Stem Cells International

Stem Cells in Heart Failure

Guest Editors: Gabriela Kania, Kenneth R. Boheler, Ulf Landmesser, and Wojciech Wojakowski

Stem Cells in Heart Failure

Stem Cells International

Stem Cells in Heart Failure

Guest Editors: Gabriela Kania, Kenneth R. Boheler, Ulf Landmesser, and Wojciech Wojakowski

Editorial Board

Contents

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 193918, 3 pages
doi:10.4061/2011/193918

Editorial

Stem Cells in Heart Failure

Gabriela Kania,[1] Kenneth R. Boheler,[2] Ulf Landmesser,[3] and Wojciech Wojakowski[4]

[1] *Cardioimmunology, Cardiovascular Research, Institute of Physiology, University of Zürich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[2] *NIH/NIA/GRC/LCS, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA*
[3] *Department of Cardiology, University Hospital, Rämistrasse 100, 8091 Zurich, Switzerland*
[4] *Division of Cardiology, Medical University of Silesia, 40-583 Katowice, Poland*

Correspondence should be addressed to Gabriela Kania, gabriela.kania@uzh.ch

Received 17 October 2011; Accepted 17 October 2011

Heart failure is a leading cause of morbidity and mortality in the developed world. Cardiac injury with a significant loss of cardiomyocytes usually leads to a common final pathway of cardiac pathological remodelling that includes fibrosis, and the development and progression of heart failure. The treatment of chronic heart failure generally relies on strategies designed to target and limit cardiac ischemia, additional tissue damage, pathological cardiac remodelling, or hemodynamic impairment and cardiac dyssynchrony. Moreover, understanding of the pathways that chauffeur pathological remodelling as opposed to myocardial repair and regeneration in the failing heart still remains challenging. The identification of stem cells has, however, opened new prospects for the development of cell-based regenerative medicine [1]. Indeed, the heart may contain a pool of resident or circulation-derived stem cells, but, following injury, these endogenous cells are not sufficiently regenerative to fully repair-damaged myocardium [2]. To overcome this insufficiency, treatments have been proposed with exogenous stem cells or their progeny, which may reduce pathological processes in the damaged heart and improve cardiac tissue regeneration in mice and humans. Despite concerted efforts to treat damaged myocardium through cell transplantation, it remains a matter of debate whether the delivery of stem cells or stem cell progeny contributes principally to new cardiac tissue formation, to the activation of endogenous repair mechanisms, or to the modulation of inflammatory processes [3]. More importantly, stem cell-based therapies have resulted in improved cardiac function, and the development of this line of research represents a new frontier in modern cardiovascular research. In this special issue of Stem Cells International, we have assembled a series of original manuscripts and review articles dealing with this research frontier. The articles describe a variety of novel strategies to obtain cells for cardiac repair or regeneration and discuss current efforts, available tools, and new methods for stem cell-based therapies.

Pluripotent stem cells represent one potential source for stem cell-based therapy in the failing heart [4]; however, this kind of therapy has some serious limitations, ranging from ethical issues in humans to the degree of heterogeneity found in cultures of "purified" embryonic stem cell-derived cardiomyocytes (ESC-CMs). Following injection into heart, previous studies demonstrated that ESC-CMs form grafts that may mediate long-term recovery of global and regional myocardial contractile function following infarction. In this issue, K. R. Boheler et al. [5] specifically addressed the question of developmental state and showed that immature hypoxia-resistant ESC-CMs can be isolated in mass *in vitro*. The authors suggested that the use of immature proliferating CMs that do not seem to form tumors *in vivo* may serve as a source of innately hypoxia-resistant CMs useful in the treatment of ischemic cardiac disorders. Such an approach might become a viable strategy for treating human cardiac disease states and injuries in the future; however, several obstacles still need to be resolved, including potential immunological responses, safety, and durable improvement of cardiac function in large animal models.

In a separate paper, S. Schmitteckert et al. [6] propose the transcription factor Lbx1 as new marker of differentiating ESC-CMs. Lbx1 plays a role in the migration of muscle progenitor cells in limb buds and determines neuronal differentiation processes [7, 8]. Since Lbx1 was largely expressed in differentiating ESC-CMs, Lbx1 might represent a novel tool for the identification of proper cell source to induce the reparative processes in the injured heart. Moreover, this finding may provide a model system of Lbx1 target genes and signaling pathways involved in early heart failure caused by Lbx1 inactivation.

An entirely new vision of stem cell-based therapy was presented by S. Liebau et al. [9]. In this paper, the authors focused on calcium-activated potassium channels (SKCas) as important inducers of stem cell differentiation. SKCas are involved in cardiac pacemaker-cell development from ESCs and morphological shaping of neural stem cells [10, 11]. SKCas are also important modulators of the cytoskeleton rearrangement [12]. Previously, these authors showed that increased SKCas channel activity resulted in a strong and fast differentiation of pluripotent cells followed by a cell-fate determination into the cardiac lineage, mainly with a phenotype of cardiac pacemaker-like cells derived from ESC and iPS cells [13]. Here, this group reported the successful generation and characterization of a murine ESC line overexpressing the subtype 4 of SKCas channels in a doxycycline-dependent manner. Overexpression of SKCas4 was increased in cardiac and pacemaker-like cells suggesting SKCas4 as a unique tool to characterize the differentiation of pluripotent cells into cardiac phenotypes. SKCas channel-mediated stem cell differentiation might also be applicable to the human system.

Although substantial efforts have been made to develop therapeutic strategies with stem cells to regenerate injured heart [3], there is increasing evidence that stem cells modulate inflammatory processes in a paracrine fashion more so than through direct cardiac tissue regeneration [14]. Recent findings have also suggested that the poor effectiveness of stem cell-based therapies in heart diseases is a result of nonphysiological microenvironment in affected cardiac tissue [14, 15]. In particular, inflamed myocardium seems to inhibit the cardio-regenerative capacity of transplanted stem cells, while promoting profibrotic processes. A growing body of evidences suggests that the specific signaling milieu of the affected heart is a key determinant of the fate and function of stem cells in the myocardium [16]. Coupling modulation of the myocardial microenvironment with patient-specific stem cells must, therefore, be considered before successful stem cell-based therapies of heart disorders will be achieved. Accordingly, our special issue offers a comprehensive comparison of different sources of stem cells for heart regeneration in basic science and in clinical trials. Moreover, there is a discussion of the potential mechanisms involved in reparative processes [17, 18]. Finally, A. Kleger et al. [19] provided a comprehensive review on the differential and developmental impact of lysophospholipids on cardiovascular development, which represents a novel approach in the field and may have relevance for the niche environment.

Taken together, the compilation of articles in this special issue of Stem Cells International, discusses the current state of stem cell-based therapies. The authors address both experimental and clinical aspects of stem cell research aimed at improving the reparative processes in the failing heart. The three research articles specifically provide novel information designed either to select for specific types of stem cells or to induce the differentiation of pluripotent cells into the phenotype of cardiac lineages. The reviews also offer a broad-based view of current efforts designed to understand the response of stem cells in a "niche" environment or in response to specific molecules. We hope that this issue will be helpful and interesting for basic researchers as well as for clinicians interested in or performing experiments designed to address relevant cardiac issues in regenerative medicine.

Gabriela Kania
Kenneth R. Boheler
Ulf Landmesser
Wojciech Wojakowski

References

[1] P. Anversa, J. Kajstura, and A. Leri, "If I can stop one heart from breaking," *Circulation*, vol. 115, no. 7, pp. 829–832, 2007.

[2] C. E. Murry, M. H. Soonpaa, H. Reinecke et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[3] M. A. Laflamme and C. E. Murry, "Regenerating the heart," *Nature Biotechnology*, vol. 23, no. 7, pp. 845–856, 2005.

[4] K. R. Boheler, "Pluripotency of human embryonic and induced pluripotent stem cells for cardiac and vascular regeneration," *Thrombosis and Haemostasis*, vol. 104, no. 1, pp. 23–29, 2010.

[5] K. R. Boheler, R. N. Joodi, H. Qiao et al., "Embryonic stem cell-derived cardiomyocyte heterogeneity and the isolation of immature and committed cells for cardiac remodeling and regeneration," *Stem Cells International*, vol. 2011, Article ID 214203, 10 pages, 2011.

[6] S. Schmitteckert, C. Ziegler, L. Kartes, and A. Rolletschek, "Transcription factor lbx1 expression in mouse embryonic stem cell-derived phenotypes," *Stem Cells International*, vol. 2011, Article ID 130970, 7 pages, 2011.

[7] H. Brohmann, K. Jagla, and C. Birchmeier, "The role of Lbx1 in migration of muscle precursor cells," *Development*, vol. 127, no. 2, pp. 437–445, 2000.

[8] S. Watanabe, S. Kondo, M. Hayasaka, and K. Hanaoka, "Functional analysis of homeodomain-containing transcription factor Lbx1 in satellite cells of mouse skeletal muscle," *Journal of Cell Science*, vol. 120, no. 23, pp. 4178–4187, 2007.

[9] S. Liebau, M. Tischendorf, D. Ansorge et al., "An inducible expression system of the calcium-activated potassium channel 4 to study the differential impact on embryonic stem cells," *Stem Cells International*, vol. 2011, Article ID 456815, 12 pages, 2011.

[10] S. Liebau, B. Vaida, C. Proepper et al., "Formation of cellular projections in neural progenitor cells depends on SK3 channel activity," *Journal of Neurochemistry*, vol. 101, no. 5, pp. 1338–1350, 2007.

[11] S. Liebau, J. Steinestel, L. Linta et al., "An SK3 channel/nWASP/Abi-1 complex is involved in early neurogenesis," *PLoS One*, vol. 6, no. 3, Article ID e18148, 2011.

[12] S. J. Kattman, E. D. Adler, and G. M. Keller, "Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development," *Trends in Cardiovascular Medicine*, vol. 17, no. 7, pp. 240–246, 2007.

[13] A. Kleger, T. Seufferlein, D. Malan et al., "Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells," *Circulation*, vol. 122, no. 18, pp. 1823–1836, 2010.

[14] J. Endo, M. Sano, J. Fujita et al., "Bone marrow-derived cells are involved in the pathogenesis of cardiac hypertrophy in response to pressure overload," *Circulation*, vol. 116, no. 10, pp. 1176–1184, 2007.

[15] M. Bujak and N. G. Frangogiannis, "The role of TGF-β signaling in myocardial infarction and cardiac remodeling," *Cardiovascular Research*, vol. 74, no. 2, pp. 184–195, 2007.

[16] G. Kania, P. Blyszczuk, and U. Eriksson, "Mechanisms of cardiac fibrosis in inflammatory heart disease," *Trends in Cardiovascular Medicine*, vol. 19, no. 8, pp. 247–252, 2009.

[17] R. Mingliang, Z. Bo, and W. Zhengguo, "Stem cells for cardiac repair: status, mechanisms, and new strategies," *Stem Cells International*, vol. 2011, Article ID 310928, 8 pages, 2011.

[18] O. M. Villet, A. Siltanen, T. Pätilä et al., "Advances in cell transplantation therapy for diseased myocardium," *Stem Cells International*, vol. 2011, Article ID 679171, 8 pages, 2011.

[19] A. Kleger, S. Liebau, Q. Lin, G. von Wichert, and T. Seufferlein, "The impact of bioactive lipids on cardiovascular development," *Stem Cells International*, vol. 2011, Article ID 916180, 13 pages, 2011.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 214203, 10 pages
doi:10.4061/2011/214203

Research Article

Embryonic Stem Cell-Derived Cardiomyocyte Heterogeneity and the Isolation of Immature and Committed Cells for Cardiac Remodeling and Regeneration

Kenneth R. Boheler,[1] Robert N. Joodi,[2,3] Hui Qiao,[3] Ondrej Juhasz,[1] Amanda L. Urick,[4,5] Sandra L. Chuppa,[4,5] Rebekah L. Gundry,[4,5] Robert P. Wersto,[6] and Rong Zhou[3,7,8]

[1] *Molecular Cardiology and Stem Cell Unit, Laboratory of Cardiovascular Sciences, National Institute of Aging, NIH, Baltimore, MD 21224, USA*
[2] *UMDNJ-Robert Wood Johnson Medical School, One Cooper Plaza, Camden, NJ 08103, USA*
[3] *Laboratories of Molecular Imaging, Department of Radiology, University of Pennsylvania, Philadelphia, PA 19104, USA*
[4] *Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA*
[5] *Biotechnology and Bioengineering Center, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA*
[6] *Resource Research Branch, National Institute on Aging, NIH, Baltimore, MD 21224, USA*
[7] *Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104, USA*
[8] *Cardiovascular Institute, University of Pennsylvania, Philadelphia, PA 19104, USA*

Correspondence should be addressed to Kenneth R. Boheler, bohelerk@grc.nia.nih.gov

Received 15 May 2011; Accepted 14 June 2011

Academic Editor: Gabriela Kania

Pluripotent stem cells represent one promising source for cell replacement therapy in heart, but differentiating embryonic stem cell-derived cardiomyocytes (ESC-CMs) are highly heterogeneous and show a variety of maturation states. In this study, we employed an ESC clonal line that contains a cardiac-restricted *ncx1* promoter-driven puromycin resistance cassette together with a mass culture system to isolate ESC-CMs that display traits characteristic of very immature CMs. The cells display properties of proliferation, CM-restricted markers, reduced mitochondrial mass, and hypoxia-resistance. Following transplantation into rodent hearts, bioluminescence imaging revealed that immature cells, but not more mature CMs, survived for at least one month following injection. These data and comparisons with more mature cells lead us to conclude that immature hypoxia resistant ESC-CMs can be isolated in mass in vitro and, following injection into heart, form grafts that may mediate long-term recovery of global and regional myocardial contractile function following infarction.

1. Introduction

The regenerative capacity of adult mammalian heart is insufficient to fully restore function following myocardial injury or heart failure. In cases of severe cardiac insufficiency, the treatment of choice is cardiac transplantation, but this approach is hampered by both a severe shortage of donor organs and the potential for organ rejection [1, 2]. Cell replacement therapy represents one promising alternative, but it is limited by the availability of transplantable human cardiomyocytes (CMs). To overcome this limitation, cardiovascular researchers have undertaken numerous studies over the past decade to identify cells in animal and human systems with cardiomyogenic potential, including cardiac derivatives isolated at various times of development and noncardiac progenitors derived from bone-marrow, fat, or skeletal muscle [3]. Subsequent preclinical studies have demonstrated that a wide variety of cardiomyogenic stem/progenitor cells can improve the cardiac function after transplantation, but it remains largely unclear whether improved cardiac function occurs through integration and coupling of new CMs with endogenous cells (reviewed by [4, 5]) or conversely through secondary events related to angiogenesis, sparing of endogenous CMs, cell fusion, or anti-inflammatory responses [6–10].

While numerous investigators have described putative stem/progenitor cells with cardiomyogenic potential, myocardial regeneration confirmed through direct functional coupling is limited to a few cell types [11, 12]. Among these, pluripotent stem cells (PSCs), including both embryonic stem (ES) and induced pluripotent stem (iPS) cells, represent the best viable cell source suitable for transplantation therapy [13]. PSCs can be passaged indefinitely and can produce all body cells; consequently, these cells are capable of treating a wide range of debilitating disease states whose underlying pathology involves cell degeneration, death, or acute injury. There are, however, significant challenges to be overcome before these approaches can be applied in the clinic. Constraints to the use of human-derived ESCs for cell therapy include ethical barriers and potential immunogenicity of ESC-progeny [14, 15]. These concerns may be overcome by iPS cells, which are generated in vitro via transcription factor-mediated reprogramming [16–18], but iPS cells also suffer from interline heterogeneity and incomplete epigenetic remodeling [19–21]. Although generation of sufficient numbers of cells for therapeutic intervention is feasible, isolation of a desired cell type in pure form, a systematic analysis of cell delivery systems, and determination of which cell type may be necessary to correct a specific pathology remain unanswered questions [14].

To deal with cell purity issues, several groups reported isolation of ES cell-derived CMs (ESC-CMs) with cardiac-restricted gene promoters (*nkx2.5*, cardiac *α-mhc*, *mlc2v*, cardiac *α*-actin, *ncx1*) from mouse, however, each has limitations. Some like *nkx2.5* are not restricted to heart, whereas others like *α-mhc* are only weakly expressed in the primary myocardium before becoming restricted to the atrial regions throughout the remainder of embryonic and early fetal development [22, 23]. In adult rodents, the *mlc2v* promoter is restricted to ventricle, but early in development, its expression is present in anterior (atrial and atrioventricular) portions of the heart tube, and at later stages, in the caval myocardium [23, 24]. The cardiac *α*-actin gene promoter is expressed both in cardiac and skeletal muscle during embryogenesis [23]. Moreover, the distal upstream region of the *ncx1* promoter is cardiac-restricted during embryonic and fetal development, but it does not distinguish among chamber-specific types of heart cells [25]. Functionally, ESC-CMs are most typical of embryonic or fetal stages of development [23, 26], and no one has successfully generated ESC-CMs in vitro with characteristics of adult-derived ventricular CMs. Moreover, none of these promoters have been employed in conjunction with stage-specific analyses to determine which cell type and maturation state may be necessary to correct a specific pathology. The goal of such a study would be to relate stage-specific CMs with their suitability to regenerate damaged heart muscle.

In the current study, we have begun to test the hypothesis that very immature but committed CMs may be suitable for cardiac regenerative medicine. We have taken advantage of murine ESCs containing a cardiac restricted *ncx1* promoter-driven puromycin-resistance gene that eliminates noncardiac cells by antibiotic treatment and permits the isolation of highly enriched and early-staged ESC-CMs [27, 28]. Developmentally, this promoter is active in proliferating myocardium, and we previously showed that it could be employed to isolate proliferative cells using a monolayer culture and that late-stage nonproliferating cells could be generated for therapeutic interventions. In this pilot study designed to determine if early dividing CMs could be isolated from a mass culture system in therapeutically relevant quantities, we present our initial in vitro findings and data following injection of these cells into the mouse heart. These data indicate that early-staged ESC-CMs can be isolated in therapeutic quantities, are hypoxic-resistant and proliferative competent and potentially suitable to treat myocardial infarctions.

2. Materials and Methods

2.1. Production of Highly Enriched ESC-CMs. Murine R1 ESCs (clone syNP4) that stably express a puromycin resistant gene cassette under the cardiac-specific promoter of sodium calcium exchanger (*ncx1*) were employed in this study. Differentiation of ESCs to CMs was performed as previously described, except where differences were noted in the text [27, 28]. To track these cells following injection into mice hearts, syNP4 cells were electroporated with an expression cassette containing a mouse EF1*α* promoter-luciferase construct and a hygromycin resistance cassette. Clonal lines containing this construct with stable expression of luciferase were isolated using standard protocols. Differentiation of ESC-CMs and their purity and maturation were confirmed using RT-PCR with primers specific for *α*-SMA, cardiac calsequestrin, and atrial natriuretic factor (ANF) [27, 28] and by immunological techniques using antibodies against *α*-smooth muscle actin (*α*-SMA, Biomeda, Foster City, CA), sarcomeric *α*-actinin (Sigma, St. Louis, MO), cTnT (Millipore, Billerica, MA), $p21^{CIP}$ (ab7960), $p27^{KIP1}$ (ab7961), and phospho-Ser10-Histone H3 (ab5176) (Abcam, Cambridge, MA). CMs in the S phase of the cell cycle were identified by 5-Bromodeoxyuridine (BrdU) (Sigma) incorporation into DNA as described previously [27].

2.2. Flow Cytometry. DNA content was determined from ice cold 70% ethanol fixed cells using a BD-Canto II after staining with propidium iodide (PI, Sigma). Cell cycle compartments were deconvoluted from single-parameter DNA histograms of 10,000 to 50,000 cells, and DNA cell cycle analysis was performed as previously described [27, 29]. Cell viability based on mitochondrial membrane potentials and mitochondrial content were determined using mitotracker dyes according to the manufacturer's instructions. Live versus dead (apoptotic) cells were analyzed on a flow cytometer using 488 nm excitation after labeling the cells using a JC-1 mitochondrial membrane potential assay kit (Invitrogen, Carlsbad, CA). In this assay, the cationic dye JC-1 spontaneously forms complexes known as J-aggregates that have intense red fluorescence. In apoptotic or unhealthy cells with a low membrane potential, JC-1 remains in the monomeric form and shows only green fluorescence. Mitochondria content was also estimated by staining cells with MitoTracker Green FM (MTG), a mitochondrial-selective

green fluorescent dye (Invitrogen, Carlsbad, CA). This probe preferentially accumulates in mitochondria regardless of mitochondrial membrane potential and is useful for determining mitochondrial mass. Cells were incubated in 200 micromolar concentrations of the MTG probe for 30 minutes followed by flow cytometry, according to the manufacturer's instructions.

2.3. Hypoxia and Nutrient Deprivation In Vitro. To induce hypoxia, Day 10 and Day 18 ESC-CM cultures in either glucose- and serum-free Dulbecco's modified Eagle's medium (DMEM) or DMEM supplemented with 5.5 mM glucose and 10% heat-inactivated fetal bovine serum for 24 or 48 hours were transferred into a humidified hypoxic incubator maintained at 37°C with 5% CO_2, 0.5% O_2, and the balance in N_2 gas. Following incubation, culture media was immediately collected to quantify total Lactate Dehydrogenase (LDH, Sigma), an indicator of cell death and injury. Cells were also harvested to measure viability via trypan blue exclusion by two observers blind to experimental conditions.

2.4. In Vivo Studies. Surgical Procedures: all animal protocols were approved by the local Institutional Animal Care and Use Committee of the University of Pennsylvania. Surgical procedures were performed on 8–10 athymic nude mice (NCI) as we have described previously [30–32]. Briefly, animals were anesthetized using an induction dose of 3% isoflurane. Mice were intubated, ventilated, and anesthesia maintained with inhaled isoflurane (1% to 2%). An aseptic left thoracotomy was performed and the pericardium was opened. Mice then received a single mid-anterior intramyocardial injection of 1-2 $\times$ 10^6 ESC-CMs in 50 μL of fibrin glue. The developmental stage of the ESC-CMs is indicated in the text.

In Vivo Bioluminescent Imaging (BLI): BLI was performed on days 1, 3, 7, 14, 21, and 30 on IVIS Lumina (Caliper LifeSciences, Hopkinton MA) as we have shown previously [33, 34]. Mice were maintained under isoflurane (1% mixed with oxygen) via a nose cone. Luciferin (250 mg/kg) was injected i.p. and continuous BLI was performed for 15 min after injection. BLI signal was standardized for exposure time and quantified in units of maximum photons per second per square centimeter per steradian (p/s/cm^2/sr). The area with the greatest signal intensity was used for region of interest (ROI) analysis at each time point.

2.5. Statistical Analysis. Data are presented as mean ± standard deviation. Student's t-test was used to compare two groups, and significance was considered for P values of < 0.05.

3. Results

To test the ability of committed but proliferating ESC-CM to form grafts in the heart, we had to demonstrate that therapeutically relevant quantities of very immature cells could be generated in vitro. For this, we took advantage of an ESC line that was manipulated to express the puromycin resistance (PacR) cassette under control of the cardiac-restricted portion of the *ncx1* promoter. Using the syNP4 clone and the classic hanging drop technique, we generated CMs from >70–80% of the embryoid bodies (EBs). The addition of ascorbic acid at day 2 of cultivation improves this efficiency such that >90% of the EBs contain contracting cells. As shown in Figure 1(a), following collagenase treatment, the cells plate as a pseudo-monolayer characterized by clusters of beating ESC-CMs found throughout the plate; however, the total number of CMs is relatively low. When selected by puromycin (2.5 μg/mL), CMs can be isolated that are ~98% pure based on α-actinin or cTnT labeling in vitro and by flow cytometric analysis of cTnT labeled cells [27]. Here, we also show that cells isolated at early time points and selected for at least 48 hours are transiently proliferative. As previously described, cell cycle attributes can be assayed by BrdU incorporation, Ser10 phosphorylation of histone H3, and by increasing cell numbers on the plate (Figure 1(a)). However, within 8–10 days of plating and selection (Day 7 + 8–10), the number of cells in G2/M decreases ($P < 0.05$), histone H3 phosphorylation at Ser10 is no longer observed, and bi-nucleation increases (not shown). Here, we also report that cell markers associated with contact inhibition (proteins—$p21^{CIP}$ and $p27^{KIP1}$) are upregulated (Figure 1(b)) with time of differentiation, which together with the cell cycle data, suggest that these cells rapidly undergo a maturation process associated with the loss of proliferative capacity. Transcripts for α-smooth muscle actin, a marker of very early cardiac differentiation, are however retained in these cells (Figure 1(b)).

When the syNP4 clone is cultured in a spinner flask system for the production of CMs, a number of experimental changes are required to achieve high yields of CMs [28]. The system requires EB formation and suspension for 8-9 days with media changes on a daily basis. At day 6, the addition of BMP2 improves the yield of CMs, and selection with puromycin begins at day 9. The partially selected EBs are then collagenase dissociated and cultivated as a monolayer culture. This method routinely produces ~35 million ESC-CM in a 16-17 day cycle after initial seeding of 25 million ESC, and we have been able to reproduce these results here (see Figure 1(c)). Consistent with our previous report, CM enrichment at day 14 of differentiation estimated by TnT staining was >85% when manually counted under microscope but ranging from 75 to 92% by flow cytometry (Figure 1(d)). The cultures are however heterogeneous, and the presence of α-smooth muscle actin protein is present in a subset of these cells [28].

To isolate immature and potentially proliferative and hypoxic resistant cells, we employed the same mass culture system, except that differentiating syNP4 ESC-CMs were dissociated at day 8 by collagenase treatment and immediately treated with puromycin for 24 to 48 hours prior to analysis. For all experiments, Day 9–11 cells were compared with Day 14–16 or Day 21 cells. At the earlier time point, the overall yield of CMs is decreased significantly, but when expanded ~10 million or more cells can be routinely isolated. The cells plated as a pseudo-monolayer, and most clusters showed asynchronous automaticity. Similar to the later staged cells described earlier, the ESC-CMs were heterogeneous and positive for α-SMA and α-actinin staining (Figure 2(a)). The

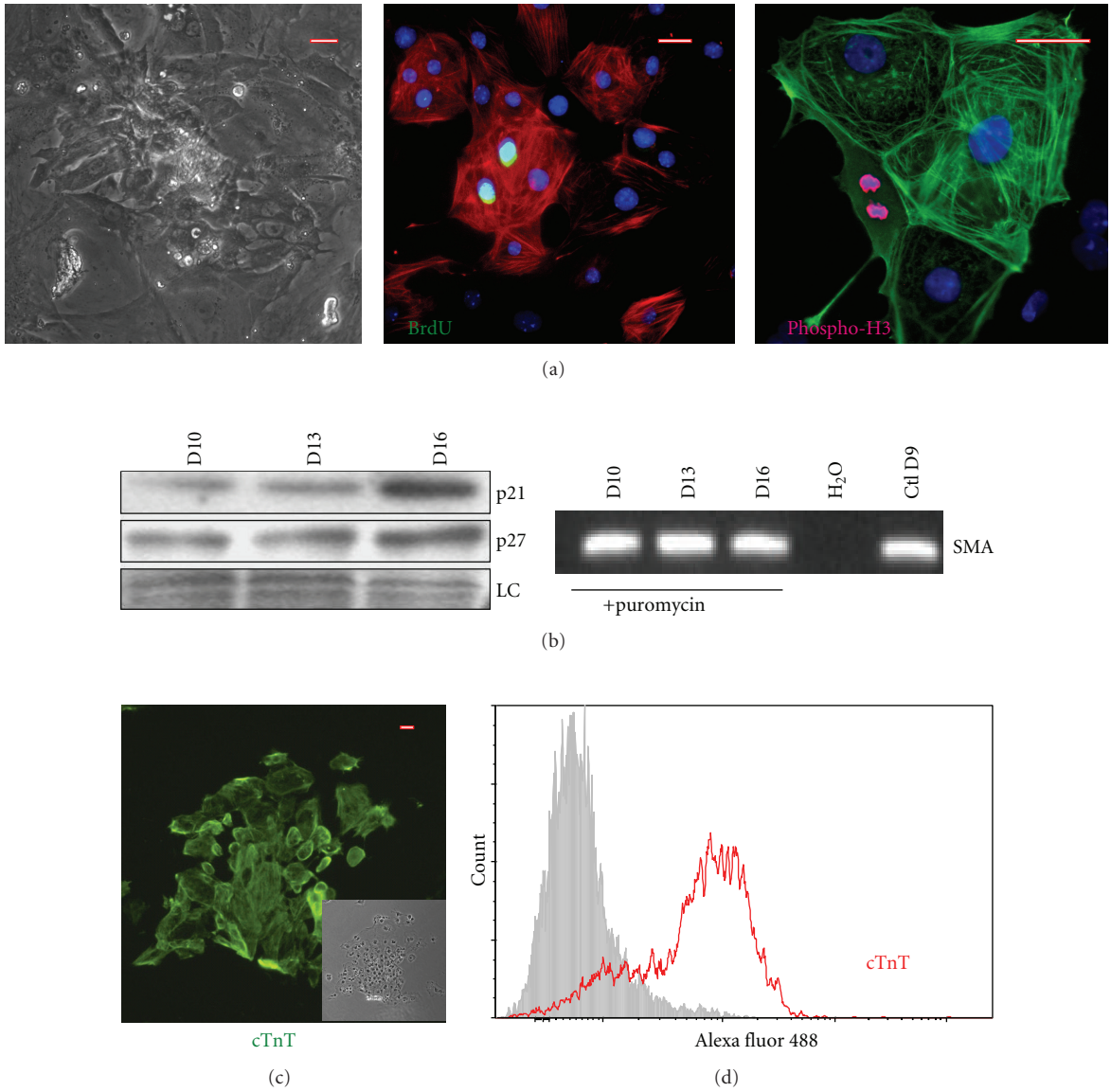
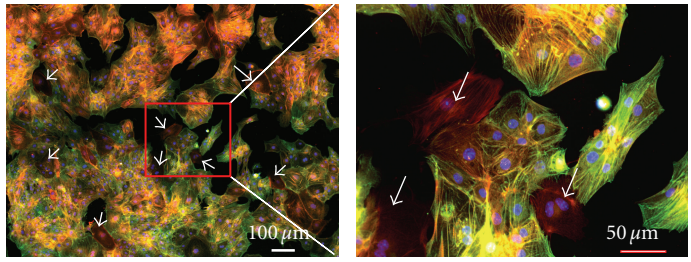


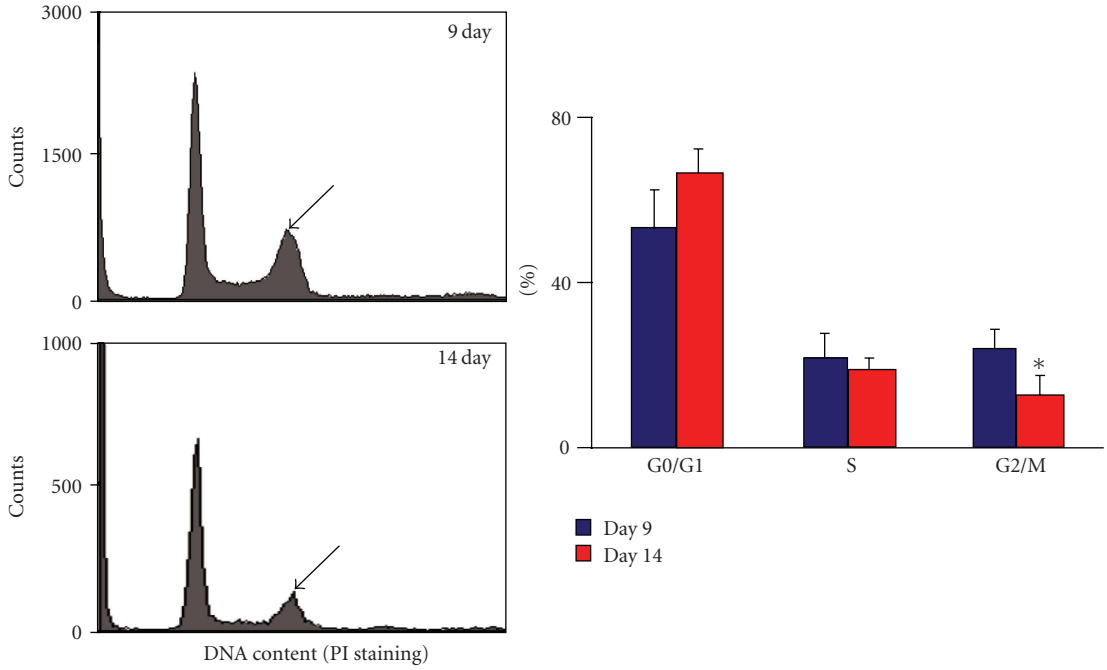
Figure 1: Characterization of syNP4 ES cell derived-CMs. (a) Traits of ESC-CM generated using the hanging drop technique. Following differentiation and plating, puromycin-resistant cells aggregate in clusters and show spontaneous contractions. Day 8-9 CMs (one or two days after plating) readily incorporate BrdU (green) and dividing cells can be readily identified by the use of an antibody against a phosphorylated form of histone H3. (b) As a function of differentiation time, syNP4 derived-CMs begin to express $p21^{CIP}$ and $p27^{KIP1}$ markers of contact inhibition and cell cycle checkpoint control, but the cells also continue to express transcripts to α-smooth muscle actin (α-SMA), a marker of immature cardiomyocytes. (c) Using the mass culture system, plated puromycin-resistant syNP4-derived CMs cluster similarly to that described in 1A above (see inset). Cells were immunostained with an antibody against cardiac TnT (cTnT), demonstrating that these clusters consist of CMs. (d) By flow cytometry, the purity of the cells has been determined to range from 75 to 92% ($n = 5$), depending on the cultivation and selection conditions. LC: loading control. Size markers = 50 μm.

degree of maturation of ESC-CM as a function of differentiation time is characterized by altered gene expression. More specifically, the ratio of genes encoding ANF (early marker) and calsequestrin (late marker) decreases with increased time of maturation ($P < 0.05$). Calsequestrin is undetectable by RT-PCR in very early Day 9-10 cells, prior to being upregulated in Day 14–16 cells.

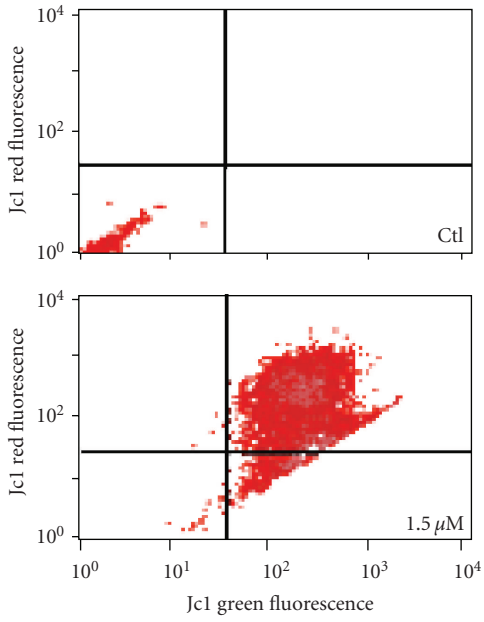
At selected time points, we measured DNA content on propidium iodide- (PI, Sigma) stained nuclei from puromycin selected ESC-CMs. Consistent with our previous results with hanging drop derived ESC-CMs, the DNA content of PI labeled cells showed an increase in the number of G0/G1 cells and a decrease in G2/M phase cells between days 9 and 14 of differentiation (Figure 2(b)). In contrast to our previous report, no significant change in the percentages of S phase cells was observed during this time period. The changes in G1 versus G2/M phase cells is, however, consistent with the isolation of a proliferating and consequently



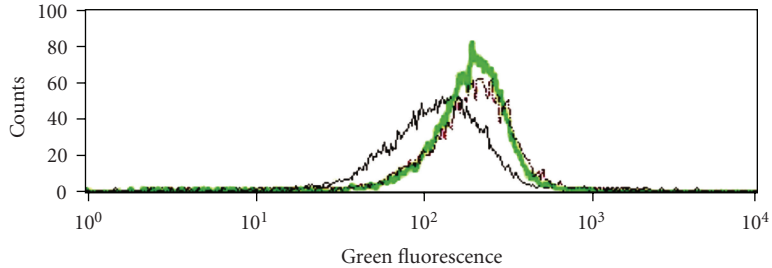
(a)



(b)



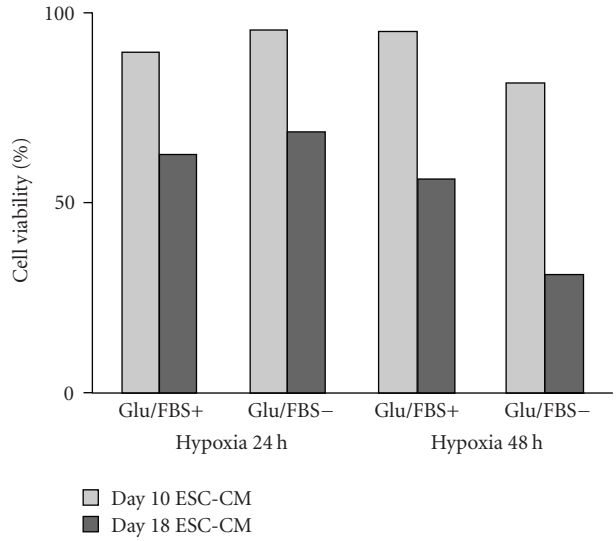
(c)



	% Total	Mean	Geo	CV	Median	Peak Ch
D8+2 (black)	75.06	145.68	119.72	84.35	125.21	129
D8+10 (green)	78.06	206.04	185.38	44.37	196.32	192
D8+17 (burgundy)	72.33	225.16	194.69	57.91	205.35	257

(d)

Figure 2: Continued.



(e)

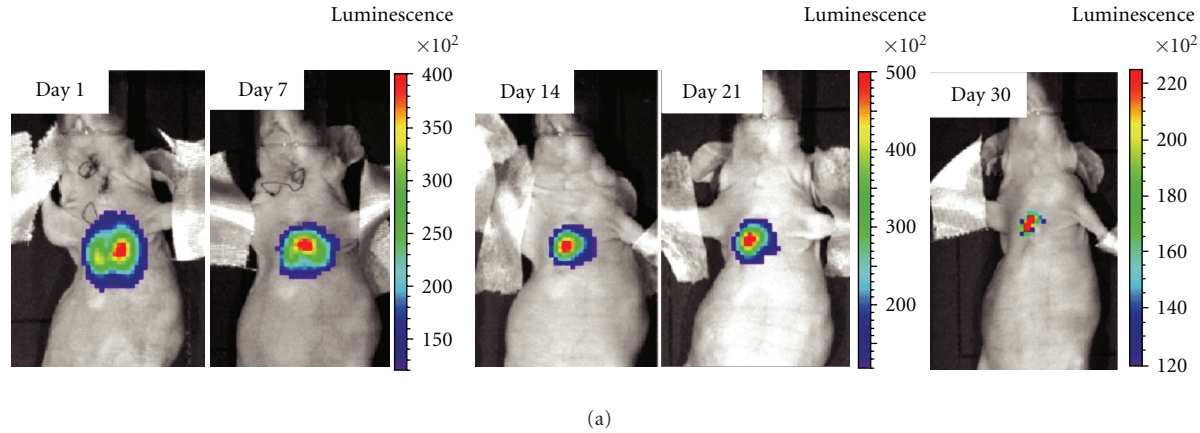
Figure 2: Characterization of immature syNP4 ES cell derived-CMs. (a) The purity and maturity of ESC-CMs at Day 11 were estimated by costaining with anti-α-SMA (red) and anti-α-actinin (sarcomeric) (green) antibodies. All cells express α-SMA, while $90 \pm 2\%$ cells are α-actinin positive. The right figure is a high magnification of the box in the left one. Arrows mark cells with α-SMA positive and α-actinin negative staining. (b) The DNA content of puromycin-selected ESC-CMs generated by mass culture was determined following PI staining. In these experiments ($n = 3$), the histogram indicates that Day 9 cells contain a higher percentage of G2/M phase cells than Day 14 cells. The number of G0/G1 cells also tends to increase with time of differentiation ($P > 0.05$). (c) Relative to controls, immature ESC-CMs incubated with 1.5 μM Jc1 had a strong increase in red fluorescence, which based on membrane potentials is indicative of live cells. Moreover, lack of change in membrane potential in either cell population indicates that the data shown from the MTG assay is directly comparable. (d) Typical results from MTG-stained cells assessed by flow cytometry. The green fluorescence signal is directly proportional to the mitochondrial content. As shown in the figure and table, the mitochondrial content increases with time of differentiation. (e) Cell viability of ESC-CMs cultivated under hypoxic and nutrient deprivation conditions. Data are presented as a percentage of control values.

less mature cell population than those isolated at Days 14–16.

