Stem Cell Biology and Regenerative Medicine

Ira S. Cohen
Glenn R. Gaudette
Editors

Regenerating the Heart

Stem Cells and the Cardiovascular System

Stem Cell Biology and Regenerative Medicine

Series Editor
Kursad Turksen, Ph.D.
kturksen@ohri.ca

For other titles published in this series, go to
www.springer.com/series/7896

Ira S. Cohen • Glenn R. Gaudette
Editors

Regenerating the Heart

Stem Cells and the Cardiovascular System

Editors
Ira S. Cohen
Department of Physiology and Biophysics
Stony Brook University
Stony Brook, NY
USA
ira.cohen@stonybrook.edu

Glenn R. Gaudette
Department of Biomedical Engineering
Worcester Polytechnic Institute
Worcester, MA
USA
gaudette@wpi.edu

ISBN 978-1-61779-020-1 e-ISBN 978-1-61779-021-8
DOI 10.1007/978-1-61779-021-8
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011922496

© Springer Science+Business Media, LLC 2011
All rights reserved. This work may not be translated or copied in whole or in part without the written permission of the publisher (Humana Press, c/o Springer Science+Business Media, LLC, 233 Spring Street, New York, NY 10013, USA), except for brief excerpts in connection with reviews or scholarly analysis. Use in connection with any form of information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed is forbidden.
The use in this publication of trade names, trademarks, service marks, and similar terms, even if they are not identified as such, is not to be taken as an expression of opinion as to whether or not they are subject to proprietary rights.
While the advice and information in this book are believed to be true and accurate at the date of going to press, neither the authors nor the editors nor the publisher can accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, express or implied, with respect to the material contained herein.

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

Contents

1 **Introduction** 1
Ira S. Cohen and Glenn R. Gaudette

Part I Stem Cells for Regeneration of Mechanical Function

2 **Inducing Embryonic Stem Cells to Become Cardiomyocytes** 7
Alexander M. Becker, Michael Rubart, and Loren J. Field

3 **Regenerating Function In Vivo with Myocytes Derived from Embryonic Stem Cells** 25
Priya R. Baraniak and Todd C. McDevitt

4 **Excitation–Contraction Coupling, Functional Properties, and Autonomic and Hormonal Regulation in Human Embryonic Stem Cell Derived Cardiomyocytes** 37
Oshra Sedan and Ofer Binah

5 **Embryonic Stem Cell Derivatives for Cardiac Therapy: Advantages, Limitations, and Long-Term Prospects** 53
Michal Weiler-Sagie and Lior Gepstein

6 **Methods for Differentiation of Bone-Marrow-Derived Stem Cells into Myocytes** 67
Shinji Makino and Keiichi Fukuda

7 **Homing, Survival, and Paracrine Effects of Human Mesenchymal Stem Cells** 83
Sergey Doronin

8 Bone Marrow Cell Therapy After Myocardial Infarction: What have we Learned from the Clinical Trials and Where Are We Going? 111
Kai C. Wollert

9 Evidence for the Existence of Resident Cardiac Stem Cells 131
Isotta Chimenti, Roberto Gaetani, Lucio Barile, Elvira Forte, Vittoria Ionta, Francesco Angelini, Elisa Messina, and Alessandro Giacomello

10 Multiple Sources for Cardiac Stem Cells and Their Cardiogenic Potential 149
Antonio Paolo Beltrami, Daniela Cesselli, and Carlo Alberto Beltrami

11 Skeletal Muscle Stem Cells in the Spotlight: The Satellite Cell 173
Zipora Yablonka-Reuveni and Kenneth Day

12 Regenerating Mechanical Function In Vivo with Skeletal Myoblasts 201
Todd K. Rosengart and Muath Bishawi B.S

13 Methods for Inducing Pluripotency 219
Raymond L. Page, Christopher Malcuit, and Tanja Dominko

14 Inducible Pluripotent Stem Cells for Cardiac Regeneration 241
Naama Zeevi-Levin and Joseph Itskovitz-Eldor

15 Induced Pluripotent Cells for Myocardial Infarction Repair 263
Timothy J. Nelson and Andre Terzic

Part II Stem Cells for Regeneration of Electrical Function

16 Substrates of Cardiac Reentrant Arrhythmias: The Possible Role of Tissue Regeneration and Replacement 283
André G. Kléber

17 Integration of Stem Cells into the Cardiac Syncytium: Formation of Gap Junctions 301
Peter R. Brink, Ira S. Cohen, and Richard T. Mathias

18 Bradyarrhythmia Therapies: The Creation of Biological Pacemakers and Restoring Atrioventricular Node Function 321
Richard B. Robinson

19 Tachyarrhythmia Therapies: Approaches to Atrial Fibrillation and Postinfarction Ventricular Arrhythmias 349
J. Kevin Donahue and Kenneth R. Laurita

20 Long-Term Prospects for Arrhythmia Treatment: Advantages and Limitations of Gene and Cell Therapies 379
Michael R. Rosen

Part III Regenerating Cardiac Tissues

21 Regenerating Blood Vessels 393
Tracy A. Gwyther and Marsha W. Rolle

22 Regenerating Heart Valves 403
Benedikt Weber and Simon P. Hoerstrup

23 Tissue Engineering Strategies for Cardiac Regeneration 443
Amandine F.G. Godier-Furnémont, Yi Duan, Robert Maidhof, and Gordana Vunjak-Novakovic

Part IV Technical Issues for Stem Cell Therapy in the Heart

24 Methods of Cell Delivery for Cardiac Repair 479
Sarah Fernandes and Hans Reinecke

25 Tracking of Stem Cells In Vivo 499
Yingli Fu and Dara L. Kraitchman

26 Assessing Regional Mechanical Function After Stem Cell Delivery 523
Jacques P. Guyette and Glenn R. Gaudette

Index 543

Contributors

Francesco Angelini
Department of Experimental Medicine,
Cenci-Bolognetti Foundation, University "Sapienza" of Rome, Rome, Italy

Priya R. Baraniak
The Wallace H. Coulter Department of Biomedical Engineering at Georgia
Institute of Technology and Emory University, Atlanta, GA, USA

Lucio Barile
Department of Biotechnology and Biosciences,
University "Bicocca" of Milan, Milan, Italy

Alexander M. Becker
The Heart Center Göttingen,
George-August University, Göttingen, Germany

Antonio Paolo Beltrami
Centro Interdipartimentale di Medicina Rigenerativa (CIME),
Università degli Studi di Udine, Udine, Italy

