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Neonatal epicardial-derived progenitors aquire myogenic traits in skeletal muscle, but not cardiac muscle

Ditte C. Andersen [a,b,d,*], Charlotte H. Jensen [a,d], Ida Skovrind [a,b,d], Rikke Helin Johnsen [a,b,d], Gunnhildur Asta Traustadottir [a,c,d], Katrine S. Aagaard [a,c,d], Suganya Ganesalingam [a,c,d], Søren P. Sheikh [a,c,d]

[a] Laboratory of Molecular and Cellular Cardiology, Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark
[b] Clinical Institute, University of Southern Denmark, Denmark
[c] Cardiovascular and Renal Research, University of Southern Denmark, Winsloewparken 21[3rd], 5000 Odense, Denmark
[d] The Danish Regenerative Center (danishcrm.com), Odense University Hospital, Sdr. Boulevard 29, 5000 Odense, Denmark

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ABSTRACT

Background/Objectives: Epicardium-derived progenitor cells (EPDCs) differentiate into all heart cell types in the embryonic heart, yet their differentiation into cardiomyocytes in the adult heart is limited and poorly described. This may be due to EPDCs lacking myogenic potential or the inert adult heart missing regenerative signals essential for directed differentiation of EPDCs. Herein, we aimed to evaluate the myogenic potential of neonatal EPDCs in adult and neonatal mouse myocardium, as well as in skeletal muscle. The two latter tissues have an intrinsic capability to develop and regenerate, in contrast to the adult heart.
Methods: Highly purified mouse EPDCs were transplanted into damaged neonatal and adult myocardium as well as regenerating skeletal muscle. Co-cultures with skeletal myoblasts were used to distinguish fusion independent myogenic conversion.
Results: No donor EPDC-derived cardiomyocytes were observed in hearts. In contrast, a remarkable contribution of EPDCs to skeletal muscle myofiber formation was evident *in vivo*. Furthermore, co-cultures of EPDCs with myoblasts showed that EPDCs became part of multinucleated fibers and appeared to acquire myogenic traits independent of a fusion event. Fluorescence activated cell sorting of EPDCs co-cultured with and without myoblasts and subsequent qRT-PCR of 64 transcripts established that the myogenic phenotype conversion was accomplished through induction of a transcriptional myogenic program.
Conclusion: These results suggest that EPDCs may be more myogenic than previously anticipated. But, the heart may lack factors for induction of myogenesis of EPDCs, a scenario that should be taken into consideration when aiming for repair of damaged myocardium by stem cell transplantation.

1. Introduction

Four million Europeans die annually of cardiovascular disease (CVD) [1]. The high mortality is because the heart muscle becomes irreversibly damaged and lacks the ability to repair itself [2]. In contrast, other organs like the skeletal muscle found in limbs are highly regenerative and repair completely from resident stem cells [3]. Current treatments of CVD merely serve to reduce symptoms, whilst heart transplants are relatively rare. Novel regenerative therapeutics are thus needed to reduce mortality rates.

Conventional stem cell therapy has been state-of-the art treatment in regenerative cardiac medicine for a decade. However, recent results [4] suggest that this strategy is inefficient, possibly because the heart lacks key regenerative signals needed to support engraftment and directed differentiation of the stem cells applied.

The lack of a regenerative capacity in the heart has been ascribed to, among other causes, an absence of endogenous cardiac stem cells (CSCs) with an ability to repair the damaged heart. CSCs are generally considered to be immature cells present in the heart, which may proliferate in response to an activating signal and then differentiate into mature heart cells including cardiomyocytes, fibroblasts, and endothelial cells. However, since the description of C-kit positive multipotent CSCs in the adult heart, numerous other CSCs have been proposed [2]. Nonetheless, limited success has been achieved with these CSC candidates [5]. Two of the most popular CSC candidates – C-kit + CSCs and cardiospheres – have been shown not to lack cardiomyogenic potential [6,7]. Epicardial progenitor cells (EPDCs) are another intriguing CSC source. In contrast to C-kit CSCs and cardiospheres, lineage tracing has demonstrated that during cardiac development EPDCs differentiate

* Corresponding author at: Laboratory of Molecular and Cellular Cardiology, Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Denmark.
E-mail address: dandersen@health.sdu.dk (D.C. Andersen).

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into all heart cell types including cardiomyocytes [8]. Thus, EPDCs exhibit a genuine ability, at least during development, to contribute to the myogenic cell lineage.

However, in adult mammalian hearts only a few studies have been performed with *in vivo* transplantation. These studies have shown that EPDCs only rarely give rise to cardiomyocytes [9,10]. This response may be augmented by thymosin-B4 stimulation [9], but it still occurs at a frequency that is insufficient for regenerating the damaged heart [9].

