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Comparative transcriptomic analysis of endothelial progenitor cells derived from umbilical cord blood and adult peripheral blood: Implications for the generation of induced pluripotent stem cells☆

Xiugong Gao *, Jeffrey J. Yourick, Robert L. Sprando

Division of Applied Regulatory Toxicology, Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Laurel, MD 20708, USA

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ABSTRACT

Induced pluripotent stem cells (iPSCs) offer the potential to generate tissues with ethnic diversity enabling toxicity testing on selected populations. Recently, it has been reported that endothelial progenitor cells (EPCs) derived from umbilical cord blood (CB) or adult peripheral blood (PB) afford a practical and efficient cellular substrate for iPSC generation. However, differences between EPCs from different blood sources have rarely been studied. In the current study, we derived EPCs from blood mononuclear cells (MNCs) and reprogrammed EPCs into iPSCs. We also explored differences between CB-EPCs and PB-EPCs at the molecular and cellular levels through a combination of transcriptomic analysis and cell biology techniques. EPC colonies in CB-MNCs emerged 5–7 days earlier, were 3-fold higher in number, and consistently larger in size than in PB-MNCs. Similarly, iPSC colonies generated from CB-EPCs was 2.5-fold higher in number than from PB-EPCs, indicating CB-EPCs have a higher reprogramming efficiency than PB-EPCs. Transcriptomic analysis using microarrays found a total of 1133 genes differentially expressed in CB-EPCs compared with PB-EPCs, with 675 genes upregulated and 458 downregulated. Several canonical pathways were impacted, among which the human embryonic stem cell pluripotency pathway was of particular interest. The differences in the gene expression pattern between CB-EPCs and PB-EPCs provide a molecular basis for the discrepancies seen in their derivation and reprogramming efficiencies, and highlight the advantages of using CB as the cellular source for the generation of iPSCs and their derivative tissues for ethnic-related toxicological applications.

1. Introduction

Induced pluripotent stem cells (iPSCs) offer the potential to become multipurpose research and clinical tools to model diseases, screen candidate drugs, and deliver cell replacement therapy to support regenerative medicine (Goldthwaite, 2016). In the field of toxicology, iPSCs afford a well-defined source for deriving tissue-specific cell types, which can provide physiologically relevant *in vitro* systems of easy access and amenable to a variety of assays (Suter-Dick et al., 2015). More importantly, since iPSCs can virtually be generated from any donor, they allow for the selection of relevant subjects in toxicity studies. Ethnic diversity in response to certain chemicals or drugs has been reported previously, which may result from differences in metabolism among various ethnic groups (Yasuda et al., 2008). By utilizing iPSCs derived from individuals with specific ethnicity, toxicity testing and risk assessment for different ethnicities could be achieved.

An important issue in iPSC generation is the choice of the starting cell source for reprogramming. Although skin fibroblasts are the first and most common cell type used (Takahashi et al., 2007; Yu et al., 2007), these cells suffer from several drawbacks such as relatively low reprogramming efficiency and especially the pain and discomfort associated with the surgical procedures in obtaining skin biopsies. Several other somatic cell types have thereafter been explored for the reprogramming of iPSCs, including renal epithelial cells from urine, keratinocytes from hair follicle, and mononuclear cells (MNCs) from blood samples (Raab et al., 2014; Singh et al., 2015). Recently, it has been reported that endothelial progenitor cells (EPCs) derived from umbilical cord blood (CB) or adult peripheral blood (PB) afford a practical and efficient cellular substrate for iPSC generation (Geti et al., 2012). Using EPCs as source cells for iPSC generation has the following advantages over other somatic cell types: (1) EPC colonies can be established from both CB and PB, which are easier to obtain than other cell types; (2) EPCs are a highly proliferative adherent cell type, therefore can be easily expanded and banked as reference cells; (3) EPCs do not exhibit genetic rearrangements associated with B-cells or T-cells; (4) EPCs are free from somatic mutations associated with blood disorders; and (5) EPCs are highly efficient and viable for RNA transfection, thus bypassing

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* Corresponding author.
E-mail address: xiugong.gao@fda.hhs.gov (X. Gao).

