A Shh coreceptor Cdo is required for efficient cardiomyogenesis of pluripotent stem cells

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A Shh coreceptor Cdo is required for efficient cardiomyogenesis of pluripotent stem cells. A multifunctional receptor Cdo functions as a Shh coreceptor together with Boc and Gas1 to activate Shh signaling and these coreceptors seem to play compensatory roles in early heart development. Thus in this study, we examined the role of Cdo in cardiomyogenesis by utilizing an in vitro differentiation of pluripotent stem cells. Here we show that Cdo is required for efficient cardiomyogenesis of pluripotent stem cells by activation of Shh signaling. Cdo is induced concurrently with Shh signaling activation upon induction of cardiomyogenesis of P19 embryonal carcinoma (EC) cells. Cdo-depleted P19 EC and Cdo−/− mouse embryonic stem (ES) cells display decreased expression of key cardiac regulators, including Gata4, Nkx2.5 and Mef2c and this decrease coincides with reduced Shh signaling activities. Furthermore Cdo deficiency causes a stark reduction in formation of mature contractile cardiomyocytes. This defect in cardiomyogenesis is overcome by reactivation of Shh signaling at the early specification stage of cardiomyogenesis. The Shh agonist treatment restores differentiation capacities of Cdo-deficient ES cells into contractile cardiomyocytes by recovering both the expression of early cardiac regulators and structural genes such as cardiac troponin T and Connexin 43. Therefore Cdo is required for efficient cardiomyogenesis of pluripotent stem cells and an excellent target to improve the differentiation potential of stem cells for generation of transplantable cells to treat cardiomyopathies.

1. Introduction

Even though the development of cardiac and skeletal muscle cells shares some homology in the regulatory networks such as transcription factors or signaling pathways regulating the specification and differentiation of myocytes and contractile functions [1–4], cardiac muscles exhibit a poor tissue regeneration capacity after injuries in comparison to greater regenerative capacities of skeletal muscles [5,6]. This is due to the poor ability of cardiomyocytes to divide and their limited regenerative capacities [7]. The potential intervention to repair the damaged heart may be through transplanting cardiomyocytes or stimulating the endogenous stem cells to divide efficiently. Recent advances have identified several sources of transplantable cardiomyocytes, including skeletal muscle stem cells, embryonic stem cells, cardiac progenitor cells and more recently, induced pluripotent stem cells [8–12]. To harness the potential and efficacy of these stem cell sources to generate transplantable cardiomyocytes and to enhance the regenerative capacities of the resident cardiac stem cells, understanding the molecular mechanisms of cardiomyocyte differentiation will be of a great importance. In this regard, identifying regulators and pathways controlling cell fate specification and differentiation during embryonic heart development will be beneficial to improve the efficiency to generate transplantable cardiomyocytes.

Key cardiac regulators such as Gata4, Nkx2.5 and Mef2c are expressed in the early cardiogenic cells and involved in the specification and differentiation of cardiac progenitor cells [13]. Mice lacking any one of these transcription factors display severe defects in heart formation [14,15]. These transcription factors function synergistically to induce cardiomyogenesis of pluripotent stem cells such as P19 embryonal carcinoma (EC) and embryonic stem (ES) cells and activate each other’s expression forming a positive feedback loop to induce efficient cardiomyogenesis [13,16,17]. One of the most important regulatory mechanisms for the above mentioned transcription factors is Sonic hedgehog (Shh) signaling. Shh signaling is proposed to play a critical role in early stages of cardiomyogenesis by regulation of Nkx2.5, a key

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transcription factor for early cardiac lineage specification in the cardiac crescent stage [18]. Upon binding of Shh to its primary receptor Patched1 (Ptc1), Smoothened (Smo) activates the Gli family transcription factors thereby inducing the expression of target genes [19]. Mice lacking Smo exhibited a decreased expression of Nkx2.5, while Shh signaling activation by loss of the inhibitory component Ptc1 results in upregulation of this gene [20].

