Part IX

Current Molecular Mechanism in Cardiovascular Development

Perspective

Osamu Nakagawa

Progenitor cell populations of multiple origins, including those from the mesodermal primary heart field, secondary heart field, and neural crest, participate in the formation of complex structures of the heart and great vessels. A variety of differentiated cell types, such as cardiomyocytes, fibroblasts, endocardial cells, and vascular cells, are coordinated to fulfill the mature functions of the heart and circulatory system. Sequential and combinatorial functions of numerous genes in these cells are necessary for proper cardiovascular development. Phenotype analyses of mutant mice and genetic studies of human patients have revealed the genes essential for cardiovascular cell differentiation, migration, proliferation, and alignment in developing embryos. A significant fraction of those genes encodes DNA-binding transcription factors and their cofactors, indicating that transcriptional regulation plays central roles in cardiovascular differentiation and morphogenesis. Mutations of the genes for inter-/intracellular signaling molecules often cause perturbation of downstream gene expression patterns. Genes associated with the laterality control and those encoding the cardiac sarcomere components were also found responsible for the congenital cardiovascular defects.

Emerging evidence indicates that additional molecules involved in the epigenetic regulation of gene expression are indispensable for cardiovascular development. Chromatin-remodeling protein complexes, SWI/SNF, ISWI, NuRD, and INO80, control the chromatin structure and accessibility of transcriptional machinery including DNA-binding proteins and RNA polymerase II. Gene deletion of a

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SWI/SNF complex factor Brg1/Smarca4 or its partner protein Baf60c/Smarcd3 caused various defects of cardiac structures and growth in mice. *CHD7*, a gene responsible for CHARGE syndrome, functions as a component of the NuRD transcriptional repressor complex. Histone modification was also shown to be important for cardiovascular development. Mutations of *MLL2*/*KMT2D*, which encodes a histone methyltransferase, are a major cause of Kabuki (Niikawa-Kuroki) syndrome. Mutations in the genes involved in histone methylation are significantly enriched in those identified in sporadic cases of congenital heart defects. Importance of histone methylation by polycomb group proteins, histone demethylation by jumonji family proteins, and histone acetylation/deacetylation by HAT/HDAC proteins was also documented using mouse models. In addition, noncoding functional transcripts, namely, microRNAs and long noncoding RNAs, possess crucial functions in the regulation of gene expression during cardiovascular development. MicroRNAs repress gene expression through the degradation of target mRNAs and/or the inhibition of their translation. Long noncoding RNAs modulate gene expression by various mechanisms including the association with epigenetic factors, transcriptional machinery, and microRNAs. Lack of miR-1, miR-133, and many other miRNAs as well as long noncoding RNAs such as Braveheart and Fendrr markedly affects differentiation and growth of cardiomyocytes in experimental models.

In this part, among these epigenetic regulators of gene expression, Shirai et al. describe the significance of polycomb group proteins in embryonic heart development, while Kataoka and Wang provide an overview on the roles of microRNAs and long noncoding RNAs in the developing heart and vasculature. Studies of new molecular mechanisms of cardiovascular development will lead to a better understanding of the etiologies of human congenital heart defects.

Combinatorial Functions of Transcription Factors and Epigenetic Factors in Heart Development and Disease

42

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Abstract

Heart malformations are the most common type of birth defect, affecting more than 2 % of newborns and causing significant morbidity and mortality. In the past two decades, studies have revealed the function and importance of cardiac transcription factors during heart development and in congenital heart disease. Transcription factors generally form complexes with other transcription factors and/or with chromatin factors to perform specific functions. This review focuses on how chromatin factors modify cardiac transcription factors during cardiovascular development and disease.

Keywords

Cardiac development • Cardiac disease • T-box genes • Epigenetic factors • SWI/SNF-type chromatin remodeling factors

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42.1 Transcription Factors in Heart Development

The heart is an organ that pumps blood to and from the body's tissues through the blood vessels. Cardiac muscle contains cardiomyocytes, which ensure the heart's contractile ability; however, cardiomyocytes alone are not sufficient for the heart to function. Other components, such as the conduction cells, fibroblasts, blood vessels, and endocardial cells, are important for maintaining the heart's systemic pumping ability. Each cell type can be identified by its expression of specific transcription factors, signaling molecules, and/or function-specific proteins (Fig. 42.1). The heart is the first organ to form in vertebrates, and it performs a vital role in distributing oxygen and nutrients throughout the embryo. The primordial heart is derived from cardiovascular mesodermal cells that transiently express *T* (*Brachyury*), *Mesp1*, and *Flk1* during gastrulation. A subset of these cardiac mesodermal cells gives rise to cardiac progenitor cells, which can differentiate into any type of cardiac cell.

T-box transcription factors compose a conserved family of genes that are important for heart development and patterning. In humans, disruption of the cardiac *T-box* genes leads to various congenital heart defects [1]. Mutations in *Tbx5* are associated with Holt-Oram syndrome [2, 3], whereas mutations in the *Tbx20* gene result in atrial septal defect [4, 5]. Interestingly, in the developing heart, Tbx5 and Tbx20 are complementarily expressed in the left and right ventricle, respectively (Fig. 42.2) [6]. The regions in which the *T-box* genes are expressed and the regions that are defective in a given disease are very similar. *Tbx5* is expressed in the inflow tract, atria, AV cushion, and left ventricle but not in the outflow tract or

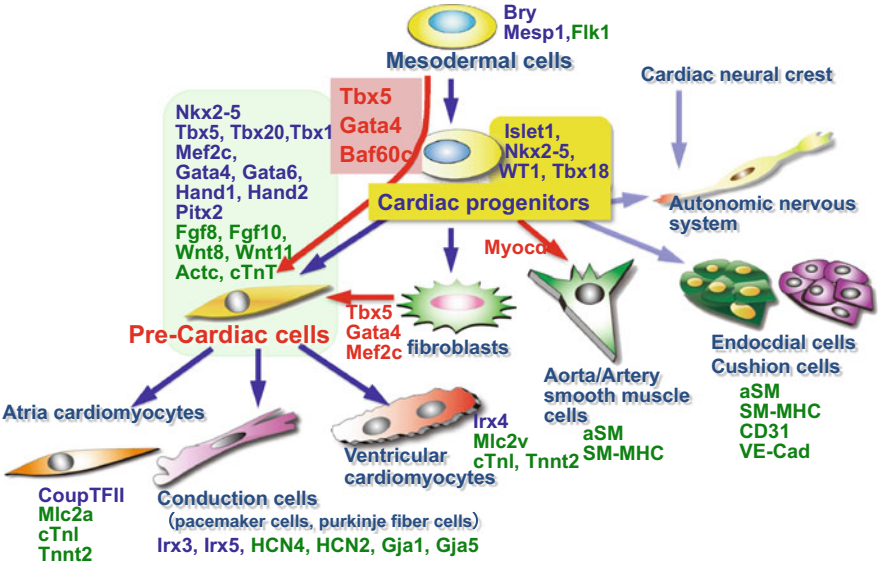

Fig. 42.1 Multiple cardiac cell types. Each cell type differentiates from mesoderm-derived cardiac progenitor cells. The major molecules associated with each cell type are indicated

