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ENDOTHELIAL CELL MONOLAYER FORMATION ON A SMALL-DIAMETER VASCULAR GRAFT SURFACE UNDER PULSATILE FLOW CONDITIONS

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Objective: to create a cell-populated small-diameter vascular graft (SDVG) using autologous endothelial cells and extracellular matrix proteins, and to evaluate the efficiency of endothelial cell monolayer formation during shear stress preconditioning in a SDVG. **Materials and methods.** PHBV/PCL tubular scaffolds of vascular grafts were made by electrospinning from a mixture of polyhydroxybutyrate-valerate (PHBV) copolymer and polycaprolactone (PCL) and modified with fibrin. To populate the graft, an endothelial cell culture was isolated from the blood of patients with coronary heart disease. Phenotyping of endothelial colony-forming cell (ECFC) culture was performed by flow cytometry and immunofluorescence microscopy. Cell proliferative and angiogenic activity were also studied. Cell-populated vascular scaffolds were cultured in a pulsatile flow setup with a final shear stress of 2.85 dyne/cm^2. The effect of pulsatile flow on monolayer formation was assessed by immunofluorescence, scanning electron microscopy, atomic force microscopy, and whole-transcriptome RNA sequencing. **Results.** Under the influence of pulsatile flow, endothelial cells that were seeded into the tubular scaffold showed an increase in the expression level of endothelial profile proteins, focal adhesion and cytoskeleton. In contrast to endothelial cell culture on a vascular graft surface under static conditions, when cultured under pulsatile flow with 2.85 dyne/cm2 shear stress, endothelial lining cells have an increased ability to adhere and are oriented along the pulsatile flow path. Whole-transcriptome RNA sequencing showed that induced shear stress increased expression levels of differentially expressed genes encoding proteins that ensure vascular development, endothelial integrity, and endothelial metabolism. A protocol for fabrication of a personalized cell-populated biodegradable SDVG under pulsatile flow conditions was developed. **Conclusion.** The use of autologous fibrin and ECFC culture, as well as shear stress preconditioning, allow to obtain a personalized cell-populated SDVG with continuous functional endothelial monolayer adapted to the flow.

Keywords: tissue engineering, autologous endothelial cells, pulsatile flow, personalized vascular graft.

INTRODUCTION

Creation of biodegradable small-diameter vascular grafts capable of being resorbed after implantation and replaced by the patient's own new vessels, frees the patient from the need for replacement. Therefore, it is a priority in tissue vascular engineering [1, 2]. Researchers are looking for an ideal culture that would have a high proliferative potential, full functional activity, and reproducibility.

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It is believed that in vitro endothelialization of vascular grafts or early stimulated in situ endothelialization of engineered constructs can be very effective in preventing thrombosis of tissue-engineered vascular grafts [3–5].

Vascular endothelial cells are constantly subjected to shear stress due to frictional force created by blood flow. Through activation of mechanosensors, they recognize changes in local shear stress and cyclic deformation, intracellular signal transduction is modulated, leading to changes in gene expression, cell morphology and structural remodeling [6].

In the straight part of the vessel, shear stress and cyclic stretch have well-defined directions; the response of endothelial cells to targeted mechanical stimuli involves remodeling of the cytoskeleton structure to minimize intracellular stress. These adaptive changes help maintain homeostasis and they have an atheroprotective effect. In the vascular network of complex geometry, multidirectional mechanical stimuli arise, which can contribute to atherogenesis in these areas [7].

In vitro endothelialization of grafts is expensive and requires cell preconditioning by shear stress in order to increase cell viability and resistance to flushing by blood flow from the surface of the cell-based graft after its implantation into the vascular bed [8–9]. It is also

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believed that it is impossible to obtain large quantities of autologous endothelial cells from peripheral blood [10–12]. Therefore, in order to colonize the inner surface of tubular scaffolds with cells, scientists have often resorted to the use of bone marrow-derived mononuclear fraction or adipose-derived stromal vascular fraction, or used induced pluripotent cells in order to observe personalization of biological material, acting as a source of own vascular cells [13–15].

