV; sinus venosus, SVC; superior vena cava, SVP: sinus venosus primordia.

Fig. 13. *Sfrp5* expressing cells derived from *T* positive lineage exist on the lateral cardiac crescent.

(A) Scheme of mouse mating to observe *Sfrp5* expressing cells derived from *T* lineage using *T*-Cre/CAG-floxed CAT-mCherry/*Sfrp5*$^{v/+}$ (B-D, n=6). Confocal images of cardiac crescents with vYFP and mCherry signals at 0 somite (B), 1 somite (C) and 2 somite (D) embryos. Arrowheads indicate double–positive cells.

L; left-hand side, R; sight-hand side.

Fig. 14. *Mesp1*-negative/*T*-positive lineage provide CCS progenitor and pacemaker cells in the SAN.

(A) eGFP and lynmRFP pattern of *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lenmRFP (see Fig. 15 A) at E10.5 (n = 5). Upper panel shows left-hand side and lower panel shows right–hand side.

(B) Magnified picture of (A) focusing on heart tubes.

(C) Immunohistochemistry of (B) using anti-HCN4 (white, CCS progenitor). (C') is magnified picture of SVP in the boxed area of (C). White arrows indicate GFP-positive/RFP-negative/HCN4-positive cells.

(D) eGFP and lynmRFP pattern of *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-

Roxed Neo-lenmRFP (see Fig. 15 A) at E16.5 (n = 9). Upper panel shows ventral side and lower panel shows dorsal side.

Immunohistochemistry of section confining the SAN (E) prepared from E16.5 embryo using anti-HCN4 (white, SAN marker) merged with confocal images of GFP and lynmRFP.

A; atrium, L; left-hand side, LA; left atrium, LV: left ventricle, OFT: outflow truct, R; right-hand side, RA; right atrium, RV: right ventricle, SVC; superior vena cava, SVP: sinus venosus primordia, V; ventricle.

Fig. 15. *Mesp1*-negative/*T*-positive lineage cell exist on posterior cardiac crescent.

(A) Scheme of mouse mating to observe cells derived from *Mesp1*-negative/*T*-positive lineage using *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP (n = 8).

(B) Confocal images of cardiac crescents with eGFP and lynmRFP signals at E7.5. White arrowheads indicate the eGFP positive/lynmRFP negative cells.

Fig. 16. Summary of CCS Developmental process.

The contribution of cardiac progenitor cell is restricted before cardiac crescent formation (A), and the restriction can be predicted by the gene expression of *Mesp1* (yellow), *Foxa2* (pink) and *T* (blue). Early formed mesoderm derived from *Mesp1* and *Foxa2* contribute to the anterior part of the cardiac crescent, while later mesoderm derived from *T* contribute to the posterior crescent (B). Anterior and posterior cardiac crescent commonly expressed cardiac progenitor gene *Sfrp5* (green) and the descendant differentiate into many cardiac components. Particularly, posterior *Sfrp5* lineage provide CCS progenitor

cells in SVP (C) expressing *Hcn4* (red) and differentiate into CCS in four-chambered heart (D).

Sup. Fig. 1. *Sfrp5* lineage from E7.5 is differentiated into myocardium.

Double immunohistochemistry of E7.5TM-E16.5 *Sfrp5*-CreERT2/CAG-CAT-eGFP heart using anti-GFP (green) and anti-actinin (red). Lower panel is magnified picture of boxed area in upper panel. White arrowheads indicate GFP/actinin double positive cell.

Sup. Fig. 2. Targeting strategy for generating *Sfrp5*-Cre, CreERT2 and Dre KI mouse.

Schematic drawing of targeting strategy. Targeting vector was introduced in TT2 ES cells with Cas9-vector designed to cut a target sequence indicated by arrow. After establishing knock-in mice, Pgk-neo cassette was removed by crossing with CAG-Cre mouse to establish Dneo-knockin mice and used for lineage tracing analyses.

Sfrp5 sgRNA: 5'-GGCCAGGAGTACGACTACTA-3

Sup. Fig. 3. Targeting strategy for *Mesp1*-Dre mouse.

Targeting vector was directly injected into nucleus of fertilized egg with Cas9 vectors designed to cut Mesp1 -5' and 3' sequences indicated by arrows.

Mesp1-5' sgRNA: 5'-GCTACAGCGGACCCAATGGTC-3'

Mesp1-3' sgRNA: 5'-GCCGATTGTGCTAGTGTCATT-3'

Sup. Fig. 4. DNA constructs used for generating CAG-Roxed *Neo*-lynmRFP and CAG-floxed CAT-mCherry transgenic mouse lines.

CAG-Roxed Neo-lynmRFP mouse line (Dre reporter) and CAG-Loxed CAT-mCherry mouse line (mCherry reporter) was established using transgenic techniques.

Sup. Fig5. The lineage contribution of *T* is gradually restricted in caudal part of embryo with embryogenesis.

mGFP patterns of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryos administrated TM at E6.5(A), E7.5 (B) and E8.5 (C). (A') and (B') are magnified picture focused boxed are of (A) and (B).

LV; left ventricle, A; atrium

Sup. Fig. 6 HCN4 is expressed in *Sfrp1/2/5* KO mouse.

Immunohistochemistry of *Sfrp1*$^{-/-}$*2*$^{-/-}$*5*$^{v/v}$ triple knockout mouse focused on the heart tube (A) at E9.5. It is stained with HCN4 (red, CCS progenitor marker) and GFP (green, *Sfrp* TKO cell) and boxed area of (A) is magnified in (A'). White arrowheads indicate GFP/HCN4 double positive cell.

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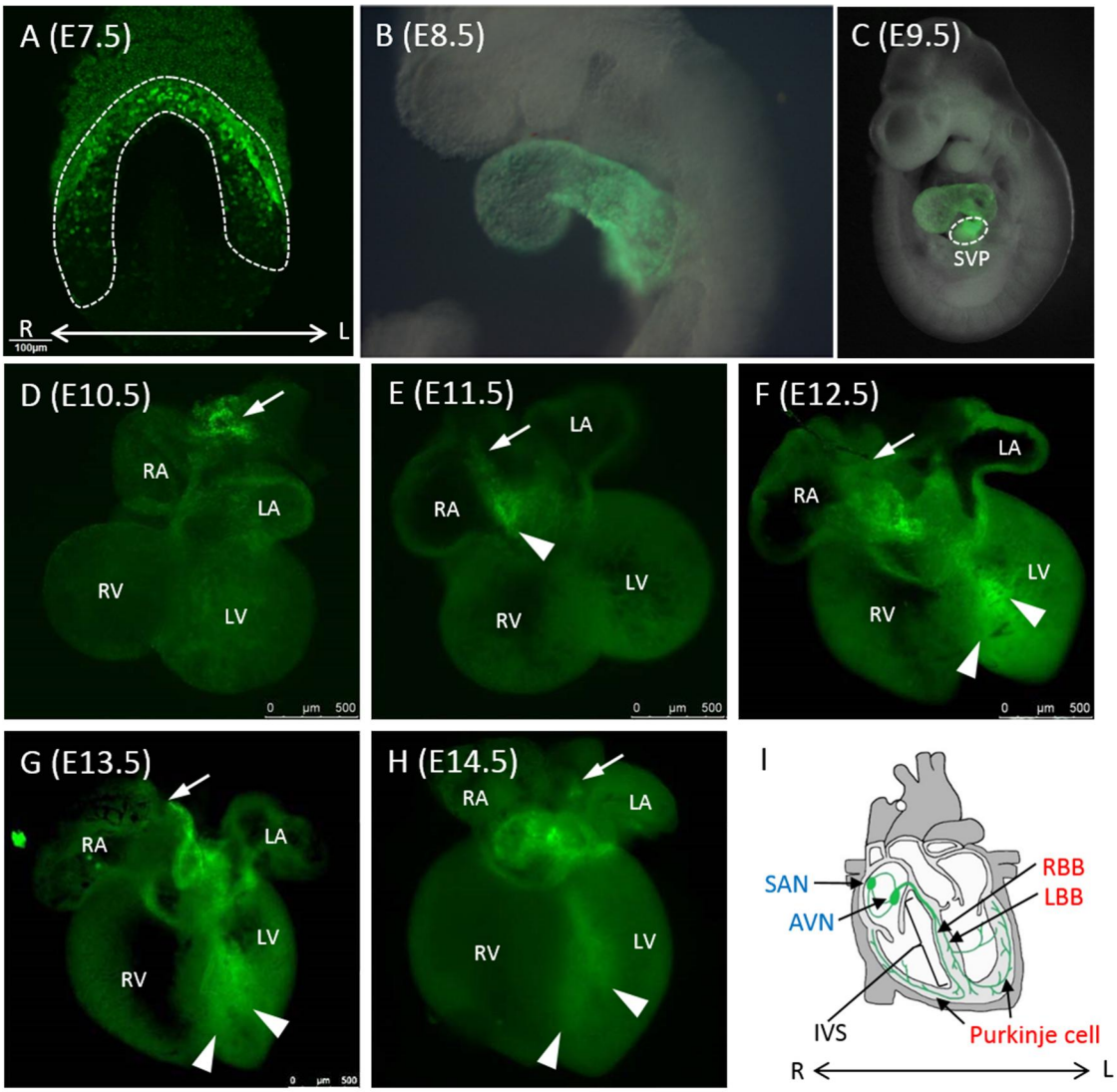


Fig. 1. *Sfrp5* is expressed from the cardiac crescent to four-chambered heart.
(A-H) vYFP pattern of *Sfrp5*$^{v/+}$ at E7.5 (A), E8.5 (B), E9.5 (C), E10.5 (D), E11.5 (E), E12.5 (F), E13.5 (G) and E14.5 (H). arrows; SVP and SV, arrow head; apex of vYFP signal in ventricle (n≥5). Dotty circle in (A) indicates the cardiac crescent.
(I) The cartoon showing the CCS networks.
AVN; atrioventricular node, IVS; interventricular septum, LA; left atrium, LV; left ventricle, LBB; Left bundle branch, RA; right atrium, RBB; right bundle branch, RV; right ventricle, SAN; sinoatrial node, SV: sinus venosus, SVP: sinus venosus primordia.

Fig. 2.

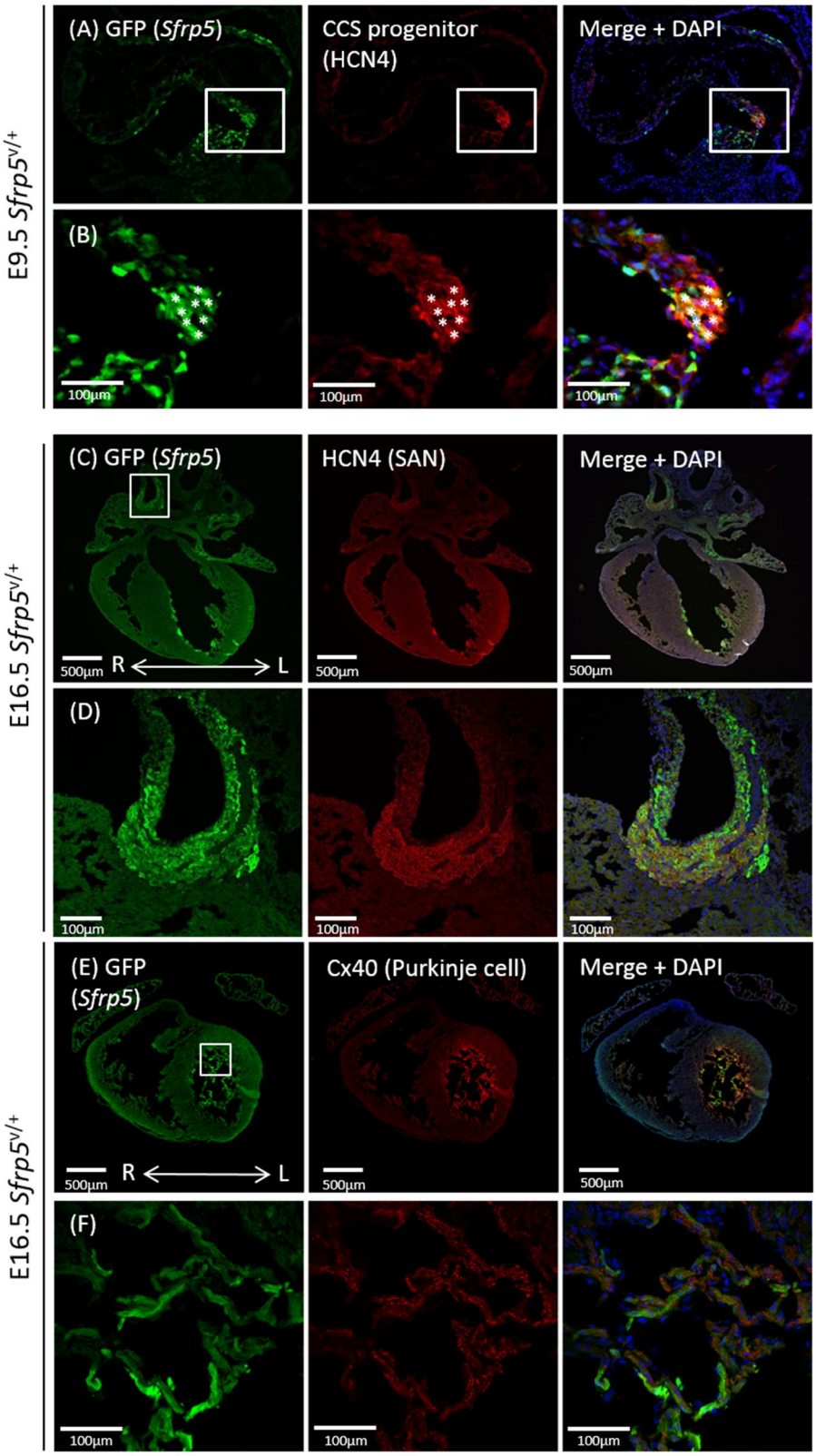

E9.5 Sfrp5v/+
(A) GFP (Sfrp5)
CCS progenitor (HCN4)
Merge + DAPI
(B)
100μm
E16.5 Sfrp5v/+
(C) GFP (Sfrp5)
HCN4 (SAN)
Merge + DAPI
500μm
R
L
(D)
E16.5 Sfrp5v/+
(E) GFP (Sfrp5)
Cx40 (Purkinje cell)
(F)

Fig. 2. Sfrp5 is expressed in CCS progenitor cells and differentiated CCS of four-chambered heart.

(A, B) Double immunohistochemistry of E9.5 *Sfrp5*$^{v/+}$ embryo using anti-HCN4 (red) and anti-GFP (green). Boxed area of (A) is magnified in (B). Asterisks indicate the co-expressing GFP and HCN4 cells.

(C-F) Double immunohistochemistry of E16.5 *Sfrp5* $^{v/+}$ heart using anti-HCN4, anti-Cx40 (red) and anti-GFP (green). Boxed area of (C, E) is magnified in (D, F).

R; right-hand side, L; left-hand side.