Carlo Alberto Beltrami
Centro Interdipartimentale di Medicina Rigenerativa (CIME),
Università degli Studi di Udine, Udine, Italy

Ofer Binah
The Sohnis Family Stem Cells Center, The Rappaport Family Institute for
Research in the Medical Sciences, The Department of Physiology, Ruth and Bruce
Rappaport Faculty of Medicine, Technion – Israel Institute of Technology,
Haifa, Israel

Muath Bishawi B.S
Department of Surgery, Stony Brook University Medical Center,
Stony Brook, NY, USA

Peter R. Brink
Department of Physiology and Biophysics, Stony Brook University,
Stony Brook, NY, USA

Daniela Cesselli
Centro Interdipartimentale di Medicina Rigenerativa (CIME),
Università degli Studi di Udine, Udine, Italy

Isotta Chimenti
Department of Experimental Medicine, Cenci-Bolognetti Foundation,
University “Sapienza” of Rome, Rome, Italy

Ira S. Cohen
Department of Physiology & Biophysics, Stony Brook University,
Stony Brook, NY, USA

Kenneth Day
Hudson Alpha Institute for Biotechnology, Huntsville, AL, USA

Tanja Dominko
Department of Biology and Biotechnology, Bioengineering Institute,
Worcester Polytechnic Institute, Worcester, MA, USA
and
Cellthera, Inc., Southbridge, MA, USA

J. Kevin Donahue
Heart and Vascular Research Center, MetroHealth Hospital,
Case Western Reserve University, Cleveland, OH, USA

Sergey Doronin
Department of Physiology and Biophysics, Stony Brook University,
Stony Brook, NY, USA

Yi Duan
Department of Biomedical Engineering,
Columbia University, New York, NY, USA

Sarah Fernandes
Center for Cardiovascular Biology,University of Washington
Medicine at South Lake Union, Seattle, WA, USA

Loren J. Field
The Riley Heart Research Center, Herman B Wells Center
for Pediatric Research, Indiana University School of Medicine,
Indianapolis, IN, USA

Elvira Forte
Department of Experimental Medicine,
Cenci-Bolognetti Foundation, University “Sapienza” of Rome,
Rome, Italy

Yingli Fu
Russell H. Morgan Department of Radiology
and Radiological Science School of Medicine,
The Johns Hopkins University, Baltimore, MD, USA

Keiichi Fukuda
Department of Cardiology, KEIO University School of Medicine, Tokyo, Japan

Roberto Gaetani
Department of Experimental Medicine, Cenci-Bolognetti Foundation,
University "Sapienza" of Rome, Rome, Italy

Glenn R. Gaudette
Department of Biomedical Engineering, Worcester Polytechnic Institute,
Worcester, MA, USA

Lior Gepstein
The Sohnis Family Research Laboratory for Cardiac Electrophysiology
and Regenerative Medicine and the Rappaport Family Institute
for Research in the Medical Sciences, The Bruce Rappaport Faculty
of Medicine, Technion – Israel Institute of Technology, Haifa, Israel

Alessandro Giacomello
Department of Molecular Medicine and Pathology,
Cenci-Bolognetti Foundation, University "Sapienza" of Rome,
Rome, Italy

Amandine F. G. Godier-Furnémont
Department of Biomedical Engineering,
Columbia University, New York, NY, USA

Jacques P. Guyette
Department of Biomedical Engineering, Worcester Polytechnic Institute,
Worcester, MA, USA

Tracy A. Gwyther
Department of Biomedical Engineering, Worcester Polytechnic Institute,
Worcester, MA, USA

Simon P. Hoerstrup
Swiss Center for Regenerative Medicine, Department of Surgical Research
and Center for Clinical Research, University of Hospital of Zurich,
Switzerland

Vittoria Ionta
Department of Experimental Medicine, Cenci-Bolognetti Foundation,
University "Sapienza" of Rome, Rome, Italy

Joseph Itskovitz-Eldor
Chairman, Department of Ob-Gyn, Rambam Health Care Campus Head,
Stem Cell Center, Technion – Israel Institute of Technology,
PoB 9602, Haifa 31096, Israel

André G. Kléber
Department of Physiology, University of Bern, Bühlplatz 5, 3012 Bern,
Switzerland

Dara L. Kraitchman
Russell H. Morgan Department of Radiology and Radiological Science,
School of Medicine, The Johns Hopkins University, Baltimore, MD, USA

Kenneth R. Laurita
Heart and Vascular Research Center, MetroHealth Hospital,
Case Western Reserve University, Cleveland, OH, USA

Robert Maidhof
Department of Biomedical Engineering, Columbia University,
New York, NY, USA

Shinji Makino
Center for Integrated Medical Research, and Department of Cardiology,
Therapeutics, KEIO University School of Medicine, Tokyo, Japan

Christopher Malcuit
Bioengineering Institution, Worcester Polytechnic Institute, Worcester, MA, USA
and
Cellthera, Inc., Southbridge, MA, USA

Richard T. Mathias
Department of Physiology and Biophysics, Stony Brook University,
Stony Brook, NY, USA

Todd C. McDevitt
The Wallace H. Coulter Department of Biomedical Engineering
at Georgia Institute of Technology and Emory University,
Petit Institute for Bioengineering and Bioscience, Atlanta, GA, USA

Elisa Messina
Department of Experimental Medicine, Cenci-Bolognetti Foundation,
University "Sapienza" of Rome, Rome, Italy

Timothy J. Nelson
General Internal Medicine and Transplant Center Mayo Clinic,
Rochester, MN, USA

Raymond L. Page
Department of Biomedical Engineering,
Department of Biology and Biotechnology, Bioengineering Institute,
Worcester Polytechnic Institute, Worcester MA, USA;
Cellthera, Inc., Southbridge, MA, USA

Hans Reinecke
Center for Cardiovascular Biology, University of Washington
Medicine at South Lake Union, Seattle, WA, USA

Richard B. Robinson
Department of Pharmacology and Center for Molecular Therapeutics,
Columbia University College of Physicians and Surgeons,
New York, NY 10032, USA

Marsha W. Rolle
Department of Biomedical Engineering, Worcester Polytechnic Institute,
Worcester, MA, USA

Michael R. Rosen
Department of Pharmacology, Department of Pediatrics, Center for Molecular
Therapeutics, Columbia University, New York, NY, USA

Todd K. Rosengart
Department of Surgery, Stony Brook University Medical Center,
Stony Brook, NY, USA

Michael Rubart
The Riley Heart Research Center, Herman B Wells Center for Pediatric Research,
Indiana University School of Medicine, Indianapolis, IN, USA

