Short communication

Sall1 transiently marks undifferentiated heart precursors and regulates their fate

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A B S T R A C T
Cardiac progenitor cells (CPCs) are a crucial source of cells in cardiac development and regeneration. However, reported CPCs are heterogeneous, and no gene has been identified to transiently mark undifferentiated CPCs throughout heart development. Here we show that Spalt-like gene 1 (Sall1), a zinc-finger transcription factor, is expressed in undifferentiated CPCs giving rise to both left and right ventricles. Sall1 was transiently expressed in precardiac mesoderm contributing to the first heart field (left ventricle precursors) but not in the field itself. Similarly, Sall1 expression was maintained in the second heart field (outflow tract/right ventricle precursors) but not in cardiac cells. In vitro, high levels of Sall1 at mesodermal stages enhanced cardiomyogenesis, whereas its continued expression suppressed cardiac differentiation. Our study demonstrates that Sall1 marks CPCs in an undifferentiated state and regulates cardiac differentiation. These findings provide fundamental insights into CPC maintenance, which can be instrumental for CPC-based regenerative medicine.

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1. Introduction
Cardiac progenitor cells (CPCs), identified from embryos or pluripotent stem cell (PSC) culture, hold tremendous regenerative potential with the unique capability to expand and differentiate into cardiac cells [1]. Cardiogenesis initiate as the basic-helix-loop-helix factor Mesp1 is expressed in precardiac mesoderm. Mesp1+ cells migrate and form the cardiac crescent (referred to as the first heart field (FHF)), which largely contribute to the left ventricle (LV). A subset of Mesp1+ cells remain undifferentiated in the second heart field (SHF), located dorsal to the FHF, and give rise to the outflow tract (OFT), right ventricle (RV), and part of atria [2]. Early CPCs can be identified by transient expression of the chromatin factor Smarcd3, prior to expression of known CPC markers [3, 4], including Islet1 (Isl1), fetal liver kinase 1 (Flk1), and Nkx2.5 [1]. While Isl1 is transiently expressed in the SHF, Smarcd3 and Nkx2.5 are continually expressed in cardiomyocytes [5, 6]. Although, CPCs can be identified by their stage-specific expression of various cell markers, no gene has been identified to mark undifferentiated pools of CPCs giving rise to four chambers throughout development. In the present study, we demonstrate that Sall1, a zinc-finger transcription factor, is transiently expressed and maintained in undifferentiated CPCs throughout early heart development in vivo. Sall1 has a biphasic role: early overexpression of Sall1 enhanced, but its late overexpression suppressed cardiomyogenesis, whereas its continued expression suppressed cardiac differentiation. Our study demonstrates that Sall1 marks CPCs in an undifferentiated state and regulates cardiac differentiation. These findings provide fundamental insights into CPC maintenance, which can be instrumental for CPC-based regenerative medicine.

2. Materials and methods
2.1. Mice and lineage tracing
Sall1GFP, Sall1CreERT2, ROSA26P mouse lines were generated as described [7, 8]. Experiments were conducted according to a protocol approved by the International Animal Care and Use Committee of IMCB, the University
of Tokyo. To induce Cre activity of Sall1CreERT2/+, depending on embryonic stage, pregnant mice were fed 150 μl/30 g of Tamoxifen (10 mg/ml: sigma) by injection into the abdominal cavity at desired time points.

2.2. PSC generation, maintenance, and differentiation

Sall1GFP/+ ESCs were derived from Sall1GFP/+ mice [9]. ESCs were maintained in Knockout DMEM supplemented with 15% KSR (Gibco), Penicillin-Streptomycin (Nacalai tesque), MEM-NEAA, GlutaMAX, Sodium Pyvlate, 2-mercaptoethanol (Gibco), Lif (WAKO) on MEF. To differentiate Sall1-GFP ESCs, embryoid bodies (EB) were generated with DMEM (KOHHIN BIO) containing 20% FBS, 2.4 mM L-glutamine, MEM NEAA, 2-mercaptoethanol (Gibco), Lif (WAKO). After 2 days, EBs were cultured in DMEM (KOHHIN BIO) containing 20% FBS, 2.4 mM L-glutamine, 2-mercaptoethanol (Gibco). The medium was changed every two days. To generate time dependent Sall1 overexpressing cells, a DOX inducible SALL1 expressing piggybac vector and a PB-EF1a-mSALL1-IREs-mcherry vector were co-electroporated with the piggybac transposase vector PBASE2 into 201B7 cells [10] with NEPA21 (NEPA GENE) [11]. DOX-SALL1 hiPSCs were maintained and differentiated as described [12].

