CELLULAE PROGENITORES CORDIS

Progenitor Cells from the Heart

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Progenitor Cells from the Heart

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(met een samenvatting in het Nederlands)

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WE STAND IN THE PRESENCE OF RIDDLES, BUT NOT WITHOUT THE HOPE OF SOLVING THEM.
AND RIDDLES WITH THE HOPE OF SOLUTION, WHAT MORE CAN A SCIENTIST DESIRE?

HANS SPEMANN

Voor Anke,
mijn ouders,
en mijn oma

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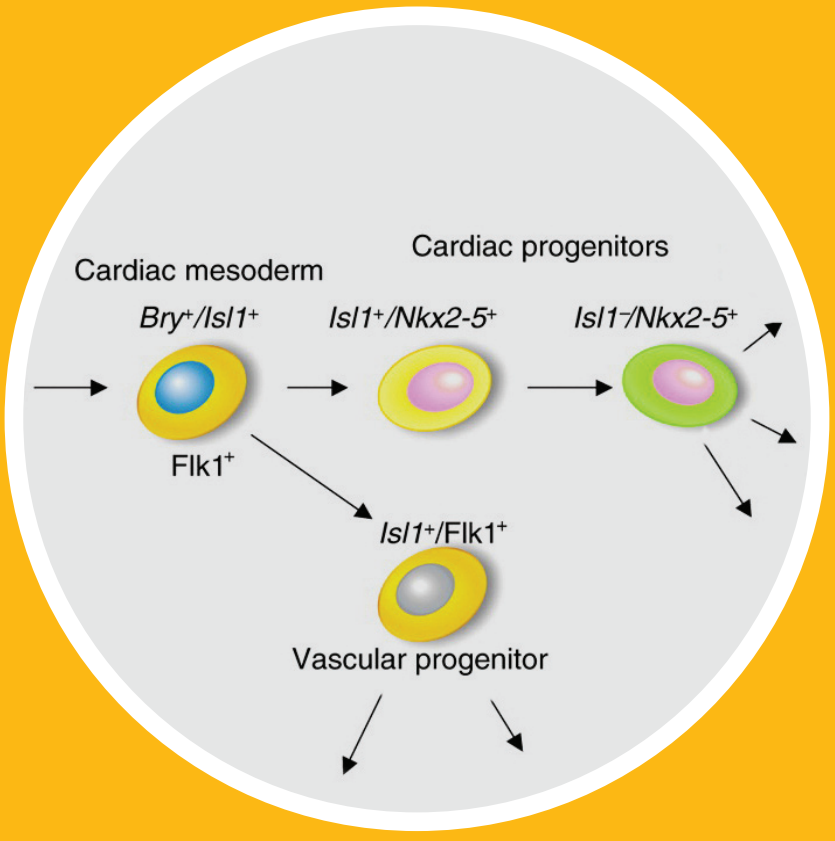
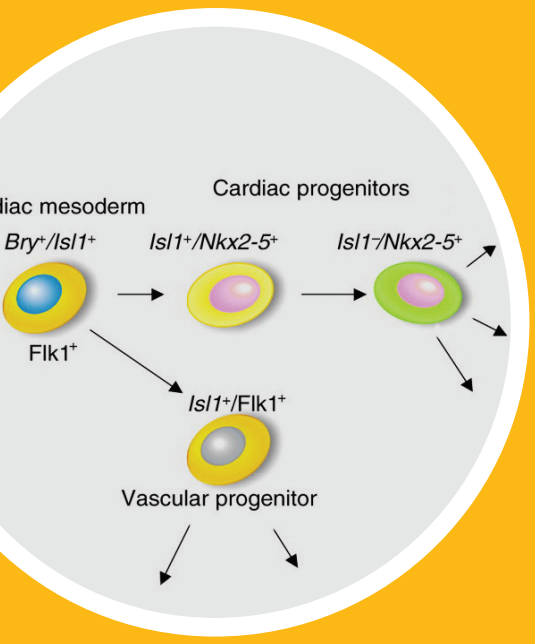
CHAPTER

1


Cardiac progenitors
Cardiac mesoderm
Bry+/Isl1+
Isl1+/Nkx2-5+
Isl1−/Nkx2-5+
Flk1+
Isl1+/Flk1+
Vascular progenitor

CHAPTER 1

Introduction



HEART DEVELOPMENT

During mammalian embryogenesis, cardiac tissue is formed soon after gastrulation. Myocardial progenitors migrate from the primitive streak to two cardiogenic regions on either side of the midline at day 6.5 post-coïtum (E6.5, see figure 1). Stimulated by cues from adjacent endoderm, the cardiogenic mesoderm develops into the primary and secondary heart fields at E7.5 in the mouse and day 16-17 in humans[1,2]. Here, transcription factors like the LIM homeodomain islet-1 (Isl1), the homeobox NK2 related locus 5 (Nkx2.5), and the zinc finger Gata4 activate several signaling pathways and direct further development of cardiac progenitor cells[3]. The primary and secondary heart fields fuse into a linear heart tube and the primary myocardium and endocardium form the first functional layers in the primitive heart. During embryonic development, neural crest cells migrate from the neural tube to different regions in the embryo, including the heart, where they contribute to the conduction system and outflow tract mesenchyme. The tubular heart becomes subject to a process called looping in which the anterior out flow tract and posterior inflow tract both become located cranially of the myocardium. Cells from the endocardium undergo epithelial-to-mesenchymal cell transformation and form endocardial cushions that develop into cardiac septa and valves. While epicardial cells contribute to cardiac fibroblasts and smooth muscle cells of coronary arteries[4], the atria and ventricles undergo further growth and specification, resulting in a multi-chambered heart with functional electromechanical properties.

CARDIOVASCULAR PROGENITOR CELLS

The isolation of progenitor cells from embryonic, fetal, or adult hearts and their subsequent characterization, differentiation, and transplantation in injury models is reviewed extensively in chapter two of this thesis. For the purpose of simplicity and because these progenitor cells have been shown to form cardiomyocytes,

smooth muscle cells, and endothelial cells, these populations will be collectively referred to as 'cardiovascular progenitor cells', unless otherwise specified.
Cardiovascular progenitor cells from human hearts have been isolated by cardiac explant culture and the subsequent formation of so-called cardiospheres[5,6], by clonal expansion of single cells from dissociated heart tissue[7], or by their expression of Isl1[8], c-Kit[9], or a yet unidentified membrane protein recognizing a mouse Sca-1 antibody (chapter three and[7]).

The developmental origin of the different cardiovascular progenitor cell populations described thus far is not completely understood, nor is it known if these populations have derived from a common precursor. Lineage tracing studies in ES cells suggest a Bry^{+}/Flk-1^{+} precursor origin from which subpopulations subsequently develop[10-12] (see figure 2, reviewed in[13]). Although in this model

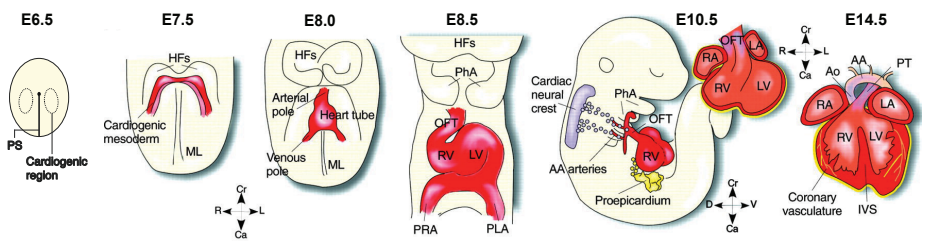


Figure 1: Early heart development

For details, see text. E6.5 to E8.5 and E14.5: ventral view, E10.5: lateral view. Cardiogenic mesoderm is depicted in red, cardiac neural crest progenitors in purple, and (pro-)epicardium in yellow. Abbreviations: AA: aortic arch, Ao: aorta, Ca: caudal, Cr: cranial, D: dorsal, HF: head fold, IVS: inter-ventricular septum, L: left, LA: left atrium, LV: left ventricle, ML: midline, PhA: pharyngeal arches, PLA: primitive left atrium, PRA: primitive right atrium, PS: primitive streak, PT: pulmonary trunk, R: right, RA: right atrium, RV right ventricle, V: ventral. Picture adapted from[1,13].

the cardiovascular precursors are depicted as Bry$^+$/Flk1$^+$/Isl1$^+$, human cardiovascular progenitor cells are not known to express Bry, suggesting the loss of this marker (and possibly others) during isolation or the existence of additional intermediate stages, like Flk1$^+$/Isl1$^+$/Nkx2.5$^+$ cardiovascular progenitor cells[11].

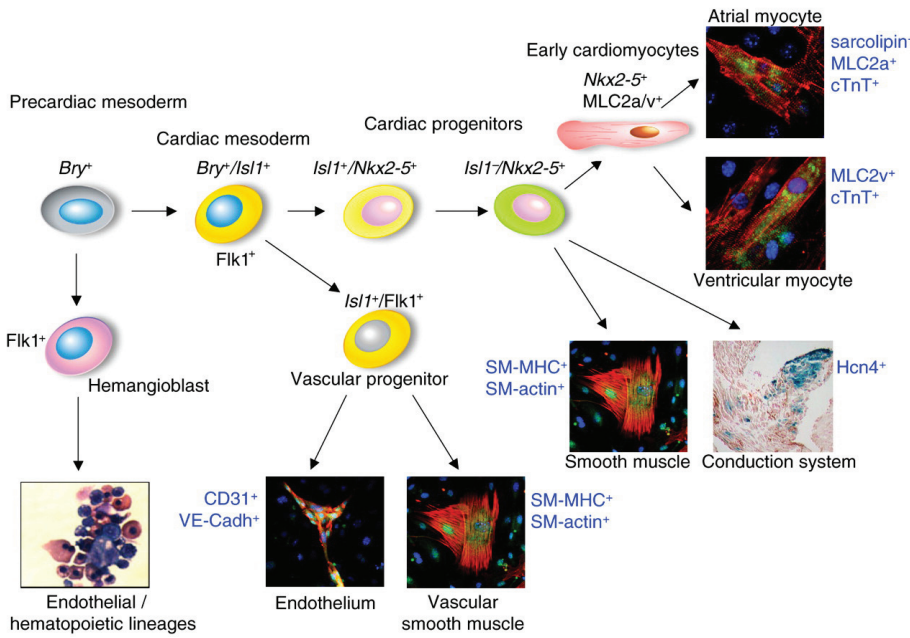


Figure 2: Linear development of different progenitor cell stages

Based on lineage tracing in mouse and ES-cell differentiation assays, different stages of cardiovascular progenitors have been identified [10-12]. In this model, mesodermal Bry$^+$ cells from the primitive streak develop into either Flk1$^+$ hemangioblasts, which form endothelial and hematopoietic cells, or Bry$^+$/Flk$^+$/Isl1$^+$ cardiovascular progenitor cells. This latter population then diverges into Isl1$^+$/Nkx2.5$^+$ cardiac progenitor cells and Isl1$^+$/Flk1$^+$ vascular progenitor cells that differentiate into the cells in the heart (cell-specific markers in blue). Picture taken from[13].

Studies on Isl1$^+$ progenitor cells suggested a continued presence of embryonic-derived, undifferentiated progenitor cells in the adult heart[8,11]. The question remains however whether other progenitor cell populations reside in the heart after development as well or if they have homed to the heart from the circulation. The expression of Isl1, Nkx2.5, and c-Kit by embryonic[11,12] as well as adult cardiovascular progenitor cells[7,8,14] may suggest an ancestral relationship. Alternatively, integration into myocardial tissue after migration from the circulation is not unlikely for c-Kit$^+$ and Sca-1$^+$ cells since these markers are also present on bone marrow-derived hematopoietic stem cells within the circulation. This is reinforced by studies on sex-mismatched heart transplants where cells from the circulation of the recipient were found in the transplanted heart[15,16]. As a consequence, these cells probably reside at places where homeostasis or repair was most necessary at the time of integration and, in time, adopted a cardiovascular progenitor cell phenotype, including the expression of Isl1 and Nkx2.5. Currently, it remains unclear whether different expression levels of Isl1, Nkx2.5, c-Kit, and Sca-1 after isolation are due to different isolation methods and culture differences or if these differences reflect a different developmental origin.

OVERVIEW OF THIS THESIS

In the following chapters, we will describe the characterization and differentiation of 'cardiomyocyte progenitor cells' (CMPCs). These cells appear to be different from earlier described cardiovascular progenitor cells (reviewed in **chapter two**), even though they do share some similarities. Direct comparison of CMPCs with other described cardiovascular progenitor cells has not been performed yet, but the detailed characterization of CMPCs and elucidation of several regulatory mechanisms described in this thesis will hopefully provide a useful platform from which to expand our current knowledge.
The identification, isolation, and characterization of human CMPCs, based on their recognition of a mouse Sca-1 antibody, are described

in **chapter three**. We show that CMPCs are able to differentiate into functional, spontaneously beating cardiomyocytes *in vitro*. In **chapter four,** we report the results from genomic profiling at different stages of CMPC differentiation into cardiomyocytes. Future validation of these screens will be important to identify and understand important genes and transcription factors that regulate cardiomyogenic differentiation in CMPCs. We show that the potential to form cardiomyocytes and other mesodermal cell lineages *in vitro* is different when CMPCs are isolated from fetal or adult heart tissue (**chapter five**), which has implications for the interpretation of both experimental studies and future clinical use. Fine-tuning of transcriptional activity by micro-RNAs (miRs) has recently been reported to play an important role during cardiac development [17,18]. We therefore screened for differential expression of miRs before and after CMPC differentiation and investigated functional roles of two interesting targets in **chapter six**. In order to explain the effect of TGFβ on CMPC differentiation, we studied the effect of membrane hyperpolarization, a mechanism that has been described to mediate myoblast differentiation into skeletal muscle (**chapter seven**). The cardiovascular differentiation potential of CMPCs makes them an interesting source to treat the injured heart. In this respect, enhancing CMPC proliferation and survival will be beneficial to their contribution to cardiac tissue. In **chapter eight,** we overexpressed a pro-survival gene in CMPCs and found that this enhanced proliferation and protected against apoptosis. The studies described in this thesis are summarized and discussed in **chapter nine**. Additionally, we describe several methods for overexpression of target genes in CMPCs in the **appendix**. Here, we also report our results regarding the establishment of cDNA expression libraries, intended to screen for factors that regulate proliferation and differentiation of CMPCs.

REFERENCES

1. Buckingham, M., Meilhac, S. & Zaffran, S. Building the mammalian heart from two sources of myocardial cells. *Nat Rev Genet* **6**, 826-35 (2005).
2. Moorman, A.F., Christoffels, V.M., Anderson, R.H. & van den Hoff, M.J. The heart-forming fields: one or multiple? *Philos Trans R Soc Lond B Biol Sci* **362**, 1257-65 (2007).
3. Zaffran, S. & Frasch, M. Early signals in cardiac development. *Circ Res* **91**, 457-69 (2002).
4. Lie-Venema, H. *et al.* Origin, fate, and function of epicardium-derived cells (EPDCs) in normal and abnormal cardiac development. *ScientificWorldJournal* **7**, 1777-98 (2007).
5. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
6. Smith, R.R. *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* **115**, 896-908 (2007).
7. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
8. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
9. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
10. Kattman, S.J., Huber, T.L. & Keller, G.M. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* **11**, 723-32 (2006).
11. Moretti, A. *et al.* Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151-65 (2006).
12. Wu, S.M. *et al.* Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137-50 (2006).
13. Laugwitz, K.L., Moretti, A., Caron, L., Nakano, A. & Chien, K.R. Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* **135**, 193-205 (2008).
14. Beltrami, A.P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763-76 (2003).

15. Quaini, F. *et al.* Chimerism of the transplanted heart. *N Engl J Med* **346**, 5-15 (2002).
16. Laflamme, M.A., Myerson, D., Saffitz, J.E. & Murry, C.E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res* **90**, 634-40 (2002).
17. Zhao, Y. & Srivastava, D. A developmental view of microRNA function. *Trends Biochem Sci* **32**, 189-97 (2007).
18. Thum, T., Catalucci, D. & Bauersachs, J. MicroRNAs: novel regulators in cardiac development and disease. *Cardiovasc Res* **79**, 562-70 (2008).

ABBREVIATIONS

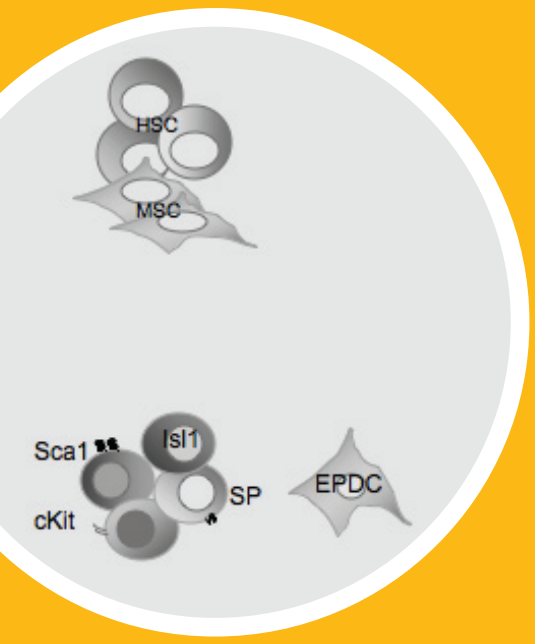
BMP	Bone morphogenetic protein
Bry	Brachyury T
CD	Cluster of differentiation surface molecule
CDK	Cyclin-dependent kinase
CMPC	Cardiomyocyte progenitor cell
CMPC-cm	CMPC-derived cardiomyocyte
EPC	Endothelial progenitor cell
ES cell	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
HDAC	Histone deacetylase
HEK 293	Human embryonic kidney cell line 293
HGF	Hepatocyte growth factor
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
I_{K1}	Inward rectifier current
iPS	Induced pluripotent stem cell
Isl1	Islet 1
Kir	Potassium inward rectifier
KWGF cell	Wild-type Kir2.1-GFP overexpressing HEK 293 cell
MAPC	Multipotent adult progenitor cell
MI	Myocardial infarction
MiR	Micro-RNA
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NFAT	Nuclear factor of activated T cells
Nkx2.5	NK2 related locus 5
RCAN	Regulator of calcineurin
RMP	Resting membrane potential
RNAi	RNA interference
Sca-1	Stem cell antigen-1
SP	Side population
TFBS	Transcription factor binding site
TGFβ	Transforming growth factor beta
UTR	Untranslated region
VEGF	Vascular endothelial growth factor

CHAPTER

2

CHAPTER 2

Progenitor cells for the heart



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ABSTRACT

Myocardial infarction (MI) and the consequent loss of functional myocardium is the leading cause of high mortality rates in Western countries[1]. After ischemia, lost cardiomyocytes are replaced by fibrotic tissue, while remaining cardiomyocytes try to compensate through a hypertrophic response, both further impairing ventricular function[2]. Current therapies consist of restoration of reperfusion and drug administration to improve chronic symptoms and prevent the development of heart failure. In recent years, stem cell transplantation therapy has been put forward as a promising approach to enhance perfusion and replace lost cardiomyocytes in the failing heart to prevent further damage and restore cardiac function[3,4]. When considering future clinical standardized practice, the question arises which stem cell population is most promising. Cell types from embryonic as well as adult origin have achieved substantial attention, but, since these differ in their potential to differentiate into cardiac cells, careful evaluation of their potential is required.

Based on:

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PLURIPOTENT STEM CELLS

Pluripotent stem cells can differentiate into cells of all three germ layers and are a viable source for transplantable cardiomyocytes (figure 1)[5-7]. The pluripotent embryonic stem (ES) cells and induced pluripotent stem (iPS) cells can both be cultured for extended periods of time and differentiated into the cardiomyocyte lineage with a fetal phenotype *in vitro*. The use of human ES cells is however ethically sensitive and their differentiation is not homogenous despite recent improvements in protocols[8]. Nonetheless, ES cell-derived cardiomyocytes have been shown to ameliorate damage resulting from myocardial infarction[9-11].

The recently described induced pluripotent stem cells (iPS cells) may be useful to overcome the ethical and possibly the immunological drawbacks of human ES cells. The introduction of a combination of specific transcription factors into skin fibroblasts turned a small subset of these cells into pluripotent ES cell-like cells[12,13]. While Oct4 seemed to be critical for the generation of these iPS cells in the studies reported thus far, different additional factors could be used to establish the fully pluripotent cell type.

The current efficiency of establishing these iPS cells needs to be improved and, as in ES cells, the potential tumorigenesis warrants caution. The source of cells used to create iPS cells also needs to be carefully evaluated. Fibroblasts from different tissues have shown different gene expression patterns and this may affect the future developmental potential and applicability of iPS cells[14]. Furthermore, cells from patients with genetic defects would require additional genetic manipulation before they can be used optimally for transplantation purposes. The treatment of sickle cell anemia in mice[15] is an important step in the right direction, but more research is needed before therapeutic application of iPS cells can be used to treat genetic defects in humans.

MULTIPOTENT SOMATIC STEM CELLS

Bone marrow stem cells

The bone marrow harbors different populations of stem cells, including stromal or mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs), hematopoietic stem cells (HSCs), side population (SP) cells, and endothelial progenitor cells (EPCs). These populations have shown varying potential with respect to multilineage differentiation and functional engraftment into host animals.

MSCs are well known for their differentiation capacity into various mesenchymal cell types including bone, tendon, cartilage, and fat (figure 1). Upon addition of the DNA demethylation agent 5-azacytidine, murine MSCs even showed a cardiomyocyte phenotype, expressed cardiac markers, and revealed spontaneous beating[16,17]. The cardiogenic potential of MSCs was further demonstrated *in vivo* by injecting early-passage human MSCs, expressing β-galactosidase, into the left ventricle of immune-deficient mice. Although only a small percentage of donor cells could be detected in the heart (0.44%), the engrafted cells did express several cardiac markers, such as cardiac troponin T, α-actinin, and phospholamban[18].

Hematopoietic stem cells (HSCs) have also been studied for their capacity to regenerate the injured myocardium, but their cardiomyogenic potential is still debated. HSC have been reported to transdifferentiate into cardiomyocytes, endothelial cells, and smooth muscle cells when transplanted directly into the heart after a myocardial infarction[19]. In contrast, Balsam and Murry tested cell transdifferentiation in genetically tagged mice and clearly demonstrated that HSCs do not differentiate into cardiomyocytes *in vivo*[20,21]. Alternatively, the small number of differentiated cells found *in vivo* after transplantation may result from fusion between donor bone marrow cells and host tissue, at least in the liver, brain, and heart (see box 1)[22,23]. Adult bone marrow-derived and circulating stem cells have already been taken into the clinic. Recent results from these trials have shown no substantial or varying degrees of enhanced functional

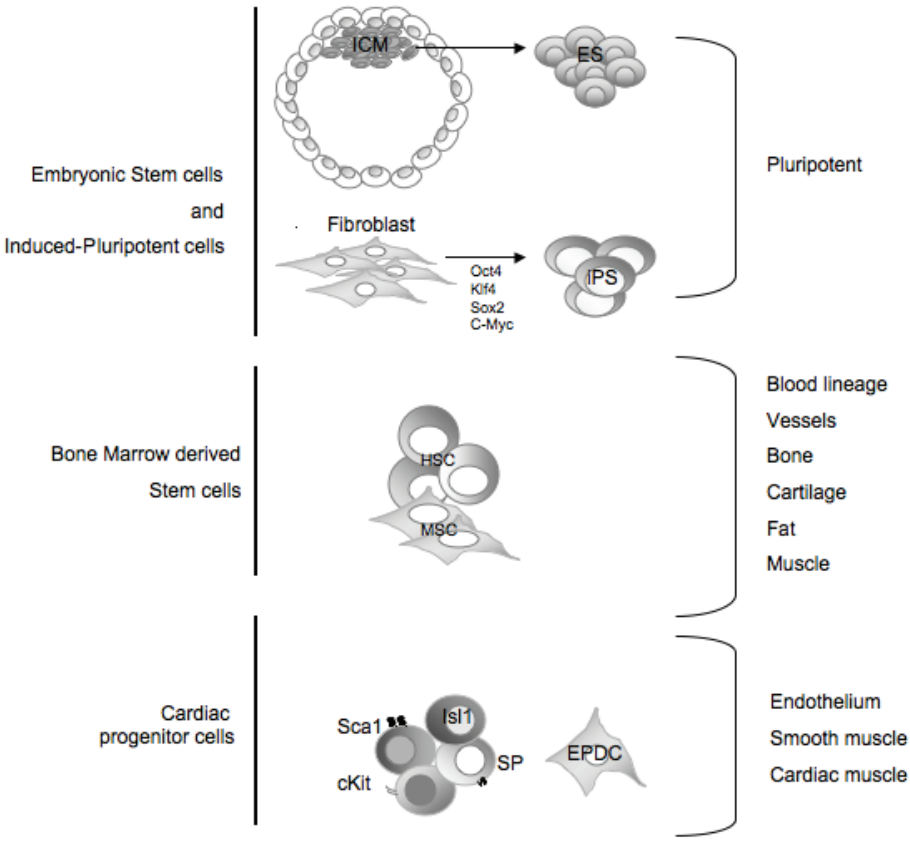


Figure 1: Stem cells sources for cardiac repair

Pluripotent stem cells can be isolated from the inner cell mass (ICM) of the blastocyst stage embryo to generate embryonic stem (ES) cells. Alternatively, adult skin fibroblasts can be reprogrammed by overexpression of specific transcription factors to create induced pluripotent stem cells (iPS).

Hematopoietic and mesenchymal stem cells derived from the bone marrow can give rise to cells from the blood (HSC) or bone, cartilage, fat, and muscle (MSC).

Cardiovascular progenitor cells have been identified by different markers and can be isolated from fetal and adult heart tissue. These progenitor cells are able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro* and *in vivo*. Epicardium-derived cells (EPDC) can be isolated from human heart as well and form fibroblasts and smooth muscle cells. For further details, see text. Picture taken from[5].

cardiac performance[24]. The predominant *in vivo* effects, at least with bone marrow or endothelial progenitor cells, may be neoangiogenesis or arteriogenesis and not cardiomyocyte differentiation to regain ventricular mass[25,26]. Cell replacement therapy may still be effective, since enhanced perfusion of cardiac tissue may be useful in early post-infarct phases, but in the late phase it is crucial that lost ventricular myocytes will be functionally replaced.

Cardiovascular progenitor cells

Although stem cells have been isolated from many adult tissues, the search for stem cells in the heart was considered futile given the accepted lack of regenerative potential of cardiomyocytes. Previous work suggested that the adult heart is a terminally differentiated organ without any regenerative capacity. However, this concept was questioned by several reports showing some evidence of mitotic division in the heart[27,28], suggesting that the adult heart contains at least some newly formed myocytes, possibly derived from resident cardiovascular progenitor cells[29]. The recently isolated cardiovascular progenitor cells may therefore be very effective in cardiomyocyte replacement.

Cardiovascular progenitor cells from animal models

Cardiovascular progenitor cells harbor specific gene expression profiles that can be used for their identification. Thus far, these progenitor cell populations have been isolated by cardiac explant culture, Hoechst dye efflux, or based on expression of Nkx2.5, Islet1 (Isl1), stem cell antigen-1 (Sca-1), or c-Kit.

Different animal species and experimental models have been used to determine the function of genes, like Isl1 and Nkx2.5, and show their importance during heart development (see chapter one and[30,31]). Besides its function during cardiogenesis, the transcription factor Nkx2.5 has also been identified as a marker for cardiovascular progenitor cells within the cardiac crescent of the developing murine heart[32]. These Nkx2.5 expressing progenitor cells were isolated by FACS sorting and microarray analysis of these cells revealed expression of genes specific for the developmental stage of the embryonic heart.

The transcription factor Isl1 has also been identified as a marker specific for progenitor cells[33,34] and shown to be highly expressed during the linear heart-tube stage. Lineage tracing revealed that Isl1$^+$ cardiovascular progenitor cells contribute to the formation of the outflow tract, atria, and ventricles. Moreover, deletion of the BMP receptor-1a disrupted expression of T-box transcription factors in murine Isl1$^+$ progenitors[34]. T-box factors play a role in early heart development[35] and their dependence on BMP signaling demonstrates the functional importance of embryonic growth factor pathways in cardiovascular progenitor cells. The identification of cardiovascular progenitor cells by Isl1 expression was used to isolate and further characterize this population in mouse, rat, and human adult hearts *in vitro* and *in vivo* (see figure 1 and table 1)[36]. Staining and Isl1-based reporter gene expression showed that Isl1$^+$ cardiovascular progenitors could be found in embryonic and postnatal hearts and that these cells differentiated into cardiomyocytes *in vivo*. Isl1$^+$ cells were then isolated from postnatal murine hearts, FACS sorted, and expanded in culture. The cultured cells revealed expression of early cardiac transcription factors and could be expanded *in vitro* without spontaneous differentiation. Co-culture experiments with non-viable neonatal mouse cardiomyocytes induced adult cardiomyocyte gene expression, suggesting differentiation without fusion (see box 1). Real-time intracellular calcium imaging and electrical stimulation of the differentiated progenitor cells suggested the presence of a functional β-adrenergic signaling pathway and excitation-contraction coupling. Further electrophysiological evaluation indicated that the differentiated progenitor cells had reached a neonatal cardiomyocyte phenotype. It will be interesting to see whether Isl1$^+$ progenitor cells can be located in and isolated from adult hearts as well.

Based on other markers known from bone marrow stem cell isolations, different cell populations were isolated from rodent hearts and tested for their cardiomyogenic potential *in vitro* and *in vivo* (see table 1). Cardiovascular progenitor cells expressing Sca-1 were isolated from mouse hearts[37-39]. The Sca-1$^+$ cardiovascular progenitor cells were CD45$^+$, CD34^{low}, and c-Kit^{low} and comprised an

estimated 0.3% of total cardiomyocyte number in the adult murine heart. These cells were able to differentiate into spontaneously beating areas after stimulation with oxytocin[37]. Further analysis revealed cardiomyocyte-specific gene expression on both RNA and protein level. Calcium current measurements in combination with isoproterenol administration enhanced the beating rate, indicating a functional β-adrenergic signaling pathway in differentiated cells. Additionally, the Sca-1$^+$ progenitor cells also developed osteocyte- and adipocyte-related characteristics when specifically stimulated with osteogenic inducers or cultured in adipogenic culture medium, respectively, suggesting an even broader differentiation potential. The murine adult heart contains Sca-1$^+$, CD31$^+$, c-Kit$^-$, CD34$^-$, and CD45$^-$ cardiovascular progenitor cells[38]. FACS analysis showed that 2% of the total heart cell population was Sca-1$^+$. This high number and thereby the discrepancy with other studies[37] suggest that other cell-types might be Sca-1$^+$ as well, probably including endothelial cells from the vasculature[40]. However, the myocyte-depleted fraction of Sca-1$^+$ progenitor cells showed expression of early cardiomyogenic transcription factors and the presence of endogenous telomerase activity, revealing the potential for self-renewal. Similar to the progenitor cells from Matsuura *et al.*[37], these cells differentiated into cardiomyocytes after stimulation with 5-azacytidine as determined by cardiomyocyte-specific staining and marker expression. *In vitro* knockout of the BMP receptor-1a decreased expression of the growth factor BMP2, the cardiac transcription factor Mef2c, and the sarcomeric protein α-MHC, confirming the involvement of auto- and/or paracrine action by BMP during differentiation. By using Cre/lox mediated heart-specific reporter gene expression *in vivo* (see box 1), derivatives of transplanted cardiovascular progenitor cells could be found in the heart shortly after myocardial infarction. It was shown that the donor cardiovascular progenitor cells either differentiated into new cardiomyocytes or fused with cardiomyocytes from the recipient. The question remains whether fusion preceded differentiation or whether progenitor cells differentiate prior to fusion with other cardiomyocytes. Furthermore, the effect of transplanted cells on cardiac function was not tested.

BOX 1: STEM CELL PLASTICITY VERSUS FUSION

In the first experiments describing stem cell plasticity, cells were claimed to transdifferentiate into a variety of cell types based on the presence of both donor-derived genes and specific differentiation markers of the host tissue wherein they resided[87]. However, several groups showed that fusion of donor and host cells *in vitro* and/or *in vivo* might have caused false-positive observations. Cells simultaneously expressing (tissue unspecific) markers from different mouse strains in co-culture or after bone marrow transplantation showed that cells had spontaneously fused whereas non-fused differentiated cells would have expressed only the donor-derived markers[22,23]. Cardiovascular progenitor cells were also shown to form new cardiomyocytes with and without fusion[38], while others have reported differentiation of cardiovascular progenitor cells without fusion[36,45]. This indicates that although conclusions on transplanted (trans)differentiated cells should be taken with caution, it is not entirely impossible to determine whether differentiation or fusion with resident cells occurred.

The presence of Y-positive cardiomyocytes in female heart transplants in male recipients suggested an extra-cardiac origin for these cells[63,64]. These mismatch experiments are a clear indication of whether the cells are derived from the donor or recipient. However, detailed examination should be performed to exclude false-positive observations of differentiated donor-derived cells[88]. In experimental models, other methods to distinguish between donor and recipient cells exist as well. These are based on site-specific recombination of specific sequences in the genome of the target cell by so-called Cre-lox recombination[89]. When the recombinase Cre is added to a cell with loxP sequences flanking a specific DNA sequence in the genome, the 'floxed' DNA fragment is removed and the loxP sites are recombined. Excising a stop codon via Cre-lox recombination can reactivate reporter genes that are inactivated by a homozygously added loxP-flanked stop codon between a promoter and the reporter gene. When donor cells expressing Cre fuse with recipient cells containing such an inactive reporter gene, Cre expression from the donor cell causes reactivated reporter gene expression in the recipient. This should give a clear distinction between fused and non-fused cells. However, fusion without recombination has been described as well[38], indicating that a combination of different techniques is required.

Side population (SP) cells are characterized and can be isolated by FACS via their capacity for Hoechst dye efflux via multidrug resistance (MDR) channels[41]. SP cells have been isolated from adult mouse hearts by several groups (see table 1)[42-45]. In addition to their isolation based on Hoechst dye efflux, SP cells could also be characterized by the expression of the ATP-binding cassette (ABC) transporter ABCG2 itself. Within the murine cardiac SP cell population there is a subpopulation positive for Sca-1 and negative for CD31[45]. This cell population already expressed cardiac, smooth muscle, and endothelial cell markers and this Sca-1$^+$/CD31$^-$ fraction was shown to have the highest differentiation potential towards the cardiomyocyte lineage. When co-cultured with adult rat cardiomyocytes, improved differentiation of the cardiac Sca-1$^+$/CD31$^-$ cells and spontaneous contractions combined with calcium transients were observed. Fusion was excluded in the majority of differentiated cells based on the absence of Cre/lox recombination (see box 1) and non-colocalization of green-fluorescent SP cells and red-fluorescent cardiomyocytes.

It is not clear how Sca-1$^+$ cardiovascular progenitor cells differ from cardiac SP cells. FACS analysis suggested that the SP cells described above are in fact a subpopulation of the Sca-1$^+$ cardiovascular progenitor cells[38]. Therefore, it might be that only Sca-1$^+$/CD31$^-$ SP cells are responsible for the observed cardiomyogenic potential of this progenitor cell population[45]. Additional support for the cardiomyogenic potential of CD31-negative progenitor cells came when Sca-1$^+$/CD31$^-$ progenitor cells isolated by magnetic cell sorting were shown to be able to differentiate into endothelial and cardiomyogenic lineages[39]. In *in vitro* assays, stimulation of Sca-1$^+$/CD31$^-$ progenitor cells with VEGF resulted in enhanced network formation and endothelial cell marker expression. Stimulation with 5-azacytidine and several growth factors induced a cardiomyocyte differentiation. As with the Sca-1$^+$/CD31$^-$ SP cells described by Pfister *et al*.[45], the differentiation of Sca-1$^+$/CD31$^-$ progenitor cells was improved when co-cultured with rat cardiomyocytes. FACS analysis of cardiac and circulating cell suspensions following left anterior descending (LAD) artery ligation in mice revealed a relative

increase and subsequent decrease of resident, but not circulating Sca-1$^+$/CD31$^-$ cells in time. This might suggest local proliferation and differentiation of resident cardiovascular progenitor cells after cardiac damage, but additional research is needed to support this. Direct intramyocardial Sca-1$^+$/CD31$^-$ progenitor cell transplantation after MI showed an improved cardiac function and enhanced efficiency in cardiac energy metabolism. However, despite increased myocardial neovascularization, endothelial cell and cardiomyocyte differentiation remained limited, suggesting that some other mechanism may be responsible for the observed effect.

A fourth population of rodent cardiovascular progenitor cells was identified based on the expression of the stem cell factor (SCF) receptor c-Kit[46]. The c-Kit$^+$ progenitor cells were found in the interstitia of rat hearts and were estimated to comprise 0.01% of total myocytes. Like Sca-1$^+$ cells, the c-Kit$^+$ cells expressed cardiomyocyte specific transcription factors like Nkx2.5, Gata4, and Mef2c[47]. *In vitro*, the c-Kit$^+$ cardiovascular progenitor cells were clonogenic and differentiated into a mixed population of primitive cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts after culture in differentiation medium containing dexamethasone. Transplantation of c-Kit$^+$ cells in rats suffering from an acute myocardial infarction resulted in increased cardiac function and survival, possibly due to the attenuation of the infarct by newly formed cardiomyocytes and smooth muscle cells. Fusion events were not explicitly tested. The c-Kit$^+$ cells share some similarities with above described cardiovascular progenitor cell populations (see table 1), but it remains to be determined whether they have been derived from a common ancestor.

To translate progenitor cell transplantation experiments into possible therapeutic applications, larger animal models with a human cardiac phenotype are essential. Likewise, isolation and characterization of cardiovascular progenitor cells from these animals will finally lead to a better understanding of their potential in cardiac regeneration in human patients. Recently, the dog heart was used to isolate c-Kit$^+$/Sca-1$^+$/MDR$^+$ progenitor cells with cardiomyogenic differentiation potential[48]. Upon

differentiation, the c-Kit$^+$ cells acquired a cardiomyocyte, smooth muscle, endothelial, or fibroblast phenotype. Interestingly, the canine cardiovascular progenitor cells expressed HGF, IGF, and their specific receptors. *In vivo* administration of HGF and IGF after MI induced an increase in cardiovascular progenitor cell number and proliferation, recruitment of progenitor cells to the infarcted area, and improvement in cardiac function, similar to what was observed in mice[49]. However, the effects of growth factor administration on other tissues need to be elucidated before it is safe to translate these experiments to a clinical setting. Besides the exertion of a paracrine effect on cardiac restoration, the canine cardiovascular progenitor cells might also be useful for autologous cell transplantation studies in animal models.

Cardiovascular progenitor cells in the human heart

Since autologous cell transplantation might prevent immune responses in patients, progenitor cell isolation from human tissues and especially the heart is very relevant. Motivated by earlier observations in animal models that the adult heart might contain replicating cells in pathologic circumstances[29], Anversa and co-workers investigated the presence of mitosis in biopsies from end-stage ischemic and idiopathic dilated cardiomyopathy patients[50]. Sections from biopsies from these patients and control hearts were used to study mitotic events in cardiomyocytes. Interestingly, different stages of mitosis were observed in non-cardiomyocyte interstitial cells and cardiomyocytes. Sections from diseased hearts showed a significant increase in mitotic cells compared to controls, suggesting that nuclear and cellular divisions were increased in diseased hearts. Cellular division was also reported in hearts from patients suffering from myocardial infarction[28]. Cell cycling was investigated in samples from the infarct border zone and more distant from the infarcted region. A profound increase in Ki67 labeling, a nuclear protein that is expressed in proliferating cells[51], and putative characteristics of karyokinesis and cytokinesis were found in cardiomyocytes in the infarcted heart, suggesting elevated levels of replicating cells in response to cardiac injury.

Table 1: Comparison of different cardiovascular progenitor cell populations

	Islet-1[36]	*Side Population cells*[42-45]	*Sca-1 cells*[37-39,57,58]	*C-Kit cells*[47,48,54]	*Cardiospheres*[55-56]
Localization	Mouse, rat, and huma atria, ventricles, and OFT	Mouse ventricle	Mouse and human heart	Rat, canine, and human heart	Mouse and human heart
Clonogenic	N.D.	N.D.	N.D./Yes	Yes	Yes
Characterization in vitro	Limited Hoechst efflux Sca-1-/ c-Kit-/ CD31- Nkx2.5+/GATA4+	Hoechst efflux Abcg2+ CD31+/- Sca1+/-	Limited Hoechst efflux CD34-/CD45- CD31+/- c-Kit+/-	c-Kit+/Sca-1+/MDR+ CD34-/CD45- GATA4+/Nkx2.5+/ Mef2c+	Clonal spheres CD34+/CD31+ Sca-1+/c-kit+
Differentiation in vitro	Co-culture with neonatal cardiomyocytes	Co-culture with cardiomyocytes improved cardiomyogenic differentiation	Cardiomyocyte differentiation induced by 5-azacytidine (+/- TGFβ) and oxytocin. Endothelial differentiation induced by VEGF	Differentiation into cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts	Spontaneous beating with or without co-culture
Characterization in vivo	Lineage tracing by Isl1-based lacZ expression	Abcg2 expression in the heart	Sca-1+, CD31+/- in mouse heart	c-Kit+/Nkx2.5+/ Mef2c+ in rats	N.D.
Differentiation in vivo	N.D.	N.D.	Limited endothelial and cardiomyocyte differentiation	Cardiomyocyte and SMC differentiation	Cardiomyocyte, SMC, and endothelial cell differentiation
Functional characterization	Ca2+ imaging, action potential recording, excitation-contraction coupling	Ca2+ imaging, excitation-contraction coupling	Ca2+ imaging, energy metabolism characterized	Excitation-contraction coupling	N.D.

Interestingly, in tissue from patients with cardiac hypertrophy, differentiating c-Kit$^+$ progenitor cells were observed to contribute to the formation of new cardiomyocytes, resulting in increased cardiac mass[52]. In this study, cells were identified as non-hematopoietic cardiac stem-, progenitor-, and precursor cells that increased in number in hypertrophic hearts compared to controls. Increased telomerase activity was observed in cells positive for stem-, progenitor-, and primitive cardiomyocyte cell-surface antigens. The increase in replicating cells in hypertrophic hearts supported earlier findings by the same group that cell replication in the pathologic heart is increased. A similar approach was used to look for progenitor cells in the acute and chronically infarcted heart[53]. Increased telomerase activity was observed in response to cardiac injury, but during chronic heart failure cell cycling appeared to be suppressed and apoptosis increased, which in turn can lead to cardiac dysfunction. Following up on these and earlier experiments with rodent cardiovascular progenitor cells[47], the group of Anversa was able to isolate a population of c-Kit$^+$ human cardiovascular progenitor cells that was self-renewing, clonogenic, and multipotent (figure 1)[54]. *In vitro*, these cells could form endothelial and smooth muscle cells, and, when co-cultured with rat neonatal cardiomyocytes, differentiated into cardiomyocytes as well. *In vivo*, the cardiovascular progenitor cells mainly developed into cardiomyocytes and attenuated cardiac remodeling after MI. Clusters of fibroblast-like cells termed 'cardiospheres' were isolated from postnatal human heart biopsies as well as murine hearts from different developmental stages[55,56]. These cardiospheres could be cultured from single cells and differentiated into cardiomyocyte-like cells when cultured in medium containing endothelial and basic fibroblast growth factors. Upon differentiation, spontaneous beating was observed in both murine and human cell clusters, even though the human equivalent only did so after co-culture and cellular coupling with adult rat cardiomyocytes. Cells from these clusters showed DNA synthesis, suggestive of cell cycling, and were positive for stem cell-, cardiomyocyte-, or endothelial cell-specific markers (see table 1). Importantly, single cells derived from the

cardiospheres could form new clusters with similar functional and phenotypic characteristics, including spontaneous beating, indicating the presence of a uniform cardiovascular progenitor cell with endothelial and cardiomyogenic differentiation potential. After transplantation of pooled cardiospheres in the infarcted heart, co-localization of donor-specific and endothelial, smooth muscle, or cardiomyocyte markers plus improved cardiac function was observed, suggesting *in vivo* differentiation and functional restoration of ischemic damage.

A more detailed *in vitro* characterization has been performed on cardiomyocyte progenitor cells (CMPCs) from human fetal tissue and adult heart specimens[57,58]. These progenitor cells can be isolated by clonal expansion of single cells or based on their recognition of a mouse Sca-1 antibody. Both populations express stem cell markers and early cardiac transcription factors, demonstrate telomerase activity, can easily be expanded in culture, and are clonogenic. These cardiomyocyte progenitor cells differentiate into spontaneously beating cells when stimulated with 5-azacytidine and develop heart-specific gene expression without the need for co-culture with adult cardiomyocytes. Cardiomyogenic differentiation efficiency can even be further enhanced (up to 90%) by TGFβ stimulation. Electrophysiological measurements revealed fetal ventricular parameters with a more mature phenotype than the differentiated cardiovascular progenitor cells described thus far by others. The more mature electrophysiological phenotype may lead to a better preservation of conduction characteristics and might sufficiently support stimulus propagation when transplanted. Additionally, CMPCs form endothelial and smooth muscle cells under angiogenic conditions, which may help to restore perfusion.

Finally, epithelial cells have been isolated from the epicardial layer of the heart. During heart development, cells migrate from the pro-epicardial organ to the outer myocardial surface to form an epicardial layer. Epicardial cells can then undergo epithelial-to-mesenchyme transition (EMT) and migrate into the myocardium to contribute to interstitial fibroblasts and coronary smooth muscle cells[59]. Human epicardium-derived cells (EPDCs) have been isolated

from the atrial appendages of adult heart and shown to undergo spontaneous EMT *in vitro*. The obtained fibroblast-like cells are then able to form smooth muscle cells as well[60]. When these cells were injected into a myocardial infarction model, vascularization was increased and cardiac remodeling reduced[61].

The isolation of progenitor cells from adult human cardiac tissue and subsequent characterization, followed by their application in experimental animal models have provided us with more insight in cardiovascular progenitor cell potential. However, the exact origin of these progenitor cells is still not clear. Many of the different progenitor cell populations share comparable marker expression patterns upon isolation, indicative of a common precursor; however, these populations have variable differentiation potentials that could be due to cell-origin, cell isolation procedures, or culture differences between different laboratories. Support for resident cardiovascular progenitor cells allocated during embryonic life has been provided by the identification and isolation of progenitor cells based on the expression of genes already expressed in the early embryonic heart[32,33,36]. Their identification in both embryonic and post-natal hearts might suggest that they persist in the adult heart as well. Alternatively, the notion that progenitor cells in the heart have resided there after their homing from the circulation is equally plausible. Especially since most cardiovascular progenitor cell populations reveal markers known from hematopoietic stem cells such as Sca-1, c-Kit, or the ABC transporter, which have then been used for specific cell isolation. Reports on stem cell homing to the heart after cytokine stimulation[62] and mismatch experiments after heart transplantation[63,64] favor a non-cardiac origin for vascular supportive structures, but evidence for localization of replaced cardiomyocytes is limited. After homing to the heart, undifferentiated progenitor cells can differentiate into cardiac cells when surrounded by the correct environment and growth factors. Therefore, endogenous and exogenous factors need to be explored that can elucidate underlying mechanisms for functional cardiovascular progenitor cell differentiation *in vitro* and *in vivo*.

FUNCTIONAL MECHANISMS DURING STEM CELL DIFFERENTIATION AND TRANSPLANTATION

Studies with bone marrow-derived and circulating stem/progenitor cells have demonstrated that injected cells are difficult to locate after their administration to the circulation or the heart. Transplanted cells may be transported away from the heart via the circulation, undergo apoptosis, or be removed by the high inflammatory response in the ischemic heart. Yet an effect can be seen following cell transplantation after myocardial infarction in experimental models and clinical studies, suggesting that progenitor cells contribute in a direct functional or supportive fashion. In the past years, research concerning the beneficial effects of stem cell transplantation has begun to unravel the responsible mechanisms. Different cytokines, chemicals, growth factors, or culture techniques promote progenitor cell differentiation into cardiomyocytes[65]. *In vitro* and *in vivo*, direct cell-cell contact or factors released by other cells are suggested to stimulate cardio- and neoangiogenesis. Co-culture experiments of embryonic stem cells with the endodermal cell line End2[66] or rat cardiomyocytes[67] stimulated differentiation of ES cells into cardiomyocytes with functional electromechanical coupling. Differentiation was also enhanced in cardiac SP cells co-cultured with cardiomyocytes compared to SP cells in monoculture[45]. Furthermore, bone marrow-derived stem cells and $Isl1^+$ cardiovascular progenitor cells failed to differentiate when not in direct contact with rat or mouse cardiomyocytes, respectively[36,68]. *In vivo*, cellular coupling will be needed for functional improvement of the failing heart and limit the occurrence of arrhythmias. Transplantation of fetal cardiomyocytes into murine hearts showed synchronous calcium transients between donor- and host-derived cardiomyocytes, suggesting electrical coupling via gap junctions[69]. Direct action potential propagation and events of fusion were not tested and only a low number of fetal cardiomyocytes survived after transplantation.

Despite the need for direct contact between some progenitor cells and their co-cultured partners, evidence arguing a paracrine effect

is accumulating. Many of the currently investigated transcription and growth factors are known from early heart development and function[35,70] and may influence cardiovascular progenitor cell proliferation and differentiation. A novel human bone marrow-derived stem cell (hBMSC) population was isolated that, based on FACS analysis, was different from MSCs, HSCs, and MAPCs. These hBMSCs showed the potential to differentiate into cells from all three germ layers *in vitro* and into cardiomyocytes, smooth muscle cells, and endothelial cells *in vivo*[71]. After MI, hearts transplanted with hBMSCs showed improved cardiac function and increased expression of several growth factors and cytokines that may have paracrine effects on angiogenesis and vasculogenesis. Similarly, bone marrow cell transplantation from wild-type mice rescued cardiac defects of c-Kit mutant mice and increased VEGF levels in the heart[72]. The exact source of increased growth factor release in the heart was not determined, but the bone marrow-derived cells themselves may have secreted these growth factors after transplantation. Conditioned medium from BMSCs showed increased levels of growth factors under hypoxia conditions as compared to normoxia[73], suggesting that transplanted cells may indeed secrete these factors when transplanted or migrated into the peri-infarct region, thereby eliciting a paracrine effect. Rat mesenchymal stem cells overexpressing the pro-survival gene Akt were shown to be less prone to apoptosis under hypoxia conditions and transplantation of these cells restored cardiac function in a dose-dependent manner[74]. However, the observed effects could not be completely attributed to MSC-derived cardiomyocytes and therefore paracrine signaling from Akt-MSCs was suggested. Subsequently, conditioned medium from cultured Akt-MSCs was shown to reduce cardiomyocyte apoptosis and infarct size via growth factors expressed by the mesenchymal stem cells[75,76]. It was later found that the frizzled related protein Sfrp2 was highly present in the Akt-MSC conditioned medium and that Sfrp2 could inhibit apoptosis in cardiomyocytes via stabilization of β-catenin[77].

Resident Sca-1$^+$ cardiovascular progenitor cells also express FGFR1 (the receptor for FGF2)[38,78], and mobilization and

differentiation of these cells was largely dependent on FGF2 *in vitro* and *in vivo*[78]. FGF has already been used in clinical trials to improve vascularization in ischemic myocardium, but resulted in mixed outcomes[79,80]. TGFβ and BMP2 were shown to regulate cardiac gene expression and differentiation of mouse embryonic stem cells cultured alone or together with a BMP2-secreting cell line. Disruption of this growth factor signaling pathway prevented differentiation *in vitro* and *in vivo*[81].

In summary, a possible paracrine effect of transplanted stem cells after MI might explain the discrepancy between the improved cardiac function after treatment and the low number of identified donor-derived cells. Whether the same mechanism will be activated after cardiovascular progenitor cell transplantation remains to be determined.

Much attention has focused on characterization of stem/progenitor cells and factors regulating their proliferation and differentiation, but their temporal gene expression program during differentiation *in vitro* remains largely enigmatic or invalidated. Recently, genome-wide transcriptional profiling was performed on human ES cells differentiating towards cardiomyocytes[82]. Genes specific for undifferentiated stem cells or early or late cardiomyocyte development were differentially expressed, comparable to temporal gene expression patterns *in vivo*[31]. Further investigation is needed to determine which genes will prove to be helpful in directed proliferation and differentiation of stem and progenitor cells.

