学位論文　（要約）

Cardiac cell fate determination and regeneration by the transcription factor *Sall1*

（ 心臓細胞運命決定および心臓再生に関わる転写因子 *Sall1* の研究 ）

平成 27 年 12 月博士(理学)申請

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Contents

Abbreviations

- ChIP Chromatin immunoprecipitation
- CKO Conditional knockout
- DKO Double knockout
- DOX Doxycycline
- E Embryonic day
- EB Embryoid body
- EMT Epithelial-mesenchymal transition
- ESC Embryonic stem cell
- FHF First heart field
- GFP Green fluorescent protein
- iPSC Induced pluripotent stem cell
- KO Knockout
- LA Left atrium
- LV Left ventricle
- NC Neural crest
- OFT Outflow tract
- PEO Proepicardial organ
- PS Primitive streak
- RA Right atrium

- RFP Red fluorescent protein
- RV Right ventricle
- *Sall1* *Spalt-like transcription factor 1*
- *Sall4* *Spalt-like transcription factor 4*
- SHF Second heart field
- SMA Smooth muscle actin
- YFP Yellow fluorescent protein

Abstract

The mammalian heart primarily consists of the anatomically and molecularly distinct first heart field (FHF) and second heart field (SHF). The FHF gives rise to the left ventricle and part of the atria, whereas the SHF gives rise to the outflow tract, the right ventricle and part of the atria. These cardiac fields consist of numerous types of cardiac progenitor cells (CPCs), which are derived from common mesoderm precursors. Previous studies have demonstrated that the transcription factor *Mesp1* precedes the establishment of the heart fields. However, $Mesp1^{+}$ cells also contribute to a broad range of derivatives, including the developing heart. Thus, little is known about the specification of mesoderm cells into cardiac cell lineages. Here, I demonstrate that *Spalt-like 1* (*Sall1*), a zinc finger transcription factor, is expressed shortly after gastrulation at the pre-crescent stage. *Sall1* is expressed prior to Isl1 and Nkx2.5 and restricted to CPCs in the distal mesoderm region before they enter the developing heart and differentiate into cardiac cells. In addition, I identified Sall1 binding sites in the regulatory regions of *Isl1, Nkx2-5* and *c-kit*, which are all transcriptionally active in $Sall1^{+}$ cells. Based on a lineage-tracing analysis in $Sall1^{CreERT2/+}$ mice, I demonstrate that cells that previously expressed *Sall1* substantially contribute to the embryonic heart; they comprise the majority of cells in the outflow tract (OFT), the right ventricle (RV), the atrium and the left ventricle (LV) upon tamoxifen treatment at E5.5 to E7.5 and become restricted to the OFT, the RV and the atrium upon tamoxifen treatment at E8.5 to E9.5. In differentiating embryonic stem cells (ESCs), the overexpression of

Sall1 promoted the differentiation of cardiomyocytes, but the depletion of *Sall1* inhibited cardiogenesis, which was accompanied by the up-regulation of non-cardiac lineage markers. Importantly, the promotion of cardiogenesis by *Sall1* was highly conserved in human induced pluripotent stem cells (iPSCs). To identify the partner of *Sall1*, I focused on the major mesoderm marker *Mesp1* because Sall1 expression was similar to Mesp1 lineage expression in $Sall1^{GFP/+};Mesp1^{Cre/+};ROSA26^{RFP/+}$ mouse embryos at E7.0. To assess the role of Sall1 in the cardiac lineage derived from non-*Mesp1* expressing cells, GFP^+ cells isolated by flow cytometry from E6.5 to 8.0 $Sall1^{GFP/+};Mesp1^{Cre/+};ROSA26^{RFP/+}$ mouse hearts were cultured for 5 days. As a result, $GFP^+;RFP^-$ cells differentiated into cardiomyocytes. Interestingly, *Sall1;Mesp1* double knockout (DKO) mice failed to form $Isl1^-$ and $Nkx2.5^+$ cardiac fields. In fact, the cooperative and highly conserved function between *Sall1* and *Mesp1* promotes the differentiation of beating cardiomyocytes in human iPSCs. These results indicated that *Sall1* specified *Mesp1* function during cardiogenesis.

During the post-natal stages, $Sall1^+$ cells were absent in the heart, except for endothelial cells. However, Sall1 expression was immediately induced around a resected area during heart regeneration. Greater than 80% of GFP^+ cells were positive for Thy1, a major immature cardiac fibroblast marker. A genetic fate mapping study revealed that *Sall1*-expressing progenitors differentiated into cardiomyocytes *in vivo* during heart regeneration. These lines of evidence indicate that *Sall1* plays a novel role in the plasticity of differentiating cells, and this gene positively promotes cardiogenesis during heart development and regeneration.

Because I identified a new cardiac cell population defined by *Sall1*, my doctorial studies shed light on the mechanisms that determine cardiovascular cell fate and suggest a common phenomenon by which cardiomyocytes regulate heart development and regeneration.

General Introduction

Embryonic stem cells are pluripotent stem cells derived from the inner mass of embryos, which give rise to specialized cells (eg. cardiac muscle cells, skeletal muscle cells, blood cells, neurons and liver cells) during development (Gilbert S., Developmental Biology 2010). Embryonic stem cells are able to self-renew, and to differentiate into all derivatives of the three germ layers: ectoderm, endoderm and mesoderm in a stepwise manner. Figure 0 illustrates how mesodermal precursor cells are determined as one cell type of three-germ layers, and are committed to the mesodermal linage (eg. heart cells, blood cells and bones). After linage commitment, a precursor cell or progenitor cell differentiates into a certain of specialized cell (differentiation). Each specialized cell has a different morphology and function to coordinate its organ structure.

Progenitor cells or precursor cells are multipotent and retain their immature characteristics before differentiating into specialized cells. I am very interested in progenitor/precursor cell biology, and how these cells are able to maintain their characteristics before they differentiate into specialized cells, and how they share the same potential to differentiate into terminal cells even if they are derived from the distinct origin. Cardiac progenitor cell (CPC) biology is a great model to investigate cell-fate determination and organogenesis during heart development. CPCs are located in the restricted area adjacent to the developing heart, and contribute to the developing

heart by differentiating into atria muscle, ventricular muscle, pacemaker cell, endothelial cell, smooth muscle (Sahara et al., EMBO J., 2015). Although the several key genes/factors expressed in CPC have been identified and described, the switch between maintenance and differentiation remains unclear.

Thus, I would like to address the “switch” mechanism of CPCs during differentiation to cardiac cells from CPC by identifying a key molecule in this thesis.

Materials and Methods

Mice

Sall1$^{GFP/+}$, *Sall1*$^{CreERT2/+}$, *ROSA-YFP, ROSA-RFP, Sall1*$^{Flox/+}$, *Sall4*$^{Flox/+}$, *Mesp1*$^{Cre/+}$, *Isl1*$^{Cre/+}$ mouse lines were generated as previously described (Inoue et al., 2010; Takasato et al., 2004; Srinivas et al., 2001; Luche et al., 2007; Yuri et al., 2009; Saga et al., 1999; Sakaki-Yumoto et al., 2006). All mouse experiments were completed according to a protocol reviewed and approved by the international Animal Care and Use Committee of the IMCB at the University of Tokyo.

Lineage trace analysis

To induce Cre activity in *Sall1*$^{CreERT2/+}$ mice depending on the embryonic stage, pregnant mice were fed 150 μl/30 g of tamoxifen (10 mg/ml: Sigma) by injection into the abdominal cavity at the desired time points (Sun et al., 2007).

Whole embryo culture

Mouse embryos were sacrificed at E7.0 and washed with phosphate-buffered saline (PBS). The embryos were then cultured in whole embryo culture medium (DMEM supplemented with 45% rat serum, 25% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol (Gibco), 2 mM L-glutamine (Gibco), and 1 mM sodium pyruvate (Gibco) at 37°C in a CO_2 incubator with rotation for 24 h.

Immunohistochemistry (IHC)

The embryos were fixed in 4% paraformaldehyde (PFA) overnight and then washed thrice in PBS and then, replaced with 10% sucrose, 30% sucrose, and a 30% sucrose:OCT=1:1 solution. The embryos were then embedded in OCT, sectioned and stained using standard protocols as previously described (Takeuchi and Bruneau, 2009; Takeuchi *et al*., 2011). The hearts of postnatal mice were cut in half and fixed in 4% PFA 2-overnight, washed thrice with PBS, and incubated in 30% sucrose for 2-overnight following the same protocol used to fix the embryos.

The following primary antibodies were used: mouse a-Sall1 (1:100; PPMX), rabbit a-Isl1 (1:200; Abcam), mouse a-Isl1 (1:100; Hybridoma Bank), goat a-Nkx2-5 (1:2000; Santa Cruz Biotechnology), rabbit a-GFP (1:400; MBL), chick a-GFP (1:400; Life Technologies), rat a-CD31 (1:100; BD Biosciences), mouse a-cTnT (1:10000; Thermo Fisher Scientific), rabbit a-HCN4 (1:2000; Alomone Lab), rabbit a-RFP (1:400; ROCKLAND), rabbit a-TNI (1:400; Abcam), mouse a-Mlc2a (1:400; Synaptic Systems), and mouse a-SSEA4 (1:200; Santa Cruz Biotechnology). Alexa Fluor secondary antibodies (Life Technologies) were used for secondary detection, and images were acquired using a KEYENCE BZ-9000 fluorescence microscope. The staining protocol for whole-mount IHC was the same as that used for IHC described above.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted with Sepasol (Nacalai Tesque, Inc.). Reverse transcription was

performed using a ReverTra Ace qPCR RT Master Mix (Toyobo Life Sciences). Real-time PCR was performed using a SYBR Green Master Mix from Takara.

Flow cytometry

The region containing the SHF was carefully dissected from mouse embryos at E9.0 to E9.5 and dissociated with collagenase (Sigma Aldrich). Embryonic stem cells (ESCs)/iPSCs were dissociated using 0.1% trypsin or Accumax (Funakoshi). The cells were re-suspended in 0.1%FBS/D-PBS (-) without Ca^{2+} and Mg^{2+} and sorted using a FACSAriaIII (BD Biosciences). To assess the expression of cTnT, Mlc2v and TNI, the cells were incubated with anti-cTnT antibodies (Thermo Fisher Scientific), anti-Mlc2v antibodies (Synaptic Systems) and an anti-TNI antibody (Abcam), followed by incubation with secondary antibodies conjugated with Alexa Fluor 647 (Invitrogen). CD31/49a/140b-PE was previously described by Dubois et al., 2011. CD90 was used to identify cardiac fibroblasts as $Thy1^{+}$ cells during heart regeneration (BD Biosciences).

ESC induction and differentiation

Sall1$^{GFP/+}$ ESCs were derived from *Sall1*$^{GFP/+}$ mice (Takasato et al., 2004). *Sall1*$^{GFP/+}$ ESCs were maintained in Knockout Dulbecco's Modified Eagle Medium (Knockout DMEM; Gibco) supplemented with 15% Knockout Serum Replacement (KSR; Gibco), penicillin-streptomycin (Nacalai Tesque), MEM-NEAA (Gibco), GlutaMAX (Gibco), sodium pyruvate (Gibco), 2-mercaptoethanol (Gibco), and LIF (WAKO) in MEF. To

differentiate Sall1-GFP ESCs, embryoid bodies (EBs) were generated with DMEM (KOHJIN BIO) containing 20% FBS (Gibco), 2.4 mM L-glutamine (Gibco), MEM NEAA (Gibco), 2-mercaptoethanol (Gibco), and LIF (WAKO). After 2 days, the EBs were cultured in DMEM (KOHJIN BIO) containing 20% FBS (Gibco), 2.4 mM L-glutamine (Gibco), and 2-mercaptoethanol (Gibco). The medium was changed every two days (Cheng *et al*., 2013). As a control, Ring1Bflox/flox ES cells and Six2 ES cells were gifted from H. Koseki (Research Center for Allergy and Immunology, RIKEN, JAPAN) as well as A. Kobayashi and A. McMahon (Harvard Stem Cell Institute, USA) (Endoh *et al*., 2008; Kobayashi *et al*., 2008).

Primary culture

Cardiac progenitors isolated from mouse embryos (E8.5-10.0) and cardiac cells from postnatal mice hearts (P0-7) were maintained in F12 (Gibco) containing 5% Knockout Serum Replacement (KSR), 0.1 mM 2-mercaptoethanol (Gibco), 1% MEM-NEAA (Gibco) and 10 ng/ml rhEGF (R&D) at 37°C. After 24 h, these cells were cultured in the primary culture medium (GMEM; Gibco) supplemented with 0.1 mM 2-mercaptoethanol (Gibco), 10% FBS, and penicillin-streptomycin (Nacalai tesque). The medium was changed every 2 days.

***In vivo* ChIP assay**

SHF cells from *Sall1*$^{GFP/+}$ mice were processed for chromatin immunoprecipitation assays using antibodies against a-trimethylated H3K27 (Cell Signaling) and a-acetylated

H3K27 (Abcam), as previously described (Endoh et al., 2008; Tsuchiya et al., 2011). Immunoprecipitated DNA was amplified using the following primer pairs:

Isl1 3.2F 5-CCAATCTAGTGAGCAGGCAAA-3 and

Isl13.2R 5-TCAAGTTTCAGGAGGAACCAAG-3;

Isl1 3.1F 5-TCAGTGGGCACTGGCTCAA-3 and

Isl1 3.1R 5-GCTAGCAGTGGATAAAGGGCATC-3;

C-kit 5-1F 5-ATTCACTCAACCACGCCAGA-3 and

C-kit 5-1R 5-CATCAGGGTTTGCTTTGTTCC-3;

C-kit 5-2F 5-TGTTCTCTGACACCCACATGAA-3 and

C-kit 5-2R 5-GGGTTCTTTAAGTCCCAAGATTGTA-3;

Nkx2-5 5-1F 5-ATATACTTTTCGCGGCTGATTG-3 and

Nkx2-5 5-1R 5-CTGTGGAGTGATTTCTTTGCCTTT-3; and

Nkx2-5 5-2F 5-GTCAACTCTGGAAGCCCTTATATCC-3 and

Nkx2-5 5-2R 5-CGCGATGACAGTTTGAAAGATTAG-3.

Knock-down/overexpression assay

Sall1 siRNA (MSS226691 Life Technologies) was used for the knockdown studies at a concentration of 100 nM. Differentiating ESCs were transfected using Lipofectamine RNAiMAX (Invitrogen) and re-plated for continuous analysis. The pL-sin-EF1a-mSall1-IRES-puromycin plasmid was generated by A. Hotta (CiRA, Kyoto University) (Hotta et al., 2009). Differentiating ESCs were transduced using Sall1 Lenti virus.

DOX-inducible hiPSC generation, maintenance, and differentiation

The human iPSC line 201B7 was previously established (Takahashi et al., 2007). To generate cells that overexpress Sall1 in a time-dependent manner, a DOX-inducible Sall1 expressing piggyback vector and a PB-EF1a-mSall1-IRES-mcherry vector were co-electroporated into 201B7 cells with the piggyback transposase vector PBASE2 with NEPA21 (NEPA GENE)(Kim et al., 2015). DOX-SALL1 hiPSCs were maintained in KSR medium (Invitrogen) with 6 ng/ml of human bFGF (Peprotech). EBs were generated in low-attachment 96-well plates using Stempro34 medium (Invitrogen) with 1% L-glutamine, 150 μg/ml transferrin, 50 μg/ml L-ascorbic acid, $4X10^{-4}$ M MTG, 10 μM Y27632, 2 ng/ml BMP4, and 0.5% Matrigel in 5% O_2. The next day, medium containing 1% L-glutamine, 150 μg/ml transferrin, 50 μg/ml L-ascorbic acid, $4X10^{-4}$ M MTG, 18 ng/ml BMP4, 10 ng/ml bFGF, and 12 ng/ml activin A was added to the StemPro34 medium in 5% O_2 for mesoderm induction. After 2 days, the EBs were transferred to a low-attachment 24-well plate, and the medium changed to Stempro34 medium containing 1% L-glutamine, 150 μg/ml transferrin, 50 μg/ml L-ascorbic acid, $4X10^{-4}$ M MTG, 10 ng/ml VEGF, and 150 ng/ml DKK1 in 5% O_2. After 4 days, the medium was exchanged for StemPro34 medium containing 1% L-glutamine, 150 μg/ml transferrin, 50 μg/ml L-ascorbic acid, $4X10^{-4}$ M MTG, 10 ng/ml VEGF, and 5 ng/ml bFGF in 5% O_2, and the medium was exchanged daily thereafter. After 5 days, the EBs were transferred to 5% CO_2, and the medium was exchanged every two days (Miki et al., 2015; Uosaki et al., 2013; Yang et al., 2008).

Field potential analysis

Field potential analyses for differentiated human iPSCs were performed using MEA2100-systems from Multi Channel systems equipped with an MEA dish (MSD BIOMEDICAL). EBs that had differentiated for 10 to 20 days were individually measured for 1 to 10 min to ensure a critical analysis.

Chapter3 に記載のため非公開

Chapter Ⅰ

Analysis of *Sall1* during heart development

Introduction

The heart is the first organ to form in the circulatory system, and it exchanges nutrients and oxygen. Heart morphogenesis (circulatory system) is vastly divergent among species, but the "heart" plays common/pivotal roles in life. In mammals, the heart consists of two major cardiac progenitor cell lineages: the first heart field (FHF) and the second heart field (SHF). These fields are thought to derive from a distinct origin: cardiac progenitors. Although numerous genes have been identified at the CPC stage, CPC markers have not been identified in any of the stages of heart development. In addition, defined factors have not been identified to specify cardiac lineages. Therefore, the regulation of cardiac lineage commitment from mesoderm cells remains unclear. As a starting point of this thesis, I will introduce 1) the origin of the heart in the mouse, 2) differences in the markers and signaling pathways between the FHF and the SHF, and 3) the history of the candidate "Spalt family" gene in my research.

1) The origin of the heart in mammals

The *tinman* gene is an Nk-homeobox gene that specifies and differentiates cardiac cells in *Drosophila* (Bodmer, 1993). Its name is derived from a *Wizard of Oz* reference because *tinman* mutants do not have a heart. The *tinman* gene is highly conserved in vertebrates. *CSX/Nkx2-5* was identified in mice (Komuro and Izumo, 1993), and *xNkx2-5* was identified in *Xenopus* (Tonissen et al., 1994). In *Drosophila*, tinman acts

as a master regulator of cardiac muscle development, and *Nkx2-5* compound knockout (KO) mice exhibit severe cardiac malformations. However, cardiomyocytes continued to develop in the KO mice. This result indicates that *Nkx2-5* plays as a key role in heart development, but the cardiogenesis of mammals includes more complex processes in terms of gene regulatory networks and morphological changes compared to *Drosophila*. In mammals, the heart is a complex and unique organ that dynamically changes its shape during development. As described in the introduction, the heart consists of two separate progenitor cell lineages: the FHF and the SHF. Differentiated cardiomyocytes are first observed in the FHF at E7.5 in mice. The FHF exhibits a crescent shape, and the E7.5 stage is consequently also known as the cardiac crescent stage. As development proceeds, the cardiac crescent fuses at the midline to form a linear heart tube at E8.0 and initiates circulation. Finally, FHF cells contribute to mainly the left ventricle (LV) and both atria (right atrium (RA) and left atrium (LA)) and differentiate into ventricular cardiomyocytes, atrial cardiomyocytes, pacemaker cells, and smooth muscle cells. The SHF was identified by Cai et al. in 2003; it is defined by the LIM/homeodomain factor *Islet1 (Isl1)* at E7.5 and adjacent to the FHF. As the linear heart tube forms, the SHF moves dorsally at E8.0, spreading both anteriorly and posteriorly. CPCs are mainly located in the SHF, and these cells migrate from the outflow tract (OFT) and the inflow tract (IFT) and mainly contribute to the RV, the RA and the LV, where they differentiate into ventricular cardiomyocytes, atrial cardiomyocytes, pacemaker cells, smooth muscle cells, and endothelial cells during later stages.

