Part X

iPS Cells and Regeneration in Congenital Heart Diseases

Perspective

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Human adult somatic cells can be reprogrammed to induced pluripotent stem (iPS) cells upon the introduction of four transcription factors that are part of the pluripotency network in embryonic stem cells. The ability to readily from patients with disease has ushered in new opportunities to understand disease mechanisms, screen for therapeutics, and consider regenerative approaches using personalized human cells. In this session, several examples of the use of iPS cells in novel ways were presented.

By making iPS cells from patients with genetically defined disease, the investigators were able to differentiate the pluripotent cells into the cell type affected by the congenital cardiovascular disorder. These cells carried the disease-causing mutation and provided a platform for understanding the cellular and molecular consequences of the mutation in the most relevant human cells. Deep interrogation of such cells promises to reveal fundamental mechanisms of disease and should point to new targets to intervene in the disease process. This is being done for diseases involving cardiomyocytes, smooth muscle cells, and endothelial cells, each of which can be easily differentiated from human iPS cells with good efficiency and purity. Once new targets for disease pathology are discovered in such cells, small molecule or biologic screens can be performed to identify lead

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candidates for new therapeutics. For those congenital diseases that have ongoing consequences after birth, there is potential to intervene postnatally in the disease evolution.

In addition to the use of iPS cells for disease modeling and drug discovery, there are robust efforts to use pluripotent stem cells for regenerative medicine. Such efforts often involve bioengineering approaches to assemble stem cell-derived cardiomyocytes into a three-dimensional structure. This can be useful for cardiomyocytes, valves, or vessels. The use of iPS cells may allow personalized tissues to be developed, as tissue could be generated with one's own cells. New approaches using efficient gene-editing techniques may allow correction of abnormal genes and subsequent use of corrected cells for transplant. Other types of progenitor cells are also being studied for their regenerative capacity and are discussed in this section.

While there is great hope that the use of iPS cells will lead to new therapeutic approaches, many hurdles must be overcome. For disease modeling, purifying specific subtypes of cells that are affected by disease will be important, as will the ability to generate more mature, adult-like cells from the iPS cells. For regenerative medicine approaches, the ability to generate mature cells that can survive and integrate upon transplantation will be critical and will likely require clever engineering strategies. Nevertheless, it is likely that iPS-based technologies will provide us a better understanding of human disease and lead to new interventions.

Human Pluripotent Stem Cells to Model Congenital Heart Disease

45

Seema Mital

Abstract

Congenital heart disease (CHD) is the most common cause of neonatal mortality related to birth defects. Etiology is multifactorial including genetic and/or environmental causes. The genetic etiology is known in less than 20 % cases. Animal studies have identified genes involved in cardiac development. However, generating cardiac phenotypes usually requires complete gene knockdown in animal models which does not reflect the haplo-insufficient model commonly seen in human CHD. Human pluripotent stem cells which include human embryonic stem cells (hESC) and human-induced pluripotent stem cells (hiPSC) provide a unique in vitro platform to study human "disease in a dish" by providing a renewable resource of cells that can be differentiated into virtually any somatic cell type in the body. This chapter will discuss the use of human pluripotent stem cells to model human CHD.

Keywords

Human embryonic stem cells • Induced pluripotent stem cells • Williams syndrome • Hypoplastic left heart syndrome • Fetal reprogramming

45.1 Introduction

Human embryonic stem cells (hESC) can give rise to all three germ layers – ectoderm, endoderm, and mesoderm – and can be used to generate differentiated cells of different lineages [1]. The Nobel prize-winning discovery by Yamanaka of the ability to reprogram somatic cells to induced pluripotent stem cells (iPSC) using

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_45

specific reprogramming factors [2] uncovered a whole new field of research focused on the use of iPSCs to model human disease, perform drug screens, and explore strategies for autologous cell-based therapies in the future. Reprogramming protocols include ectopic expression of four transcription factors [2, 3] that induce reprogramming of somatic cells into an embryonic state. Viral integration-free protocols are also used albeit are less efficient. These cells can then be expanded and differentiated into several somatic cell types including cardiac lineages such as cardiomyocytes, vascular smooth muscles cells (SMCs), and endothelial cells. The process of cardiac differentiation of hESCs and hiPSCs recapitulates cardiac embryogenesis thereby providing a unique opportunity to explore the impact of gene or environmental defects on early cardiac development and gain novel insights into disease mechanisms [4]. Strategies for modeling cardiac malformations are discussed.

45.2 Modeling Fetal Cardiac Reprogramming in Hypoplastic Left Heart Syndrome (HLHS)

HLHS is one of the most severe cardiac malformations characterized by poor growth of left-sided cardiac structures. This is commonly associated with endocardial fibroelastosis (EFE). The mechanism of LV growth failure and fibrosis is poorly understood. We studied 29 normal and 30 HLHS fetal hearts during second trimester [5]. We found increased nuclear expression of hypoxia-inducible factor 1α (HIF1α) in fetal HLHS compared to normal LVs, a central hypoxia-responsive gene that promotes activation of angiogenic, metabolic, and other genes to facilitate cardiac adaptation to hypoxia. However, expression of vascular endothelial growth factor (VEGF) was downregulated. The failure of hypoxia-induced angiogenesis was likely related to cell senescence as shown by DNA damage (nuclear γH2AX activation and p53 upregulation) and of cell senescence (β-galactosidase upregulation). Senescent cells, although functional, do not produce growth factors essential for the survival and proliferation of stem/progenitor cells thereby compromising tissue renewal capacity. Not surprisingly, HLHS hearts showed fewer cardiac progenitor markers, as well as reduced differentiated cardiomyocyte and endothelial cells. DNA damage was most prominent in endothelial cells followed by myocytes, with SMCs being least susceptible. Additionally, there was increased transforming growth factor (TGFβ1) expression, increased myofibroblast transformation, and increased interstitial and perivascular fibrosis in fetal HLHS compared to controls. Together this suggested that the fetal LV may be susceptible to chronic hypoxia or reduced blood flow (a phenomenon that occurs in HLHS due to reduced antegrade flow through the diminutive ascending aorta) resulting in DNA damage and cell senescence and consequent loss of cell replication and growth capacity as well as fibrosis.

To clarify the role of hypoxia in fetal cardiac differentiation, we exposed hESC-derived cardiac lineages to 1 % hypoxia for 72 h. This was associated with recapitulation of the fetal HLHS phenotype including increased HIF1α; reduced

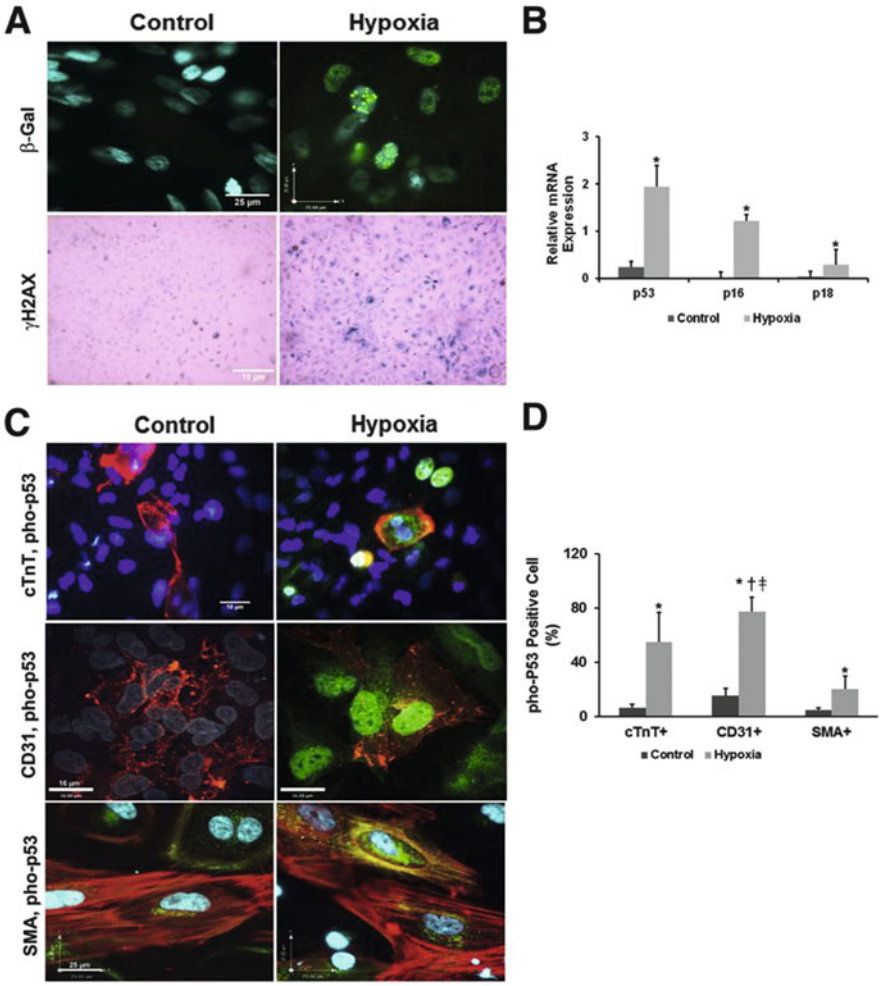

Fig. 45.1 Effect of hypoxia on DNA damage and oncogene upregulation in hPSC-derived cardiac lineages. (**a**) DNA damage-related marker γH2AX (*green nuclear foci*) and senescence marker β-gal (*blue*) are increased in hypoxic cells. *Blue* represents nuclear staining with DAPI. (**b**) qPCR results revealed higher mRNA expression of the tumor suppressor oncogene p53 and the G1 cell cycle inhibitors p16 and p18 in hypoxic (*gray bars*) compared with control cells (*black bars*). (**c**) Double immunostaining revealed co-localization of ph-p53 (*green*) with cTnT+ myocytes (*red*), CD31+ endothelial cells (*red*), and SMA+ SMCs (*red*), indicating DNA damage in all three lineages. *Blue* represents nuclear staining with TO-PRO-3. (**d**) Cellomics quantification confirmed the higher number of phospho-p53+ cardiac lineages in hypoxic cells (*gray bars*) compared with controls (*black bars*), with most severe injury in ECs followed by myocytes and then SMCs. $*P < 0.01$ versus controls; $\dagger P < 0.05$ versus SMA+ cells; $\ddagger P < 0.05$ versus cTnT+ cells ($n = 3$ experiments in each group). Original magnification: ×1,000 (γH2AX); ×600 (β-gal) (**a**); ×1,000 (**c**) (Reproduced with permission) [5]. Reprinted from Gaber et al. [5], Copyright (2013), with permission from Elsevier [5])

VEGF; TGFβ1 upregulation; DNA damage (highest in endothelial cells followed by myocytes followed by SMCs); cell senescence; reduced cell proliferation, resulting in a reduction in myocyte and endothelial lineages but increase in SMC lineages; and reduced contractility (Fig. 45.1). Treatment with TGFβ1 inhibitor reversed this abnormal phenotype. This suggests that immature cardiac lineages may be susceptible to hypoxic injury and that this may be mediated in part by TGFβ1 activation. This may contribute to the phenotype of LV growth failure and fibrosis in cardiac malformations like fetal HLHS.