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the need for DNA and avoiding the dangers associated with DNA integration in iPSC reprogramming (Hayes and Zavazava, 2013).

Currently, there is no consensus definition of an EPC. First discovered by Asahara and coworkers in 1997 (Asahara et al., 1997), EPCs outgrow from the MNC fraction of blood under endothelial-selective conditions (Lin et al., 2000). EPCs have been previously reported useful for angiogenic therapies or as biomarkers to assess cardiovascular disease risk (Kalka et al., 2000; Vasa et al., 2001). Similar to mature endothelial cells, there may be a significant heterogeneity among EPCs; in addition, different sources may produce distinct subtypes of EPCs (Ingram et al., 2004). However, differences between EPCs from different blood sources have rarely been studied. In the current study, we derived EPCs from MNCs and reprogrammed EPCs into iPSCs, and also compared CB and PB for EPC derivation and iPSC generation. Next, we explored differences between CB-EPCs and PB-EPCs at the molecular level through transcriptomic analysis using microarrays and provided mechanistic explanations for the discrepancies seen in their reprogramming efficiencies.

2. Materials and methods

2.1. Derivation of EPCs from CB and PB

Frozen human MNCs from umbilical CB and adult PB of healthy individuals were obtained from AllCells (Alameda, CA). The cells were thawed, washed, and resuspended in EGM-2 medium (Lonza, Walkersville, MD) containing 20% (v/v) HyClone embryonic stem cell (ESC) grade fetal bovine serum (FBS) (GE Healthcare, Logan, UT). A total of 5.0×10^7 cells were seeded in a T75 tissue culture flask coated with type 1 rat tail collagen (Corning, Corning, NY). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and culture medium was changed every 2 days. EPC colonies appeared between 8 and 17 days of culture and were identified as well-circumscribed monolayers of cobblestone-like cells. EPC colonies were enumerated by visual inspection under an inverted microscope. Following generation, EPCs were released from the original tissue culture flask using 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA), resuspended in EGM-2 media (Lonza) containing 10% (v/v) HyClone FBS (GE Healthcare), and plated onto a T25 tissue culture flask without collagen coating for further passaging. The cells were used for the experiments at passages 4 or 5.

2.2. Immunophenotyping of EPC surface markers using flow cytometry

A total of 5×10^5 cells were incubated at room temperature for 30 min with varying concentrations of primary or isotype control antibodies, as listed below, in 200 μl phosphate-buffered saline (PBS) containing 2% FBS. Stained cells were then washed 3 times with PBS containing 2% FBS, filtered through a 30 μm filter, and analyzed by a Guava easyCyte 8HT Flow Cytometer from EMD Millipore (Billerica, MA). The primary antibodies used include: mouse monoclonal [B-B38] antibody against human CD31 conjugated to phycoerythrin (PE); mouse monoclonal [4H11(APG)] antibody against human CD34 conjugated to allophycocyanin (APC); mouse monoclonal [KDR/EIC] antibody against human VEGF Receptor 2 conjugated to fluorescein isothiocyanate (FITC); and rabbit monoclonal [EPSISR15] antibody against human von Willebrand Factor conjugated to Alexa Fluor 488. All the antibodies and their isotype controls were obtained from Abcam (Cambridge, MA). Data collection and analysis was performed using the InCyte program included in the guavaSoft software suite (ver. 3.1.1), and instructions from the manufacturer were followed.

2.3. Endothelial tube formation assay and uptake of DiI-acetylated low-density lipoprotein (DiI-Ac-LDL)

To assess *in vitro* angiogenesis through capillary tube formation, EPCs were seeded onto 24-well tissue culture plates coated with Matrigel (Corning) at a cell density of 1.5×10^5 cells per well. Cells were cultured in EGM-2 medium at 37 °C and 5% CO_2 in a humidified atmosphere, and were observed after 24 h under an inverted microscope at 40× magnification for capillary-like formations.

