Part I

From Molecular Mechanism to Intervention for Congenital Heart Diseases, Now and the Future

Perspective

Toshio Nakanishi

Srivastava and his team have long since been consistently contributing to research on the molecular mechanisms underlying early heart development, especially on the signaling and transcriptional cascades that regulate cell fate in the heart tissue of the embryo. Early on, Srivastava recognized the importance of microRNA in regulating gene transcripts in the developing heart. Through their research, they were successful in directly converting fibroblasts into cardiomyocytes, a process in which microRNA may play an important role. Srivastava emphasizes the importance of the molecular biology of cardiac morphogenesis for the development of regenerative medicine. He also emphasizes the importance of cardiac fibroblasts as a source of cells for restoring damaged cardiac cells. There seems to be great difficulty in the conversion of fibroblasts to cardiac cells noninvasively in humans. It will be a while before the clinical use of these technologies becomes a reality in saving the lives of pediatric and adult patients with heart failure due to congenital heart disease.

Gittenberger-de Groot and her team emphasize the potential importance of epicardium in repairing damaged myocardium. They report that epicardial-derived cells can differentiate into various cell types such as fibroblasts, arterial smooth muscle cells, endothelial cells, and cardiomyocytes. Epicardial-derived cells contribute to the formation of the coronary arteries, semilunar valves, atrioventricular valves, and myocardium. It is thought that since epicardial-derived cells have the

T. Nakanishi (✉)
Department of Pediatric Cardiology, Heart Institute, Tokyo Women's Medical University, Tokyo, Japan

International Molecular Cellular Immunological Research Center, Tokyo, Japan
e-mail: pnakanis@hij.twmu.ac.jp

potential to develop into various types of cells, it may be possible to use them for the repair of ischemic myocardium. The methodology of using the epicardium or epicardial-derived cells to rescue the damaged heart tissue remains to be investigated.

Sekine, Shimizu, and Okano report a technology to generate a cell sheet efficiently by using a temperature-responsive culture dish. The cell sheet can then be layered to form the cardiac tissue. Clinical trials using this cardiac tissue to repair the damage caused by myocardial infarction or cardiomyopathy are ongoing. Long-term results of this technology will need to be studied in order to apply it to pediatric patients with dilated cardiomyopathy and severely reduced contractile function.

Tohyama and Fukuda report a technology to efficiently purify cardiomyocytes differentiated from induced pluripotent stem (iPS) cells derived from human T lymphocytes. They were able to transplant these cardiomyocytes into the heart. They have also made disease models, including that of long QT syndrome, using these cardiomyocytes. iPS technology has great potential because it can synthesize the cardiac tissue from a patient's own blood cells. However, even for diagnostic purposes, the establishment of stable techniques to generate cardiomyocytes reproducibly from iPS cells is difficult, at least at this moment. For clinical use, safety issues of iPS cells remain to be clarified.

Markwald and his team emphasize that central signaling pathways (or hubs) in which mutations disrupt fundamental cell functions play a key role in the development of congenital heart disease. More specifically, they note two signaling pathways as "hubs": the nodal kinase activated by extracellular ligands (such as periostin) and the cytoskeletal regulatory protein, filamin A. They report that disruption of nodal kinase pathways causes septal defects and malformation of atrioventricular valves. However, if we can manipulate these pathways after birth, we may be able to rescue these defects. Fibroblasts, which may be derived from the bone marrow, play an important role in forming the ventricular septum and atrioventricular valves, and their activity may be regulated by periostin and filamin A. Manipulation of periostin or filamin A may modify fibroblast activity and may be able to rescue septal and valve malformation.

1 Reprogramming Approaches to Cardiovascular Disease: From Developmental Biology to Regenerative Medicine

Deepak Srivastava

Abstract

Heart disease is a leading cause of death in adults and children. We, and others, have described complex signaling, transcriptional, and translational networks that guide early differentiation of cardiac progenitors and later morphogenetic events during cardiogenesis. We found that networks of transcription factors and miRNAs function through intersecting positive and negative feedback loops to reinforce differentiation and proliferation decisions. We have utilized a combination of major cardiac regulatory factors to induce direct reprogramming of cardiac fibroblasts into cardiomyocyte-like cells with global gene expression and electrical activity similar to cardiomyocytes. The in vivo efficiency of reprogramming into cells that are more fully reprogrammed was greater than in vitro and resulted in improved cardiac function after injury. We have also identified a unique cocktail of transcription factors and small molecules that reprogram human fibroblasts into cardiomyocyte-like cells and are testing these in large animals. Knowledge regarding the early steps of cardiac differentiation in vivo has led to effective strategies to generate necessary cardiac cell types for regenerative approaches and may lead to new strategies for human heart disease.

Keywords

Cardiac • Reprogramming • Regeneration • Transcription factors

D. Srivastava (✉)
Gladstone Institute of Cardiovascular Disease, San Francisco, CA 94158, USA

Department of Pediatrics and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158, USA
e-mail: dsrivastava@gladstone.ucsf.edu

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1.1 Introduction

Heart disease remains the leading cause of death worldwide, despite improved treatments that have decreased death rates from cardiovascular diseases. Congenital heart malformations, the most common of all human birth defects, occur in nearly 1 % of the population worldwide, regardless of race. With more than one million survivors of congenital heart disease (CHD) in the United States, it is becoming apparent that genetic disruptions that predispose to developmental defects can have ongoing consequences in maintenance of specific cell types and cellular processes over decades. A more precise understanding of the causes of CHD is imperative for the recognition and potential intervention of progressive degenerative conditions among survivors of CHD.

Because cardiomyocytes (CMs) rarely regenerate in postnatal hearts, survivors of congenital and acquired heart disease often develop chronic heart failure. Unfortunately, end-stage heart failure can only be addressed by heart transplantation, which is limited by the number of donor organs available, particularly in children. Alternative solutions include cellular therapy that replaces lost CMs either by transplanting CMs or inducing new CMs in situ at areas affected by the infarction.

In recent years it has become apparent that adult somatic cells can be converted into other types of cells through epigenetic reprogramming. With this technology, for example, somatic cells, such as fibroblasts, can be dedifferentiated into pluripotent stem cells by nuclear transfer [1] or with defined transcription factors [2]. Direct reprogramming of fibroblasts into the chief functional cells of different organs, including CMs, neurons, hepatocytes, hematopoietic cells, and endothelial cells, has been accomplished and holds great promise for regenerative medicine [3]. In particular, cardiac fibroblasts represent a large pool of cells in the adult heart [4] and thus may provide a reservoir of cells from which to generate new CMs through epigenetic reprogramming.

In 2010, we reported that mouse cardiac and dermal fibroblasts could be directly reprogrammed into induced CM-like cells (iCMs) in vitro by a combination of three developmental cardiac transcription factors, Gata4, Mef2c, and Tbx5 (GMT) [5]. Since then, other labs around the world have reported success in reprogramming mouse fibroblasts into iCMs with similar cocktails of reprogramming factors [6–10]. We and others have also directly converted human cardiac and dermal fibroblasts into cardiac cells [11–13]. Several review papers published from different labs illustrate the potential and challenges of this new avenue for cardiac regenerative medicine [14–19].

Here, we discuss the cardiac developmental biology discoveries that underpin direct reprogramming approaches and consider the opportunities and challenges of this technology for addressing cardiac regeneration.

1.2 Molecular Networks Regulate Cardiac Cell Fate

In vertebrate embryos, the heart is the first functional organ to form. The morphological development of the heart has been summarized in several reviews. During development, numerous signaling and transcriptional cascades regulate cell fate decisions in distinct heart fields. In addition, the Gata, Mef2, Hand, Nkx, and T-box family of transcription factors control expression of cardiac genes and direct the specification and differentiation of cardiac myocytes. These transcription factors have been frequently included in studies that directly convert fibroblasts into cardiac cells.