These findings have several implications. They suggest that antenatal intervention for HLHS may be more effective in promoting LV growth if performed before irreversible tissue injury. However, complementary strategies to provide missing growth factors and/or inhibit TGFβ1 either pre- or postnatally may be needed to promote LV growth and ameliorate progressive fibrosis.

45.3 hiPSCs to Model Williams-Beuren Syndrome (WBS)

WBS is a genetic disorder caused by deletion of 26–28 genes in the 7q11.23 region. Cardiac manifestations are common and are related primarily to haploinsufficiency of the elastin gene in the deleted region. Elastin insufficiency causes vascular SMC proliferation resulting in either generalized arteriopathy or discrete arterial stenoses including supravalvar aortic stenosis, coronary stenosis, pulmonary stenosis, and renal artery stenosis [6]. Surgical correction is often associated with recurrence of stenosis, and there are no medical therapies to prevent or reduce vascular stenoses. Mouse models require complete elastin gene knockdown to reproduce supravalvar aortic stenosis. We therefore generated iPSCs from a patient with WBS with supravalvar aortic stenosis to provide a more human-relevant model for study. Skin fibroblasts obtained at the time of surgery were reprogrammed using four factor retroviral reprogramming. Four iPSC lines were characterized for pluripotency and subjected to SMC differentiation using a published protocol [7]. SMCs generated from iPSCs from normal BJ fibroblasts showed high elastin expression, with 90 % positive for SM22α (a marker of SMC differentiation). These cells showed a good contractile response (Ca^{2+} flux) to a vasoactive agonist like endothelin and tube-forming capacity on Matrigel assay. In contrast, WBS iPSC-derived SMCs showed low elastin expression, had fewer SM22α-positive cells, were highly proliferative, showed poor tube-forming capacity on Matrigel, and did not show a contractile response to endothelin (Fig. 45.2) [8]. Treatment with rapamycin, a mTOR inhibitor and antiproliferative agent, showed partial rescue of the abnormal phenotype in WBS-SMCs by enhancing differentiation, reducing proliferation, and improving tube-forming capacity. However, it did not restore contractile response to endothelin. Ge et al. used a similar approach to generate and study SMCs from a patient with supravalvar aortic stenosis with WBS and another with elastin loss-of-function mutation that showed a similar phenotype that was rescued by ERK1/2 inhibition [9]. To identify additional compounds that not only improve SMC differentiation but also promote functional maturation and

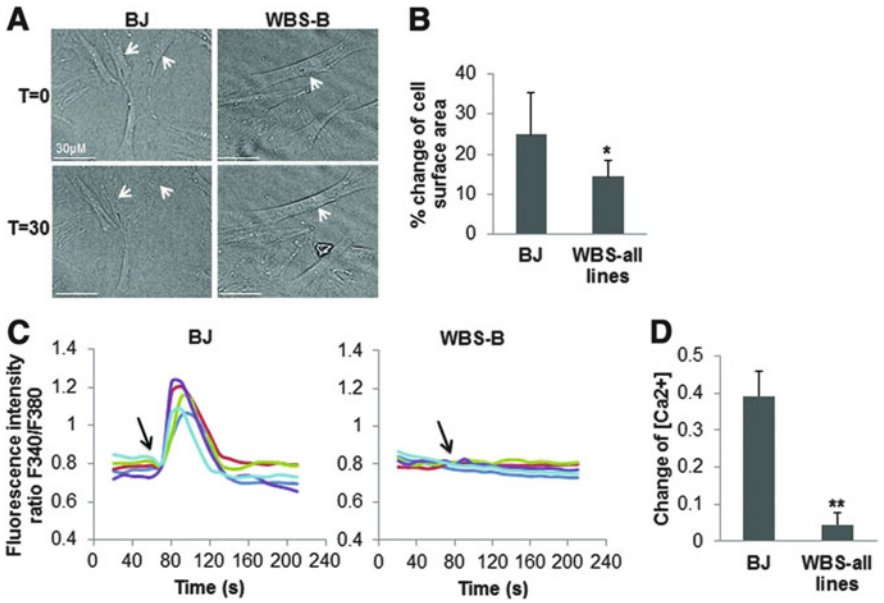

Fig. 45.2 Functional characterization of BJ-smooth muscle cells (SMCs) and WBS-SMCs. (**a**) BJ and WBS-SMCs (line B shown) were treated with 10 mM carbachol, a muscarinic agonist, and phase-contrast live-cell imaging was done every 30 s. Change in cell surface area (*white arrows*) was calculated from 0 min (*top panel*) to 30 min (*bottom panel*). (**b**) BJ-SMCs showed a 25 % reduction in cell surface area compared with 14 % reduction in WBS-SMCs (average of all four lines). *, $p < 0.05$ BJ versus WBS. (**c**) Calcium flux $[Ca^{2+}]_i$ was measured in response to endothelin-1 treatment (*arrow*) in BJ and WBS-SMCs (five cells each). The fluorescence intensity ratio ($F_{340\ nm}/F_{380\ nm}$) showed a transient rise in $[Ca^{2+}]_i$ after activation by endothelin-1 in BJ-SMCs but not in WBS-B SMCs. (**d**) Graph showing the changes in $[Ca^{2+}]_i$ following endothelin-1 treatment in BJ and all the WBS lines. Changes of $[Ca^{2+}]_i$ = peak $[Ca^{2+}]_i$ − resting $[Ca^{2+}]_i$. *, $p < 0.01$ BJ versus WBS. *WBS* Williams-Beuren syndrome (Reprinted from Kinnear et al. [8], Copyright (2013), with permission from Alpha Med Press [8] (pending))

vasoactive responsiveness, we are developing a high-throughput high-content screening assay to facilitate screening of drug libraries using WBS-SMCs. Compounds that fully rescue the abnormal SMC phenotype in WBS may guide the development of new drugs to relieve vascular stenoses in WBS and, by extension, in other vascular disorders including atherosclerosis, stent restenosis, and transplant graft vasculopathy.

45.4 Future Directions and Clinical Applications

These studies provide proof of principle that hESCs and iPSCs can generate in vitro models to study CHD. However, the cardiac lineages generated using this approach are relatively immature, i.e., fetal stage. While fetal stage cells may be well suited to study developmental cardiac disorders [10], maturation protocols that generate

more functionally mature lineages may be more useful to study late-onset disease phenotypes and accurately evaluate drug responses [11–14]. Our study further suggests that the technology can be expanded to study not just genetic influences, particularly in the rapidly emerging era of genome editing [15], but also environmental teratogens (toxins, chemicals, drugs, infections) to define the mechanisms by which they impact fetal cardiac development or differentiation. This may facilitate delineating the combined role of genetic and environmental factors in CHD causation in the near future [16]. The ability to differentiate pluripotent stem cells into many different organ or cell types may allow the study not only of cardiac but also of extracardiac phenotypes particularly in syndromic disorders as recently shown in a patient with Timothy syndrome [17, 18].

In summary, pluripotent stem cell-derived models are revolutionizing our understanding of disease pathogenesis and are positioned to expedite drug screening and discovery particularly for rare cardiac disorders with a genetic basis for which no therapies are available and where clinical studies are challenging. The technology provides a renewable source of functional cardiomyocytes and other cardiac lineages with genetic and epigenetic variation that are likely to be more human relevant. While the use of these cells for in vivo therapies is several years away, this platform is well positioned to study the molecular underpinnings of genetic cardiac disorders and help identify new therapies for personalized care of the affected child.

Acknowledgments The work was funded by the Canadian Institute of Health Research (MOP 126146), SickKids Labatt Family Heart Centre Innovations fund, and the Ontario Ministry of Research and Innovation GL2 award.

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Engineered Cardiac Tissues Generated from Immature Cardiac and Stem Cell-Derived Cells: Multiple Approaches and Outcomes

46

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Abstract

The translation of in vitro engineered cardiac tissues (ECTs) from immature cardiac and stem cell-derived cells toward clinical therapies is benefiting from the following major advances: (1) rapid progress in the generation of immature cardiac cells from the cardiac and noncardiac cells of multiple species including normal and disease human cells, (2) incorporation of multiple cell lineages into 3D tissues, (3) multiple scalable 3D formulations including injectable gels and implantable tissues, and (4) insights into the regulation of cardiomyocyte proliferation and functional maturation. These advances are based on insights gained from investigating the regulation of cardiac morphogenesis and adaptation. Our lab continues to explore this approach, including changes in gene expression that occur in response to mechanical loading and tyrosine kinase inhibition, the incorporation of vascular fragments into ECTs, and the fabrication of porous implantable electrical sensors for in vitro conditioning and postimplantation testing. Significant challenges remain including optimizing ECT survival postimplantation and limited evidence of ECT functional coupling to the recipient myocardium. One clear focus of current research is the optimization and expansion of the cellular constituents, including CM, required for clinical-grade ECTs. Another major area of investigation will be large animal preclinical models that more accurately represent human CV failure and that can generate data in support of regulatory approval for phase I human clinical trials. The generation of reproducible human ECTs creates the opportunity to develop in vitro myocardial surrogate tissues for novel drug therapeutics and toxicity assays.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_46

Keywords
Cardiomyocytes • Cardiac repair and regeneration • Engineered cardiac tissues • Stem cells

46.1 Introduction

Following significant cardiac injury, the postnatal human heart lacks the ability to restore lost myocardium, resulting in an adaptive response that often ultimately leads to progressive cardiac dysfunction, morbidity, and mortality. There are currently many strategies for cardiac "cellular therapy" undergoing both preclinical and clinical trials [1–4]. While there has been modest success with improvement in cardiac function in some of the early human clinical trials, it is clear that injected or implanted cells do not survive, and functional improvement occurs via paracrine mechanisms. In contrast, rapid advances in tissue engineering over the past two decades have resulted in the generation of functional, multicellular, 3D cardiac tissues with the potential for translation to human cardiac repair and regeneration [5–8]. This chapter provides a concise overview of some of the key issues in the generation, maturation, and translation of these engineered cardiac tissues (ECTs).

46.2 A Broad View of Bioengineering Cardiac Tissues

The bioengineering process for complex tissues begins with an understanding of the cellular and noncellular constituents of the target tissue [9]. For replacement myocardium, the major cellular constituents include cardiomyocytes (CM), fibroblasts, and vessel-associated cells. There are also numerous extracellular matrix (ECM) constituents including collagen, fibronectin, laminin, and multiple growth factors bound within the ECM. Of course, the neonatal myocardium and the adult myocardium have vastly different profiles for cellular and noncellular constituents, architecture, and biomechanical properties. While all currently successful ECTs are constructed using immature cells and simplified ECM components, the target tissue is usually mature myocardium. The success of ECT survival, integration, and functional maturation depends on the ability of these ECT constituents to acquire "mature" fates.