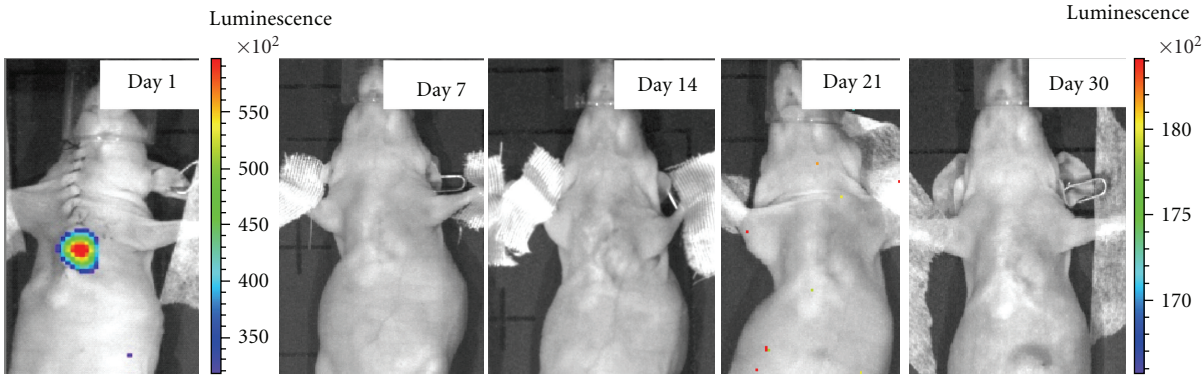
Mitochondria content/activity as a function of differentiation time was estimated in the ESC-CMs by staining with fluorescent dyes and analysis by flow cytometry (Figures 2(c) and 2(d)). The cells were initially analyzed by flow cytometer to assess live versus apoptotic cells based on mitochondrial membrane potentials. As shown in Figure 2(c), JC1 fluorescence indicates that the vast majority (>90%) of Day 8 + 3 cells are alive (based on red versus green fluorescence) following selection. Mitochondria content as a function of differentiation time was also estimated by staining ESC-CMs with MTG, a mitochondrial-selective green fluorescent dye. ESC-CMs appear relatively rich in mitochondria, but importantly, the mitochondria content increases as a function of differentiation time. The fluorescence signals at Days 10, 18, and 25 of differentiation are 145 ± 84, 206 ± 44, and 225 ± 57, respectively (Figure 2(d)). This result shows that the content of mitochondria in cells isolated at Day 10 is less than that isolated at Day 18 or 25; and consequently, these cells should be less sensitive to changes in oxygen content.

To test this possibility, we induced hypoxia and nutrient depletion in these cells. Under hypoxic and glucose and serum-deprived (double stress) conditions, early stage ESC-CMs, which were isolated at Day 10, survived remarkably better than those mature ESC-CMs isolated at Day 18 (Figure 2(e)). In these experiments, over 80% of Day 10 ESC-CMs were viable, which is on average 36% greater than Day 18 cells at 48 hrs. Consistently, under double stress conditions, Day 18 ESC-CMs released much more total LDH into the media than Day 10 cells. In fact, within 24 hours the increase in LDH release from Day 18 cells was twofold greater and at 48 hours threefold greater than that observed from Day 10 cells.

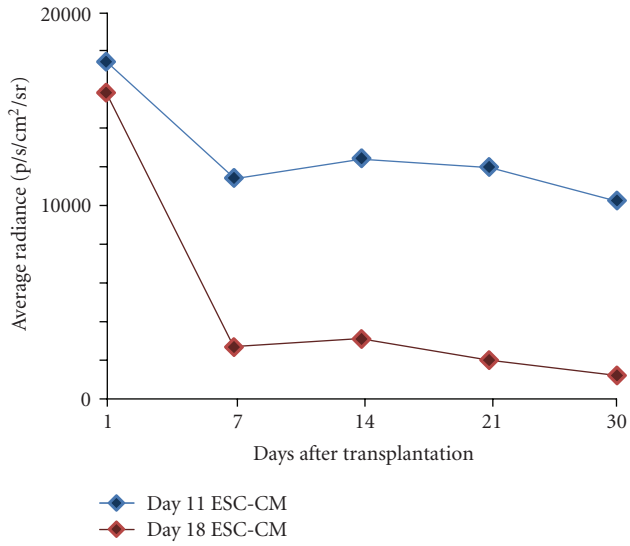
Finally, to monitor survival of CMs grafted in the heart, a subclone of syNP4 cells that stably express firefly luciferase (Fluc) was established following selection with hygromycin. Here, only one Fluc-expression sub-clone was employed for in vivo analyses. In these experiments, 1-2 million ESC-CMs (Day 8 + 3) selected by puromycin and expressing Fluc were injected into the mid ventricular wall of a normal (noninfarcted) heart in nude mice. Bioluminescent imaging (BLI) was performed at days 1, 3, 7, 14, 21, and 30 post transplantation, and the signal was quantified from an ROI as described previously [35, 36]. As shown in (Figures 3(a) and 3(b)), BLI signal from very immature ESC-CMs was detectable for at least 30 days, whereas the signal from more mature cells was lost within one week of injection. The signal intensity observed in Figure 3(a) falls substantially during the first week, achieves a plateau from day 7 to 21, and then declines further by 1 month. Perhaps more importantly, Fluc signal was only detected in the thoracic region during



(a)



(b)



(c)

Figure 3: Image analysis of immature syNP4-derived cardiomyocytes injected into heart. (a, b) Representative longitudinal bioluminescence imaging (BLI) of athymic nude mice following intramyocardial transplantation of 2×10^6 syNP4-derived CMs stably expressing a firefly luciferase reporter construct. The images shown in (a) involved CMs isolated at day 11 in culture, while those shown in (b) involved CMs isolated at day 18. (c) Analysis of BLI data from two sets of ESC-CMs shows a robust cardiac signal on day1, which decreases substantially in the first week, achieves a plateau from days 7 to 21, and then declines at 1 month, whereas those cells, which are slightly more mature, have minimal signals within 7 days of injection.

the entire experiments, indicating that injected cells were retained primarily at the site of injection. At least within the detection limits of the method, the "wash out" cells either did not take up residence elsewhere or did not survive in noncardiac (e.g., liver) locations [37]. Finally, these mice were examined for teratoma formation to ensure against false Fluc signals due to tumor formation. In these animals, no teratoma formation was observed in either experimental group one month following injection ($n = 2$), confirming that early and proliferating CMs are not tumorgenic.

4. Discussion

During myocardial infarction, heart muscle is damaged by ischemia due to a loss of blood flow and a lack of oxygen. Some if not most of the CMs in the affected area may survive brief and transient episodes of ischemia; however, the longer the injury without treatment the more likely the probability of significant cell death. This occurs in part to hypoxic stresses, mitochondrial dysfunction, pH changes, and the accumulation of reactive oxygen species. If however the ischemic episode is severe and is followed by reperfusion as typically occurs following many coronary occlusions, CMs die in mass due to the opening of the membrane permeability transition pore of the mitochondria. A spreading wave of reactive oxygen species causes further damage. An inflammatory response is mounted, and the process of left ventricular remodeling begins shortly thereafter. This latter process involves expansion of the infarcted area, ventricular dilatation, extensive fibrosis, and thinning of the ventricular wall [38–41].

For cell-based therapies designed to treat heart failure to become reality, problems associated with immunogenicity, cell differentiation, and isolation, and delivery systems must be overcome. While there is general agreement that ventricular defects should be treated with ventricular cells, the stage of cell maturation is also an area of active debate. This is particularly true with regards to the ability of transplanted cells to tolerate ischemic conditions. Cell replacement therapy of a myocardial infarction, particularly after extensive remodeling, is going to require the introduction of cells into poorly vascularized and therefore hypoxic conditions. Under these conditions, mature CMs are unlikely to survive; however, immature CMs with innate hypoxic resistance should survive to repopulate damaged tissue. Consistent with this view, Reinecke et al. have shown that highly differentiated adult CMs do not form viable grafts, which makes adult hearts unsuitable as a source of donor cells [42]. Moreover, in animal models, the vast majority of neonatal CMs die within one week of grafting; however, a modest improvement in survival is seen following grafting into vascularized tissue [43]. Reinecke and coworkers also documented that grafted fetal CMs survive and proliferate in the impaired myocardium as well as in the normal myocardium [42, 44]. Significant effort has therefore been made to find CMs that have the ability to improve left ventricular function in damaged heart, and currently it appears that fetal CMs are the best cell candidates for myocardial repair.

For ethical reasons, fetal tissue sources cannot be used to treat human heart failure; however, the derivation of CMs from embryonic or adult stem cells with embryonic or fetal-like characteristics represents one promising alternative [14, 26, 45, 46]. These cells show a wide range of functional attributes in part due to the state of maturation. Some researchers have therefore advocated the use of more mature stem cell-derived CMs that contain a developed contractile apparatus and can integrate through gap junctions and intercalated disks with host myocardium. When functionally coupled, these cells should work to increase force generation and decrease the stresses on surviving CMs [47]. Alternatively, we and others have argued for the use of progenitor cells or very immature but committed CMs that have the capacity to integrate and mature in vivo. These immature cells should ultimately form work-producing cells that couple with endogenous cells. While there are merits to both arguments, the appropriate cell type is likely to be disorder (e.g., myocardial infarction, hypertrophic or dilated cardiomyopathy) specific as well as location dependent; and to date, no research efforts have been designed to address this critical issue.

Here, we report the isolation of therapeutic quantities of ESC-CMs that are functionally immature based on automaticity, cell cycle analysis, hypoxia resistance, lower mitochondrial mass, and the presence of markers typical of immature embryonic or fetal heart cells. Because the puromycin resistant cells are all CMs, based on previous electrophysiological analyses and immunostaining, we conclude that these cells are fully committed CMs, albeit nonchamber specific [27]. Fijnvandraat et al. previously reported that Day 7 to 13 ESC-CMs corresponded most closely to E8.75–9 mouse heart cells based on a variety of chamber-specific markers and electrophysiological properties [26]. The ESC-CMs analyzed by patch clamp in that study all showed automaticity, similar to what we observed here. While contraction is observed in cells present in older cultures (Day 14–21), it is not cell autonomous. Instead, synchronous contractions are driven by the presence of pacemaker-like cells [45], which has led investigators to conclude that later-staged cells were most analogous to fetal or even neonatal stages of development. Based on these published findings, the presence of smooth muscle actin, time of differentiation, and the dynamic proliferative capacity, we conclude that the immature ESC-CMs described here correspond most closely to cells present in mouse primary heart tube stage prior to E8.75–9.

Moreover, we present the first evidence to indicate that these cells can in fact be introduced into myocardium, and that the cells survive for at least one month in mouse heart. Moreover, these cells survive preferentially to those isolated at slightly later times of differentiation. We further demonstrate that the purified ESC-CMs take up residence in the myocardium without teratoma formation, at least during the time-period analyzed, and that Fluc signals can be followed as a function of time to test therapeutic potentials. Together with our recent publication utilizing more mature cells [28], the development of this model system means that we can begin addressing the possibility that committed and

proliferating CMs will be therapeutically superior to more mature cells in the treatment of myocardial infarction.

5. Conclusions

In conclusion, we present data showing that the mass culture system employed here can be adapted to generate immature proliferating CMs tolerant of hypoxia. With the development of this system, ESC-CMs at defined differentiation stages can now be tested in vivo to determine their value for treatment of myocardial infarctions. Techniques like cell surface capturing technologies [48] will however be required to isolate pure ventricular populations, but once accomplished, we predict that the results will be analogous to those generated with fetal, neonatal, and adult ventricular cells isolated directly from heart. Early stage-specific CMs should lead to increased cell survival and regeneration of damaged heart, and once confirmed in mouse, this approach could become a powerful strategy for treating human cardiac disease states and injuries.

Acknowledgments

The research was supported by NIH Grants R21EB-2473 (RZ), 4R00HL094798-03 (RLG), R01-HL081185 (RZ), R01-HL081185S1 (RZ) the Innovation Center at the Medical College of Wisconsin (RLG), and the Intramural Research Program (IRP) of the NIH, National Institute on Aging (KRB, RPW). The authors also thank Dr. M. Celeste Simon for helping with hypoxic studies.

References

[1] K. R. Boheler and M. Y. Fiszman, "Can exogenous stem cells be used in transplantation?" *Cells Tissues Organs*, vol. 165, no. 3-4, pp. 237–245, 1999.

[2] R. A. Li, J. C. Moore, Y. S. Tarasova, and K. R. Boheler, "Human embryonic stem cell-derived cardiomyocytes: therapeutic potentials and limitations," *Journal of Stem Cells*, vol. 1, no. 2, pp. 109–124, 2006.

[3] M. G. Perino, S. Yamanaka, J. Li, A. M. Wobus, and K. R. Boheler, "Cardiomyogenic stem and progenitor cell plasticity and the dissection of cardiopoiesis," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 4, pp. 475–494, 2008.

[4] K. C. Wollert and H. Drexler, "Cell-based therapy for heart failure," *Current Opinion in Cardiology*, vol. 21, no. 3, pp. 234–239, 2006.

[5] M. Rubart and L. J. Field, "Stem cell differentiation: cardiac repair," *Cells Tissues Organs*, vol. 188, no. 1-2, pp. 202–211, 2008.

[6] C. E. Murry, M. H. Soonpaa, H. Reinecke et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[7] L. B. Balsam, A. J. Wagers, J. L. Christensen, T. Kofidis, I. L. Weissmann, and R. C. Robbins, "Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium," *Nature*, vol. 428, no. 6983, pp. 668–673, 2004.

[8] Q. L. Ying, J. Nichols, E. P. Evans, and A. G. Smith, "Changing potency by spontaneous fusion," *Nature*, vol. 416, no. 6880, pp. 545–548, 2002.

[9] N. Terada, T. Hamazaki, M. Oka et al., "Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion," *Nature*, vol. 416, no. 6880, pp. 542–545, 2002.

[10] M. Alvarez-Dolado, R. Pardal, J. M. Garcia-Verdugo et al., "Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes," *Nature*, vol. 425, no. 6961, pp. 968–973, 2003.

[11] M. Rubart and L. J. Field, "Cell-based approaches for cardiac repair," *Annals of the New York Academy of Sciences*, vol. 1080, pp. 34–48, 2006.

[12] M. Rubart and L. J. Field, "Cardiac regeneration: repopulating the heart," *Annual Review of Physiology*, vol. 68, pp. 29–49, 2006.

[13] K. R. Boheler, "Pluripotency of human embryonic and induced pluripotent stem cells for cardiac and vascular regeneration," *Thrombosis and Haemostasis*, vol. 104, no. 1, pp. 23–29, 2010.

[14] A. M. Wobus and K. R. Boheler, "Embryonic stem cells: prospects for developmental biology and cell therapy," *Physiological Reviews*, vol. 85, no. 2, pp. 635–678, 2005.

[15] J. Nussbaum, E. Minami, M. A. Laflamme et al., "Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response," *FASEB Journal*, vol. 21, no. 7, pp. 1345–1357, 2007.

[16] K. Okita, T. Ichisaka, and S. Yamanaka, "Generation of germline-competent induced pluripotent stem cells," *Nature*, vol. 448, no. 7151, pp. 313–317, 2007.

[17] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[18] J. Yu, M. A. Vodyanik, K. Smuga-Otto et al., "Induced pluripotent stem cell lines derived from human somatic cells," *Science*, vol. 318, no. 5858, pp. 1917–1920, 2007.

[19] S. Yamanaka, "A Fresh Look at iPS Cells," *Cell*, vol. 137, no. 1, pp. 13–17, 2009.

[20] G. Q. Daley, M. W. Lensch, R. Jaenisch, A. Meissner, K. Plath, and S. Yamanaka, "Broader implications of defining standards for the pluripotency of iPSCs," *Cell Stem Cell*, vol. 4, no. 3, pp. 200–201, 2009.

[21] K. Kim, A. Doi, B. Wen et al., "Epigenetic memory in induced pluripotent stem cells," *Nature*, vol. 467, no. 7313, pp. 285–290, 2010.

[22] S. M. Wu, Y. Fujiwara, S. M. Cibulsky et al., "Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart," *Cell*, vol. 127, no. 6, pp. 1137–1150, 2006.

[23] D. Franco, W. H. Lamers, and A. F. M. Moorman, "Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model," *Cardiovascular Research*, vol. 38, no. 1, pp. 25–53, 1998.

[24] V. M. Christoffels, P. E. Habets, D. Franco et al., "Chamber formation and morphogenesis in the developing mammalian heart," *Developmental Biology*, vol. 223, no. 2, pp. 266–278, 2000.

[25] M. U. Koban, S. A. Brugh, D. R. Riordon et al., "A distant upstream region of the rat multipartite Na^+—Ca^{2+} exchanger NCX1 gene promoter is sufficient to confer cardiac-specific expression," *Mechanisms of Development*, vol. 109, no. 2, pp. 267–279, 2001.

[26] A. C. Fijnvandraat, A. C. G. van Ginneken, P. A. J. de Boer et al., "Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube," *Cardiovascular Research*, vol. 58, no. 2, pp. 399–409, 2003.

[27] S. Yamanaka, I. Zahanich, R. P. Wersto, and K. R. Boheler, "Enhanced proliferation of monolayer cultures of embryonic stem (ES) cell-derived cardiomyocytes following acute loss of retinoblastoma," *PLoS ONE*, vol. 3, no. 12, Article ID e3896, 2008.

[28] H. Qiao, H. Zhang, S. Yamanaka et al., "Long-term improvement in postinfarct left ventricular global and regional contractile function is mediated by embryonic stem cell-derived cardiomyocytes," *Circulation: Cardiovascular Imaging*, vol. 4, no. 1, pp. 33–41, 2011.

[29] K. V. Tarasov, Y. S. Tarasova, W. L. Tam et al., "B-MYB is essential for normal cell cycle progression and chromosomal stability of embryonic stem cells," *PLoS ONE*, vol. 3, no. 6, Article ID e2478, 2008.

[30] R. Zhou, D. H. Thomas, H. Qiao et al., "In vivo detection of stem cells grafted in infarcted rat myocardium," *Journal of Nuclear Medicine*, vol. 46, no. 5, pp. 816–822, 2005.

[31] H. Qiao, S. Surti, S. R. Choi et al., "Death and proliferation time course of stem cells transplanted in the myocardium," *Molecular Imaging and Biology*, vol. 11, no. 6, pp. 408–414, 2009.

[32] H. Qiao, H. Zhang, Y. Zheng et al., "Embryonic stem cell grafting in normal and infarcted myocardium: serial assessment with MR imaging and PET dual detection," *Radiology*, vol. 250, no. 3, pp. 821–829, 2009.

[33] P. J. Gruber, Z. Li, H. Li et al., "In vivo imaging of MLC2v-luciferase, a cardiac-specific reporter gene expression in mice," *Academic Radiology*, vol. 11, no. 9, pp. 1022–1028, 2004.

[34] H. Zhang, H. Qiao, A. Bakken et al., "Utility of dual-modality bioluminescence and MRI in monitoring stem cell survival and impact on post myocardial infarct remodeling," *Academic Radiology*, vol. 18, no. 1, pp. 3–12, 2011.

[35] P. J. Gruber, Z. Li, H. Li et al., "In vivo imaging of MLC2v-luciferase, a cardiac-specific reporter gene expression in mice," *Academic Radiology*, vol. 11, no. 9, pp. 1022–1028, 2004.

[36] H. Zhang, H. Qiao, A. Bakken et al., "Utility of dual-modality bioluminescence and MRI in monitoring stem cell survival and impact on post myocardial infarct remodeling," *Academic Radiology*, vol. 18, no. 1, pp. 3–12, 2011.

[37] F. Cao, S. Lin, X. Xie et al., "In vivo visualization of embryonic stem cell survival, proliferation, and migration after cardiac delivery," *Circulation*, vol. 113, no. 7, pp. 1005–1014, 2006.

[38] S. Frantz, J. Bauersachs, and G. Ertl, "Post-infarct remodelling: contribution of wound healing and inflammation," *Cardiovascular Research*, vol. 81, no. 3, pp. 474–481, 2009.

[39] J. S. Silvestre, Z. Mallat, A. Tedgui, and B. I. Lévy, "Post-ischaemic neovascularization and inflammation," *Cardiovascular Research*, vol. 78, no. 2, pp. 242–249, 2008.

[40] M. N. Sack, "Mitochondrial depolarization and the role of uncoupling proteins in ischemia tolerance," *Cardiovascular Research*, vol. 72, no. 2, pp. 210–219, 2006.

[41] G. Ertl and S. Frantz, "Healing after myocardial infarction," *Cardiovascular Research*, vol. 66, no. 1, pp. 22–32, 2005.

[42] H. Reinecke, M. Zhang, T. Bartosek, and C. E. Murry, "Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts," *Circulation*, vol. 100, no. 2, pp. 193–202, 1999.

[43] M. Zhang, D. Methot, V. Poppa, Y. Fujio, K. Walsh, and C. E. Murry, "Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 5, pp. 907–921, 2001.

[44] C. E. Murry, M. L. Whitney, and H. Reinecke, "Muscle cell grafting for the treatment and prevention of heart failure," *Journal of Cardiac Failure*, vol. 8, supplement 6, pp. S532–S541, 2002.

[45] K. R. Boheler, J. Czyz, D. Tweedie, H. T. Yang, S. V. Anisimov, and A. M. Wobus, "Differentiation of pluripotent embryonic stem cells into cardiomyocytes," *Circulation Research*, vol. 91, no. 3, pp. 189–201, 2002.

[46] A. C. Fijnvandraat, A. C. G. van Ginneken, C. A. Schumacher et al., "Cardiomyocytes purified from differentiated embryonic stem cells exhibit characteristics of early chamber myocardium," *Journal of Molecular and Cellular Cardiology*, vol. 35, no. 12, pp. 1461–1472, 2003.

[47] K. Musunuru, I. J. Domian, and K. R. Chien, "Stem cell models of cardiac development and disease," *Annual Review of Cell and Developmental Biology*, vol. 26, pp. 667–687, 2010.

[48] R. L. Gundry, K. R. Boheler, J. E. van Eyk, and B. Wollscheid, "A novel role for proteomics in the discovery of cell-surface markers on stem cells: scratching the surface," *Proteomics—Clinical Applications*, vol. 2, no. 6, pp. 892–903, 2008.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 130970, 7 pages
doi:10.4061/2011/130970

Research Article

Transcription Factor Lbx1 Expression in Mouse Embryonic Stem Cell-Derived Phenotypes

Stefanie Schmitteckert,[1] Cornelia Ziegler,[1] Liane Kartes,[1,2] and Alexandra Rolletschek[1]

[1] *Institute for Biological Interfaces 1, Karlsruhe Institute of Technology (KIT) Campus North, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany*
[2] *Zoological Institute Department of Ecology and Parasitology, Karlsruhe Institute of Technology (KIT) Campus South, Kornblumenstraße 13, 76131 Karlsruhe, Germany*

Correspondence should be addressed to Alexandra Rolletschek, alexandra.rolletschek@kit.edu

Received 15 April 2011; Accepted 10 July 2011

Academic Editor: Gabriela Kania

Transcription factor Lbx1 is known to play a role in the migration of muscle progenitor cells in limb buds and also in neuronal determination processes. In addition, involvement of Lbx1 in cardiac neural crest-related cardiogenesis was postulated. Here, we used mouse embryonic stem (ES) cells which have the capacity to develop into cells of all three primary germ layers. During *in vitro* differentiation, ES cells recapitulate cellular developmental processes and gene expression patterns of early embryogenesis. Transcript analysis revealed a significant upregulation of *Lbx1* at the progenitor cell stage. Immunofluorescence staining confirmed the expression of Lbx1 in skeletal muscle cell progenitors and GABAergic neurons. To verify the presence of Lbx1 in cardiac cells, triple immunocytochemistry of ES cell-derived cardiomyocytes and a quantification assay were performed at different developmental stages. Colabeling of Lbx1 and cardiac specific markers troponin T, α-actinin, GATA4, and Nkx2.5 suggested a potential role in early myocardial development.

1. Introduction

Lbx1 is a member of the *Ladybird*-like homeobox gene family that encodes a homeodomain transcription factor. This mouse counterpart of *Drosophila m. ladybird* gene was first discovered by Jagla et al. [1]. In vertebrates, expression of Lbx1 has been described in the CNS and in migrating muscle precursor cells. During early mouse embryonic development, the presence or absence of Lbx1 distinguishes two major neuronal classes generated in the dorsal spinal cord [2]. Specifically, Lbx1 is essential for determining a somatosensory instead of a viscerosensory fate in relay neurons in the hindbrain [3]. At later stages of mouse neurogenesis, expression of Lbx1 defines a basal GABAergic differentiation state for dorsal horn neurons [4].

Furthermore, it was found that Lbx1 plays an important role in the migration of hypaxial muscle precursor cells during development. It was suggested by Brohmann et al. [5] that Lbx1 controls the expression of genes that are essential for the recognition or interpretation of cues that guide migrating muscle precursors and maintain their migratory potential. Watanabe et al. [6] detected Lbx1 in activated but not quiescent satellite cells of adult mice. They suggested that Lbx1 plays important roles in the differentiation and maintenance of satellite cells of mature myofibers.

In addition to its relevance in neuronal [7] and muscle cell [8] development in *Drosophila ladybird* genes have also been reported to be expressed in a specific subset of cardioblasts, required for the diversification of heart precursor cells [9]. Until today, there are only few data about the involvement of Lbx1 in mouse cardiogenesis. Inactivation of *Lbx1* in mice mainly resulted in defects in heart looping and increased cell proliferation leading to myocardial hyperplasia [10]. Obviously there are striking morphological and functional differences between the tubular *Drosophila* heart and the four-chambered mammalian heart. However, the specification of cardiac primordia in both *Drosophila*

and vertebrate embryos is under the control of conserved core cardiac transcription factors encoding, for example, Nkx2.5/Tinman, GATA/Pannier, Mef2, and Hand family members [11].

Murine embryonic stem (ES) cells are characterized by the capacity to differentiate into virtually any cell type of an organism, including neurons, skeletal, and cardiac muscle cells [12]. *In vitro* differentiation of mouse ES cells into cardiomyocytes recapitulates the programmed expression of cardiac genes observed in the mouse embryo in a time-controlled manner [13]. During ES cell differentiation, cardiac-specific genes are up- or downregulated dependent on extracellular signals and cell-cell interactions, thus providing an excellent model system to study early embryonic development at the cellular level.

Therefore, the aim of this study was the identification of Lbx1 expression at the transcript and protein level in ES cell-derived neurons and muscle cell progenitors to ensure the presence of Lbx1 in our *in vitro* model system. Specifically, we investigated whether Lbx1 is also expressed in ES cell-derived cardiac myocytes. The presence of Lbx1 was clearly demonstrated in a small subpopulation of ES cell-derived cardiomyocytes by immunocytochemistry.

2. Materials and Methods

2.1. Cell Culture and Differentiation. ES cells of line R1 [14] were cultured on a mitotically inactivated embryonic fibroblast feeder layer in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) and supplements as described [15]. The two-step differentiation protocol included (i) the formation of embryoid bodies (EBs) and (ii) after EB plating on adhesive substrate (0.1% gelatin) the expansion of multilineage progenitor cells and spontaneous differentiation to form differentiated phenotypes. In short, ES cells ($n = 600$ cells/20 μL drop) were plated on the lids of Petri dishes (∅ 10 cm), cultured as "hanging drops" for 2 days and on bacteriological plates (∅ 6 cm) in suspension for additional 3 days to form EBs (five days in total = 5 d). The differentiation medium consisted of Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FCS (selected batches), L-glutamine/penicillin/streptomycin (1 : 100 of stock solution), nonessential amino acids (1 : 100 of stock solution, all from Invitrogen), and α-monothioglycerol (final concentration 450 μM; Sigma-Aldrich, Taufkirchen, Germany). At day 5, EBs were plated in IMDM with the supplements mentioned above and cultivated for further two (= 5 + 2 d) up to 35 days (= 5 + 35 d) onto gelatin-coated 6 cm culture dishes for RT-PCR analysis and cardiomyocyte isolation (30 EBs). 30 EBs were plated onto gelatin-coated 6 cm culture dishes containing cover slips for immunocytochemistry, and 100 EBs onto 10 cm culture plates for protein isolation. Cultures were maintained in a 37°C/5% CO_2 incubator. Although this differentiation approach is preferentially used to obtain cardiomyocytes and skeletal muscle cell progenitor cells, a small population of neuronal cells can be generated, too.

2.2. RNA Extraction and RT-PCR. Control mouse heart and brain tissues at day E12.5, ES cells, EBs, or differentiated cells collected at days 5 + 2, 5 + 4, 5 + 7, 5 + 9, 5 + 11, 5 + 14, 5 + 18, 5 + 25, 5 + 30, and 5 + 35 were suspended in lysis buffer (peqGOLD RNAPure, peqlab, Erlangen, Germany). Total RNA was isolated by the single-step extraction method according to Chomczynski and Sacchi [16]. RNA was reverse transcribed using Oligo $d(T)_{16}$ primers (Applied Biosystems, Darmstadt, Germany) and amplified using oligonucleotide primers specific for the following genes (oligonucleotide sequences are given in brackets in the order forward-, reverse-primer followed by the annealing temperature used for PCR, the length of the amplified fragments in base pairs, and the number of cycles): Lbx1 (CAGACCTCGCCTCTCTGC, CTCCTC-TAGGTCCCGCTTG; 60°C; 318 bp; 32), Gapdh (CAGC-CTCGTCCCGTAGAC, CGCTCCTGGAAGATGGTG; 60°C; 253 bp; 32). Reverse transcription was performed with MuLV reverse transcriptase (Invitrogen) for 10 min at 25°C and 90 min at 42°C, followed by denaturation for 10 min at 70°C and cooling to 8°C according to the protocol supplied by the manufacturer. For semiquantitative determination of mRNA levels, PCR analyses were carried out with Taq DNA polymerase (peqGOLD Taq-Polymerase, peqlab, Erlangen, Germany). For determination of relative mRNA levels, two separate PCR reactions, either using primers of the analyzed gene or primers specific for Gapdh, were performed.

The PCR reactions were performed on a 25 μL reaction volumes. One-third of each PCR reaction was electrophoretically separated on 2% agarose gels containing GelRed nucleic Acid Gel Stain (Biotium, Hayward, USA). Gels were illuminated with UV light, and the GelRed fluorescence signals were analyzed by the TINA2.08e software (Raytest Isotopenmeßgeräte, Straubenhardt, Germany). Data of the target genes were plotted as percentage changes in relation to the expression of the housekeeping gene Gapdh. Gels of ten independent experiments were analyzed. For statistical evaluation, data were compared using analysis of variance (ANOVA).

2.3. Western Blotting. EBs, cells at differentiation stages 5 + 2, 5 + 4, 5 + 7, 5 + 11, 5 + 14, and 5 + 18, embryonic mouse hearts and brains at days E12.5 and E15.5, and adult heart and brain tissue were washed three times with PBS and homogenized in 300–500 μL cold disruption buffer (PARIS Kit, Applied Biosystems, Darmstadt, Germany). The samples were stored at −80°C until use. The total protein content was determined spectrophotometrical using the BCA Protein Acid Kit (Sigma-Aldrich) and NanoDrop (peqlab). Sixty μg protein were solubilized in Laemmli buffer and heated at 95°C for 5 min and electrophoresed on 15% SDS-PAGE. Proteins were electrotransferred to PVDF membrane and blocked in Tris-buffered saline containing 0.2% Tween and 10% nonfatty milk powder for 1 h. Blots were incubated in 10% nonfatty milk powder/TBS-Tween with rabbit anti mouse Lbx1 antibody (1 : 1000, gift from Dr. T. Müller, Berlin, Germany) or rabbit anti mouse Gapdh antibody (1 : 2000, Abcam, Cambridge, UK) at room temperature (RT) for 1 h. Afterwards, the blots were washed three times

for 10 min in TBS-Tween and then incubated with goat anti rabbit IgG (1 : 80000) conjugated to horseradish peroxidase (Sigma-Aldrich) in 10% nonfatty milk powder/TBS-Tween at RT for 1 h. After washing, immunoreactive signals were visualized by enhanced chemiluminescence detection (ECL plus, Amersham Biotech, Freiburg, Germany). Apparent molecular weights were determined by comparison with standard molecular weight markers (PageRuler Plus Prestained Protein Ladder, Fermentas, St. Leon-Rot, Germany).

2.4. Immunofluorescence Analysis. Indirect immunofluorescence (IF) analysis of EB outgrowths was carried out at days 5 + 11 for neuronal phenotypes and 5 + 14 for skeletal muscle cell progenitors. To quantify the amount of Lbx1-positive cardiac cells in EB outgrowths at various differentiation stages, beating cardiac clusters were isolated as described [15] at days 5 + 4, 5 + 7, 5 + 9, 5 + 11, and 5+15. Clusters were replated onto gelatin-coated cover slips (∅10 mm) in wells of 4-well plates and allowed to attach overnight. Cells were rinsed with PBS and fixed with PBS containing 4% paraformaldehyde at RT for 20 min. After washing, cells were permeabilized with 0.1% Triton in PBS at RT for 10 min. Preparations were incubated with 1% bovine serum albumine in PBS for 1 h followed by incubation with the primary antibodies at RT in a humidified chamber for 1 h. The following antibodies were applied: rabbit anti mouse Lbx1 (1 : 1000, gift from Dr. T. Müller), mouse anti mouse sarcomeric alpha actinin (1 : 200), mouse anti mouse cardiac Troponin T (1 : 200; all from Abcam), mouse anti mouse β III tubulin isoform (1 : 150), guinea pig anti mouse gamma-aminobutyric acid (GABA, 1 : 500; all from Chemicon-Millipore, Schwalbach, Germany), goat anti mouse GATA4 (1 : 100), and goat anti mouse Nkx2.5 (1 : 100; all from Santa Cruz Biotechnology, Heidelberg, Germany), respectively. After washing (3x) with PBS, cells were incubated at RT for 1 h with fluorescence-labelled secondary antibodies which were either anti rabbit, anti guinea pig, anti goat Cy^3 (1 : 700), anti mouse Cy5 (1 : 700; all from Dianova, Hamburg, Germany) or anti mouse, anti rabbit Alexa 488, (1 : 100) or anti mouse Alexa 350 (1 : 100; all from Molecular Probes-Invitrogen). After counterstaining with DAPI, cover slips were rinsed (3x) with PBS and with A. tridest (1x). After being embedded in Vectashield mounting medium (Vector Lab.—Biozol, Wertheim-Bettingen, Germany), specimen were analyzed with the fluorescence microscope (Axiovert; Zeiss, Germany).

The quantitative estimation based on the evaluation of approximately 1000 cardiac cells ($n = 10$ experiments) positively stained for troponin T at the given differentiation stages. Lbx1-immunopositive cells displaying a strong fluorescence signal coexpressing troponin T (Lbx1+, troponin T+) and cells positive for troponin only T (Lbx1−, troponin T+) were counted. Lbx1 expressing cells not costained by troponin T antibody in close proximity to cardiac clusters were not counted. Most likely, these cells did not rearrange the sarcomeric apparatus properly after dissociation and replating.

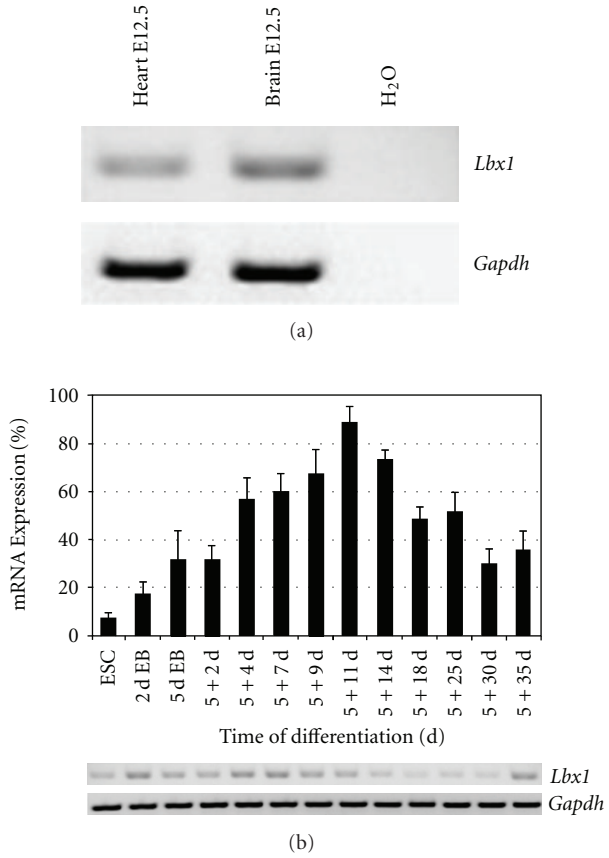


Figure 1: RT-PCR analysis of mRNA levels of *Lbx1* gene in embryonic tissues and ES cell-derived progeny. (a) Representative gel of semiquantitative RT-PCR *Lbx1* products in brain and heart tissue at E12.5. (b) Representative gel and *Lbx1* transcript levels at given differentiation stages of ES cell differentiation. The data were plotted (in %) in relation to the transcript levels of the housekeeping gene *Gapdh* as means ($n = 10$ experiments). Error bars represent standard error of the mean. The P value is <0.0001.

3. Results

3.1. RT-PCR Analysis of Lbx1 Expression. Embryonic mouse heart and brain tissues as well as ES cells and differentiated progeny of 2 to 5+35 days of cultivation were analyzed by RT-PCR. Heart and brain tissue from E12.5 revealed transcript levels with slightly higher levels in brain tissue (Figure 1(a)). Transcript levels of *Lbx1* were low in undifferentiated ES cells (8.2%) but showed a significant transient upregulation at the progenitor cell stage from 5 + 4 d (55.9%) to 5 + 14 d (72.3%; Figure 1(b)). Maximum level of 86.7% was reached at day 5+11. At the terminal differentiation stage 5+30 d, *Lbx1* was significantly downregulated to 30.3%.