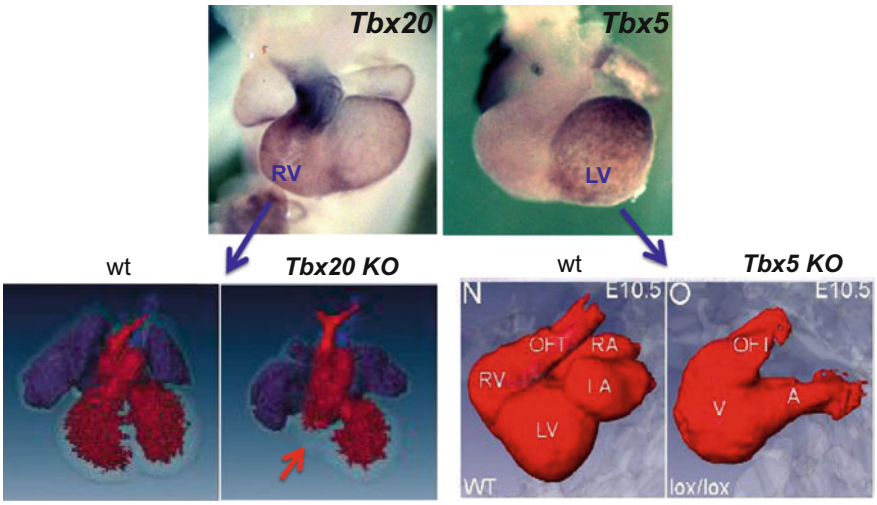

Fig. 42.2 The expression patterns of *Tbx5* and *Tbx20* in the mouse heart. These genes show complementary expression patterns in the ventricles. The knockout mice for each gene show hypoplasia in the same region in which the gene is normally expressed (Adapted from Bruneau et al. [8] and Takeuchi et al. [9])

the right ventricle; *Tbx5* expression appears to be restricted to the first heart field (FHF)-derived region [7]. *Tbx5* knockout mice experience severe left ventricle hypoplasia and die at approximately E9 without their hearts ever beating (Fig. 42.2) [8]. By contrast, *Tbx20* is primarily expressed in the outflow tract and the right ventricle, which are derived from the second heart field (SHF). *Tbx20* knockdown mice develop a single ventricle and show severe hypoplasia of the right ventricle (Fig. 42.2) [9]. These facts indicate that Tbx5 and Tbx20 may specify the identity of each ventricle. Tbx5 also acts in association with Sall4 in ventricular septum formation. Sall4 is a zinc-finger transcription factor that, when mutated, causes Okihiro syndrome (Duane-radial ray syndrome, DRRS) in humans [10, 11]. The heart and limb phenotypes of Okihiro syndrome are very similar to those of Holt-Oram syndrome. In fact, some Holt-Oram patients lack mutations in *TBX5* and instead have mutations in *SALL4* [12]. Tbx5 and Sall4 participate in protein-protein interactions and synergistically regulate downstream gene expression [13]. Furthermore, Tbx5 is a key gene involved in the acquisition of the ventricular septum during vertebrate evolution [14]. During vertebrate evolution from aquatic to terrestrial life, the morphology of the heart has changed. As a result, avian and mammalian hearts contain four chambers—two atria and two ventricles—and their circulatory systems contain two loops, the pulmonary and systemic loops, that separate the oxygen-rich and oxygen-poor blood. In vertebrates with four-chambered hearts, *Tbx5* expression is restricted to the left ventricle, whereas in animals with a single ventricle, *Tbx5* expression is observed throughout the ventricle. Reptiles show a unique *Tbx5* expression pattern that is associated with

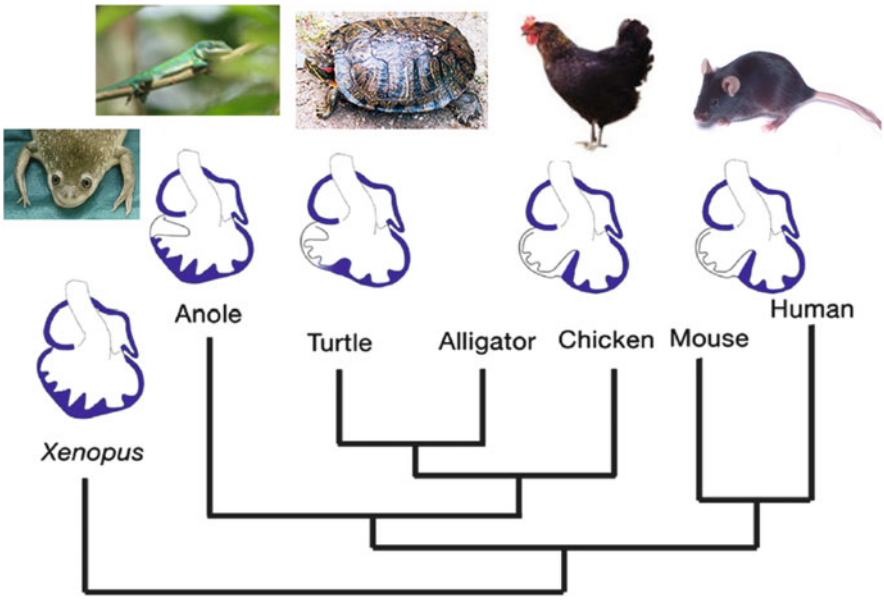

Fig. 42.3 *Tbx5* expression and heart morphology in vertebrates. Note that animals with two ventricles express *Tbx5* on the left side (Adapted from Koshiba-Takeuchi et al. [14])

their ventricular morphology. *Anolis*, which are a type of squamate, have a single ventricle that expresses *Tbx5* throughout this chamber and throughout development. Interestingly, turtles show a left-high to right-low gradient of *Tbx5* expression during late developmental stages, and a septum-like structure forms in the middle of the ventricle (Fig. 42.3). To confirm the precise interaction between *Tbx5* expression patterns and ventricular septum formation, we performed Tbx5 mis-expression experiments using transgenic mice. Transgenic mice that express Tbx5 throughout the ventricle fail to form a ventricular septum. These results strongly indicate that *Tbx5* expression in the left ventricle is important for the development of two-chambered ventricles. We hypothesized that the regulatory region of *Tbx5* might have been modified during vertebrate evolution, thereby changing the *Tbx5* expression pattern and the ventricular morphology, as shown in Fig. 42.3.