46.3 Immature Cells for Engineered Cardiac Tissues

Because the goal for cardiac regeneration is the restoration of functionally coupled, working myocardium, a variety of cell sources with the potential to generate CM are under investigation. Immature CM can be isolated from the hearts of developing chick, mouse, and rat embryos to generate ECTs for preclinical investigation (Fig. 46.1) [5–7]. These cells mature in vivo or in vitro along timelines proportional

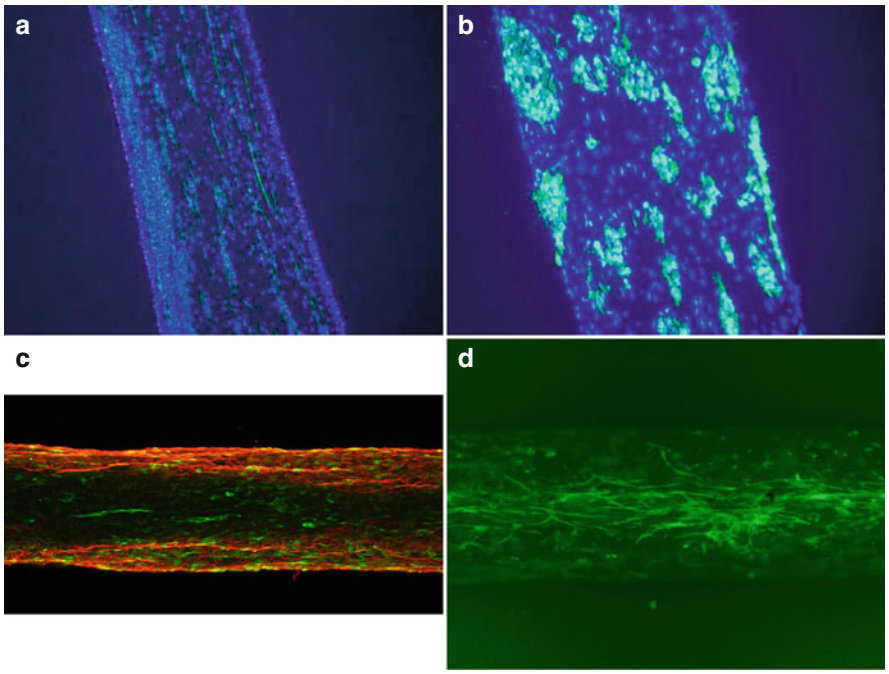

Fig. 46.1 Representative engineered cardiac tissues (ECTs) derived from (**a**) embryonic chick heart cells; (**b**) human-iPS-derived cardiomyocytes; (**c**) embryonic rat heart cells and rat adipose vascular fragments; and (**d**) enlarged image of vascular fragments within a rat ECT. Staining for (**a**, **b**) are *blue* (DAPI, nuclei), *green* (cardiac troponin T), and *red* (EdU). Staining for (**c**, **d**) are *red* (alpha actinin) and *green* (GFP+vascular fragments). Images (**a**, **c**) are 20× magnification; images (**b**, **d**) are 40× magnification (Keller lab, unpublished)

to the gestational length of their species of origin. A variety of stem cell sources (embryonic stem cells, induced pluripotent stem cells, cardiac stem cells, adipose stem cells, etc.) have also been used to generate immature CM using a variety of CM lineage specification and selection protocols [10–14]. Not surprisingly, stem cell populations can be rapidly expanded in vitro along with the induction of cardiac lineages; however, their functional maturation remains a major technical challenge [15–17]. Because human cells are required for clinical translation, the optimization of protocols that can generate large quantities of functional human CM is a high priority for cardiac repair strategies. Further, there may be advantages to generating ECTs that contain both cardiac and vascular lineage cells to accelerate angiogenesis and vascular perfusion of implanted ECTs [11–13].

46.4 Various Formulations for Engineered Cardiac Tissues

The constructs used for cardiac tissue repair include the implantation of multicellular cardiospheres [18], various formulations of 2D cellular sheets [19–22], and various formulations of 3D tissues [5–8, 13]. The composition of the noncellular constituents varies from minimal constituents for cardiosphere clusters to a range of ECM components [23, 24] and growth factors [25–27] selected for their ability to facilitate CM survival and functional maturation. Some of the ECT formulations allow for in vitro preconditioning strategies that can stimulate cell proliferation and/or maturation [5, 6]. While there can be wide variation in the formulation of ECTs used for preclinical studies, all constituents used to generate ECTs for human use are required to conform to strict FDA regulatory guidelines that include the elimination of all sources for potential infectious agents and/or toxins and the generation of clinical-use materials using good manufacturing practices [28].

46.5 In Vitro ECT Findings

Immature CM survive, proliferate, and functionally mature rapidly within ECTs as quantified by standard measures of cell number, gene expression profiling (Fig. 46.2) [29], sarcomeric protein content, electrophysiologic properties, and the ability to generate substantial force [5–7, 13].

Maturing functional syncytia can include CM, myofibroblasts, and vascular cells with a functional advantage noted for multiple lineage constructs over pure CM-derived ECTs [11–13]. Conditioning protocols with the intent of accelerating

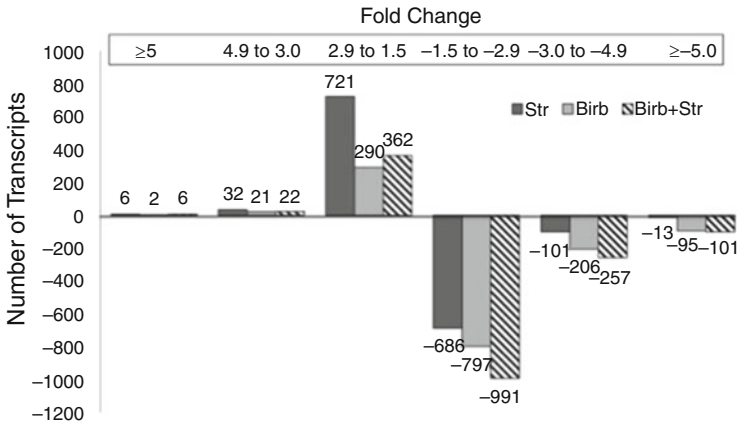

Fig. 46.2 Changes in rat ECT gene expression in response to mechanical loading and/or p38MAPK inhibition. ECT transcript expression changes at least 1.5-fold measured by microarray in response to stretch (*dark solid bar*), the p38MAPK inhibitor BIRB796 (*gray solid bar*), or stretch+BIRB796 (*dashed bar*). Note that most transcripts increased by less than threefold (above the X-axis) or decreased by less than fivefold (below the X-axis) [29]

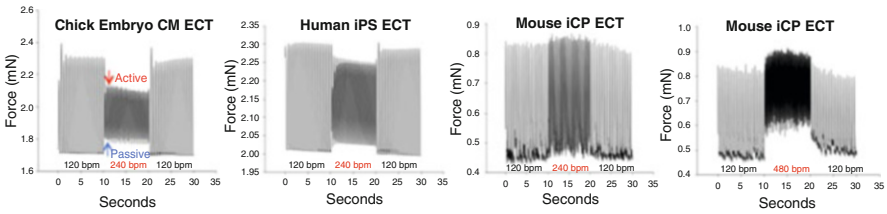

Fig. 46.3 Representative force-frequency relations for chick embryo-derived ECT and human-iPS-CM-derived ECT. Force-frequency relations quantify force generation in response to increased beat rate and reflect ECT maturational ability to release and restore Ca^{2+}. Chick embryo and h-iPS ECT showed increased passive force and reduced active force as beat rate increased from 120 to 240 bpm consistent with immature Ca^{2+} release during contraction and limited Ca^{2+} sequestration at faster rates (Keller lab, unpublished)

cell proliferation and/or maturation include the use of exogenous paracrine factors [15, 26], electrical stimulation [17, 27], and/or mechanical conditioning [6, 13]. Structural CM maturation is readily documented using immunohistochemical stains for sarcomeric proteins, gap junctions, and the presence of ion channels [30]. Functional CM maturation is documented by decreasing pacing voltage thresholds to initiate pacing, intrinsic rates and maximal beat rates in response to electrical stimulation, increasing rates of electrical conduction across ECTs, developed force in response to pacing, and both force-length and force-frequency relations reflecting increased contractility and increased calcium cycling efficiency (Fig. 46.3). ECTs generated from human-derived cells are now proposed as in vitro models for human diseases as well as surrogate models to detect drug toxicity prior to clinical trials [31, 32].

46.6 In Vivo ECT Findings

Ultimately, ECTs require in vivo implantation to assess survival, structural integration to the recipient myocardium, functional integration, and evidence for recovery of lost cardiac function. Preclinical implantation models have been primarily small animals (rodents); however, there is an increasing experience with the preclinical testing of ECTs using large animal models including pigs [22] and, eventually, nonhuman primates. The in vivo results have been very encouraging and confirm the capacity of implanted ECTs to survive, functionally couple, and recover damaged myocardium within the context of the experimental design [33]. There are several challenges to the interpretation of in vivo ECT studies. First, many of the studies involve the implantation of ECTs into immune-compromised animals. These studies are required to validate the capacity of implanted ECTs to functionally couple to recipient myocardium but underestimate the rapid inflammatory degradation that occurs as evidenced by the presence of macrophage-associated arginase and reduced ECT post-implant cellularity, even in syngeneic animals (Fig. 46.4) [13, 30].

