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Genetic profiling and surface proteome analysis of human atrial stromal cells and rat ventricular epicardium-derived cells reveals novel insights into their cardiogenic potential

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ABSTRACT

Epicardium-derived cells (EPDC) and atrial stromal cells (ASC) display cardio-regenerative potential, but the molecular details are still unexplored. Signals which induce activation, migration and differentiation of these cells are largely unknown. Here we have isolated rat ventricular EPDC and rat/human ASC and performed genetic and proteomic profiling. EPDC and ASC expressed epicardial/mesenchymal markers (WT-1, Tbx18, CD73, CD90, CD44, CD105), cardiac markers (Gata4, Tbx5, troponin T) and also contained phosphocreatine. We used cell surface biotinylation to isolate plasma membrane proteins of rEPDC and hASC, Nano-liquid chromatography with subsequent mass spectrometry and bioinformatics analysis identified 396 rat and 239 human plasma membrane proteins with 149 overlapping proteins. Functional GO-term analysis revealed several significantly enriched categories related to extracellular matrix (ECM), cell migration/differentiation, immunology or angiogenesis. We identified receptors for ephrin and growth factors (IGF, PDGF, EGF, anthrax toxin) known to be involved in cardiac repair and regeneration. Functional category enrichment identified clusters around integrins, PI3K/Akt-signaling and various cardiomyopathies. Our study indicates that EPDC and ASC have a similar molecular phenotype related to cardiac healing/regeneration. The cell surface proteome repository will help to further unravel the molecular details of their cardio-regenerative potential and their role in cardiac diseases.

1. Introduction

Epicardium-derived cells (EPDC) play a fundamental role in embryonic heart development and cardiac disease (Ruiz-Villalba and Pérez-Pomares, 2012). After birth, epicardial cells are in a relatively dormant state as a single cell-layer covering the myocardium. However, following myocardial infarction (MI), the ventricular epicardium becomes reactivated and is characterized by EMT, a thickening of the epicardial layer and the re-expression of embryonic epicardial genes such as WT1 (Wilms tumor protein 1) and Tbx18 (T-box 18 transcription factor) (Zhou et al., 2011). These adult EPDC can migrate into the injured myocardium to differentiate into distinct cardiovascular cells (Smart et al., 2011; Zhou et al., 2011). Adult EPDC are also involved in the *de novo* formation of cardiomyocytes, however, the number of EPDC which are transformed into cardiomyocytes is quite low and therefore, their contribution appears to be insufficient for effective myocardial regeneration (Masters and Riley, 2014).

While EPDC have been intensively studied in rodents *in vivo* and *in vitro*, studies on human EPDC (hEPDC) are less numerous. Primary hEPDC from patient-derived atrial appendage biopsies can be isolated by stripping the epicardial layer (Clunie-O'Connor et al., 2015). Alternatively, cells types which strongly resemble hEPDC can be isolated from the human atrium by enzymatic techniques and were termed either "cardiac stromal cells" (Rossini et al., 2011) or "cardiac atrial appendage stem cells (CASC)" (Fanton et al., 2016; Koninckx et al., 2013; Windmolders et al., 2015). They show a similar mesenchymal morphology, express mesenchymal markers and have been shown to display strong regenerative potential upon intramyocardial implantation after MI: Implantation of these cells improved cardiac performance (Fanton et al., 2015; Rossini et al., 2011; Winter et al., 2007), attenuated adverse remodeling (Rossini et al., 2011), promoted angiogenesis (Fanton et al.,

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2016) and a fraction of the cells differentiated into cardiomyocytes (Fanton et al., 2015; Koninckx et al., 2013; Rossini et al., 2011).

Given the remarkable similarity of EPDC and stromal cells, the aim of the present study was to characterize the molecular phenotype of human ASC (hASC) and rat ventricular EPDC (rEPDC) formed after MI by genetic profiling and systematic analysis of cell surface proteins.

2. Materials and methods

2.1. Isolation and cultivation of hASC, rASC and rEPDC

Right or left atrial biopsy samples were obtained from 40 patients (66.4 ± 10.2 years of age; 31 men, 9 women) who underwent different cardiovascular surgical interventions (valve replacement/reconstruction, cardiac transplantation, aortic-coronary bypass). The study was approved by the institutional ethics committee (reference number 4125, 4412R und 4646) according to the principles outlined in the Declaration of Helsinki. Human atrial biopsies were immediately transferred into ice-cold PBS. After removal of fat tissue, epicardial and myocardial tissue was separated by a scalpel and cut into 1 mm^3 pieces which were transferred to PBS containing 1200 IU/ml collagenase II (Biochrom AG) for 3 h at 37 °C. Cell suspension was filtered (70 μm cell-strainer), centrifuged at 350g and resuspended in culture medium (DMEM supplemented with 20% FCS, 10 ng/ml bFGF (Sigma Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM Glutamax). The filtrate was transferred into a culture flask and incubated for 3 h at 37 °C to eliminate fibroblasts. The supernatant was transferred into a new culture flask and experiments were performed after 2–5 passages.

Animal experiments were performed in accordance with the European Union guidelines described in the directive 2010/63/EU and were approved by the local authorities (reference number 84-02.04.2014.A174). Male Wistar rats (220–280 g body weight, 12–16 weeks of age) used in this study were obtained from Janvier (Le Genest-Saint-Isle, France), and were fed with a standard chow diet receiving tap water *ad libitum*. MI in the rat (60 min ischemia/reperfusion) and isolation of EPDC 5 days after MI was conducted as recently described (Ding et al., 2016). For isolation of rASC, left and right atrial tissue issue (from MI and non-MI rats) was carefully prepared, minced and processed as described above for the human material.