Cdo (CAM-related/downregulated by oncogenes) is cell surface protein which functions as a Shh coreceptor together with a related protein Boc (brother of Cdo) and Gas1 to activate the signaling [21–23]. During embryonic development, Cdo is expressed with a dynamic pattern in a broad range of tissues [24]. Mice lacking Cdo singly display defects in neural tube development without alteration in early heart development [25]. The removal of all three Shh-binding proteins, Cdo, Boc and Gas1 results in a near complete loss of hedgehog signaling, including a failure of heart looping, ventral neural tube patterning and early embryonic lethality, similar to Smo−/− embryos [18,23], suggesting that these coreceptors play redundant and compensatory roles. In this study, we examined the role and mechanisms of Cdo in cardiomyogenesis of pluripotent stem cells. Here, we provide the evidence supporting for an important role of Cdo in induction of cardiomyogenesis of pluripotent stem cells. Cdo is expressed in the early stages of cardiomyogenesis of P19 EC and mES cells. Cdo deficiency caused a reduction in Shh signaling activities and the expression of key cardiac regulators. Cdo−/− mESCs exhibited delayed cardiomyogenesis, which were rescued by Shh agonist treatment, suggesting that Cdo regulates cardiomyogenesis via controlling Shh signaling.

2. Materials and methods

2.1. Cell culture, transfection and cardiomyocyte differentiation

P19 EC cells (ATCC CRL-1825™) were maintained as previously described [26]. Briefly, P19 EC cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS). To analyze the function of Cdo in cardiomyogenesis, P19 EC cells were transfected with the previously characterized expression vectors for pBabePuro-Cdo to overexpress or pSuper-Cdo shRNA to knockdown [26,27] by using LipoFectamin 2000 (Life technologies). Generally the transfection efficiency reached to almost 90%. To induce cardiomyogenesis, 3 × 10⁵ P19 EC cells per 60 mm bacterial grade petri dish were plated, aggregated for 4 days in the presence of 0.8% DMSO to induce cardiomyogenesis (Induction) and re-plated on mammalian cell culture dishes, followed by further culturing cells for differentiation (Differentiation).

The Cdo−/− and Cdo−/+ mES cells were maintained as previously described [28]. ES cells were maintained in ES cell medium containing 1000 units/ml of Leukemia inhibitory factors (LIF, Millipore) on γ-irradiated mouse embryonic fibroblasts (MEFs). For in vitro cardiomyogenic differentiation, MEFs were removed by 2 h of incubation on uncoated culture dishes after trypsinization, unattached ES cells were transferred in ES cell medium containing 0.5% DMSO without LIF and further cultured as hanging-droplets to generate the even sized embryo bodies (EBs). In this study, 300 single ES cells were aggregated in the presence of DMSO and for the Shh agonist treatment, cells were treated either the vehicle DMSO or 2 μM pumorphamine (Pur.). To induce differentiation, 48 h later, 72 EBs are individually re-planted into three 24-well dishes coated with 0.1% gelatin in the same culture medium without DMSO, with the medium change for every second days followed by 3 days of attachment for EBs. These experiments were repeated three times. To characterize the contractile behavior, contractile behavior Cdo−/− and Cdo−/+ EBs were observed and imaged with a Nikon eclipse Ti. Asynchronous contractile property of differentiated EBs was defined with “dose not contract at the same time in linked clusters” and “beating with randomness in single contractile clusters within 20 s” [29]. To determine the contracting area, contractile Cdo−/+ and Cdo−/− EBs were photographed with a Nikon eclipse Ti, followed by analysis using Nikon Elements software.

2.2. Immunocytochemistry, immunoblotting and antibodies

Immunostaining of P19 and mES cells was carried out as previously described [28,30]. Briefly, trypsinized cells are grown on 0.1% gelatin coated cell culture slide (SPL life science) for 2 days followed by fixation with 4% paraformaldehyde for 15 min, permeabilization with 0.5% triton-X 100 in PBS and blocking with 5% goat serum. Primary and secondary antibodies were diluted with 1:250 or 1:500 in blocking solution for overnight at 4 °C or 1 h at RT, respectively. Images were analyzed with an LSM-510 META confocal microscopy system (Carl Zeiss) or an Nikon ECLIPSE TE-2000 U and NIS-Elements F software (Nikon). Immunoblotting was performed as previously described [31]. Differentiated ESCs were harvested with cell lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 10 mM NaF, 1 mM sodium vanadate, 0.1 mM PMSE, 1X protease inhibitor cocktail). After gel-electrophoresis and transfer, membranes were blocked with 5% skim milk in TBST buffer for 30 min and incubated with primary antibodies at 4 °C for overnight. Primary antibodies used in this study are α-Actin (Sigma-Aldrich), β-tubulin (BD Bioscience), Cdo (R&D system), Connexin 43 (Cell Signaling), cardiac troponin T (cTnT, Abcam), GATA4 (Santa Cruz), N-cadherin (Abcam), Mef2c (Abcam), MHC (MF20, Hybridoma Bank) and ZO-1 (Invitrogen).

