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

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Background. **In recent years, resident cardiac 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 cardiac progenitor cell populations show immature development and need co-culture with neonatal rat cardiomyocytes in order to differentiate in vitro. Here we describe the localisation, isolation, characterisation, and differentiation of cardiomyocyte progenitor cells (CMPCs) isolated from the human heart.**

Methods. **hCMPCs were identified in human hearts based on Sca-1 expression. These cells were isolated, and FACS, RT-PCR and immunocytochemistry were used to determine their baseline characteristics. Cardiomyogenic differentiation was induced by stimulation with 5-azacytidine.**

Results. **hCMPCs were localised within the atria, atrioventricular region, and epicardial layer of the foetal and adult human heart. In vitro, hCMPCs could be induced to differentiate into cardiomyocytes and formed spontaneously beating aggregates, without the need for co-culture with neonatal cardiomyocytes.**

Conclusion. **The human heart harbours a pool of resident cardiomyocyte progenitor cells, which can be expanded and differentiated in vitro. These cells may provide a suitable source for cardiac regeneration cell therapy. (*Neth Heart J* 2008;16: 163-9.)**

Keywords: human cardiac progenitor cell, cardiomyocytes, differentiation

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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 ischaemic 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,[5-8] cardiac stem cells,[5] embryonic stem (ES) cells[9,10] and foetal 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 foetal 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,[5] stem cell antigen-1 (Sca-1),[23] homeodomain transcription factor islet-1 (isl-1),[24] side population cells (SP),[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 in vivo, fusion of transplanted progenitor cells with resident adult cardiomyocytes can occur, which may lead to a misinterpretation of the cardiomyogenic differentiation of the stem 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 vitro.

In the present study, we isolated cardiomyocyte progenitor cells (CMPCs) from human heart tissue using an anti-Sca-1 antibody. Although an Sca-1 epitope in human cells is disputed, the cells we selected using the Sca-1 antibody from both foetal 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 artefacts 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.

Material and methods

Isolation and culture of cardiomyocyte progenitor cells from human hearts

To collect human foetal 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. Foetal 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% foetal 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).

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 synthesised 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 normalised for expression of β-actin. Results were analysed on 10% acrylamide gel stained with ethidium bromide.

Flow cytometric analysis

Cultured CMPCs (passage 7) were trypsinised 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/0.05M 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 analysed 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 permeabilised (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 recognised 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. Immunolabelling was performed using Texas Red (TR)- or FITC-conjugated secondary antibodies (Jackson Laboratories). Hoechst dye was used to localise 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, o/n 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.[27] Detection was by ECL (Amersham). P-H3 and H3 antibodies that specifically recognise phosphorylated histone 3 or total histone 3, respectively, were used 1:5000. Beta-actin detection (1:10,000, Chemicon) was used as a loading control.

Results

Localisation and characterisation of human cardiomyocyte progenitor cells

To identify CMPCs in foetal and adult human heart, we used an anti-Sca-1 antibody that has been shown to recognise mouse cardiac 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