MicroRNAs (miRs)—small single-stranded noncoding RNAs that negatively regulate the stability of gene transcripts—also regulate cardiac gene expression [20, 21]. Transcription factors regulate miR expression, and in turn, miRs can modulate the activities of transcription factors through positive and negative feedback loops. One of the major regulators of cardiac lineage determination during heart development is miR-1. Expression of miR-1 in either mouse or human embryonic stem cells (ESCs) caused them to favor the muscle cell fate. In contrast, miR-133 promoted muscle progenitor expansion and prevented terminal differentiation, while another miRNA, miR-499, promoted the ventricular cell fate in human ESC-differentiated CMs and caused cardiac hypertrophy and enlarged hearts in miR-499 transgenic mice. These studies demonstrate that miRs cooperate with transcription factors to form an intertwined network that reinforces specific cell fate decisions and differentiation during cardiac development.

1.3 Cardiac Fibroblasts in the Normal and Remodeling Heart

Fibroblasts are mesenchymal cells that produce many extracellular matrix components in organs. Fibroblasts show heterogeneity based on morphology, glycogen pools, collagen production, cell surface markers, and global gene-expression profiles. Although the percentage of fibroblasts among the total cells in the heart varies between species, the large population of fibroblasts is quiescent and abundantly distributed in the interstitial and perivascular matrix in the normal heart.

Cardiac fibroblasts synthesize extracellular matrix to provide a 3D network for myocytes and other cells of the heart; they also regulate the biological and electrophysiological response of CMs during physiological and pathological development. In embryonic mouse hearts, cardiac fibroblasts induced proliferation of CMs via paracrine signals of fibronectin, collagen, and heparin-binding EGF-like growth factor; however, in adult mouse hearts, cardiac fibroblasts promoted hypertrophic maturation of CMs via beta1-integrin signaling [22]. Cardiac fibroblasts were also found to form intracellular electrical coupling and communicate with myocytes through gap junctions, suggesting that cardiac fibroblasts could conduct electric signaling between different regions of myocytes that are electrically isolated by connective tissue in the normal heart.

1.4 Direct Cardiac Reprogramming *In Vitro*

To identify the combination of reprogramming factors that could convert a cardiac fibroblast toward a cardiomyocyte-like state, we generated a transgenic mouse in which enhanced green fluorescent protein (EGFP) was driven by the alpha myosin heavy chain (αMHC) promoter [5, 22], providing a tool to screen through many potential regulators. We ultimately found that a combination of three transcription factors—Gata4, Mef2c, and Tbx5 (GMT)—could convert ~15 % of cardiac fibroblasts into αMHC-EGFP-positive cells, which we called induced CM-like cells (iCMs) [5]. iCMs formed sarcomere structures and displayed whole-transcriptome expression profiles that were shifted significantly toward the profile of CMs, even at the single-cell level. Although most in vitro iCMs were only partially reprogrammed, many of them could generate Ca^{2+} transients, and some started beating spontaneously 4–6 weeks after reprogramming. Using a lineage-tracing strategy in mice (e.g., Isl1-Cre-YFP and Mesp1-Cre-YFP), we did not observe activation of cardiac progenitor markers during GMT cardiac reprogramming [5], suggesting that GMT directly converted fibroblasts toward the cardiac cell fate without dedifferentiation back into a progenitor status. Song et al. [8] found that a basic helix-loop-helix transcription factor, Hand2, could help GMT to convert TTFs into functional beating iCMs.

1.5 Direct Cardiac Reprogramming *In Vivo*

The ultimate goal in generating new iCMs is to improve systolic function of damaged hearts and restore its normal structure and function. Therefore, we tested direct cardiac reprogramming in vivo with the hypothesis that the heart's native microenvironment would promote direct reprogramming of fibroblasts to CMs. We expected that direct cardiac reprogramming would be enhanced in the native heart and could improve the function of the damaged heart.

We delivered GMT into mouse hearts after acute MI via retroviruses. These viruses can only infect actively dividing cells and, thus, could deliver the reprogramming factors into non-myocytes (mostly fibroblasts), but not into CMs that exit the cell cycle after differentiation. Four weeks after introducing GMT, we found numerous genetically labeled reprogrammed cells within the scar area of mouse hearts, indicating newly born iCMs reprogrammed from cardiac fibroblasts (Fig. 1.1). We found that more than 50 % of in vivo-derived iCMs closely resembled endogenous ventricular CMs, had a rod shape, were binucleate, assembled sarcomeres, generated Ca^{2+} transients, and elicited ventricular-like action potentials and beating activity [23]. By microarray analysis, in vivo mouse GMT-iCMs showed similar global gene-expression profiles with mouse adult CMs, such that they were clustered as one type of cells [11]. The iCMs reprogrammed in vivo were electrically coupled with endogenous CMs, and no arrhythmias were observed in mice that received GMT reprogramming factors. Most importantly, introducing GMT in vivo reduced scar size and cardiac

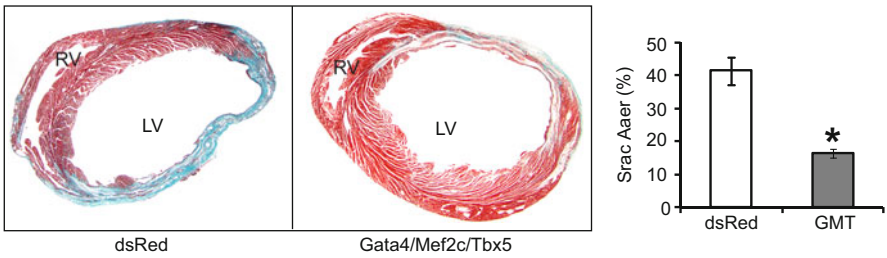

Fig. 1.1 In situ reprogramming of fibroblasts to cardiomyocytes. Representative histologic sections from mouse hearts treated with dsRed or Gata4/Mef2c/Tbx5 containing retroviral vectors injected into the myocardium after coronary ligation. Scar area quantification is indicated

dysfunction up to 12 weeks after coronary ligation [23]. Similarly, Inagawa et al. [24] successfully reprogrammed cardiac fibroblasts into iCMs in vivo by introducing GMT into the heart of immunosuppressed mice with single-polycistronic retrovirus, which contains GMT with self-cleaving 2A peptides. Furthermore, Song et al. [8] found that introducing GMT and Hand2 in vivo could directly convert cardiac fibroblasts into iCMs and also improved function and decreased scar.

1.6 Direct Cardiac Reprogramming in Human Fibroblasts

Establishing the technology of cardiac reprogramming in human cells was a necessary step toward considering clinical application. However, neither GMT nor GHMT, which reprogrammed iCMs from mouse fibroblasts, was able to reprogram human fibroblasts into iCMs in vitro [11–13]; however, inclusion of additional reprogramming factors resulted in successful reprogramming. Nam et al. [12] found that GHT, without MEF2C, but with another transcription factor, myocardin, and two muscle-specific miRNAs, miR-1 and miR-133, could reprogram human fibroblasts into iCMs. These reprogrammed iCMs expressed multiple cardiac genes, developed sarcomere-like structures, and generated Ca^{2+} transients with a small subset of the cells exhibiting spontaneous contractility after 11 weeks in culture. Wada R. et al. [13] reported that GMT with MESP1 and myocardin could activate cardiac gene expression in human neonatal and adult cardiac fibroblasts. In our study, pairing GMT with ESRRG and MESP1 induced global expression of cardiac genes and shifted the phenotype of human fibroblasts toward the CM-like state. Reprogrammed human iCMs were epigenetically stable and formed sarcomere structures, and some could generate Ca^{2+} transients and action potentials [11]. By comparing whole-transcriptome expression of 4-week and 12-week iCMs, we found that reprogramming human cells takes longer than mouse cells because of their progressive repression of fibroblast genes. Nonetheless, our analysis of orthologous gene expression indicated that at the global gene-expression

level, human iCMs were reprogrammed at a level similar to mouse iCMs reprogrammed by GMT in vitro [11].