42.2 Chromatin Factors and Cardiac Differentiation

Recent studies have shown that chromatin factors are essential for determining cell fate in several organs. In heart development, SWI/SNF-type chromatin remodeling factors play key roles in the differentiation of cardiomyocytes by interacting with heart-specific transcription factors [15, 16]. Cardiac transcription factors alone are not sufficient to induce cardiomyocyte differentiation in vivo or in vitro (Figs. 42.4 and 42.5). This result suggests that chromatin accessibility is important for

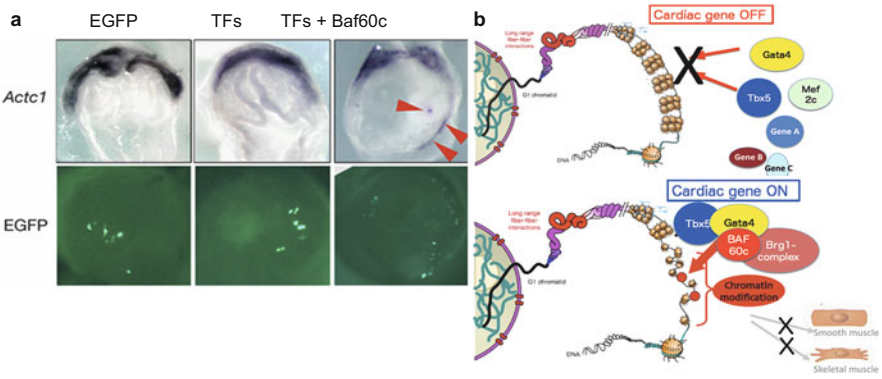

Fig. 42.4 (**a**) A mixture of transcription factors (*TFs*), Baf60c and EGFP, but not the control (EGFP) or TFs + EGFP, ectopically induce *Actc1* in the lateral plate mesodermal region. (**b**) A schematic of cardiac gene regulation. The SWI/SNF complex-mediated change in chromatin conformation is important for the activation of cardiac gene transcription (Adapted from Takeuchi and Bruneau [18] and Van Weerd et al. [15])

Tbx5	Gata4	Gata1	Nkx2-5	Baf60c	Baf60b	*Actc1,Myl7* expression	Beating
+	−	−	−	−	−	×	×
−	+	−	−	−	−	×	×
−	−	+	−	−	−	×	×
+	+	−	−	−	−	×	×
+	−	+	−	−	−	×	×
−	−	−	+	−	−	×	×
+	−	−	+	−	−	×	×
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−	+	−	−	−	+	○	×
−	−	+	−	+	−	○	×
−	−	+	−	−	+	×	×
+	+	−	+	+	−	○	○
+	+	−	−	+	−	○	○

Fig. 42.5 Schematic diagram shows that ectopic expression of major cardiac contracted genes (Actc1 and Myl7) are observed by combinatorial transfection of cTF and Baf60c into ex vivo mouse

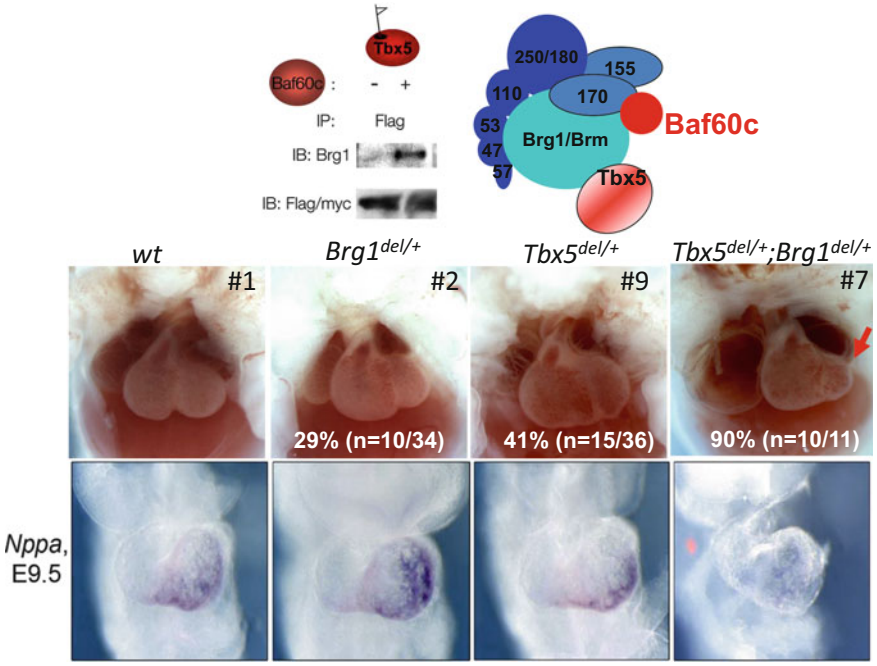

Fig. 42.6 The combinatorial functions of cardiac transcription factors and Baf chromatin remodeling factors. Only the transcription factors can induce cardiac markers, but the differentiation of beating cardiomyocytes requires both transcription factors and chromatin remodelers

transcription factors to bind to their target sites. Therefore, we searched for chromatin remodeling factors that are expressed in the cardiac region at early stages of heart development. A previous study showed that a component of the SWI/SNF-type chromatin remodeling complex, Baf60c (also known as *Smarcd3*), has specific roles in heart development [17]. When a mixture of cardiac transcription factors and Baf60c was injected into the lateral plate mesoderm of mouse embryos, alpha cardiac actin-positive cells were ectopically induced (Fig. 42.4) [18]. These ectopically induced cardiac cells could beat, which showed that they were functional cardiomyocytes. Baf60c can directly associate with the Tbx5, Nkx2-5, Gata4, and RBPjk proteins and regulate the transcription of downstream genes [17, 19]. Mutations in chromatin factors cause abnormal cardiac function in both mice and humans. Baf60c determines a cell's fate by not only loosening the chromatin structure but also synergistically interacting with specific factors. When associated with Tbx5, Brg1, a core protein of the SWI/SNF-type chromatin remodeling complex, synergistically regulates cardiac differentiation in the presence of Baf60c [20]. Mice heterozygous for both *Brg1* and *Tbx5* had more severe defects than did single mutants, particularly in the left ventricle (Fig. 42.6). These

results indicate that the dosage of epigenetic factors affects the severity of the *Tbx5* mutant phenotype (i.e., left ventricular hypoplasia). The severity of congenital heart disease in humans may be related to the level of expression of epigenetic factors and/or of partner factors. To address this possibility, we need to elucidate the relationship between the expression level of epigenetic factors and the penetration of heart failure.