3.2. Detection of Lbx1 by Western Blot Analysis. To confirm the presence of Lbx1 at the protein level, heart and brain tissues from different developmental stages were analyzed. Lbx1 was moderately detectable in comparative brain tissues (Figure 2(a)). Lbx1 could also be detected in E12.5, E15.5, and adult hearts. In differentiated ES cell derivatives, Lbx1

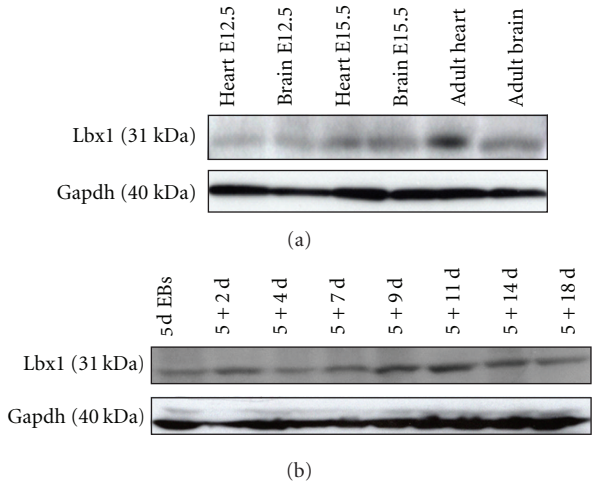


Figure 2: Western blot analysis of Lbx1 in mouse tissues and ES cell-derived cultures. (a) Representative gels of analyzed embryonic heart and brain tissue at E12.5 and E15.5 as well as adult tissue compared to internal standard Gapdh. (b) Representative gels of Lbx1 and Gapdh at the indicated differentiation stages of ES cell differentiation.

was expressed at all stages of differentiation at moderate levels (Figure 2(b)).

3.3. Immunocytochemical Analysis of Lbx1 in Differentiated ES Cell Progeny. Immunocytochemical analysis was first performed with skeletal muscle progenitors and neuronal cells to confirm the involvement of Lbx1 in early developmental processes in ES cell-derived cultures *in vitro*. Costaining with sarcomeric α-actinin revealed a small number of Lbx1-positive skeletal muscle cell progenitors (Figure 3(a)). Lbx1 immunoreactivity was also detected in numerous β III tubulin-positive neuronal cells (Figure 3(b) and at higher magnification Figure 3(c)). To identify the neuronal subtype, costaining with anti-GABA antibody was performed. As shown in Figures 3(d′) and 3(d‴), Lbx1 was expressed in the nuclei of GABA-positive neuronal cells. Immunocytochemical analysis of Lbx1 in undifferentiated ES cells revealed its absence (not shown).

3.4. Lbx1 Is Colocated in ES Cell-Derived Cardiomyocytes. In order to discover whether Lbx1 could also be detected in ES cell-derived cardiomyocytes, triple immunofluorescence staining including sarcomeric α-actinin and early cardiac transcription factors was performed in isolated beating clusters. Lbx1 was found in a small subpopulation of cardiomyocytes in coexpression with GATA4 (Figure 4(a‴)) and Nkx2.5 (Figure 4(b‴)).

To further investigate the amount of Lbx1-expressing cardiomyocytes, a quantification assay was performed at different developmental stages. Immunocytochemistry revealed that about 10% of troponin T-positive cardiomyocytes stained positive for Lbx1 as early as day 5 + 4 (Figure 5(a)). This amount slightly changed at days 5 + 7 (7.8%) and 5 + 9 (8.2%) to 10.9% at 5 + 11 d and 9.6% at day 5+15, but changes were not significant. Figures 5(b)–5(d) showed representative images of immunofluorescence staining of isolated beating cardiomyocytes partially coexpressing Lbx1 at given time points.

Because there was no Lbx1-specific purification or enrichment procedure included, approximately less than five percent of ES cell-derived cells expressed Lbx1. Expression was restricted to skeletal myocyte progenitors, cardiomyocytes, and neuronal cells.

4. Discussion

Analysis of Lbx1 at the transcript and protein level in ES cell-derived progeny revealed the expression in neurons as well as skeletal muscle progenitors and in a small subpopulation of cardiomyocytes. To our knowledge, this is the first time that a coexpression of Lbx1 and several cardiac-specific markers could be demonstrated in ES cell-derived cardiomyocytes.

RT-PCR analysis showed an expression signal in embryonic heart tissue. While Schäfer et al. [10] detected Lbx1-LacZ-positive cells in embryonic mouse hearts but failed to detect Lbx1 mRNA, Chao et al. [17] found moderate expression levels of *Lbx1* gene in porcine hearts. *Lbx1* was significantly upregulated at the progenitor cell stage around day 5 + 11 of ES cell differentiation. Western blot analysis of embryonic hearts tissue samples and several ES cell differentiation stages confirmed the presence of Lbx1 protein in our model system. Because there are progenitors from several lineages including the mesodermal as well as ectodermal lineage present at this time of ES cell differentiation [18], double immunocytochemistry using cell type-specific antibodies was performed to assign Lbx1 signal to specific phenotypes. As expected, Lbx1 immunoreactivity could be detected in skeletal muscle cell precursors and GABAergic neurons, reflecting the tissue expression pattern in mice as described previously [4, 19].

Costaining of Lbx1 with cardiac transcription factors (GATA4, Nkx2.5) and the continuous expression in a subpopulation of troponin T-positive cardiomyocytes suggested a potential role in myocardial differentiation and function. During mouse cardiogenesis at E11.0, single Lbx1-LacZ-positive cells were detected in the myocardium of the left ventricle [10]. The authors stated that this small population of Lbx1-LacZ-positive cells might originate from the neural tube migrating to the caudal branchial arch and the truncus arteriosus between E9.0 and E9.5 or reflect a *de novo* Lbx1 expression in the myocardium. Our data provide first evidence for an expression of Lbx1 in murine cardiomyocytes independent from the cardiac neural crest system.

Long-term expression of the Lbx1 homolog Ladybird was also found in *Drosophila* [20]. The authors performed a muscle and heart-targeted genome-wide transcriptional profiling and a chromatin-immunoprecipitation- (ChIP-)on-chip search for direct Ladybird targets. They concluded that Ladybird contributes to specifying the identity of cardiac precursors, regulates genes required for the acquisition of cell-type specific properties (e.g. motility, shape, and size),

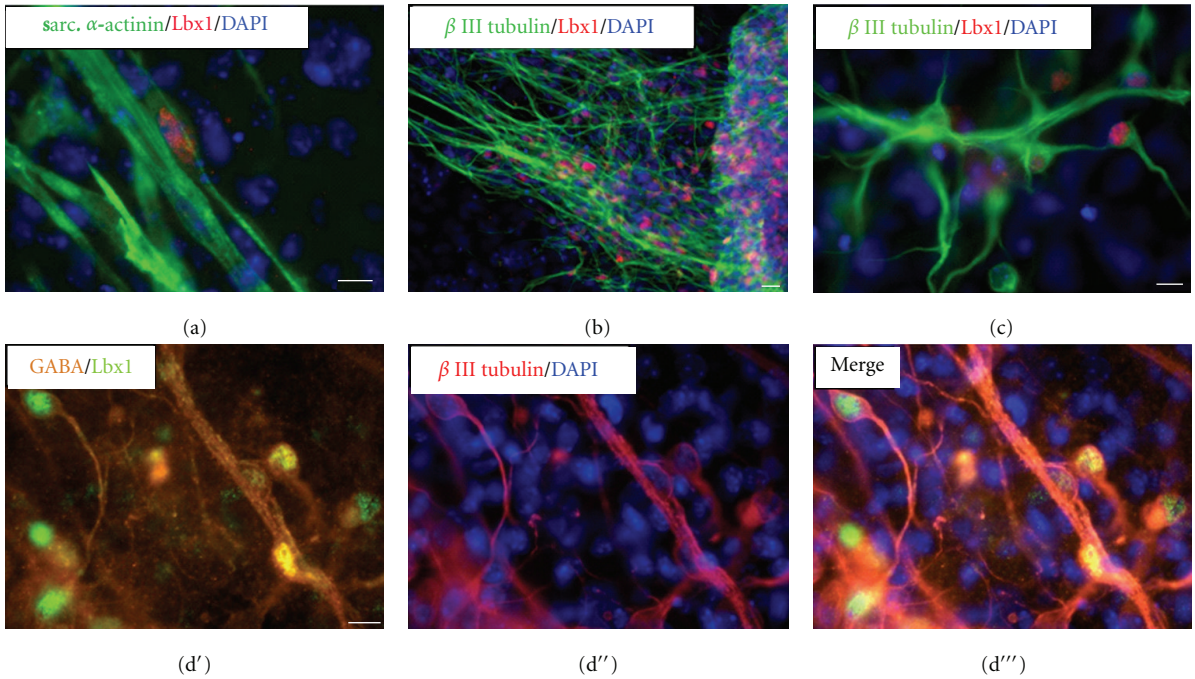


Figure 3: Immunofluorescence analysis of Lbx1 expression in ES cell-derived cells. (a) Sarcomeric α-actinin- (green)-labeled skeletal muscle cell progenitors partially costained for Lbx1 (red) at day 5 + 14 of spontaneous ES cell differentiation. (b) β III tubulin- (green)-positive neuronal cells partially coexpressed Lbx1 (red) at day 5 + 11. (c) Lbx1 staining (red) was clearly located to the nuclear region of β III tubulin- (green)-positive neurons. Triple immunofluorescence staining revealed the coexpression of (d′) Lbx1 (green) and GABA (orange) in (d″) β III tubulin- (dark red)-positive neurons. (d‴) Merged image. Nuclei were labeled with fluorescent marker DAPI (blue). Bar = 20 μm.

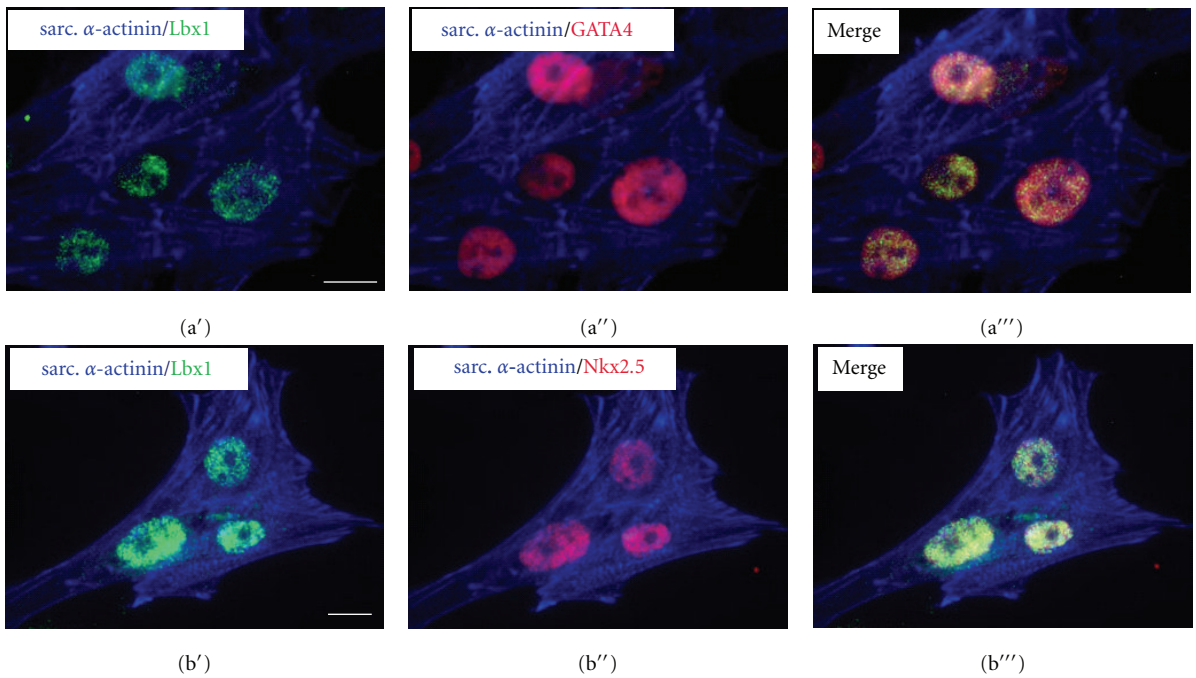


Figure 4: Immunofluorescence analysis of Lbx1 expression in ES cell-derived cardiomyocytes. (a′,b′) Sarcomeric α-actinin- (blue)-stained cardiac myocytes coexpressed Lbx1 (green) and (a″) GATA 4 (red) or (b″) Nkx2.5 (red). (a‴,b‴) Merged images. Bar = 20 μm.

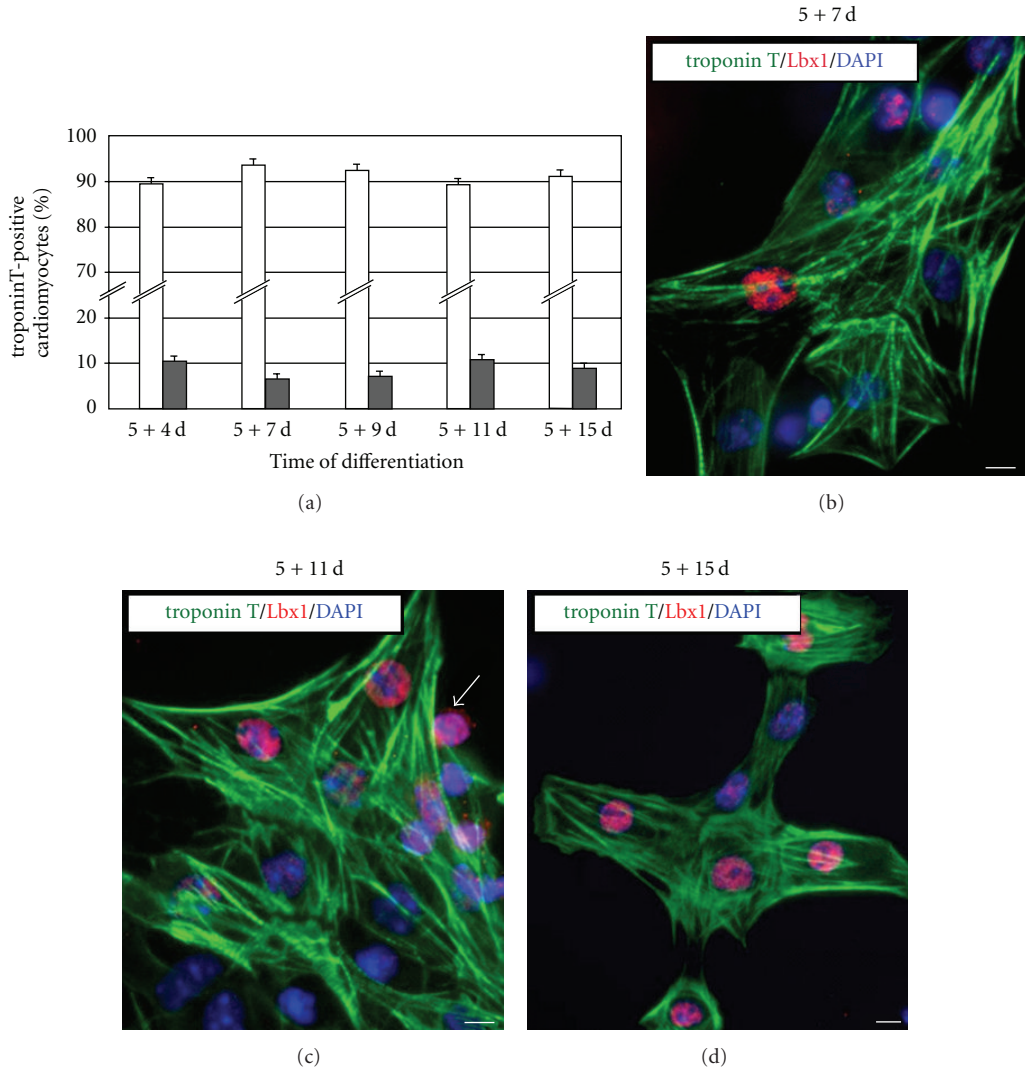


FIGURE 5: Quantification of Lbx1-expressing cardiomyocytes. (a) Percentage of Lbx1/troponin T double positive cardiomyocytes (filled bars) in relation to all troponin T-positive cardiomyocytes (open bars; approximately 1000 cells per time point) at the indicated differentiation stages. Data are shown as means ($n = 10$ experiments). Error bars represent standard error of the mean. (b)–(d) Representative double immunostainings of troponin T (green) and Lbx1 (red) at given time points. Nuclei were labeled with fluorescent marker DAPI (blue). White arrow in (c) indicates an Lbx1 expressing cell close to a cardiac cluster not costained by troponin T antibody. Bar =10 μm.

and might be involved in the regulation of genes required for terminal differentiation and the functional properties of cardiac cells [20]. A similar approach in mice would help to discover the entire function of Lbx1 in vertebrate heart development.

Taken together, our findings clearly demonstrated the expression of Lbx1 in embryonic stem cell-derived cardiomyocytes, thus providing a model system for the identification of Lbx1 target genes and signaling pathways involved in early heart failure caused by *Lbx1* inactivation.

Acknowledgment

The authors wish to thank Dr. T. Müller for providing them with the anti-Lbx1 antibody.

References

[1] K. Jagla, P. Dolle, M. G. Mattei et al., "Mouse Lbx1 and human LBX1 define a novel mammalian homeobox gene family related to the Drosophila lady bird genes," *Mechanisms of Development*, vol. 53, no. 3, pp. 345–356, 1995.

[2] T. Müller, H. Brohmann, A. Pierani et al., "The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord," *Neuron*, vol. 34, no. 4, pp. 551–562, 2002.

[3] M. A. Sieber, R. Storm, M. Martinez-de-la-Torre et al., "Lbx1 acts as a selector gene in the fate determination of somatosensory and viscerosensory relay neurons in the hindbrain," *Journal of Neuroscience*, vol. 27, no. 18, pp. 4902–4909, 2007.

[4] L. Cheng, O. A. Samad, Y. Xu et al., "*Lbx1* and *Tlx3* are opposing switches in determining GABAergic versus glutamatergic

transmitter phenotypes," *Nature Neuroscience*, vol. 8, no. 11, pp. 1510–1515, 2005.

[5] H. Brohmann, K. Jagla, and C. Birchmeier, "The role of Lbx1 in migration of muscle precursor cells," *Development*, vol. 127, no. 2, pp. 437–445, 2000.

[6] S. Watanabe, S. Kondo, M. Hayasaka, and K. Hanaoka, "Functional analysis of homeodomain-containing transcription factor Lbx1 in satellite cells of mouse skeletal muscle," *Journal of Cell Science*, vol. 120, no. 23, pp. 4178–4187, 2007.

[7] F. de Graeve, T. Jagla, J.-P. Daponte et al., "The *ladybird* homeobox genes are essential for the specification of a subpopulation of neural cells," *Developmental Biology*, vol. 270, no. 1, pp. 122–134, 2004.

[8] T. Maqbool, C. Soler, T. Jagla et al., "Shaping leg muscles in Drosophila: role of landybird, a conserved regulator of appendicular myogenesis," *PLoS ONE*, vol. 1, no. 1, article e122, 2006.

[9] K. Jagla, M. Frasch, T. Jagla, G. Dretzen, F. Bellard, and M. Bellardi, "ladybird, a new component of the cardiogenic pathway in Drosophila required for diversification of heart precursors," *Development*, vol. 124, no. 18, pp. 3471–3479, 1997.

[10] K. Schäfer, P. Neuhaus, J. Kruse, and T. Braun, "The homeobox gene *Lbx1* specifies a subpopulation of cardiac neural crest necessary for normal heart development," *Circulation Research*, vol. 92, no. 1, pp. 73–80, 2003.

[11] E. N. Olson, "Gene regulatory networks in the evolution and development of the heart," *Science*, vol. 313, no. 5795, pp. 1922–1927, 2006.

[12] A. M. Wobus and K. R. Boheler, "Embryonic stem cells: prospects for developmental biology and cell therapy," *Physiological Reviews*, vol. 85, no. 2, pp. 635–678, 2005.

[13] K. R. Boheler, J. Czyz, D. Tweedie, H.-T. Yang, S. V. Anisimov, and A. M. Wobus, "Differentiation of pluripotent embryonic stem cells into cardiomyocytes," *Circulation Research*, vol. 91, no. 3, pp. 189–201, 2002.

[14] A. Nagy, J. Rossant, R. Nagy, W. Abramow-Newerly, and J. C. Roder, "Derivation of completely cell culture-derived mice from early-passage embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 18, pp. 8424–8428, 1993.

[15] M. Wobus, K. Guan, H. T. Yang, and K. R. Boheler, "Embryonic stem cells as a model tostudy cardiac, skeletal muscle, and vascular smooth muscle cell differentiation," in *Methods in Molecular Biology*, K. Turksen, Ed., vol. 185, pp. 127–156, Humana Press, Totowa, NJ, USA, 2002.

[16] P. Chomczynski and N. Sacchi, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction," *Analytical Biochemistry*, vol. 162, no. 1, pp. 156–159, 1987.

[17] Z. Chao, J. Wu, R. Zheng, F.-E. Li, Y.-Z. Xiong, and C.-Y. Deng, "Molecular characterization and expression patterns of Lbx1 in porcine skeletal muscle," *Molecular Biology Reports*, vol. 38, no. 6, pp. 3983–3991, 2011.

[18] A. Rolletschek, I. S. Schroeder, H. Schulz, O. Hummel, N. Huebner, and A. M. Wobus, "Characterization of mouse embryonic stem cell differentiation into the pancreatic lineage in vitro by transcriptional profiling, quantitative RT-PCR and immunocytochemistry," *International Journal of Developmental Biology*, vol. 54, no. 1, pp. 41–54, 2010.

[19] M. Buckingham, L. Bajard, T. Chang et al., "The formation of skeletal muscle: from somite to limb," *Journal of Anatomy*, vol. 202, no. 1, pp. 59–68, 2003.

[20] G. Junion, L. Bataillé, T. Jagla, J. P. da Ponte, R. Tapin, and K. Jagla, "Genome-wide view of cell fate specification: ladybird acts at multiple levels during diversification of muscle and heart precursors," *Genes and Development*, vol. 21, no. 23, pp. 3163–3180, 2007.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 916180, 13 pages
doi:10.4061/2011/916180

Review Article

The Impact of Bioactive Lipids on Cardiovascular Development

Alexander Kleger,[1,2] Stefan Liebau,[3] Qiong Lin,[4] Götz von Wichert,[5] and Thomas Seufferlein[6]

[1] *Department of Internal Medicine I, University of Ulm, 89081 Ulm, Germany*
[2] *Institute of Molecular Medicine and Max-Planck-Research Group on Stem Cell Aging, University of Ulm, 89081 Ulm, Germany*
[3] *Institute for Anatomy and Cell Biology, Ulm University, 89081 Ulm, Germany*
[4] *Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University Medical School, 52074 Aachen, Germany*
[5] *Department of Internal Medicine I, Martin Luther University Halle-Wittenberg (Saale), 06120 Halle, Germany*
[6] *Department of Internal Medicine I, Division of Medicine, Martin Luther University Halle-Wittenberg (Saale), 06120 Halle, Germany*

Correspondence should be addressed to Alexander Kleger, alexander.kleger@uni-ulm.de
and Thomas Seufferlein, thomas.seufferlein@uk-halle.de

Received 15 April 2011; Accepted 5 June 2011

Academic Editor: Gabriela Kania

Lysophospholipids comprise a group of bioactive molecules with multiple biological functions. The cardinal members of this signalling molecule group are sphingosylphosphorylcholine (SPC), lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P) which are, at least in part, homologous to each other. Bioactive lipids usually act via G-protein coupled receptors (GPCRs), but can also function as direct intracellular messengers. Recently, it became evident that bioactive lipids play a role during cellular differentiation development. SPC induces mesodermal differentiation of mouse ES cells and differentiation of promyelocytic leukemia cells, by a mechanism being critically dependent on MEK-ERK signalling. LPA stimulates the clonal expansion of neurospheres from neural stem/progenitor cells and induces c-fos via activation of mitogen- and stress-activated protein kinase 1 (MSK1) in ES cells. S1P acts on hematopoietic progenitor cells as a chemotactic factor and has also been found to be critical for cardiac and skeletal muscle regeneration. Furthermore, S1P promotes cardiogenesis and similarly activates Erk signalling in mouse ES cells. Interestingly, S1P may also act to maintain human stem cell pluripotency. Both LPA and S1P positively regulate the proliferative capacity of murine ES cells. In this paper we will focus on the differential and developmental impact of lysophospholipids on cardiovascular development.

1. Origin and Synthesis

1.1. Sphingosylphosphorylcholine: Origin and Synthesis. Lysosphingomyelin or sphingosylphosphorylcholine (SPC) has been initially identified in the brain of Niemann Pick type A patients [1]. SPC acts as both an extracellular and intracellular signalling molecule. Although the origin of circulating SPC is not well characterized, activation of platelets during clotting is one likely mechanism since serum contains higher concentrations of SPC than plasma [2–4]. SPC is synthesized from either sphingomyelin or other so far unknown molecules by different metabolic pathways. In atopic dermatitis, sphingomyelin deacylase activity is enhanced generating SPC and resulting in sphingomyelin depletion. This, in turn, leads to decreased ceramide levels that may be the cause of barrier dysfunction in patients suffering from atopic dermatitis [5]. In addition, certain diseases, for example, Niemann-Pick type a that exhibit pathologic SPC accumulation in different organs are characterized by a lack of acid sphingomyelinase activity [1]. One possible explanation for this effect might be that high levels of sphingomyelin lead to an enhanced degradation by N-deacylation [3].

1.2. Sphingosine-1-Phosphate: Origin and Synthesis. Sphingosine-1-phosphate (S1P) consists of the 18-carbon base sphingosine and a phosphate group at the C1 position. Sphingosine is phosphorylated to S1P by either sphingosine kinase 1 or 2. S1P can then be metabolized by the S1P lyase to phosphoethanolamine and hexadecenal, which are subsequently metabolized into glycerophosph lipids and

phosphatidylethanolamine, respectively. Conversely, S1P-phosphohydrolase, an ectoenzyme localized in the plasma membranes of cells, regenerates sphingosine by dephosphorylating S1P [6–9]. On the other hand, sphingosine can be rephosphorylated to S1P [10]. One major source of circulating S1P is activated platelets which contain a highly active sphingosine kinase but no S1P lyase [11]. Besides platelets, red blood cells have been identified to contain high amounts of S1P [12–14]. Further S1P sources are provided by direct cellular uptake [8, 15]. Clair and coworkers demonstrated thatautotaxinhydrolyzes SPC to S1P. This exoenzyme potently induces tumor cell motility and enhances experimental (*in vitro*) metastasis as well as angiogenesis [16]. However, the physiological relevance of the conversion of SPC to S1P by this mechanism is as yet unclear, since there is no detectable S1P in mice lacking sphingosine kinase 1 and 2 [17].

1.3. Lysophosphatidic Acid: Origin and Synthesis. Lysophosphatic acid (LPA) is a glycerophospholipid with a glycerol backbone. Variability in the satellite groups generates distinct subforms of LPA with different biological functions [18–21]. LPA can be de novo synthesized either by esterification of glycerol-3-phosphate via glycerol phosphate acyltransferase (GPAT) or by 4 other metabolic pathways: (i) reduction of acyl dihydroxy acetone phosphate, (ii) phosphorylation of monoacylglycerol (MAG) by the monoacylglycerol kinase, (iii) deacylation of phosphatidic acid (PA) by PLA1 or PLA2, or (iv) via autotaxin hydrolysis of SPC [22–24]. Degradation of LPA is mediated through either dephosphorylation to MAG by lipid phosphohydrolases of the PAP type 2 family (LPP1–3) or conversion to PA by LPA acyltransferase (LPAAT) or to glycerol-3-phosphate by lysophospholipase [9], respectively.

2. Bioactive Lipid Receptors and Signalling

Bioactive lipid receptors belong to the family of G-protein coupled receptors (GPCRs) and are termed LPAR1–5, S1PR1–5, OGR1, GPR4, and GPR12, respectively [25–27]. Recently, the LPA receptor family has been extended to the GPCRs P2Y5, GPR87, and P2Y10 which have been shown to bind both S1P and LPA [28–32]. Especially, OGR1 and GPR4 are believed to have a dual function in both lipid signalling and proton sensing [33]. All GPCRs are proteins containing seven transmembrane domains with an extracellular N-terminus and an intracellular C-terminus [34, 35]. There are two arginines in the extracellular loops one and three of the S1P and LPA receptors, forming an ion pair with the phosphates of LPA and S1P. SPC receptors must have a basic and acidic residue in order to interact with the ammonium and the phosphate residues of SPC. An additional acidic residue may be required to interact with the choline base. till now, three high-affinity receptors have been identified: OGR1, GPR4, and GPR12. Most likely, a ligand-binding pocket is formed by basic and acidic amino acids in the second and third extracellular loop similar to the S1P and LPA receptors [36]. In addition, lipid GPCRs also form heterodimers with each other [37]. For further review of lipid receptor function please refer to [25]. Downstream signalling of these receptors is linked to all types of G-proteins G_s, G_i, G_q, and $G_{12/13}$, respectively [38]. Distinct G-proteins activate a variety of signalling cascades, predominantly, the MEK-ERK signalling cascade [39–41] but also crosstalk with other pathways that are ascribed with respect to bioactive lipid signalling [42].

3. Lysophospholipids and Their Impact on Cardiovascular Development

Little is known about bioactive lipids and their functions in cardiogenesis despite the fact that several lipid receptors are expressed in the cardiac derivates. Most of the so far published data regarding this link focus on acute effects mediated by the lipids. Several studies have addressed the effect of bioactive lipids on cardiac function at the signal transduction level, for example, by modulating cAMP signalling [43] and ion conduction followed by changes in intracellular ion concentrations in the heart. However, the effects of ion conduction on the sarcoplasmatic reticulum and cell membrane differ between bioactive lipids such as S1P and SPC [44]. A study by Nofer et al. has shown that lysophospholipids stimulate endothelial nitric oxide production thereby regulating the vascular tone [45]. Nitric oxide also plays a crucial role in the mobilization and function of endothelial progenitor cells, an important bone marrow-derived cell type responsible for endothelial damage repair,neovascularization and generation of collaterals [46–48]. Formation of new blood vessels is crucial for both embryonic development and homeostasis in the adult organism. Regarding the expression pattern of lipid receptors and further development of receptor knockout models, it has been revealed that bioactive lipids play an important role during blood vessel formation [49]. Therefore the following passage will focus on the effects of SPC, S1P, and LPA on mesodermal development and differentiation, predominantly cardiogenesis and angiogenesis.

3.1. Sphingosylphosphorylcholine

3.1.1. Lipid Receptors in Pluripotent Embryonic Stem Cells of Mice and Men. With respect to stem cells in particular pluripotent cells the expression of each particular receptor type varies in distinct cell lines. Our own data show expression of all S1P and SPC receptors including OGR1 and GPR4 in R1 ES cells. S1PR1–4 and GPR4 are expressed at high levels. There were no major differences in receptor expression between undifferentiated ES cells, different EB stages, or different stages of ES cell outgrowths apart from a slight decrease in the S1PR5 expression level at EB day 2. However, Rodgers and colleagues did not detect S1PR4 in CGR8 and D3 ES cells [40, 41]. To clarify this issue, we analysed several sets of published transcriptome sets for the expression of several bioactive lipid receptors in ES and iPS cells derived from mice and men. As shown in Figures 1 and 2 virtually all bioactive lipid receptors are expressed in both iPS and ES cells from both species. There is no relevant difference

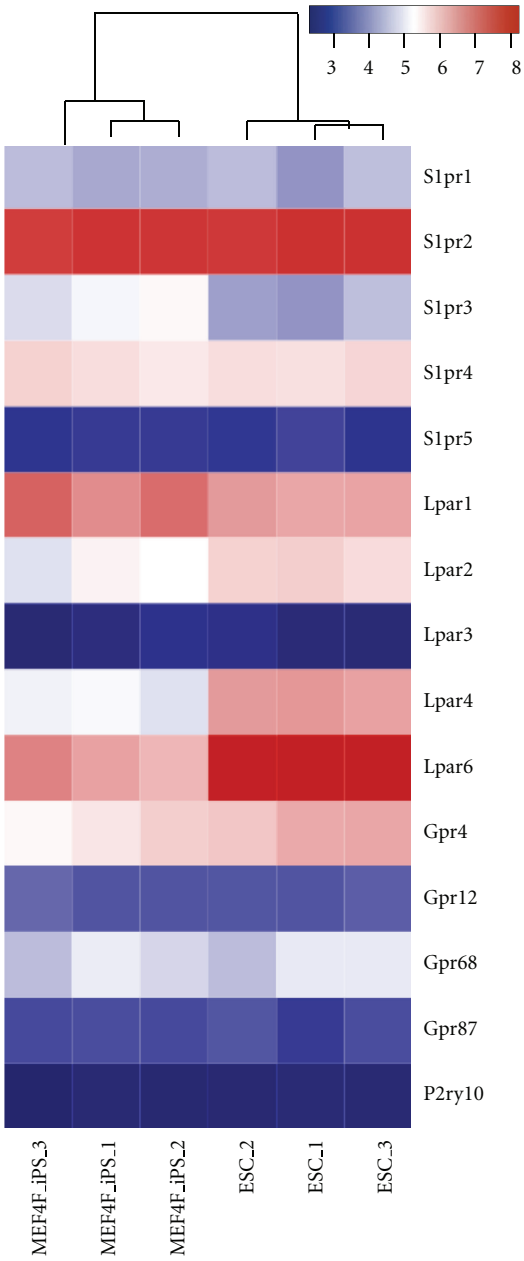


Figure 1: Lysophospholipid receptor expression in murine pluripotent stem cells. Heatmap of microarray gene expression of a list of mouse bioactive lipid receptors. Gene expression color key is shown in log2 scale. Microarray data were downloaded from the GEO database; accession numbers GSE10806.

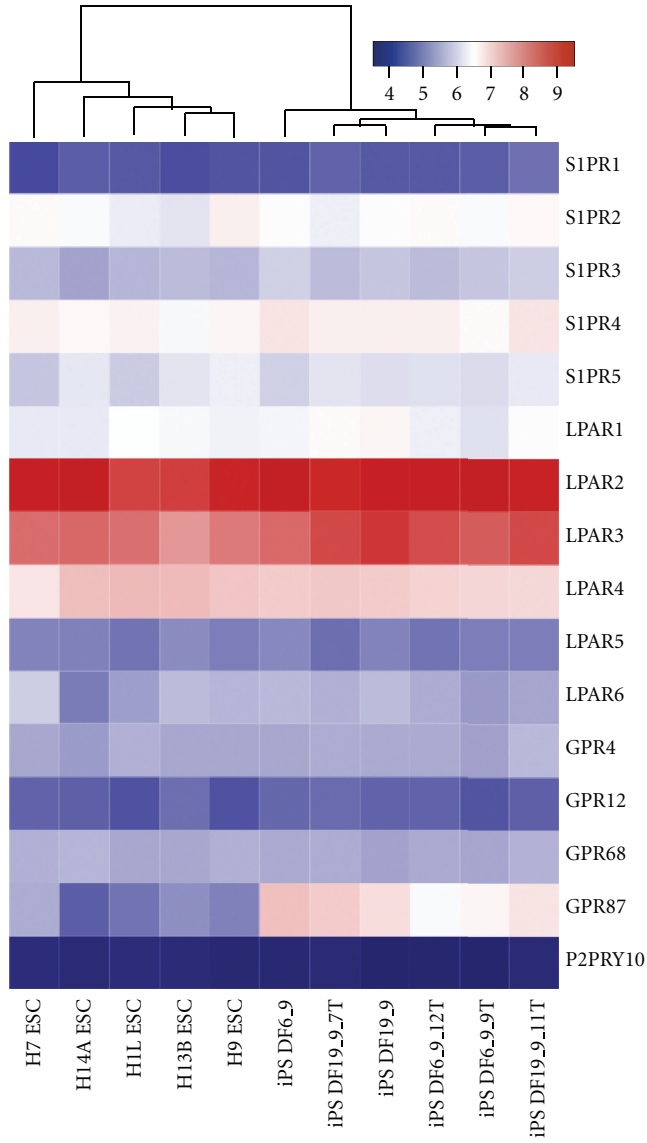


Figure 2: Lysophospholipid receptor expression in human pluripotent stem cells. Heatmap of microarray gene expression of a list of human bioactive lipid receptors. Gene expression color key is shown in log2 scale. Microarray data were downloaded from the GEO database; accession numbers GSE151716.

between the ES and iPS cells pointing to the identity of both pluripotent cell types. However, the distinct expression levels of the particular subtypes differ in pluripotent cells of either murine or human origin. Summarized, bioactive lipid receptors are widely expressed throughout pluripotent cells of mice and men. Nevertheless, the precise function of each subtype remains unclear so far.

3.1.2. SPC Induces Cardiac Differentiation of Murine Embryonic Stem Cells. To date, there are very limited data linking SPC to mesodermal or cardiac differentiation. Besides our own study using the embryonic stem cell differentiation model to characterize the role of SPC in cardiac development [40], there are few further studies showing that SPC differentiates pluripotent and multipotent stem cells [50, 51]. SPC similar to S1P stimulates cardiac ES cell differentiation at the level of both morphology and gene expression. However, there was no obvious difference regarding cardiac subtype formation since SPC increased cardiogenesis during all IVD stages [40]. Skeletal muscle cells and cardiac cells arise from the same germ layer [52]. Consequently whole germ layer induction by SPC is unlikely since otherwise both myogenic and cardiac cells would have been increased, suggesting a defined role in later stages of cardiac development, for

example, on progenitor cells. A possible candidate is the recently identified multipotent cardiovascular progenitor which gives rise to cardiomyocytes, endothelial, and vascular smooth muscle cells [52]. These cells are defined by the expression of the vascular endothelial growth factor receptor 2 (VEGFR2/FLK1) which was recently shown to crosstalk with SPC signalling especially during tube formation [42]. This assumption is supported by the finding that SPC supports vessel formation in an EB sprouting assay and furthermore induces expression of endothelial markers (A. Kleger, unpublished observations). SPC also displays proliferation inducing properties by influencing ERK-signalling [53, 54]. Also, in ES cells, SPC leads to ERK-phosphorylation, suggesting that the increased number of cardiac cells could be caused by progenitor cell proliferation (Figure 3). This is unlikely as the SPC-induced gene expression profiles were both atrial (alpha-myosin heavy chain) and ventricular (myosin light chain, MLC-2v). Realtime PCR analysis of both transcripts exhibited similar expression profiles supporting the progenitor cell hypothesis. Simply increased proliferation would not increase specialized cardiac cells marked by increased MLC-2v expression. However, there are data that both SPC and S1P share a common cardiac phenotype.

Similarly, as discussed in detail below, S1P treatment of EBs increases cardiac differentiation [55]. In addition, a developmental study using the zebra fish model revealed a S1P receptor as a relevant candidate during cardiogenesis (see also S1P and cardiac development). This implicates a kind of "family affair" with respect to cardiac differentiation.