2) Differences in the markers and signaling pathways in the FHF and SHF

The FHF and the SHF differ in terms of differentiation timing, the components of cardiac progenitors, the regulatory networks of transcription factors and signaling pathways. Whereas FHF progenitors already differentiate into cardiac cells at E7.5, SHF progenitors remain in a proliferative and undifferentiated state until E9.5 to 10.0. Given the position of the FHF, its progenitor cells are exposed to FGF and BMP signaling, which regulate cell proliferation and differentiation and inhibit canonical Wnt/b-catenin signaling (Marvin et al., 2001; Tzahor and Lassar., 2001; Noseda et al., 2011). These signals induce the expression of key transcription factors, such as *Nkx2-5, Tbx5, Gata4* and the *myosin light chain-2a (Mlc2a)* cardiac contractility gene in the crescent at E7.5. *Hand1/2* and *Mef2c* are also expressed by differentiated cardiac cells in the crescent. *Gata4, Tbx5*, and the chromatin-remodeling factor *Smarcd3/Baf60c* are master regulators that specify cardiac lineage differentiation from mesoderm cells (Takeuchi and Bruneau, 2009).

Although key factors that specify FHF progenitors have not been identified, SHF progenitors are defined by the LIM-homeodomain transcription factor 1 *Islet1* (*Isl1)*. Progenitors in the SHF are time- and dose-dependently controlled by several secreted molecules. Wnt signaling from the neural tube and Shh signaling from the endoderm are important for CPCs to inhibit differentiation and maintain proliferation. FGF signaling within the SHF promotes the proliferation of CPCs (Zaffran and Kelly, 2012), whereas BMP signaling, Notch signaling and non-canonical Wnt signaling from the lateral plate mesoderm promote cardiac differentiation (Vincent and Buckingham, 2010). Retinoic

acid signaling is also important for patterning and limiting the posterior boundary of the SHF (Ryckebush et al; 2008; Sirbu et al., 2008). Thus, the SHF is the target of numerous signals.

Additionally, *Fgf10* (Kelly et al., 2001), *Isl1* (Cai et al., 2003), *Tbx1* (Xu et al., 2004), *Fgf8* (Ilagan et al., 2006), *Hox* genes (Bertrand et al., 2011), *Six1* and *Eya1* (Guo et al., 2011) are expressed in the pharyngeal mesoderm of the SHF and have been identified as functional genes. Although a number of transcription factors and signaling pathways have been identified in the FHF and the SHF, a common/key factor that regulates CPCs and cardiac specification from mesoderm cells has not been identified. Thus, I attempted to identify this functional factor and focused on the mechanism that regulates cardiac cell fate in mesoderm cells.

3) The history of the candidate gene "Spalt family"

The spalt family, which was originally identified in *Drosophila*, consists of *spalt major* (*salm*) and *spalt related* (*salr*). These genes play important roles in photoreceptor specification (Domingos et al., 2004), tracheal system development (Kuhnlein and Schuh, 1996), sensory organ development (de Celis et al., 1999), wing patterning (de Celis and Barrio, 2000) and fibrillar flight muscle fate (Schonbauer et al., 2011). In vertebrates, homologs of *spalt* have are also involved in the formation of the kidney, limbs and several other organs, including the nervous system and the heart, and *spalt* gene mutations are associated with congenital diseases.

Specifically, *SALL1* mutations in humans cause Townes Brocks Syndrome (TBS),

which is characterized by triphalangeal thumbs, external ear defects, sensorineural hearing loss, kidney defects, an imperforate anus and heart defects, including ventricular septal defects. Furthermore, mutations in *SALL4* have been associated with Okihiro syndrome, which is characterized by limb malformations and eye retraction (Kohlhase., 2000; Kohlhase et al., 1998, Kohlhase et al., 2002a; Kohlhase et al., 2002b). Although mutations in or the KO of *Spalt* genes results in defects in several organs, such as the limbs, eyes, kidney and heart, *Spalt* genes exhibit highly conserved functions and are expressed in tissue-specific progenitors to control organ development. In fact, Spalt proteins physically interact with each other or other transcription factors to specifically facilitate organ development (Nishinakamura et al., 2001; Kohlhase, 2002a; Kohlhase *et al*., 2002b; Koshiba-Takeuchi et al., 2006; Sakai-Yumoto et al., 2006). Moreover, recent studies reported that *Spalt-like 1 (Sall1)* acts as a key player in the differentiation and maintenance of nephrons, cortical progenitors and ESCs (Basta et al., 2014; Kanda *et al., 2*014, Harrison et al., 2012; Karantzali et al., 2011). In addition, *spalt* gene mutations in *Drosophila* result in phenotypes similar to that of TBS in humans, which is caused by *SALL1* mutations but not *SALL4* mutations or mutations in other *SALL* genes (Dong et al., 2003; Kohlhase et al., 1999; Kelberman et al., 2014). Thus, the functions of *Spalt* family genes, especially the function of *Sall1*, may be highly conserved from *Drosophila* to humans and are key to understand the maintenance and CPC differentiation into cardiac cells. These genes may also serve as novel markers of CPCs or plastic cells.

Results

Sall1 and Isl1 expression overlap during early cardiac development

To identify a common/key factor among cardiovascular lineages, I first performed a microarray analysis of YFP^{+};$Flk1^{+}$ cells and YFP^{-};$Flk1^{+}$ cells in E9.0 embryos from $Isl1^{Cre/+}$;$R26R^{YFP/+}$ mice (Figure 3) and identified 15 candidate genes. To assess the role of *Sall1* during heart development, I then analyzed the relative expression of candidate genes in a differentiating mESC system (Figure 4). In this system, the major mesoderm markers *Mesp1* and *Flk1* are expressed starting on day 3. One of the major CPC genes, *Isl1,* is transiently expressed starting 4.5 to 6.0 days before the expression of *Tbx5* and *Nkx2-5,* which are cardiac transcriptional factors, or *Actc1*, which is a marker of cardiomyogenesis. Interestingly, *Sall1* and *Sall4* are transiently expressed prior to *Isl1*. Thus, *Sall1* and *Sall4* might be strong candidate genes for cardiovascular specification. To assess endogenous expression pattern in mouse development, I then performed whole-mount immunostaining with anti-Sall1, Nkx2.5 and Isl1 antibodies. Sall1 expression was initially observed as early as embryonic day 7 (E7.0) at the pre-crescent stage in the mesodermal region (data not shown). At the crescent stage (E7.5), the Sall1 expression domain did not overlap with that of Nkx2-5, which is a major marker of the FHF during the early developmental stages (Figures 5A-C and A'-C'). Similarly, Sall1 expression did not overlap with Nkx2-5 expression at E8.5 (Figure 5D and D'). By contrast, the Sall1 expression domain overlaps with Isl1, which is a major SHF marker

at this stage (Figure 6: TOP). A histological analysis of serial sections revealed an overlapping expression pattern between Sall1 and Isl1 (Figure 6: BOTTOM). At E8.5, the Sall1 expression domain was continuous in the developing outflow tract (OFT) and overlapped with Isl1 and Nkx2.5 expression in the OFT (Sall1^{+}; Islet1^{+}; Nkx2-5^{+}) but was absent in the myocardial region where Nkx2.5 was expressed (Sall1^{-}; Islet1^{-}; Nkx2-5^{+}). In the mesodermal region adjacent to the OFT, Isl1 partially overlapped with the Sall1 domain (Sall1^{+}; Islet1^{+}; Nkx2-5^{-}), marking Isl1^{+} CPCs in the SHF. However, Sall1 expression extended beyond the Isl1 expression domain to the distal mesodermal region (Sall1^{+}; Islet1^{-}; Nkx2-5^{-}) (Figure 7). These data suggest that Sall1 is expressed prior to Isl1 in developing CPCs and that CPC development is a stage-specific process defined by the expression of cardiac transcriptional factors as previously described (Shenje, Andersen *et al*, eLife, 2014) (Figure 7).

Sall4 was previously detected in the ventricular myocardium at E9.5 to 10.5 (Koshiba-Takeuchi et al., 2006), Thus, I analyzed other members of the Sall-like family and examined their involvement in early cardiogenesis. However, *Sall2* and *Sall3* were not expressed in any region of the heart at E7.5 to 8.5 (data not shown). However, *in situ* hybridization experiments revealed overlapping *Sall1* and *Sall4* expression in the precardiac mesoderm region, suggesting a possible redundant or synergistic function between Sall1 and Sall4 (data not shown). To examine the roles of *Sall1* and *Sall4*, I generated *Sall1* and *Sall4* DKO mice (*Sall1/4* DKO) by crossing *Sall1*$^{flox/flox}$;*Sall4*$^{flox/flox}$ mice with *Sall1*$^{flox/+}$;*Sall4*$^{flox/+}$;*Mesp1*$^{Cre/+}$ mice (Sakaki-Yumoto et al., 2006; Saga et al., 1999). *Sall1* heterozygotes, *Sall4* KO and *Sall1* KO/*Sall4* heterozygotes exhibited mild

defects in the OFT and RV during heart formation, whereas heart formation was significantly abnormal in *Sall1/4* DKO embryos, as evidenced by the lack of an outflow tract (OFT) structure at E8.5 to 9.5 (Figure 8). The phenotype was similar to that of *Isl1* KO embryos, suggesting that *Sall*, similar to *Isl1*, functions in SHF formation during cardiogenesis in mice. Mild defects in the SHF, OFT and IFT were detected in *Sall1* compound KO mice, and immunofluorescence staining for Nkx2-5 revealed decreased Nkx2-5 expression in some Sall1 KO mice. To examine the abnormalities in *Sall1/Sall4* mutants, I then counted the number of CPCs and cardiac cells in the OFT of each KO mouse (Figure 9). Figure 9 shows that $Isl1^{+}$ or $Isl1^{+}/Nkx2\text{-}5^{+}$ CPC cells were significantly decreased in KO mice, indicating that *Sall1* and *4* synergistically regulate CPC development and proliferation. In addition, ventricular formation in *Sall1/4 KO* mice was also severely abnormal, and the ventricle was smaller than that of normal wild-type hearts (Figures 8A, A', D and D'; Figure 9A). These data suggest that *Sall1* and *Sall4* are essential for proper heart formation during the early stages of development.

Sall1 is expressed in cardiac progenitors during early cardiogenesis

To determine the role of Sall1 during cardiogenesis, I first examined the characteristics and lineage of Sall1+ cells *in vitro* and *in vivo*. Sall1+ and Sall1- cells were isolated from $Sall1^{GFP/+}$ knock-in mice (Takasato et al., 2004) by dissecting the distal mesodermal region (SHF) of E9.5 embryos, followed by FACS. The expression of CPC factors was then analyzed (Figure 10A, B). Several CPC genes, such as *Isl1*,

Six1/2, *Foxa2/c2, Nkx2-5* and *Sall1*, were all up-regulated in GFP^+ cells, whereas the expression of cardiac contractile genes (*Tnnt2*, *Tnni3*, *Nppa, Nppb, Myl7*, *Myh6,* and *Acta2*) was significantly reduced compared with that in GFP^- cells (Figure 10C). This finding demonstrates that isolated $Sall1^+$ cells mark CPCs but not differentiating cardiomyocytes .

To determine the cardiogenic potential of $Sall1^+$-expressing cells, I cultured GFP^+ cells for 2-5 days after sorting and performed IHC using the cTnT antibody (Figure 11A). After 5 days in culture, compared with after 2 days in culture, I observed $cTnT^+$ cardiomyocytes in GFP^+ cells (Figure 11A) accompanied by increased contractile gene expression (*Tnnt2*, *Tnni3*, and *Myl7*) (Figure 11B). Conversely, the expressions of CPC genes (*Isl1*, *Flk1*, *c-kit* and *Tbx1*) were down-regulated in 5 $Sall1^+$ cells after 5 days in culture compared with cells cultured for 2 days, indicating that Sall1/GFP^+-expressing cells ultimately differentiate into cardiac cells (Figure 11B). In addition, the expression of non-cardiac lineage marker genes (*Pax2, Tal1, MyoD, NeuroD, Hnf4a* and *Pdx1*) did not differ between cells cultured for 2 and 5 days in this experiment (Figure 11C). Thus, E9.5-derived $Sall1^+$ cells from the anterior mesodermal region are immature CPCs (Sall1+ CPCs), and these cells have the potential to differentiate into cardiac cells.

Sall1 progeny give rise to distinct anatomical structures within the heart

To further investigate the role of $Sall1^+$ CPCs during heart development *in vivo*, I performed lineage-tracing experiments by crossing $Sall1^{CreERT2/+}$ to $R26R^{YFP/+}$ lineage reporter mice (Inoue et al.; 2011; Srinvas et al.; 2001) (Figure 12). Although GFP^+ cells

from *Sall1*$^{GFP/+}$ mice were specifically observed in the SHF and the OFT at E10.5 in *Sall1*$^{GFP/+}$ mice, the lineage tracing of Sall1^{+}-expressing cells identified YFP^{+} cells in the whole heart of *Sall1*$^{CreERT2/+}$*;R26R*$^{YFP/+}$ mice upon tamoxifen injection at E7.0 and E8.0 (Figure 13A and B). To analyze the time-limited migration of Sall1-derived cells, tamoxifen was then injected at several stages to induce Cre activity (E5.5-E9.5), and YFP expression was analyzed in the hearts at E10.5 (Figure 14A). Cre activation prior to gastrulation (E5.5) resulted in the widespread distribution of YFP^{+} cells throughout the heart. However, when Cre activity was induced during the crescent stage (E7.5), the number of YFP^{+} cells was reduced in the LV compared with that in the OFT and the RV. Upon Cre activation after E8.5, Sall1-expressing cells were primarily restricted to the OFT (Figure 14B). These observations demonstrate that Sall1-expressing cells differentiate into cardiac cells and that the migration of Sall1+ CPCs is gradually restricted as development proceeds. This lineage analysis result (Figure 14B) suggests that Sall1-expressing cells already function as CPCs during the cardiac crescent stage prior to the expression of Isl1^{+} in CPCs of the SHF. Therefore, I generated *Isl1*$^{cre/+}$*;R26R*$^{YFP/+}$ mice for comparison with Sall1-expressing cells. Tamoxifen injection resulted in YFP^{+} cells during the early stages (E7.0/8.0) throughout the hearts of *Sall1*$^{CreERT2/+}$*;R26R*$^{YFP/+}$ mice (Figure 15, right). Conversely, Isl1-expressing cells were specifically observed in the OFT, the RV and both atria derived from the SHF (Figure 15 left). In *Sall1*$^{CreERT2/+}$*;R26R*$^{YFP/+}$ mice injected with tamoxifen at E7.0/8.0, a histological analysis of hearts revealed YFP^{+} cells in atrial and ventricular cardiomyocytes (Figure 16B-1, B-1-1, B-1-2), endothelial cells (Figure 16B-2, B-2-1),

and pacemaker cells in the sinoatrial node (SAN) (Figure 16B-3, B-3-1), indicating that Sall1 is also expressed in progenitors from the proepicardium. Cre induction at E9.0 resulted in a pronounced reduction of YFP^+ cells within the left ventricle (Figure 14), with YFP^+ cells primarily restricted to the OFT, RV and atria. Taken together, these results demonstrate that Sall1-expressing cells contribute to heart cells and that Sall1-expressing cells already function as CPCs for cardiogenesis during gastrulation.

Sall1 regulates Isl1 and Nkx2.5 expression and promotes cardiogenesis in mESCs

To examine whether Sall1 plays an instructive role and promotes cardiogenesis, I then used *Sall1*$^{GFP/+}$ knock-in embryonic stem cells (ESCs) (Takasato et al., 2004) (Figure 17A) and isolated GFP^+ cells with an ESC differentiation system that recapitulates early cardiogenesis (according to a modified protocol described in Shenje et al., 2014). After mesoderm induction, I isolated GFP^+/GFP^- cells by FACS based on the strongest expression of GFP in differentiating mESCs (Figure 17B). The cells were then re-plated and cultured for an additional 8 days to allow differentiation (Figure 17A). Compared with GFP^- cells, the relative gene expression of isolated GFP^+ cells revealed enrichment for *PDGFRa* and *Flk1* (these genes are major progenitors in several organs during development), as well as for *Mesp1* (Figure 17C), indicating that *Sall1* marks a previously reported population of multipotent CPCs in the ES system (Kattman et al.,2006; David et al., 2008; Bondue et al., 2008; Lindsley et al., 2008; Takahashi et al., 2007; Kim et al., 2015). *Isl1* and *Gata4* were likewise enriched, together with the forkhead transcription factors *Foxa2*, *Foxc2* and *Six2*, which are involved in regulating

nephron progenitor renewal (Kobayashi et al., 2008; Park, 2012) (Figure 17C), but these genes are in also CPCs during development (data not shown). After 8 additional days of culture (Figure 18A), GFP^+ cells efficiently differentiated into $cTnT^+$ cardiomyocytes compared with GFP^- cells (Figure 18B), and beating colonies efficiently developed from GFP^+ EBCs (Figure 18C). Furthermore, the expression of contractile genes (*Tnnt2* and *Mlc2a*) was significantly increased compared with that of differentiated GFP^- cells (Figure 18D). By contrast, the expression of markers of non-cardiac lineages (*NeuroD*, *MyoD* and *Pdx1)* decreased in GFP^+ cells compared with that in GFP^- cells (Figure 18D), indicating that Sall1 regulates the expression of CPC-genes during early cardiogenesis.

To assess whether Sall1 directly stimulates the expression of known CPC genes, I analyzed known CPC genes for Sall1-binding sites (ATAAaa, ATAAta, ATAAtt, and ATAAat) (Kanda et al.; 2014,Yamashita et al.; 2007) (Figure 19A). Using bioinformatics analyses, I identified two Sall1-binding sites in the 3' untranslated region (UTR) of *Isl1* and two sites in each of the 5' UTRs of *c-kit* and *Nkx2-5* (Figure 19A), suggesting that Sall1 may directly regulate the expression of these genes.

I then performed chromatin immunoprecipitation (ChIP) analyses. GFP^+/GFP^- cells were isolated from the SHF of $Sall1^{GFP/+}$ mice at E9.5 using FACS. Following cell lysis and sonication, DNA fragments from both populations were histone-immunoprecipitated (ChIP) using antibodies against histone H3K27ac or histone H3K27me3. ChIP using antibodies against H3K27me3 revealed that the expression of all Sall1-binding sites on the promoters of *Isl1* and *c-kit* loci as well as

that on the promoter of *Nkx2-5* loci were significantly decreased (5- to 30-fold) in GFP+ cells compared with GFP- cells. Conversely, the expression of these binding sites significantly increased (5- to 30-fold) following ChIP with antibodies against H3K27ac, demonstrating that Sall1-binding sites within *Isl1*, *Nkx2.5* and *c-kit* are de-methylated and active in Sall1-GFP+ CPCs (Figure 19B: red).

To further understand the role of Sall1 during cardiogenesis, I used siRNA to knock-down *Sall1* in differentiating mESCs (the siRNA knock-down method is described in Figure 20). Reduced cardiac differentiation following siRNA knock-down was confirmed by immunostaining with cTnT (Figure 21A). The knock-down efficiency in each cell was also confirmed by qPCR, along with expression of other cardiac and non-cardiac genes. Figure 21B shows that the knock-down of *Sall1* results in the down-regulation of CPC genes and cardiac lineage markers, such as *Isl1*, *Nkx2-5*, *Fgf10*, *Mef2c*, *Gata4* and *Tbx20*), contractile genes (*Myl2*, *Myl7*, *Tnnt2* and *Tnni3*) and the vascular marker *CD31*, by approximately 50% but caused the up-regulation of the non-cardiac lineage markers *NeuroD* (neurogenesis), *Etv2* (blood and vessel development) and *Pdx1* (pancreas). These changes indicate that *Sall1* is involved in cardiac lineage specification during the early mesodermal developmental stages (Figure 21B).