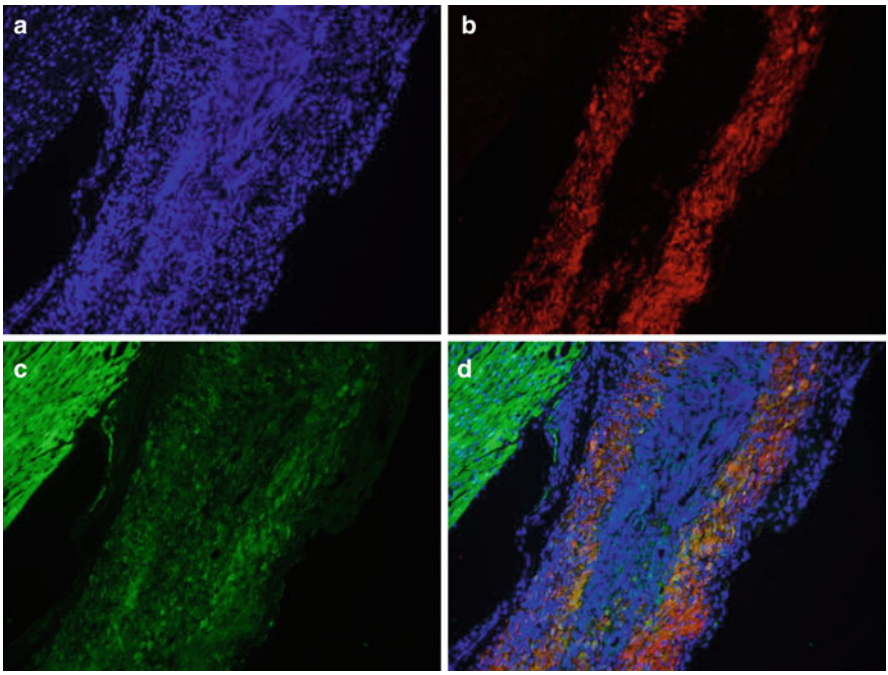

Fig. 46.4 Representative postimplantation histology for rat embryo-derived ECT implanted onto the epicardial surface of a syngeneic adult male rat 2 weeks after coronary artery ligation-induced myocardial infarction. (**a**) *Blue* (DAPI, nuclei), (**b**) *red* (arginase marker for macrophages), (**c**) *green* (GFP+ for recipient myocardium and inflammatory cells), (**d**) merged image. Images are 10× magnification. Note the high cellularity of the recipient myocardium (*upper left corner*) and the implanted ECT. Note the increased presence of arginase along the outer margins of the implanted ECT, consistent with the higher cell density noted in ECT (Keller lab, unpublished)

Second, the in vivo results are often reported after relatively short periods that may not reflect long-term, sustained functional recovery [13]. Finally, the acute surgical models of cardiac injury in preclinical models often do not fully represent the human disease state with medical comorbidities and recurrent episodes of ischemia/injury. To date there are too few studies in large animal models to validate the feasibility of scaling up CM and ECT production for cardiac repair and to compare direct cell delivery strategies to ECT implantation strategies for short- and longer-term efficacy.

46.7 Future Directions

One clear focus of current and future research is the optimization and expansion of the cellular constituents, including CM, required for clinical-grade ECTs. Ultimately, these cells will need to be from human sources and have minimal immunogenic profiles. Another major area of investigation will be large animal preclinical

models that more accurately represent human CV failure and that can generate data in support of regulatory approval for phase I human clinical trials. Related to these large animal models will be innovations in the ability to manufacture large-scale ECTs and to implant them with minimally invasive techniques. Although it is beyond the scope of this chapter, the generation of reproducible human ECTs creates the opportunity to develop in vitro myocardial surrogate tissues for novel drug therapeutics and toxicity assays.

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Dissecting the Left Heart Hypoplasia by Pluripotent Stem Cells

47

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Keywords
Hypoplastic left heart syndrome • Pluripotent stem cells • Disease modeling

The genetic background of hypoplastic left heart syndrome (HLHS) is still unknown. Cardiac differentiation from pluripotent stem cells (PSCs) can recapitulate the cardiogenesis in vitro, and PSC technology could be useful to dissect the diseases with the complex mechanisms. In the past few years, some researches were reported to seek the pathogenesis of HLHS by using PSCs. This paper reports the achievements.

1. Gaber N et al. showed that human embryonic stem cells (hESCs) during cardiovascular lineage with hypoxia recapitulated the phenotype of the HLHS heart, which was characterized by increased expression of the oncogenes and TGF-ß1, damaged DNA, and senescence with cell cycle arrest [1]. The phenotypes were rescued by TGF-ß1 inhibition.
2. Jiang Y et al. generated disease-specific induced pluripotent stem cells (iPSCs) from a patient with HLHS [2]. HLHS-iPS-derived cardiomyocytes demonstrated repression of MESP1, TNNT2, and delayed expression of GATA4 compared with hESCs and control-iPSCs. HLHS-iPS-derived cardiomyocyte showed calcium oscillation under caffeine and inositol trisphosphate receptor upregulation, presumably as a result of ryanodine receptor dysfunction.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_47

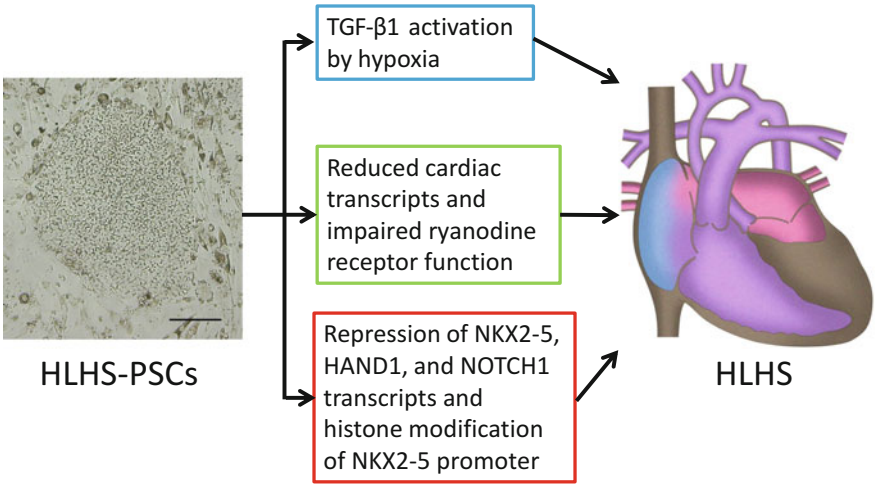

Fig. 47.1 PSC technology models HLHS. Three possible mechanisms of HLHS were unveiled by PSCs. Bar, 200 μm

3. Kobayashi J et al. generated five HLHS-iPSC lines and found repression of the transcripts such as NKX2-5, HAND1, HAND2, NOTCH1, HEY1, HEY2, and TBX2 in HLHS-iPS-derived cardiomyocytes [3]. The promoter activities of SRE, TNNT2, and NPPA were suppressed in HLHS-derived cardiac progenitor cells and iPSCs compared with those from bi-ventricle (BV). All promoter activities of both cell types could be fully restored by co-transfection of NKX2-5, HAND1, and NOTCH1, and co-transfection of the shRNAs into BV-derived cells reduced the promoter activation. HLHS-derived cardiomyocytes demonstrated repressed H3K4me2 and acH3 and increased H3K27me3 in NKX2-5 promoter, implying suppressed NKX2-5 promoter activity.

Taken together, the PSC technology can be useful to dissect the complex heart diseases. Further investigation using this technique is necessary to determine the pathogenesis of HLHS (Fig. 47.1).

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Lentiviral Gene Transfer to iPS Cells: Toward the Cardiomyocyte Differentiation of Pompe Disease-Specific iPS Cells

48

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Keywords
iPS cells • Lentivirus • Pompe disease

Pompe disease is an inherited neuromuscular disorder caused by a genetic deficiency of acid-glucosidase-alpha (GAA). The clinical symptoms of Pompe disease include progressive weakness, respiratory failure, and ventricular hypertrophy. Enzyme replacement therapy has been shown to ameliorate these symptoms. Cardiomyocytes derived from patient/disease-specific iPS cells (iPS-CMs) have been used for pathophysiological analyses, drug screening, and cell therapy. Our research goal was to generate cardiomyocytes that can be differentiated from gene-corrected Pompe disease-specific iPS cells.

We obtained iPSC (TkDA3-4) generated from human dermal fibroblasts [1]. GAA was cloned into cDNA expressing third-generation lentiviral vectors (CS2-EF1α-GAA). To assess the transfection efficacy, Venus, a YFP variant protein, was also cloned into the vector (CS2-EF1α-Venus). Then, we transfected lentiviral vectors containing GAA to iPSCs at three different concentrations to

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_48

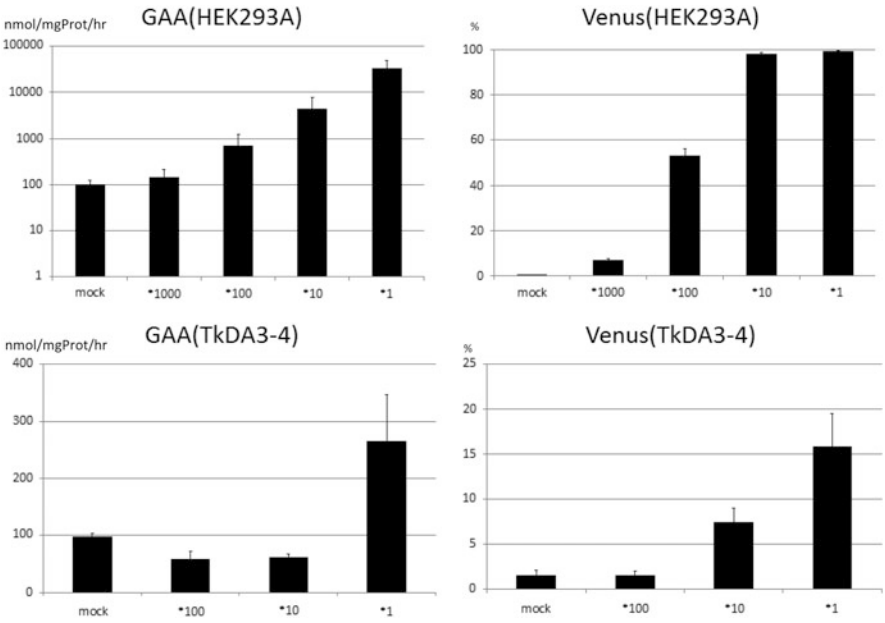

Fig. 48.1 Gene transfer to HEK293A cells and TkDA3-4. Dose-dependent expressions of GAA and Venus were observed in each cell line

determine the optimized titer for gene correction. We showed that dose-dependent expression of both GAA and Venus was observed in iPSCs, even though the expression levels were relatively low compared to HEK293A cells.

Cardiomyocyte differentiation of iPS cells is the most important procedure for replicating the disease hallmarks of Pompe disease. In fact, there is no single best protocol for obtaining cardiomyocytes derived from iPS cells. The functional assessment of iPSC-derived cardiomyocytes is another critical aspect of our research. The differences between the function of iPSC-derived cardiomyocytes obtained from normal control cells and those obtained from Pompe disease cells should therefore be strictly evaluated in order to thoroughly discuss the efficacy of gene therapy for iPSC (Fig. 48.1).

Acknowledgments The author thanks Dr. M. Otsu at Tokyo University for providing iPSC TkDA3-4 cell lines. Third-generation lentiviral vector was a kind gift from Dr. H. Miyoshi at RIKEN BRC, and Venus was kindly provided from A. Miyawaki at RIKEN BSI.