Oshra Sedan
The Sohnis Family Stem Cells Center, The Rappaport Family Institute for
Research in the Medical Sciences, The Department of Physiology, Ruth &
Bruce Rappaport Faculty of Medicine, Technion – Israel Institute of Technology,
Haifa, Israel

Andre Terzic
Marriott Heart Disease Research Program, Division of Cardiovascular Diseases,
Department of Medicine, Molecular Pharmacology
and Experimental Therapeutics and Medical Genetics,
Mayo Clinic, Rochester, MN, USA

Gordana Vunjak-Novakovic
Department of Biomedical Engineering, Columbia University,
New York, NY, USA

Benedikt Weber
Swiss Center for Regenerative Medicine,
Department of Surgical Research and Center for Clinical Research,
University of Hospital of Zurich, Zurich, Switzerland

Michal Weiler-Sagie
The Sohnis Family Research Laboratory for Cardiac Electrophysiology
and Regenerative Medicine, The Rappaport Family Institute
for Research in the Medical Sciences, The Bruce Rappaport Faculty of Medicine,
Technion – Israel Institute of Technology, Haifa, Israel

Kai C. Wollert
Hans-Borst Center for Heart and Stem Cell Research,
Department of Cardiology and Angiology, Hannover Medical School,
Hanover, Germany

Zipora Yablonka-Reuveni
Department of Biological Structure, University of Washington
School of Medicine, Seattle, WA, USA

Naama Zeevi-Levin
Sohnis and Forman Families Center for Stem Cell and Tissue Regeneration
Research, Ruth & Bruce Rappaport, Faculty of Medicine, Technion, Haifa, Israel

Introduction

Ira S. Cohen and Glenn R. Gaudette

The twentieth century witnessed many positive changes for heart disease. In particular, our ability to treat myocardial ischemia was greatly improved. Developments from coronary artery bypass surgery to drug-eluding stents restored blood flow and helped keep coronary blood vessels open, thereby extending patients lives. Electrophysiology also made great strides, with implantable electronic pacemakers and automatic internal defibrillators providing patients with electrical rhythm control. Public awareness of heart disease also increased over the past 100 years. Multiple groups have formed to fight heart disease and educate the general public with regard to this debilitating disease. Automatic external defibrillators are showing up in public locations, demonstrating public awareness of the seriousness of heart disease. As we look forward to the twenty-first century and consider the next great challenges, we see the potential of cell therapy to address many cardiovascular diseases. From heart failure to atrioventricular nodal dysfunction, the young but promising field of cell therapy is likely to play a significant role in developing a cure during this century.

Both of us entered the stem cell field less than a decade ago; one of us an electrophysiologist, the other as a mechanical engineer. Like others we were attracted by the opportunity for a real breakthrough. Arrhythmias and heart failure had one thing in common: neither pharmacology nor devices were a panacea. Instead, therapies represented the best that modern medicine had to offer, but certainly were far short of a cure. With our backgrounds, we both faced the same problem: How do we accumulate sufficient knowledge in this burgeoning field to think creatively? Together we attended meetings sponsored by the National Heart, Lung, and Blood Institute, and by the American Heart Association and found them helpful, but ultimately we continuously found ourselves at a disadvantage, almost as if we were entering in the middle of a long conversation without a good source to quickly catch up on the field. A book to document where the field has been, where it is, and where

I.S. Cohen (✉)
Department of Physiology and Biophysics,
Stony Brook University, SUNY, Stony Brook, NY, USA
e-mail: ira.cohen@sunysb.edu

I.S. Cohen and G.R. Gaudette (eds.), *Regenerating the Heart*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-61779-021-8_1,
© Springer Science+Business Media, LLC 2011

it is heading would be helpful. To our surprise, we were recently invited to write a book on stem cells and the cardiovascular system. Fortunately, we had sufficient self-insight to “know what we know and also know what we don’t know” and declined the offer. However, a renewed offer to edit such a book held much more interest. First knowing what we did not know, this text offered us the opportunity to learn from the experts we invited. Second, we could organize this missing knowledge in a manner that would afford others like us an opportunity to learn as well.

Our major challenge for this book was to organize the field into a tractable body of knowledge. We decided to organize the text into four major sections. The first section considers mechanical regeneration. When the heart fails as a pump, the major cause is the loss of contractile elements (possibly more than a billion myocytes). In this first section of the book, we consider approaches to mechanical regeneration of cardiac function. Despite the large number of patients suffering from myocardial infarction, currently, the only clinical method available to add contractile myocytes is whole heart transplantation. However, the demand for hearts for transplant far exceeds the supply. Here, the potential for cell therapy is large. Multiple stem cells have demonstrated cardiogenic potential and so these cell types had to be reviewed individually. Embryonic stem cells, bone-marrow-derived cells, cardiac stem cells, and induced pluripotent stem cells are all considered. Both the basic properties of these cells and the methods to drive them toward cardiac lineages are considered. Skeletal myocytes may not be cardiogenic but do contract and are also included in this section because of their early role in clinical attempts at cardiac cellular myoplasty. However, not all cardiac regeneration by these cells occurs through cardiac differentiation and thus other regenerative mechanisms are considered. Further, the ability to differentiate stem cells into myocytes in vitro is necessary but not sufficient to achieve regeneration of mechanical function in vivo and so translational efforts in both animal and human trials are reviewed.

We next consider electrical regeneration. The mechanical function of the heart is triggered by the orderly electrical activation of each of its myocytes through a predefined electrical pathway. Each myocyte is electrically connected to all others, creating a functional electrical syncytium. Here the problem is somewhat different. It is not the massive loss of myocytes that creates the problem, but the punctuate loss of electrical connectivity or decreased excitability that is at fault. To consider the therapeutic potential of stem cells it is necessary to understand the genesis of arrhythmias and the basis of electrical connectivity in biological systems. Each of these topics is considered in individual chapters. Arrhythmias are classified into two types: bradyarrhythmias due to excessive slowing of heart rate, and tacchyarrhythmias due to excessive speeding of heart rate. Stem cell approaches to each of these common problems are discussed. Finally, it is worth considering what future stem cells have in the panoply of alternative therapies for electrical dysfunction and a chapter looking to the future concludes this section.

The heart is a complex tissue that subserves its mechanical function with various tissue types. The third section of our book considers cardiac tissues. These include heart valves which separate the upper and lower chambers as well as the lower chambers and the systemic and pulmonic circulations. One chapter reviews attempts

to create biologic solutions to their replacement. Vessels which carry the blood throughout the systemic and pulmonic circulations frequently fail and exciting new approaches to vessel replacement are also considered. Finally, it is a dream of all cardiac researchers to replace not only individual myocytes or blood vessels but also complete cardiac tissue, and approaches to engineer such tissue are also considered.