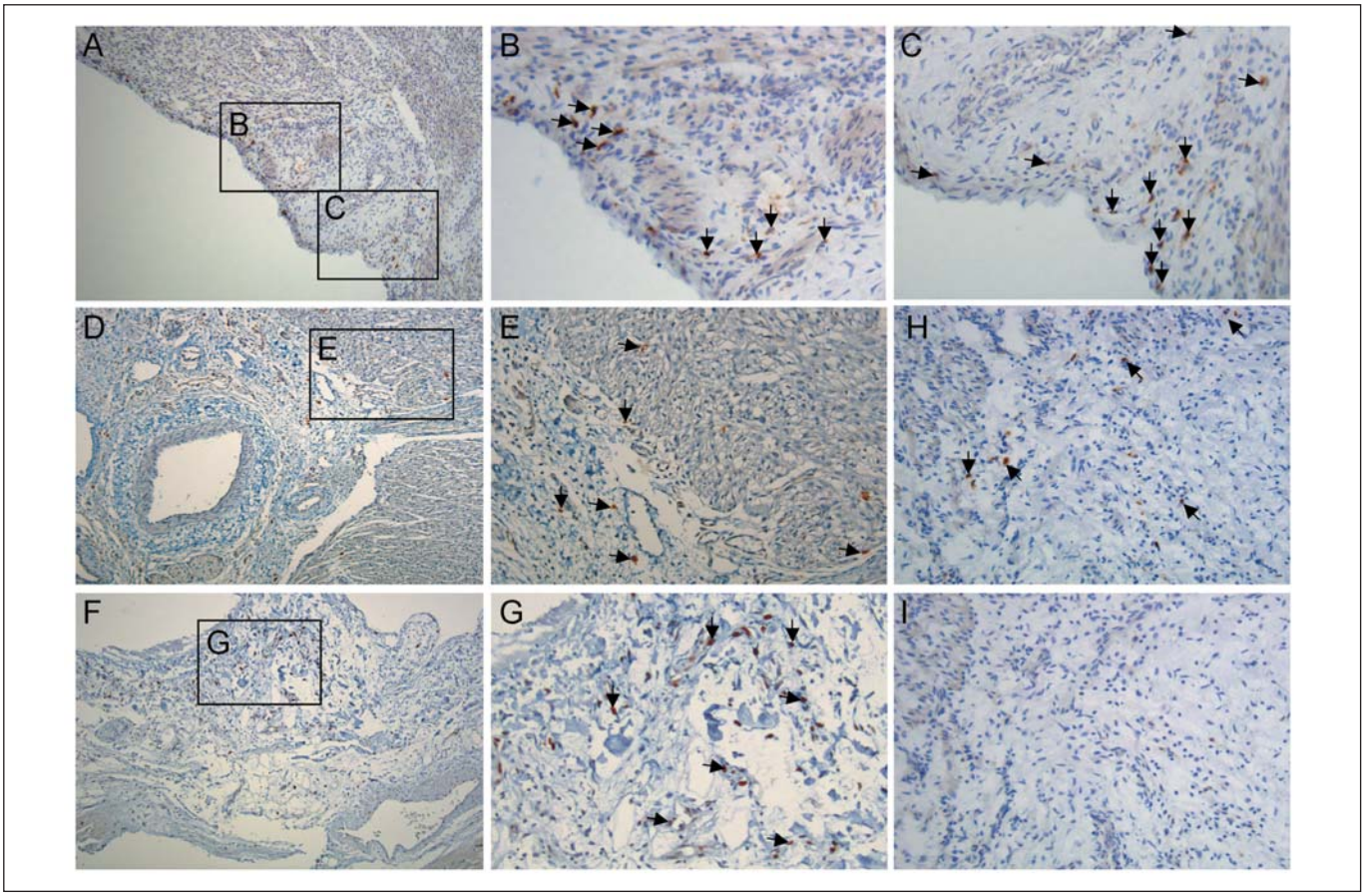

Figure 1. hCMPC in the human heart. (A-G) Immunohistochemistry for Sca-1 in foetal and adult heart. (B, C) High power magnification of areas in A. (D) Atrial ventricular boundary. (E) High power magnification of area in D. (F, H) hCMPCs in biopsy from adult patient. (G) High power magnification of area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

