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# Autologous cell sources in therapeutic vasculogenesis: *In vitro* and *in vivo* comparison of endothelial colony–forming cells from peripheral blood and endothelial cells isolated from adipose tissue

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**Abstract**

*Background aims.* Autologous endothelial cells are promising alternative angiogenic cell sources in trials of therapeutic vasculogenesis, in the treatment of vascular diseases and in the field of tissue engineering. A population of endothelial cells (ECs) with long-term proliferative capability, endothelial colony–forming cells (ECFCs), can be isolated from human peripheral blood. ECFCs are considered an endothelial precursor population. They can be expanded in cell factories in sufficient numbers for clinical applications, but because the number of isolated primary ECs is low, the culture period required may be long. Another EC population that is easily available in the autologous setting and may be expanded *in vitro* through several population doublings are ECs from adipose tissue (AT-ECs). *Methods.* Through extensive comparisons using whole-genome microarray analysis, morphology, phenotype and functional assays, we wanted to evaluate the potential of these EC populations for use in clinical neovascularization. *Results.* Global gene expression profiling of ECFCs, AT-ECs and the classical EC population, human umbilical vein ECs, showed that the EC populations clustered as unique populations, but very close to each other. By cell surface phenotype and vasculogenic potential *in vitro* and *in vivo*, we also found the ECFCs to be extremely similar to AT-ECs. *Conclusions.* These properties, together with easy access in the autologous setting, suggest that both AT-ECs and ECFCs may be useful in trials of therapeutic neovascularization. However, AT-ECs may be a more practical alternative for obtaining large quantities of autologous ECs.

**Key Words:** *adipose tissue-derived endothelial cells, cell therapy, endothelial colony–forming cells, microarray analysis*

## Introduction

Therapeutic angiogenesis, the treatment of tissue ischemia by promoting the proliferation of new blood vessels, has recently emerged as a promising treatment option. However, such pro-angiogenic therapy is not limited to vascular disease; it is also highly relevant in the field of tissue engineering, where clinical use of engineered tissues and tissue substitutes is often hampered by a lack of vascular perfusion. One approach promoting angiogenesis in ischemic diseases and vascularization of tissue-engineered constructs is the use of cells with angiogenic potential. Human umbilical vein derived endothelial cells (HUVEC) have been one of the most important sources of vascular endothelial cells since the first successful culture by Jaffe et al. in 1973 [1]. However, HUVECs are not suited for therapeutic use because of their allogenic nature. A source of autologous endothelial cells (ECs) with potential for use in therapeutic applications are

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the endothelial progenitor cells (EPCs), which were first isolated from human peripheral blood (PB) by Asahara et al. [2]. EPCs exhibit a spindle-like morphology and are able to differentiate into ECs and incorporate into sites of active angiogenesis in animal models [2]. Later, based on new clonogenic assays, two distinct EPC populations with different growth characteristics were observed, referred to as early- and late-outgrowth EPCs [3–5]. Early-outgrowth EPCs appeared in culture after 4 to 7 days, had low proliferation potential and have been found to be hematopoietic in origin [6]. Late-outgrowth EPCs are now also known as endothelial colony–forming cells (ECFCs) or outgrowth endothelial cells (OEC) [3,6]. Colonies of these cells appear after 14 to 21 days in culture, have a cobblestone appearance in monolayer culture and have high proliferative potential [7].

EPCs/ECFCs have been considered extremely rare in human PB, with reported frequencies as low as 0.05 EPCs/mL of blood [6,8,9]. However, culturing unfractionated PB, Reinisch et al. recently quantified ECFCs at four colonies per milliliter of blood and showed how these cells could be expanded to clinically relevant numbers in cell factories [10]. This strategy would enable the use of autologous ECFCs in trials of therapeutic angiogenesis. Another EC population that is easily accessible in the autologous setting and may be expanded *in vitro* through relatively few population doublings (PDs) to yield $\geq 10^8$ cells are the ECs from adipose tissue (AT) [11–13]. In the context of clinical applications, other EC populations may not be expanded to sufficient numbers because of they can supply only small amounts of starting material (dermal microvascular cells), are not accessible (ECs from the arterial side) or are allogeneic (HUVECs and foreskin microvascular cells) and therefore likely to be rejected by alloimmune mechanisms. Thus, at this time, the ECs most likely to have a place in trials of therapeutic angiogenesis are ECFCs and AT-ECs.