Finally, this text would not be complete without considering the approaches employed to evaluate stem cell therapies in vivo. In the last section, there are chapters which consider methods for stem cell delivery to the myocardium, methods to track the delivered stem cells, and finally methods for how to assess their contributions to mechanical function.

We have learned greatly from the preparation of this text. We thank the authors for their fine contributions and hope that it contributes to the education of newly committed and veteran stem cell researchers alike.

Part I
Stem Cells for Regeneration of Mechanical Function

Inducing Embryonic Stem Cells to Become Cardiomyocytes

Alexander M. Becker, Michael Rubart, and Loren J. Field

Abstract Many forms of heart disease are associated with a decrease in the number of functional cardiomyocytes. These include congenital defects (e.g. hypoplastic and noncompaction syndromes) as well as acquired injuries (e.g. exposure to cardiotoxic agents or injuries resulting from coronary artery disease, hypertension, or surgical interventions). Although the adult mammalian heart retains some capacity for cardiomyocyte renewal (resulting from cardiomyocyte proliferation and/or cardiomyogenic stem cell activity), the magnitude of this regenerative process is insufficient to effect repair of substantively damaged hearts. It has become clear that exogenous cardiomyocytes transplanted into adult hearts are able to structurally and functionally integrate. It has also become clear that embryonic stem cells (ESCs), as well as induced progenitors with ESC-like characteristics, are able to generate bona fide cardiomyocytes in vitro and in vivo. These cells thus constitute a potential source of donor cardiomyocytes for therapeutic interventions in damaged hearts. This chapter reviews spontaneous cardiomyogenic differentiation in ESCs, methods used to generate enriched populations of ESC-derived cardiomyocytes, and current results obtained after engraftment of ESC-derived cardiomyocytes or cardiomyogenic precursors.

Keywords Cardiac differentiation • Intracardiac engraftment • Cell therapy

1 Introduction

The structure and cellular composition of the adult mammalian heart are complex; consequently, myocardial disease can manifest itself at many different levels, and can impact multiple structures and cell types (valves, coronary arteries, capillaries,

L.J. Field (✉)
The Riley Heart Research Center, Herman B Wells Center for Pediatric Research,
Indiana University School of Medicine,
Indianapolis, Indiana, USA
e-mail: ljfield@iupui.edu

I.S. Cohen and G.R. Gaudette (eds.), *Regenerating the Heart*, Stem Cell Biology and Regenerative Medicine, DOI 10.1007/978-1-61779-021-8_2,
© Springer Science+Business Media, LLC 2011

endothelial cells, veins, interstitial fibroblasts, nodal cells, conduction system cells, working cardiomyocytes, etc.). Advances in surgical and pharmacologic interventions, as well as the development of electrophysiologic and mechanical devices, have steadily advanced and currently provide a wide variety of viable treatments for many forms of heart disease. The elucidation of the molecular underpinnings of cell lineage commitment and morphogenesis provide additional avenues of treatment, particularly in the area of angiogenesis. Unfortunately, the ability to promote widespread replacement of lost contractile units (i.e. cardiomyocyte replacement) has remained elusive.

Developmental and molecular studies have identified progenitor cells which give rise to cardiomyocytes in the developing heart. Proliferation of immature but contracting cardiomyocytes is a major contributor to the increase in cardiac mass observed during fetal development. The proliferative capacity of cardiomyocytes decreases markedly in postnatal life. Additionally, several progenitor cell populations with cardiomyogenic activity identified during development are depleted or have lost their ability to form new cardiomyocytes in neonatal life. Nonetheless, evidence for cardiomyocyte proliferation and/or apparent cardiomyogenic stem cell activity has been reported in the adult heart. For example, quantitation of radioisotope incorporation into cardiomyocyte nuclei of individuals alive during atmospheric atomic bomb detonations suggested an annual cardiomyocyte renewal rate of approximately 1% in young adults [1], a value remarkably similar to that extrapolated from shorter pulse/chase tritiated thymidine incorporation studies in mice [2]. Although the findings of these studies collectively are more consistent with the notion of cardiomyocyte renewal via proliferation, they do not rule out potential contributions from cardiomyogenic stem cells. Indeed, studies employing an elegant conditional reporter transgene system suggested stem-cell-based regeneration following injury in adult mice [3]. The notion of cardiomyocyte renewal in the adult heart has been with us for a long time – at issue is the magnitude of the regenerative response, a point which is the subject of intense research and debate among cardiomyocyte aficionados. What is clear is that the adult heart lacks the ability to reverse damage following the loss of large numbers of cardiomyocytes.

Studies from the 1990s demonstrated that donor cells could successfully engraft the hearts of recipient animals. Proof-of-concept experiments showed that cardiomyocytes from enzymatically dispersed fetal mouse hearts structurally integrated into the hearts of adult recipients following direct intracardiac injection [4, 5]. Subsequent analyses demonstrated that the donor cardiomyocytes formed a functional syncytium with the host myocardium, using the presence of intracellular calcium transients as a surrogate marker for contractile activity [6]. Although promising, it was soon apparent that only a small fraction of the injected cardiomyocytes survived and engrafted [7], a problem which remains a major obstacle for clinical efficacy of this approach. Nonetheless, several studies have reported that intracardiac injection of fetal cardiomyocytes could preserve cardiac function following experimental injury in rodents [8–10].

In light of these observations, considerable effort has been invested to identify potential sources of donor cardiomyocytes, or alternatively progenitor cells with

cardiomyogenic activity. Toward that end, many cells with apparent cardiomyogenic activity have been reported in the recent literature, a remarkable observation given that the intrinsic regenerative rate in the adult myocardium is quite low. Many factors likely contribute to this phenomenon. For example, the presence of multiple markers, or alternatively the transient expression of different markers, could result in an individual cell or cell lineage being categorized as multiple cells/lineages. The ability of some cell types to fuse with cardiomyocytes [11] could result in their false identification as cardiomyogenic stem cells. The relative rigor of the assays employed to detect cardiomyogenic activity could also contribute to the identification of false positives. It is also possible that reprogramming during in vitro propagation unmasked or enhanced cardiomyogenic potential. Given the intense activity in the field, it is likely that the true in vitro and in vivo cardiomyogenic activity of the various progenitor cells identified to date will rapidly be either validated or repudiated.

