Biochemical and Biophysical Research Communications 443 (2014) 700–705

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of functional endothelial-like cells from adult mouse germline-derived pluripotent stem cells

Julee Kim [a], Sarah Eligehausen [b], Martin Stehling [a], Sigrid Nikol [b,1], Kinarm Ko [a,2], Johannes Waltenberger [b,*], Rainer Klocke [b,*]

[a] Department of Cell and Developmental Biology, Max Planck Institute of Molecular Biomedicine, Röntgenstr. 20, D-48149 Münster, Germany
[b] Department of Cardiovascular Medicine, University Hospital of Münster, Albert-Schweitzer-Campus 1, Bldg. A1, D-48149 Münster, Germany

ARTICLE INFO

Article history:
Received 3 December 2013
Available online 12 December 2013

Keywords:
Germline-derived pluripotent stem cells
Endothelial cells
Ischemia
Cell-based therapy
Arteriogenesis
Angiogenesis

ABSTRACT

Functional endothelial cells and their progenitors are required for vascular development, adequate vascular function, vascular repair and for cell-based therapies of ischemic diseases. Currently, cell therapy is limited by the low abundance of patient-derived cells and by the functional impairment of autologous endothelial progenitor cells (EPCs). In the present study, murine germline-derived pluripotent stem (gPS) cells were evaluated as a potential source for functional endothelial-like cells.

Cells displaying an endothelial cell-like morphology were obtained from gPS cell-derived embryoid bodies using a combination of fluorescence-activated cell sorting (FACS)-based selection of CD31-positive cells and their subsequent cultivation on OP9 stromal cells in the presence of VEGF-A. Real-time reverse transcriptase polymerase chain reaction, FACS analysis and immunofluorescence staining showed that the gPS cell-derived endothelial-like cells (gPS-ECs) expressed endothelial cell-specific markers including von Willebrand Factor, Tie2, VEGFR2/Flk1, intercellular adhesion molecule 2 and vascular endothelial-cadherin. The high expression of ephrin B2, as compared to Eph B4 and VEGFR3, suggests an arterial rather than a venous or lymphatic differentiation. Their capability to take up Dil-conjugated acetylated low-density lipoprotein and to form capillary-like networks on matrigel confirmed their functionality.

We conclude that gPS cells could be a novel source of endothelial cells potentially suitable for regenerative cell-based therapies for ischemic diseases.

1. Introduction

Vascular development, adequate vascular function and vascular repair are depending on endothelial cells. Accordingly, for vascular regenerative cell-based therapies, irrespective whether these are relying on single cell preparations [1] or tissue-engineered vascular grafts [2] functional endothelial cells are required. Currently, such applications are predominantly based on endothelial progenitor cells (EPCs) [1]. However, the purification of EPCs from peripheral blood or bone marrow is laborious. Furthermore, patient-derived EPCs [3,4] required for immunocompatible cell-based therapies for ischemic diseases such as coronary heart disease and peripheral artery disease are often functionally impaired, low in number and have a limited proliferation potential *in vitro* [5,6]. Therefore, the evaluation of alternative sources of therapeutically suitable endothelial cells is required.

The prototypical pluripotent embryonic stem (ES) cells have been demonstrated already to be a suitable source for the derivation of endothelial cells. The transplantation of such cells generated from murine [7] as well as human embryonic stem cells [8] improved blood perfusion in mouse models of cardiac [7] and hindlimb [8] ischemia. However, concerns remain with respect to their use in clinical applications due to ethical implications and immunological problems. Germline-derived pluripotent stem (gPS) cells [9] could be an alternative source for endothelial cells. The transplantation of gPS cell-derived cells is supposed to be basically devoid of the risk of immune rejection, as their establishment would be based on immunocompatible donor testicular tissue or even autologous testicular biopsies. Therefore, we investigated whether functional endothelial-like cells potentially suitable for therapeutic applications can be derived from murine gPS cells as a model for the human counterparts which still remain to be established.

* Corresponding authors. Fax: +49 251 83 43204 (J. Waltenberger). Address: Department of Cardiovascular Medicine, University Hospital of Münster, Albert-Schweitzer-Campus 1, Bldg. D3, D-48149 Münster, Germany. Fax: +49 251 83 55747 (R. Klocke).

E-mail addresses: johannes.waltenberger@ukmuenster.de (J. Waltenberger), rainer.klocke@ukmuenster.de (R. Klocke).

[1] Present address: Klinische und Interventionelle Angiologie, ASKLEPIOS Klinik St. Georg, Lohmühlenstr.5, D-20099 Hamburg, Germany.

[2] Present address: Center for Stem Cell Research, Institute of Advanced Biomedical Science, Konkuk University, Seoul 143-701, Korea.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.035

2. Materials and methods

2.1. Cell culture

Murine gPS cells and OP9 cells were cultivated as previously described [9,10]. To induce differentiation, embryoid bodies (EBs) were generated. On day 0 of differentiation, gPS cells were seeded at a density of 1×10^6 cells/ml in 10-cm bacteriological dishes (Sarstedt, Nümbrecht, Germany) in differentiation medium consisting of Iscove's medium (PAA, Cölbe, Germany) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France), L-glutamine with penicillin/streptomycin (stock solution: 100×; PAA, Cölbe, Germany), 10 μM β-mercaptoethanol (Gibco, Berlin, Germany), and 1% (v/v) NEA non-essential amino acids stock solution (100×; Gibco, Berlin, Germany).