FROM CULTURE DISH TO BEDSIDE

Progenitor cells with cardiomyogenic potential have been isolated from the hearts from different species. To fully understand their potency and usefulness for cardiac repair, several issues need to be addressed. First, we need to identify the ideal type of cell or combination thereof to use. Even though progenitor cells can be isolated from the heart, their true origin or a common precursor has not been defined yet. Second, similar isolation and culture techniques are needed to compare the different progenitor cell

types. Third, a thorough characterization of these cells will better predict their differentiation and function after transplantation and prevent hazardous side effects after clinical application. Although improved understanding from *in vitro* experiments is needed regarding the need for cell-to-cell contact and induced signaling pathways during differentiation, more *in vivo* research will be needed to reveal the potential of transplanted cells in disease models. A potential candidate for routine cell transplantation in patients will need to be able to highly proliferate and fully differentiate into multiple lineages such as cardiomyocytes and endothelial and smooth muscle cells and functionally integrate into the host environment. So far, no single progenitor cell population has demonstrated the capacity for complete, long-term cardiac repair, probably due to incomplete stimulation or differentiation conditions. Possibly, transplantation of multiple stem/progenitor cell types such as cardiovascular progenitor cells, endothelial progenitor cells, and smooth muscle cells may be needed to completely restore functional cardiac tissue. And although several progenitor cell populations have been described in rodents, the ultimate goal will be the use of cells from human origin and therefore research should be focused on the human cardiovascular progenitor cells. Moreover, delivery techniques and cell maintenance will have to be optimized as well. While it is important that patients suffering from acute myocardial infarction can be treated immediately, direct or indirect administration of cardiovascular progenitor cells for cardiac repair is still inefficient and time-consuming. A combination of improving perfusion in early stages after myocardial infarction to prevent further loss of viable cells, followed by long-term replacement of lost tissue by progenitor cells may be an alternative. The application of signals to enhance perfusion and stem cell mobilization has already been performed in several clinical trials. Unfortunately, this has not resulted in consistent and substantial improvements[80,83]. Since this might be due to the ineffectiveness of administration, other tools may be needed, such as drug-eluting stents[84] or polymeric carriers[85]. Alternatively, the use of engineered heart tissue grafts may improve cardiac function[86], albeit not in acute settings.

KEY ISSUES

- Pluripotent ES and iPS cells have shown the capacity for cardiomyocyte differentiation, but differentiation efficiency is relatively low and ES cells will probably not be applicable in clinical settings due to immunogenic, arrhythmogenic, and ethical issues.
- Bone marrow-derived and circulating stem cells might be useful during early post-infarct phases by attenuation of cardiac vascular tissue, but their differentiation into functional cardiomyocytes remains disputed.
- Resident cardiovascular progenitor cells can be isolated from rodent, canine, and human hearts and differentiate into new cardiac cells, including cardiomyocytes needed for long-term repair of ischemic damage.
- Different isolation methods and other experimental procedures to characterize cardiovascular progenitor cells have confounded the identification of a common precursor.
- Growth factor signaling promotes cardiovascular progenitor cell differentiation into cardiomyocytes, indicating the importance of paracrine pathways on adult cardiovascular progenitor functionality.
- Future clinical cardiac cell replacement therapy may probably benefit most from a combination of different progenitor cells and growth factors to promote their integration and function.

REFERENCES

1. Rosamond, W. *et al.* Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 117, e25-146 (2008).
2. Holmes, J.W., Borg, T.K. & Covell, J.W. Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng* 7, 223-53 (2005).
3. Smits, A.M., van Vliet, P., Hassink, R.J., Goumans, M.J. & Doevendans, P.A. The role of stem cells in cardiac regeneration. *J Cell Mol Med* 9, 25-36 (2005).
4. Zwaginga, J.J. & Doevendans, P. Stem cell-derived angiogenic/vasculogenic cells: possible therapies for tissue repair and tissue engineering. *Clin Exp Pharmacol Physiol* 30, 900-8 (2003).
5. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* 50, 19-30 (2008).
6. Passier, R. & Mummery, C. Origin and use of embryonic and adult stem cells in differentiation and tissue repair. *Cardiovasc Res* 58, 324-35 (2003).
7. Passier, R., van Laake, L.W. & Mummery, C.L. Stem-cell-based therapy and lessons from the heart. *Nature* 453, 322-9 (2008).
8. Passier, R. *et al.* Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells* 23, 772-80 (2005).
9. Laflamme, M.A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 25, 1015-24 (2007).
10. van Laake, L.W. *et al.* Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. *Stem Cell Res* 1, 9-24 (2007).
11. van Laake, L.W., Passier, R., Doevendans, P.A. & Mummery, C.L. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ Res* 102, 1008-10 (2008).
12. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-72 (2007).
13. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917-20 (2007).
14. Chang, H.Y. *et al.* Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A* 99, 12877-82 (2002).
15. Hanna, J. *et al.* Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 318, 1920-3 (2007).

16. Makino, S. *et al.* Cardiomyocytes can be generated from marrow stromal cells in vitro. *J Clin Invest* 103, 697-705 (1999).
17. Hakuno, D. *et al.* Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. *Circulation* 105, 380-6 (2002).
18. Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J. & Kessler, P.D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105, 93-8 (2002).
19. Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* 410, 701-5 (2001).
20. Balsam, L.B. *et al.* Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 428, 668-73 (2004).
21. Murry, C.E. *et al.* Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 428, 664-8 (2004).
22. Alvarez-Dolado, M. *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 425, 968-73 (2003).
23. Nygren, J.M. *et al.* Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat Med* 10, 494-501 (2004).
24. Abdel-Latif, A. *et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med* 167, 989-97 (2007).
25. Dimmeler, S., Zeiher, A.M. & Schneider, M.D. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 115, 572-83 (2005).
26. Murry, C.E., Field, L.J. & Menasche, P. Cell-based cardiac repair: reflections at the 10-year point. *Circulation* 112, 3174-83 (2005).
27. Soonpaa, M.H. & Field, L.J. Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circ Res* 83, 15-26 (1998).
28. Beltrami, A.P. *et al.* Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* 344, 1750-7 (2001).
29. Anversa, P. & Nadal-Ginard, B. Myocyte renewal and ventricular remodelling. *Nature* 415, 240-3 (2002).
30. Olson, E.N. & Schneider, M.D. Sizing up the heart: development redux in disease. *Genes Dev* 17, 1937-56 (2003).
31. Franco, D., Lamers, W.H. & Moorman, A.F. Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model. *Cardiovasc Res* 38, 25-53 (1998).
32. Masino, A.M. *et al.* Transcriptional regulation of cardiac progenitor cell

populations. *Circ Res* 95, 389-97 (2004).

33. Cai, C.L. *et al.* Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. *Dev Cell* 5, 877-89 (2003).
34. Yang, L. *et al.* Isl1Cre reveals a common Bmp pathway in heart and limb development. *Development* 133, 1575-85 (2006).
35. Zaffran, S. & Frasch, M. Early signals in cardiac development. *Circ Res* 91, 457-69 (2002).
36. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* 433, 647-53 (2005).
37. Matsuura, K. *et al.* Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem* 279, 11384-91 (2004).
38. Oh, H. *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A* 100, 12313-8 (2003).
39. Wang, X. *et al.* The role of the sca-1+/CD31- cardiac progenitor cell population in postinfarction left ventricular remodeling. *Stem Cells* 24, 1779-88 (2006).
40. van de Rijn, M., Heimfeld, S., Spangrude, G.J. & Weissman, I.L. Mouse hematopoietic stem-cell antigen Sca-1 is a member of the Ly-6 antigen family. *Proc Natl Acad Sci U S A* 86, 4634-8 (1989).
41. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S. & Mulligan, R.C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 183, 1797-806 (1996).
42. Zhou, S. *et al.* The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 7, 1028-34 (2001).
43. Hierlihy, A.M., Seale, P., Lobe, C.G., Rudnicki, M.A. & Megeney, L.A. The post-natal heart contains a myocardial stem cell population. *FEBS Lett* 530, 239-43 (2002).
44. Martin, C.M. *et al.* Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. *Dev Biol* 265, 262-75 (2004).
45. Pfister, O. *et al.* CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res* 97, 52-61 (2005).
46. Ronnstrand, L. Signal transduction via the stem cell factor receptor/c-Kit. *Cell Mol Life Sci* 61, 2535-48 (2004).

47. Beltrami, A.P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 114, 763-76 (2003).
48. Linke, A. *et al.* Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate infarcted myocardium, improving cardiac function. *Proc Natl Acad Sci U S A* 102, 8966-71 (2005).
49. Urbanek, K. *et al.* Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival. *Circ Res* 97, 663-73 (2005).
50. Kajstura, J. *et al.* Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A* 95, 8801-5 (1998).
51. Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 182, 311-22 (2000).
52. Urbanek, K. *et al.* Intense myocyte formation from cardiac stem cells in human cardiac hypertrophy. *Proc Natl Acad Sci U S A* 100, 10440-5 (2003).
53. Urbanek, K. *et al.* Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. *Proc Natl Acad Sci U S A* 102, 8692-7 (2005).
54. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* 104, 14068-73 (2007).
55. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 95, 911-21 (2004).
56. Smith, R.R. *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* 115, 896-908 (2007).
57. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* 1, 138-149 (2008).
58. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* 16, 163-9 (2008).
59. Lie-Venema, H. *et al.* Origin, fate, and function of epicardium-derived cells (EPDCs) in normal and abnormal cardiac development. *ScientificWorldJournal* 7, 1777-98 (2007).
60. van Tuyn, J. *et al.* Epicardial cells of human adults can undergo an epithelial-to-mesenchymal transition and obtain characteristics of smooth muscle cells in vitro. *Stem Cells* 25, 271-8 (2007).
61. Winter, E.M. *et al.* Preservation of left ventricular function and attenuation

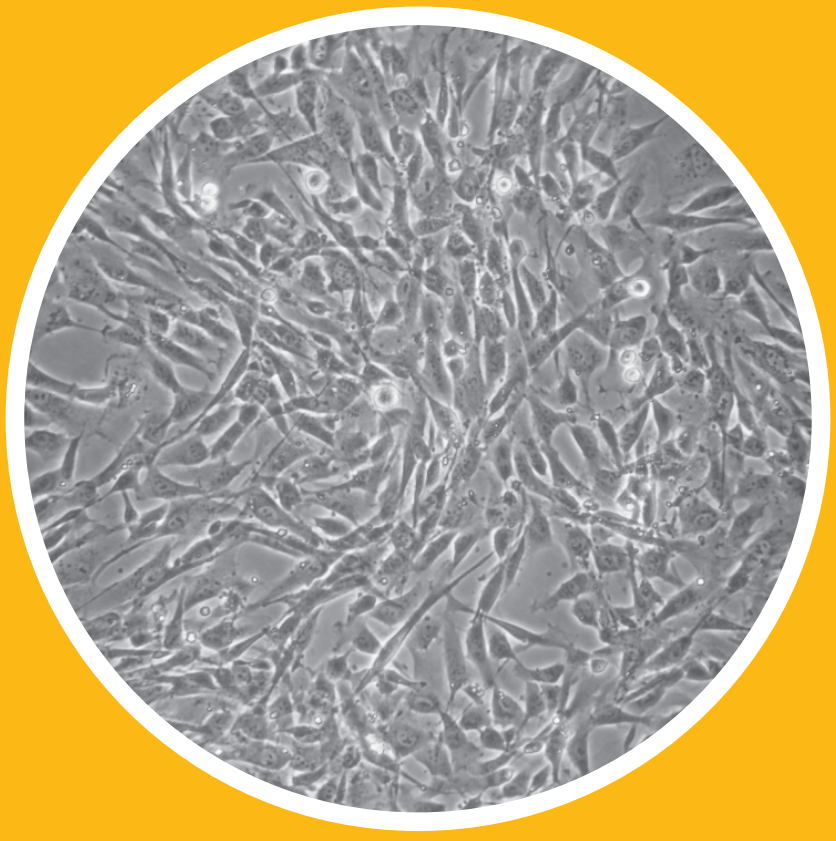
of remodeling after transplantation of human epicardium-derived cells into the infarcted mouse heart. *Circulation* 116, 917-27 (2007).

62. Orlic, D. *et al.* Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A* 98, 10344-9 (2001).
63. Quaini, F. *et al.* Chimerism of the transplanted heart. *N Engl J Med* 346, 5-15 (2002).
64. Laflamme, M.A., Myerson, D., Saffitz, J.E. & Murry, C.E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res* 90, 634-40 (2002).
65. Heng, B.C., Haider, H., Sim, E.K., Cao, T. & Ng, S.C. Strategies for directing the differentiation of stem cells into the cardiomyogenic lineage in vitro. *Cardiovasc Res* 62, 34-42 (2004).
66. Mummery, C. *et al.* Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 107, 2733-40 (2003).
67. Kehat, I. *et al.* Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. *Nat Biotechnol* 22, 1282-9 (2004).
68. Fukuhara, S. *et al.* Direct cell-cell interaction of cardiomyocytes is key for bone marrow stromal cells to go into cardiac lineage in vitro. *J Thorac Cardiovasc Surg* 125, 1470-80 (2003).
69. Rubart, M. *et al.* Physiological coupling of donor and host cardiomyocytes after cellular transplantation. *Circ Res* 92, 1217-24 (2003).
70. Azhar, M. *et al.* Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev* 14, 391-407 (2003).
71. Yoon, Y.S. *et al.* Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. *J Clin Invest* 115, 326-38 (2005).
72. Fazel, S. *et al.* Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J Clin Invest* 116, 1865-77 (2006).
73. Uemura, R., Xu, M., Ahmad, N. & Ashraf, M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. *Circ Res* 98, 1414-21 (2006).
74. Mangi, A.A. *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 9, 1195-201 (2003).

75. Gnecchi, M. *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 11, 367-8 (2005).
76. Gnecchi, M. *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J* 20, 661-9 (2006).
77. Mirotsou, M. *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* 104, 1643-8 (2007).
78. Rosenblatt-Velin, N., Lepore, M.G., Cartoni, C., Beermann, F. & Pedrazzini, T. FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. *J Clin Invest* 115, 1724-33 (2005).
79. Simons, M. & Ware, J.A. Therapeutic angiogenesis in cardiovascular disease. *Nat Rev Drug Discov* 2, 863-71 (2003).
80. Nordlie, M.A., Wold, L.E., Simkhovich, B.Z., Sesti, C. & Kloner, R.A. Molecular aspects of ischemic heart disease: ischemia/reperfusion-induced genetic changes and potential applications of gene and RNA interference therapy. *J Cardiovasc Pharmacol Ther* 11, 17-30 (2006).
81. Behfar, A. *et al.* Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* 16, 1558-66 (2002).
82. Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C. & Passier, R. Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. *Stem Cells* 24, 1956-67 (2006).
83. Yoon, Y.S., Johnson, I.A., Park, J.S., Diaz, L. & Losordo, D.W. Therapeutic myocardial angiogenesis with vascular endothelial growth factors. *Mol Cell Biochem* 264, 63-74 (2004).
84. Lemos, P.A., Serruys, P.W. & Sousa, J.E. Drug-eluting stents: cost versus clinical benefit. *Circulation* 107, 3003-7 (2003).
85. Babensee, J.E., McIntire, L.V. & Mikos, A.G. Growth factor delivery for tissue engineering. *Pharm Res* 17, 497-504 (2000).
86. Zimmermann, W.H. *et al.* Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med* 12, 452-8 (2006).
87. Krause, D.S. Plasticity of marrow-derived stem cells. *Gene Ther* 9, 754-8 (2002).
88. Laflamme, M.A. & Murry, C.E. Regenerating the heart. *Nat Biotechnol* 23, 845-56 (2005).
89. Metzger, D. & Feil, R. Engineering the mouse genome by site-specific recombination. *Curr Opin Biotechnol* 10, 470-6 (1999).

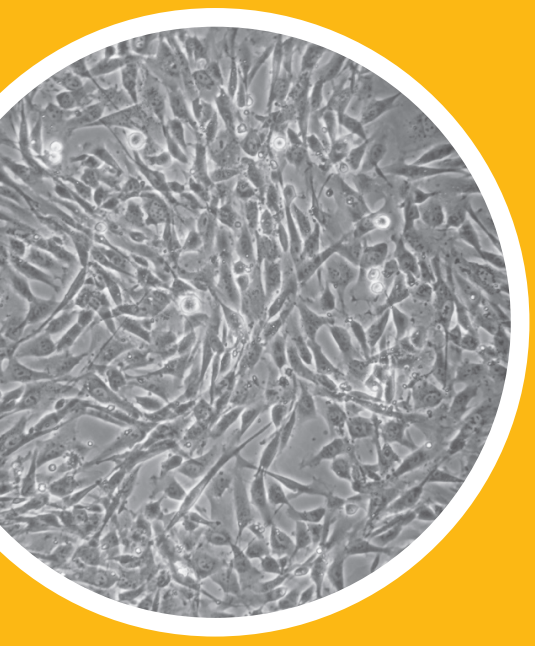
CHAPTER

3



CHAPTER 3

Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy



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ABSTRACT

In recent years, resident cardiovascular progenitor cells have been identified in and isolated from the rodent heart. These cells show the potential to form cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro* and *in vivo* and could potentially be used as a source for cardiac repair. However, previously described cardiovascular progenitor cell populations show immature development and need co-culture with neonatal rat cardiomyocytes in order to differentiate *in vitro*. Here we describe the localization, isolation, characterization, and differentiation of cardiomyocyte progenitor cells (CMPCs) isolated from the human heart.

CMPCs were identified in human hearts based on Sca-1 expression. CMPCs were localized within the atria, atrioventricular region, and epicardial layer of the fetal and adult human heart. CMPCs were isolated, and FACS, RT-PCR and immunocytochemistry were used to determine their baseline characteristics. *In vitro*, CMPCs could be induced by 5-azacytidine to differentiate into spontaneously beating cardiomyocytes, without the need for co-culture with neonatal cardiomyocytes.

In conclusion, the human heart harbors a pool of resident cardiomyocyte progenitor cells, which can be expanded and differentiated *in vitro*. These cells may provide a suitable source for cardiac cell therapy.

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INTRODUCTION

Cardiovascular disease is an important cause of mortality in the Western world[1]. The central cellular mechanism underlying the development of myocardial dysfunction is a decrease in the number of viable cardiomyocytes as a result of ischemic injury or ongoing apoptosis, and the inability of remaining cardiomyocytes to compensate the cell loss by cardiomyocyte regeneration. Stem cells have been studied intensively as a source of new cardiomyocytes to ameliorate injured myocardium and improve cardiac function[2-4]. The potential therapeutic benefit of stem cell transplantation has been investigated in animal models using bone marrow-derived cells[5-7], cardiovascular progenitor cells[8], embryonic stem (ES) cells[9,10], and fetal cardiomyocytes[11,12] by injecting them at the site of cardiac injury. The encouraging results reported in these animal studies led to the initiation of several clinical trials in which bone marrow derived cells and skeletal myoblasts were investigated[4,13-16]. However, the developmental plasticity of bone marrow cells to differentiate into cardiomyocytes has been questioned[17,18] and the predominant *in vivo* effect of bone marrow injection may be neoangiogenesis instead of muscle regeneration. Furthermore, autologous transplantation of skeletal myoblasts is confounded by the possible induction of life-threatening arrhythmias despite partial integration, survival, and contribution to cardiac contractility[15]. Another source of transplantable cardiomyocytes is human embryonic stem cell (hES)-derived cardiomyocytes. Although hES cells can be directed into the cardiomyocyte lineage, with a fetal phenotype[19], their differentiation is not homogenous despite recent improvements in culture methods[20,21]. Furthermore, immunogenic, arrhythmogenic, and especially ethical problems will limit their clinical use. These obstacles underscore the need to search for new sources of autologous adult cells to generate cardiomyocytes for regeneration of the failing myocardium.

Among the potential candidates are several different cardiomyocyte progenitor cell populations that have been identified in the rodent and human heart[22]. Cells expressing stem cell factor receptor c-Kit[8], stem cell antigen-1 (Sca-1)[23], homeodomain transcription

factor islet-1 (isl-1)[24], side population (SP) cells[25], and cells able to grow in cardiospheres[26] have been suggested to be capable of differentiation into cardiomyocytes, either *in vivo* or *in vitro*. However, recent reports indicate that fusion of transplanted progenitor cells with resident adult cardiomyocytes can occur *in vivo*, which may lead to a misinterpretation of the cardiomyogenic differentiation of the progenitor cells[23]. Until now, *in vitro* differentiation of stem cells into cardiomyocytes has only been achieved by co-culturing the cells with neonatal cardiomyocytes. To avoid misreading the *in vitro* differentiation capacity, other culture methods are needed to identify true cardiomyocyte generation.

In the present study, we isolated cardiomyocyte progenitor cells (CMPCs) from human heart tissue using an anti-Sca-1 antibody. Although a Sca-1 epitope in human cells is disputed, the cells we selected using the Sca-1 antibody from both fetal and adult human heart consistently proved to be a homogenous population and amenable to expansion in culture. We show that CMPCs are able to differentiate into mature cardiomyocytes *in vitro* after 5-azacytidine treatment, even after prolonged passage, thereby excluding artifacts that may result from co-culture. This report demonstrates the existence of human CMPCs in prenatal and postnatal human hearts and their capacity for cardiomyocyte differentiation *in vitro*.

RESULTS

Localization and characterization of human cardiomyocyte progenitor cells

To identify CMPCs in fetal and adult human heart, we used an anti-Sca-1 antibody that has been shown to recognize mouse cardiovascular progenitor cells[23]. Human CMPCs identified on this basis were found within the atrium, the intra-atrial septum, the atrium-ventricular boundary, and scattered within the epicardial layer (figure 1). To isolate CMPCs, cardiac tissue was enzymatically dissociated, followed by cardiomyocyte depletion. Using a Ferro coupled anti Sca-1 antibody, a cell fraction with a diameter of <50 µm was isolated and subsequently characterized by flow

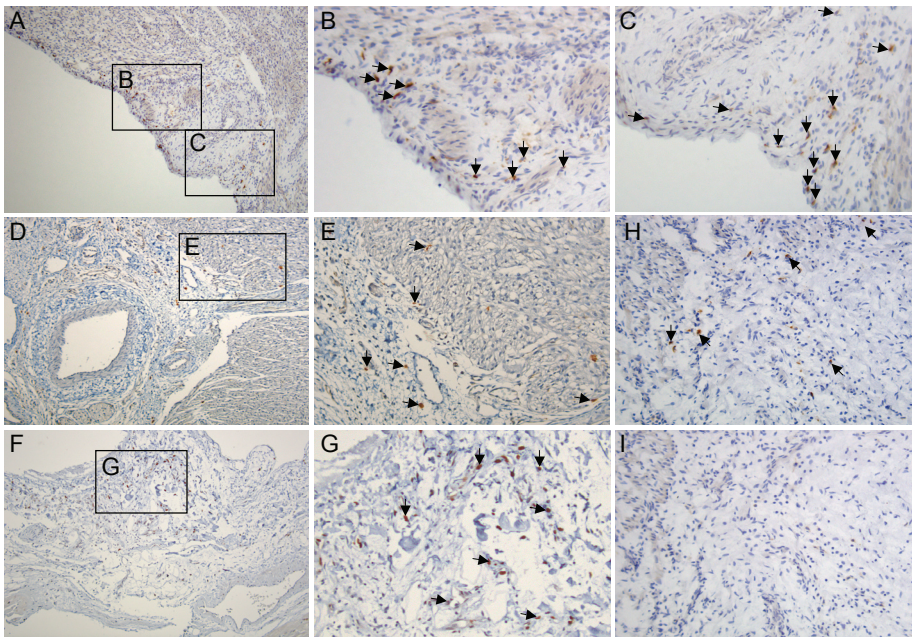

Figure 1: Localization of CMPCs in the human heart.

A-H | Immunohistochemistry for Sca-1 in fetal and adult heart. B, C | High power magnification of areas in A. D | Atrial ventricular boundary. E | High power magnification of area in D. F, H | CMPCs in biopsy from adult patient. G | High power magnification of area in F. I | IgG control. Arrows designate some of the CMPCs. Magnification: A, D, F, H, I: 100x, B, C, E, G: 200x.

cytometry (figure 2). Fetal CMPCs were negative for CD45, CD34, CD133, and CD14, and positive for CD105 and Sca-1. After isolation, fetal and adult progenitor cells were able to proliferate *in vitro* as spindle-shaped cells with a high nucleus-to-cytoplasm ratio (figure 3A and B). RT-PCR analysis of fetal progenitor cells revealed that they do not express Oct4, a marker for pluripotent ES cells (figure 3C). However, they did show expression of early cardiac transcription factors Gata4 and Nkx2.5, while cardiomyocyte-specific genes were not expressed. Adult progenitor cells showed a similar expression pattern (not shown).

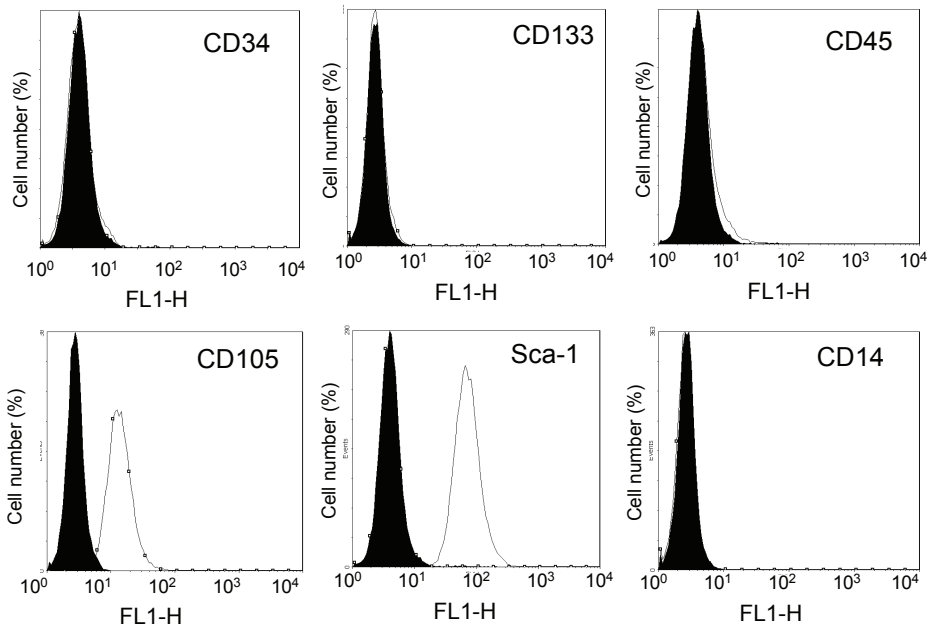

Figure 2: Flow cytometric analysis.

Histogram plots for (stem) cell marker expression in fetal CMPCs are shown with the isotype control in black and the specific signal in white.

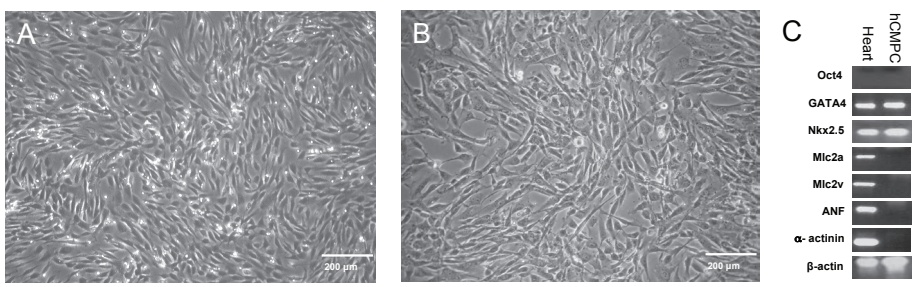

Figure 3: Characteristics of undifferentiated CMPCs

Bright field images of fetal (A) and adult (B) CMPCs. C | Semi-quantitative RT-PCR on RNA isolated from undifferentiated CMPCs probed for the expression of the indicated genes.

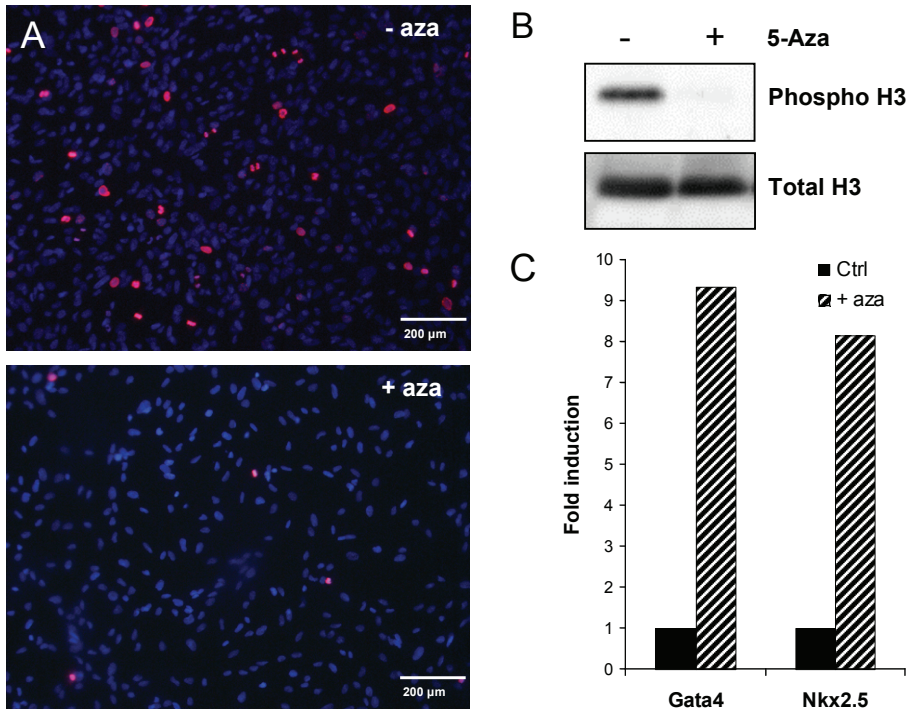

Figure 4: Induction of cardiomyogenic differentiation

A | Immunocytochemistry on CMPCs before (upper image) and after (lower image) 5-azacytidine stimulation. Phosphorylated-histone 3 is indicated in red, nuclei in blue. B | Western blot analysis for phospho-H3 and total H3 on protein from CMPCs with or without 5-azacytidine stimulation. C | Quantitative RT-PCR on RNA from CMPCs with (striped bars) or without (black bars) 5-azacytidine stimulation. Expression was normalized for β-actin and fold expression was calculated compared with control.

Cardiomyogenic differentiation of human CMPCs

To initiate differentiation of CMPCs towards a cardiomyogenic lineage, the proliferation of the cells should be arrested. In P19 embryonic carcinoma cells and mouse Sca-1^{+} cardiovascular progenitor cells, cardiomyogenic differentiation can be induced by stimulation with the demethylating agent 5-azacytidine[23,27]. Five-

azacytidine inhibited cell proliferation of CMPCs, as shown by the reduced number of mitotic figures, staining positive for phospho-histone 3 (ser10) (figure 4A), and by Western blot of the same samples (figure 4B). We subsequently found a strongly increased expression of the cardiomyogenic transcription factors Gata4 and Nkx2.5 (figure 4C).
After stimulation with 5-azacytidine and culture in differentiation medium for several weeks, CMPCs developed into spontaneously beating aggregates. RT-PCR showed increased expression of several cardiomyocyte-specific genes (figure 5A). Differentiated cells also showed expression of troponin I and α-actinin (figure 5B), indicating that they had become cardiomyocytes. The gap junctional proteins connexin 40 and 43 were expressed at the cell membrane border of CMPC-derived cardiomyocytes (figure 5C and D), suggesting that these cells are able to functionally couple with each other and other cardiomyocytes, which is necessary to form a functional syncytium.

DISCUSSION

In this study, we report the isolation and cardiomyogenic differentiation of resident cardiovascular progenitor cells. We show that, upon isolation, these human CMPCs are already committed to the cardiac lineage, as shown by their expression of early cardiac transcription factors. These cells can be efficiently propagated *in vitro* and differentiated into spontaneously beating cardiomyocytes after 5-azacytidine stimulation, excluding the need for co-culture with neonatal cardiomyocytes.
In the past years, several groups have reported the identification of rodent and human cardiovascular progenitor cells[28]. Due to different methods for isolation and subsequent culture it is difficult to make a direct comparison between these progenitor cell populations. It is very likely however that they have been derived from a common mesodermal precursor[29,30] and that current isolation methods result in cell populations that are at a different developmental stage. These cells were shown to

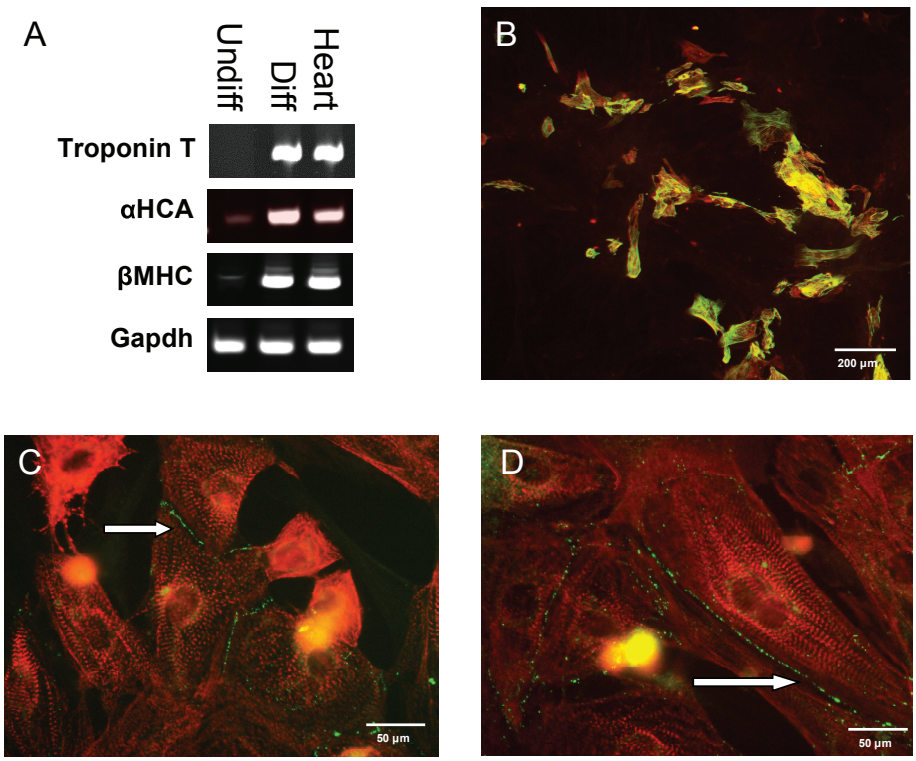

Figure 5: CMPC-derived cardiomyocytes

A | Semiquantitative RT-PCR on RNA isolated from differentiated CMPCs. B | Immunolabeling against troponin I (green) and α-actinin (red) in CMPCs differentiated into cardiomyocytes after 5-azacytidine stimulation. C, D | Immunolabeling against the connexin isoforms Cx40 (left, green), Cx43 (right, green), and α-actinin (red). Arrows indicate cell membrane localization of connexin isoforms.

differentiate *in vitro* into cardiomyocytes. However, none of these populations showed spontaneous beating without co-culture with rat neonatal cardiomyocytes. Moreover, they were shown to differentiate towards the endothelial and smooth muscle lineage. *In vivo*, cardiovascular progenitor cells also show the capacity to form different cardiac cell types. It still remains unknown which

signals are required to drive differentiation. Furthermore, *in vivo* differentiation remains inefficient, indicating the need to elucidate the fate of cardiovascular progenitor cells under normal and pathological circumstances. Possibly, the reactivation of the fetal gene expression program after myocardial infarction or the release of growth factors play an important role in guiding these cells towards their optimal potential. In a separate study, we show that growth factor addition during differentiation greatly enhances cardiomyocyte formation and maturation *in vitro*[31]. The potential of CMPCs to differentiate into endothelial cells and smooth muscle cells as well greatly enhances their putative clinical application. The unexpectedly high frequency with which we were able to isolate and culture CMPCs from atrial biopsies of adult patients undergoing cardiac surgery opens perspectives for autologous transplantation at a later date than the initial surgery if cultures were carried out under clinically compatible conditions.

Analysis of the differentiation potential of the fetal-derived CMPCs showed that addition of the demethylating agent 5-azacytidine induced the expression of cardiac and contractile genes and spontaneous beating. Expression of gap junctional proteins is almost exclusively found on the sarcolemma of CMPC-derived cardiomyocytes. It should, however, be confirmed whether these gap junction channels are functional, e.g. by testing metabolic and electrical coupling. Especially since transplantation of poorly coupled skeletal myoblasts in human hearts in a clinical trial resulted in ventricular tachyarrhythmias in some patients[15]. Proper intercellular coupling with host heart cells will therefore be necessary in order to preserve conduction characteristics and will be among the most important criteria for determining whether CMPCs can be taken forward to clinical trials. A detailed electrophysiological characterization of CMPC-derived cardiomyocytes may be required to predict their behavior after transplantation and integration into host tissue. The potential of CMPCs to differentiate into endothelial cells and smooth muscle cells as well, greatly enhances their putative clinical application.

In conclusion, human CMPCs provide a useful tool for cardiomyocyte differentiation studies and drug screening. Eventually they may serve as a suitable source for cellular therapy in failing hearts.

MATERIALS AND METHODS

Isolation of cardiomyocyte progenitor cells from human hearts

To collect human fetal tissue and atrial biopsies, individual permission was obtained using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht. Fetal hearts were collected after elective abortion followed by Langendorff perfusion with Tyrode's solution containing collagenase and protease. Atrial biopsies were minced into small pieces followed by collagenase treatment. After cardiomyocyte depletion of the cell suspension, cardiomyocyte progenitor cells were isolated by magnetic cell sorting (MACS, Miltenyl Biotec, Sunnyvale, CA) using Sca-1-coupled magnetic beads, according to the manufacturer's protocol. Sca-1^+ cells were eluted from the column by washing with PBS supplemented with 2% fetal calf serum (FCS) and cultured on 0.1% gelatin-coated dishes in M199 (Gibco)/EGM (3:1) supplemented with 10% FCS (Gibco), 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml epithelial growth factor (EGF), 5 ng/ml insulin-like growth factor (IGF-1) and 5 ng/ml hepatocyte growth factor (HGF)[31,32].

Cardiomyogenic differentiation in CMPCs

To induce differentiation, cells were treated with 5 μM 5-azacytidine (Sigma) for 72 hours in differentiation medium (Iscove's Modified Dulbecco's Medium /Ham's F12 (1:1) (Gibco)) supplemented with L-glutamine (Gibco), 2% horse serum, non-essential amino acids, insulin-transferrin-selenium supplement, and 10^{-4} M ascorbic acid (Sigma). After induction, the medium was changed every three days.

RNA isolation and RT-PCR

RNA was isolated using TriPure (Roche) as described by the

manufacturer. cDNA was synthesized with the iScript cDNA synthesis kit (Biorad). Primers for quantitative reverse transcriptase polymerase chain reaction (RT-PCR) were designed with Beacon Designer 4.0 (Premier Biosoft International). Primer sequences and annealing temperatures are available on request. Quantitative expression of genes was normalized for expression of β-actin. Results were analyzed on 10% acrylamide gel stained with ethidium bromide.

Flow cytometric analysis

Cultured CMPCs (passage 7) were trypsinized and 200,000 cells per sample were used for fluorescence-activated cell sorting (FACS) analysis. The cells were washed twice in wash-buffer (wb: 1% FCS/PBS/0.05% azide) and resuspended in 100 µl wb containing antibody. The cells were incubated on ice in the dark for 30 minutes, washed four times with cold wb, resuspended in 250 µl wb and analyzed using a Beckman Coulter Cytomics FC500 FACS. Antibodies used were fluorescein isothiocyanate (FITC) or phycoerthrin conjugated against CD14, CD34, CD45, CD133, CD105 (endoglin), Sca-1, and isotype control IgGs, all from Pharmingen BD.

Immunocytochemistry

For immunocytochemistry, coverslips with cultured cells were fixed in 4% paraformaldehyde at room temperature or methyl alcohol at -20 °C. Cells were permeabilized (0.2% Triton X-100/PBS) and blocked (2% bovine serum albumin (BSA), 15-30 minutes). Subsequently, coverslips were incubated overnight at 4 °C with primary antibody in PBS/10% normal goat serum (NGS). The antibodies used recognized Cx40 (Chemicon), Cx43 (Zymed), α-actinin (Sigma), troponin I (Chemicon), and phospho-histone 3 (Abcam). The following day, coverslips were blocked again and incubated with secondary antibody in PBS/10% NGS for two hours. Immunolabeling was performed using Texas Red (TR)- or FITC-conjugated secondary antibodies (Jackson Laboratories). Hoechst dye was used to localize nuclei. All incubation steps were performed at room temperature and in

between all incubation steps cells were washed with PBS. Finally, coverslips were mounted in Vectashield (Vector Laboratories) and examined with a Nikon Optiphot-2 light microscope equipped for epifluorescence.

Immunohistochemistry

Cryo sections (7 µm) were blocked with 1.2% hydrogen peroxide in methanol for 15 min, air dried, and after washing with PBS, blocked with 2% BSA in PBS for 30 minutes. The sections were incubated with the anti-Sca-1 antibody (Pharmingen), diluted 1:100 in blocking solution, overnight at 4 °C. PowerVision Poly-HRP-Conjugates (ImmunoVision Technologies) was used as secondary antibody with the Fast 3,3'-diaminobenzidine tablet set (DAB, SIGMA). The sections were counterstained with Meyer's haematoxylin and mounted in Entellan.

Western blot analysis

Western blot analysis was performed as described previously[33]. Detection was by ECL (Amersham). P-H3 and H3 antibodies that specifically recognize phosphorylated histone 3 or total histone 3, respectively, were used 1:5000. Beta-actin detection (1:10000, Chemicon) was used as a loading control.

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REFERENCES

1. Rosamond, W. *et al.* Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **117**, e25-146 (2008).
2. Hassink, R.J., Passier, R., Goumans, M.J., Mummery, C.L. & Doevendans, P.A. New and viable cells to replace lost and malfunctioning myocardial tissue. *Minerva Cardioangiol* **52**, 433-45 (2004).
3. Smits, A.M., van Vliet, P., Hassink, R.J., Goumans, M.J. & Doevendans, P.A. The role of stem cells in cardiac regeneration. *J Cell Mol Med* **9**, 25-36 (2005).
4. Van Laake, L.W., Van Hoof, D. & Mummery, C.L. Cardiomyocytes derived from stem cells. *Ann Med* **37**, 499-512 (2005).
5. Fukuda, K. & Yuasa, S. Stem cells as a source of regenerative cardiomyocytes. *Circ Res* **98**, 1002-13 (2006).
6. Mangi, A.A. *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* **9**, 1195-201 (2003).
7. Orlic, D. *et al.* Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A* **98**, 10344-9 (2001).
8. Beltrami, A.P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763-76 (2003).
9. Menard, C. *et al.* Transplantation of cardiac-committed mouse embryonic stem cells to infarcted sheep myocardium: a preclinical study. *Lancet* **366**, 1005-12 (2005).
10. Behfar, A. *et al.* Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* **16**, 1558-66 (2002).
11. Rubart, M. *et al.* Physiological coupling of donor and host cardiomyocytes after cellular transplantation. *Circ Res* **92**, 1217-24 (2003).
12. Muller-Ehmsen, J. *et al.* Effective engraftment but poor mid-term persistence of mononuclear and mesenchymal bone marrow cells in acute and chronic rat myocardial infarction. *J Mol Cell Cardiol* **41**, 876-84 (2006).
13. Meyer, G.P. *et al.* Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation* **113**, 1287-94 (2006).

14. Assmus, B. *et al.* Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* **106**, 3009-17 (2002).
15. Menasche, P. Skeletal myoblast transplantation for cardiac repair. *Expert Rev Cardiovasc Ther* **2**, 21-8 (2004).
16. Smits, J.M. *et al.* Mortality rates after heart transplantation: how to compare center-specific outcome data? *Transplantation* **75**, 90-6 (2003).
17. Balsam, L.B. *et al.* Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428**, 668-73 (2004).
18. Laflamme, M.A., Myerson, D., Saffitz, J.E. & Murry, C.E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res* **90**, 634-40 (2002).
19. Mummery, C. *et al.* Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* **107**, 2733-40 (2003).
20. Passier, R. *et al.* Increased cardiomyocyte differentiation from human embryonic stem cells in serum-free cultures. *Stem Cells* **23**, 772-80 (2005).
21. Xu, C., Police, S., Rao, N. & Carpenter, M.K. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* **91**, 501-8 (2002).
22. Anversa, P., Kajstura, J., Leri, A. & Bolli, R. Life and death of cardiac stem cells: a paradigm shift in cardiac biology. *Circulation* **113**, 1451-63 (2006).
23. Oh, H. *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A* **100**, 12313-8 (2003).
24. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
25. Pfister, O. *et al.* CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res* **97**, 52-61 (2005).
26. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
27. Ohtsu, Y. *et al.* Stimulation of P19CL6 with multiple reagents induces pulsating particles in vivo. *Curr Med Res Opin* **21**, 795-803 (2005).
28. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).

29. Wu, S.M. *et al.* Developmental origin of a bipotential myocardial and smooth muscle cell precursor in the mammalian heart. *Cell* **127**, 1137-50 (2006).
30. Laugwitz, K.L., Moretti, A., Caron, L., Nakano, A. & Chien, K.R. Islet1 cardiovascular progenitors: a single source for heart lineages? *Development* **135**, 193-205 (2008).
31. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
32. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
33. Goumans, M.J. *et al.* Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J* **21**, 1743-53 (2002).

CHAPTER

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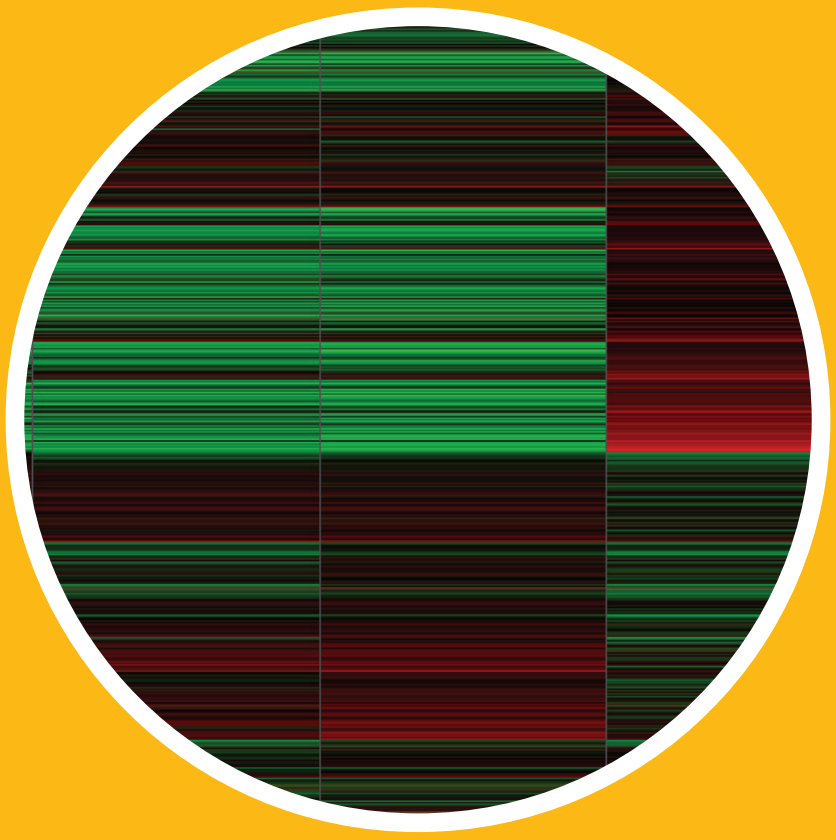
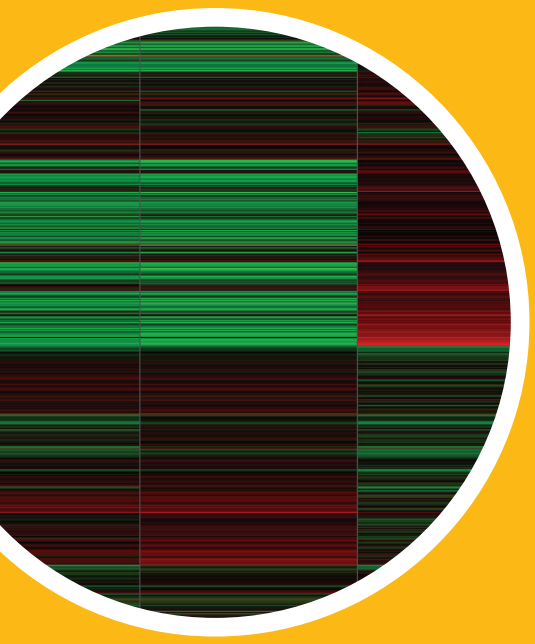


CHAPTER 4

Temporal genomic profiling of human cardiomyocyte progenitor cell differentiation



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ABSTRACT

The isolation and characterization of resident cardiovascular progenitor cells has enhanced our understanding of their developmental potential. However, the exact mechanisms that drive proliferation and differentiation in these cells are not well understood. We have recently isolated human cardiomyocyte progenitor cells (CMPCs) and shown that these cells can differentiate into several cardiac cell types like cardiomyocytes, smooth muscle cells, and endothelial cells. To gain more insight into how CMPCs become cardiomyocytes, we investigated differential gene expression during CMPC cardiomyogenic differentiation using microarray analysis at one-week intervals. We identified several cell cycling genes that were decreased shortly after induction of differentiation, while genes required for muscle development and contraction became highly upregulated during later stages. Genes related to DNA transcription, metabolism, and an immunogenetic phenotype were also differentially expressed. Further analysis of these genes and the transcription factors influencing their expression could help to better define the processes that are involved in CMPC proliferation and differentiation.

INTRODUCTION

In the past years, the adult mammalian heart has been shown to contain populations of multipotent progenitor cells that can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells[1,2]. These progenitor cells have been suggested as a source for cardiac cell replacement therapy. Up to date however, cardiomyogenic differentiation of progenitor cells needed co-culture with neonatal rat cardiomyocytes and remains incomplete, resulting in immature, fetal-like cardiomyocytes. Since immature and asynchronous contracting cardiomyocytes could induce arrhythmias, the capacity of progenitor cells to differentiate into mature cardiomyocytes eventually needs to be shown. Investigation of regulatory mechanisms involved in cardiomyogenesis is therefore important to improve differentiation efficiency of cardiovascular progenitor cells.

We have recently isolated multipotent cardiomyocyte progenitor cells (CMPCs) from fetal and adult human heart tissue and shown that these cells can differentiate into fetal/early neonatal, ventricular-like cardiomyocytes *in vitro*[3,4]. Morphologically, proliferating CMPCs are small and have a spindle-shaped phenotype. After induction of differentiation by 5-azacytidine, CMPCs first show a slightly stressed phenotype. However, when differentiation is further stimulated, cells regain their normal morphology and start to become larger[5]. After 5-azacytidine treatment, CMPCs show downregulated expression of phosphorylated histone 3, normally associated with mitosis[6], indicating inhibition of proliferation[4]. Meanwhile, Mef2c, Nkx2.5, and Gata4 are upregulated and Islet-1 downregulated[3,4]. Between weeks one and two of differentiation, expression of several sarcomeric proteins could already be observed. After three to four weeks, cells start to beat spontaneously, leading to simultaneously contracting clusters within several days[3,5].

In order to elucidate the biological processes that regulate CMPC proliferation and differentiation, we performed microarray analysis at different time points during differentiation. Identification and manipulation of involved signaling cascades could help our

understanding of cardiomyogenesis, improve proliferation and differentiation efficiency, and, subsequently, potential repair of damaged tissue when CMPCs are used for transplantation into the heart.

RESULTS

Temporally regulated gene expression in CMPC differentiation

Microarray analysis performed at one-week intervals of differentiation revealed that CMPC differentiation is highly regulated. From the 24357 genes that were detected, we analyzed the expression of genes that were at least two-fold differentially expressed at one time point of differentiation compared to at least one other time point (figure 1). When we clustered these 2231 genes based on expression profile during the three weeks of differentiation, a hierarchy of similarity between patterns could be observed (figure 2). To further analyze these processes, temporal gene expression profiles were divided into 11 different clusters with 93% similarity between the two subnodes within each cluster (figure 3 and supplemental tables 1-11). With respect to a better understanding of CMPC proliferation and differentiation, we mainly focused on clusters that showed relevant up- and downregulation of genes involved in cell cycling or cardiomyogenesis.

Genomic profiling early during CMPC differentiation

In the first week after induction of differentiation, the cardiac transcription factors Gata4 (1.79x) and Mef2a (1.49x) were upregulated (week one, not shown). Several cell cycle genes (Cdc42 and Ccnd3, cluster three, and Cdc14b, cluster eight) were initially upregulated and subsequently downregulated. The cyclin-dependent kinase (CDK) inhibitors Cdkn2a ($p16^{INK4a}$) and Cdkn1a ($p21^{CIP}$) were downregulated in week one and three (cluster five), respectively.