It is well established that embryonic stem cells (ESCs) are able to generate bona fide cardiomyocytes [12]. ESCs are derived from the inner cell mass (ICM) of preimplantation embryos [13, 14]. ESCs can be propagated in vitro in an undifferentiated state, and when allowed to differentiate can form endodermal, ectodermal, and mesodermal derivatives in vitro and in vivo. ESCs thus constitute a potential source of donor cardiomyocytes (or alternatively, donor cardiomyogenic progenitors) for therapeutic interventions targeting diseased hearts. In this chapter we review the spontaneous cardiomyogenic differentiation in ESCs, the various methods which have been developed to generate enriched populations of ESC-derived cardiomyocytes, and the current status of preclinical studies aimed at regenerating myocardial tissue via engraftment of ESC-derived cardiomyocytes or cardiomyogenic precursors. We then consider the challenges which must be overcome for successful translation to the clinic.

2 ESCs and Spontaneous Caridomyogenic Differentiation

After fertilization, initial growth of the preimplantation mammalian embryo is characterized by rapid cell division. Cells within the embryo begin to differentiate at the 16-cell stage (morula). As development proceeds, cells on the periphery of the morula give rise to trophoblasts (which, together with maternal endometrium, form the placenta) and cells in the center of the morula give rise to the ICM (which forms the embryo). The resulting blastocyst remains surrounded by the zona pellucida. Blastocysts can be cultured on feeder layers of mitomycin-treated mouse embryonic fibroblasts (MEFs). In the example shown in Fig. 1a, the MEFs were derived from transgenic mice carrying a transgene encoding leukemia inhibitory factor (LIF). LIF activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase pathways and suppresses differentiation in mouse ESCs (but is not required for generating human ESCs).

After several days of culture, the zona pellucida of the preimplantation embryo will rupture, allowing the outgrowth of both trophoblasts and ICM cells (Fig. 1b–d). The two cell types were readily distinguished by phase-contrast microscopy, with the

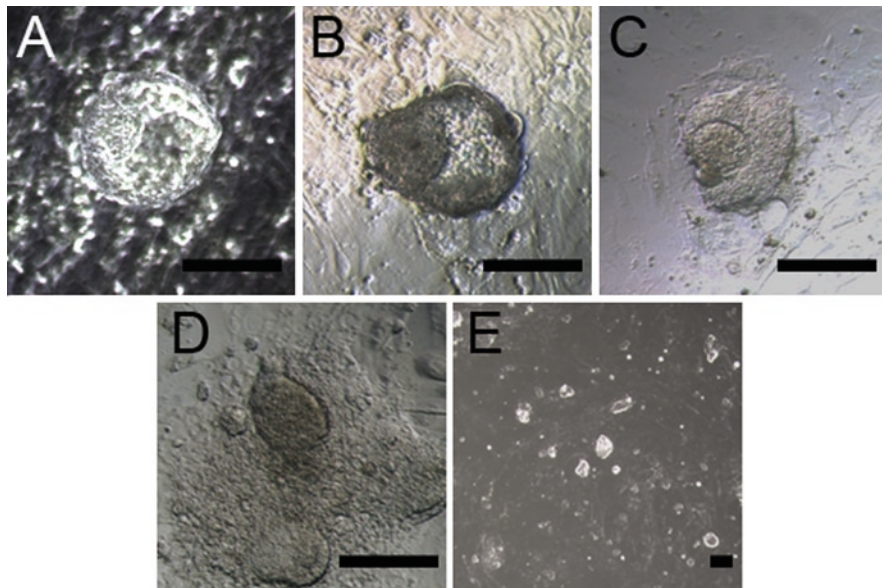

Fig. 1 Derivation of mouse embryonic stem cell (ESC) lines. (**a**) Blastocyst isolated at 3.5 days post coitus and culture for 3 days on a mouse embryonic fibroblast (MEF) feeder layer. Note the presence of the zona pellucida (a refractile ring surrounding the embryo) and the inner cell mass. (**b**–**d**) Blastocysts after 6, 7, and 8 days of culture on the MEF feeder layer. Note that the zona pellucida ruptures with time, releasing inner cell mass and trophoblast cells. (**e**) ESC lines after three passages. *Bar* 100 μm

ICM derivatives exhibiting a very dense, refractile morphology. Clusters of refractile cells were then physically isolated, dispersed, and replated onto MEF feeder layers. This process was repeated until clonal ESC lines were established (Fig. 1e). Mouse ESC lines can be propagated extensively in an undifferentiated state as long as care is exercised to maintain high levels of LIF and to limit colony size.

Early studies demonstrated that, when cultivated in suspension, ESCs form multicellular aggregates which have been termed “embryoid bodies” (EBs) [14]. Stochastic signaling between different cell types within the EBs mimics in vivo developmental induction cues, and upon further differentiation (either in suspension or adherent culture) the EBs give rise to ecto-, endo-, and mesodermal derivatives. Wobus and colleagues [15] developed a very useful technique to generate EBs with reproducible ESC content (which in turn resulted in more reproducible patters of differentiation). This entailed placing microdrops of medium seeded with a fixed number of undifferentiated ESCs on the inner surface of a tissue culture dish lid. The lid was then gently inverted so as to prevent mixing of the microdrops, and was placed on a tissue culture dish containing medium. The resulting “hanging drops” provide an ideal environment for the ESCs to coalesce and form EBs in a highly reproducible manner. Subsequent studies by Zweigerdt and colleagues demonstrated that EBs with reproducible ESC content could be generated in bulk in tissue culture dishes on rotating devices [16] or in stirred bioreactors [17].

To document cardiomyogenic differentiation using the hanging drop approach, ESCs were generated using blastocysts derived from myosin heavy chain (MHC)-enhanced green fluorescent protein (EGFP) transgenic mice. These mice carry a transgene comprising the cardiomyocyte-restricted α-MHC promoter and an EGFP reporter. The transgene targets EGFP expression in cardiomyocytes [6], and thus provides a convenient reporter to trace cardiomyogenic activity in differentiating ESC cultures, as illustrated in Fig. 2 (Fig. 2a–c shows phase-contrast images of the EBs and adherent cultures, and Fig. 2d–f shows epifluorescence images of the same field). Individual dispersed ESCs were plated in hanging drops; after several days in culture, the ESCs formed EBs which continued to grow and differentiate. No EGFP epifluorescence was apparent, consistent with the absence of cardiomyogenic differentiation at this stage (Fig. 2a). The EBs were transferred from