I then overexpressed Sall1 in differentiating mESCs to evaluate whether Sall1 directly promotes cardiogenesis (Figure 22A). As a result, $cTnT^{+}$ cardiomyocytes were efficiently induced in the Sall1-overexpressing EBs (Figure 22B). The overexpression of Sall1 resulted in increased levels of *Isl1*, *Tnnt2*, *Tnni3*, *Myl2* and *Hcn4* (Figure 22C),

indicating that *Sall1* robustly promotes cardiogenesis. The overexpression of Sall1 did not affect the levels of *CD31* and other genes in this tissue (Figure 22C), suggesting that increased Sall1 levels create a differentiation bias toward the cardiomyogenic lineage.

Sall1 promotes cardiogenesis in the human system

Because the gene network in mammalian cardiogenesis is highly conserved, *Sall1* might play a similar role in human embryonic stem cells (ESCs) and human induced pluripotent stem cells (hiPSCs) during the early stages of differentiation. Mammalian Sall1 expression is specific in CPCs *in vivo* and strong in differentiating mESCs only 2 to 4 days before cardiac induction, and its expression is abrogated after cardiomyogenesis. These expression patterns suggest that Sall1 also plays a critical/specific role during early development in humans. To address this question, I generated hiPSCs that stably expressed a reverse tetracycline-controlled trans-activator (rtTA) and a doxycycline (DOX)-inducible expression cassette that contained *Sall1* and *mCherry* (*Sall1*-TGhiPSCs) (Kim et al.; 2015) (Figure 23A). The expression of exogenous *Sall1* and mCherry was observed within 12 h of DOX treatment (Figure 23B, 23C). Cardiac differentiation was performed as previously described (Dubois et al.; 2011,Miki et al.; 2015) (Figure 24A) and followed a step-wise developmental progression from a primitive streak–like population defined by *T* (BRACHYURY) expression (days 2–4) to the development of the early mesoderm (*MESP1*; days 3 and 4) and the emergence of ISL^{+} and $NKX2\text{-}5^{+}$ cardiac precursors (days 4–8). Cardiomyocytes were detected between days 8 and 10 of differentiation. My lineage

tracing experiments revealed that *Sall1/SALL1* is expressed shortly after the initiation of gastrulation and prior to the expression of *Isl1/ISL1* and *Nkx2-5/NKX2-5* (Figure 4E, 24B, and data not shown). Therefore, I started DOX treatment on day 1 and continued until day 7 during the mesodermal and cardiac progenitor induction of differentiating *Sall1*-TGhiPSCs (Figure 24A: red; de Celis et al.; 2009). After 10 days of differentiation, the cells were analyzed by qPCR and flow cytometry. *Sall1* induction markedly up-regulated the CPC markers *ISL1* and *NKX2-5,* as well as several contractile markers, including *TNNT2, TNNI3, MYH6* (also known as α*MHC*), *MYH7* (also known as *βMHC*) and *MYL7* (also known as *MLC2A*) (Figure 25A). *MYL2* (also known as *MLC2v*) expression was observed, which corroborates previous reports demonstrating that *MYL2* is expressed during the later stages of development in ESC-derived cardiomyocytes (Dubois et al.; 2011). Flow cytometry analyses revealed a >7-fold increase in cardiac Troponin T (cTNT) DOX-treated cells (Figure 25). To understand the importance of *Sall1* function during cardiogenesis, I then treated differentiating hiPSCs with DOX (1) from days 1 to 3 (Figure 26A: blue), which corresponds to the *Sall1* expression window during early cardiogenesis in mice; (2) from days 3 to 7, which corresponds to CPC induction (Figure 26A: green); and (3) during days 3-10 to maintain continuous *Sall1* expression during cardiomyogenesis (Figure 26A: orange). Interestingly, CPC genes and cardiomyocyte genes were specifically induced only when DOX was administered during days 1 to 3 (Figure 26B: blue), whereas DOX treatment after day 3 prevented the transcription and translation of cardiomyogenesis factors in hiPSCs. (Figure 26B: green and orange). To identify

increases in the number of cardiomyocytes, I sorted DOX-treated differentiating hiPSCs several times with FACS using cTNT and TNI (Troponin I) (Figure 26C). Figure 26C shows that Sall1-overexpressing hiPSCs contained more cardiomyocytes during induction 1 (I1) than the control (IN). Interestingly, cardiomyogenesis was down-regulated in differentiating hiPSCs in response to the I2 treatment after CPC induction. Section IHC reveals that Isl1+ cells are efficiently induced only when DOX was administered during days 1 to 3, and cells expressing SSEA4, a marker of immature cells, were not observed (Figure 27). Conversely, Isl1 expression was induced in cells not treated with DOX (IN); SSEA4+ cells were also present, suggesting that cardiac differentiation is insufficient and that CPC or stem cell fate is maintained (Figure 27). Interestingly, SSEA4+ cells remained prevalent DOX for induction 2 (I2) and DOX for induction 2+3 (I2+3). Taken together, these results demonstrate that *Sall1* promotes cardiogenesis in humans in a time-dependent manner.

Discussion

In this chapter, I first identified Spalt family members as common/key factors for cardiac specification/induction that act upstream of cardiac progenitors. To identify a novel key gene, *Sall1*, I utilized *Isl1*$^{YFP/+}$ mice and the Flk1 antibody to focus on *Isl1* and *Flk1* because *Isl1* was identified as a marker of CPCs in the SHF and because *Flk1* is an important effector of cardiovascular lineage differentiation during cardiogenesis. Using a combinatorial analysis between Isl1^{+} cells and Flk1^{+} cells, I isolated unique genes in cardiac specific-lineage cells derived from Flk1^{+} and Isl1^{+} cells while excluding Flk1^{+} vascular lineage cells. In this gene profile, I isolated 15 candidate genes with unique functions in other organs during development or homeostasis, including two types of *Sall* genes, *Sall1* and *Sall4*.

Sall has highly conserved functions during the genesis of several organs. Specifically, Spalt function is conserved between insects and mammals, despite the evolutionary separation that occurred 280 million years ago (Schonbauer et al.; 2011). In *Drosophila*, *Spalt major* (*Salm*) and *Spalt related* (*Salr*) are Spalt zinc finger transcription factors, and *Salm* functions as a master regulator of fibrillar flight muscle fate (Schonbauer; 2011). In mice and humans, *Sall1-4 (SALL1-4)* are known as orthologs. Specifically, *Sall1* plays an important role in the maintenance and differentiation of ESCs and nephron progenitors and is associated with human congenital heart defects, suggesting that *Sall1* may act as a key factor for stem/progenitor behavior in each tissue and is

involved in early heart development. However, its role in heart development has not been addressed. In this chapter, I focused on *Sall1* during early cardiac development and demonstrated the mechanism by which Sall1 regulates cardiac cell fate in mice/humans.

In the next chapter, I will identify a co-activator of Sall1 and demonstrate how this factor coordinates with Sall1 to regulate early heart development.

Sall1+ CPCs differentiate into cardiomyocytes

I demonstrated that Sall1 is expressed in the SHF prior to the expression of Isl1 and Nkx2-5, which are important CPC genes. Sall1 expression is maintained in the SHF and partially overlaps with Isl1 expression, and it is abrogated as CPCs differentiate into cardiac cells. Sall1 expression was not observed in the FHF during cardiomyocyte differentiation or in Nkx2-5+;Isl1- cells in the FHF during early cardiogenesis. Although Sall1 CKO mice exhibited mild defects of the heart, *Sall1/4* DKO mice exhibited severe phenotypes characterized by loss of OFT and RV, similar to the phenotype of *Isl1* CKO mice (Cai et al., 2003). The number of CPCs ($Isl1^+$, $Isl1^+$;$Nkx2\text{-}5^+$) and immature cardiomyocytes ($Nkx2\text{-}5^+$) was markedly reduced, suggesting that Sall1/4 coordinately regulates OFT and RV formation. The lineage trace analysis demonstrated that Sall1 was expressed prior to the crescent stage and that Sall1-expressing cells migrated and contributed to all regions in the heart, where they differentiated into cardiac cells. An *in vitro* analysis revealed that GFP^+ cells from the SHF of $Sall1^{GFP/+}$ mouse embryos differentiated into cardiomyocytes. Based on these results, Sall1 fulfills the definition of a CPC factor during early cardiac development,

and it functions upstream of Isl1/Nkx2-5/Flk1/PDGFRa, which are major markers of CPCs during cardiogenesis. These results indicate that these CPCs (Sall1^{+}, Sall1^{+};Isl1^{+}, Sall1^{+};Isl1^{+};Nkx2-5^{+}, and Nkx2-5^{+}) may have several differentiating steps or exhibit various characteristics during cardiac development. The individual characteristics of each CPC must be addressed to understand the differentiation of CPCs into cardiomyocytes, especially ventricular cardiomyocytes, atrial cardiomyocytes or pacemaker cells and to study the differentiation timing of cardiomyocytes.

Sall1 **specifies the cardiac lineage**

Future work aims to elucidate how Sall1 expression is specified and when it is initiated *in vivo* in mouse embryos during organogenesis. Amazingly, the differentiating *Sall1*$^{GFP/+}$ mESC system demonstrated that CPC factors are enriched in the GFP+ population and that Sall1-expressing cells effectively differentiated into functional cardiomyocytes and heart cells but not other organs. Although Sall1 acts in a time-dependent manner, this result suggests that *Sall1* already specifies cardiac lineage at the mesoderm stage. Thus, Sall1+ mesoderm cells efficiently differentiate into cardiac cells. In this analysis, I also found that the timing of cardiogenesis was important in Sall1-expressing cells (E7.5~9.5 in mice or d0~d3 in mESCs; Figure 26/27). This findings suggests that *Sall1*-mediated cardiogenesis is restricted during organogenesis, which correlates with CPC localization and maintenance in mammals. These lines indicate that functional regulators upstream of Sall1 and the isolation of functional partners of Sall1 for the specification of cardiogenesis will be necessary to

understand CPC differentiation.

CPC factors are activated in Sall1+ cells

I identified tentative Sall1 binding elements on the enhancer/promoter of major CPC genes in mice. The molecular ChIP analysis clearly showed that Sall1 regulates *Isl1, Nkx2-5* and *ckit* expression via histone modification on their respective promoters, including cardiac enhancers in *Nkx2-5*, suggesting that Sall1 is a direct regulator of these genes. A genome-wide ChIP analysis using a Sall1 antibody during heart development will provide further insight into the specific roles of Sall1 (Kanda et al., 2014). Nishinakamura and colleagues note that Sall1 also acts as a nephron progenitor after E8.5, suggesting *Sall1* may time-dependently regulate a network that decides cardiac or nephron cell fate (Taguchi et al., 2014). Furthermore, the partner of Sall1 that modulates cardiac cell fate *in vivo* must be isolated to understand the time-limited function of Sall1 during cardiogenesis. To this end, a proteome analysis may be useful. Clarifying these mechanisms will elucidate why CPCs exist during early cardiac development and why CPCs are maintained in the SHF at approximately E10.0. A gain-of-function analysis also demonstrated that *Sall1* directly promoted cardiogenesis *in vitro*, and its function in cardiogenesis was highly conserved between mouse ES cells and human iPSCs. Thus, Sall1 serves as a novel factor for CPCs upstream of other major genes in CPCs.

Sall1 function is conserved in mice and humans and induces cardiogenesis

I demonstrated that Sall1-mediated cardiogenesis via the regulation of *Isl1/ISL1* was conserved between mESCs and hiPSCs. Utilizing a DOX-inducible piggyback system to induce Sall1 expression in a DOX-dependent manner, I demonstrated that Sall1 plays two key roles in regulating CPC-gene expression. First, Sall1 induces the expression of several cardiac transcription factors and cardiac contractile genes in humans. This result has a strong potential for clinical application in the future. Second, Sall1-mediated cardiogenesis was time-limited in hiPSCs as shown in Figure 26. The identification of the most effective time window for cardiomyogenesis via CPC induction would also be useful for clinical applications in humans. Our findings define Sall1 as an early marker of cardiac cells and as a regulator upstream of key genes (*Islet1, Nkx2-5,* and *c-kit*) expressed in CPCs. Defining these early progenitors will aid in the production of cardiac cells for future cell therapies or to model cardiogenesis *in vitro*.

Identifying secreted molecules regulated by *Sall1* or regulating *Sall1* will be useful to produce CPCs for clinical application and improving the efficiency of cardiomyocytes induction in iPSC technologies.

第 2 章

本章については、3 年以内に雑誌等で刊行予定のため、非公開

第 3 章

本章については、3 年以内に雑誌等で刊行予定のため、非公開

Figures

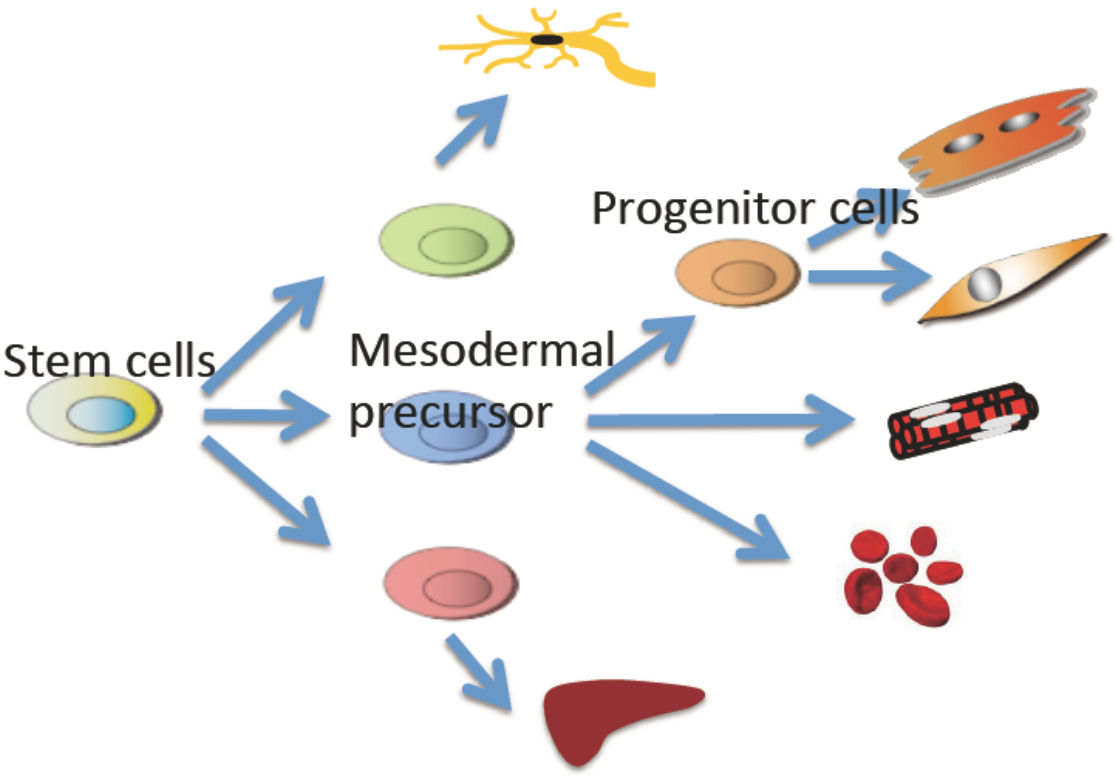


Figure 0. Cell type specification

Schematic showing the differentiation of specialized cells from stem cells via precursor cells or progenitor cells. Each terminal cell is differentiated from a cell fate committed, undifferentiated cell termed a progenitor or precursor cell.

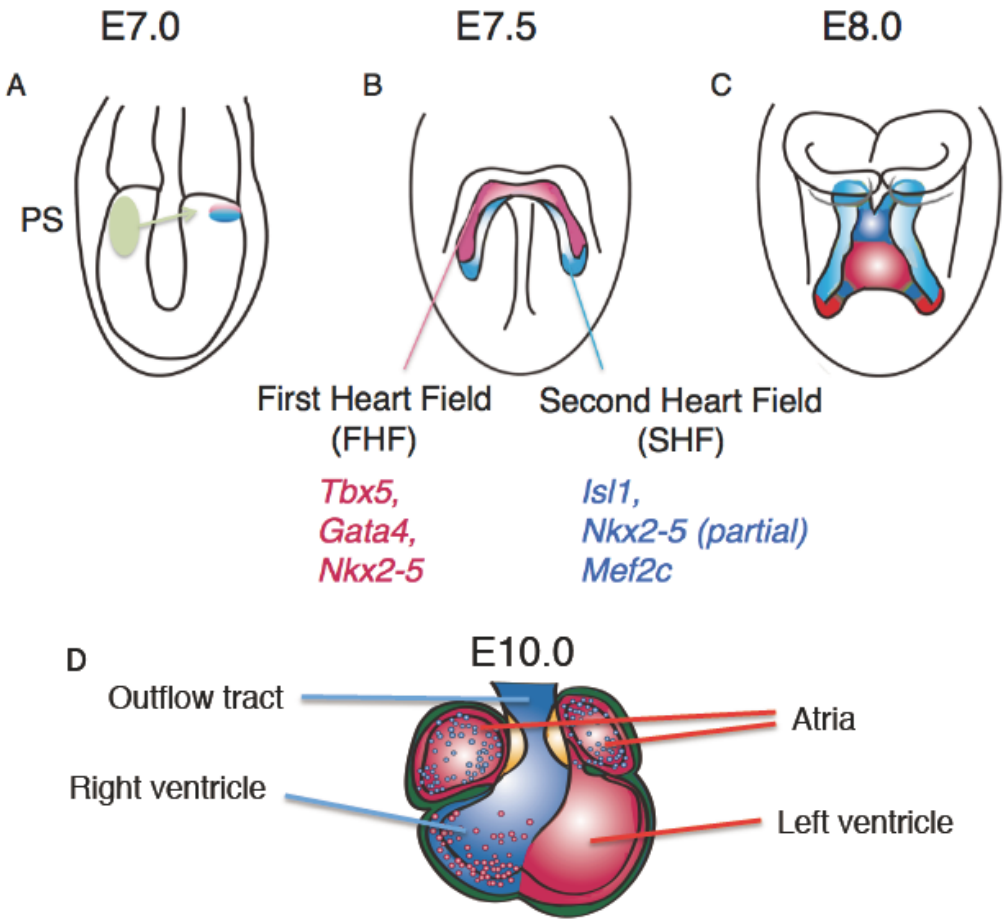


Figure 1. Atlas of the heart field during early organogenesis

Two major sources give rise to early heart formation. Nkx2-5^{+}Tbx5^{+} cells in the first heart field (FHF: red) contribute to the left ventricle and a part of both atria, and Isl1^{+} cells in the second heart field (SHF: blue) contribute to the outflow tract and right ventricle and a part of both atria. The primary heart field (red/blue) is observed at E7.0 (A) of the primitive streak stage, and these two heart fields are separated anatomically as the FHF/SHF at E7.5 of the early headfold stage (B). Cells of the SHF (blue) migrate to the FHF at E8.0 of the linear heart tube stage (C), and a primitive 4-chambered heart is formed at E10.0 (D).