2.2. Cell-based assays

Cell proliferation: Approximately ~20.000 hASC, or rEPDC of $n = 3$ individual isolates were plated in six-well plates as triplets. After 2–4 days, viable cells were counted using trypan blue (4%).

Quantitative real time PCR: To analyze mRNA expression, total RNA was isolated using the RNeasy Micro Kit and cDNA was synthesized applying the QuantiTect Reverse Transcription Kit (Qiagen GmbH) according to the manufacturer's instructions. We used predesigned TaqMan Gene Expression Assays for human and rat samples (Supp. materials and methods) and the StepOnePlus™ System (Life Technologies) following the manufacturer's protocol. Gene expression was normalized to β-actin.

UPLC: For ultra-performance liquid chromatography (UPLC), extraction and separation of various purine compounds was carried out as described (Hesse et al., 2017). Creatine derivatives were measured as described (Timohhina et al., 2009) using a Waters Acquity UPLC system.

Flow cytometry and immunofluorescence: For flow cytometry, cells were detached from the culture dish using PBS/5 mM EDTA (PBS/EDTA) and washed with FACS buffer (PBS, 0.5% BSA, 2 mM EDTA). After blocking with PBS/5% BSA, and Fc-block, cells were stained with antibodies against CD73, CD90, c-Kit, CD105, CD44 and Pecam-1/CD31and appropriate secondary antibodies (Suppl. Materials and Methods).

For immunofluorescence, cells were seeded on coverslips, fixed with Zamboni's fixative (4% PFA, 0.2 M picric acid (saturated aqueous), 0.1 M NaH_2PO_4/Na_2HPO_4, pH = 7.3) and permeabilized with PBS/0.1% Triton-X100 (Sigma) or left non-permeabilized. After blocking with PBS/5% normal serum, cells were stained with antibodies for WT-1, Tbx18, Gata4, Tbx5, troponin T, α-SMA, Pecam-1/CD31 and appropriate secondary antibodies. Nuclei were counterstained with DAPI. Samples were analyzed using a fluorescence microscope (BX61, Olympus) and recorded using high resolution digital cameras [F-ViewII (fluorescence) and UC30 (bright field), Olympus] or a confocal laser scanning microscope (LSM710 meta, Zeiss).

Identification of WT-1 positive cells within the human atrial tissue was performed in air-dried and fixed (10 min Zamboni's fixative) cyrosections (8 μm). Samples were incubated with antibodies for WT-1 and troponin T and with Cy3- or FITC-labeled secondary antibodies.

2.3. Isolation and analysis of cell surface proteins

A detailed description of protein isolation and analysis can be found in Supplementary Materials and Methods. In brief, human atrial or rat ventricular cells of three individual isolates (three rat ischemia/reperfusion experiments; three different patient biopsies) were washed with ice-cold PBS and incubated with EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher). After biotinylation was stopped, cells were lysed, incubated with Neutravidin-agarose and resuspended in elution buffer to detach biotinylated proteins. For mass spectrometry, three individual samples were processed as described recently (Poschmann et al., 2014) and the resulting peptides were separated by liquid chromatography coupled to the mass spectrometer (Orbitrap Elite (Thermo Fisher) ion trap mass spectrometer or a Q Exactive (Thermo Fisher) quadrupole-orbitrap mass spectrometer). Spectra were searched against proteome datasets downloaded from UniProtKB with a precursor mass tolerance of 20 ppm for the first and 4.5 ppm for the second search and a fragment mass tolerance of 20 ppm (Q Exactive) and 0.5 Da (Orbitrap Elite), respectively. Protein identification was carried out within the MaxQuant framework using standard parameters. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD005013. Bioinformatics functional enrichment analysis was performed using Cytoscape plugin ClueGO. Proteins overlapping between rat and human were analyzed for biological GO processes, pathways from KEGG and REACTOME.

3. Results

3.1. Human atrial stromal cells and rat EPDC display a similar morphology and express WT-1 and Tbx18

Isolation of stromal cells from human atrial appendage biopsies (hASC) was performed in 40 patients undergoing various surgical interventions using collagenase digestion. Cell preparations, regularly displayed adherent cells with a spindle shaped-morphology (Fig. 1A, left). Cell yield after six days of culture was 1156 ± 1105 cells ($n = 4$) per mg tissue sample. The *in vitro* doubling time was 54 ± 2 h (n = 3) and cells could be cultivated for up to 10 passages. To obtain stromal cells from the atrium of healthy rats (rASC), we used the same protocol and again obtained a homogenous cell population with spindle-shaped appearance (Fig. 1A, middle). Rat ventricular EPDC (rEPDC) were retrieved by collagenase treatment of the heart surface after five days following myocardial infarction (Ding et al., 2016). This procedure permitted the selective removal of the epicardial layer (thickness: ~150 μm 3-4d post MI) and resulted in the cultivation of cells with spindle-shaped structure (Fig. 1A, right). Doubling time was 35 ± 8 h (n = 3) and cells could be kept in culture for up to 20 passages. Cell yield was 307,500 ± 60,140 cells (n = 4) per processed heart after 4d of expansion.

Given the morphological similarity of rEPDC, rASC and hASC, we used flow cytometry to analyze cell surface expression of marker