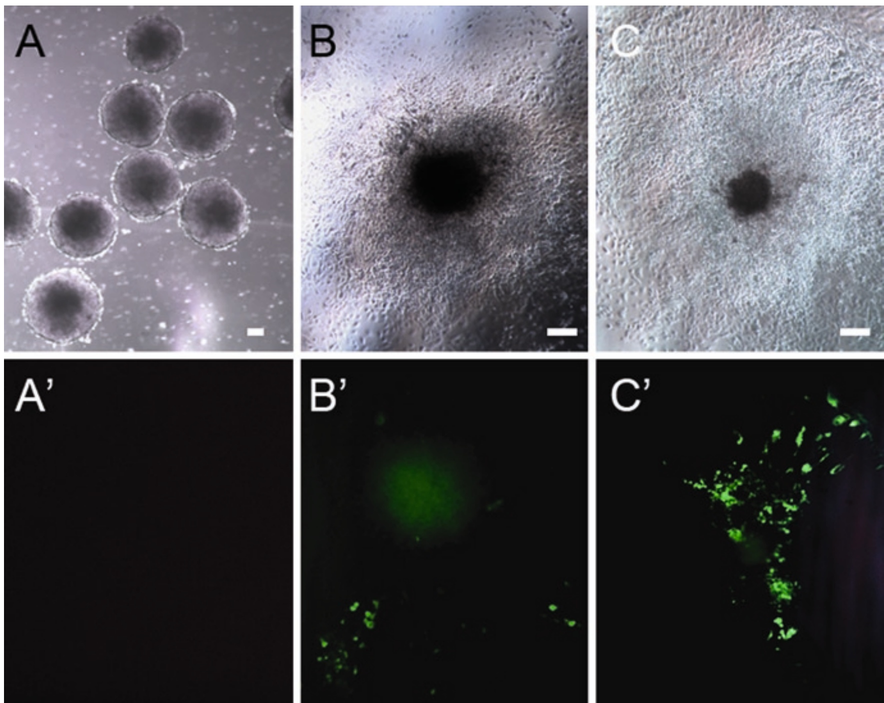

Fig. 2 Timeline of cardiomyogenic differentiation in mouse ESCs carrying a myosin heavy chain (MHC)-enhanced green fluorescent protein (EGFP) reporter transgene. (**a**, **a**′) Phase-contrast and epifluorescence images, respectively, of embryoid bodies (EBs) generated by the hanging drop procedure after 4 days of suspension culture. The absence of EGFP epifluorescence indicates that cardiomyocyte differentiation has not yet occurred. (**b**, **b**′) Phase-contrast and epifluorescence images, respectively, of an EB after 5 days of suspension culture and 2 days of adherent culture. A few scattered cells with EGFP epifluorescence indicates the initial onset of cardiomyocyte differentiation. (**c**, **c**′) Phase-contrast and epifluorescence images, respectively, of an EB after 5 days of suspension culture and 5 days of adherent culture. Most cardiomyogenic differentiation has occurred by this time. *Bar* 100 μm

suspension culture to adherent culture after 5 days of differentiation. Expression of the cardiomyocyte-restricted reporter transgene was first detected after 7 days of differentiation (i.e. 5 days of suspension culture and 2 days of adherent culture; Fig. 2b); however, contractile activity was not apparent until 3 days of differentiation. This reflected the time differential between the induction of myofiber structural protein gene expression (and, consequently, activation of the reporter transgene expression) and the assembly of functional myofibers and the requisite intracellular machinery for the generation and propagation of action potentials and calcium transients. It was also apparent that cardiomyocytes constituted only a small fraction of the total cell population during spontaneous ESC differentiation (Fig. 2c). With the development of human ESC lines [18], in vitro cardiomyocyte differentiation was rapidly observed and characterized [19].

3 Inducing ESCs to Produce Cardiomyocytes

Numerous approaches have been developed to generate enriched cultures of ESC-derived cardiomyocytes (Table 1). Perhaps the most obvious approach entails the identification of growth factors which enhance cardiomyocyte differentiation. Indeed,

Table 1 Approaches to enhance cardiomyocyte yield from embryonic stem cells (*ESCs*)

Approach	Comments	References
Growth factors	Retinoic acid enhanced cardiomyocyte differentiation in mouse ESCs	[20]
	Exogenous glucose, amino acids, vitamins, and selenium enhanced cardiomyocyte differentiation in mouse ESCs	[21]
	LIF enhances and inhibits cardiomyocyte commitment and proliferation in mouse ESCs in a developmental stage-dependent manner	[22]
	Reactive oxygen species enhanced cardiomyocyte differentiation in mouse ESCs	[23, 24, 25, 26]
	Endoderm enhanced cardiomyocyte differentiation in mouse ESCs	[27, 28]
	A TGF/BMP paracrine pathway enhanced cardiomyocyte differentiation in mouse ESCs	[29]
	Activation of the MEK/ERK pathway enhanced cardiomyocyte differentiation in mouse ESCs	[30]
	Verapamil and cyclosporine enhanced cardiomyocyte differentiation in mouse ESCs	[31]
	5-Aza-2′-deoxycytidine enhanced cardiomyocyte differentiation in human ESCs	[32]
	Endoderm cell lines enhanced cardiomyocyte differentiation in human ESCs	[33, 34]
	Ascorbic acid enhanced cardiomyocyte differentiation in human ESCs	[35]
	Directed differentiation with activin A and BMP4 in monolayers of human ESC	[36]

(continued)

Table 1 (continued)

Approach	Comments	References
Genetic engineering	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from mouse ESCs	[37, 17, 38]
	Highly purified cardiomyocyte cultures generated by FACS of mouse ESCs expressing a lineage-restricted EGFP reporter	[39]
	Targeted expression of α-1,3-fucosyltransferase enhanced cardiomyocyte differentiation in mouse ESCs	[40]
	Coexpression of EA1, dominant negative p53, and dominant negative CUL7 enhanced cell cycle in mouse ESC-derived cardiomyocytes	[41]
	Expression of SV40 T antigen enhanced cell cycle in mouse ESC-derived cardiomyocytes	[42]
	Antagonization of Wnt/β-catenin enhanced cardiomyocyte differentiation in mouse ESCs	[43]
	Lineage-restricted drug resistance gene resulted in highly purified cardiomyocyte cultures from human ESCs	[44, 45]
Miscellaneous	A single 90-s electrical pulse applied to day 4 EBs increased cardiomyocyte differentiation in mouse ESCs	[46]
	Application of mechanical loading enhanced cardiomyocyte differentiation in mouse ESCs	[47, 48]
	FACS for transient Flk-1 isolated cardiomyogenic progenitors from mouse ESCs	[49]
	Cardiomyocyte enrichment using density centrifugation and cultures of cell aggregates in human ESCs	[50]
	Activin A, BMP4, bFGF, VEGF, and DKK1 treatment, followed by KDR^{+}/c-kit^{-} FACS, identified cardiovascular progenitor cells in human ESCs	[51]