Embryonic and adult EPDCs appear to be very different, with the latter being less potent with respect to differentiation capacity [11]. Indeed, the low occurrence in general of CSCs for cardiomyocyte differentiation [2] is a major obstacle for developing CSC-based stem cell therapy for heart disease. The low extent of EPDC-to-cardiomyocyte conversion [9], may be a result of non-embryonic EPDCs having a genuine muted cardiomyocyte differentiation capability, but it is also tempting to speculate that important regenerative factors required for this process are missing in the inert adult heart. More detailed basic knowledge of the differentiation potential of EPDCs is required to establish if these cells remain a robust candidate as a CSC source for cell replacement therapy in heart disease.

In this study, we tested whether neonatal EPDCs are myogenic in damaged adult myocardium or when exposed to more remodeling myogenic environments like the developing neonatal myocardium [12,13] and regenerating skeletal muscle [14].

2. Materials and methods

2.1. Animals

C57BL/6 mice were housed in plastic cages with an alternating 12 h light/dark cycle. They were fed *ad libitum* with a chow appropriate for pregnant or normal mice. The original stock of C57BL/6 mice was obtained from Taconic Europe. All animal experiments were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, as approved by the Danish Council for Supervision with Experimental Animals (#2011/561-1966 and #2010/561-1792).

2.2. EPDC isolation and culture

Throughout the study we used neonatal EPDCs that were isolated as recently described [4]. Briefly, neonatal (day 1–3 postnatal) mice from one litter were sacrificed by decapitation. Their hearts were carefully dissected without disturbing the epicardium and pooled. EPDCs were dissociated by gentle treatment with 0.3% Trypsin/DNase solution followed by centrifugation and red blood cell lysis. Harvested EPDCs were plated on ECM (E1270; Sigma-Aldrich) coated plates and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 20% FBS (Fetal Bovine Serum)/1% PS (Penicillin–Streptomycin) (all from Lonza). Purity was checked by flow cytometry as previously described [4].

2.3. Cell counting

Total cell numbers were determined using a Beckman Coulter Counter Z2 fitted with a 100 μm aperture. The size range of particles counted varied according to cell type. Counting was performed in triplicate.

2.4. Labeling with fluorescent dye and co-culture

For all co-culture and transplantation studies, EPDCs at high confluence were labeled as previously described [4] the day before the experiment with optimal amounts (determined to be 4 μM; Supplemental Fig. S1) of fluorescent CellTracker CM-DiI (Life Technologies) that binds cellular thiols and is retained inside the cells during subsequent processing. As such, CM-DiI freely passes cell membranes but then is transformed into a cell-impermeant reaction product. Cells were extensively washed to remove excess dye and cultured for 24 h until co-culture or transplantation studies were initiated. CM-DiI labeling was verified after 24- and 72 h by microscopy and flow cytometry and fixation did not affect fluorescence intensity (Supplemental Fig. S1). Co-culture studies used either mouse C2C12 skeletal myoblasts (ATCC) or rat embryonic heart derived myoblasts (ATCC). Notably, the latter cell line behaves like skeletal myoblasts, even though the cells have been isolated from the heart. Cell lines were cultured as recommended by suppliers and plated to obtain confluence at day 3. CM-DiI labeled EPDCs were added to cultured myoblasts at a an EPDC:myoblast ratio of 1:5 to ensure a myogenic environment. Myogenic differentiation was induced by 2% horse serum as previously described [14–16].

2.5. Immunofluorescence

Cultured cells or cryosections were fixed in 4% NBF (normal buffered formaldehyde) for 10 min, permeabilized for 10 min in 0.5% Triton X-100/TBS (Tris buffered saline), blocked in 2% BSA (Bovine serum albumin)/TBS for 10 min, and incubated for 2 h with primary antibodies or isotype/serum IgG controls diluted in 1% BSA/TBS. Secondary antibodies used were Alexa 488, 555, or 647 donkey, α-mouse, rat, goat, or rabbit IgG (1:200; Molecular Probes) and mounting medium containing DAPI (Vectashield; Vector Labs). Microscopic examinations were performed using a DMI4000B Cool Fluo Package microscope (Leica) instrument equipped with a DFC340 FX digital camera (Leica). In all experiments, exposure (camera settings) and picture processing (brief adjustment of contrast/brightness and color balance using Adobe Photoshop, Version 11.0.2) were identical for sample and controls specimens (isotypes or whole IgGs).

2.6. Flow cytometry, cell sorting, and low density arrays (qRT-PCR)

For flow cytometry analysis, cells were detached using 0.25% trypsin-EDTA, fixed in 1% NBF, and washed extensively before permeabilization in HBSS (Hank's Balanced Salt Solution)/5% FBS/BD Perm Wash (BD Biosciences). Cells were then immunostained with primary antibodies for 60 min on ice, washed three times in HBSS/5% FBS/BD Perm Wash, and incubated with Alexa 488 or 647 donkey α-mouse, rat, or rabbit IgG (1:200, Molecular Probes) before a final three washes.

