to recognize the signals. Cell events were collected using a FACS Calibur flow cytometer and data were analyzed by Cell Quest (BD Biosciences) and FlowJo software (Tree Star).

Real-Time RT-PCR

Total RNA was extracted from the cells using TRIzol, and RT-PCR was performed with a QuantiTect Reverse Transcription Kit (QIAGEN). The primer sequences are provided in Online Tables II and III in the Supplemental Materials (Applied Biosystems).

Gated Single Photon Emission Computed Tomography (SPECT) Study

All participants receiving CDC infusion were examined by a quantitative gated SPECT algorithm before and 3 and 12 months after treatment to measure RVEF on the basis of data during myocardial perfusion studies.[3] Patients were injected with a weight-adjusted dose (185-296 MBq) of Tc-99m-methoxyisobutylisonitrile (MIBI) under resting conditions. Data acquisition was performed with a double-head SPECT system (GCA7200A/DI, Toshiba, Japan) equipped with a low-energy, high-resolution collimator, and centered on the 140-keV energy peak with a 20% symmetrical energy window. A protocol consisting of a 64×64 matrix, 30 projections per head over a 180^0 circular orbit, and 16 frames per cycle was applied. The images were reconstructed using field back projection (Butterworth filter: order 8; cut-off frequency: 0.25 cycles/pixel). Tomographic images were displayed as short-axis, horizontal, and vertical long-axis slices. Gated short-axis images were processed using quantitative SPECT software and single-ventricular ejection fractions for rest were automatically calculated as described previously.[4, 5] Each study was analyzed by experienced radiologists with specialization on pediatric cardiology who were blinded to all clinical data including patients' trial enrollment.

Physiological and Functional Health Evaluation

Somatic growth for each patient was evaluated by height and weight converted to z-score for analysis with the Center for Disease Control normative population data, as previously reported.[6] The pathophysiological status of heart failure was defined by BNP measurement and Ross heart failure classification.[7] The severity of heart failure was further characterized based on physiological indicators and medical treatment and scored as New York University Pediatric Heart Failure Index (NYUPHFI).[8, 9] The condition of physical, mental, and social well-being of the enrolled patients at 18 months of follow-up was evaluated by The Infant Toddler Quality of Life Questionnaire™ (ITQOL-SF47).[10] Parenting stress in mothers of patients was evaluated by Abidin's Parenting Stress Index Short Form.[11]

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Online Table I. Angiographic assessments at baseline in enrolled patients

	Control group (n=7)	CDC-treated group (n=7)	*P* value
Right distal PA diameter (mm)	7.4 ± 2.3	9.2 ± 2.0	0.20
Left distal PA diameter (mm)	7.1 ± 1.9	8.0 ± 2.3	0.52
Nakata index (mm^2/m^2)	279.1 ± 82.4	261.4 ± 74.3	0.72
Mean right PA pressure (mmHg)	10.8 ± 1.2	9.8 ± 3.0	0.46
Mean left PA pressure (mmHg)	11.3 ± 2.1	10.0 ± 2.5	0.36
PVRI (Wood unit·m^2)	1.6 ± 0.5	1.3 ± 0.3	0.19
SVC pressure (mmHg)	6.5 ± 3.1	9.0 ± 3.5	0.22
IVC pressure (mmHg)	5.3 ± 1.2	4.8 ± 1.3	0.51
RA pressure (mmHg)	5.0 ± 1.0	4.8 ± 1.3	0.82
RV end-diastolic pressure (mmHg)	6.5 ± 2.1	5.7 ± 1.2	0.41
Right PV pressure (mmHg)	7.0 ± 1.3	5.6 ± 1.8	0.17
Left PV pressure (mmHg)	7.0 ± 1.3	5.8 ± 1.5	0.18
Systolic aortic pressure (mmHg)	79.1 ± 14.1	77.5 ± 7.1	0.80
LA pressure (mmHg)	6.4 ± 1.8	4.5 ± 2.0	0.13
Qp/Qs	0.8 ± 0.3	0.6 ± 0.1	0.26

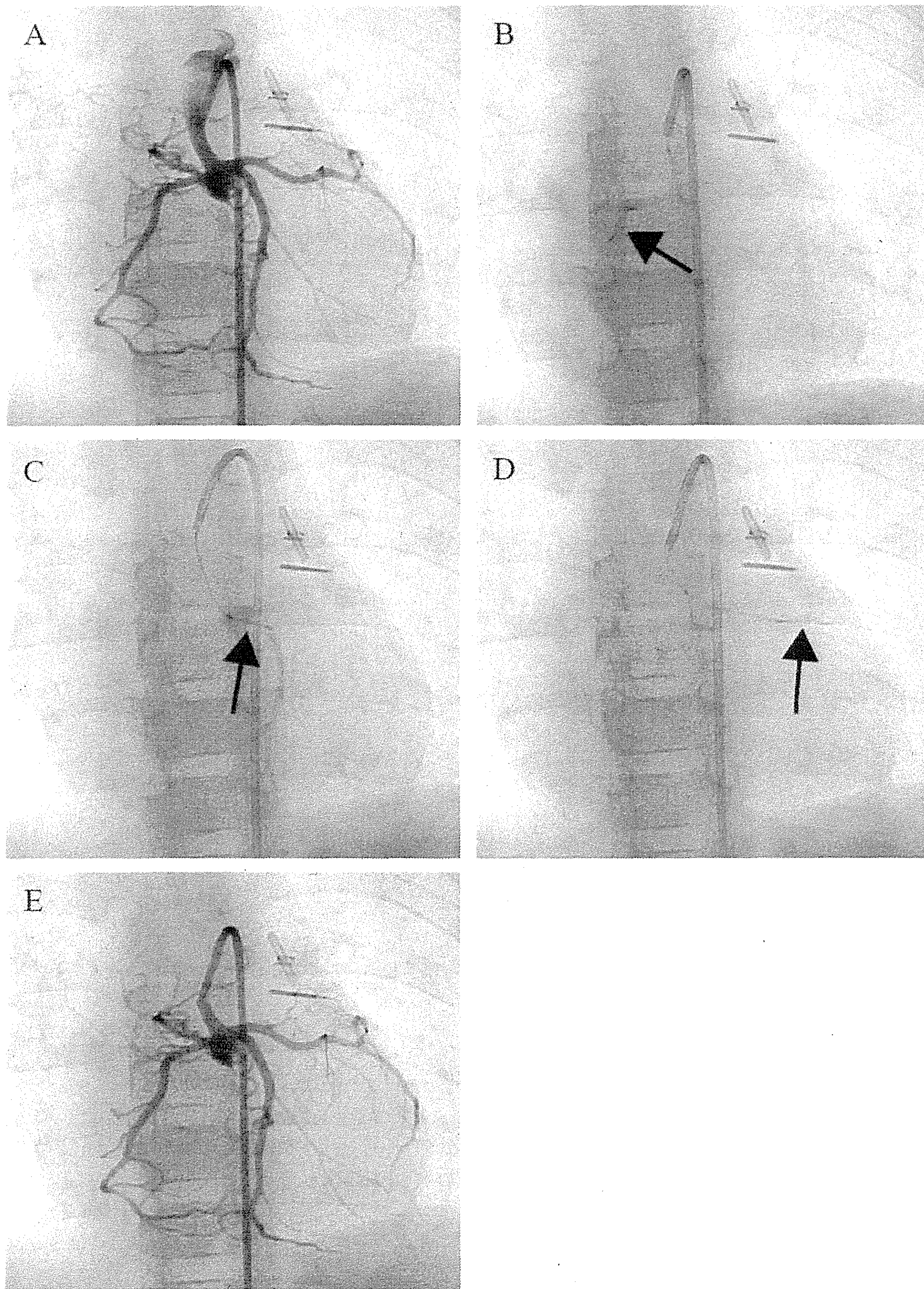
Abbreviations: Nakata index is calculated by the cross-sectional area of right and left PA divided by BSA; PVRI, pulmonary vascular resistance index calculated by mean PA pressure minus mean pulmonary capillary wedge pressure divided by systemic pulmonary blood flow and corrected by BSA; SVC, superior vena cava; IVS, inferior vena cava; RA, right atrium; PV, pulmonary vein; LA, left atrium; Qp/Qs, total pulmonary blood flow/total systemic blood flow ratio. Data are expressed as mean ± SD.