2.3. Immunohistochemistry and flow cytometry

Antibodies used: mouse a-Sall1 (1:100, PPMX), rabbit a-Isl1 (1:200, Abcam), mouse a-Is1 (1:100, hybridroma 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 (1:10,000, Thermo Fisherscientific), rabbit a-HCN4 (1:2000, alomone lab). Alexa Fluor secondary antibodies (Life technologies) were used for secondary detection and images were acquired with a KEYENCE BZ-9000 Fluorescence Microscope. For flow cytometry, ESCs/iPSCs were dissociated using 0.1% Trypsin or Accumax (Funakoshi). Cells were re-suspended in 0.1%FBS/D-PBS(−) without Ca2+ and Mg2+ and sorted using a FACSariaII (BD Biosciences). Cells were incubated with primary antibodies against secondary antibodies conjugated with Alexa Fluor 647 (Invitrogen).

2.4. Chromatin immunoprecipitation (ChiP)

ChiP was done using antibodies against a-trimethylated H3K27 (Cell Signaling), and a-acetylated H3K27 (Abcam) as described [13]. Immunoprecipitated DNA was amplified using the primer pairs:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isl1 3.2F</td>
<td>5-CCAATCTAGTGACAGCCAAA-3</td>
</tr>
<tr>
<td>Isl1 3.2R</td>
<td>5-TCACTGATTCGACCGACAACCAAG-3</td>
</tr>
<tr>
<td>Isl1 3.1F</td>
<td>5-TCACTGATTCGACCGACAACCAAG-3</td>
</tr>
<tr>
<td>Isl1 3.1R</td>
<td>5-GCTAGCTGGATTAAGGGCATC-3</td>
</tr>
<tr>
<td>Flk1 F</td>
<td>5-CAGGATGGAAGCAGCTTGAG-3</td>
</tr>
<tr>
<td>Flk1 F</td>
<td>5-CACCATGCCACATTCT-3</td>
</tr>
</tbody>
</table>

3. Results

3.1. Sall1 is expressed in early CPCs during development

To examine Sall1 expression during heart development, we used Sall1GFP mice [9] and compared GFP expression with Mesp1-lineage cells by generating Sall1CreERT2; R26GFP line-age reporter mice [7, 8] (Fig. 1E). Cre activity was induced at different stages (E5.5–E9.5) by tamoxifen, and hearts were analyzed for YFP expression at E10.5 (Fig. 1F). Cre activation prior to gastrulation (E5.5) resulted in widespread distribution of YFP positive cells in the heart. However, when Cre activity was induced at crescent stages (E7.5), YFP positive cells were mostly confined to the OFT and RV. Later stages of Cre activation (E8.5 and E9.5) resulted in further restriction of YFP positive cells to OFT/RV cells (Fig. 1F). Next, we induced Cre activation at E7.0 and E9.0, collected hearts at E14.5, and analyzed YFP positive cells. Cre activation at the pre-crescent stage (E7.0) resulted in abundant appearance of YFP cells in the entire heart (Fig. 1G), with YFP cells primarily restricted to the RV. These results suggest that Sall1 is expressed in precursors of FHF and SHF cells during gastrulation, and remains expressed in the SHF before giving rise to the OFT/RV.

3.2. Sall1 + cells give rise to distinct anatomical structures of the heart in vivo

To determine the fate of Sall1 + CPCs during heart development, we performed lineage-tracing experiments with Sall1CreERT2; R26GFP lineage reporter mice [7, 8] (Fig. 1E). Cre activity was induced at different stages (E5.5–E9.5) by tamoxifen, and hearts were analyzed for YFP expression at E10.5 (Fig. 1F). Cre activation prior to gastrulation (E5.5) resulted in widespread distribution of YFP positive cells in the heart. However, when Cre activity was induced at crescent stages (E7.5), YFP positive cells were mostly confined to the OFT and RV. Later stages of Cre activation (E8.5 and E9.5) resulted in further restriction of YFP positive cells to OFT/RV cells (Fig. 1F). Next, we induced Cre activation at E7.0 and E9.0, collected hearts at E14.5, and analyzed YFP positive cells. Cre activation at the pre-crescent stage (E7.0) resulted in abundant appearance of YFP cells in the entire heart (Fig. 1G), with YFP cells primarily restricted to the RV. These results suggest that Sall1 is expressed in precursors of FHF and SHF cells during gastrulation, and remains expressed in the SHF before giving rise to the OFT/RV.