1.7 Challenges and Future Directions

The studies summarized here demonstrate that forced expression of three or four developmentally critical transcription factors can directly convert mouse cardiac fibroblasts into CM-like cells in vitro, although most of these iCMs were partially reprogrammed. These same factors generated more mature CM-like cells in the native heart, improved heart function, and reduced scar size in the mouse heart post-MI. Similarly, expression of five to seven reprogramming factors converted human fibroblasts into non-perfect iCMs in vitro. By comparing in vitro versus in vivo iCMs in mice [8, 23] and considering the similarities between human and mouse iCMs in vitro [11], we speculate that the cocktails recently identified in human fibroblasts may be sufficient to reprogram adult CM-like cells that are fully functional in the in vivo environment, such as in the pig or nonhuman primate heart, as is the case in mice. These studies have been driven by the deep knowledge of cardiac development gained over the last two decades and represent a valuable application of this knowledge for potential clinical development postnatally.

Successfully achieving cardiac reprogramming requires high expression and proper stoichiometry of reprogramming factors, healthy and non-senescent fibroblasts, and optimal conditions for cell culture. In addition, culture conditions, such as electric stimulation, might help facilitate and maintain the functional maturation of iCMs at late stages (i.e., after cardiac cell fate conversion), and the process may require some small-molecule compounds and growth factors to overcome epigenetic barriers at the early stages of in vitro reprogramming. The epigenetic barriers that prevent cardiac reprogramming in vitro remain unknown; however, the in vivo environment of the heart appears to overcome these epigenetic blocks. We speculate that secreted factors and direct cell-cell interactions, including mechanical and electrical, from myocytes and non-myocytes may work together to improve direct cardiac reprogramming.

Another elusive concept is the molecular mechanism that underlies direct cardiac reprogramming. What are the DNA targets of those reprogramming factors? How are those transcriptional changes epigenetically stabilized during reprogramming? By combining mechanism assays at whole-population and single-cell levels, we can gain a more integral and comprehensive understanding of how core transcription factors establish a self-reinforcing molecular network that controls cardiac cell fate.

While many challenges and hurdles remain in this blossoming research field, the high demand for regenerative medicine strategies for the heart emphasizes the significance of these efforts in discovering new therapeutic strategies. Observing the functional benefits of in vivo reprogramming in mouse heart and the promising and similar degree of reprogramming in mouse and human in vitro iCMs, we are

endeavoring to translate direct cardiac reprogramming for future clinical applications.

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The Arterial Epicardium: A Developmental Approach to Cardiac Disease and Repair

2

Adriana C. Gittenberger-de Groot, E.M. Winter, M.J. Goumans, M.M. Bartelings, and R.E. Poelmann

Abstract

The significance of the epicardium that covers the heart and the roots of the great arteries should not be underestimated as it is a major component with impact on development, disease, and repair. The epicardium differentiates from the proepicardial organ located at the venous pole (vPEO). The differentiation capacities of the vPEO into epicardium-derived cells (EPDCs) have been extensively described. A hitherto escaped part of the epicardium derives from a second proepicardial organ located at the arterial pole (aPEO) and covers the intrapericardial part of the aorta and pulmonary trunk. In avian and mouse embryos, disturbance of epicardium differentiation causes a spectrum of cardiac anomalies including coronary artery abnormalities, deficient annulus fibrosis with rhythm disturbances, valve malformations, and non-compaction cardiomyopathies. Late in prenatal life the epicardium becomes dormant, losing the activity of many genes.

In human cardiac diseases, both arterial and venous epicardium can be activated again into EPDCs. The epicardial reactivation observed after experimental

A.C. Gittenberger-de Groot (✉)
Departments Cardiology, Leiden University Medical Center, Leiden, The Netherlands

Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands
e-mail: a.c.gittenberger_de-groot@lumc.nl

E.M. Winter • M.M. Bartelings
Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands

M.J. Goumans
Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

R.E. Poelmann
Departments Cardiology, Leiden University Medical Center, Leiden, The Netherlands

Institute of Biology, Leiden University, Leiden, The Netherlands

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myocardial infarction and during aneurysm formation of the ascending aorta provides clinical relevance. EPDCs applied for cell therapy demonstrate repair processes synergistic with the resident cardiac progenitor stem cells that probably share an embryonic origin with EPDCs. Future therapeutic strategies might be possible addressing cell autonomous-based and signaling capacities of the adult epicardium.

Keywords
Epicardium • Venous pole • Arterial epicardium • Cardiac disease • Congenital anomlies

2.1 Origin of the Epicardium

The cardiogenic mesoderm is referred to as first heart field, flanked medially by second heart field (SHF) mesoderm. The addition of SHF-derived cardiac mesoderm enables the formation of all cardiac components. A secondary layer will cover the complete myocardial tube (Fig. 2.1) and the developing roots of the great arteries. At the venous pole, the vPEO develops from which the epicardium (cEP) spreads over the cardiac tube up to the ventriculo-arterial junction [1]. Here, the cEP meets the PEO located at the arterial pole (Fig. 2.2) [2] forming arterial epicardium (aEP) that is continuous with the pericardium. Both PEO structures and the spreading cEP and aEP express Wilms' tumor-1 suppressor gene (WT-1) among others and harbor endothelial and mesenchymal cells.

2.2 Epicardium-Derived Cells (EPDCs)

Epicardial cells lose epithelial contacts by epithelial–mesenchymal transition (EMT) and EPDCs relocate subepicardially [3, 4]. Early EPDCs invade the thin myocardial wall. Proliferation of the myocardium depends on both endocardial- and epicardial-derived signals, including Raldh2 [5] with a spatiotemporal difference between right and left ventricle (Fig. 2.1). The aEP starts EMT slightly later than cEP, and ensuing EPDCs can be detected in the outer layers of the developing great arteries and at the myocardial–endocardial cushion interface (Fig. 2.2). Epicardial heterogeneity with a subset of cells taking part in the initial EMT wave provides the myocardium with the main number of the future interstitial fibroblasts. The next wave of EMT correlates with the formation of the fibrous atrioventricular annulus [6] and contributes to part of the atrioventricular cushion cells. At the ventriculo-arterial junction aEP migrates into the outflow tract forming the future arterial annuli and partake in the semilunar valves [7].

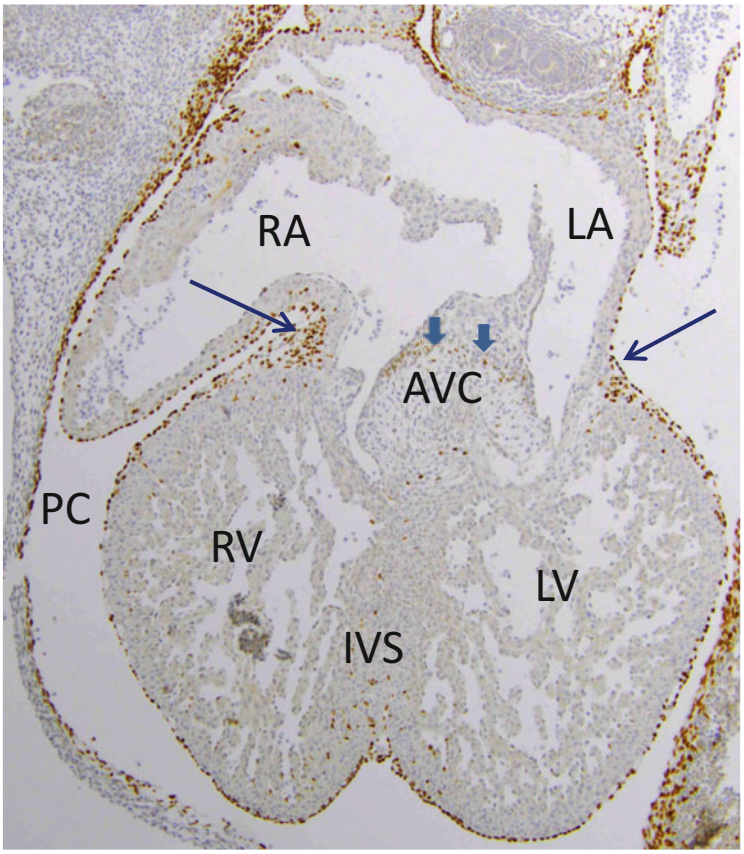

Fig. 2.1 Four-chamber view of a mouse heart (ED12.5) immunostained for WT1. Note brown cells lining the pericardial cavity (PC), including the epicardium. The AV groove is indicated (*arrows*). The left ventricular (LV) wall contains hardly EPDCs, whereas some are present in the RV and in the interventricular septum (IVS). Note the presence of EPDCs at the border of the AV cushion and the interatrial septum (*short arrows*)

2.3 Heterogeneity of Epicardial Cells

2.3.1 The Cardiac Fibroblast

The epicardium is the main source of the interstitial, the fibrous annulus, and the coronary adventitial fibroblasts [7, 8]. The endocardial cells lining the cushions are the other source of the valve fibroblasts [3].