Fig. 3

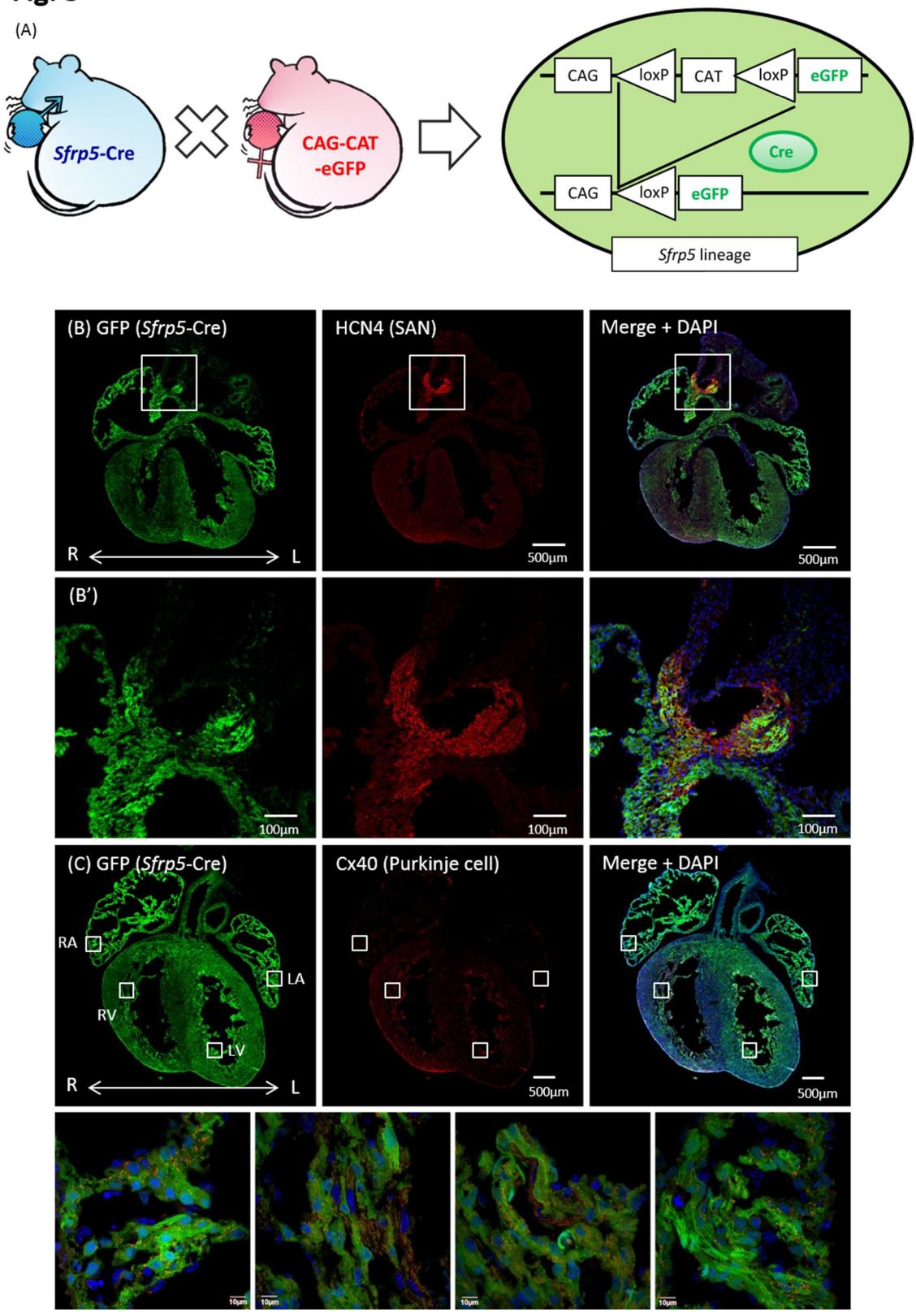

(A)
Sfrp5-Cre
CAG-CAT
-eGFP
CAG
loxP
CAT
loxP
eGFP
Cre
CAG
loxP
eGFP
Sfrp5 lineage
(B) GFP (Sfrp5-Cre)
HCN4 (SAN)
Merge + DAPI
R
L
500μm
(B')
100μm
(C) GFP (Sfrp5-Cre)
Cx40 (Purkinje cell)
RA
LA
RV
LV
10μm

Fig. 3. *Sfrp5* lineage contribute to SAN and Purkinje cells in all cardiac chambers.

(A) The scheme of lineage tracing analysis using *Sfrp5*-Cre/CAG-floxed CAT-eGFP.

(B-C') Double immunohistochemistry of E16.5 *Sfrp5* Cre/CAG-floxed CAT-eGFP heart using anti-HCN4, anti-Cx40 (red) and anti-GFP (green). Boxed area of (B, C) is magnified in (B', C').

L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle.

Fig. 4

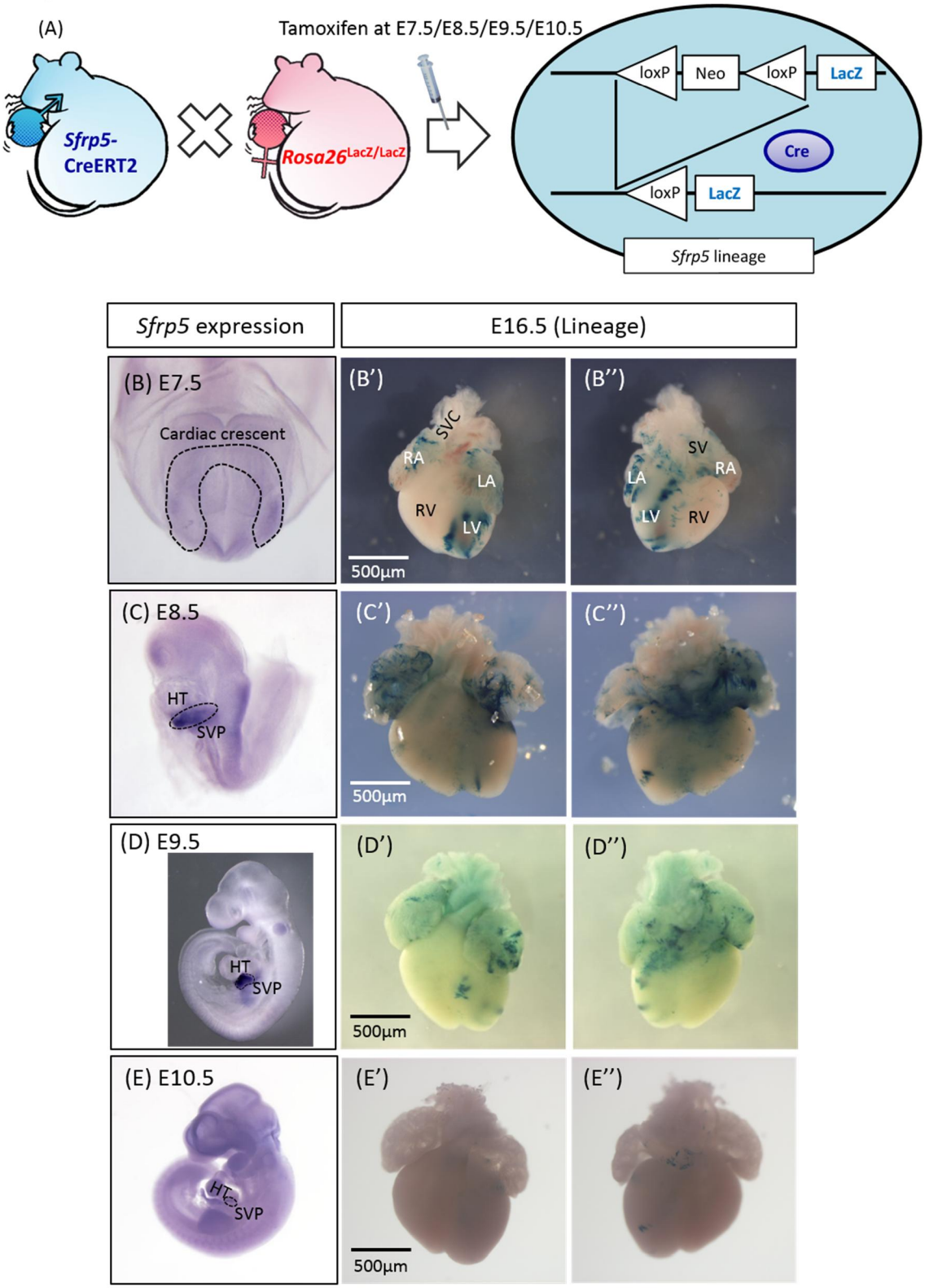

(A)
Tamoxifen at E7.5/E8.5/E9.5/E10.5
Sfrp5-CreERT2
Rosa26LacZ/LacZ
loxP
Neo
loxP
LacZ
Cre
loxP
LacZ
Sfrp5 lineage
Sfrp5 expression
E16.5 (Lineage)
(B) E7.5
Cardiac crescent
(B')
SVC
RA
LA
RV
LV
500μm
(B'')
SV
RA
LA
LV
RV
(C) E8.5
HT
SVP
(C')
500μm
(C'')
(D) E9.5
HT
SVP
(D')
500μm
(D'')
(E) E10.5
HT
SVP
(E')
500μm
(E'')

Fig. 4. *Sfrp5* lineage contribute to all chambers except for RV.
(A) The scheme of lineage tracing analysis using *Sfrp5*-CreERT2/R26R mouse.
(B-E) Whole-mount *in situ* hybridization using *Sfrp5* probe. The expression pattern correspond to the timing of TM administration (n≥4).
(B'-E') Ventral view of LacZ stained *Sfrp5* CreERT2/ Rosa26-LacZ E16.5 hearts.
(B''-E'') Dorsal view of LacZ stained *Sfrp5* CreERT2/ Rosa26-LacZ E16.5 hearts.
HT; Heart tube, L; left-hand side, LA; right atrium, LV; left ventricle. R; right-hand side, RA; right atrium, RV; right ventricle, SVP; sinus venosus primordia

Fig. 5

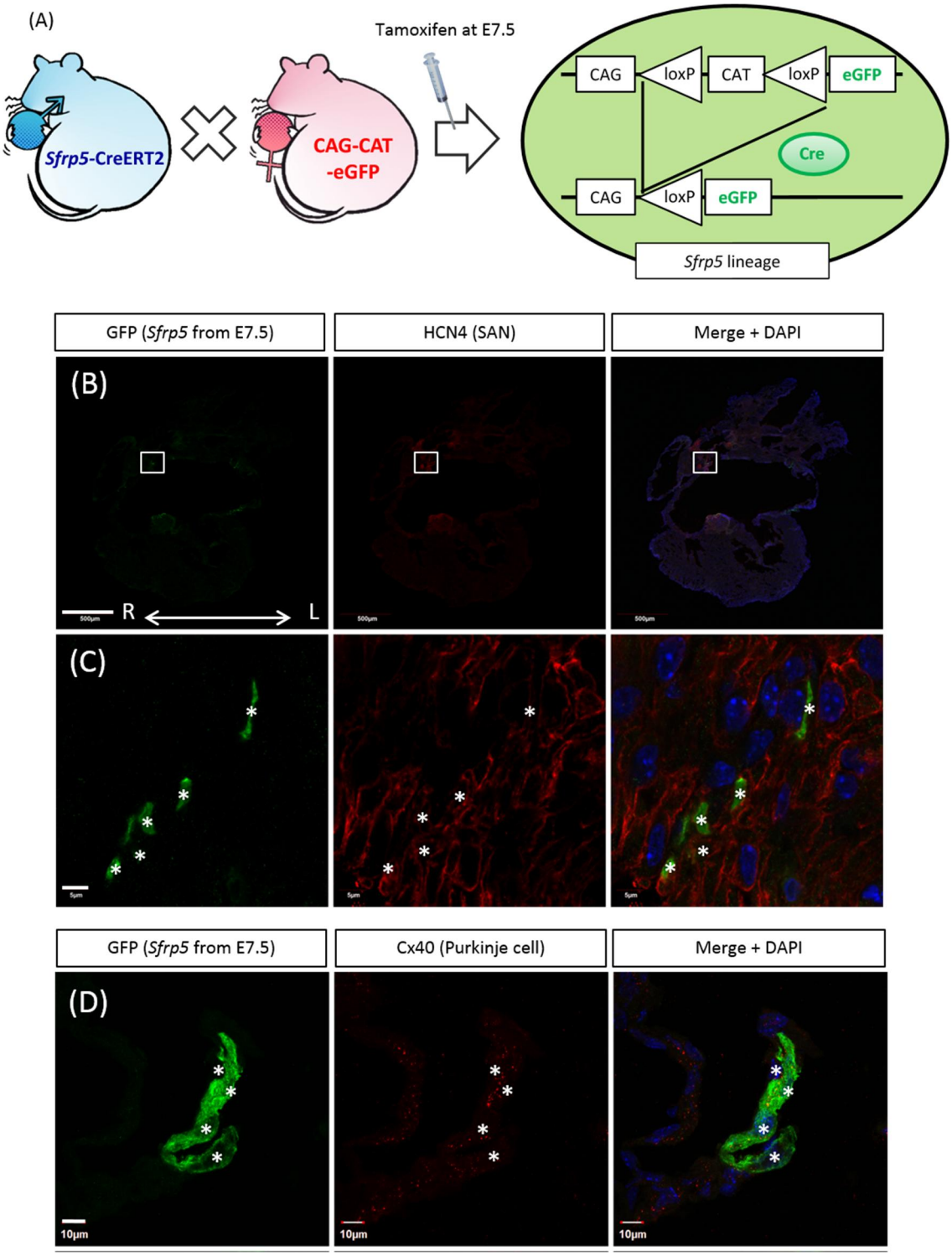
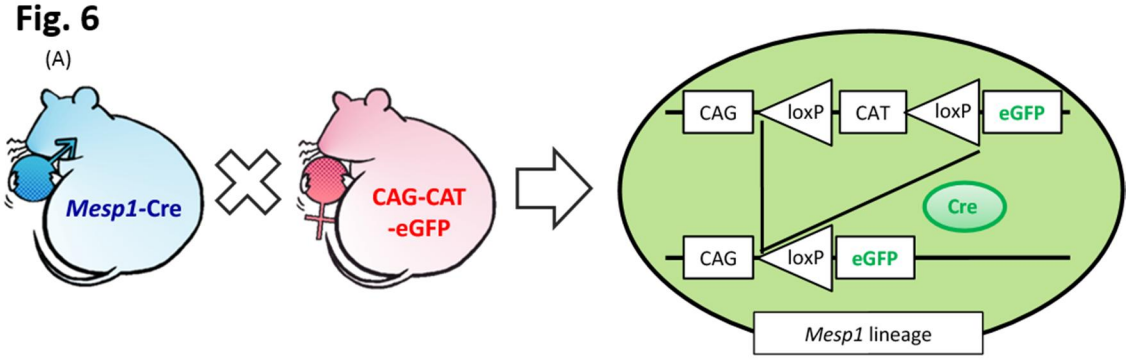
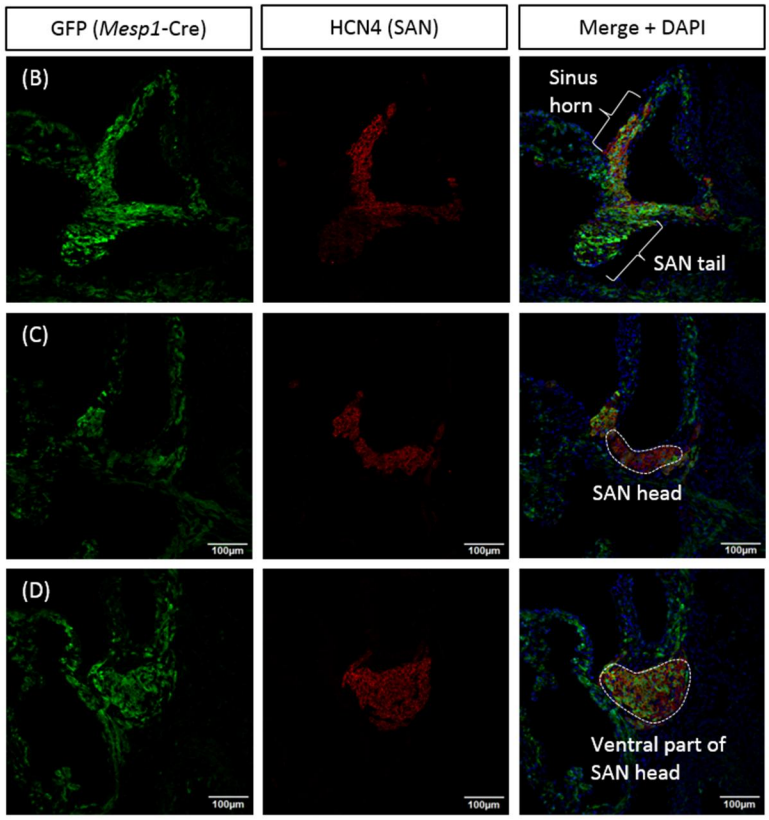

(A)
Tamoxifen at E7.5
Sfrp5-CreERT2
CAG-CAT
-eGFP
CAG
loxP
CAT
loxP
eGFP
Cre
CAG
loxP
eGFP
Sfrp5 lineage
GFP (Sfrp5 from E7.5)
HCN4 (SAN)
Merge + DAPI
(B)
R
L
(C)
GFP (Sfrp5 from E7.5)
Cx40 (Purkinje cell)
Merge + DAPI
(D)
10μm

Fig. 5. *Sfrp5* lineage from E7.5 differentiated into SAN and chamber CCS cell.
(A) The scheme of lineage tracing analysis using *Sfrp5*-CreERT2/CAG-floxed CAT -eGFP.
(B-D) Double immunohistochemistry of E16.5 *Sfrp5* CreERT2/CAG-floxed CAT -eGFP heart using anti-GFP (green), anti-HCN4 (red) or anti-Cx40 (red). Asterisks; cells co-expressing GFP and HCN4/Cx40 cells.
L; left-hand side, R; right-hand side.