42.3 Future Directions and Clinical Implications

We have analyzed the functions of Baf60c, a component of the SWI/SNF-type chromatin remodeling complex, in heart development in vivo and in vitro, but the mechanism by which Baf60c is regulated is still unknown. An important question is whether the functions of Brg1 or Baf60c are altered in each type of tissue. If their functions do not vary, they may be regulated in a partner-dependent manner. One approach to confirm this hypothesis is to use ChIP-sequencing to compare Baf60c and Brg1 target genes in different tissues. Another question that must be addressed is how Baf60c's expression pattern and dosage are regulated and which molecule (s) participate in this regulation. We found a candidate transcription factor that directly regulates Baf60c expression, but this molecule alone could not explain the dynamic change in Baf60c's expression pattern. Further analysis is required to determine the molecular mechanisms of Baf60c regulation.

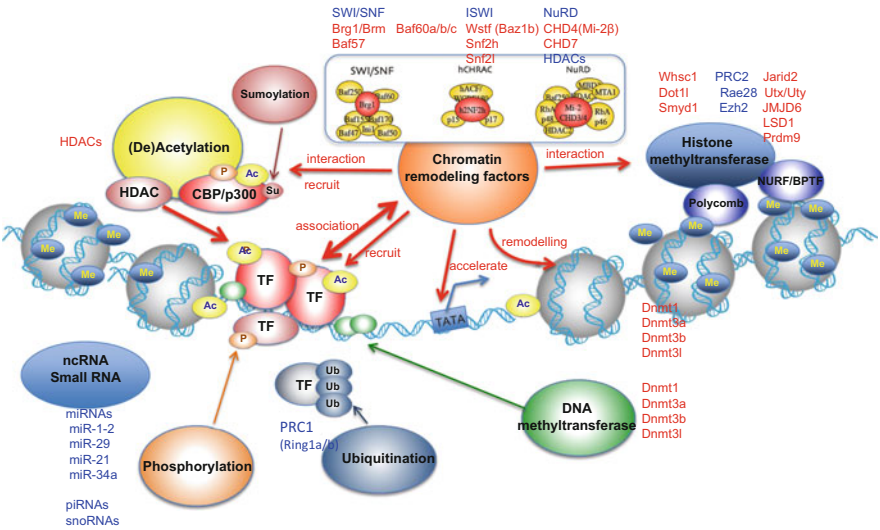

Fig. 42.7 Immunoprecipitation experiments indicate that Brg1 can strongly bind to Tbx5 with Baf60c. The lower panel shows the morphology of *Brg1del*/+, *Tbx5del*/+, and double heterozygote mouse hearts. The heart of the double heterozygote shows severe hypoplasia of the *left* ventricle (Adapted from Takeuchi et al. [20])

Over the last 20 years, cardiac researchers have elucidated many causes of congenital heart disease and have identified many genes that are involved. However, it is not sufficient to only understand the diversity or severity of the disease. In the future, we must also determine the role of epigenetic factors in heart failure because these factors regulate cardiac gene transcription (Fig. 42.7).

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Pcgf5 Contributes to PRC1 (Polycomb Repressive Complex 1) in Developing Cardiac Cells

43

Manabu Shirai, Yoshihiro Takihara, and Takayuki Morisaki

Abstract

Polycomb-group (PcG) proteins maintain transcriptional silencing through specific histone modification and are essential for cell-fate transition and proper development of embryonic and adult stem cells. Recent advances in molecular analysis of PcG proteins have revealed that the distinct subunit composition of PRC1 confers specific and nonoverlapping functions for regulation of embryonic and adult stem cells. Here, we provide an overview of recent findings regarding the role of PcG proteins in cardiac development, with focus on the diversity of PcG complexes.

Keywords

Polycomb-group protein • Cardiac development • Transcriptional silencing • Histone modification

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43.1 Introduction

Cardiac development is a complex and ordered process that requires cellular specification, proliferation, and differentiation, as well as further migration of cell populations from diverse sites. The primary heart field (PHF) originates in the anterior splanchnic mesoderm, then gives rise first to the cardiac crescent, later to the linear heart tube, and ultimately contributes to parts of the left ventricular (LV) region. The second cardiogenic region, known as the second heart field (SHF), lies in the anterior, posterior, and dorsal to the linear heart tube and is derived from the pharyngeal mesoderm located medial and anterior to the cardiac crescent. Cells from the SHF are added to the developing heart tube and give rise to the outflow tract (OFT), right ventricular (RV) region, and main parts of atrial tissues [1].

Congenital heart defects (CHDs) represent the most common anomaly seen in human newborns, with a prevalence of approximately 1 % of all births [1]. Traditionally, focus on causes of CHD has involved transcriptional networks during cardiogenesis, because correct alignment and septation of cardiac structures regulated by cardiac specific transcriptional factors, such as *Tbx1*, *Tbx5*, *Tbx20*, *Gata4*, and *Nkx2-5*, are essential for cardiac morphogenesis [1]. In addition to these multiple genetic factors, recent studies have shown that some chromatin remodeling factors moderate gene expression to control cardiogenesis and are also involved in the molecular pathogenesis of CHD [2–7].

Polycomb-group (PcG) proteins maintain transcriptional silencing by regulating chromatin configuration [8, 9]. There are two principal PcG repressive complexes (PRCs), PRC1 and PRC2. Mammalian PRC2 contains four core proteins (Ezh1/2, Eed, Suz12, Rbbp4/7) and trimethylates histone H3 at lysine 23 (H3K27), while the other complex, PRC1, consists of a combination of several protein families, including chromobox (Cbx), Ring, polyhomeotic (Ph), and posterior sex combs (Psc), and induces mono-ubiquitination of histone H2A at lysine 119 (Fig. 43.1). In recent studies, PRC1 have been divided to Cbx-PRC1 (canonical PRC1) and Rybp-PRC1 (noncanonical PRC1) [10, 11]. Since development of high-throughput techniques for analyzing the genome in the past decade, PcG-mediated transcriptional repression has resulted in increased molecular information regarding its role in a number of important biological activities such as cell cycle progression, differentiation, and cell-fate transition in multiple cell types and tissue contexts, including embryonic, adult, and cancer stem cells. This PcG-mediated transcriptional repression can vary during development and among cell types. However, the precise role in the context of cell conditions remains unclear.