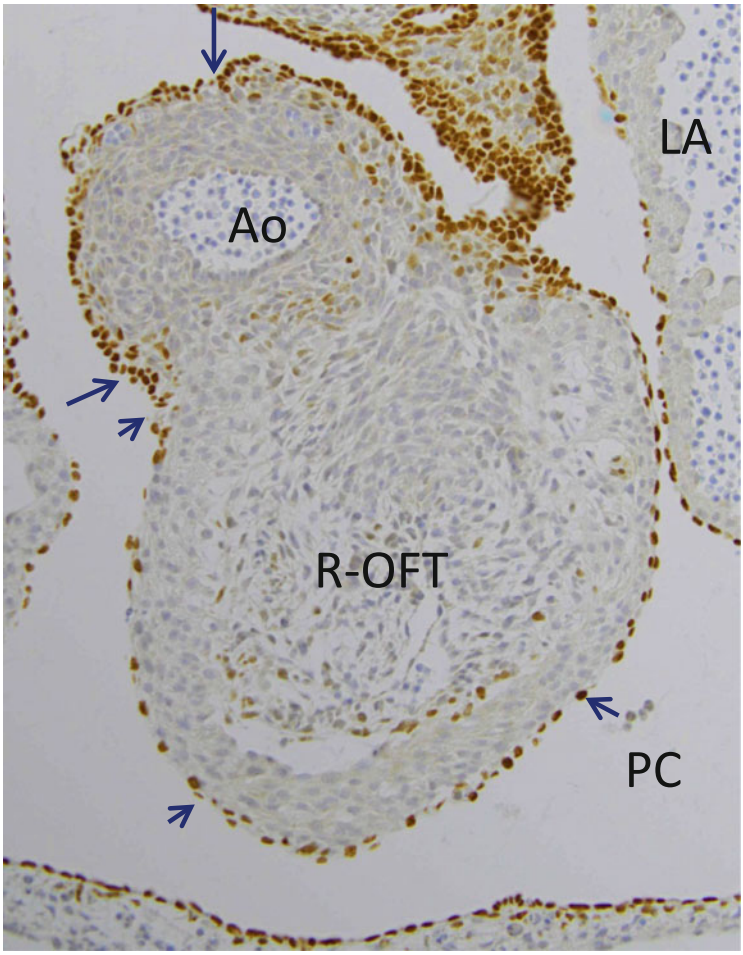

Fig. 2.2 Cross section of aorta (Ao) and right ventricular outflow tract (R-OFT). Aortic epicardium is densely packed and cuboid (*arrows*), whereas cEP is squamous (*short arrows*). Note EPDCs at the border of R-OFT cushions and myocardium and between Ao and R-OFT

2.3.2 Arterial Smooth Muscle Cell

After ingrowth of the peritruncal coronary capillary plexus into the aorta, EPDCs surround the main coronary vessels and differentiate into smooth muscle cells (SMC). Differentiation into SMCs is regulated by many genes including FGF, VEGF, Notch, SRF, and PDGFRb and their ligands [8]. Quail–chicken chimera studies demonstrated the timing of EMT and required cell interactions [4].

2.3.3 Endothelial Cells

The origin of the coronary endothelium is still under debate. Using a quail vPEO transplanted into the isochronous chick as reviewed in [10] pointed out that coronary ECs do not derive from the coelomic lining but from endothelial cells from the sinus venosus sprouting into the stalk of the vPEO. This is also supported by studies using transgenic mice [11]. We have shown that the sinus venosus-derived endothelial cells express the "arterial" Notch1, underlining the plasticity of these cells [9]. The discussion on the origin of the coronary endothelium is kept alive as specific compartments produce restricted numbers of ECs [12, 13].

2.3.4 Cardiomyocytes

Conditional reporter mice studies, using WT-1 and Tbx18 as epicardial marker, suggested that a population of EPDCs might differentiate into a myocardial phenotype. However, based on interaction between BMP and FGF, some of the progenitors of cardiomyocytes in the SHF share the same markers with cells in the underlying mesoderm of the SHF and the epicardial population [14]. Other data, including quail–chicken chimera, also do not support EPDC–cardiomyocyte transition [3].

2.3.5 The Purkinje Fiber

The Purkinje fiber is a specialized cardiomyocyte induced by endothelin produced by endocardium and endothelium. We have postulated an essential interaction between EPDCs and endocardial-/endothelial-derived factors after vPEO tracing and inhibition experiments [15].

2.4 Congenital and Adult Cardiac Disease

In a large-screen microarray [16], no epicardium-specific gene has been encountered. Therefore, it is challenging to attribute specific cardiac malformations and diseases to epicardial malfunctioning. However, in animal models it is possible to link the epicardium to certain cardiac defects and diseases. We are dealing with complex tissues in which epicardial cells are essential.

2.4.1 Non-compaction

The most relevant cardiomyopathy resulting from abnormal EPDC function is the primary left ventricular non-compaction cardiomyopathy [4] demonstrating a spongious myocardium usually including the ventricular septum. Differences in

amount and timing of LV and RV invasion by EPDCs might account for predilection of the LV. With respect to congenital heart disease, a spongious ventricular septum can be connected with muscular VSDs.

2.4.2 Conduction System Anomalies

The main components of the conduction system are myocardial in origin. Clinically, it has been hypothesized that the genetically determined long QT syndrome is linked to abnormal Purkinje fiber function. Indirectly, the abnormal formation of the fibrous annulus with persisting accessory pathways can result in reentry tachycardias. PEO inhibition in chick embryos showed defective atrioventricular isolation, delaying the shift from a base-to-apex to an apex-to-base conduction [17].

2.4.3 Valvulopathies

PEO inhibition can lead to deficient AV valve formation [18, 19]. Furthermore, abnormal differentiation including defective undermining of the valve leaflet is similar to Ebstein's anomaly of the tricuspid valve as observed in combination with accessory pathways [4]. EPDCs of aEP origin are found in the outflow tract cushions (Fig. 2.2) probably acting via Notch signaling and hence influencing bicuspid aortic valve formation.

2.4.4 Coronary Vascular Anomalies

Experimental studies disturbing normal coronary development result in a number of malformations that associate human congenital pattern variations with abnormal ventriculo-coronary-arterial communications (fistulae). Fistulae found in avian models with absent coronary arterial orifices in the aorta [20] resembling coronary malformations found in pulmonary atresia without VSD are hypothesized to be a primary coronary vascular disease [21].

2.5 Cardiovascular Repair

Myocardial infarction. Different approaches have focused on the potential of the adult epicardial cell after myocardial infarction (MI). A c-kit-positive subepicardial population indicates renewed epicardial activity with acquisition of stem cell characteristics [22]. Using a retrovirally induced fluorescent Katushka labeling of dormant epicardium showed EPDCs that migrated into the myocardium and differentiated into a myofibroblast phenotype. The activated epicardium and EPDCs reexpressed WT1 not only in the MI border zone but also in remote areas. Another approach is represented by epicardial cells cocultured with

cardiomyocytes in which EPDCs change myocardial alignment and contraction [23]. A direct approach was provided by grafting human adult atrial epicardial cells [24] cultured in vitro from a cobblestone epithelium into spindle shape, thereby acquiring characteristics of mesenchymal stem cells. Injection of these adult human EPDCs into immune-incompetent mice resulted in a marked improvement of cardiac function [24] indicative of repair. Combined injection with adult human cardiomyocyte progenitors (CMPCs) aimed at induction of cardiomyocyte regeneration [25] which showed an additive effect on remodeling, although no new cardiac cell types (endothelial cells, fibroblasts, SMCs, or cardiomyocytes) could be traced to human origin. The capacities, expressed within the normal embryonic state, seem to be preserved in adult life and in disease states.

