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Human cardiomyocyte progenitor cells: a short history of nearly everything

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Abstract

The high occurrence of cardiac disease in the Western world has driven clinicians and cardiovascular biologists to look for alternative strategies to treat patients. A challenging approach is the use of stem cells to repair the heart, in itself an inspiring thought. In the past 10 years, stem cells from different sources have been under intense investigation and, as a result, a multitude of studies have been published on the identification, isolation, and characterization, of cardiovascular progenitor cells and repair in different animal models. However, relatively few cardiovascular progenitor populations have been identified in human hearts, including, but not limited to, cardiosphere-derived cells [1], cKit+ human cardiac stem cells [2], Isl1+ cardiovascular progenitors [3], and, in our lab, cardiomyocyte progenitor cells (CMPCs) [4, 5]. Here, we aim to provide a comprehensive summary of the past findings and present challenges for future therapeutic potential of CMPCs.

Keywords: cardiac stem cells • regeneration • microRNA • miRNA • cell therapy • apoptosis • necrosis

Location, isolation and characterization of CMPCs

Similar to the Isl1+ cardiovascular progenitors [3], CMPCs can be found in the atria, atrial appendages, and, in addition, atrioventricular region, intra-atrial septum and scattered within the subepicardial layer of the ventricles [4]. In particular, atrial appendages from adult human hearts, obtained as surgical waste, are routinely used to obtain adult CMPCs [5, 6]. To isolate CMPCs from human foetal and adult hearts, we have developed two different protocols, based on enzymatic dissociation of cardiac tissue followed by clonal expansion or by magnetic activated cell sorting (MACS) using an epitope that is recognized by a Sca-1 antibody [6]. We obtained a highly proliferating population of

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cells that express Isl1, cKit, Nkx2.5, Gata4, Mef2c, CD31, Endoglin (CD105), and telomerase, but do not express haematopoietic or mesenchymal stem-cell markers or cardiomyocyte sarcomeric proteins [4, 5]. *In vitro*, CMPCs appear as spindle-shaped cells with a high nucleus-to-cytoplasm ratio, which is typical for progenitor cells (Fig. 1A).

Cardiovascular potential of CMPCs *in vitro*

To test the cardiomyogenic potential of CMPCs *in vitro*, we treated CMPCs with 5-azacytidine for three consecutive days, followed by culture in medium containing a mix of growth factors [4, 6]. With regard to future standard clinical practices, it is important that we do not need to co-culture CMPCs with fully developed cardiomyocytes, which is often required for cardiomyogenesis of other cardiovascular progenitor populations. Within 3–4 weeks, spontaneously beating cells could be observed and mRNA and protein analysis showed expression of cardiomyocyte sarcomeric proteins and gap junction proteins [4] (Fig. 1B). Despite its potent effect, the efficiency of cardiomyogenic differentiation with 5-azacytidine treatment is relatively low (13.5%) and therefore we exposed cells to cardiogenic inducing growth factors. When we added TGFbeta1 and/or BMP6 after 5-azacytidine treatment, the CMCP differentiation efficiency was greatly increased (up to 95% [5]). TGFbeta1 or BMP alone, without 5-azacytidine, was not that effective. CMPC-derived cardiomyocytes (CMPC-cm) express sarcomeric proteins in the typical striated pattern. In addition, all the ion channels required for a functional action potential and phosphorylated connexin proteins at the cell membrane to form gap junctions are present. Moreover, adherens junction and desmosomal proteins for cell–cell interactions were detected. Electrophysiological analyses showed that CMPC-cm have functional gap junctions, excitation-contraction coupling, a foetal ventricular cardiomyocyte-like action potential, and the capacity to react to adrenergic agents Interestingly, CMPC-cm from foetal cells generally have a more immature phenotype than CMPC-cm form adult cells and often show spontaneous beating, whereas adult CMPC-cm are able to form rod-shaped cardiomyocytes that do not contract spontaneously, probably because of a more stable resting membrane potential [5, 7, 8]. This suggests that foetal and adult CMPCs are either intrinsically different upon isolation (maybe due to the age of the tissue used), differentially affected by the culture conditions, or both [8]. Based on their cardiomyogenic potential *in vitro*, we termed the cells 'cardiomyocyte progenitor cells'.