3.1.3. SPC Plays an Important Role in Angiogenesis. Further evidence linking SPC to mesodermal development and differentiation is given by the effect of SPC on angiogenesis. SPC induces differentiation of endothelial cells into capillary-like structures *in vitro* in addition to migration and chemotaxis of vascular smooth muscle cells [56, 57]. Supporting evidence for a crucial role of SPC in angiogenesis arises from studies investigating its receptor GPR4. SPC induces GPR4-dependent vessel formation *in vitro* from HUVEC cells [42]. GPR4 deficiency leads to leaky blood vessels (e.g. spontaneous hemorrhages, dilated, tortuous subcutaneous blood vessels, and defective vascular smooth muscle cell coverage) during development and the receptor functions in blood vessels as a pH sensor [49]. S1P and SPC activate distinct MAP kinase isoforms and increase $[Ca^{2+}]_i$ via different mechanisms in rat cerebral arteries. This does not affect the ability of both compounds to activate CREB, although this occurs via different pathways [58]. For further information please refer to [59] where the involved signalling cascades are discussed in more detail. Piao and colleagues used a rat aortic ring assay. SPC significantly stimulated the sprouting of endothelial cells from the aortic ring and markedly enhanced the chemotactic migration and capillary-like tube formation. This effect was dependent on a urokinase-type plasminogen activator (uPA), an important regulator of angiogenesis [60].

3.1.4. SPC Modulates Differentiation of Several Stem Cell Populations. Besides end-differentiation of already committed endothelial and vascular smooth muscle cells, SPC also modulates proliferation and differentiation of multipotent adult stem cells. Jeon and colleagues investigated SPC effects in a series of manuscripts on human adipose-tissue-derived mesenchymal stem cells (hATSCs). In the first study Jeon et al. showed induction of apoptosis under involvement of the mitochondrial death pathway by SPC in a concentration-dependent manner (>10 μM SPC). This effect was critically dependent on ERK activity [61]. In lower concentrations the compound induced proliferation of the same cell type in a JNK-dependent manner without any involvement of the ERK pathway [51]. A further study revealed SPC as a differentiation-inducing agent for hATSCs to smooth muscle cells. The authors discovered a new signalling pathway for SPC through Gi/o-ERK-dependent autocrine secretion of TGF-beta, which activates a Smad2-SRF/myocardin-dependent pathway (Figure 3). Despite the fact that hATSCs do not express any known SPC receptors, in all studies SPC effects seemed to be receptor dependent. This suggests either a crosstalk with further signalling cascades (e.g., TGF-beta or VEGF signalling) or involvement of so far unknown SPC receptors. Another possible explanation would be an SPC-stimulated release of other bioactive lipids like S1P or LPA via, for example, autotoxin [50]. The most recent study of the group identified fibronectin (FN) as an agent produced by hATSC after SPC treatment via the pathway described above. Since fibronectin is essential for cell recruitment and adhesion during wound healing and angiogenesis, the authors concluded that SPC-induced FN expression plays a pivotal role in wound healing by stimulating adhesion and recruitment of leukocytes [62]. Furthermore, SPC-mediated TGF-beta release seems not to be restricted to hATSCs, as also endothelial cells react with release of the anti-inflammatory cytokine after HDL treatment including SPC and S1P as active compounds [63]. Therefore different local SPC concentrations in distinct microenvironments and niches seem to regulate the effect of the bioactive lipid.

In summary there are many studies suggesting that SPC plays a pivotal role in mesodermal development with respect to cardiogenesis and angiogenesis. Furthermore the key role of SPC in angiogenesis is defined by coordinated migration of both endothelial cells and vascular smooth muscle cells in response to the changing gradients of this bioactive lipid messenger. SPC effects on both cardiac and vascular development could be caused by modulation of a common progenitor as described in [52].

3.2. Sphingosine-1-Phosphate

3.2.1. Cardiovascular Receptor Expression. Expression studies of the S1P receptor family suggest the particular importance of S1P in the cardiovascular system. Lung and heart show the highest overall expression of S1PR1, S1PR2, and S1PR3 [64, 65]. S1PR2 and S1PR3 receptors expression is generally lower than that of S1PR1 receptors in the heart, although distribution within cardiac subregions (e.g., ventricle, septum and atrium) is similar [66, 67]. Beyond that, S1P receptors are expressed with a distinct expression pattern in the different aortic cell types. High expression levels of S1P

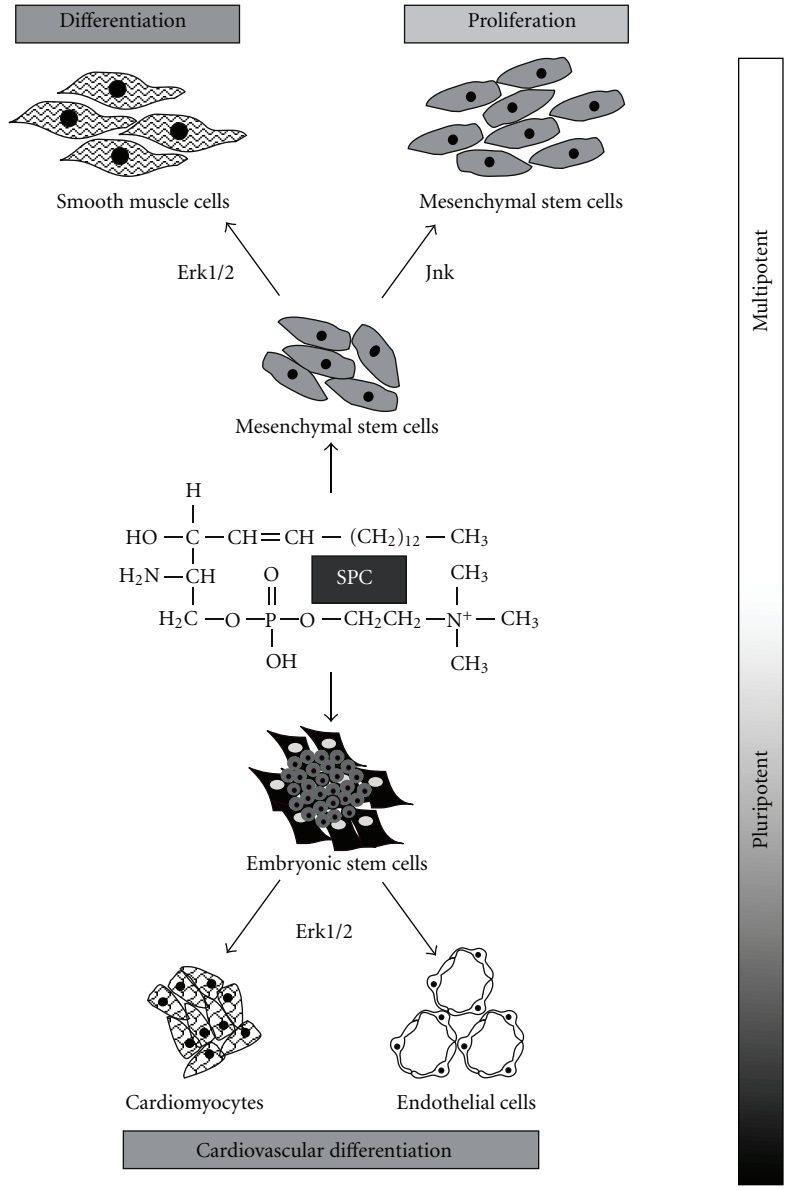


Figure 3: Schematic illustration of SPC-induced differentiation in murine multipotent and pluripotent stem cells. Signaling pathways mediating the respected phenotype are noted next to black arrows.

receptors seem to be a general characteristic of endothelial cells [67–69]. Most of the work was done to clarify acute effects of S1P and other bioactive lipids on the cardiovascular system, for example, the role of S1P during protection of heart ischemia has been evaluated in several studies [70–72]. Besides developmental functions and ion channel modulation, S1P induces cardiomyocyte hypertrophy mainly via the S1PR1 receptor and subsequently via a G protein through the ERK pathway and via the Rho pathway [73].

3.2.2. S1P and Cardiac Development In Vivo. A developing action field of bioactive lipids is their role in promoting cardiomyocyte survival and their contribution to ischemic preconditioning. There are several lines of evidence suggesting that *in vivo* levels of bioactive lipids influence cardiomyocyte survival and regeneration. For example, the stress-activated enzyme S1P lyase (SPL) which metabolizes S1P has been identified as therapeutic target for ischemia/reperfusion injury of the heart [74, 75]. Inhibition of SPL results in elevated levels of S1P [74]. In aging hearts S1P exhibits cardioprotective effects during cardiac injury in a pre- and postconditioning experimental setup [75]. Indeed, local S1P levels seem to regulate not only acute but also developmental aspects of S1P. The first clue for a link between S1P and cardiac development/differentiation was given by a study from Kupperman et al. in the zebrafish model. The authors

investigated the zebrafish analogon of S1PR2, miles apart (mil), and found that mil specifically affects the migration of heart precursors to the midline resulting in cardia bifida. Cross-transplantation studies suggest that S1P provides clues for the fusion of the bilateral heart progenitors by generating an environment permissive for migration [76]. This was further investigated by Kawahara et al. who found that a mutant of the sphingolipid transporter 2 (spns2) leads to a similar cardiac defect as the mil mutant. They also could show that spns2 is involved in the secretion of S1P and thereby regulating myocardial precursor migration (Figure 4, [77]). Further evidence is given by means of a primary culture system of embryonic zebrafish cells. The interaction of cardiac precursors with fibronectin, that is, a major component of the extracellular matrix, seems to be critical for precursor migration [78]. Recently, a report using a mouse *ex vivo* culture system underlined inalienability of S1P for cardiac development in a mammalian system. Wendler and Rivkees showed that both increased and decreased S1P levels alter cardiac development by reduced mesenchyme formation. While decreased S1P levels induce cell death, increased S1P levels inhibit cellular migration, both resulting in a reduced amount of mesenchymal transformed cells. S1PR2 was found to be the most likely candidate for mediating these effects [79]. Interestingly, Osborne et al. detected another player involved in the signalling and trafficking of S1P, namely, two of hearts (toh), a spinster homologue. They found that the mutations are causative of a cardiac phenotype comparable to that of S1P dysfunction [80]. In general, developmental acquisition of tolerance to environmental stress may be contingent upon acquisition of protective biochemical mechanisms (e.g., S1P-mediated cellular survival) or simply due to increased cell numbers (e.g., S1P-induced cellular proliferation) [81]. Both issues are eventually targeted by S1P during development [44, 82–84]. Regarding intracellular signalling VEGF could be a possible target of S1P. VEGF activates the sphingosine kinase and increases S1P levels in human umbilical vein endothelial cells [85]. Chang and colleagues showed that a distinct regulation pattern of VEGF is essential for regular valve formation and morphogenesis [86]. These data indicate that S1P could be a possible candidate for indirect mediation of VEGF signalling by induction of sphingosine kinase. Therefore, lipid signalling and especially accurate regulation of local concentration in this particular niche are indispensable for proper function during mammalian heart development and cellular differentiation into cardiac tissue.

3.2.3. S1P Signalling during Vasculogenesis-Importance of VEGF Crosstalk. VEGF is probably the most important regulator of angiogenesis in both physiological and pathological vessel formation. There are several studies supporting a crosstalk of VEGF and bioactive lipid signalling. However, this process seems to be rather complicated: several mechanisms, for example, transactivation, upregulation, or downstream activation, are propagated [44]. Igarashi and colleagues observed that VEGF induces S1PR1 expression. This process consequently leads, to enhanced intracellular signalling responses to S1P and the potentiation of S1P-mediated vasorelaxation. S1P and VEGF signalling leads to, for example, eNOS regulation which in turn sensitizes the vascular endothelium to the effects of lipid mediators by promoting the induction of S1P1 receptors [87]. Furthermore, sphingosine kinase induction has been reported by VEGF with subsequently altered Ras signalling [85]. Endo et al. found that S1P induces membrane ruffling of human umbilical vein endothelial cells via the vascular endothelial growth factor receptor 2 (VEGFR-2) [88]. Most recently, another study proposed that VEGF induces S1PR3 induction which is necessary for downstream AKT3 signalling [89]. In summary, there is a well-documented, but still unclear crosstalk between two angiogenic signalling pathways, which is not surprising, given the fact that hypoxia, a consequence of insufficient vessel formation, induces VEGF. This issue can be clearly followed in a study of Chae et al. They showed that S1PR1 knockout mice suffer from defect limb formation due to insufficient vessels formation caused by impaired S1P signalling via S1PR1 leading to hypoxia and VEGF induction with secondary insufficient hypervascularisation [90].

3.2.4. S1P Receptors Decide on the Angiogenic Phenotype In Vivo. S1P seems to function on all levels of vascular development. Lee and colleagues showed S1P induced tube formation in umbilical vein endothelial cells by means of a matrigel assay [91]. In several further studies this effect has been proven in different model systems and with distinct endothelial cell types. Tube formation was critically dependent on ERK signalling [44, 92, 93]. Recently, HDL proteins have been implicated in serving as a platform for the delivery of S1P. Using reconstituted high-density lipoprotein (rHDL), Matsuo and colleagues investigated the same effects as induced by direct incubation with S1P [94].

The last step of angiogenesis is the recruitment of mural cells, for example, pericytes in capillaries and vascular smooth muscle cells of arteries [95]. Distinct functions for S1P in both smooth muscle cells and mural cells have been reported: S1P modulates and increases smooth muscle cell migration and differentiation by Rho Kinase [96] and by RhoA-mediated activation of serum response factor and involvement of the SRF cofactor, myocardin-related transcription factor A (MRTF-A). S1P also moderately stimulated SMC proliferation, a process that was dependent upon ERK and involved activation of another SRF cofactor, Elk-1 [97]. S1PR1 null mice exhibited embryonic haemorrhage leading to intrauterine death around embryonic day 13 and incomplete limb development. Interestingly, these animals displayed normal vasculogenesis and angiogenesis but failed to recruit both vascular smooth muscle cells to arteries and pericytes to capillaries [98]. In the limbs, lack of S1PR1 results in aberrant chondrocyte condensation and irregular digit morphogenesis. The authors concluded that defective vascular development induced by lack of S1PR1 caused hypoxia. Secondary to hypoxia, HIF1 alpha and VEGF are induced and lead to hypervascularisation, ultimately disturbing limb morphogenesis [90]. In a later study, Allende and colleagues using a conditional S1PR1 knockout mouse model targeting endothelial cells found that S1PR1 null endothelium is defective in mural cell recruitment [99].

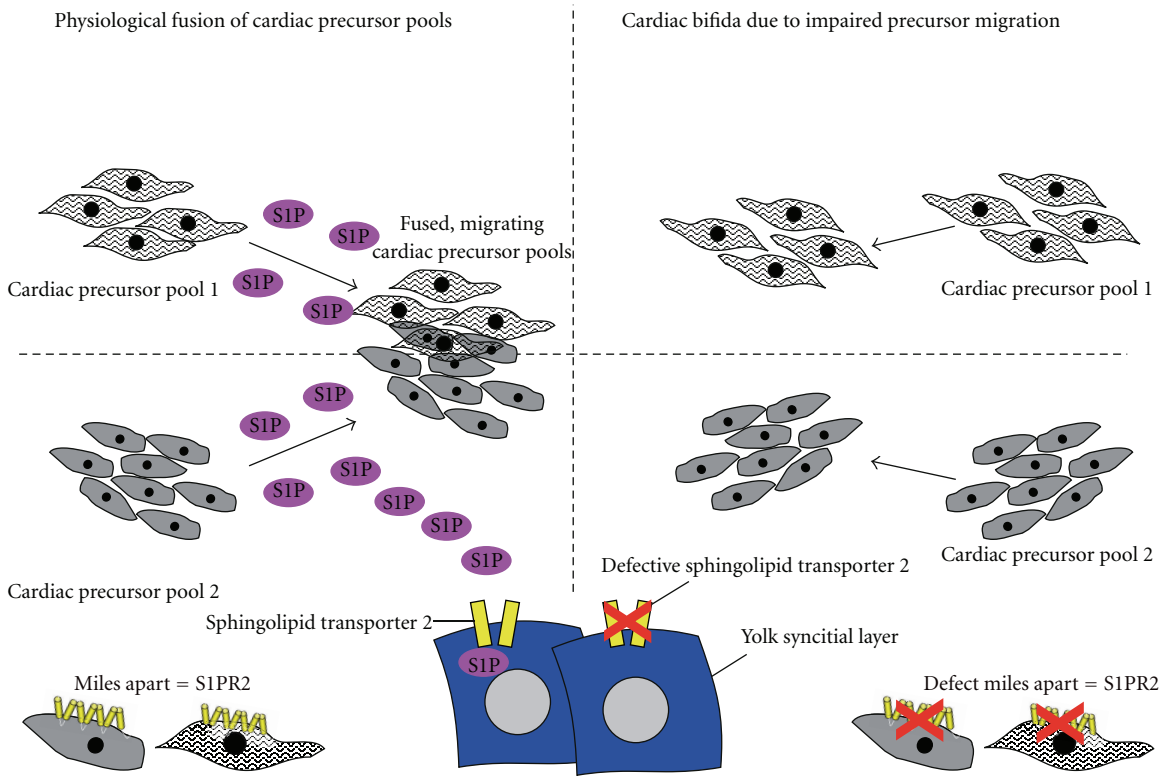


FIGURE 4: *In vivo* model of S1P function and its respective cofactors in the developing zebrafish.

S1P signalling on endothelial cells and bone marrow-derived stromal cells *in vitro* results in differentiation, proliferation, migration, and tube formation. Different studies revealed that especially S1PR1 but also S1PR2 and -3 to be crucial for these effects [94, 100]. Since S1PR1 null animals displayed normal vasculogenesis and angiogenesis, it is likely that there is functional redundancy among members of the S1P receptor. This hypothesis is underlined by the fact that S1PR2 and -3 null mice reveal no apparent anatomical or physiological defects [64, 101]. Kono and colleagues faced this issue and created subsequently different combinations of S1PR1–3 double null mice and triple null mice. They found that S1PR1 S1PR2 double null and S1PR1 S1PR2 S1PR3 triple null embryos displayed a substantially more severe vascular phenotype than embryos lacking only S1PR1. All these embryos completed vasculogenesis regularly but displayed immature vessels in the brain indicating that the S1PR1 and S1PR2 receptors are required for some aspects of vessel development during angiogenesis. In addition, the researchers found partial embryonic lethality and vascular abnormalities in S1PR2 S1PR3 double null embryos. Ultrastructural analysis of microvessels in the S1PR2 S1PR3 double null mice showed abnormal endothelial cells with thin cell bodies, suggesting that these vessels may be fragile and prone to rupture [102]. However, despite no obvious anatomical defects in either S1PR2 or S1PR3 null mice, S1PR2 null mice exhibit early life deafness which is associated with pathologic changes within the cochlea. Most likely this phenotype is caused by vascular disturbance within the stria vascularis. Additionally, it was reported, that S1PR2 knockout mice display a vascular dysfunction affecting the renal, mesenterical, and, furthermore, aortic hemodynamics [103]. S1PR2 S1PR3 double null mice display stronger defects even leading to head tilt while S1PR3 null mice do not share this phenotype [104]. These data further support the hypothesis of overlapping S1P receptor function with both partial redundancy and unique receptor properties. Apart from that there is growing evidence that S1PR2 also participates in pathological neovascularisation. Skoura et al. [105] found that S1PR2 null mice display reduced neovascularisation in response to ischemia-driven retinopathy. Furthermore, the inflammatory reaction that is usually concomitant to ischemic retinopathy was suppressed in mice lacking S1PR2. Therefore S1PR2 antagonism could be a potential target for treatment of pathologic neovascularisation [105].

3.2.5. S1P and the Recruitment of Stem/Progenitor Cells. Homing is the first process in which circulating hematopoietic cells actively cross the blood-bone-marrow-endothelium barrier and stay at least temporarily in the bone marrow compartment to undergo self-renewal. There is overwhelming evidence that circulating progenitor cells are responsible for vascular healing and remodelling under physiological and pathological conditions [106]. Usually stem/progenitor cells persist in a quiescent state in their niche. In case of angiogenesis or neovascularisation specific signals initiate differentiation, further mobilization, and homing of vascular progenitor cells [107]. Seitz et al. could already show

that S1P is acting on hematopoietic progenitor cells as chemoattractant via S1PR1. Given the fact that activated platelets represent a major source of extracellular S1P, stem cell homing may occur at sites of tissue injury in addition to the bone marrow [108]. Walter et al. found that incubation of patient-derived endothelial progenitor cells (EPCs) with S1P or its synthetic analogue FTY720 improved blood flow recovery in ischemic hind limbs. This phenomenon was critically dependent on S1PR3 activity since it was abolished in S1P3 null mice [109]. In a recent study from Donati and colleagues, S1P was found to be essential for mesoangioblast proliferation and survival [110]. In summarize, S1P is likely to act on different immature cell types regarding the mesendodermal germ layer.

3.3. Lysophosphatidic Acid. Similar to the other bioactive lipids LPA seems to be involved in modulation of cardiomyocyte signalling. Recently, elevated serum LPA concentrations in patients with myocardial infarction have been reported, indicating a role of LPA in cardiac pathophysiology [111]. In cultured neonatal cardiomyocytes, LPA induces via LPA_1/LPA_3 hypertrophy under involvement of AKT and NFκB signalling [112, 113]. Furthermore, it prevents hypoxia-induced apoptosis in cardiomyocytes similar to S1P and SPC [82]. Hence, LPA is likely to play a role in left ventricular remodelling. Nevertheless, differentiation inducing effects of LPA in terms of cardiac development remain to be established. So far, a developmental role of LPA is restricted to the nervous system where LPA plays an elementary function during neurogenesis.

3.3.1. LPA Functions Similarly But Not Equally as S1P during Vasculogenesis. Regarding vessel formation LPA seems to induce similar but not equal effects compared to S1P. For example, LPA has been shown to induce expression of MMPs and migration of endothelial cells, both steps known to be critical for vessel formation [114, 115]. However, LPA affects MMP induction and migration to a much lower extent than S1P though it most likely uses the same signalling pathways. Given the fact that LPA is a low-affinity ligand of, for example, the S1P receptor [116], it could be possible that LPA effects on endothelial cells are driven by S1P receptors [100, 114, 115, 117]. In mesenchymal stem cells LPA fails to induce tube formation but promotes vascular network formation in murine E8.5 allantois explants, still, in both studies less efficaciously than S1P [100, 118]. Despite controversial *in vitro* data, *in vivo* studies of receptor or enzyme knockouts affecting LPA signalling showed gross abnormalities including in angiogenesis. Homozygous LPAR1 null mice showed 50% neonatal lethality and reduced size caused by impaired suckling behaviour. Beside the neuronal phenotype LPAR1 null mice also suffered from craniofacial dysmorphism, uniformly notable by short snouts and widely spaced eyes. This phenotype is most likely due to loss of LPAR1 during development of facial bone tissue, where expression is usually strong [119]. In addition, a small group of the knock out animals displayed frontal hematoma. This could be explained by improper vasculogenesis, similar to the phenotype of S1P-receptor knock out studies showing abnormal limb/bone development [90]. However, given the small number of LPAR1 null pups developing frontal hematoma, functional angiogenic redundancy is likely also true for LPA receptors. This issue is underlined by data achieved from LPAR1 and LPAR2 double null animals showing a more severe phenotype. Therefore LPAR1 and LPAR2 are not essential for proper development but most likely involved in mediation of LPA-induced angiogenic signals [120]. In contrast, a recent study identified LPAR4 as a critical regulator of the structure and function of blood and lymphatic vessels [121]. The overall not severe phenotype of all single LPA receptor null animals stands in striking contrast with autotaxin null animals [122]. Lack of autotaxin leads to embryonic lethality around E9.5 with profound vascular defects in yolk sac and the embryos resemble the phenotype of animals lacking both small G-protein G_{12} and G_{13}. These two G-proteins are major mediators of LPA receptor signalling [123]. Furthermore, ATX-deficient embryos showed allantois malformation, neural tube defects, and asymmetric headfolds [122]. However, developmental dysfunction is not only due to lack of LPA but also to excess shown by knock out studies of lipid phosphate phosphatases (LPPs), a group of enzymes involved in lipid phosphate biosynthesis. Escalante-Alcalde and colleagues disrupted the LPP3 gene leading to increased LPA levels. LPPR3 null mice failed to form a chorioallantoic placenta and yolk sac vasculature. Furthermore, some embryos exhibited shortening of the anterior-posterior axis and frequent duplication of axial structures [124]. To summarize this section, LPA and its distinct receptors are most likely, similar to the S1P-S1P receptor axis, functionally redundant and therefore only triple or quadruple knock outs would resemble the phenotype of ATX null animals and mirror, as major LPA synthesizer, an LPA-deficient phenotype. Hence, it seems crystal clear that developmental importance of LPA is not restricted to the neuronal systems but rather widely affects mammalian development.

4. Conclusion

There are several lines of evidence pointing to lysophospholipids or bioactive lipids as important regulators of stem cell differentiation *in vitro* and cardiovascular development *in vivo*. This has been established by a variety of studies using either knock out mouse models or embryonic stem cells as tools to recapitulate cardiovascular development. In particular S1P seems to be crucial for this particular system while LPA seems to be more pronounced in the nervous system. However, the SPC role in this process seems to be underdeveloped so far and needs further studies to dissect its developmental impact more precisely.

Abbreviations

SPC: Sphingosylphosphorylcholine
LPA: Lysophosphatidic acid
S1P: Sphingosine 1-phosphate
S1PR: Sphingosine 1-phosphate receptor
GPCRs: G-protein coupled receptors
MAG: Monoacylglycerol

LPAR: Lysophosphatidic acid receptor
LPPs: Lipid phosphate phosphatases
ATX: Autotaxin
hATSC: Human adipose-tissue-derived mesenchymal stem cell
HUVEC: Human umbilical vein endothelial cell.

Authors' Contribution

A. Kleger and S. Liebau contributed equally to this paper.

References

[1] C. Rodriguez-Lafrasse and M. T. Vanier, "Sphingosylphosphorylcholine in Niemann-Pick disease brain: accumulation in type A but not in type B," *Neurochemical Research*, vol. 24, no. 2, pp. 199–205, 1999.

[2] K. Liliom, G. Sun, M. Bünemann et al., "Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors," *Biochemical Journal*, vol. 355, no. 1, pp. 189–197, 2001.

[3] D. Meyer zu Heringdorf, H. M. Himmel, and K. H. Jakobs, "Sphingosylphosphorylcholine—biological functions and mechanisms of action," *Biochimica et Biophysica Acta*, vol. 1582, no. 1–3, pp. 178–189, 2002.

[4] N. Murata, K. Sato, J. Kon, H. Tomura, and F. Okajima, "Quantitative measurement of sphingosine 1-phosphate by radioreceptor- binding assay," *Analytical Biochemistry*, vol. 282, no. 1, pp. 115–120, 2000.

[5] J. Hara, K. Higuchi, R. Okamoto, M. Kawashima, and G. Imokawa, "High-expression of sphingomyelin deacylase is an important determinant of ceramide deficiency leading to barrier disruption in atopic dermatitis," *Journal of Investigative Dermatology*, vol. 115, no. 3, pp. 406–413, 2000.

[6] B. M. Buehrer and R. M. Bell, "Sphingosine kinase: properties and cellular functions," *Advances in Lipid Research*, vol. 26, pp. 59–67, 1993.

[7] H. Le Stunff, S. Milstien, and S. Spiegel, "Generation and metabolism of bioactive sphingosine-1-phosphate," *Journal of Cellular Biochemistry*, vol. 92, no. 5, pp. 882–899, 2004.

[8] A. Olivera and S. Spiegel, "Sphingosine kinase: a mediator of vital cellular functions," *Prostaglandins and Other Lipid Mediators*, vol. 64, no. 1–4, pp. 123–134, 2001.

[9] J. D. Saba and T. Hla, "Point-counterpoint of sphingosine 1-phosphate metabolism," *Circulation Research*, vol. 94, no. 6, pp. 724–734, 2004.

[10] D. N. Brindley, "Lipid phosphate phosphatases and related proteins: signaling functions in development, cell division, and cancer," *Journal of Cellular Biochemistry*, vol. 92, no. 5, pp. 900–912, 2004.

[11] Y. Yatomi, F. Ruan, S. I. Hakomori, and Y. Igarashi, "Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets," *Blood*, vol. 86, no. 1, pp. 193–202, 1995.

[12] C. Bode, S. C. Sensken, U. Peest et al., "Erythrocytes serve as a reservoir for cellular and extracellular sphingosine 1-phosphate," *Journal of Cellular Biochemistry*, vol. 109, no. 6, pp. 1232–1243, 2010.

[13] R. Pappu, S. R. Schwab, I. Cornelissen et al., "Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate," *Science*, vol. 316, no. 5822, pp. 295–298, 2007.

[14] P. Hanel, P. Andreani, and M. H. Graler, "Erythrocytes store and release sphingosine 1-phosphate in blood," *The FASEB Journal*, vol. 21, pp. 1202–1209, 2007.

[15] L. C. Boujaoude, C. Bradshaw-Wilder, C. Mao et al., "Cystic fibrosis transmembrane regulator regulates uptake of sphingoid base phosphates and lysophosphatidic acid: modulation of cellular activity of sphingosine 1-phosphate," *Journal of Biological Chemistry*, vol. 276, no. 38, pp. 35258–35264, 2001.

[16] T. Clair, J. Aoki, E. Koh et al., "Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate," *Cancer Research*, vol. 63, no. 17, pp. 5446–5453, 2003.

[17] K. Mizugishi, T. Yamashita, A. Olivera, G. F. Miller, S. Spiegel, and R. L. Proia, "Essential role for sphingosine kinases in neural and vascular development," *Molecular and Cellular Biology*, vol. 25, no. 24, pp. 11113–11121, 2005.

[18] K. Bandoh, J. Aoki, A. Taira, M. Tsujimoto, H. Arai, and K. Inoue, "Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors," *FEBS Letters*, vol. 478, no. 1-2, pp. 159–165, 2000.

[19] A. Tokumura, Y. Kanaya, M. Kitahara, M. Miyake, Y. Yoshioka, and K. Fukuzawa, "Increased formation of lysophosphatidic acids by lysophospholipase D in serum of hypercholesterolemic rabbits," *Journal of Lipid Research*, vol. 43, no. 2, pp. 307–315, 2002.

[20] A. Tokumura, K. Tominaga, K. Yasuda, H. Kanzaki, K. Kogure, and K. Fukuzawa, "Lack of significant differences in the corrected activity of lysophospholipase D, producer of phospholipid mediator lysophosphatidic acid, in incubated serum from women with and without ovarian tumors," *Cancer*, vol. 94, no. 1, pp. 141–151, 2002.

[21] E. J. Van Corven, A. Van Rijswijk, K. Jalink, R. L. Van Der Bend, W. J. Van Blitterswijk, and W. H. Moolenaar, "Mitogenic action of lysophosphatidic acid and phosphatidic acid on fibroblasts. Dependence on acyl-chain length and inhibition by suramin," *Biochemical Journal*, vol. 281, part 1, pp. 163–169, 1992.

[22] C. Pagès, M. F. Simon, P. Valet, and J. S. Saulnier-Blache, "Lysophosphatidic acid synthesis and release," *Prostaglandins and Other Lipid Mediators*, vol. 64, no. 1–4, pp. 1–10, 2001.

[23] C. Pagès, M. F. Simon, P. Valet, and J. S. Saulnier-Blache, "Lysophosphatidic acid synthesis and release," *Prostaglandins and Other Lipid Mediators*, vol. 64, no. 1–4, pp. 1–10, 2001.

[24] M. Umezu-Goto, Y. Kishi, A. Taira et al., "Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production," *Journal of Cell Biology*, vol. 158, no. 2, pp. 227–233, 2002.

[25] D. Meyer zu Heringdorf and K. H. Jakobs, "Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism," *Biochimica et Biophysica Acta*, vol. 1768, no. 4, pp. 923–940, 2007.

[26] W. H. Moolenaar, L. A. Van Meeteren, and B. N. G. Giepmans, "The ins and outs of lysophosphatidic acid signaling," *BioEssays*, vol. 26, no. 8, pp. 870–881, 2004.

[27] S. Spiegel and S. Milstien, "Sphingosine-1-phosphate: an enigmatic signalling lipid," *Nature Reviews Molecular Cell Biology*, vol. 4, no. 5, pp. 397–407, 2003.

[28] Y. Shimomura, M. Wajid, Y. Ishii et al., "Disruption of P2RY5, an orphan G protein-coupled receptor, underlies

autosomal recessive woolly hair," *Nature Genetics*, vol. 40, no. 3, pp. 335–339, 2008.

[29] S. M. Pasternack, I. Von Kügelgen, K. A. Aboud et al., "G protein-coupled receptor P2Y5 and its ligand LPA are involved in maintenance of human hair growth," *Nature Genetics*, vol. 40, no. 3, pp. 329–334, 2008.

[30] K. Yanagida, K. Masago, H. Nakanishi et al., "Identification and characterization of a novel lysophosphatidic acid receptor, p2y5/LPA6," *Journal of Biological Chemistry*, vol. 284, no. 26, pp. 17731–17741, 2009.

[31] K. I. Tabata, K. Baba, A. Shiraishi, M. Ito, and N. Fujita, "The orphan GPCR GPR87 was deorphanized and shown to be a lysophosphatidic acid receptor," *Biochemical and Biophysical Research Communications*, vol. 363, no. 3, pp. 861–866, 2007.

[32] M. Murakami, A. Shiraishi, K. Tabata, and N. Fujita, "Identification of the orphan GPCR, P2Y10 receptor as the sphingosine-1-phosphate and lysophosphatidic acid receptor," *Biochemical and Biophysical Research Communications*, vol. 371, no. 4, pp. 707–712, 2008.

[33] H. Tomura, C. Mogi, K. Sato, and F. Okajima, "Proton-sensing and lysolipid-sensitive G-protein-coupled receptors: a novel type of multi-functional receptors," *Cellular Signalling*, vol. 17, no. 12, pp. 1466–1476, 2005.

[34] R. M. Eglen, "Emerging concepts in GPCR function—the influence of cell phenotype on GPCR pharmacology," *Proceedings of the Western Pharmacology Society*, vol. 48, pp. 31–34, 2005.

[35] R. M. Eglen, R. Bosse, and T. Reisine, "Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening," *Assay and Drug Development Technologies*, vol. 5, no. 3, pp. 425–451, 2007.

[36] A. Ignatov, J. Lintzel, I. Hermans-Borgmeyer et al., "Role of the G-protein-coupled receptor GPR12 as high-affinity receptor for sphingosylphosphorylcholine and its expression and function in brain development," *Journal of Neuroscience*, vol. 23, no. 3, pp. 907–914, 2003.

[37] A. Zaslavsky, L. S. Singh, H. Tan, H. Ding, Z. Liang, and Y. Xu, "Homo- and hetero-dimerization of LPA/S1P receptors, OGR1 and GPR4," *Biochimica et Biophysica Acta*, vol. 1761, no. 10, pp. 1200–1212, 2006.

[38] H. Rosen, P. J. Gonzalez-Cabrera, M. G. Sanna, and S. Brown, "Sphingosine 1-phosphate receptor signaling," *Annual Review of Biochemistry*, vol. 78, pp. 743–768, 2009.

[39] E. Afrasiabi, T. Blom, E. Ekokoski, R. K. Tuominen, and K. Törnquist, "Sphingosylphosphorylcholine enhances calcium entry in thyroid FRO cells by a mechanism dependent on protein kinase C," *Cellular Signalling*, vol. 18, no. 10, pp. 1671–1678, 2006.

[40] A. Kleger, T. Busch, S. Liebau et al., "The bioactive lipid sphingosylphosphorylcholine induces differentiation of mouse embryonic stem cells and human promyelocytic leukaemia cells," *Cellular Signalling*, vol. 19, no. 2, pp. 367–377, 2007.

[41] A. Rodgers, D. Mormeneo, J. S. Long, A. Delgado, N. J. Pyne, and S. Pyne, "Sphingosine 1-phosphate regulation of extracellular signal-regulated kinase-1/2 in embryonic stem cells," *Stem Cells and Development*, vol. 18, no. 9, pp. 1319–1330, 2009.

[42] K. S. Kim, J. Ren, Y. Jiang et al., "GPR4 plays a critical role in endothelial cell function and mediates the effects of sphingosylphosphorylcholine," *FASEB Journal*, vol. 19, no. 7, pp. 819–821, 2005.

[43] M. R. Steiner, J. R. Urso, J. Klein, and S. M. Steiner, "Multiple astrocyte responses to lysophosphatidic acids," *Biochimica et Biophysica Acta*, vol. 1582, no. 1–3, pp. 154–160, 2002.

[44] A. E. Alewijnse, S. L. M. Peters, and M. C. Michel, "Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites," *British Journal of Pharmacology*, vol. 143, no. 6, pp. 666–684, 2004.

[45] J. R. Nofer, M. Van Der Giet, M. Tölle et al., "HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3," *Journal of Clinical Investigation*, vol. 113, no. 4, pp. 569–581, 2004.

[46] P. D. Lambiase, R. J. Edwards, P. Anthopoulos et al., "Circulating humoral factors and endothelial progenitor cells in patients with differing coronary collateral support," *Circulation*, vol. 109, no. 24, pp. 2986–2992, 2004.

[47] J. M. Isner, C. Kalka, A. Kawamoto, and T. Asahara, "Bone marrow as a source of endothelial cells for natural and iatrogenic vascular repair," *Annals of the New York Academy of Sciences*, vol. 953, pp. 75–84, 2001.

[48] U. Landmesser, N. Engberding, F. H. Bahlmann et al., "Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase," *Circulation*, vol. 110, no. 14, pp. 1933–1939, 2004.

[49] L. V. Yang, C. G. Radu, M. Roy et al., "Vascular abnormalities in mice deficient for the G protein-coupled receptor GPR4 that functions as a pH sensor," *Molecular and Cellular Biology*, vol. 27, no. 4, pp. 1334–1347, 2007.

[50] E. S. Jeon, H. J. Moon, M. J. Lee et al., "Sphigosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like through a TGF-β-dependent mechanism," *Journal of Cell Science*, vol. 119, no. 23, pp. 4994–5005, 2006.

[51] E. S. Jeon, H. Y. Song, M. R. Kim et al., "Sphingosylphosphorylcholine induces proliferation of human adipose tissue-derived mesenchymal stem cells via activation of JNK," *Journal of Lipid Research*, vol. 47, no. 3, pp. 653–664, 2006.

[52] S. J. Kattman, T. L. Huber, and G. Keller, "Multipotent Flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages," *Developmental Cell*, vol. 11, no. 5, pp. 723–732, 2006.

[53] T. Seufferlein and E. Rozengurt, "Sphingosylphosphorylcholine rapidly induces tyrosine phosphorylation of p125(FAK) and paxillin, rearrangement of the actin cytoskeleton and focal contact assembly. Requirement of p21(rho) in the signaling pathway," *Journal of Biological Chemistry*, vol. 270, no. 41, pp. 24343–24351, 1995.

[54] T. Seufferlein and E. Rozengurt, "Sphingosylphosphorylcholine activation of mitogen-activated protein kinase in Swiss 3T3 cells requires protein kinase C and a pertussis toxin-sensitive G protein," *Journal of Biological Chemistry*, vol. 270, no. 41, pp. 24334–24342, 1995.

[55] A. Sachinidis, C. Gissel, D. Nierhoff et al., "Identification of plateled-derived growth factor-BB as cardiogenesis- inducing factor in mouse embryonic stem cells under serum-free conditions," *Cellular Physiology and Biochemistry*, vol. 13, no. 6, pp. 423–429, 2003.

[56] G. Boguslawski, J. R. Grogg, Z. Welch et al., "Migration of vascular smooth muscle cells induced by sphingosine 1-phosphate and related lipids: potential role in the angiogenic response," *Experimental Cell Research*, vol. 274, no. 2, pp. 264–274, 2002.