Fig. 6



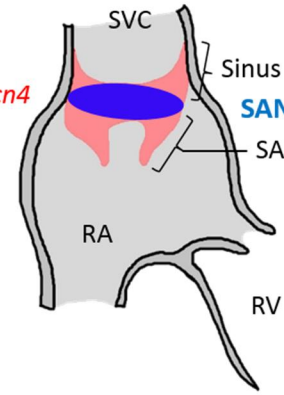


$$\frac{\text{HCN4+/GFP+}}{\text{HCN4+}} = 27.2\%$$

$\therefore$ *Mesp*1 negative linea
$= 72.8\%$

(E)

Tbx18, *Hcn4*
Tbx18, *Tbx3*, *Hcn4*
Hcn4, *Nkx2-5*



Sinus horn (*Mesp1*-positive lineage)
SAN head (72.8% *Mesp1*-negative lineage)
SAN tail (*Mesp1*-positive lineage)

(F)

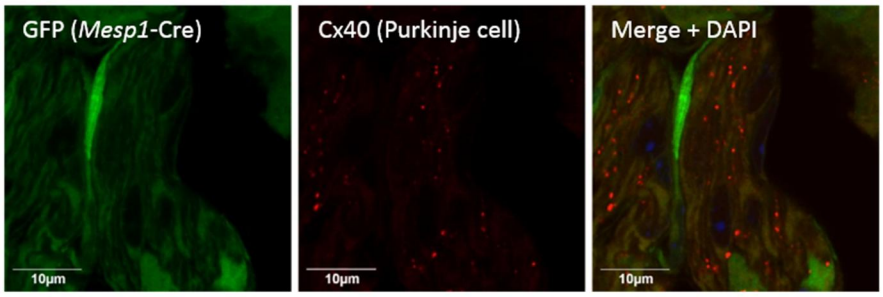


Fig. 6. Contribution of *Mesp1*-negative lineage to the CCS.
(A) The scheme of lineage tracing analysis using *Mesp1*-Cre/CAG-floxed CAT-eGFP mouse.
(B-D) Double immunohistochemistry of E16.5 *Mesp1*-Cre/CAG-floxed CAT-eGFP heart using anti-GFP (green) and anti-HCN4 (red). (B) A section of dorsal part SAN tail and sinus horn. (C) A section of middle part of SAN head. (D) A section of ventral part of SAN head .
(E) Cartoon indicate the contribution of *Mesp1* lineage in the SAN.
(F) Double immunohistochemistry of E16.5 *Mesp1*-Cre/CAG-floxed CAT-eGFP heart using anti-GFP (green) and anti-Cx40 (red).

Fig. 7

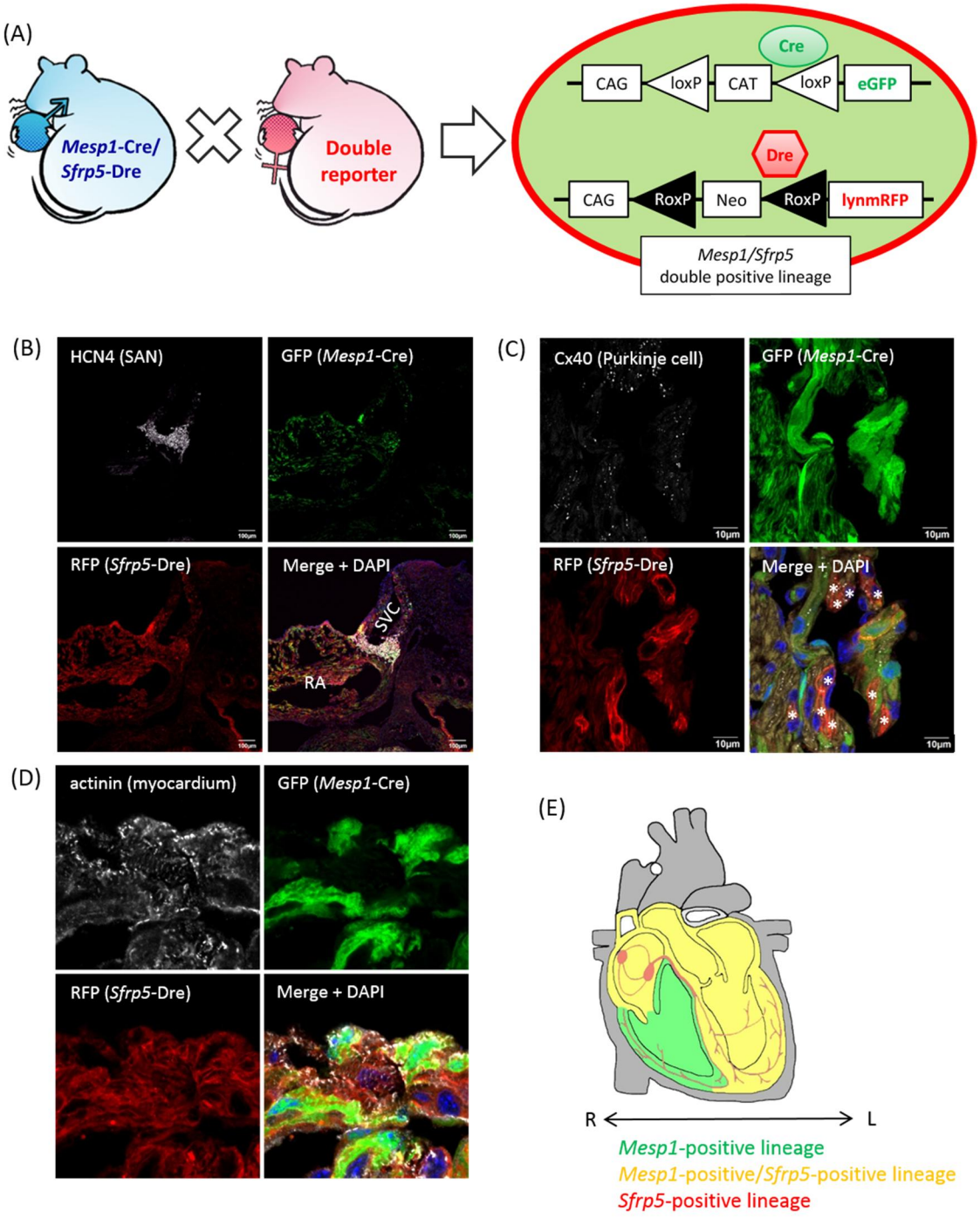
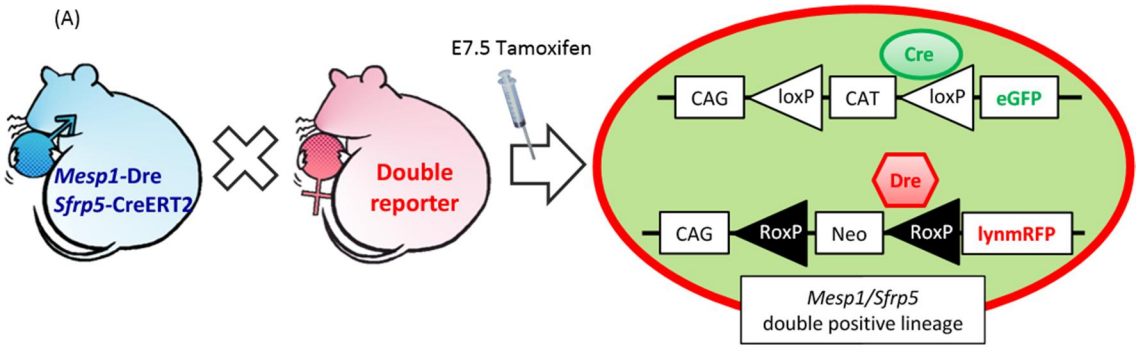
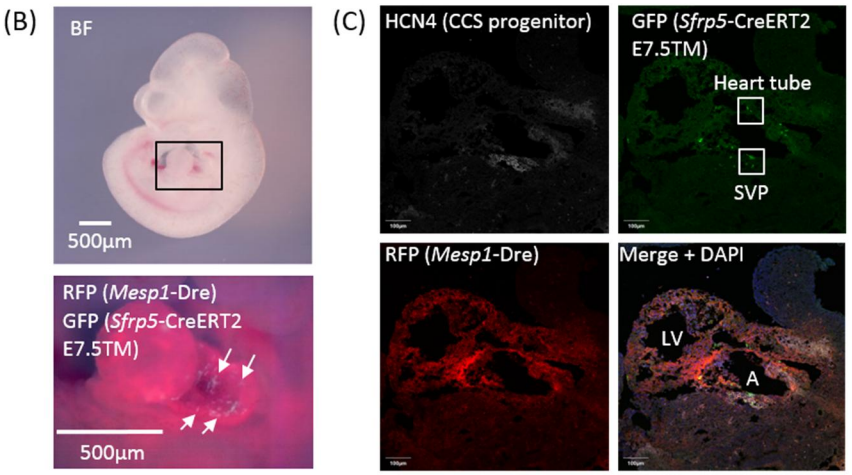

(A)
Mesp1-Cre/
Sfrp5-Dre
Double
reporter
Cre
CAG
loxP
CAT
loxP
eGFP
Dre
CAG
RoxP
Neo
RoxP
lynmRFP
Mesp1/Sfrp5
double positive lineage
(B)
HCN4 (SAN)
GFP (Mesp1-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI
SVC
RA
(C)
Cx40 (Purkinje cell)
GFP (Mesp1-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI
10μm
(D)
actinin (myocardium)
GFP (Mesp1-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI
(E)
R
L
Mesp1-positive lineage
Mesp1-positive/Sfrp5-positive lineage
Sfrp5-positive lineage

Fig.7. Contribution of *Sfrp5*-positive/*Mesp1*-negative lineage to the SAN.
(A) The scheme of double lineage tracing analysis using *Mesp1*-Cre/*Sfrp5*-Dre mouse and double reporter mouse.
(B-D) The immunohistochemistry of E16.5 *Mesp1*-Cre/*Sfrp5*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP heart using anti-HCN4 (B, SAN), anti-Cx40 (C, Purkinje cell) or anti-actinin (D, myocardium). GFP (*Mesp1* lineage) and lynmRFP (*Sfrp5* lineage) were directly observed without antibody. Asterisk indicate GFP-negative/RFP-positive/Cx40-positive cell.
(E) The cartoon showing the lineage contribution of *Mesp1* and *Sfrp5* lineages.
L; left-hand side, R; right-hand side, RA; right atrium, SVC; superior vena cava.

Fig. 8


(A)
E7.5 Tamoxifen
Mesp1-Dre
Sfrp5-CreERT2
Double reporter
Cre
CAG
loxP
CAT
loxP
eGFP
Dre
CAG
RoxP
Neo
RoxP
lynmRFP
Mesp1/Sfrp5
double positive lineage


(B)
BF
500μm
RFP (Mesp1-Dre)
GFP (Sfrp5-CreERT2 E7.5TM)
500μm
(C)
HCN4 (CCS progenitor)
GFP (Sfrp5-CreERT2 E7.5TM)
Heart tube
SVP
RFP (Mesp1-Dre)
Merge + DAPI
LV
A

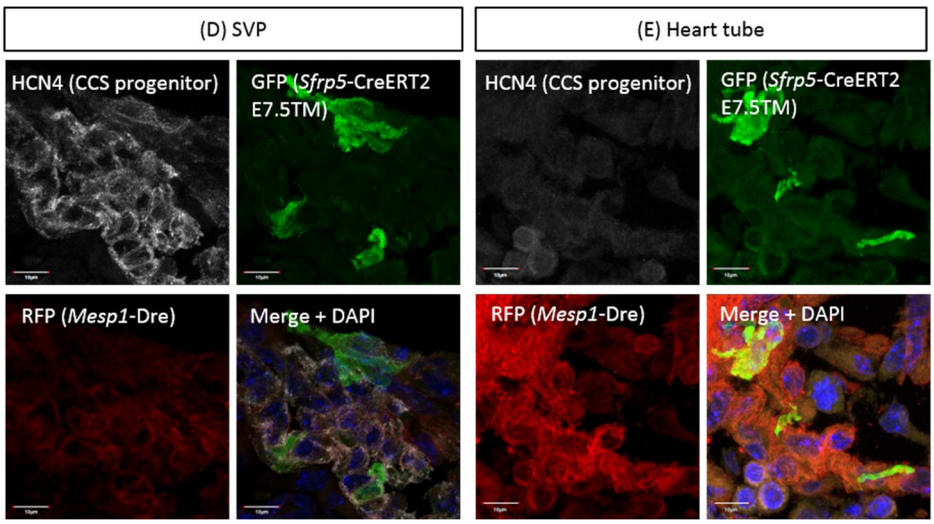

(D) SVP
(E) Heart tube
HCN4 (CCS progenitor)
GFP (Sfrp5-CreERT2 E7.5TM)
HCN4 (CCS progenitor)
GFP (Sfrp5-CreERT2 E7.5TM)
RFP (Mesp1-Dre)
Merge + DAPI
RFP (Mesp1-Dre)
Merge + DAPI

Fig. 8

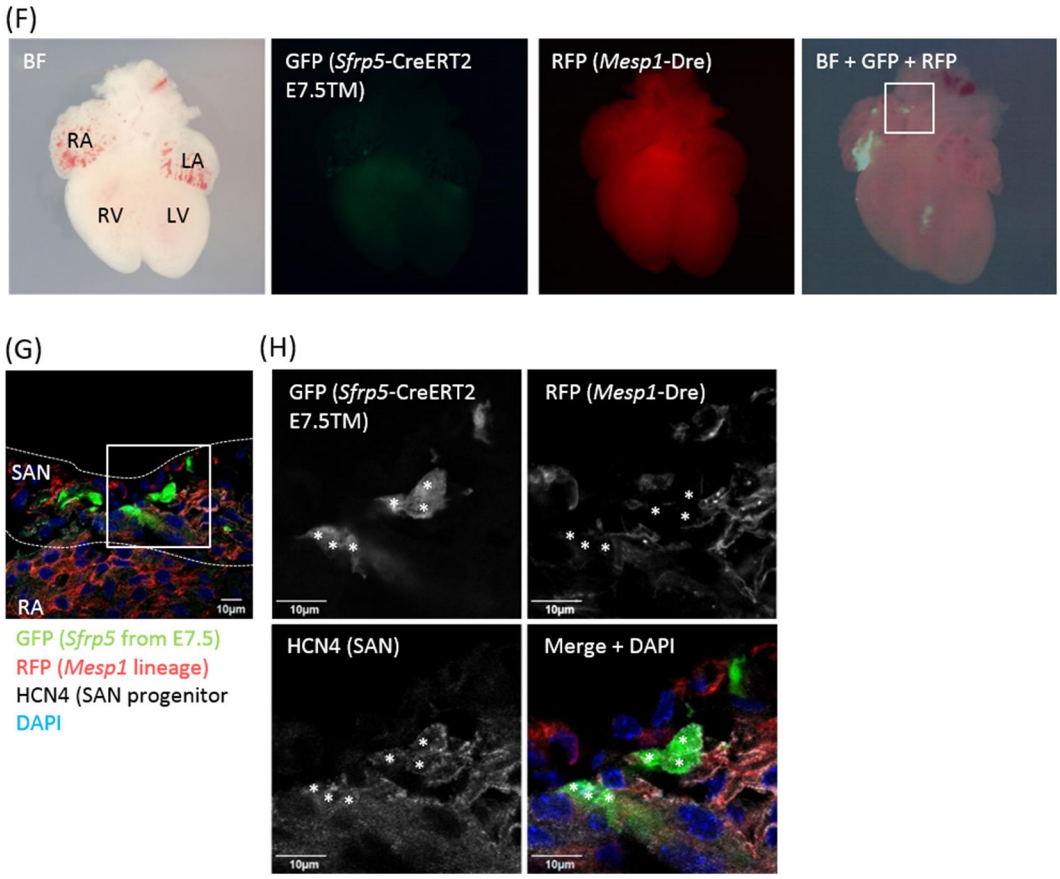


Fig. 8. Cells derived from *Sfrp5*-positive/*Mesp1*-negative lineage provide CCS progenitor cells and differentiate into SAN.