43.2 PcG Functions in Cardiac Development

In this review, we primarily focused on the functions of PcG proteins in cardiac development. Their roles have been elucidated via generation of knockout (KO) mice for each of the PcG components. Among the PRC2 components, loss

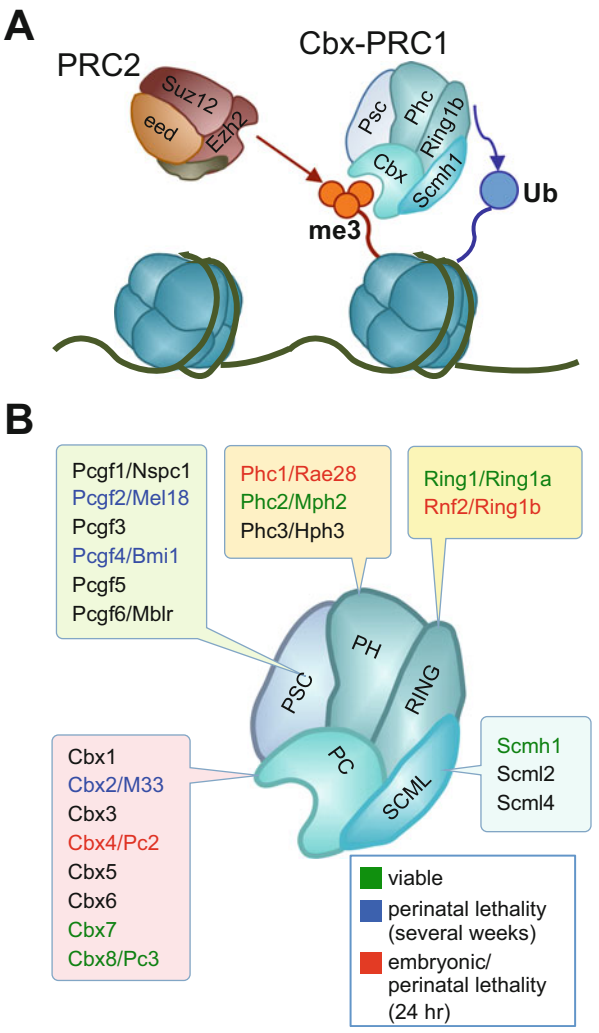

Fig. 43.1 PcG complexes in mammals. (**A**) Molecular functions of PcG complexes (PRCs). PRC2 trimethylates lysine 27 of histone H3. Canonical PRC1 binds to the H3K27me3 mark and mediates the mono-ubiquitination of histone H2A at lysine 119. (**B**) Canonical PRC1 components and results of KO of each in mice

of Suz12, Ezh2, or Eed results in embryonic lethality during the early postimplantation stage [12–14]. To address the role of PRC2 in cardiac development, Ezh2 and Eed were conditionally inactivated in specified cardiac cells using Nkx2-5:Cre or TnT:Cre [3, 4]. Inactivation of Ezh2 by Nkx2-5:Cre ($Ezh2^{NK}$) and Eed by TnT:Cre (Eed^{TnT}) led to embryonic lethality and several cardiac defects including compact myocardial hypoplasia, whereas inactivation of Ezh2 by TnT:Cre ($Ezh2^{TnT}$) did not result in severe defects in cardiogenesis despite a modest upregulation of some cardiac genes, probably because of the redundant functions of Ezh1 and Ezh2.

Embryos deficient of *Ring1b*, a core component of PRC1, also displayed early embryonic lethality caused by gastrulation arrest [15]. Although early developmental arrest in *Ring1b* KO embryos was partially restored by inactivation of Cdkn2a

(Ink4a/ARF), cardiac tissue did not develop in double-KO embryos. Unlike early developmental defects seen in KO mice lacking some of the core PRC1 and PRC2 components, deficiency of other components has been shown to give rise to restricted effects. For example, loss of Rae28/Phc1 resulted in perinatal lethality with cardiac anomalies, double outlet right ventricle, and tetralogy of Fallot [6, 7]. In addition to cardiac defects, *Rae28/Phc1* deficient mice also showed craniofacial developmental defects, as well as thymus and parathyroid gland defects as seen in human DiGeorge syndrome.

Among Cbx proteins, Cbx4 may play an important role in cardiogenesis. SUMO-specific protease 2 (SENP2) was reported to regulate transcription of *Gata4* and *Gata6*, mainly through alteration of the occupancy of Cbx4 on their promoters [5]. In *SENP2*-deficient embryos, sumoylated Cbx4 accumulates on the promoters of target genes, leading to transcriptional repression of *Gata4* and *Gata6*. Furthermore, *Cbx4* mutant mice displayed postnatal lethality with severe hypoplasia of the developing thymus as a result of reduced thymocyte proliferation. However, the function of Cbx4 in cardiogenesis has not been clearly elucidated [16]. Thus, PcG proteins are essential for molecular regulation of the expression of several cardiac genes during embryogenesis and important for cardiac morphogenesis.

43.3 Diversity of PcG Proteins

In our recent studies, expression of the PRC1 components Ring1b, Bmi1/Pcgf4, and Rae28/Phc1 was detected in embryonic hearts containing both the PHF and SHF embryonic cardiac fields (Fig. 43.2), though their expression patterns were not restricted in cardiac cells. Despite the ubiquitous expression of PRC1 components, mice with single PcG KO except for the core proteins show more distinct and restricted phenotypes. Among Psc proteins, genetic deletion of *Mel18/Pcgf2* or *Bmi1/Pcgf4* resulted in postnatal lethality caused defects in anterior-posterior specification, while there was no effect on cardiogenesis [17, 18]. Also, *Mel18/Bmi1* double-KO mice died around E9.5 and exhibited more severe developmental defects than those with KO of either alone, suggesting that Mel18/Pcgf2 and Bmi1/Pcgf4 have partially redundant functions [18]. In addition to functional redundancy, recent studies have shown that distinct Cbx and Pcgf proteins confer specific and nonoverlapping functions of PRC1 in embryonic and adult stem cells [10, 11]. Exchanging Cbx protein in canonical PRC1 is involved in the switch from self-renewal to a differentiation state, whereas Pcgf and other noncanonical components, such as Rybp, Kdm2b, and L3mbtl2, also confer restricted functions to PRC1.