More global analysis of differentially expressed genes during differentiation showed a gradual downregulation of synthases, aminoacyl-tRNA synthetases (AARS[7,8]), and extracellular matrix proteins (like tenascin C (TNC), laminin (LAMB2), and several

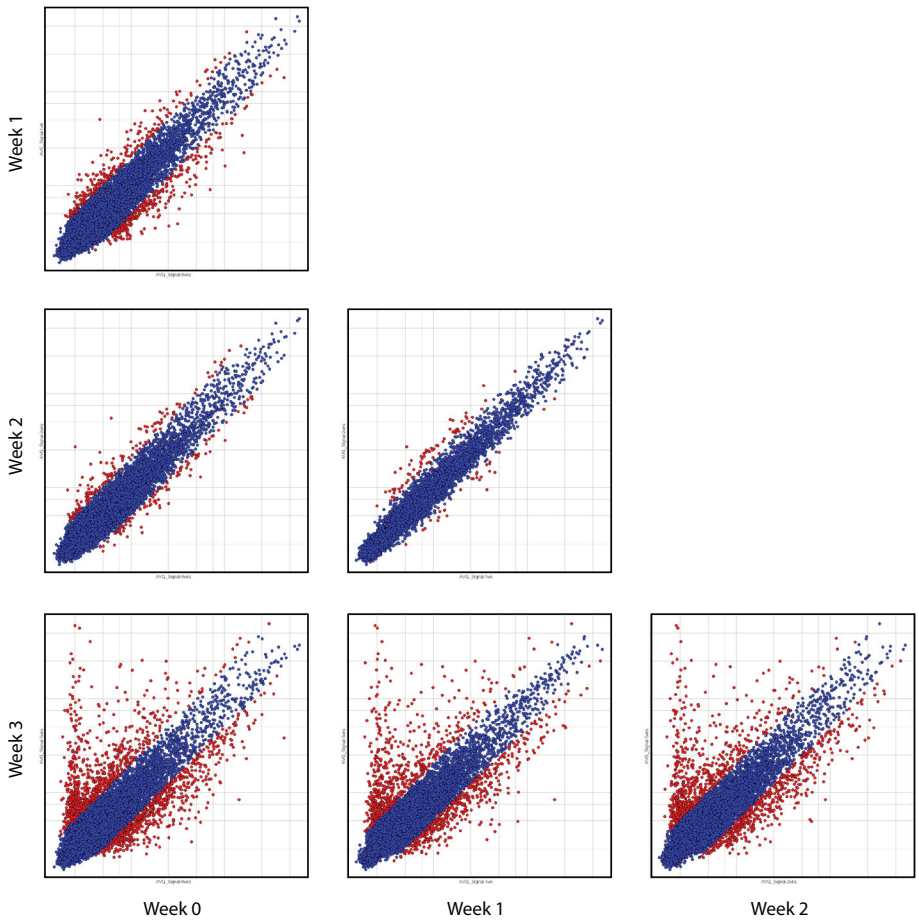

Figure 1: Scatter plots showing differential gene expression in week 0, 1, 2, and 3 of CMPC differentiation

Blue: <2x, red: >2x differential expression between time points indicated.

collagen types) involved in aminoacid activation, cell structure and mobility, and mesoderm development (cluster five) (figure 4 and supplemental tables 5 and 12). In contrast, cytoskeletal proteins, calcium binding proteins, and isomerases, involved mainly in glycolysis (such as enolase (ENO)-1 and -2) as well as developmental processes (like mesenchyme homeobox (MEOX)-2),

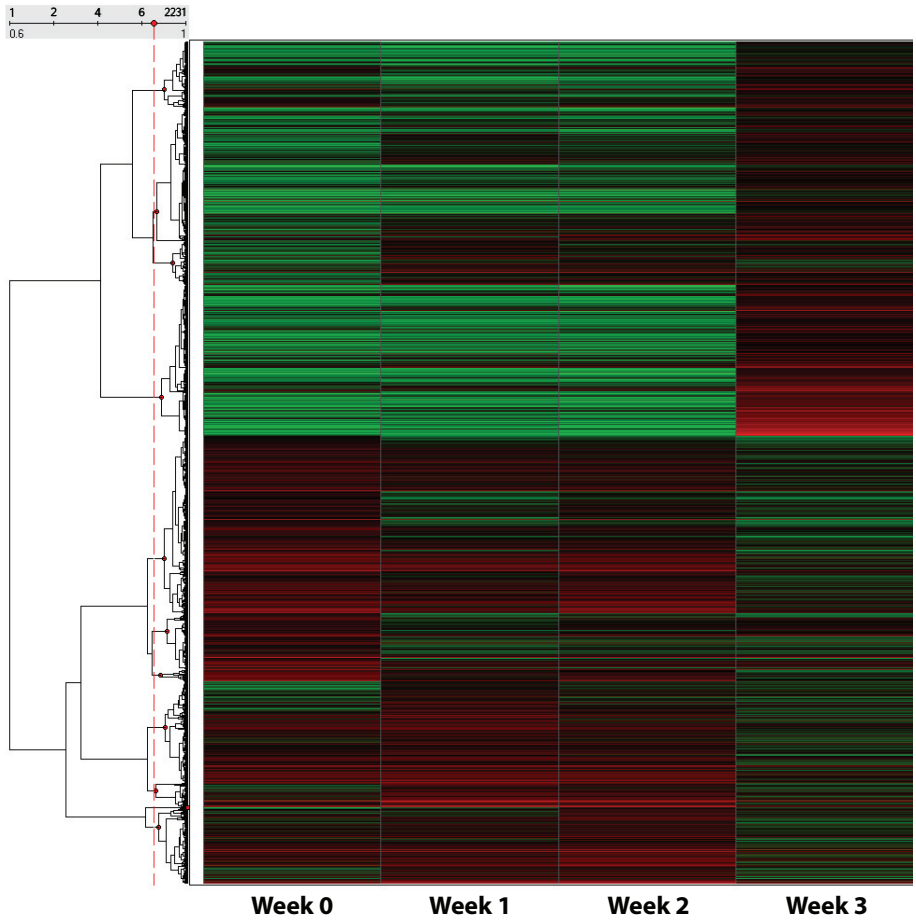

Figure 2: Hierarchical clustering of differentially expressed genes during CMPC differentiation

Hierarchical clustering of genes that were at least two-fold differentially expressed at any time point during CMPC differentiation. Differentially expressed genes were clustered in 11 temporal gene expression profiles, with 93% similarity between the subnodes within a cluster.

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were found to be over-represented at one week post-induction of differentiation compared to proliferating CMPCs in week 0 (cluster nine) (figure 5 and supplemental tables 9 and 12).

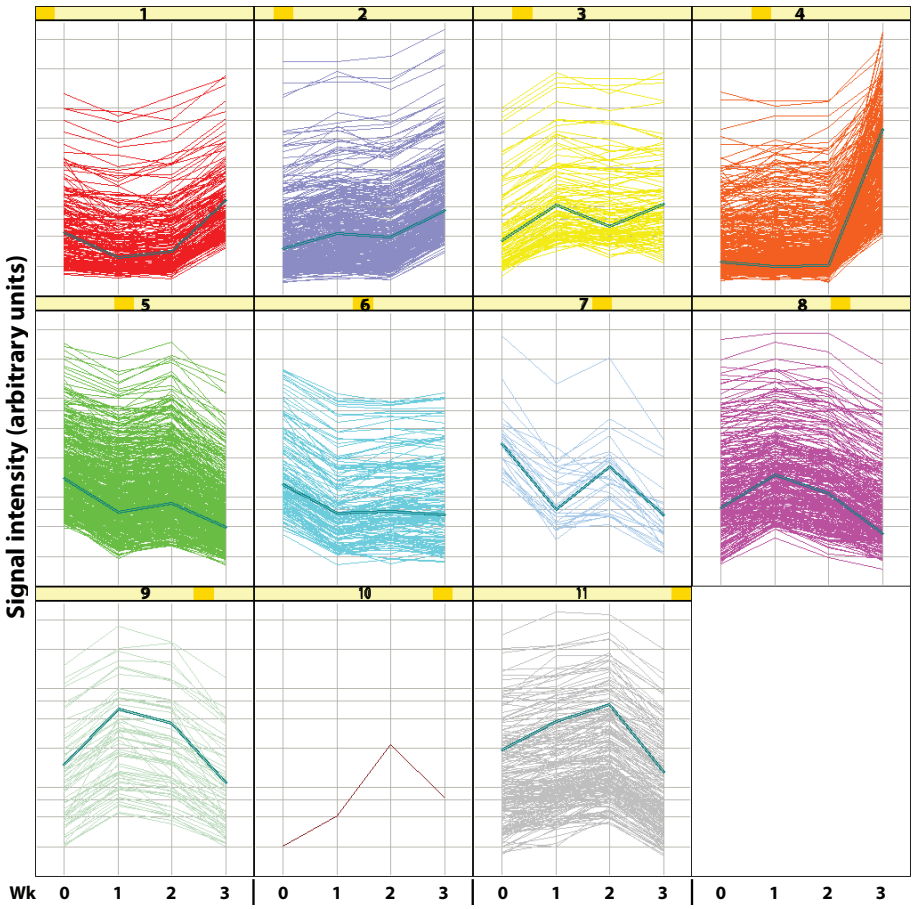

Figure 3: Hierarchical clustering resulted in 11 temporal gene expression profiles during CMPC differentiation

Bold line represents an example of temporal gene expression in that cluster.

Genomic profiling during terminal CMPC differentiation

After three weeks of differentiation, we observed increased expression of Nkx2.5 (1.94x, week three, not shown) and Mef2c (3.75x, week three, cluster four). Furthermore, mostly cytoskeletal proteins, actin binding motor proteins, and ATP synthases involved in muscle development and contraction (including several

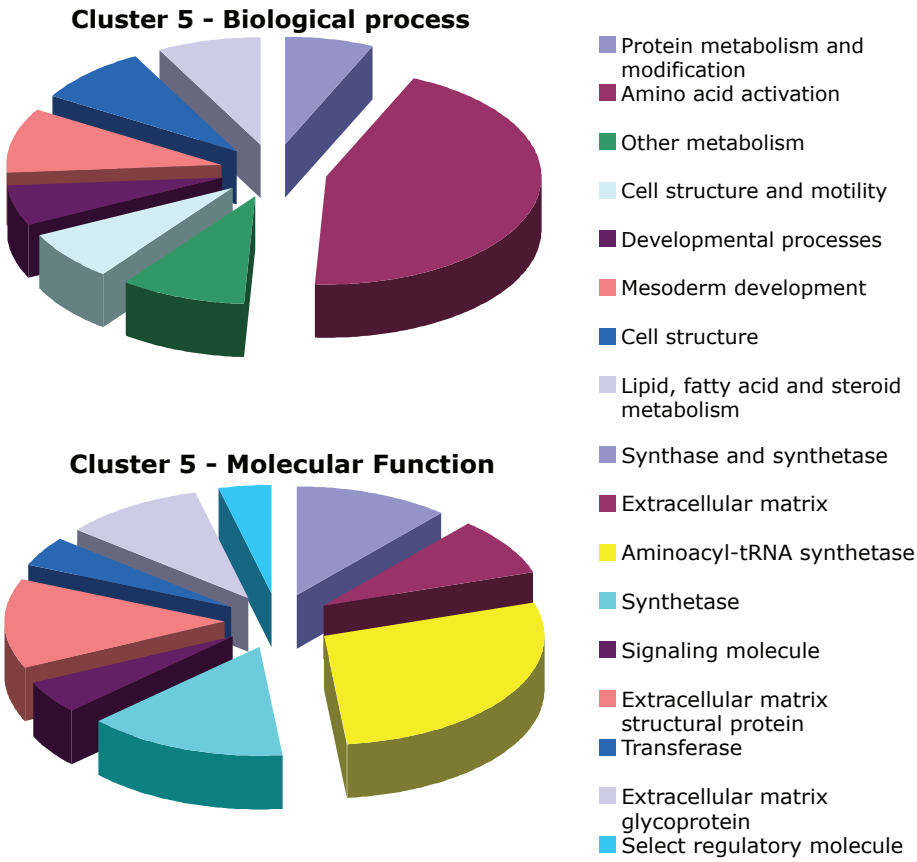

Figure 4: Panther analysis of differentially expressed genes in cluster five

troponins and myosin heavy and light chains), oxidative phosphorylation (NADH dehydrogenases and cytochome c oxidases), and lipid and fatty acid binding (FABP-3, -4, and -5) were found to be upregulated (cluster four) (figure 6 and supplemental tables 4 and 12).

Major histocompatibility complex (MHC) antigens involved in immunity were also differentially regulated (clusters one and four) (figures 4 and 7 and supplemental tables 1, 4 and 12). This could indicate that, during differentiation, CMPCs may acquire a different immunogenic phenotype.

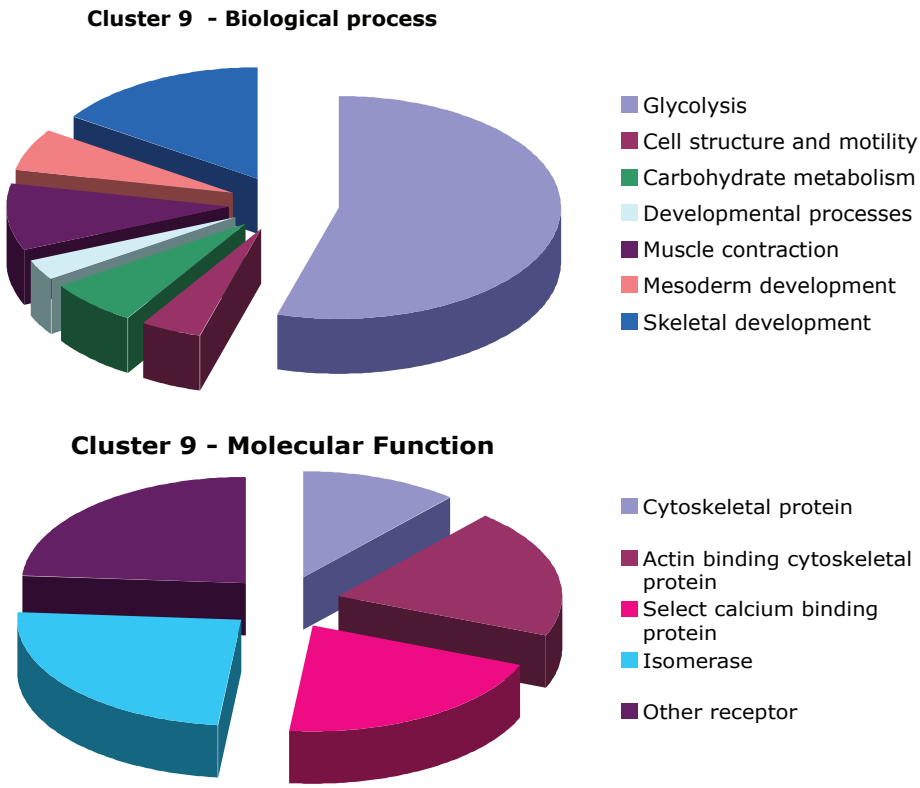

Figure 5: Panther analysis of differentially expressed genes in cluster nine

Identification of transcription factors regulating temporal gene expression patterns

To identify the upstream factors that regulate the temporal gene expression profiles within one cluster, we performed whole genome rVISTA analysis for putative transcription factor binding sites (TFBS) in conserved upstream regions of the genes in that cluster.

Genes that were either upregulated or downregulated early during CMPC differentiation (week one) had a predicted TFBS for eg. NFκB, RREB1, or HIF1 in common (figure 8 and cluster five, eight, and nine in supplemental table 13).

At three weeks of CMPC differentiation, genes with a TFBS for Mef2a, Smad3, NFκB, and Pitx2 were upregulated (figure 8 and

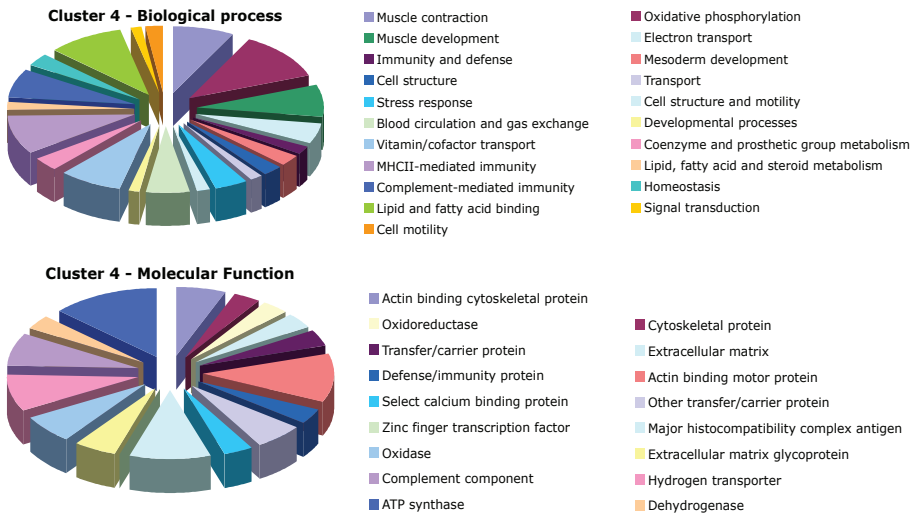

Figure 6: Panther analysis of differentially expressed genes in cluster four

cluster four supplemental table 13). Genes with a TFBS for Nanog, Myogenin, RREB1, and Meis1 were downregulated at week one (cluster one and five, supplemental table 13), but upregulated again at week three (cluster one and four).

DISCUSSION

Temporal genomic profiling is a useful approach to identify factors that regulate different steps during progenitor cell differentiation. In this study, we have performed microarray analysis at one-week intervals of cardiomyogenic differentiation of CMPCs. Identification of upstream signaling may help to understand complex networks of stage-specifically expressed genes. Therefore, we also performed rVISTA analysis for putative transcription factor binding sites to determine which transcription factors may regulate the temporal gene expression profiles observed in this study. With regard to future improvement of CMPC proliferation, differentiation, and transplantation, especially cell cycle regulators, cardiomyogenic

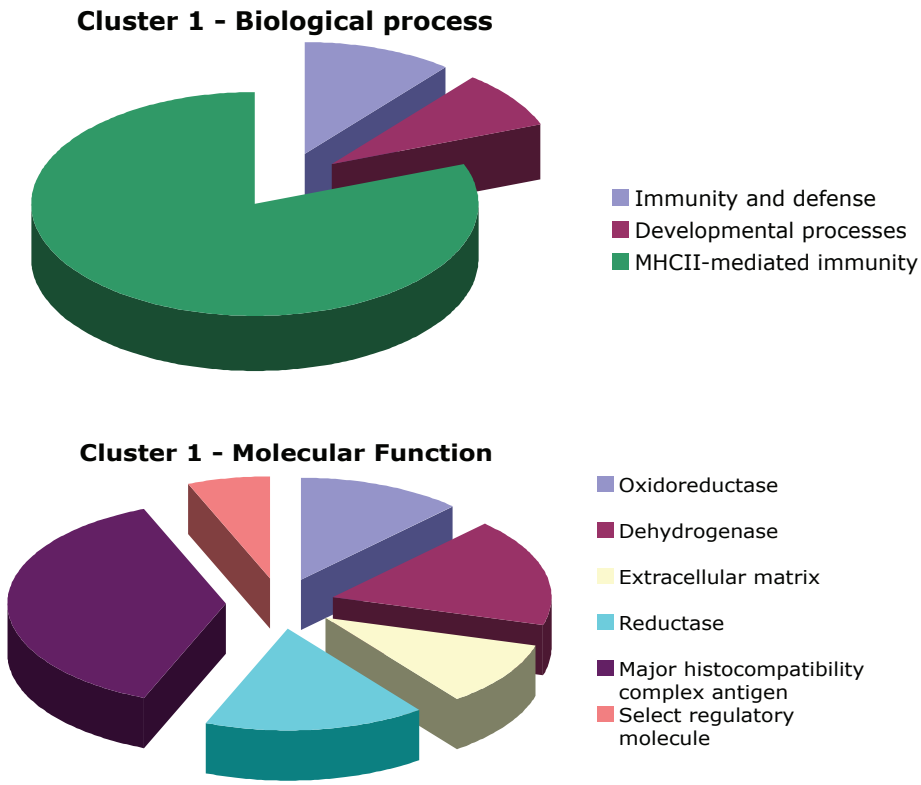

Figure 7: Panther analysis of differentially expressed genes in cluster one

stimulators, and immunogenic factors are of great interest.

Differential expression of cell cycle regulators

We observed downregulated expression of CDK inhibitors p16 and p21 (cluster five) in week one and three. Normally, p16 and p21 inhibit cell cycling[9,10] and their downregulation is therefore in contrast with a previously observed decreased mitosis upon induction of differentiation[4]. Different CDKs and E2F transcription factors, which regulate cell proliferation, were moderately upregulated or downregulated. This expression pattern may suggest that upon treatment of CMPCs with 5-azacytidine and TGFβ, transition from cell proliferation to differentiation is not

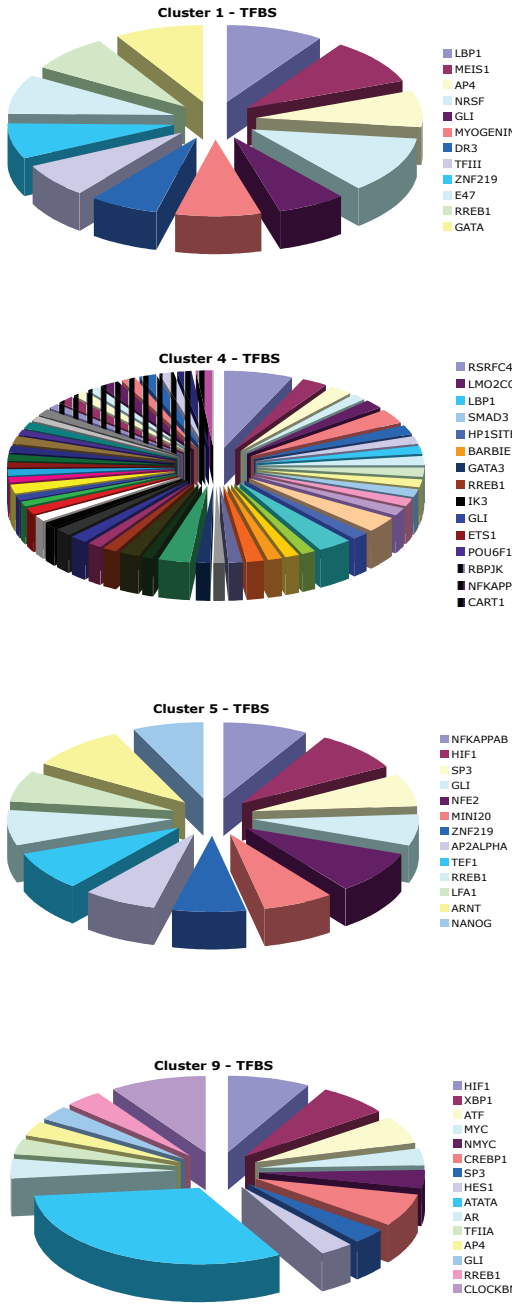

Figure 8: rVISTA analysis of transcription factor binding sites

immediate. Instead, both cycling and early differentiating cells could be present in the same population. However, cell cycle modulator activity often depends on (de)phosphorylation, while E2F transcription factors can act as activators as well as repressors (reviewed in[9]). This indicates that functional validation of these cell cycle proteins will be needed to determine their significance in CMPCs.

Cardiomyogenesis-related gene expression

In a previous study, we reported increased expression of Nkx2.5 and Mef2c already one week after inducing differentiation in CMPCs[3]. In the analysis performed here, we observed the highest increase in Nkx2.5 and Mef2c expression around week three (cluster four). Nkx2.5 and Mef2c act together with several cofactors like Gata4 and SRF, while Mef2c activity is regulated by kinases and HDACs (reviewed in[11-13]). Possibly, the low abundance of Nkx2.5 and Mef2c during the first two weeks of CMPC differentiation represents a basal expression level of mostly inactive transcription factors. Only when the stage for terminal cardiomyogenic differentiation is reached, an enhanced transcription of Nkx2.5 and Mef2c target genes is required and, in order to facilitate this process, transcription factor expression and activity are enhanced. The regulation of transcription factor activity should therefore be taken into account when interpreting the role of Nkx2.5 and Mef2c during CMPC cardiomyogenesis.

The CMPC immunophenotype changes during differentiation

MHC class 2 molecules were differentially expressed during CMPC differentiation (cluster one and four). Normally, most cell types only express MHC class 1 molecules, while only a few specialized antigen-presenting cells (APCs) like macrophages, dendritic cells, activated T cells, and B cells express MHC class 2 molecules[14]. The detection of MHC class 2 molecules on CMPCs and CMPC-derived cardiomyocytes was therefore unexpected. Other cells than APCs generally do not express MHC class 2 molecules, unless they are exposed to inflammatory signals like IFN-γ. However, the IFN-γ-induced response can be inhibited by TGFβ stimulation (reviewed

in[14]). Possibly, the exposure to TGFβ in the first and second week of CMPC differentiation resulted in the observed downregulation of MHC class 2 proteins, followed by an upregulation in the third week, when CMPCs were no longer stimulated by TGFβ. Further investigation will be needed to establish whether TGFβ stimulation indeed affects the CMPC immunophenotype and determine whether a different phenotype will initiate a host immune response, which may lead to rejection of transplanted CMPCs.
In summary, cardiomyogenic differentiation in CMPCs is a highly regulated process and temporal profiling has provided interesting targets for further investigation. The regulation of multiple signaling cascades by a variety of transcription factors offers the opportunity to manipulate them simultaneously. In the end, this may lead to a more efficient improvement of CMPC proliferation and differentiation.

MATERIALS AND METHODS

CMPC culture and differentiation

Informed consent procedures were followed and prior approval of the ethics committee of the University Medical Center Utrecht was obtained. CMPCs from human fetal hearts were MACS isolated. Culture and induction of cardiomyogenic differentiation with 5-azacytidine and TGFβ was performed as described previously[3,5]. Briefly, CMPCs were plated on day 0 and exposed to 5 μM 5-azacytidine (Sigma) on day 1, 2, and 3 in differentiation medium. From day 6 onwards, 10^{-4} M ascorbic acid (Sigma) was added to the differentiation medium, which was refreshed thrice weekly. TGFβ (1 ng/ml, PeproTech) was added on day 7, 10, and 13 post-induction.

Microarray

Fetal undifferentiated CMPCs or CMPCs one, two, or three weeks after induction of differentiation were lyzed in Tripure (Roche). Total RNA was isolated according to the manufacturer's instruction and subsequently purified with Nucleospin columns (Macherey-

Nagel). Five hundred ng of total RNA was used for cDNA synthesis and subsequent cRNA synthesis with the Illumina® TotalPrep RNA amplification kit (Ambion). The Illumina Expression BeadChip (HumanRef-8 v2) was hybridized, labeled, washed, and scanned following manufacturers instruction.

Microarray analysis

Gene expression values from the bead array were quantile normalized in BeadStudio (version 3.2 Illumina) and analyzed for similar temporal gene expression profiles during CMPC differentiation in Spotfire DecisionSite for Functional Genomics (TIBCO Spotfire Europe, Goteborg, Sweden). Genes were considered differentially expressed if the absolute fold-change between one time point and at least one other time point was more than two ('max ratio', see supplemental tables 1-11).

Functional analysis of the selected gene sets was done using Panther (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org). Classification was listed if detected genes were significantly overrepresented or underrepresented as compared to the human reference.

Identification of conserved transcription factor binding sites (TFBS) was performed with whole genome regulatory VISTA (rVISTA[15]). TFBS over-represented in the 500 bp upstream of genes with similar temporal expression profiles were compared to the total number of these TFBS in the 500 bp regions of Reference Sequence genes in the whole genome (http://www.ncbi.nlm.nih.gov/RefSeq). P-values were calculated based on frequency of putative transcription factor binding sites in the 500 bp upstream sequences of a target gene set compared to a similar size random gene set in the total genome.

REFERENCES

1. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).
2. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* **50**, 19-30 (2008).
3. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
4. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
5. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
6. Hans, F. & Dimitrov, S. Histone H3 phosphorylation and cell division. *Oncogene* **20**, 3021-7 (2001).
7. Woese, C.R., Olsen, G.J., Ibba, M. & Soll, D. Aminoacyl-tRNA synthetases, the genetic code, and the evolutionary process. *Microbiol Mol Biol Rev* **64**, 202-36 (2000).
8. Knight, R.D., Freeland, S.J. & Landweber, L.F. Rewiring the keyboard: evolvability of the genetic code. *Nat Rev Genet* **2**, 49-58 (2001).
9. Johnson, D.G. & Walker, C.L. Cyclins and cell cycle checkpoints. *Annu Rev Pharmacol Toxicol* **39**, 295-312 (1999).
10. Zhu, L. & Skoultchi, A.I. Coordinating cell proliferation and differentiation. *Curr Opin Genet Dev* **11**, 91-7 (2001).
11. McKinsey, T.A., Zhang, C.L. & Olson, E.N. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem Sci* **27**, 40-7 (2002).
12. Olson, E.N. & Schneider, M.D. Sizing up the heart: development redux in disease. *Genes Dev* **17**, 1937-56 (2003).
13. Evans, S.M. Vertebrate tinman homologues and cardiac differentiation. *Semin Cell Dev Biol* **10**, 73-83 (1999).
14. Reith, W., LeibundGut-Landmann, S. & Waldburger, J.M. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* **5**, 793-806 (2005).
15. Loots, G.G., Ovcharenko, I., Pachter, L., Dubchak, I. & Rubin, E.M. rVista for comparative sequence-based discovery of functional transcription factor binding sites. *Genome Res* **12**, 832-9 (2002).

SUPPLEMENTAL TABLES

Supplemental tables 1 to 11

Complete lists of differential gene expression at week 0, 1, 2, or 3 of CMPC differentiation. Data for each expression profile cluster shown in figure 3 is listed as a separate table (1 to 11). Avg Signal is average signal of eight dots per annotation on the array. 'Max ratio' is the maximal ratio between one time point and at least one other time point. Fold change treshold (minimally two-fold) is based on maximal ratio.

Supplemental Table 12: Panther analysis of differentially expressed genes during CMPC differentiation

Complete list of biological processes, molecular functions, and signaling pathways of genes with similar temporal expression profiles. Clusters are the same as in figure 3. Data for clusters 7 and 10 were not significant and therefore not listed.

Supplemental Table 13: rVISTA analysis of transcription factor binding sites.

Complete list of significantly overrepresented TFBS ($p<0.005$). Clusters are the same as in figure 3.

CHAPTER

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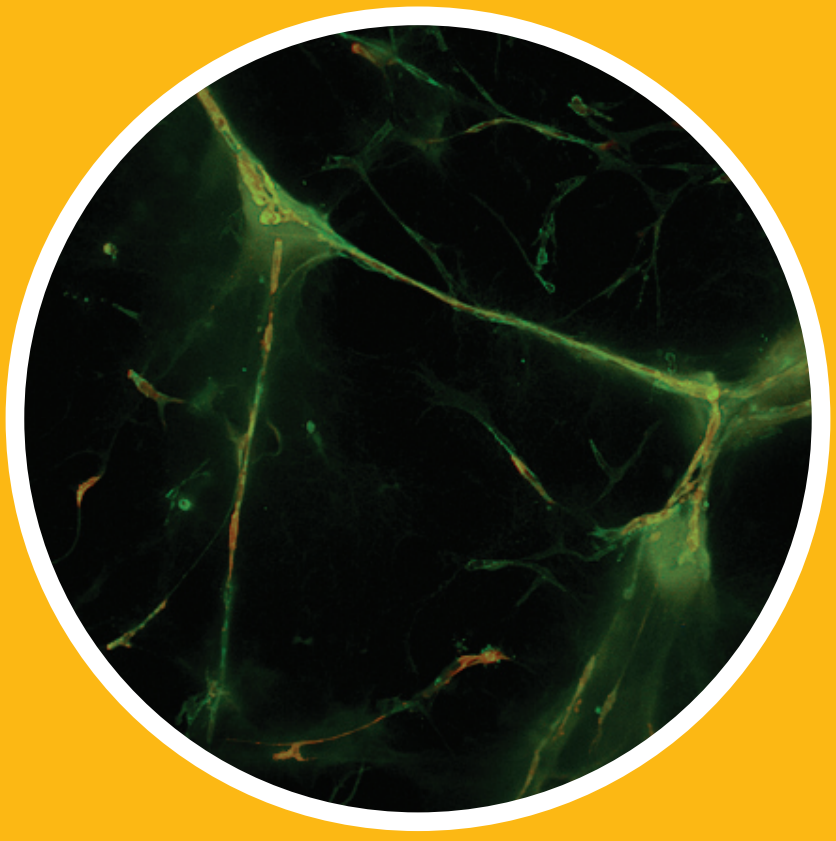
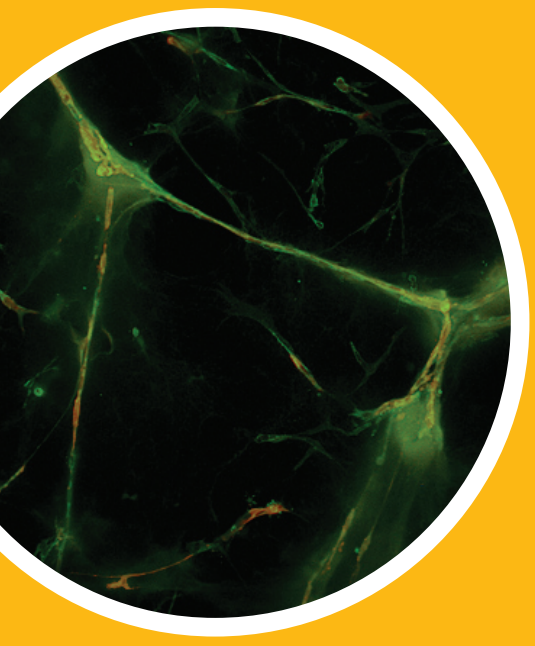


CHAPTER 5

Fetal and Adult Cardiomyocyte Progenitor Cells Have Different Developmental Potential



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ABSTRACT

In the past years, efforts have been made to characterize and isolate different progenitor cell populations from the heart. Direct comparison of the different cardiovascular progenitor populations is difficult due to different methods for isolation, culture, differentiation, and profiling. Up to date, no studies have been reported in which the potential of fetal and adult cardiovascular progenitor populations were tested simultaneously. Here, we report a direct comparison between human fetal and adult heart-derived cardiomyocyte progenitor cells (CMPCs). We show that fetal CMPCs are generally less restricted in their potential to differentiate into different mesodermal lineages than adult CMPCs. Adult CMPCs cells predominantly form quiescent and electrophysiologically more mature cardiomyocytes. Under angiogenic conditions, fetal CMPCs form more endothelial cells, but less smooth muscle cells than adult CMPCs. Fetal CMPCs can also develop into adipocytes, while neither fetal nor adult CMPCs show significant osteogenic differentiation. Taken together, fetal and adult CMPCs have a distinct differentiation potential. These differences may influence future conclusions and directions regarding progenitor cell potential and application of CMPCs for regenerative medicine.

INTRODUCTION

Multipotent progenitor cell types, i.e. cells that have the ability to form different cell types within one developmental lineage, have been identified in a variety of adult tissues, albeit in very low numbers. The number of progenitor cells in developing tissues is high until organs have fully differentiated, after which their numbers decrease tremendously. As has been shown for the adult liver, skin, gut, and skeletal muscle[1], only a small subset of progenitor cells routinely repair lost or damaged tissue in the adult. Ageing of progenitor cells is often associated with a decline in function and plasticity[2]. For instance, the prevalence of mesenchymal stem cells (MSCs) in fetal bone marrow, liver, and blood was significantly higher than in adult tissues[3] and a later study showed that in fetal MSCs proliferation and differentiation efficiency were higher than in adult MSCs[4]. While adult bone marrow derived MSCs can be forced along osteogenic, chondrogenic and adipogenic lineages, fetal MSCs can additionally differentiate into neurons, skeletal muscle and sometimes even blood cells[5].

For several decades, the adult heart was considered a post-mitotic organ, devoid of progenitor cells contributing to homeostasis of cardiac tissue. However, the identification of dividing cells in the adult heart[6] has led to the isolation and characterization of several adult cardiovascular progenitor cell populations. These cells have been proposed as an ideal source for cardiac stem cell therapy to repair the damaged heart[7,8]. Recently, we have isolated cardiomyocyte progenitor cells (CMPCs) from human heart tissue[9-11]. Fetal and adult heart-derived CMPCs showed similar phenotypes and expression patterns of early cardiac transcription factors. Stimulation with 5-azacytidine and TGFβ resulted in the formation of cardiomyocytes within three to four weeks with high efficiency (93-98% α-actinin-positive cardiomyocytes with fetal CMPCs compared to 84-93% when using adult CMPCs[9]). However, fetal CMPC-derived cardiomyocytes (fCMPC-cm) more frequently showed spontaneous beating than adult CMPC-cm (seven versus two out of ten experiments, respectively). CMPCs were also able to form endothelial and smooth muscle cells, but their capacity

to form other cell types within the mesodermal lineage remained unexplored. These differences between fetal and adult CMPC multipotency are important when deciding on the optimal cell population to investigate mechanisms regulating proliferation or differentiation, cellular behavior in response to drug screening, or their clinical applicability. Therefore, we investigated the differentiation potential of fetal versus adult CMPCs towards cardiomyocytes, vascular cells, fat, and bone.

RESULTS

Proliferation and cardiomyogenic differentiation of CMPCs

CMPCs derived from both fetal and adult human heart tissue show similar spindle-shaped morphology and high nucleus to cytoplasm ratio (figure 1A). After isolation, cultured fetal CMPCs (fCMPCs) revealed a slightly higher, exponential proliferation rate than adult CMPCs (aCMPC, figure 1B).

Upon stimulation by 5-azacytidine and TGFβ, both fetal and adult CMPCs differentiate into cardiomyocytes. Spontaneous contractions appear on average between 19-25 or 25-30 days in fetal and adult CMPC-derived cardiomyocytes (CMPC-cm), respectively. Both populations express a striated pattern of sarcomeric proteins[9]. Double labeling of fetal and adult CMPC-cm for α-actinin and the gap junction protein Connexin 43 (Cx43) is shown in figure 1C. Both fetal and adult CMPC-cm show robust staining for Cx43, which is in line with the high degree of intercellular coupling as reported previously[9]. Interestingly, in aCMPC-cm, Cx43 labeling was not only found as intense staining all around the cells but also in a polarized fashion in cells that presented an elongated phenotype. In such cells, labeling was most intense at the longitudinal cell border.

Electrophysiologically, CMPC-cm resemble fetal/early neonatal ventricular cardiomyocytes[9]. Both fCMPC and aCMPC-derived cardiomyocytes are quiescent in the absence of fetal calf serum and field stimulation. When stimulated, fCMPC-cm and aCMPC-cm show action potentials of comparable overshoot (figure 1D). Compared to their fetal counterparts, aCMPC-cm have a more

mature action potential shape with a spike and dome morphology accompanied by a longer lasting plateau phase.

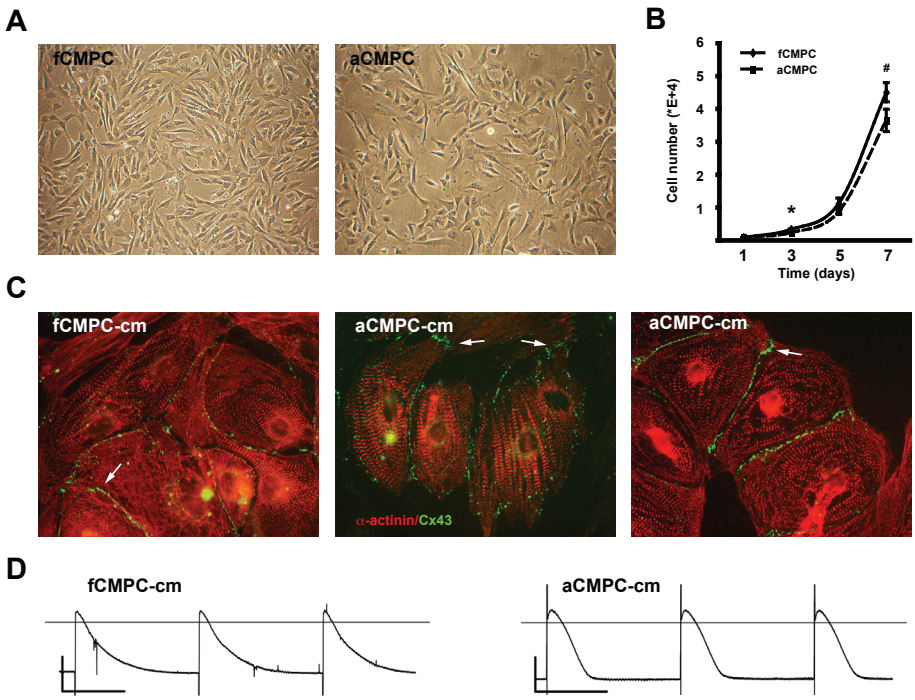

Figure 1: Proliferation and differentiation of CMPCs

A | Bright field images showing fetal (fCMPC, left) and adult CMPCs (aCMPC, right) during normal culture conditions (100x magnification). Note the similarities in spindle-shaped phenotype. B | Growth curve of fetal and adult CMPCs (* p=0.01 and # p=0.04 versus aCMPC, n=3). C | Immunostaining for α-actinin (red) and Connexin 43 (green) in fetal (fCMPC-cm, left) and adult CMPC-derived cardiomyocytes (aCMPC-cm, middle and right). White arrows indicate the presence of gap junctions at the plasma membrane borders (400x magnification). D | Action potentials recorded from fCMPC-cm (left) and aCMPC-cm (right) using sharp microelectrodes. Monolayers were field stimulated at 1 Hz. Scale bars: vertical = 50 mV, horizontal = 500 ms.

Genetic profiling of CMPC-derived cardiomyocytes

To gain more insight into the differences between fetal and adult CMPC-derived cardiomyocytes on a molecular level, we analyzed their gene expression by microarray. The majority of detected genes were less than three-fold differentially expressed in both fetal and adult CMPC-cm, including myosin light chain (MYL)-5, 6, and 9, myosin heavy chain (MYH)-10, 13, and 14, tropomyosin (TPM)-2 and 4, troponin (TNN)-T1, titin (TTN), and connexin 43 (GJA1) (figure 2A and supplemental table 1).

About 500-600 genes were at least three-fold differentially expressed in fetal versus adult CMPC-cm (figure 2A and supplemental tables 2 and 3). However, instead of differential expression of single genes, clustering of regulated genes based on biological process or molecular function is more likely to reveal relevant differences in the overall phenotype and developmental state of the CMPC-cm (figure 2B and C).

Both fetal and adult CMPC-cm expressed genes that were involved in protein metabolism, developmental processes, and extracellular matrix, even though specific genes were differentially expressed (supplemental tables 4 and 5). Examples include metalloproteases ADAMTS1 versus AEBP1, MMP1 versus MMP2, 14, and 17, CaM kinases CAMK2B versus CAMKK2, and different protein kinases C in fetal and adult CMPC-cm, respectively.

Compared to aCMPC-cm, fCMPC-cm showed more than three-fold differential expression of several cell structure and motility-related genes like cardiac muscle α-actin (ACTC), desmin (DES), myosin heavy chain α as well as β (MYH6 and MYH7, respectively), MYL2, TPM-1 and 3, TNNC1, TNNI3, TNTT2 and 3, skelemin (MYOM-1), myomesin (MYOM-2), phospholamban (PLN), and sarcoplasmic reticulum Ca^{2+}-ATPase (ATP2A2 or Serca2a) transcript variant 2.

In adult CMPC-cm, kinases and transporters required for signal transduction, protein modification, and transport process-related genes were highly present. Here, ATP2A2 transcript variant 1, synthaxin-1, spermidine synthase, mitogen-activated protein kinase kinase kinase (MAP3K), and several ABC transporters and tyrosine protein kinase receptors were more than three-fold upregulated compared to fCMPC-cm.

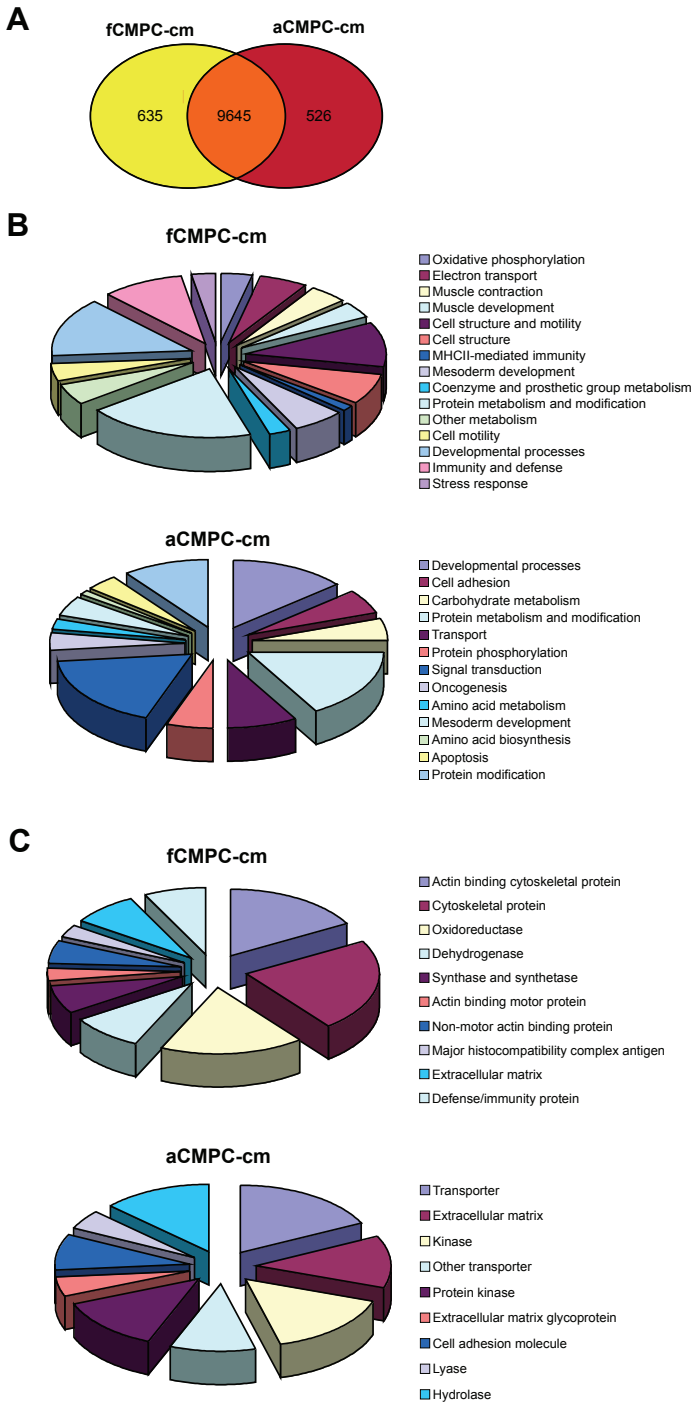
A
fCMPC-cm
aCMPC-cm
635
9645
526
B
fCMPC-cm
Oxidative phosphorylation
Electron transport
Muscle contraction
Muscle development
Cell structure and motility
Cell structure
MHCII-mediated immunity
Mesoderm development
Coenzyme and prosthetic group metabolism
Protein metabolism and modification
Other metabolism
Cell motility
Developmental processes
Immunity and defense
Stress response
aCMPC-cm
Developmental processes
Cell adhesion
Carbohydrate metabolism
Protein metabolism and modification
Transport
Protein phosphorylation
Signal transduction
Oncogenesis
Amino acid metabolism
Mesoderm development
Amino acid biosynthesis
Apoptosis
Protein modification
C
fCMPC-cm
Actin binding cytoskeletal protein
Cytoskeletal protein
Oxidoreductase
Dehydrogenase
Synthase and synthetase
Actin binding motor protein
Non-motor actin binding protein
Major histocompatibility complex antigen
Extracellular matrix
Defense/immunity protein
aCMPC-cm
Transporter
Extracellular matrix
Kinase
Other transporter
Protein kinase
Extracellular matrix glycoprotein
Cell adhesion molecule
Lyase
Hydrolase

Figure 2: Gene expression profiling in fetal and adult CMPC-derived cardiomyocytes

A | Comparison of gene expression in fetal (fCMPC-cm) and adult CMPC-derived cardiomyocytes (aCMPC-cm). B | Pie diagram showing gene expression based on biological process in fetal (top) and adult CMPC-cm (bottom). C | Pie diagram showing gene expression based on molecular function in fetal (top) and adult CMPC-cm (bottom).

Spontaneous beating in CMPC-derived cardiomyocytes is determined by resting membrane potential stability

Patch clamp recordings confirmed that adult CMPC-derived cardiomyocytes have a more negative resting membrane potential (RMP) compared to fetal CMPC-cm (figure 3A). To determine whether this difference in RMP influenced electrical (in)stability and occurrence of spontaneous beating in fCMPC-cm and aCMPC-cm, we blocked potassium inward rectifying channels with barium. This resulted in a less negative RMP for both fCMPC-cm and aCMPC-cm, although the effect was more pronounced in aCMPC-cm (+18.2 mV versus +13.5 mV in fCMPC-cm, figure 3A). This suggests higher functional expression of potassium inward rectifying channels in aCMPC-cm, which may underlie the more mature electrical phenotype of aCMPC-cm as illustrated by the more negative RMP, faster repolarization of the action potential, and lesser occurrence of spontaneous beating in these cardiomyocytes.

Previously, we showed that coculture and subsequent gap junctional coupling of a Kir2.1GFP-overexpressing HEK 293 cell line (KWGF cells) with neonatal rat cardiomyocytes resulted in a more negative, stable resting membrane potential[12]. We therefore determined if coculture with KWGF cells also affected RMP and spontaneous beating in fCMPC-cm. KWGF cells induced a RMP of -82.3 (+/- 2.4) mV in fCMPC-cm ($p<0.05$ versus fCMPC-cm alone, $n=5$). This was similar to the RMP seen in aCMPC-cm alone (figure 3A), which demonstrates that with our differentiation protocol, aCMPC-cm have a RMP that equals the potassium equilibrium potential. The lower RMP induced by KWGF cells subsequently inhibited spontaneous beating (figure 3B-E and supplemental movies 1, 2, and 3).

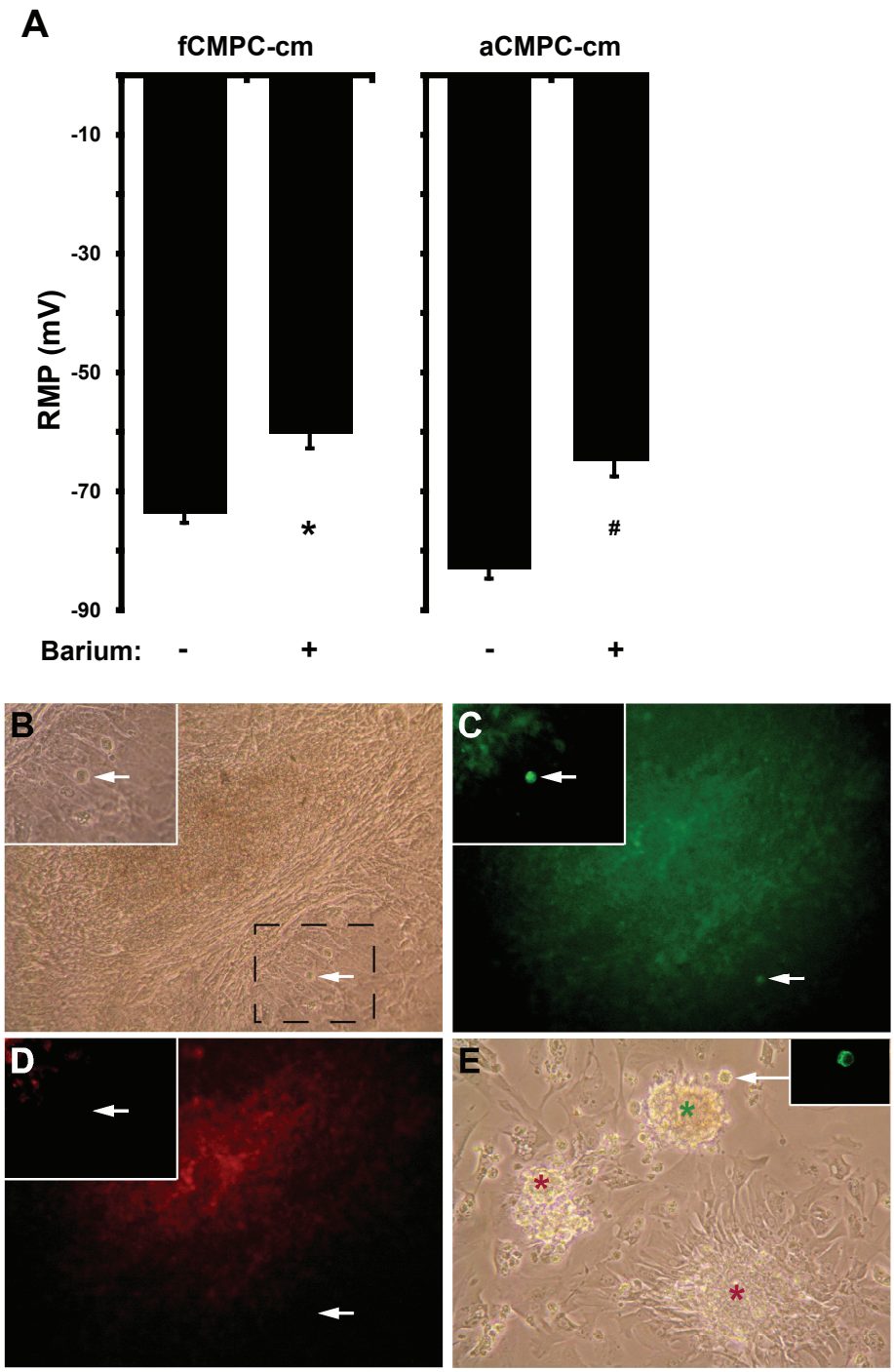
A
fCMPC-cm
aCMPC-cm
RMP (mV)
-10
-30
-50
-70
-90
*
#
Barium: - + - +
B
C
D
E

Figure 3: Membrane potential stability of fCMPC-cm and aCMPC-cm

A | Resting membrane potential of fCMPC-cm and aCMPC-cm with (+) or without (-) 1 mM $BaCl_2$. Barium induced a large depolarization in fCMPC-cm (min barium n=6, plus n=4, * $p<0.01$) and aCMPC-cm (min barium n=7, plus n=7, # $p<0.01$). B | Bright field image of fCMPC-cm cocultured with KWGF cells (white arrows). Insets B-D: magnification of boxed area in B. C | FITC image showing GFP fluorescence in a KWGF cell (arrow) and autofluorescence in a beating cluster of fCMPC-cm. D | TRITC image showing autofluorescence in a beating cluster, but not the KWGF cell. E | Bright field image of beating clusters (red asterisks) and a quiescent cluster (green asterisk) of fCMPC-cm. White arrow indicates a KWGF cell adjacent to the quiescent cluster (FITC image in inset). All images are at 100x magnification. Same areas are shown in supplementary movies 1-3.