(A) The scheme of double lineage tracing analysis using *Mesp1*-Dre/*Sfrp5*-CreERT2 and the reporter CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mice.

(B) eGFP and lynmRFP pattern of E10.5 *Sfrp5*-CreERT2/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mouse (E7.5 TM, n=8). Upper panel: A bright field image of the embryo. Lower panel: A magnified confocal image of the heart tube. Arrows indicate GFP-positive *Sfrp-5* lineage.

(C-E) Immunohistochemistry of (B) using anti-HCN4 (white, CCS progenitor cells). Boxed areas of heart tube (C) are magnified in panels (D and E).

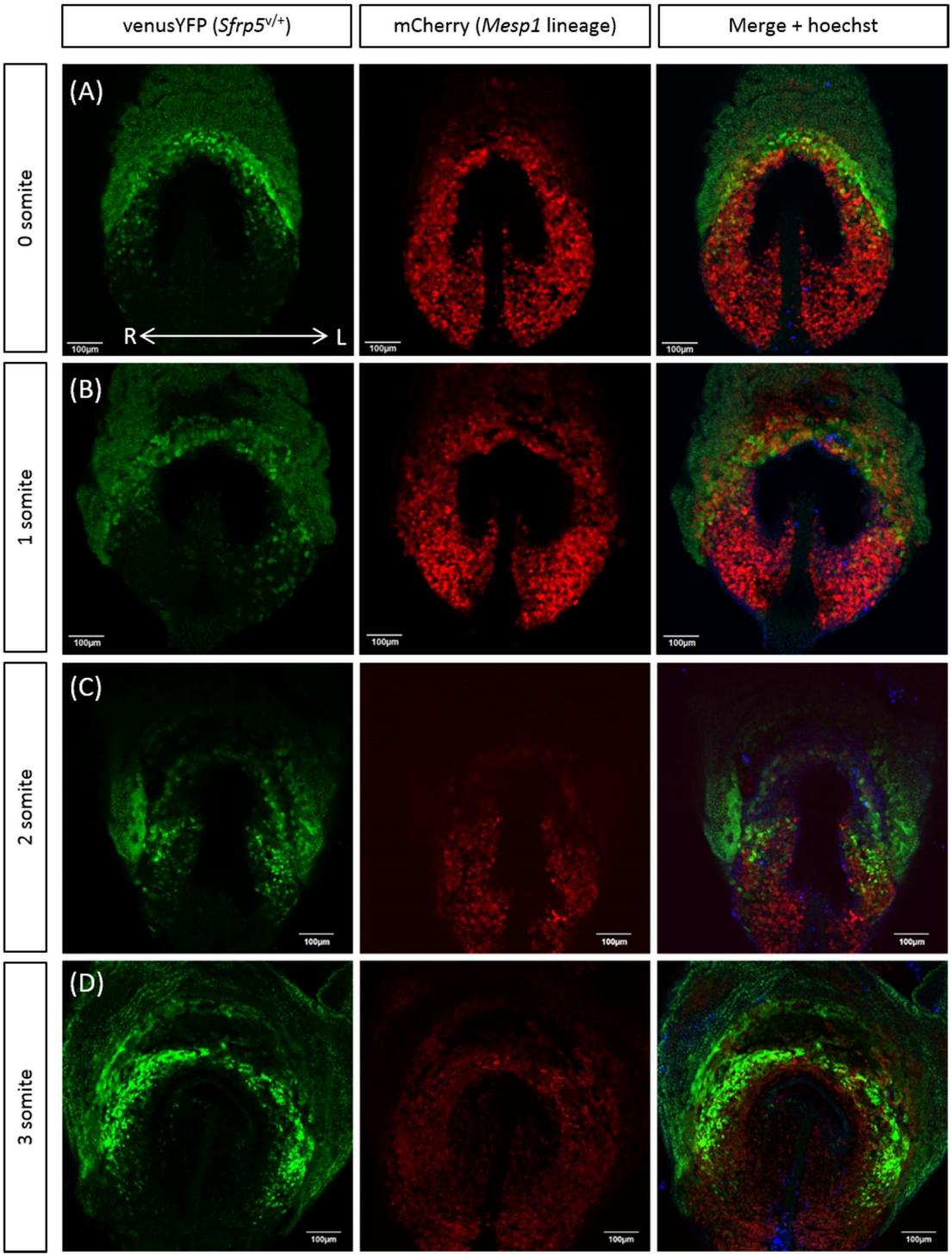
(F) The eGFP and lynmRFP pattern of E16.5 *Sfrp5*-CreERT2/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP heart (E7.5 TM, n=13).

(G) Immunohistochemistry of (F) using anti-HCN4 (white, SAN) merged with GFP and mRFP signals. Nuclei were stained with DAPI.

(H) The magnified pictures of the boxed area in (G). Asterisks indicate eGFP-positive /lynmRFP-negative/HCN4-positive (blue) cells.

L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle, SVP; sinus venosus primordia.

Fig. 9


venusYFP (*Sfrp5*$^{v/+}$)
mCherry (*Mesp1* lineage)
Merge + hoechst
0 somite
1 somite
2 somite
3 somite
(A)
(B)
(C)
(D)
R
L
100μm

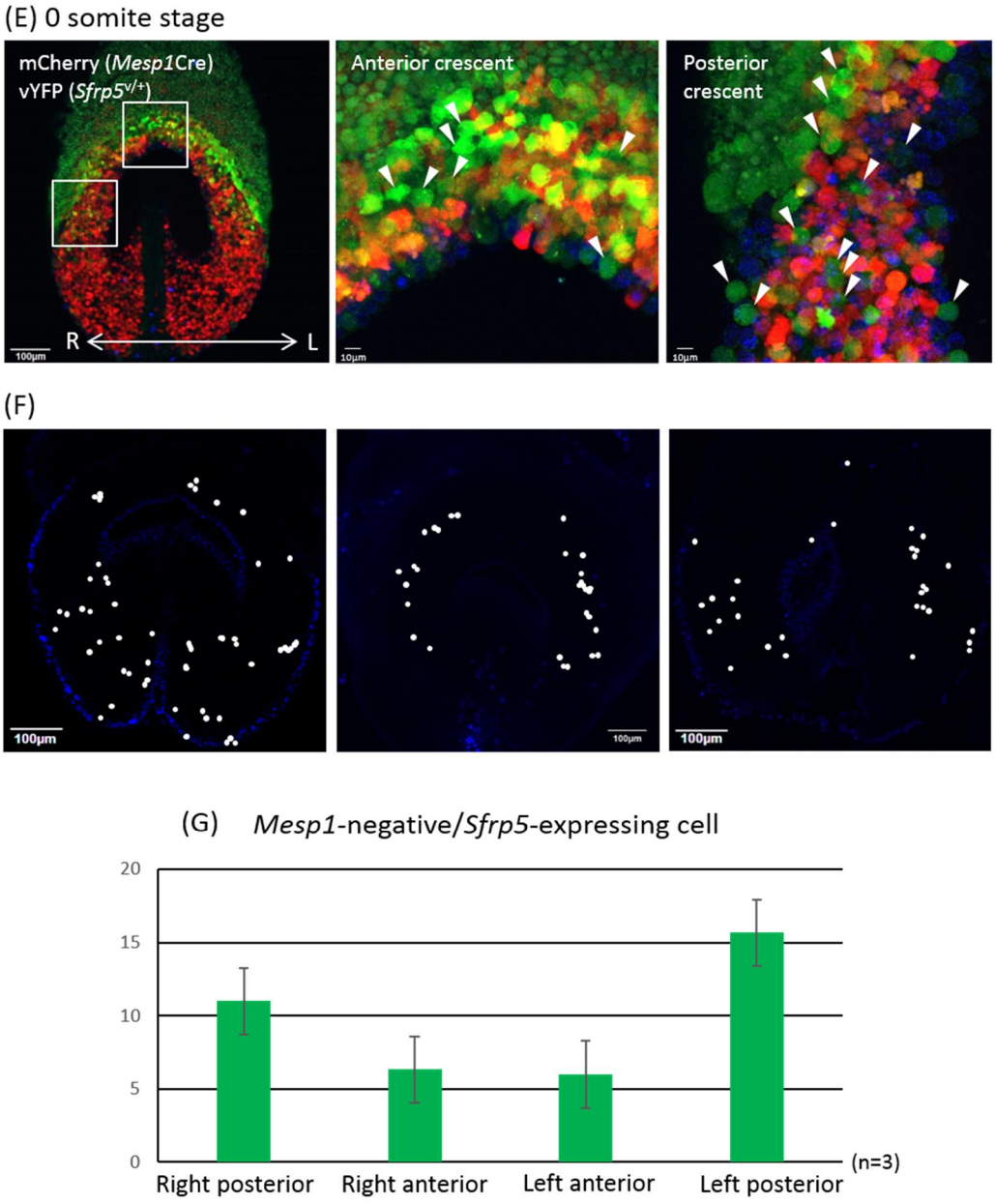


Fig.9. *Sfrp5* expressing cells derived from *Mesp1* negative lineage on the cardiac crescent.

(A-D) vYFP and mCherry pattern of *Mesp1*Cre/CAG-floxed CAT-mCherry/*Sfrp5*$^{v/+}$ at 0 somite (A), 1 somite (B), 2 somite (C) and 3 somite (D) stage embryos (n=5).

(E) The magnified picture of (A) focused on the mCherry-negative/vYFP-positive cells in the anterior and posterior cardiac crescent. Arrows indicate *Sfrp5* expressing cells derived from *Mesp1*-negative lineage.

(F) The mapping of mCherry-negative/vYFP-positive cells (indicated by white dots) observed in (E).

(G) The quantification of (F). Anterior: 6.16 ± 0.74, Posterior: 13.3 ± 2.82 (P=0.057)

R; right-hand side, L; left-hand side.

Fig. 10

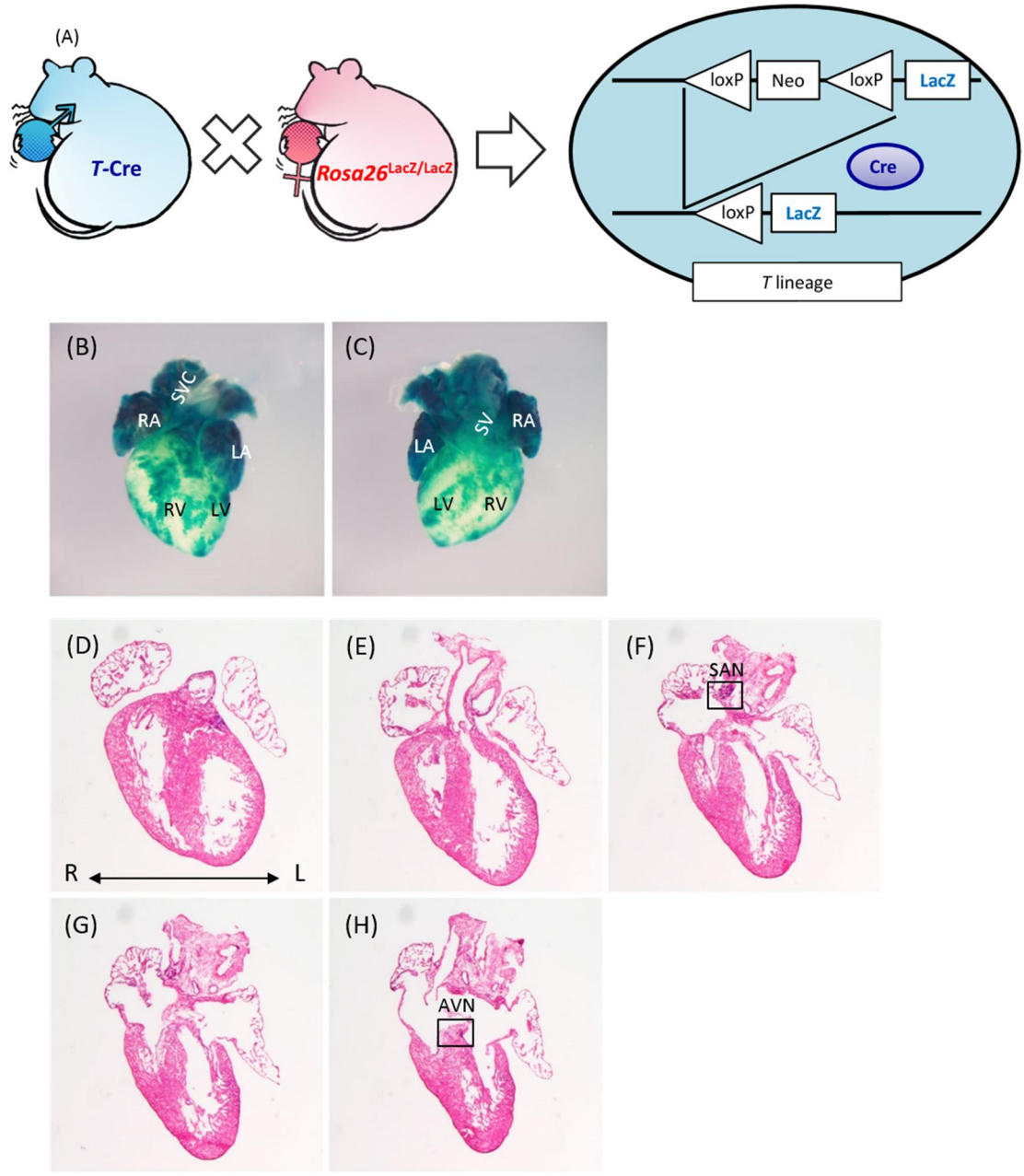

(A)
T-Cre
Rosa26LacZ/LacZ
loxP
Neo
loxP
LacZ
Cre
loxP
LacZ
T lineage
(B)
SVC
RA
LA
RV
LV
(C)
SV
RA
LA
LV
RV
(D)
R
L
(E)
(F)
SAN
(G)
(H)
AVN