Online Table II. PCR primers used for sample validation

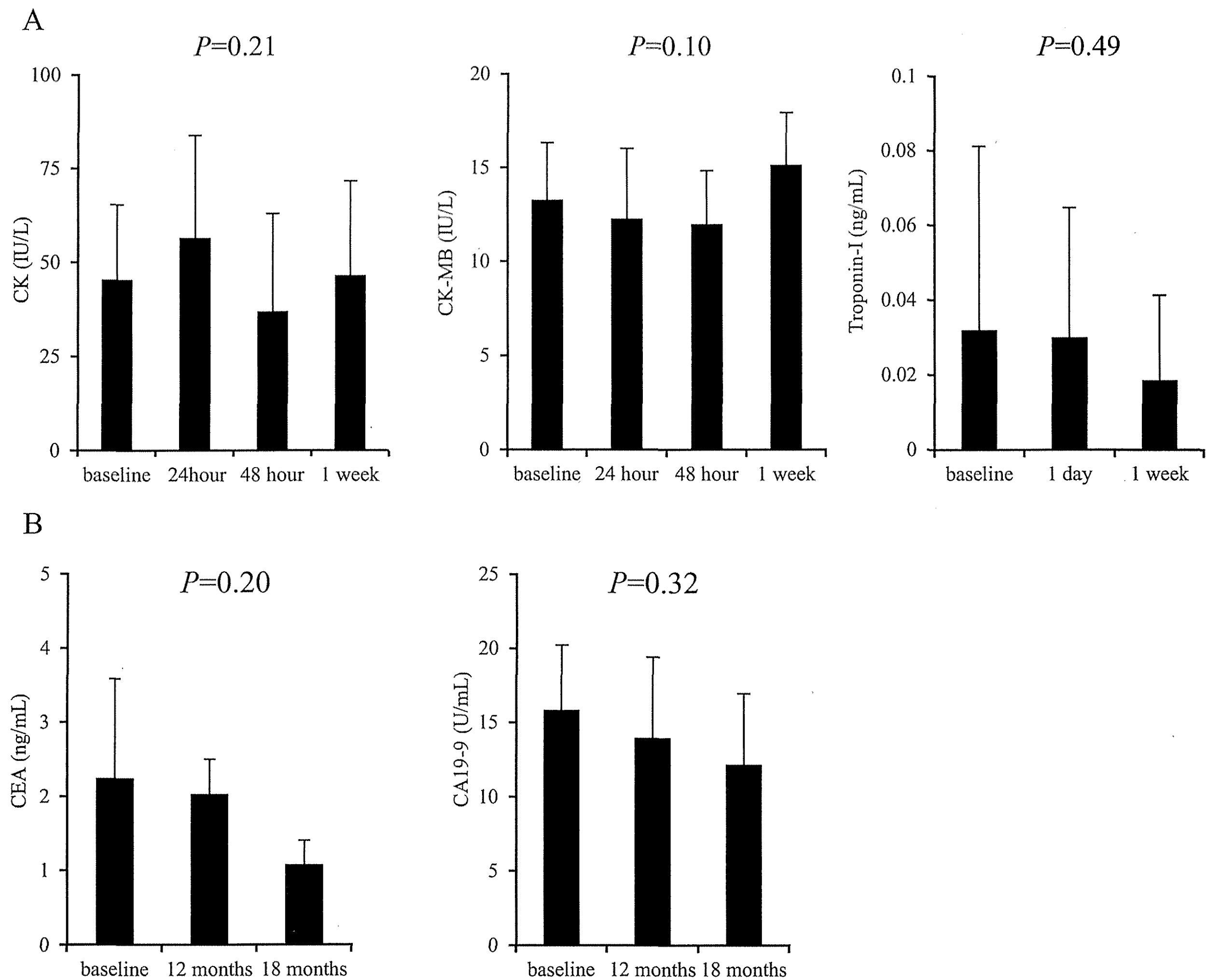
Gene	Forward primers	Reverse primers
GATA4	GGAAGCCCAAGAACCTGAAT	GTTGCTGGAGTTGCTGGAA
Mef2c	ATCTGCCCTCAGTCAGTTGG	GGGGTGGTGGTACGGTCT
Tbx5	TGATCATAACCAAGGCTGGAA	GATTAAGGCCCGTCACCTTC
Hand2	TCAAGAAGACCGACGTGAAA	GTTGCTGCTCACTGTGCTTT
Myocd	TCTCATGAGCAGCCTGAATG	TTGTCCTTCTCGGAGTCCA
Mesp1	CTGTTGGAGACCTGGATGC	CGTCAGTTGTCCCTTGTCAC
Nkx2.5	CTAAACCTGGAACAGCAGCA	CGTAGGCCTCTGGCTTGA
Flk1	GGTTGCATTACTGTACCCATCA	TTTTAGGTGTCGGCCACTGT
SCN5a	CGGGAGCTTTGAGACAGGT	CTAAGGGTTGGGTCTAGCTCAC
Kir2.1	GGTGGAAGCTCATGTTCGAG	AGAGGGATATACTCCCCTTCAGA
Kir2.2	CCCTCTTGGAGGTCTGTGC	GCCTTTGGGGAGATGAGG
α1c	GGGGGTGTTTTCACATTTCTT	CATTGACCATGGACCAAAAA
Ryr2	CAGTAGTTTTGGGTTTTGCTCA	TGGCCTGACAAGAAGTCCTTA
SERCA2	AACGTCGGGGAAGTTGTCT	GAATCAAAGCCTCGGGAAAT
IP3R-1	TCATTCTTCTGGATTAGTGACTGC	AAAAGCAGAAGAGACAGGAGATTT
NPPA	TTCTCCACCACCACCGTGA	GGCTCCAATCCTGTCCATCC
β2MG	GTGCTCGCGCTACTCTCTCT	TCAATGTCGGATGGATGAAA

Online Table III. TaqMan primers used for sample validation

Gene	Assay
ELN	Hs00355783
TNNT2	Hs00165960
MYH6	Hs00411908
MYL2	Hs00166405
β2MG	Hs99999907

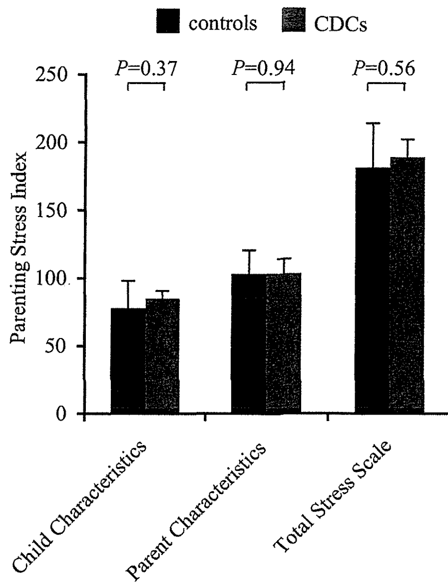


Online Figure I. Angiographic images of transcoronary infusion of CDCs into children with HLHS.
(A) Primary coronary angiography was performed prior to the CDC infusion. A 5 French guiding catheter Launcher (Medtronic) was placed in the hypoplastic ascending aorta, which was connected to the newly reconstructed aortic arch by the Norwood procedure. Selective CDC transfer was performed into right (B), left ascending (C), and left circumferential (D) coronary arteries by a stop-flow technique (arrows). (E) Hand injection of contrast medium demonstrates that the coronary arteries were patent after CDC infusion.