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Molecular Analysis of Long-Term Cultured Cardiac Stem Cells for Cardiac Regeneration

49

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Keywords
Cardiac stem cell • Myocyte • Regeneration • c-Kit • IGF-1

A c-Kit (CD117) is a well-known cell surface marker for adult somatic stem cells. We harvested c-Kit-positive cardiac stem cells (CSCs) from adult rat hearts by performing magnetic-activated cell sorting (MACS) and subjected them to long-term bulk culture more than 40 times. We made 11 attempts to obtain c-Kit-positive cells from adult (6–8-month-old) rats. Our initial expectation was of obtaining cells with homogenous cardiac phenotypes. However, each CSC bulk culture expressed varying degrees of the genes and cell surface markers belonging to cardiac and other mesenchymal lineages. The results suggested that these CSCs retained multiple developmental potential to some extent. Consequently, we investigated these CSCs in detail, hoping to establish the regeneration method by using c-Kit-positive cardiac cells [1–12].

- CSC-21E maintained the cell shape, yielding spherical aggregates under a culture condition. The aggregate shape did not facilitate cell adherence to the dish surface. Interestingly, the proteomic analysis of these two morphological statuses revealed the drastic change of the protein profiles with the spherical aggregates showing protein profiles characteristic of stem cells and the flat cells

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_49

showing the profile indicative of differentiated cells, especially of the distinct differences in stress proteins and metabolic enzymes [2, 3, 7].

- CSC5 differentiated into cells with a myocyte/adipocyte mixed phenotype. Members of the transforming growth factor (TGF)-β superfamily were identified as significant regulators of the differentiation of these cells into either adipocytes or myocytes [1–6, 9, 10].
- CSC4A exhibited the ability for sustained contractibility shown by cardiomyocytes that were cocultured with a membrane filter separating cardiomyocytes from CSC4A. This suggests that the CSC4A cells release factors that support cardiomyocyte contraction. Among the cytokines measured in the cocultured medium, insulin-like growth factor-1 (IGF-1) levels appeared to correlate with cardiomyocyte sustenance. However, CSC4A cells do not express IGF-1. This suggests that some other unknown factors are released from CSC4A that can induce IGF expression in cardiomyocytes [1–6, 8, 12].

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Minor Contribution of Cardiac Progenitor Cells in Neonatal Heart Regeneration

50

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Keywords
Heart regeneration • Cardiomyocyte proliferation • Cardiac progenitor cells

The adult mammalian heart is incapable of regeneration after injury, as shown by the limited amount of cardiomyocyte proliferation and poor neovascularization. We recently showed that neonatal mice have a remarkable ability to regenerate damaged heart after apical resection or myocardial infarction (MI), which includes complete reconstruction of myocardial wall with vascular network [2, 3]. Although lineage tracing showed that the main source of newly formed cardiomyocyte is preexisting cardiomyocytes, it is still possible that there is a minor contribution of other types of cells to the cardiomyocyte. In addition, lineage origin of the newly formed vasculature during postnatal cardiac maturation and neonatal heart regeneration remains unclear (Fig. 50.1).

In order to trace the lineage of non-myocyte-derived cells during neonatal heart regeneration, we utilized Rosa26-tdTomato reporter mouse line crossed with capsulin-merCremer line in which epicardial cells and interstitial fibroblasts are labeled specifically and irreversibly after induction with tamoxifen [1]. At postnatal day 0 (P0), Cre was activated by intraperitoneal injection of tamoxifen, and then MI was induced 2 days later (P2). Subsequently the hearts were harvested at 21 days after MI and tdTomato expression was examined. tdTomato+ cells were detected in the epicardium, interstitial fibroblasts, vascular endothelium, and smooth muscle in the regenerated heart. Remarkably, we could detect a very small

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_50

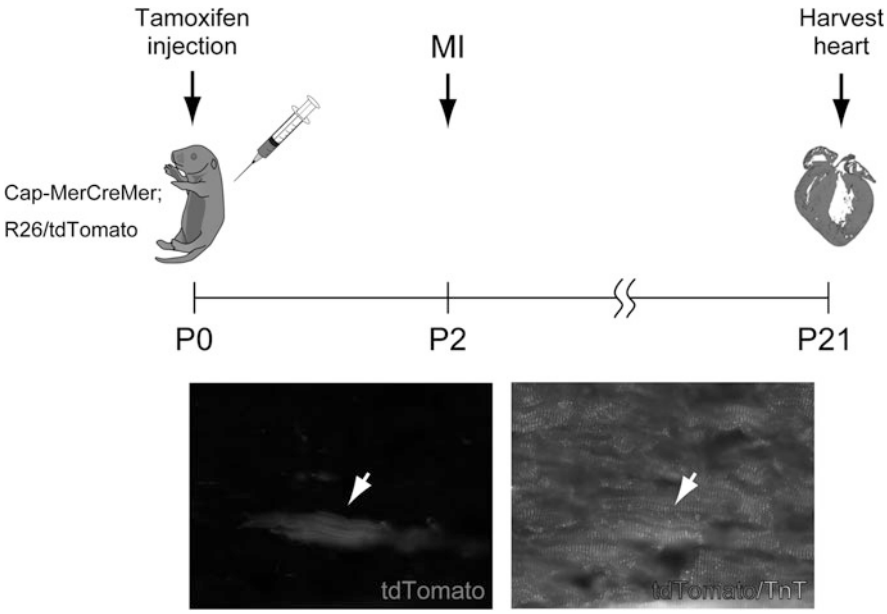

Fig. 50.1 Capsulin-positive cardiac progenitor cells contribute to myocardial lineages during neonatal heart regeneration. Schematics show experimental outline for genetic fate mapping after neonatal MI. *Lower panels* show immunofluorescence on section of 21 days post-MI heart. tdTomato-labeled cells are found in cardiac troponin T (TnT)-positive cardiomyocytes

population (1–2 cells/section) of tdTomato+ cardiomyocyte in the regenerated neonatal heart. No tdTomato+ cardiomyocyte was detected at P21 without inducing MI. These results strongly suggest that capsulin-positive cardiac progenitor cells play important roles during neonatal heart regeneration, primarily in neovasculogenesis by contributing directly to the endothelial/smooth muscle progenitor cells and to a much lesser extent rare myocytes.

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Part XI

Current Genetics in Congenital Heart Diseases

Perspective

Hiroyuki Yamagishi

Congenital heart disease (CHD) occurs in nearly 1 % of all live births and is the major cause of infant mortality and morbidity; about three per 1,000 live births will require some intervention during the first year of life. Additionally, 95 % of CHD patients survive to adulthood in these days, resulting in a growing population of adult CHD. Despite their clinical importance, the underlying genetic etiology of most CHD remains unknown, so-called "multifactorial" disease.

Identifying genetic cause of CHD is important not only to well understand the disease but also to enhance current knowledge about the molecular biology and genetics involved in the human cardiovascular development. Such knowledge may lead to new preventive and/or therapeutic strategies. Identification of disease genes would benefit the genetic counseling for CHD that is particularly important for the growing population of adult CHD. Deeper understanding of factors and pathways involved in differentiation of the cardiac stem cell and morphogenesis of the cardiovascular system would also provide the development of regenerative therapy for CHD.

During the last two decades, linkage analysis has been used to successfully identify disease genes involved in isolated CHD or genetic syndromes where CHD is part of the phenotype. However, this traditional approach is not generally suitable for CHD because it requires many large families with multiple affected individuals. During the same period, some of syndromes with chromosomal abnormalities have been well studied to identify disease genes in syndromic CHD. CHD is commonly a

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characteristic part of the clinical spectrum in a significant number of syndromes caused by chromosomal abnormalities, including submicroscopic deletions or duplications. To date, these genetic approaches have led to the identification of more than 50 human genes although they have limitation where large familial cases and chromosomal abnormalities account for relatively small portion of CHD.

Recently, a technique of genome-wide association studies (GWAS) has provided common genetic variations that can influence population-attributable risks of certain types of CHD although the information cannot be directly related to the affected individual. Finally, application of the next-generation sequencing (NGS) technologies is revolutionary in the field of genetics in CHD. In contrast to GWAS, the results of NGS are directly applicable to the affected individual. NGS can be targeted or nontargeted. Exome sequencing and whole-genome sequencing, scanning the whole exome of ~20,000 base pairs and the whole genome of ~3,000,000,000 base pairs, respectively, are well suited to the study of complex, heterogeneous diseases such as CHD and the current best technique for discovery of novel genetic causes for CHD. The biggest obstacle during any NGS analysis is, however, to single out the causal variant from the thousands of variants identified during sequencing. Follow-up animal studies, particularly in mice, for candidate genes discovered by genetic analyses have been successful in validating the candidates and uncovering the function of their gene products for the cardiovascular development. More recently, fine mapping of genomic copy number variants (CNVs) by NGS in patients with isolated or syndromic CHD has been used to identify candidate disease genes.

In this part, authors describe the current advance in genetics in CHD using linkage analysis, chromosomal studies, and CNVs studies by NGS, combined with animal experiments that verified novel genetic causes of CHD and provided new insights into the molecular and functional analyses of the cardiovascular development. Current understandings about molecular pathways associated with CHD involve numerous transcription factors and cofactors, including chromatin modifiers, and signaling molecules from ligands to receptors.

Genetic Discovery for Congenital Heart Defects

51

Bruce D. Gelb

Abstract

Congenital heart disease (CHD) behaves like a complex genetic trait in most instances. Recent advances in genomics have provided tools for uncovering genetic variants underlying complex traits that are now being applied to study CHD. Massively parallel DNA sequencing has shown that de novo mutations contribute to ~10 % of severe CHD and implicated chromatin remodeling in pathogenesis. Genome scanning methods for copy number variants (CNVs) identify likely pathogenic genomic alterations in 10 % of infants with hypoplastic left heart syndrome and related single ventricle forms of CHD. The growth and neurocognitive development of children with CHD and those CNVs is worse, and clinical examination is relatively insensitive for detecting those CNVs. In sum, new opportunities for preventing and ameliorating CHD and its comorbidities are anticipated as its genetic architecture is elaborated through the use of state-of-the-art genomic approaches.

Keywords

Congenital heart disease • Copy number variants • Exome sequencing • Genetics

51.1 Introduction

Congenital heart defects (CHD), with an estimated incidence of 2–3 % when bicuspid aortic valve (BAV) is included, are widely believed to have strong genetic underpinnings. Epidemiologic studies have shown considerable consistency in the

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_51

distribution of CHD lesions across time and geographic location. The landmark study by Ruth Whittemore, in which she examined recurrence risks for offspring of women with CHD, revealed a 16 % rate with a 60 % concordance in the form of CHD between mother and child [1]. Estimates of heritability for BAV and hypoplastic left heart syndrome (HLHS) are 89 % and 95 %, respectively [2, 3]. Recent studies of CHD from Denmark, where the highly organized medical system enables population studies, have provide estimates of the relative risks of various forms of CHD among first-degree relatives that significantly increased, often to >5 [4]. Taken as a whole, these epidemiologic findings point to genetic defects contributing importantly to CHD etiology.