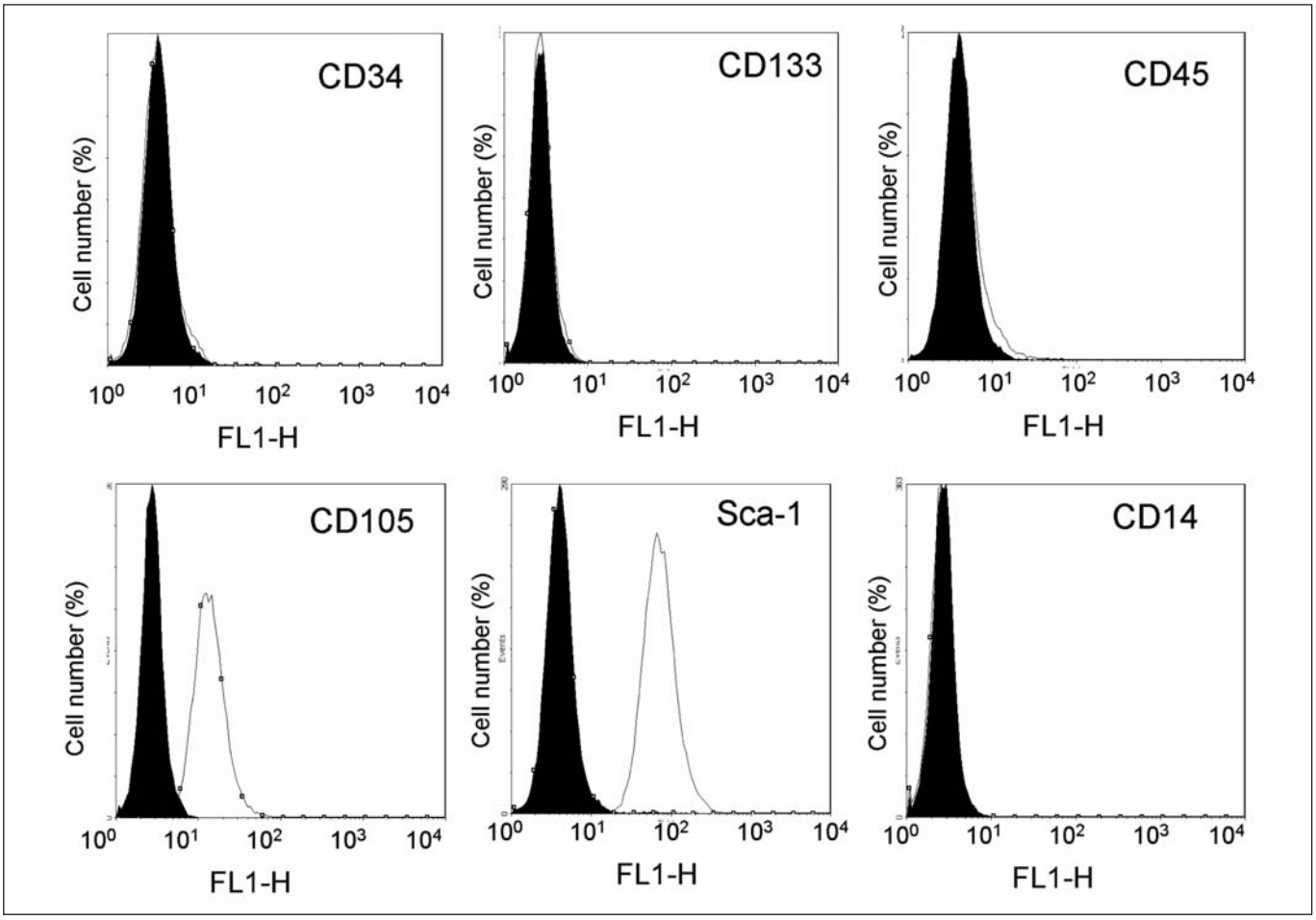

Figure 2. Flow cytometric analysis of (stem) cell marker expression on cultured foetal hCMPCs. Histogram plots are shown with the isotype control in black and the specific signal in white.

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 characterised by flow cytometry (figure 2). Foetal CMPCs were negative for CD45, CD34, CD133, and CD14, and positive for CD105 and Sca-1. After isolation, foetal 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 foetal 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 Nkx 2.5, while cardiomyocyte-specific genes were not expressed. Adult progenitor cells showed a similar expression pattern (not shown).