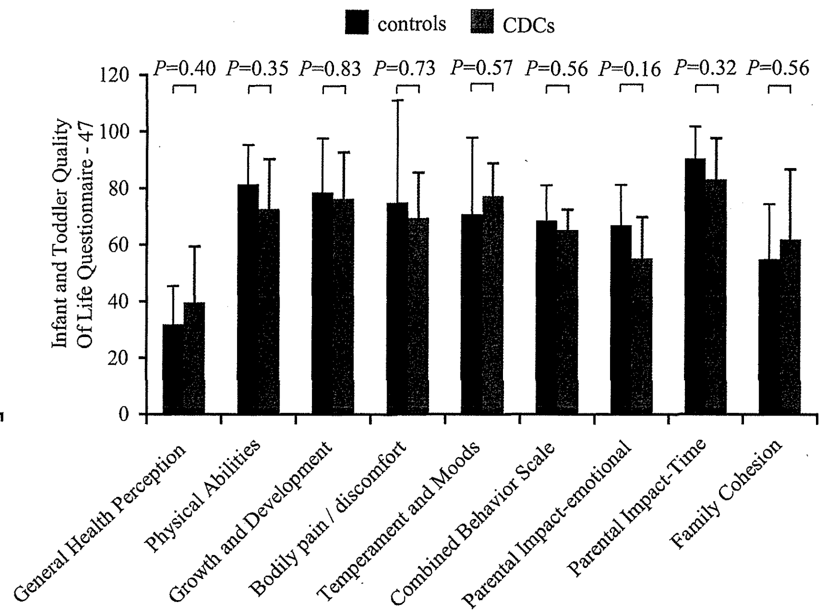


Online Figure II. Myocardial ischemia and tumor marker analyses after CDC infusion.
(A) Total creatinine kinase (CK) and its muscle subtype (MB) were examined at baseline, 24 hours, 48 hours, and 1 week after cell infusion. Cardiac troponin-I (~ 0.04 ng/mL) was measured at baseline, 24 hours, and 1 week post infusion. One patient had severe heart failure even 1 month after stage 2 shunt procedure (BNP=163 pg/mL) and showed abnormal cardiac troponin-I levels at baseline through 1 week after cell infusion. (B) Specific tumor markers such as CEA and CA19-9 were assessed at baseline, 12 months, and 18 months in CDC-treated patients. Data are presented as mean ± SD. One-way ANOVA repeated measures were used for analysis. All parameters measured showed no statistically significant changes between baseline and after CDC infusion.

A



B



Online Figure III. Quality of life assessed in mother of children with HLHS who enrolled in TICAP study. Parenting stress test (A) and The Infant Toddler Quality of Life Questionnaire™ (B) were obtained from mothers of children with HLHS those were allocated to receive CDC infusion or controls after standard surgical palliations. Data were analyzed by using 2-tailed unpaired Student's t-test.

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Intracoronary Autologous Cardiac Progenitor Cell Transfer in Patients With Hypoplastic Left Heart Syndrome: The TICAP Prospective Phase 1 Controlled Trial

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REVIEW

Epigenetic modification in congenital heart diseases by using stem cell technologies

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Congenital heart diseases are the most common birth defects, occurring in more than 1% of newborns. The gene regulatory network of cardiac development has been revealed and some cases of congenital heart diseases have been shown to be associated with cardiac-specific gene mutations or related chromosomal abnormalities; however, almost all cases of congenital heart diseases are sporadic and the pathogenesis of heart malformations still remains unknown. Recently, studies of epigenetic regulation have been making progress in the field of cardiac development and the accumulated knowledge helps us understand more about cardiogenesis and congenital heart diseases. Interestingly, pluripotent stem cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells have attracted attention as tools to dissect epigenetic regulation during differentiation. Reprogramming and differentiation of ES cells and iPS cells can be induced by changes of gene expression patterns and epigenetic regulation, not by changing DNA sequences. We can also modify histone patterns and chromatin structures using chemical reagents. Hence, pluripotent stem cells enable us to dissect and modify epigenetic regulation during cardiogenesis in vitro. Moreover, in terms of iPS cells, they have become disease models because they can be generated from the somatic cells of patients. In this review, we summarize the recent findings of epigenetic regulation in cardiac development and congenital heart diseases. We also review the epigenetic modification during the reprogramming and cardiac differentiation of ES cells and iPS cells, and introduce our study showing that the disease-specific iPS cells of hypoplastic left heart syndrome, one of the severest congenital heart diseases with single ventricular circulation, exhibit unique epigenetic regulation during differentiation. Furthermore, we briefly focus on modifications of epigenetic regulation in cardiac development using chemical reagents, which suggest the possibility of treating congenital heart diseases using drugs. Lastly, we discuss the epigenetic memory of iPS cells, a specific feature that often complicates or prevents study of the differentiation of iPS cells.

Keywords: Epigenetic regulation, hypoplastic left heart syndrome; congenital heart disease; stem cell technologies; cardiac development

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Introduction

Patients with congenital heart defects often need surgical treatment. Serious heart anomalies lead to high mortality and require sequential operations or heart transplantation. Recently, cardiovascular development has been well studied

and numerous genes related to cardiogenesis have been confirmed [1]. Some gene mutations or chromosomal abnormalities were also found in patients with congenital heart diseases; however, almost all cases of congenital heart diseases are sporadic and the genetic background remains unknown. The lack of animal models for congenital heart diseases hampers the study of the pathogenesis. Recent studies have revealed the epigenetic regulation during cardiovascular development, and have also shown that impaired epigenetic modification during cardiogenesis causes congenital heart diseases [2,3]. To understand cardiac development and the pathogenesis of congenital heart diseases, it might be necessary to understand epigenetic regulation. To investigate epigenetic modification during cardiac development, pluripotent stem cells such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells might be powerful tools because we can recapitulate cardiogenesis in the process of differentiation from pluripotent stem cells into cardiomyocytes in vitro. In terms of iPS cells, we can reset the disease phenotypes in the process of reprogramming when we generate these cells. In the processes of reprogramming and differentiation, genetic information remains unchanged, while the epigenetic regulation and expression pattern of genes change dynamically. Therefore, we can investigate the epigenetic changes during cardiogenesis in vitro by using pluripotent stem cells. This review focuses on the recent findings of epigenetic modification during cardiac development and congenital heart diseases, as well as investigation of epigenetic regulation using stem cell technologies. As an example to study the epigenetic regulation of congenital heart diseases by using pluripotent stem cells, we introduce our study of iPS cells from patients with hypoplastic left heart syndrome (HLHS), one of the severest congenital heart diseases with single ventricle circulation. Furthermore, we mention the possibilities of new therapy for cardiac malformations by using chemical reagents that modify epigenetic regulation, and discuss the recently emerged problem in studies on the differentiation of iPS cells: epigenetic memory.

Epigenetic regulation of cardiovascular development and congenital heart diseases

Epigenetic regulation mainly involves DNA methylation, ATP-dependent chromatin remodeling, and covalent histone modification. Recent studies have revealed that the regulation of ATP-dependent chromatin remodeling and covalent histone modification is related to cardiogenesis and the formation of congenital heart defects [2,3] (Table 1). The ATP-dependent chromatin-remodeling complexes consist of switching defective/sucrose nonfermenting (SWI/SNF), imitation switch (ISWI), chromodomain, helicase, DNA binding (CHD), and inositol requiring 80 (INO80) complexes. Other chromatin-modifying enzymes that catalyze the covalent modification of histones include histone deacetylases (HDACs), histone acetyltransferases (HATs), and histone methyltransferases (HMTs). These chromatin remodelers and histone modifiers coordinate to regulate cardiac development, and impaired regulation could cause the failure of cardiogenesis, leading to congenital heart diseases. In this section, we summarize the epigenetic regulation related to cardiogenesis and congenital heart diseases.