2.6 Future Directions and Clinical Applications

Many positive effects of EPDCs either after injection or by stimulation of the dormant native epicardial covering of the heart are due to a paracrine mechanism [22, 24, 25]. These findings bear important potential for drug and cell-based therapeutic approaches to stimulate the native epicardium in repair of the ischemic cardiac wall. An underdeveloped area is the priming of grafts taken from the pericardium for use in cardiac or arterial repair.

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Cell Sheet Tissue Engineering for Heart Failure

3

Hidekazu Sekine, Tatsuya Shimizu, and Teruo Okano

Abstract

In recent years, regenerative medicine using cells for treating tissue defects has been in the spotlight as a new treatment for severe heart failure. Direct injection of bone marrow-derived cells and isolated skeletal myoblasts has already been used clinically as a method to improve cardiac function by regenerating cardiac muscle cells and blood vessels. The research on reconstructing functional three-dimensional (3D) cardiac grafts using tissue engineering methods has also now been addressed as a treatment for the next generation. Our laboratory has proposed an original tissue engineering technology called "cell sheet engineering" that stacks cell sheets to reconstruct functional 3D tissues. Transplantation of cell sheets has already shown it can cure damaged hearts, and it seems clear that the field of cell sheet tissue engineering can offer realistic treatment for patients with severe cardiac disorders.

Keywords

Cardiac tissue engineering • Cell sheet • Regenerative medicine

3.1 Introduction

Heart transplantation is the last hope for treatment of patients with severe heart failure due to ischemia-related disease and dilated cardiomyopathy. However, the lack of donor organs for transplantation continues to be a serious problem around the world. Although there have been many developments in artificial heart systems such as mechanical temporary assist devices or left ventricular assist devices (LVADs), there are also problems in conjunction with thromboembolism, infection, and finite

H. Sekine • T. Shimizu • T. Okano (✉)
Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, 162-8666 Tokyo, Japan
e-mail: tokano@twmu.ac.jp

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durability. Given these challenges with current technologies, regenerative therapies are being investigated as an alternative approach and present new possibilities for the repair of a damaged heart. Recently, the direct injection of either autologous skeletal myoblasts or bone marrow-derived cells has been examined in clinical studies as an alternative cell source to cardiac muscle cells [1–3]. The direct injection of the dissociated cells has shown to be slightly effective, but it is often difficult to control the form, dimensions, or the position of implanted cells. In an attempt to solve these problems, research on advanced therapies using functional tissue produced by engineered cardiac grafts has started. Over the past decade, several studies have proved that bioengineered cardiac tissues could improve cardiac function in animal models of impaired heart [4]. In this review, we discuss the progress of research on myocardial regeneration with a focus on our own original approach using cell sheet engineering.

3.2 Cell Sheet Engineering

We have developed our own "cell sheet engineering" method using temperature-responsive culture dishes created by the covalent grafting of a temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), to normal cell culture dishes [5]. Under normal culture conditions at 37 °C, the dish surface is relatively hydrophobic, and cells can attach, spread, and proliferate similar to commercially available tissue culture surfaces. However, when the temperature is reduced to below the polymer's lower critical solution temperature of 32 °C, the polymer surface becomes hydrophilic and swells, forming a hydration layer between the dish surface and the cultured cells. This allows the cells to detach as a single sheet without the need of enzymatic treatments such as trypsinization. Since the use of proteolysis measures is unnecessary, critical cell surface proteins and cell-to-cell junction proteins remain intact, so that the cells can be harvested noninvasively as an intact sheet while retaining their extracellular matrix (ECM) (Fig. 3.1). Consequently, we can recreate 3D structures such as cardiac tissue by repeated layering of individual cell sheets [6].

3.3 Cardiac Tissue Reconstruction

Harvested cell sheets consist of only confluently cultured cells with their biological ECM on the basal side of the cell sheets, which acts as an adhesive agent for promoting an intimate attachment between each layered cardiac cell sheet. Within layered cardiac constructs, gap junctions are formed which rapidly establish an electrical connection between the cell sheets, leading to 3D cardiac tissues that synchronously pulsatile [7]. Additionally, when these tissues were transplanted onto the subcutaneous tissue of nude rats, the grafts were macroscopically observed to beat synchronously [8]. Importantly, these implanted tissues also showed long-term survival up to 1 year and 8 months, and the grafts also contained elongated sarcomeres, gap junctions, and well-organized vascular networks within the bioengineered cardiac tissues [9].

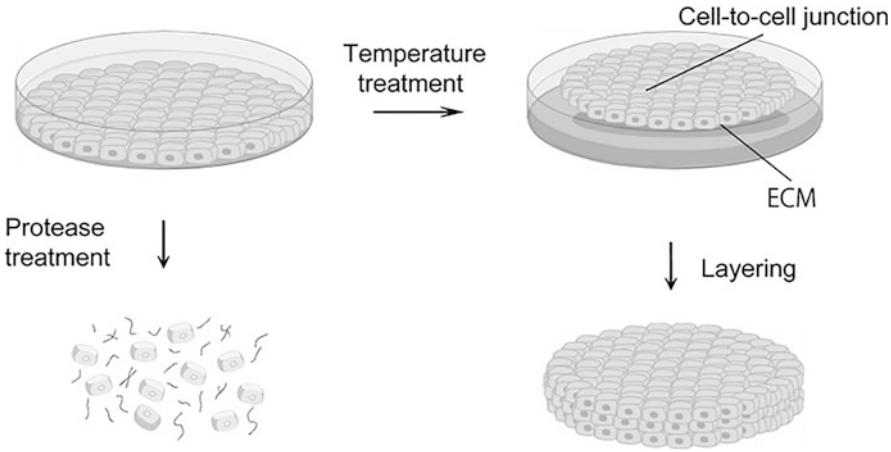

Fig. 3.1 Cell sheet engineering. Using temperature-responsive dishes, cultured cells can be harvested as intact contiguous sheets by simple temperature reduction without proteolytic treatment

3.4 Cell Sheet Transplantation in Small Animal Models

Transplantation of engineered tissue such as cardiac grafts onto infarcted rat hearts (Fig. 3.2) demonstrated morphological and functional connections via bridging cardiomyocytes that migrated from the transplanted grafts to the host heart [10]. Cardiac graft transplantation also improved damaged heart function with significant improvements in the host ejection fraction [11]. We have also demonstrated that control of EC densities in engineered cardiac tissues induces enhanced neovascularization and leads directly to improved function of the ischemic myocardium [12]. Moreover, when compared to direct cell injection, the cardiac graft transplantation exhibited superior cell survival and engraftment [13]. Similarly, skeletal myoblast grafts were able to improve left ventricular contraction, reduce fibrosis, and prevent remodeling via the recruitment of hematopoietic stem cells through the release of various growth factors [14]. The implantation of myoblast grafts also induced the restoration of left ventricular dilatation and prolonged life expectancy in dilated cardiomyopathic hamster [15]. Additionally, mesenchymal stem cell grafts demonstrated improved cardiac function in impaired rat hearts, with the reversal of cardiac wall thinning and prolonged survival after myocardial infarction. This recovery after myocardial infarction suggests that the improvement in cardiac function may be primarily due to the effects of growth factor-mediated paracrine and/or a decrease in left ventricle wall stress, which in turn result from the relatively thick mesenchymal stem cell sheets [16].