[57] G. Boguslawski, D. Lyons, K. A. Harvey, A. T. Kovala, and D. English, "Sphingosylphosphorylcholine induces endothelial cell migration and morphogenesis," *Biochemical and Biophysical Research Communications*, vol. 272, no. 2, pp. 603–609, 2000.

[58] F. A. Mathieson and G. F. Nixon, "Sphingolipids differentially regulate mitogen-activated protein kinases and intracellular Ca^{2+} in vascular smooth muscle: effects on CREB activation," *British Journal of Pharmacology*, vol. 147, no. 4, pp. 351–359, 2006.

[59] D. G. Hemmings, "Signal transduction underlying the vascular effects of sphingosine 1-phosphate and sphingosylphosphorylcholine," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 373, no. 1, pp. 18–29, 2006.

[60] Y. J. Piao, C. H. Lee, M. J. Zhu et al., "Involvement of urokinase-type plasminogen activator in sphingosylphosphorylcholine-induced angiogenesis," *Experimental Dermatology*, vol. 14, no. 5, pp. 356–362, 2005.

[61] E. S. Jeon, Y. J. Kang, H. Y. Song et al., "Role of MEK-ERK pathway in sphingosylphosphorylcholine-induced cell death in human adipose tissue-derived mesenchymal stem cells," *Biochimica et Biophysica Acta*, vol. 1734, no. 1, pp. 25–33, 2005.

[62] H. J. Moon, E. S. Jeon, Y. M. Kim, M. J. Lee, C. K. Oh, and J. H. Kim, "Sphingosylphosphorylcholine stimulates expression of fibronectin through TGF-β1-smad-dependent mechanism in human mesenchymal stem cells," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 6, pp. 1224–1234, 2007.

[63] G. D. Norata, E. Callegari, M. Marchesi, G. Chiesa, P. Eriksson, and A. L. Catapano, "High-density lipoproteins induce transforming growth factor- β2 expression in endothelial cells," *Circulation*, vol. 111, no. 21, pp. 2805–2811, 2005.

[64] I. Ishii, B. Friedman, X. Ye et al., "Selective loss of sphingosine 1-phosphate signaling with No obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP B3/EDG-3," *Journal of Biological Chemistry*, vol. 276, no. 36, pp. 33697–33704, 2001.

[65] Q. Zhang, O. Peyruchaud, K. J. French, M. K. Magnusson, and D. F. Mosher, "Sphingosine 1-phosphate stimulates fibronectin matrix assembly through a Rho-dependent signal pathway," *Blood*, vol. 93, no. 9, pp. 2984–2990, 1999.

[66] H. M. Himmel, D. Meyer Zu Heringdorf, E. Graf et al., "Evidence for Edg-3 receptor-mediated activation of I(K.ACh) by sphingosine-1-phosphate in human atrial cardiomyocytes," *Molecular Pharmacology*, vol. 58, no. 2, pp. 449–454, 2000.

[67] D. Mazurais, P. Robert, B. Gout, I. Berrebi-Bertrand, M. P. Laville, and T. Calmels, "Cell type-specific localization of human cardiac S1P receptors," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 5, pp. 661–670, 2002.

[68] T. Kimura, T. Watanabe, K. Sato et al., "Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3," *Biochemical Journal*, vol. 348, part 1, pp. 71–76, 2000.

[69] F. Wang, J. R. Van Brooklyn, J. P. Hobson et al., "Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis," *Journal of Biological Chemistry*, vol. 274, no. 50, pp. 35343–35350, 1999.

[70] C. K. Means, C. Y. Xiao, Z. Li et al., "Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 6, pp. H2944–H2951, 2007.

[71] G. Theilmeier, C. Schmidt, J. Herrmann et al., "High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor," *Circulation*, vol. 114, no. 13, pp. 1403–1409, 2006.

[72] J. Zhang, N. Honbo, E. J. Goetzl, K. Chatterjee, J. S. Karliner, and M. O. Gray, "Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 293, no. 5, pp. H3150–H3158, 2007.

[73] P. Robert, P. Tsui, M. P. Laville et al., "EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 9, pp. 1589–1606, 2001.

[74] P. Bandhuvula, N. Honbo, G. Y. Wang et al., "S1P lyase (SPL): a novel therapeutic target for ischemia/reperfusion injury of the heart," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 300, no. 5, pp. H1753–H1761, 2011.

[75] D. A. Vessey, M. Kelley, L. Li, and Y. Huang, "Sphingosine protects aging hearts from ischemia/reperfusion injury: superiority to sphingosine 1-phosphate and ischemic pre- and post-conditioning," *Oxidative Medicine and Cellular Longevity*, vol. 2, no. 3, pp. 146–151, 2009.

[76] E. Kupperman, S. An, N. Osborne, S. Waldron, and D. Y. R. Stainier, "A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development," *Nature*, vol. 406, no. 6792, pp. 192–195, 2000.

[77] A. Kawahara, T. Nishi, Y. Hisano, H. Fukui, A. Yamaguchi, and N. Mochizuki, "The sphingolipid transporter Spns2 functions in migration of zebrafish myocardial precursors," *Science*, vol. 323, no. 5913, pp. 524–527, 2009.

[78] T. Matsui, A. Raya, C. Callol-Massot et al., "Miles-apart-Mediated regulation of cell-fibronectin interaction and myocardial migration in zebrafish," *Nature Clinical Practice Cardiovascular Medicine*, vol. 4, supplement 1, pp. S77–S82, 2007.

[79] C. C. Wendler and S. A. Rivkees, "Sphingosine-1-phosphate inhibits cell migration and endothelial to mesenchymal cell transformation during cardiac development," *Developmental Biology*, vol. 291, no. 2, pp. 264–277, 2006.

[80] N. Osborne, K. Brand-Arzamendi, E. A. Ober et al., "The spinster homolog, two of hearts, is required for sphingosine 1-phosphate signaling in zebrafish," *Current Biology*, vol. 18, no. 23, pp. 1882–1888, 2008.

[81] J. L. Edwards, W. A. King, S. J. Kawarsky, and A. D. Ealy, "Responsiveness of early embryos to environmental insults: potential protective roles of HSP70 and glutathione," *Theriogenology*, vol. 55, no. 1, pp. 209–223, 2001.

[82] J. S. Karliner, N. Honbo, K. Summers, M. O. Gray, and E. J. Goetzl, "The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes," *Journal of Molecular and Cellular Cardiology*, vol. 33, no. 9, pp. 1713–1717, 2001.

[83] S. Pyne and N. J. Pyne, "Sphingosine 1-phosphate signalling in mammalian cells," *Biochemical Journal*, vol. 349, no. 2, pp. 385–402, 2000.

[84] K. Inniss and H. Moore, "Mediation of apoptosis and proliferation of human embryonic stem cells by sphingosine-1-phosphate," *Stem Cells and Development*, vol. 15, no. 6, pp. 789–796, 2006.

[85] X. Shu, W. Wu, R. D. Mosteller, and D. Broek, "Sphingosine kinase mediates vascular endothelial growth factor-induced activation of ras and mitogen-activated protein kinases," *Molecular and Cellular Biology*, vol. 22, no. 22, pp. 7758–7768, 2002.

[86] C. P. Chang, J. R. Neilson, J. H. Bayle et al., "A field of myocardial-endocardial NFAT signaling underlies heart valve morphogenesis," *Cell*, vol. 118, no. 5, pp. 649–663, 2004.

[87] J. Igarashi, P. A. Erwin, A. P. V. Dantas, H. Chen, and T. Michel, "VEGF induces S1P1 receptors in endothelial cells: implications for cross-talk between sphingolipid and growth factor receptors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 19, pp. 10664–10669, 2003.

[88] A. Endo, K. I. Nagashima, H. Kurose, S. Mochizuki, M. Matsuda, and N. Mochizuki, "Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells via vascular endothelial growth factor receptor and CrkII," *Journal of Biological Chemistry*, vol. 277, no. 26, pp. 23747–23754, 2002.

[89] C. B. Fieber, J. Eldridge, T. A. Taha, L. M. Obeid, and R. C. Muise-Helmericks, "Modulation of total Akt kinase by increased expression of a single isoform: requirement of the sphingosine-1-phosphate receptor, Edg3/S1P3, for the VEGF-dependent expression of Akt3 in primary endothelial cells," *Experimental Cell Research*, vol. 312, no. 7, pp. 1164–1173, 2006.

[90] S. S. Chae, J. H. Paik, M. L. Allende, R. L. Proia, and T. Hla, "Regulation of limb development by the sphingosine 1-phosphate receptor S1p1/EDG-1 occurs via the hypoxia/VEGF axis," *Developmental Biology*, vol. 268, no. 2, pp. 441–447, 2004.

[91] M. J. Lee, S. Thangada, K. P. Claffey et al., "Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate," *Cell*, vol. 99, no. 3, pp. 301–312, 1999.

[92] K. J. Bayless and G. E. Davis, "Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices," *Biochemical and Biophysical Research Communications*, vol. 312, no. 4, pp. 903–913, 2003.

[93] S. I. Miura, H. Tanigawa, Y. Matsuo, M. Fujino, A. Kawamura, and K. Saku, "Ras/Raf1-dependent signal in sphingosine-1-phosphate-induced tube formation in human coronary artery endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 306, no. 4, pp. 924–929, 2003.

[94] Y. Matsuo, S. I. Miura, A. Kawamura, Y. Uehara, K. A. Rye, and K. Saku, "Newly developed reconstituted high-density lipoprotein containing sphingosine-1-phosphate induces endothelial tube formation," *Atherosclerosis*, vol. 194, no. 1, pp. 159–168, 2007.

[95] W. Risau and I. Flamme, "Vasculogenesis," *Annual Review of Cell and Developmental Biology*, vol. 11, pp. 73–91, 1995.

[96] K. A. Harvey, Z. Welch, D. Sliva, and R. A. Siddiqui, "Role of Rho kinase in sphingosine 1-phosphate-mediated endothelial and smooth muscle cell migration and differentiation," *Molecular and Cellular Biochemistry*, vol. 342, no. 1-2, pp. 7–19, 2010.

[97] K. Lockman, J. S. Hinson, M. D. Medlin, D. Morris, J. M. Taylor, and C. P. Mack, "Sphingosine 1-phosphate stimulates smooth muscle cell differentiation and proliferation by activating separate serum response factor co-factors," *Journal of Biological Chemistry*, vol. 279, no. 41, pp. 42422–42430, 2004.

[98] Y. Liu, R. Wada, T. Yamashita et al., "Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation," *Journal of Clinical Investigation*, vol. 106, no. 8, pp. 951–961, 2000.

[99] M. L. Allende, T. Yamashita, and R. L. Proia, "G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation," *Blood*, vol. 102, no. 10, pp. 3665–3667, 2003.

[100] B. Annabi, S. Thibeault, Y. T. Lee et al., "Matrix metalloproteinase regulation of sphingosine-1-phosphate-induced angiogenic properties of bone marrow stromal cells," *Experimental Hematology*, vol. 31, no. 7, pp. 640–649, 2003.

[101] A. J. MacLennan, P. R. Carney, W. J. Zhu et al., "An essential role for the H218/AGR16/Edg-5/LPB2 sphingosine 1-phosphate receptor in neuronal excitability," *European Journal of Neuroscience*, vol. 14, no. 2, pp. 203–209, 2001.

[102] M. Kono, Y. Mi, Y. Liu et al., "The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis," *Journal of Biological Chemistry*, vol. 279, no. 28, pp. 29367–29373, 2004.

[103] J. N. Lorenz, L. J. Arend, R. Robitz, R. J. Paul, and A. J. MacLennan, "Vascular dysfunction in S1P2 sphingosine 1-phosphate receptor knockout mice," *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology*, vol. 292, pp. R440–R446, 2007.

[104] M. Kono, I. A. Belyantseva, A. Skoura et al., "Deafness and stria vascularis defects in S1P2 receptor-null mice," *Journal of Biological Chemistry*, vol. 282, no. 14, pp. 10690–10696, 2007.

[105] A. Skoura, T. Sanchez, and K. Claffey, "Essential role of sphingosine 1-phosphate receptor 2 in pathological angiogenesis of the mouse retina," *The Journal of Clinical Investigation*, vol. 117, no. 9, pp. 2506–2516, 2007.

[106] M. Sata, "Role of circulating vascular progenitors in angiogenesis, vascular healing, and pulmonary hypertension: lessons from animal models," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, pp. 1008–1014, 2006.

[107] M. Hristov and C. Weber, "Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance," *Journal of Cellular and Molecular Medicine*, vol. 8, no. 4, pp. 498–508, 2004.

[108] G. Seitz, A. M. Boehmler, L. Kanz, and R. Möhle, "The role of sphingosine 1-phosphate receptors in the trafficking of hematopoietic progenitor cells," *Annals of the New York Academy of Sciences*, vol. 1044, pp. 84–89, 2005.

[109] D. H. Walter, U. Rochwalsky, J. Reinhold et al., "Sphingosine-1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR4-dependent signaling pathway via the S1P3 receptor," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, pp. 275–282, 2007.

[110] C. Donati, F. Cencetti, P. Nincheri et al., "Sphingosine 1-phosphate mediates proliferation and survival of mesoangioblasts," *Stem Cells*, vol. 25, no. 7, pp. 1713–1719, 2007.

[111] X. Chen, X. Y. Yang, N. D. Wang et al., "Serum lysophosphatidic acid concentrations measured by dot immunogold filtration assay in patients with acute myocardial infarction," *Scandinavian Journal of Clinical & Laboratory Investigation*, vol. 63, pp. 497–503, 2003.

[112] J. Chen, Y. Chen, W. Zhu et al., "Specific LPA receptor subtype mediation of LPA-induced hypertrophy of cardiac myocytes and involvement of Akt and NFkappaB signal pathways," *Journal of Cellular Biochemistry*, vol. 103, no. 6, pp. 1718–1731, 2008.

[113] R. Hilal-Dandan, C. K. Means, A. B. Gustafsson et al., "Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho," *Journal of Molecular and Cellular Cardiology*, vol. 36, no. 4, pp. 481–493, 2004.

[114] T. S. Panetti, D. F. Hannah, C. Avraamides et al., "Extracellular matrix molecules regulate endothelial cell migration stimulated by lysophosphatidic acid," *Journal of Thrombosis and Haemostasis*, vol. 2, pp. 1645–1656, 2004.

[115] W. T. Wu, C. N. Chen, C. I. Lin, J. H. Chen, and H. Lee, "Lysophospholipids enhance matrix metalloproteinase-2 expression in human endothelial cells," *Endocrinology*, vol. 146, no. 8, pp. 3387–3400, 2005.

[116] M. J. Lee, S. Thangada, C. H. Liu, B. D. Thompson, and T. Hla, "Lysophosphatidic acid stimulates the G-protein-coupled receptor EDG-1 as a low affinity agonist," *Journal of Biological Chemistry*, vol. 273, no. 34, pp. 22105–22112, 1998.

[117] S. Langlois, D. Gingras, and R. Béliveau, "Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation," *Blood*, vol. 103, no. 8, pp. 3020–3028, 2004.

[118] K. M. Argraves, B. A. Wilkerson, W. S. Argraves, P. A. Fleming, L. M. Obeid, and C. J. Drake, "Sphingosine-1-phosphate signaling promotes critical migratory events in vasculogenesis," *Journal of Biological Chemistry*, vol. 279, no. 48, pp. 50580–50590, 2004.

[119] J. J. A. Contos, N. Fukushima, J. A. Weiner, D. Kaushal, and J. Chun, "Requirement for the lp(A1) lysophosphatidic acid receptor gene in normal suckling behavior," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 24, pp. 13384–13389, 2000.

[120] J. J. A. Contos, I. Ishii, N. Fukushima et al., "Characterization of lpa2 (Edg4) and lpa1/lpa2 (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa2," *Molecular and Cellular Biology*, vol. 22, no. 19, pp. 6921–6929, 2002.

[121] H. Sumida, K. Noguchi, Y. Kihara et al., "LPA4 regulates blood and lymphatic vessel formation during mouse embryogenesis," *Blood*, vol. 116, no. 23, pp. 5060–5070, 2010.

[122] L. A. Van Meeteren, P. Ruurs, C. Stortelers et al., "Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development," *Molecular and Cellular Biology*, vol. 26, no. 13, pp. 5015–5022, 2006.

[123] S. Offermanns, V. Mancino, J. P. Revel, and M. I. Simon, "Vascular system defects and impaired cell chemokinesis as a result of Gα13 deficiency," *Science*, vol. 275, no. 5299, pp. 533–536, 1997.

[124] D. Escalante-Alcalde, L. Hernandez, H. Le Stunff et al., "The lipid phosphatase LPP3 regulates extra-embryonic vasculogenesis and axis patterning," *Development*, vol. 130, no. 19, pp. 4623–4637, 2003.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 310928, 8 pages
doi:10.4061/2011/310928

Review Article

Stem Cells for Cardiac Repair: Status, Mechanisms, and New Strategies

Ren Mingliang, Zhang Bo, and Wang Zhengguo

Department 4, State Key Laboratory of Trauma, Burn and Combined Injury, Research Institute of Surgery and Daping Hospital, Third Military Medical University, Chongqing 400042, China

Correspondence should be addressed to Zhang Bo, zhangbo67184@gmail.com

Received 11 January 2011; Revised 3 March 2011; Accepted 10 April 2011

Academic Editor: Gabriela Kania

Faced with the end stage of heart disease, the current treatments only slow worsening of heart failure. Stem cells have the potential of self-renewal and differentiation. It is expected to replace and repair damaged myocardium. But many clinical trials have shown that the stem cell therapy of heart failure is modest or not effective. The possible causes for the limited effects of stem cell in curing heart failure are the stem cells which have been transplanted into the ischemic heart muscle may suffer low survival rate, affected by inflammatory molecules, proapoptotic factor, and lack of nutrients and oxygen, and then the stem cells which home and have been completely transplanted to the site of myocardial infarction become very small. Therefore, through preconditioning of stem cells and appropriate choice of genes for mesenchymal stem cell modification to improve the survival rate of stem cells, ability in homing and promoting angiogenesis may become the newly effective strategies for the application of stem cells therapy in heart failure.

1. Introduction

With the changes in lifestyle and aging of population, the morbidity of hypertension, coronary heart disease, and other common cardiovascular disease has shown a continuous rising tendency. As the end stage of cardiovascular disease, heart failure suffers high morbidity and poor prognosis. Heart failure was mentioned on 277 193 death certificates and was the underlying cause in 56 565 of those deaths in the United States in 2007 [1, 2]. According to a random sampling survey to 15,518 residents aging from 35 to 74 in China in 2003, the prevalence of heart failure was 0.9%. It was lower than developed countries. However, the total prevalence was still up to 4,000,000, and the incidence was increasing ceaselessly [3]. Current drug treatment can only improve symptoms without preventing the ventricular remodeling and the deterioration of progressive heart function. Heart transplantation is an effective means of treating patients with heart failure. But the vast majority of patients are restricted by the age, the donor, surgical complications, medical costs, and so forth.

Stem cells are the origin cells of various mature cells. They have the potential of self-renewal and differentiation. Either immediately after isolation or after expansion in vitro, stem cells are transplanted into a specific region of the heart, and ultimately replace, repair the myocardial necrosis or pathological cells; then the aim of curing heart failure can be achieved and it has brought a bright prospect for the treatment of heart failure. Although the basic research on the differentiation of stem cell transplantation has not yet achieved consistent results, many clinical trials regarding the stem cell transplantation for acute and chronic heart failure have been carried out [4]. The results suggest that stem cells therapy improve the clinical symptoms modestly, but almost have no effect in preventing ventricular remodeling and long-term prognosis.

2. Clinical Experience

Currently, the main types of stem cells used for clinical treatment include bone marrow-derived cells (BMCs), adipose-derived stem cells (ADSCs), cardiac stem cells (CSCs), peripheral blood derived cells, embryonic stem cells (ES), and induced pluripotent stem cells (Figure 1). Autologous skeletal myoblast is one of the stem cells which have been

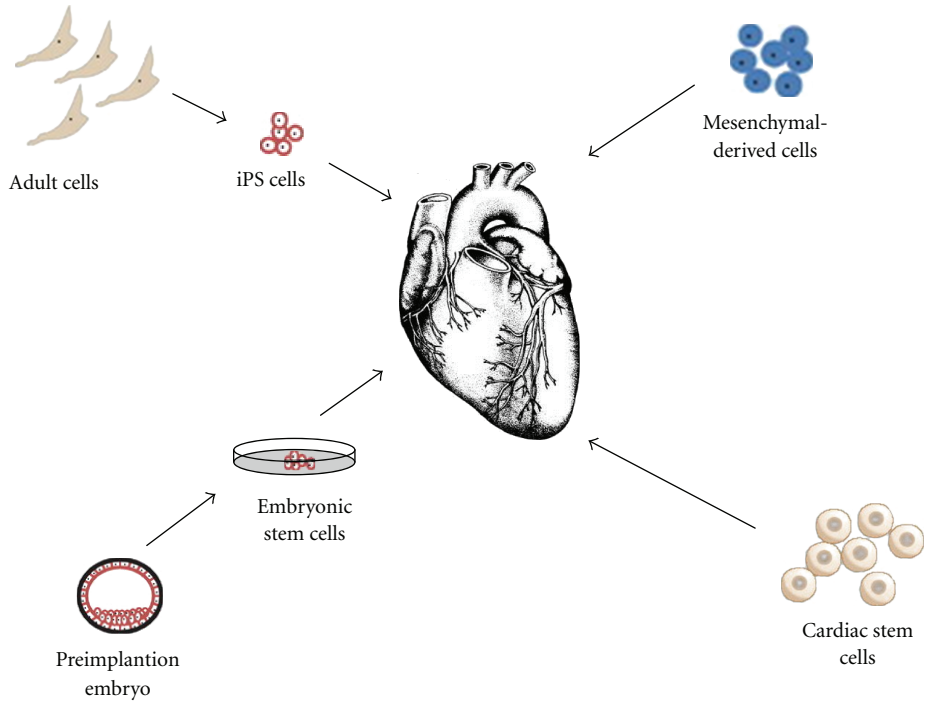


Figure 1: The cell types used in heart repair.

firstly used in myocardial regeneration. It can counteract ischemia and has the ability of regeneration after damage. However, skeletal myoblasts cannot form an effective electrical coupling synchronization with the living myocardial cells and may lead to malignant arrhythmia [5]. It has been shown that ES transplantation can improve cardiac contractile function and still have proliferative capacity. However, ethical debate of embryonic stem cells, immune rejection, and the risk of tumorigenicity still hinder its application.

2.1. Bone Marrow-Derived Cells. BMC is currently the most widely used cells in clinical trials (Table 1). It mainly contains mesenchymal stem cells (MSCs), hematopoietic stem cells, border cells, endothelial progenitor cells, and so forth. Although, the acquisition of BMC induces pain and the number of BMCs decreases dramatically with age, it is relatively simple to separate and large numbers of cells can be obtained without the need for ex vivo expansion. It provides initial cells with many mixed components and mutidifferentiation potential. Furthermore, transplantation of autologous cells avoids immune rejection and ethical disputes caused by embryonic stem cells transplantation. REPAIR-AMI [6] multicenter randomized controlled trial included 204 patients with acute myocardial infarction. After 3~7 days' reperfusion therapy, they received the autologous bone marrow-derived mononuclear cells (BMMNCs) intracoronary or placebo (medium) treatment. 4 months later, the treatment group left ventricular ejection fraction (LVEF) improved slightly. Compared with the control group, there were statistically significant differences. However, in one-year-followup, the cardiac indicators associated with remodeling had no significant changes. Subgroup analysis showed that only patients with large myocardial infarction had a marked effect. European STAR-heart [7] examined the stem cells treatment for chronic heart failure. 391 patients with chronic heart failure patients due to ischemic heart disease (myocardial infarction) were included, diagnostic criteria for LVEF $\leqslant$ 35%. BMMNCs directly infused to related artery infarction through angioplasty balloon catheter. Effects on the patients were evaluated after treatment of 3 months to 5 years. LVEF, cardiac index, exercise capacity, oxygen uptake, and left ventricular contractility had improved significantly. In addition, in the first assessment after 3-month treatment the beneficial effects observed lasted to 12 months and 60 months, while there was a phenomenon of deterioration of left ventricular function in the control group at the same time. Compared with the control group, long-term mortality of patients treated with stem cells significantly decreased.

Although the results of many experiments show that BMC transplantation can improve LVEF (compared with the control group, only about 3%), there are no obvious long-term effects [17]. Recent meta-analysis of a series of published clinical trials before 2009 also found that although coronary artery transplanted stem cells can significantly reduce left ventricular end systolic volume in patients with acute myocardial infarction, they have no significant effect on left ventricular end-diastolic volume [19]. In the randomized controlled BOOST trial [12], patients with ST segment elevation after acute myocardial infarction received an intracoronary BMC infusion. Results showed that LVEF had improved after 6 months, but there were no significant differences compared with the control group after 18 months. In 5-year followup, all indicators did not find differences with the control group. However, according to the current trials, the rate of adverse effect with autologous BMC transplantation is relatively low, and compared with conventional treatment, the

Table 1: Randomized controlled bone marrow-derived cell trials in myocardial infarction.

Studies	Mean age (years)	Patients randomized (patients followup)	LVEF baseline (%)	Followup (month)	Dose	Assessment method	Outcome
Chen et al. (2004) [8]	58	69 (69)	49	6	6×10^{10}	Echo	EF increased 18%
REPAIR-AMI (2006) [9]	56	204 (187)	47.6	12	2.4×10^{8}	Angiography	EF increased 2.5%
ASTAMI (2006) [10]	57.4	100 (100)	46.3	6	8.7×10^{7}	Echo/SPECT/MRI	No effect
TCT-STAMI (2006) [11]	58.5	20 (20)	56	6	4×10^{7}	Echo/SPECT	EF ↑ 6.7%
BOOST (2009) [12]	56.3	60 (60)	50.7	60	2.5×10^{9}	MRI	No effect
Janssens et al. (2006) [13]	58.7	67 (66)	47.7	4	1.7×10^{8}	MRI	No effect
Meluzín et al. (2006) [14]	55	66 (66)	41.7	3	$10^{8}/10^{7}$	SPECT/Echo	EF increased 3% (10^{8}). No effect (10^{7})
Huikuri et al. (2008) [15]	59.5	80 (77)	60.5	6	3.6×10^{8}	Echo/Angiography	EF increased 4% (Echo)/7.1% (angiography)
Plewka et al. (2009) [16]	56	60 (56)	37	6	1.44×10^{8}	Echo	EF increased 10%
REGENT (2009) [17]	57	200 (199)	37	6	1.78×10^{8}/ 1.9×10^{6} (sorted)	MRI	EF increased 3%
Wöhrle et al. (2010) [18]	61	42 (42)	54	6	3.81×10^{8}	MRI	EF increased 5.7%
STAR-heart [7]	59.5	391 (391)	32.83	60	6.6×10^{7}	Angiography	EF increased 6.2%

All studies demonstrated satisfactory patient matching. EF: ejection fraction; Echo: echocardiography; SPECT: single-photon-emission computed tomography; MRI: magnetic resonance imaging.

incidence of malignant arrhythmias, restenosis did not significantly elevate. The safety of BMC transplantation has been initially verified.

Clinical studies on intracoronary BMC infusion in patients with myocardial infarction revealed mixed results, partly because of marked heterogeneity between trials [12]. The preclinical studies were performed in young healthy animals with a single coronary occlusion. The clinical trials were performed in old patients with extensive comorbidities. The numbers and proliferation of stem cells decline in elderly patients. This may be the reason that the clinical trials were not as beneficial as the animal studies. But a meta-analysis suggests that BMC therapy is likely more effective in ageing and diabetic individuals [20]. A speculation is that the patients who are ageing, postmenopausal female, or diabetic are likely to suffer from impaired endothelium and to have inadequate physiological angiogenesis response to ischemia, therefore tend to gain beneficial effects from the supplementation of BMC. Many current clinical trials are not double-blind studies, and there could be a "placebo effect". The long-term effectiveness of BMC treatment of heart failure needs the evidence of large-scale double-blind randomized controlled trials.

2.2. Cardiac Stem Cells. Many kinds of CSCs which were taken from adult heart tissue can be isolated and identified including c-kit+ cells and Sca-1+ cells [21]. Regardless of the gender or age of the patient, or of diabetes, David et al. [22] isolate in all of them a pool of functional CSCs. Although less so in older or diabetic patients, they found that cells had long telomeres, or "caps," on their chromosomal ends indicating that expanded CSCs retained a significant growth reserve. CSCs can differentiate into three kinds of major heart cell precursors: myocardial cells, smooth muscle cells and epithelial cells [23]. Although isolation of these cells requires access to cardiac tissue, CSCs have been successfully isolated from right ventricular endomyocardial biopsy [24], a technique that could be used clinically. Some Phase I clinical trials are being conducted to observe the safety and feasibility of using CSCs in patients (http://www.clinicaltrials.org/NCT00474461).

2.3. Induced Pluripotent Stem Cells. In 2006, Takahashi and Yamanaka [25] reported the first successful reprogramming by delivering four stem cell-related genes (Oct3/4, Sox2, Klf4, c-Myc) into skin fibroblasts. The adult cells conversed into an ES-like characteristic of pluripotent stem cells,

called induced pluripotent stem cells (iPSs). iPSs avoid a moral controversy, and can be patient-specific stem cells. Human iPSs have been implanted in mouse models of myocardial infarction, and it was indicated that they regenerated myocardium, smooth muscle, and endothelial tissue, restoring postischemic contractility performance and electric stability [26]. As with ES, iPSs-derived cells may be contaminated with potentially tumorigenic cells. Ieda et al. [27] reported that a combination of three developmental transcription factors rapidly and efficiently reprogrammed postnatal cardiac or dermal fibroblasts directly into functional cardiomyocytes. Reprogramming of endogenous or explanted adult cells might provide a source of cardiomyocytes for regenerative approaches.

2.4. Adipose-Derived Stem Cells. Numerous studies have provided evidence that ADSCs contain a population of adult multipotent mesenchymal stem cells and endothelial progenitor cells. The similarities between bone marrow-derived cells and the ADSCs suggest the potential of the adipose tissue to act as an alternative, and perhaps preferable, cell source for repairing damaged myocardium. ADSCs are able to differentiate into multiple cell lineages including cardiomyocytes [28]. ADSCs can effectively improve LVEF in animal models of acute and chronic myocardial infarction. According to a small, first-of-its-kind study [29], ADSCs can be safely obtained and infused inside the hearts of patients following an acute heart attack. ADSCs are emerging as a new source of adult stem cells for cardiovascular repair. Certainly, more clinical trials are needed to demonstrate the long-term efficacy and safety.

3. Mechanism of Stem Cells Therapy

The stem cells including ES, CSCs, and iPSs can be differentiated into cardiomyocytes after transplantation and restore contractile function. They also can be differentiated into endothelial cells and promote angiogenesis, turn part of the damaged heart muscle alive, and limit scar expansion. Stem cells including BMC and ADSCs transdifferentiate into cardiomyocytes in vivo, but no one has yet observed that MSCs give rise to fully differentiated and functional cardiomyocytes in vivo [30, 31]. With the discovery of paracrine effect of the stem cell, many studies have confirmed that stem cell therapy of heart failure depends on the mechanism, mainly in the promotion of angiogenesis, against myocardial apoptosis, immune regulation, and so on [32].

(1) The autocrine or paracrine growth factor such as vascular endothelial growth factor (VEGF) promotes reconstruction of myocardial vascular network [33]. VEGF can increase permeability of capillary wall, activate matrix metalloproteinase, and promote endothelial cell proliferation and migration. It is one of the most important angiogenesis factors. Research has shown that sustained high expression of VEGF, cooperated with the other angiogenesis factors (such as bFGF), may promote the formation of smooth muscle cells, participate in the "arteriogenesis" process, and improve myocardial ischemia. Tang et al. [34] confirmed capillary proliferation in the areas of acute myocardial infarction and surrounding area after stem cell transplantation.

(2) MSCs transplantation inhibits the activation of NF-κB, attenuates the protein production of TNF-α and IL-6, and increases anti-inflammatory cytokines IL-10 expression [35]. As proinflammatory cytokines, TNF-α and IL-6 have a toxic effect on myocardial cells, can inhibit the cardiac contractile function, and induce apoptosis of cardiomyocyte. In addition, they can regulate the expression of monocyte chemoattractant protein, vascular endothelial cellular adhesion molecule to chemotaxis of inflammatory cells into myocardial tissue, increase myocardial tissue inflammatory responses, and thus promote the progress of ventricular remodeling after AMI [36]. As an anti-inflammatory cytokine, IL-10 may be expressed by monocytes, macrophages, cardiac cells, and so on. Through inhibition of NF-κB activity to decrease TNF-α and IL-6 expression, it can also inhibit the inflammatory response to some degree [37].

(3) Cardiomyocyte hypertrophy and the extracellular matrix deposition play major roles in the remodeling of non-infarcted myocardium. Pathologic increase in extracellular collagen leads to interstitial fibrosis, and although this can be useful in limiting ventricular enlargement, it decreases the compliance of ventricular wall and affects heart function [38]. MSCs transplantation improves cardiac function in part through regulation of cardiac fibroblasts proliferation and transcriptional downregulation of types I and III collagen syntheses [39]. This may be one of the mechanisms through which they inhibit the ventricular remodeling.

4. New Strategy and Direction of Stem Cell Therapy for Heart Failure

The stem cell transplantations for treatment of heart failure have modest effect or no effect. In addition to problems in the best method of cell delivery, the treatment time, and patient selection, an important common issue the transplantation of stem cells faces is that the survival rate in the host is very low. Because they are transplanted in an ischemia, hypoxia, and proapoptotic niche, most stem cells cannot survive after transplantation [40]. The results of real-time PCR and TUNEL staining after the stem cells transplant showed that more than 90% of the stem cells die within 24 hours after transplantation [41]. Another reason is the amount of cells which migrate to regions of myocardial infarction is too small. PET showed that only 1.3% to 2.6% of the ^{18}F-FDG-labeled stem cells which were intracoronary injected migrated to the myocardium 2 hours after injection, while the majority of the cells moved to the tissue outside heart muscle, including liver, spleen, lung, bladder, and brain [42]. 20 hours later, the stem cells settled in the myocardium are only about 1.49% [43]. In response to these problems, there are some new strategies now.

5. Preconditioning

In order to improve the viability of stem cells after trans plantation and counteract the hypoxia-induced apoptosis, it is an

effective protection strategy to use various methods of preconditioning of stem cells before transplanting the cells into the damaged myocardium. Hypoxic preconditioning indicates that the cell is cultivated under hypoxic conditions before transplantation. Hypoxic preconditioning has been found to be able to start the PI3K/AKT signaling pathway and enhance the stability of HIF-1 to increase the antiapoptotic ability of MSCs [44]. Hypoxic preconditioning of peripheral blood mononuclear cells increased the expression of various genes related to antioxidant and survival signals remarkably [45]. Hypoxic preconditioning also enhances the benefit of CSCs therapy for treatment of myocardial infarction by SDF/CXCR4 axis [46]. In addition to hypoxic preconditioning, with some growth factors preconditioning on MSCs, it can improve the ability of cell resistance to apoptosis, too. By using stromal cell-derived factor-1 (SDF-1α) to precondition MSCs, Pasha et al. [47] found that SDF-1 preconditioning through SDF/CXCR4 activated multiple signaling pathways, including the PI3K/AKT signaling pathway. Via using MSCs with SDF-1 preconditioning for the treatment of myocardial infarction in rats, it is found that the viability of MSCs which were transplanted significantly increased, and they got a better effect of myocardial repair. By Using diazoxide to precondition MSCs, Afzal et al. found that diazoxide preconditioning can enhance protective role of the MSCs through the NF-κB signaling pathway [48].

6. Combination Drug Therapy

After cell transplantation, administration of drugs combination therapy can also improve the viability of MSCs, too. Statins used to reduce blood lipids in the past were considered to have good protective action. Yang et al. [49] used simvastatin to conduct the combination therapy, and Xu et al. [50] conducted the combination therapy with lovastatin. They both got remarkable curative effects. Then they thought that simvastatin and lovastatin could have a cytoprotective effect by inhibiting the mitochondrial apoptotic pathway to activate the signaling pathway of PI3K/Akt and ERK1/2. In addition, Zhang et al. [51] found that Chinese herbs Berberine can inhibit the hypoxia-induced apoptosis in vitro. The mechanism is also related to the inhibition of the signaling pathway of mitochondrial apoptosis.

7. Gene Modified Stem Cells

7.1. Increase the Capacity of Antiapoptotic. The early apoptosis of the majority of stem cells after transplantation into the ischemic heart imposes restrictions on their repair functions. Li et al. [52] applied anti-apoptotic gene Bcl-2 to modify MSCs and found that the anti-apoptotic ability of modified MSCs increased; they can also promote VEGF secretion under the hypoxic condition; the number of surviving cells after transplantation in vivo significantly increased, and improvement in cardiac function appeared significantly. Lim et al. [53] modified MSCs with Akt and found that Akt-MSCs tolerated more about hypoxia-induced apoptosis. And after hypoxia, extracellular signal regulated ERK activation, VEGF expression increased, and survival of Akt-MSCs increased after transplantation. Compared with MSCs transplantation alone, it further repaired the damaged myocardium and improved cardiac function. Through enhancing the MSCs's ability of antihypoxia, it can also play the role of increasing the survival rate of the transplanted cells. Tang et al. [40] modified MSCs with heme oxygenase -1 (HO-1), and found tolerance of modified MSCs to hypoxia significantly increased, and the survival rate in the ischemic heart also significantly increased. Seven days after the transplantation, survival rate of HO-1-modified MSCs was as 5 times as that of the control group in vivo. With the modified MSCs transplantation therapy of myocardial infarction, cardiac function was further improved. These genes enhance the viability of MSCs from such perspectives as anti-apoptotic, promoting survivals, antioxidant protection, and so forth.

7.2. Promoting Angiogenesis. To promote angiogenesis effectively and improve myocardial blood flow may be one of the effective ways of the treatment of ischemic heart disease. Genes related to angiogenesis include VEGF, Ang-1, FGF-2, IGF, and hepatocyte growth factor (HGF). Yang et al. [54] used liposome-mediated method transfected pcDNA-hVEGF to rat MSCs and used intramyocardial injections method to inject the myocardium of the rat two weeks after myocardial infarction. After four weeks, the results showed that the cardiac function, infarct size, and angiogenesis of the VEGF modified group were significantly better than the other groups, MSCs treatment group. Four weeks after infarction, Yang et al. [55] transplanted HGF-modified MSCs through the non-infarct-related coronary artery into pig heart. The results showed a significant increase in angiogenesis. Sun et al. [56] intramyocardially injected human Ang1-modified MSCs (hAng1-MSCs) to treat rats acute myocardial infarction. The results showed that hAng1-MSCs could survive in the local and express hAng-1 mRNA and protein. Vascular density of hAng1-MSCs and MSCs group was significantly higher than PBS control group, ventricular remodeling and cardiac function were significantly improved. What is more, compared with MSCs, the increase of angiogenesis and arteriogenesis and the decrease of the infarct size and thickening of the left ventricular wall were more significant in hAng1-MSCs group.