Fig. 10

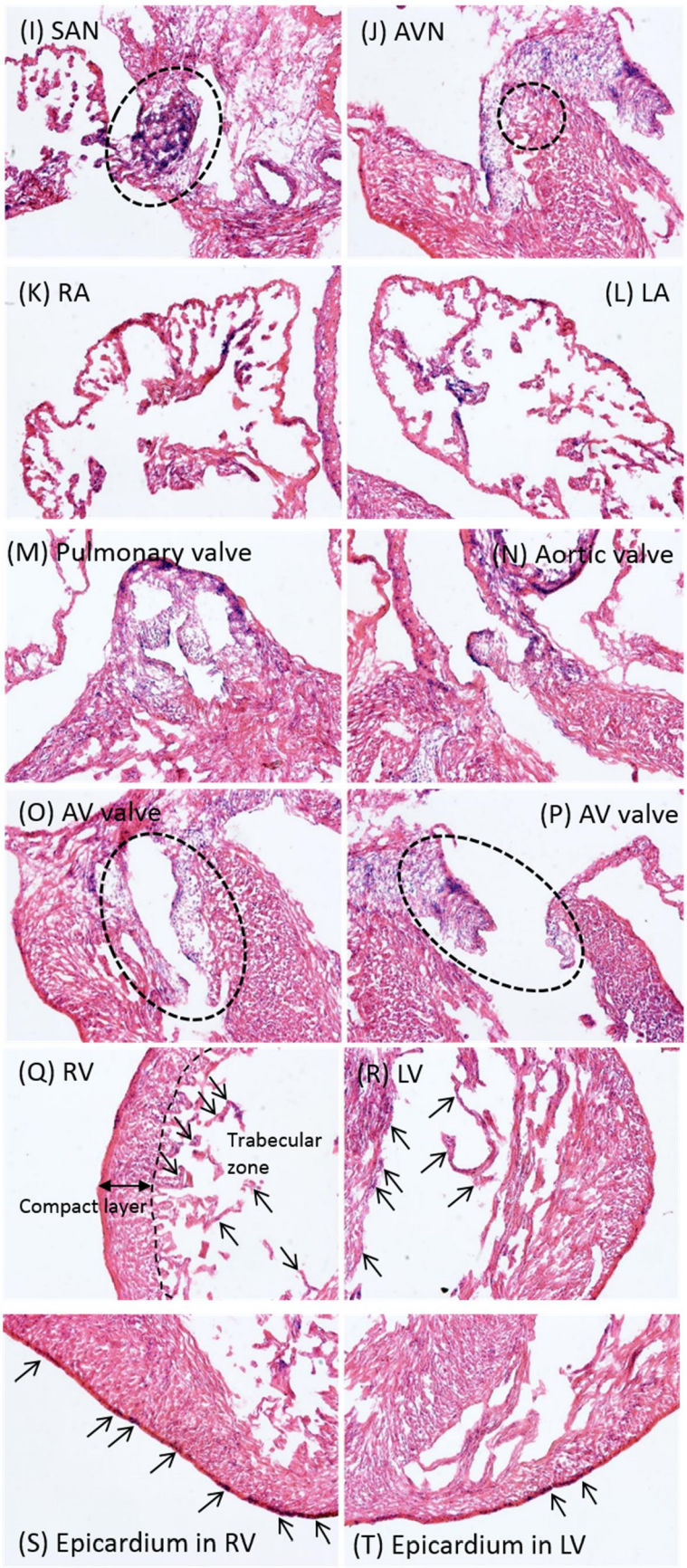

(I) SAN
(J) AVN
(K) RA
(L) LA
(M) Pulmonary valve
(N) Aortic valve
(O) AV valve
(P) AV valve
(Q) RV
Trabecular zone
Compact layer
(R) LV
(S) Epicardium in RV
(T) Epicardium in LV

Fig. 10. *T*-lineage contributes to SAN, Purkinje cells and atrium.
(A) Scheme of the lineage tracing analysis using *T*-Cre/R26R mouse.
(B-C) Ventral view (B) and dorsal view (C) of LacZ stained *T*-Cre/R26R heart at E16.5 (n=4).
(D-H) Frontal section of LacZ stained *T*-Cre/R26R heart at E16.5.
(I-T) Magnified picture of (D)-(H). SAN (I) and AVN (J) is shown in dotted circles. LacZ-positive cells were observed in right (K) and left (L) atria, pulmonary valve (M), aortic valve (N), AV valve (O, P), myocardial layer of right (Q) and left (R) ventricle and epicardium (S and T). Black arrow indicate β-gal positive cell.
L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand, RA; right atrium, RV; right ventricleside, SV; sinus venosus, SVC; superior vena cava.

Fig. 11

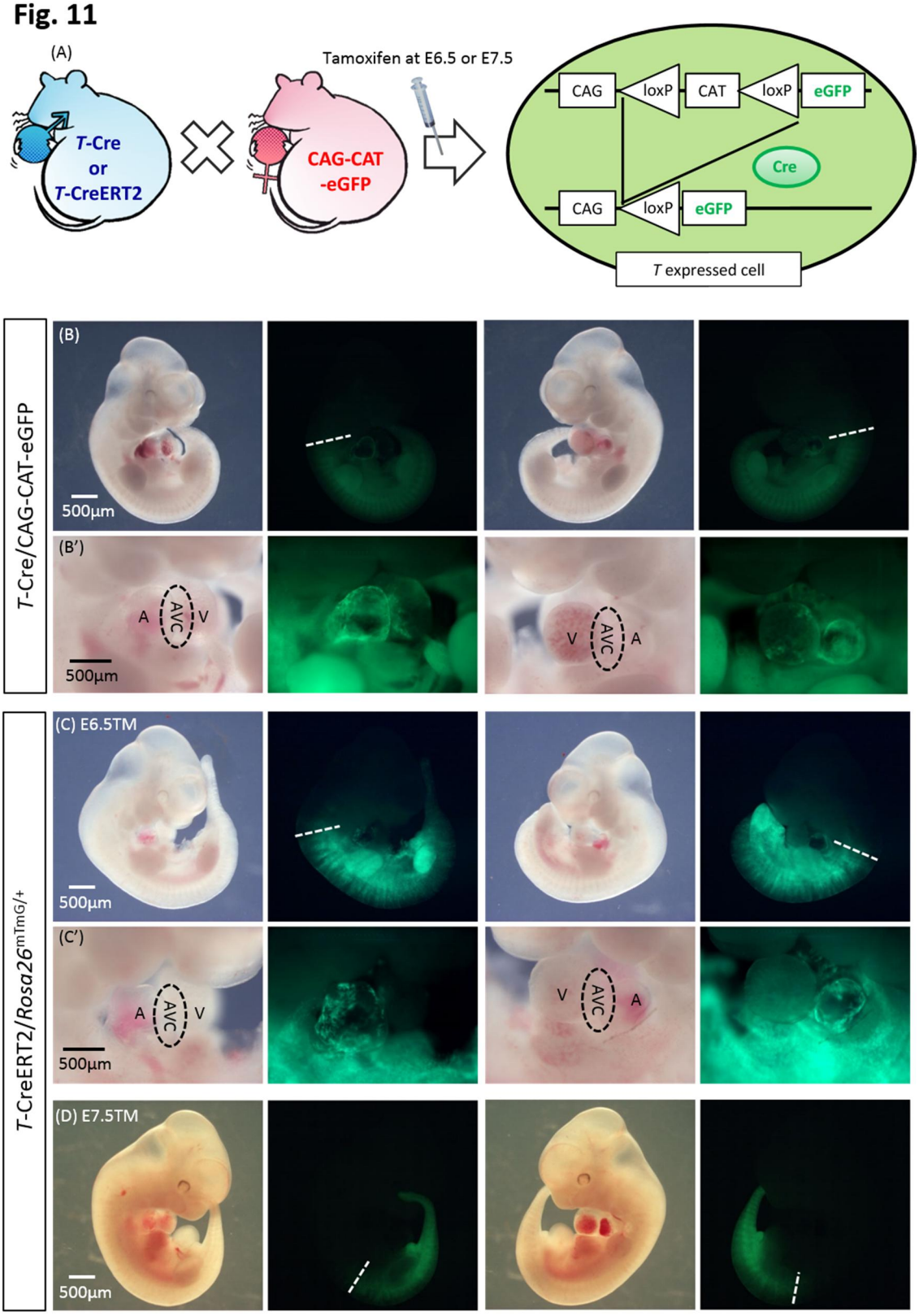

(A)
T-Cre
or
T-CreERT2
CAG-CAT
-eGFP
Tamoxifen at E6.5 or E7.5
CAG
loxP
CAT
loxP
eGFP
Cre
CAG
loxP
eGFP
T expressed cell
T-Cre/CAG-CAT-eGFP
(B)
500μm
(B')
A
AVC
V
500μm
V
AVC
A
T-CreERT2/Rosa26mTmG/+
(C) E6.5TM
500μm
(C')
A
AVC
V
500μm
V
AVC
A
(D) E7.5TM
500μm

Fig. 11

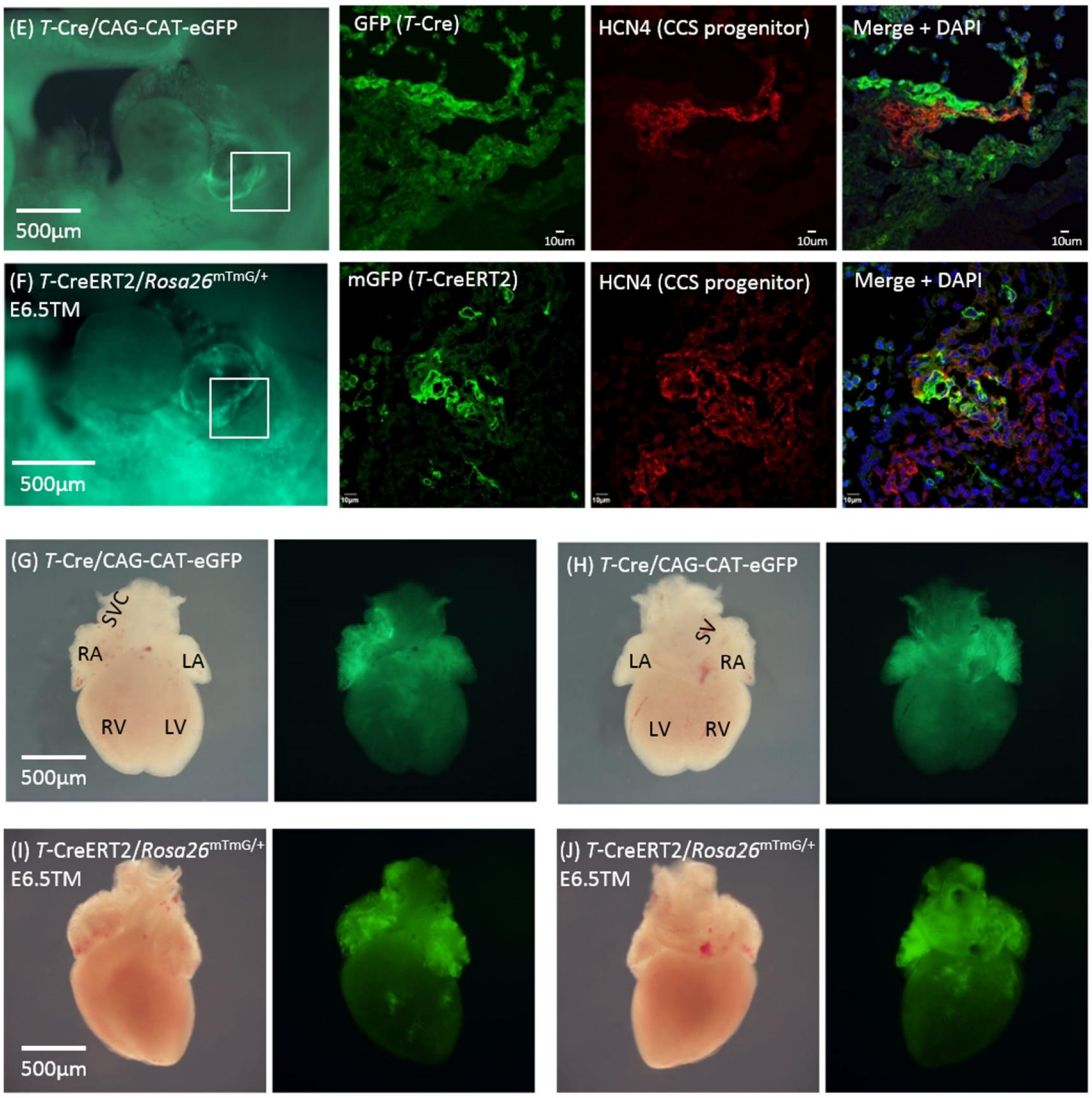
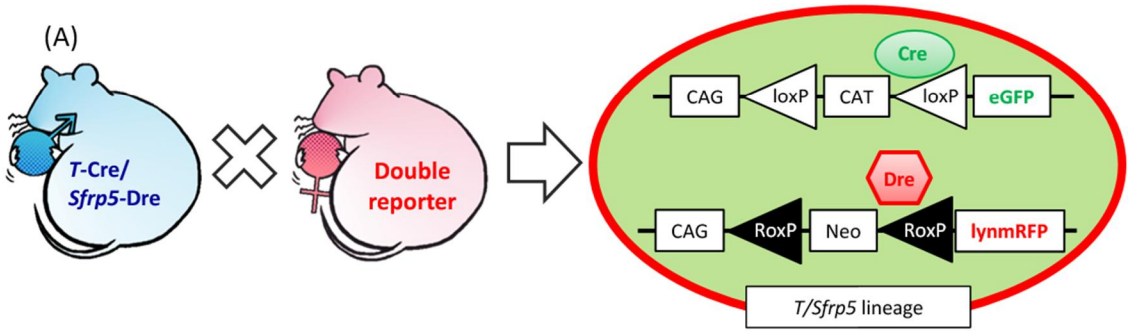

(E) T-Cre/CAG-CAT-eGFP
500μm
GFP (T-Cre)
10um
HCN4 (CCS progenitor)
10um
Merge + DAPI
10um
(F) T-CreERT2/Rosa26mTmG/+
E6.5TM
500μm
mGFP (T-CreERT2)
HCN4 (CCS progenitor)
Merge + DAPI
(G) T-Cre/CAG-CAT-eGFP
SVC
RA
LA
RV
LV
500μm
(H) T-Cre/CAG-CAT-eGFP
SV
LA
RA
LV
RV
(I) T-CreERT2/Rosa26mTmG/+
E6.5TM
500μm
(J) T-CreERT2/Rosa26mTmG/+
E6.5TM

Fig. 11. *T* lineage from E6.5 contribute to the CCS and atrium.
(A) Scheme of the lineage tracing analysis using *T*-Cre/CAG-floxed CAT-eGFP or *T*-CreERT2/*Rosa26*$^{mTmG/+}$. When *T*-CreERT2 mouse was crossed with *Rosa26*$^{mTmG/+}$, TM was administrated at E6.5 or E7.5.
(B, B') eGFP pattern of E10.5 *T*-Cre/CAG-floxed CAT-eGFP embryo (n=11). (B') is magnified pictures of heart tube of (B).
(C, C') mGFP pattern of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryo (E6.5 TM, n=18). (C') is magnified pictures of heart tube of (C). Dotted lines in (B) and (C) indicate the anterior limit of GFP signal.
(D) mGFP pattern of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryo (E7.5 TM, n=3).
(E) Immunohistochemistry of (B) using anti-HCN4 (red, CCS progenitor cell) and anti-GFP (green, *T* lineage reporter).
(F) Immunohistochemistry of (C) using anti-HCN4 (red, CCS progenitor cell) and anti-GFP (green, *T* lineage reporter).
(G, H) eGFP pattern of E16.5 *T*-Cre/CAG-CAT-eGFP heart (n=21). (G) is the ventral view and (H) is the dorsal view.
(I, J) mGFP pattern of E16.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ heart (E6.5 TM, n=18). (I) is the ventral view and (J) is the dorsal view.
A; atrium, AVC; atrio-ventricular canal, L; left-hand side, LA; right atrium, LV; left ventricle, R; right-hand side, RA; right atrium, RV; right ventricle, SV; sinus venosus, SVC; superior vena cava, V; ventricle.