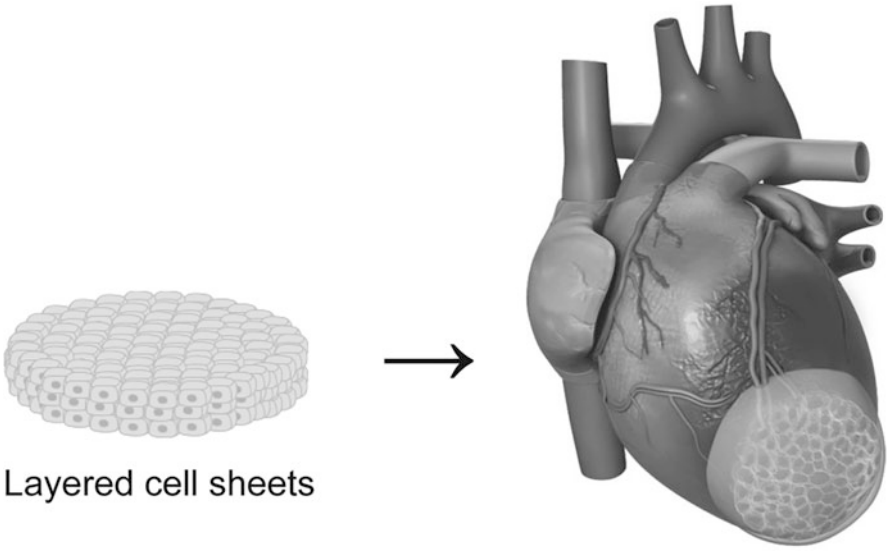

Fig. 3.2 Transplantation of the cardiac grafts onto infarcted rat hearts

3.5 Cell Sheet Transplantation in Preclinical and Clinical Studies

Our latest work has been to transplant engineered tissue in clinically relevant large-animal models. In a pacing-induced canine dilated cardiomyopathy model, transplantation of skeletal myoblast grafts has shown improved cardiac function with reduction of fibrosis and apoptosis [17]. In a porcine cardiac infarction model, skeletal myoblast graft transplantation provided improvement of cardiac function with attenuation of cardiac remodeling [18]. Transplantation of skeletal myoblast grafts has also demonstrated that it is an appropriate and safe treatment for chronic myocardial infarction without increasing the risk of ventricular arrhythmias. Most recently, the transplantation of cardiac progenitor cell grafts derived from embryonic stem cells improved cardiac function without teratoma formation and induced cardiomyogenic differentiation in a simian impaired heart model. Our latest work provides evidence of the safety and efficacy of using embryonic stem cells for myocardial regeneration [19].

Based on the promising results in various animal models, there is a clinical study using cell sheet transplantation currently underway. Autologous skeletal myoblast sheet therapy has demonstrated that it is possible to improve cardiac function to such a degree that LVADs are no longer required for patients with dilated cardiomyopathy [20].

3.6 Conclusions

The field of tissue engineering presents an exciting approach to regenerative therapies. The future solutions scaling up give more powerful construct creation, resulting in the developments of remarkable tissue-engineered cardiac assist devices or organ replacement. Overall, cell sheet tissue engineering is a novel approach for cardiac treatment that promises efficient and effective alternative therapies in regenerative medicine.

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Future Treatment of Heart Failure Using Human iPSC-Derived Cardiomyocytes

4

Shugo Tohyama and Keiichi Fukuda

Abstract

Heart transplantation can drastically improve survival in patients with a failing heart; however, the shortage of donor hearts remains a serious problem with this treatment strategy and the successful clinical application of regenerative medicine is eagerly awaited. To this end, we developed a novel method to generate human induced pluripotent stem cells (iPSCs) from circulating human T lymphocytes using Sendai virus containing Yamanaka factors. To establish an efficient cardiac differentiation protocol, we then screened factors expressed in the future heart site of early mouse embryos and identified several growth factors and cytokines that can induce cardiomyocyte differentiation and proliferation. Subsequent transcriptome and metabolome analysis on undifferentiated stem cells and cardiomyocytes to devise a specific metabolic environment for cardiomyocyte selection revealed completely different mechanisms of glucose and lactate metabolism. Based on these findings, we succeeded in metabolically selecting cardiomyocytes using glucose-free and lactate-supplemented medium, with up to 99 % purity and no teratoma formation. Using our aggregation technique, we also showed that >90 % of the transplanted cardiomyocytes survived in the heart and showed physiological growth after transplantation. We expect that combining these techniques will achieve future heart regeneration.

Keywords

Induced pluripotent stem cell • Purification • Cardiomyocyte • Transplantation • Human

S. Tohyama • K. Fukuda (✉)
Department of Cardiology, Keio University School of Medicine, Tokyo, Japan
e-mail: kfukuda@a2.keio.jp

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4.1 Introduction

Heart disease remains a leading cause of death despite recent medical advances, and heart transplantation remains the ultimate treatment for severe heart failure. However, limited donor numbers remain an unsolved problem for transplantation therapy, and both patients and clinicians hold great hope for the future success of heart regenerative cell therapies as an alternative strategy [1]. Pluripotent stem cells (PSCs) including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can self-renew infinitely and are potential mass production sources for therapeutic cardiomyocytes. In particular, human iPSCs have the huge advantage of avoiding immunological rejection after cell transplantation.

Human iPSCs were first generated from dermal fibroblasts by Takanashi and Yamanaka in 2007 [2], using a retroviral transduction system. Subsequently, the methodology for generating iPSCs has dramatically improved. We previously reported that integration-free iPSCs could be easily and rapidly generated from terminally differentiated circulating T lymphocytes in peripheral blood using Sendai virus [3]. Our method makes it possible to generate iPSCs from any patients including children, girls, and the very elderly by blood sampling alone.

Such cumulative advances in iPSC generation techniques should accelerate the development of applications for iPSCs generated from patients. However, many hurdles remain in realizing such applications in human heart regeneration, due largely to the assumption that one patient will require at least 1×10^9 cardiomyocytes to recover cardiac function (Fig. 4.1). The important steps to overcome are as follows: (1) cardiac differentiation efficiencies should be stably improved regardless of the source cell lines; (2) large-scale cultivation systems will be required to obtain the billions of differentiated cells required; (3) approximately 1×10^9 cardiomyocytes should be efficiently collected and residual undifferentiated stem cells should be eliminated from large-scale, mixed, differentiated cell populations; (4) purified cardiomyocytes should be functionally and electrophysiologically characterized; (5) tissue engineering technologies might need to be used prior to transplantation because dispersed transplanted cardiomyocytes have not yet achieved high survival rates [4]; and (6) confirmation of safety and efficiency with these techniques is essential in large animal models before clinical application. In this chapter, we discuss these hurdles to realizing heart regenerative therapy in more detail.

4.2 Cardiac Differentiation from Human iPSCs

Many approaches using ESCs have been investigated to induce cardiac differentiation. In general, the differentiation of ESCs into any cell lineage is based on the mechanism of normal early development [5]. The visceral endoderm is known to play a key role in the differentiation of cardiac precursors that are present in the adjacent mesoderm during development, and Mummery et al. [6] previously reported that human ESCs effectively differentiate into cardiomyocytes when