Samples were analyzed using a LSR II flow cytometer (BD Biosciences) and flow data were analyzed by FACSDiva™ software, version 5.0.1 (BD Biosciences). Debris and cell doublets were excluded from the analysis by gating in the forward and sideward scatter as previously described [15].

Fluorescence activated cell sorting (FACS) was performed as previously described [15,16]). Briefly, co-cultured CM-DiI + EPDCs (n = 4) and CM-DiI stained muscle cells were detached by trypsin digestion and resuspended in HBSS/10% FBS at a cell density of 10^6/mL before separation based on CM-DiI fluorescence. As a control for later RNA analysis, single cultured CM-DiI + EPDCs (n = 4) and CM-DiI stained muscle cells were mixed at the aforementioned 1:5 EPDC:myoblast ratio immediately before cell sorting, and cell fractions were sorted in an identical setup to that used for the co-cultured cells. FACS was performed on a BD FACSVantageSE/DiVa cell sorter (BD Bioscience). FACSDiva™ software, version 5.0.1 (BD Biosciences) was used for sort gating strategies and for FACS analysis as previously described [15]. Cells from four independent experiments (four EPDC isolations, co-cultures, and four corresponding sortings) were sorted directly into 2× RNA Lysis buffer (Applied Biosystems) and used for relative qRT-PCR (Quantitative real time polymerase chain reaction) by Taqman low density arrays (Applied Biosystems) of 64 genes as previously described [14–16]. The purity of sorted CM-DiI positive and negative samples was tested by a second round of flow cytometry using the sort setup.

2.7. Antibodies

Antibodies used in immunocytochemistry and flow cytometry included: rabbit α-WT1 (1:100, Abcam), rabbit α-Gata4 (1:100, Santa Cruz Biotechnology), mouse α-Smooth muscle actin (aSMA; 1:200, Sigma-Aldrich), mouse anti-Vimentin (1:100, Abcam), rat anti-Cytokeratin 19 (1:50, Developmental Studies Hybridoma Bank), rat α-CD31 1:100 (BD Pharmingen), Rat α-CD45 1:100 (BD Pharmingen), mouse α-Pax7 (1:30, Developmental Studies Hybridoma Bank), mouse α -Myosin (MF20; 1:200; Developmental Studies Hybridoma Bank), rat α-Myogenin (1:50, Dako), rat α-Laminin (1:50, Abcam), mouse α-Cardiac muscle myosin heavy chain (MHC) (1:400, Abcam), mouse α-non-muscle MHC (1:400, Abcam), goat α-Desmin (1:50, Santa Cruz Biotechnology), rabbit IgG (Santa Cruz Biotechnology), rat IgG2b (BD Pharmingen), rat IgG2a (BD Pharmingen), mouse IgG2a (Sigma-Aldrich), and mouse IgG1 (Sigma-Aldrich).

2.8. CM-DiI + EPDC transplantation

EPDCs were enumerated using a Coulter counter and resuspended at densities of 0.7×10^4, 1.4×10^4, 2.1×10^4, 2.8×10^4, 3.5×10^4, 5×10^4, 1×10^5, 2×10^5 cells per 2.5 μL PBS (Phosphate buffered salt solution). For intracardiac transplantation, recipient neonatal P1 (Postnatal day 1) mice were anesthetized by hypothermia for 4 min on an ice-bed as previously described [4]. The hearts were then exposed by thoracotomy at the 5th intercostal space, and exactly 2.5 μL (1×10^5) of CM-DiI + EPDC/PBS solution was injected into the left ventricle near the apex using a custom designed 33-gauge Hamilton syringe. For apex-resected mice [12], approximately 10% of the heart was removed prior to EPDC injection. The chest was then carefully sutured with 1–2 stiches (Silk 8-0) and the skin was further closed by a skin adhesive. Following recovery under a heat lamp, the cubs were returned to their mother.

For EPDC transplantation into adult myocardium, adult C57Bl/6 females were anesthetized using isoflurane (Baxter, Deerfield, IL, USA) and endotracheally intubated using the BioLite system (Braintree Scientific, Braintree, MA, US). The heart was accessed by parting of the lower ribs and opening of the pericardial sac. Permanent ligation of the LAD (Left anterior descending artery) was performed by placement of a single suture in the myocardium surrounding the LAD using 8-0 prolene suture (Ethicon). Ligation of the LAD was confirmed visually by paling of myocardium distal to the suture. Thereafter $1–3 \times 10^5$ EPDCs (2.5 μL) were injected into the myocardium of the left ventricle wall, using a Hamilton syringe. The EPDCs were injected into three different locations along the border zone, defined as the border between pale and the red well-perfused area. The thorax wall and skin were sutured and the thoracic cavity was drained using a 23G