7.3. Promote Migration. Most of the transplanted stem cells cannot effectively home in on the damaged heart; how to improve the migration of stem cells is one of the research directions in recent years. A series of signals caused by necrosis after myocardial infarction leads to their own MSCs mobilization into peripheral blood pool. The damaged tissue expressed specific receptor or ligand, to guide corresponding stem cells to move and adhere to the injury. SDF-1/CX2CR4 is currently known to promote the homing of MSCs in on the injured tissue [57]. SDF-1/CXCR4 cannot only promote the transplanted MSCs to migrate to the damaged tissue, but also inhibit apoptosis of MSCs, increase the survival rate and proliferation of MSCs [58], and promote the homing efficiency of MSCs from many aspects. 24 hours after the rat coronary artery occlusion-reperfusion, Cheng et al. [59] transplanted retrovirus-mediated CXCR4-modified MSCs

(CXCR4-MSCs) by intravenous injection. Results found that the amount of CXCR4-MSCs's homing in on the infarcted myocardium was far more than that of the control group, and 30 days after transplantation, cardiac function and ventricular remodeling indicators gained the upper hand of the MSCs or saline transplantation group.

7.4. Anti-Inflammatory. The nonspecific inflammation of the body is one important reason that causes loss of transplanted cells [60]. Tumor necrosis factors (TNF) are an important kind of inflammatory mediators and act as a major factor to the mediated apoptosis of the receptor. Bao et al. [61] studied the anti-inflammatory and cardiac function improvement effects of TNF receptor- (TNFR-) modified MSCs transplantation in the treatment of myocardial infarction. Two weeks after transplantation of TNFG-transfected MSCs into the ischemic myocardium, they found that the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 reduced, myocardial apoptosis decreased and the left ventricular function improved significantly.

7.5. Multigene Modified MSCs Exploration. The ideal intervention therapy for heart damage should at least include the survival of transplanted cells and myocardial reperfusion. The effect of single gene is limited. Some researchers were exploring a joint approach to meet different demands of therapy by applying to a variety of genes combination, and they have made some achievements. Yau et al. [62] used MSCs which were transfected by VEGF and IGF-1 plasmid to treat myocardial infarction. They found that the MSCs with combined genes had the highest survival rate after one week, while its improvement in cardiac function also showed the best condition after 3 weeks. All suggest that multigene combination therapy can play a therapeutic effect of the superposition. Some other studies have applied both Ang-1 and Akt to modify MSCs transplantation cooperatively to treat myocardial infarction to meet the demands of angiogenesis and antiapoptosis. They also achieved the expected results. The cardiac function of rats has been further improved, and having shown the long-term therapy effects, the results lasted for 3 months [63]. But the effect of mutigene transfection to biological behavior of stem cells is not clear. Is there a single gene that can have both of the functions in the meantime? Some researchers think that HGF has the ability in angiogenesis, anti-apoptosis, and promoting cell migration, but there is still no acknowledged best gene modification program existing at present. The tissue engineering research of genetic enhancement with MSCs as the carrier has brought new hope to repair damaged hearts.

8. Conclusion

Although in some clinical trials stem cells have achieved effectiveness for ischemic heart disease, the effectiveness of stem cells is still lacking of consistency conclusion. Clinical trials should try to unify stem cells separation, cultural method, and transplantation approach, to set reasonable control, and to have enough followup time. The design destination should go beyond the alternative indicators, so as to obtain sufficient convincing evidence to clarify the differences in clinical endpoints, such as survival rate, hospitalization rate, recurrent myocardial infarction. Stem cell transplantation brings about hope, but the study of stem cell therapy in cardiac repair is still in the initial stage. We should be cautious about the study and application of this technology. Improving the survival rate after stem cell transplantation and prompt homing of stem cells may be effective strategies for stem cell therapy.

Conflict of Interests

None of the authors has a conflict of interests to declare.

References

[1] J. Lloyd, "Executive summary: heart disease and stroke statistics-2010 update:a report from the American heart association," *Circulation*, vol. 121, pp. 948–954, 2010.

[2] J. Lloyd, "Executive summary: heart disease and stroke statistics-2010 update: a report from the American heart association," *Circulation*, vol. 121, no. 12, p. E259, 2010.

[3] D. F. Gu, G. Y. Huang, J. He et al., "Investigation of prevalence and distributing feature of chronic heart failure in Chinese adult population," *Zhonghua Xin Xue Guan Bing Za Zhi*, vol. 31, no. 1, pp. 3–6, 2003.

[4] R. Sanz-Ruiz, E. Gutierrez Ibanes, A. V. Arranz et al., "Phases I-III clinical trials using adult stem cells," *Stem Cells International*, vol. 2010, Article ID 579142, 2010.

[5] S. Durrani, M. Konoplyannikov, M. Ashraf, and K. H. Haider, "Skeletal myoblasts for cardiac repair," *Regenerative Medicine*, vol. 5, no. 6, pp. 919–932, 2010.

[6] K. J. Osterziel, "Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1 year results of the REPAIR-AMI trial," *European Heart Journal*, vol. 28, no. 5, p. 638, 2007.

[7] B. E. Strauer, M. Yousef, and C. M. Schannwell, "The acute and long-term effects of intracoronary stem cell transplantation in 191 patients with chronic heart failure: the STAR-heart study," *European Journal of Heart Failure*, vol. 12, no. 7, pp. 721–729, 2010.

[8] S. L. Chen, W. W. Fang, F. Ye et al., "Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction," *American Journal of Cardiology*, vol. 94, no. 1, pp. 92–95, 2004.

[9] V. Schächinger, S. Erbs, A. Elsässer et al., "Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial," *European Heart Journal*, vol. 27, no. 23, pp. 2775–2783, 2006.

[10] K. Lunde, S. Solheim, S. Aakhus et al., "Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction," *New England Journal of Medicine*, vol. 355, no. 12, pp. 1199–1209, 2006.

[11] J. Ge, Y. Li, J. Qian et al., "Efficacy of emergent transcatheter transplantation of stem cells for treatment of acute myocardial infarction (TCT-STAMI)," *Heart*, vol. 92, no. 12, pp. 1764–1767, 2006.

[12] G. P. Meyer, K. C. Wollert, J. Lotz et al., "Intracoronary bone marrow cell transfer after myocardial infarction: 5-year

follow-up from the randomized-controlled BOOST trial," *European Heart Journal*, vol. 30, no. 24, pp. 2978–2984, 2009.

[13] S. Janssens, C. Dubois, J. Bogaert et al., "Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial," *Lancet*, vol. 367, no. 9505, pp. 113–121, 2006.

[14] J. Meluzín, J. Mayer, L. Groch et al., "Autologous transplantation of mononuclear bone marrow cells in patients with acute myocardial infarction: the effect of the dose of transplanted cells on myocardial function," *American Heart Journal*, vol. 152, no. 5, pp. 975.e9–975.e15, 2006.

[15] H. V. Huikuri, K. Kervinen, M. Niemelä et al., "Effects of intracoronary injection of mononuclear bone marrow cells on left ventricular function, arrhythmia risk profile, and restenosis after thrombolytic therapy of acute myocardial infarction," *European Heart Journal*, vol. 29, no. 22, pp. 2723–2732, 2008.

[16] M. Plewka, M. Krzemińska-Pakuła, P. Lipiec et al., "Effect of intracoronary injection of mononuclear bone marrow stem cells on left ventricular function in patients with acute myocardial infarction," *American Journal of Cardiology*, vol. 104, no. 10, pp. 1336–1342, 2009.

[17] M. Tendera, W. Wojakowski, W. Ruzyllo et al., "Intracoronary infusion of bone marrow-derived selected CD34(+)CXCR4(+) cells and non-selected mononuclear cells in patients with acute STEMI and reduced left ventricular ejection fraction: results of randomized, multicentre myocardial regeneration by intracoronary infusion of selected population of stem cells in acute myocardial infarction (REGENT) trial," *European Heart Journal*, vol. 30, no. 11, pp. 1313–1321, 2009.

[18] J. Wöhrle, N. Merkle, V. Mailänder et al., "Results of intracoronary stem cell therapy after acute myocardial infarction," *American Journal of Cardiology*, vol. 105, no. 6, pp. 804–812, 2010.

[19] L. Sun, T. Zhang, X. Lan, and G. Du, "Effects of stem cell therapy on left ventricular remodeling after acute myocardial infarction: a meta-analysis," *Clinical Cardiology*, vol. 33, no. 5, pp. 296–302, 2010.

[20] Y. Bai, T. Sun, and P. Ye, "Age, gender and diabetic status are associated with effects of bone marrow cell therapy on recovery of left ventricular function after acute myocardial infarction: a systematic review and meta-analysis," *Ageing Research Reviews*, vol. 9, no. 4, pp. 418–423, 2010.

[21] C. Bearzi, M. Rota, T. Hosoda et al., "Human cardiac stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 35, pp. 14068–14073, 2007.

[22] D. A. David, U. Konrad, H. Toru et al., "Aged cardiac stem cells could treat heart failure," *American Heart Association Meeting Report*, 2010.

[23] A. Leri, J. Kajstura, and P. Anversa, "Cardiac stem cells and mechanisms of myocardial regeneration," *Physiological Reviews*, vol. 85, no. 4, pp. 1373–1416, 2005.

[24] C. Bearzi, A. Leri, F. Lo Monaco et al., "Identification of a coronary vascular progenitor cell in the human heart," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15885–15890, 2009.

[25] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[26] T. J. Nelson, A. Martinez-Fernandez, S. Yamada, C. Perez-Terzic, Y. Ikeda, and A. Terzic, "Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells," *Circulation*, vol. 120, no. 5, pp. 408–416, 2009.

[27] M. Ieda, J. D. Fu, P. Delgado-Olguin et al., "Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors," *Cell*, vol. 142, no. 3, pp. 375–386, 2010.

[28] R. Madonna and R. De Caterina, "Adipose tissue: a new source for cardiovascular repair," *Journal of Cardiovascular Medicine*, vol. 11, no. 2, pp. 71–80, 2010.

[29] M. D. Duckers, "Treating heart attack with fat-derived stem cells may be safe in humans be safe in humans," *American Heart Association Meeting Report*, p. P6021, 2010, abstract no. 12225.

[30] C. E. Murry, M. H. Soonpaa, H. Reinecke et al., "Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts," *Nature*, vol. 428, no. 6983, pp. 664–668, 2004.

[31] J. Kajstura, M. Rota, B. Whang et al., "Bone marrow cells differentiate in cardiac cell lineages after infarction independently of cell fusion," *Circulation Research*, vol. 96, no. 1, pp. 127–137, 2005.

[32] R. Uemura, M. Xu, N. Ahmad, and M. Ashraf, "Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling," *Circulation Research*, vol. 98, no. 11, pp. 1414–1421, 2006.

[33] U. Testa, G. Pannitteri, and G. L. Condorelli, "Vascular endothelial growth factors in cardiovascular medicine," *Journal of Cardiovascular Medicine*, vol. 9, no. 12, pp. 1190–1221, 2008.

[34] Y. L. Tang, Q. Zhao, X. Qin et al., "Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction," *Annals of Thoracic Surgery*, vol. 80, no. 1, pp. 229–237, 2005.

[35] Y. Y. Du, S. H. Zhou, T. Zhou et al., "Immuno-inflammatory regulation effect of mesenchymal stem cell transplantation in a rat model of myocardial infarction," *Cytotherapy*, vol. 10, no. 5, pp. 469–478, 2008.

[36] C. Stumpf, K. Seybold, S. Petzi et al., "Interleukin-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction," *European Journal of Heart Failure*, vol. 10, no. 8, pp. 733–739, 2008.

[37] Y. Onai, J. I. Suzuki, Y. Maejima et al., "Inhibition of NF-κB improves left ventricular remodeling and cardiac dysfunction after myocardial infarction," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 292, no. 1, pp. H530–H538, 2007.

[38] T. Kinnaird, E. Stabile, S. M. Burnett et al., "Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms," *Circulation*, vol. 109, no. 12, pp. 1543–1549, 2004.

[39] S. Ohnishi, H. Sumiyoshi, S. Kitamura, and N. Nagaya, "Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions," *FEBS Letters*, vol. 581, no. 21, pp. 3961–3966, 2007.

[40] Y. L. Tang, Y. Tang, Y. C. Zhang, K. Qian, L. Shen, and M. I. Phillips, "Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector," *Journal of the American College of Cardiology*, vol. 46, no. 7, pp. 1339–1350, 2005.

[41] S. I. Hodgetts, M. W. Beilharz, A. A. Scalzo, and M. D. Grounds, "Why do cultured transplanted myoblasts die in vivo? DNA quantification shows enhanced survival of donor male myoblasts in host mice depleted of CD4+ and CD8+ cells

or NK1.1+ cells," *Cell Transplantation*, vol. 9, no. 4, pp. 489–502, 2000.

[42] M. Hofmann, K. C. Wollert, G. P. Meyer et al., "Monitoring of bone marrow cell homing into the infarcted human myocardium," *Circulation*, vol. 111, no. 17, pp. 2198–2202, 2005.

[43] J. K. Won, H. J. Kang, H. S. Kim, J. K. Chung, C. L. Myung, and S. L. Dong, "Tissue distribution of 18F-FDG-labeled peripheral hematopoietic stem cells after intracoronary administration in patients with myocardial infarction," *Journal of Nuclear Medicine*, vol. 47, no. 8, pp. 1295–1301, 2006.

[44] X. Hu, S. P. Yu, J. L. Fraser et al., "Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis," *Journal of Thoracic and Cardiovascular Surgery*, vol. 135, no. 4, pp. 799–808, 2008.

[45] M. Kubo, T. S. Li, R. Suzuki et al., "Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 2, pp. H590–H595, 2008.

[46] Y. L. Tang, W. Zhu, M. Cheng et al., "Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression," *Circulation Research*, vol. 104, no. 10, pp. 1209–1216, 2009.

[47] Z. Pasha, Y. Wang, R. Sheikh, D. Zhang, T. Zhao, and M. Ashraf, "Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium," *Cardiovascular Research*, vol. 77, no. 1, pp. 134–142, 2008.

[48] M. R. Afzal, H. K. Haider, N. M. Idris, S. Jiang, R. P. H. Ahmed, and M. Ashraf, "Preconditioning promotes survival and angiomyogenic potential of mesenchymal stem cells in the infarcted heart via NF-κB signaling," *Antioxidants and Redox Signaling*, vol. 12, no. 6, pp. 693–702, 2010.

[49] Y. J. Yang, H. Y. Qian, J. Huang et al., "Combined therapy with simvastatin and bone marrow-derived mesenchymal stem cells increases benefits in infarcted swine hearts," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 12, pp. 2076–2082, 2009.

[50] R. X. Xu, J. Chen, X. F. Cong, S. Hu, and X. Chen, "Lovastatin protects mesenchymal stem cells against hypoxia- and serum deprivation-induced apoptosis by activation of PI3K/Akt and ERK1/2," *Journal of Cellular Biochemistry*, vol. 103, no. 1, pp. 256–269, 2008.

[51] W. Zhang, X. Su, Y. Gao et al., "Berberine protects mesenchymal stem cells against hypoxia-induced apoptosis in vitro," *Biological and Pharmaceutical Bulletin*, vol. 32, no. 8, pp. 1335–1342, 2009.

[52] W. Li, N. Ma, L. L. Ong et al., "Bcl-2 engineered MSCs inhibited apoptosis and improved heart function," *Stem Cells*, vol. 25, no. 8, pp. 2118–2127, 2007.

[53] S. Y. Lim, Y. S. Kim, Y. Ahn et al., "The effects of mesenchymal stem cells transduced with Akt in a porcine myocardial infarction model," *Cardiovascular Research*, vol. 70, no. 3, pp. 530–542, 2006.

[54] J. Yang, W. Zhou, W. Zheng et al., "Effects of myocardial transplantation of marrow mesenchymal stem cells transfected with vascular endothelial growth factor for the improvement of heart function and angiogenesis after myocardial infarction," *Cardiology*, vol. 107, no. 1, pp. 17–29, 2006.

[55] Z. J. Yang, D. C. Ma, W. Wang et al., "Experimental study of bone marrow-derived mesenchymal stem cells combined with hepatocyte growth factor transplantation via noninfarct-relative artery in acute myocardial infarction," *Gene Therapy*, vol. 13, no. 22, pp. 1564–1568, 2006.

[56] L. Sun, M. Cui, Z. Wang et al., "Mesenchymal stem cells modified with angiopoietin-1 improve remodeling in a rat model of acute myocardial infarction," *Biochemical and Biophysical Research Communications*, vol. 357, no. 3, pp. 779–784, 2007.

[57] M. S. Penn, "Importance of the SDF-1: CXCR4 axis in myocardial repair," *Circulation Research*, vol. 104, no. 10, pp. 1133–1135, 2009.

[58] Q. Yin, P. Jin, X. Liu et al., "SDF-1α inhibits hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells through PI3K/Akt and ERK1/2 signaling pathways," *Molecular Biology Reports*, vol. 38, no. 1, pp. 9–16, 2010.

[59] Z. Cheng, L. Ou, X. Zhou et al., "Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance," *Molecular Therapy*, vol. 16, no. 3, pp. 571–579, 2008.

[60] V. H. Fan, K. Tamama, A. Au et al., "Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells," *Stem Cells*, vol. 25, no. 5, pp. 1241–1251, 2007.

[61] C. Bao, J. Guo, G. Lin, M. Hu, and Z. Hu, "TNFR gene-modified mesenchymal stem cells attenuate inflammation and cardiac dysfunction following MI," *Scandinavian Cardiovascular Journal*, vol. 42, no. 1, pp. 56–62, 2008.

[62] T. M. Yau, C. Kim, G. Li, Y. Zhang, R. D. Weisel, and R. K. Li, "Maximizing ventricular function with multimodal cell-based gene therapy," *Circulation*, vol. 112, no. 9, pp. I123–I128, 2005.

[63] J. Shujia, H. K. Haider, N. M. Idris, G. Lu, and M. Ashraf, "Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair," *Cardiovascular Research*, vol. 77, no. 3, pp. 525–533, 2008.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 679171, 8 pages
doi:10.4061/2011/679171

Review Article

Advances in Cell Transplantation Therapy for Diseased Myocardium

Outi M. Villet,[1] Antti Siltanen,[2] Tommi Pätilä,[1] M. Ali A. Mahar,[1,2] Antti Vento,[1] Esko Kankuri,[2] and Ari Harjula[1]

[1] *Department of Cardiothoracic Surgery, University of Helsinki Meilahti Hospital, P.O. Box 340, FIN-00029 HUS, Finland*
[2] *Department of Pharmacology, Institute of Biomedicine, University of Helsinki, 00014 Helsinki, Finland*

Correspondence should be addressed to Outi M. Villet, outivillet@gmail.com

Received 15 January 2011; Accepted 2 April 2011

Academic Editor: Wojciech Wojakowski

The overall objective of cell transplantation is to repopulate postinfarction scar with contractile cells, thus improving systolic function, and to prevent or to regress the remodeling process. Direct implantation of isolated myoblasts, cardiomyocytes, and bone-marrow-derived cells has shown prospect for improved cardiac performance in several animal models and patients suffering from heart failure. However, direct implantation of cultured cells can lead to major cell loss by leakage and cell death, inappropriate integration and proliferation, and cardiac arrhythmia. To resolve these problems an approach using 3-dimensional tissue-engineered cell constructs has been investigated. Cell engineering technology has enabled scaffold-free sheet development including generation of communication between cell graft and host tissue, creation of organized microvascular network, and relatively long-term survival after *in vivo* transplantation.

1. Introduction

Cardiac repair by cell therapy offers hope to improve performance of diseased heart by reconstituting or maintaining cardiac specific tissue [1]. First studies were performed with cells such as myoblasts [2], but later the field expanded to several cell types including bone marrow cells [3], endothelial progenitors [4], mesenchymal stem cells (MSCs) [5], resident cardiac stem cells [6], and embryonic stem cells [7]. Numerous preclinical studies have shown improved cardiac function in animal models of heart failure, but the underlying mechanisms of this improvement have remained obscure.

Nevertheless, the hypothesis that cardiac function in heart failure can benefit from cell therapy has gained extensive attention and preliminary clinical trials have been launched [8–14]. Miyagawa et al., reported their first clinical trial with combined cell therapy instead of single cell therapy for a patient based on their finding that combined cell therapy (both myoblasts and bone marrow mononuclear cells) has a more synergistic effect on severely damaged myocardium [15]. In this case recovery of cardiac function and histological changes were observed. However, they could not establish if the dramatic functional improvement was attributable to decreased left ventricle (LV) distension by a left ventricular assist system (LVAS) or to the cell transplantation due to lack of an appropriate control. The brain natriuretic peptide (BNP) levels were significantly lower after cell transplantation than under LVAS before cell transplantation [15]. However, for those patients with ischemic cardiomyopathy, LVAS implantation alone does not achieve sufficient recovery of myocardial function. After LVAS implantation, decreased LV distension contributed to the reduced cell diameter and lower BNP [15]. They also detected other changes such as improved regional diastolic function and vascular density in the targeted region, in addition to the changes evoked by LVAS implantation. This indicates that cell transplantation had a positive effect on the distressed ischemic myocardium. Yet, the mixed results received from the clinical studies have regained the interests for laboratory work.

Table 1: Cell transplantation by intramyocardial injection and vascular infusion in clinical studies.

Cell injection to myocardium				
Cell type	$\times 10^6$ cells	Function	Remarks	
Myoblast	...	↑	Arrhythmogenic potential	[20]
Myoblast	206	No effect	Arrhythmogenic potential	[21]
Myoblast	400–800	No effect	Arrhythmia	[14]
Myoblast	870	...	Arrhythmogenic potential	[22]
Myoblast, BMSC	300, 100	↑		[15]
Vascular infusion				
Cell type	$\times 10^6$ cells	Function	Remarks	
BMSC	~1.5–9	...	Restoration of coronary flow reserve	[12]
BMSC	39	↑		[13]
BMSC	68	No effect		[11]
BMSC	~100–300	↑	Lower mortality	[8]
BMSC	200–400	No effect	Infarct remodelling	[10]
BMSC	56–60 000	↑		[9]

BMSC: bone marrow stem cell.

2. Cell Delivery by Injection

One of the crucial issues in cell therapy for heart failure has been the cell delivery route. Injection of the cells has been the most typical method in clinical feasibility studies (Table 1). The cells have been injected directly into the myocardium or into the coronary vasculature. However, several underlying issues make this a challenging technique to use clinically. The injected cells can be washed out through channel leakage and the vascular system, creating a significant loss of grafted cells [16–18]. Additional loss of the grafted cells is generated by the disruption of the extracellular matrix and the subsequent loss of signals that modulate cell survival, differentiation, and patterning [19].

In addition, arrhythmogenicity of intramyocardial myoblast transplantation has aroused debate. Episodes of ventricular tachycardia and fibrillation have been noted after several feasibility studies. Due to small number of patients in nonrandomized series and the arrhythmogenic nature of the heart disease itself, direct causality is difficult to conclude. In an early study of Menasché et al., 4 out of 10 patients experienced sustained ventricular tachycardia [22], and in the study by Smits et al., 4 out of 15 patients experienced clinically significant arrhythmias in the early phase [20]. A four-year surveillance of the patients showed more intracardiac defibrillator (ICD) interventions when compared to a matched patient population [21]. According to the preliminary data, all patients in MAGIC trial received an ICD implant. At one year after the myoblast injections, no significant difference was found between the myoblast-treated group or the control group regarding arrhythmogenicity [14]. To overcome this evident problem, several strategies for preventing cell therapy-associated arrhythmias have been introduced. Genetic engineering by overexpressing connexin-43 by the transplanted cells might improve the cell graft integration to the host [23]. Myocardial damage resulting from multiple needle injections is potentially a significant cause to ventricular arrhythmias. Avoiding the injections by using intracoronary delivery might decrease arrhythmia [24], although cell loss has been described [25]. Still, even more important aspect would be to improve the method of cell delivery. Various cell types and their delivery methods into damaged myocardium are compared in Table 2.

3. Cell Delivery by Injection versus Cell Sheets

Another approach for cell delivery as opposed to injection involves tissue-engineered constructs. The main advantage of this technology over standard cell implantation lies on the preservation of microcellular communication and matrix, which is lost upon trypsin treatment in the typical procedure of cell preparation. Epicardial deposition of cell sheets might be a solution to prevent significant cell loss and arrhythmia after cell transplantation [26]. Memon et al., reported that myoblast sheet implantation improved global cardiac function to a greater extent than the injection of cell suspension [27]. Similarly, in a recent study, myoblast cell sheets were compared to direct injections of myoblast cells in ischemic heart failure (Patila et al., submitted). Holter monitoring showed more ventricular premature contractions in the cell injection group. Furthermore, epicardial electropotential mapping showed areas of electrical reentry in the injection sites. RT-PCR showed more inflammatory markers and more inflammatory cells accumulated at the infarct border area in the injection group, when compared to the cell sheet-treated animals (Patila et al., submitted).

4. Cell Sheets in Diseased Heart

Current tissue engineering methods allow us to reconstruct myocardial tissue grafts for clinical applications, though human fetal and neonatal cardiomyocytes are difficult to

Table 2: Cell types and delivery methods in cardiac functional outcome.

Cell injection to myocardium					
Model	Cell type	$\times 10^6$ cells	Function	Remarks	
Mouse	BMSC	0.0175–0.1	↑	Regeneration	[1]
Rat	BMSC	10	↑	Arrhythmogenicity	[24]
Rat	BMSC	20	↑		[23]
Rat	CM	0.2	↑	Regeneration	[6]
Rat	CM	5	…	Cell washout	[16]
Rat	Myoblast	1	↑	Improved exercise capacity	[28]
Rat	Myoblast	2.3	↑		[29]
Rat	Myoblast	10	↑	Arrhythmogenicity	[23]
Rabbit	FB			↑ diastolic, ↓systolic performance	[30]
Rabbit	Myoblast		↑		[30]
Sheep	Myoblast	…	↑		[31]
Swine	BMSC	100	…	Cell washout	[17]
Swine	BMSC	100	…	High cell homing	[25]
Vascular infusion					
Model	Cell type	$\times 10^6$ cells	Function	Remarks	
Mouse	BMSC	20	…	Regeneration	[3]
Rat	BMSC	10	↑		[24]
Swine	BMSC	100	…	Cell washout	[25]
Cell sheets					
Model	Cell type	Nr of sheets	Function	Remarks	
Hamster	Myoblast	2 layers	↑	Higher life expectancy	[32]
Rat	AdMSC	Monolayer	↑	Paracrine pathways—angiogenesis	[33]
Rat	AdMSC	Monolayer	↑		[34]
Rat	CM, SC	Monolayer	↑	Cell survival, proliferation, differentiation	[35]
Rat	CM, EC	"Monolayer"	↑	Higher vessel density	[36]
Rat	CM	2 monolayers	↑	Integration of graft and myocardium	[37]
Rat	CM	Bilayer		Functional integration	[26]
Rat	CM	3 monolayers	…	Integration of graft and myocardium	[38]
Rat	CM	3 monolayers	…	Thick grafts, multiple surgeries	[39]
Rat	FB, EC	3 monolayers	↑	Less fibrosis	[40]
Rat	Myoblast	1–5 monolayers	↑	Dose-response	[41]
Rat	Myoblast	2 monolayers	↑	Less fibrosis, remodeling	[27]
Rat	Myoblast	2 layers	↑	Less fibrosis, higher vessel density	[42]
Rat	Myoblast	2 × 5 layers	↑	Higher vessel density	[43]
Rat	Myoblast	2 × 5 layers	↑	Paracrine pathways—angiogenesis	[44]
Swine	Myoblast	2–10 layers	↑		[45]

AdMSC: adipose tissue-derived mesenchymal stem cell, BMSC: bone marrow stem cell, CM: cardiomyocyte, EC: endothelial cell, FB: fibroblast, SC: stromal cell.

obtain. Therefore, several classes of stem cells are being investigated as a potential cell source. Despite their attractive potential to differentiate into various cell types, several issues remain, including difficulties in obtaining and amplifying the cells and the lack of understanding of the mechanisms for differentiation and proliferation. Consequently, the clinical cell sheet transplantation has mainly focused on utilization of myoblasts.

Memon et al., [27] reported the use of skeletal myoblast sheets in a rat myocardial infarction model. Preclinical data

have shown that autologous skeletal myoblasts are capable not only of fusion and differentiation into striated muscle cells within damaged myocardium [31] but also of augmenting systolic and diastolic performance in animal models of acute myocardial infarction and heart failure [28–30, 46]. Inhibition of apoptosis in myoblast sheets by expression of antiapoptotic *bcl2* was shown to enhance the efficacy of sheet transplantation therapy in acute myocardial infarction [43]. Moreover, Siltanen et al. demonstrated that prevention of graft apoptosis by *bcl2* improved myoblast sheet transplantation therapy also in chronic myocardial infarction model [44].

Dilated cardiomyopathy (DCM) is characterized by global myocardial remodeling, which mainly consists of myocardial fibrosis associated with changes in the cytoskeletal and sarcolemmal proteins in cardiomyocytes, leading to a reduction in the number and function of these cells [47]. Consequently, cardiac remodeling chronically progresses with ventricular dilation and thinning, leading to progressive congestive heart failure. Kondoh et al. used human hereditary DCM representative model of TO-2 hamster strain [32]. In this hamster strain, the number of cardiomyocytes decreases progressively because of apoptosis after birth, and cardiac remodeling, which mainly consists of myocardial fibrosis, occurs with ventricular dilation and thinning, leading to progressive congestive heart failure. Transplantation of myoblast sheet grafts showed reorganization of the cytoskeletal proteins, reduction of myocardial fibrosis, and prevention of dilation of the left ventricle, leading to prolonged life expectancy and a longer preservation of cardiac performance in the impaired heart.

Hoashi et al., [42] showed that myoblast sheet transplantation improved right ventricular diastolic dysfunction. Briefly, animals underwent pulmonary artery banding thus created chronic pressure overload resulting in right ventricular failure. Chronic pressure overload is one of the major risk factors of right ventricular dysfunction. In this situation the right ventricle is hypertrophied and systolic function is initially preserved, whereas diastolic function gradually deteriorates. Prolonged exposure to excessive pressure overload results in irreversible right ventricular failure. Thus they successfully demonstrated improvement in diastolic dysfunction and suppressed ventricular fibrosis with increased capillary density in a rat model of a pressure-overloaded right ventricle by implanting myoblast sheets.

Preclinical studies in impaired porcine heart using single monolayer skeletal muscle cell sheet demonstrated improved cardiac performance accompanied with increased myocardial perfusion and viable myocardial tissue [45].

Further, Miyahara et al., used mesenchymal stem cells (MSCs) derived from adipose tissue in a rat myocardial infarction model [33]. MSC sheets incorporated into the host myocardium and improved cardiac function and increased survival. Similarly, Okura et al., showed that transplanted sheets of adipose tissue-derived MSCs differentiated into cardiomyoblast-like cells and resulted in recovery of cardiac function and improved survival rate of rat with infarcted heart [34].

5. Development of Complex Sheet Structures

Difficulties still exist in the outcome of cell therapy, as it is challenging to control the cell growth and localization of the grafted cells and to deliver a cell sheet patch that significantly aids the function of the damaged myocardium. To overcome these problems research has begun on fabricating three-dimensional cardiac grafts composed of multilayered cell sheets. Several methods have been studied with reconstructed tissues based on biodegradable scaffolds, such as poly(lactic-co-glycolic acid) and gelatin or extracellular matrix components [48, 49].

5.1. Fabrication of Scaffold-Free Cell Sheets. In native cardiac tissue the cell density is considerably high, cells being tightly interconnected with gap junctions facilitating electrical communication. The use of scaffolds can lead to abnormal tissue development, electrical communication caused by insufficient cell-to-cell connections, inflammatory responses, and fibrous tissue formation. Alternatively, fabrication of scaffold-free cell sheets requires means of cell detachment from the culture surface that will preserve cell morphology, orientation within the scaffold, and adhesion to surrounding cells and the extracellular matrix. One way to achieve such detachment is to covalently employ a temperature-responsive polymer on cell culture surface. Poly(N-isopropylacrylamide) (PIPAAm) is a hydrophobic polymer at temperatures above 32°C which—after grafting to cell culture dishes—allows cell adhesion and proliferation [50]. At temperatures below 32°C, PIPAAm grafted surfaces change their properties and become hydrophilic to allow cell detachment as intact sheets which harbor the ECM on the basal surface [51]. These sheets retain their cell-to-cell as well as cell-to-ECM adhesions [51], while cell viability is not compromised [50]. Further, PIPAAm-grafted surfaces can be used to engineer monolayer [52] as well as three-dimensional sheet structures comprised of several cell layers [53].

Stevens et al., and Itabashi et al., described other methods for creating scaffold-free sheets. Stevens et al., created embryonic stem cell-derived cardiomyocyte sheets utilizing Teflon-coated low-attachment tissue culture dishes combined with rotating orbital shaker [54]. The diameter of these sheets was dependent on cell number, and the thickness was approximately 300–600 μm. These sheets, however, were subject to necrosis due to limited oxygen supply. The same method was then used to create prevascularized sheets composed of cardiomyocytes, endothelial cells, and fibroblasts. These sheets effectively integrated with the coronary circulation after implantation and evaded necrosis. Furthermore, Itabashi et al., fabricated cardiomyocyte sheets using polymerized fibrin-coated culture dishes [55]. This method is based on the proteolytic activity of the cardiomyocytes that degrades the underlying fibrin coating and allows harvesting of intact sheets mechanically using a cell scraper.

5.2. Cellular Communication in Cell Sheets. In addition to cell-to-cell communication, the layers need to establish a connection with each other and with the host tissue. Using

a multielectrode extracellular recording system, Haraguchi et al., demonstrated that the electrical coupling between 2 sheets starts approximately 34 minutes after initial layering and is completed by about 46 minutes. They also showed small molecule exchange through gap junctions and presence of connexin-43 within 30 minutes of layering [56].

When Shimizu et al., implanted a 4-layered neonatal rat cardiomyocyte sheet into the subcutaneous space of nude rats, synchronous beating [57] and survival up to 1 year [58] were observed. The implanted graft showed characteristic structures of heart tissue, including elongated cardiomyocytes, well-differentiated sarcomeres, and gap junctions. Additionally, conduction velocity, contractile force, and size of implanted grafts increased in proportion to the host's growth [58]. Hata et al., further showed synchronous contraction with defined direction of neonatal rat cardiomyocyte sheets on decellularised porcine small-intestinal submucosa [59].

Miyagawa and colleagues showed that neonatal cardiomyocyte sheets fabricated on temperature-responsive culture dishes attached to the infarcted myocardium and led to an improvement in cardiac performance and improved vascular density [37]. The implanted sheets communicated with the host myocardium as indicated by the presence of connexin-43 and changes in the QRS wave and action potential amplitude.

Another study demonstrated a similar electrical integration between a neonatal myocyte sheet and the host myocardium without serious arrhythmia [26]. Furthermore, histological analyses in infarcted rat hearts with a transplanted 3D tissue graft showed bridging of the cardiomyocytes with functional gap junctions and intercalated disks [38].

5.3. Recreation of Microvessels and Cell Sheet Survival. Heart is a metabolically active organ that requires virtually constant oxygen supply in order to function in a normal fashion. The major limitation of the multilayered cell grafts is insufficient circulation causing hypoxia, nutrient insufficiency, and accumulation of waste products. Cells in living tissues receive oxygen supply through a capillary network, whereas the cultured cell aggregates *in vitro* rely on diffusion. In order to reconstruct thicker and metabolically active tissue grafts sufficient blood supply network has to be created.

One strategy could be to generate capillary-like networks *in vitro* in tissue-engineered constructs before transplantation. Levenberg et al., demonstrated the induction of endothelial vessel networks in engineered skeletal muscle tissue constructs using a 3D culture system consisting of myoblasts, embryonic fibroblasts, and endothelial cells coseeded on porous, biodegradable polymer scaffolds [60]. Similar spontaneous 3D prevascular network formation has been shown also in *in vitro* bone coculture model with human MSCs, human umbilical vein endothelial cells (HUVECs) [61, 62], and in human endothelialized reconstructed skin (ERS), including keratinocytes, fibroblasts, and endothelial cells in a collagen sponge [63]. As the reconstructed skin was transplanted to a nude mouse, Tremblay et al., concluded that the early vascularization observed in the ERS was most likely the result of inosculation of the capillary-like structures with the host's capillaries, rather than neovascularisation, which is a slower process.

Sasagawa et al., developed a novel cell sheet stacking manipulation technique to create multilayered cell sheets from human skeletal muscle myoblasts [64]. They placed a hydrogel-coated plunger onto a confluently cultured cell layer in a temperature-responsive culture dish. To harvest the cell layer the temperature was decreased to 20°C after which the plunger with the cell layer was transferred onto another confluent myoblast monolayer in another dish and incubated at 37°C to promote the cell layer adhesion. After 30–50 minutes in 20°C the plunger was lifted up with a double-layer myoblast sheet. They were able to reach a 5-layered construct with this procedure, which did no harm to the cells confirmed by cell viability assay. To further develop a viable cell dense tissue construct Sasagawa et al., sandwiched HUVECs into the 5-layer myoblast sheet construct. Four days after culture, the HUVECs had started to develop into capillary-like structures. One week after the constructs were engrafted on the dorsal subcutaneous tissue of nude rat, newly formed microvessels connected to the host vessels were found.

A single myoblast layer is about 45 μm thick [57]. Accordingly, most previous studies used multilayer constructs up to about 250 μm thick. In order to progress the human cell sheet applications the greater thickness of the construct would be of great advantage. Sekiya et al., investigated the relationship between the number of transplanted cell layers and cardiac function. They found a significant improvement of cardiac function, induction of angiogenesis, more elastic fibers, and less fibrosis with implantation of 3- and 5- layered myoblast sheets compared to single layer [41].

Shimizu et al., showed that the 1-, 2-, and 3-layer constructs transplanted into the dorsal subcutaneous tissue of nude rats thoroughly survived without necrosis [39]. However, parts of the 4- and 5-layer constructs showed disordered vasculature and connective tissue, indicative of necrosis. Subsequently, transplantation of two triple-layer grafts at 1-day intervals permitted whole tissue survival with a well-organized microvascular network, whereas 2- and 3-day transplantation intervals had poor outcome. Shimizu et al., further developed the polysurgical method by transplanting up to 10 triple-layer cell sheet grafts at 1- or 2-day intervals. Interestingly, at one week after the final transplantation about 0.9 mm thick cell-dense myocardium graft revealed vigorous myocardium-like pulsation with well-organized microvessels throughout the graft. This polysurgical method would encounter difficulties in clinical replacement therapy as each procedure has a relatively high risk of complications. To overcome this obstacle, Shimizu et al., developed an ectopic construct repeatedly transplanting the layered cell sheets over an exposed superficial caudal epigastric, and femoral artery and vein of a nude rat. After 2 weeks the pulsating graft was resected together with the femoral artery and vein and further connected to carotid artery and the jugular vein in a new host. The grafts survived and maintained their characteristic beating 2 weeks after the procedure.

5.4. Angiogenesis in Cell Sheets. One way to enhance the cell sheet graft survival is to promote angiogenesis. Enhanced angiogenesis and functional improvement were achieved by using cocultured cell sheets with fibroblasts and endothelial progenitor cells [40]. Another study showed an accelerated secretion of angiogenic factors *in vitro* and increased blood perfusion *in vivo* by using a coculture of fibroblasts and human smooth muscle cells [65]. In addition, Sekine et al., showed that a coculture of cardiomyocytes and endothelial cells in a cell sheet enhanced vascularization and that the implanted sheet improved cardiac performance compared with a cardiomyocyte-only sheet [36].