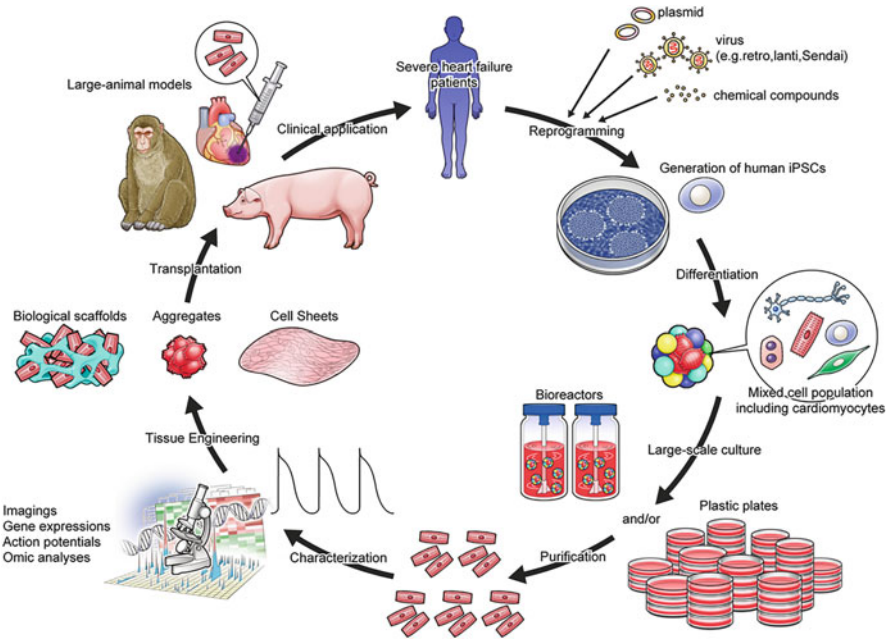

Fig. 4.1 Overview of steps to overcome for realization of heart regenerative therapy. The critical hurdles to overcome are as follows: (1) improvement of cardiac differentiation efficiencies, (2) establishment of large-scale cultivation systems, (3) purification of large-scale cardiomyocytes, (4) electrophysiological characterization, (5) utilization of appropriate tissue engineering technologies, and (6) confirmation of safety and efficiency in large animal models

cocultured with mouse visceral endoderm-like (END-2) cells. In an attempt to improve cardiac differentiation efficiencies inexpensively and easily, Takahashi et al. [6] screened a chemical compound library approved by the United States Food and Drug Administration (FDA) and found that ascorbic acid efficiently induced cardiac differentiation. However, this chemical alone might be not enough to induce cardiac differentiation from iPSCs.

Several studies have shown that various combinations of heart development-related proteins including BMP, activin, Wnt, BMP inhibitor, and Wnt inhibitor induce cardiomyocytes from ESCs [7–10]. We reported that the context-dependent differential action of BMPs in cardiomyocyte induction is explained by the local action of Noggin and other BMP inhibitors and, accordingly, developed a protocol to induce cardiac differentiation of mouse ESCs through transient administration of Noggin [9]. However, to obtain hundreds of millions of cardiomyocytes, it is necessary to establish a cardiac differentiation method that is both efficient and cost-effective due to the many expensive recombinant protein factors used. To address this problem, Minami et al. [11] screened small-molecule compounds to identify those that significantly increase cardiac differentiation induction, and they revealed some inhibitors of canonical Wnt signaling as candidates. In addition,

recently novel efficient protocols using small molecules and/or chemically defined media have been reported (Lian et al. Nat Protocols 2013;8:162–175, Burridge et al. Nat Methods. 2014;11:855–60).

Several studies then showed that induction techniques in ESCs could also be applied to iPSCs, although differentiation efficiencies were suggested to be inferior compared to ESCs. However, as such differentiation efficiencies vary greatly with different cell lines [12], further investigation is needed in the future. Furthermore, to efficiently obtain large quantities of cardiomyocytes inexpensively, it is necessary to continue refining efficient cardiac differentiation systems combined with the use of small-molecule compounds. However, despite improved cardiac differentiation efficiencies, it is inevitable that human PSC derivatives will contain not only cardiomyocytes but also undifferentiated stem cells and/or noncardiac cells because all PSCs cannot differentiate into cardiomyocytes. Therefore, to confirm safety after transplantation, it is necessary to remove noncardiac cells and undifferentiated stem cells that could cause tumors.

4.3 Nongenetic Methods for Purifying Cardiomyocytes

One of the biggest risks with in vitro-generated cardiomyocytes for clinical use is teratoma formation due to residual PSC contamination [13]. Current procedures for eliminating such contamination and boosting cardiomyocyte enrichment involve genetic modification [14, 15] and nongenetic methods using a mitochondrial dye [16] or antibodies to specific cell-surface markers [17]. However, none of these methods are ideal for the therapeutic application of PSC-derived cardiomyocytes due to insufficient stability, genotoxicity, and the use of fluorescence-activated cell sorting (FACS). To address this issue, we sought to purify cardiomyocytes efficiently and inexpensively, based on differences among cell-specific nutrition sources.

To create a metabolic environment where “residual undifferentiated stem cells cannot survive and only cardiomyocytes can survive,” we performed metabolome and transcriptome analysis in neonatal cardiomyocytes and PSCs. We found that these PSCs mainly depended on activated glycolysis and actively discharged lactate into the extracellular media. In addition, biomass needed for proliferation such as amino acids and nucleic acids were actively synthesized in PSCs compared to cardiomyocytes. On the other hand, cardiomyocytes mainly depended on oxidative phosphorylation in mitochondria to obtain adenosine triphosphate (ATP) efficiently. We also performed metabolome analysis to demonstrate that other noncardiac proliferating cells also depended on glycolysis, like PSCs. Thus, cardiomyocytes and proliferating noncardiac cells including PSCs showed differences in metabolism (Fig. 4.2) that we then successfully exploited to select cardiomyocytes from human PSCs efficiently and inexpensively simply by changing the cell-specific medium to one that is glucose depleted and lactate supplemented [18]. The cardiomyocytes selected by metabolism showed normal electrophysiological properties and did not form teratoma after transplantation.

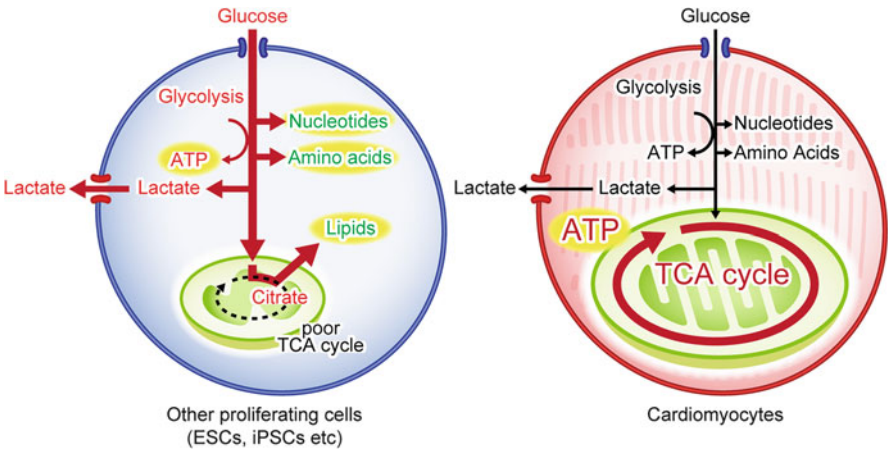

Fig. 4.2 Distinct metabolic differences between cardiomyocytes and other proliferating cells including undifferentiated stem cells. Cardiomyocytes efficiently obtain ATP mainly via oxidative phosphorylation, while other proliferating cells including PSCs obtain ATP, nucleotide, and amino acids via activated glycolysis

Furthermore, we succeeded to establish a practical culture system for generating substantial numbers of purified cardiomyocytes by combining a massive suspension culture system with a metabolic selection medium (Hemmi et al. Stem Cells Transl Med. 2014;3:1473–83).

4.4 Transplantation of Human PSC-Derived Cardiomyocytes

Many studies have been conducted regarding cell transplantation therapies in animal models using human PSC-derived cardiomyocytes. The survival of dispersed transplanted cardiomyocytes is reportedly very low [4], and some ingenuity is required in the method of transplantation. Our group previously reported that transplanted cell survival was dramatically improved by transplantation after formation of cardiomyocyte aggregates [16], while Laflamme et al. [9] showed a similar effect using a prosurvival cocktail that inhibited apoptosis.

With regard to the effectiveness of cell transplantation, Laflamme et al. [9] also demonstrated that transplanted human ESC-derived cardiomyocytes could improve cardiac function after 4 weeks in a rat myocardial infarction model. In addition, Shiba et al. [20] demonstrated that transplanted human ESC-derived cardiomyocytes electrically coupled to the host cardiomyocytes and suppressed arrhythmias in a guinea pig myocardial infarction model. Furthermore, with respect to cell transplantation in large animals, Kawamura et al. [19] recently reported that cardiac cell sheets comprising purified human iPSC-derived cardiomyocytes generated using our method [18] improved cardiac function in a pig myocardial infarction model. Thus, efficacies have been achieved in cell transplantation

therapies using human iPSC-derived cardiomyocytes, although many such studies showed only short-term effectiveness. Thus, careful evaluation of the efficacy and safety of human iPSC-derived cardiomyocytes in cell transplantation over the longer term must be ongoing.

