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Generation of functional endothelial cells with progenitor-like features from murine induced pluripotent stem cells

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ABSTRACT

Induced pluripotent stem cells (iPSCs) have shown great potential in regenerative medicine and research applications like disease modeling or drug discovery. Endothelium is indispensable for vascular homeostasis, whereas endothelial dysfunction could lead to different diseases. Therefore, generating autologous cells, able to restore the endothelial lining, can be crucial for slowing or reversing certain pathological processes. In the current study we show efficient differentiation of murine iPSCs into endothelial cells (ECs) with stable CD34+/Tie-2+/Sca-1+/CD45− phenotype and proven functionality. iPS-derived ECs (iPS-ECs) were positive for phospho-eNOS and von Willebrand factor, and responded to shear stress with up-regulation of KLF-2, KDR, HO-1, and increased nitric oxide and VEGF production. These cells reacted to cytokine stimulation through increase in VCAM-1 and inflammatory cytokine secretion. iPS-ECs showed also certain progenitor features, like expression of progenitor markers (CD34, Sca-1, c-kit) and high clonogenic potential. The angiogenic capacity of iPS-ECs in spheroid sprouting assay was similar to primary ECs, whereas on Matrigel, tube structures could be formed only in the presence of other support cells. Angiogenic potential of iPS-ECs *in vivo*, was similar to murine endothelial cell line MS-1. Summarizing, our approach enabled generation of functional progenitor-like ECs, which can be used as a research model.

1. Introduction

The endothelium is involved in a wide variety of processes like angiogenesis, vascular structure formation, capillary transport and maintaining of cardiovascular homeostasis [1]. Through these, it is also linked to the progression of diseases like atherosclerosis and diabetes [1,2]. Stem cell-based vascular regeneration could offer broad potential in treatment of various diseases, which still represent largely unmet clinical needs [3]. Induced pluripotent stem cells (iPSCs), obtained from patient's own somatic cells upon reprogramming with defined transcription factors, can be differentiated to any cell type providing a source of autologous cell populations for regenerative medicine with minimized risk of their rejection after transplantation. Therefore, successfully creating a functional endothelium from iPSCs would not only bring the possibility of replacing damaged one, but could also serve as a patient and disease specific model system for translational studies.

Cell differentiation from pluripotent stem cells (PSCs) is a complex and poorly defined process, triggered by a variety of stimulations [3]. Currently there is no standardized protocol for generation of vascular cells and methods used for differentiation toward endothelium from murine or human PSCs were summarized recently [4,5]. One approach exploits co-culture of iPSCs with OP-9 (murine stromal cell line from op/op mice) cells, leading to differentiation toward hematopoietic and endothelial lineage [6] or lymphatic endothelium [7]. Another possibility requires the intermediate step of embryoid bodies (three dimensional clusters of differentiating PSCs) formation and subsequent culturing together with different growth factors like fibroblast growth factor 4 (FGF-4) or vascular endothelial growth factor (VEGF) [8,9]. Furthermore, one may try to recreate the natural environment of endothelial

Abbreviations: ECs, endothelial cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stromal cells; PSCs, pluripotent stem cells; SS, shear stress; VEGF, vascular endothelial growth factor; FGF4, fibroblast growth factor 4; IL, interleukin; KLF-2, Kruppel-like factor 2.

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cells (ECs) by using plates coated with collagen IV or fibronectin. This, in combination with culturing of the cells in the presence of growth factors, also shoves iPSCs toward endothelial lineage [10]. The iPS-generated ECs (iPS-ECs) show expression of different endothelial specific markers and their functionality has been proven using different experimental models [11–13]. Similar approaches for differentiation of embryonic stem cells (ESCs) toward endothelium have been used. Effective differentiation process could be achieved by culturing on collagen IV and application of shear stress [14], and/or sorting for certain cell marker [15,16]. There were also studies, where ECs were generated through formation of embryonic bodies [17,18].

MicroRNAs (miRNAs), which are small non-coding RNAs, have been found to play a central role in all pivotal cellular processes. In PSCs there are miRNAs regulating pluripotency state, while others promote differentiation process [19]. It was reported that over-expression of miR-21 in differentiating mouse ESCs led to increase in numerous endothelial lineage markers [20]. Additionally, miR-199a was found to be involved in ECs differentiation from iPSCs and its expression was increased in later stages of this process [21]. Moreover, the proper functioning of mature ECs is also regulated by miRNAs.

Successful generation of ECs was shown also for partially reprogrammed cells [22] or using direct conversion of somatic cells [23–25]. Generated cells were termed as induced ECs (iEC) and their major advantage over iPS-ECs seems to be the safety of use (iPSCs are predisposed to teratoma formation). Using this method also progenitor CD34+ cells with multiple differentiation capacity were formed [26].

Recently, a new appreciation of the possibility to create simultaneously different cell types needed for generation of complete blood vessel, emerged. Using human PSCs, ECs were generated together with smooth muscle cells [27–29], pericytes [30] or hematopoietic progenitors [6]. However, regardless of the protocol used, one of the most prominent problem in generating iPS-ECs is the rather low percentage of differentiated cells and the need for cell sorting on certain endothelial marker for enrichment and purification of the population. Also, limited growth rates and early senescence were reported [8].