To assess the ability of cells to incorporate acetylated low-density lipoprotein (Ac-LDL), EPCs at 70–90% confluence were incubated with 10 μg/ml of Ac-LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL; Molecular Probes, Eugene, OR) in EGM-2 medium containing 10% (v/v) HyClone FBS (GE Healthcare) for 4 h at 37 °C. Cells were washed 3 times and then examined for uptake of DiI-Ac-LDL using a phase-contrast fluorescence microscope.

2.4. iPSC generation by reprogramming EPCs using self-replicative RNA (srRNA)

iPSCs were generated from EPCs using the StemRNA-SR Reprogramming Kit from Stemgent (Cambridge, MA) following the manufacturer's protocol. Briefly, 2.0×10^5 EPCs were plated into one well of a 6-well plate coated with Matrigel (Corning) on day 0, and cultured at 37 °C and 5% CO_2 overnight in EGM-2 medium (Lonza) containing 10% (v/v) HyClone FBS (GE Healthcare). Culture medium was changed every day. The cells were transfected with microRNA and srRNA on day 1 and day 2, respectively. B18R was supplemented in the medium from day 1 and continued for 24 days. Puromycin was added to the medium between day 3 and day 14, and bFGF from day 8 on until the end of the experiment. The cells were transitioned to NutriStem XF/FF medium (Stemgent) on day 20, around which time iPSC colonies emerged. Colonies were identified and mechanically picked between day 26 and day 30, and further passaged in NutriStem XF/FF medium.

2.5. Immunofluorescence staining

Cells were fixed using 4% (v/v) paraformaldehyde (Alfa Aesar; Tewksbury, MA), washed three times with PBS containing 0.2% (v/v) Tween (PBST) (Fisher Scientific; Waltham, MA) and permeabilized using 0.15% (v/v) TritonX-100 (Sigma-Aldrich; St. Louis, MO) in PBS for 1 h at 25 °C. After permeabilization, cells were blocked with 1% (v/v) BSA (Invitrogen; Carlsbad, CA) in PBST (PBSTB) for 30 min at 25 °C. After gentle removal of PBSTB, cells were incubated with primary antibody in PBSTB overnight at 4 °C. Primary antibodies used in this study include SSEA4 (Biolegend; San Diego, CA), TRA-1-60 (Biolegend), Oct4 (Cell Signaling; Beverly, MA), Sox2 (R&D Systems; Minneapolis, MN), Pax6 (R&D Systems), a-SMA (Sigma-Aldrich), and SOX17 (R&D Systems). After the overnight incubation, cells were washed three times with PBST and stained with secondary antibody (Alexa Fluor 488 or 594; Invitrogen) diluted 1:500 in PBSTB for 1 h at 25 °C. The cells were washed three times in PBST and stained with Hoechst dye (Invitrogen). Images of the stained cells were taken under Leica DMi8 fluorescence microscope with CCD camera.

2.6. Induction of iPSC differentiation through embryoid body (EB) formation

The iPSCs were subjected to suspension culture in EB maintenance media (iXCells Biotechnologies; San Diego, CA) to form EBs. The EBs were maintained in suspension culture for 1–2 weeks with daily media change and attached to gelatin-coated dishes in the same media for another 1–2 weeks before antibody staining.

2.7. Total RNA isolation and quality assurance

EPCs were harvest at passage 5 and were immediately lysed in Buffer RLT (Qiagen; Valencia, CA) supplemented with β-mercaptoethanol (Sigma-Alderich). The lysate was then homogenized using QIAshredder spin column (Qiagen). Total RNA was isolated on the EZ1 Advanced XL (Qiagen) automated RNA purification instrument using the EZ1 RNA Cell Mini Kit (Qiagen) following the manufacturer's protocol, including