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Macrophage precursor cells from the left atrial appendage of the heart spontaneously reprogram into a C-kit +/CD45 − stem cell-like phenotype

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ABSTRACT

Background: The developmental origin of the c-kit expressing progenitor cell pool in the adult heart has remained elusive. Recently, it has been discovered that the injured heart is enriched with c-kit^{+} cells, which also express the hematopoietic marker CD45.
Methods and results: In this study, we characterize the phenotype and transcriptome of the c-kit +/CD45 +/CD11b +/Flk-1 +/Sca-1 ± (B-type) cell population, originating from the left atrial appendage. These cells are defined as cardiac macrophage progenitors. We also demonstrate that the CD45 + progenitor cell population activates heart development, neural crest and pluripotency-associated pathways in vitro, in conjunction with CD45 down-regulation, and acquire a c-kit +/CD45 −/CD11b −/Flk-1 −/Sca-1 + (A-type) phenotype through cell fusion and asymmetric division. This putative spontaneous reprogramming evolves into a highly proliferative, partially myogenic phenotype (C-type).
Conclusions: Our data suggests that A-type cells and cardiac macrophage precursor cells (B-type) have a common lineage origin, possibly resolving some current conundrums in the field of cardiac regeneration.

1. Introduction

Cardiac regenerative biology has been under intense research during the last decade, but the exact biological mechanism by which resident cells may regenerate cardiac tissue has remained elusive. [1,2] Recent studies have suggested cardiomyocyte dedifferentiation [3] and c-kit + cardiac stem cells [4] as key players in cardiac regenerative responses. The disparity of these results is striking, although it can be explained partly by the different methods of myocardial injury used.

We identified a vast population of diverse cardiac progenitor cells (CPCs) in the left atrial appendages of adult mice [5]. One of the populations was c-kit +/CD45 −/Sca-1 +/Gata-4 +/Nkx2.5 + (A-type) and the other c-kit +/CD45 +/Sca-1 −/Gata-4 +/Nkx2–5 + (B-type). The B-type CPC population has been identified in the failing human hearts [6], and has been recognized as the main cell population attracted to infarct sites after stem-cell factor (the c-kit receptor ligand) gene therapy [7]. Interestingly, some c-kit + CPCs used in cardiac cell therapy clinical trials were acquired from right atrial appendage tissue. [8] No lineage tracing has been established for these A-type CPCs, although a possible regulating role in embryonic cardiomyogenesis has been identified [9].

Macrophages have been found to have important trophic function in the embryonic development and injury repair. [10] Tissue resident macrophages are not derived from the bone-marrow in normal situation [11]: Microglia, Langerhans cells and Kuppfer cells originate from the yolk sac and are genetically distinct from the bone-marrow derived hematopoietic stem cell progeny. Yolk Sac derived macrophages have been shown to persist in the adult heart. [12] Hematopoietic tissue of yolk sac origin can acquire a cardiomyogenic fate. [13] To this date the mechanism of self-renewal of the resident macrophage pool is unknown, as it does not share the hematopoietic developmental tree inherent to bone-marrow derived cells [14]. A cardiac tissue macrophage population was recently found in the ventricle of the adult murine heart [15], raising intriguing questions about their function and origin.

In our previous article [5], we described a method to grow two phenotypically distinct c-kit + cardiac progenitor cell types from the left atrial appendage, enzymatically removing the epicardial cells in the early digestion fractions [16]. We found that CD45 + B-type cells underwent a spontaneous differentiation, when they were cultured for prolonged periods of time. In this study, we strive to identify and characterize an atrial appendage-enriched macrophage progenitor

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population (B-type), and demonstrate that it can reprogram into a c-kit+/Sca-1+/CD45− cell state (A-type), and can be further cultured in a highly proliferative c-kit-stem cell-like phenotype (C-type).

2. Methods

2.1. Ethics

The Hebrew University Animal Ethics Committee approved all animal studies, and experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

2.2. Mice strains, tissue extraction and handling

Adult (8–12 weeks old) C57BL/6, Pax3 cre-Rosa YFP, Cx3cr1 cre-SCS YFP, Tie2cre Rosa tdTomato or MHC cre-ROSA tdTomato mice were deeply anesthetized with isoflurane and left atrial appendages were removed. The explants were then digested three times (5 min) before culture with trypsin & collagenase D. Strong enzyme digestion was performed with trypsin 0.25% and collagenase D 0.1% and alternatively, weak enzyme digestion with trypsin 0.05% and collagenase D 0.1%.

2.3. Cell culture

Left atrial appendage explants were placed on 24-well plates (tissue culture treated) in culture medium (CEM: Iscove's Modified Dulbecco's Medium with 25 mM HEPES, supplemented with 10% FBS (fetal bovine serum), 1% L-glutamine, 1% penicillin–streptomycin & 0.1 mM 2-mercaptoethanol). Proliferation rate of B-type cells is low until the C-type cells are formed (typically at p5). First passage is made after 30 days, followed by passages every 2–3 weeks depending on cell number/confluence. After C-type cell formation, the proliferation rate is exponential and passages have to be done every 3–4 days.