In addition to the cardiomyogenic capacity, CMPCs can also form endothelial cells and smooth muscle cells in an *in vitro* angiogenesis model [5]. However, foetal- and adult-derived CMPCs show a different angiogenic potential, with foetal CMPCs forming relatively more endothelial cells and less smooth muscle cells than adult CMPCs [8]. The underlying mechanism for the differences in foetal and adult CMPC-cm discussed above remains unclear. Of note is the limited potential of foetal CMPCs to undergo adipogenic differentiation *in vitro* [8].

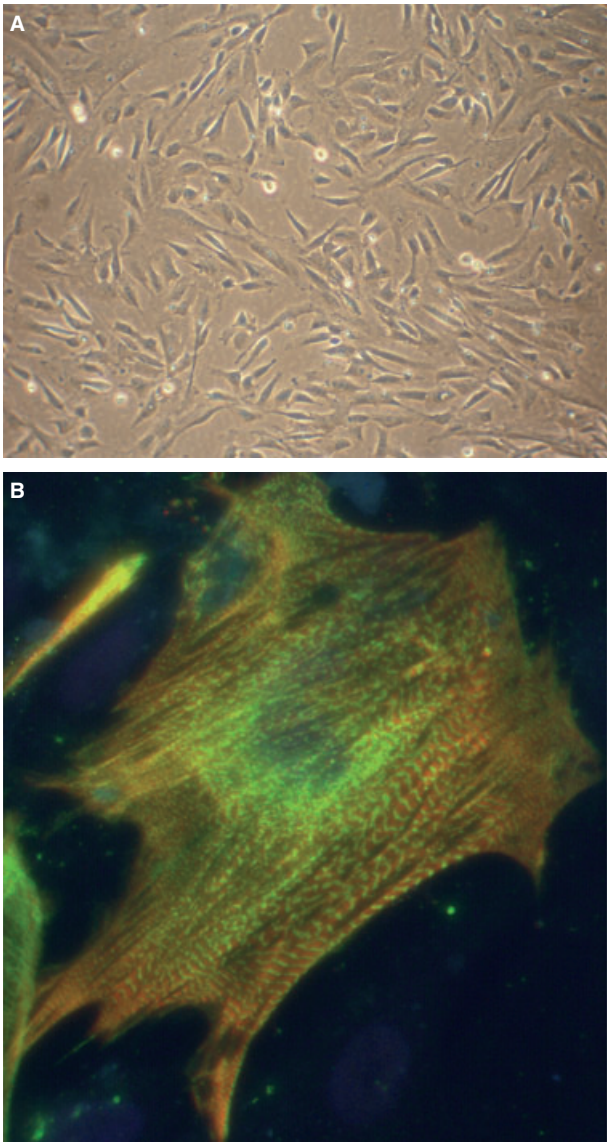

Fig. 1 Human foetal-derived cardiomyocyte progenitor cells (CMPCs) cultures under proliferating conditions (**A**), and after differentiating by 5-aza and TGF-beta stimulation into beating cardiomyocytes (**B**). Beating cells were stained for cardiac actinin (red), troponin T (green) and hoechst (nuclei, blue).

Cardiovascular potential of CMPCs *in vivo*

To confirm the potential therapeutic effects *in vivo*, CMPCs and predifferentiated CMPM-cm were injected into the infarct border zone of infarcted mouse hearts [9]. Twenty-eight days post-injection, cardiac function was markedly improved compared with the vehicle control in both groups, which continued up to 3 months post-injection. CMPCs

were able to differentiate into cardiomyocytes (~55%), SMCs (~11%) and endothelial cells (~10%). CMPCs showed enhanced VEGF secretion as compared with CMPC-cm, which resulted in greater vessel density of the injured tissue. Otherwise, there were no substantial differences upon CMPCs or CMPC-cm transplantation, indicating that CMPCs do not need *in vitro* differentiation prior to injection. Importantly, CMPCs were able to migrate throughout the infarcted area upon injection, which allows them to be most effective in places where they are needed. Although these results are very promising, only limited number of injected cells could be (4%) observed after 3 months. This opens the discussion for the true mechanisms for observed functional improvements and allows further improvements for transplantation strategies.