In the present study, we performed an extensive comparison of these two EC populations to evaluate their potential for use in clinical applications. For comparison of expression profiles between the two EC populations, we include HUVECs as an endothelial reference cell population. We show that ECFCs and AT-ECs are extremely similar in morphology, phenotype and their expression of EC-relevant genes. Furthermore, both EC populations showed similar potential for *in vivo* angiogenesis, as demonstrated using a mouse model.

## Methods

All reagents were purchased from Sigma Aldrich unless otherwise stated.

### *Ethics statement*

Biological samples were collected after written informed consent in accordance with the guidelines of the Declaration of Helsinki. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway, Section A, and by the Institutional Review Board of the Medical University of Graz (protocol numbers 19–252 ex 07/08 and 18–243 ex 06/07). Animal experiments were approved by the Animal Care and Use Committee at the Veterinary University of Vienna on behalf of the Austrian Ministry of Science and Research according to the Guide for the Care and Use of Laboratory Animals (BMWF-66.010/0082-II/10b/2009).

### *Cells*

AT-ECs from liposuction material, ECFCs from PB and HUVECs from HUVs were isolated as described previously [10,12,14], with details also described later in the article. All comparisons were performed using cells from three donors for each EC population.

### *Isolation and in vitro humanized expansion of AT-ECs*

AT was obtained from liposuction material from abdominal regions of three healthy female donors (aged 22–35 years; BMI: 23–30) undergoing cosmetic surgery. The stromal vascular fraction (SVF) was separated from AT as described previously [15]. Briefly, lipoaspirate was washed and digested using 0.1% collagenase A type 1. After centrifugation, the cell pellet was filtered through 100-μm and then 40-μm cell sieves (Becton Dickinson). SVF cells were obtained from the interface after Lymphoprep gradient separation (Axis Shield).

The acquisition of AT-ECs from the SVF has been described previously [12]. The strategy chosen included removal of $CD44^+$ cells from SVF because this gave superior purity and yield compared with other isolation procedures. $CD44^+$ cells were removed using Dynabeads (Dynabeads Pan Mouse IgG, Invitrogen Dynal AS) according to the manufacturer's description. Dynabeads were washed then pre-coated with monoclonal anti-CD44 antibody (Southern Biotech) at the concentration of 0.6 μg antibody per 25 μL Dynabeads and incubated for 2 h with gentle tilting and rotation. Dynabeads were then washed again, and approximately $400 \times 10^6$ magnetic CD44 beads were added to $100 \times 10^6$ SVF cells.

AT-ECs were plated at $2 \times 10^6$ cells per 75-$cm^2$ tissue culture flask (Nunc). Cells were maintained at 37°C in an atmosphere of 5% $CO_2$ in humid air using EC growth medium (MCDB 131, Gibco) supplemented with 1% L-glutamine, 1 ng/mL basic fibroblast growth factor, 1 μg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 50 μg/mL gentamicin,

250 ng/mL amphotericin B and 10% pooled human platelet lysate (pHPL) with 10 U/mL heparin (Wockhardt UK Ltd). The pHPL was prepared from pooled platelet-rich plasma as previously described [16]. Cells still in suspension were removed on day 4–5, and the medium was replaced. When the cells reached 80–90% confluence, cell adherence was interrupted by trypsin-ethylenediaminetetraacetic acid (EDTA) solution for ECs, and the cells were inoculated into new flasks at 10,000 cells per $cm^2$. Additional purging of contaminating cells was performed using anti-CD90 (BD Biosciences) coated Dynabeads if required at the first passage. Cells were routinely passaged every 3–4 days. Viable cells were counted at each passage.