2.3. RNA analysis

Total RNAs from P19 and ES cells were isolated with easy-BLUE reagent (Intron Biotechnology), cDNA of each samples was generated with 0.5μg of total RNA as templates. For gene expression profiling, the templates were analyzed by reverse transcription polymerase chain reaction using nTaq DNA polymerase (Enzynomics). Quantitative RT-PCR was performed using SYBR premix Ex taq (TakaRa) following Thermal Cycler Dice Real Time system analysis. Gapdh was used as a reference of gene expression. Primer sequences used in this study are listed below.

For Shh signaling genes: Shh, F; 5′-CTGGCAGATGTGTTTCTGCT-3′, R; 5′-GATCCTGCGGTGTATGG-3′, Patched1, F; 5′-TTTTGTGGTGTGGTCTCCC-3′, R; 5′-TCAACCTGAAAGGAGCTTA-3′, Glii F; 5′-GAAGGATATGTCGCGCTGAGCT-3′, R; 5′-GGATCTGTCGAGGCTGGTC-3′, Cdo F; 5′-GCGGCTGCTGGGTTTAATGTC-3′, R; 5′-CTCAATTTATGTTTCCACT-3′; Boc F; 5′-TGCTCTGGTGCTCCTCATA-3′, R; 5′-ATGGCATGATCGATGTTG-3′, Gas1 F; 5′-GCAACACTGTCACCCACACT-3′, R; 5′-AAAGACCCACCGTTAC-3′. For cardiovascular progenitor markers: Brty-F; 5′-CGCGTTGCTGAAAGTAAACTG-3′, R; 5′-TCACTTGAGCTGTTGTA-3′, Bmp4 F; 5′-TGATACCTGAGACCCGGGAAG-3′, R; 5′-AGCCGGTAAAGATCCCTCCAT-3′. For mesodermal differentiation markers: Brty-F; 5′-CGCGTTGCTGAAAGTAAACTG-3′, R; 5′-TCACTTGAGCTGTTGTA-3′, Bmp4 F; 5′-TGATACCTGAGACCCGGGAAG-3′, R; 5′-AGCCGGTAAAGATCCCTCCAT-3′. For vascular cell markers: CD31, F; 5′-TGGGCATGTTGCTGCTCTGACGTA-3′, R; 5′-TCACTTGAGCTGTTGTA-3′, Bmp4 F; 5′-TGATACCTGAGACCCGGGAAG-3′, R; 5′-AGCCGGTAAAGATCCCTCCAT-3′.
2.4. β-galactosidase staining and whole-mount in situ hybridization

For wholemount β-galactosidase staining of Cdo+/− and Cdo−/− embryos, embryonic day 8.5 and 9.5 embryos were harvested, followed by β-galactosidase staining essentially as previously described [26]. Images were obtained with a Nikon SMZ-1500 microscope. RNA in-situ hybridization of E9.5 mouse embryo was carried out as previously described [21,32]. Briefly, harvested embryos were fixed, dehydrated and embedded in waxes. Embryos were then dehydrated into PTW buffer (0.1% Tween-20 in PBS) and bleached with 6% hydrogen peroxide (H2O2). After three times of washing, embryos were treated with proteinase K (10 μg/ml in PTW, Qiagen) for 20 min. Embryos were then rinsed, post-fixed, and equilibrated in the hybridization solution (50% formamide, 1.3X SSC, 5 mM EDTA, 50 μg/ml yeast tRNA, 0.2% Tween-20, 0.5% Chaps, 100 μg/ml heparin) at 65 °C followed by incubation of digoxigenin-labeled RNA probe overnight. After five times of washing, embryos were blocked and incubated overnight at 4 °C with alkaline-phosphatase-conjugated antidigoxigenin antibody (Roche) in blocking buffer (2% Blocking Reagent (Roche), 20% heat-inactivated sheep serum in MABT (100 mM maleic acid at pH 7.5, 150 mM NaCl, 0.1% Tween-20)). After washing the embryos with MABT and NTMT (500 mM NaCl, 100 mM Tris/HCl (pH 9.5), 50 mM MgCl2, 1% Tween-20), signals were developed using BM Purple AP substrate (Roche). In situ probe for Nkx2.5 is kindly provided by Dr. Guntram Suske at IMT.