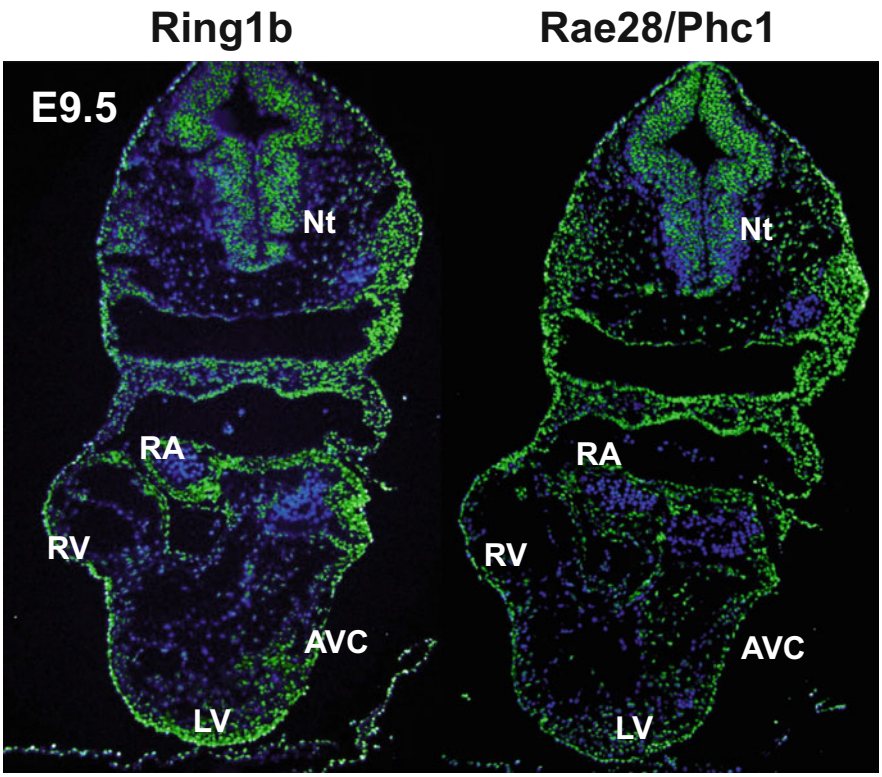

Fig. 43.2 Expression patterns of Ring1b and Rae28/Phc1 in E9.5 mouse embryos. Anti-Ring1b and anti-Rae28/Phc1 antibody-positive cells are ubiquitously distributed on E9.5. AVC, atrioventricular canal, LV, left ventricle, Nt, neural tube, RA, right atrium, RV, right ventricle

43.4 Pcgf5 Expression in the Developing Heart

Recently, we identified *Pcgf5* as an upregulated gene during cardiomyocyte differentiation of ES cells as well as strong expression of *Pcgf5* in cardiac fields during the early embryonic stages as compared to other embryonic tissues [19] (Fig. 43.3). Unlike *Bmi1*/*Pcgf4*, *Pcgf5* expression, patterns were more restricted to early mouse embryos. Our hypothesis states that exchanging Pcgf components within PRC1 determines the specificity of their function in the developing heart.

43.5 Conclusions

Recent increasing evidence obtained in experiments with embryonic and adult stem cells indicates that the diversity of PRC components, particularly PRC1, contributes to specification of their function (Fig. 43.4). Furthermore, transcriptional repression

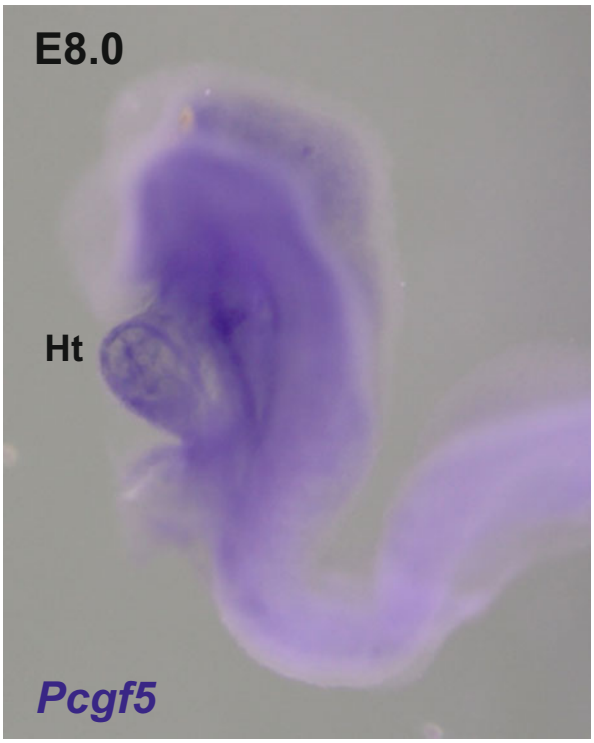

Fig. 43.3 Expression pattern of *Pcgf5* in E8.0 mouse embryos. Whole-mount in situ hybridization (WISH) showing expression of *Pcgf5* at E8.0. High Pcgf5 expression was observed in developing hearts. Ht, heart

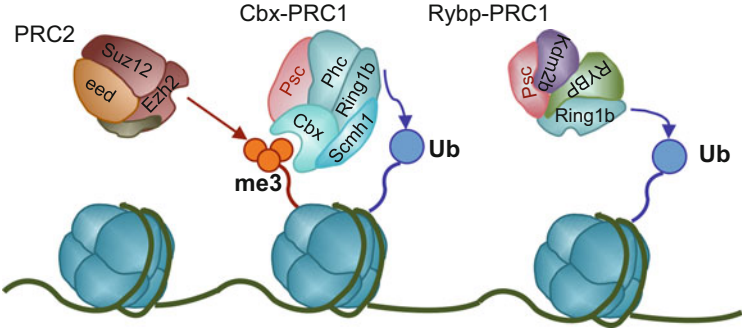

Fig. 43.4 Recent insight regarding PRC1 complexes. Canonical PRC1 (Cbx-PRC1) is recruited to H3K27me3 and causes mono-ubiquitination of lysine 119 of histone H2A. Noncanonical PRC1 (Rybp-PRC1) mediates the mono-ubiquitination of H2A in a PRC2-independent manner

by PcG is essential for cardiogenesis. Understanding PcG functions and more detailed characterization of the PcG complex during cardiogenesis may be crucial for elucidating the molecular mechanisms regulating cardiac development.