Regulation of vascular formation

Recent studies have demonstrated the epigenetic regulation during vascular development. Brg1, encoding the ATPase subunit of the BAF complex, is indispensable for yolk sac vascular development [4]. The deletion of Brg1 in smooth muscle cells of mice leads to persistence of patent ductus arteriosus (PDA) [5]. In addition, Baf180, a subunit of the BAF complex, is essential for coronary vessel formation [6]. Hdac7 as a class II HDAC is also related to vascular development, and Hdac7-disrupted embryo cannot maintain vascular integrity [7]. In vitro, HDAC7 silencing in endothelial cells was also shown to be associated with altered morphology, migration, and capillary structures [8].

Regulation of heart formation

Numerous chromatin remodelers and histone modifiers have important roles in regulating heart formation. Brg1 in the endocardium regulates the establishment of cardiac jelly necessary for myocardial trabeculation via repressing Adamts1 [9]. The deletion of Brg1 in the myocardium causes ventricular septum defect (VSD) [10]. Deletion of Brg1 in the secondary heart field also causes hypoplastic outflow tract and right ventricle [10]. Knockdown of Baf60c, a component of BAF complexes, results in impaired secondary heart field formation [11]. Germline deletion of Baf180 in the mouse embryo is associated with hypoplastic ventricle and VSD [12], as well as coronary vessel formation [6]. Deletion of both Hdac1 and Hdac2 in the myocardium is associated with dilated cardiomyopathy and arrhythmia [13]. In addition, doubly null mice of Hdac5 and Hdac9 show VSD [14]. The phenotypes of Sirt1 in class III HDAC deletion in mice show atrial septal defect (ASD), VSD, and heart valve defect [15]. Furthermore, point mutation of p300, a member of the HATs, leads to heart defects such as ASD and VSD [16]. The germline deletion of jumonji, a member of the jumonji family of histone demethylases, results in double outlet right ventricle (DORV) and hypertrabeculation [17]. Germline deletion of Smyd1, which encodes HMTs, causes ventricular hypoplasia [18]. Wolf-Hirschhorn syndrome candidate 1

(Whsc1) knockout mice exhibit ASD and VSD [19]. Finally, in patients with Kabuki syndrome, a mutation of mixed lineage leukemia 2 (MLL2) was found [20].

Table 1. The epigenetic regulation in cardiac development and congenital heart diseases

Role	Classification	Factor	Modification	Phenotype
Vascular formation	SWI/SNF	Brg1	Mutation in endothelium in mice	Abnormal vascular remodeling in yolk sac
			Deletion in smooth muscle cells in mice	Persistent ductus arteriosus (PDA)
		Baf180	Deletion in germline in mice	Failure of coronary vessel formation
	Class II HDAC	Hdac7	Deletion in germline in mice	Vascular abnormality
			Silenced in the endothelium	Altered endothelial morphology, migration, and structure
Heart formation	SWI/SNF	Brg1	Deletion in the endocardium	Loss of cardiac jelly leading to hypotrabeculation
			Deletion in the myocardium	Ventricular septal defects
			Deletion in the secondary heart field	Hypoplastic outflow tract and right ventricle
		Baf60c	Knockdown in mouse embryo	Impaired secondary heart field formation
		Baf180	Germline deletion	Hypoplastic ventricle, VSD, coronary vessel defects
	Class I HDAC	Hdac1,2	Both Hdac1 and Hdac2 deletion in the myocardium	Dilated cardiomyopathy, arrhythmia
	Class II HDAC	Hdac5,9	Both Hdac5 and Hdac9 deletion in germline	VSD
	Class III HDAC	Sirt1	Germline deletion	ASD, VSD, abnormal atrioventricular valves
	HAT	p300	Mutation which erases HAT activity	ASD, VSD
	Histone demethylase	Jumonji	Germline deletion	DORV, hypertrabeculation
	HMT	Smyd1	Germline deletion	Ventricular hypoplasia
		Whsc1	Germline deletion	ASD, VSD
		MLL2	Mutation in Kabuki syndrome patients	ASD, VSD, aortic coarctation

Interaction between chromatin-remodeling/histone-modifying factors and transcription factors

These chromatin-remodeling and histone-modifying factors also interact with transcription factors that are related to cardiac development. For example, Brg1 regulates the expression of SRF/MRTFA-dependent genes involved in smooth muscle contraction [21]. Brg1 also interacts with cardiac transcription factors such as Nkx2.5, Tbx5, and Tbx20 in a dose-dependent manner [22]. In addition, combinatorial induction of Tbx5, Gata4, and Baf60c is necessary for reprogramming of beating cardiomyocytes [23]. Hdac2 interacts with Hop to inhibit the transcriptional activities of Gata4 [24]. p300 is also a co-activator of Gata4 via acetylating Gata4, leading to the promotion of DNA binding and transcriptional activities [25]. Another example involves Jarid2/Jumonji, which suppresses Notch1 expression in the endocardium [26]. In addition, Rae28, a polycomb repressive complex 1 (PRC1) member, is necessary for Nkx2.5 transcriptional activity [27]. Finally, Whsc1 associates with Nkx2.5 to regulate heart development [19]. As seen in this section, chromatin-remodeling and histone-modifying factors have multiple functions in the regulation of cardiogenesis.

Investigation of epigenetic modification using stem cell technologies

As shown in the previous section, numerous chromatin remodelers and histone modifiers regulate cardiac development and the failure of this regulation might cause congenital heart diseases. In order to investigate epigenetic modification during cardiac development in vitro, pluripotent stem cells such as ES cells and iPS cells might be useful. We can reset the disease phenotype in the process of reprogramming, and recapitulate cardiogenesis during differentiation in vitro without changing genetic information. Through investigation of the epigenetic regulation in pluripotent stem cells, we can research molecular mechanisms during reprogramming and cardiac differentiation (Figure 1). In the following section, we summarize the epigenetic regulation in the process of reprogramming and cardiac development.

Epigenetic modification during reprogramming into iPS cells

In the process of generating iPS cells, the vectors encoding Yamanaka 4 factors such as Oct4, Klf4, Sox2, and Myc (called 'OKSM') are originally induced into somatic cells [28]. During cellular reprogramming, OKSM change the gene expression pattern and the epigenetic state of somatic cells into those of pluripotent stem cells without changing the DNA sequences. OKSM mainly achieve two functions

epigenetically during reprogramming: releasing the program for somatic cells and initiation/maintenance of the state of pluripotent stem cells [29]. To perform these epigenetic modifications, OKSM bind to their target components [30-32], interact with histone-modifying enzymes [33-35], regulate the 3D structures of chromatins [36], and trigger DNA methylation.

Chromatin dynamics during cardiomyocyte differentiation

We accumulated knowledge about the combinatorial expression of transcriptional factors during cardiac development; however, the epigenetic modifications in pluripotent stem cells during cardiac lineage induction are still poorly understood. However, recent studies have started to unveil the mechanisms involved [37,38]. Paige *et al.* performed directed differentiation of human ES cells into cardiomyocytes and investigated the chromatin modification in this process of differentiation [37]. They investigated the modification of trimethylated histone H3-lysine 4 (H3K4me3), trimethylated histone H3-lysine 27 (H3K27me3), and trimethylated histone H3-lysine 36 (H3K36me3) along the time course of directed differentiation, including pluripotent stem cells (day-0), mesodermal progenitors (day-2), specified tripotential cardiovascular progenitors (day-5), committed cardiovascular cells (day-9), and definitive cardiovascular cells (day-14), by chromatin immunoprecipitation (ChIP)–sequencing. In terms of the results, most of the transcription factors that regulate cardiac development demonstrated increased H3K27me3 and decreased H3K4me3, H3K36me3, and RNA expression (indicating inactive promoters) at day-0, although H3K27me3 was gradually suppressed and H3K4me3, H3K36me3, and RNA expression increased (indicating active promoters) in the process of differentiation. H3K4me3, H3K36me3, and RNA expression for genes encoding cardiomyocyte structural proteins also increased along the time course of differentiation; however, H3K27me3 remained at undetectable levels throughout [39,40]. Wamstad *et al.* differentiated mouse ES cells into cardiomyocytes and determined chromatin regulation at the phases of ES cells, mesoderm, cardiac precursors, and cardiomyocytes [38]. They showed that the functionally related genes expressed specific chromatin patterns, although the gene expression patterns were similar. For example, among the genes expressed at the cardiomyocyte stage, metabolism-related genes showed increased H3K4me3 and no H3K27me3, the signaling-related genes demonstrated increases of both H3K4me3 and H3K27me3, and in terms of the genes for muscle contraction, there was increased expression of H3K4me3 but no increase of H3K27me3. They also identified that monomethylated histone H3-lysine 4 (H3K4me1), related to the opening of chromatin at distal enhancers preceding activation [41,42], was deposited at transcriptional start sites preceding the increase of H3K4me3 and transcriptional activation in cardiac regulatory genes [38].