2.5. FACS analysis

To analyze the number of cTnT positive ESCs, 25 EBs of each genotypes were plated and induced cardiac differentiation for 9 days. ESCs were permeabilized, and incubated with anti-cTnT antibody followed by blocking with 2% FBS in PBS on ice. After 1 h incubation, anti-mouse FITC conjugated secondary antibody was treated for 20 min. Cells stained with mouse IgG control antibody with anti-mouse FITC conjugated secondary antibody were used as control. Flow cytometric analysis was performed with a FACScanto II (BD Biosciences), and the acquired data were analyzed using the FlowJo software (Tree Star).

2.6. Microinjection of Lucifer Yellow

Dye transfer through gap junction channels was investigated using beating ESCs cluster. Lucifer Yellow (LY) (Molecular Probes) was dissolved in the pipette solution to reach a concentration of 2 mmol/l [33]. A micropipette (tip resistance 2.5 ± 0.5 MΩ) was used to inject LY into cell, after a gigaohm seal was established, the cell membrane at the tip of the pipette was ruptured by applying brief suction. Microinjection and image recording were performed using confocal microscopy (Carl Zeiss).

2.7. Statistical analysis

Statistical analyses of the results are expressed as mean ± SD from at least three independent experiments. Error bars represent means ± SD. For comparison between multiple groups, statistical significance was tested by ANOVA test using SPSS (12.0 version; SPSS).

3. Results

3.1. Cdo depletion reduces cardiomyogenesis of P19 EC cells

To examine the function of Cdo in cardiomyogenesis of pluripotent stem cells, the initial study was carried out with P19 EC cells. P19 cells can differentiate efficiently into mesodermal cell lineages, such as smooth, skeletal and cardiac muscles when cells are induced to form aggregates and treated with DMSO [34]. The protocol for P19 EC cell differentiation into cardiomyocytes is schematically depicted in Fig. 1A, using for a 4 day aggregation protocol. Changes in gene expression during cardiomyogenesis were assessed by semi-quantitative RT-PCR for cardiac regulatory genes (Fig. 1B). A mesodermal marker, Brachyury T (BryT) and cardiac regulators, Flk-1, Bmp4, Nkx2.5 and Gata4 are induced from the induction day 1 (I1) while Pdgfrα and Isl-1 (I5-I) were induced from I2 or I3, respectively. Alpha-Smooth muscle actin (αSMA, an immature cardiomyocyte marker and a smooth muscle marker) and cardiac Troponin T (cTnT, a cardiomyocyte marker) started to be expressed weakly at I3 and the robust expression was observed from differentiation day 1 (D1). Next we have examined the expression of Cdo and Shh signaling components during cardiomyogenesis. Three Shh coreceptors, Cdo, Boc and Gas1, the primary receptor Ptc1, and GlI1 were induced during the induction period with different kinetics (Fig. 1C). Cdo was induced concomitantly with GlI1 and Ptc1, two of Shh target genes at I1, while the expression of two other coreceptors Boc and Gas1 was initiated 2 days later. These data suggest that Cdo may play a primary role in activation of Shh signaling during specification and differentiation of P19 EC cells into cardiogenic lineages. Wnt/β-catenin signaling has been implicated in cardiac development and cardiomyogenesis [35–38]. Wnt/β-catenin signaling promotes cardiac cell commitment at the early stage however inhibit cardiomyocyte differentiation in the late stage. Thus, we have also assessed the expression of components of Wnt signaling, Wnt3, Wnt3a and a Wnt target gene Axin2 (Fig. 1C). Similarly to Shh signaling components, the expression of Wnt3a, Axin2 and GlI1 was transiently elevated upon cardiac induction and decreased in the late differentiation stage, which is consistent with the proposed biphasic function of Wnt signaling in cardiomyogenesis [35].