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Noncoding RNAs in Cardiovascular Disease 44

Masaharu Kataoka and Da-Zhi Wang

Abstract

For decades, it has been recognized that proteins, which are encoded by our genomes via transcription and translation, are building blocks that play vital roles in almost all biological processes. Mutations identified in many protein-coding genes are linked to various human diseases. However, this "protein-centered" dogma has been challenged in recent years with the discovery that majority of our genome is "noncoding" yet transcribed. Noncoding RNA has become the focus of "next generation" biology. Here, we review the emerging field of noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), and their function in cardiovascular biology and disease.

Keywords

Cardiac disease • Heart development • Long noncoding RNAs • MicroRNAs

44.1 Introduction

When the human genome project was completed, it was surprising that only about 20,000–25,000 protein-coding genes exist in our species, with less than 2 % of the human genome used for coding proteins. What are the functions of noncoding sequences, which make up more than 98 % of our genome? We are now finding answers with the recognition that the majority of the genome is actively transcribed to produce thousands of noncoding transcripts, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs), in many cell types and tissues. miRNAs are a class of small noncoding RNAs (~22 nucleotides) and were first discovered in

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C. elegans two decades ago. More than 2,000 miRNAs have been found in humans, and many of them are evolutionarily conserved. By imperfect base pairing with mRNAs in a sequence-dependent manner, miRNAs repress gene expression by degrading target mRNAs and/or inhibiting their translation. Roles for miRNAs have been demonstrated in the regulation of a broad range of biological activities and diseases [1]. More recently, thousands of lncRNAs, which are transcribed noncoding RNAs greater than 200 nucleotides, were discovered and implicated in a variety of biological processes [2]. Clearly, investigating and understanding of how miRNAs and lncRNAs regulate gene expression during cardiovascular development and function will greatly facilitate therapeutic treatment of cardiovascular disease.

44.2 miRNAs in Cardiac Development

Global disruption of all miRNA expression in the heart is the first step to understanding the function of miRNAs in cardiac development and physiology. Dicer, a RNase III endoribonuclease, is a critical enzyme for the maturation of most miRNAs. Conventional deletion of Dicer causes early embryonic lethality in mice [3]. Disrupting miRNA expression in the early embryonic stage using Nkx2.5-Cre leads to improperly compacted ventricular myocardium in mutant embryos [4], and α-MHC-Cre-mediated conditional deletion of Dicer causes postnatal lethality due to dilated cardiomyopathy and heart failure [5]. These studies suggest that many miRNAs have crucial roles in cardiac development. miR-1 is tissue-specifically expressed in the heart and skeletal muscle, and genetic deletion of both miR-1-1 and miR-1-2 indicated that miR-1 is required for cardiomorphogenesis and the expression of many cardiac contractile proteins [6].

44.3 Cardiac Regeneration, Remodeling, and Ischemia Regulated by miRNAs

Mammalian adult cardiomyocytes are terminally differentiated cells with very limited regenerative ability. A recent report identified about 40 miRNAs that strongly increased cell proliferation in neonatal mouse and rat cardiomyocytes. Two of these miRNAs, miR-590 and miR-199a, were further demonstrated to induce cardiomyocyte proliferation both in vitro and in vivo [7]. Using both gain- and loss-of-function approaches in transgenic and knockout mice models, we demonstrated that the miR-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation. More specifically, we identified miR-19a/b as the major contributors among the miR-17-92 cluster to the regulation of the cardiomyocyte proliferation [8]. These studies suggest that miRNAs are key regulators of cardiomyocyte proliferation and heart regeneration, suggesting their significant therapeutic potential to treat cardiac-degeneration-associated heart disease.

Cardiac remodeling, which is defined as an alteration in the structure (dimensions, mass, shape) of the heart, is one of the major responses of the heart to biomechanical stress and pathological stimuli. Numerous studies have demonstrated the functional involvement of many miRNAs during cardiac remodeling [9]. Recently, we and others demonstrated that miR-22, a miRNA enriched in cardiomyocytes but only mildly upregulated during cardiac hypertrophy, significantly promotes cardiac hypertrophy in vitro and in vivo [10, 11].

Ischemia is an independent risk factor of cardiovascular events, which leads to myocardial infarction (MI) and ischemia-reperfusion (I/R) injury. Several miRNAs participate in the regulation of these pathologic processes, especially cardiomyocyte apoptosis following MI and I/R injury. miR-92a, a member of the miR-17-92 cluster involved in cardiomyocyte proliferation, also participates in the control of cardiomyocyte survival by targeting integrin subunit α5 and eNOS. Inhibition of miR-92a by antagomir has improved cardiac function and reduced cardiomyocyte apoptosis after MI in mice [12]. miR-21 serves as an anti-apoptotic factor in MI animal models by targeting PDCD4 and repressing its expression. Interestingly, miR-21 seems to target cardiac fibroblasts, not cardiomyocytes, in the heart [13]. Conversely, miR-320 is downregulated after I/R injury. Gain- and loss-of-function studies demonstrated that miR-320 promotes cardiomyocyte apoptosis via maintaining HSP20 levels [14]. Together, these studies establish miRNAs as key regulators of cardiomyocyte survival and cardiac remodeling in response to pathophysiological stresses.

44.4 LncRNAs in Cardiac Development

While many lncRNAs have recently been discovered, relatively little is known about their function. A novel lncRNA, *Braveheart*, has been defined as a critical regulator of cardiovascular commitment from embryonic stem cells (ESCs) [15]. *Braveheart* activates a cardiovascular gene network and functions upstream of mesoderm posterior 1, a master regulator of a common multipotent cardiovascular progenitor. *Braveheart* mediates the epigenetic regulation of cardiac commitment by interacting with SUZ12, a component of the polycomb repressive complex 2 (PRC2). *Braveheart* therefore represents the first lncRNA that defines cardiac cell fate and lineage specificity, linking lncRNAs to cardiac development and disease. It remains to be seen if *Braveheart* is required for normal heart development in vivo. Equally critically, it will be important to determine whether genetic mutation of the *Braveheart* gene is linked to human cardiovascular disorders. Nevertheless, the discovery of *Braveheart* will significantly impact the cardiovascular research field and link lncRNAs to human cardiovascular disease.

Fendrr, another novel lncRNA, has been defined as an essential regulator of heart and body wall development. *Fendrr* is expressed in the mouse lateral plate mesoderm, from which precursors for the heart and body wall are derived, and the knockout of *Fendrr* resulted in defects in heart development [16]. Like *Braveheart*, *Fendrr* interacts with the PRC2 complex to regulate gene expression. It is expected

that many more lncRNAs will be found to play important roles in cardiovascular development and function.