4.5 Future Directions

The discovery and refinement of human iPSCs generation is expected to advance not only regenerative medicine but also drug discovery and analyses of genetic disorders using patient-specific iPSCs [19]. The major and common problem remaining in this quest is securing sufficient numbers of mature and functional cardiomyocytes with high purity. To solve this problem, it is essential to develop a stable and efficient mass culture system and to establish a simple system to analyze the electrophysiological function of the generated cardiomyocytes (Fig. 4.1). Furthermore, the future realization of clinical applications using human iPSCs will necessitate a better understanding of the cell biology and techniques involved in tissue engineering generally. Only then will we be able to achieve long-term safety and efficacy in the heart failure models of large animals and finally realize human heart regenerative therapies.

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Congenital Heart Disease: In Search of Remedial Etiologies

5

Roger R. Markwald, Shibnath Ghatak, Suniti Misra, Ricardo A. Moreno-Rodríguez, Yukiko Sugi, and Russell A. Norris

Abstract

In searching for remedial etiologies for congenital heart disease (CHD), we have focused on identifying interactive signaling pathways or "hubs" in which mutations disrupt fundamental cell biological functions in cardiac progenitor cells in a lineage-specific manner. Based on the frequency of heart defects seen in a clinical setting, we emphasize two signaling hubs – *nodal kinases activated by extracellular ligands* (e.g., *periostin*) *and the cytoskeletal regulatory protein*, *filamin A* (*FLNA*). We discuss them in the context of valve and septal development and the lineages which give origin to their progenitor cells. We also explore developmental windows that are potentially amenable to remedial therapy using homeostatic mechanisms like those revealed by a chimeric mice model, i.e., *irradiated animals whose bone marrow had been reconstituted with GFP+ hematopoietic stem cells*, that shows bone marrow-derived cells track to the heart, engraft, and give rise to bona fide fibroblasts. We propose to use this model to deliver genetic payloads or protein cargos during the neonatal period to override biochemical or structural deficits of CHD associated with valve and septal signaling hubs or fibroblast/myocyte interactions. Preliminary tests of the model indicate remedial potential for cardiac injuries.

Keywords

Heart defects • Valves • Septa • Matricellular proteins • Periostin • Cell signaling • Kinases • Filamin A • Hematopoietic stem cells • Fibroblast • Lineage • Genetic engineering

R.R. Markwald, Ph.D. (✉) • S. Ghatak • S. Misra • R.A. Moreno-Rodríguez • Y. Sugi • R.A. Norris
Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, 173 Ashley Avenue, Room BSB-648B, P.O. Box 250508, Charleston, SC 29425, USA
e-mail: Markwald@musc.edu

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5.1 Introduction

5.1.1 Emerging Concepts

Etiologies of congenital heart defects have been difficult to identify and explain, often invoking discussions of genetics and environment or both. After 40+ years of searching for etiologies, especially ones that might be potentially remedial, a few conceptual observations are emerging (although by no means is there consensus). These include suppositions that:

1. Genetics more than environment is responsible for CHD based on the ability to simulate human heart congenital defects in animal genetic models (*see also Bhattacharya* [1]).
2. The evolution of genetic thought is toward trans-heterozygous, multigenetic interactions vs. single gene hits.
3. Mutations in the downstream intracellular signaling targets of growth factor, transcription factors, or matricellular proteins are more likely to be the root cause of heart defects seen in children by pediatric cardiologists (*as the loss of both alleles for upstream early regulatory genes is usually lethal*).
4. Intersecting regulatory networks or signaling hubs coordinate fundamental biological processes in cardiac progenitor cells in a lineage-restricted manner.
5. Based on the frequency of different types of heart defects, the lineages most likely to be modified by genetic mutations or environmental stressors in CHD are the non-cardiomyocyte lineages, primarily fibroblasts.

5.1.2 Hub Hypothesis

In trying to provide answers to the questions most often asked by parents of children born with heart defects – "why did it happen?" and "what can be done about it?" – we have endeavored to integrate these five emerging concepts around two examples of central signaling hubs or platforms that intersect with multiple gene regulatory networks (like the spokes of a wheel) to regulate or "tune" behavioral changes in the progenitor cells engaged in valvuloseptal morphogenesis (or myocardial remodeling). In so doing, we propose that intersecting signaling hubs explain why so many different genes, if mutated or deleted, can engender similar anatomical dysmorphic phenotypes, e.g., ventricular septal defects. This suggests that there are only so many ways progenitor cells can respond to normal or abnormal signaling inputs: e.g., they can proliferate, activate, or suppress apoptotic pathways; transport ions; secrete, endocytose, adhere/migrate, and generate contractile forces; change polarity or shape; and differentiate. Thus, the conceptual appeal of common final pathways or intersecting signaling hubs/nodes is that they provide potential for exploring "shared" remedial therapies for CHD that do not require an individual approach for correcting each abnormal gene.

5.2 Searching for Candidate Signaling Hubs in Heart Development

5.2.1 Nodal Signaling Kinases

In patients with Down, Marfan, or Noonan Syndromes, there is increasing evidence that genes encoding nodal signaling kinases like FAK/AKTkt/PI3K, RAS, MEKK/ERK1/2, PTPN11, etc., are likely candidates for CHD if they are mutated or overexpressed [2–4]. Such genes are not usually lethal (*as there exist molecular or functional redundancies*), yet as indicated for syndromic heart defects, they have potential to change functional behaviors in progenitor cells that normally mold and remodel the simple tubular heart into a four-chambered organ. As indicated in Fig. 5.1, intracellular signaling kinases (and small regulatory GTPases) are the direct and indirect downstream targets of growth factors (e.g., TGFβ and BMP 2&4) that are normally secreted by the embryonic endocardium or myocardium [5], or in the case of extracellular ligands like matricellular proteins (periostin, the CCN family), they are secreted by the mesenchymal progenitor cells of valves and connective septa which are derived from both endocardium and epicardium [6]. In the case of matricellular and other extracellular proteins (Fig. 5.2), binding to integrin receptors triggers integrin-dependent, downstream signaling kinases/GTPases (FAK/AKT/PI3k) which activate effector mechanisms of growth, survival, and differentiation into the fibrous structures (valves and septa) that assure coordinated and unidirectional blood flow through the right and left sides of the developing heart. Epicardial-derived mesenchymal cells also express periostin as they invade the ventricular myocardium and, like endothelial-derived mesenchyme, secrete collagen and differentiate into ventricular connective tissue but also contribute to the parietal leaflets of the AV valves [7, 8]. Disruption of these signaling pathways by either silencing one or more of the kinases shown in Fig. 5.1, inhibiting β-integrin functions, or deleting the periostin gene itself resulted in septal defects, abnormal (poorly differentiated, hypertrophic) valves, arrhythmias associated with a reduction in the AV fibrous connective tissue, and a reduction in ventricular elastic modulus due to loss of interstitial collagen [9, 10]. These findings are consistent with the relevance of central or nodal signaling kinases to heart development and how different genes, if mutated or inhibited, could produce similar abnormal anatomical phenotypes as a result of either binding to, activating, or encoding a kinase or GTPase component of an interactive signaling pathway. Conceivably, these same interactive signaling pathways could also be used to explore remedial therapies for CHD. Any one or combination of the signaling kinases or effector proteins shown in Fig. 5.1 could be a candidate therapeutic target that could be used to bypass or circumvent a genetic or biochemical block associated with a particular CHD, *if a way could be found to administer drugs or small molecules that silence*, *simulate*, *or activate them*. Identifying an in utero approach using orally administered or injected signaling inhibitors, lithium, or retinoids is appealing but lacks target specificity (potentially engendering a broad spectrum of side effects), or it can create a catch-22 in the sense that treatments may