Stimulation of angiogenesis has also been shown in single cell-type cell sheets. Miyagawa et al., demonstrated that human HGF gene transfection enhanced the cellular cardiomyoplasty likely by stimulating angiogenesis, restoring the impaired extracellular matrix, and promoting the integration of the dissociated grafted myocytes [66]. Zakharova et al., fabricated sheets from cardiac progenitor cells and showed that these sheets improved cardiac function, suppressed wall thinning, and increased vascular density [35].

To understand the molecular mechanisms of cell sheet angiogenesis Sekiya et al., studied both *in vitro* and *in vivo* models and demonstrated that cardiac cell sheets express VEGF, Cox-2, and Tie-2 and exhibit endothelial cell organisation and microvessel formation [67]. Kitabayashi et al., showed that myoblast sheets express proangiogenic VEGF and placental growth factor and that implantation of these sheets induces angiogenesis *in vivo*. Expression of these proangiogenic genes was further induced by preventing graft apoptosis with antiapoptotic gene therapy [43]. Moreover, Siltanen et al., showed that the proangiogenic effect of myoblast sheets is mediated via the Flt1/Flk-1 pathway [44]. Memon et al., reported expression of SDF-1, HGF, and VEGF in the myocardium after myoblast sheet transplantation [27]. Finally, expression of human HGF in myoblast sheets further enhanced the proangiogenic potential of myoblast sheet therapy [68]. In this study, HGF-expressing sheet therapy increased vascular density in the infarct and border area, as well as in the noninfarcted myocardium.

6. Conclusion

Difficulties in reproducibility in cell injection therapy, including low survival and function of the cells, have led to search for more robust methods. Engineering of 3D cell constructs has currently been under extensive investigation. Preassembled cell constructs might provide effective tools for the future cell therapy research. Establishment of programmable materials used in the cell engineering technology has enabled the creation of scaffold-free cell sheets in a rather simple and inexpensive method. The main aspects of cell sheet construction that must be met for successful regenerative therapy are dynamic, electrical, and histological integration. Increased cell-to-cell communication and survival in cell sheets warrant further attention. The reviewed studies demonstrate the existing potential to produce viable, functional myocardial tissue implantable constructs well beyond the current diffusion-limited thickness regime.

References

[1] D. Orlic, J. Kajstura, S. Chimenti et al., "Bone marrow cells regenerate infarcted myocardium," *Nature*, vol. 410, no. 6829, pp. 701–705, 2001.

[2] R. C. J. Chiu, A. Zibaitis, and R. L. Kao, "Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation," *Annals of Thoracic Surgery*, vol. 60, no. 1, pp. 12–18, 1995.

[3] R. E. Bittner, C. Schöfer, K. Weipoltshammer et al., "Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice," *Anatomy and Embryology*, vol. 199, no. 5, pp. 391–396, 1999.

[4] T. Asahara, T. Murohara, A. Sullivan et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[5] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[6] A. P. Beltrami, L. Barlucchi, D. Torella et al., "Adult cardiac stem cells are multipotent and support myocardial regeneration," *Cell*, vol. 114, no. 6, pp. 763–776, 2003.

[7] M. V. Westfall, K. A. Pasyk, D. I. Yule, L. C. Samuelson, and J. M. Metzger, "Ultrastructure and cell-cell coupling of cardiac myocytes differentiating in embryonic stem cell cultures," *Cell Motility and the Cytoskeleton*, vol. 36, no. 1, pp. 43–54, 1997.

[8] B. Assmus, U. Fischer-Rasokat, J. Honold et al., "Transcoronary transplantation of functionally competent BMCs is associated with a decrease in natriuretic peptide serum levels and improved survival of patients with chronic postinfarction heart failure: results of the TOPCARE-CHD registry," *Circulation Research*, vol. 100, no. 8, pp. 1234–1241, 2007.

[9] S. L. Chen, WU. W. Fang, F. Ye et al., "Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction," *American Journal of Cardiology*, vol. 94, no. 1, pp. 92–95, 2004.

[10] S. Janssens, C. Dubois, J. Bogaert et al., "Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial," *The Lancet*, vol. 367, no. 9505, pp. 113–121, 2006.

[11] K. Lunde, S. Solheim, S. Aakhus et al., "Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction," *The New England Journal of Medicine*, vol. 355, no. 12, pp. 1199–1209, 2006.

[12] V. Schächinger, B. Assmus, J. Honold et al., "Normalization of coronary blood flow in the infarct-related artery after intracoronary progenitor cell therapy: intracoronary Doppler substudy of the TOPCARE-AMI trial," *Clinical Research in Cardiology*, vol. 95, no. 1, pp. 13–22, 2006.

[13] K. C. Wollert, G. P. Meyer, J. Lotz et al., "Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial," *The Lancet*, vol. 364, no. 9429, pp. 141–148, 2004.

[14] P. Menasché, O. Alfieri, S. Janssens et al., "The myoblast autologous grafting in ischemic cardiomyopathy (MAGIC) trial: first randomized placebo-controlled study of myoblast transplantation," *Circulation*, vol. 117, no. 9, pp. 1189–1200, 2008.

[15] S. Miyagawa, G. Matsumiya, T. Funatsu et al., "Combined autologous cellular cardiomyoplasty using skeletal myoblasts and bone marrow cells for human ischemic cardiomyopathy with left ventricular assist system implantation: report of a case," *Surgery Today*, vol. 39, no. 2, pp. 133–136, 2009.

[16] J. Dow, B. Z. Simkhovich, L. Kedes, and R. A. Kloner, "Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy," *Cardiovascular Research*, vol. 67, no. 2, pp. 301–307, 2005.

[17] H. Zhang, P. Song, Y. Tang et al., "Injection of bone marrow mesenchymal stem cells in the borderline area of infarcted myocardium: heart status and cell distribution," *Journal of Thoracic and Cardiovascular Surgery*, vol. 134, no. 5, pp. 1234–1240, 2007.

[18] W. Hudson, M. C. Collins, D. deFreitas, Y. S. Sun, B. Muller-Borer, and A. P. Kypson, "Beating and arrested intramyocardial injections are associated with significant mechanical loss: implications for cardiac cell transplantation," *Journal of Surgical Research*, vol. 142, no. 2, pp. 263–267, 2007.

[19] F. N. Smets, Y. Chen, L. J. Wang, and H. E. Soriano, "Loss of cell anchorage triggers apoptosis (anoikis) in primary mouse hepatocytes," *Molecular Genetics and Metabolism*, vol. 75, no. 4, pp. 344–352, 2002.

[20] P. C. Smits, C. Nienaber, A. Colombo et al., "Myocardial repair by percutaneous cell transplantation of autologous skeletal myoblast as a stand alone procedure in post myocardial infarction chronic heart failure patients," *EuroIntervention*, vol. 1, no. 4, pp. 417–424, 2006.

[21] C. E. Veltman, O. I. I. Soliman, M. L. Geleijnse et al., "Four-year follow-up of treatment with intramyocardial skeletal myoblasts injection in patients with ischaemic cardiomyopathy," *European Heart Journal*, vol. 29, no. 11, pp. 1386–1396, 2008.

[22] P. Menasché, A. A. Hagège, J. T. Vilquin et al., "Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction," *Journal of the American College of Cardiology*, vol. 41, no. 7, pp. 1078–1083, 2003.

[23] S. R. Coppen, S. Fukushima, Y. Shintani et al., "A factor underlying late-phase arrhythmogenicity after cell therapy to the heart: global downregulation of connexin43 in the host myocardium after skeletal myoblast transplantation," *Circulation*, vol. 118, no. 14, pp. S138–S144, 2008.

[24] S. Fukushima, A. Varela-Carver, S. R. Coppen et al., "Direct intramyocardial but not intracoronary injection of bone marrow cells induces ventricular arrhythmias in a rat chronic ischemic heart failure model," *Circulation*, vol. 115, no. 17, pp. 2254–2261, 2007.

[25] J. Mäkelä, V. Anttila, K. Ylitalo et al., "Acute homing of bone marrow-derived mononuclear cells in intramyocardial vs. intracoronary transplantation," *Scandinavian Cardiovascular Journal*, vol. 43, no. 6, pp. 366–373, 2009.

[26] A. Furuta, S. Miyoshi, Y. Itabashi et al., "Pulsatile cardiac tissue grafts using a novel three-dimensional cell sheet manipulation technique functionally integrates with the host heart, in vivo," *Circulation Research*, vol. 98, no. 5, pp. 705–712, 2006.

[27] I. A. Memon, Y. Sawa, N. Fukushima et al., "Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets," *Journal of Thoracic and Cardiovascular Surgery*, vol. 130, no. 5, pp. 1333–1341, 2005.

[28] M. Jain, H. DerSimonian, D. A. Brenner et al., "Cell therapy attenuates deleterious ventricular remodeling and improves cardiac performance after myocardial infarction," *Circulation*, vol. 103, no. 14, pp. 1920–1927, 2001.

[29] B. Pouzet, S. Ghostine, J.-T. Vilquin et al., "Is skeletal myoblast transplantation clinically relevant in the era of angiotensin-converting enzyme inhibitors?" *Circulation*, vol. 104, supplement 1, pp. i223–i228, 2001.

[30] K. A. Hutcheson, B. Z. Atkins, M. T. Hueman, M. B. Hopkins, D. D. Glower, and D. A. Taylor, "Comparison of benefits on myocardial performance of cellular cardiomyoplasty with skeletal myoblasts and fibroblasts," *Cell Transplantation*, vol. 9, no. 3, pp. 359–368, 2000.

[31] S. Ghostine, C. Carrion, L. C.G. Souza et al., "Long-term efficacy of myoblast transplantation on regional structure and function after myocardial infarction," *Circulation*, vol. 106, no. 12, supplement 1, pp. I131–I136, 2002.

[32] H. Kondoh, Y. Sawa, S. Miyagawa et al., "Longer preservation of cardiac performance by sheet-shaped myoblast implantation in dilated cardiomyopathic hamsters," *Cardiovascular Research*, vol. 69, no. 2, pp. 466–475, 2006.

[33] Y. Miyahara, N. Nagaya, M. Kataoka et al., "Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction," *Nature Medicine*, vol. 12, no. 4, pp. 459–465, 2006.

[34] H. Okura, A. Matsuyama, C. M. Lee et al., "Cardiomyoblast-like cells differentiated from human adipose tissue-derived mesenchymal stem cells improve left ventricular dysfunction and survival in a rat myocardial infarction model," *Tissue Engineering Part C*, vol. 16, no. 3, pp. 417–425, 2010.

[35] L. Zakharova, D. Mastroeni, N. Mutlu et al., "Transplantation of cardiac progenitor cell sheet onto infarcted heart promotes cardiogenesis and improves function," *Cardiovascular Research*, vol. 87, no. 1, pp. 40–49, 2010.

[36] H. Sekine, T. Shimizu, K. Hobo et al., "Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts," *Circulation*, vol. 118, no. 14, pp. S145–S152, 2008.

[37] S. Miyagawa, Y. Sawa, S. Sakakida et al., "Tissue cardiomyoplasty using bioengineered contractile cardiomyocyte sheets to repair damaged myocardium: their integration with recipient myocardium," *Transplantation*, vol. 80, no. 11, pp. 1586–1595, 2005.

[38] H. Sekine, T. Shimizu, S. Kosaka, E. Kobayashi, and T. Okano, "Cardiomyocyte bridging between hearts and bioengineered myocardial tissues with mesenchymal transition of mesothelial cells," *Journal of Heart and Lung Transplantation*, vol. 25, no. 3, pp. 324–332, 2006.

[39] T. Shimizu, H. Sekine, J. Yang et al., "Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues," *FASEB Journal*, vol. 20, no. 6, pp. 708–710, 2006.

[40] H. Kobayashi, T. Shimizu, M. Yamato et al., "Fibroblast sheets co-cultured with endothelial progenitor cells improve cardiac function of infarcted hearts," *Journal of Artificial Organs*, vol. 11, no. 3, pp. 141–147, 2008.

[41] N. Sekiya, G. Matsumiya, S. Miyagawa et al., "Layered implantation of myoblast sheets attenuates adverse cardiac remodeling of the infarcted heart," *Journal of Thoracic and Cardiovascular Surgery*, vol. 138, no. 4, pp. 985–993, 2009.

[42] T. Hoashi, G. Matsumiya, S. Miyagawa et al., "Skeletal myoblast sheet transplantation improves the diastolic function of a pressure-overloaded right heart," *Journal of Thoracic and Cardiovascular Surgery*, vol. 138, no. 2, pp. 460–467, 2009.

[43] K. Kitabayashi, A. Siltanen, T. Pätilä et al., "Bcl-2 expression enhances myoblast sheet transplantation therapy for acute myocardial infarction," *Cell Transplantation*, vol. 19, no. 5, pp. 573–588, 2010.

[44] A. Siltanen, K. Kitabayashi, T. Pätilä et al., "Bcl-2 improves myoblast sheet therapy in rat chronic heart failure," *Tissue Engineering Part A*, vol. 17, no. 1-2, pp. 115–125, 2011.

[45] S. Miyagawa, A. Saito, T. Sakaguchi et al., "Impaired myocardium regeneration with skeletal cell sheets-a preclinical trial for tissue-engineered regeneration therapy," *Transplantation*, vol. 90, no. 4, pp. 364–372, 2010.

[46] D. A. Taylor, "Cell-based myocardial repair: how should we proceed?" *International Journal of Cardiology*, vol. 95, supplement 1, pp. S8–S12, 2004.

[47] N. E. Bowles, K. R. Bowles, and J. A. Towbin, "The 'final common pathway' hypothesis and inherited cardiovascular disease: the role of cytoskeletal proteins in dilated cardiomyopathy," *Herz*, vol. 25, no. 3, pp. 168–175, 2000.

[48] R. Langer and J. P. Vacanti, "Tissue engineering," *Science*, vol. 260, no. 5110, pp. 920–926, 1993.

[49] R. K. Li, Z. Q. Jia, R. D. Weisel, D. A. G. Mickle, A. Choi, and T. M. Yau, "Survival and function of bioengineered cardiac grafts," *Circulation*, vol. 100, no. 19, pp. II63–II69, 1999.

[50] T. Okano, N. Yamada, H. Sakai, and Y. Sakurai, "A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide)," *Journal of Biomedical Materials Research*, vol. 27, no. 10, pp. 1243–1251, 1993.

[51] AI. Kushida, M. Yamato, C. Konno, A. Kikuchi, Y. Sakurai, and T. Okano, "Decrease in culture temperature releases monolayer endothelial cell sheets together with deposited fibronectin matrix from temperature-responsive culture surfaces," *Journal of Biomedical Materials Research*, vol. 45, no. 4, pp. 355–362, 1999.

[52] T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, "Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude," *Tissue Engineering*, vol. 7, no. 2, pp. 141–151, 2001.

[53] T. Shimizu, M. Yamato, T. Akutsu et al., "Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets," *Journal of Biomedical Materials Research*, vol. 60, no. 1, pp. 110–117, 2002.

[54] K. R. Stevens, K. L. Kreutziger, S. K. Dupras et al., "Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 39, pp. 16568–16573, 2009.

[55] Y. Itabashi, S. Miyoshi, H. Kawaguchi et al., "A new method for manufacturing cardiac cell sheets using fibrin-coated dishes and its electrophysiological studies by optical mapping," *Artificial Organs*, vol. 29, no. 2, pp. 95–103, 2005.

[56] Y. Haraguchi, T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, "Electrical coupling of cardiomyocyte sheets occurs rapidly via functional gap junction formation," *Biomaterials*, vol. 27, no. 27, pp. 4765–4774, 2006.

[57] T. Shimizu, M. Yamato, Y. Isoi et al., "Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces," *Circulation Research*, vol. 90, no. 3, p. e40, 2002.

[58] T. Shimizu, H. Sekine, Y. Isoi, M. Yamato, A. Kikuchi, and T. Okano, "Long-term survival and growth of pulsatile myocardial tissue grafts engineered by the layering of cardiomyocyte sheets," *Tissue Engineering*, vol. 12, no. 3, pp. 499–507, 2006.

[59] H. Hata, A. Bär, S. Dorfman et al., "Engineering a novel three-dimensional contractile myocardial patch with cell sheets and decellularised matrix," *European Journal of Cardio-thoracic Surgery*, 2010.

[60] S. Levenberg, J. Rouwkema, M. Macdonald et al., "Engineering vascularized skeletal muscle tissue," *Nature Biotechnology*, vol. 23, no. 7, pp. 879–884, 2005.

[61] J. Rouwkema, J. de Boer, and C. A. van Blitterswijk, "Endothelial cells assemble into a 3-dimensional prevascular network in a bone tissue engineering construct," *Tissue Engineering*, vol. 12, no. 9, pp. 2685–2693, 2006.

[62] N. Asakawa, T. Shimizu, Y. Tsuda et al., "Pre-vascularization of in vitro three-dimensional tissues created by cell sheet engineering," *Biomaterials*, vol. 31, no. 14, pp. 3903–3909, 2010.

[63] P. L. Tremblay, V. Hudon, F. Berthod, L. Germain, and F. A. Auger, "Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice," *American Journal of Transplantation*, vol. 5, no. 5, pp. 1002–1010, 2005.

[64] T. Sasagawa, T. Shimizu, S. Sekiya et al., "Design of prevascularized three-dimensional cell-dense tissues using a cell sheet stacking manipulation technology," *Biomaterials*, vol. 31, no. 7, pp. 1646–1654, 2010.

[65] K. Hobo, T. Shimizu, H. Sekine, T. Shin'oka, T. Okano, and H. Kurosawa, "Therapeutic angiogenesis using tissue engineered human smooth muscle cell sheets," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 4, pp. 637–643, 2008.

[66] S. Miyagawa, Y. Sawa, S. Taketani et al., "Myocardial regeneration therapy for heart failure: hepatocyte growth factor enhances the effect of cellular cardiomyoplasty," *Circulation*, vol. 105, no. 21, pp. 2556–2561, 2002.

[67] S. Sekiya, T. Shimizu, M. Yamato, A. Kikuchi, and T. Okano, "Bioengineered cardiac cell sheet grafts have intrinsic angiogenic potential," *Biochemical and Biophysical Research Communications*, vol. 341, no. 2, pp. 573–582, 2006.

[68] A. Siltanen, K. Kitabayashi, P. Lakkisto et al., "hHGF overexpression in myoblast sheets enhances their angiogenic potential in rat chronic heart failure," *PLoS One*, vol. 6, no. 4, Article ID e19161, 2011.

SAGE-Hindawi Access to Research
Stem Cells International
Volume 2011, Article ID 456815, 12 pages
doi:10.4061/2011/456815

Research Article

An Inducible Expression System of the Calcium-Activated Potassium Channel 4 to Study the Differential Impact on Embryonic Stem Cells

Stefan Liebau,[1] Michael Tischendorf,[2] Daniel Ansorge,[2] Leonhard Linta,[1] Marianne Stockmann,[1] Clair Weidgang,[2] Michelina Iacovino,[3] Tobias Boeckers,[1] Götz von Wichert,[2] Michael Kyba,[3] and Alexander Kleger[2,4]

[1] *Institute for Anatomy and Cell Biology, Ulm University, 89081 Ulm, Germany*
[2] *Department of Internal Medicine I, Ulm University, 89081 Ulm, Germany*
[3] *Department of Pediatrics, Lillehei Heart Institute, University of Minnesota, Minneapolis, MN, USA*
[4] *Institute of Molecular Medicine and Max-Planck-Research Group on Stem Cell Aging, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany*

Correspondence should be addressed to Alexander Kleger, alexander.kleger@uni-ulm.de

Received 15 April 2011; Accepted 14 June 2011

Academic Editor: Gabriela Kania

Rationale. The family of calcium-activated potassium channels consists of four members with varying biological functions and conductances. Besides membrane potential modulation, SK channels have been found to be involved in cardiac pacemaker cell development from ES cells and morphological shaping of neural stem cells. *Objective*. Distinct SK channel subtype expression in ES cells might elucidate their precise impact during cardiac development. We chose SK channel subtype 4 as a potential candidate influencing embryonic stem cell differentiation. *Methods*. We generated a doxycycline inducible mouse ES cell line via targeted homologous recombination of a cassette expressing a bicistronic construct encoding SK4 and a fluorophore from the murine HPRT locus. *Conclusion*. We characterized the mouse ES cell line iSK4-AcGFP. The cassette is readily expressed under the control of doxycycline, and the overexpression of SK4 led to an increase in cardiac and pacemaker cell differentiation thereby serving as a unique tool to characterize the cell biological variances due to specific SK channel overexpression.

1. Introduction

Calcium-activated potassium channels (SKCas) of small and intermediate conductance represent a family of pore proteins which selectively transduce transmembranous potassium ion currents with differing conductance capacity [1]. Throughout the organism, these channels are expressed in tissues derived from all three germ layers, the central nervous tissue [2, 3], hematopoietic and muscle cells [4–6], or intestine epithelial cells [7], with distinct expression patterns. In excitable cells, SKCas are mostly involved in the generation of the hyperpolarization following the action potential [1]. Besides the modulation of the membrane potential in various tissues and cell populations, these ion channels have been found to be involved in a number of biological processes, for example, proliferation, cell differentiation, and cell morphology. As these mechanisms are present in stem or progenitor cells of different origin and potency, a role in developmental processes can be hypothesized [8–11]. Although the distinct mechanisms have not been fully understood yet, studies have shown on the one hand that the modulation of the intracellular calcium signaling by this channel family and its protein partner calmodulin might play a substantial role. On the other hand we showed in earlier studies that SKCas are important players as modulators of the cytoskeleton via specific protein-protein interaction [9, 12]. To elucidate the developmental involvement of SK channels we utilized embryonic stem cells (ESCs) representing a bona fide model to investigate the specific role of distinct proteins during development and cell differentiation [13, 14]. Moreover,

SKCas have shown to be involved in the regulation of proliferation in several types of stem cells [10, 15–17]. Recently, we were able to describe the abundance of SK channels subtypes in ESCs with SK4 being predominantly expressed. These findings were extended by our group to murine pluripotent stem cells, namely, ES and iPS cells (Liebau, unpublished data and [5]). We found in consequent experiments that increased SK channel activity resulted in a strong and fast differentiation of ES cells followed by a cell fate determination to the cardiac lineage. Precise characterization of the arising cardiomyocytes revealed the generation of a predominant population of cardiac pacemaker-like cells upon SKCa activation in both ES and iPS cells (Liebau, unpublished data and [5]). In fact, SK4 appeared to play a superior role during this process as its knockdown in embryonic stem cells led to a significantly decreased rate of cardiac differentiation [5]. Nevertheless, the impact of each particular SKCa subtype remains elusive. Apart from that, it remained unclear whether the inhibition of proliferation via SK channel activity in ES cells was solely due to a direct impact on the mitotic cell cycle or whether enhanced differentiation diminished proliferation of stem cells. This might be explained by the fact that the activation via the SKCa activator EBIO activates not only one, but all 4 subtypes, most likely having overlapping functions within the SKCa family. Unfortunately, loss of function experiments usually do not allow a stage-specific analysis and chemical inhibitors commonly harbor off-target effects. Thus, a clean and bias-free genetic system is required to dissect the contribution of each SK subtype towards the cardiac program.

Cardiac development follows a specific developmental cascade that is initiated by early mesendoderm and mesoderm formation while the heart fields arise from cells in the anterior lateral plate mesoderm of the early embryo. These fields include the precursors of both myocardial and endocardial cells, although there is apparently no common pool of bipotential precursors for these two heart cell lineages [18, 19]. This is followed by the anlagen of a cardiogenic plate and formation of cardiac progenitor cells with varying potential to differentiate into the various cardiac subtypes. The next step is the specification of cardiomyocytes during the formation of the cardiac tissue [20, 21], shortly followed by expression of differentiation markers [22]. The early cardiac zone exhibits inducing activities on cardiac differentiation. This activity is not only driven by the cardiac zone but also from the anterior endoderm [19, 23–25]. Genes encoding factors of the NK homeodomain, GATA, T-box, and other families were found to exert the functions of inducive signals during specification, patterning, and differentiation of the heart [18]. Subsequently, structural and functional proteins are required during late tissue formation and the long functional period of cardiomyocytes and their respective subtypes, such as pacemaker cells of the conduction system are expressed.

The role of SK channels during this process remains to be determined as their mechanistic position within the cardiac program is still underdeveloped. Genetic engineering of ES cells, a commonly used developmental *in vitro* model for studying cardiac development, is often limited by several factors. This includes, for example, low or varying transfection efficiencies in transiently overexpressing constructs or, in the case of stable transfection, random insertions of the gene of interest, leading to varying insertion numbers and unforeseen side effects in the cell. Apart from that, a controllable expression of the inserted construct is highly desirable [26, 27]. Here we report on the generation and characterization of an ESC line expressing SK4 with a doxycycline-dependent expression level and its potential impact on cardiac development from embryonic stem cells.

2. Material and Methods

2.1. ES Cell Culture. Embryonic stem (ES) cells were cultivated in the undifferentiated state on primary embryonic mouse fibroblasts. The mouse embryonic fibroblasts (MEFs) were inactivated by mitomycin C (Sigma-Aldrich, St. Louis, USA; final concentration 0.01 mg/mL) incubation for 2.5 h [28, 29] before using them for ES cell culture. MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), L-glutamine (Invitrogen; final concentration 2 mM), nonessential amino acid mix (Invitrogen; stock solution diluted 1 : 100), sodium pyruvate (Invitrogen; final concentration 1 mM), and β-mercaptoethanol (Sigma-Aldrich; final concentration 10^{-5} M). ES cell culture was performed in Knockout DMEM (Invitrogen) supplemented with 15% FCS (Invitrogen; stem cell tested), 1000 units/mL LIF (ESGRO), and the same additions as used in the fibroblast medium [30]. ES cells were trypsinized (0.1% Trypsin/0.02% EDTA mixed 1 : 1) and replated every 24–36 h on inactivated mouse embryonic fibroblasts growing on tissue culture dishes (Nunc, Wiesbaden, Germany).

2.2. Generation of iSK4 Cells. One day before the nucleofection procedure, A2lox.cre cells were imposed to 1 μg/mL of doxycycline to induce Crerecombinase. ES cells were nucleofected using the Nucleofector Technology (Lonza) according to the manufacturer procedures. The Nucleofector Kit for Mouse Embryonic Stem Cells has been used upon further optimization together with program A1 and 10 μg of DNA (SK4-IRES-acGFP p2lox vector) per 5 million parental A2lox.cre embryonic stem cells. Nucleofected cells were plated on neomycin-resistant, mitomycin-C-inactivated murine embryonic fibroblasts. Two days after nucleofection the assortment in ES cell medium supplemented with neomycin (400 μg/mL) was started. Neomycin-resistant clones were picked on inactivated feeder cells and expanded for further analysis after 10 days of selection.

2.3. In Vitro Differentiation of ES Cells. In vitro differentiation of ES cells was carried out according to the standard protocol using the hanging drop method as described in [30, 31]. Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% FCS (Lonza, Basel, Switzerland), GlutaMax, NEAA, and freshly prepared monothioglycerol (Sigma-Aldrich; final concentration 450 μM) were used for differentiation. Briefly, 400 cells per 20 μL differentiation

medium were placed on the inside of lids of Petri dishes filled with 10 mL PBS and were cultivated for 2 days in hanging drops. After another two days of cultivation on bacteriological dishes, these 4-day-old (4 d) embryoid bodies (EBs) ($n = 20$) were plated on (0.2%) gelatin-coated 6-well dishes or cover slips for RNA or immunofluorescence analysis and assayed at specific time points. RNA samples were collected 10 days after EB plating. For doxycycline (Sigma-Aldrich) treatment a 1 mg/mL stock solution was prepared in sterile water and EBs were treated at final concentration of 1 μg/mL doxycycline in differentiation medium from day 0 until day 10 after EB plating. Doxycycline was added to the culture medium every second day with regular medium changes. All experiments were performed at least three times. For monolayer conditions, doxycycline was added to ES cells which were kept under pluripotency conditions for three days of culture in a final concentration of 1 μg/mL. Afterwards, cells were collected for RNA and immunostaining analysis.

2.4. Genomic PCR. Genomic ES cell DNA was isolated by the Blood & Tissue Kit (QIAGEN, Hilden, Germany). For PCR reaction recombinant Taq DNA polymerase (Invitrogen) was used. The reaction was performed in 25 μL containing 1 μL DNA, 0.25 μL Dream Taq/Herculase and the following final supplements: 2.5 μL PCR buffer (10X), 2 μL 1.5 mM $MgCl_2$, 0.5 μL of each 10 mM dNTP, 0.3 μL of each primer. PCR conditions for ES cell analysis started with denaturation at 94°C for 3 min followed by a primer-dependent number of cycles with denaturation at 94°C for 30 s, annealing temperature at 61°C for 45 s, and product elongation at 72°C for 1 min. The following gene locus specific primer sequences have been used: LOXIN R 5′-ATA CTT TCT CGG CAG GAG CA-3′, and LOXIN F 5′-CTA GAT CTC GAA GGA TCT GGA G-3′.

2.5. Quantitative One Step Real-Time PCR. One-step real time PCR was carried out according to [9, 32] the Rotor-Gene Q usage instructions (QIAGEN). Amplification was monitored and analyzed by measuring the binding of the fluorescence dye SYBR Green I to the minor groove of double-stranded DNA. 1 μL of total RNA was reverse-transcribed and subsequently amplified using QuantiFAST SYBR Green RT-PCR Master mix (QIAGEN). QuantiTect primer assays (QIAGEN) were used in all experiments. Each RNA preparation was tested for genomic DNA contamination in a LightCycler RT-PCR by replacing reverse transcriptase with water. Tenfold dilutions of total RNA were used as an external standard curve. PCR efficiency results from the slope of these standard curves. Internal standards (house-keeping gene) and samples were simultaneously amplified. RT-PCR conditions started with 20 min of RT at 50°C, followed by activation of Taq-polymerase at 95°C for 15 min. 45 cycles with denaturation at 94°C for 15 s, 58°C for 20 s, and 72°C for 16 s amplified all described products with a PCR efficiency close to 3.3 (exact doubling of PCR product). To verify the specificity of the PCR amplification products, melting curve analysis was performed using the following thermal cycling profile: 95°C for 0 s, 65°C for 15 s, and ramping to 95°C with stepwise signal acquisition. The concentration of each transcript was calculated by reference to the respective standard curve. Relative gene expression was expressed as a ratio of target gene concentration to housekeeping gene hydroxymethylbilane synthase (HMBS) concentration.

2.6. Immunocytochemistry. Immunofluorescence analysis was carried out as shown in [33, 34] with either ES cells or differentiating EBs cultivated on 0.2% gelatin-coated coverslips. The cells were rinsed in PBS and fixed with PBS containing 4% paraformaldehyde at room temperature for 20 min. For Oct4 staining cells were incubated for 10 min in 0.2% Triton X (Sigma-Aldrich) followed by 30 min incubation in 5% goat serum in 0.5% fish skin gelatin (Sigma) to block unspecific binding. Then preparations were incubated with the primary antibody against Oct3/4 (Santa Cruz; Santa Cruz, USA; diluted 1 : 400) at RT in a humidified chamber for 1 h. In case of SSEA1 staining (Developmental Hybridoma Banks, 1 : 500) the permeabilization step was skipped. After washing three times in PBS, cells were incubated for another 1 h at 37°C with the respective Alexa fluorophore-labeled secondary antibodies. Samples were subsequently embedded in VECTASHIELD mounting medium (Vector, Burlingame USA) and analyzed by fluorescence microscopy.

2.7. Immunoblotting. iSK4 cells were pelleted and lysed in RIPA lysis buffer (50 mM tris-HCl pH 8.8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2.5 mM $MgCl_2$, protease, and phosphatase inhibitor cocktail) (Roche, Mannheim, Germany). After centrifugation at 14,000 rpm for 10 min, protein concentrations were measured in the lysates. 100 μg of total protein was resuspended in Laemmli buffer and subjected to SDS-PAGE. The gels were blotted onto a PVDF membrane and blocked with 5% milk in 0.1% TBS-Tween buffer (TBS-T). After blocking, SK4-specific antibody was applied (1 : 1000 4°C over night incubation) (Cell Application, San Diego, USA), beta-actin (1 : 1000). After washing with TBS-T samples were incubated with secondary horseradish peroxidase- (HRP-) labeled antimouse (1 : 4000) (Roche) or antirabbit IgG (1 : 5000) (Bio-Rad, Munich, Germany) antibodies in blocking solution for 1 h at room temperature. ECL signals were visualized and quantified using a Fujifilm LAS with the corresponding quantification software.

2.8. FACS Analysis. A single-cell suspension was generated from iSK4 using trypsin/EDTA. Finally the washed pellet was dissolved in 5% FCS/PBS and analyzed on an LSR FACS machine (BD Bioscience, San Jose, USA) for GFP fluorescence encoded from the AcGFP cassette in iSK4 cells upon doxycycline exposure. Data were analyzed using the WinMDI software version 2.8.

2.9. Proliferation Assay. Cell proliferation assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA) according to the manufacturer's protocol.

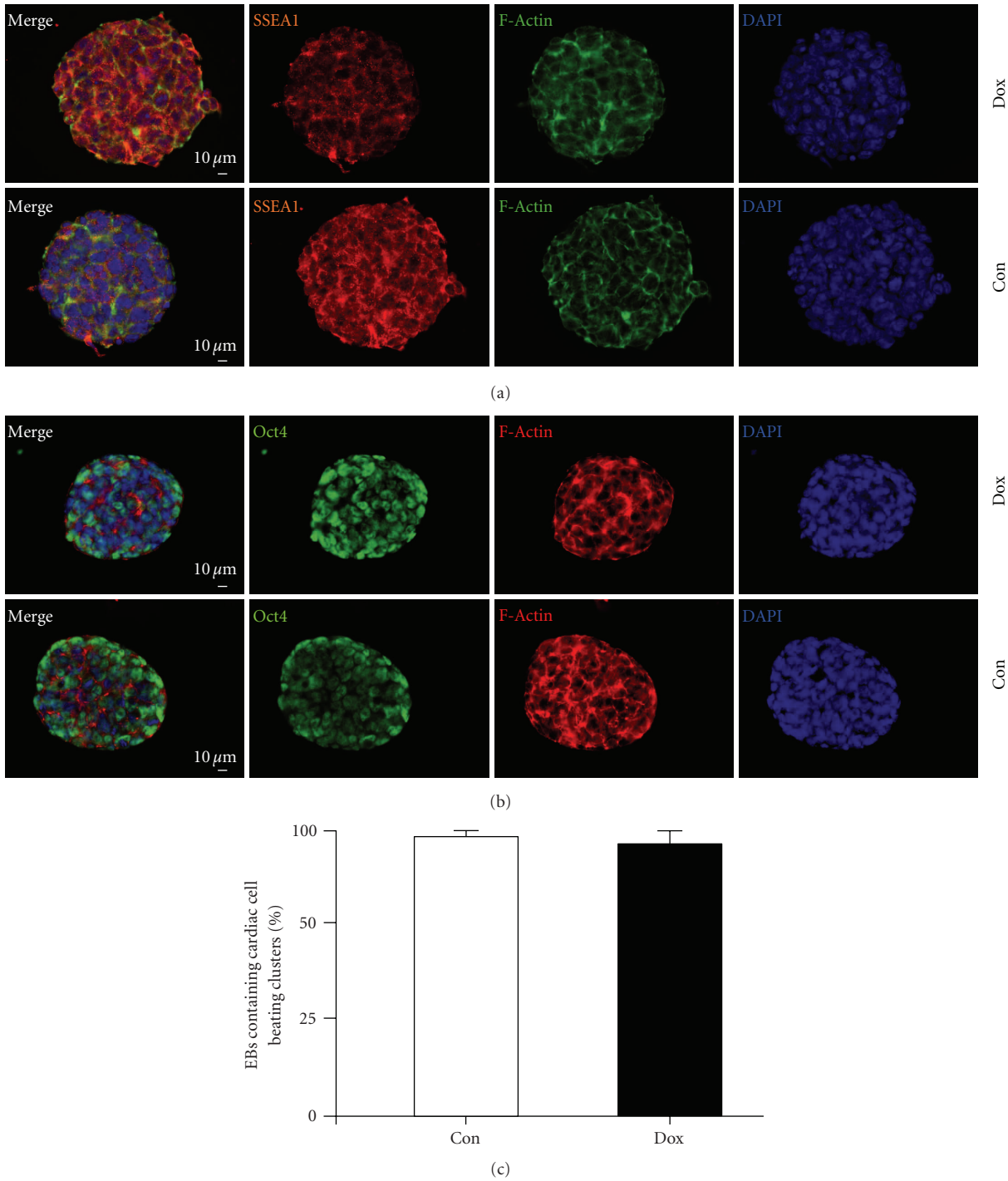


Figure 1: Effects of doxycycline treatment on A2lox.cre ES cells. (a, b) SSEA-1 (a) and Oct4 (b) staining upon 3 days of treatment with 1 μg/mL of doxycycline revealed no difference in expression levels. (c) EBs were plated, and the percentage of EBs containing cardiac cell beating clusters was determined in dox-treated versus untreated cultures on day 10 after EB plating on day 4.