To examine the role of Cdo in cardiomyogenesis of P19 EC cells, control or Cdo shRNA expressing P19 cells were induced to differentiate and analyzed by RT-PCR for the expression of cardiac markers, Gata4, cTnT and αSma and by immunostaining for α-Axin, Mef2c, Myosin heavy chain (MHC) and Nkx2.5. The expression of Cdo shRNA resulted in the efficient depletion of Cdo expression during cardiomyogenic induction and differentiation. Cdo depletion in P19 cells decreased the expression of Gata4, cTnT and α-SMA (Fig. 1D). In addition, the immunostaining analysis of these cells at D6 revealed that Cdo depletion starkly decreased the number of cells positive for expression of cardiomyocyte markers, α-Axin, Mef2c, MHC and Nkx2.5 (Fig. 1E and F). Consistently, the expression of Nkx2.5 and Mef2c was drastically decreased in Cdo-depleted P19 EC cells, compared to the control cells (Fig. 1G). Taken together, these data suggest that Cdo is induced upon cardiomyogenic induction and required for cardiomyogenesis.

3.2. Cdo-deficient mES cells exhibit decreased expression of cardiac regulators

To further examine, we utilized Cdo+/+ and Cdo−/− mES cells that we have established and characterized previously [28]. The differentiation protocol used in this study is depicted in Fig. 2A. To generate even-sized cell aggregates, 300 cells were aggregated as a hanging droplet for 2 days in the presence of 0.5% DMSO and further cultured on the gelatin-coated plates as the individual embryoid bodies (EBs) for cardiac differentiation generally for 14 days to 20 days. The morphologies of EBs from hanging droplet cultures and differentiating EBs were similar between Cdo+/+ and Cdo−/− mES cells (Supplementary Fig. 1), suggesting that Cdo deficiency does not affect greatly cell growth. Consistent with data obtained with P19 EC cells, Cdo-deficient EBs showed a decreased expression of cardiac markers, including Gata4, Gata6, Nkx2.5, Flk1, Isl1, cTnT,
Mef2c (Fig. 2B). In addition, the expression of Shh was significantly decreased in Cdo−/− EBs from D3 to D14, compared to Cdo+/+ EBs. Moreover Cdo+/+ EBs were immunostained intensely for cTnT and Mef2c at D10 while Cdo−/− EBs displayed decreased staining intensities for these genes (Fig. 2C). Furthermore, FACS analysis shows roughly 46.3% of cells in Cdo+/+ EBs were positive for cTnT at D9 while only 7.8% of cells were positive for cTnT in Cdo−/− EBs (Fig. 2D). To further verify, the individual Cdo+/+ and Cdo−/− EBs at D14 were dissociated and plated on gelatin-coated slide chambers for 2 days, followed by immunostaining with GATA4 and Mef2c antibodies. Similarly to the gene expression data, Cdo−/− EBs had a lower percentile of cells positive for GATA4 and Mef2c expression, relative to Cdo+/+ EBs (Fig. 2E and F). Interestingly, the expression level of vascular progenitor markers such as CD31 and Tie1 was significantly increased in Cdo-deficient EBs at D7 and D14 (Fig. 2G). Wnt signaling was also examined in EBs between D5 and D14. As seen in Supplementary Fig. 2, the expression of Wnt3a was greatly elevated in Cdo-deficient EBs at D10 and D14, compared to the wildtype EBs. These data suggest that Cdo expression is critical for the expression of cardiac regulators and cardiomyocyte differentiation.