Identification of the precise mutations has been challenging [5]. We have known for some time that a modest percentage of CHD (~5 %) is attributable to aneuploidies such as trisomy 21. With the advent of molecular genetic approaches, point mutations with apparently strong effects have been identified in rare families inheriting CHD in Mendelian or near-Mendelian fashion. More recently, the role of larger genomic events generating pathologic copy number variants (CNVs) for CHD has become apparent. This started with the recognition of 22q11 deletions underlying DiGeorge, Takao conotruncal face, and velocardiofacial syndromes. Several surveys have implicated a wide range of gain and loss CNVs in various forms of CHD.

Based on the author's oral presentation at the 2013 Takao Symposium, two recent studies that further elaborate the genetic etiology of CHD will be reviewed here.

51.2 De Novo Mutations

Through recent advances in molecular genetic technologies, it is now possible to sequence the roughly 1 % of the human genome that contains the coding regions for all genes (called the exome), representing approximately 180,000 exons and 30 megabases (Mb), in a relatively rapid and affordable manner. While exome sequencing was initially used to discover mutations underlying Mendelian disorders, current efforts are increasingly focusing on unraveling complex genetic traits.

The Pediatric Cardiac Genomics Consortium (PCGC) [6], a National Heart, Lung, and Blood Institute-funded research enterprise, recently completed a first-of-kind study to determine the role of de novo mutations in the etiology of severe forms of CHD [7]. Exome sequencing was performed for 362 parent-offspring trios, in which the offspring had a sporadic conotruncal defect, left ventricular outflow track obstructive lesion, or heterotaxy, and compared to comparable data from 264 control trios. While the overall rate of de novo point and small insertion/deletion (indel) changes was equivalent between CHD cases and controls, there was an excess burden of protein-altering mutations in genes highly expressed during heart development (odds ratio (OR) of 2.53). Excess mutations had a role in 10 % of CHD cases and led to the estimate that ~400 genes underlie these birth defects. After filtering to retain variants most likely to be deleterious (nonsense,

splice site, and frameshift defects), the burden among CHD cases increased, attaining an OR of 7.50.

Next, the PCGC investigators asked whether the burden of de novo protein-altering mutations among the CHD cases preferentially targeted particular biologic processes [7]. Indeed, they observed a highly significant enrichment of mutation among genes encoding proteins relevant for chromatin biology, specifically the production, removal, or reading of methylation of Lys4 of histone 3 (H3K4me) (Fig. 51.1). The phenotypes of the eight subjects harboring H3K4me de novo mutations were diverse, both with respect to the form of CHD and the involvement of extracardiac tissues. In addition, two independent de novo mutations were found in *SMAD2*, which encodes a protein with relevance for demethylation of Lys27 of histone 3 (H3K27me). SMAD2 contributes to the development of the left-right body axis; both subjects harboring *SMAD2* mutations had dextrocardia with unbalanced complete atrioventricular canal defects with pulmonic stenosis. While the contribution of chromatin remodeling to cardiovascular development generally and

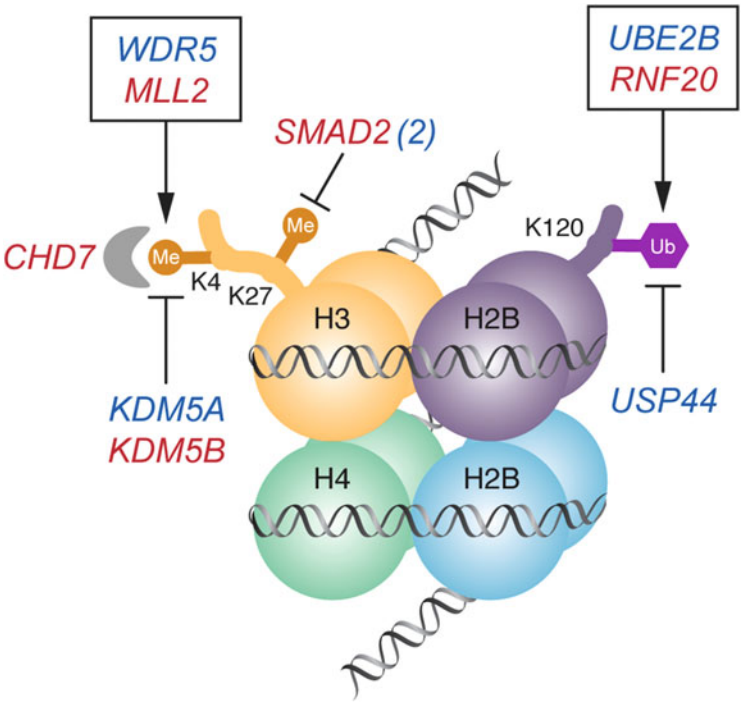

Fig. 51.1 De novo mutations in the H3K4 and H3K27 methylation pathways. Nucleosome with histone octamer and DNA, H3K4 methylation bound by CHD7, and H3K27 methylation and H2BK120 ubiquitination is shown. Genes mutated in CHD that affect the production, removal, and reading of these histone modifications are shown; genes with damaging mutations are shown in red, and those with missense mutations are shown in blue. SMAD2 (2) indicates there are two patients with a mutation in this gene. Genes whose products are found together in a complex are enclosed in a box (Reprinted without modification from Ref. [7])

certain rare genetic syndromes with CHD like Kabuki syndrome had been recognized previously, this study exposed a far broader role in CHD pathogenesis. The finding also suggests a fascinating potential link to other birth defects as de novo chromatin remodeling mutations have also been implicated in autism [8].

51.3 Copy Number Variants

CNVs, which are gains or losses of DNA ranging in size from 1 kb to several Mbs, affect roughly 10 % of the human genome [9]. CNVs are typically detected on a genome-wide basis using SNP microarrays or array comparative genomic hybridization (aCGH). Although differentiating pathogenic CNVs from benign polymorphic one remains challenging, it has become clear that pathologic CNVs contribute significantly to the pathogenesis of CHD as rare large CNVs are observed in 5–15 % of affected individuals [10–14].

To address the issue of how pathogenic CNVs affected outcomes for children with CHD, Carey and colleagues studied children who had previously been subjects in one or both of two clinical trials undertaken by the National Heart, Lung, and Blood Institute-funded Pediatric Heart Network [15]. The two studies were the Infants with Single Ventricle (ISV) and Single Ventricle Reconstruction (SVR) trials, both focusing primarily on infants with HLHS. Carey et al. used aCGH to identify CNVs in 223 subjects from the ISV and SVR trials for whom genomic DNA was available. Because the DNA samples were procured late in those trials, subjects who died earlier, particularly perioperatively, could not be studied. For the CNV work, CNVs were $\geq$300 kb and not identified as genetic polymorphisms based on existing databases.

Carey and co-workers observed pathogenic CNVs in 13.9 % of the children, which was significantly more than the 4.4 % rate in the controls (preexisting data from blood samples from subjects with solid cancers from The Cancer Genome Atlas project) [15]. For the CHD cases, there was a roughly 4:1 ratio of duplications to deletions and the median sizes were 674 kb and 1.5 Mb, respectively. Parental genomic DNAs were available for 12 of the subjects with pathogenic CNVs; analysis of those showed that slightly more than have of the pathogenic CNVs were inherited.

For the ISV and SVR studies, careful assessment of growth (weight-, height- and head circumference-for-age z-scores) and neurocognitive function (Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) measured with the Bayley Scales of Infant Development II) were determined at 14 months of age [15]. Comparison of the CHD subjects harboring pathogenic CNVs to those who did not revealed that the former were significantly shorter by an average of 0.65 z-score. Subgroup analysis showed that those with deletion CNVs had significantly lower PDI scores.

Among the 31 pathogenic CNVs found among the CHD subjects, 13 had previously been associated with genomic disorders. The children harboring these known CNVs had the worst outcomes with globally reduced neurocognitive development (MDI and PDI) as well as the slowest growth (Table 51.1).

Table 51.1 Fourteen-month outcomes for subgroups based on genetic examination

	N^a	MDI	PDI	Weight *Z*	Length *Z*	HC *Z*
CNV- Syndrome- Dysmorphic- Extracardiac-	69	89.1	77.5	−0.71	−1.13	−0.34
		(18.0)	(20.2)	(1.07)	(1.32)	(1.24)
CNV+	14	85.4	65.1*	−0.94	−1.61	−0.04
		(20.1)	(17.6)	(0.88)	(1.08)	(1.38)
CNV+ or syndrome	18	83.2	67.9*	−1.19*	−1.99*	−0.16
		(18.7)	(19.4)	(1.11)	(1.73)	(1.27)
Dysmorphic/extracardiac	29	89.3	78.1	−0.63	−1.18	−0.10
		(18.1)	(20.0)	(1.25)	(1.30)	(1.28)
CNV syndrome	98	89.4	77.8	−0.73	−1.18	−0.25
		(17.5)	(19.8)	(1.10)	(1.43)	(1.32)

All data shown as mean (standard deviation)
MDI mental developmental index, *PDI* psychomotor developmental index, *Weight Z* weight-for-age Z score at 14 months, *Length Z* length-for-age Z score at 14 months, *HC Z* head circumference Z score at 14 months
*$p < 0.05$ compared to CNV/syndrome/dysmorphic/extracardiac
[a]Size of each cohort. Incomplete data for outcomes resulted in lower Ns. Reprinted without modification from Ref. [15]

Finally, Carey and co-workers looked at the sensitivity of clinical examination in detecting children with CHD and pathogenic CNVs, which was possible for the subjects from the SVR study [15]. Of 116 children examined, 3.4 % were diagnosed with a defined genetic syndrome, none associated with a pathogenic CNV, and 25 % had one or more dysmorphic features and/or extracardiac malformations, which were not enriched among those with CNVs. Most strikingly, more than 70 % of the children with CNVs previously associated with genomic disorders had no dysmorphic feature or extracardiac anomaly. Taken as a whole, this analysis showed that clinical examination was relatively insensitive for determining which children with CHD harbored pathogenic CNVs.

The findings from this study support the routine use of CNV testing in newborns with single ventricle forms of CHD to enable better prognostication and early intervention. Similarly, the poorer linear growth associated with all pathogenic CNVs, the worse neurocognitive outcomes with deletions, and particularly the globally poor outcomes with CNVs associated with known genomic disorders could impact clinical trial outcomes depending on the designated endpoints.

51.4 Future Directions

Recent advances in genomics have enabled the elucidation of the architecture of CHD genetics. As that project proceeds, the next challenge will be translating those gene discoveries into actionable clinical approaches. Improved prognostication with respect to the heart disease and extracardiac comorbidities can be used most immediately. Finding strategies that reduce CHD incidence or alter its natural

history will require the elucidation of pathogeneses, and a careful balancing of potential benefits with adverse effects as fundamental biological process like chromatin remodeling will probably predominate.

Acknowledgments The author declares that he has no conflict relevant to this manuscript. Funding for this work was partly from a grant from NIH (U01 HL071207) to B.D.G.