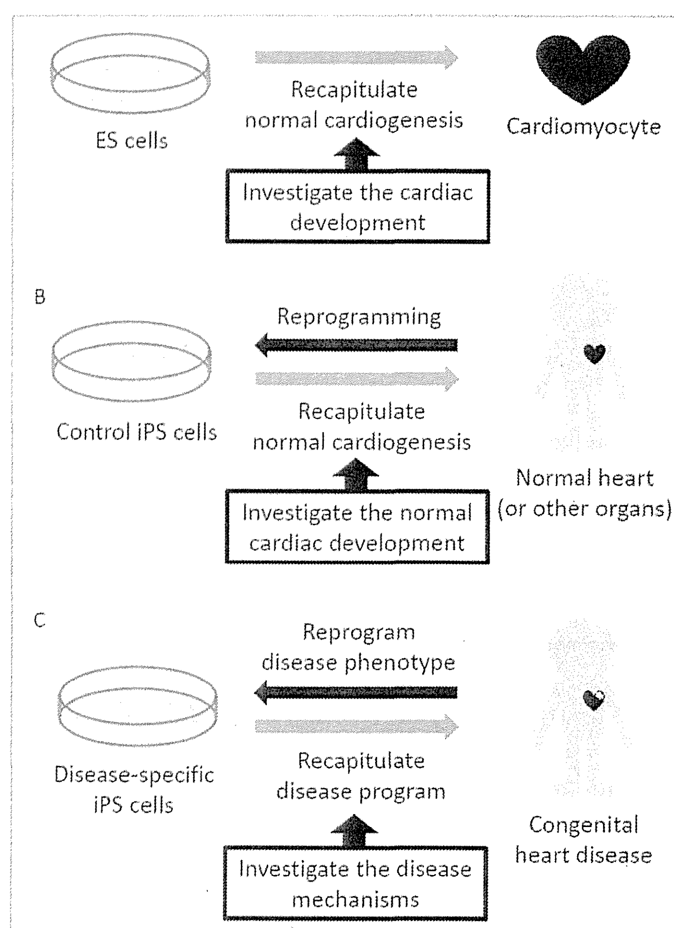

Figure 1. The studied features of epigenetic regulation using pluripotent stem cells. (A) The differentiation of ES cells into cardiomyocytes can be used to investigate cardiogenesis during differentiation. (B) In study using control iPS cells, we can analyze the mechanism involved in the normal processes of both reprogramming and differentiation into cardiomyocytes. (C) Disease-specific iPS cells can be practically reset the disease phenotype during reprogramming and recapitulate the disease program during differentiation. This could enable us to investigate the pathogenesis of congenital heart diseases.

enrichment of H3K4me3 and suppression of H3K27me3 on the promoters of ventricular myosin light chain (MYL2), atrial myosin light chain (MYL7), cardiac troponin T (TNNT2), and natriuretic peptide A (NPPA) compared with human ES cells. Upregulated H3K4me3 levels were also seen on the promoters of Ca^{2+}-handling proteins, sodium and potassium ion channels, such as phospholamban (PLN), dihydropyridine receptor (DHPR), junction (ASPH/JCTN), triadin (TRDN), SCN5A, and KCND3 in human ES cell-derived ventricular cardiomyocytes compared with those in human ES cells.

Investigation of epigenetic modification in hypoplastic left heart syndrome by using patient-specific iPS cells

As shown in the previous section, we have started to understand the epigenetic regulation of pluripotent stem cells during cardiomyocyte differentiation. Investigations of epigenetic regulation in patient-specific iPS cells during cardiac development might enable us to dissect the pathogenesis of congenital heart diseases. By using patient-specific iPS cells, we attempted to determine the epigenetic regulation during the cardiogenesis of HLHS, which is one of the severest congenital heart diseases with single ventricular circulation [44]. Some cases of HLHS are related to the mutation of cardiac-specific genes or associated chromosomal abnormalities; however, most of the cases are sporadic and the genetic basis has remained unknown. We generated five clones of iPS cells from the cardiac progenitor cells (CPCs) of patients with HLHS and investigated histone modifications in the process of cardiomyocyte differentiation. We performed ChIP assay on NKX2-5 promoter lesion of undifferentiated iPS cells, CPCs, and cardiomyocytes differentiated from HLHS-derived iPS cells and compared the results with those from biventricular heart (BV)-derived cells. In the undifferentiated iPS cells and CPCs, there were no significant differences in dimethylated histone H3-lysine 4 (H3K4me2), H3K27me3, and acetylated histone H3 (acH3) on NKX2-5 promoter lesion between HLHS-derived cells and BV-derived cells. However, the differentiated iPS cells demonstrated significant decreases of H3K4me2 and acH3 and a significant increase of H3K27me3 on NKX2-5 promoter lesion of HLHS-derived iPS cells compared with BV-derived iPS cells. These results indicated that HLHS-iPS-derived cardiomyocytes had suppressed promoter activity of NKX2-5 compared with BV-iPS-derived cardiomyocytes. Correlated with these results, the gene expression of NKX2-5 was significantly downregulated in HLHS-iPS-derived cardiomyocytes compared with that of BV-iPS-derived ones, as determined by quantitative RT-PCR. Furthermore, reporter assay demonstrated that the induction of NKX2-5 into HLHS-derived CPCs and iPS cells contributed to the restoration of cardiac-specific promoter activities such as serum response elements, TNNT2, and NPPA to levels equivalent to those of BV-derived cells. These findings suggested that NKX2-5 might be an essential transcription factor for the pathogenesis of HLHS. Interestingly, suppression of H3K4me2 and acH3 and an increase of H3K27 on NKX2-5 promoter lesion were found only in the differentiated HLHS-iPS-derived cardiomyocytes, not in the undifferentiated iPS cells or CPCs, and the significant suppression of NKX2-5 gene expression was only seen in the HLHS-iPS-derived cardiomyocytes, not in HLHS-derived iPS cells. Considering that NKX2-5 is a cardiac-specific transcription factor [45], these findings suggest that histone modification of NKX2-5 is specific at the stage of differentiation and impaired transient epigenetic activation of NKX2-5 might cause the suppressed NKX2-5 expression of HLHS-iPS-derived cardiomyocytes.