Fig. 12


(A)
T-Cre/
Sfrp5-Dre
Double
reporter
Cre
CAG
loxP
CAT
loxP
eGFP
Dre
CAG
RoxP
Neo
RoxP
lynmRFP
T/Sfrp5 lineage

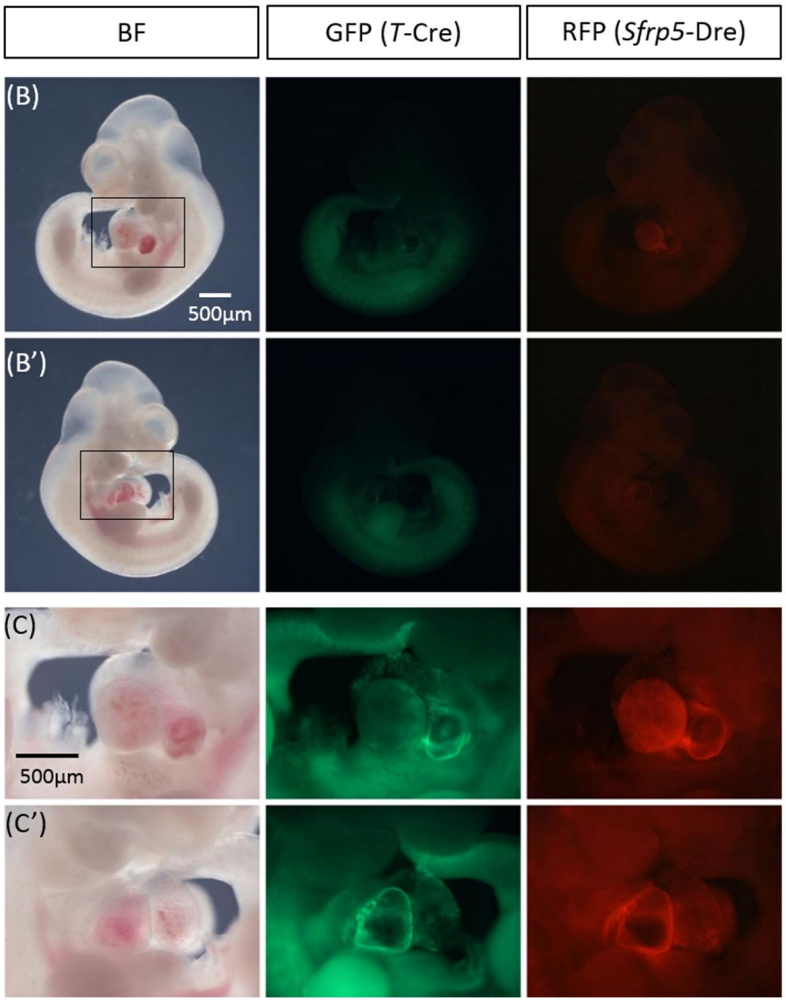
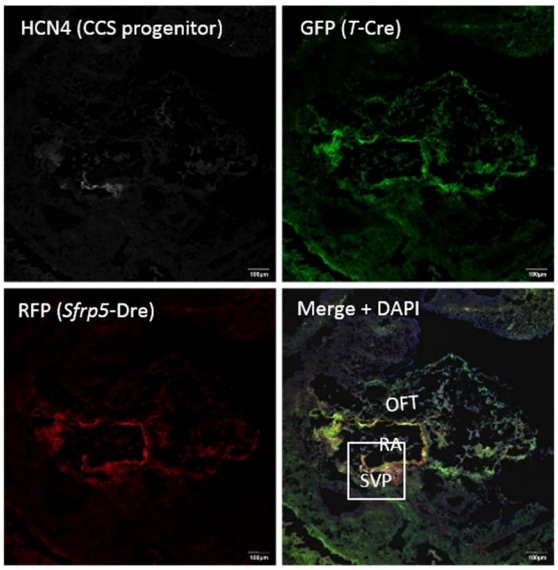
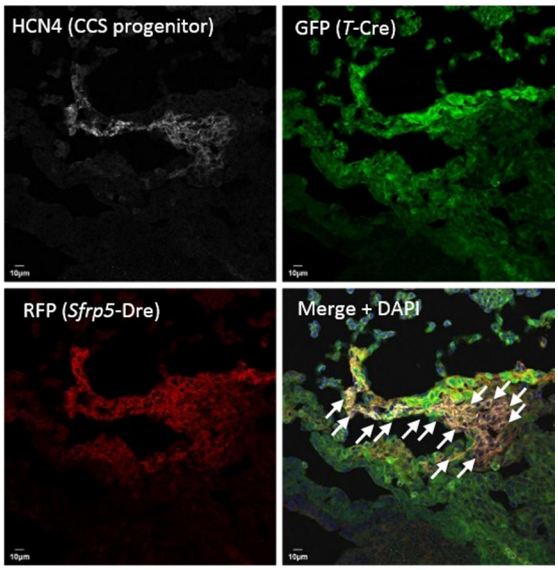

BF
GFP (T-Cre)
RFP (Sfrp5-Dre)
(B)
500μm
(B')
(C)
500μm
(C')

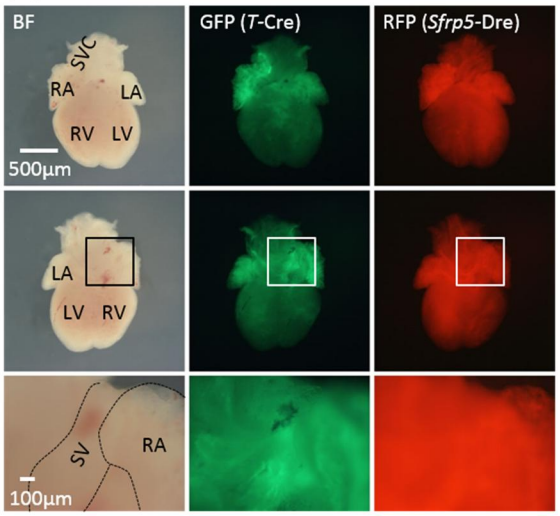
Fig. 12 (D)


HCN4 (CCS progenitor)
GFP (T-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI
OFT
RA
SVP

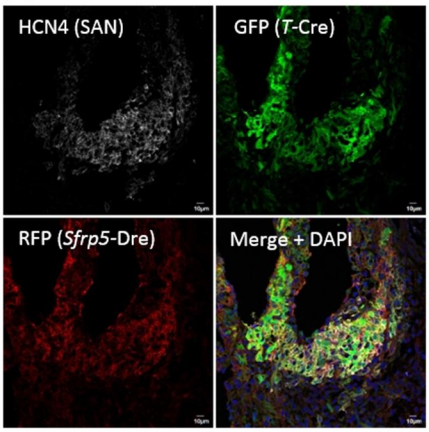
(D')


HCN4 (CCS progenitor)
GFP (T-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI

(E)


BF
SVC
RA
LA
RV
LV
500μm
GFP (T-Cre)
RFP (Sfrp5-Dre)
LA
LV
RV
SV
RA
100μm

(F)


HCN4 (SAN)
GFP (T-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI

(G)

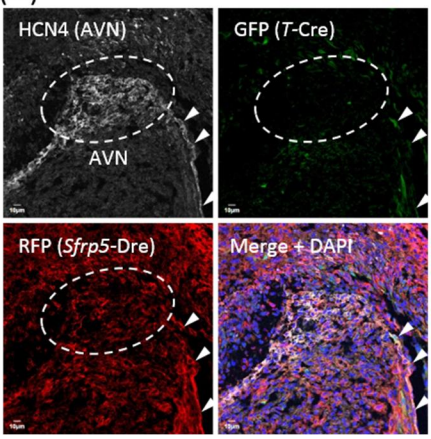

HCN4 (AVN)
AVN
GFP (T-Cre)
RFP (Sfrp5-Dre)
Merge + DAPI

Fig. 12. *T*-positive/*Sfrp5*-positive lineage provide the CCS progenitor cells.
(A) Scheme of the double lineage tracing analysis using *T*-Cre/*Sfrp5*-Dre and CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP mouse.
(B-C') eGFP and lynmRFP pattern of double reporter at E10.5 (n= 6). (B) is left side view and (B') is right view of the embryo and (C) and (C') are magnified picture of boxed area in (B) and (B').
(D, D') Immunohistochemistry of the E10.5 embryonic heart using anti-HCN4 (white, CCS progenitor cell). Boxed area of (D) is magnified in (D'). White arrow indicate GFP/RFP/HCN4 triple positive cell.
(E) eGFP and lynmRFP pattern of *T*-Cre/*Sfrp5*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP at E16.5 (n = 12). Upper panel is the ventral view, middle panel is the dorsal view and lower panel is magnified images of SV (boxed area of the middle panels).
(F, G) Immunohistochemistry of section confining the SAN (F) and AVN (G) prepared from E16.5 embryo using anti-HCN4 (white, SAN and AVN marker) merged with confocal images of GFP and lynmRFP. White arrowhead indicate GFP/RFP/HCN4 triple positive cell, Dotty circle indicates the AVN.
AVN; atrio-ventricular node, L; left-hand side, LA; left atrium, LV: left ventricle, OFT: outflow tract, R; right-hand side, RA; right atrium, RV: right ventricle, SV; sinus venosus, SVC; superior vena cava, SVP: sinus venosus primordia.

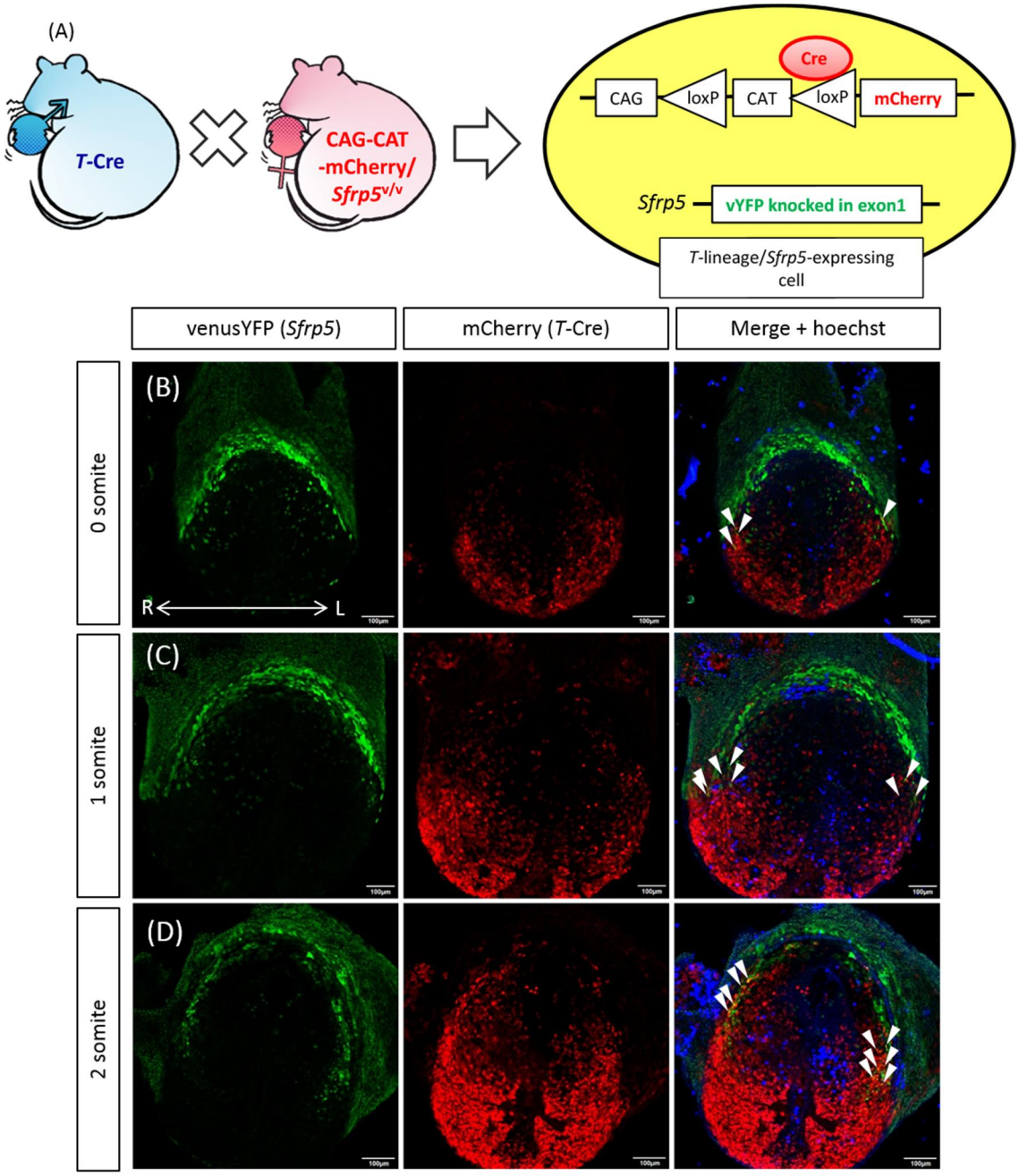


Fig. 13. *Sfrp5* expressing cells derived from *T* positive lineage exist on the lateral cardiac crescent.

(A) Scheme of mouse mating to observe *Sfrp5* expressing cells derived from *T* lineage using *T*-Cre/CAG-floxed CAT-mCherry/*Sfrp5*$^{v/+}$ (B-D, n=6). Confocal images of cardiac crescents with vYFP and mCherry signals at 0 somite (B), 1 somite (C) and 2 somite (D) embryos. Arrowheads indicate double–positive cells.
L; left-hand side, R; sight-hand side.