(This figure was modified from Morita Y et al., Vascular Biology & Medicine 2012)

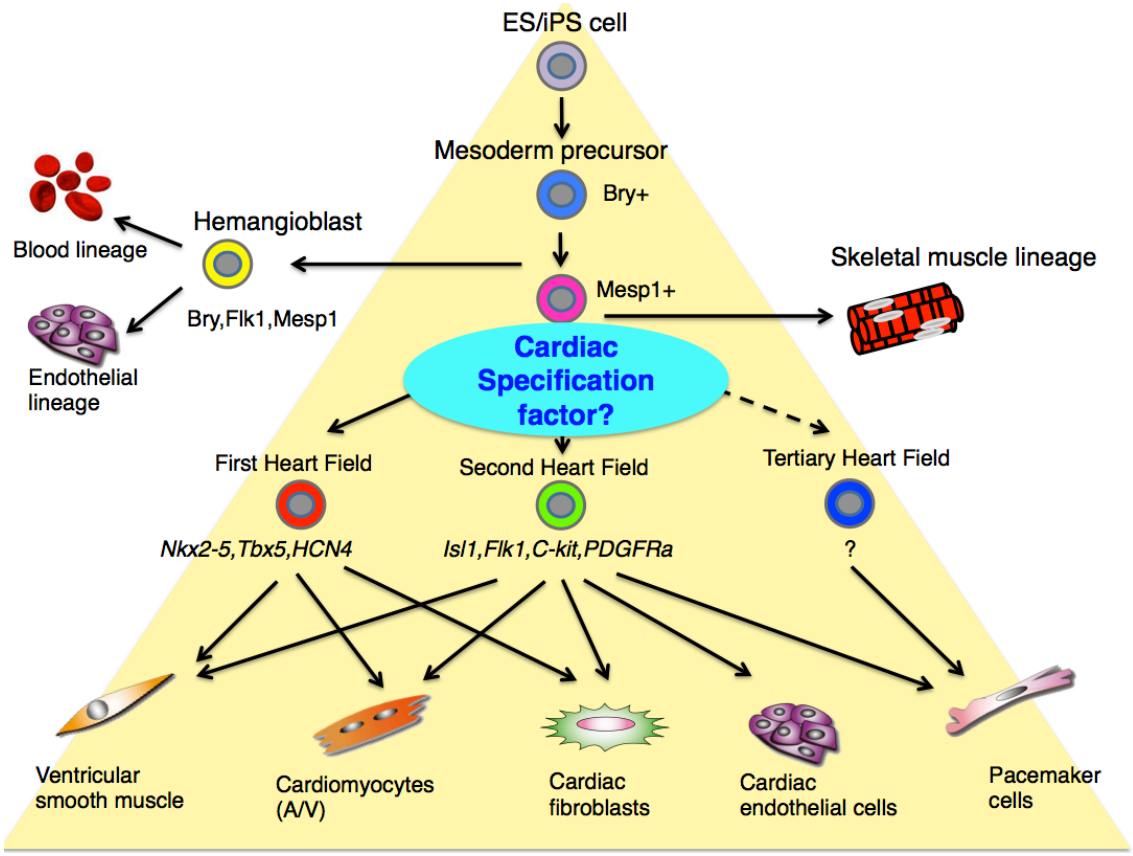


Figure 2. Cellular hierarchy of cardiovascular cell lineages during development

This cartoon shows the stepwise differentiation of heart cells from embryonic/induced pluripotent stem (ES/iPS) cells via several progenitor/precursor stages in response to defined factors (modified from Sehara et al., The EMBO J. 2014). Heart cells in the early stages are composed of FHF and SHF cells. Pacemaker cells are derived from the tertiary heart field. These heart cells are mainly derived from $Mesp1^{+}$ mesoderm cells. However, $Mesp1^{+}$ cells also contribute to the hematopoietic and skeletal muscle lineages, suggesting that Mesp1 acts as a multifunctional regulator for mesoderm-derived cells.

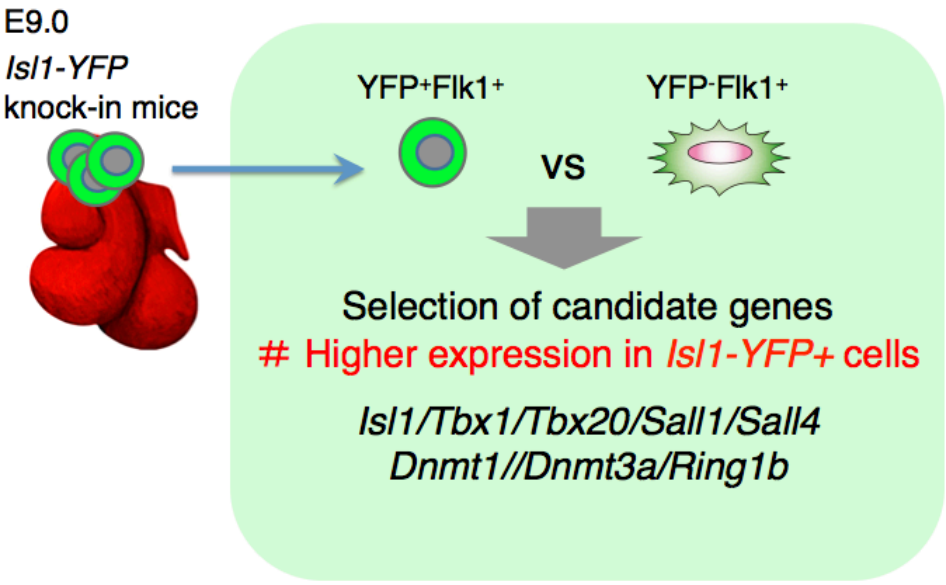


Figure 3. Screening of functional/key factors of cardiovascular lineage specification

To identify common factors of cardiac progenitors (CPCs), I performed microarray analysis of $YFP^{+}Flk1^{+}$ and $YFP^{-}Flk1^{+}$ populations from the SHF at E9.0 of *Isl1*-YFP knock-in mice (collaboration with Drs. C. Kwon and S. Evans). In a screen for targets of the Isl1+Flk population, I identified 15 candidate genes with high expression or repression, including the transcription factor *Sall1*.

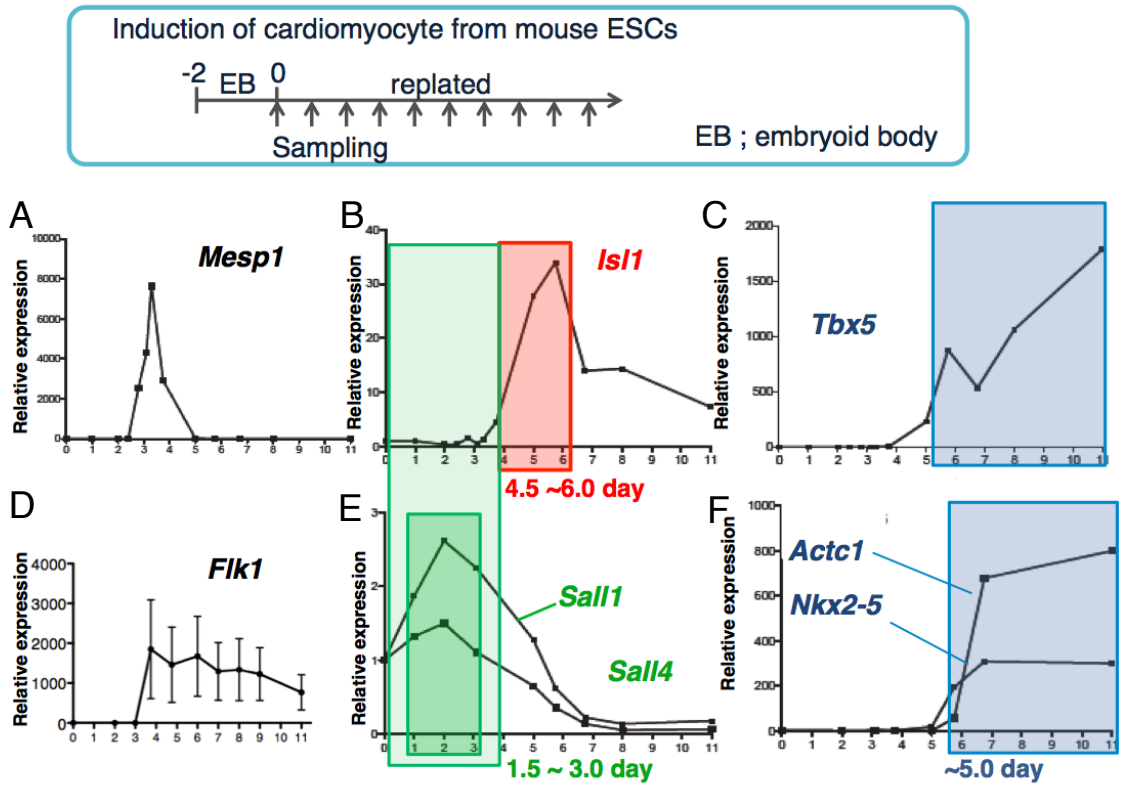


Figure 4. Time course expression analysis of stage-specific markers and novel CPC factors in the mouse ESC differentiation system

Gene expression analysis was performed by quantitative PCR in differentiating ESCs from day 0 to day 11.

(A, D) The expression of *Mesp1 and Flk1* as major mesoderm markers starts at day 3 of the ESC differentiating stage.

(B, E) The expression of *Sall1, Sall4, and* novel CPC candidate genes is observed at days 2-4, prior to *Isl1,* and this expression disappears during cardiomyogenesis (after 5 days).

(C, F) *Sall1* expression disappears before the expression of cardiac transcriptional factors (*Tbx5/Nkx2-5)* and cardiac contracting genes (*Actc1)*.

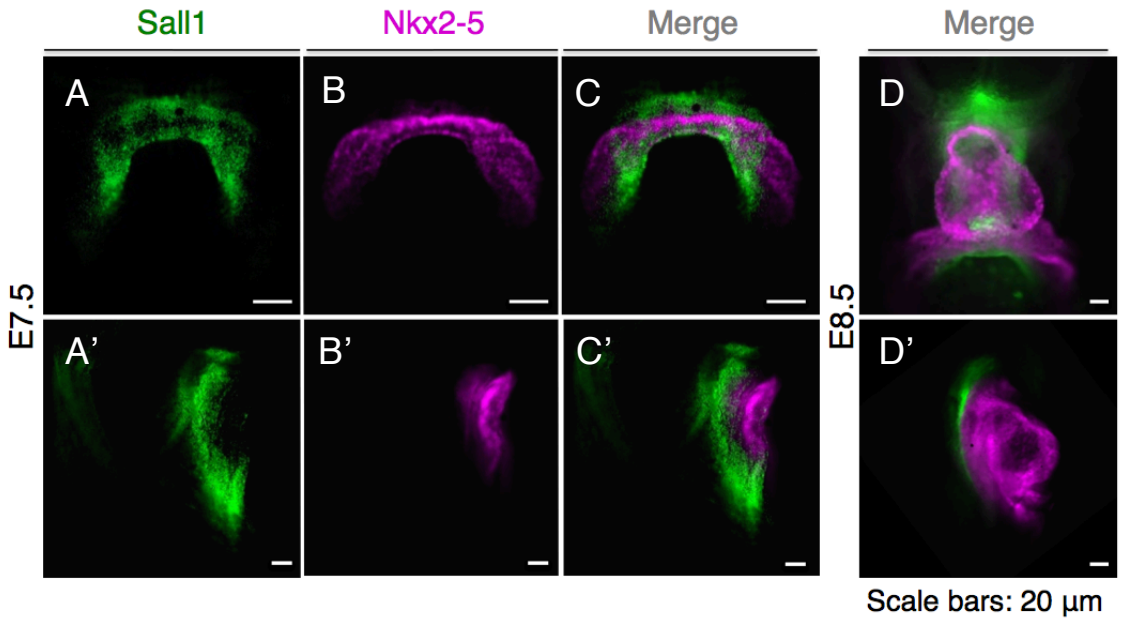


Figure 5. Sall1 is not expressed in the FHF during cardiogenesis

Whole-mount immunohistochemistry of Sall1 and Nkx2-5 with the corresponding antibodies.

(A, B, C) Front view of Sall1 and Nkx2-5 expression patterns at E7.5 of a wild-type (WT) mouse embryo. Sall1 expression does not overlap with Nkx2-5 expression.

(A', B', C') Lateral view of Sall1 and Nkx2-5 expression patterns.

(D, D') Front and lateral view of Sall1 and Nkx2-5 expression patterns at E8.5 of a WT mouse embryo. These immunohistostaining results show that the Sall1 expression area (green) does not colocalize with Nkx2-5 (magenta), a major marker of the FHF at the E7.5-8.5 stage.

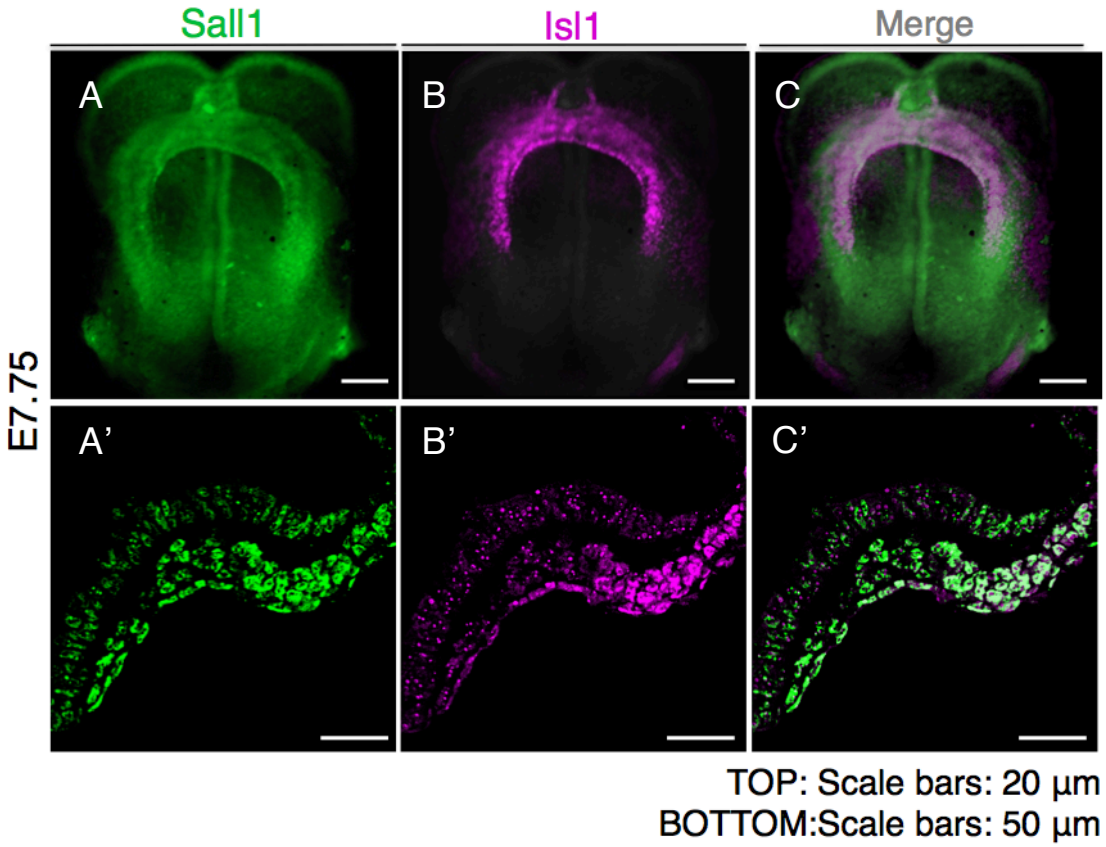


Figure 6. Sall1 is strongly expressed in the SHF during early heart development

Whole-mount immunohistochemistry of Sall1 and Isl1 with the corresponding antibodies.

(A, B, C) Front view of Sall1 and Isl1 expression patterns at E7.75 of a WT mouse embryo. Sall1 expression is overlapped with Isl1 (magenta) as a major marker of the SHF at this stage.

(A', B, C') Section immunohistochemistry of Sall1 and Isl1 with the corresponding antibodies.

Sall1-expressing cells (green) colocalize with $Isl1^{+}$ cells (magenta).

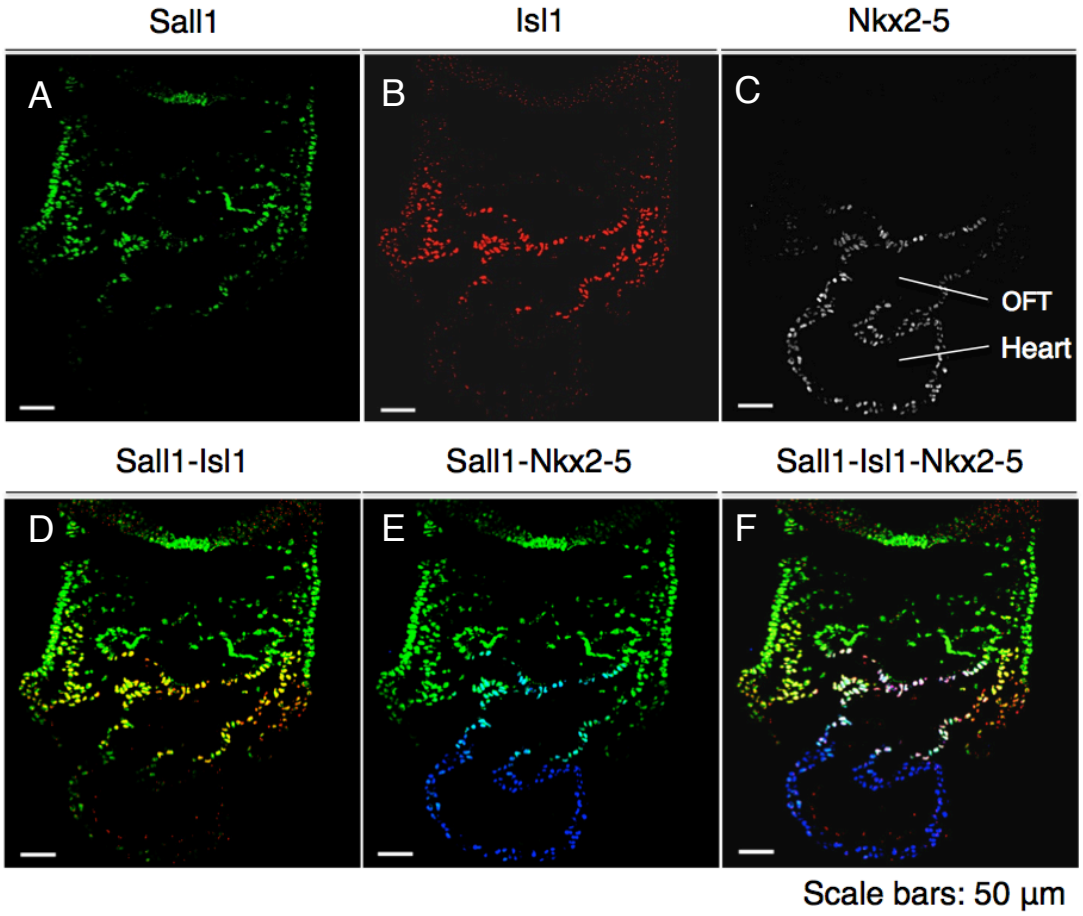


Figure 7. Sall1 is expressed in Isl1^{+} and Isl1^{+}Nkx2-5^{+} cells in the SHF at the heart looping stage during early heart formation

(A-F) Section of a WT mouse embryo at E8.75 immunostained to

(A, B, C) Single-color staining of Sall1 (A; green), Isl1 (B; red), and Nkx2-5 (C; white). Sall1 and Isl1 are mainly expressed in the SHF, but not in the FHF. Nkx2-5 is mainly expressed in the cardiomyogenic region (Heart) and outflow tract (OFT).

(D, E) Double-color staining of Sall1/Isl1 (D) and Sall1/Nkx2-5 (E). Sall1 colocalizes with Isl1 in the SHF (yellow). A portion of Sall1 expression colocalizes with Nkx2-5 in the OFT (right blue), but not in the FHF (dark blue).

(F) Triple-color staining of Sall1, Isl1 and Nkx2-5. These factors show overlapping expression in the OFT, but not in the FHF (heart forming region).