Cardiomyogenic differentiation of human CMPCs

To initiate differentiation of hCMPCs towards a cardiomyogenic lineage, the proliferation of the cells should be arrested. In P19 embryonal carcinoma cells and mouse Sca-1$^+$ cardiac progenitor cells, cardiomyogenic differentiation can be induced by stimulation with the demethylating agent 5-azacytidine.[23,28] 5-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 Nkx 2.5 (figure 4C).

After stimulation with 5-azacytidine and culture in differentiation medium for several weeks, hCMPCs 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 hCMPC-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.

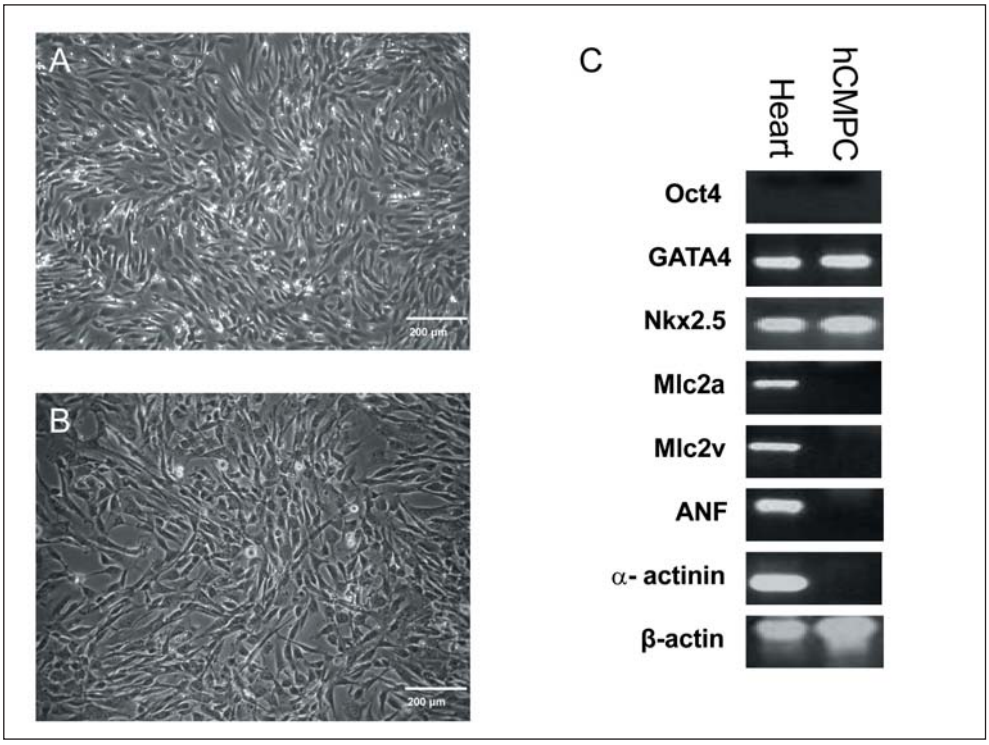

Figure 3. Bright field images of foetal (A) and adult (B) hCMPCs. (C) Semiquantitative RT-PCR on RNA isolated from undifferentiated hCMPCs probed for the expression of the indicated genes.