44.5 Noncoding RNAs in Cardiac Disease

The expression and function of multiple miRNAs have been associated with human cardiovascular disease. Recent studies also linked several lncRNAs to heart disease. *ANRIL*, a lncRNA, was identified as a risk factor for coronary disease [17]. Though it is still not fully understood how ANRIL functions, evidence suggests that this lncRNA may participate in the regulation of histone methylation [18]. Another lncRNA MIAT (myocardial infarction-associated transcript) (or *Gomafu/RNCR2*) was identified as a risk factor associated with patients with myocardial infarction [19]. However, how MIAT controls MI status remains largely unknown. Intriguingly, the genetic loci that encode MYH6 and MYH7, the main myosin heavy chain genes in cardiac muscle, appear to produce a noncoding antisense transcript (*Myh7-as*). *Myh7-as* transcription may regulate the ratio of *Myh6* and *Myh7*, altering the function of muscle contraction [20].

We have just started the era of "noncoding." We are looking forward to see more and more reports on the roles of noncoding RNAs in the regulation of a variety of essential biological processes. Furthermore, with efficient strategies for gain- and loss-of-function investigations, more fruitful work about the molecular mechanism and therapeutic application of noncoding RNAs in cardiovascular disease will emerge.

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Part X

iPS Cells and Regeneration in Congenital Heart Diseases

Perspective

Deepak Srivastava

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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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

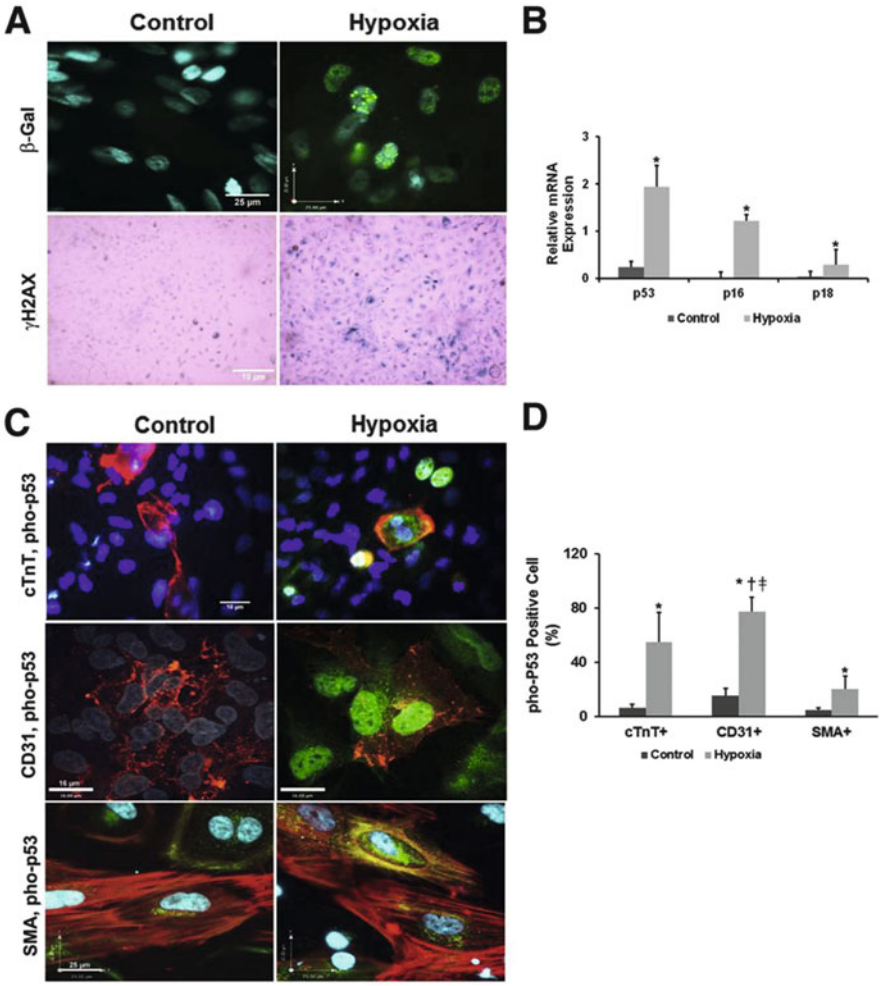

Fig. 45.1 Effect of hypoxia on DNA damage and oncogene upregulation in hPSC-derived cardiac lineages. (**a**) DNA damage-related marker γH2AX (*green nuclear foci*) and senescence marker β-gal (*blue*) are increased in hypoxic cells. *Blue* represents nuclear staining with DAPI. (**b**) qPCR results 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

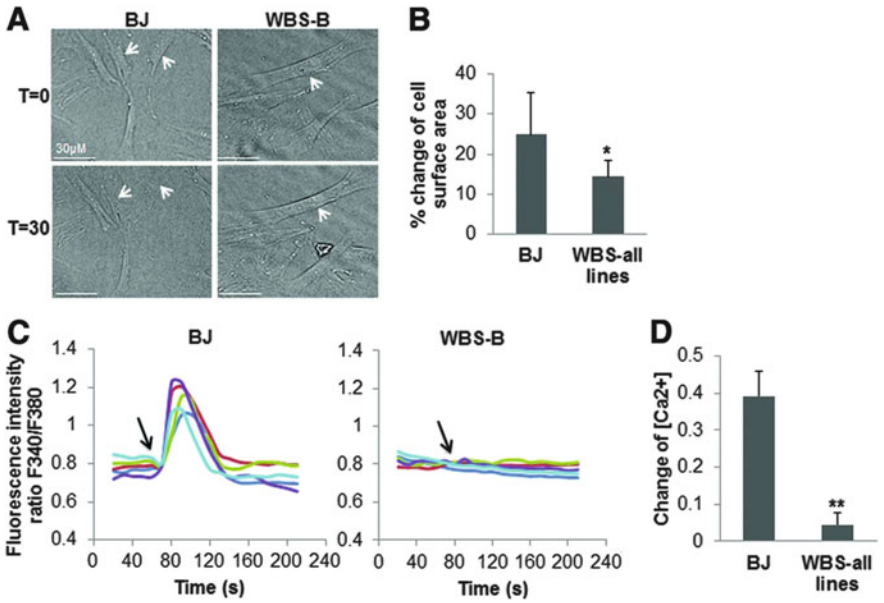

Fig. 45.2 Functional characterization of BJ-smooth muscle cells (SMCs) and WBS-SMCs. (**a**) BJ and WBS-SMCs (line B shown) were treated with 10 mM carbachol, a muscarinic agonist, and phase-contrast live-cell imaging was done every 30 s. Change in cell surface area (*white arrows*) was calculated from 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.

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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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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