In our previous works, we showed the possibility of obtaining autologous human endothelial colony-forming cells (ECFCs) with high proliferative potential from the mononuclear blood fraction of the majority of patients with coronary heart disease [16].

Adhesion of endothelial cells to the polymer base is impeded by hydrophobicity of the surface, its foreign molecular composition and absence of cell adhesion sites. In order to eliminate these factors, the surface is modified by various extracellular matrix proteins. In tissue engineering, extracellular matrix proteins (collagen, fibronectin, gelatin) from animal sources or allogeneic proteins are usually used as a substrate, which increases the risk of negative immune response after graft implantation into the vascular bed. The use of autologous fibrin, obtained from the patient's peripheral blood as a feeder layer, reduces immunogenicity of protein-coated biodegradable polymeric vascular graft. With autologous fibrin and ECFC culture, one can design a personalized small-diameter vascular graft, while cultivation in the pulsatile flow unit allows to adapt endothelial cells to the active mechanical stimuli existing in natural blood flow.

Our objective is to create a cell-based small-diameter vascular graft using autologous endothelial cells and extracellular matrix proteins and evaluate the efficiency of endothelial monolayer formation under shear stress preconditioning.

MATERIALS AND METHODS

Fabrication of biodegradable tubular scaffolds

Biodegradable polymer tubular scaffolds from a mixture of 5% poly(3-hydroxybutyrate-co-3-hydroxyvalerate) solution, (PHBV, Sigma-Aldrich, USA) and 10% poly(ε-caprolactone) solution, (PCL, Sigma-Aldrich, USA) dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich, USA) in a 1:2 ratio were made by electrospinning on a Nanon-01A machine (MECC, Japan). 22G-gauge needles were used to make the scaffolds. Needle voltage was 23 kV, polymer solution feed rate was 0.3 mL/h, collector rotation speed was 200 rpm, distance from needle to the winding collector was 15 cm, and the winding collector diameter was 4 mm.

Fibrin modification of the inner surface of tubular scaffolds

The inner surface of tubular scaffolds was modified with fibrin. Fibrinogen was isolated from peripheral blood of patients with coronary heart disease. Blood sampling was performed after obtaining voluntary informed consent to use biological material for research purposes. The study was approved by the local ethics committee (protocol No. 20 of December 9, 2020). Blood collected in vacuum tubes with sodium citrate was centrifuged at 2000 g for 10 min, and the resulting plasma was cooled to 4 °C. Cold ethanol (70 vol%, 4 °C) was added to the plasma at a 4:1 ratio (plasma/ethanol) with constant stirring. Immediately thereafter, the solution was centrifuged at 600 g at 4 °C for 5 min. The supernatant plasma was decanted and the fibrinogen precipitate was dissolved in 0.9% $NaCl_2$ with HEPES at 37 °C (to 30–40 mg/mL fibrinogen concentration) [17]. Fibrinogen was polymerized using thrombin and $CaCl_2$. Polymer grafts were immersed and impregnated with the resulting fibrinogen solution. Next, a solution of thrombin 500 IU/ml (Thrombin from human plasma, T7009, Sigma-Aldrich, USA) and $CaCl_2$ 40 mmol/L were applied to the surface of the tubular scaffold for fibrin polymerization. After polymerization, the tubular scaffold was immersed in sodium phosphate buffer solution (PBS) with ε-aminocaproic acid 2 mg/ml until subsequent cellular seeding.

Obtaining ECFCs

A culture of endothelial colony-forming cells of the peripheral blood mononuclear fraction with high proliferative activity and full functionality was used for populating the vascular grafts. Blood was taken from patients with coronary heart disease (a group of patients who, with the further development of the disease, would require prosthetic repair to the extent of coronary artery bypass grafting) after obtaining voluntary informed consent to use biological material for research purposes. ECFC culture was obtained based on culturing blood mononuclear fraction (BMF) in media containing growth factors. The studies were performed at the Research Institute for Complex Issues of Cardiovascular Diseases and were approved by the local ethics committee (protocol No. 20 of December 9, 2020). The cultivation technique, immunophenotyping and study of the functional properties of the resulting culture are presented earlier in a paper by our team [16]. A comparative study of the gene expression profile of ECFC culture with mature coronary artery cell culture was conducted [18].