*Isolation and in vitro expansion of ECFCs*

Cell isolation was started within 2 h after blood collection. To minimize initial cell loss, red blood cell lysis or density gradient centrifugation were strictly avoided. Blood was directly diluted in endothelial growth medium (EGM) in a 1:4 to 1:10 ratio depending on the surface area of the culture vessel used. Non-adherent cells were removed after overnight culture at 37°C, 5% $CO_2$ in humidified atmosphere by washing three to six times with excess 37°C pre-warmed phosphate buffered saline before adding new 37°C pre-warmed EGM. Thereafter, the medium was replaced three times weekly until a visible outgrowth of cobblestone-type colonies appeared. For further expansion adherent ECFCs were passaged into four-layered cell factories (CF-4; Nunc) at a maximum seeding density of 100 ECFCs/$cm^2$ and cultured at 5% $CO_2$, 37°C, 95% air humidity. All ECFC cultures were maintained by replacing one-third of the medium with new pHPL-supplemented EGM twice weekly until cells reached near confluence. After 11 to 25 days, ECFCs were harvested using 0.25% trypsin/1 mmol/mL EDTA (70 mL per CF-4, 1–5 min, 37°C; Gibco Cell Culture, Invitrogen Corporation). Nucleated cell numbers were determined using a hemocytometer as the mean of four measurements and viability by trypan blue exclusion.

*Isolation and in vitro expansion of adipose tissue derived mesenchymal stem cells (AT-MSCs)*

To obtain AT-MSCs/pericytes for the Matrigel vascularization studies, the $CD44^+$ cells positively selected from the SVF from the same AT donors were cultured in Dulbecco's modified eagle medium/F12 (Gibco) containing 10% pHPL and antibiotics.

*Microarray analysis and bioinformatics*

For the microarray analyses, ECFCs, AT-ECs and HUVECs were cultured in the same medium (EGM-2 medium, Lonza) under identical conditions. We used cells from three donors for each of the three EC populations. For RNA isolation cells were pelleted, frozen in liquid nitrogen and stored at −80°C until used. Total RNA was extracted from cells using RNAqueous—4PCR Kit (Applied Biosystems/Ambion) according to the manufacturer's protocol and treated with DNase I (Applied Biosystems/Ambion). Total RNA was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The integrity of the total RNA samples was assessed by Agilent 2100 Bioanalyser and scanned by Illumina Whole-Genome Gene Expression Direct Hybridization Assay (BeadChip technology) at the Norwegian Microarray Consortium according to the manufacturer's protocols. Briefly, biotin-labeled cRNA was synthesized from 500 ng total RNA using the Illumina TotalPrep-96 RNA Amplification Kit (Ambion). Next, 750 ng of cRNA was hybridized to Illumina HumanHT-12v4 Expression BeadChip containing >48,000 probes at 58°C overnight. The hybridized chip was stained with streptavidin-Cy3, washed, dried and scanned by Illumina BeadArray Reader software. The signal intensity files were imported into Illumina GenomeStudio software V2011.1, Gene Expression module V1.9.0, for quality control, extraction and quintile normalization. The array annotation file "HumanHT-12_V4_0_R2_15002873_B.bgx" was used.

The microarray results were exported to J-Express 2009 (Molmine) for expression analysis [17]. The data were mean normalized and log2 transformed. Rank product was used to find differentially expressed genes [18]. A change in expression of greater than twofold and $P < 0.05$ were used as cutoff levels for differentially expressed genes. Gene ontology (GO) annotation was performed using DAVID bioinformatics resources on the significantly differentially expressed genes to analyze their functional characteristics [19,20]. Benjamini correction was applied to correct for multiple analyses. Gene set enrichment analysis was performed and the GO terms were sorted by enrichment [21,22].

All array data are available from Gene Expression Omnibus (accession number: GSE55695).

*Real-time reverse transcription quantitative polymerase chain reaction analysis*

Total RNA isolation and reverse transcription (RT) was performed as described previously [12] using 200 ng total RNA per 20-μL RT reaction. Information about the TaqMan assays are provided in Supplementary Table S1. After verifying the stable expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), this gene was selected as the endogenous control. Relative mRNA levels were calculated by the comparative CT method [23]. Differential expres-