3.3. Cdo-deficient ES cells exhibit defects in maturation of contractile cardiomyocytes

A small fraction of EBs from the wildtype cells started to contract around 5 days of differentiation (D5) which increased progressively and roughly 63% EBs contracted at D20, while Cdo−/− cells generated a small fraction of contracting EBs at D14 and roughly 25% of EBs contracted at D20 (Fig. 3A). Among the contracting EBs, around 70% of Cdo−/− EBs displayed an asynchronous contraction behavior, while only 10% of wildtype EBs showed such characteristics (Fig. 3B and C; Supplementary Video Files 1 and 2). Connexin 43 (Cx43) is a major gap junctional protein and plays a critical role for the cell coupling in adult myocardium [39]. Furthermore, Cx43 is critical for normal cardiac development [40] and the immature cardiomyocyte can trigger the pro-arrhythmogenic phenotype by low coupling [41]. Thus we
examined whether Cx43 is normally expressed in Cdo−/− cardiomyocytes by immunostaining. To do so, the contracting Cdo+/+ and Cdo−− colonies at D14 were dissociated and plated as a monolayer for 2 days followed by immunostaining for α-Actinin and Cx43. As shown in Fig. 3D and E, ~90% of Cdo+/+ cardiac cells with a typical tubular α-Actinin staining exhibited the immunopositivity for Cx43 at the cell-cell junctions while roughly 43% of Cdo−− cardiomyocytes had decreased Cx43 signals at the cell junctions, suggesting that an abnormal regulation of Cx43 may reflect delayed cardiomyogenesis. However, we failed to detect any changes in expression levels or modification of key gap junctional proteins such as Cx43, N-cadherin and ZO-1 (Fig. 3F).

3.4. The shh agonist treatment restores cardiomyocyte differentiation of cdo-deficient ES cells

Next we examined whether the defects in cardiomyogenesis caused by Cdo deficiency are due to its role in Shh signaling. To assess the Shh signaling activities, RNAs isolated from Cdo+/+ and Cdo−− EBs at D5 were analyzed by qRT-PCR. The expression of Shh and Gli1 was substantially decreased in Cdo-deficient EBs, while Ptc1 was not significantly altered, compared to the Cdo+/+ EBs (Fig. 4A), suggesting that Shh signaling is decreased in Cdo−− EBs during cardiomyogenesis. To assess whether the reactivation of Shh signaling in Cdo-deficient EBs can restore cardiomyogenesis, Cdo+/+ and Cdo−− EBs were treated with either vehicle DMSO or a Shh agonist Purmorphamine (Pur) with a final concentration of 2 μM and examined its effect on cardiomyogenesis. First we have tested 3 different conditions of Pur treatment to determine the optimal time point and duration of treatment; the treatment either for the initial 2 days during aggregation, for the initial 3 days upon differentiation or for the combined 5 days, and observed the best results from the Pur treatment for the initial 5 days of cardiogenic induction (data not shown). Therefore cells were treated with Pur for the initial 5 days followed by analysis for the expression of Shh signaling components and cardiac markers. The Pur treatment restored the expression of Gli1 at D2, Ptc1 at D2 or Shh at D5 in Cdo−− EBs. Furthermore the expression of cardiac markers, such as Gata4, Gata6...
and MeF2c was restored to a similar level of wildtype EBs at D10 (Fig. 4B). The qRT-PCR analysis of DMSO or Pur-treated Cdo<sup>+/+</sup> EBs from D5 showed that the ectopic Shh signaling activation increased only slightly the expression of Shh, Gata4, Gata6, MeF2c, Flk1 and Pdgfrα compared to the DMSO control. However, the Pur treatment of Cdo<sup>−/−</sup> EBs restored the expression of Gata4, Gata6 and Pdgfrα almost to the level of control Cdo<sup>+/+</sup> EBs (Fig. 4C). In addition, control or Pur-treated Cdo<sup>+/+</sup> and Cdo<sup>−/−</sup> EBs at D10 were immunostained for the expression of Gata4A, MHC and Nkx2.5. The Pur treatment enhanced the expression of Gata4A, MHC and Nkx2.5 in Cdo<sup>−/−</sup> EBs (Fig. 4D). Together, these data suggest that the defects in cardiomyogenesis in Cdo<sup>−/−</sup> EBs are caused by decreased Shh signaling activation.