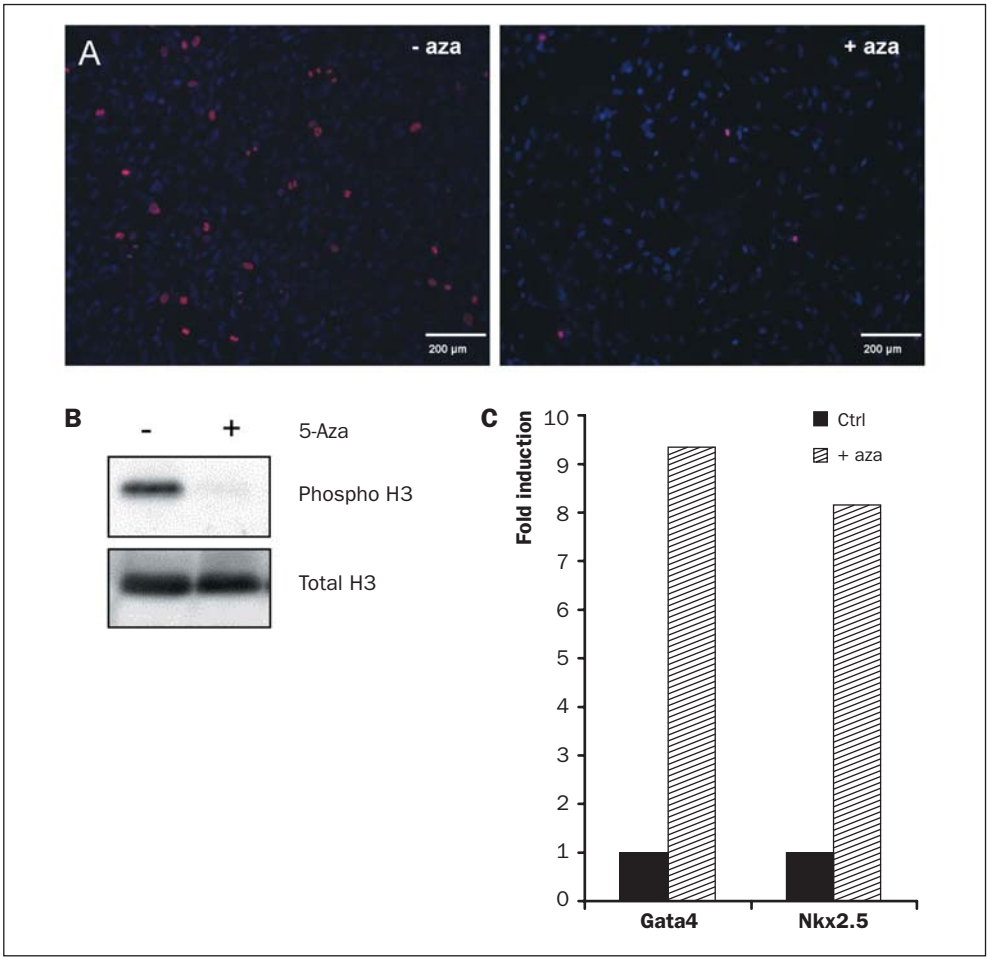

Figure 4. (A) Immunocytochemistry on hCMPCs before (left) and after (right) 5-azacytidine stimulation. Phosphorylated-histone 3 is indicated in red, nuclei in blue. (B) Western blot analysis for phospho-H3 and total H3 on protein from hCMPCs with or without 5-azacytidine stimulation. (C) Quantitative RT-PCR on RNA from hCMPCs with (striped bars) or without (black bars) 5-azacytidine stimulation. Expression was normalised for β-actin and fold expression was calculated compared with control.