The possibility of improving epigenetic regulation during cardiogenesis

Some experimental data have shown that impaired epigenetic regulation affects cardiac development. In fact, some reported cases of congenital heart diseases were caused by the mutation of genes controlling epigenetic regulation. In addition to the gene regulatory network during cardiac development, we should take the epigenetic regulation into consideration when we think about the pathogenesis of congenital heart diseases. Furthermore, by utilizing the knowledge of epigenetic regulation during cardiac development, we could have a new approach to improve cardiac development and treat congenital heart diseases. When it comes to the therapies of congenital heart diseases, the major strategy is operation; however, we might treat such diseases during and after cardiac development if we can change the impaired epigenetic regulation. Some studies using pluripotent stem cells reported the possibility of this approach. Otsuji *et al.* reported that human ES cell-derived cardiomyocytes in 3D culture demonstrated an increased level of acH3 and increased expression of cardiac-specific genes, such as alpha myosin heavy chain (MYH6), ERG1b, and KCNQ1, compared with those in adhesion culture [46]. They also reported that trichostatin A (TSA), a selective inhibitor of the class I and II mammalian HDAC families [47,48], significantly upregulated the acH3 level of human ES cell-derived cardiomyocytes compared with that of dimethyl sulfoxide (DMSO)-treated controls. TSA also increased the expression of ERG1b and KCNQ1 in human ES cell-derived cardiomyocytes, as determined by quantitative RT-PCR. Global gene expression analysis showed that human ES cell-derived cardiomyocytes treated with TSA exhibited significant upregulation of two of eight sodium ion channel-related genes, two of sixteen calcium ion channel-related genes, and sixteen of forty-nine potassium ion channel-related genes in human ES cell-derived cardiomyocytes. TSA also increased the electrophysiological function of human ES cell-derived cardiomyocytes in a multi-electrode array. Chow *et al.* showed that valproic acid (VPA), an HDAC inhibitor like TSA [49], augmented the levels of H3K4me3 on the promoter of MYH6, NPPA, Ca^{2+}-handling protein, and sodium and potassium ion channels, and the expression of these genes in human ES cell-derived ventricular cardiomyocytes, although VPA did not improve the electrophysiological properties of the cells in Ca transient analysis [43]. Ohtani *et al.* created mouse ES cells with the knockout of jumonji domain-containing protein 3

(Jmjd3), which is also known as H3K27 demethylase [50,51], and differentiated these cells into cardiomyocytes [52]. They showed the reduced expression of Brachyury, a mesoderm marker of the T-box genes [53], and Mixl1, a mesoderm marker classified into the Mix/Bix family of paired-like homeobox genes [54], in the Jmjd3$^{-/-}$ embryoid bodies (EBs) during differentiation, compared with those in wild-type EBs. The reduced expression of Brachyury and Mixl1 in Jmjd3$^{-/-}$ EBs was caused by the enrichment of H3K27me3 marks due to the knockout of Jmjd3. They also demonstrated the significant suppression of genes for cardiac development, such as Mesp1, Pdgfa, Nppa, Mef2c, Tnnt2, and Myh6, and lower contractility in Jmjd3$^{-/-}$ EBs compared with those of wild-type EBs. Horrillo *et al.* reported that zebularine, a cytidine analogue that inhibits DNA methyltransferases [55] and which is more stable and less toxic than 5'-aza-2'-deoxycytidine (AzadC) [56,57], induced demethylation of Nkx2.5 promoter in mouse ES cells during cardiac differentiation, which in turn induced the upregulation of cardiac-specific genes, such as Nppa, Flk1, Hrt1, Mef2c, Actc, Tnnt2, Myh7, Myl2, and Myl7, in zebularine-treated EBs compared with those in untreated EBs. Zebularine also increased the number of more contractile EBs that responded well to the cardiotropic agents [58]. These reports suggest the possibility of treating impaired cardiac development using chemical reagents that modify epigenetic regulation.

A barrier to epigenetic study using iPS cells: epigenetic memory

By using stem cell technologies, we can recapitulate the cardiogenesis during differentiation in vitro, which enables us to investigate cardiogenesis more safely and intensively. When it comes to pluripotent stem cells, we have two main types: ES cells and iPS cells. These pluripotent stem cells are known to have almost the same properties: they express similar gene expression patterns, proliferate unlimitedly, and differentiate into any designed type of cell. However, iPS cells have a special feature: epigenetic memory. Epigenetic memory is the phenomenon by which iPS cells still have the epigenetic signature of the donor somatic cells, even after reprogramming [59,60]. This epigenetic memory is caused by incomplete silencing of the donor cell-specific gene expression patterns; the majority of this is attributed to DNA methylation [61,62] because histone modifications were shown to be similar between iPS cells and ES cells [63,64]. Numerous studies have reported that epigenetic memory is seen in the early passage phases of iPS cells and could cause variable differentiation potential; that is, iPS cells are more likely to differentiate into their original cell types [61,65,66]. Some reports have shown that epigenetic memory remained in iPS cells even after passages, but others demonstrated that repeated passages could eliminate the epigenetic differences among iPS cells derived from their original different types of somatic cells. Kim *et al.* compared the epigenetic modification during differentiation between umbilical cord blood-derived iPS cells and neonatal keratinocyte-derived iPS cells [66]. They concluded that the umbilical cord blood-derived iPS cells more potently differentiated into hematopoietic cells than keratinocyte-derived iPS cells, and the keratinocyte-derived iPS cells had better potential to differentiate into keratinocytes than the umbilical cord blood-derived iPS cells, from the results of DNA methylation patterns and microarray analyses. They also commented that the epigenetic memory did not disappear after passages. Polo *et al.* generated mouse iPS cells derived from tail tip-derived fibroblasts, splenic B cells, bone marrow-derived granulocytes, and skeletal muscle precursors and compared them [67]. The iPS cells from the different cell types demonstrated distinct epigenetic modifications and differential potentials, which were specific to their original cell types. In contrast to the findings of Kim *et al.*, Polo *et al.* reported that the repeated passage of iPS cells eliminated their transcriptional, epigenetic, and functional differences among those with different original cell types. In addition, Sanchez-Freire *et al.* generated iPS cells from CPCs and skin fibroblasts from the same donors and differentiated them into cardiomyocytes [68]. They showed that there were significantly higher rates of TNNT2-positive cells and beating EBs in the CPC-iPS-derived cardiomyocytes than in the fibroblast-iPS-derived ones. The CPC-derived iPS cells also demonstrated significantly upregulated expression of cardiac-specific genes such as NKX2-5, MESP1, ISL1, HAND2, MYOCD, MEF2C, and GATA4 during cardiomyocyte differentiation compared with the fibroblast-derived ones. They also showed that the fibroblast-iPS-derived cardiomyocytes had significantly higher DNA methylation at the NKX2-5 promoter than the CPC-iPS-derived ones. These findings indicate that the CPCs-derived iPS cells had more cardiomyogenic potential than the fibroblast-derived ones. In agreement with the work of Polo *et al.*, Sanchez-Freire *et al.* demonstrated that the differences in cardiomyogenic potential between CPC-derived iPS cells and fibroblast-derived ones disappeared upon further passages. In terms of the influence of epigenetic memory on the variability between iPS cells and ES cells, recent studies have reported that epigenetic memory could explain the small level of variability. Bock *et al.* performed DNA methylation assay by genome-scale bisulfite sequencing, microarrays, and quantitative differentiation assay for 20 human ES cell lines, 14 human iPS cell lines, and 6 primary fibroblast cell lines [69]. As a result, they reported that the epigenetic memory in iPS cells accounted for the small rate of variability between iPS cells and ES cells. Rouhani *et al.* generated 9 fibroblast-derived

iPS cells, 6 keratinocyte-derived iPS cells, and 10 endothelial progenitor cell-derived iPS cells from 4 adult donors and analyzed the iPS cells, the donor cells, and 2 human ES cell lines by RNA sequencing [70]. They concluded that epigenetic memory from the donor cells is rare in human iPS cells, and the variation between iPS lines is more likely to occur due to the changing genetic backgrounds.