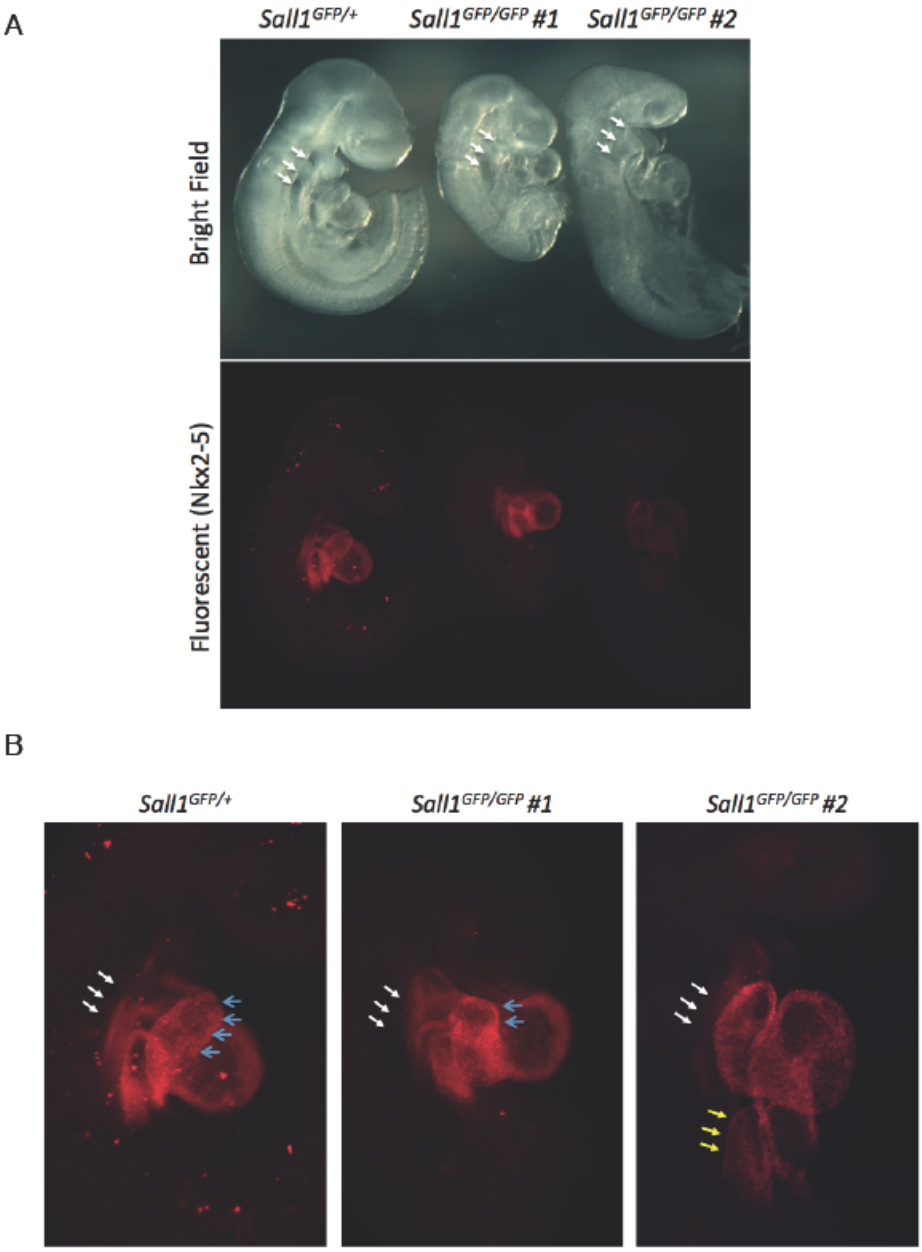


Figure 8. A-B. Sall1-deficient mice show mild defects in the pharyngeal arch, the SHF, the OFT and the IFT during heart formation

(A; TOP) Bright-field image of *Sall1*$^{GFP/+}$ and *Sall1*$^{GFP/GFP}$ mouse embryos.

(A; BOTTOM) Immunostaining with Nkx2-5 in those embryos. A reduction in Nkx2-5 expression was observed in some of the Sall1 knockout (KO) embryos.

(B) Magnified images of the fluorescent image in Fig. 8A. The exposure time is different in each figure to identify heart malformation more clearly. White arrows indicate the SHF, blue arrows indicate the OFT, and yellow arrows indicate the inflow tract (IFT).

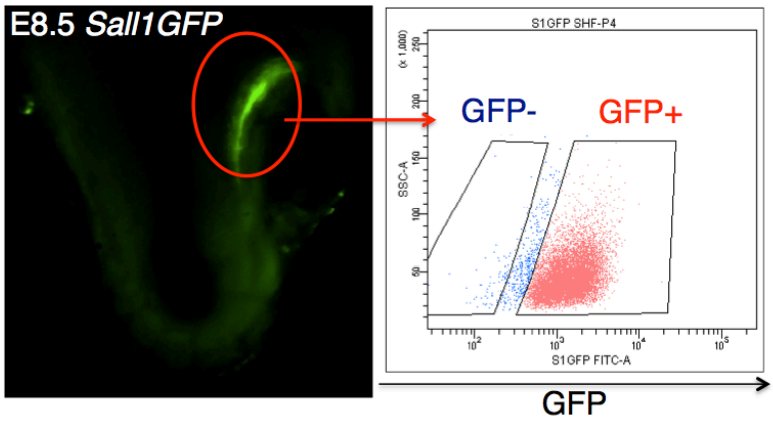
Figure 8C-F

インターネット公表に関する共著者全員の同意が得られていないため、非公開

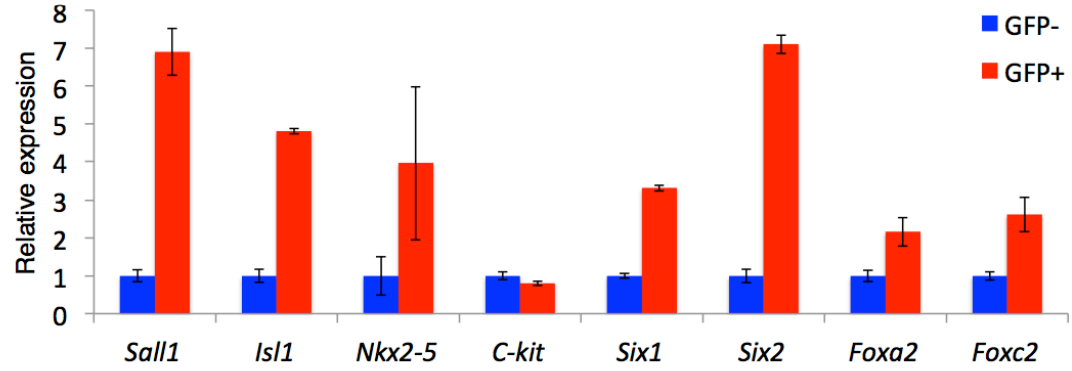
Figure 9

インターネット公表に関する共著者全員の同意が得られていないため、非公開

A


E8.5 Sall1GFP
S1GFP SHF-P4
GFP-
GFP+
SSC-A
S1GFP FITC-A
GFP

B


Relative expression
GFP-
GFP+
Sall1
Isl1
Nkx2-5
C-kit
Six1
Six2
Foxa2
Foxc2

C

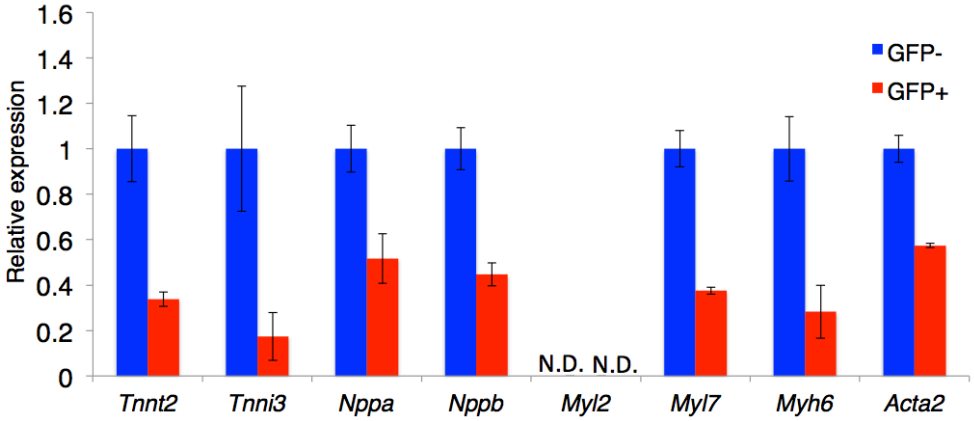

Relative expression
GFP-
GFP+
N.D. N.D.
Tnnt2
Tnni3
Nppa
Nppb
Myl2
Myl7
Myh6
Acta2

Figure 10. CPC genes are expressed in sorted Sall1^{+} cells in the SHF

(A) Left panel: GFP expression in E8.5 Sall1-GFP knock-in (KI) mice. GFP expression in this KI mouse is almost the same as endogenous Sall1 expression, suggesting that this KI mouse is useful for isolating live cells with endogenous Sall1 expression from the SHF.

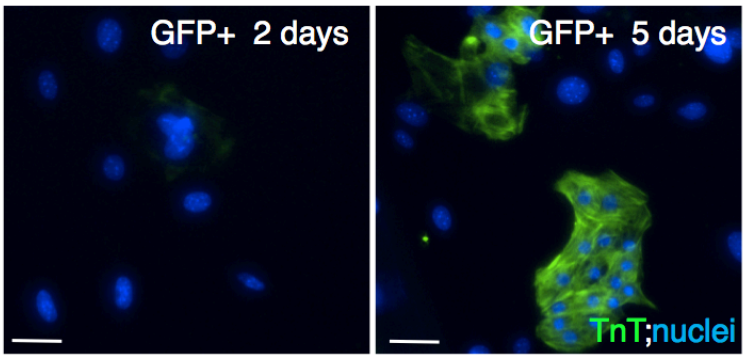
Right panel: Isolation of GFP$^{+/-}$ cells in the SHF from E8.5 Sall1-GFP KI mice by flow cytometry.

(B) qPCR-based gene expression analysis from sorted GFP^{+} and GFP^{-} fractions in the SHF at E8.5 of Sall1GFP mice. This analysis shows that CPC genes are enriched in Sall1^{+} cell fractions.

(C) qPCR analysis from sorted GFP+ or GFP- fractions in the SHF at E8.5 in Sall1GFP mice. This analysis shows that expression of cardiac contractile genes (*Tnnt2, Tnni3, Nppa, Nppb, Myh6* and *Acta2*) is lower in GFP+ fractions compared with GFP+ fractions.

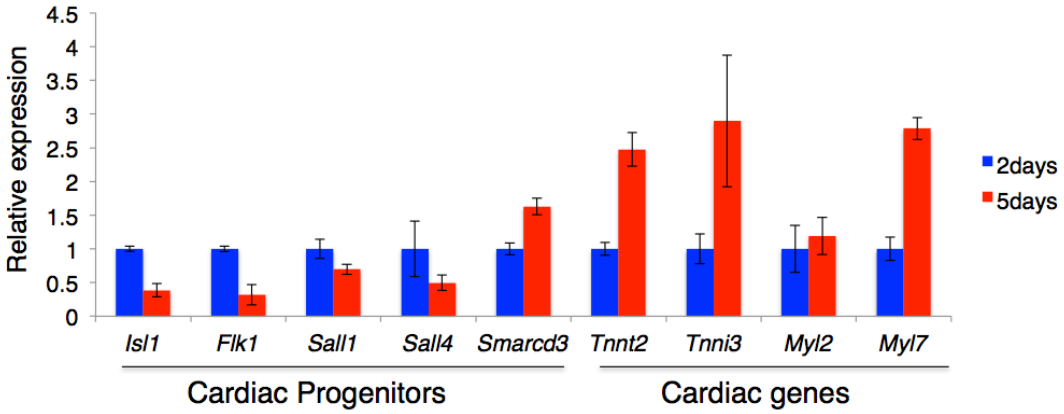
(B, C) The expression of each gene in GFP+ fractions is shown relative to the expression in GFP- fractions.

A



Scale bars: 50 µm

B



C

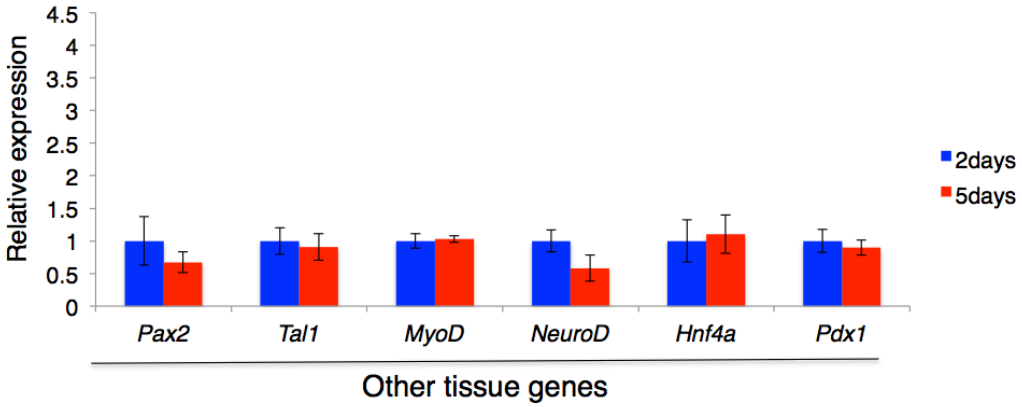


Figure 11. Sall1-expressing cells differentiate into cTnT^{+} cardiomyocytes

(A) Immunostaining with cTnT during the differentiation of Sall1-expressing cells in culture for 2 or 5 days after sorting from the SHF of *Sall1-GFP KI* mice.

(B) qPCR analysis of CPC gene expression in re-cultured Sall1^{+} cells at day 2 (blue) or day 5 (red) after sorting. After culturing for 2 days, CPC genes *(Isl1, Flk1, Sall1, Sall4)* are highly expressed. These CPC genes are inhibited, whereas cardiac contracted genes (*Tnnt2, Tnni3, Myl2, Myl7*) are up-regulated after culturing for 5 days.

(C) qPCR analysis of several gene expression profiles during early organogenesis (*Pax2*; kidney, *Tal1*; blood, *MyoD*; skeletal muscle, *NeuroD*; neuron, *Hnf4a*; Hepatocyte, *Pdx1*; pancreas) in primary Sall1^{+} cells cultured for 2 or 5 days. The expression levels of these factors do not change between days 2 and 5 in culture.

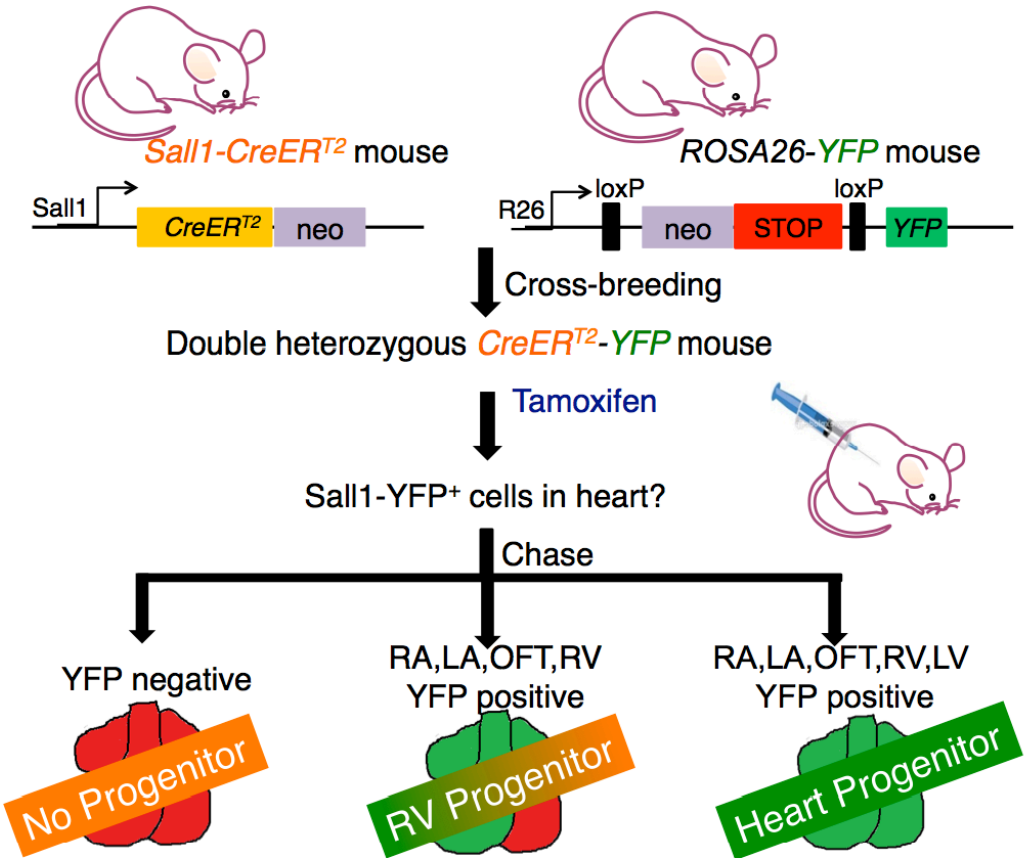


Figure 12. Schematic of the genetic fate mapping study design

Schematic diagram of lineage analysis of Sall1-expressing cells with tamoxifen treatment. *Sall1CreER^{T2}* transgenic mice were crossed with *Rosa26-YFP* reporter mice. The presence of YFP^{+} cells within the heart suggests that cells that previously expressed Sall1 migrate to the heart and differentiate into cardiac cells. (right: no progenitor). If YFP^{+}-derived cells are not observed in the heart, this means that Sall1 does not mark a cardiac progenitor. (middle: RV progenitor). If YFP^{+}-derived cells are observed in the OFT, right ventricle (RV), and part of the atria, this means that Sall1 is a novel factor regulating the SHF progenitor. (left: heart progenitor). If YFP^{+}-derived cells are observed in the whole heart, this means that Sall1 acts as a novel regulator of FHF/SHF progenitors in the heart.

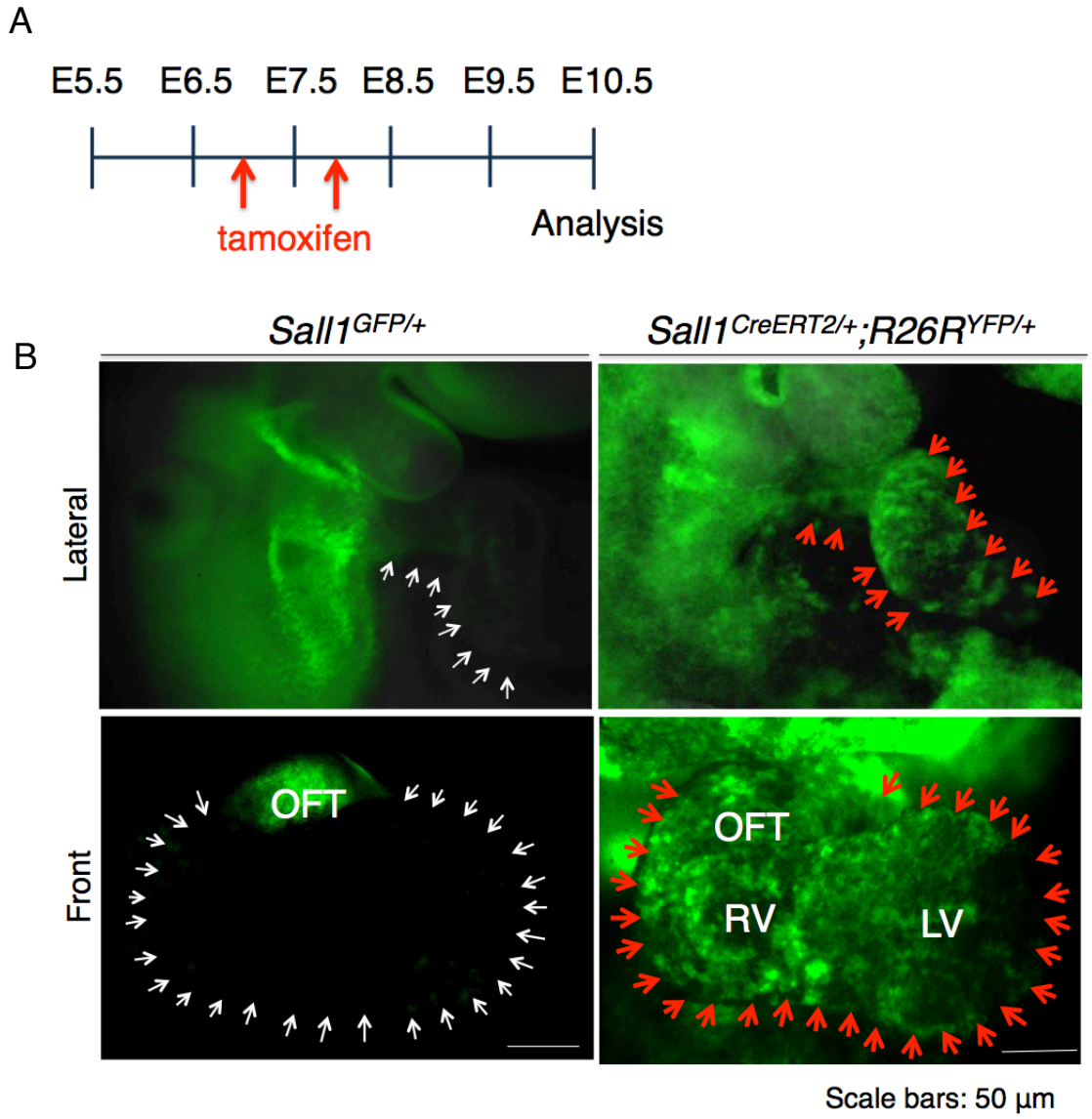


Figure 13. Sall1-expressing progenitors contribute to the whole heart

(A) Schematic representation of the experimental procedure for detecting Sall1-expressing cells between E7.0 and E8.0 following tamoxifen injection.