3.3. Sall1 positively regulates CPC genes

To examine whether Sall1 affects cardiogenesis in vitro, we differentiated Sall1GFP ESCs into precardeal mesodermal cells and isolated GFP + /GFP − cells (Fig. S1A, B histogram). The GFP + cells showed enrichment for Flk1 and Pdgfra along with Mesp1 compared to GFP − cells (Fig. S1D), indicating that the Sall1 + cells contain early precardeal mesodermal cells. Is1l was also enriched along with Foxa2/Foxo2 and Six2, which are involved in regulating progenitor renewal, suggesting the ESC-derived Sall1 + cells contain both early and SHF progenitors. The GFP + cells were efficiently differentiated into cardiomyocytes (Fig. S1B, C) with increased levels of contractile genes (Fig. S1E). Isl1 levels were maintained even after 8 days of culture, but this is likely due to an ESC culture effect [14]. Through bioinformatics analyses, we identified putative Sall1-binding sites near loci of Isl1 and Flk1 (Fig. S1F). Our ChiP assay showed that the sites were poorly associated with H3K27me3 or showed no change in GFP + cells as compared to GFP − cells (Fig. S1F). In contrast, the sites were highly associated with H3K27ac and in SHF cells from heart fields.

3.4. Mesodermal SALL1 expression promotes cardiac fate but its prolonged expression suppresses cardiac differentiation

To temporally control Sall1 expression, we generated human PSCs that stably express a reverse tetracycline-controlled transactivator (rtTA) and a doxycycline (DOX)-inducible expression cassette containing Sall1 and mCherry (Sall1-TChiPSCs) [11] (Fig. 2A). Expression of exogenous Sall1 and mCherry was observed within 12 h (maintaining its expression for 2 days) following DOX treatment (Fig. 2B, C). Cardiac differentiation was performed with a step-wise developmental progression from a primitive streak-like population (days 1–3) to early precardeal mesoderm (days 3–4), CPC specification (days 4–7), and cardiomyocyte formation (after day 7) (Fig. S2A). Molecular profiling showed that SALL1 is initially upregulated during mesodermal
stages but downregulated near the end of CPC stages (Fig. S2B). We found that DOX treatment during CPC stages markedly increased expression of cardiac genes (Fig. 2D). However, continued DOX treatment at later stages strongly prevented cardiac gene expression (Fig. 2E). Consistently, the number of cardiomyocyte was markedly increased with early DOX treatment and decreased with late DOX treatment (Fig. 2E).

4. Conclusion

The present study shows that Sall1 is a previously unrecognized CPC gene expressed specifically in undifferentiated cells during CPC development. The absence of Sall1 expression in the FHF suggests that Sall1 is a maker of undifferentiated CPCs as, unlike SHF cells, FHF cells express sarcomere genes [15]. Sall1 expression was also maintained in the SHF...
from mesodermal stages. These findings suggest that Sall1+ cells represent an undifferentiated population of CPCs. In agreement with this, a temporal increase in Sall1 levels at early stages enhanced the cardiogenic potential, whereas its prolonged expression suppressed cardiac differentiation. Genome-wide ChIP analysis may allow us to identify a set of CPC genes crucial for maintaining CPCs in an undifferentiated state.

Fig. 2. Mesodermal SALL1 expression promotes cardiac fate but its continued expression in CPCs suppresses cardiac differentiation. (A) DOX-inducible Sall1 construct and strategy for temporal SALL1 overexpression in human PSCs (Sall1-TGhPSCs). (B, C) mCherry (B) or exogenous Sall1 (C) expression with/without DOX (after 2 days). This level of SALL1 expression is about six-fold higher than endogenous levels (Fig. S2B), which may be appropriate for the assay. Scale bars: 200 μm. (D) Relative expression of cardiac transcription factors and myocardial genes after temporal DOX treatment shown in (A). (E) Flow cytometry analysis showing percentages of cTNT/TNI/KDR positive cells after temporal DOX treatment shown in (A).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.02.008.

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