Fetal CMPC-cm cocultured with wildtype HEK 293 cells continued to beat spontaneously (supplemental movie 4), indicating that inhibition of spontaneous beating by KWGF cells is not due to coculture effects.

Angiogenic properties of fetal versus adult CMPCs

Under angiogenic conditions, CMPCs are able to form tube-like structures with endothelial cells surrounded by smooth muscle cells[9]. To compare their angiogenic potential, fetal and adult CMPCs were cultured on Matrigel and stimulated with VEGF, resulting in network formation in both populations (figure 4A). Immunostaining for α-smooth muscle actin (αSMA) and platelet/endothelial cell adhesion molecule (PECAM) revealed the presence and colocalization of endothelial and smooth muscle cells (figure 4B). Interestingly, fCMPCs showed pronounced staining for PECAM, while aCMPCs show a stronger staining for αSMA. Quantification of the tube-like structures revealed that aCMPC formed longer and thicker structures with less junctions (figure 4C). On a gene expression level, adult CMPCs showed higher expression of αSMA (not shown) and smooth muscle myosin heavy chain (SM-MHC), but lower expression of Tie-2 and VE-Cadherin, compared to fCMPCs (figure 4D). This confirms that, following angiogenic induction, aCMPCs seem more prone to form smooth muscle-like cells than endothelial cells when compared to fCMPCs.

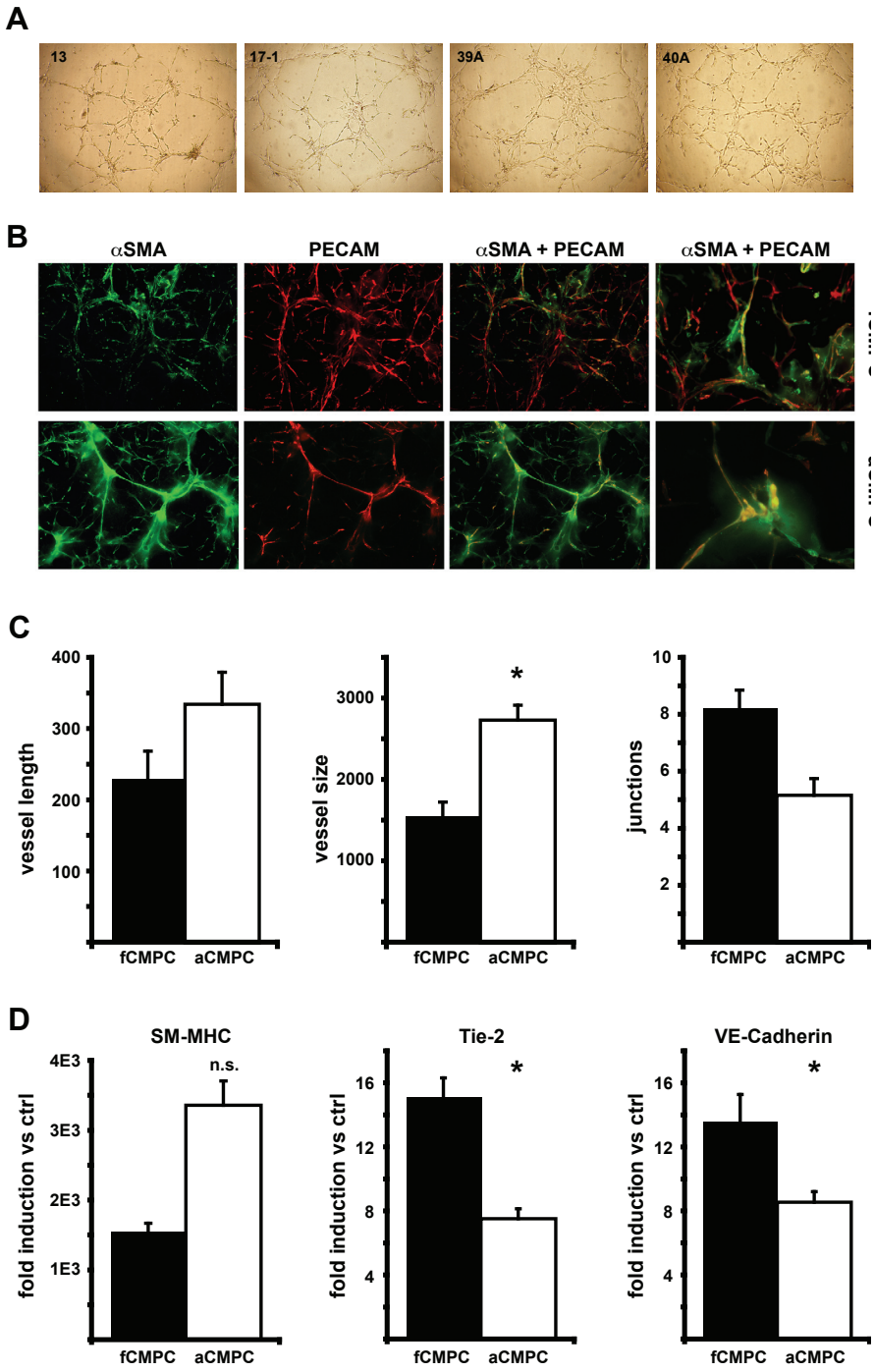
A
13
17-1
39A
40A
B
αSMA
PECAM
αSMA + PECAM
αSMA + PECAM
C
vessel length
400
300
200
100
fCMPC
aCMPC
vessel size
3000
2000
1000
*
fCMPC
aCMPC
junctions
10
8
6
4
2
fCMPC
aCMPC
D
SM-MHC
n.s.
fold induction vs ctrl
4E3
3E3
2E3
1E3
fCMPC
aCMPC
Tie-2
*
fold induction vs ctrl
16
12
8
4
fCMPC
aCMPC
VE-Cadherin
*
fold induction vs ctrl
16
12
8
4
fCMPC
aCMPC

Figure 4: Angiogenic potential of fetal and adult CMPCs

A | Representative bright field images of Matrigel assays with fetal (week 13 and 17) and adult (clones 39 and 40) CMPCs (40x magnification). B | Immunofluorescence images of the same assay as in A with staining for αSMA (green) and PECAM (red, 40x magnification). Images most right are at 100x magnification. C | Angioquant quantification of average vessel length and size and number of junctions from images in B. Data is from one of three separate experiments performed in triplo (* p=0.02). D | Quantitative RT-PCR analyses indicating fold induction of fetal and adult CMPCs from the angiogenesis assays in A-C compared to controls cultured in normal culture medium. SM-MHC: not significant, p=0.08, n=2. Tie-2: p=0.01, n=5. VE-Cadherin: p=0.04, n=5.

Adipogenic potential of fetal versus adult CMPCs

To assess the potential of CMPCs to form other mesodermal cell types, CMPCs were subjected to an adipogenic differentiation protocol. MSCs were used as positive control. Under control conditions, MSCs, fCMPCs and aCMPCs formed lipoprotein-containing vacuoles positive for Oil Red O (figure 5A). Quantification showed that, upon adipogenic differentiation, fCMPCs contained significantly more lipoprotein than aCMPCs (figure 5B). Stimulated aCMPCs did not contain significantly more lipoprotein than their controls. Higher adipogenic differentiation capacity in fCMPCs compared to aCMPCs was confirmed on gene expression level by higher levels of leptin, adipsin, and PPARγ2, and lower expression of CCN1 (figure 5C).

Osteogenic potential of fetal versus adult CMPCs

After induction of osteogenesis, MSCs, but not fetal or adult CMPCs, stained positive for alkaline phosphatase and Alizarin Red S (figure 6A). Expression of the osteogenic transcription factor Runx2 was 584x upregulated in fCMPCs compared to control, while neither CTGF nor osteocalcin were increased in fetal or adult CMPC (figure 6B).

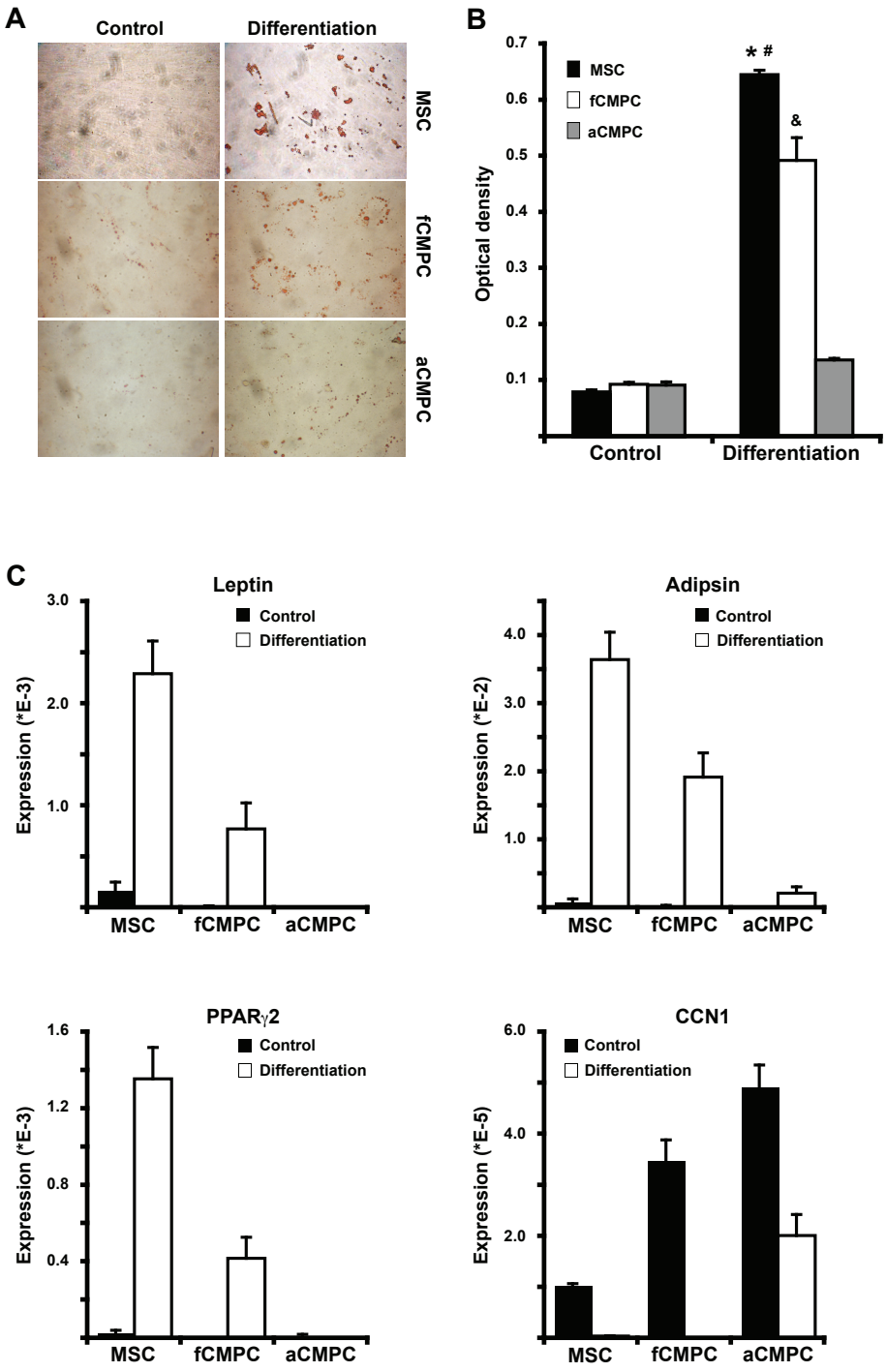
A
Control
Differentiation
MSC
fCMPC
aCMPC
B
MSC
fCMPC
aCMPC
Optical density
0.7
0.6
0.5
0.4
0.3
0.2
0.1
* #
&
Control
Differentiation
C
Leptin
Control
Differentiation
Expression (*E-3)
3.0
2.0
1.0
MSC
fCMPC
aCMPC
Adipsin
Control
Differentiation
Expression (*E-2)
4.0
3.0
2.0
1.0
MSC
fCMPC
aCMPC
PPARγ2
Control
Differentiation
Expression (*E-3)
1.6
1.2
0.8
0.4
MSC
fCMPC
aCMPC
CCN1
Control
Differentiation
Expression (*E-5)
6.0
4.0
2.0
MSC
fCMPC
aCMPC

Figure 5: Adipogenic potential of fetal and adult CMPCs

A | MSCs and CMPCs cultured in 0.5% culture medium (Control) or adipogenic medium (Differentiation) and stained for Oil Red O after 5 days (40x magnification). B | Quantification of Oil Red O in A, indicating a large increase of Oil Red O in MSCs (n=2) and fCMPCs (n=4) but only a small increase in aCMPCs (n=4, * p=0.000 vs all Controls and aCMPC Differentiation, # p=0.007 vs fCMPC Differentiation, & p=0.000 vs all Controls and aCMPC Differentiation). C | Quantitative RT-PCR analysis for Leptin, Adipsin, PPARγ2, and CCN1 in MSCs, fCMPCs, and aCMPCs cultured in normal culture medium (Control) versus adipogenic medium (Differentiation, n=2).

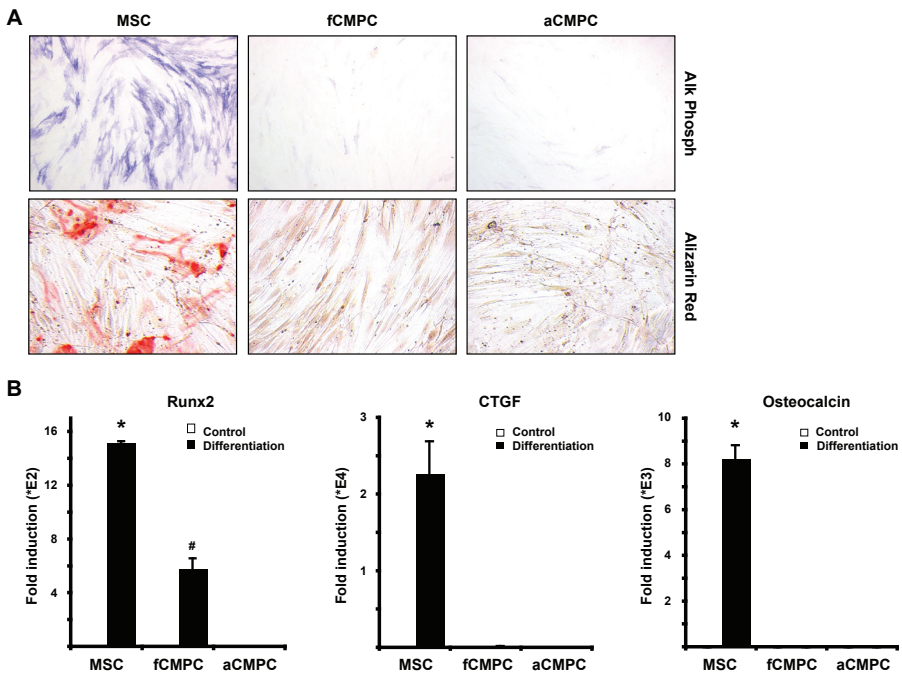

Figure 6: Osteogenic potential of fetal and adult CMPCs

A | MSCs, fCMPCs, and aCMPCs cultured in osteogenic differentiation medium and stained for Alkaline Phosphatase or Alizarin Red S (40x magnification).

B | Quantitative RT-PCR analysis for Runx2, CTGF, and osteocalcin in MSCs (n=2), fCMPCs (n=3), and aCMPCs (n=3) cultured in normal culture medium (Control) versus osteogenic medium (Differentiation, * and # p=0.000 vs all other conditions).

DISCUSSION

The multipotency of cardiac progenitor cells is of great interest developmentally and clinically. Many different mammalian cardiovascular progenitor cell populations have been isolated from the fetal and/or adult heart and shown to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells[7,8]. However, comparisons between cells from different developmental origins have not been performed thoroughly. Here, we show that human fetal and adult heart-derived CMPCs can be expanded in approximately similar rates and that both can differentiate into cardiomyocytes. However, fCMPCs differentiate into electrophysiologically less mature, spontaneously beating cardiomyocytes than aCMPCs. Spontaneous beating was inhibited in fCMPC-cm when the resting membrane potential became similar to that of aCMPC-cm, which have a membrane potential equal to the potassium equilibrium potential.
Fetal CMPCs also form longer, endothelial tube-like structures during an angiogenesis assay. So far, only one other study reported adipogenic and osteogenic differentiation of (murine) cardiac progenitor cells[14], Here we show that fCMPCs have more adipogenic potential than aCMPC and that neither fetal nor adult CMPCs showed substantial osteogenic differentiation.

Gene expression profiles in CMPC-derived cardiomyocytes

Several sarcomere-related genes that were higher expressed in fCMPC-cm when compared to aCMPC-cm are also differentially expressed during development in the heart[15,16]. Since sarcomere proteins are thought to be very stable, the high expression of so many of these genes suggests that fCMPC-cm still express a somewhat more immature cardiac program in order to develop a more robust contractile apparatus. The high expression of transporters and kinases involved in signal transduction and protein modification in the more mature and electrophysiologically stable aCMPC-cm suggests that they already achieved a more terminally differentiated state.
Validation of our microarray analyses will be needed however to

interpret the importance of these differentially expressed genes. The lack of profound differences in for instance ion channel expression suggests that modulation of protein activity may play an equally important role. Therefore, detailed analysis of fetal and adult CMPC-cm electrophysiology will be needed to further explain their distinct phenotypes.

Gene expression and developmental potential

Microarray comparison of undifferentiated fetal and adult CMPCs will be helpful to shed more light on the differences in developmental potential between fetal and adult CMPCs that were found in our study. The predictive value of transcription profiling was shown in human MSCs from first trimester fetal blood, liver, and bone marrow compared to adult bone marrow[4]. Even though the immunophenotype of the different cell populations was similar, higher basal expression of osteogenic genes in MSCs from fetal bone marrow resulted in a higher level of differentiation. Likewise, high basal expression of the mitogenic CCN1[17,18] in aCMPCs may have prevented significant adipogenic and osteogenic differentiation. The contrast in angiogenic differentiation between fCMPCs and aCMPCs may have been caused by differential basal gene expression as well.

In summary, we have compared the proliferation and differentiation potential of human fetal and adult CMPCs. Fetal CMPCs seem more versatile and may be very suitable for cardiomyogenic and angiogenic development studies. Adult CMPCs form more mature cardiomyocytes and lack adipo- and osteogenic potential. This suggests a decreased risk for arrhythmogenesis after transplantation into the heart. Adult CMPCs may therefore be useful to replace lost cardiac tissue in clinical settings.

MATERIALS AND METHODS

Cell isolation

Informed consent procedures were followed and prior approval of the ethics committee of the University Medical Center Utrecht

was obtained. CMPCs from human fetal and adult hearts were MACS isolated as described[9,11]. Whole fetal hearts were obtained after elective abortion of 13-17 week-old fetuses. Adult heart tissue was obtained from the auricles from patients undergoing cardiac surgery. The biopsies were anonymously transported to the laboratory for further processing.
MSCs were obtained from bone marrow aspirates from the sternum of patients undergoing cardiac surgery. MSCs were isolated by density gradient centrifugation (Ficoll-paque, 1.077g/ml, GE Health Care Bio-Sciences AB) and were subsequently plated on a plastic surface.

Cell culture and differentiation

Independent isolations of CMPC and MSC cultures were used for all experiments. Differentiation of CMPCs into cardiomyocytes with 5-azacytidine and TGFβ was performed as described previously[9,11]. MSCs were maintained in M199 medium (Gibco) supplemented with 10% FCS (Gibco), penicillin-streptomycin (PenStrep, 100U/ml each, Gibco), 20 mg/ml ECGF (Roche diagnostics) and 8 IU/ml heparin (Leo Pharma). Wildtype HEK 293 cells and HEK 293 cells stably overexpressing murine wild-type Kir2.1-GFP fusion protein (KWGF cells[12]) were maintained in DMEM medium (Gibco) containing 5% FCS.

Coculture experiments

For co-culture experiments with wildtype HEK293 cells or KWGF cells, CMPC-derived cardiomyocytes were first cultured in differentiation medium[9] and KWGF cells were plated on top the next day.

Angiogenesis assays

To determine their angiogenic capacity, CMPCs were plated on Matrigel (Chemicon) and stimulated with 25 ng/ml VEFG as described[9]. Following overnight culture, cells were fixed in 4% paraformaldehyde (MP Biomedicals) for immunocytochemistry. The number and characteristics of the formed tube-like structures were analyzed using Angioquant software[13].

Adipogenic differentiation

To induce adipogenic differentiation, CMPCs and MSCs were cultured in DMEM containing 10% FCS, 4.5 g/L glucose, 1 µM dexamethasone, 1 mM sodium pyruvate, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 10 µg/ml insuline, 0.2 mM indomethacin, and PenStrep. Medium was refreshed twice weekly. Cells were cultured until fat deposits became visible under light microscopy (MSCs 2-3 days, CMPCs 5 days). As negative control, cells were cultured in normal growth medium containing 0.5% FCS. After differentiation, cells were lyzed for RNA isolation or stained for Oil Red O.

For Oil-Red-O staining, differentiated cells were fixed in 4% paraformaldehyde, washed with 60% isopropanol, and dried. Oil Red O solution, prepared by dissolving 3.5 mg/ml Oil Red O (Sigma) in isopropanol and H_2O, was added to the cells. For quantification of Oil Red O content, cells were lyzed in isopropanol. Optical density was measured at 500 nm.

Osteogenic differentiation

CMPCs and MSCs were cultured in DMEM containing 10% FCS, 2 mM L-Glutamine, 4.5 g/L glucose, 0.1 µM dexamethasone, 50 µg/ml ascorbic acid, and PenStrep. After the first week, 5 mM β-Glycerophosphate was added to the medium. Negative controls were cultured in normal growth medium containing 0.5% FCS. CMPCs and MSCs were lyzed for RNA isolation or stained for alkaline phosphatase or Alizarin Red S after and 21 days.

For alkaline phosphatase staining, cells were washed once with PBS, fixed for 5 minutes in 37% formaline (Klinipath) at room temperature, and washed trice with PBS. Subsequently, fixed cells were stained with staining solution (0.2 mg/ml Naphthol AS-MX Phosphate (Sigma), 0.6 mg/ml Fast Blue (Sigma), 0.1 M Tris-HCl (pH 8.8), and 0.01% (w/v) $MgSO_4$) and washed twice with PBS before taking pictures.

To stain for Alizarin Red S, cells were washed once with PBS, fixed for one hour in ice cold 70% ethanol, and washed twice with H_2O. Fixed cells were stained with 0.1% (w/v) Alizarin Red S (Fluka) for 30 minutes in the dark at room temperature and washed four times before taking pictures.

RNA isolation and quantitative RT-PCR

Cells were lyzed in TriPure (Roche). Total RNA was isolated and DNAse treated (Amersham Biosciences). Five hundred ng total RNA was used for cDNA synthesis with iScript cDNA synthesis kit (BioRad). For qRT-PCR, 10 µl cDNA (1:20 diluted) was mixed with 10 µl SYBR-Green mix (BioRad) and forward plus reverse primers (final concentration 0.5 µM each) and run on a MyiQ iCycler (BioRad). PCR conditions were: 2 min at 94 °C followed by 40 cycles of: 30 sec at 94 °C, 30 sec at annealing temperature (see table 1), and 30 sec at 72 °C. Amplicon quantities were determined by comparison with known quantities of cloned PCR products and are expressed in pg/pg or as fold induction ($2^{-\Delta\Delta Ct}$). Specificity of amplification for the detection with SYBR-Green was visually checked on PAGE gels or melting curve analysis after the PCR run. Primers were designed with Beacon Designer 4.0 (Premier Biosoft International). Primer sequences and annealing temperatures are given in table 1. Expression levels were normalized to β-actin expression.

Immunocytochemistry

Immunocytochemistry was performed as described previously[9]. Primary antibodies used were: PECAM (Santa Cruz), αSMA (Dako), α-actinin (Sigma), and Connexin 43 (Zymed). Secondary antibodies were Cy3 donkey-anti-goat (Dako), 488 nm goat-anti-mouse (Invitrogen), or 555 nm goat-anti-rabbit (Invitrogen).

Electrophysiology

To measure resting membrane potentials in CMPCs, cells were cultured in media appropriate for their state (undifferentiated or differentiated)[9,11]. Determination of membrane potential was performed as described earlier[9]. Briefly, patch clamp microelectrodes were used to measure the membrane potential of differentiated monolayers at 37°C, first in control medium and then in medium containing 1 mM $BaCl_2$. In addition, sharp microelectrodes were used to measure action potentials in response to bipolar field stimulation at 1 Hz.

Microarray

Fetal and adult CMPC-derived cardiomyocytes were lyzed in Tripure (Roche). Total RNA was isolated according to the manufacturer's instruction and subsequently purified with Nucleospin columns (Macherey-Nagel). Five hundred ng of total RNA was used for cDNA synthesis and subsequent cRNA synthesis with the Illumina total prep RNA amplification kit (Ambion).

The Illumina bead arrays (human ref.6, n=2 for fCMPC, n=1 for aCMPC) were hybridized, labeled, washed, and scanned following manufacturer's instruction. Gene expression values of the bead array were quantile normalized in BeadStudio (version 3.2 Illumina) and used for further analysis. Functional analysis of the selected gene sets was done using Panther (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org).

Statistics

All data are presented as average ± SEM. Number of replicates is indicated in the figure legends. Data was analyzed with a Student's T-test or ANOVA for group comparisons. Significance was assumed when $P<0.05$.

Acknowledgements

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REFERENCES

1. Blau, H.M., Brazelton, T.R. & Weimann, J.M. The evolving concept of a stem cell: entity or function? *Cell* **105**, 829-41 (2001).
2. Rando, T.A. Stem cells, ageing and the quest for immortality. *Nature* **441**, 1080-6 (2006).
3. Campagnoli, C. *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* **98**, 2396-402 (2001).
4. Guillot, P.V. *et al.* Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation* DOI: 10.1111/j.1432-0436.2008.00279.x (2008).
5. O'Donoghue, K. & Fisk, N.M. Fetal stem cells. *Best Pract Res Clin Obstet Gynaecol* **18**, 853-75 (2004).
6. Beltrami, A.P. *et al.* Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* **344**, 1750-7 (2001).
7. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).
8. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* **50**, 19-30 (2008).
9. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
10. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
11. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
12. de Boer, T.P. *et al.* Inhibition of cardiomyocyte automaticity by electrotonic application of inward rectifier current from Kir2.1 expressing cells. *Med Biol Eng Comput* **44**, 537-42 (2006).
13. Niemisto, A., Dunmire, V., Yli-Harja, O., Zhang, W. & Shmulevich, I. Robust quantification of in vitro angiogenesis through image analysis. *IEEE Trans Med Imaging* **24**, 549-53 (2005).
14. Matsuura, K. *et al.* Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem* **279**, 11384-91 (2004).

15. Franco, D., Lamers, W.H. & Moorman, A.F. Patterns of expression in the developing myocardium: towards a morphologically integrated transcriptional model. *Cardiovasc Res* **38**, 25-53 (1998).
16. Masino, A.M. *et al.* Transcriptional regulation of cardiac progenitor cell populations. *Circ Res* **95**, 389-97 (2004).
17. Perbal, B., Brigstock, D.R. & Lau, L.F. Report on the second international workshop on the CCN family of genes. *Mol Pathol* **56**, 80-5 (2003).
18. Schutze, N., Noth, U., Schneidereit, J., Hendrich, C. & Jakob, F. Differential expression of CCN-family members in primary human bone marrow-derived mesenchymal stem cells during osteogenic, chondrogenic and adipogenic differentiation. *Cell Commun Signal* **3**, 5 (2005).

Table 1: Primer sequences and annealing temperatures

Name	*Sequence*	*Annealing temperature*
αSMA For	ACT GGG ACG ACA TGG AAA AG	60 ^{0}C
αSMA Rev	TAG ATG GGG ACA TTG TGG GT	
SM-MHC For	TCA ACA TGC AGG CGC TCA	60 ^{0}C
SM-MHC Rev	CGT CTC ATA CTC GTG AAG CTG TCT	
Tie-2 For	CAC CAT CCA AAC ATC ATC AAT CTC	60 ^{0}C
Tie-2 Rev	TGC GAA GGA AGT CCA GAA G	
VE-Cadherin For	GCC AAC ATC ACA GTC AAG	60 ^{0}C
VE-Cadherin Rev	GCC ATA TCC TCG CAG AA G	
Leptin For	GTG CGG ATT CTT GTG GCT TT	60 ^{0}C
Leptin rev	GGA ATG AAG TCC AAA CCG GTG	
Adipsin For	CAA GCA ACA AAG TCC CGA GC	60 ^{0}C
Adipsin Rev	CCT GCG TTC AAG TCA TCC TC	
PPARγ2 For	GCT GTT ATG GGT GAA ACT CTG	60 ^{0}C
PPARγ2 Rev	ATA AGG TGG AGA TGC AGG CTC	
CCN1 For	ACC AGT CAG GTT TAC TTA CG	60 ^{0}C
CCN1 Rev	TGC CTC TCA CAG ACA CTC AT	
Runx2 For	GGA GTG GAC GAG GCA AGA GTT T	60 ^{0}C
Runx2 Rev	AGC TTC TGT CTG TGC CTT CTG G	
CTGF (CCN2) For	AAC ACC ATA GGT AGA ATG TAA AGC	60 ^{0}C
CTGF (CCN2) Rev	CTG ATC AGC TAT ATA GAG TCA CTC	
Osteocalcin For	CGC AGC CAC CGA GAC ACC AT	60 ^{0}C
Osteocalcin Rev	GGG CAA GGG CAA GGG GAA GA	
β-Actin For	GAT CGG CGG CTC CAT CCT G	60 ^{0}C
β-Actin Rev	GAC TCG TCA TAC TCC TGC TTG C	

SUPPLEMENTAL DATA

Supplemental movie 1
Cluster of contracting, fetal CMPC-derived cardiomyocytes shown in figure 3B the day before coculture with KWGF cells.

Supplemental movie 2
Same cluster of fetal CMPC-derived cardiomyocytes as in movie 1, the day after coculture with KWGF cells leading to inhibition of spontaneous contractions.

Supplemental movie 3
Several clusters of (replated) fetal CMPC-derived cardiomyocytes shown in figure 3E the day after coculture with KWGF cells. Note the lack of contractions in the cluster adjacent to a KWGF cell and continued contractions in clusters not adjacent to KWGF cells. After 41 seconds, filter is switched from bright field to FITC channel. GFP fluorescence from the KWGF cell is visible after 50 seconds; channel is switched to TRITC after 57 seconds and switched back to FITC after 62 seconds, and turned back to bright field after 95 seconds.

Supplemental movie 4
Cluster of contracting, fetal CMPC-derived cardiomyocytes the day after coculture with normal HEK 293 cells. The same relative number of cocultured cells was used as in movie 2 (5%).

Supplemental table 1
List of non-differentially expressed genes in fetal and adult CMPC-cm.

Supplemental table 2
List of genes with three-fold increased expression in fetal versus adult CMPC-cm ($p<0.01$).

Supplemental table 3
List of genes with three-fold increased expression in adult versus fetal CMPC-cm ($p<0.01$).

Supplemental table 4

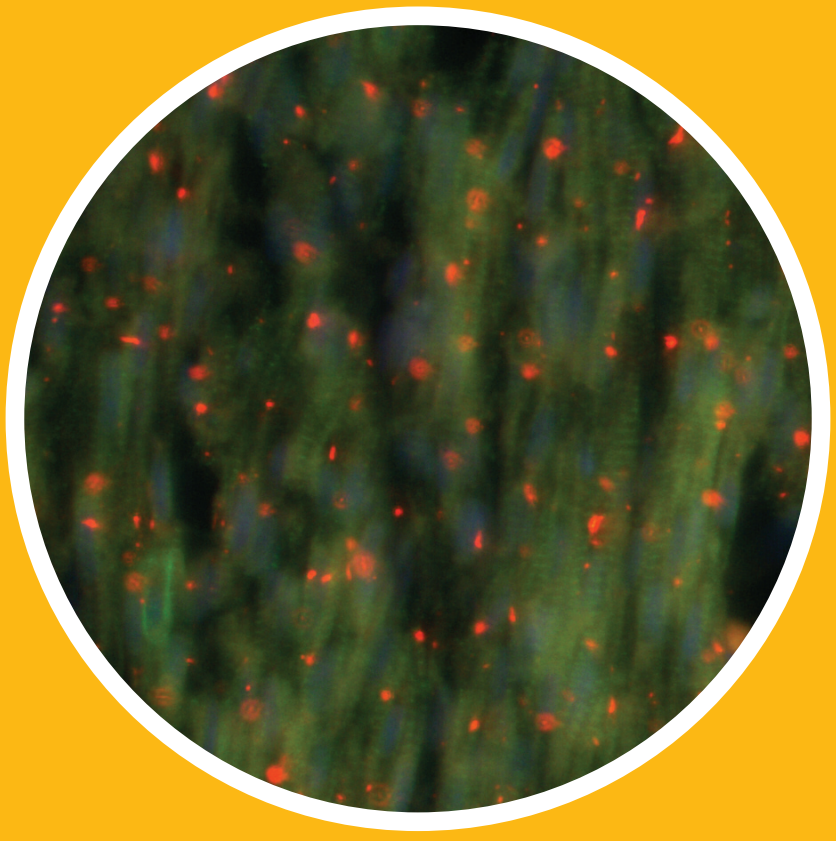
Gene expression in fetal CMPC-cm clustered based on biological process or molecular function.

Supplemental table 5

Gene expression in adult CMPC-cm clustered based on biological process or molecular function.

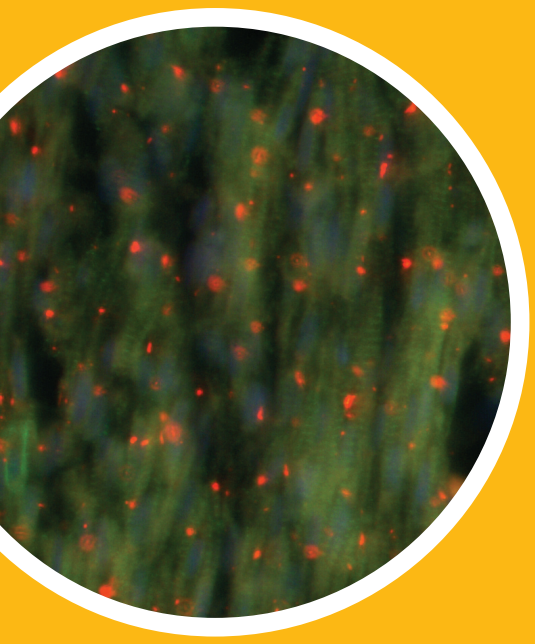
CHAPTER

6



CHAPTER 6

MICRO-RNA-1 AND 499 REGULATE DIFFERENTIATION AND PROLIFERATION IN HUMAN-DERIVED CARDIOMYOCYTE PROGENITOR CELLS



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ABSTRACT

To improve regeneration of the injured myocardium, it is necessary to enhance the intrinsic capacity of the heart to regenerate itself and/or replace the damaged tissue by cell transplantation. One promising cell population for cell replacement therapy is heart-derived progenitor cells. Cardiomyocyte progenitor cells (CMPCs) are obtained from human fetal hearts and from adult human biopsies, easily expanded in culture, and efficiently differentiated into beating cardiomyocytes. Recently, several studies have demonstrated that microRNAs (miRNAs) are important for transcriptional regulation during heart development, and maintenance of stem cell populations by translational repression. We hypothesize that, since miRNAs regulate stem cell maintenance and differentiation, miRNAs are also involved in proliferation and differentiation of the human cardiomyocyte progenitor cells *in vitro*.

Human fetal CMPCs were isolated, cultured, and efficiently differentiated into beating cardiomyocytes. MiRNA expression was profiled in proliferating and differentiated CMPCs. We observed that the muscle-specific miRNA-1 and miRNA-499 were highly upregulated upon differentiation. Transient transfection of miRNA-1 and 499 in undifferentiated CMPCs reduced their proliferation rate by 25 and 15%, respectively. Moreover, introducing miRNA-1 and 499 in CMPCs and embryonic stem cells enhanced differentiation into cardiomyocytes. This is potentially mediated via repression of HDAC4 and Sox6, respectively, as indicated by reduced protein levels. Functional knockdown of Sox6 strongly induced myogenic differentiation.

Our results demonstrate a strong regulatory role of miRNAs in human CMPC proliferation and differentiation into cardiomyocytes. By modulating miRNA-1 and 499 expression levels, CMPC function can be modulated and differentiation directed, thereby enhancing cardiomyogenic differentiation.

INTRODUCTION

The old paradigm that the heart is a terminally differentiated organ and that it lacks the capacity for regeneration of damaged tissue is being challenged since the identification of resident cardiovascular progenitor cells[1,2]. These progenitor cells are able to differentiate into cardiomyocytes as well as other cardiac cell types, such as endothelial cells or fibroblasts[3-6]. The progenitor cells can be isolated, expanded *in vitro*, and transplanted into the damaged rodent myocardium, thereby improving cardiac performance. Recently, we have isolated human cardiomyocyte progenitor cells (CMPCs) from human fetal hearts and adult human biopsies that can be expanded in culture and efficiently differentiated into beating cardiomyocytes, without the need for co-culture with rat neonatal cardiomyocytes[7,8]. However, little is known about what drives proliferation and differentiation *in vitro* and their exact role and regulation *in vivo* are still unexplored.

Several studies have demonstrated that microRNAs (miRNAs) are important for the post-transcriptional regulation of target genes and serve important regulatory functions in a range of biological processes, including maintenance of stemcellness and modulation of differentiation[9]. MiRNAs are short (19-23 nucleotides) non-coding small regulatory RNAs that are loaded into the RNA-induced silencing complex (RISC), recognize the 3'- untranslated region (UTR) of target mRNAs and thereby regulate their expression by translational repression or mRNA degradation[10]. Recently, several papers focused on the expression of miRNAs in cardiac pathology, reporting clusters of differentially expressed miRNAs among different human cardiomyopathies[11,12]. Furthermore, the contribution of specific miRNAs in muscle differentiation and cardiac hypertrophy was described[13,14].

Here, we analyzed the miRNA expression in CMPCs during proliferation and after differentiation towards cardiomyocytes and explored the potential function of two miRNAs, miR-1 and the previously unexplored miR-499 in progenitor cells. We show that, in CMPCs, miR-1 and 499 repress proliferation and induce cardiomyogenic differentiation *in vitro*.

RESULTS

Expression of micro-RNAs in proliferating and differentiated CMPC

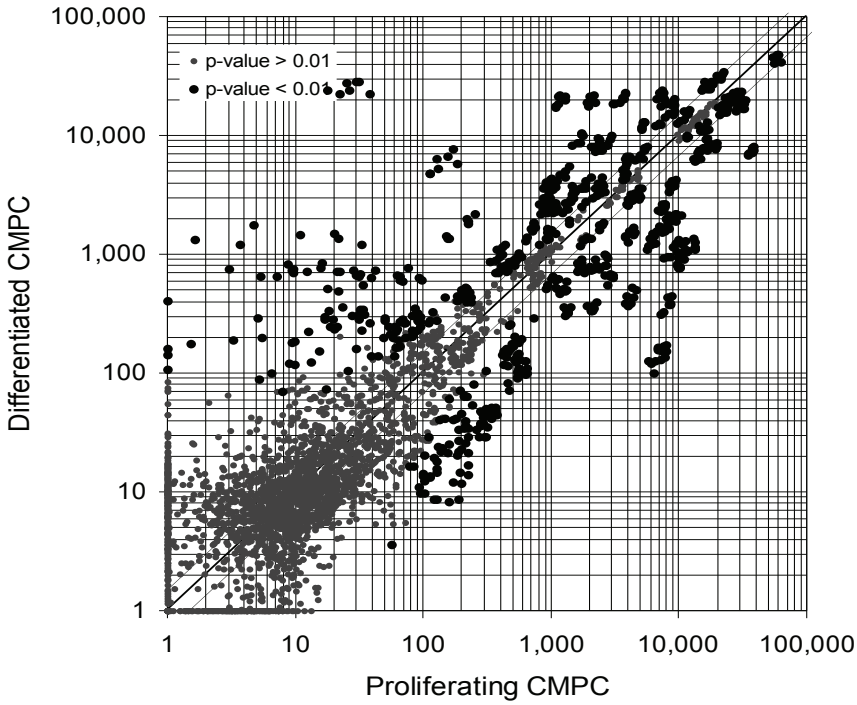
Micro-RNA expression was determined in both proliferating and differentiated CMPCs by miR microarray analysis. From all human targeted miRs (total 453), 188 miRs (42%) were detectable in proliferating CMPCs and 195 (43%) in differentiated CMPCs, of which 19 miRs show a four-fold or higher increase (figure 1A, B) upon differentiation. Among the highly upregulated miRs (figure 1B), several cardiac and skeletal muscle specific miRs were present, such as miR-1, miR-133a, and miR-133b. MiR-206 and miR-208, also described in cardiomyocytes, were not detectable by the LC Sciences platform in our differentiated CMPCs. In addition to these known muscle specific miRs, other miRs were highly regulated as well, including miR-499, a so far not studied miR.

To confirm increased miR expression, we performed quantitative Taqman based RT-PCR (figure 2). After normalization to RNU19, miR-1, miR133a, miR-499, and miR-214 expression was significantly increased in differentiated CMPCs (figure 2). We selected the strongest upregulated miRs, miR-1 and miR-499, for functional follow-up studies in CMPCs.

Localization and tissue distribution of miR-499

MicroRNA-1 is a muscle-specific miRNA, that is known to control myogenic differentiation in embryonic mouse heart[15]. However, to our current knowledge, tissue distribution and function of miR-499 is not known. This highly conserved miRNA (supplemental figure 1A) is located in intronic region 20 of human MYH7B (myosin heavy chain 7B, cardiac muscle, beta) on chromosome 20 (supplemental figure 1B). To confirm the presence of miR-499 in cardiomyocytes *in vivo*, we performed *in situ* hybridization for miR-499 in fetal and adult human hearts. MiR-499 is present in the heart and is located in cardiomyocytes as demonstrated by co-staining with cardiac α-actinin (figure 3A, C, and F), but not present in endothelial cells (figure 3E). To determine whether this miRNA is expressed in multiple tissues, we explored the expression of the mature miR-499 in mouse heart, brain, spleen, liver, lung,

A



B

	miRNA ID	Proliferating CMPC	Differentiated CMPC	log2	fold induction
1	hsa-miR-1	26.1	24058.8	9.91	962.3
2	hsa-miR-499	10.9	1372.2	7.07	133.9
3	hsa-miR-133a	9.7	715.5	6.28	77.6
4	hsa-miR-133b	15.6	727.8	5.72	52.9
5	hsa-miR-335	128.9	5829.0	5.42	42.8
6	hsa-miR-450	32.2	643.7	4.40	21.2
7	hsa-miR-542-3p	12.5	245.1	4.21	18.5
8	hsa-miR-143	1143.5	19731.0	4.04	16.5
9	hsa-miR-217	8.8	122.1	3.79	13.8
10	hsa-miR-204	27.9	330.7	3.68	12.8
11	hsa-miR-203	20.0	181.2	3.54	11.6
12	hsa-miR-210	31.3	295.4	3.35	10.2
13	hsa-miR-145	2015.3	19156.3	3.34	10.2
14	hsa-miR-422b	68.4	630.5	3.21	9.3
15	hsa-miR-218	221.0	1798.7	3.10	8.6
16	hsa-miR-214	3645.1	20597.9	2.55	5.9
17	hsa-miR-152	1709.7	10020.7	2.51	5.7
18	hsa-miR-30d	918.3	4009.8	2.07	4.2
19	hsa-miR-374	59.6	249.2	2.06	4.2

Figure 1: miRNA profiling of proliferating CMPCs and differentiated to cardiomyocytes.

A | Each miRNA is located 7 times on the array and these are all plotted in the figure, in black $p<0.01$ and in grey $p>0.01$ (Axes are arbitrary expression units). B | MiRNAs are presented that are highly upregulated in differentiated CMPCs (>log2 difference) (Numbers in proliferating and differentiated CMPCs are arbitrary expression units).

quadriceps muscle, kidney and gut. MiR-499 is abundantly expressed in cardiac tissue and almost absent in other tissues, including the skeletal muscles (figure 3G). We observed no differences in the expression levels of miR-499 when comparing human fetal and adult, and mouse embryonic (E12), neonatal and adult heart (data not shown). The mRNA expression of MYH7B-beta is also restricted to heart muscle (figure 3H), indicating that MYH7B-beta and miR-499 have a heart specific expression pattern.

Functional analysis of pre-miR-1and 499 in vitro

Since expression of miR-1 and miR-499 increased upon differentiation, we used a gain-of-function approach to study their roles in proliferating CMPCs by transfection of miR precursor molecules (pre-miRs). A FAM-labeled negative control pre-miR was used to optimize transfection procedures. A dose dependent signal was observed upon transfection of FAM-pre-miRs (figure 4A-D) and these were visible up to 12 days after transfection in proliferating CMPCs.

To verify the transfection efficiency and functional cellular processing of the pre-miR into the mature miR, we analyzed the mature miR-1 expression by Taqman based RT-PCR. We observed an increase in expression of mature miR-1 with increased transfection concentrations, as compared to endogenous miR-1 expression levels in control CMPCs (figure 4E). The amount of mature, transfected miR-1 was 31x (3nM), 750x (30nM), and 5221x (100nM) higher, as compared to endogenous levels.

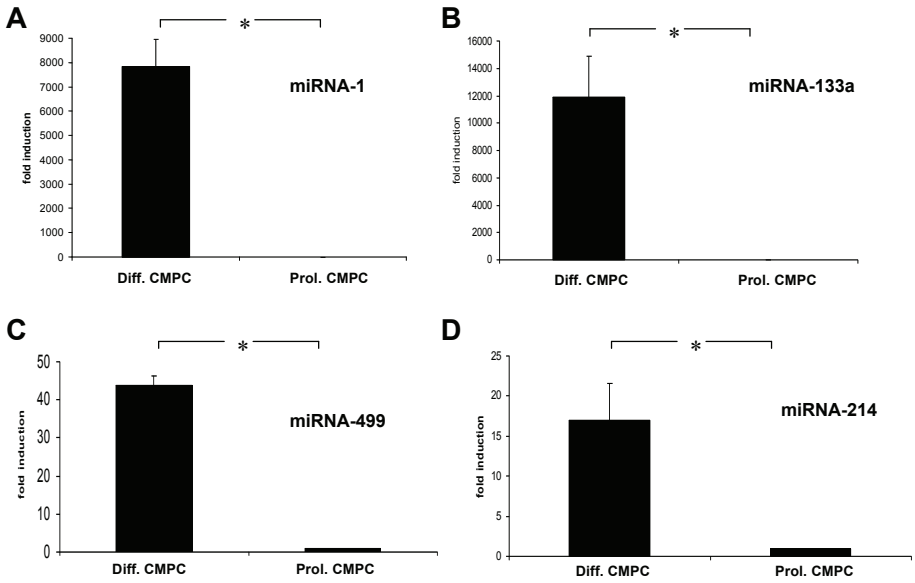


Figure 2: Quantitative RT-PCR analysis

MiRNA-1 (A), miRNA-133a (B), miRNA-499 (C), and miRNA-214 (D) in proliferating CMPCs (prol. CMPC) and differentiated to cardiomyocytes (diff. CMPC). Data is presented as fold induction +/- sem, * = $p<0.05$.

Four days after transfection, increased concentration of pre-miR-1 resulted in a significant reduction in cellular proliferation up to 25% (100nM) when compared to control cells (figure 5A), as determined by MTT assay. Transfection of miR-499 in CMPCs resulted in a significant reduction in cell proliferation as well (15% with 100nM, figure 5B). When using similar concentrations of the scrambled control miR, no significant effect on proliferation was observed (figure 5C).

CMPC differentiation into cardiomyocytes

A prerequisite for differentiation is an inhibition of cell growth. Since addition of miR-1 and 499 reduced CMPC proliferation, and increased levels of these miRs are present in differentiated CMPCs, we determined if we could enhance cardiomyocyte

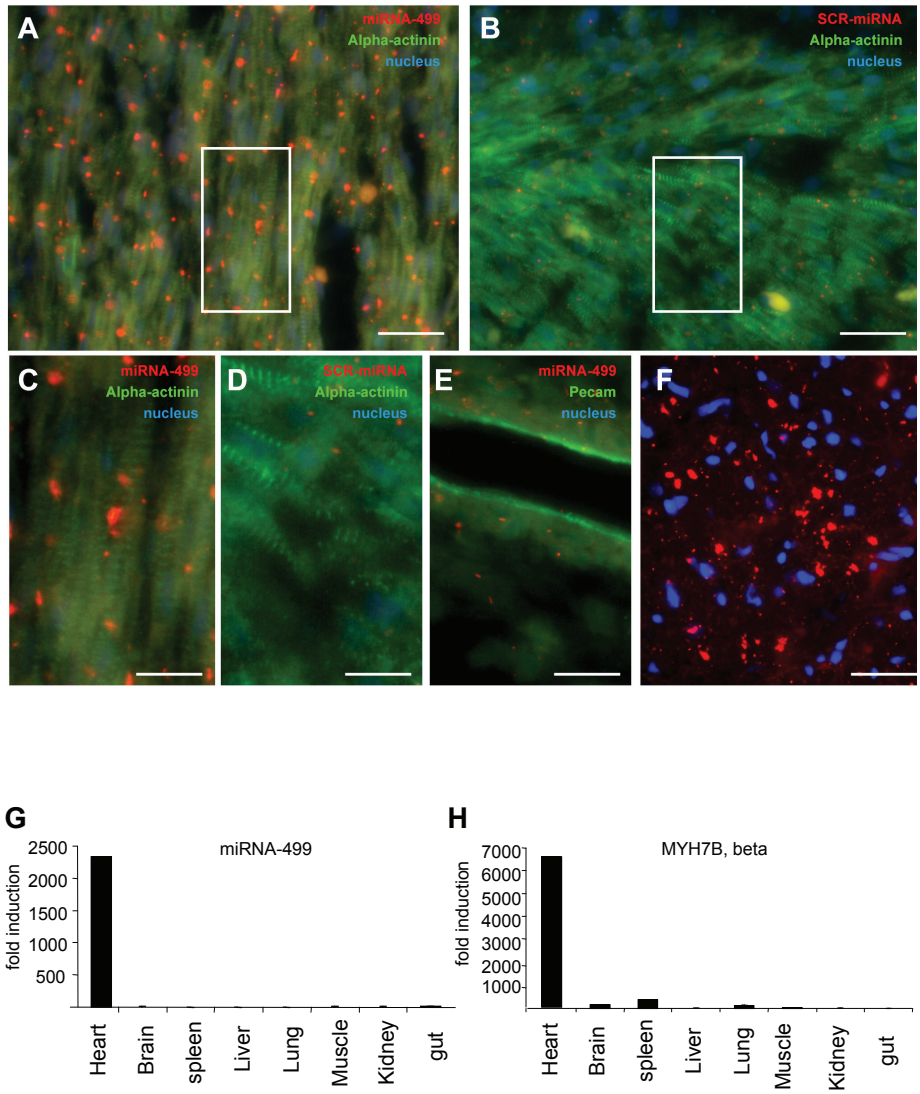


Figure 3: Localization of miRNA-499

In situ hybridization for miRNA-499 (A, C) (red dots) and a scrambled control probe (B, D) in human fetal heart and double stained for α-actinin (A-D, green) or PECAM (E, green). F | MiRNA-499 localization in adult human heart. Nuclei are stained blue. Bar=50 μm in A,B, E, and F and Bar=15 μm in C and D. Quantitative RT-PCR for miR-499 (G) and MYH7B-beta (H) in different mouse samples. Expression is presented as fold induction +/- sem and compared to lowest expression (N=5).

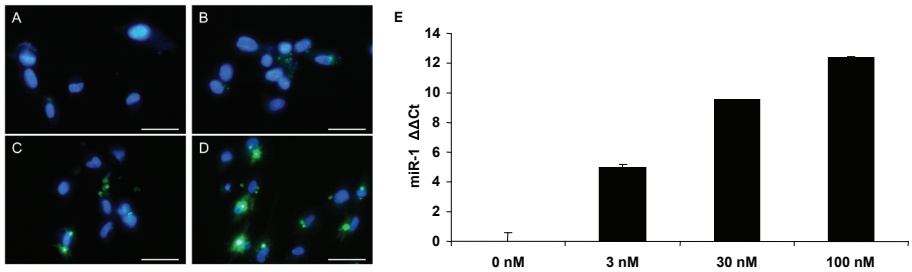


Figure 4: MiRNA transfection optimalization

Immunofluorescent detection of 0 nM (A), 3 nM (B), 30 nM (C), and 100 nM (D) FAM labeled control miR, transfected in CMPCs. Nuclei are stained blue (Bar=25 μm). E | Transfection with increased concentration of miR-1 pre-molecules resulted in increased detectable expression of mature miR-1 by quantitative RT-PCR. Data is expressed as ΔΔCt +/- sem.