3.5. The Shh agonist treatment restores the synchronous contraction and junctional Cx43 localization in Cdo-deficient ES cells

To further examine the effect of Pur treatment on cardiomyogenesis, the percentile of contractile EBs was analyzed from D8 to D16. Roughly 10% of EBs from control or Pur-treated Cdo<sup>+/+</sup> EBs had contractile cells at D8 and this was further increased to 62–65% at D16. In contrast, only a small fraction of Cdo<sup>−/−</sup> EBs in the vehicle-treated cultures had contractile cells even at D16 however the Pur treatment restored the efficiency of contractile EB formation to the comparable levels of the control wildtype cells (Fig. 5A). In addition, the contractile area in individual EBs was quantified (Fig. 5B). The contractile area in Cdo<sup>−/−</sup> EBs was enlarged by the Pur treatment, but not significantly, compared to control-treated EBs. Furthermore the Pur treatment restored the contractile area of Cdo<sup>−/−</sup> EBs comparable to that of control wildtype EBs (Fig. 5B; Supplementary Video Files 3–6). Among contractile EBs, roughly 15% of control-treated Cdo<sup>−/−</sup> EBs at D12 displayed an asynchronous contraction which was not altered by the Pur treatment (Fig. 5D). However the Pur-treatment of Cdo<sup>−/−</sup> EBs reduced significantly the proportion of asynchronously contracting EBs, from ~55% to ~32% of total contracting EBs, compared to the control treatment. We analyzed Cx43 distribution in Cdo<sup>−/−</sup> EBs with or without Pur-treatment. Control and Pur-treated Cdo<sup>−/−</sup> EBs from D12 cultures were dissociated, cultured as a monolayer on slide chambers for 2 days, followed by immunostaining for Cx43 and α-Actinin. The Pur treatment restored the junctional localization of Cx43 in Cdo<sup>−/−</sup> cardiomyocytes (marked with red arrowheads) (Fig. 5E and F). Next we analyzed the gap junction activity by using Lucifer Yellow, a transportable dye through gap junctions in Cdo<sup>−/−</sup> EBs at D12 with or without Pur-treatment. As shown in Fig. 5G, the control DMSO-treated Cdo<sup>−/−</sup> EBs exhibit slower dye transfer while Pur-treated Cdo<sup>−/−</sup> EBs exhibited accelerated dye transfer. These data suggest that Pur-treatment restores the delayed cardiomyogenesis in Cdo<sup>−/−</sup> EBs with contractile characteristics and enhanced gap junctional function.

3.6. A modest reduction in Shh signaling activity in developing hearts of Cdo null mice

The signal inflow of Shh from foregut endoderm to cardiac mesoderm is critical for early heart development, such as heart looping and left-right asymmetry [42]. While Cdo<sup>−/−</sup> ES cells exhibit delayed cardiomyogenesis and decreased Shh signaling activity, Cdo null mice did not exhibit overt defects in early heart development [25]. To clarify its role in heart development, we have examined the expression of Cdo
in developing heart by assessing the activity of the transgene β-galactosidase, as previously described [26,32]. A weak blue staining for Cdo expression was observed in developing primitive heart ventricle and outflow tract in E8.5 and E9.5 mouse embryos, in addition to other tissues, such as somites and neural tubes (Supplementary Fig. 3A and B). The β-galactosidase staining of E13.5 Cdo+/- heart sections and E18.5 heart showed a strong expression of Cdo in the outflow tract, epicardium (Supplementary Fig. 3C and D). To determine Shh signaling activity in Cdo null mice, the RNA in situ hybridization for Shh signaling molecules, Shh and Gli1, and Nkx2.5, a cardiac marker was performed in E9.5 mouse embryos. In Cdo−/− embryos, Shh and Gli1 were modestly reduced in foregut endoderm and aortic sac, compared to control littermate embryos (Supplementary Fig. 4A and B). Moreover, the level of Nkx2.5 was also modestly deceased in Cdo−/− embryo, compared to Cdo+/- littermate (Supplementary Fig. 4C). These data suggest that Cdo deficiency decreases modestly Shh signaling in developing heart. Taken together, Cdo is required for efficient cardiomyogenesis of pluripotent stem cells through activation of Shh signaling.