Fig. 14

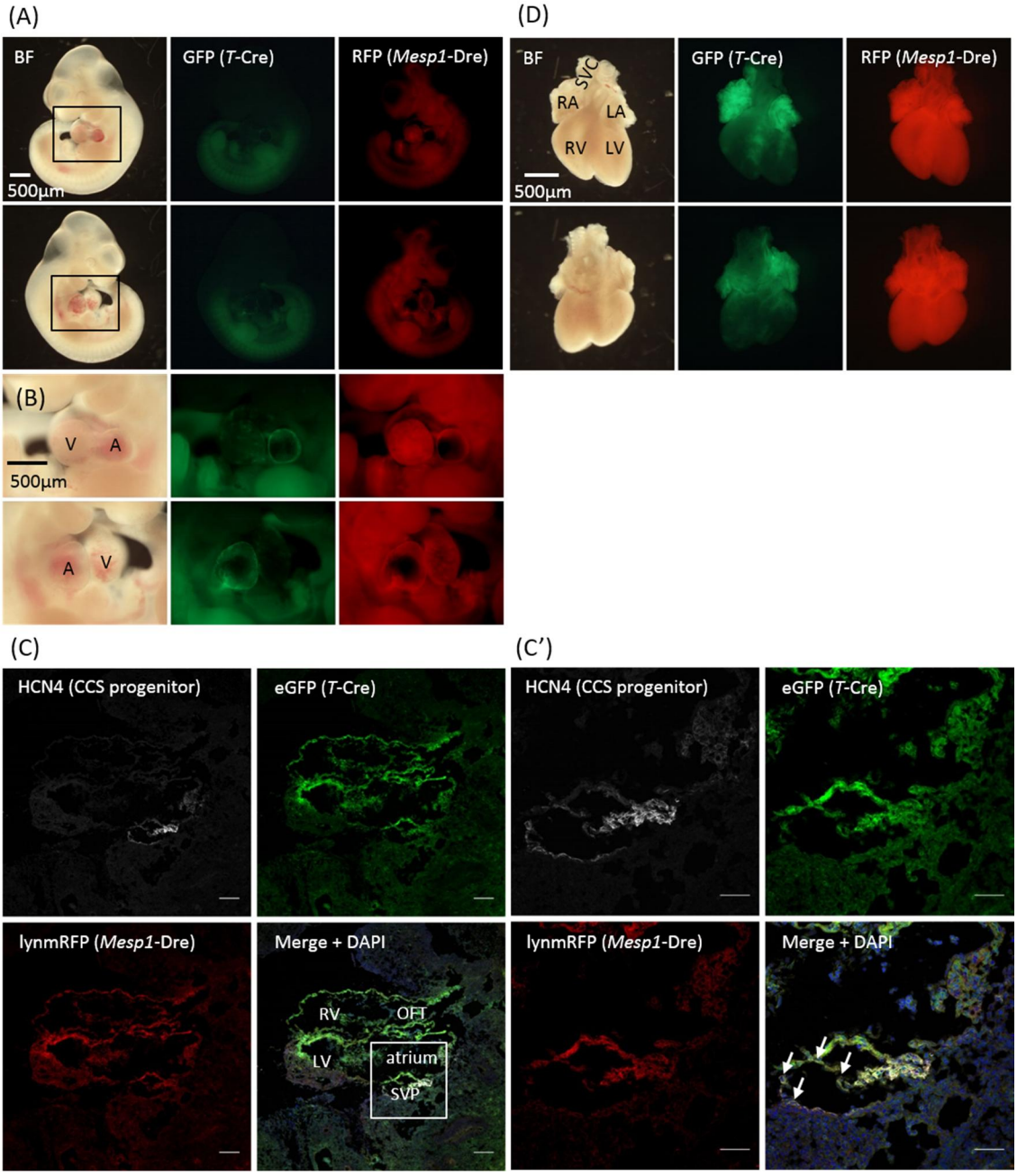

(A)
BF
GFP (T-Cre)
RFP (Mesp1-Dre)
500μm
(B)
V
A
500μm
A
V
(D)
BF
SVC
RA
LA
RV
LV
500μm
GFP (T-Cre)
RFP (Mesp1-Dre)
(C)
HCN4 (CCS progenitor)
eGFP (T-Cre)
lynmRFP (Mesp1-Dre)
Merge + DAPI
RV
OFT
LV
atrium
SVP
(C')
HCN4 (CCS progenitor)
eGFP (T-Cre)
lynmRFP (Mesp1-Dre)
Merge + DAPI

Fig. 14

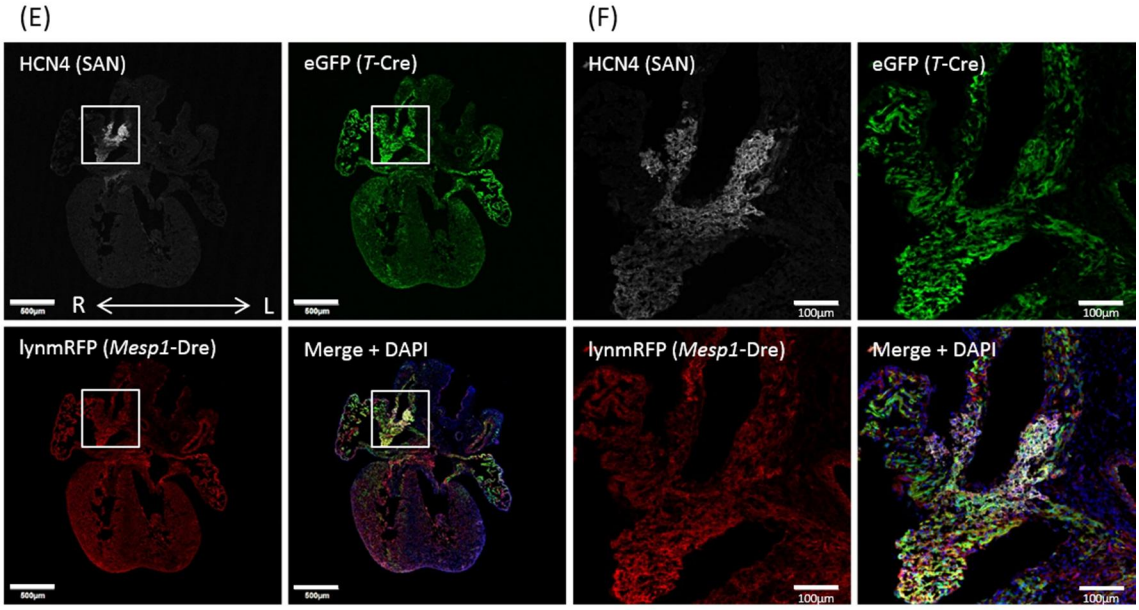


Fig. 14. *Mesp1*-negative/*T*-positive lineage provide CCS progenitor and pacemaker cells in the SAN.

(A) eGFP and lynmRFP pattern of *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lenmRFP (see Fig. 15 A) at E10.5 (n = 5). Upper panel shows left-hand side and lower panel shows right–hand side.

(B) Magnified picture of (A) focusing on heart tubes.

(C) Immunohistochemistry of (B) using anti-HCN4 (white, CCS progenitor). (C') is magnified picture of SVP in the boxed area of (C). White arrows indicate GFP-positive/RFP-negative/HCN4-positive cells.

(D) eGFP and lynmRFP pattern of *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lenmRFP (see Fig. 15 A) at E16.5 (n = 9). Upper panel shows ventral side and lower panel shows dorsal side.

Immunohistochemistry of section confining the SAN (E) prepared from E16.5 embryo using anti-HCN4 (white, SAN marker) merged with confocal images of GFP and lynmRFP.

A; atrium, L; left-hand side, LA; left atrium, LV: left ventricle, OFT: outflow truct, R; right-hand side, RA; right atrium, RV: right ventricle, SVC; superior vena cava, SVP: sinus venosus primordia, V; ventricle.

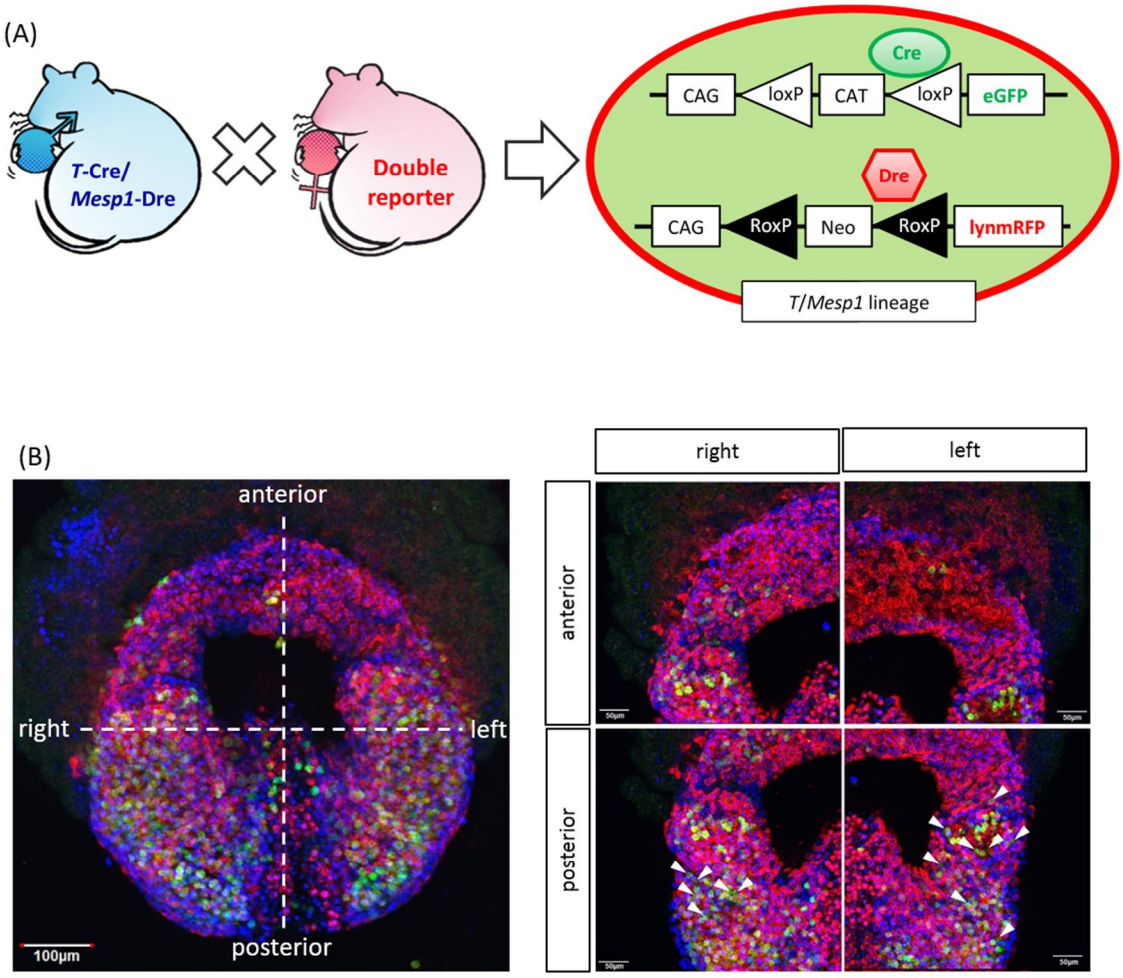


Fig. 15. *Mesp1*-negative/*T*-positive lineage cell exist on posterior cardiac crescent.
(A) Scheme of mouse mating to observe cells derived from *Mesp1*-negative/*T*-positive lineage using *T*-Cre/*Mesp1*-Dre/CAG-floxed CAT-eGFP/CAG-Roxed Neo-lynmRFP (n = 8).
(B) Confocal images of cardiac crescents with eGFP and lynmRFP signals at E7.5. White arrowheads indicate the eGFP positive/lynmRFP negative cells.

Fig. 16. Summary of CCS Developmental process.

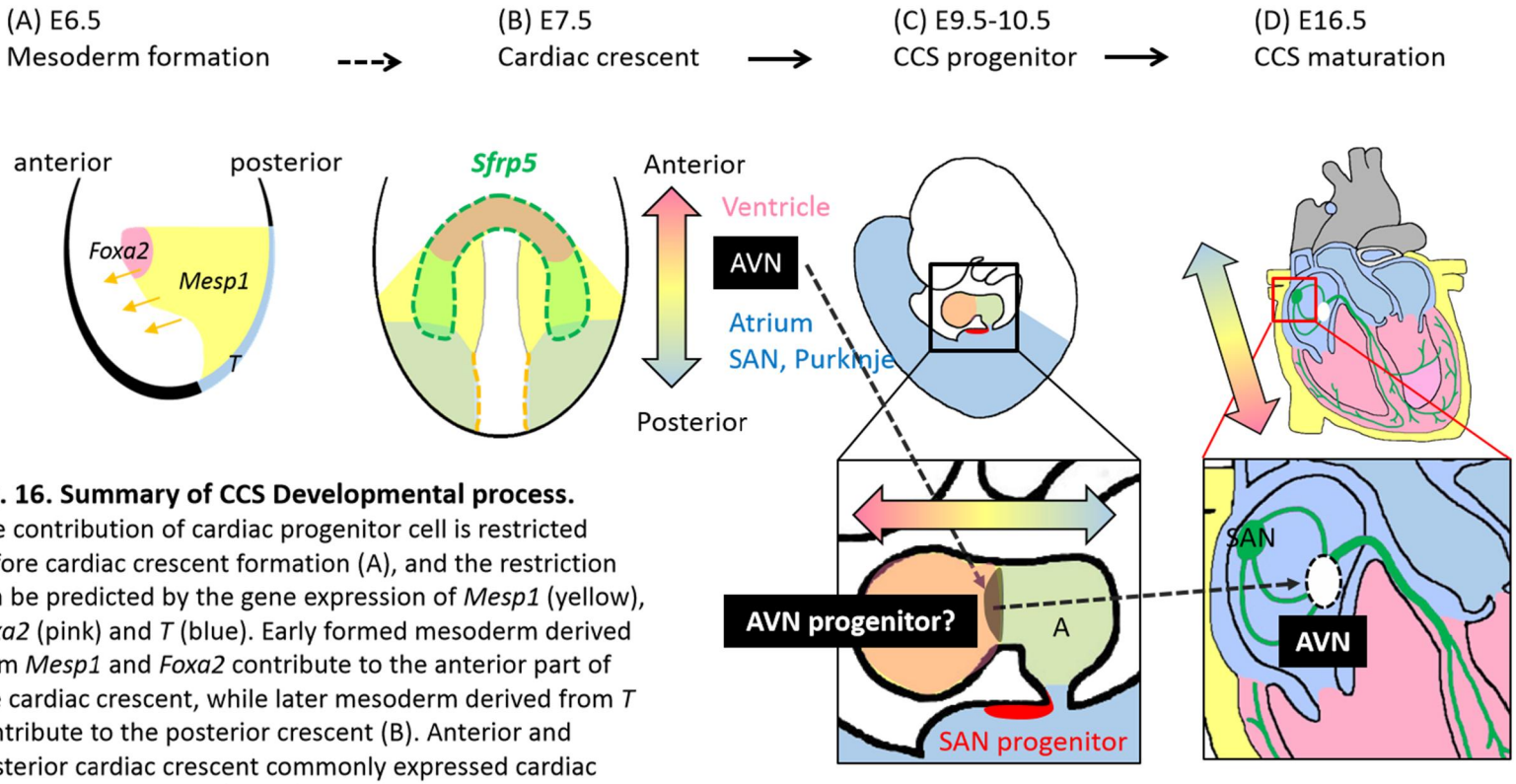
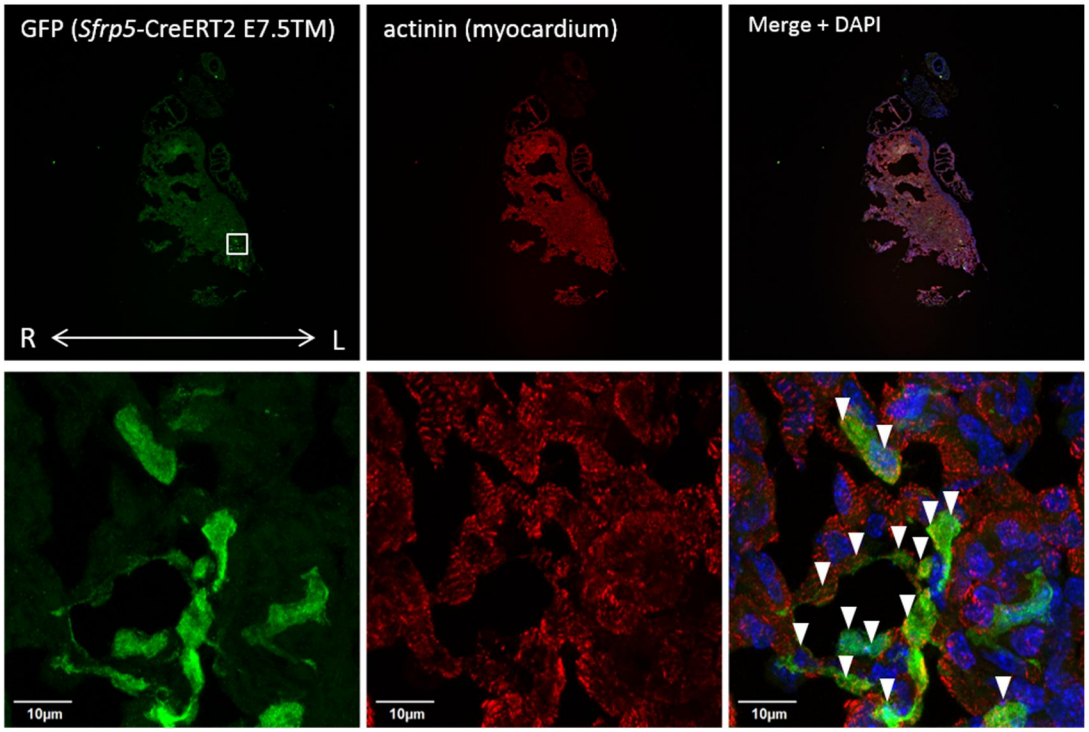
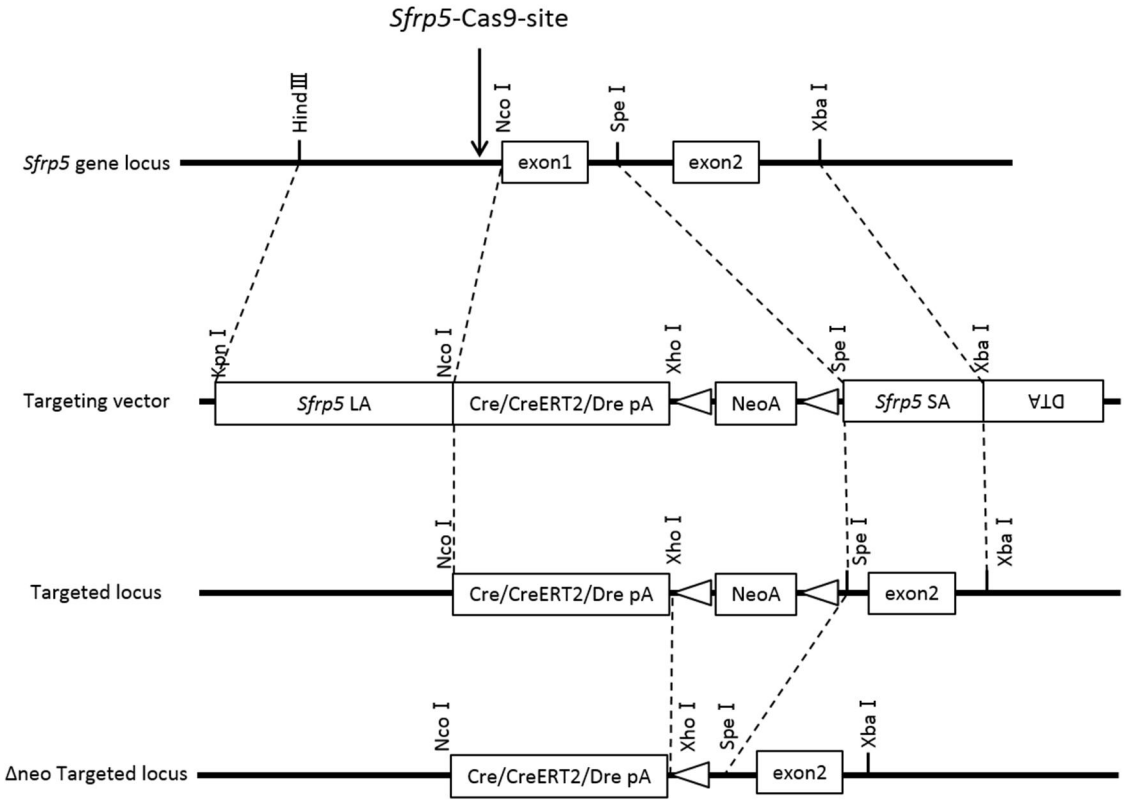