Phenotyping culture by flow cytometry

For staining, 1×10^5 cells of the resulting culture washed with PBS were selected. We used a combination of monoclonal antibodies conjugated with various

dyes: with fluorescein isothiocyanate (FITC) – CD3 (BC, A07746), CD34 (BC, IM1870U), vWF (abcam, ab8822); with phycoerythrin (PE) – CD309 (BD, 560494), CD14 (BC, A07764); with allophycocyanin (APC) – CD133 (MACS, 130-090-826), CD31 (BL, 303115); with phycoerythrin-cyanine 7 (PC7) – CD146 (BL, 361008); with Pacific Blue 450 (PB 450) – HLA DR (BL, 307633); with Krome Orange (KrOr) – CD45 (BC, A96416).

Sample preparation was performed according to the protocols of the manufacturers for two panels: 1) CD3, CD14, HLADR, CD45; 2) CD34, KDR, CD146, CD133, CD31, CD45. Cell culture with cobblestone morphology was additionally stained with vWF, CD146. A culture stained with antibodies of the corresponding isotypic control, CD146 – PC7 Mouse IgG1 (BL, 400127), CD31, was used as a control, CD133 – APC Mouse IgG1 (BL, 400121), CD309 – PE Mouse IgG1 (BD, 550617), CD3, CD3, CD34 – FITC Mouse IgG1 (BC, A07795), CD14 – PE Mouse IgG2α (BC, 559319), CD45 – KrOr Mouse IgG1 (BC, A96415), HLA DR – PB Mouse IgG2α (BL, 400235). When intracellular vWF protein was stained, cells were fixed and permeabilized using the IntraPrep kit (BC, A07803, USA). Samples were then resuspended in PBS and analyzed on a CytoFlex laser flow cytometer (BC, USA) using the CytExpert software. The instrument was set up for each panel using control samples stained with the corresponding isotypes; further analysis of all samples was performed using the same instrument settings.

Cell seeding of a biodegradable tubular scaffold

ECFC suspension was injected into PHBV/PCL scaffolds at 700,000/ml concentration. For the first 8 hours after seeding, the vascular grafts were inverted every 30 minutes to evenly distribute the cells over the surface. The culture medium was replaced after 24 hours. The vascular grafts were cultivated under static conditions for a total of 2 days. After that, the vascular prostheses were connected to the pulsatile flow unit (Harvard Apparatus, USA) at preset settings, providing voltage of 1.27 dyne/cm^2 for 24 hours. Then, the voltage was gradually increased to 2.85 dyne/cm^2. The operating mode of culturing in the pulsatile flow unit included the following parameters: ejection rate 20 beats/min, ejection volume 0.7 mL, and a final shear voltage of 2.85 dyne/cm^2. Culturing was continued in this mode for 5 days under sterile conditions in a CO_2 incubator at 37 °C. For the control, similar vascular grafts were cultured under static conditions.

Scanning electron microscopy

The inner surface of tubular scaffolds was examined by scanning electron microscopy at various stages (before and after fibrin modification of PHBV/PCL scaffolds, after ECFC seeding) on a Hitachi S-3400N microscope (Hitachi, Japan) under high vacuum conditions. Sample preparation consisted of fixation in glutaraldehyde (Sigma, USA) for one day followed by freezing and lyophilization in Freezone 2.5 machine (Labonco, USA) at –40 °C and pressure <0.133 mbar. After that, the samples were mounted on special tables and the conductive gold-palladium coating with a 7 nm thickness was formed on their surface by ion sputtering in the EM CE200 machine (Leika Mikro-systeme GmbH, Austria).