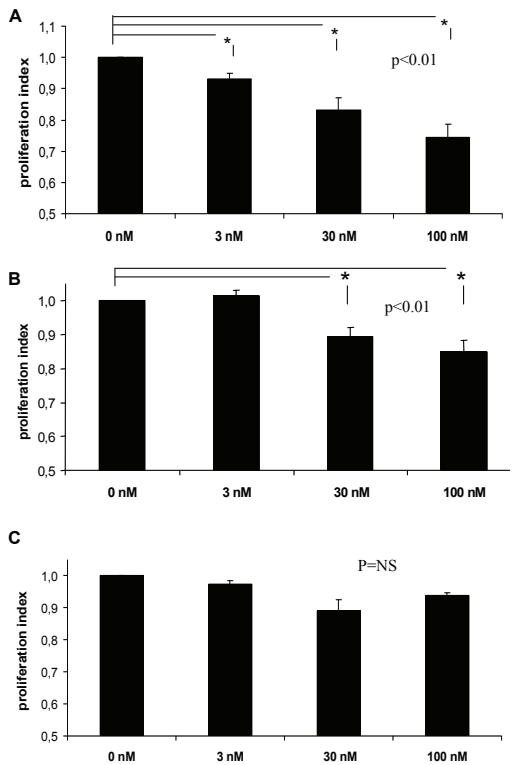


Figure 5: Effects of miR overexpression on CMPC proliferation

Proliferation of CMPCs is determined after transfection of increased concentrations pre-miR-1 (A), pre-miR-499 (B) and scrambled miR (C). Proliferation is significantly decreased after transfection of pre-miR-1 and 499, but not after the control miR. (*=p<0.01).

differentiation by addition of miR-1 and -499 to our differentiation protocol. Normally, spontaneous beating clusters of differentiated cells are observed after 21 days. However, when adding both miRs we observed spontaneous beating areas already after 6 days (supplementary movie 1). This suggests that the differentiation of our progenitor cells is greatly enhanced by transfection of miR-1 or miR-499. To quantify this, we studied mRNA levels for different cardiomyocyte markers by quantitative RT-PCR after 4 weeks of differentiation. We observed that the expression of cardiac troponin T, α-cardiac actinin, and Mlc-2v was increased upon addition of miR-1 and 499 (figure 6).

Interestingly, miR-1 and -499 also enhanced cardiac differentiation of mESCs cells. After treatment with pre-miRs, percentage of beating embryoid bodies (EBs) increased from 19% in non–treated controls to 23% in SCR, 56% in miR-499, and 95% in miR-1-treated cells (figure 7A). Enhanced differentiation was confirmed by increased immunofluorescent α-actinin staining (figure7B-E), and increased levels of GATA4 mRNA expression at day 4 (figure 7F) and MLC-2V at day 11 (figure 7G).

MiR targets and potential mechanism

MicroRNA-1, shown to enhance CMPC differentiation into cardiomyocytes, has previously been reported to promote myogenesis by targeting histone deacetylase 4 (HDAC4), a negative regulator of muscle gene expression. Upon transfection of miR-1 in CMPCs, we confirmed that HDAC4 protein levels were indeed reduced by miR-1 transfection (figure 8A).

Since miR-499 was not studied before, we used online logarithms to predict potential targets that could be involved in myogenic

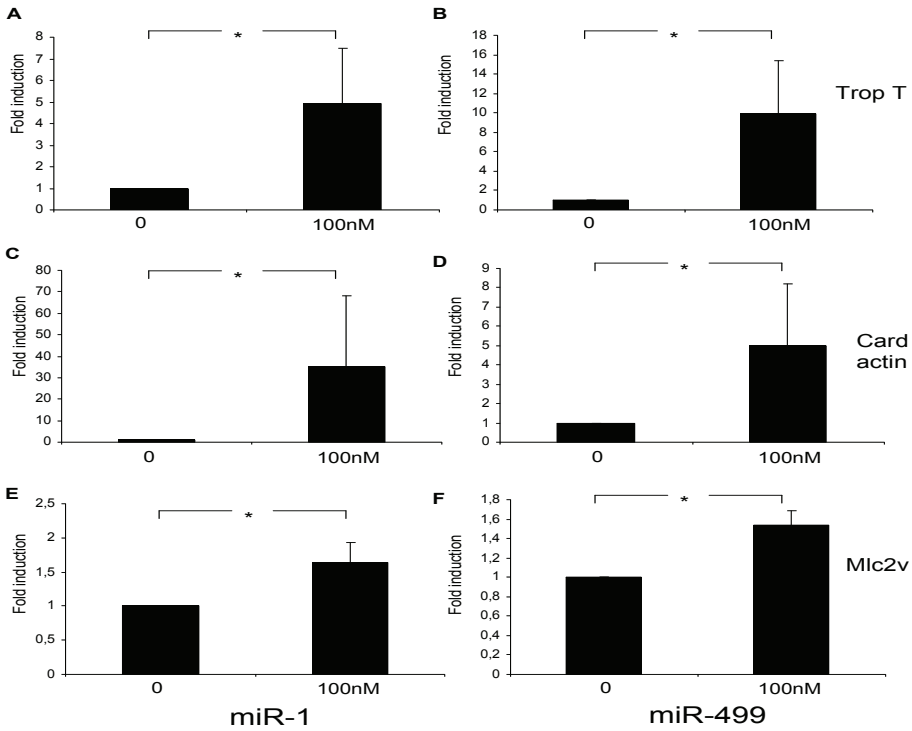


Figure 6: Effects of miR overexpression on CMPC differentiation

CMPCs were differentiated into cardiomyocytes and additionally transfected with 100 nM miR-1 (A, C, E), or miR-499 (B, D, F). mRNA expression was determined by RT-PCR for troponin T (A, B), cardiac actinin (C, D), and Mlc-2v (E, F). The expression of cardiac markers was significantly increased upon transfection of miR-1 and 499. (Data is presented as mean fold increase +/- sem, N=4).

differentiation (TargetScan and Sanger). Both algorithms predicted three conserved seed sites in SOX6 (SRY (sex determining region Y)-box 6), involved in transcription regulation and muscle differentiation. We cloned the 3'UTR of mouse Sox6 in the multiple cloning site of pMIR-REPORT™ Luciferase (Ambion) behind CMV-driven luciferase, to verify the potential inhibitory effect of miR-499 on Sox6 translation. We co-transfected this plasmid into HEK293 cells, with premiR-499 and inhibitors of miR-499, and

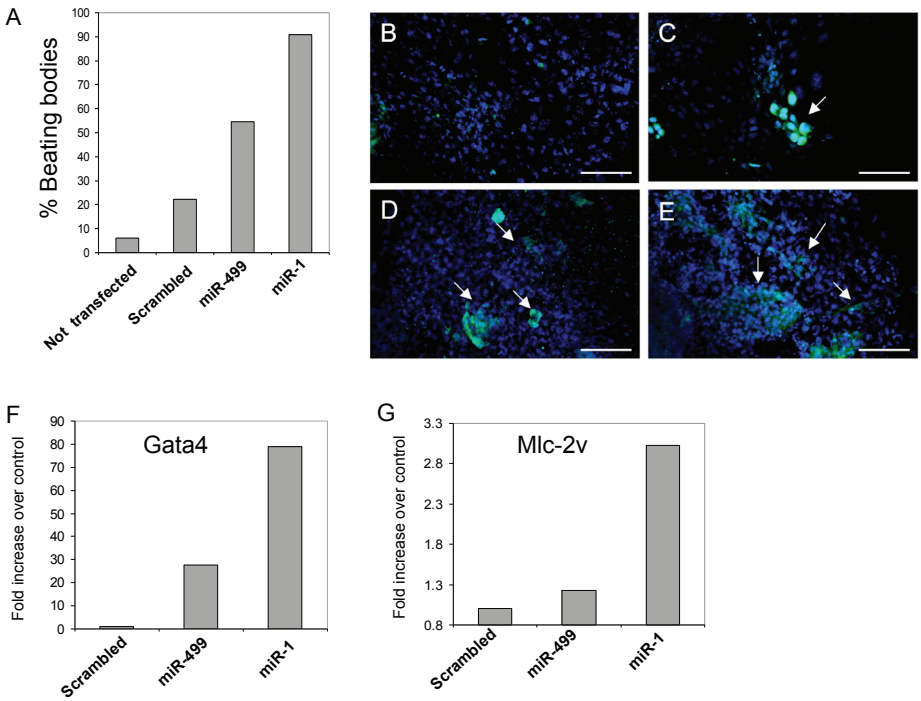


Figure 7: Effects of miR overexpression on ES cell differentiation

Mouse embryonic stem cells are transfected with or without 100nM miRNAs and embryoid bodies are generated via hanging drops. A | After 7 days, the number of beating aggregates was counted. Introduction of miR-499 and 1 increased the number of beating bodies significantly. B-D | Embryoid bodies are plated after 7 days, grown for additional 4 days and stained for α-actinin (green, arrows). B | Untransfected. C | Scrambled. D | MiR-499. E | MiR-1. With miR-499 and 1 α-actinin staining was increased (bar=50 μm, nuclei are stained with Hoechst). RNA was isolated 4 and 11 days after embryoid body generation. Gata 4 expression (F) was increased after 4 days, and Mlc-2v expression (G) after 11 days of introducing miR-499 and 1.

observed a reduction in luciferase activity by miR-499 that could be restored using a specific miR-499 inhibitor (figure 8B). Interestingly, the 3'UTR of Sox6 also contains a putative site targeted by miR-1 (supplementary figure 2) and co-transfection

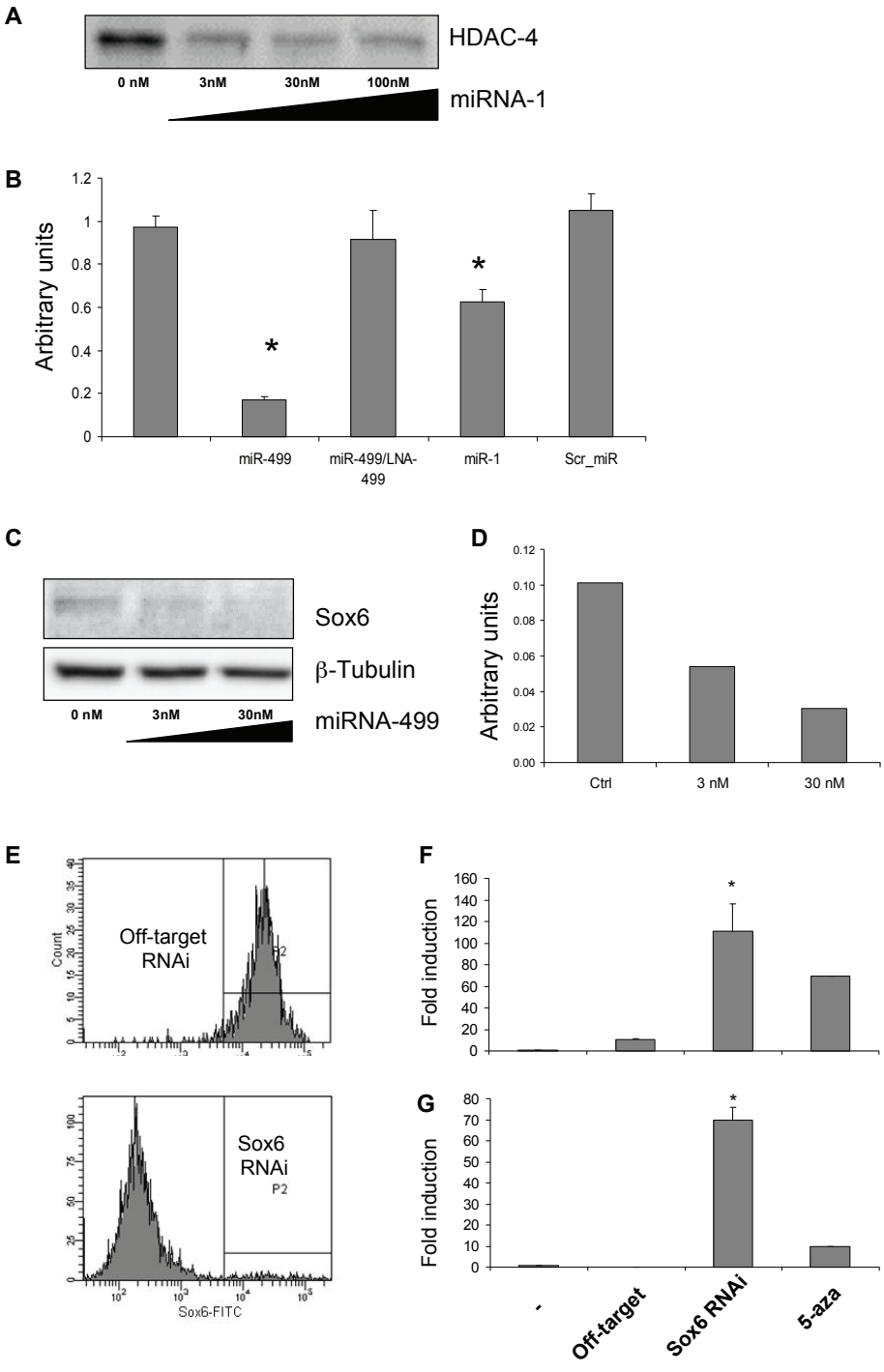

A
HDAC-4
0 nM
3nM
30nM
100nM
miRNA-1
B
Arbitrary units
1.2
1
0.8
0.6
0.4
0.2
0
*
*
miR-499
miR-499/LNA-499
miR-1
Scr_miR
C
Sox6
β-Tubulin
0 nM
3nM
30nM
miRNA-499
D
Arbitrary units
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ctrl
3 nM
30 nM
E
Off-target RNAi
Count
P2
Sox6 RNAi
P2
Sox6-FITC
F
Fold induction
160
140
120
100
80
60
40
20
0
*
G
Fold induction
80
70
60
50
40
30
20
10
0
*
-
Off-target
Sox6 RNAi
5-aza

Figure 8: Downstream targets of miR-1 and 499

A | Proliferating CMPCs were transfected with increased concentrations of miRNA-1, resulting in decreased concentrations of HDAC-4 protein expression. B | A CMV-Luciferase plasmid was transfected in HEK293 cells, containing the 3'UTR of mouse Sox6. Cells were co-transfected with miR-499 (30 nM), a specific inhibitor of miR-499 (LNA-499, 50 nM), miR-1, or a scrambled miR (30 nM). By transfection of pre-miR-499, luciferase expression was reduced and could be restored upon specific inhibition of miR-499 (LNA-499). C | Sox6 protein expression was reduced upon transfection with increased concentrations of miRNA-499 (0, 3, 30 nM). D | Densometric quantification of Sox6 protein expression, normalized for β-tubulin. By repressing Sox6 expression via RNAi in CMPCs (E), as indicated via Sox6 expression by FACS analysis, muscle differentiation was induced as indicated by increased expression of Mlc-2v (F) and cardiac actinin (G) after 7 days of differentiation. The induction was even higher than with standard 5-aza treatment.

of miR-1 and CMV-Luc-3'UTR Sox6 led to a mild reduction in luciferase expression. When using a scrambled miR, this effect was not observed (Figure 8B).
Sox6 was expressed in our proliferating CMPCs. To confirm that Sox6 is a target of miR-499, we transfected miR-499 in the CMPCs and observed reduced Sox6 protein levels (Figure 8C, D). To confirm that Sox6 is important for myogenic differentiation of CMPCs, Sox6 expression was knocked down (KD) in CMPCs via lentiviral transduction of shRNA (Figure 8E). CMPCs, KD for Sox6, stopped proliferating and displayed enhanced muscle markers (Figure 8F, G) after 7 days of differentiation. HDAC4-KD did not stop proliferating (data not shown).

DISCUSSION

The transcriptional regulation of cardiomyocyte differentiation is highly conserved and requires sequential activation and/or repression of different genetic programs[16,17]. Specific disruption of Dicer, essential for processing of mature miRs, in mouse

cardiovascular progenitor cells resulted in embryonic lethality due to cardiac failure, pointing to an essential role of miRs in cardiac development and cardiomyocyte differentiation[15]. We observed that CMPCs express many different miRs; some of them highly regulated upon differentiation. Here, we focused on miR-1 and 499[18] to study whether they could be used *in vitro* to induce cardiomyocyte differentiation.

MiR-1 is a highly conserved miR with a cardiac and skeletal-muscle specific expression pattern and has binding sites in its promoter region for several essential cardiac transcription factors, like Mef2, SRF, Nkx2.5, and GATA4[14]. Several miR-1 target genes play a role in cardiac development or function and are experimentally confirmed, such as Hand[14], TMSB4X[14], HDAC4[19], GJA1[20], and KCNJ2[20]. In miR-1 transgenic mice, the total number of cycling myocardial cells was decreased[14], whereas in miR-1-2 null mice an increased proliferation of cardiomyocytes was observed, resulting in severe heart defects[15]. This suggests that miR-1 fine-tunes the balance between cardiomyocyte proliferation and differentiation, thereby repressing mouse cardiovascular progenitor cell proliferation. Here, we demonstrated that introducing miR-1 into human heart-derived CMPCs resulted in a reduction in proliferation rate.

In Drosophila, loss of dmiR-1 is lethal, because it is required for determination or differentiation of cardiac or somatic muscle progenitor cells[21]. Similar observations were reported in embryonic stem cell differentiation, demonstrating that miR-1 and miR-133 promote mesoderm formation but have opposing functions during further differentiation into cardiac muscle progenitors[22]. Furthermore, introducing miR-1 in C2C12 myoblasts promoted myogenesis by targeting histone deacetylase 4 (HDAC4)[19], a transcriptional repressor of muscle gene expression through binding of Mef2 transcription factors. Accordingly, we were able to induce cardiac differentiation via miR-1 in our human CMPCs and mESCs, which may also be mediated through repression of HDAC4 protein levels.

In addition to its role in cardiomyocyte differentiation, miR-1 function in adult cardiomyocytes was recently explored. MiR-1 was increased in structural diseased human hearts and in the

border zone of infarcted hearts, thereby inhibiting the expression of connexin 43 (Cx43) and Kir2.1. Excessively prolonged repolarization, due to inhibition of Kir2.1, and reduced electrical coupling of cardiomyocytes, by inhibiting Cx43, could lead to premature contractions and arrhythmias. Blocking miR-1 with antisense oligonucleotides prevented this prolongation and reduced the susceptibility to arrhythmias[20]. By transfection of miR-1 into our differentiated spontaneous beating CMPCs, we observed a reduction in beating rate and more individually beating cells instead of clusters of beating cells (data not shown), suggesting uncoupling of cells through a similar mechanism.

Taking these data together, miR-1 seems to be a highly conserved miRNA that plays a role in muscle differentiation and maintaining cardiomyocyte homeostasis, not only in mouse and Drosophila, but also in human-derived CMPCs.

Our results suggest that miR-499 and MYH7B are strongly enriched in cardiac tissue and in differentiated cardiomyocytes, suggesting a role in cardiomyocyte homeostasis or maintenance of their differentiation state. Indeed, by transfection of miR-499 into our CMPCs and mESCs, we could enhance differentiation into cardiomyocytes. Since the function of miR-499 was not studied before, we identified possible target genes. Sox6, which is expressed in the heart and skeletal muscle[23], was predicted to contain 3 seed regions (see supplemental figure 2). Sox6 is a member of the Sox gene family of transcription factors (Sry related HMG box) that play a key role in embryonic development and cell fate determination.

Mice homozygote for a Sox6 null mutation, show delayed growth and die within 2 weeks after birth, having abnormal muscle architecture and developing cardiomyopathies[24]. In P19Cl6 cells, a cell line that can differentiate into beating cardiomyocytes, Sox6 is only expressed when cells are committed to differentiate into beating cardiomyocytes and is regulated by BMP (Bone Morphogenic Protein), suggesting an association with the initiation of the cardiomyogenic program. Furthermore, the L-type Ca^{2+} channel, critical for cardiomyocyte contraction, is repressed by Sox6[25] and therefore this protein needs to be tightly regulated in developing

and differentiated cardiomyocytes. In skeletal muscle of Sox6 null mice, an isoform-specific change in muscle gene expression is observed; including increased cardiac troponin and MHC genes, suggesting that Sox6 is involved in muscle development and the tight balance between different muscle isoforms[24]. As indicated by our results, induction of miR-499 represses Sox6 in CMPCs, leading to a reduction in cell proliferation and enhanced myocyte differentiation, confirming the direct role of Sox6 in muscle differentiation. Thus, our data suggest that when cells are committed to the cardiac lineage and start to express MYH7B, miR-499 is co-expressed; thereby repressing Sox6 to induce differentiation further and modulating/fine-tuning fiber expression in developing cardiomyocytes (supplemental figure 3).

In summary, miRNA levels are highly regulated in cultured and differentiated human heart-derived cardiomyocyte progenitor cells (CMPCs), with miR-1 and miR-499 expression being significantly induced upon CMPC differentiation. Our results demonstrate that a single miRNA, miR-1, induces muscle differentiation via repression of HDAC4. Furthermore, miR-499, a thus far unexplored miR, is highly enriched in cardiac tissue and targets Sox6, and enhances cardiomyocyte differentiation in CMPCs *in vitro.* These results demonstrate that miRNAs are powerful regulators driving CMPC differentiation and they can be used to influence cell fate. In addition, CMPCs can be used as a model to study human *in vitro* developmental processes in order to better understand cardiac development and cardiomyocyte homeostasis.

MATERIALS AND METHODS

Human cardiomyocyte progenitor cells (CMPCs)

Human fetal heart tissue was collected after elective abortion, followed by Langendorff perfusion with Tyrode's solution, collagenase, and protease treatment. Single cells were obtained and CMPCs were isolated and characterized as described[7,26]. Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical

Center Utrecht were obtained. Cells were cultured as described and differentiated into cardiomyocytes by treatment with 5 μM 5'-azacytidine for 72 hours in differentiation medium, followed by TGF-β stimulation[7,26]. In addition, SOX6 and HDAC4 RNAi was used to block expression prior to the start of differentiation. RNAi effect was confirmed by flow cytometry as explained below.

Analysis of miR expression by microarray

Low-molecular-weight RNA was isolated from proliferating and differentiated CMPCs by using the mirVana™ RNA Isolation Kit (Ambion). MicroRNA (miR) expression profile was determined by microarray analysis using the μParaflo™ microfluidic chip (MiHuman_8.2-Based on Sanger miRBase Release 8.2, LC Sciences), according the manufacturer.

Quantitative RT-PCR for miR expression

Total RNA was isolated with mirVana™ RNA Isolation Kit or Tripure isolation reagent (Roche Applied Science). In brief, 10 ng purified total RNA was used for reverse transcriptase (Taqman MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with Taqman MicroRNA Assays for quantification of miR-1, 133a, 214, 499, and RNU19 control transcripts (Applied Biosystems: 4373161, 4378064, 4373142, 4373124, 4373085, 4373224, 4373378), according to the manufacturers conditions. Amplification and detection of specific products were performed in a MyIQ single-color real-time PCR system (Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to control RNU19 (ΔCt value). The relative difference in expression levels of each miR in proliferating and differentiated CMPCs (ΔΔCt) was calculated and presented as fold induction ($2^{-\Delta\Delta Ct}$).

CMPC transfection

Pre-miR™ miR Precursor molecules for miR-1 (5'-UGGAAUGUAAAGAAGUAUGUA-3', PM10660), miR-499 (5'-UUAAGACUUGCAGUGAUGUUUAA-3', PM10496), and a

scrambled miR control (AM17121) with or without FAM™ dye-label were obtained from Ambion. CMPCs were transfected with siPORT™ NeoFX™ Transfection Agent (Ambion) and pre-miRs (0, 3, 30 and 100 nM), according to the manufacturers conditions.
Transfection efficiency of pre-miR was confirmed by RT-PCR (Taqman miR assay) and visually by means of FAM-labeled negative control pre-miR. For fluorescence microscope analysis of FAM-labeled pre-miR expression, cells were washed with PBS and fixated with 4% paraformaldehyde in PBS for 15 min at RT. Cells were stained with 0.2 µg/ml Hoechst (Invitrogen) and mounted in VectaShield (Molecular Probes, Amsterdam).

CMPC proliferation assay

Cells were plated in a 0.1% gelatin-coated 96-well plate and transfected as described above. Fresh medium was added when necessary. After 4 and 6 days, culture medium was removed and cells were treated with 0.5 mg/ml of Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma-Aldrich) in RPMI serum-free medium (Gibco) and incubated at 37°C for 3 h. Subsequently, purple formazan crystals, formed by mitochondrial reductase enzymes, were solubized in 0.1 ml DMSO and absorbance was quantified at 540 nm with a reference filter of 690 nm in a photometric plate reader (Labsystems Multiskan Ascent).

Flow cytometry assay

HEK 293T cells were transfected with pLKO.1-puro-shRNA-Sox6 (Mission library, Sigma) to produce lentiviral transduction particles. CMPCs were transduced with shRNA-Sox6 or an off-target control for 24 hours, washed and grown in the presence of puromycin (2 ug/ml) for 7 days, followed by flow cytometry analysis for the expression of Sox6. Selected cells were trypsinized, fixed in 2% PFA for 15 minutes, permeabilized for 15 minutes in 0.5% NP-40 in PBS, washed with PBS and incubated with anti-Sox6 antibody (1:200, S7193, Sigma) for 1 hour at 4 degrees, washed and incubated with anti-FITC secondary antibody (1:600, R&D systems), and measured using a flow cytometer (BD Biosciences).

Embryoid body assay

Mouse embryonic stem cells (E14-IB10 mESCs), were cultured in BRL-conditioned medium as described before[27]. Mouse ESCs were differentiated by generating embryoid bodies (EBs) in hanging drops. Mouse ESCs were transfected with pre-miRNA-1, -499, and SCR prior to body formation. Cells were monitored at day 4 and 7 for percentage of beating EBs, plated to visualize α-actinin staining via immunohistochemistry, and 50 EBs were collected per condition after 11 days to determine differentiation rate by RT-PCR, as described below.

Western blot

Total protein was extracted using TriPure (Roche), and 33 µg, reduced with DTT, was separated by PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked (5% non-fat-dry milk in PBS-0.1% Tween), probed with polyclonal rabbit-anti-human HDAC4 (12171, GeneTex), and Sox6 (S7193, Sigma) and goat anti-rabbit IgG secondary antibodies (P0448, Sigma-Aldrich) in blocking-buffer. Signal was visualized with enhanced chemiluminescence (Amersham) and bands were scanned using a ChemiDoc XRS system (Bio-Rad).

Quantitative RT-PCR

Total RNA was isolated with TriPure reagent (Roche), cDNA was prepared using the SuperScript First-Strand Synthesis System for RT-PCR (170-8890, Bio-Rad), and qRT-PCR amplification was detected in a MyIQ single-color real-time PCR system (Bio-Rad). Amplifications were performed with iQ™ SYBR Green Supermix (170-8884, Bio-Rad). Used qRT-PCR conditions are: 5 min at 95 °C, followed by 40 cycles of 15s at 95 °C, 30s at specific annealing temperature (Supplemental table A), and 45s at 72 °C, followed by melting curve analysis to confirm single product amplification. Expression levels were normalized to β-actin expression (ΔCt value), relative differences were calculated (ΔΔCt value) and presented as fold induction ($2^{-\Delta\Delta Ct}$).

In Situ hybridization

In situ hybridization to determine hsa-miR-499 localization was performed as previously described[28]. In brief, 10 µm thick sections of human fetal heart tissue were hybridized overnight at 46 ºC with 5 nM LNA DIG-labeled probes in denaturizing hybridization buffer (Scramble-miR, Exiqon, 99001-01 and hsa-miR-499, Exiqon, 38306-01), incubated with an anti-DIG-alkaline phosphatase antibody (1:1500, Roche 11093274910), an anti-PECAM (1:200, Santa Cruz, sc-1505), and an anti-α-actinin (Sigma) at RT for 2 hours. After washing, sections were incubated with fluorescent-labeled secondary antibodies, exposed to Fast-Red Substrate-Chromogen (DAKO, K0597) overnight at RT and mounted in VectaShield (Molecular Probes, Amsterdam).

Luciferase experiments

Primers were generated for mouse 3'UTR of Sox6, a putative target of miR-499, and extended with SpeI restriction sites (see table 1). After RT-PCR, the 3'UTR of Sox6 was inserted into pMIR-REPORT™ miRNA Expression Reporter Vector (#5795, Ambion), by SpeI directed ligation, at the 3'UTR of CMV-Luc. In a 24-wells plate, HEK 293 cells were transfected with 200 ng plasmid, containing the CMV-Luc and 3'UTR of Sox6, with and without 30 nM of miR-499, miR-1, or Scr-miR, and 200 ng CMV-lacZ for normalization. To block miR-499 activity, the LNA-probe for miR-499 was used at 30 nM. After 48 hours, Luciferase and β-Galactosidase activity was measured with the Luciferase Assay System and β-Galactosidase Enzyme Assay System (Promega), respectively.

Statistical analysis

Data is presented as mean ± SEM of at least three independent experiments and were compared using the two-tailed paired Student's T-test or one-way ANOVA. A difference with $p<0.05$ was considered to be statistically significant.

Acknowledgements

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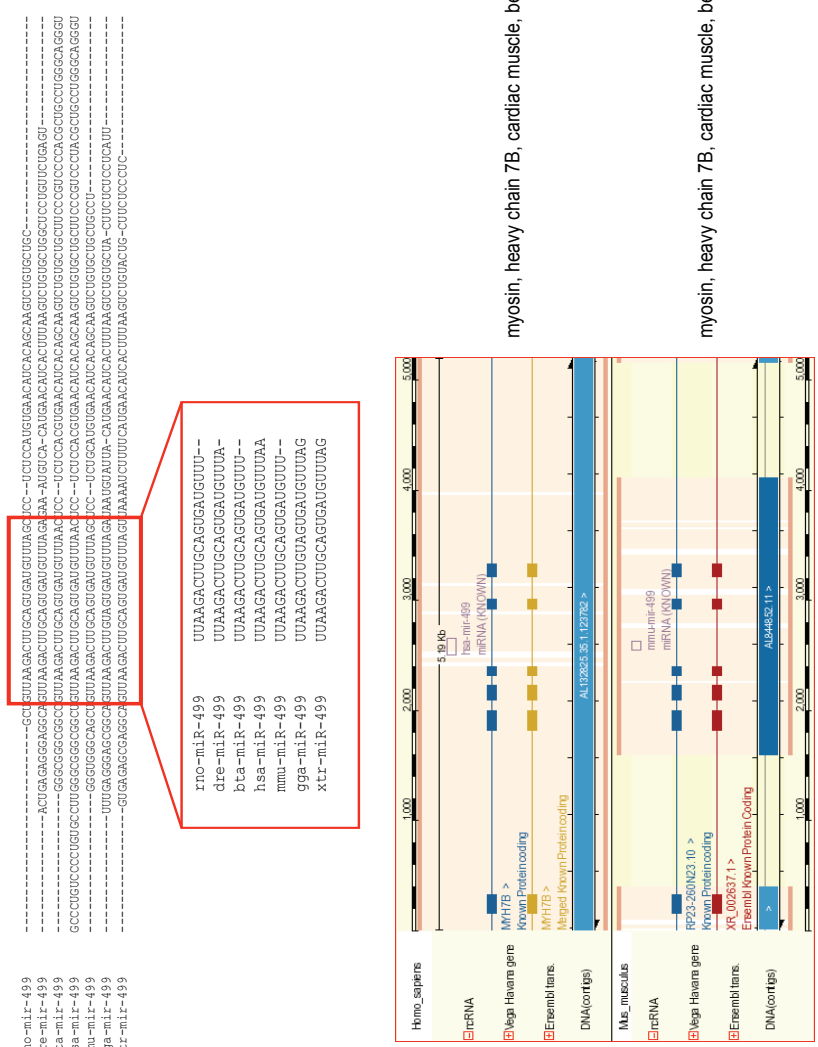
REFERENCES

1. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
2. Hierlihy, A.M., Seale, P., Lobe, C.G., Rudnicki, M.A. & Megeney, L.A. The post-natal heart contains a myocardial stem cell population. *FEBS Lett* **530**, 239-43 (2002).
3. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
4. Moretti, A. *et al.* Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. *Cell* **127**, 1151-65 (2006).
5. Kattman, S.J., Huber, T.L. & Keller, G.M. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* **11**, 723-32 (2006).
6. Beltrami, A.P. *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**, 763-76 (2003).
7. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
8. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
9. Kanellopoulou, C. *et al.* Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev* **19**, 489-501 (2005).
10. Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P. & Burge, C.B. Prediction of mammalian microRNA targets. *Cell* **115**, 787-98 (2003).
11. Thum, T. *et al.* MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* **116**, 258-67 (2007).
12. Ikeda, S. *et al.* Altered microRNA expression in human heart disease. *Physiol Genomics* **31**, 367-73 (2007).
13. van Rooij, E. *et al.* Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**, 575-9 (2007).
14. Zhao, Y., Samal, E. & Srivastava, D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* **436**, 214-20 (2005).
15. Zhao, Y. *et al.* Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* **129**, 303-17 (2007).

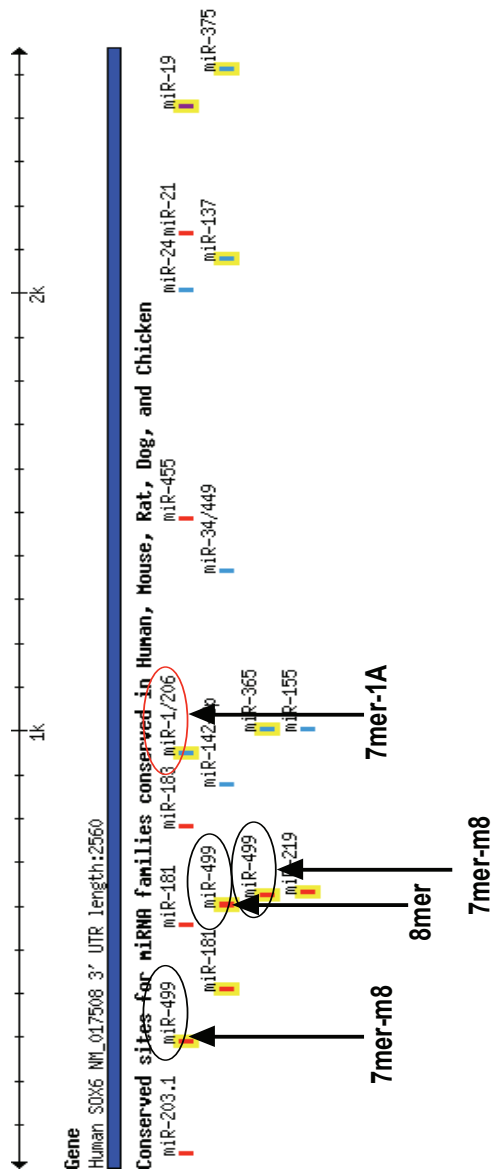
16. Doevendans, P.A. & van Bilsen, M. Transcription factors and the cardiac gene programme. *Int J Biochem Cell Biol* **28**, 387-403 (1996).
17. Srivastava, D. & Olson, E.N. A genetic blueprint for cardiac development. *Nature* **407**, 221-6 (2000).
18. Griffiths-Jones, S. The microRNA Registry. *Nucleic Acids Res* **32**, D109-11 (2004).
19. Chen, J.F. *et al.* The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* **38**, 228-33 (2006).
20. Yang, B. *et al.* The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nat Med* **13**, 486-91 (2007).
21. Kwon, C., Han, Z., Olson, E.N. & Srivastava, D. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc Natl Acad Sci U S A* **102**, 18986-91 (2005).
22. Ivey, K.N. *et al.* MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell* **2**, 219-29 (2008).
23. Hagiwara, N. *et al.* Sox6 is a candidate gene for p100H myopathy, heart block, and sudden neonatal death. *Proc Natl Acad Sci U S A* **97**, 4180-5 (2000).
24. Hagiwara, N., Ma, B. & Ly, A. Slow and fast fiber isoform gene expression is systematically altered in skeletal muscle of the Sox6 mutant, p100H. *Dev Dyn* **234**, 301-11 (2005).
25. Cohen-Barak, O. *et al.* Sox6 regulation of cardiac myocyte development. *Nucleic Acids Res* **31**, 5941-8 (2003).
26. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
27. Monteiro, R.M., de Sousa Lopes, S.M., Korchynskyi, O., ten Dijke, P. & Mummery, C.L. Spatio-temporal activation of Smad1 and Smad5 in vivo: monitoring transcriptional activity of Smad proteins. *J Cell Sci* **117**, 4653-63 (2004).
28. Obernosterer, G., Martinez, J. & Alenius, M. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc* **2**, 1508-14 (2007).

SUPPLEMENTAL FIGURES



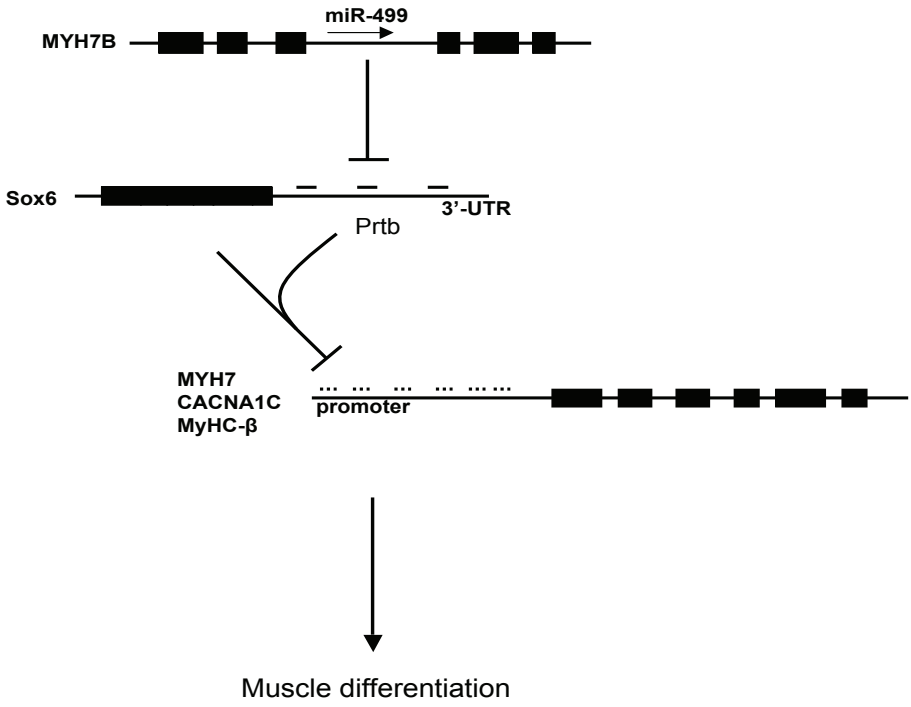
Supplemental figure 1

A | The sequence of miR-499 is well conserved among different species. B | MiR-499 localization within intron 20 of MYH7B (myosin heavy chain, cardiac muscle beta isoform 7B) is conserved between human and mice.



Supplemental figure 2

The untranslated region (UTR) of human and mouse Sox6 has three predicted binding sites for miR-499, one 8-mer and two 7mer-m8. Moreover, one additional miR-1 binding site is present, one 7mer-1A.

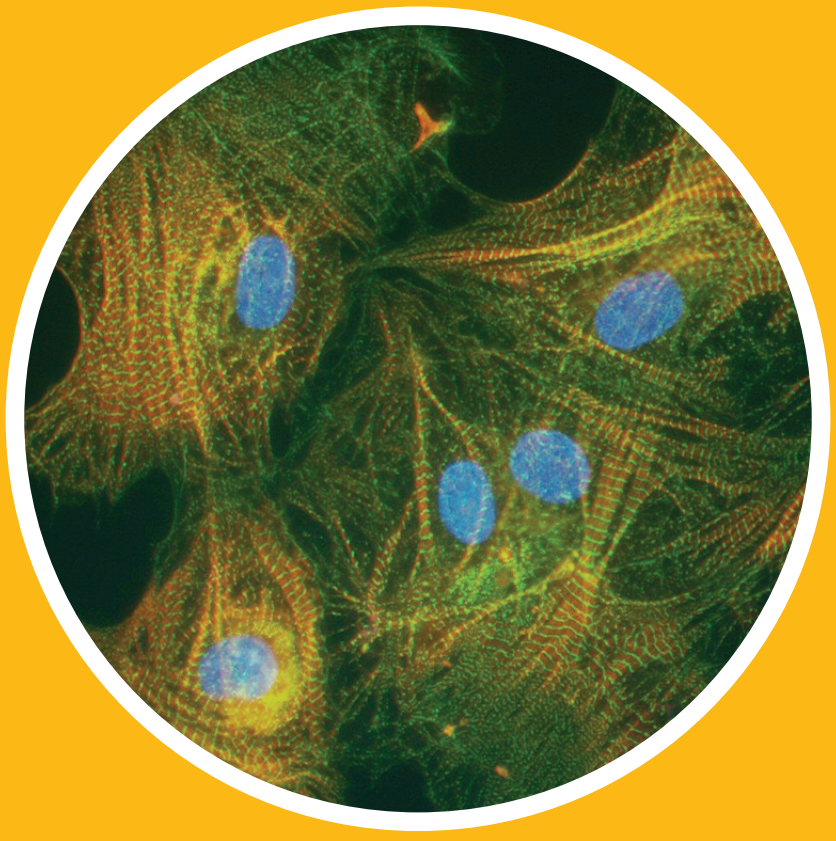


Supplemental figure 3

MiR-499 is transcribed from the intron of MYH7B and is able to suppress the translation of Sox6, via 3 potential sites in the untranslated region (UTR). This transcription factor is involved in the regulation of different muscle genes, such as myosin heavy chain 7 (MYH7), myosin heavy chain-β, and the L-type Ca2+ channel (CACNA1C). Sox6 represses different genes, probably via binding of the promoter region, interacting with other factors such as Proline-rich transcript of the brain (Prtb). Our results suggest that by repressing Sox6 expression via miR-499, proliferation is decreased and muscle differentiation is induced.

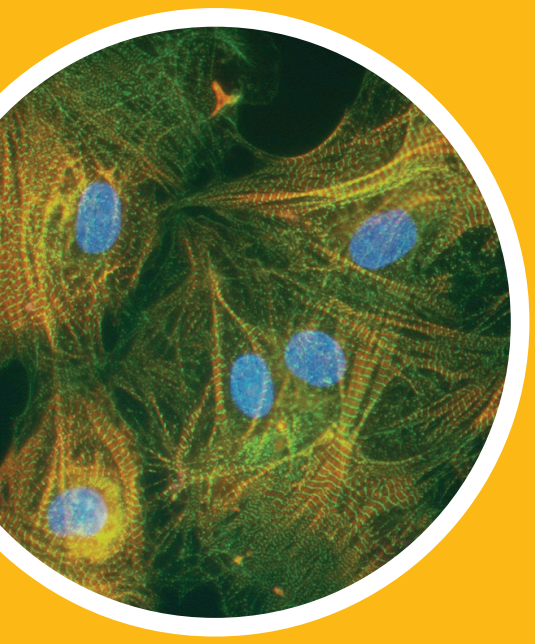
CHAPTER

7



CHAPTER 7

Hyperpolarization induces differentiation in human cardiomyocyte progenitor cells



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ABSTRACT

Stem cell-based therapy is being suggested as treatment for cardiac repair. Several different cardiovascular progenitor cell types have been isolated, characterized, and tested for their *in vitro* and *in vivo* differentiation potential and their regenerative capacity upon transplantation. Recently, we isolated cardiomyocyte progenitor cells (CMPCs) from human hearts. CMPCs differentiate efficiently into cardiomyocytes when cultured in the presence of TGFβ. Although TGFβ is known to induce differentiation in several stem and progenitor cell types, the exact mechanisms driving cardiomyogenic differentiation have not been elucidated. Interestingly, TGFβ stimulation results in hyperpolarization of CMPCs. Therefore we tested whether hyperpolarization is sufficient to induce cardiomyogenic differentiation of CMPCs. We induced hyperpolarization in CMPCs by either co-culturing them with a Kir2.1-overexpressing cell line or overnight culture in medium containing low potassium concentrations. Hyperpolarization led to activation of calcineurin signaling, increased cardiac-specific gene and protein expression levels, and ultimately to the formation of spontaneously beating cardiomyocytes. Thus, hyperpolarization is sufficient to induce differentiation of CMPCs, showing a novel mechanism for cardiomyogenic differentiation of heart-derived progenitor cells.

INTRODUCTION

During embryonic development and possibly in adult life, cardiovascular progenitor cells are needed for the formation and homeostasis of heart tissue[1]. Recently, several cardiovascular progenitor cell populations have been isolated from human hearts and shown to form cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro* and *in vivo*[2,3]. We have isolated cardiomyocyte progenitor cells (CMPCs) from human fetal and adult heart tissue[4,5]. These cells can be differentiated efficiently into functional, spontaneously beating cardiomyocytes when stimulated with TGFβ. TGFβ also induces cardiomyogenic differentiation in other stem and progenitor cell types[6-10] and increases inward rectifier currents (I_{K1}) in c-kit$^+$ cells[11]. Since an I_{K1}-mediated hyperpolarization is required for myoblast differentiation into skeletal muscle cells[12,13], we tested if TGFβ-enhanced differentiation in CMPCs is mediated via hyperpolarization. Here we show that hyperpolarization alone is sufficient to induce cardiomyogenic differentiation of CMPCs.

RESULTS

TGFβ signaling induces hyperpolarization in CMPCs

Stimulation of CMPCs with TGFβ increased the phosphorylation of the transcription factor Smad2 (figure 1A), leading to the activation of the downstream signaling cascade as shown by activation of CAGA-Luciferase reporter activity (figure 1B). Activation of the TGFβ pathway subsequently resulted in a more negative membrane potential (figure 1C), indicating that TGFβ signaling induces hyperpolarization in these cells.

I_{K1}-mediated hyperpolarization stimulates differentiation of CMPCs

To test whether cardiomyogenic differentiation of CMPCs[4] is mediated via hyperpolarization, we used KWGF cells. KWGF cells have previously been shown to induce hyperpolarization of rat neonatal cardiomyocytes by electrotonic application of Kir2.1-

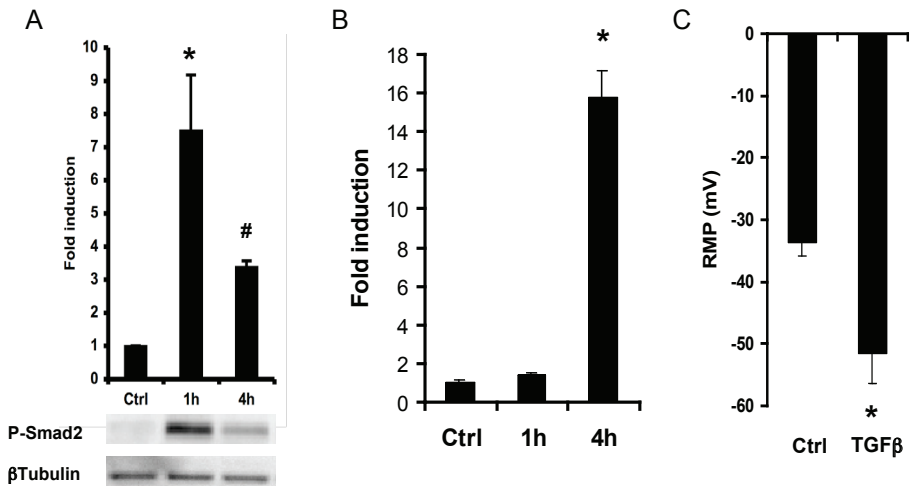

Figure 1: TGFβ signaling induces hyperpolarization

A | Western blot analysis showing Smad2 phosphorylation in CMPCs after 1 to 4 hours TGFβ stimulation (n=3, * P=0.018, # P=0.000 vs. Ctrl). B | TGFβ stimulation significantly increases activation of CAGA-luciferase reporter activity in CMPCs (P=0.008). C | TGFβ stimulation resulted in significant hyperpolarization (from -34 mV to -52 mV, n=32 and n=11, p=0.006) within 4 hours.

mediated I_{K1} currents[14]. CMPCs were stimulated with 5-azacytidine and co-cultured with KWGF cells in differentiation medium. Transfer of Lucifer Yellow dye from a KWGF cell to adjacent CMPCs indicated functional coupling (figure 2A). When coupled, KWGF cells induced a stable, strong hyperpolarization in CMPCs (figure 2B), which could be inhibited by the gap junction blocker halothane (figure 2C), demonstrating that hyperpolarization was induced by electrotonic application of I_{K1}. Two weeks after hyperpolarization, CMPCs showed a significant, dose-dependent increase in expression of the sarcomeric genes troponin T and β-myosin heavy chain (βMHC) (figure 2D). HEK 293 cells, KWGF cells alone, or CMPCs co-cultured with HEK 293 cells did not show increased expression of these markers when subjected to a similar differentiation protocol (data not shown). These results

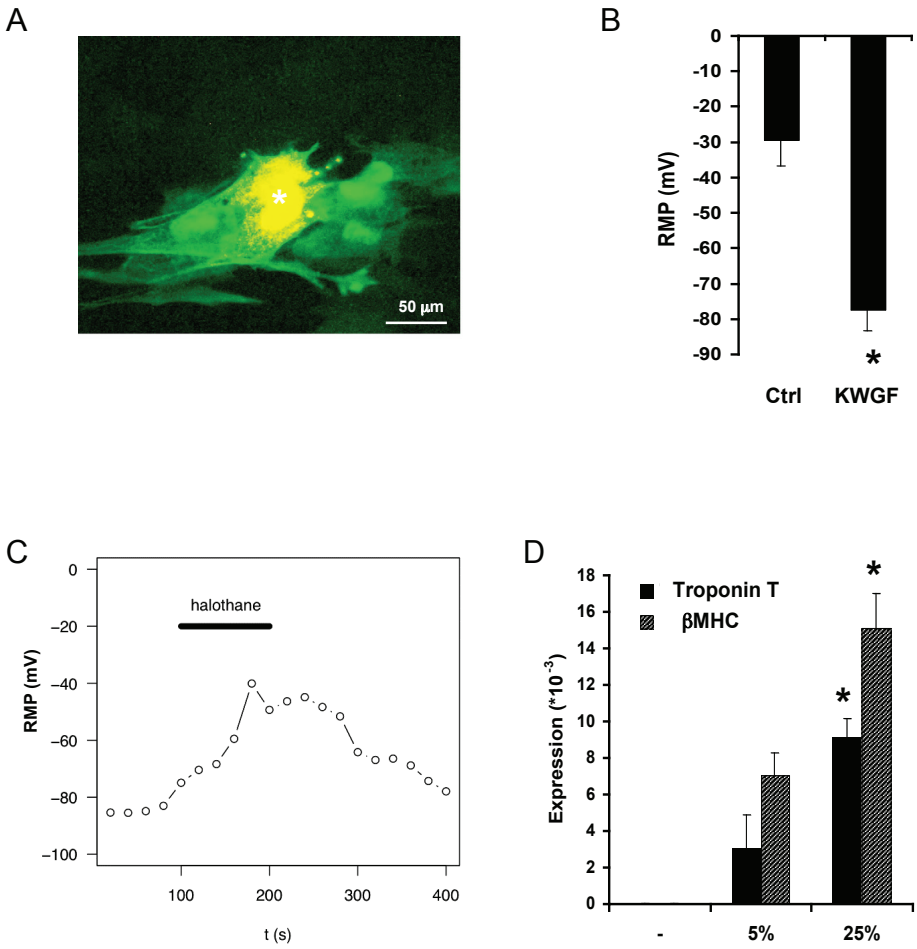

Figure 2: I_{K1}-mediated differentiation in CMPCs

A | KWGF cells (white asterisk) were identified by GFP fluorescence and co-injected with dextran (red) and Lucifer Yellow dye (green), which led to spreading of Lucifer Yellow to neighboring CMPCs (n=6). B | Resting membrane potential decreased from -29 mV (CMPCs alone, n=4) to -78 mV in CMPCs co-cultured with KWGF cells (n=4, p=0.003). C | Inhibition of gap junctional coupling by halothane resulted in restoration of normal RMP in CMPCs. CMPC hyperpolarization is restored upon halothane washout (n=4). D | Co-culture of CMPCs with an increased relative number of KWGF cells (from 0 to 5 or 25%) resulted in a dose-dependent increased expression of troponin T and βMHC mRNA two weeks later.

indicate that hyperpolarization of CMPCs by KWGF cells enhances cardiomyocyte differentiation of CMPCs.

Hyperpolarization induces cardiomyogenic differentiation of CMPCs

To test whether CMPCs could be differentiated without 5-azacytidine or co-culture with KWGF cells, we hyperpolarized CMPCs by decreasing the extra-cellular potassium concentrations (figure 3A). When CMPCs were subsequently cultured in differentiation medium, expression of troponin T, βMHC, α-human cardiac actin (αHCA), and ryanodine receptor 2 (RyR2) was significantly increased in hyperpolarized CMPCs after five weeks (figure 3B). Interestingly, increasing the number of overnight hyperpolarizations in the first week of differentiation leads to increased cardiac gene expression after several weeks, confirming the dose-dependent effect of hyperpolarization by electrotonic application of I_{K1} (figure 2D). Differentiated cells revealed a striated sarcomeric pattern (figure 3C), typical for cardiomyocytes. Notably, CMPC-derived cardiomyocytes also started to beat spontaneously (figure 3D and supplemental video 1) and had a resting membrane potential similar to cardiomyocytes derived after differentiation induced by 5-azacytidine and TGFβ (figure 3E and[4]). Therefore, hyperpolarization alone is sufficient to induce differentiation of CMPCs into functional, spontaneously beating cardiomyocytes.