LIF leukemia inhibitory factor, *TGF* transforming growth factor, *BMP* bone morphogenetic protein, *MEK* mitogen-activated protein kinase, *ERK* extracellular-signal-regulated kinase, *FACS* fluorescence-activated cell sorting, *EGFP* enhanced green fluorescent protein, *EBs* embryoid bodies, *bFGF* basic fibroblast growth factor, *VEGF* vascular endothelial growth factor

many studies have reported modest to moderate increases in cardiomyocyte yield in differentiating ESC cultures. Perhaps the most impressive work was from Murry and colleagues [36], who demonstrated that treatment of monolayers of human ESCs with a combination of activin A and bone morphogenetic protein 4, followed by gradient centrifugation, resulted in an average final cardiomyocyte content of 82%. The degree to which this approach can be scaled up for the production of large numbers of donor cardiomyocytes (and, in particular, if directed differentiation is effective in suspension as opposed to in monolayer cultures) remains to be determined.

One of the earliest approaches to enhance cardiomyocyte yield entailed introduction of a lineage-restricted selectable marker. In one example, the cardiomyocyte-restricted MHC promoter was used to target expression of aminoglycoside phosphotransferase (MHC-neo transgene). After spontaneous differentiation, cultures were enriched for cardiomyocytes by simple treatment with G418 [37]. Cultures with more than 99% cardiomyocyte content can routinely be obtained.

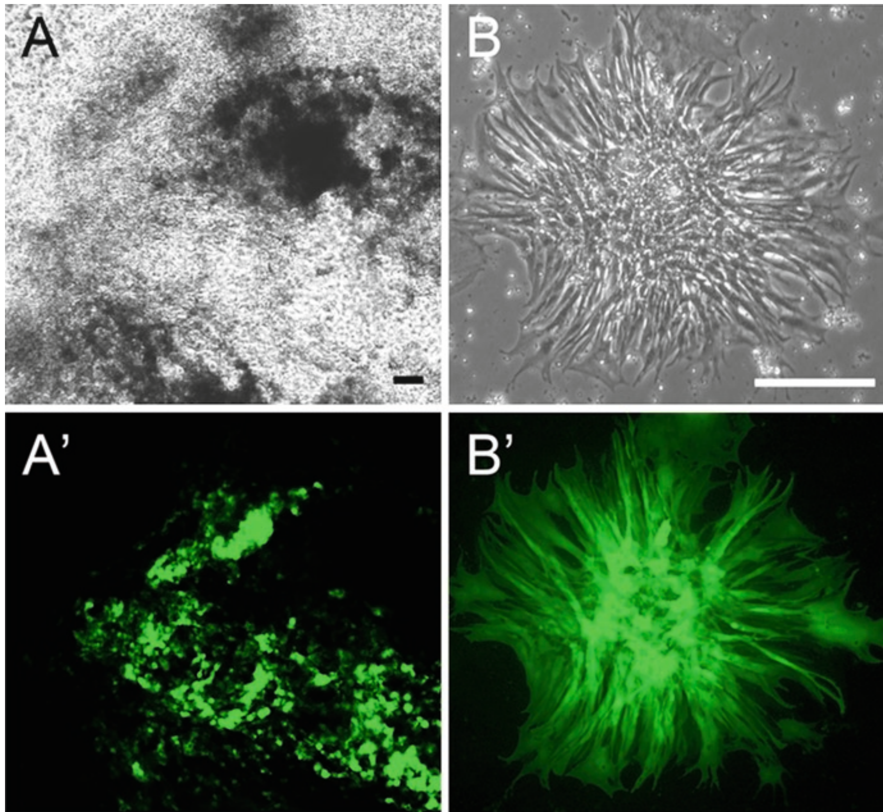

Fig. 3 Adherent culture of EBs generated from ESCs carrying the MHC-EGFP and MHC-neo transgenes after a total of 23 days of differentiation in the absence (**a**, **a′**) or presence (**b**, **b′**) of 11 days of G418 selection. (**a**, **b**) Phase-contrast images; (**a′**, **b′**) epifluorescence images. Note the marked cardiomyocyte enrichment in the G418-treated sample (**b**, **b′**). *Bar* 100 μm

To illustrate this approach, ESCs carrying the MHC-EGFP reporter transgene described earlier as well as the MHC-neo transgene were generated. The ESCs were allowed to differentiate spontaneously, and were then cultured in the absence or presence of G418. In the absence of G418, cardiomyocyte constituted only a small portion of the cultures, in agreement with the data presented above (Fig. 3a). In contrast, G418 treatment effectively eliminated the noncardiomyocytes, resulting in highly enriched cultures (Fig. 3b). This selection approach was readily scalable to bioreactors [52], and could yield more than 10^9 cardiomyocytes per 2-L reaction vessel in preparations seeded with dispersed ESC cultures [17]. Similarly, lineage-restricted expression of an EGFP reporter has been employed in conjunction with fluorescence-activated cell sorting (FACS) to generate highly enriched cardiomyocyte cultures [39]. Importantly, both the selection-based and the FACS-based approaches were readily used for the generation of human ESC-derived cardiomyocytes [44, 45, 53].

4 Intracardiac Transplantation of ESCs or ESC-derived Cardiomyocytes

Given that the ability to form teratomas in syngeneic or immune-compromised hosts is a major criterion for ESC identification, one would a priori expect that delivery of undifferentiated ESCs into the heart would also give rise to teratomas. Indeed, teratomas were reported following ESC injection into normal [37] or infarcted [54] myocardium. Nonetheless a number of studies have delivered undifferentiated ESCs and failed to report teratoma formation (Table 2). This could reflect compromised differentiation capacity in the ESCs being tested, or alternatively the insufficient