Immunofluorescence study

After culturing, the endothelial layer formed on the inner surface of the vascular prosthesis was subjected to immunofluorescent staining. The vascular prosthesis was fixed in 4% paraformaldehyde solution for 10 minutes, then cut lengthwise and fixed open on slides with the inner surface upward. Cells on the inner surface were stained for endothelial cell markers: CD31, VEGFR2 (CD309), CD144, vWF, adhesion marker F-actin, and Talin. Intracellular markers (vWF, F-actin) were permeabilized with 0.01% Triton X-100 solution before staining. F-actin was stained with phalloidin conjugated with Alexa Fluor 568 fluorescent dye (Alexa Fluor™ 568 Phalloidin, A12380, Thermo Fisher, USA) according to the manufacturer's instructions. Primary antibodies used for the remaining markers were Mouse anti-CD31 (ab119339), Rabbit anti-VEGFR2 (ab2349), Rabbit anti-CD144 (ab33168), Sheep anti-vWF – FITC (ab8822), Rabbit anti-Talin (ab71333) and secondary antibodies: Donkey anti-Mouse IgG Highly Cross-Adsorbed – AF555 (A-31570), Donkey anti-Rabbit IgG Highly Cross-Adsorbed – AF488 (A-21206) (Thermo Fisher, USA). Incubation with primary antibodies was performed at 4 °C for 16 hours, with secondary antibodies at room temperature for one hour. A 1% bovine serum albumin solution was used to block nonspecific binding. The samples were counterstained with DAPI (Sigma, USA). Prepared glasses were embedded in ProLong (Life technologies, United States) under glass. The preparations were analyzed using an LSM 700 laser scanning microscope (Zeiss, Germany).

Assessment of cell adhesion and viability

Adhesion and viability of endothelial cells on the surface of vascular grafts were assessed by fluorescence microscopy. Samples were washed in PBS, serially injected with Hoechst 33342 (10 μg/mL, 14533, Sigma Aldrich, USA), followed with ethidium bromide (30 μg/mL, 46067, Sigma Aldrich, USA), and incubated for 10 minutes and 1 minute, respectively. Samples were analyzed on an LSM 700 laser scanning microscope (Zeiss, Germany). Cell adhesion was assessed by counting cell nuclei stained with Hoechst 33342 in the field of view. At least 20 randomly selected fields of view

were analyzed at ×200 magnification. The results were recalculated and presented as units/mm^2.

The relative number of dead cells was calculated using the formula: absolute number of dead cells × 100% / absolute number of all adherent cells. The relative number of live cells was determined by subtracting the proportion of dead cells from 100% of adherent cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. Data are presented as medians (Me) and quartiles (25% and 75%). Statistical significance of differences between the two independent groups was assessed using the nonparametric Mann–Whitney U test. Differences were considered significant at a significance level of $p < 0.05$.

Whole transcriptome sequencing

An ECFC culture of 10 million cells was used for whole transcriptome sequencing (RNA-seq). Cells were lysed with Trizol (15596018, Invitrogen, USA) followed by isolation of total RNA using the Purelink RNA Micro Scale Kit (12183016, Invitrogen, USA) with accompanying DNAase treatment (DNASE70, Sigma-Aldrich, USA). RNA quality was monitored using the RNA 6000 Pico Kit (5067-1513, Agilent, USA) on a Bioanalyzer 2100 (Agilent, USA) using the RNA integrity index (RIN). Amount of isolated RNA was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and a Qubit 4 fluorometer (Invitrogen, USA). For 1 μg of isolated RNA, rRNA was depleted using using the RiboCop rRNA Depletion Kit V1.2 (037.96, Lexogen, Austria) with further formation of DNA libraries (SENSE Total RNA-Seq Library Prep Kit, 042.96, Lexogen, Austria). The quality of obtained DNA libraries was analyzed using the High Sensitivity DNA Kit (5067-4626, Agilent, USA) on a Bioanalyzer 2100 (Agilent, USA). DNA libraries were analyzed quantitatively by quantitative polymerase chain reaction (RT-qPCR) on a CFX96 Touch amplifier (Bio-Rad, USA). Next, DNA libraries were mixed in an equimolar manner and sequenced on a HiSeq 2000 platform (Illumina) with a 2 × 125 nucleotide paired-end read length.