(B) (Left panels) Fluorescent image of endogenous Sall1 expression at E9.5 of *Sall-GFP KI* mice. Green signals clearly show that the expression of Sall1 is in the SHF and OFT, but not in the developing heart region (white arrows). (Right panels) Green signals with YFP+ fluorescence show that cells that previously expressed *Sall1* migrated to the whole heart at E10.5 in *Sall1*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mouse embryos following tamoxifen injection at E7.0~8.0 (red arrows).

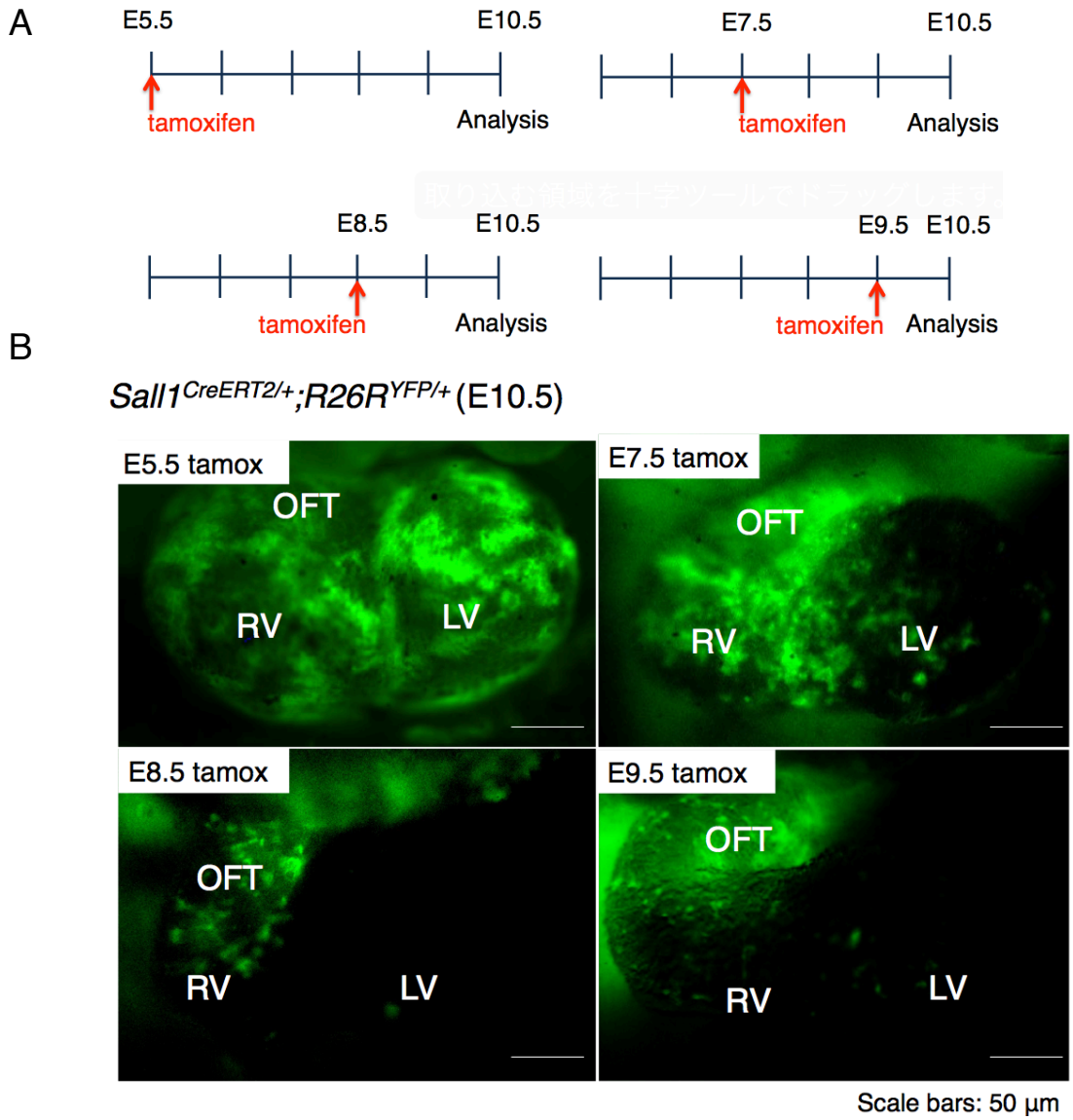


Figure 14. Stage-specific migration of Sall1-expressing progenitors

(A) Schematic diagram of tamoxifen injections into the abdominal cavity at several stages between E5.5, 7.5, 8.5, and 9.5.

(B) Fluorescent image of Sall1^{+} lineage cells in E10.5 of *Sall1*$^{CreERT2/+}$*; R26R*$^{YFP/+}$ mouse embryos with a tamoxifen injection at each time point.

(B; left top panels) YFP+ cells derived from Sall1^{+} cells contribute to the whole heart.

(B; right top panels) YFP+ cells migrate to the OFT, RV and a part of the LV.

(B; left bottom panels) YFP+ cells contribute to the OFT and a part of the RV.

(B; right bottom panels) YFP+ cells contribute to the OFT.

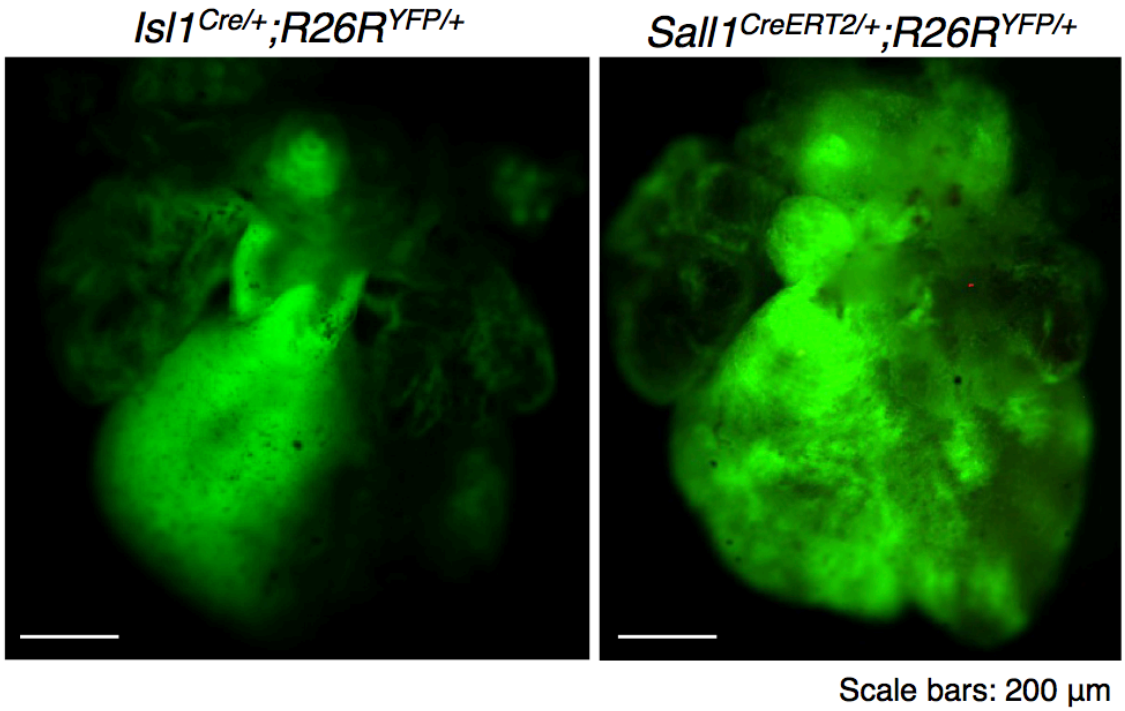


Figure 15. Distinct contributions to the embryonic heart from Isl1+ and Sall1+ progenitors

(Left) Fluorescent image of cells that previously expressed *Isl1* from an *Isl1*$^{Cre/+}$;*R26R*$^{YFP/+}$ mouse at E14.5. YFP+ cells contribute to the OFT, RV, and both atria.

(Right) Fluorescent image of cells that previously expressed *Sall1* from *a* *Sall1*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mouse at E14.5, with tamoxifen administered at E7.5. YFP+ cells contribute to the whole heart.

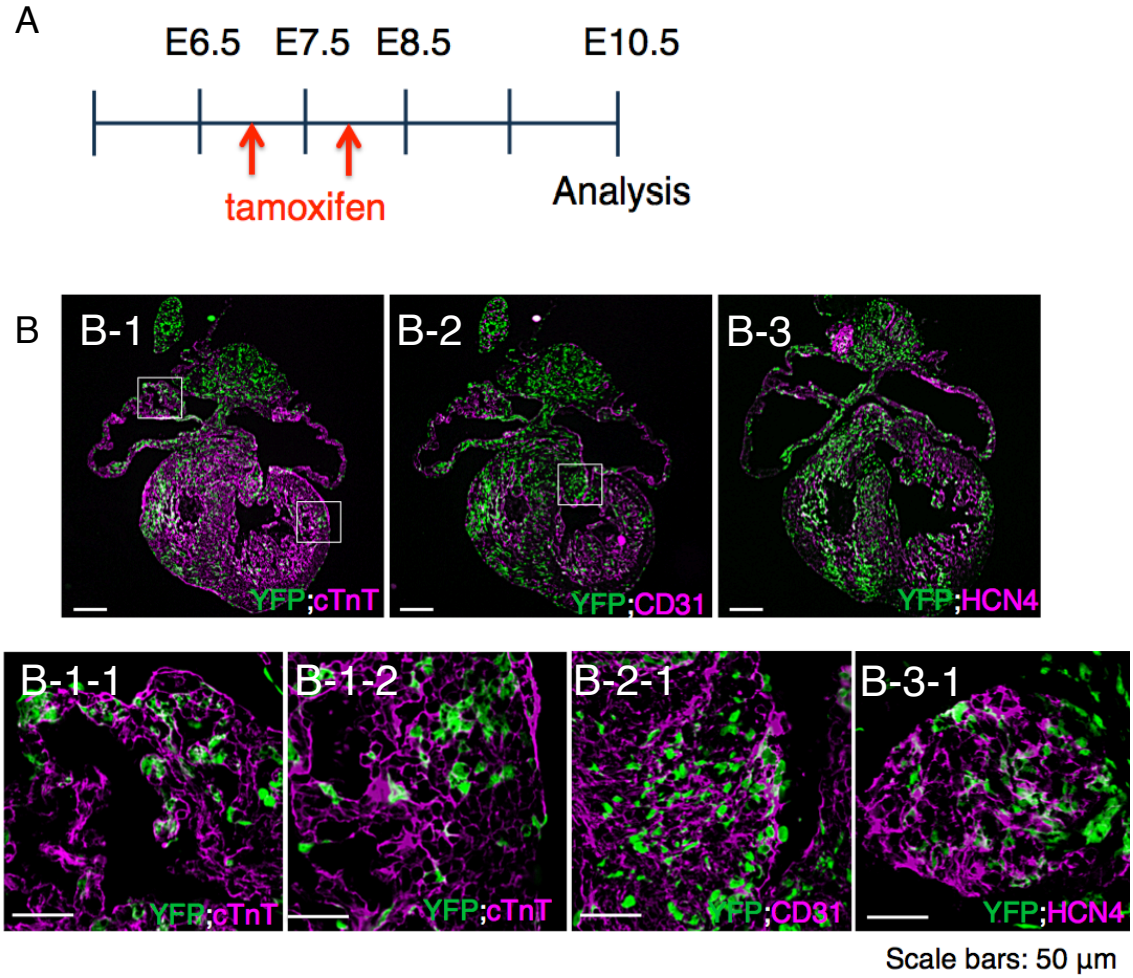


Figure 16. Cells derived from Sall1-expressing cells differentiate into several types of cardiac cells

(A) Schematic diagram of tamoxifen injection at E7.0 and E8.0.

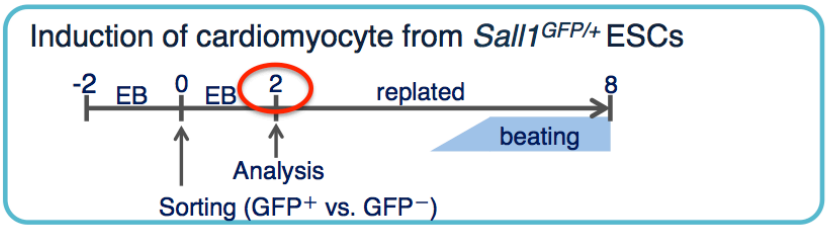
(B) Section images from *Sall1*$^{CreERT2/+}$;*R26R*$^{YFP/+}$ mice at E14.5 with YFP, cTnT, CD31, and HCN4 antibodies.

(B-1, B-1-1, B-1-2) Double staining with YFP and cTnT antibodies shows that YFP^{+} cells differentiate into atria cardiomyocytes and ventricle cardiomyocytes.

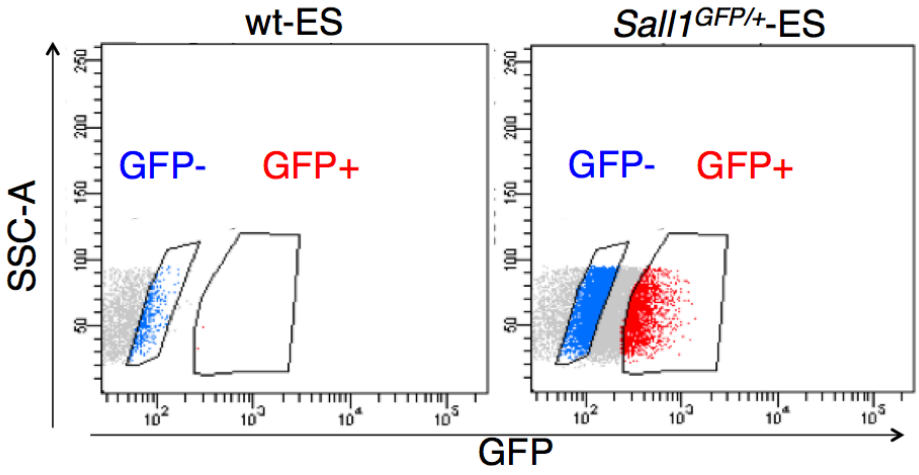
(B-2, B-2-1) Double staining with YFP and CD31 antibodies shows that YFP^{+} cells differentiate into endothelial cells.

(B-3, B-3-1) Double staining with YFP and HCN4 antibodies shows that YFP^{+} cells differentiate into pacemaker cells.

A


Induction of cardiomyocyte from Sall1GFP/+ ESCs
-2
EB
0
EB
2
replated
8
beating
Analysis
Sorting (GFP+ vs. GFP−)

B


wt-ES
Sall1GFP/+-ES
GFP-
GFP+
GFP-
GFP+
SSC-A
GFP

C

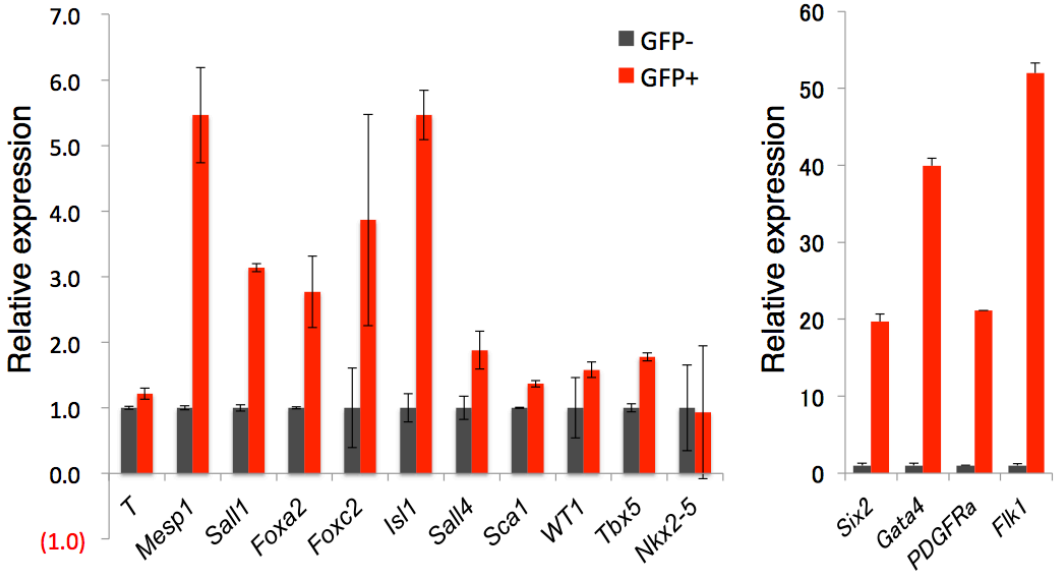

Relative expression
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
(1.0)
GFP-
GFP+
T
Mesp1
Sall1
Foxa2
Foxc2
Isl1
Sall4
Sca1
WT1
Tbx5
Nkx2-5
Relative expression
60
50
40
30
20
10
0
Six2
Gata4
PDGFRa
Flk1

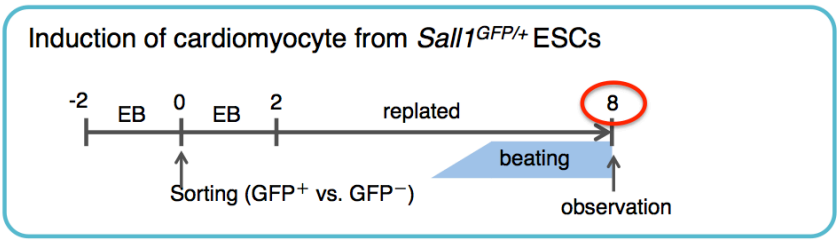
Figure 17. Enrichment of pre-cardiac mesoderm/CPC genes in differentiating Sall1$^+$ ES cells

(A) Schematic diagram showing the isolation of GFP$^+$ cells from differentiating Sall1-GFP ES cells. Embryoid bodies (EBs) were generated from a hanging drop at day 2. Isolation of Sall1$^+$ and Sall1$^-$ populations was performed at day 0 by flow cytometry, and re-aggregation of these isolated cells was performed with a hanging drop for culture. After 2 days, these populations were analyzed by qRT-PCR.