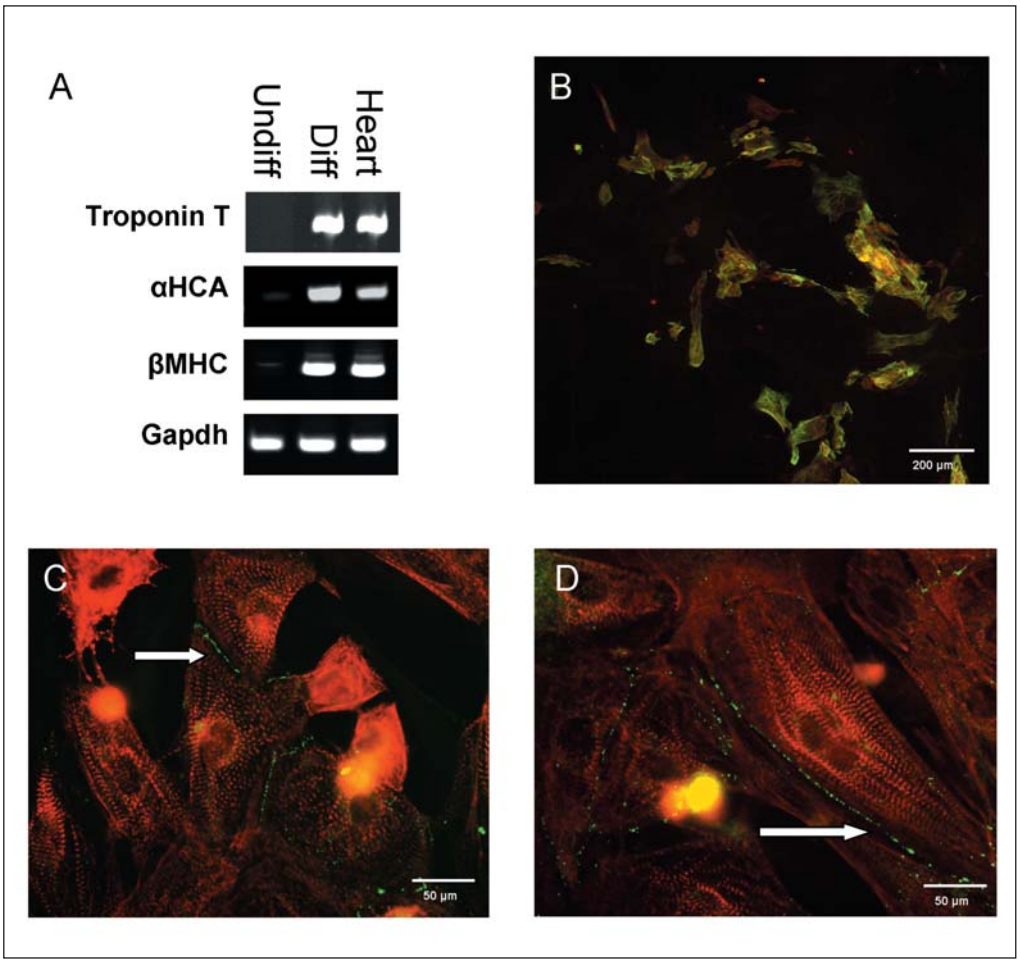

Figure 5. (A) Semiquantitative RT-PCR on RNA isolated from differentiated hCMPCs. (B) Immunolabelling against troponin I (green) and α-actinin (red) in hCMPCs differentiated into cardiomyocytes after 5-azacytidine stimulation. (C and D) Immunolabelling against the connexin isoforms Cx40 and Cx43 (green) and α-actinin (red). Arrows indicate cell membrane localisation of connexin isoforms.

Discussion

In this study, we report the isolation and cardiomyogenic differentiation of resident cardiac 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 cardiac progenitor cells.[29] 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,[30,31] and that current isolation methods result in cell populations that are at a different developmental stage. These cells were shown to 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, cardiac progenitor cells also show the capacity to form different cardiac cell types. It still remains unknown, however, which signals are required to drive differentiation. Furthermore, in vivo differentiation remains inefficient, indicating the need to elucidate the fate of cardiac progenitor cells under normal and pathological circumstances. Possibly, the reactivation of the foetal gene expression programme 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.[32] The potential of hCMPCs 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 hCMPCs 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 foetal-derived hCMPCs 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 hCMPCs can be taken forward to clinical trials. A detailed electrophysiological characterisation of hCMPC-derived cardiomyocytes may be required to predict their behaviour after transplantation and integration into host tissue. Human CMPCs, characterised in this study, provide a useful tool to study human cardiomyocyte differentiation and could be used for drug screening. Eventually they may serve as a suitable source for cellular therapy in failing hearts.

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