2.2. Enrichment of gPS-ECs

On day 5 of differentiation, the EBs were incubated with acutase (PAA, Cölbe, Germany) to create a single-cell suspension. CD31-positive cells were isolated from the cell suspension by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) using a phycoerythrin (PE)-conjugated anti-CD31 antibody (Biozol, Eching, Germany). Subsequently, the isolated cells were plated onto OP9 cells and maintained in alpha-Minimum Essential Medium supplemented with 10% FBS, L-glutamine with penicillin/streptomycin (100× stock solution), 10 μM β-mercaptoethanol, and 50 ng/ml of recombinant mouse vascular endothelial growth factor-A (VEGF-A_{164}) (Prospec, Rehovot, Israel). Endothelial colonies were then plated onto gelatin- or collagen IV-coated cell culture dishes and maintained in the above-mentioned medium.

2.3. Real-time RT-PCR

RNA was extracted from gPS cells, 2-day-old EBs, 5-day-old EBs, and gPS-ECs using the RNeasy Mini-kit (Qiagen, Hilden, Germany) and reverse transcribed using a cDNA synthesis kit (Applied Biosystems, CA, USA). The amplification was performed on the ABI prism 7500 Fast Sequence Detection System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Real-time RT-PCR analysis was carried out for each sample in triplicates. GAPDH was used as an internal control. Relative gene expression values were calculated by normalizing Ct (threshold cycle) values of the target genes with that of the housekeeping gene (GAPDH) using the $\Delta\Delta C_t$ method. Specific primers used are listed in Table 1.

2.4. Flow cytometric analysis

Flow cytometric analysis of the cells was performed on a FACS Calibur (BD Bioscience, Heidelberg, Germany). The antibodies used in these FACS experiments were: PE-conjugated anti-stage-specific embryonic antigen1 (SSEA1; BD Bioscience, Heidelberg, Germany), PE-conjugated anti-CD31 (Biozol, Eching, Germany), PE-conjugated anti-Flk1 (eBioscience, Hatfield, United Kingdom), anti-vascular endothelial-cadherin (VE-Cadherin; gift from Prof. Vestweber, Max-Planck-Institute for Molecular Biomedicine; Münster, Germany), anti-Tie2 (gift from Prof. Vestweber), anti-von Willebrand Factor (vWF; DAKO, Glostrup, Denmark), PE-conjugated IgG control, rat IgG control (BD Bioscience, Heidelberg, Germany), and Alexa 488-conjugated anti-rat IgG antibody (Invitrogen, Karlsruhe, Germany).

2.5. Immunofluorescence staining

The cells were fixed with 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.5% triton-X for 5 min at room temperature (RT). The fixed cells were incubated with 3% BSA in PBS for 30 min to block non-specific binding and stained with the primary antibodies anti-VE-Cadherin (gift from D. Vestweber, Münster) and anti-vWF (DAKO, Glostrup, Denmark) for 1 h at RT. Bound antibodies were visualized using Alexa 488-conjugated goat anti-rat IgG secondary antibody (Invitrogen, Karlsruhe, Germany). Incubations with the secondary antibody were carried out for 1 h at RT. The stained cells were then examined under a Leica microscope (Leica Microsystems, Heidelberg, Germany).

2.6. In vitro angiogenesis assay in matrigel and staining with BS-1 lectin

To induce tube formation, the cells were plated in matrigel-coated 24-well culture dishes [11] and cultivated in medium containing 50 ng/ml of VEGF. Cell morphology was observed under a Leica microscope and cell staining was performed using tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Bandeiraea simplicifolia*-1 (BS-1) lectin (Sigma–Aldrich, Schnelldorf, Germany) [12].

2.7. Cellular uptake of Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) [13]

The cells were incubated with 10 μg/ml of acetylated low-density lipoprotein labeled with 1, 1′ dioctadecyl-3, 3, 3′,

Table 1
Sequences of oligonucleotide primers.

Gene	Forward primer	Reverse primer
GAPDH	TGGTTCCAGTATGACTCCACTCAC	GATGACAAGCTTCCCATTCTCG
Tie2	TTGAAGTGACGAATGAGAT	ATTTAGAGCTGTCTGGCTT
VE-Cadherin	GAATGACAACCCTCCGGAAT	TCCTCGTTCTTCAGGGCAAA
CD31	TGTCATTGGAGTGGTCATCG	GGCTTCCACACTAGGCTCAG
Flt1	CTCTGATGGTGATCGTGG	CATGCGTCTGGCCACTTG
vWF	AGGGCTGGAGTGTGCTAAGA	TACCAATGGCAGAtGCAAGTG
ICAM2	ACTCCACAGACCCCACAGAC	ATGGCAAAAGAAGACCGTGT
Flk1	CCCGCATGAAATTGAGCTAT	AAACATCTTCGCCACAGTCC
VEGFR3	CGATGCCCTGTACCTGCAGTG	CCGCTGGCAGAACTCCTCATTG
EphB4	GTCCACCGAGACCTGGCTG	GGGCCGGGCATTCCGGTC
Ephrin B2	AACCAGGAGGGAGGGGTGTG	GACAGCGTGGTCGTGTGCTG
Nkx2.5	CAAGTGCTCTCCTGCTTTCC	CATCCGTCTCGGCTTTGT
SM22α	CACCTGGCACTCTCCACCTTC	GATTTCATCCCACTACCGAAAG
Oct4	AGTATGAGGCTACAGGGACACCTTTC	GGACTGAGTAGAGTGTGGTGAAGTGG
Nanog	CTGCTCCGCTCCATAACTTCG	AATGCGCATGGCTTTCCCTA
CD34	ATGTCCGGCCTTCTCCTATT	CCCAAAGGTCAGAGATTGGA