Hyperpolarization activates calcineurin signaling in CMPCs

In myoblasts, differentiation towards skeletal muscle is mediated by hyperpolarization-induced Ca^{2+}-calcineurin signaling[15]. In CMPCs, hyperpolarization resulted in increased intracellular calcium levels (figure 4A) and increased NFAT-reporter activity (figure 4B). This led to increased expression of the calcineurin target gene RCAN1-4 (figure 4C), indicating enhanced calcineurin signaling.

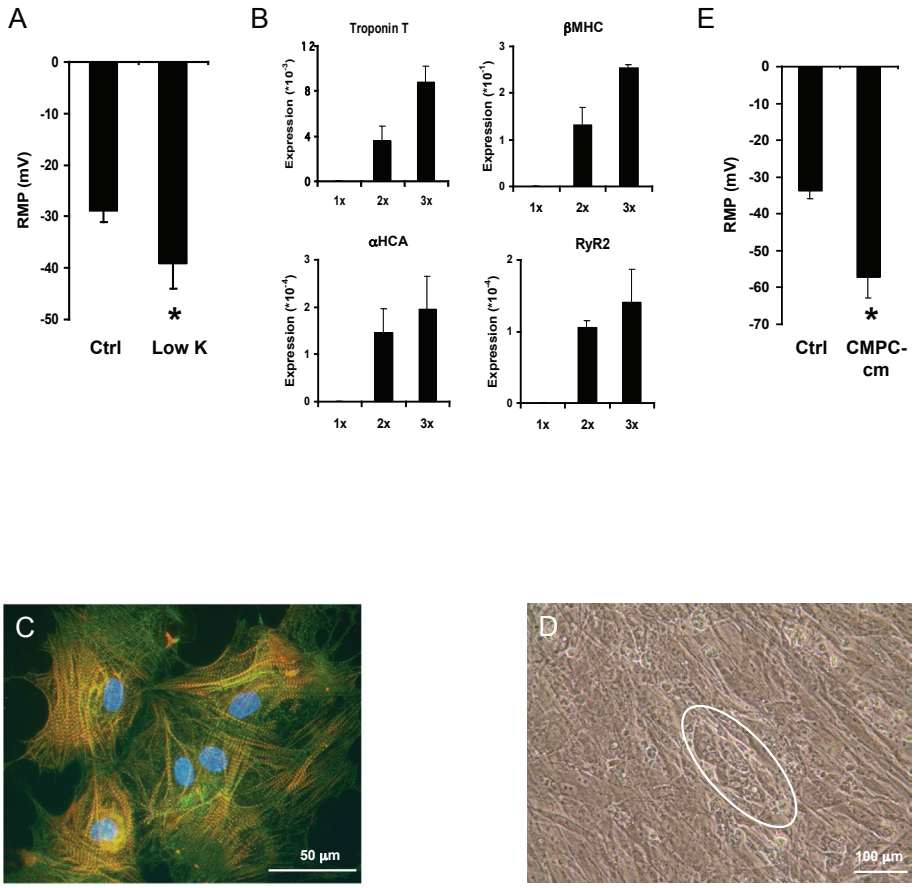

Figure 3: Hyperpolarization induces CMPC differentiation into cardiomyocytes

A | Decreasing extracellular potassium levels from 5 mM (ctrl) to 1.5 mM resulted in a hyperpolarized membrane potential in CMPCs (from -29 mV to -39 mV, n=5, p=0.04). B | Quantitative RT-PCR analysis on RNA isolated from CMPCs that underwent hyperpolarization in week 1, revealed a dose-dependent increased expression of troponin T, βMHC, αHCA, and RyR2 in CMPC-derived cardiomyocytes five weeks later. C | Immunolabeling of the contractile proteins α-actinin (green) and troponin I (red) with nuclei in blue. CMPC-derived cardiomyocytes showed a typical striated sarcomeric staining pattern. D | Bright-field image of beating area. White circle indicates a beating cluster of CMPC-derived cardiomyocytes. E | CMPC-derived cardiomyocytes (CMPC-cm) showed a RMP of -57 mV, typical for fetal-like ventricular cardiomyocytes. Ctrl: undifferentiated CMPCs (n=6, p=0.006).

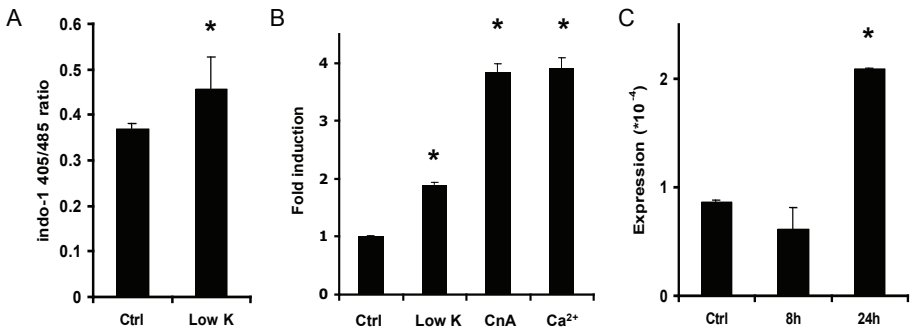

Figure 4: Hyperpolarization enhances calcineurin signaling

A | Ratiometric determination of intracellular calcium levels in CMPCs. Hyperpolarization resulted in significantly increased intracellular calcium (n=48, $p<0.0001$). B | NFAT-luciferase reporter activity in CMPCs. Hyperpolarization resulted in an almost two-fold induction of reporter activity ($p=0.002$). The positive controls, constitutive active calcineurin or calcium ionophore, resulted in an approximately four-fold induction ($p=0.002$ and 0.003, respectively). C | Quantitative RT-PCR analysis on RNA isolated from CMPCs, 8 to 24 hours after hyperpolarization. Compared to control, hyperpolarization induced a two-fold increase of the calcineurin downstream target RCAN1-4.

DISCUSSION

TGFβ has previously been shown to stimulate cardiomyogenic differentiation in CMPCs and a variety of other stem and progenitor cell types[4,7,8,10]. In this study, we showed that TGFβ induces hyperpolarization in CMPCs. This hyperpolarization is sufficient to induce calcineurin signaling and differentiation into functional, contracting cardiomyocytes.

In skeletal progenitor cells, an I_{K1}-mediated hyperpolarization results in activation of the calcineurin pathway, thereby enhancing the expression of myogenic transcription factors that further promote differentiation[15]. Therefore, induction of hyperpolarization may be important for (cardio)myogenic differentiation in general.

Cardiomyogenic differentiation of cardiovascular progenitor cells

The low resting membrane potential (RMP) in KWGF cells induces hyperpolarization in CMPCs via gap junctional communication. In other studies, co-culture with neonatal cardiomyocytes is required for cardiomyogenic differentiation of human cardiovascular progenitor cells[16-19]. Similar to KWGF cells, rat cardiomyocytes have a rather negative RMP[14]. Additionally, expression of gap junction proteins was shown in progenitor cells in co-culture experiments[17,18]. Possibly, gap junction-mediated hyperpolarization and, subsequently, induction of calcineurin signaling plays a role in the induction of differentiation in these cardiovascular progenitor cell-cardiomyocyte co-culture studies. However, simultaneous paracrine signaling cannot be excluded in these co-culture settings. Cardiovascular progenitor cells also express gap junction proteins *in vivo*[19]. It would therefore be interesting to test whether hyperpolarization-induced differentiation occurs *in vivo* as well.

In conclusion, we provide evidence for a novel mechanism for differentiation, mediated via hyperpolarization, in human CMPCs. Hyperpolarization may be sufficient for (cardio)myogenic differentiation in different stem and progenitor cell types.

MATERIALS AND METHODS

Cell culture

Human fetal CMPCs were isolated as described[4,20]. Informed consent procedures were followed and prior approval of the ethics committee of the University Medical Center Utrecht was obtained. To stimulate differentiation of CMPCs by hyperpolarization, cells were 1) stimulated with 5-azacytidine for three consecutive days, followed by co-culture in differentiation medium with HEK 293-KWGF cells[14] or 2) cultured overnight in a modified Tyrode's solution (containing 8 mg/ml phenol red, 140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM HEPES, 35 mM $NaHCO_3$, and 6 mM glucose, pH 7.20/NaOH) supplemented with 0.5% BSA, MEM essential and non-essential amino acids, L-Glutamine, 10^{-4} M ascorbic acid, and potassium concentrations as indicated. Afterwards, cells

were washed with PBS and cultured in differentiation medium as described previously[4,20].
For stimulation experiments, cells were washed with PBS and cultured for several hours in M199 medium with 0.5% BSA and MEM non-essential amino acids. Cells were washed and fresh medium containing TGFβ1 (1 ng/ml, PeproTech) was added according to the indicated time periods.

Immunohistochemistry and Western blot analysis
Immunohistochemistry and Western blot analysis were performed as described previously[4]. β-Tubulin was used as loading control.

Electrophysiology
For electrophysiology measurements, cells were cultured in DMEM/Ham's F12, supplemented with L-Glutamine and 0.5% FCS. Determination of membrane potential by whole-cell patch clamp and Lucifer Yellow microinjection were performed as described[4]. To mark KWGF cells in CMPC-KWGF co-culture, KWGF cells were injected with tetramethylrhodamine 10,000 MW Dextran (Invitrogen). Gap junctional communication was blocked by 2 mM halothane. Intracellular calcium levels were determined by ratiometric imaging of indo-1 fluorescence (labeling was done with 1 μM indo-1-AM (Invitrogen) for 30 minutes).

RNA isolation and quantitative RT-PCR
Cells were lyzed in TriPure (Roche). Total RNA was isolated and DNAse treated (Amersham Biosciences). Five hundred ng total RNA was used for cDNA synthesis with iScript cDNA synthesis kit (BioRad). For qRT-PCR, 10 μl cDNA (1:20 diluted) was mixed with 10 μl SYBR-Green mix (BioRad) and forward plus reverse primers (endconcentration 0.5 μM each) and run on a MyiQ iCycler (BioRad). PCR conditions were: 2 min at 94 °C followed by 40 cycles of: 30 sec at 94 °C, 30 sec at annealing temperature (see supplemental table 1), and 30 sec at 72 °C. Amplicon quantities were determined by comparison with known quantities of cloned PCR products and are expressed in pg. Specificity of amplification for the detection with SYBR-Green was visually checked on PAGE gels or melting

curve analysis after the PCR run. Primers were designed with Beacon Designer 4.0 (Premier Biosoft International). Sequences and annealing temperatures are given in supplemental table 1. Expression levels were normalized to β-actin expression.

Luciferase assays

CMPCs were transfected with a $(CAGA)_9$-Luciferase reporter construct[21] by standard calcium phosphate transfection or adenovirally transduced with a $(NFAT)_9$-luciferase reporter. For NFAT-luciferase assays, transduced cells were washed the following day and hyperpolarized overnight. As positive control, cells were co-transduced with constitutively active calcineurin A (virus was kindly provided by Dr. Leon de Windt, Dept. of Medical Physiology, UMC Utrecht, the Netherlands) or stimulated for eighteen hours with a calcium ionophore (4-bromo A-23187, 5 μM). Luciferase assays were performed with the Luciferase Assay System (Promega) and Luciferase activity was normalized for β-Galactosidase expression.

Statistics

All data are presented as average ± SEM. Experiments were performed at least twice in duplo (qRT-PCR), triplo (luciferase reporter assays), or as indicated (electrophysiology measurements). Representative experiments are shown. Significance was determined with a Student's T-test or ANOVA. Significance was assumed when $P<0.05$.

Acknowledgements

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REFERENCES

1. Harvey, R.P. Patterning the vertebrate heart. *Nat Rev Genet* **3**, 544-56 (2002).
2. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).
3. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* **50**, 19-30 (2008).
4. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
5. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
6. Zentella, A. & Massague, J. Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. *Proc Natl Acad Sci U S A* **89**, 5176-80 (1992).
7. Behfar, A. *et al.* Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* **16**, 1558-66 (2002).
8. Li, T.S. *et al.* TGF-beta induces the differentiation of bone marrow stem cells into immature cardiomyocytes. *Biochem Biophys Res Commun* **366**, 1074-80 (2008).
9. Abdel-Latif, A. *et al.* TGF-beta1 enhances cardiomyogenic differentiation of skeletal muscle-derived adult primitive cells. *Basic Res Cardiol* **103**, 514-24 (2008).
10. Lim, J.Y., Kim, W.H., Kim, J. & Park, S.I. Involvement of TGF-beta1 signaling in cardiomyocyte differentiation from P19CL6 cells. *Mol Cells* **24**, 431-6 (2007).
11. Lagostena, L. *et al.* Electrophysiological properties of mouse bone marrow c-kit+ cells co-cultured onto neonatal cardiac myocytes. *Cardiovasc Res* **66**, 482-92 (2005).
12. Fischer-Lougheed, J. *et al.* Human myoblast fusion requires expression of functional inward rectifier Kir2.1 channels. *J Cell Biol* **153**, 677-86 (2001).
13. Konig, S. *et al.* Membrane hyperpolarization triggers myogenin and myocyte enhancer factor-2 expression during human myoblast differentiation. *J Biol Chem* **279**, 28187-96 (2004).

14. de Boer, T.P. *et al.* Inhibition of cardiomyocyte automaticity by electrotonic application of inward rectifier current from Kir2.1 expressing cells. *Med Biol Eng Comput* **44**, 537-42 (2006).
15. Konig, S., Beguet, A., Bader, C.R. & Bernheim, L. The calcineurin pathway links hyperpolarization (Kir2.1)-induced Ca2+ signals to human myoblast differentiation and fusion. *Development* **133**, 3107-14 (2006).
16. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
17. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
18. Smith, R.R. *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* **115**, 896-908 (2007).
19. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
20. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
21. Dennler, S. *et al.* Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* **17**, 3091-100 (1998).

SUPPLEMENTAL DATA

Supplemental table 1: Primer sequences and annealing temperatures

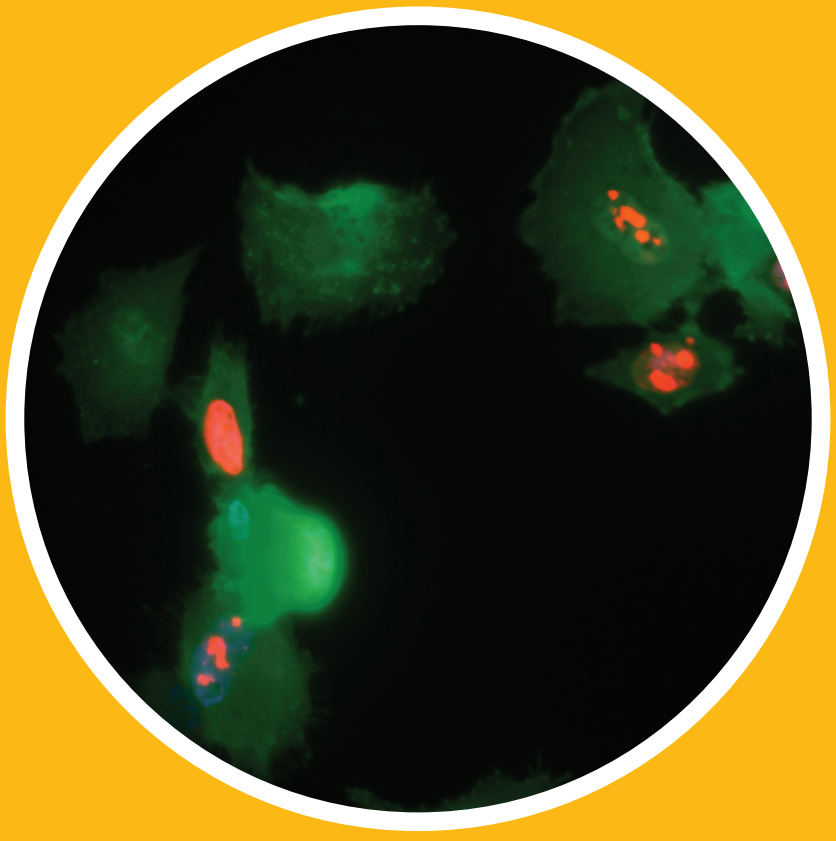
Name	*Sequence*	*Annealing temperature*
Troponin T For	GTG GGA AGA GGC AGA CTG AG	62 ^{0}C
Troponin T Rev	ATA GAT GCT CTG CCA CAG C	
β-MHC For	TCT TTC CCT GCT GCT CTC	60 ^{0}C
β-MHC Rev	GAC TGC CAT CTC CGA ATC	
αHCA For	ACC GAC CTT GCT GTG AAT C	60 ^{0}C
αHCA Rev	AAT TGT GCT CCG AAA CTA ACC	
RyR2 For	ACA AGC AAG AAA GAA CTT ACA C	58 ^{0}C
RyR2 Rev	AGA TGA GGA GGC AAC ACG	
RCAN1-4 For	GCA TTT TAG AAA CTT TAA CTA C	59 ^{0}C
RCAN1-4 Rev	TGA TGT CCT TGT CAT ACG	
β-Actin For	GAT CGG CGG CTC CAT CCT G	60 ^{0}C
β-Actin Rev	GAC TCG TCA TAC TCC TGC TTG C	

Supplemental Video 1

Spontaneous beating clusters of CMPC-derived cardiomyocytes five weeks after induction of differentiation by hyperpolarization.

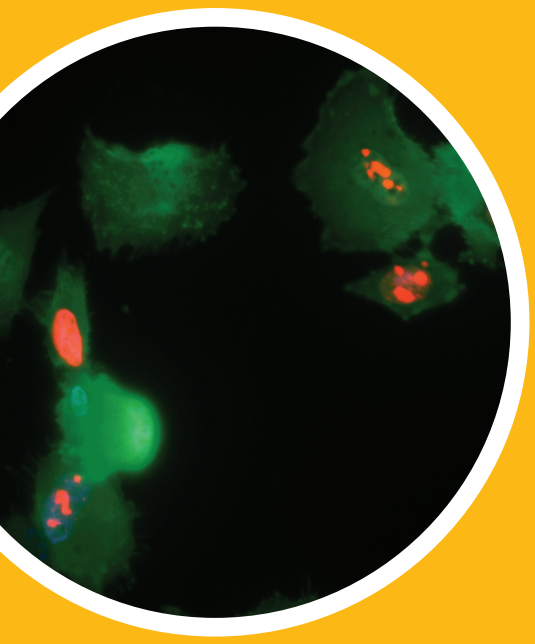
CHAPTER

8



CHAPTER 8

Survivin increases cardiomyocyte progenitor cell proliferation and survival



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ABSTRACT

In the past years, a multitude of studies have shown different levels of improvement after stem cell transplantation in the heart, depending on the cell type used and additional genetic modification of the transplanted cells. We have recently reported the isolation and characterization of human heart-derived cardiomyocyte progenitor cells (CMPCs). These cells can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells and may therefore be suitable to restore cellular loss after myocardial ischemia. The hypoxic environment in the infarcted heart may however result in premature death of transplanted cells, thereby decreasing their contribution to new cardiac tissue and improved perfusion.
To determine the effect hypoxia has on CMPCs, we exposed them to low oxygen levels *in vitro* and found that their proliferation was increased compared to normoxic conditions. Enhanced proliferation was mediated by the pro-survival factor Survivin. Moreover, adenoviral Survivin overexpression resulted in enhanced proliferation and decreased apoptosis, but also decreased angiogenesis in CMPCs. However, CMPCs overexpressing Survivin stimulate angiogenesis in endothelial cells *in vitro*. Possibly, an enhanced CMPC number and survival may be beneficial to stimulate perfusion *in vivo* as well.

INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality in the world[1]. The loss of cardiomyocytes and other cell types after myocardial infarction (MI) induces remodeling of the heart and deposition of extra-cellular matrix proteins that further impair cardiac function[2]. In order to restore the loss of contractility, stem cell replacement therapy has emerged as a potential treatment and already several clinical trials with bone marrow-derived cells have been performed[3]. Unfortunately, long-term improvement was limited, probably due to the removal of transplanted cells via the circulation and the lack of cardiomyocyte formation *in vivo*. Furthermore, mesenchymal stem cells (MSCs) have been reported to form bone after transplantation into infarcted myocardium, which may induce arrhythmias[4]. Therefore, progenitor cells with endogenous cardiomyogenic potential may be more suitable to treat cardiac dysfunction[5-7]. Cardiovascular progenitor cells have been identified in the human adult heart and, after isolation, shown to be able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro*[8-12]. Although these progenitor cells are able to form cardiac cells *in vivo* as well and improve cardiac function after transplantation into rodent models, the efficiency is still very low. The hostile and hypoxic host environment may result in cell death due to the lack of sufficient oxygen and nutrients[13] and thereby decrease the contribution of transplanted cells to new tissue and enhanced perfusion. Thus, inhibition of apoptosis and stimulation of survival is necessary to improve the efficiency of stem cell transplantation.

The baculoviral IAP repeat-containing 5 (BIRC5) protein, also known as Survivin, is a member of the inhibitor of apoptosis (IAP) gene family[14] and mediates mitosis and prevents apoptosis[15-17]. Gene expression is ubiquitously present in the embryo around day 11.5 post-coitum, but becomes more restricted to highly proliferating tissues later during embryonic development and is eventually mostly absent in adult tissue, where cell cycle progression is low[18]. Survivin is important during embryonic angiogenesis in zebrafish and mouse[19,20]. It has been implicated

in cell proliferation and inhibition of apoptosis in cardiomyocytes, smooth muscle cells, and endothelial cells[21-23]. Loss of Survivin expression results in hypoplasia and cardiac failure[23].
Survivin is known to be a downstream target of Akt[24], which has been shown to enhance the beneficial effect of MSC transplantation on cardiac perfusion in a myocardial infarction model[25]. In the future, modulation of Survivin expression in CMPCs may provide a valuable tool to enhance cell proliferation and survival after transplantation into ischemic tissue. We therefore examined the effect of hypoxia and Survivin functionality in human cardiomyocyte progenitor cells (CMPCs) *in vitro*.

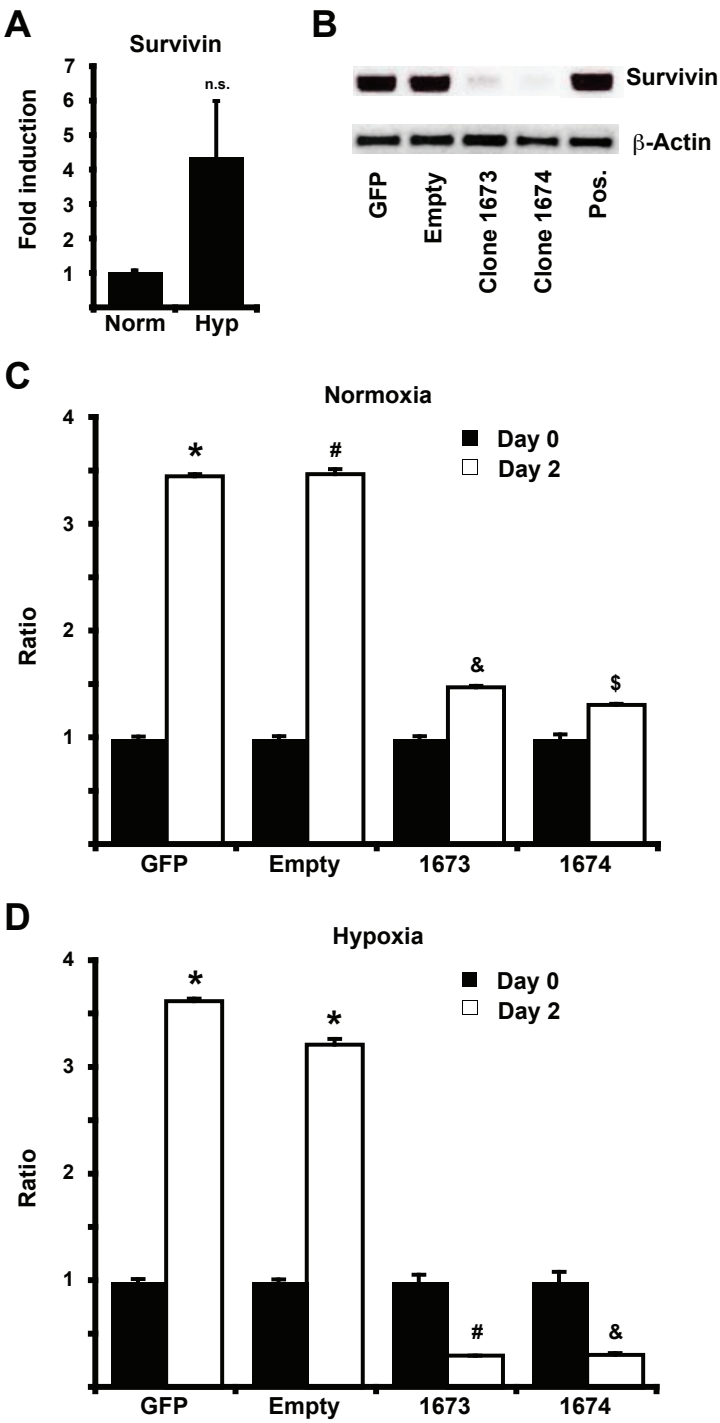
RESULTS

Hypoxia enhances Survivin-mediated proliferation in CMPCs

Hypoxia and hypoxia-inducible factors (HIFs) have been shown to regulate progenitor cell behavior[26]. Since we previously observed that hypoxia increased the proliferation of CMPCs *in vitro* (AAM van Oorschot, unpublished data), we tested whether this increased proliferation was mediated by Survivin. CMPCs cultured for four days under hypoxic conditions showed a four-fold increase in Survivin expression when compared to CMPCs cultured under normoxia (figure 1A). Degradation of Survivin mRNA by RNA interference (figure 1B) inhibited the growth of CMPCs under normoxia (figure 1C). Under hypoxia, the absence of Survivin even resulted in a decrease compared to day 0 (figure 1D), likely due to cell death, indicating that Survivin expression is required for CMPC proliferation and survival under hypoxic conditions.

Survivin overexpression enhances proliferation of CMPCs

Since a lack of Survivin blocks CMPC proliferation, we determined if Survivin overexpression could further enhance their growth. CMPCs were transduced with adenovirus either containing a GFP control or Survivin-GFP (figure 2A). This resulted in increased expression of Survivin protein compared to non- and GFP-transduced cells (figure 2A). GFP fluorescence and Survivin expression


A
Survivin
Fold induction
7
6
5
4
3
2
1
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Norm
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B
Survivin
β-Actin
GFP
Empty
Clone 1673
Clone 1674
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Day 0
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1
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GFP
Empty
1673
1674

Figure 1: Hypoxia-induced Survivin expression mediates CMPC proliferation
A | Quantification of Survivin mRNA expression in CMPCs cultured for four days under 20% O_2 (normoxia) or 1% O_2 (hypoxia). Not significant (n=3, p=0.17). B | Gel analysis of RT-PCR on CPMCs transduced with RNA interference plasmids for GFP, empty plasmid, Survivin shRNA clone 1673, or clone 1674. Positive control is from un-transduced CMPCs. C | Representative results from one of two MTT assays, performed in triplo on different CMPC clones cultured under normoxia and with or without Survivin RNAi. Downregulation of Survivin expression inhibited CMPC proliferation (ratios are versus respective controls, * p=0.000 vs all other conditions except 'Empty day 2', # p=0.000 versus all other conditions except 'GFP day 2', & p=0.000 versus all other conditions, except '1674 day 2' where p=0.005, $ p=0.000 versus all other conditions except '1673 day 2'). D | Similar assay as in C on CMPCs cultured under hypoxia with or without Survivin RNAi. Downregulation of Survivin expression completely abolished CMPC proliferation (ratios are versus respective controls, * p=0.000 versus all other conditions, # p=0.000 versus all other conditions except '1674 day 2', which is not significantly different, & p=0.000 versus all other conditions except '1673 day 2').

co-localized (figure 2B), confirming correct expression of the virus in CMPCs. Subsequently, Survivin overexpression resulted in more CMPCs positive for BrdU (figure 2C) or the cell proliferation marker Ki-67[27] (figure 2D), compared to GFP controls. This indicates that Survivin enhances mitosis in CMPCs.

Survivin inhibits apoptosis in CMPCs

Since Survivin has been reported to inhibit apoptosis in cardiomyocytes, smooth muscle cells, and endothelial cells[21-23], we analyzed the effect of Survivin expression on apoptosis in cardiomyocyte progenitor cells. GFP- and Survivin-GFP-transduced CMPCs were exposed to anisomycin, previously shown to induce apoptosis in proliferating cancer cells[28,29]. FACS analysis showed a 1.84-fold increase in Annexin V-positive/7-AAD-negative, early apoptotic cells after anisomycin treatment in GFP-transduced CMPCs (figure 3). Overexpression of Survivin resulted in less apoptotic cells (1.36x compared to Survivin control), indicating

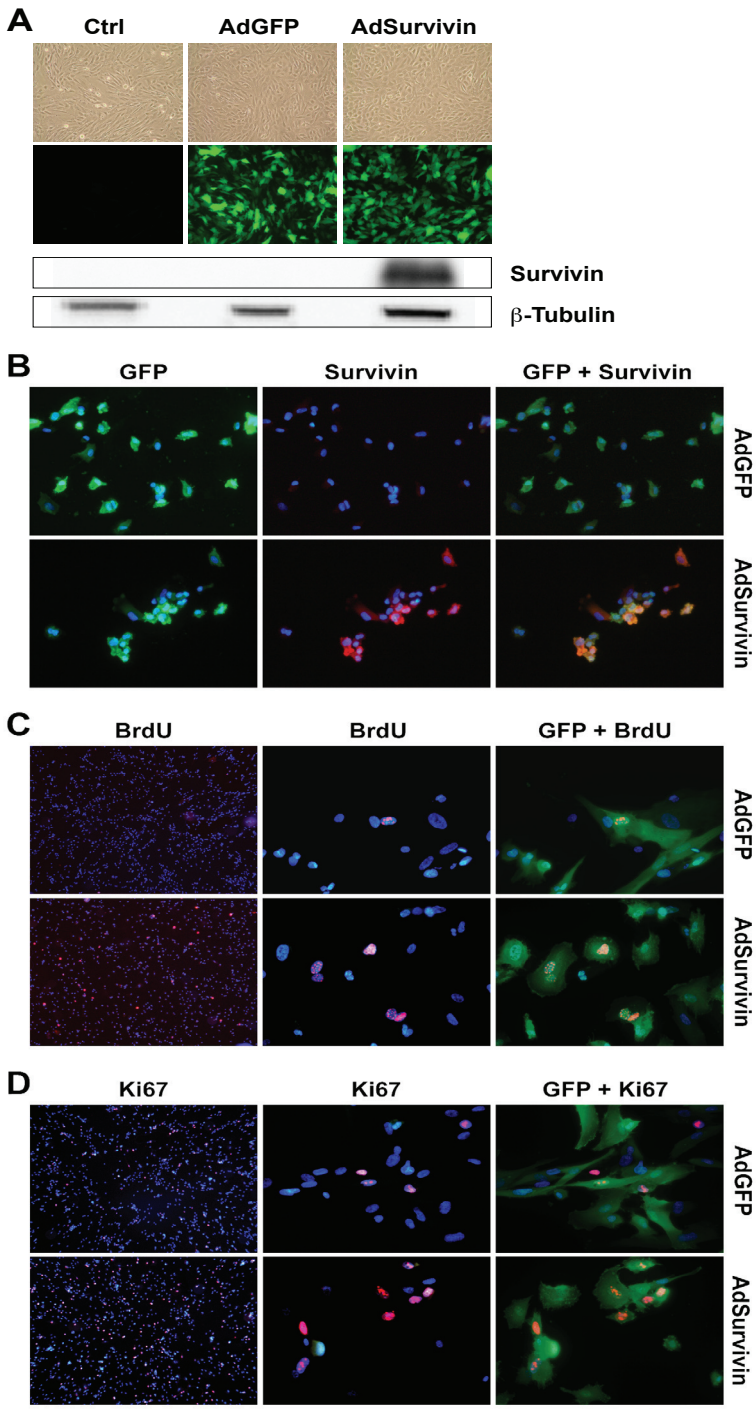

A
Ctrl
AdGFP
AdSurvivin
Survivin
β-Tubulin
B
GFP
Survivin
GFP + Survivin
AdGFP
AdSurvivin
C
BrdU
BrdU
GFP + BrdU
AdGFP
AdSurvivin
D
Ki67
Ki67
GFP + Ki67
AdGFP
AdSurvivin

Figure 2: Survivin enhances proliferation of CMPCs

A | Top: from left to right, non-transduced CMPCs, CMPCs transduced with AdGFP or AdSurvivin-GFP. Bottom: Western blot analysis of Survivin protein expression in the same cells. β-Tubulin was used as loading control. B | Immunostaining of transduced CMPCs showing GFP fluorescence (green) and staining for Survivin (red), with nuclei in blue (200x magnification). C | Immunostaining of transduced CMPCs (left: 4x magnification, middle and right: 400x). BrdU incorporation (red) was increased in Survivin-transduced cells. D | Immunostaining of transduced CMPCs. Survivin overexpression resulted in a higher number of cells expressing Ki-67 (red).

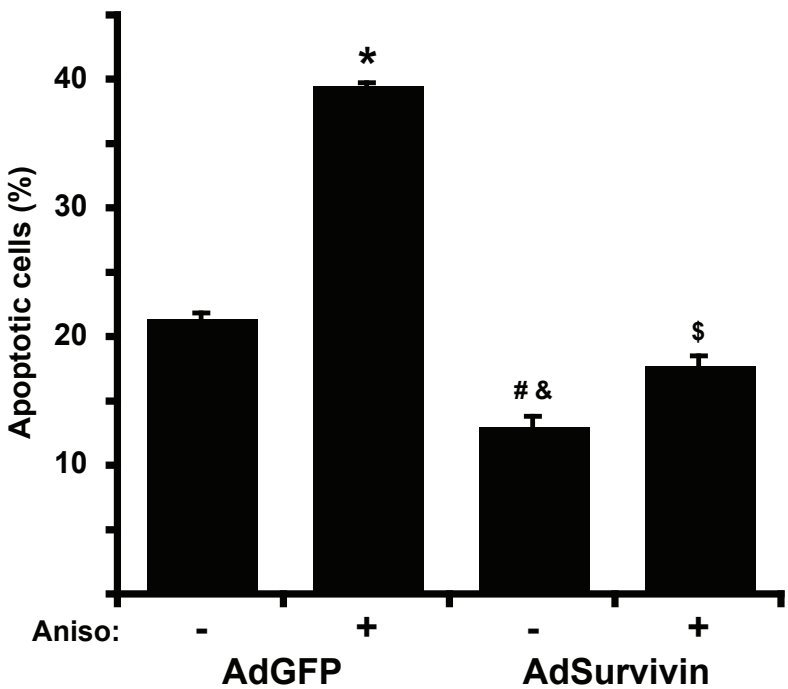
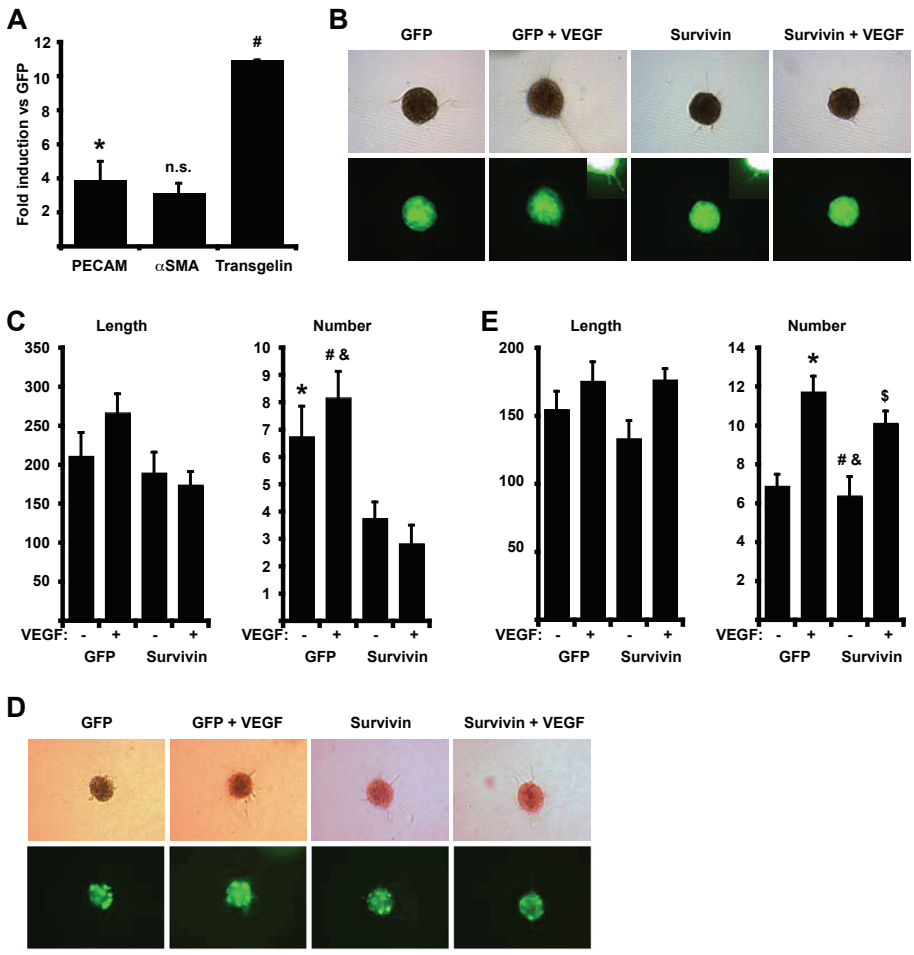


Figure 3: Survivin inhibits apoptosis in CMPCs

Relative number of early apoptotic, Annexin V-positive/7-AAD negative CMPCs with (+) or without (-) anisomycin treatment (performed in duplo). * P=0.000 vs all other conditions, # p=0.002 vs GFP (-), & p=0.018 vs Survivin (+), $ p=0.046 vs GFP (-).



that Survivin inhibits the induction of apoptosis. Similar results were obtained with H_2O_2 treatment (not shown).

Survivin decreases sprouting frequency, but not capacity in CMPCs

For optimal stem cell therapy, increased proliferation and survival of transplanted cells in the infarct or peri-infarct region is as important as the potential of transplanted cells to contribute to enhanced blood flow. Previously, we have shown that CMPCs are able to form tube-like structures in a Matrigel assay with increased expression of α-smooth muscle actin (αSMA) and vascular

Figure 4: Survivin affects sprouting in CMPCs

A | Quantification (in duplo) showing increased mRNA expression of PECAM (3.9x), αSMA (3.1x) and Transgelin (11x) in Survivin-overexpressing CMPCs (* p=0.022, $^{n.s.}$ not significant (p=0.093), and $^{\#}$ p=0.000 vs respective GFP controls). B | CMPCs transduced with AdGFP or AdSurvivin-GFP in a sprouting assay with or without VEGF (top: brightfield, bottom: GFP fluorescence, 100x magnification). Insets: longer exposure to GFP fluorescence shows migration of GFP-positive cells into the 3D-matrix. C | Quantification of B (n=9). VEGF enhances sprouting frequency in GFP control, but not in Survivin-overexpressing CMPCs. Survivin decreases average sprout number per aggregate compared to GFP-CMPCs. * P=0.035 vs Survivin (+), $^{\#}$ p=0.005 vs Survivin (-), $^{\&}$ P=0.002 vs Survivin (+). D | HUVECs transduced with AdGFP or AdSurvivin-GFP in a sprouting assay with or without VEGF. E | Quantification of D (n=13). Survivin did not affect average sprout length of number, while VEGF increased both in GFP and Survivin transduced cells. * P=0.000 vs GFP (-), $^{\#}$ p=0.000 vs GFP (+), $^{\&}$ P=0.003 vs Survivin (+), $^{\$}$ p=0.015 vs GFP (-).

endothelial (VE)-cadherin[11]. To test if Survivin overexpression influenced the angiogenic potential of CMPCs, we performed quantitative RT-PCR on Survivin-transduced CMPCs. Compared to GFP control, Survivin overexpression resulted in increased expression of platelet/endothelial cell adhesion molecule (PECAM), αSMA, and Transgelin (also known as SM22α, figure 4A), suggesting an increased angio- and arteriogenic potential in Survivin overexpressing CMPCs[30].

To functionally confirm a Survivin-induced increase in angiogenic potential, we performed a 3D-sprouting assay[31]. As shown in figure 4B, CMPCs transduced with GFP-control or GFP-Survivin formed compact aggregates that were able to sprout in different directions when seeded into a 3D collagen matrix. Quantification of the sprouts shows that VEGF moderately enhances the average sprout length and number per aggregate in the GFP control (figure 4C). However, Survivin transduced cells did not show this VEGF-induced increase in sprouting. Interestingly, Survivin overexpression reduced sprout number, but not length, compared to GFP control.

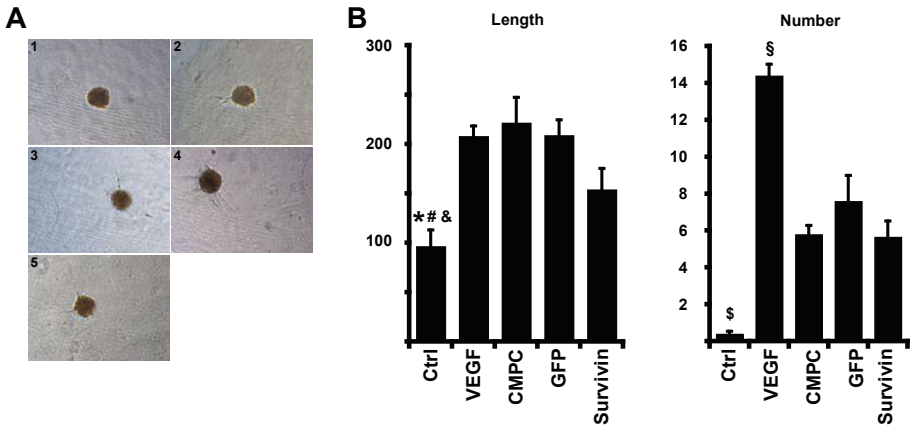


Figure 5: Paracrine effects of Survivin-transduced CMPCs

A | Brightfield images of a HUVEC sprouting assay. During the assay, HUVECS were either not stimulated (1, Ctrl) or stimulated with VEGF (2), conditioned medium from non-transduced CMPCs (3: CMPC), or CMPCs transduced with AdGFP (4) or AdSurvivin-GFP (5). B | Quantification of A (n=10). Compared to AdGFP-CMPCs, conditioned medium from AdSurvivin-CMPCs did not significantly affect HUVEC sprouting. * P=0.027 vs VEGF, # p=0.009 vs CMPC, & P=0.003 vs GFP. $ p=0.000 vs all other conditions, § p=0.000 vs all other conditions.

To determine if the effect Survivin has on CMPC behavior is a general mechanism or specific for the CMPCs, we transduced HUVECs with GFP or Survivin-GFP and tested their sprouting capacity. In contrast to CMPCs, Survivin overexpression did not significantly affect the average sprout length or number of HUVEC-derived spheroids, while VEGF treatment still resulted in increased sprouting in both GFP control and Survivin overexpressing cells (figure 4D, E).

Paracrine signaling by Survivin overexpression in CMPCs

Previous studies indicated that paracrine signaling is an important mechanism for the beneficial effects seen after transplantation of genetically engineered stem cells[32,33]. To determine if Survivin overexpression affects paracrine signaling by CMPCs, conditioned

medium from transduced CMPCs was used to stimulate HUVECs in 3D sprouting assays. VEGF as well as conditioned medium from CMPC, CMPC plus AdGFP, and CMPC plus AdSurvivin enhanced sprouting (figure 5A, B). Although a slightly lower average HUVEC sprout length could be seen with conditioned medium from Survivin-CMPCs compared to GFP-CMPCs, this was not significant. There was also no significant difference in average HUVEC sprout number with conditioned medium from GFP- compared to Survivin-transduced CMPCs.

DISCUSSION

Stem and progenitor cells have been intensively studied in order to repair the damaged heart after myocardial infarction[5-7,34]. However, transplanted cells often do not survive and are either removed via the circulation or go into apoptosis due to the hypoxic and hostile environment in post-MI tissue[13].

In CMPCs, hypoxia enhanced proliferation via the induction of Survivin. Previously, rat bone marrow-derived MSC showed increased proliferation *in vitro* under hypoxic conditions as well[35], similar to embryonic hematopoietic progenitors[36]. Additionally, hypoxic cultured hematopoietic stem cells (HSCs) engraft and repopulate the hematopoietic system more efficiently than normoxic cultured HSCs[37]. Together, this may suggest that preconditioning CMPCs under hypoxic conditions may help to improve post-transplant survival and contribution to tissue repair as well.

We further showed that Survivin overexpression enhances proliferation and prevents apoptosis in cardiovascular progenitor cells. However, we also observed a decreased average number, but not length of sprouts in CMPCs during an angiogenesis assay. Probably, increasing proliferation reduces the frequency, but not capacity of CMPCs to sprout. For comparison, MSCs stably expressing the pro-survival gene Akt show reduced expression of several genes related to matrix remodeling, especially when cultured under hypoxia[38], suggesting that Akt-MSCs may also have

an impaired migration capacity. This effect and the responsible mechanisms should be investigated in more detail however.
Akt-MSCs have been shown to increasingly attenuate cardiac remodeling compared to MSCs alone[25]. It was subsequently shown that growth factors secreted by Akt-MSCs actually mediated the improvement in cardiac function[32,33]. The key factor that was secreted by Akt-MSCs was identified as the secreted frizzled related protein Sfrp2[38]. Interestingly, Sfrp2 also increased expression of the IAP protein BIRC1b/NAIP2 in hypoxic rat cardiomyocytes *in vitro*. Whether BIRC5/Survivin was also differentially expressed was not reported. Our results in CMPCs support that the beneficial effect of Akt-MSC transplantation is mainly mediated by increased paracrine signaling and not enhanced expression of intrinsic mitogenic and anti-apoptotic factors like Survivin.
In summary, we show that hypoxia induces Survivin overexpression in CMPCs, which subsequently enhances their proliferation and prevents apoptosis. Sprouting frequency, but not capacity, was reduced in Surivivin-CMPCs under angiogenic conditions. Although CMPCs can enhance HUVEC sprouting via paracrine signaling, Survivin overexpression did not result in a synergistic effect. However, an increased number of CMPCs due to enhanced proliferation and survival may still have a beneficial effect on angiogenesis via prolonged paracrine signaling.

MATERIALS AND METHODS

Cell isolation and culture

CMPCs from human fetal hearts were isolated by MACS and cultured as described[11,39]. Informed consent procedures were followed and prior approval of the ethics committee of the University Medical Center Utrecht was obtained. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and cultured on fibronectin-coated plates in EGM-2 medium (Cambrex), supplemented with 10% FCS (Gibco) and penicillin-streptomycin (PenStrep, 100U/ml each, Gibco).
For hypoxia experiments, CMPCs were cultured under normoxia

(20% O_2) or hypoxia (1% O_2) for four days in culture medium containing 0.5 % FCS, after which cells were used for MTT assays or RNA isolation followed by qRT-PCR.

Adenoviral transduction

Cells were transduced with AdGFP control or AdSurvivin-GFP. The AdSurvivin-GFP plasmid contains separate CMV promoters for GFP and Survivin[40] (kindly provided by Prof. D. Altieri and Dr. T. Dohi, University of Massachusetts Medical School, Worcester, USA). Transduced cells were isolated, fixated, or used for subsequent experiments minimally two days post-transduction. Conditioned medium was obtained by culturing (transduced) cells for two days in 0.5% culture medium, after which the medium was isolated and filter-sterilized.

Proliferation assays

To determine proliferation, transduced cells were incubated overnight with 10 μM BrdU (Calbiochem) in culture medium containing 2% FCS and subsequently fixed for immunocytochemistry.

For MTT assays, CMPCs were similarly cultured for two days, after which cells were cultured for three hours in serum-free medium containing 500 μg/ml Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma). Next, cells were washed in PBS and lyzed in Dimethylsulfoxide (DMSO, MP Biomedicals) for 15 minutes at roomtemperature. Optical density was measured at 540 nm with reference at 690 nm on a microplate reader (Model 550, BioRad).

Sprouting assays

In vitro sprouting assays were performed as described previously[31], with minor adaptations. Aggregates were formed by overnight culture of 400 (previously transduced) cells per well in a non-adherent round-bottom 96-wells plate (Greiner). The following day, aggregates were embedded in collagen/methylcellulose gels as described[31]. Following polymerization of the gel (approximately 30 minutes), serum free medium with or without 50 ng/ml VEGF was added. After overnight culture, sprouts were fixed for

30 minutes in 10% formaldehyde and pictures were taken for quantification of sprout length and number. The level of sprouting per aggregate was determined by drawing lines over sprouts in Cell^P (Olympus) and quantifying the number of pixels.

Apoptosis induction and FACS analysis

Two days after adenoviral transduction of CMPCs with AdGFP or AdSurvivin-GFP, apoptosis was induced by 24h exposure to 10 ug/ml anisomycin (Sigma) in culture medium containing 2% FCS. The following day, CMPCs were washed with PBS plus 2 mM EDTA, trypsinized briefly, and resuspended in culture medium. Trypsinized CMPCs were washed with PBS and resuspended in Annexin V binding buffer (BD Pharmingen). Subsequently, CMPCs were stained with Annexin V-PE antibody (BD Pharmingen) and 7-Amino-actinomycin (7-AAD, BD Pharmingen) for >15 minutes in the dark and analysed using a Beckman Coulter Cytomics FC500 FACS.

Immunocytochemistry and Western blot analysis

Immunocytochemistry and Western blot analysis were performed as described previously[11]. Used antibodies were for BrdU (Calbiochem), human Ki-67 (DAKO), Survivin (R&D Systems, kindly provided by Dr. Lens, Dept. of Medical Oncology, UMC Utrecht, the Netherlands[41]), and β-Tubulin (Cell Signaling). Hoechst was used for staining of nuclei.

RNA interference

CMPCs were transduced with lentivirus expressing shRNA for human Survivin from the MISSION RNAi library (Sigma) and cultured overnight in the presence of 4 µg/ml polybrene. The following day, the medium was refreshed and 1 µg/ml puromycin was added for selection of transduced cells. After selection for four days, the expression of Survivin was analyzed by RT-PCR and their growth was analyzed using an MTT assay.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

Cells were lyzed in TriPure (Roche). Total RNA was isolated and DNAse treated (Amersham Biosciences). Five hundred ng total

RNA was used for cDNA synthesis with iScript cDNA synthesis kit (BioRad). For qRT-PCR, 10 μl cDNA (1:20 diluted) was mixed with 10 μl SYBR-Green mix (BioRad) and forward plus reverse primers (final concentration 0.5 μM each) and run on a MyiQ iCycler (BioRad). PCR conditions were: 2 min at 94 °C followed by 40 cycles of: 30 sec at 94 °C, 30 sec at annealing temperature (see table 1), and 30 sec at 72 °C. Amplicon quantities were determined by comparison with known quantities of cloned PCR products and are expressed in pg. Specificity of amplification for the detection with SYBR-Green was visually checked on PAGE gels or melting curve analysis after the PCR run. Primers were designed with Beacon Designer 4.0 (Premier Biosoft International). Primer sequences and annealing temperatures are given in table 1. Expression levels were normalized to β-actin expression.

Statistics

All data are presented as average ± SEM. Number of replicates is indicated in the figure legends. Data was analyzed with a Student's T-test or ANOVA with Bonferroni post-hoc analysis for group comparisons. Significance was assumed when $P<0.05$.