Apart from primary and second heart lineage progenitor cells, epicardial cells (the cells on the outer layer of the heart) contribute to cardiogenesis as well [10]. We hypothesized that human CMPCs and epicardium-derived progenitor cells (EPDCs) together would provide an even stronger approach to treat an injured myocardium. When co-cultured *in vitro*, CMPCs enhanced EPDC proliferation, they stimulated each other's migration under normoxia, but not hypoxia, and increased paracrine signalling under hypoxic conditions [11]. By injecting the two populations together, the interactions resulted in increased cardiac function upon cardiac injury as compared with either cell type alone. This was partly due to a more pronounced paracrine stimulation of angiogenesis in the CMPC-EPDC mixed group.

CMPCs *in vitro*; a cell model to improve understanding and test human cardiomyogensis

The cardiovascular potential of CMPCs *in vitro* makes them ideal tools to investigate underlying mechanisms and/or develop alternative protocols to modulate proliferation, migration, paracrine signalling, or cardiomyo- and vasculogenesis *in vitro*.

Cardiomyogenic differentiation

To develop an alternative to the use of 5-azacytidine, which may have unknown side-effects, we have used a modulation of the cell membrane potential, which is known to affect cellular proliferation and differentiation [12, 13]. We showed that by co-culturing CMPCs with HEK293 cell lines that have an engineered low membrane potential (KWGF cells, −75 mV) [14], CMPCs differentiated into cardiomyocytes [15]. Disrupting functional gap junction coupling between KWGF cells and CMPCs suggested that the electrotonic application of a low membrane potential in CMPCs, thereby hyperpolarizing the membrane, was responsible for this effect. Indeed, when CMPCs were exposed to medium with a low potassium concentration alone, which effectively hyperpolarized their resting membrane potential, cardiomyogenesis was induced, resulting in spontaneously contracting cardiomyocytes after several weeks. The increase in intracellular calcium and enhanced nuclear factor of activated T cells (NFAT) activity after exposure to medium with a low potassium concentration, thereby hyperpolarizing the membrane, suggests that calcineurin signalling was involved in activating myogenic transcription factors, similar to hyperpolarization-mediated myogenesis in skeletal myoblasts [16]. Interestingly, TGFbeta1 stimulated hyperpolarization in CMPCs as well, which suggests that hyperpolarization-mediated cardiomyogenesis plays a role in our standard 5-azacytidine/TGFbeta1 differentiation protocol.

In an attempt to understand the cardiomyogenic differentiation of the CMPCs, gene expression and micro RNA (miRNAs) arrays were performed. When differentiated into CMPC-cm, cells expressed an enrichment of known cardiac transcription factors and cardiac/structural genes. Interestingly, during differentiation, several mesodermal and developmental genes are (re-)expressed and novel transcription factors could be observed.

Several myogenic inducing miRNAs, such as miR-1, 133a, and 133b, could not be observed in our proliferating CMPCs; however, they become highly enriched in the CMPC-cm. In addition, we observed that miR-499 was highly enriched; a miRNA with a previously unknown function [17]. In addition to its presence in CMPC-cm, miR-499 was highly expressed in human and mouse cardiomyocyte. By introducing miR-1 and 499 into our CMPCs, we could enhance their myogenic differentiation by increased appearance of spontaneous beating and increased expression levels of Nkx2.5, Gata4, cardiac actinin, Mlc-2v and troponin T. By using miRNA inhibitors, cardiomyogenic differentiation could completely be prevented, demonstrating the prerequisite of these miRNAs for differentiation. HDAC4 and Sox6 were identified as potential targets for miR-1 and 499, respectively, and by using a RNAi knock-down approach, we observed that Sox6 efficiently drives cardiomyocyte differentiation [17].

Cellular proliferation and survival

CMPCs are exposed to a hypoxic environment upon transplantation into infarcted hearts and hypoxia is known to have different effects on cell proliferation, migration, paracrine signalling, differentiation and engraftment [18–20], consistently with studies in mesenchymal and haematopoietic stem cells and skeletal muscle satellite cells [21–23]; increased proliferation was observed in CMPCs exposed to hypoxia [24]. CMPCs did not differentiate into cardiomyocytes or vascular cells in response to hypoxia, but secretion of the pro-inflammatory cytokines MCP-1, TGFbeta and Il-8 was decreased, while pro-angiogenic VEGF-A secretion was increased. Further investigation revealed that thrombospondin-1 and -2, were up-regulated in hypoxic CMPCs, thereby allowing these enzymes to facilitate CMPC migration through a collagen matrix.