Conclusions

Some cases of congenital heart diseases are caused by the mutation of definite cardiac-specific genes or related chromosomal abnormalities [1], but the causes of most cases are unknown. The impaired expression of multiple genes seemed to be involved in the pathogenesis of congenital heart diseases and the lack of animal models has hampered studies. Recently, we identified a new approach to dissect the complicated pathogenesis: epigenetic regulation. Recent studies have accumulated knowledge on the epigenetic regulation during cardiac development, and impaired epigenetic regulation has been shown to cause congenital heart defects [2,3]. To study epigenetic regulation, stem cell technologies could be a powerful tool. We can recapitulate the cardiogenesis during the differentiation from stem cells into cardiomyocytes without changing DNA sequences, and we can investigate and also modify the epigenetic regulation of cardiac development in vitro during these processes. Several studies using stem cell technologies have revealed the relationship between stage-specific epigenetic regulation and stage-specific cardiac gene expression [37,38], and also succeeded in improving the altered epigenetic regulation and gene expression during cardiac development in vitro by using chemical reagents that modify chromatins [43,46,52,58]. These results suggest that we could treat some congenital heart diseases by using chemical reagents. However, for iPS cells, we have to take the issue of epigenetic memory into consideration, namely, the retention of the epigenetic signature of somatic cells from which iPS cells originated [59,60]. Numerous studies have reported that iPS cells retained epigenetic memory and that they tended to differentiate into their original cell types [61,65,66]. Epigenetic memory has thus complicated study of the differentiation of iPS cells. In terms of this phenomenon, variable findings have been reported: some studies claimed that epigenetic memory accounted for only a small proportion of the variability [69,70] and disappeared after repeated passages [67,68], while others reported that epigenetic memory remained [66]. Indeed, there are certain difficulties associated with the use of iPS cells, but they have fascinating features compared with ES cells, so they could be used as disease models. In contrast to ES cells, which are created from embryos, iPS cells are generated from somatic cells. Thus, we can generate iPS cells from patients with specific diseases in order to model and recapitulate the disease phenotypes during differentiation. This might enable us to dissect the complicated pathogenesis in vitro. With regard to the use of iPS cells, some suggestions can now be proposed. When we differentiate iPS cells into a designed type of cell, it would be better to select iPS cells generated from that particular type of somatic cell. Some studies have proved the significant differences in differentiation potential among designed cells to differentiate into other types of cell [66-68]. In our experiments, we generated iPS cells from CPCs and differentiated them into cardiomyocytes [44]. This strategy might be reasonable, especially for research using patient-specific iPS cells, which are often resistant to differentiation. Of course, the obtainment of somatic cells designed to differentiate into certain cell types, like neurons, would be difficult. In such cases, we should use iPS cells for differentiation study after repeated passages. By overcoming and utilizing epigenetic memory, iPS cells could become a more promising cell source for stem cell research. Knowledge of epigenetic regulation would enable us to understand cardiac development and the pathogenesis of congenital heart diseases in more detail, and for the investigation of epigenetic modification, stem cell technologies would be necessary.

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MURC/Cavin-4 facilitates recruitment of ERK to caveolae and concentric cardiac hypertrophy induced by α1-adrenergic receptors

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The actions of catecholamines on adrenergic receptors (ARs) induce sympathetic responses, and sustained activation of the sympathetic nervous system results in disrupted circulatory homeostasis. In cardiomyocytes, α1-ARs localize to flask-shaped membrane microdomains known as "caveolae." Caveolae require both caveolin and cavin proteins for their biogenesis and function. However, the functional roles and molecular interactions of caveolar components in cardiomyocytes are poorly understood. Here, we showed that muscle-restricted coiled-coil protein (MURC)/Cavin-4 regulated α1-AR–induced cardiomyocyte hypertrophy through enhancement of ERK1/2 activation in caveolae. MURC/Cavin-4 was expressed in the caveolae and T tubules of cardiomyocytes. MURC/Cavin-4 overexpression distended the caveolae, whereas MURC/Cavin-4 was not essential for their formation. MURC/Cavin-4 deficiency attenuated cardiac hypertrophy induced by α1-AR stimulation in the presence of caveolae. Interestingly, MURC/Cavin-4 bound to α1A- and α1B-ARs as well as ERK1/2 in caveolae, and spatiotemporally modulated MEK/ERK signaling in response to α1-AR stimulation. Thus, MURC/Cavin-4 facilitates ERK1/2 recruitment to caveolae and efficient α1-AR signaling mediated by caveolae in cardiomyocytes, which provides a unique insight into the molecular mechanisms underlying caveola-mediated signaling in cardiac hypertrophy.

caveola | signal transduction | heart | plasma membrane

Caveolae are plasmalemmal invaginations enriched in cholesterol, glycosphingolipids, and lipid-anchored proteins relative to the bulk of the plasma membrane (1, 2). Owing to their specific lipid composition, caveolae concentrate several signaling molecules involved in cellular processes or trafficking events from the cell surface; therefore, they are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses (3–5). Accumulating evidence has demonstrated that caveola biogenesis and function depend on two distinct caveolar components: caveolins and cavins (6, 7). Caveolin (Cav)-1 and Cav-2 are expressed in most cell types, including adipocytes, endothelial cells, fibroblasts, and smooth myocytes, whereas Cav-3 is expressed exclusively in smooth, skeletal, and cardiac myocytes. Caveolin deficiency leads to caveolar loss, which is accompanied by alterations in signaling responses (8, 9). Cavins are also structural components of caveolae and assume four isoforms, namely, polymerase I and transcript release factor (PTRF)/Cavin-1, serum deprivation protein response (SDPR)/Cavin-2, SDR-related gene product that binds to C kinase (SRBC)/Cavin-3, and muscle-related coiled-coil protein (MURC) (6), which is also known as "Cavin-4" because of its sequence homology with other cavins and localization to the caveolae (10). Previously, we reported the association of MURC/Cavin-4 with SDPR/Cavin-2 and identified MURC/Cavin-4 mutations in dilated cardiomyopathy patients (11, 12). PTRF/Cavin-1 and SDPR/Cavin-2 are expressed in various cell types, including myocytes (10, 11), and SRBC/Cavin-3 is expressed in many cell types except for muscle cells (13), whereas MURC/Cavin-4 is expressed exclusively in myocytes, similar to Cav-3 (11). Recent studies have shown that cavins and caveolins form a complex, called the "caveolin–cavin complex," which modifies caveolar biogenesis and function (14, 15). PTRF/Cavin-1 and SDPR/Cavin-2 are required for caveolar invagination and SRBC/Cavin-3 for caveolar budding to form caveolar vesicles (13, 16, 17). However, the functional role of MURC/Cavin-4 in caveolar morphology is not known.

Alpha-1 adrenergic receptors (α1-ARs) are members of the G protein-coupled receptor (GPCR) family and have been demonstrated to accumulate in caveolar fractions of the myocardium (5). GPCRs are well-known representatives of receptors concentrated in caveolae and transduce several signals from substrates to downstream effectors in caveolae (18). Because disruption of the caveolae affects the response to several GPCRs, caveolae are considered important plasma membrane structures that coordinate GPCRs and their downstream signaling components (5). In our previous study, we showed that MURC/Cavin-4 knockdown suppressed α1-AR agonist-induced atrial natriuretic peptide expression and myofibrillar organization in cardiomyocytes and that transgenic mice overexpressing MURC/Cavin-4 in cardiac tissue

Significance

Caveolae are recognized as a platform for preassembled complexes of receptors, signal components, and their targets, facilitating efficient and specific cellular responses at the plasma membrane. ERK is activated at the plasma membrane and an important molecule that has been well studied for its integral role in signal transduction events during physiological adaptation and pathological manifestation. Here we show that although muscle-restricted coiled-coil protein (MURC)/Cavin-4, a muscle-specific caveola component, is dispensable for caveolar formation in cardiomyocytes, MURC/Cavin-4 serves as an ERK-recruiting protein in the caveolae within cardiomyocytes. The recruiting function of MURC/Cavin-4 is necessary to elicit efficient signaling of the α1-adrenergic receptor–ERK cascade in concentric cardiac hypertrophy. Our findings provide unique insight into the molecular mechanisms underlying caveola-mediated signaling in cardiac hypertrophy.

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(MURC-Tg) developed cardiomyocyte hypertrophy at 5 wk of age (11). These results suggest that MURC/Cavin-4 is involved in α1-AR signaling and cardiac hypertrophy.