2.4. Immunocytochemistry and immunohistochemistry

Immunocytochemistry was performed using Ibidi μ-slide 8-well plates (IbiTreat coating). Fixation was performed with formaldehyde 4% and permeabilization with Triton 0.2% or with absolute methanol (2 min, −20 °C). Blocking was performed with 1% BSA in PBS. Primary antibodies were incubated overnight in 4 °C or 1–2 h in room temperature (S1 Text). Secondary antibodies were incubated for 40 min in room temperature. Immunohistochemistry was performed on snap-frozen left atrial appendages cut to 10 μm thickness. The samples were fixed with acetone (5 min, −20 °C) and blocked with Casblock (10 min, Invitrogen). Tissue was fixed with 4% PFA/30% sucrose, when fluorescent reporter mice were analyzed. Antibody incubation times were same as described above. LSM 710 confocal microscope was used (Carl Zeiss) and the pictures were processed with Zen 2009 software.

2.5. Flow cytometry and cell sorting

Flow cytometry was performed with BD LSR II (BD Bioscience) or BD FACSAria III (BD Bioscience) apparati. The cells were first detached with non-enzymatic Cell Dissociation Solution (Sigma) (30 min, 37 °C) and centrifuged (400 *g*, 5 min). Cell amount was adjusted using a hemocytometer on the test tubes. Anti-Mouse CD16/CD32 (14-0161-82, eBioscience) was added for each test tube for 10 min to inhibit unspecific binding of fluorescent antibodies. Wash buffer was PBS + 1% BSA and for sorting buffer was PBS + 2% FBS + 1 mM EDTA + 25 mM Hepes. Antibodies (Supplemental experimental procedures) were incubated for 30 min. Cells for RNA extraction were sorted to PBS and for culturing to medium. At least 10,000 events were collected per sample.

2.6. Live microscopy and time lapse experiments

For live microscopy the cells were stained with CD11b (11-0112-81, eBioscience) and Sca-1 (553336, BD Bioscience) antibodies according to a previously published protocol [52]. The antibodies were first cleaned from sodium azide using Amicon Ultra 10 k centrifugal filters. Final concentration of the antibodies in the medium was 20 ng/ml, which was added an hour before the experiments. The right exposure for the markers was adjusted with an isotype control stained well before the experiment. Time-lapse pictures were taken every 10 min initially for 3 h. All time-lapse-experiments were performed using a Nikon Eclipse Ti inverted microscope equipped with a humidified CO_2 incubator.

2.7. RNA extraction

Total RNA of cultured cells was extracted with a High Pure RNA isolation kit (Roche) according to the manufacturer's instructions. RNA-sample quality and integrity was tested with Bioanalyzer 2100 (Agilent Technologies). RNA from control tissue samples was isolated using a TRI-reagent.

2.8. RNA labeling, hybridization and array scanning

200 ng of total RNA for each sample was amplified and labeled with fluorescent dyes (Cy3) using a Low RNA Input Linear Amplification & Labeling kit (Agilent Technologies). The quality of the resulting labeled cRNA was measured using a Nanodrop ND-100 spectrophometer. The Cy3 labeled cRNA was hybridized to Agilent Whole Human Genome Oligo Microarray V2 (design 026,652, Agilent Technologies, USA) for 17 h at 65 °C in an Agilent DNA-Microarray Hybridization Oven. The arrays were later washed using the Gene Expression Wash Buffer Kit. The Microarray was scanned using a Agilent High-Resolution Microarray Scanner and the data was extracted from the resulting images using Feature Extraction 10.7 software.

2.9. RNA preparation for sequencing, sample sequencing

500 ng of total RNA for each sample was prepared to a ds cDNA library according to TruSeq RNA Sample Preparation v2 LS Workflow using a unique 6 bp adapter for each sample. Steps were RNA purification and fragmentation, first strand cDNA synthesis, second strand cDNA synthesis, end repair, 3′ end adenylation, adapter ligation, PCR amplification and library validation using Qubit (Life Technologies) and Bioanalyzer 2100 (Agilent Technologies). Samples were sequenced using HiSeq 2500 (1 × 50 bp, Illumina). Average number of reads/sample was 17×10^6. Mean Quality Score (PF) range = 34.38–34.62). Data was converted to bam-format in Galaxy server with Tophat for Illumina (version 1.5.0) using mm10 reference genome.

2.10. Transcriptome data analysis

Array and sequencing data (bam-files) was analyzed using Genespring 12-software (Agilent Technologies). Normalization for sequencing data was done using a DESeq algorithm [53]. Statistical testing was performed by One way ANOVA (p-value cut-off: 0.05, p value computation: Asymptotic, Multiple Testing Correction: Benjamini-Hochberg) in all of the experiments before fold change analysis. Data is available as NCBI GEO Series GSE5716.

2.11. Statistical methods

Two group comparison were analyzed with two-tailed unpaired t-test, p-values < 0.05 were considered significant. Data is presented as mean + SD.