In the current study we describe generation of functional murine iPS-derived ECs, with some progenitor-like features, obtained without cell sorting and retaining stable phenotype over 2-month culture period.

2. Materials and methods

2.1. Cell culture

2.1.1. Generation of murine iPS cells and differentiation toward endothelium

iPS cells were generated as described in Stepniewski et al. [31]. Pluripotency of the resulting cells was confirmed *in vitro* by staining for SSEA-1 and murine protein Nanog and *in vivo* by teratoma formation after subcutaneous transplantation to the nude SCID mice as shown earlier [31]. iPS cells were subsequently differentiated toward endothelium (iPS-ECs) as described previously [31]. Briefly, iPS cells were cultured for four days on collagen IV coated plates in α-MEM (Lonza) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), 1% non-essential amino acids and 0.1 mM 2-mercaptoethanol (EDM, endothelial differentiation medium). Subsequently, cells were transferred on fibronectin-coated plates and cells were cultured in EDM medium supplemented with 50 ng/mL VEGF (Sigma-Aldrich). After achieving stable phenotype (CD34+/Tie-2+) cells were cultured in EGM-2MV medium (Lonza).

2.1.2. Isolation of murine mesenchymal stromal cells (MSCs) and differentiation toward endothelium

MSCs were isolated from bones of C57BL-GFP or C57BL mice after flushing out the bone marrow. Digestion of the bones was performed with 1 mg/mL collagenase II (Gibco) for 90–120 min/37 °C and the resulting cell suspension was cultured in α-MEM complete medium (CM), supplemented with 10% FBS, 1× penicillin/streptomycin (PEST) and 2 mM L-Glutamine (Glu), for three passages. Then CD45 negative cells were separated using MACS separator (Miltenyi) and used further. MSCs showed expression of CD90, CD105, CD29, CD49e, with no endothelial markers like CD31 or CD34 (Fig. S1). Differentiation toward osteoblasts and adipocytes was performed according to the established protocols [32]. Briefly, for the differentiation to adipocytes MSCs were cultured for 3 weeks with α-MEM CM supplemented with 1.0 μmol/L dexamethasone, 50 μmol/L 3-isobutyl-1-methylxanthine (IBMX) and 10 ng/mL insulin (all from Sigma-Aldrich) and verified with OilRedO staining (Fig. S2). Osteoblast differentiation was induced for 3 weeks with α-MEM CM supplemented with 0.1 μmol/L dexamethasone, 10 mmol/L β-glycerol phosphate and 50 μmol/L ascorbate-2-phosphate (all from Sigma-Aldrich) and verified with AlizarinRed S staining (Fig. S2). For differentiation toward endothelium CD45-negative cells were seeded on gelatin/fibronectin in EGM-2MV medium and cells (MSC-ECs) were cultured for one week [33]. As non-differentiated control CD45-negative cells were cultured in α-MEM CM.

2.1.3. Other cell lines

Human aortal endothelial cells (HAoEC) were purchased from Gibco (Invitrogen) and cultured in EGM-2MV medium (Lonza), MS-1 cell line was a kind gift from prof. Jack Arbiser (Emory University, Atlanta, USA) and was cultured in DMEM low glucose supplemented with 10% FBS (Lonza), 1 x PEST (Sigma) and 2 mM L-Glu (Sigma-Aldrich) [34].

2.2. Gene expression

For the analysis of the gene expression profile of the cells during the process of differentiation and after that, part of the cells in different time points were used for RNA isolation. RNA was reverse transcribed with NCode VILO miRNA cDNA Synthesis Kit (Invitrogen) following the manufacturer's instruction guide. Quantitative RT-PCR was performed in a Step One Plus real-time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Sigma-Aldrich). Expression of mouse genes was tested with the following protocol: denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, primer annealing at 60 °C for 60 s, 72 °C for 45 s and final elongation at 72 °C for 10 min. As reference gene *EF2* was used. The purity of the product was estimated with the melt curve analysis after the final PCR step. The sequences of the primers used are listed in Table 1. The primers for murine KLF-2 were described earlier [35].

For miRNA analysis the total RNA was reversed transcribed with NCode VILO (Invitrogen) and the expression was assessed using the following protocol: 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. As a reference miRNA mU6 was used. Sequences of all primers used for miRNA expression are presented in Table 2.

2.3. Staining of surface markers

Briefly, cells were detached with accutase (Cytogen), washed with PBS and resuspended in PBS with 2% FBS. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 10 min/4 °C and afterwards specific antibodies were added. CD34, CD31, CD106, CD144, CD45 and Sca-1 were obtained from BD Biosciences, CD105 and Tie-2 were from Biolegend, CD117 from eBioscience. The unspecific binding was ruled out with gating on fluorescent minus one (FMO) controls, whereas compensation controls were used for excluding the signal overlap between the channels. The staining was performed for 30 min/4 °C and afterwards the unbound antibodies were washed and cells resuspended in PBS with 2% FBS. The percentage of positive cells was assessed on LSR II with Diva software (BD).