In addition, hypoxia enhanced expression of the pro-survival and mitogenic factor Survivin in CMPCs. By introducing or knocking-down Survivin in CMPCs, we could confirm that Survivin was indeed stimulating and required for CMPC proliferation (P. van Vliet, A. M. van Oorschot, A. M. Smits, Z. Liu, J. P. G. Sluijter, I. E. Hoefer, P. A.

Doevendans, and M. J. Goumans, unpublished data), respectively. Lack of Survivin resulted also in early apoptosis, likely due to a cell cycling defect. Unexpectedly, adenoviral Survivin overexpression also resulted in reduced migration and paracrine signalling in CMPCs, but not in HUVECs, probably *via* a reduced VEGF-A secretion.

Despite the differential expression of miR-155 in CMPCs [17], we did not find evidence that miR-155 influenced CMPCs proliferation, differentiation or angiogenesis in several follow-up studies. However, upon oxidative-stress, miR-155 is up-regulated as well, indicating a potential role of miR-155 in the stress response in CMPCs [25]. By ectopic introduction of miR-155 into CMPCs, oxidative stress-stimulated necrotic cell death *via* exposure to H_2O_2 is significantly reduced. In addition, we could observe that a death domain protein, receptor interacting protein1 (RIP1), is required for activating necrosis in CMPCs and a target for miR-155. Interestingly, miR-155 protects CMPCs from necrotic cell death independently of common PI3K-Akt survival and apoptosis pathways [25]. These findings demonstrate the cyto-protective role of miR-155 in CMPCs and suggest the possibility of utilizing miR-155 or target analogues to optimize cell engraftment for cellular transplantation therapy.

Paracrine effects

In addition to the true regenerative potential of CMPCs by replacing damaged myocardium, forming new cardiomyocytes, endothelial and smooth muscle cells, also the high secretory potential of the cells could be beneficial. One of the potential mechanisms of the observed *in vivo* effects upon transplantation of the CMPCs could be the paracrine release of growth factors, cytokines and chemokines, which are known to be strong modulators of tissue growth, angiogenesis and inflammatory responses. Injection of conditioned medium from ESC-derived MSCs was shown to reduce infarct size and improve cardiac function in a porcine model of ischaemia/reperfusion [26]. The activity of the conditioned medium was mediated by cell-released exosomes [27]. Exosomes are small membranous vesicles with a lipid bilayer, secreted by many, if not all, cells, and described to be involved in numerous processes, including immune modulation, angiogenesis and migration of cells. Exosomes contain many different proteins, including growth factors and cytokines, and coding and non-coding RNA molecules. Recently, we demonstrated that CMPCs do release exosomes into their environment, which are functionally active and can stimulate migration of endothelial cells in an *in vitro* scratch wound assay [28].

Perspective

Cardiac-derived progenitor cells, like the CMPCs, are a promising cell type that can be obtained, cultured and used for cell transplantation therapy to restore function of damaged myocardium. They have been shown to have true differentiation potential *in vitro* and *in vivo* towards cardiomyocytes and vascular cells, without the need for co-culture. In addition, these cells can be used to study human cardiomyogenesis *in vitro* or for testing new approaches to improve cell transplantation studies, like the use of tissue engineering approaches, to stimulate cardiac repair.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. **Elisa M, Luciana DA, Giacomo F, *et al.*** Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res.* 2004; 95: 911–21.
2. **Bearzi C, Rota M, Hosoda T, *et al.*** Human cardiac stem cells. *Proc Natl Acad Sci USA.* 2007; 104: 14068–73.
3. **Bu L, Jiang X, Martin-Puig S, *et al.*** Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature.* 2009; 460: 113–7.
4. **van Vliet P, Roccio M, Smits AM, *et al.*** Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Netherlands Heart J.* 2008; 16: 163–9.
5. **Goumans M-J, de Boer TP, Smits AM, *et al.*** TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes *in vitro*. *Stem Cell Res.* 2007; 1: 138–49.
6. **Smits AM, van Vliet P, Metz CH, *et al.*** Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an *in vitro* model for studying human cardiac physiology and pathophysiology. *Nat Protoc.* 2009; 4: 232–43.
7. **Boer TPD, Veen TABV, Jonsson MKB, *et al.*** Journal of Molecular and Cellular Cardiology Human cardiomyocyte progenitor cell-derived cardiomyocytes display a maturated electrical phenotype. *J Mol Cell Cardiol.* 2010; 48: 254–60.
8. **van Vliet P, Smits AM, de Boer TP, *et al.*** Foetal and adult cardiomyocyte progenitor cells have different developmental potential. *J Cell Mol Med.* 2010; 14: 861–70.
9. **Smits AM, Laake LWV, Ouden KD, *et al.*** Human cardiomyocyte progenitor cell transplantation preserves long-term function of the infarcted mouse myocardium. *Cardiovasc Res.* 2009; 83: 527–35.
10. **Evans SM, Yelon D, Conlon FL, *et al.*** Myocardial lineage development. *Circ Res.* 2010; 107: 1428–44.