Table 2 Intracardiac transplantation of ESCs or ESC-derived cardiomyocytes

Donor/host species	Comments	References
Mouse/mouse	Genetically selected cardiomyocytes engrafted in normal myocardium	[37]
Mouse/mouse	In vivo cardiomyocyte differentiation of ESCs required TGF and BMP2	[29]
Mouse/mouse	Intravenous ESC delivery improved cardiac function during viral myocarditis	[55]
Mouse/mouse	Cardiomyocyte-enriched cells plus VEGF enhanced postinfarct function	[56]
Mouse/mouse	Growth factors enhanced ESC engraftment in infarcted hearts	[57, 58]
Mouse/mouse	ESC-seeded synthetic scaffolds improved postinfarct function	[59]
Mouse/mouse	Allogenic ESCs evoked an immune response following heart transplant	[60, 61]
Mouse/mouse	Matrigel enhanced ESC seeding in infarcted hearts	[62]
Mouse/mouse	Genetically selected cardiomyocytes improved postinfarct function	[63]
Mouse/mouse	ESCs improved function in infarcted hearts	[64, 65]
Mouse/mouse	TNF enhanced cardiomyocyte differentiation and lessened teratoma potential	[66]
Mouse/mouse	Cardiomyocytes improved postinfarct function via paracrine mechanisms	[67]
Mouse/mouse	In vivo MR imaging of transplanted cardiomyocytes in infarcted hearts	[68]
Mouse/mouse	Allogenic ESCs formed teratomas when transplanted into infarcts	[54]
Mouse/mouse	Cardiomyocyte engraftment blocked adverse post-MI remodeling	[69]
Mouse/rat	ESC transplantation improved function following myocardial infarction	[70]
Mouse/rat	Density-gradient-enriched cardiomyocytes improve postinfarct function	[71]
Mouse/rat	Differentiated ES cultures survived in immune-suppressed normal heart	[72]
Mouse/rat	ESC-seeded synthetic scaffolds improved postinfarct function	[73]
Mouse/rat	ESCs improved cardiac function in aging hearts	[74]

(continued)

Table 2 (continued)

Donor/host species	Comments	References
Mouse/rat	GCSF enhanced cardiomyocyte engraftment in infarcted hearts	[75]
Mouse/rat	Intravenously delivered ESCs homed to infarcted myocardium	[76]
Mouse/rat	ESCs formed teratomas when transplanted into infarcted hearts	[77]
Mouse/rat	Chitosan hydrogel enhanced ESC seeding and postinfarct function	[78]
Mouse/sheep	Enriched cardiomyocytes improved postinfarct function	[79, 80]
Human/mouse	Allopurinol/uricase/ibuprofen increased postinfarct cardiomyocyte survival	[81]
Human/mouse	Cardiomyocyte impact on adverse post-MI remodeling is transient	[82, 83]
Human/mouse	KDR progenitors for 3 lineages in vivo improved post-MI function	[51]
Human/rat	In vivo MR imaging of transplanted ESCs	[84]
Human/rat	Microdissected cardiomyocytes improved function in infarcted hearts	[85]
Human/rat	Cardiomyocytes engrafted athymic hearts after ischemia/reperfusion	[86]
Human/rat	Cardiomyocyte engraftment blocked adverse post-MI remodeling	[36, 87]
Human/rat	Cardiomyocytes from BMP2 treatment engrafted infarcted hearts	[88]
Human/rat	ESCs do not form teratomas when engrafted into infarcted hearts	[89]
Human/rat	Physically enriched cardiomyocytes engrafted normal athymic rat heart	[90]
Human/guinea pig	Mixed SAN and cardiomyocyte transplants provided pacemaker activity	[91]
Human/pig	Mixed SAN and cardiomyocyte transplants provided pacemaker activity	[92]

MR magnetic resonance, *MI* myocardial infarction, *GCSF* granulocyte colony stimulating factor, *SAN* sinoatrial node

histologic analyses of the engrafted hearts. It has also been suggested that the milieu of the normal or infarcted heart may be sufficient to drive lineage-restricted differentiation of progenitor cells. Nonetheless, the bulk of available data suggest that this is not the case for transplanted ESCs.

Since the initial observation that ESC-derived cardiomyocytes could successfully engraft recipient hearts [37], a large number of experiments have been performed to examine the impact of injecting ESCs or ESC-derived cardiomyocytes into normal or injured hearts (Table 2). Of note, many of these studies indicated that animals receiving ESCs or ESC-derived cardiomyocytes following experimental injury exhibited superior cardiac function as compared with those which did not receive cells. In almost all instances, cardiac function was not improved in the engrafted hearts. Rather, the process of engraftment appeared to attenuate the

deleterious postinjury ventricular remodeling and concomitant decreases in cardiac function. Similar results have been reported with a number of donor cell types. In particular, work by Dzau and colleagues using mesenchymal stem cells strongly suggests that the benefit of cell transplantation in their studies likely reflects the secretion of proangiogenic and antiapoptotoic factors from donor cells [93–97]. Such a mechanism would readily explain how engraftment of a relatively small number of ESC-derived cells could impact function in injured hearts. Unfortunately, studies from the Mummery laboratory suggest that this improvement in postinjury remodeling may be transient in nature [82, 83].

Although there are direct data at the cellular level supporting the functional engraftment of fetal cardiomyocytes in recipient hearts [6], the current data available with ESC-derived cardiomyocytes are more circumstantial in nature. Gepstein and colleagues [92] demonstrated that ectopic pacemaker activity originated at the site of engraftment of human ESC-derived cells following atrioventricular node blockade in swine, consistent with the notion that the donor cells were functionally integrated. Similar results were obtained with guinea pig [91]. Despite these promising observations, it would be prudent to directly assess at the cellular level the ability of ESC-derived cardiomyocytes to functionally integrate following engraftment, as formation of a functional syncytium is an absolute requirement for regenerative repair. This is particularly important for studies wherein human ESC-derived cells promoted better function when engrafted into rodent hearts, as it is not at all clear that human cells can sustain rapid rates for extended periods of time. Indeed, rapid pacing is often used to induce heart failure in larger experimental animals [98].

5 Future Challenges

The discussions herein suggest that donor cardiomyocytes likely functionally integrate following transplantation into recipient hearts, that methods are available to eliminate the risk of teratoma formation following transplantation of ESC-derived cardiomyocytes, and that approaches to the large scale generation of ESC-derived cells are in hand. Perhaps the greatest challenge facing the use of ESC-derived cardiomyocytes for myocardial regeneration is the limitation in graft size using current approaches. Arguably the best study to date, by Murry and colleagues [36], utilized a combination of materials to enhance survival of donor cells after engraftment. This intervention permitted on average 4% replacement of an infarct which constituted 10% of the left ventricle (which correlates to only 0.4% of the ventricular mass). Thus, we have a long way to go before we will be able to replace transmural myocardial defects.

A number of approaches can be explored to attempt to enhance graft size. For example, many cardiomyocyte prosurvival pathways have been identified [99]. Targeting these pathways in donor ESC-derived cardiomyocytes, either by genetic intervention prior to cardiomyocgenic differentiation or via pharmacologic interventions, may facilitate enhancement of donor cell survival, as exemplified by the work