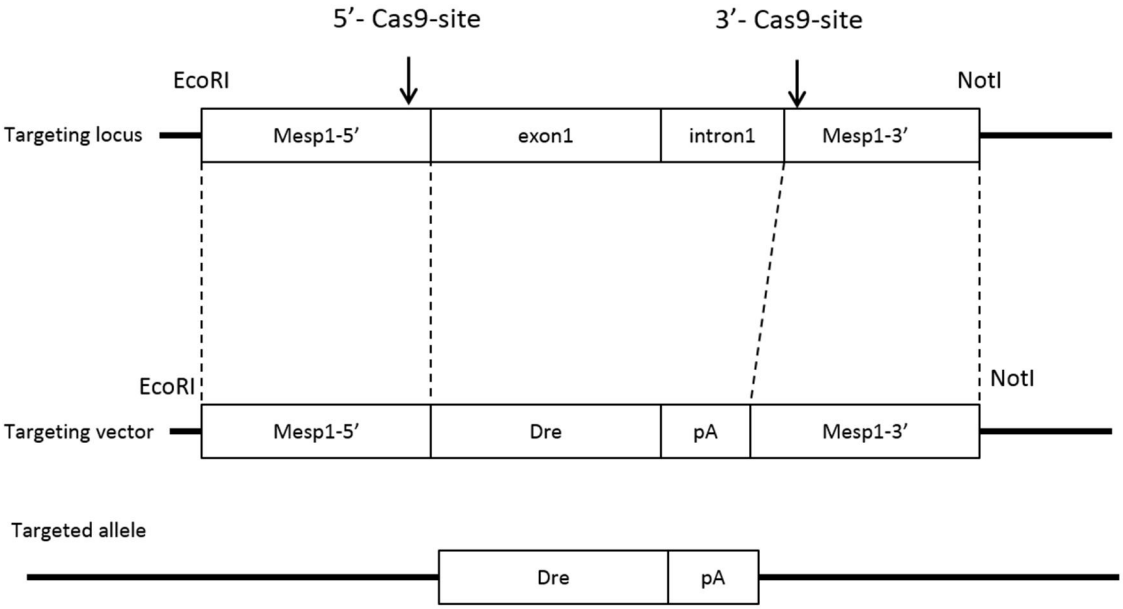
Fig. 16. Summary of CCS Developmental process.
The contribution of cardiac progenitor cell is restricted before cardiac crescent formation (A), and the restriction can be predicted by the gene expression of *Mesp1* (yellow), *Foxa2* (pink) and *T* (blue). Early formed mesoderm derived from *Mesp1* and *Foxa2* contribute to the anterior part of the cardiac crescent, while later mesoderm derived from *T* contribute to the posterior crescent (B). Anterior and posterior cardiac crescent commonly expressed cardiac progenitor gene *Sfrp5* (green) and the descendant differentiate into many cardiac components. Particularly, posterior *Sfrp5* lineage provide CCS progenitor cells in SVP (C) expressing *Hcn4* (red) and differentiate into CCS in four-chambered heart (D).



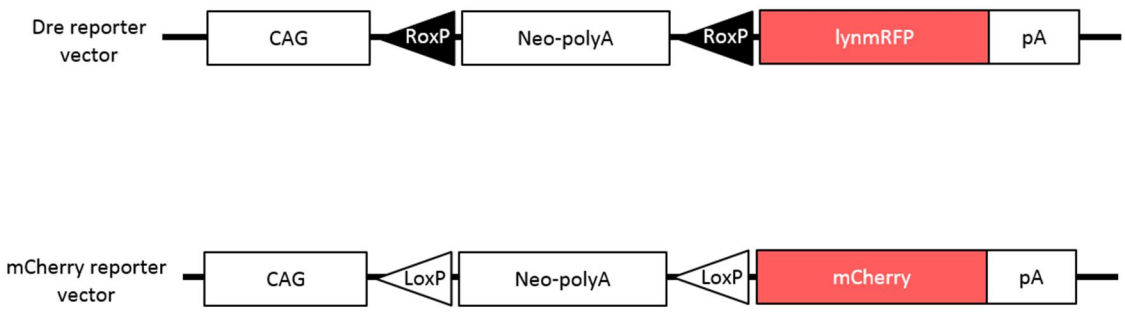
Sup. Fig. 1. *Sfrp5* lineage from E7.5 is differentiated into myocardium.
Double immunohistochemistry of E7.5TM-E16.5 *Sfrp5*-CreERT2/CAG-CAT-eGFP heart using anti-GFP (green) and anti-actinin (red). Lower panel is magnified picture of boxed area in upper panel. White arrowheads indicate GFP/actinin double positive cell.



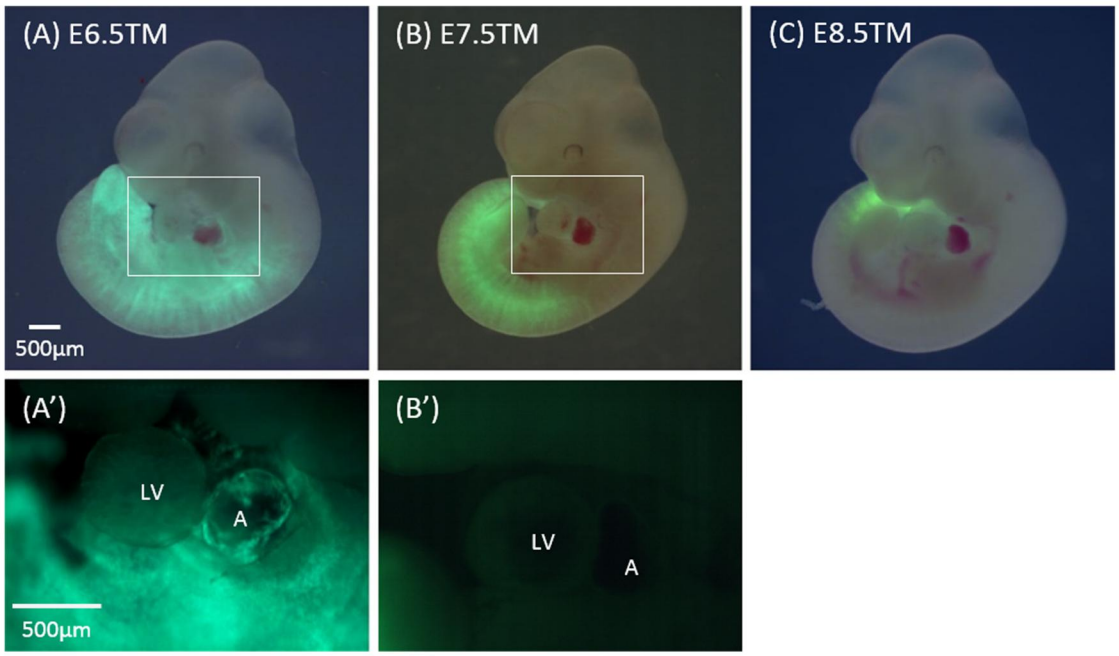
Sup. Fig. 2 Targeting strategy for generating *Sfrp5*-Cre, CreERT2 and Dre KI mouse. Schematic drawing of targeting strategy. Targeting vector was introduced in TT2 ES cells with Cas9-vector designed to cut a target sequence indicated by arrow. After establishing knock-in mice, Pgk-neo cassette was removed by crossing with CAG-Cre mouse to establish Dneo-knockin mice and used for lineage tracing analyses. *Sfrp5* sgRNA: 5'-GGCCAGGAGTACGACTACTA-3



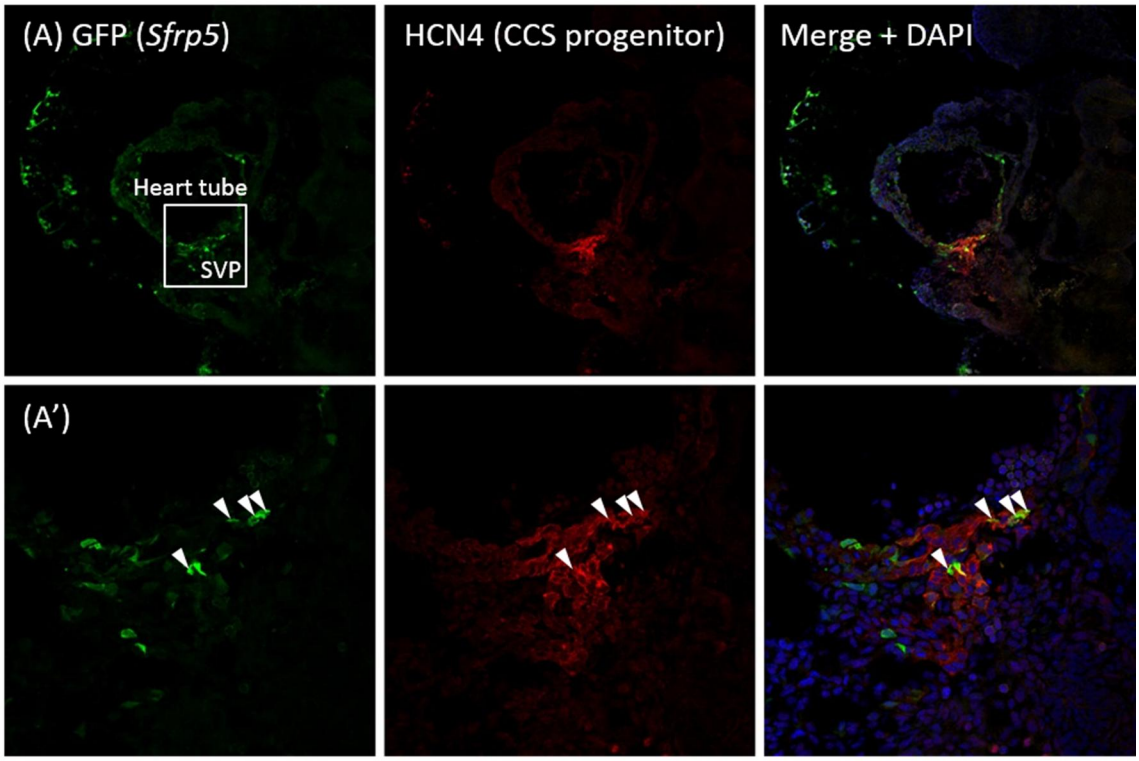
Sup. Fig. 3. Targeting strategy for *Mesp1*-Dre mouse.
Targeting vector was directly injected into nucleus of fertilized egg with Cas9 vectors designed to cut Mesp1 -5' and 3' sequences indicated by arrows.
Mesp1-5' sgRNA: 5'-GCTACAGCGGACCCAATGGTC-3'
Mesp1-3' sgRNA: 5'-GCCGATTGTGCTAGTGTCATT-3'



Sup. Fig. 4. DNA constructs used for generating CAG-Roxed *Neo*-lynmRFP and CAG-floxed CAT-mCherry transgenic mouse lines.
CAG-Roxed Neo-lynmRFP mouse line (Dre reporter) and CAG-Loxed CAT-mCherry mouse line (mCherry reporter) was established using transgenic techniques.



Sup. Fig5. The lineage contribution of *T* is gradually restricted in caudal part of embryo with embryogenesis.
mGFP patterns of E10.5 *T*-CreERT2/*Rosa26*$^{mTmG/+}$ embryos administrated TM at E6.5(A), E7.5 (B) and E8.5 (C). (A') and (B') are magnified picture focused boxed are of (A) and (B).
LV; left ventricle, A; atrium



Sup. Fig. 6 HCN4 is expressed in *Sfrp1/2/5* KO mouse.
Immunohistochemistry of *Sfrp1*$^{-/-}$*2*$^{-/-}$*5*$^{v/v}$ triple knockout mouse focused on the heart tube (A) at E9.5. It is stained with HCN4 (red, CCS progenitor marker) and GFP (green, *Sfrp* TKO cell) and boxed area of (A) is magnified in (A'). White arrowheads indicate GFP/HCN4 double positive cell.

antibody	dilution	company, catalog code	fixative	tissue preparation	secondary antibody
GFP	1:800	aves, GFP-1010	4% PFA	OCT-frozen	Alexa488
actinin	1:1200	SIGMA, A-7811	4% PFA	OCT-frozen	Alexa594, Alexa647
HCN4	1:800	alamone labs, APC-052	4% PFA	OCT-frozen	Alexa594, Alexa647
Cx40	1:500	Alpha Diagnostic, Cx40-A	4%PFA	OCT-frozen	Alexa594, Alexa647

Table1. Antibody conditions for immunohistochemistry.