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52 Evidence That Deletion of ETS-1, a Gene in the Jacobsen Syndrome (11q-) Cardiac Critical Region, Causes Congenital Heart Defects through Impaired Cardiac Neural Crest Cell Function

Maoqing Ye, Yan Yin, Kazumi Fukatsu, and Paul Grossfeld

Abstract

Jacobsen syndrome (11q-) is a rare chromosomal disorder characterized by multiple problems including congenital heart defects, behavioral problems, intellectual disability, dysmorphic features, and bleeding problems. Septal defects, including double outlet right ventricle (DORV), are among the most common CHDs that occur in 11q-. One possible mechanism underlying the CHDs and other problems in 11q- is a defect in neural crest cell function. The E26 avian leukemia 1, 5′ domain (ETS-1) gene is a member of the ETS-domain transcription factor family. ETS-1 is deleted in every 11q- patient with CHDs, and gene-targeted deletion of the ETS-1 gene in C57/B6 mice causes DORV with 100 % penetrance. Normal murine cardiac development requires precisely regulated specification of the cardiac neural crest cells (cNCCs). To begin to define the role of ETS-1 in mammalian cardiac development, we have demonstrated that ETS-1 is strongly expressed in mouse cNCCs during early heart development. Sox10 is a key regulator for the neural crest cell gene regulatory network. It is also an early marker for NCCs, and its expression can facilitate the analysis of cNCC function during embryonic development. We have demonstrated that loss of ETS-1 causes decreased migrating Sox10-expressing cells in E10.5 C57/B6 mouse embryos. These results suggest a NCC migration defect in ETS-1 mutants. Our data support the hypothesis that ETS-1 is required for specification and migration of cNCCs and for regulating a cNCC-specific gene regulatory network that is required for normal cardiac development.

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T. Nakanishi et al. (eds.), *Etiology and Morphogenesis of Congenital Heart Disease*,
DOI 10.1007/978-4-431-54628-3_52

Keywords
Jacobsen syndrome • Cardiac neural crest • ETS-1 • Double outlet right ventricle • Genetic modifier

52.1 Introduction

Congenital heart defects (CHDs) are the most common birth defect in live-born infants, occurring in 0.7 % of the general population. Although there are numerous genetically engineered mouse models for CHDs, only a small number of these genes are currently associated with CHDs in humans.

Conotruncal defects (CTDs), including double outlet right ventricle (DORV), are among the most common CHDs in the general population and usually require surgical repair to ensure a normal life expectancy. Little is known about the molecular and cellular mechanisms underlying the development of CTDs in humans. Normal murine cardiac development requires precisely regulated specification of the cardiac neural crest cells (cNCCs) and subsequent migration to the developing outflow tract. In animal models, impairment of NCCs causes CTDs [1].

The 11q terminal deletion disorder (11q-, Jacobsen syndrome) (OMIM # 1477910) is caused by heterozygous deletions in distal 11q (Fig. 52.1).

Fifty-six percent of patients have CHDs (Table 52.1). Septal defects, including DORV, account for about half of all CHDs that occur in 11q- patients.

As shown in Fig. 52.2, we have identified a Jacobsen syndrome cardiac "critical" region in distal 11q containing only five known genes, including the ETS-1 transcription factor.

The ETS-1 gene is a member of the ETS-domain transcription factor family. ETS factors have important roles in a host of biological functions, including the regulation of cellular growth and differentiation as well as organ development.

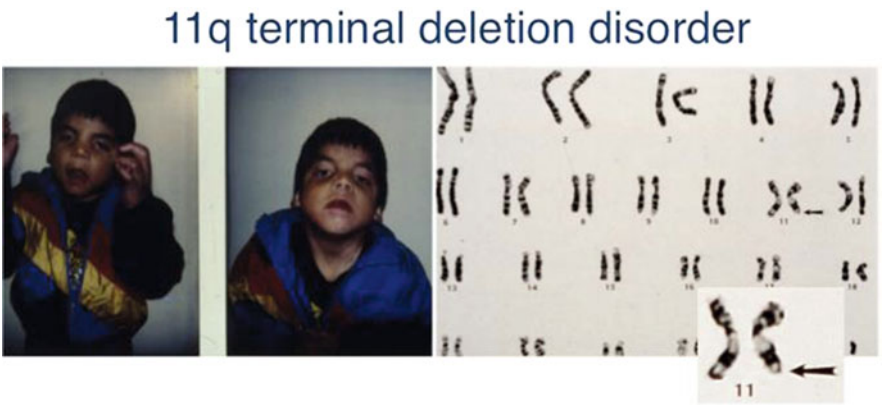


Fig. 52.1 Patient with Jacobsen syndrome. Karyotype demonstrates large terminal deletion in 11q

Table 52.1 CHDs in 11q-

Left-sided/flow lesions (two-thirds)
Hypoplastic left heart syndrome[a]
Shone's complex
Coarctation
Bicuspid aortic valve
Aortic valve stenosis
Mitral valve stenosis
Ventricular septal defect
Less common heart defects (one-third)
Secundum atrial septal defect
Aberrant right subclavian artery
Atrioventricular septal canal defect
D-transposition of the great arteries
Dextrocardia
Left-sided superior vena cava
Tricuspid atresia
Type B interruption of the aortic arch/truncus arteriosus
Pulmonary atresia/intact ventricular septum
TAPVR
Ebstein anomaly
Tetralogy of Fallot

[a]~10 % born with HLHS; ~1–2 % of all HLHS pts

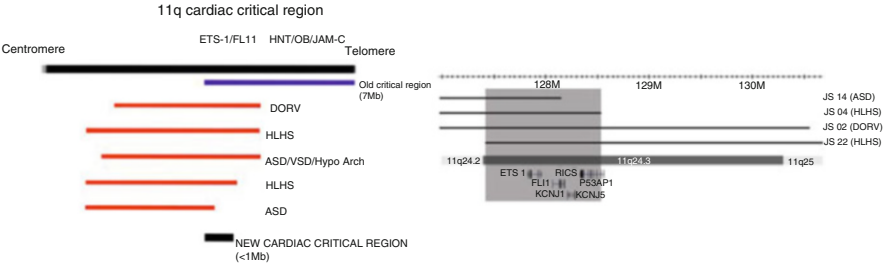


Fig. 52.2 Cardiac "critical" region in 11q, defined by region of overlap between smallest terminal deletion and interstitial deletions in patients with Jacobsen syndrome clinical phenotype, including congenital heart defects

Until recently, nothing was known about the function of ETS-1 in mammalian heart development or its possible role in causing human congenital heart disease.

Although little is known about the mechanisms underlying ETS-1 in mammalian heart development, recent studies in the ascidian *Ciona intestinalis* have demonstrated that ETS-1 regulates two critical aspects of heart development: heart progenitor cell migration and heart cell differentiation. Interestingly, loss of ETS-1 abolishes normal heart cell migration during development, resulting in an ectopically located heart chamber. Taken together, these results indicate that ETS-1

is required for normal cell migration in heart development, although the mechanism (s) underlying these cell migration defects remains to be elucidated.

52.2 Evidence for a Role for ETS-1 in the Cardiac Neural Crest in Mice

52.2.1 Expression of ETS-1 in Cardiac Lineages During Murine Heart Development

To begin to define the role of ETS-1 in mammalian cardiac development, we have performed in situ hybridization studies on mouse embryos and have shown that ETS-1 is strongly expressed in murine cNCCs as well as the endocardium during early heart development (Fig. 52.3).

52.2.2 ETS-1 Mutant Mice Have a Double Outlet Right Ventricle (DORV) Phenotype

To determine if loss of ETS-1 causes congenital heart defects, we have analyzed gene-targeted ETS-1 deletion mice. As shown in Fig. 52.4, ETS-1 homozygous null mice in a C57/B6 background exhibit DORV with 100 % penetrance, resulting in perinatal lethality [2].

52.2.3 Lost of ETS-1 Causes Decreased Expression of Sox10

We have previously demonstrated that ETS-1 expression is expressed in cNCCs and endocardium during murine embryonic development. Sox10 is a key regulator in the NCC gene regulatory network. It is critical for migration and specification of NCC fate. To examine the role of ETS-1 in murine cardiac NCC migration, we examined Sox10 expression in ETS-1−/− mutant and control C57/B6 embryos at E10.5 by using whole-mount in situ hybridization analysis. Expression of Sox10 in the NCCs in the pharyngeal arch region and dorsal root ganglia was reduced in ETS-1−/− mutant embryos, suggestive of a cNCC migration defect as shown in Fig. 52.5 (left). The result was confirmed independently by quantitative RT-PCR analysis (right).

52.3 Establishment of an Explanted cNCC "Ex Vivo" Culture System

We hypothesized that defects in cNCCs migration should be able to be reproduced in an "ex vivo" culture system. Toward that end, we have utilized an explanted culture system to observe cNCC migration [3]. Mouse embryos were collected from

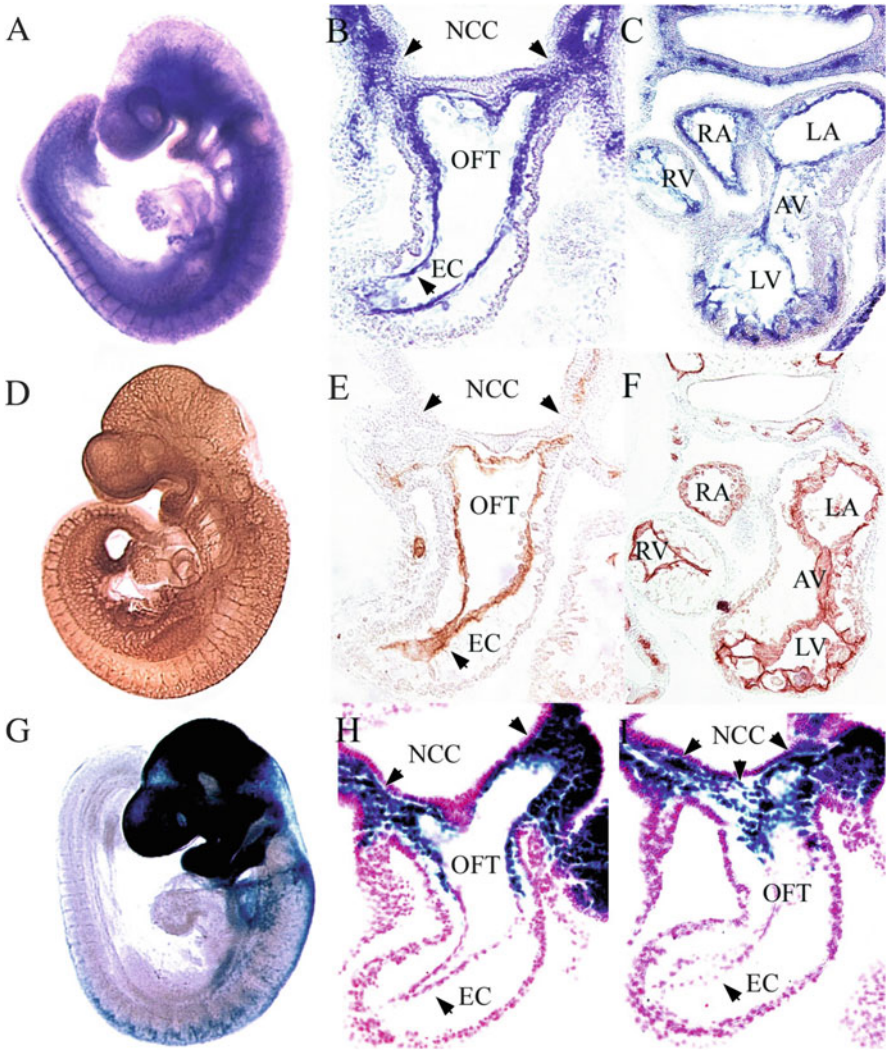


Fig. 52.3 Expression of ETS-1 in the heart in ED9.5 embryos: in situ hybridizations are shown in (**a**) (whole mount) and in sections (**b**) (anterior coronal section) and (**c**) (posterior coronal section). Immunohistochemistry indicating endothelial expression using a PECAM (CD31) antibody is shown in (**d**) (whole mount) and in sections (**e**) (anterior coronal) and (**f**) (posterior coronal). LacZ staining of neural crest using a Wnt1-Cre; ROSA26 LacZ indicator strain is shown in (**g**) (whole mount), (**h**) (anterior coronal), and (**i**) (posterior coronal)