In the present study, we manipulated MURC/Cavin-4 expression to investigate the role of MURC/Cavin-4 in caveolar morphology and α1-AR–induced cardiac hypertrophy. Overexpression and deletion of MURC/Cavin-4 showed the roles of MURC/Cavin-4 in the caveolar morphology of cardiomyocytes. Furthermore, we found that MURC/Cavin-4 facilitated ERK1/2 recruitment to caveolae and ERK activation in α1-AR–induced concentric cardiomyocyte hypertrophy.

Results

MURC/Cavin-4 Forms Caveolin–Cavin Complexes in the Caveolae and T Tubules and Modulates Caveolar Morphology in Cardiomyocytes. To reveal the functional significance of MURC/Cavin-4 as a caveolar component in cardiomyocytes, we examined the association of MURC/Cavin-4 with other cavins and caveolins. Expression plasmids encoding MURC/Cavin-4, Cav-3, and PTRF/Cavin-1 were transfected into CV-1 (simian) in origin, and carrying the SV40 genetic material (COS) cells, a fibroblast-like cell line derived from monkey kidney tissue. Immunoblot analysis showed that MURC/Cavin-4 was coimmunoprecipitated with Cav-3 and PTRF/Cavin-1 (Fig. S1 *A* and *B*). MURC-HA and Cav-3-T7 expressions were not reduced in supernatants immunoprecipitated by anti-T7 and anti-HA antibodies, respectively (Fig. S1*A*), whereas MURC-FLAG expression was reduced in the supernatant immunoprecipitated by the anti-HA antibody, although PTRF/Cavin-1-HA expression was not reduced in the supernatant immunoprecipitated by the anti-FLAG antibody (Fig. S1*B*). These results suggest that MURC/Cavin-4 binds to PTRF/Cavin-1 with high affinity, and that MURC/Cavin-3 does not entirely bind to Cav-3.

The bimolecular fluorescence complementation (BiFC) assay confirmed that MURC/Cavin-4, Cav-3, PTRF/Cavin-1, and SDPR/Cavin-2 interact in living cardiomyocytes (Fig. S1 *C* and *D*). Immunoelectron microscopy revealed that MURC/Cavin-4 was expressed in caveolae and T tubules in cardiomyocytes of adult mice (Fig. 1*A*). These observations are in accordance with our previous finding showing that MURC/Cavin-4 was localized to the Z line in cardiomyocytes (11) because the T-tubule system is in register with the Z lines and the immunostaining pattern of Cav-3 has been shown to coincide with the Z line in the heart (19). Because it was confirmed that MURC/Cavin-4 was expressed by caveolae, we assessed whether MURC/Cavin-4 affected caveolar morphology in cardiomyocytes. In cardiomyocytes of 13-wk-old MURC-Tg mice, the caveolae were significantly distended compared with those of wild-type (WT) mice (Fig. 1*B*). The effects of MURC/Cavin-4 on caveolae were supported by the results of an in vitro study in which MURC/Cavin-4 overexpression significantly increased the caveolar area and perimeter in cardiomyocytes compared with β-galactosidase (LacZ) overexpression (Fig. 1*C*). These results indicated that MURC/Cavin-4 modified the morphology of formed caveolae in cardiomyocytes.

MURC/Cavin-4 Is Associated with α1-ARs at Caveolae in Cardiomyocytes. We next investigated the localization of α1-ARs in cardiomyocytes. α1-AR exists as three molecular subtypes: α1A, -B. and -D. The α1A and -B subtypes are expressed in the myocardium, whereas the α1D subtype is expressed in vascular muscle (20). Because antibodies for α1-AR subtypes, which are frequently cited, have been shown to be nonspecific (21), we used plasmids encoding red fluorescent protein mCherry-conjugated α1A-AR (ADRA1A) and α1B-AR (ADRA1B). ADRA1A and ADRA1B signals were observed predominantly at the plasma membrane and partly within the cytoplasm (Fig. 2*A*). ADRA1A and ADRA1B signals were colocalized with endogenous Cav-3 and MURC/Cavin-4 at the plasma membrane and partly within the cytoplasm. Immunoprecipitation and BiFC assays revealed that both ADRA1A and ADRA1B were bound to MURC/Cavin-4 in COS cells and cultured rat cardiomyocytes, respectively (Fig. 2 *B* and *C*).

Because Cav-3 has also been demonstrated to bind to α1-ARs (22), we investigated whether Cav-3 could influence the localization of MURC/Cavin-4 and α1-ARs in cardiomyocytes. Cav-3 knockdown impaired the plasma membrane localization of MURC/Cavin-4, resulting in the accumulation of MURC/Cavin-4 in the cytosol of cardiomyocytes (Fig. S2 *A*–*C*). However, α1-ARs were retained at the plasma membrane in Cav-3–knocked down cardiomyocytes (Fig. S2*A*).

MURC/Cavin-4 Deficiency Attenuates α1-AR–Induced ERK Activation and Cardiac Hypertrophy. The above-mentioned observations that α1-ARs bound to MURC/Cavin-4 at caveolae in cardiomyocytes led us to examine whether MURC/Cavin-4 influenced the response to α1-AR stimulation in vivo. To this end, we subjected WT and

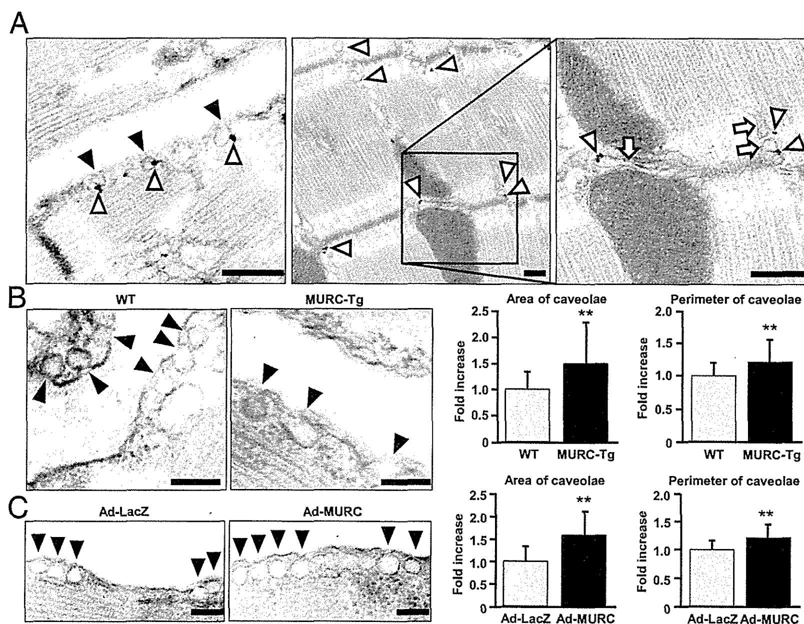

Fig. 1. MURC/Cavin-4 exists in the caveolae and T tubules and modulates caveolar morphology in cardiomyocytes. Representative electron microscopic images of mouse heart tissue and adult cultured cardiomyocytes. (*A*) Immunogold staining of MURC/Cavin-4 (white arrowheads) was observed at caveolae (black arrowheads) and T tubules (white arrows) of cardiomyocytes. (*B* and *C*) Representative shapes of caveolae in mouse heart tissue (*B*, *Left*) and rat cultured cardiomyocytes with or without MURC/Cavin-4 overexpression (*C*, *Left*). Caveolae were identified by their characteristic flask shapes and locations at or near the plasma membrane. Relative caveolar areas and perimeters were measured in cardiomyocytes from heart tissue (*B*, *Right*) and cultured cardiomyocytes (*C*, *Right*). Data are presented as mean ± SEM. $^{**}P < 0.01$ compared with controls. (Scale bars: 500 nm in *A*; and 200 nm in *B* and *C*.)