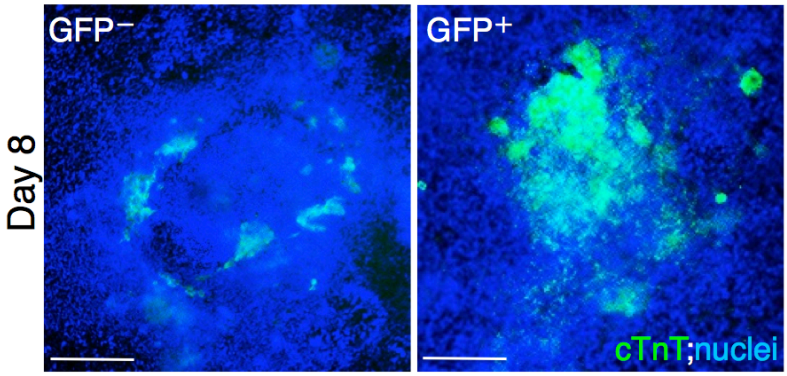
(B) Flow cytometry analysis of GFP expression on day 0 in differentiating Sall1-GFP ES cells. WT-ES cells were used as a control for this analysis.

(C) Relative mRNA expression of mesoderm genes (*T, Mesp1, Foxa2, Foxc2*), CPC genes (*Isl1, Sca1, Six2, PDGFRa, Flk1*), cardiac transcription factors (*Tbx5, Nkx2-5, Gata4*), and proepicardial genes (*WT1*: a novel gene for CPC) in isolated GFP$^+$ and GFP$^-$ populations. There is strong up-regulation of CPC genes such as *Six2, PDGFRa, and Flk1* (and *Gata4*) in the GFP$^+$ population. The expression of each gene in GFP+ cells is shown relative to the expression in GFP- cells.

A

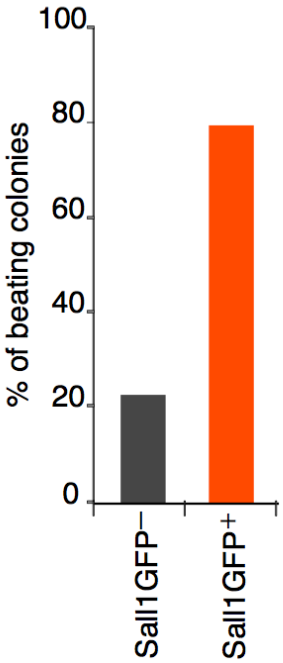


B



Scale bars: 200 μm

C



D

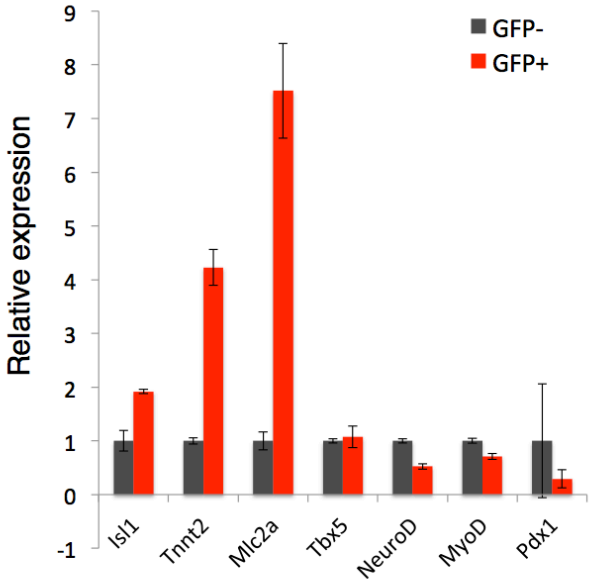


Figure 18. Sall1-expressing cells isolated at the mesoderm/pre-cardiac stage efficiently differentiate into cardiomyocytes

(A) Schematic diagram showing the isolation of GFP^+ and GFP^- EBs, their differentiation, and evaluation by immunohistochemistry, beating colony counts and qRT-PCR.

(B) Immunohistochemistry with cTnT (green) in GFP^- or GFP^+ differentiating colonies at day 8.

(C) Number of beating foci per 100 colonies in cultured cells after sorting GFP^- and GFP^+ EBs.

(D) Relative mRNA expression of cardiac genes and other tissue genes (*NeuroD* for neuron, *MyoD* for skeletal muscle, *Pdx1* for pancreas) in differentiating ES cells from GFP^+ and GFP^- EBs at day 8.

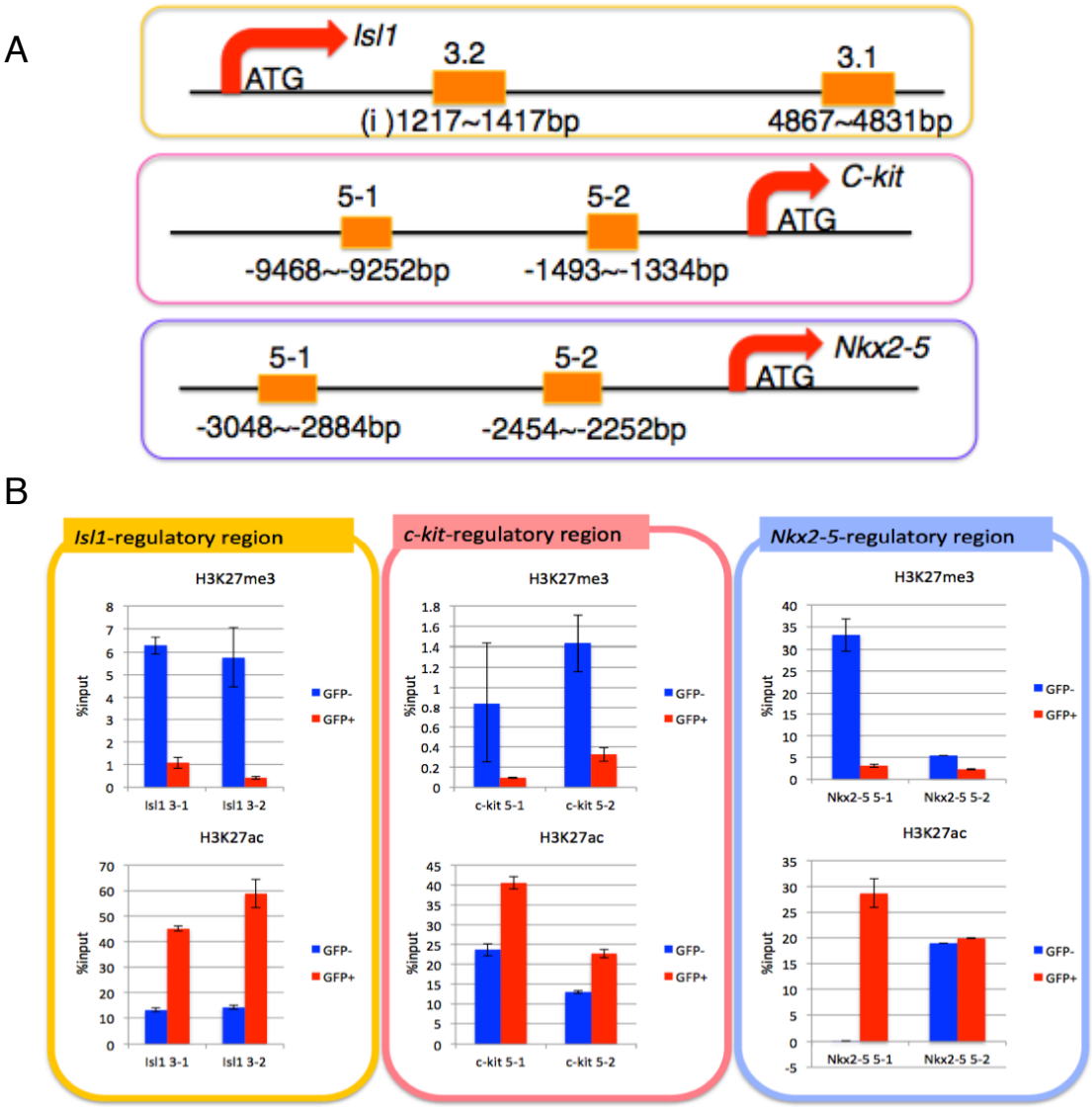


Figure 19. Histone modulation at the regulatory regions of CPC genes including *Islet1/c-kit/Nkx2-5* in Sall1^{+} cells

(A) The regulatory regions of major CPC genes (*Isl1,c-kit,Nkx2-5*) contain Sall1 binding sites (orange square).

(B) ChIP-qPCR analysis with H3K27me3 and H3K27ac antibodies in Sall1^{+} and Sall1^{-} cells sorted from the SHF of E9.0 Sall1-GFP KI mouse embryos.

Blue bars (GFP^{-} fraction) and red bars (GFP^{+} fraction) show the activities of histone modulation. In the GFP^{+} fraction, H3K27ac is strongly modulated in CPC regulatory regions.

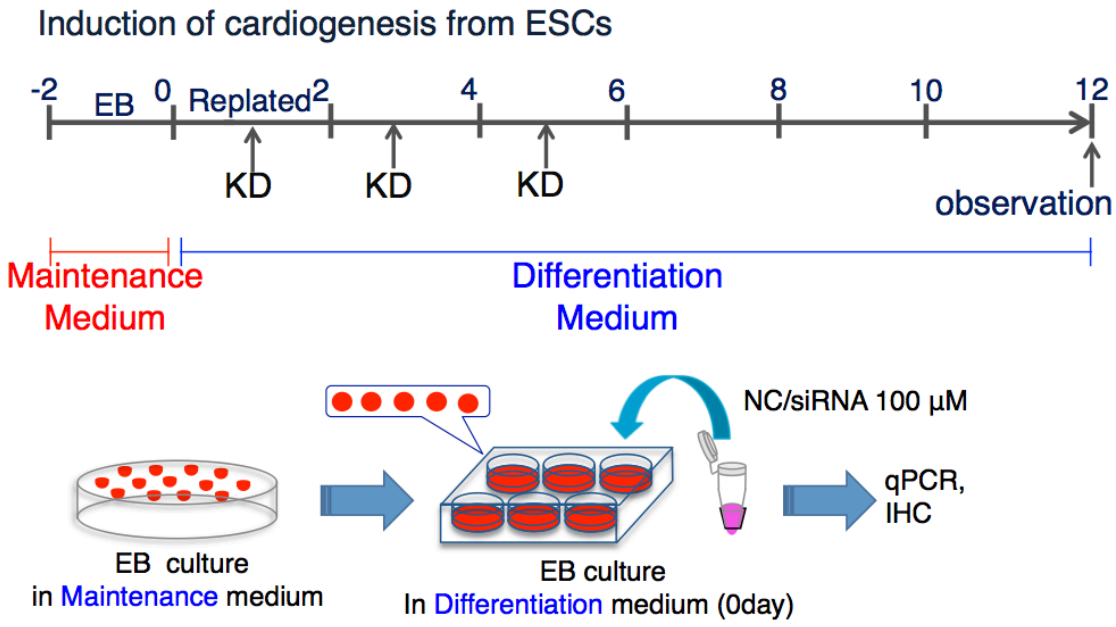


Figure 20. Loss-of-function analysis of Sall1 in differentiating mESCs

(Top) Schematic diagram showing the differentiation of ES cells using siRNA-based *Sall1* knockdown (*siSall1*). siSall1 was administered 3 times every 2 days (KD; 1st, 3rd, 5th days) during cardiac differentiation.

(Bottom) EBs from the hanging drop were cultured in maintenance medium for 2 days. Next, 5 EBs were inoculated into each well of a 12-well plate in differentiation medium following Sall1 or negative control siRNA treatment at day 1, day 3, and day 5. The samples were analyzed at day 12 of differentiation.

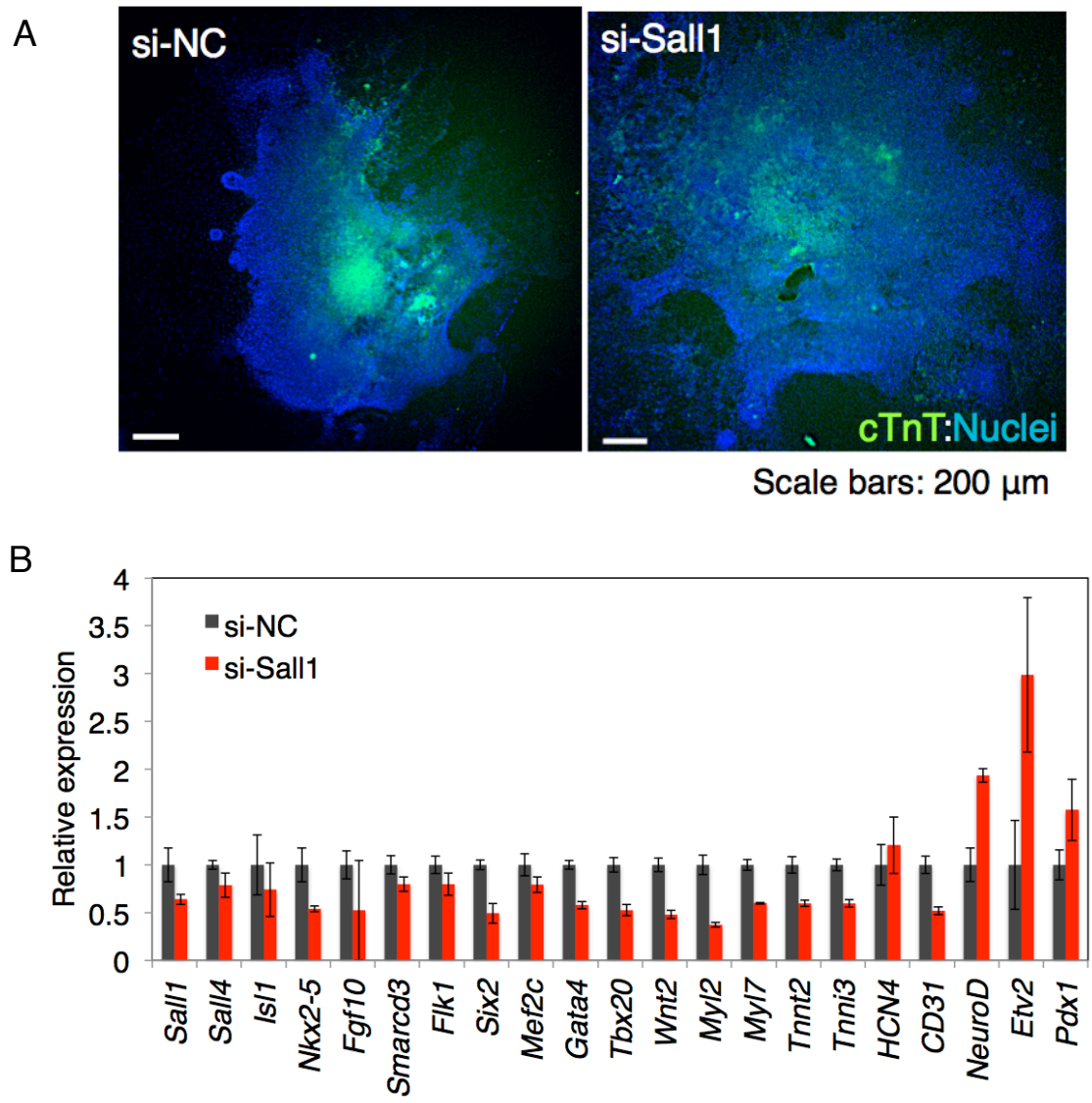
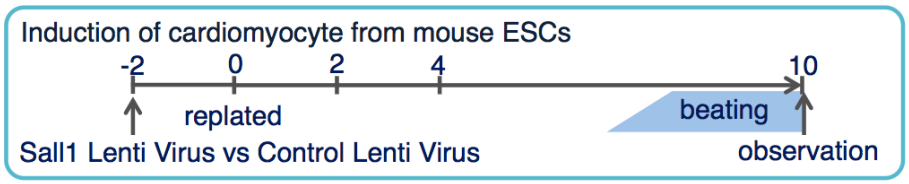


Figure 21. Knockdown of Sall1 prevents cardiogenesis

(A) Immunofluorescent staining with cTnT (green) and DAPI (blue) on mouse ES cells with siRNA for negative control (NC siRNA) or knockdown of Sall1 (Sall1 siRNA) after differentiation for 12 days.

(B) Relative expression of CPC genes (*Isl1, fgf10, Flk1, Six2, (Nkx2-5)*), cardiac genes (*Nkx2-5, Mef2c, Gata4, Tbx20, Wnt2, Myl2, Myl7, Tnnt2, Tnni3 and HCN4*), an endothelial gene (*CD31*) and other tissue genes (*NeuroD, Etv2, Pdx1*). The expression levels of CPC genes and cardiac genes are down-regulated in Sall1 knockdown EBs. Other tissue markers such as NeuroD, Etv2 and Pdx1 are up-regulated.

A



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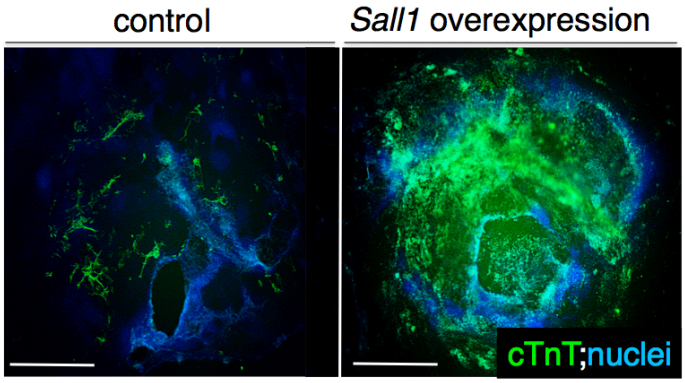

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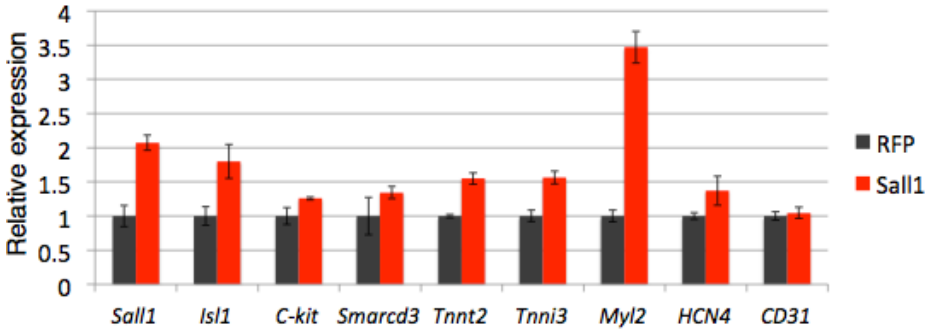
C



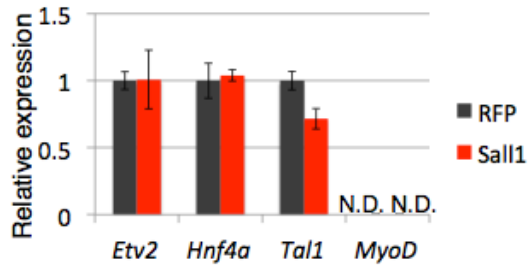


Figure 22. Sall1 promotes cardiac differentiation in mESCs based on gain-of-function analysis

(A) Schematic diagram of the cardiac differentiation of mouse ES cells with Lenti Virus infection for Sall1 overexpression.

(B) Immunofluorescent staining for cTnT (green) and DAPI (blue) in mES-derived cells with Sall1 or RFP Lenti Virus infection. Sall1-overexpressed ESCs efficiently differentiated into cTnT+ cardiomyocytes, as opposed to those of RFP-overexpressed ESC derived cells used as a control.

(C) Relative expression of CPC genes (*Isl1, c-kit, Smarcd3*), cardiac contract genes (*Tnnt2, Tnni3, Myl2*), a conduction gene (HCN4), an endothelial gene (*CD31*), a differentiating vessel gene (*Etv2*), a hepatocyte gene (*Hnf4a*), a lymphocyte gene (*Tal1*) and a myogenic gene (*MyoD*) in mES-derived cells with Sall1 Lenti Virus after differentiation at 10 days. Sall1 Lenti Virus was injected (0 μl, 0.5 μl, or 2 μl) at the hanging drop stage (day 2). This result indicates that Sall1 promotes the expression of CPC factors and cardiac genes, but it does not stimulate the expression of other tissue genes. The expression of each gene in Sall1-overexpressed mESCs is shown relative to RFP-overexpressed mESCs.