Acknowledgements

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Table 1: Primer sequences and annealing temperatures

Name	*Sequence*	*Annealing temperature*
Survivin For	GAG ACA GAA TAG AGT GAT AGG	60 ^{0}C
Survivin Rev	GAC AGA TGT GAA GGT TGG	
PECAM For	GCA GTG GTT ATC ATC GGA GTG	60 ^{0}C
PECAM Rev	TCG TTG TTG GAG TTC AGA AGT G	
αSMA For	ACT GGG ACG ACA TGG AAA AG	60 ^{0}C
αSMA Rev	TAG ATG GGG ACA TTG TGG GT	
Transgelin For	ATC ATC AGT TAG AGC GGA GAG G	68 ^{0}C
Transgelin Rev	ATG CTT TCG GGT AAG AAG TTG G	
β-Actin For	GAT CGG CGG CTC CAT CCT G	60 ^{0}C
β-Actin Rev	GAC TCG TCA TAC TCC TGC TTG C	

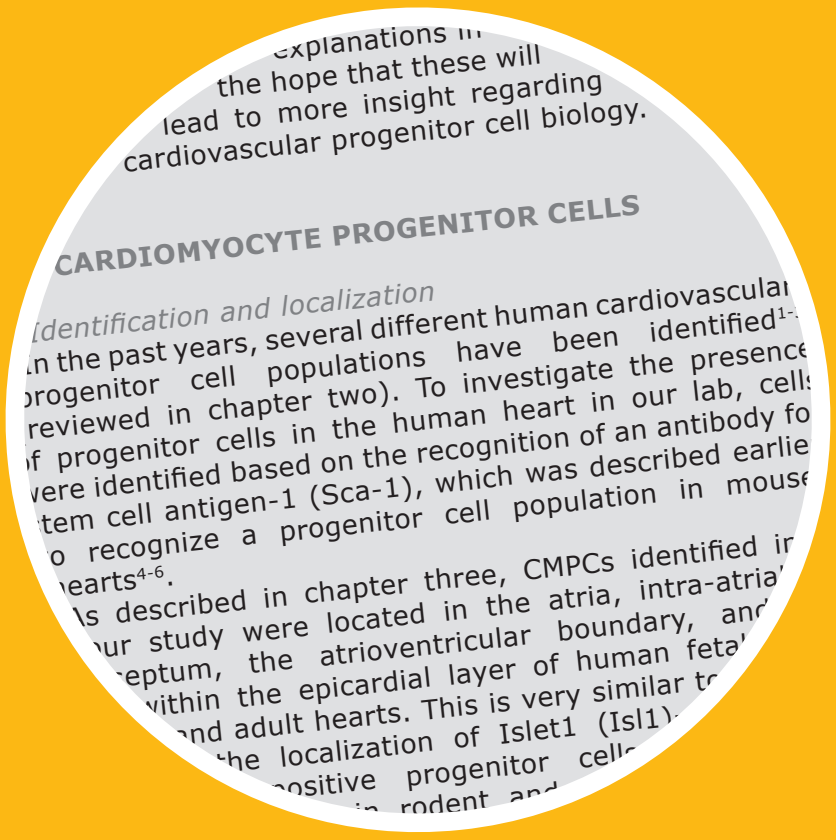
REFERENCES

1. Rosamond, W. *et al.* Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **117**, e25-146 (2008).
2. Jugdutt, B.I. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? *Circulation* **108**, 1395-403 (2003).
3. Abdel-Latif, A. *et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med* **167**, 989-97 (2007).
4. Breitbach, M. *et al.* Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* **110**, 1362-9 (2007).
5. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).
6. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* **50**, 19-30 (2008).
7. Segers, V.F. & Lee, R.T. Stem-cell therapy for cardiac disease. *Nature* **451**, 937-42 (2008).
8. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
9. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
10. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
11. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
12. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
13. Zhang, M. *et al.* Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol* **33**, 907-21 (2001).
14. Salvesen, G.S. & Duckett, C.S. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* **3**, 401-10 (2002).
15. Johnson, M.E. & Howerth, E.W. Survivin: a bifunctional inhibitor of apoptosis protein. *Vet Pathol* **41**, 599-607 (2004).

16. Lens, S.M., Vader, G. & Medema, R.H. The case for Survivin as mitotic regulator. *Curr Opin Cell Biol* **18**, 616-22 (2006).
17. Altieri, D.C. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer* **8**, 61-70 (2008).
18. Adida, C. *et al.* Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *Am J Pathol* **152**, 43-9 (1998).
19. Ma, A. *et al.* The role of survivin in angiogenesis during zebrafish embryonic development. *BMC Dev Biol* **7**, 50 (2007).
20. Zwerts, F. *et al.* Lack of endothelial cell survivin causes embryonic defects in angiogenesis, cardiogenesis, and neural tube closure. *Blood* **109**, 4742-52 (2007).
21. O'Connor, D.S. *et al.* Control of apoptosis during angiogenesis by survivin expression in endothelial cells. *Am J Pathol* **156**, 393-8 (2000).
22. von Wnuck Lipinski, K. *et al.* Integrin-mediated transcriptional activation of inhibitor of apoptosis proteins protects smooth muscle cells against apoptosis induced by degraded collagen. *Circ Res* **98**, 1490-7 (2006).
23. Levkau, B. *et al.* Survivin determines cardiac function by controlling total cardiomyocyte number. *Circulation* **117**, 1583-93 (2008).
24. Papapetropoulos, A. *et al.* Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* **275**, 9102-5 (2000).
25. Mangi, A.A. *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* **9**, 1195-201 (2003).
26. Simon, M.C. & Keith, B. The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Biol* **9**, 285-96 (2008).
27. Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**, 311-22 (2000).
28. Santibanez, J.F. & Hurtado, C. Ha-Ras sensitizes transformed mouse skin cells to Anisomycin-induced apoptosis. *FEBS Lett* **579**, 6459-64 (2005).
29. Abayasiriwardana, K.S. *et al.* Malignant mesothelioma cells are rapidly sensitized to TRAIL-induced apoptosis by low-dose anisomycin via Bim. *Mol Cancer Ther* **6**, 2766-76 (2007).
30. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* **6**, 389-95 (2000).
31. Korff, T. & Augustin, H.G. Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci* **112 (Pt 19)**, 3249-58 (1999).

32. Gnecchi, M. *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* **11**, 367-8 (2005).
33. Gnecchi, M. *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J* **20**, 661-9 (2006).
34. Passier, R., van Laake, L.W. & Mummery, C.L. Stem-cell-based therapy and lessons from the heart. *Nature* **453**, 322-9 (2008).
35. Lennon, D.P., Edmison, J.M. & Caplan, A.I. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol* **187**, 345-55 (2001).
36. Adelman, D.M., Maltepe, E. & Simon, M.C. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes Dev* **13**, 2478-83 (1999).
37. Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A. & Simon, M.C. Expansion of human SCID-repopulating cells under hypoxic conditions. *J Clin Invest* **112**, 126-35 (2003).
38. Mirotsou, M. *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* **104**, 1643-8 (2007).
39. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
40. Mesri, M., Wall, N.R., Li, J., Kim, R.W. & Altieri, D.C. Cancer gene therapy using a survivin mutant adenovirus. *J Clin Invest* **108**, 981-90 (2001).
41. Lens, S.M. *et al.* Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *Embo J* **22**, 2934-47 (2003).

CHAPTER 9


explanations in
the hope that these will
lead to more insight regarding
cardiovascular progenitor cell biology.

CARDIOMYOCYTE PROGENITOR CELLS

Identification and localization

In the past years, several different human cardiovascular
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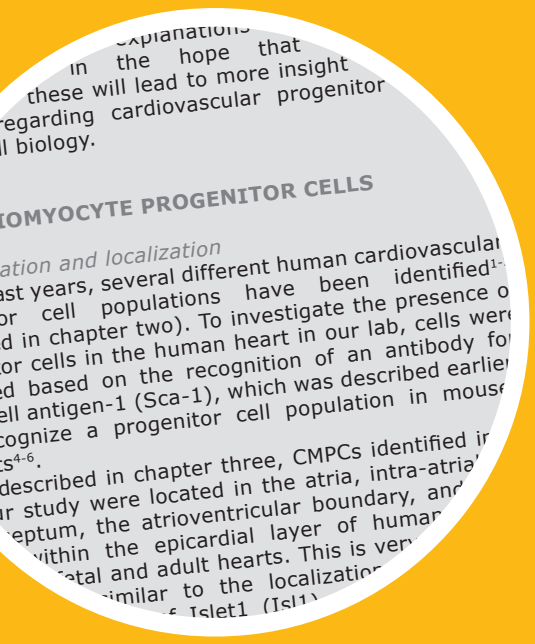
CHAPTER 9

General discussion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The identification and isolation of human cardiomyocyte progenitor cells (CMPCs) has allowed us to characterize this population and study several mechanisms involved in progenitor cell proliferation and myocyte differentiation. The results from these experiments are reported in this thesis. Here, we will put those studies in perspective and propose several explanations in the hope that these will lead to more insight regarding cardiovascular progenitor cell biology.

CARDIOMYOCYTE PROGENITOR CELLS

Identification and localization

In the past years, several different human cardiovascular progenitor cell populations have been identified[1-3] (reviewed in chapter two). To investigate the presence of progenitor cells in the human heart in our lab, cells were identified based on the recognition of an antibody for stem cell antigen-1 (Sca-1), which was described earlier to recognize a progenitor cell population in mouse hearts[4-6].

As described in chapter three, CMPCs identified in our study were located in the atria, intra-atrial septum, the atrioventricular boundary, and within the epicardial layer of human fetal and adult hearts. This is very similar to the localization of Islet1 (Isl1)-positive progenitor cells in rodent and human heart[2].

Isolation, characterization, and cardiomyogenic differentiation

By dissociating human heart tissue and either clonal expansion of single cells or labeling dissociated cells with a mouse Sca-1 antibody, we were able to isolate a population that phenotypically and genetically resembles a progenitor cell type (chapter three and[7]). By using culture medium with high serum concentrations plus a mix of several growth factors, these cells could be studied without the need for co-culture with a feeder layer of fibroblasts or rat cardiomyocytes, thereby preventing confounding and unidentified effects of co-cultured cells when studying the potential of these progenitor cells. After isolation, a high percentage of

CMPCs expressed Isl1 and c-Kit, previously used by other groups to define cardiovascular progenitor cells[2,3]. Additionally, they expressed early cardiac transcription factors Nkx2.5, Gata-4, and Mef2c, suggesting that they are already committed to a cardiac lineage. CMPCs also expressed telomerase, required for telomere extension after cell cycling and prevention of senescence. FACS analysis showed that they were not hematopoietic or endothelial progenitor cells. Although we routinely isolated progenitor cells from whole fetal hearts or adult atrial tissue, isolation of CMPCs from adult ventricular tissue was unsuccessful.

CMPCs underwent mitosis approximately every five hours (based on the proliferation index in chapter five), enabling us to grow these cells in sufficiently high numbers for subsequent experimental studies and/or transplantation into the heart. However, to contribute to cardiac tissue after cell transplantation, these progenitor cells should still able to differentiate into cardiomyocytes. Ideally, cardiomyocytes should express functional sarcomeric proteins for contraction, gap junction proteins for cellular coupling, and ion channels for maintaining their resting membrane potential and mediating the action potential. To determine the cardiomyogenic potential of CMPCs, the cells were exposed to a demethylating agent, 5-azacytidine, which has been shown to induce cardiomyogenic differentiation in P19 embryonic carcinoma cells[8], human ES cells[9], human MSCs[10], and mouse cardiac progenitor cells[4]. When subsequently placed in differentiation medium containing low serum levels, the cells differentiated into cardiomyocytes expressing sarcomeric proteins in a typical striated pattern and gap junction proteins connexin 43 and 45 at the cell membrane border, enabling functional coupling between cells.

To further improve differentiation efficiency, we explored the effect of stimulation by members of the TGFβ superfamily. TGFβ signaling induces cardiomyogenic differentiation in P19 embryonic carcinoma cells[11], ES cells[12], and bone marrow stem cells[13]. In CMPCs, TGFβ stimulation alone resulted in 31% α-actinin-positive cardiomyocytes (determined by FACS analysis) in four out of six separate differentiation experiments. After sequential stimulation of CMPCs by 5-azacytidine and TGFβ, differentiation into

cardiomyocytes was greatly enhanced, with more than 90% of cardiomyocytes in 78 out of 79 experiments[7]. The spontaneously beating cardiomyocytes showed excitation-contraction coupling, an action potential resembling fetal/early neonatal ventricular cardiomyocytes, and responded to β-adrenergic stimulation by isoproterenol[7]. Additional electrophysiological analysis showed increased functional expression of sodium, calcium, and potassium channels and pumps, mediating a rather mature ventricular action potential (TP de Boer, unpublished data). Together, this suggests that CMPC-derived cardiomyocytes can functionally couple with host cardiomyocytes after transplantation and contract synchronously. Because of their differentiation into functional cardiomyocytes, these progenitor cells were termed cardiomyocyte progenitor cells (CMPCs).

The overall epigenetic changes that occur after 5-azacytidine treatment are substantial. Although we observed a lower number of phosphorylated histone 3-positive CMPCs (indicating decreased mitosis[14]), and increased expression of several cardiac transcription factors (chapter three), it remained unclear to what extend overall gene expression was altered. In cancer cells, 5-azacytidine enhances TGFβ-receptor expression[15]. However, in CMPCs, 5-azacytidine results in decreased TGFβ-receptor expression (unpublished data), indicating that increased susceptibly to TGFβ-stimulation is probably not involved in enhanced CMPC differentiation. Instead, 5-azacytidine may result in a more general chromatin remodeling and, subsequently, dedifferentiation of CMPCs towards a more pluripotent state as was previously shown for partially differentiated ES cells[16]. This then may lead to more efficient differentiation into cardiomyocytes in response to TGFβ.

Another common determinant of progenitor cell function is BMP signaling, which is known to play a major role during heart development[17] and stem cell differentiation[12,18]. Isl1-specific knock out of the BMP receptor 1a (BMPR1a) resulted in proliferative and cardiomyogenic defects, leading to premature death *in utero*[19]. Differentiation of Sca-1^{+}/BMPR1a-negative progenitors was partially

disrupted[4], indicating an upstream requirement for BMP. In CMPCs, BMP induced differentiation with 28% efficiency[7]. Identification of downstream effects of TGFβ and BMP signaling may help to improve CMPC differentiation efficiency (see chapter seven).
Other factors may affect CMPCs as well. Oxytocin, reported to induce cardiomyogenic differentiation in Sca-1$^+$ mouse progenitor cells[5], induced differentiation in CMPCs with 3-5% efficiency (MJ Goumans, unpublished data). Stimulation with the HDAC inhibitor Trichostatin A (TSA) enhances the initial phase of cardiomyogenic differentiation in P19 and ES cells[20,21], but not in CMPCs (M Roccio, unpublished data).

Global gene expression analysis during CMPC cardiomyogenic differentiation

Elucidation of important mechanisms regulating proliferation and differentiation is needed in order to gain a better understanding of CMPC potential. Microarray analysis at one-week intervals of CMPC differentiation was performed and showed differential expression of transcriptional and translational regulators, cell cycle mediators, and cardiomyocyte-related genes (chapter four). Expected patterns regarding cardiomyogenesis, such as the substantial increase of sarcomere-related gene expression between week two and three post-induction, could be observed, confirming that CMPCs develop a cardiomyocyte-specific gene expression profile.
However, some less conventional patterns were observed as well. Previous experiments showed an increase in Mef2c and Nkx2.5 expression early during differentiation[7], while in the analysis described in chapter four, the biggest increase was observed between week two and three. Although this supports a transcription factor-induced increase in sarcomere expression at that time-point, a different temporal expression in the different studies suggests that the timing of CMPC differentiation may be variable, even though the end result seems to be similar. The direct cause for this phenomenon is currently unknown, but may be influenced by differences between isolations, serum, or even minor changes in culture conditions. Thus, the standardization of

CMPC isolation, culture, and differentiation[22] as well as validation of the observations described above are of great importance.
The clustering of genes per biological process or molecular function, as well as analysis of transcription factor binding sites in genes that were differentially expressed sheds light on signaling cascades that may determine CMPC differentiation. It is likely that several pathways act simultaneously at specific time points, while others require sequential activation. Elucidation of their synergism or competition may help to further improve CMPC proliferation, differentiation, and applicability for cell transplantation.

Cardiomyocyte progenitor cell multipotency

Progenitor cells attain different characteristics during embryonic development and post-natal and adult life. Since CMPCs are isolated from human heart tissue, it is important to gain insight into the clonal variation between different donors and possible differences resulting from tissue origin. As discussed in chapter five, adult CMPCs differentiated into electrophysiologically more mature and quiescent cardiomyocytes compared to fetal CMPC-derived cardiomyocytes. Block of potassium inward rectifiers, which are required for repolarization and maintenance of a stable resting membrane potential, resulted in more spontaneous beating adult CMPC-derived cardiomyocytes (chapter five). Co-culture with a potassium inward rectifier-overexpressing embryonic kidney cell line (KWGF cells[23]) showed that hyperpolarization of fetal CMPC-derived cardiomyocytes inhibited spontaneous contractions. Together, this suggests that spontaneous beating may result, in part, from an unstable resting membrane potential.
The differentiation potential towards non-cardiomyocyte mesodermal cell types is higher in fetal heart-derived CMPCs than in adult. When CMPCs were cultured under angiogenic conditions, tube-like structures containing endothelial cells and smooth muscle cells were formed[7] (chapters five and eight). Fetal CMPCs also demonstrated more adipogenic potential than adult, while both failed to differentiate into bone (chapter five). The variation between different populations from a similar donor age was low, indicating that our isolation protocol[22] yields similar populations.

Whether the discrepancy in developmental potential of fetal and adult CMPCs has an intrinsic molecular basis is currently unknown, but not unlikely, since also basal gene expression in MSCs obtained from different developmental stages has shown to be predictive for the developmental potential of these cells[24]. Higher basal osteogenic gene expression in fetal MSCs resulted in increased calcium deposition, representative for bone formation, when compared to adult MSCs. Microarray analyses of undifferentiated fetal versus adult CMPCs may therefore shed more light on the differences between these populations and hint towards signaling cascades that determine their potential.

MECHANISMS OF DIFFERENTIATION

The characterization of CMPCs and their potential to differentiate into several mesodermal cell types has been important to appreciate the value of this cell population (chapters two to five), However, to further improve culture and differentiation methods and understand the processes that are involved, more detailed examination of regulatory mechanisms needed to be performed. We have therefore investigated the role of micro-RNAs (chapter six) and a mechanism that may help to explain the enhanced differentiation by TGFβ stimulation (chapter seven).

Micromanaging CMPCs and cardiomyogenic differentiation

The role of non-coding RNA molecules that affect translation of messenger RNA towards protein was shown to be of great importance during development. These so-called micro-RNAs (miRs, see figure 1) are involved in balancing gene expression. Altered expression of miRs leads to profound defects, especially in stressful conditions (reviewed in[25]).

Several miRs have been described that play a role in cardiogenesis (reviewed in[26,27]). For instance, miR-1 regulates cardiomyocyte proliferation by targeting the transcription factor Hand2. Subsequently, miR-1 overexpression[28] and knockout[29] lead to cardiac hypoplasia and hyperplasia, respectively.

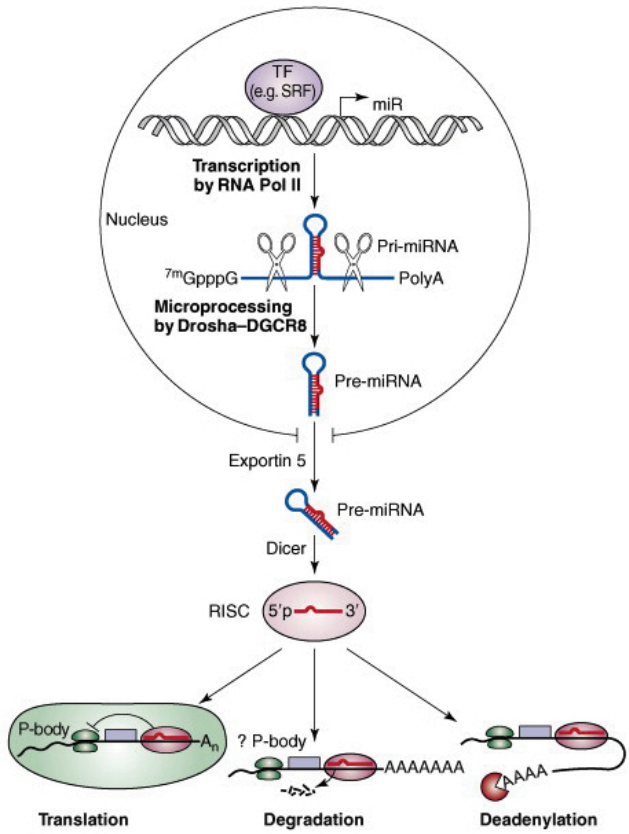


Figure 1: Micro-RNA generation and processing

Micro-RNAs are transcribed by RNA polymerases generating long (over 1000 basepairs) pri-miRs, which are processed by a nuclease complex containing Drosha to form 60-80 basepair long double stranded hairpin precursors called pre-miRs. After active translocation to the cytoplasm via exportin-5, these pre-miRs are processed by another nuclease complex containing Dicer to form single stranded, 20-24 nucleotides long mature miRs. When incorporated into a RNA-Induced Silencing Complex (RISC), these mature miRs bind to complementary mRNA sequences, mostly in the 3' untranslated region (UTR). This then leads to inhibition of mRNA translation to protein via different mechanisms[65]. Although miRs can be co-transcribed with the gene sequence wherein they are located, some miRs have their own promoters, with transcription factors and cofactors regulating their expression[28]. This places miRs within a complex signaling cascade with upstream and downstream regulation. Picture taken from[26].

To determine which micro-RNAs may be involved in differentiation of CMPCs, a screen was performed to analyze miR expression in proliferating, undifferentiated CMPCs versus CMPC-derived cardiomyocytes (chapter six). Several miRs were differentially expressed and to test whether these miRs were able to drive CMPC differentiation, the two highest differentially expressed miRs, miR-1 and miR-499, were selected for further analysis.

MiR-1 is encoded on two separate chromosomes expressing miR-1-1 and miR-1-2. Analysis of the miR-1-1 and miR-2-2 promoters showed that they are both activated by serum response factor (SRF) but different cofactors, leading to differential expression in the atrial and ventricular cardiomyocytes of the developing heart[28]. In CMPCs, miR-1 overexpression resulted in reduced proliferation, but increased cardiomyogenic gene expression. The array used in our study did not discriminate between miR-1-1 and miR-1-2, but selective stimulation of these isoforms may direct cardiomyogenic differentiation of CMPCs into atrial versus ventricular cardiomyocytes as well, further establishing opportunities for directed differentiation of CMPCs.
Protein expression of the miR-1 downstream target histone deacetylase 4 (HDAC4) was reduced in CMPCs after miR-1 overexpression. In skeletal myoblasts, HDAC4 repression of Mef2c is alleviated by miR-1, resulting in myogenic differentiation[30]. We therefore propose a similar mechanism of miR-1-enhanced differentiation in CMPCs.

The function of miR-499 was unknown. MiR-499 is located in an intron of MYH7Bβ and transcribed simultaneously. Like miR-1, miR-499 enhances cardiomyogenic gene expression. The transcription factor sex determining region Y-box 6 (Sox6) was identified as a miR-499 target based on three putative recognition sites in the 3' UTR of Sox6 and this was confirmed through repressed protein and 3'UTR reporter expression by miR-499 overexpression. RNA interference-mediated inhibition of Sox6 expression induced sarcomeric gene expression. Sox6 can function as both activator and repressor, depending on

which cofactors are bound to the target gene promoter, and has been implicated in terminal differentiation of P19 embryonic carcinoma cells[31]. We therefore propose that miR-499 enhances terminal differentiation in CMPCs through downregulation of Sox6, allowing for differential expression of Sox6 target genes. Since the Sox6 3'UTR also contains a functional miR-1 recognition sequence, a possible complementary role can be expected.

As mentioned earlier, TGFβ enhances cardiomyogenic differentiation in CMPCs[7]. TGFβ and BMP signaling also regulate processing of several pri-miRs via an interaction of regulatory Smad proteins with the Drosha complex[32]. Hypothetically, TGFβ stimulation may therefore lead to increased miR-1 and miR-499 maturation, which subsequently enhances differentiation via repression of HDAC4 and Sox6. Unfortunately, an effect of TGFβ signaling on pri-miR-1 and pri-miR-499 processing specifically was not reported, but is definitely worth investigating.

MiR-133a and miR-133b were also found to be upregulated in CMPC-derived cardiomyocytes, suggesting a similar role during differentiation as for miR-1 and -499 as described in chapter six. However, miR-133a has an inhibitory role on differentiation in mouse skeletal myoblasts by targeting SRF, leading to lower activation of miR-1[30]. In mouse and human ES cells, miR-1 and miR-133 stimulate mesoderm formation, but have opposing effects on cardiac and skeletal muscle transcription factor expression at later stages[33]. Investigation of a functional role for miR-133 in human CMPCs remains to be performed.

Finally, cardiomyogenic differentiation of CMPCs after 5-azacytidine and TGFβ treatment did not result in skeletal myocyte formation, as demonstrated by the lack of MyoD expression[7]. Lack of skeletal muscle differentiation was not tested in miR-1 overexpressing CMPCs. However, miR-1 could enhance the formation of smooth muscle cells by CMPCs in an angiogenesis assay (JPG Sluijter, unpublished data), suggesting that induction of muscle cell formation by CMPCs may require a combination of specific stimuli and the correct culture conditions.

Hyperpolarization-mediated differentiation

Different types of stimuli can lead to cardiomyogenic differentiation in CMPCs. Besides intracellular epigenetic changes by 5-azacytidine (chapter three, four, and five) and post-transcriptional regulation of gene expression by miRs (chapter six), extrinsic signals play an important role.

Human cardiovascular progenitor cells described by other labs require co-culture with neonatal rat cardiomyocytes for cardiomyogenic differentiation. Whether the cellular contact alone, secreted growth factors, or a combination thereof mediates this differentiation remained unexplored thus far. CMPCs do not need co-culture for differentiation, while growth factor signaling by TGFβ greatly enhances differentiation[7].

To understand why TGFβ enhanced differentiation in CMPCs[7], we investigated a novel mechanism (chapter seven). TGFβ was found to induce hyperpolarization in undifferentiated cells and we determined if this hyperpolarization could mediate cardiomyogenic differentiation. Undifferentiated CMPCs were co-cultured with KWGF cells[23]. This resulted in gap junctional communication with and hyperpolarization of CMPCs. Expression of sarcomeric genes was increased in CMPCs after two weeks, suggesting differentiation. Co-culture with control embryonic kidney cells did not result in increased expression, indicating that induction of differentiation was not due to co-culture effects. Induction of hyperpolarization without co-culture also resulted in increased sarcomeric gene expression and even spontaneous beating. Enhanced calcineurin signaling, shortly after hyperpolarization, suggests that this pathway may mediate the hyperpolarization-induced differentiation. We did not observe an additional effect of combined hyperpolarization and TGFβ stimulation (unpublished data), suggesting that these stimuli have no synergistic effect.

The link between hyperpolarization and calcineurin signaling should be further explored. We have tried to identify the ion channel or pump that mediates hyperpolarization. Preliminary experiments suggested that hyperpolarization is not mediated by increased gene expression of Kir2.1, Kir2.2, or Herg (unpublished data). Instead, functional modulation of ion channels or pumps

already present in the cell membrane is more likely to be involved. Dephosphorylation of the inward rectifying potassium channel Kir2.1 leads to hyperpolarization in myoblasts[34]. However, we did not observe increased inward rectifier current after TGFβ-stimulation (unpublished data), suggesting that other channels and/or pumps are required in CMPCs. Likewise, the calcium channels mediating the subsequent intracellular Ca^{2+} increase await elucidation. To increase differentiation efficiency, the relation between the actual number of hyperpolarized cells and differentiated cardiomyocytes should be clarified; if possible, increasing the number of affected cells will enhance differentiation frequency. Since hyperpolarization is relatively easy to induce, this may lead to effective pretreatment of cells before transplantation, if necessary combined with a defined set of growth factors. Finally, it will be important to determine the conservation of this mechanism in cardiovascular progenitor cells isolated by other groups.

PROGENITOR CELL PROLIFERATION AND SURVIVAL

Although investigating mechanisms regulating proliferation and differentiation is intriguing from a developmental point of view, one needs to evaluate the gained knowledge in the light of future clinical application. When we started the CMPC differentiation experiments (this thesis and[7]), our hypothesis was that for optimal cardiac muscle regeneration, we would need to differentiate CMPCs into functional cardiomyocytes prior to their transplantation. In the adult heart, progenitor cells do not respond to injury adequately, thus we expected that ischemic myocardium would not be able to instruct transplanted CMPCs to form a new ventricular wall. However, recent studies have shown that both undifferentiated and differentiated stem/progenitor cells attenuate cardiac remodeling after MI ([35-37] and AM Smits, submitted). In these and other stem cell transplantation models, several millions of cells have been transplanted in rat and mouse hearts (reviewed in[38]). In clinical trials, using bone marrow or

circulation-derived cells, up to some billions of cells have been transplanted (reviewed in[39,40]). Extrapolating these numbers and taking into account the exponential growth curves in chapter five, CMPCs need to be cultured for at least three weeks to obtain one billion cells. Thus, enhanced proliferation capacity will be needed when autologous cells are required in acute settings. Also, the ischemic heart is a hostile environment resulting in apoptosis and necrosis of transplanted cells[41], further complicating the transplantation of progenitor cells. A combination of stem cell and gene therapy may therefore have a beneficial effect on survival and prove more efficient than cell transplantation alone[42,43]. To improve proliferation *in vitro* and increase survival post-injection, inducing the expression of pro-survival factors in CMPCs is a logical step.

Heat shock preconditioning has already been shown to improve survival of transplanted cardiomyocytes[41]. Since CMPCs would be transplanted in a hypoxic area, we subjected the cells to hypoxia *in vitro* and found that proliferation was increased and mediated by the pro-survival gene Survivin (chapter eight and A van Oorschot, unpublished data). Currently, more research is being performed to determine additional effects of hypoxia on CMPCs and has already shown to induce enhanced expression of vascular endothelial growth factor (VEGF)-A and endothelial TEK receptor tyrosine kinase (Tie-2), which are known to be involved in cell survival, migration, and angiogenesis (A van Oorschot, unpublished data).

Survivin has previously been shown to play a role in endothelial cells and cardiomyocytes. Zebrafish lacking Survivin show impaired angiogenesis, but not vasculogenesis, and increased apoptosis in vascular endothelium during development[44]. In mouse, ablation of Survivin expression in Tie1-expressing endothelial cells leads to decreased epithelial-to-mesenchyme transformation, hypoplastic endocardial cushions, and *in utero* heart failure[45]. Deletion of Survivin in αMHC-positive cardiomyocytes resulted in decreased cardiomyocyte proliferation and cardiac hypoplasia *in vivo*[46].

Clearly, Survivin is important for proliferation in endothelial cells and cardiomyocytes, both of which can be formed by CMPCs.

To test whether we could enhance proliferation, survival, and angiogenesis in CMPCs, we overexpressed Survivin by adenoviral transduction. As expected, Survivin overexpression resulted in enhanced mitosis and increased resistance to apoptosis. Although Survivin overexpression did not affect sprout length of CMPCs, sprouting frequency was reduced. Possibly, continued Survivin-enhanced proliferation in CMPCs competes with induction of migration. To determine the effect of Survivin expression on the secretome, we tested if paracrine signaling would lead to increased sprouting of endothelial cells. Unfortunately, we did not observe an enhanced effect on sprouting of HUVECs when grown in the presence of Survivin-CMPC conditioned medium compared to non-transduced CMPC-conditioned medium.
Studies with MSCs have shown that overexpressing the pro-survival gene Akt further enhanced the effect of MSC transplantation post-MI[47-49]. Survivin-enhanced survival of CMPCs in hypoxic areas may extend their presence after transplantation and prolong subsequent paracrine signaling. Enhancing expression of pro-survival factors like Survivin may therefore be useful to improve the effect of CMPCs on cardiac perfusion.

CONCLUSIONS

The identification and isolation of cardiovascular progenitor cells has been a great step forward in our understanding of heart development and the role of progenitor cells therein. Their isolation by different groups has resulted in an incredible amount of studies exploring the developmental potential of these cells and the mechanisms responsible. The isolation and characterization of CMPCs has lead to the studies described in this thesis, as well as application in myocardial infarction models (AM Smits, submitted, EM Winter, submitted). The full extent of the developmental potential of these cells is still under investigation. Current research mainly focuses on the role of micro-RNAs, the influence of hypoxia, and the effects of co-culture and transplantation with other cell types.

In our standard differentiation protocol[22], CMPCs are stimulated with the demethylating agent 5-azacytidine, but its unknown impact on gene expression prevents clinical application of treated CMPCs. Hypoxia (chapter eight) and hyperpolarization (chapter seven) may be useful tools to improve proliferation or differentiation before transplantation. The validation of developmentally important transcription factor combinations and the signaling cascades that are involved (chapter four) will hopefully lead to more efficient differentiation protocols. Furthermore, adult CMPCs will be isolated from patients with known genetic defects. This will allow us to study the effects of these genetic factors in experimental models and hopefully lead to a better understanding of these defects on progenitor cell function and implications during heart development and, finally, myocyte performance in patients.

We were able to isolate CMPCs by clonal expansion and have shown that these cells are similar to CMPCs that are isolated based on the recognition of a mouse Sca-1 antibody[7]. However, an ongoing topic of debate is the identity of the surface protein that is recognized on human cells. This is unlikely to be human Sca-1 since no convincing evidence has been reported regarding a human homologue[50]. Clarification of this issue will be needed and will probably also shed more light on the developmental origin of these cells. Upon isolation, CMPCs express Isl1 and several other transcription factors typical for cardiovascular progenitor cells with a cardiac embryonic origin. Using the same Sca-1 antibody, we were able to isolate proliferating cells from bone marrow or peripheral or umbilical cord blood, but these had a different phenotype and did not differentiate into cardiomyocytes. This indicated that CMPCs are different than blood-derived cells, at least upon isolation.

FUTURE DIRECTIONS

Clinical application of cardiovascular progenitor cells

Stem and progenitor cells have been transplanted in clinical settings for some years now[39] and it is tempting to 'jump the

bandwagon' with cardiovascular progenitor cells. However, before the therapeutic use of progenitor cells can become reality, several issues need to be addressed. These include what cell-type or combinations thereof should be used, how, where, and when they should be applied, whether they can integrate long enough to have any substantial long-term effects, whether their continued presence is even necessary, how they interact and couple with their host environment, and how adverse side effects can be avoided. If anything, previous clinical trials have provided clues regarding safety and optimal route and timing of application, but they have also clearly shown that more knowledge is needed regarding the potential of transplanted cells and mechanisms that help to improve what sometimes seems an already established stem cell treatment. Different stem and progenitor cells have shown the capacity for cardiomyogenic differentiation (reviewed in chapter two). However, besides restoration of lost cardiomyocytes, improved circulation is needed to prevent further ischemic damage and support the subsequent growth of new myocardium.

The optimal cell type has not been identified yet, but in light of their tissue of origin and supposed role in the heart, cardiovascular progenitor cells are a promising option. The synergistic effect of simultaneous injection of CMPCs and EPDCs after MI was shown to prevent cardiac remodeling more than either cell type alone (EM Winter, unpublished data), indicating that a strategic combination of different cell types may be most efficient.

Isolation and expansion of a significant number of cells will be needed and even if substantial improvements are made with regard to proliferation and survival, 'off the shelve' cell populations are preferable in several ways. First of all, a worldwide pool of the optimal cell type can be established, enabling a more focused investigation of important mechanisms and future clinical use. Second, cells can be used much faster since the isolation and propagation, as well as genetic modification, can be performed in advance.

Autologous cell transplantation can help to overcome immunological problems. However, genetic defects present in patients themselves might prevent an efficient use of their own cells. Furthermore, a possible change in immunogenic phenotype during cell culture

(chapter four) should be carefully studied to predict whether autologous cells might pose similar problems as allogeneic cells.

Induced pluripotent stem cells

The use of the recently described induced pluripotent stem (iPS) cells may solve some of these issues. These cells can be derived from somatic cell types by overexpressing a combination of specific pluripotency- and cell cycle-related transcription factors[51,52] (reviewed in[53]). Like ES cells, the iPS cells can develop into cells of all three germ layers and even contribute to tissue formation when transplanted at blastocyst stages to create chimeric mice.

Several groups have analyzed the *in vitro* cardiomyogenic differentiation of iPS cells in more detail. Although differences in timing and efficiency between ES and iPS cells were reported in one study[54], results from other groups[55,56] suggests that this may resemble similar differences found between different ES cell lines[52,57].

Although the efficiency of establishing iPS cells is far from optimal (approximately 0.01% of all targeted cells), a few cells may be enough to establish populations for developmental studies or clinical treatment. Since these cells can be isolated from patients, autologous cell transplantation would be feasible. Establishing iPS cell populations with inherent genetic defects[58] can be used to investigate regulatory mechanisms *in vitro* and *in vivo*. Subsequently, these defects can be targeted in order to treat genetic diseases[59]. Creating non-immunogenic populations may help to prevent rejection of transplanted cells. Such cells may even be prepared before the patient is admitted to the hospital, although other approaches will still be needed in acute settings.

At this moment it is still difficult to decide at which developmental state the iPS cell-derivatives should be harvested when considering transplantation into the heart. Fetal and neonatal cardiomyocytes have been reported to effectively integrate into host tissue[60], but their contribution to vascular cells will be limited. Residual pluripotent cells may form tumors[61,62], so probably a cardiovascular progenitor stage may be optimal in order to achieve the highest degree of vascularization and tissue repair with minimal risk for

deleterious effects. In two studies, iPS-derived Flk-1^{+} cells were differentiated into cardiomyocytes[55,56]. Flk-1^{+} progenitor cells have been reported to form different cardiac cell types[63] and it will therefore be interesting to see whether the isolation of iPS-derived cardiovascular progenitor cells from other developmental stages will lead to more efficient differentiation into cardiac cell types. Alternatively, directed differentiation as in ES cells[64] may be useful to improve the differentiation of iPS cells as well.

In conclusion, the isolation of pluripotent stem cells and multipotent (cardiovascular) progenitor cells has provided the scientific community with great opportunities to study developmental processes. Comparative studies between pluripotent stem cells and tissue-specific progenitor cells such as CMPCs are needed to reveal general mechanisms regulating cell potential and behavior. In the end, this will lead to a better understanding of cardiovascular cell biology and, possibly, therapeutic application of stem/progenitor cells.

REFERENCES

1. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
2. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
3. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
4. Oh, H. *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A* **100**, 12313-8 (2003).
5. Matsuura, K. *et al.* Adult cardiac Sca-1-positive cells differentiate into beating cardiomyocytes. *J Biol Chem* **279**, 11384-91 (2004).
6. Pfister, O. *et al.* CD31- but Not CD31+ cardiac side population cells exhibit functional cardiomyogenic differentiation. *Circ Res* **97**, 52-61 (2005).
7. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
8. Choi, S.C., Yoon, J., Shim, W.J., Ro, Y.M. & Lim, D.S. 5-azacytidine induces cardiac differentiation of P19 embryonic stem cells. *Exp Mol Med* **36**, 515-23 (2004).
9. Xu, C., Police, S., Rao, N. & Carpenter, M.K. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* **91**, 501-8 (2002).
10. Xu, W. *et al.* Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Exp Biol Med (Maywood)* **229**, 623-31 (2004).
11. Lim, J.Y., Kim, W.H., Kim, J. & Park, S.I. Involvement of TGF-beta1 signaling in cardiomyocyte differentiation from P19CL6 cells. *Mol Cells* **24**, 431-6 (2007).
12. Behfar, A. *et al.* Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* **16**, 1558-66 (2002).
13. Li, T.S. *et al.* TGF-beta induces the differentiation of bone marrow stem cells into immature cardiomyocytes. *Biochem Biophys Res Commun* **366**, 1074-80 (2008).
14. Hans, F. & Dimitrov, S. Histone H3 phosphorylation and cell division. *Oncogene* **20**, 3021-7 (2001).

15. Ammanamanchi, S. & Brattain, M.G. 5-azaC treatment enhances expression of transforming growth factor-beta receptors through down-regulation of Sp3. *J Biol Chem* **276**, 32854-9 (2001).
16. Tsuji-Takayama, K. *et al.* Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. *Biochem Biophys Res Commun* **323**, 86-90 (2004).
17. van Wijk, B., Moorman, A.F. & van den Hoff, M.J. Role of bone morphogenetic proteins in cardiac differentiation. *Cardiovasc Res* **74**, 244-55 (2007).
18. Monzen, K. *et al.* Smads, TAK1, and their common target ATF-2 play a critical role in cardiomyocyte differentiation. *J Cell Biol* **153**, 687-98 (2001).
19. Yang, L. *et al.* Isl1Cre reveals a common Bmp pathway in heart and limb development. *Development* **133**, 1575-85 (2006).
20. Karamboulas, C. *et al.* HDAC activity regulates entry of mesoderm cells into the cardiac muscle lineage. *J Cell Sci* **119**, 4305-14 (2006).
21. Kawamura, T. *et al.* Acetylation of GATA-4 is involved in the differentiation of embryonic stem cells into cardiac myocytes. *J Biol Chem* **280**, 19682-8 (2005).
22. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
23. de Boer, T.P. *et al.* Inhibition of cardiomyocyte automaticity by electrotonic application of inward rectifier current from Kir2.1 expressing cells. *Med Biol Eng Comput* **44**, 537-42 (2006).
24. Guillot, P.V. *et al.* Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation* DOI: 10.1111/j.1432-0436.2008.00279.x (2008).
25. Tanaka, E.M. & Weidinger, G. Micromanaging regeneration. *Genes Dev* **22**, 700-5 (2008).
26. Zhao, Y. & Srivastava, D. A developmental view of microRNA function. *Trends Biochem Sci* **32**, 189-97 (2007).
27. Thum, T., Catalucci, D. & Bauersachs, J. MicroRNAs: novel regulators in cardiac development and disease. *Cardiovasc Res* **79**, 562-70 (2008).
28. Zhao, Y., Samal, E. & Srivastava, D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* **436**, 214-20 (2005).
29. Zhao, Y. *et al.* Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* **129**, 303-17 (2007).

30. Chen, J.F. *et al.* The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* **38**, 228-33 (2006).
31. Cohen-Barak, O. *et al.* Sox6 regulation of cardiac myocyte development. *Nucleic Acids Res* **31**, 5941-8 (2003).
32. Davis, B.N., Hilyard, A.C., Lagna, G. & Hata, A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454**, 56-61 (2008).
33. Ivey, K.N. *et al.* MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell* **2**, 219-29 (2008).
34. Hinard, V., Belin, D., Konig, S., Bader, C.R. & Bernheim, L. Initiation of human myoblast differentiation via dephosphorylation of Kir2.1 K+ channels at tyrosine 242. *Development* **135**, 859-67 (2008).
35. Laflamme, M.A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* **25**, 1015-24 (2007).
36. van Laake, L.W. *et al.* Human embryonic stem cell-derived cardiomyocytes survive and mature in the mouse heart and transiently improve function after myocardial infarction. *Stem Cell Res* **1**, 9-24 (2007).
37. van Laake, L.W., Passier, R., Doevendans, P.A. & Mummery, C.L. Human embryonic stem cell-derived cardiomyocytes and cardiac repair in rodents. *Circ Res* **102**, 1008-10 (2008).
38. Passier, R., van Laake, L.W. & Mummery, C.L. Stem-cell-based therapy and lessons from the heart. *Nature* **453**, 322-9 (2008).
39. Abdel-Latif, A. *et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. *Arch Intern Med* **167**, 989-97 (2007).
40. Segers, V.F. & Lee, R.T. Stem-cell therapy for cardiac disease. *Nature* **451**, 937-42 (2008).
41. Zhang, M. *et al.* Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol* **33**, 907-21 (2001).
42. Yau, T.M. *et al.* Enhanced myocardial angiogenesis by gene transfer with transplanted cells. *Circulation* **104**, I218-22 (2001).
43. Askari, A. *et al.* Cellular, but not direct, adenoviral delivery of vascular endothelial growth factor results in improved left ventricular function and neovascularization in dilated ischemic cardiomyopathy. *J Am Coll Cardiol* **43**, 1908-14 (2004).
44. Ma, A. *et al.* The role of survivin in angiogenesis during zebrafish embryonic development. *BMC Dev Biol* **7**, 50 (2007).

45. Zwerts, F. *et al.* Lack of endothelial cell survivin causes embryonic defects in angiogenesis, cardiogenesis, and neural tube closure. *Blood* **109**, 4742-52 (2007).
46. Levkau, B. *et al.* Survivin determines cardiac function by controlling total cardiomyocyte number. *Circulation* **117**, 1583-93 (2008).
47. Mangi, A.A. *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* **9**, 1195-201 (2003).
48. Gnecchi, M. *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* **11**, 367-8 (2005).
49. Gnecchi, M. *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J* **20**, 661-9 (2006).
50. Holmes, C. & Stanford, W.L. Concise review: stem cell antigen-1: expression, function, and enigma. *Stem Cells* **25**, 1339-47 (2007).
51. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-72 (2007).
52. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-20 (2007).
53. Nishikawa, S., Goldstein, R.A. & Nierras, C.R. The promise of human induced pluripotent stem cells for research and therapy. *Nat Rev Mol Cell Biol* **9**, 725-9 (2008).
54. Mauritz, C. *et al.* Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation* **118**, 507-17 (2008).
55. Narazaki, G. *et al.* Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* **118**, 498-506 (2008).
56. Schenke-Layland, K. *et al.* Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. *Stem Cells* **26**, 1537-46 (2008).
57. Osafune, K. *et al.* Marked differences in differentiation propensity among human embryonic stem cell lines. *Nat Biotechnol* **26**, 313-5 (2008).
58. Park, I.H. *et al.* Disease-specific induced pluripotent stem cells. *Cell* **134**, 877-86 (2008).
59. Hanna, J. *et al.* Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* **318**, 1920-3 (2007).

60. Reinecke, H., Zhang, M., Bartosek, T. & Murry, C.E. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation* **100**, 193-202 (1999).
61. Nussbaum, J. *et al.* Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *Faseb J* **21**, 1345-57 (2007).
62. Behfar, A. *et al.* Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. *J Exp Med* **204**, 405-20 (2007).
63. Kattman, S.J., Huber, T.L. & Keller, G.M. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell* **11**, 723-32 (2006).
64. Yang, L. *et al.* Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* **453**, 524-8 (2008).
65. Wu, L. & Belasco, J.G. Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. *Mol Cell* **29**, 1-7 (2008).

Summary

SATISFACTION OF ONE'S CURIOSITY IS ONE OF THE GREATEST SOURCES OF HAPPINESS IN LIFE.

LINUS CARL PAULING

The cells that are required for the structure and functionality of the heart are mostly derived from cardiovascular progenitor cells. Although these cells were generally believed to be present only during embryonic, fetal, and early neonatal development, the identification of dividing cells in adult human hearts led to the isolation and culture of cardiovascular progenitor cells *in vitro*. These cells were subsequently reported to be able to differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells *in vitro* and *in vivo* (reviewed in **chapter two**). In response to these exciting observations, the possibility of using these cells for stem cell therapy in patients suffering from cardiac disease has become a hot topic in the cardiovascular scientific community.

We have recently isolated cardiomyocyte progenitor cells (CMPCs) from fetal and adult human cardiac tissue. Upon isolation, CMPCs can be easily expanded and express several stem cell markers and cardiac transcription factors (**chapter three**). Stimulation with specific chemicals and growth factors induces differentiation of CMPCs into several cardiac cell types, like cardiomyocytes, smooth muscle cells, and endothelial cells, indicating that CMPCs are indeed cardiovascular progenitor cells.

To better understand CMPC cardiomyogenesis and identify signaling pathways that regulate this process, we performed microarray analyses at one-week intervals during cardiomyogenic differentiation *in vitro* (**chapter four**). We identified several cell cycle-related genes that were downregulated while genes related to muscle development and contraction became upregulated. Further investigation of our findings may help to increase our knowledge regarding the regulation of cardiomyogenesis.

A comparison between fetal and adult heart-derived CMPCs was made to determine whether these two populations had different developmental potential (**chapter five**). While fetal CMPCs can form spontaneously beating cardiomyocytes, adult CMPCs appear to differentiate into more mature, quiescent cardiomyocytes *in vitro*. The adult CMPC-derived cardiomyocytes

may therefore be more suitable for clinical application than their fetal counterparts. In contrast, the broader potential of fetal CMPCs to differentiate into other mesodermal cell types as well indicates that this is an interesting population for studies on progenitor cell development.

To further elucidate mechanisms that regulate CMPC proliferation and differentiation, we investigated the role of microRNAs (miRs, **chapter six**). MiRs are non-coding, small RNA molecules that affect translation of messenger RNA towards protein and have been reported to regulate developmental processes. We found that both miR-1 and miR-499 were highly expressed in CMPC-derived cardiomyocytes. Increased expression of miR-1 has been described to target histone deacetylase 4 and thereby relieve repression of myogenic gene expression in skeletal muscle. Our results suggest that a similar mechanism may regulate CMPC cardiomyogenesis. Overexpression of miR-499 results in decreased protein expression of the transcription factor sex-determining region Y-box 6 (Sox6). Subsequently, downregulation of Sox6 results in increased sarcomeric gene expression and enhanced cardiomyogenic differentiation.

We have previously reported that cardiomyogenic differentiation of CMPCs can be enhanced by stimulation with the growth factor TGFβ. In **chapter seven** we show that TGFβ-induced hyperpolarization of undifferentiated CMPCs leads to increased intracellular calcium content, activation of calcineurin signaling, increased cardiac-specific gene and protein expression levels, and ultimately to the formation of spontaneously beating cardiomyocytes. This novel mechanism may help to improve cardiomyogenic differentiation protocols and reduce the need for chemicals or growth factors to induce differentiation in cardiovascular progenitor cells.

Finally, we investigated the effect of hypoxia-induced Survivin expression in CMPCs *in vitro* (**chapter eight**). In CMPCs, Survivin overexpression results in increased proliferation and inhibition of apoptosis. These are both valuable traits when considering

transplantation of CMPCs into the hypoxic border zone in infarcted hearts, especially since overexpression of pro-survival factors has already been shown to enhance the beneficial effect of transplanted stem cells on cardiac function. Potentially, a higher CMPC number and survival rate after transplantation may result in increased perfusion and restoration of cardiac tissue.

In conclusion, the isolation and characterization of CMPCs has allowed us to closely study their potential for both scientific research and clinical application. The identification and investigation of signaling pathways important for progenitor cell proliferation and differentiation will help to elucidate the mechanisms that are involved. Future exploration of these mechanisms may lead to a better understanding of cardiovascular cell biology and, possibly, therapeutic application of CMPCs.

Samenvatting

ALS JE HET NIET SIMPEL KUNT UITLEGGEN, HEB JE HET NIET GOED GENOEG BEGREPEN.

ALBERT EINSTEIN

In dit proefschrift beschrijven we de karakterisatie van progenitor (voorloper) cellen uit het hart en de mechanismen die een rol spelen tijdens hun ontwikkeling naar verschillende celtypen. Progenitor cellen zijn cellen die qua ontwikkeling een stadium verder zijn dan pluripotente stamcellen. Pluripotente stamcellen kunnen zichzelf oneindig blijven vernieuwen door middel van celdeling. Daarnaast zijn ze in staat zich te specialiseren tot alle celtypen die voorkomen in de drie kiemlagen van een embryo: het ectoderm, mesoderm, en endoderm. Progenitor cellen zijn multipotent: ze kunnen zichzelf ook vele malen vernieuwen, maar vormen in het algemeen alleen celtypen van het orgaan waaruit de progenitor cellen geïsoleerd zijn. Daarnaast wordt er een onderscheid gemaakt aan de hand van de verschillende ontwikkelingsstadia van het organisme ten tijde van de isolatie van deze cellen: embryonaal, feutaal, neonataal, en volwassen (adult).

In de afgelopen jaren hebben verschillende onderzoeksgroepen progenitor cellen geïsoleerd uit dierlijk of humaan hartweefsel. In **hoofdstuk twee** hebben we reeds bestaande literatuur over deze verschillende typen (mesodermale) hart progenitor cellen samengevat en bespreken we mogelijke effecten en mechanismen die een rol spelen na transplantatie in het hart. De mogelijkheid om schade in het hart te herstellen door middel van stamcel dan wel progenitor cel transplantatie is namelijk voor zowel wetenschappers als clinici een belangrijk onderzoeksgebied gebleken. In tegenstelling tot bijvoorbeeld de huid, wordt beschadigd hartweefsel niet vervangen door gezond nieuw weefsel, maar door veel stugger littekenweefsel. De progenitor cellen die zich in het adulte hart bevinden, reageren onvoldoende op signalen uit het beschadigde deel van het hart. Als adulte progenitor cellen echter geïsoleerd en in kweek gebracht worden, blijken ze wel degelijk in staat om hartspiercellen en vaatcellen te kunnen vormen.

In **hoofdstuk drie** beschrijven we de isolatie en karakterisatie van hart progenitor cellen uit humaan hartweefsel. Deze

progenitor cellen zijn in staat nieuwe, kloppende hartspiercellen te vormen en zijn daarom 'cardiomyocyte progenitor cells' (hartspiercel voorloper cellen) genoemd. Tevens zijn deze cellen in staat vaatceltypen zoals gladde spiercellen en endotheelcellen te vormen. Dit maakt deze cel populatie mogelijk zeer geschikt om doorbloeding in het hart en herstel van beschadigd hartspierweefsel te verbeteren.

Voordat hart progenitor cellen daadwerkelijk gebruikt kunnen worden bij patiënten is er meer onderzoek nodig naar de eigenschappen van deze cellen en de beste manier om ze te stimuleren om nieuw hartweefsel te vormen. In **hoofdstuk vier** hebben we gekeken naar genexpressie tijdens verschillende stadia van het ontwikkelingsproces van progenitor cel naar hartspiercel. Hieruit bleek dat de expressie van genen die een rol spelen bij celdeling omlaag gaan, terwijl genen die nodig zijn voor eiwitten die onderdeel uitmaken van het contractie apparaat van een hartspiercel hoger tot expressie kwamen. Daarnaast zagen we ook verschillen in expressie van genen die gerelateerd zijn aan metabolisme of de immunogeniteit, i.e. het opwekken van een immuun reactie bij de ontvanger van deze cellen. Verder onderzoek naar de signaal transductie paden die betrokken zijn bij hartspiercel ontwikkeling, metabolisme, en immunogeniteit kunnen het belang van deze processen verduidelijken en ons begrip van progenitor cel eigenschappen vergroten.