4. Discussion

In this study, we examined the role of Cdo, a Shh coreceptor in cardiomyogenesis of pluripotent stem cells. Collectively, our data suggest that Cdo is critical for the induction of efficient cardiomyogenesis. Cdo deficiency causes defective cardiomyogenesis of ES cells and this defect can be overcome by reactivation of Shh signaling at the initial 5 days of cardiogenic induction of ES cells. Thus, our study further supports for the critical role of the optimal Shh signaling activation to induce the efficient cardiomyogenesis and formation of contractile cardiomyocytes. The importance for Shh signaling in cardiomyogenesis...
has been underlined by several previous studies [17,43]. Data obtained from this study is in agreement with previous reports showing that the inhibition of Shh signaling resulted in defects in myocardial progenitor specification and reduced number of ventricular and arterial cardiomyocytes [44]. The expression of Gli2 promotes cardiomyogenesis of P19 EC cells accompanied by the induction of cardiac regulators through a synergistic action with Mef2C [43]. The recent study has shown that the small molecule cocktail containing a Wnt antagonist, a Shh agonist and a TGFβ/Nodal receptor kinase antagonist can increase the efficiency of cardiomyocyte differentiation of human ES cell-derived primitive streak cells [45]. In agreement with previous studies, we demonstrate that the expression of key cardiac regulators, such as Nkx2.5, GATA4 and MEF2c, is significantly reduced in Cdo-depleted cells accompanied by the decreased expression of Shh and Gli1, suggesting that Cdo is required for Shh signaling activation during cardiomyogenesis. In addition, Cdo-deficient EBs showed a marked reduction in cardiomyocyte differentiation and the formation of contracting colonies. Since the Pur treatment restored both the expression of early cardiac regulators and the formation of contracting colonies in Cdo-deficient EBs, Cdo deficiency appears to delay the differentiation and maturation of cardiomyocytes via reduction of Shh signaling strength. Pur treatment also restored junctional expression of Cx43, which is accompanied by the increase of synchronously contractile cardiomyocytes.

The importance of Shh coreceptors, Cdo, Boc and Gas1, in cardiac development was underlined by early developmental defects and the embryonic lethality observed in triple knockout mice [23]. Even though mice null for Cdo displayed defects in ventral patterning of ventral forebrain and neural tube associated with decreased Shh signaling [26], these mice had no overt defects in early heart development [25].
However, it appears that Cdo deficiency causes a modest reduction in expression of Nkx2.5, Gli1 and Shh in developing cardiac tissue or foregut endoderm and detailed analysis will be required to address whether this reduction has any functional consequence. In contrast, Cdo seems to be important for cardiomyogenesis of ES cells and the optimal activity of Shh signaling required for efficient cardiomyogenesis. Although the reason for this requirement of Cdo in cardiomyogenesis of pluripotent stem cells is currently unclear, Cdo expression is detected prior to the expression of other coreceptors Boc and Gas1 in cells upon cardiogenic induction which might be important to activate Shh signaling to initiate the cardiogenic commitment.

Due to limited regenerative capacities of heart, stem cell therapy is an attractive strategy in addition to heart transplantation to treat heart failures. The manipulation of Shh signaling activation is advantageous to increase the efficiency in the favor of cardiogenic fate specification from undifferentiated stem cells. Considering Shh agonists are often included in induction of other coreceptors Boc and Gas1 in cells upon cardiogenic induction, Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly, Dev. Cell 10 (2006) 657–667.

Therefore, it seems that Cdo can be important for cardiomyogenesis of ES cells and the optimal activity of Shh signaling required for efficient cardiomyogenesis. Although the reason for this requirement of Cdo in cardiomyogenesis of pluripotent stem cells is unclear, Cdo expression is detected prior to the expression of other coreceptors Boc and Gas1 in cells upon cardiogenic induction which might be important to activate Shh signaling to initiate the cardiogenic commitment.

Disclosures
Not declared.

Author contributions
M.H.J., Y.E.L. and H.J.K. contributed to the experimental design, research and data analysis. H.J.K. and J.S.K. contributed to the experimental design and data analysis. H.J. and J.S.K. wrote the manuscript.

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