11. **Winter EM, van Oorschot AAM, Hogers B, *et al.*** A new direction for cardiac regeneration therapy: application of synergistically acting epicardium-derived cells and cardiomyocyte progenitor cells. *Circ Heart Failure.* 2009; 2: 643–53.
12. **Adams DS.** A new tool for tissue engineers: ions as regulators of morphogenesis during development and regeneration. *Tissue Eng. Part A.* 2008; 14: 1461–8.
13. **Sundelacruz S, Levin M, Kaplan DL.** Role of membrane potential in the regulation of cell proliferation and differentiation. *Stem Cell Rev.* 2009; 5: 231–46.
14. **de Boer TP, van Veen TAB, Houtman MJC, *et al.*** Inhibition of cardiomyocyte automaticity by electrotonic application of inward rectifier current from Kir2.1 expressing cells. *Med Biol Eng Comput.* 2006; 44: 537–42.
15. **van Vliet P, de Boer TP, van der Heyden MAG, *et al.*** Hyperpolarization induces differentiation in human cardiomyocyte progenitor cells. *Stem Cell Rev.* 2010; 6: 178–85.
16. **Konig S, Béguet A, Bader CR, *et al.*** The calcineurin pathway links hyperpolarization (Kir2.1)-induced Ca^{2+} signals to human myoblast differentiation and fusion. *Development.* 2006; 133: 3107–14.
17. **Sluijter JPG, van Mil A, van Vliet P, *et al.*** MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. *Arterioscler Thromb Vasc Biol.* 2010; 30: 859–68.
18. **Csete M.** Oxygen in the cultivation of stem cells. *Ann N Y Acad Sci.* 2005; 1049: 1–8.
19. **Degterev A, Hitomi J, Germscheid M, *et al.*** Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol.* 2008; 4: 313–21.
20. **Grayson WL.** NIH Public Access. *Artif Cells Blood Substit Immobil Biotechnol.* 2010; 25: 32–42.
21. **Fukuda S, Foster RG, Porter SB, *et al.*** The antiapoptosis protein survivin is associated with cell cycle entry of normal cord blood CD34(+) cells and modulates cell cycle and proliferation of mouse hematopoietic progenitor cells. *Blood* 2002; 100: 2463–71.
22. **Grayson WL, Zhao F, Bunnell B, *et al.*** Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun.* 2007; 358: 948–53.
23. **Lennon DP, Edmison JM, Caplan AL.** Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol.* 2001; 187: 345–55.
24. **van Oorschot AA, Smits AM, Pardali E, *et al.*** Low oxygen tension positively influences cardiomyocyte progenitor cell function. *J Cell Mol Med.* 2011; 15: 2723–34.
25. **Liu J, van Mil A, Vrijsen K, *et al.*** MicroRNA-155 prevents necrotic cell death in human cardiomyocyte progenitor cells *via* targeting RIP1. *J Cell Mol Med.* 2011; 15: 1474–82.
26. **Timmers L, Lim SK, Arslan F, *et al.*** Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res.* 2007; 1: 129–37.
27. **Lai RC, Arslan F, Lee MM, *et al.*** Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2010; 4: 214–22.
28. **Vrijsen KR, Sluijter JPG, Schuchardt MWL, *et al.*** Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *J Cell Mol Med.* 2010; 14: 1064–70.