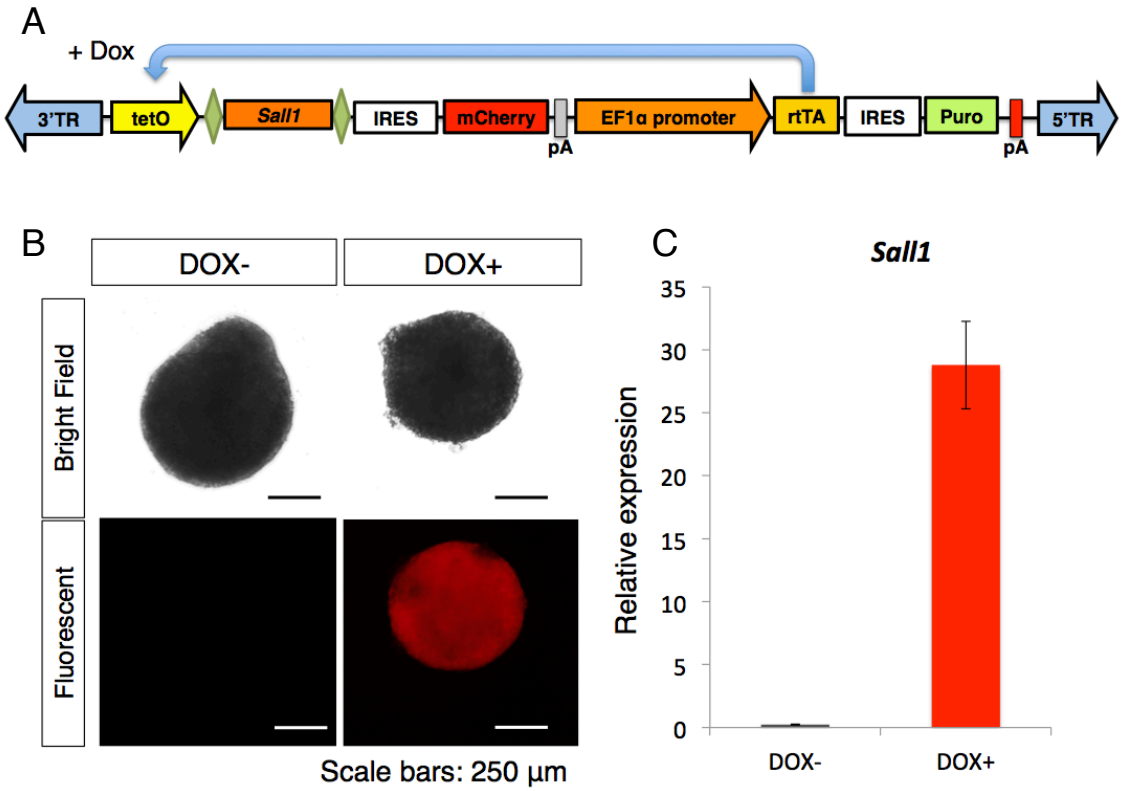


Figure 23. Strategy for DOX-inducible *Sall1* overexpression system in human iPS cells (iPSCs)

(A) Strategy for generating DOX-inducible *Sall1* overexpression with a PiggyBac system in human iPSCs.

(B) Bright-field and mCherry fluorescent images of DOX+ and DOX- in differentiating human iPSCs. Expression of the mCherry signal (red) in a DOX-dependent manner.

(C) Relative expression of exogenous *Sall1* in a DOX-dependent manner. The expression of exogenous Sall1 in DOX+ hiPSCs is shown relative to that in DOX- hiPSCs.

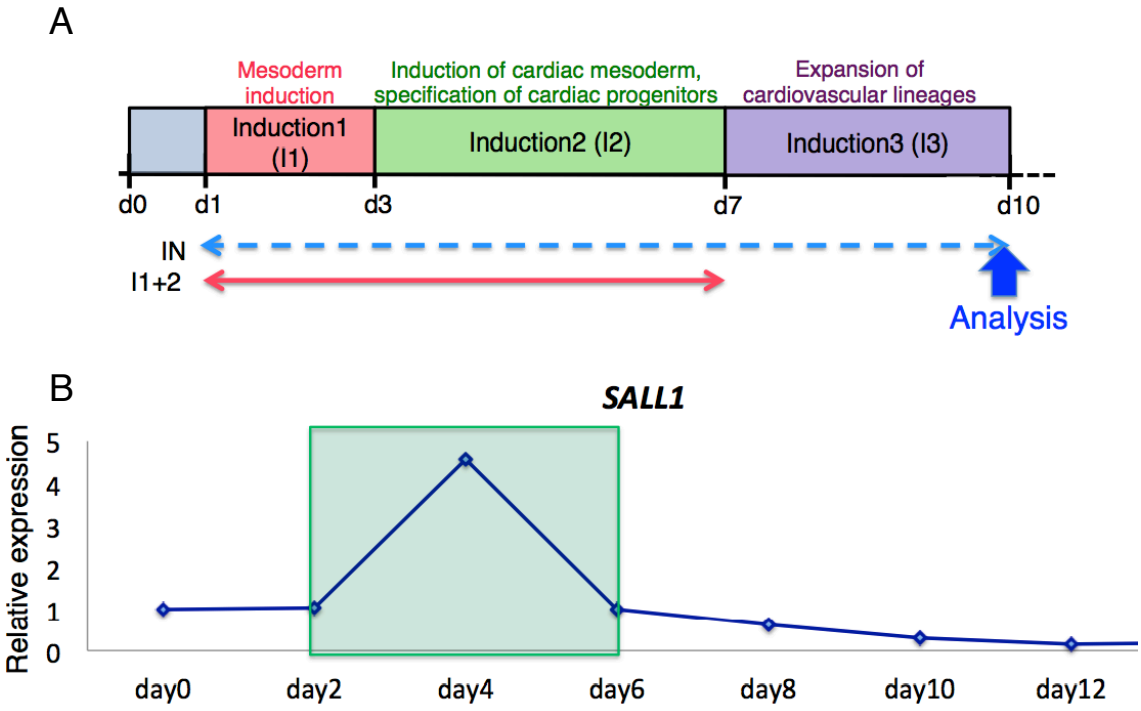


Figure 24. *SALL1* is transiently expressed during the induction of cardiac mesoderm

(A) Schematic diagram of the procedure for cardiac induction in human iPS cells. Cardiomyocytes are induced via three steps: Induction 1 (I1) with activinA/BMP/bFGF for 3 days; Induction 2 (I2) with WNT-inhibitor/VEGF for 4 days; and Induction 3 (I3) with VEGF/bFGF for maturation after day 7.

(B) Expression pattern of endogenous *SALL1* during differentiation of human iPSCs from day 0 to day 12. Expression of *SALL1* from day 2 to day 12 is shown relative to that of day 0.

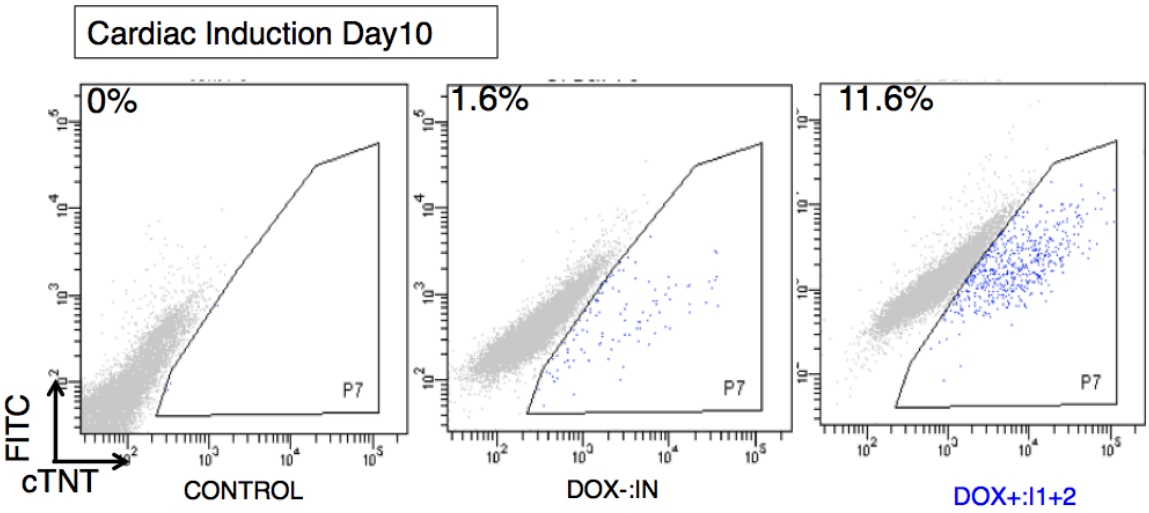


Figure 25. Overexpression of *Sall1* accelerates cardiogenesis in human iPSCs

Flow cytometry analysis for the quantification of the number of cTnT+ cells in DOX+ and DOX- hiPS cells at day 10. The control condition was not subjected to the antibody reaction.

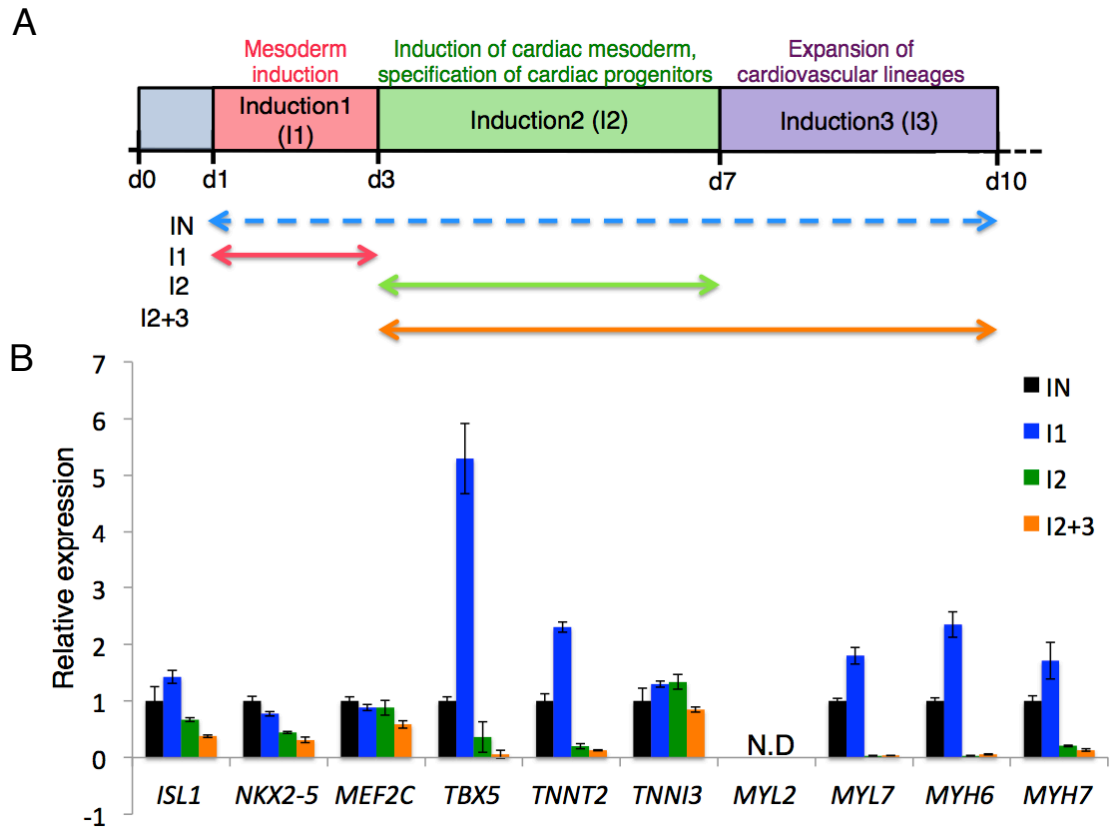


Figure 26 A/B. The timing of *Sall1* expression is crucial for cardiogenesis

(A) Schematic diagram of the timing of DOX treatment at several stages in the differentiation of human iPSCs.

(B) Relative expression of cardiac transcription factors (*ISL1, NKX2-5, MEF2C, TBX5*) and cardiac contracted genes (*TNNT2, TNNI3, MYL2, MYL7, MYH6, MYH7*) in several conditions of DOX treatment as shown in Fig. 26 (A). Expression of these genes in I1, I2, and I2+3 is shown relative to IN.

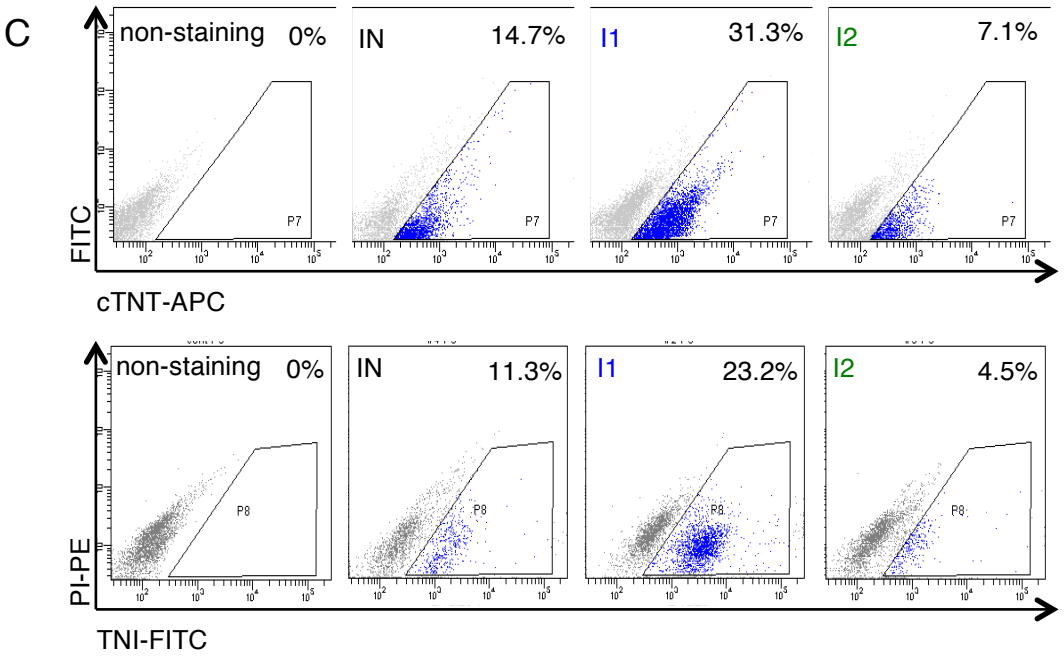


Figure 26 C. The timing of *Sall1* expression is crucial for cardiogenesis

(C) Flow cytometry analysis was performed to quantify the number of cTnT+ cells with DOX during I1 or I2 and without DOX (IN) in Sall-hiPS cells at day 10.

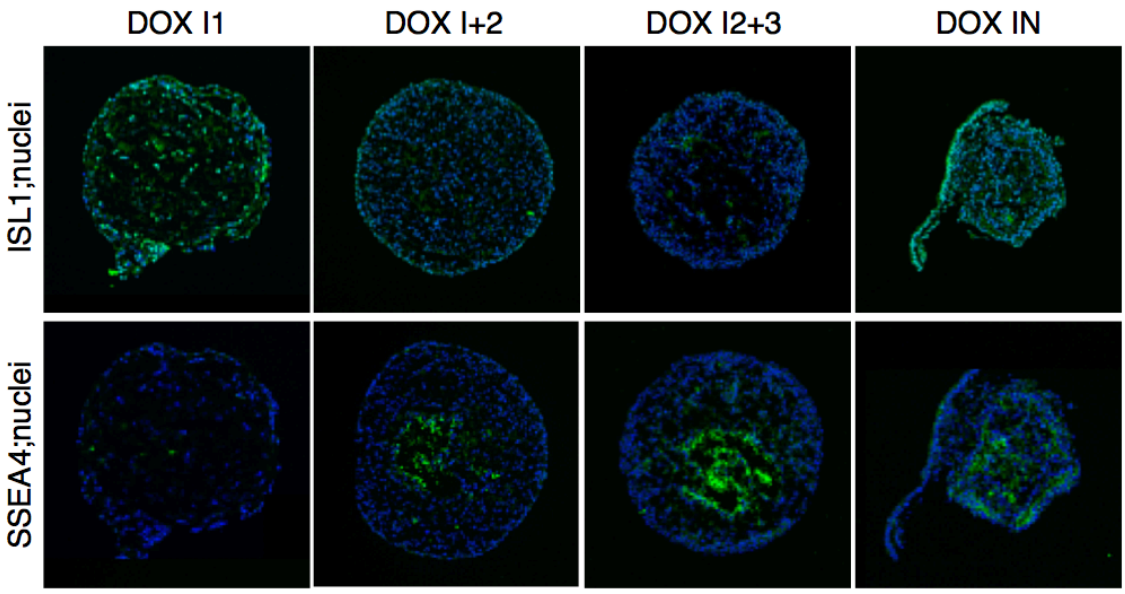


Figure 27. Appropriate timing of Sall1 expression is necessary for the induction of cardiac progenitors

(TOP) Immunofluorescent staining with Isl1 (green) and DAPI (blue) in differentiating human iPSCs that were subjected to various DOX treatments.

(BOTTOM) Immunofluorescent staining with a pluripotent marker; SSEA4 expression in differentiating human iPSCs.

第 2,3 章　Figure

本章の Figure については、3 年以内の雑誌等で刊行予定のため、非公開

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Acknowledgments

First, I would like to express the deepest appreciation to my supervisor, Dr. Jun K. Takeuchi (The University of Tokyo), for giving me the greatest environment, encouragement and continuous support for my Ph.D. study and related research. I cannot imagine having a better advisor and mentor for my scientific research.

I would also like to express my appreciation to Dr. Kazuko Koshiba-Takeuchi (The University of Tokyo) for breeding *Sall1*$^{GFP/+}$ transgenic mice and Dr. Yuko Tsukahara for helping tissue culture experiments; to Dr. Chulan Kwon (Johns Hopkins University) and Dr. Peter Andersen (Johns Hopkins University) for providing the screening data of *Isl1*$^{YFP/+}$ mice and giving me insightful suggestions for my scientific paper; to Dr. Akitsu Hotta (CiRA, Kyoto University) and Dr. Yoshinori Yoshida (CiRA, Kyoto University) for teaching me hiPSC maintenance and culture; to Dr. Ryuichi Nishinakamura (CARD, Kumamoto University) for providing *Sall1*-related ESC and mouse lines; to Mrs. Naoko Hayashida (The University of Tokyo) for helping the maintenance of mESC/hiPSC in vitro culture experiments; to Mrs. Hiroe Sugizaki and Mrs. Chizuko Koga for providing the data of FACS sorting and checking consistency of the data; to Dr. Ryo Nakamura (The University of Tokyo) for helping ChIP analysis; to Dr. Yumiko Saga (NIG) for providing the *Mesp1*$^{Cre/+}$ mouse line; to Dr. Sylvia Evans (UC San Diego) for providing the *Isl1*$^{Cre/+}$ mouse line; and to Dr. Yoshitaka Oka (The

University of Tokyo), Dr. Takeo Kubo (The University of Tokyo), Dr. Hiroyuki Takeda (The University of Tokyo) and Dr. Hiroki Kurihara (The University of Tokyo) for advising my experiments.

I would also like to thank the Japan Society for the Promotion of Science (JSPS) for providing scientific funding.

I thank the members of the Takeuchi Laboratory (Division of Cardiovascular Regeneration, Institute of Molecular and Cellular Bioscience, Graduate School of Science, The University of Tokyo) for their support and discussion of my research.

Finally, I am greatly thankful to my family for their heartfelt support and encouragement; without their help, I would not have been able to accomplish this study.