Eventuele verschillen in de ontwikkelingspotentie van feutale en adulte hart progenitor cellen zijn van groot belang voor de interpretaties van reeds behaalde en toekomstige onderzoeksresultaten en mogelijke klinische toepassingen. Hoewel beide populaties in staat zijn om hartspiercellen te vormen, blijken vooral feutale progenitor cellen zich tot spontaan kloppende cellen te ontwikkelen, terwijl adulte progenitor cellen rustende, volwassen hartspiercellen vormen (**hoofdstuk vijf**). De relatief volwassen hartspiercellen zullen mogelijk geschikter zijn voor behandeling van patiënten, omdat deze cellen waarschijnlijk beter integreren en gelijkmatiger zullen kloppen

met de hartspiercellen van de patiënt. Daarnaast hebben we verschillen gevonden in de voorkeur tot vaatceltype ontwikkeling. Hoewel het belang van dit verschil nog niet helemaal duidelijk is, zal het waarschijnlijk invloed hebben op de bijdrage van de progenitor cellen op doorbloeding van het hart. Verder kunnen feutale progenitor cellen in beperkte mate andere celtypen uit de mesodermale kiemlaag vormen, zoals vetcellen. Noch feutale of adulte progenitor cellen lijken botcellen te vormen. Deze observaties zijn van belang bij de mogelijke toepassing van adulte hart progenitor cellen. De vorming van vet- en botcellen zou namelijk kunnen leiden tot een verslechterde geleiding van signalen die aanzetten tot contractie van het hart en daarmee hartritme stoornissen kunnen veroorzaken. De grotere diversiteit van feutale progenitor cellen maakt ze zeer geschikt voor experimentele studies naar ontwikkeling van verschillende celtypes en de mechanismen die daarbij betrokken zijn.

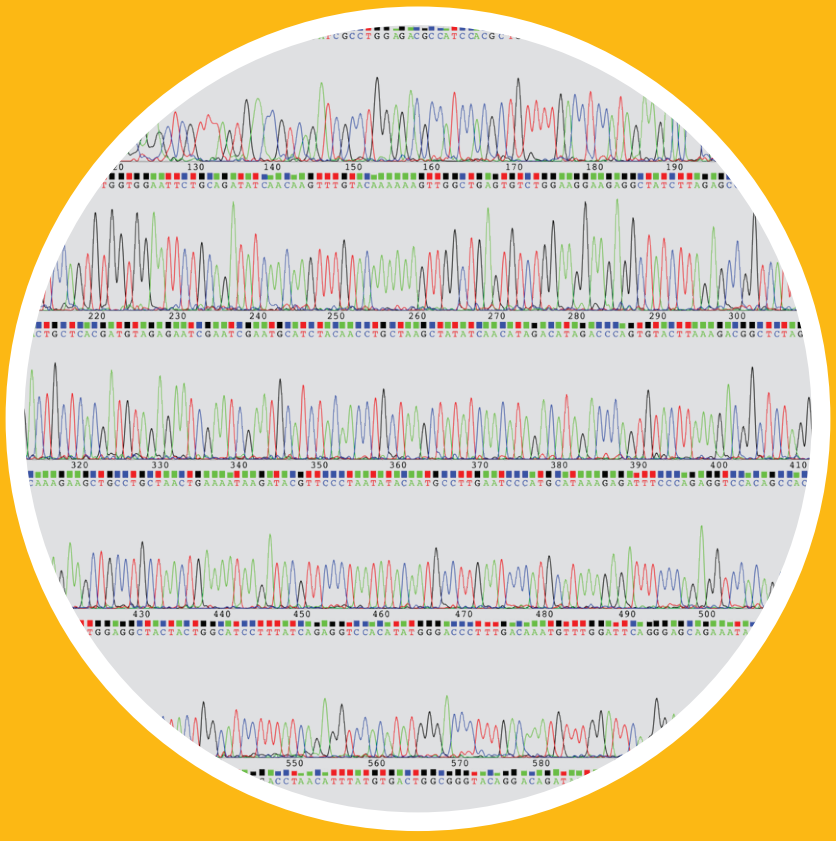
Twee van die mechanismen zijn beschreven in hoofdstuk zes en zeven. In **hoofdstuk zes** hebben we onderzocht of en hoe micro-RNAs de functie van progenitor cellen beïnvloeden. Micro-RNAs zijn kleine stukjes RNA die door binding aan gen transcripten (messenger RNA) de productie van eiwitten kunnen remmen. Het bestaan van deze stukjes RNA was al langer bekend, maar omdat hun rol niet duidelijk was, werd aangenomen dat het niet-functionele bijproducten waren van gen expressie, het zogenaamde 'junk DNA'. Inmiddels is bekend dat micro-RNAs wel degelijk een belangrijke invloed kunnen hebben op verschillende processen in een cel, waaronder de vorming van hartspiercellen. Twee van deze micro-RNAs, miR-1 en miR-499, stimuleren de hartspiercel ontwikkeling via repressie van verschillende remmers. Andere micro-RNAs spelen mogelijk een vergelijkbare rol en op dit moment wordt daar meer onderzoek naar gedaan.

Eerder onderzoek heeft uitgewezen dat de groeifactor TGFβ de vorming van hartspiercellen kan bevorderen in verschillende soorten stamcellen en progenitor cellen, inclusief de hart

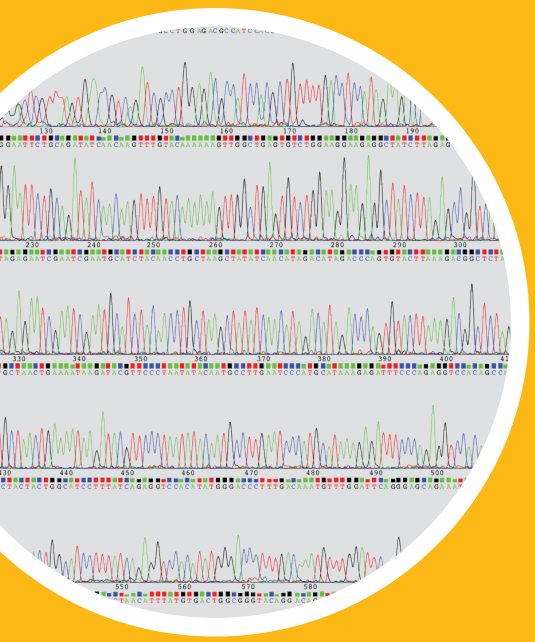
progenitor cellen die worden beschreven in dit proefschrift. Mogelijke mechanismen die betrokken konden zijn bij deze TGFβ-geïnduceerde ontwikkeling waren echter nog niet onderzocht. Het onderzoek dat beschreven is in **hoofdstuk zeven** laat zien dat TGFβ een negatievere membraanpotentiaal kan veroorzaken in hart progenitor cellen. De membraanpotentiaal van een cel wordt veroorzaakt door een onevenwichtige verdeling van positief en negatief geladen deeltjes aan weerszijden van de celmembraan, i.e. binnen en buiten de cel. De negatievere lading die wordt veroorzaakt door TGFβ zorgt vervolgens voor activatie van signalen die hartspiercel ontwikkeling bevorderen en dit leidt uiteindelijk tot de formatie van functionele, kloppende cellen. Verder onderzoek naar dit mechanisme kan hopelijk bijdragen tot verbeterde protocollen voor de vorming van hartspiercellen.

Na een hartinfarct is er in het beschadigde gebied een gebrek aan doorbloeding waardoor er minder voedsel en zuurstof beschikbaar is. Om te voorkomen dat getransplanteerde progenitor cellen sterven voordat ze hebben kunnen bijdragen aan herstel, is het van belang om ze een vehoogde kans op overleving te geven. In **hoofstuk acht** hebben we daarom gekeken naar het effect van zuurstof tekort (hypoxia) op hart progenitor cellen. We hebben hierbij gevonden dat hypoxia zorgt voor een actievere celdeling. Hierbij speelt de hogere activiteit van Survivin een belangrijke rol. Onder normale omstandigheden zorgt de afwezigheid van Survivin voor verminderde celdeling. De afwezigheid van Survivin onder hypoxia omstandigheden resulteert zelfs in een afname van het aantal cellen, waarschijnlijk veroorzaakt door een hogere sterfte van de cellen. Verhoogde expressie van Survivin zorgde echter voor een toename van het aantal cellen en verminderde sterfte wanneer celdood werd geïnduceerd. Een verhoogde Survivin expressie in hart progenitor cellen zou daarom kunnen helpen om de overleving van progenitor cellen in een hypoxisch gebied van het hart te verbeteren. Hierdoor kunnen getransplanteerde cellen mogelijk langer bijdragen aan herstel van hartschade.

Tot conclusie, het onderzoek dat wordt beschreven in dit proefschrift heeft ons begrip betreffende progenitor cellen uit het hart en hun potentie om nieuwe hartspiercellen en vaatcellen te maken verbeterd. Deze cellen zijn een goed model gebleken om onderzoek te doen naar verschillende mechanismen die hierbij een rol spelen en een aantal van daarvan zijn inmiddels bestudeerd. Verder onderzoek naar de rol van belangrijke genen en micro-RNAs in hart progenitor cellen zal een klinische toepassing van deze cellen hopelijk dichterbij brengen.



Appendix: cDNA expression libraries for genetic screening



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ABSTRACT

The potential of stem and progenitor cells to improve perfusion and contribute to new tissue after transplantation into the injured heart has received great attention in the past years. We have recently reported the isolation and characterization of human cardiomyocyte progenitor cells (CMPCs). These cells can differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells and may therefore be useful for cardiac stem cell therapy. In order to investigate the effect of known and unknown factors from post-infarct tissue on CMPC survival, proliferation, and differentiation, we established cDNA expression libraries based on gene expression after myocardial infarction. The rationale for this approach is that the infarcted heart expresses different cytokines and growth factors, some of which have already been shown to affect progenitor cell behavior *in vitro*. Identification of the most relevant targets for genetic modification of CMPCs may help to improve proliferation and differentiation protocols. Subsequently, their potential for clinical application may be enhanced.

INTRODUCTION

After chronic or acute myocardial infarction (MI), loss of viable cardiomyocytes and replacement by fibrotic tissue results in an impaired cardiac function[1,2]. Although cardiovascular progenitor cells have been identified in the human heart (reviewed in[3-5]), these cells do not appear to respond to cardiac injury adequately in order to restore damaged tissue *in vivo*. Upon isolation however, cardiovascular progenitor cells are able to proliferate efficiently *in vitro*. During co-culture with rat neonatal cardiomyocytes or after stimulation with specific chemicals and/or growth factors, these progenitor cells can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells[6-10]. Therefore, these progenitor cells have the potential to become a great tool to restore cardiac tissue via stem cell therapy. However, progenitor cell survival in the hypoxic host-tissue, as well as their differentiation into mature cell types, may need to be optimized to achieve maximal stem cell transplantation efficiency.

In this study, we intend to identify signals in the injured heart that may enhance the survival, proliferation, and/or differentiation of human cardiomyocyte progenitor cells (CMPCs[9,10]). In response to injury or increased stress, the heart switches from a mature to fetal gene expression pattern in order to preserve cardiac tissue under hypoxic conditions[11]. The infarct border zone expresses numerous chemotactic and proliferative signals, including cytokines and growth factors[12], like SDF-1, that have been shown to enhance recruitment/migration of stem cells (S. Post, unpublished data, and[13]). Furthermore, growth factors such as TGFβ are upregulated 24 hours post-MI and can enhance cardiomyogenic differentiation in stem and progenitor cells *in vitro*[9,14-16]. We have selected an open approach to identify other known and unknown factors that may play equally important roles.

To enable isolation, identification, and functional validation of interesting targets, we created cDNA expression libraries based on gene expression in the infarcted heart. When introduced into CMPCs or a P19 embryonic carcinoma reporter cell line [17], screening for spontaneous beating or analysis of stage-specific

gene expression can then be used to determine which cells contain genes responsible for the observed effect. Subsequently, amplification and identification of these genes will provide relevant targets for further investigation. Increased understanding of the identified genes and downstream signaling pathways may ultimately be used to genetically modify progenitor cells before transplantation and improve their contribution to perfusion and tissue restoration in the heart.

RESULTS

Comparison of transfection and transduction methods

$CaPO_4$ transfection with a CMV-GFP plasmid resulted in approximately 30-40% GFP-expressing CMPCs (figure 1A). Adenoviral transduction resulted in a dose-dependent overexpression of lacZ (not shown), with an optimum of 70-90% of β-Galactosidase-positive CMPCs at an MOI of 25 virus particles per cell (figure 1B). The different levels of blue staining are probably caused by a different number of virus transductions per cell.

Comparison of 2nd and 3rd generation packaging for lentivirus production showed that 2nd generation packaging generally resulted in a higher number of GFP-positive cells (figure 1C): 91.3-91.5% with 2nd and 67.0-88.1% with 3rd generation packaging (FACS analysis not shown). Introduction of 2nd generation lentivirus containing the lacZ gene (pLenti6.2/V5-GW/LacZ) showed that the cDNA expression library expression control plasmid could be functionally expressed (figure 1C). FACS analysis showed that lentiviral overexpression at different MOIs resulted in an optimum of 80-90% of GFP-positive CMPCs at MOI=5 (figure 1D). Higher MOIs did not result in substantially more GFP-positive cells.

Creation of cDNA entry libraries

To determine if signals present in infarcted hearts could influence progenitor cell survival, proliferation, and/or differentiation, we isolated RNA from mouse hearts six and 24 hours post-MI to establish two separate cDNA entry libraries (figure 2A).

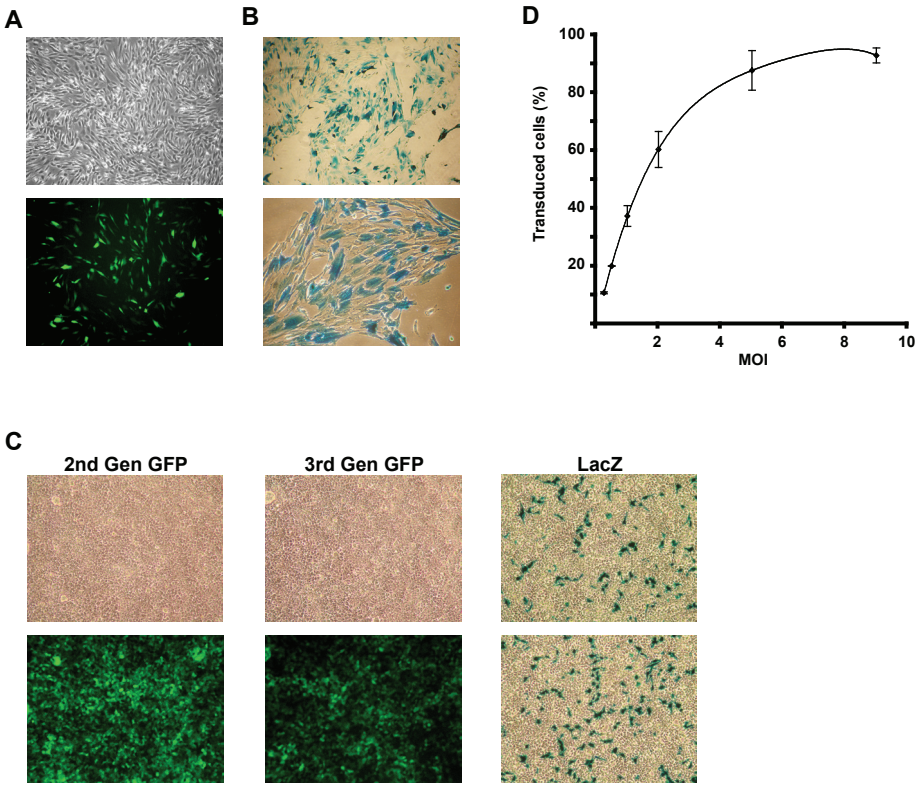


Figure 1: Optimalization of transfection and transduction methods

A | $CaPO_4$ transfection of CMPCs with a CMV-GFP plasmid. Upper panel: bright field image. Lower panel: FITC image (100x magnification). B | X-Gal staining of CMPCs after AdLacZ transduction (MOI=25). Upper panel: 100x magnification. Lower panel: 200x magnification. C | HEK 293T cells transduced with 2nd or 3rd generation GFP or lacZ virus. GFP: MOI=5, upper panel: bright field, lower panel: FITC. LacZ: MOI=1, upper panel: 2nd generation, lower panel 3rd generation (100x magnification). D | FACS analysis of lentivirally transduced CMPCs tested at an MOI of 0.25, 0.5, 1, 2, 5, or 9 (n=2). An optimum of 80-90% GFP-positive cells was achieved at an MOI of 5.

Subsequently, mRNA-derived cDNA fragments were cloned into pDONR-222 entry vectors (figure 2A-B) via site-specific

recombination of *att* sequences[18,19]. The two resulting pools of vectors containing different cDNA fragments are referred to as the six and 24-hours post-MI cDNA entry libraries.
Both entry libraries were transformed in E. coli cells, propagated, and serial dilutions of each library were plated. Counting the number of colonies on each plate revealed high titers for both libraries: $0.88.10^6$ and $1.6.10^6$ pfu/ml for the six and 24 hours post-MI libraries, respectively, indicating successful transformation and propagation. The average cDNA insert size in each library was determined by restriction analysis on randomly picked E. coli clones. All isolated clones contained a cDNA fragment, indicating high recombination efficiency. The six and 24 hours post-MI entry libraries showed an average cDNA insert size of 2023 (+/- 1348) and 1577 (+/- 836) base pairs (bp), respectively (figure 3A-B), consistent with the expected lengths based on the CloneMiner manual.

Creation of cDNA expression libraries

The cDNA fragments within an entry library can be cloned into a variety of expression vectors. For the purpose of our study, stable and long-term expression of cDNA fragments would be needed. Therefore, we isolated plasmid DNA from the entry libraries and cloned the cDNA fragments from the entry vectors into lentiviral pLenti6.2/V5-DEST plasmids (figure 2C), resulting in two pools of lentiviral expression plasmids containing the different cDNA fragments. These pools are referred to as the six and 24 hours post-MI cDNA expression libraries. Both libraries were transformed again and propagated in E. coli cells. A fraction of each E. coli pool was plated in order to pick clones for restriction analysis, while the rest was further processed to isolate expression vectors that could be used for lentivirus production. Restriction analysis of expression library clones showed an average cDNA insert size of 2026 (+/- 1192) and 1521 (+/- 717) bp in the six and 24 hours post-MI libraries, respectively (figure 3C-D). This indicated that cloning of cDNA fragments from the entry libraries to the expression libraries did not result in a bias towards shorter transcripts.

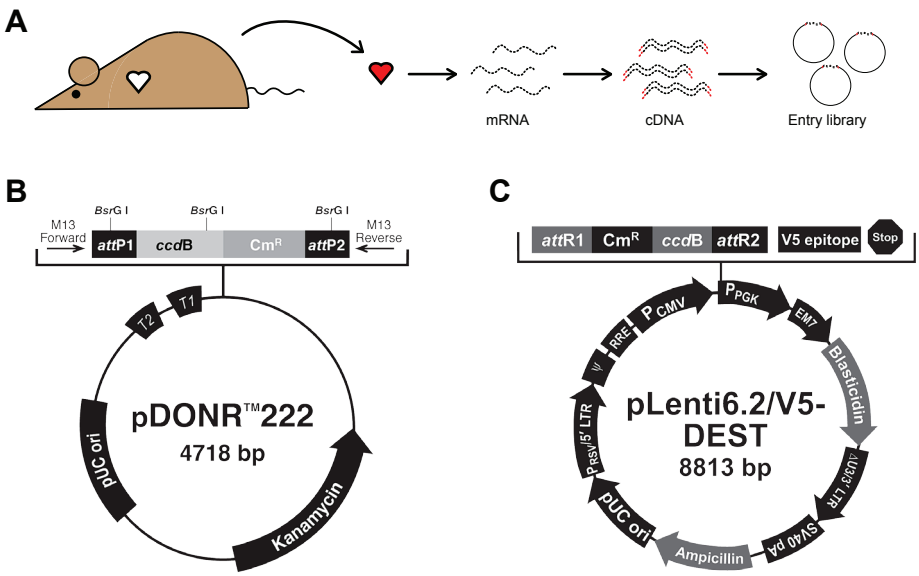


Figure 2: Overview of study design

A | Six and 24 hours after induction of myocardial infarction, the left ventricles were isolated and used for mRNA isolation. During cDNA synthesis, *att* adapters (red) are ligated to the cDNA fragment for site-specific cloning into entry vectors. The resulting pool of vectors, which each contain a different cDNA fragment, is referred to as the entry library. B | The pDONR-222 vector that was used for creation of the entry libraries (CloneMiner manual, Invitrogen). M13 Forward and Reverse primers can be used for sequencing or PCR. Two *att* sites are used for site-specific cloning with cDNA fragments. BsrG1 restriction sites are present in both *att* sequences and, potentially, also in the cDNA fragment. The ccdB gene inhibits growth of E. coli cells and is used for negative selection of non-recombined plasmids, while in recombined plasmids the ccdB gene is replaced by a cDNA fragment. Cm^R indicates a chloramphenicol resistance gene that can be used for specific propagation of the original pDONR vector in ccdB resistant E. coli cells. C | The pLenti6.2/V5-DEST vector that was used for the expression libraries (Gateway vector kit manual, Invitrogen). When introduced as virus, the region between the 5' and 3' long term repeats (LTRs) will integrate into the cell gDNA. Subsequently, the CMV promoter (P_{CMV}) permits high, constitutive expression of the cDNA fragment. For abbreviations, see B. BsrG1 restriction sites are present in the *att* sequence and, potentially, in the recombined cDNA fragment

(not indicated). The CMV Forward priming site is located approximately 50-70 bp 5′ of the transcriptional start and 150-170 bp 5′ of the *att* sequence. The V5-Reverse primer sequence is located in the V5-epitope. For further details, see text or the Gateway vector kit manual.

Functional validation of cDNA expression

After lentiviral transduction, integration of the introduced promoter and gene(s) of interest into the cell genome generally results in substantial overexpression (figure 1C-D). To test if the cDNA expression libraries could be functionally expressed in CMPCs, lentivirus containing plasmids from the expression libraries was produced with 2nd generation packaging plasmids and used for transduction of CMPCs. After recombination, the lentiviral expression plasmids (figure 2C) contain a V5 epitope sequence 3′ of the inserted cDNA fragment, which should result in the expression of cDNA fragment-V5 fusion proteins. These fusion proteins should be easily identifiable by Western blot analysis. Therefore, we transduced CMPCs with the pLenti6.2/V5-GW/LacZ virus or virus from the expression libraries and isolated protein two days post-transduction. The lacZ control clearly showed substantial overexpression of the lacZ-V5 fusion protein (figure 4A). Since the expression libraries should contain a variety of cDNA fragments with different sizes (figure 3C-D), we expected a range of bands representative for the different cDNA fragment-V5 fusion proteins. However, we only observed aspecific background signals (figure 4A).

To investigate whether the lack of V5 fusion proteins could be explained by abnormalities present in the cDNA fragments, we performed sequence analysis with primers 5′ or 3′ of the cDNA fragments in the clones that were used earlier for restriction analysis (tables 1 and 2). Analysis with reverse primers showed that, in all three reading frames, many stop codons were present in the 3′ untranslated region (UTR) of the cloned cDNA fragments (figure 4B), which prevent expression of the V5 epitope and thereby formation of cDNA fragment-V5 fusion proteins.

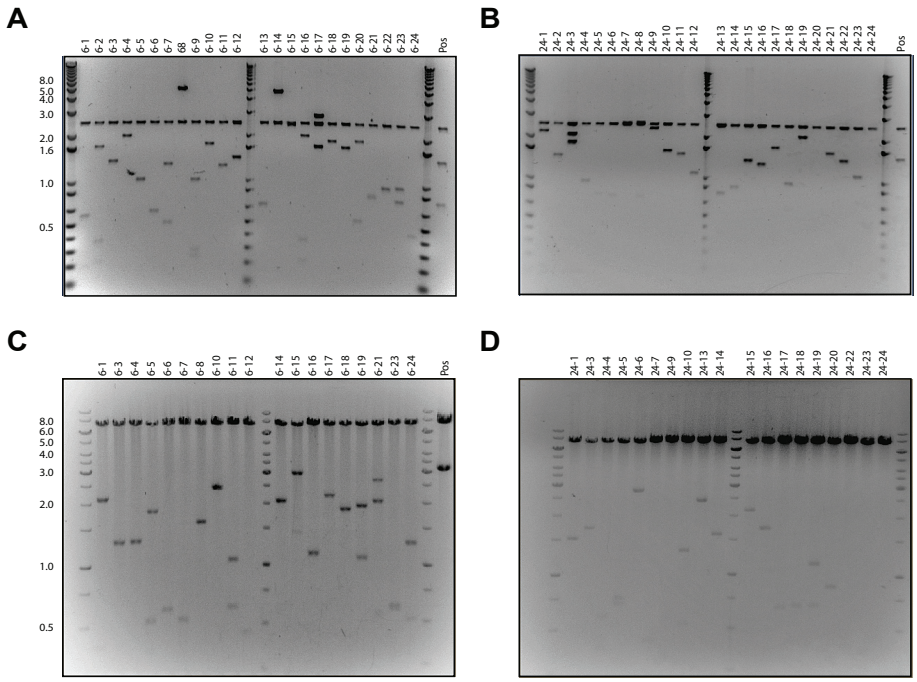


Figure 3: Restriction analysis of entry and expression libraries

A, B | BsrG1 restriction analysis showed that cDNA fragment size ranged from 450 to 5100 bp, with an average of 2023 bp, in the six hour pMI entry library (A) and from 400 to 3900 bp, with an average of 1577 bp, in the 24 hours post-MI entry library (B). The 2.5 kbp band indicates the pDONR-222 backbone, while other bands indicate restricted cDNA fragments. Note that some clones have multiple bands, indicating the presence of one or more BsrG1 restriction sites in the cDNA fragment. Pos indicates restriction of the pDONR-222 vector, resulting in a 2.5 kbp backbone and 1.4 and 0.8 kbp fragments resulting from restriction of the ccdB gene (see figure 2B). Numbers on the left of the gel shown in A indicate the sizes (in kpb) of the DNA ladder fragments. C, D | BsrG1 restriction analysis showed that cDNA fragment size ranged from 500 to 4800 bp, with an average of 2026 bp, in the six hour pMI expression library (C) and from 600 to 2500 bp, with an average of 1521 bp, in the 24 hours post-MI expression library (D). Clone numbers are different from entry library clone numbers. The 7.1 kbp band indicates the pLenti6.2 backbone, while other bands indicate restricted cDNA fragments. Pos indicates restriction of the pLenti6.2/V5-GW/LacZ control vector, resulting in a 7.1 kbp backbone and a 3.1 kpb lacZ fragment.

Unexpectedly, we also found that in 74% and 68% of the clones (tables 1 and 2, respectively), a part of the 5' coding sequence was missing (figure 4C), suggesting that a similar fraction of expression plasmids in the rest of the libraries cannot be expressed due to the lack of a start codon. The observed BsrG1 restriction patterns (figure 3C-D) were in concordance with the predicted restriction sites and band sizes based on sequence analysis of the expression clones (not shown). This confirmed that the 5' part of the mRNA sequence was indeed absent in most of the cDNA fragments.

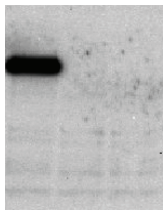
DISCUSSION

The isolation and propagation of CMPCs *in vitro* has provided us with a promising tool to study progenitor cell function and development[9,10,20]. In this study, we tested several methods for overexpression of reporter gene s and established cDNA expression libraries in order to screen for genes or combinations thereof that would help to improve cell survival, proliferation, and/or differentiation.

Mammalian transfection by $CaPO_4$ was not very efficient and resulted in a relatively low number of GFP-positive cells. Viral transduction resulted in a dose-dependent number of cells overexpressing the gene of interest. Although adenoviral plasmids do not integrate into the cell genome and are easily diluted in rapidly cycling cells, this method can be used for differentiation experiments where cell cycling is low[10]. In one experiment, adenoviral expression in CMPCs was observed up to four weeks post-transduction (not shown). For proliferation assays, lentiviral overexpression is recommended, since genomically integrated genes will remain present in daughter cells.

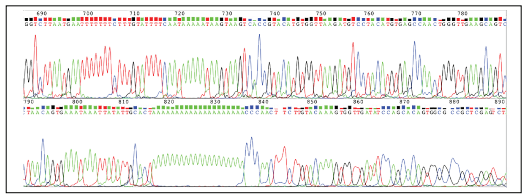
Introduction of cDNA expression libraries based on gene expression post-MI would allow us to screen for factors that affect CMPC homeostasis or development. This approach has several advantages: 1. Introduction of a random set of cDNA fragments prevents bias towards known signaling pathways and may help to identify unknown factors that might play a role in progenitor

A



B

Clone 6-5



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1

Stop C A K Stop H K L L V Stop G D V E W S Stop Stop I F F F V F S I K I S K S P Y Met W L R C P T C
E P T G L K Q S N S E I N Y I A L K K K K K K T Q L L V Q S G Stop Y P A Q W R R S S L E P P Stop

2

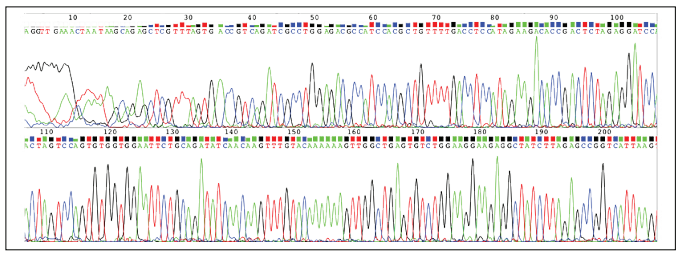
L N N T S C Stop C K G Met W N G L N E F F S L Y F Q Stop K Stop V S H R T C G Stop D V L H V S Q
L G Stop S S L T V K Stop I I L H Stop K K K K K K P N F L Y K V V D I Q H S G A A R V Stop SPRE

3

C Stop I T Q A V S V R G C G Met V L Met N F F L C I F N K N K Stop V T V H V V K Met S Y Met Stop
A N W V E A V Stop Q Stop N K L Y C T K K K K K K N P T S C T K W L I S S T V A P L E S R A P V

C

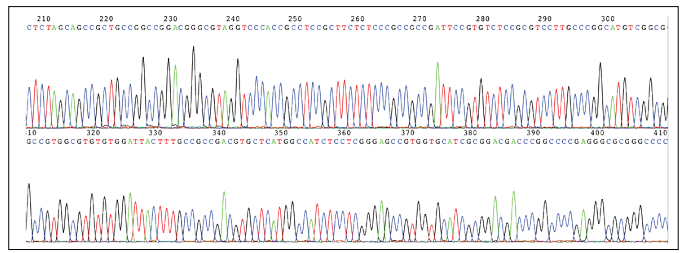
Clone 6-5



```
cDNA   163   CTGAGTGTCTGGAAGGAAGAGGCTATCTTAGAGCCGGTCATTAAAGTGGACTGCTCACGAT   222
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mRNA    745   CTGAGTGTCTGGAAGGAAGAGGCTATCTTAGAGCCGGTCATTAAAGTGGACTGCTCACGAT   804
```

AGGTTGAAACTAATAAGCAGAGCTCGTTTAGTGACCGTCAGATCGCCTGGAGACGCCATC
CACGCTGTTTTGACCTCCATAGAAGACACCGACTCTAGAGGATCCACTAGTCCAGTGTGG
TGGAATTCTGCAGATATCAACAAGTT**TGTACA**AAAAAGTTGGCTGAGTGTCTGGAAGGAA
GAGGCTATCTTAGAGCCGGTCATTAAAGTGGACTGCTCACGATGTAGAGAATCGAATCGAA

Clone 24-6



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cDNA   204   GCACTCTAGCAGCCGCTGCCGGCCGGACGGGCGTAGGTCCCACCGCCTCCGCTTCTCTCC   263
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mRNA     1    GCACTCTAGCAGCCGCTGCCGGCCGGACGGGCGTAGGTCCCACCGCCTCCGCTTCTCTCC    60
```

TTCTGCAGATATCAACAAGTT**TGTACA**AAAAAGTTGGCGGCGGCGCGCCGGGGCCTCCTC
CTCCCTTCCCTCCTCCGCCTCCCGCACTCTAGCAGCCGCTGCCGGCCGGACGGGCGTAGG
TCCCACCGCCTCCGCTTCTCTCCCGCCGCCGATTCCGTCTCCGCGTCCTTGCCCGGCA
TGTCGGCGGCCGTGGCGTGTGTGGATTACTTTGCCGCCGACGTGCTCATGGCCATCTCCT

Figure 4: Determination of cDNA expression library functionality

A | Western blot analysis of protein isolated from CMPCs previously transduced with the lacZ control or six or 24 hours post-MI expression library lentivirus. The lacZ-V5 fusion protein (121 kDa) is strongly expressed, while only background signal (vague bands) could be observed in the six and 24 hour pMI expression library lanes. B | Reverse sequence analysis (left, upper panel) of the 3' UTR of the cDNA fragment from expression clone 6-5 (Mus musculus kelch-like 23, Klhl23). The left lower panel shows part of the cDNA sequence, the *att* recombination site, and the vector. Explanation: underlined: vector sequence, not underlined: cDNA fragment sequence, italic: non-coding sequence, bold: BsrG1 restriction site. Sequence translation showed multiple stop codons, irrespective of the chosen reading frame (right). C | Examples of forward sequence analysis (upper panels) and multiple sequence alignments (middle panels) on 5' ends of expression clone sequences (lower panels). Shown are clone 6-5, in which the 5' part of the mRNA sequence is missing and clone 24-6 (Mus musculus Kruppel-like factor 16, Klf16) in which it is present. According to the NCBI Entrez Nucleotide database mRNA sequence, the Klhl23 coding sequence runs from nucleotide 172 to 1848. However, sequence alignment (left, middle panel) shows that the first cDNA fragment nucleotide of clone 6-5 corresponds to mRNA nucleotide 745 in the NCBI database, suggesting that nucleotides 1-744 from the Klhl23 mRNA sequence are missing in this expression clone (left, lower panel). Alignment of the Klf16 cDNA sequence and NCBI mRNA sequence (right, middle panel) shows that the first mRNA nucleotide is present in the cDNA fragment, suggesting that the complete coding sequence (nucleotides 97-852) is present in this cDNA expression clone (right, lower panel).

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cell growth, survival, and differentiation; 2. These cDNA expression libraries will allow us to include both highly expressed genes as well as low copy genes, normally hard to detect in e.g. microarrays; 3. Differential gene expression patterns that are detected with microarray analysis are mostly the result of preceding events. In contrast, the genes identified in our approach will have a gain-of-function and causative effect; 4. Few processes are regulated by single genes. More often, combinations of genes or even different signaling pathways are needed for optimal regulation of

stemness, cell cycling, or differentiation. By varying the number of expression sequences that are introduced in each cell, functional combinations of important factors can be detected that drive the process of interest more efficiently than single genes.

Unfortunately, sequence analysis of randomly selected cDNA expression clones revealed that most of the cDNA fragments in our expression libraries contained incomplete coding sequences. This observation could be due to several factors. First, the quality and quantity of mRNA is important for proper cDNA synthesis. However, the mRNA used for this study was isolated with kits suggested by the same manufacturer as for the CloneMiner cDNA Library Construction kit and tested with nanochip assays (not shown). The mRNA was found to be of high quality with minimal breakdown or contamination by rRNAs or tRNAs. Furthermore, we used the suggested amounts of mRNA for cDNA synthesis according to the manual. Therefore, we believe that mRNA quality and quantity were sufficient.

Second, reverse transcription of mRNA should be optimal in order to create cDNA fragments with intact coding sequences. The cDNA synthesis by the reverse transcriptase and DNA polymerases from the CloneMiner cDNA Library Construction kit starts at the oligo-dT primer that binds to the 3' poly-A tail on mRNA. Although all reactions were performed according to the protocol, cDNA synthesis appears to be abrogated in our experiments, resulting in incomplete cDNA fragments. The exact cause remains unexplored, but could be explained by insufficient reverse transcriptase activity. Previous studies showed that only 13% and 7% of cDNA fragments contained the full-length sequence of a 7.8 kb mRNA template before and after recombination, respectively[19]. This suggests that establishment of cDNA expression libraries with a high number of full-length cDNA fragments is very difficult.

Comparison with the NCBI Entrez Nucleotide database showed that the complete mRNA sequences for the genes identified in our six and 24 hours post-MI expression libraries have an average length of 4.1 and 3.0 kbp, respectively. The cDNA fragments in our expression libraries were on average 2.0 and 1.5 kbp long, as expected according to the manufacturers' protocol. In

hindsight however, cDNA fragments with an average length of >3-4 kbp may have been more representative for average gene transcript length. Selection of cDNA fragments with large sizes by agarose gel electrophoresis[19] might therefore be preferable to size-fractionation by column chromatography, in which the size of cDNA fragments is unknown at the time of isolation.
In summary, CMPCs can be genetically modified very efficiently and are therefore suitable for investigation of specific genes or signaling pathways. Unfortunately, the large fraction of incomplete coding sequences in the cDNA expression libraries greatly diminishes the chances of functional gene expression in our cells and decreases efficient use of these libraries to screen for targets of interest.

MATERIALS AND METHODS

Myocardial infarction

All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*, prepared by the institute of Laboratory Animal Resources and with prior approval by the Animal Ethical Experimentation Committee, Utrecht University.
Male B6-129 and BALB/c mice (Charles rivers), aged 10-12 weeks, were used in these experiments. Myocardial infarction was induced by ligation of the left anterior descending coronary artery following left-sided thoracotomy under isoflurane anesthesia, as described previously[21]. The ligation was placed 1-2 mm below the inferior of the left auricle to obtain reproducible infarct size. Infarction was confirmed by observation of blanching and akinesia of the tissue. Six and 24 hours post-MI, mice were sacrificed and the hearts were flushed with PBS via the right ventricle. The hearts were dissected; the left ventricle was isolated and immediately snap-frozen in liquid nitrogen.

cDNA expression libraries

Total RNA was isolated from the left ventricles from mouse hearts six and 24 hours post-MI (ten hearts per condition) with

the RNeasy Mini kit (Qiagen) according to protocol and checked for quality with the RNA 6000 Nanochip (Agilent). Messenger-RNA was isolated from total RNA with the Fast-Track 2.0 mRNA isolation kit (Invitrogen) according to protocol and checked for quality with the Nanochip as well. Five μg mRNA was used to establish the six and 24 hour post-MI cDNA expression libraries with the CloneMiner cDNA Library Construction kit (Invitrogen). During cDNA synthesis, cDNA fragments were labeled with biotin and bacteriophage λ-derived DNA sequences (called *att* adapters) for site-specific recombination[18,19]. Subsequently, cDNA fragments were size-fractionated by column chromatography and quantified by the radiolabeling method. Isolated cDNA fragments were cloned into the pDONR-222 entry vector (Invitrogen) and transformed in ElectroMAX DH10B T1 Phage Resistant E. coli cells (Invitrogen). The transformed E. coli cells were propagated in S.O.C. medium (Invitrogen) + 50 μg/ml kanamycin. The titer of each cDNA entry library was determined by plating serial dilutions of transformed E. coli cells and counting the number of colonies on each plate. The average cDNA insert size was determined by isolating plasmid DNA from 24 colonies with the NucleoSpin Plasmid QuickPure miniprep kit (Macherey-Nagel) and restriction analysis with BsrG1 (Fermentas). Digested plasmid DNA was analyzed on a 1% agarose gel.

The entry libraries were propagated and plasmid DNA was isolated with the PureLink HiPure Plasmid DNA Midiprep kit (Invitrogen). The cDNA fragments present in the pDONR-222 entry vectors were cloned into pGL6.2/V5-DEST lentiviral expression plasmids (Invitrogen) with the Gateway LR Clonase II Enzyme mix (Invitrogen) to create the cDNA expression libraries. Recombined plasmids were transformed into One Shot Stbl3 chemically competent E.coli cells (Invitrogen) and propagated in Luria Bertani (LB) medium + 100 μg/ml ampicillin. A fraction of the cDNA expression libraries was used for plating and isolation of colonies for restriction analysis and sequencing of cDNA inserts. The rest of the cDNA expression libraries was used to isolate plasmid DNA with the PureLink HiPure Plasmid DNA Filter Maxiprep kit (Invitrogen) for subsequent lentivirus production.

Cell isolation and culture

Informed consent procedures were followed and prior approval of the ethics committee of the University Medical Center Utrecht was obtained. CMPCs from human fetal hearts were isolated by MACS and cultured as described[9,20]. HEK 293T cells were cultured in DMEM (Gibco), supplemented with 10% FCS (Gibco) and penicillin-streptomycin (PenStrep, 100U/ml each, Gibco).

Adenovirus production

The Ad-Easy XI vector (Stratagene) containing the lacZ sequence (AdCMV-LacZ) was described previously[22]. HEK 293T cells were plated at 50% confluency and transfected with the AdCMV-LacZ plasmid with the ViraPack Transfection Kit (Stratagene). Primary and secondary virus production was performed as described in the AdEasy manual (Stratagene). Cesium chloride purification was performed on amplified virus stocks. Titers (plaque forming units (pfu) per ml) were determined by repeated plaque assays[23].

Lentivirus production

HEK 293T cells were plated at 50% confluency in T162 flasks and transfected the following day. A calcium-phosphate precipitation mix was prepared by combining a total of 40 μg plasmid DNA (lentiviral expression plasmid and 2nd (psPAX2 and pLP-VSVG) or 3rd (pLP1, pLP2, and pLP-VSVG, Invitrogen) generation packaging plasmids in a molar ratio of 1:1:1 or 2:1:1:1, respectively) in sterile mQ-H_2O plus $CaCl_2$ (end concentration 0.25 M). DNA-$CaCl_2$ mix was added to 2xHBS (280 mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4, pH 7.12) and incubated at room temperature for 30-45 minutes. The $CaPO_4$ precipitate was added to 20 ml culture medium containing 5% FCS and added to the cells. The following day, cells were gently washed with PBS and medium was refreshed. Two and three days post-transfection, medium was isolated, cleared from cell debris, filtered (0.45 μm), and stored as aliquots at -80 °C.

Lentiviral titer was determined by transducing HEK 293T cells with serial dilutions of virus. For pL-CMV-Luc2-IRES-GFP-lentivirus, the number of transduced cells was quantified by FACS analysis (see below). The titer (transducing units (TU) per ml) was calculated

with the formula (P*N/100*V)/DF, where P is the percentage of GFP-positive cells, N is the number of cells at the time of transduction, V is the volume of dilution added to the cells (in ml), and DF is the dilution factor.
To determine pLenti6.2/V5-GW/LacZ (Invitrogen) and cDNA expression library lentiviral titer, the amount of p24 antigen, representative for the amount of physical viral particles (PP), was determined with an enzyme-linked immuno assay (ELISA) kit (Zeptometrix) according to protocol. The titer was estimated by comparing FACS and p24 ELISA results from Luc2-IRES-GFP-transduced cells (generally resulting in one TU per 100-1000 PP).

Calcium-phosphate transfection and viral transduction

CMPCs were plated in culture medium at 70% confluency. The following day, one hour before transfection, medium was refreshed. Two μg pCMV-IRES-EGFP plasmid DNA (Invitrogen) was diluted in Tris (1.0 mM, pH 8.0)-EDTA (0.1 mM) plus $CaCl_2$ (end concentration 0.25 M). DNA-$CaCl_2$ mix was added in a drop-wise manner to 2xHBS and incubated at room temperature for 15 minutes. The calcium-phosphate precipitate was added to the cells in a drop-wise manner. The following day, cells were gently washed with PBS and medium was refreshed.
For viral overexpression experiments, cells were transduced in growth medium with a multiplicity of infection (MOI) as indicated. For lentiviral transduction, 6 μg/ml polybrene (Hexadimethrine bromide, Sigma) was added to the medium. Transduced cells were used for subsequent experiments minimally two days post-transduction. MOI optimalization was determined by FACS on Luc2-IRES-GFP-transduced cells.

FACS analysis

Two days after lentiviral transduction, cells were washed with PBS plus 2 mM EDTA, trypsinized briefly, and resuspended in culture medium. Trypsinized cells were washed with PBS, fixed with 4% PFA for 15 minutes at room temperature, washed again, and analysed using a Beckman Coulter Cytomics FC500 FACS.

X-Gal staining

Cells were washed with PBS and fixated in fixing solution (1% formaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4) for 15 minutes at room temperature. Cells were washed again and stained with X-Gal staining solution (1 mg/ml X-Gal, 5 mM $Ke_3Fe(CN)_6$, 5 mM $Ke_4Fe(CN)_6$-$3H_2O$, and 2 mM $MgCl_2$ in PBS, pH 7.4) in the dark at room temperature until the cells stained blue.

Western blot analysis

Western blot analysis was performed as described previously[9]. Antibodies were for mouse-anti-V5 (Invitrogen) and goat-anti-mouse-HRP (DAKO).

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REFERENCES

1. Jugdutt, B.I. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? *Circulation* **108**, 1395-403 (2003).
2. Holmes, J.W., Borg, T.K. & Covell, J.W. Structure and mechanics of healing myocardial infarcts. *Annu Rev Biomed Eng* **7**, 223-53 (2005).
3. van Vliet, P., Sluijter, J.P., Doevendans, P.A. & Goumans, M.J. Isolation and expansion of resident cardiac progenitor cells. *Expert Rev Cardiovasc Ther* **5**, 33-43 (2007).
4. Roccio, M., Goumans, M.J., Sluijter, J.P. & Doevendans, P.A. Stem cell sources for cardiac regeneration. *Panminerva Med* **50**, 19-30 (2008).
5. Segers, V.F. & Lee, R.T. Stem-cell therapy for cardiac disease. *Nature* **451**, 937-42 (2008).
6. Messina, E. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* **95**, 911-21 (2004).
7. Laugwitz, K.L. *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* **433**, 647-53 (2005).
8. Bearzi, C. *et al.* Human cardiac stem cells. *Proc Natl Acad Sci U S A* **104**, 14068-73 (2007).
9. Goumans, M.J. *et al.* TGF-β1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. *Stem Cell Res* **1**, 138-149 (2008).
10. van Vliet, P. *et al.* Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* **16**, 163-9 (2008).
11. Rajabi, M., Kassiotis, C., Razeghi, P. & Taegtmeyer, H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev* **12**, 331-43 (2007).
12. Birdsall, H.H. *et al.* Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion. *Circulation* **95**, 684-92 (1997).
13. Kucia, M. *et al.* Cells expressing early cardiac markers reside in the bone marrow and are mobilized into the peripheral blood after myocardial infarction. *Circ Res* **95**, 1191-9 (2004).
14. Behfar, A. *et al.* Stem cell differentiation requires a paracrine pathway in the heart. *Faseb J* **16**, 1558-66 (2002).
15. Lim, J.Y., Kim, W.H., Kim, J. & Park, S.I. Involvement of TGF-beta1 signaling in cardiomyocyte differentiation from P19CL6 cells. *Mol Cells* **24**, 431-6 (2007).

16. Li, T.S. *et al.* TGF-beta induces the differentiation of bone marrow stem cells into immature cardiomyocytes. *Biochem Biophys Res Commun* **366**, 1074-80 (2008).
17. Moore, J.C. *et al.* A P19Cl6 GFP reporter line to quantify cardiomyocyte differentiation of stem cells. *Int J Dev Biol* **48**, 47-55 (2004).
18. Landy, A. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annu Rev Biochem* **58**, 913-49 (1989).
19. Ohara, O. & Temple, G. Directional cDNA library construction assisted by the in vitro recombination reaction. *Nucleic Acids Res* **29**, E22 (2001).
20. Smits, A.M. *et al.* Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology. *Nature Protocols* **4** (2009).
21. van Laake, L.W. *et al.* Monitoring of cell therapy and assessment of cardiac function using magnetic resonance imaging in a mouse model of myocardial infarction. *Nat Protoc* **2**, 2551-67 (2007).
22. Sluijter, J.P. *et al.* Involvement of furin-like proprotein convertases in the arterial response to injury. *Cardiovasc Res* **68**, 136-43 (2005).
23. Fallaux, F.J. *et al.* Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* **7**, 215-22 (1996).


THANX

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IF WE KNEW WHAT IT WAS WE WERE DOING, IT WOULD NOT BE CALLED RESEARCH, WOULD IT?

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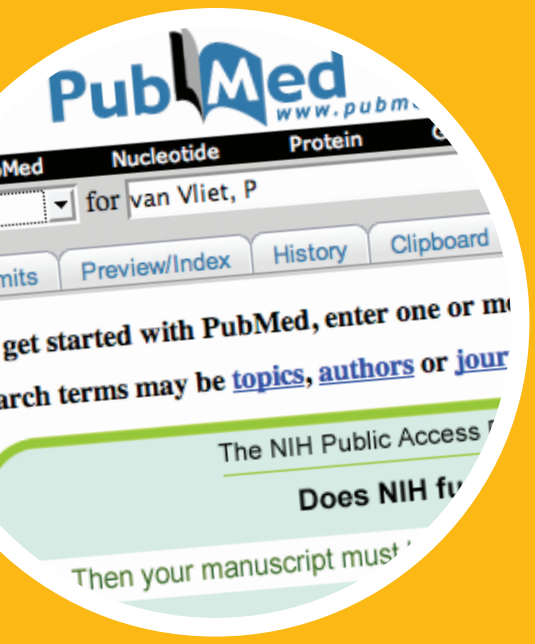

Smits AM, **van Vliet P**, Metz CH, Korfage TH, Sluijter JPG, Doevendans PA, Goumans MJ.
Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes; an in vitro model for studying human cardiac physiology and pathophysiology.
Nature Protocols 2009; 4(2): 232-243.

Bot PTG, Hoefer IE, Sluijter JPG; **van Vliet P**, Smits AM, Lebrin F, Moll F, de Vries JP, Doevendans PA, Piek JJ, Pasterkamp G, Goumans MJ.
Increased expression of the transforming growth factor-beta signaling pathway, endoglin, and early growth response-1 in stable plaques.
Stroke 2009; 40(2): 439-447

van Vliet P, Roccio M, Smits AM, van Oorschot AAM, Metz CHG, van Veen TAB, Sluijter JPG, Doevendans PA, Goumans MJ.
Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy.
Netherlands Heart Journal 2008; 16(5): 163-169.

Goumans MJ, de Boer TP, Smits AM, van Laake LW, **van Vliet P**, Metz CHG, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MAG, de Kleijn D, Mummery CL, van Veen TAB, Sluijter JPG, Doevendans PA.
TGFβ-1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro.
Stem Cell Research 2008; 1(2): 138-149.

van Vliet P, Sluijter JPG, Doevendans PA, Goumans MJ.
Isolation and expansion of resident cardiac progenitor cells.
Expert Reviews of Cardiovascular Therapy 2007; 5(1): 33-43. Review.

Smits AM, **van Vliet P**, Hassink RJ, Goumans MJ, Doevendans PA.
The role of stem cells in cardiac regeneration.
Journal of Cellular and Molecular Medicine 2005; 9(1): 25-36. Review.

Oosterveen T, **van Vliet P**, Deschamps J, Meijlink F.
The direct context of a hox retinoic acid response element is crucial for its activity.
Journal of Biological Chemistry 2003; 278(26): 24103-24107.

van Vliet P, Smits AM, de Boer TP, Korfage TH, Metz, CHG, Roccio M, van der Heyden MAG, van Veen TAB, Sluijter JPG, Doevendans PA, Goumans MJ.
Fetal and adult cardiomyocyte progenitor cells have different developmental potential.
Submitted.

Sluijter JPG, **van Vliet P**, van Mil A, Metz CHG, Doevendans PA, Goumans MJ.
MicroRNA-1 and 499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells.
Submitted.

van Vliet P, de Boer TP, van der Heyden MAG, Sluijter JPG, Doevendans PA, Goumans MJ.
Hyperpolarization induces differentiation of human cardiomyocyte progenitor cells.
Submitted.

van Vliet P, van Oorschot AAM, Smits AM, Liu Z, Sluijter JPG, Hoefer IE, Doevendans PA, Goumans MJ.
Survivin increases cardiomyocyte progenitor cell proliferation and survival.
Submitted.

Curriculum vitae

I REJECT YOUR REALITY AND SUBSTITUTE MY OWN.

ADAM SAVAGE, MYTHBUSTER

Patrick van Vliet was born on March 9, 1977 in Nieuwegein, the Netherlands. After graduating from secondary school in 1995 (Cals College, Nieuwegein), he very much enjoyed his study Biology at the University of Utrecht, the Netherlands, during which he became more commonly known as Piet. During this study, he became very interested in Developmental Biology and his first internship was therefore performed in the group of Dr. Frits Meijlink and Dr. Jacqueline Deschamps at the Hubrecht Institute in Utrecht, under the supervision of Dr. Tony Oosterveen. He then followed the Erasmus ICP Course for Marine Cell Biology in Banyuls-sur-Mer, France, given by Dr. Wim Dictus, University of Utrecht. He performed his second internship under the supervision of Dr. ir. Leen Blok and Dr. ir. Josien Oosterhoff, at the department of Reproduction and Development, Erasmus University Rotterdam. Finally, he returned to the Hubrecht laboratory to write his Master's thesis under the supervision of Dr. Bas Defize.

In September 2004, Piet started as a graduate student in the group of Prof. Dr. Pieter Doevendans and Dr. Marie-José Goumans at the laboratory of Experimental Cardiology, University Medical Center Utrecht. The results of his research are described in this thesis.
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