3. Results

3.1. Generation of SK4-AcGFP Inducible Knock in ES Cell Lines (iSK4). First, we assessed whether a doxycycline inducible system fits our requirements of being neutral with respect to differentiation of embryonic stem cells. To this end, we used wild-type ES cells and treated them with the respected concentration of doxycycline for three days. We could not observe any differences neither on the expression of common pluripotency markers such as SSEA1 or Oct4 (Figures 1(a) and 1(b)) nor on the numbers of cardiac beating clusters (Figure 1(c)). Next, we started out with the construction of

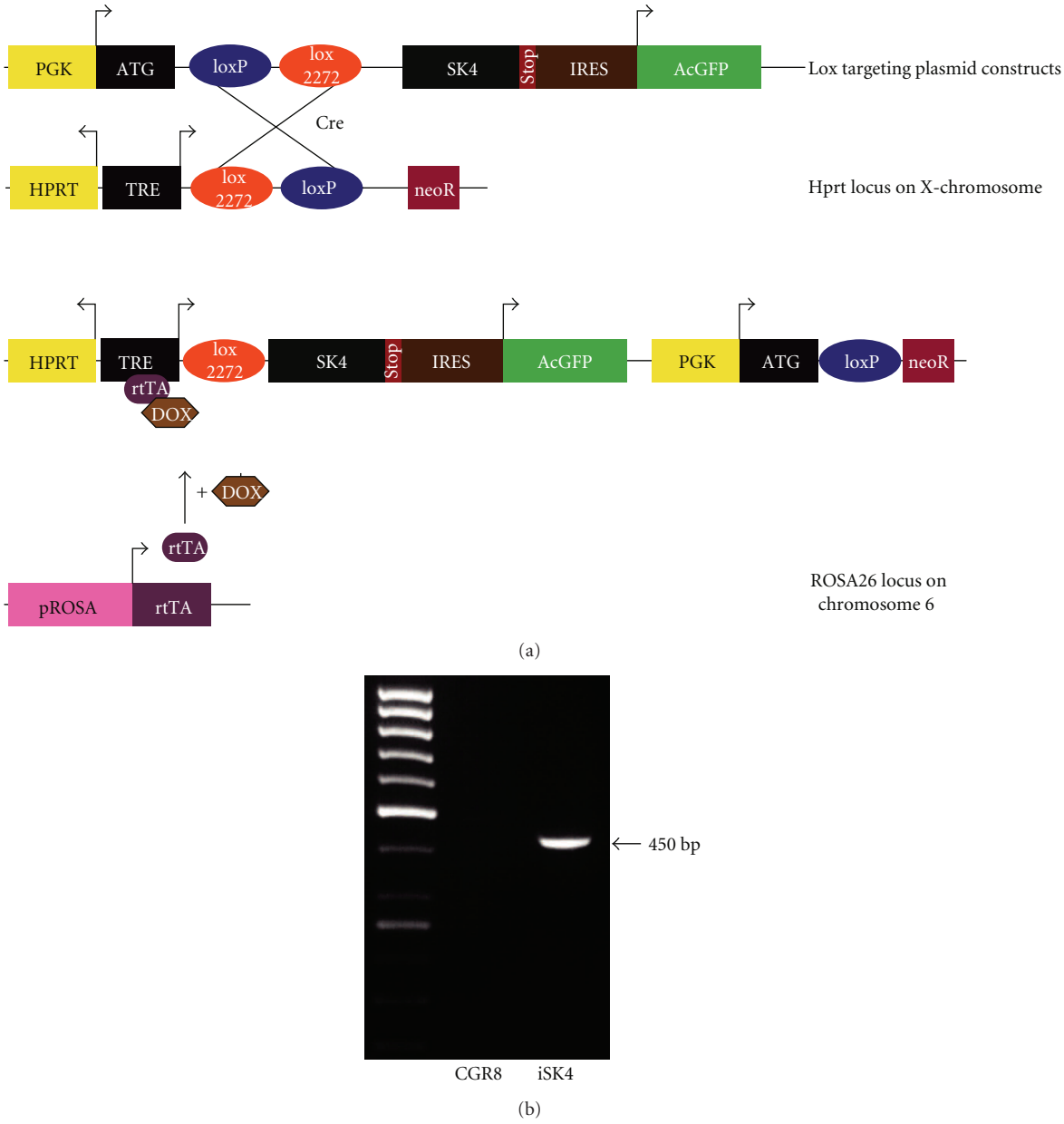


Figure 2: Construction of the iSK4 cell line. (a) Scheme of the expression cassette integrated into the desired loci. The ES cell line A2lox.cre harbors an rtTA in the constitutive ROSA26 locus on chromosome 6. A neomycin (noeR) resistance is recombined upon integration of the targeting vector. Recombination of the construct into the prepared site on the X-chromosome is facilitated by Cre. Transgenic cells can subsequently be selected by G418 (neo). tetOP: tetracycline response element; PGK: phosphoglycero kinase promoter; ATG: methionine initiation codon; black triangle: lox recognition sequence for Cre recombinase; GFP: green fluorescent protein; Neo: truncated neomycin (G418) resistance gene; pA: polyadenylation sequence. (b) Lox-in PCR verifies the correct integration of the expression cassette at determined loci on chr6 by amplifying a product at the size of ~450 bp. Untargeted ES cells were used as a control.

the desired SK4 expressing plasmid. The SK4 construct (a kind gift of Dr. Begenisich) was originally cloned into the pcDNA3.1 vector [35]. The construct was amplified by PCR introducing the required restriction sites to the sequence and was subsequently ligated into the pIRES2-AcGFP vector. In this vector, the green fluorophore is encoded by the same RNA that harbors the channel protein code but separated by an internal ribosome entry sequence (IRES) leading to the translation of a GFP which is not fused to the gene of interest. This was desirable as fluorophores are large proteins of approximately 30 kD and, fused to other proteins, may cause misfolding, wrong localization, or impaired function of the protein of interest [36]. This vector harbors the green fluorophore found in the jellyfish *Aequorea coerulescens (Ac)*. For the controlled and defined expression of the constructs in ES cells we utilized a genetically modified ES cell line

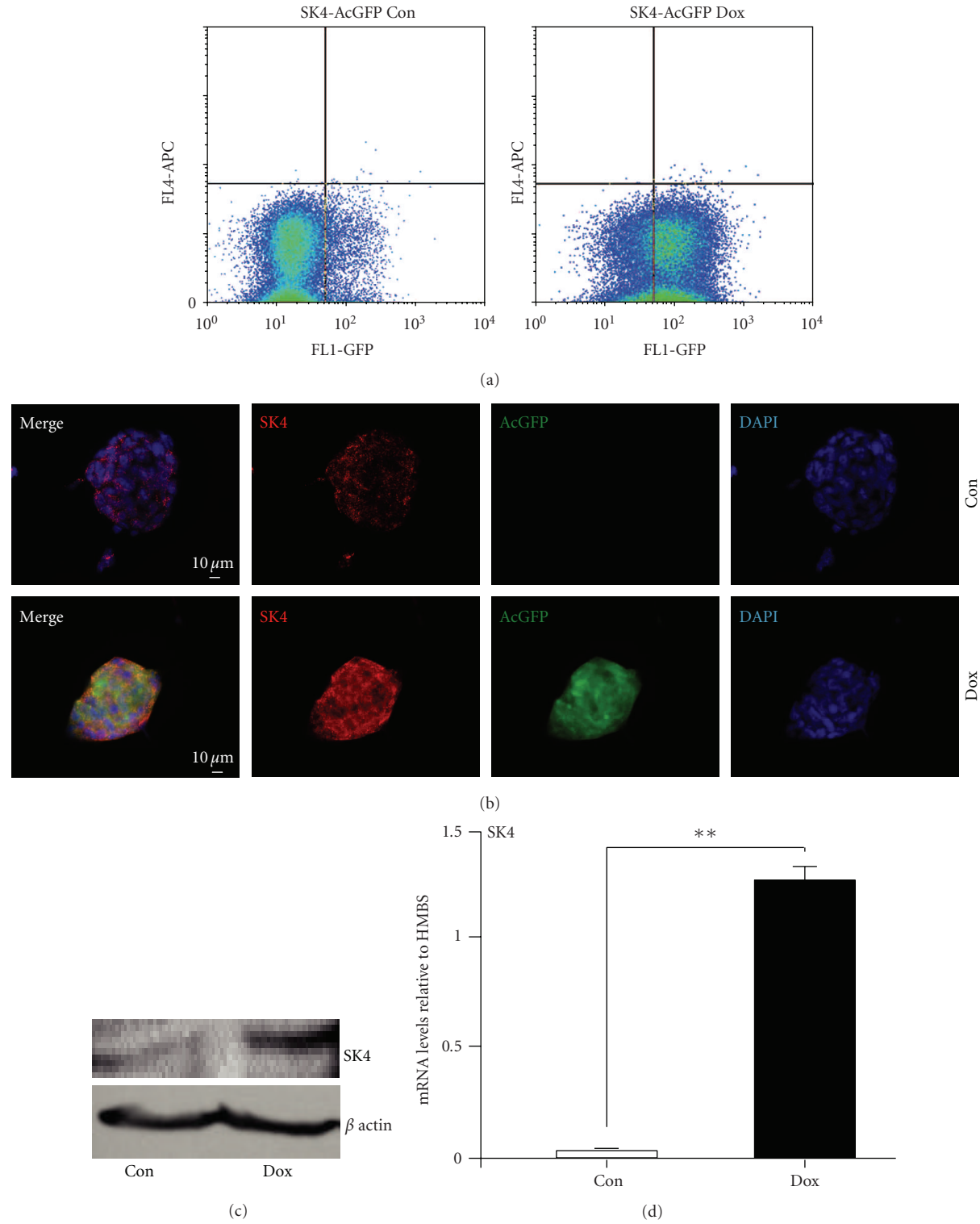


Figure 3: Characterization of iSK4 cells. (a) Flow cytometry by FACS shows a major proportion of the doxycycline-treated cells to exhibit green fluorescence (>75%). (b, c) Neomycin-selected iSK4 clonal cultures show dox-dependent elevation of SK4 proteins. (b) Iimmunocytochemistry using specific SK4 antibodies shows highly elevated protein levels of SK4 in the colonies after 1 day of doxycycline treatment. Dox-treated cells exhibit the AcGFP fluorescence (green) in contrast to controls. Both treated and untreated cells express SK4 proteins (red) with a strong increase in the dox-treated cells. Elevated SK4 proteins upon dox treatment was underlined by (c) by immunoblot. Nuclei are stained by DAPI (blue). Scale bars as indicated. (d) SK4 transcript increase upon doxycycline treatment is validated by real-time one-step RT-PCR.

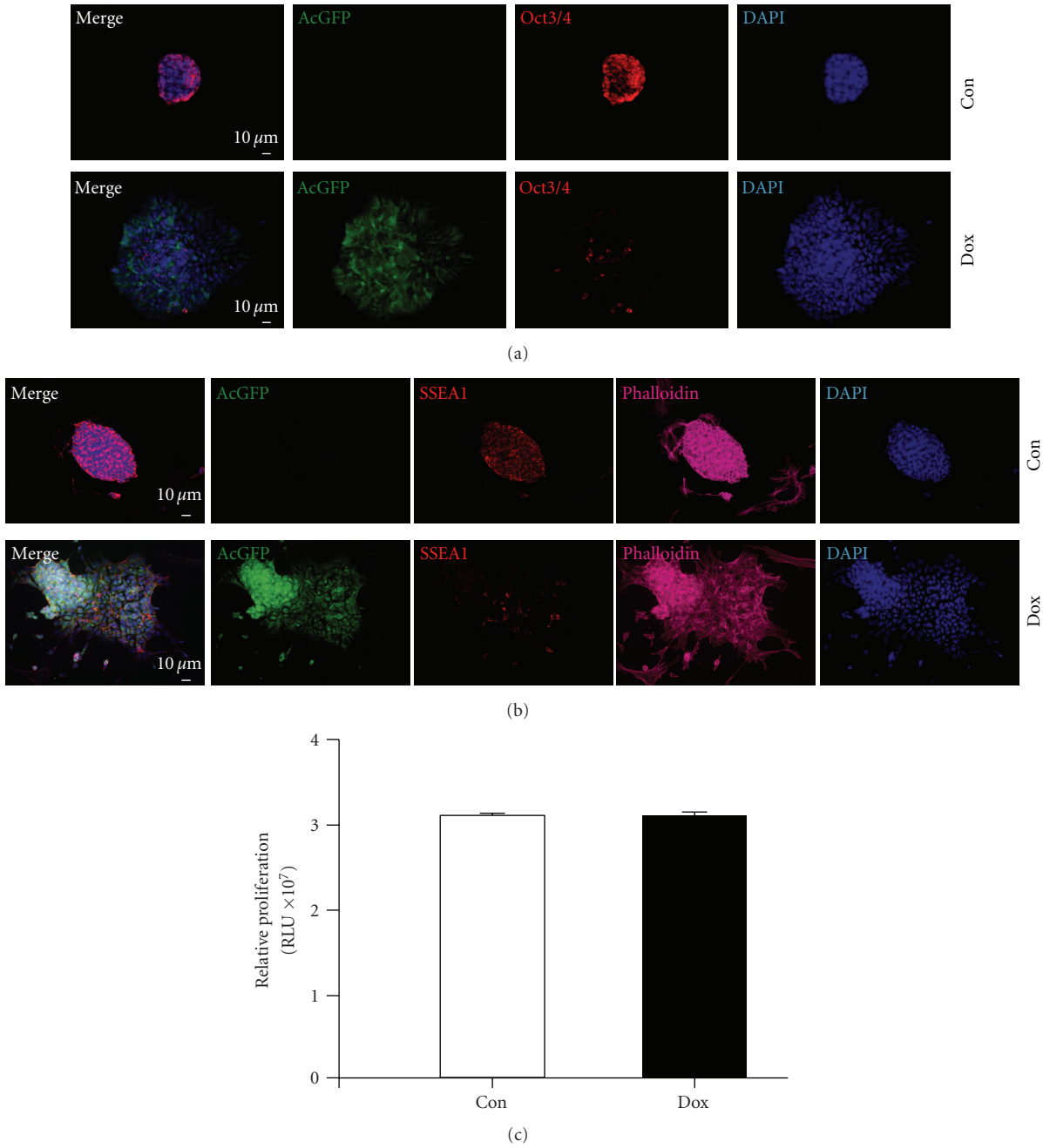


Figure 4: Doxycycline treatment alters cell behavior. (a) Elongated dox treatment for 3 days alters the pluripotency and morphology of the ES cells. Oct3/4 proteins (red) are decreased, and green fluorescence is solely expressed by dox-treated cells. (b) In line, the ES cell marker SSEA1 is lost during dox treatment (red), and the actin cytoskeleton, visualized by the f-actin marker phalloidin, is altered upon dox treatment. (c) Proliferation of iSK4 cells is not changed by dox-treatment shown in a proliferation assay. ($n = 4$) for all experiments. Nuclei are stained by DAPI (blue). Scale bars as indicated.

and the corresponding vector system initially generated by Iacovino et al. (submitted). The murine ES cell line (called A2lox.cre) allows the targeting of the requested transgene to an X-chromosomal locus, upstream of HPRT, in a controllable manner, as the pROSA26 locus already harbors a reverse tetracycline transactivator. The Crerecombinase encoding cDNA has been inserted into the HPRT locus in between *lox*P and *lox*M within A2lox.cre cells and is self-excised [37]. The cre-cassette is exchanged for the plasmid of interest (here SK4-pIRES AcGFP), upon induction of the cre protein by doxycycline (Figure 2(a)). This system has been shown to be suitable to dissect the impact of certain factors on cardiac differentiation in ES cells [38, 39]. For SK4, the complete cassette consisting of the SK4 coding sequence, the pIRES site, and the GFP was excised by restriction enzymes suitable for the p2lox system. Doxycycline inducible SK4 ES cells

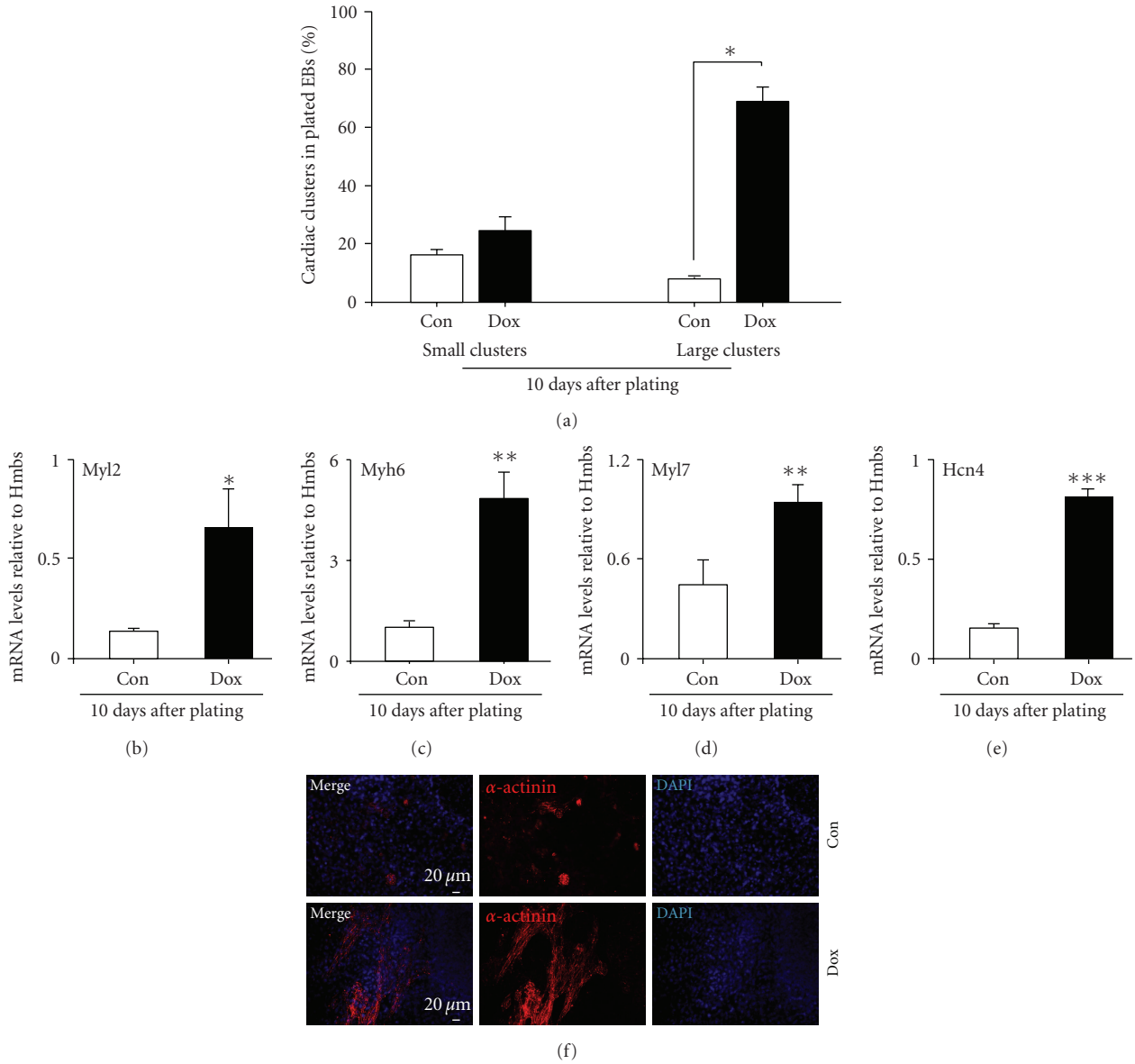


Figure 5: Cardiac differentiation is increased upon doxycycline treatment. (a) The number and size of cardiac clusters arising in dox-treated and untreated cultures were evaluated and counted. Dox treatment leads to a strong increase in both number and size of spontaneously beating cell clusters 10 days after plating. Small clusters: <100 μM; big clusters >100 μM. (b–e) Expression of cardiac marker genes upon induction of SK4. On mRNA level, the cardiac specific transcripts Myh2 (b), Myh6 (c), Myl7 (d), and Hcn4 (e) are significantly elevated upon dox treatment ($n = 4$). (f) This is also evident in immunocytochemical stainings using cardiac specific antibodies directed against α-actinin (red). The size of cardiac clusters is highly increased 10 days after plating. Nuclei are stained by DAPI (blue). Scale bars as indicated.

were generated by subcloning the SK4 cDNA into p2Lox [40] and using the derivative p2Lox-SK4 to target the inducible locus of A2Lox.cre ES cell (Iacovino et al., submitted). This cell line is an improved version of A2Lox [40] in which a floxed cre-cassette has been engineered downstream of a doxycycline inducible promoter upstream of HPRT on the X chromosome. A2Lox.cre cells were treated with 500 ng/mL doxycycline for 24 hours and harvested by trypsinization. Cre expression catalyzed replacement of the cre-cassette by the incoming SK4 cassette from p2Lox-SK4. Summarized, we chose a doxycycline controlled expression system which on the one hand harbors a cassette generating an untagged full length channel protein. On the other hand this system allowed us to select stable transfected ES cells by either FACS sorting for the soluble green fluorescing protein or via the ES cell-line-specific neomycin resistance that is solely expressed in the case of successful transfection. First, we transfected A2lox.cre cells with the according construct. Subsequently, neomycin-resistant clones were manually picked and subcultured for further analysis. Next, we checked whether the inducible cassette exchange was successful via analyzing the genomic locus. The band at 450 bp marks the successful targeting event in the cell line while no band was visible in nontransgenic CGR8 ES cells (Figure 2(b)).

3.2. Characterization of iSK4 ES Cells. In a primary approach we sought to verify the appropriate features of the transgenic cells. Therefore, we tested the desired expression of the green fluorophore in a doxycycline-dependent manner. In comparison to the untreated cultures FACS analysis of doxycycline-treated iSK4 cells showed a robust expression of the AcGFP regarding most of the subcultured clones (Figure 3(a)). Secondly, as expected, the induction of the transgene upon application of doxycycline treatment was detectable on protein levels and mRNA. Protein levels upon dox treatment were investigated either by immunofluorescence or by immunoblotting. We could detect both the endogenous expression of the channel protein and a specific increase of the expected transgenic channel proteins (Figures 3(b)–3(c)). This was further underlined by the detection of both the endogenous and overexpressed channel transcripts at doxycycline-dependent quantities (Figure 3(d)), investigated by qPCR. Further experiments were performed with several clones expressing comparable levels of SK4 under doxycycline (not shown).

3.3. Role of SK4 Overexpression in iSK4 Cell Lines. Although we observed in earlier studies that SK4 activity is crucially involved in ES cell differentiation and subsequent generation of cardiac and cardiac pacemaker-like cells, we did not yet know what impact the simple overexpression of this channel would have on the generated iSK4 ES cell line under several conditions. This is of particular interest as expression does not necessarily mean activity in case of ion channels. In fact, this would point to signaling events which are not directly linked to the membrane potential itself, as we previously reported for neural stem cells [12]. In a first step we looked for both initiation of differentiation and changes of the cell cycle upon doxycycline supplementation. In immunocytochemical stainings we observed a clear change of ES cell colony morphology and loss of pluripotency markers in iSK4 cells upon doxycycline-induced expression of SK4. This was true for the common markers Oct3/4 and SSEA1, respectively. The GFP protein seen in the green channel marks the transgene expression of the SK4 protein during this assay (Figures 4(a)–4(b)). ES cells are characterized by a unique cell cycle regulation which comprises fast self-renewing of this cell system and differentiation is usually accompanied by changes in the proliferation rates in ES cells. Nevertheless, cell titer glow assays did not show a change in proliferation capacity upon SK4 overexpression (Figure 4(c)).

3.4. Cardiac Differentiation Capacity of iSK4 ES Cells. SK channel activity strongly drives the cell fate of differentiating ES cells towards the cardiac lineage. In particular this was the case upon embryoid body-induced germ layer formation and subsequent increased SK channel activity after EB plating. Different doxycycline-treatment regimens were applied during differentiation, and cultures were analyzed regarding their cardiac differentiation potential, according to morphological criteria at several time points (data not shown). In the end, we discovered that continuous doxycycline treatment during differentiation had the strongest cardiac inducing effect. Obviously, increased SK4 expression led to highly elevated numbers of cardiac clusters with an additional increase in the size of the beating areas as shown by the high abundance of small clusters and a significant switch to form large clusters in comparison to control cells (Figure 5(a)). Next, we wanted to know whether this cardiac inducing effect is further mirrored by increased cardiac gene expression levels. A strong upregulation of the general cardiac marker gene Myl2 (4.6 fold; Figure 5(b)) was observed upon SK4 expression. Also atrial-specific genes such as Myh6 [41, 42] (5.6 fold) and Myl2a (2.07 fold) were strongly up-regulated (Figures 5(c) and 5(d)). This suggested us to investigate the expression levels of HCN4, belonging to the family of channels responsible for the hyperpolarization activated current, I_f. HCN4 plays a major role in the pacemaker potential of the sinoatrial node (SAN). It labels pacemaker tissues including the SAN, serves as an excellent nodal marker, and was strongly up-regulated upon SK4 induction (5.48 fold, Figure 5(e)). This was further underlined by the immunocytochemical detection of large α-actinin-positive clusters in the dox-treated cultures compared with only a few positive cells under control conditions (Figure 5(f)).

4. Discussion

Here we report the successful generation and characterization of a murine embryonic stem cell line overexpressing the subtype 4 of calcium-activated potassium channels in a doxycycline dependent manner. These cells were investigated concerning their impact of SK4 on cardiac differentiation derived from ES cells.

In an earlier study we were able to show that murine ES-cells change their cell fate determination in an SK activity dependent manner. Increased SK activity led to the differentiation of ES cells into a high proportion of cardiomyocytes with the majority of the cells showing functional and morphological properties of cardiac pacemaker cells. These experiments were mainly based on the potentiation of SK channel activity mediated by the commonly used chemical compound 1-EBIO (1-ethyl-2-benzimidazolinone). Mechanistically, the EBIO-induced increase in Ca^{2+}-sensitivity is suggested to result from the interaction of the benzimidazolinone with the Ca^{2+} gating apparatus of SK channels [43]. EBIO is known to nonselectively activate all SK subtypes [44] which makes it difficult to dissect the distinct biological role of the individual SK channel subtypes. In addition, the functional potassium gating pore is composed of four subunits which can consist of either 4 similar SK channel proteins (homomeric) or can be a mix of several different SK subunits (heteromeric) *in vitro*, leading to functional alterations [45], and *in vivo* [46, 47]. Furthermore, all SK channel subtypes display a variety of splice variants with yet unclear functional diversity [47–50]. In our primary studies we tried to dissect cardiac inducing effects upon SKCa activity by applying different kinds of inhibitors. However, these experiments were partly biased by cytotoxicity of the inhibitors. Therefore, we generated SK4 knockdown cells which showed

that depletion of SK4 "rescues" this inductive effect by strongly diminishing cardiac differentiation in these cultures [5]. Still, the mechanistic role of SK4 channel remained unclear. This led us to the idea of systematically exploring the exact role of this subtype during ES cell differentiation by applying a clean and straightforward genetic system allowing stage-specific expression of SK4 in ES cells. Several gain of function systems, for example, lentiviral transduction or transfection followed by selection for stable clones were considered for these approach. However, none of this approaches fulfilled all the respective criteria in one system, namely, (i) inducible and (ii) dose-dependent expression of the inserted cDNA (iii) from a defined genetic locus (iv) without random insertion-based bias of data interpretation [37]. These requirements led us to use A2lox.cre cells due to the following superior characteristics. First of all, this system allows a simple cloning of the gene of interest in a stable plasmid vector which comprises easy-to-use restriction sites for ligation. Secondly, the targeting strategy to a specific locus circvents insertion-based gene dysfunction. Together with the doxycycline inducible transcript expression of SK4 all mentioned problems were solved and allowed us to study the cell biological and developmental role of this SK channel subtype in ES cells. We found a decrease of pluripotency marker proteins upon overexpression for several days. Still, in contrast to direct increased SK channel activity by 1-EBIO in nontransgenic ES cells, we could not observe a change in proliferation rates under SK4 overexpressing conditions in iSK4 cells. Several reasons might be responsible for that finding, for example, the influence of other SK channel subtypes, heteromeric channel composition or ES-cell-specific splice variants. As for the next step, we investigated several regimens of stage-specific overexpression of SK4, starting with doxycycline treatment during distinct phases of cardiac in vitro differentiation (day 0–2: mesendoderm formation; day 2–4: mesoderm formation; day 4–9: formation of cardiac progenitors). We found that concerning cardiac differentiation, continuous transgene activation was successfully inducing cardiac myocyte generation. We could proof that by increased mRNA levels for cardiac transcripts and on protein levels. This clearly underlined the specific role of SK4 channel proteins during ES cell differentiation towards a cardiac cell fate. The ability to modulate expression times, expression in various cell stages and in a quantitative manner finally gives us the possibility to study these channel proteins under further conditions. Furthermore, it comprises the possibility to investigate participating signals which support or mediate SK channel-induced mechanisms. Solely under circumstances which combine understatement of SK channel behavior with their influence on cellular mechanisms we might be able to translate SK channel-mediated stem cell differentiation into the human system.

Authors' Contribution

S. Liebau and M. Tischendorf contributed equally to the paper.

References

[1] M. Stocker, "Ca^{2+}-activated K^+ channels: molecular determinants and function of the SK family," *Nature Reviews Neuroscience*, vol. 5, no. 10, pp. 758–770, 2004.

[2] M. Stocker and P. Pedarzani, "Differential distribution of three Ca^{2+}-activated K^+ channel subunits, SK1, SK2, and SK3, in the adult rat central nervous system," *Molecular and Cellular Neurosciences*, vol. 15, no. 5, pp. 476–493, 2000.

[3] M. K. Boettger, S. Till, M. X. Chen et al., "Calcium-activated potassium channel SK1- and IK1-like immunoreactivity in injured human sensory neurones and its regulation by neurotrophic factors," *Brain*, vol. 125, no. 2, pp. 252–263, 2002.

[4] S. Ohya, S. Kimura, M. Kitsukawa, K. Muraki, M. Watanabe, and Y. Imaizumi, "SK4 encodes intermediate conductance Ca^{2+}-activated K^+ channels in mouse urinary bladder smooth muscle cells," *Japanese Journal of Pharmacology*, vol. 84, no. 1, pp. 97–100, 2000.

[5] A. Kleger, T. Seufferlein, D. Malan et al., "Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells," *Circulation*, vol. 122, no. 18, pp. 1823–1836, 2010.

[6] J. F. Hoffman, W. Joiner, K. Nehrke, O. Potapova, K. Foye, and A. Wickrema, "The hSK4 (KCNN4) isoform is the Ca^{2+}-activated K^+ channel (Gardos channel) in human red blood cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7366–7371, 2003.

[7] L. A. Simms, J. D. Doecke, R. L. Roberts et al., "KCNN4 gene variant is associated with ileal crohn's disease in the Australian and New Zealand population," *American Journal of Gastroenterology*, vol. 105, no. 10, pp. 2209–2217, 2010.

[8] H. Jäger, T. Dreker, A. Buck, K. Giehl, T. Gress, and S. Grissmer, "Blockage of intermediate-conductance Ca^{2+}-activated K^+ channels Inhibit human pancreatic cancer cell growth in vitro," *Molecular Pharmacology*, vol. 65, no. 3, pp. 630–638, 2004.

[9] S. Liebau, B. Vaida, C. Proepper et al., "Formation of cellular projections in neural progenitor cells depends on SK3 channel activity," *Journal of Neurochemistry*, vol. 101, no. 5, pp. 1338–1350, 2007.

[10] S. P. Wang, J. A. Wang, R. H. Luo, W. Y. Cui, and H. Wang, "Potassium channel currents in rat mesenchymal stem cells and their possible roles in cell proliferation," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 9, pp. 1077–1084, 2008.

[11] D. L. Tharp, B. R. Wamhoff, J. R. Turk, and D. K. Bowles, "Upregulation of intermediate-conductance Ca^{2+}-activated K^+ channel (IKCa1) mediates phenotypic modulation of coronary smooth muscle," *American Journal of Physiology*, vol. 291, no. 5, pp. H2493–H2503, 2006.

[12] S. Liebau, J. Steinestel, L. Linta et al., "An SK_3 channel/nWASP/Abi-1 complex is involved in early neurogenesis," *PLoS ONE*, vol. 6, no. 3, p. e18148, 2011.

[13] S. Irion, M. C. Nostro, S. J. Kattman, and G. M. Keller, "Directed differentiation of pluripotent stem cells: from developmental biology to therapeutic applications," *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 73, pp. 101–110, 2008.

[14] S. J. Kattman, E. D. ler, and G. M. Keller, "Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development," *Trends in Cardiovascular Medicine*, vol. 17, no. 7, pp. 240–246, 2007.

[15] X. Bai, J. Ma, Z. Pan et al., "Electrophysiological properties of human adipose tissue-derived stem cells," *American Journal of Physiology*, vol. 293, no. 5, pp. C1539–C1550, 2007.

[16] X. L. Deng, C. P. Lau, K. Lai, K. F. Cheung, G. K. Lau, and G. R. Li, "Cell cycle-dependent expression of potassium channels and cell proliferation in rat mesenchymal stem cells from bone marrow," *Cell Proliferation*, vol. 40, no. 5, pp. 656–670, 2007.

[17] R. Tao, C. P. Lau, H. F. Tse, and G. R. Li, "Regulation of cell proliferation by intermediate-conductance Ca^{2+}-activated potassium and volume-sensitive chloride channels in mouse mesenchymal stem cells," *American Journal of Physiology*, vol. 295, no. 5, pp. C1409–C1416, 2008.

[18] S. Zaffran and M. Frasch, "Early signals in cardiac development," *Circulation Research*, vol. 91, no. 6, pp. 457–469, 2002.

[19] P. P. Tam, M. Parameswaran, S. J. Kinder, and R. P. Weinberger, "The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation," *Development*, vol. 124, no. 9, pp. 1631–1642, 1997.

[20] P. B. Antin, R. G. Taylor, and T. Yatskievych, "Precardiac mesoderm is specified during gastrulation in quail," *Developmental Dynamics*, vol. 200, no. 2, pp. 144–154, 1994.

[21] M. O. Montgomery, J. Litvin, A. Gonzalez-Sanchez, and D. Bader, "Staging of commitment and differentiation of avian cardiac myocytes," *Developmental Biology*, vol. 164, no. 1, pp. 63–71, 1994.

[22] Y. Han, J. E. Dennis, L. Cohen-Gould, D. M. Bader, and D. A. Fischman, "Expression of sarcomeric myosin in the presumptive myocardium of chicken embryos occurs within six hours of myocyte commitment," *Developmental Dynamics*, vol. 193, no. 3, pp. 257–265, 1992.

[23] T. Inagaki, V. Garcia-Martinez, and G. C. Schoenwolf, "Regulative ability of the prospective cardiogenic and vasculogenic areas of the primitive streak during avian gastrulation," *Developmental Dynamics*, vol. 197, no. 1, pp. 57–68, 1993.

[24] T. M. Schultheiss, S. Xydas, and A. B. Lassar, "Induction of avian cardiac myogenesis by anterior endoderm," *Development*, vol. 121, no. 12, pp. 4203–4214, 1995.

[25] T. A. Yatskievych, A. N. Ladd, and P. B. Antin, "Induction of cardiac myogenesis in avian pregastrula epiblast: the role of the hypoblast and activin," *Development*, vol. 124, no. 13, pp. 2561–2570, 1997.

[26] M. Lewandoski, "Conditional control of gene expression in the mouse," *Nature Reviews Genetics*, vol. 2, no. 10, pp. 743–755, 2001.

[27] M. Gossen and H. Bujard, "Studying gene function in eukaryotes by conditional gene inactivation," *Annual Review of Genetics*, vol. 36, pp. 153–173, 2002.

[28] A. M. Wobus, H. Holzhausen, P. Jakel, and J. Schoneich, "Characterization of a pluripotent stem cell line derived from a mouse embryo," *Experimental Cell Research*, vol. 152, no. 1, pp. 212–219, 1984.

[29] A. M. Wobus, G. Wallukat, and J. Hescheler, "Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers," *Differentiation*, vol. 48, no. 3, pp. 173–182, 1991.

[30] A. M. Wobus, K. Guan, H. T. Yang, and K. R. Boheler, "Embryonic stem cells as a model to study cardiac, skeletal muscle, and vascular smooth muscle cell differentiation," *Methods in Molecular Biology*, vol. 185, pp. 127–156, 2002.

[31] A. Kleger, T. Busch, S. Liebau et al., "The bioactive lipid sphingosylphosphorylcholine induces differentiation of mouse embryonic stem cells and human promyelocytic leukaemia cells," *Cellular Signalling*, vol. 19, no. 2, pp. 367–377, 2007.

[32] S. Liebau, B. Vaida, A. Storch, and T. M. Boeckers, "Maturation of synaptic contacts in differentiating neural stem cells," *Stem Cells*, vol. 25, no. 7, pp. 1720–1729, 2007.

[33] S. Liebau, C. Proepper, T. Schmidt, M. Schoen, J. Bockmann, and T. M. Boeckers, "ProSAPiP2, a novel postsynaptic density protein that interacts with ProSAP2/Shank3," *Biochemical and Biophysical Research Communications*, vol. 385, no. 3, pp. 460–465, 2009.

[34] S. Liebau, C. Pröpper, T. Böckers et al., "Selective blockage of $K_v1.3$ and $K_v3.1$ channels increases neural progenitor cell proliferation," *Journal of Neurochemistry*, vol. 99, no. 2, pp. 426–437, 2006.

[35] K. Nehrke, C. C. Quinn, and T. Begenisich, "Molecular identification of Ca^{2+}-activated K^+ channels in parotid acinar cells," *American Journal of Physiology*, vol. 284, no. 2, pp. C535–C546, 2003.

[36] C. E. Gustafson, S. Levine, T. Katsura et al., "Vasopressin regulated trafficking of a green fluorescent protein-aquaporin 2 chimera in LLC-PK1 cells," *Histochemistry and Cell Biology*, vol. 110, no. 4, pp. 377–386, 1998.

[37] D. Bosnakovski, Z. Xu, E. J. Gang et al., "An isogenetic myoblast expression screen identifies DUX4-mediated FSHD-associated molecular pathologies," *EMBO Journal*, vol. 27, no. 20, pp. 2766–2779, 2008.

[38] A. Holtzinger, G. E. Rosenfeld, and T. Evans, "*Gata4* directs development of cardiac-inducing endoderm from ES cells," *Developmental Biology*, vol. 337, no. 1, pp. 63–73, 2010.

[39] A. Bondue, S. Tännler, G. Chiapparo et al., "Defining the earliest step of cardiovascular progenitor specification during embryonic stem cell differentiation," *Journal of Cell Biology*, vol. 192, no. 5, pp. 751–765, 2011.

[40] M. Iacovino, C. Hernandez, Z. Xu, G. Bajwa, M. Prather, and M. Kyba, "A conserved role for hox paralog group 4 in regulation of hematopoietic progenitors," *Stem Cells and Development*, vol. 18, no. 5, pp. 783–791, 2009.

[41] M. G. Perino, S. Yamanaka, J. Li, A. M. Wobus, and K. R. Boheler, "Cardiomyogenic stem and progenitor cell plasticity and the dissection of cardiopoiesis," *Journal of Molecular and Cellular Cardiology*, vol. 45, no. 4, pp. 475–494, 2008.

[42] Y. H. Ching, T. K. Ghosh, S. J. Cross et al., "Mutation in myosin heavy chain 6 causes atrial septal defect," *Nature Genetics*, vol. 37, no. 4, pp. 423–428, 2005.

[43] P. Pedarzani, J. Mosbacher, A. Rivard et al., "Control of electrical activity in central neurons by modulating the gating of small conductance Ca^{2+}-activated K^+ channels," *Journal of Biological Chemistry*, vol. 276, no. 13, pp. 9762–9769, 2001.

[44] C. Hougaard, B. L. Eriksen, S. Jørgensen et al., "Selective positive modulation of the SK3 and SK2 subtypes of small conductance Ca^{2+}-activated K^+ channels," *British Journal of Pharmacology*, vol. 151, no. 5, pp. 655–665, 2007.

[45] A. S. Monaghan, D. C. Benton, P. K. Bahia et al., "The SK3 subunit of small conductance Ca^{2+}-activated K^+ channels interacts with both SK1 and SK2 subunits in a heterologous expression system," *Journal of Biological Chemistry*, vol. 279, no. 2, pp. 1003–1009, 2004.

[46] D. Tuteja, S. Rafizadeh, V. Timofeyev et al., "Cardiac small conductance Ca^{2+}-activated K^+ channel subunits form heteromultimers via the coiled-coil domains in the C termini of the channels," *Circulation Research*, vol. 107, no. 7, pp. 851–859, 2010.

[47] T. Strassmaier, C. T. Bond, C. A. Sailer, H. G. Knaus, J. Maylie, and J. P. Adelman, "A novel isoform of SK2 assembles

with other SK subunits in mouse brain," *Journal of Biological Chemistry*, vol. 280, no. 22, pp. 21231–21236, 2005.

[48] B. E. Shmukler, C. T. Bond, S. Wilhelm et al., "Structure and complex transcription pattern of the mouse SK1 K*Ca*channel gene, KCNN1," *Biochimica et Biophysica Acta*, vol. 1518, no. 1-2, pp. 36–46, 2001.

[49] H. Tomita, V. G. Shakkottai, G. A. Gutman et al., "Novel truncated isoform of SK3 potassium channel is a potent dominant-negative regulator of SK currents: implications in schizophrenia," *Molecular Psychiatry*, vol. 8, no. 5, pp. 524–535, 2003.

[50] C. Barmeyer, C. Rahner, Y. Yang, F. J. Sigworth, H. J. Binder, and V. M. Rajendran, "Cloning and identification of tissue-specific expression of KCNN4 splice variants in rat colon," *American Journal of Physiology*, vol. 299, no. 2, pp. C251–C263, 2010.