C57/B6 background embryos at E8.5, coinciding with the onset of cNCC migration toward the heart. E8.5 embryos were collected and treated by dispase to dissociate the tissue gently. After treatment, neural tubes from somite one to three region (cardiac neural crest) were dissected out and cut into 100×300 um pieces. Each

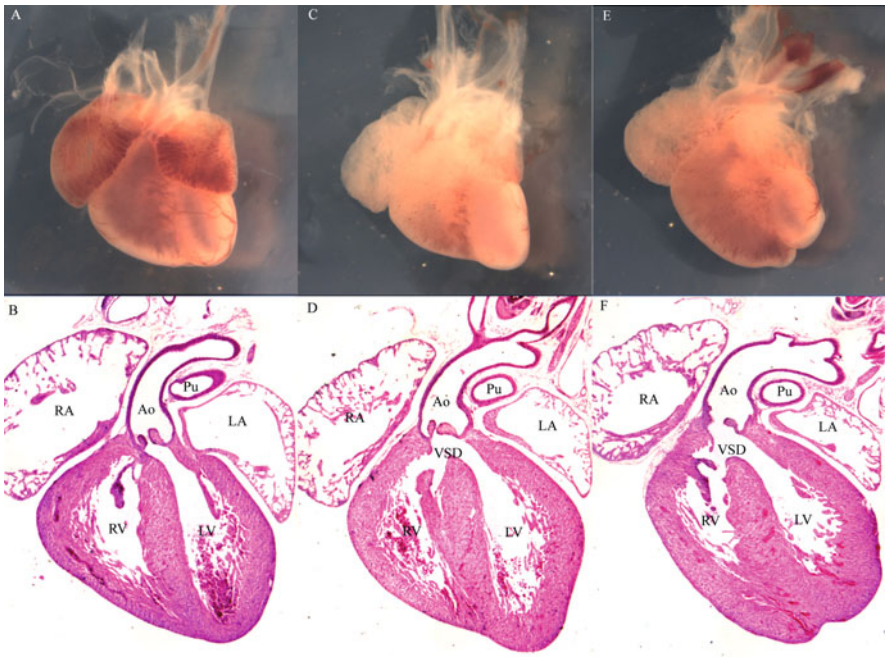


Fig. 52.4 Gene-targeted knockout of ETS-1 in C57/B6 E16.5 mice, showing double outlet right ventricle with normally related great arteries. Wild type is shown in (**a**) and (**b**); two mutant hearts are shown in panels (**c–f**). *RA* right atrium, *LA* left atrium, *RV* right ventricle, *LV* left ventricle, *Pu* pulmonary artery, *Ao* aorta, *VSD* ventricular septal defect

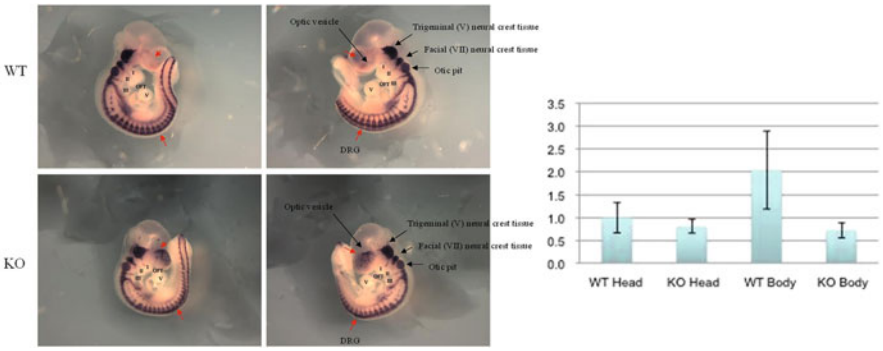
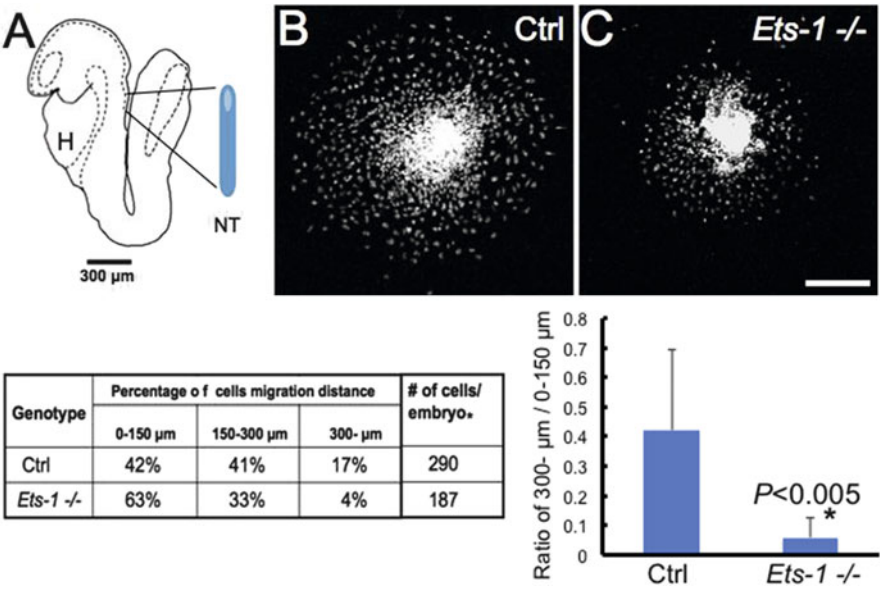


Fig. 52.5 Whole mount study demonstrating decreased Sox10 expression in E10.5 ETS-1−/− C57/B6 embryos (left, lower panel) compared to wild type (left, upper panel). Real-time quantitative PCR demonstrates decreased Sox10 expression in the body of E10.5 embryos in ETS-1−/− embryos, compared to wild type (right)

piece was placed on fibronectin-coated glass bottom slides and incubated in culture media in 5 % CO_2 and 21 % O_2. After 24 h incubation, we performed DAPI staining and obtained images of the migrating cells. Representative results from WT and ETS-$1^{-/-}$ mice are shown in Fig. 52.6.

52.3.1 Loss of ETS-1 in C57/B6 Mice Causes Decreased NCC Numbers and Decreased Migration

To analyze the migration distance using our ex vivo system, we counted the number of migrating cells in each explanted culture. The migration distance was divided into three distanced from the neural tube edge: 0–150 μm, 150–300 μm, and over 300 μm edge. The total number of cells that had migrated for each distance was manually counted, and the percentage of the total for each migration distance was determined. As shown in Fig. 52.6, cNCCs from ETS-1−/− C57/B6 embryos were fewer in number and had decreased migration distance. The percentage of total cells migrating >300 μm 24 h after explantation in ETS-1−/− mutant embryos is fourfold lower than control. The cell number per embryo was also significantly decreased in ETS-1−/− mutants.



Genotype	Percentage o f cells migration distance			# of cells/ embryo.
	0-150 μm	150-300 μm	300- μm	
Ctrl	42%	41%	17%	290
Ets-1 -/-	63%	33%	4%	187

Fig. 52.6 Cardiac neural crest cells migration in explant culture. (*A*) Schematic of E8.5 embryo. *Blue bar* shows the neural tubes somite one to three region. *H* heart, *NT* neural tube. (*B*) Representative images with DAPI staining of 24 h cultured cardiac neural crest cells in control (*B*) and ETS-1−/− mutants (*C*). *Scale bar*, 300 μm. Both images were taken by an inverted confocal microscope FV-1000 using a 10× objective lens. (*D*) Quantification of migration distances, demonstrating impaired migration ability in ETS-1−/− cells compared to wild type

We then calculated the ratio of 300+ μm over 0–150 μm of cultured migration cells. As shown in Fig. 52.6, the ratio was significantly reduced in ETS-1−/− mutants.

52.4 Cardiac Neural Crest Cell Number and Migration Are Preserved in ETS-1−/− Mice in an FVBN-1 Background

The cardiac phenotype in ETS-1 knockout mice is dependent on the genetic background. In contrast to C57/B6 mice, ETS-1−/− mutant mice in an FVBN-1 background have normal hearts. Consistent with a neural crest cell autonomous mechanism for causing DORV in the C57/B6 strain, ex vivo studies demonstrate normal cNCC numbers and migration in ETS-1−/− FVBN-1 embryos (data not shown).

52.5 Summary, Future Directions, and Clinical Implications

Using human and murine genetics systems, we have identified the ETS-1 transcription factor gene as the likely causative gene for CHDs in Jacobsen syndrome. Our expression data implicate an important role for ETS-1 in the cardiac neural crest during murine heart development. Based on the known function of the ETS-1 homologue in the ascidian *Ciona intestinalis* and using an ex vivo cell migration system, we hypothesize that ETS-1 is essential for early cNCC fate determination and migration in mammalian heart development. Future studies will include performing in vivo real-time imaging and lineage fate mapping studies in the neural crest to delineate how loss of ETS-1 causes decreased cNCCs in the developing heart [4], whether there is a NCC-autonomous mechanism and whether there is a migration defect. Importantly, loss of ETS-1 in FVBN-1 mice does not cause congenital heart defects, suggesting the presence of a genetic modifier(s) that can prevent the development of CHDs in the absence of ETS-1. To address this, determination of a neural crest cell autonomous mechanism would implicate a neural crest cell-specific modifier. Identification of such a genetic modifier could have important implications for the prevention of certain congenital heart defects.