The resulting reads were filtered by quality (QV >20) and length (>20), and adaptor sequences were removed using the TrimGalore v.0.4.4 software. After filtering, the average number of reads exceeded 10 million. They were mapped to the human genome (hg38) with Ensembl annotation (v.38.93) using the CLC GW 11.0 program (Qiagen) with the following parameters: Similarity fraction = 0.8, Length fraction = 0.8, Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3; the resulting files were obtained in *.bam* format. To assess differential gene expression, we used multivariate statistical analysis in the CLC GW 11.0 software based on the negative binomial model used in the EdgeR and DESeq2 programs.

The RNA integrity index (RIN) isolated from the ECFC culture was at least 8, indicating that it was of high quality and could be used for RNA-seq (RIN ≥7 is recommended). The amount of total RNA obtained in the samples was at least 29 μg, which was more than enough for rRNA depletion (≥1 μg is recommended).

Statistical analysis of whole transcriptome sequencing results

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software). The data are presented as median, 25th and 75th percentiles, and minimum and maximum. The groups were compared by Mann–Whitney U test. When analyzing differences between cell cultures, statistically significant differentially expressed genes (DEGs) were determined by the frequency of change ≥2 and a p value <0.05 corrected for the mean false discovery rate (FDR). Gene Ontology (GO) gene set enrichment analysis was performed in the categories of molecular functions, biological processes, and cellular components using the Gene Set Test in CLC GW. When comparing cell groups, categories with $p < 0.05$ (FDR) support and a ratio of DEGs to the total number of genes greater than 50% were considered.

RESULTS

Characteristics of autologous endothelial colony-forming cells

The phenotype of the resulting ECFC culture corresponded to that of mature endothelial cells: CD31+CD309+vWF+CD146+, no expression of progenitor markers CD34, CD133 (Fig. 1) [16]. The culture had high proliferative activity. Full-fledged functional activity, including angiogenic activity, was detected [16].

Surface structure of PHBV/PCL scaffolds before and after fibrin modification

The unmodified PHBV/PCL scaffolds exhibited a homogeneous highly porous structure with chaotic filament interlacing. The average fiber diameter was 2.8 [1.9; 3.3] μm, and the pore size was 36.1 [30.2; 38.7] μm. Modification of the polymer scaffold surface with fibrin significantly changed its architectonics (Fig. 2). It was found that during polymerization, fibrin flattened the surface of the polymer scaffold, forming a uniform fine-pored coating on their finest branched fibrils with a fiber diameter of 125 [94.0; 152.0] nm and a pore size of 273 [176.0; 333.0] nm.

On the inner surface of the PHBV/PCL/fibrin cell-populated vascular grafts, well-disseminated endothelial cells were found, indicating high-quality adhesion. Meanwhile, a highly irregular distribution of ECFCs

was found on unmodified PHBV/PCL scaffolds. In the absence of a feeder layer carrying cell adhesion sites, there was no sufficiently dense cell attachment.

Regime of cultivation of cell-populated vascular grafts under pulsatile flow conditions

The optimal pulsatile flow regime for formation and preservation of endothelial lining on the surface of PHBV/PCL/fibrin vascular grafts was selected.

It was found that increasing the endothelial cell culture time on the graft surface does not lead to increased seeding efficiency. Apparently, this is due to the fact that mature endothelial cells have moderate proliferative activity. After 2 days of culturing endothelial cells in static conditions, most of the graft inner surface was colonized. After 7 days in static conditions, moderate proliferation occurred with the formation of an almost continuous cell layer. When cells were cultured on the prosthetic graft surface in static conditions for more than 7 days (14 and 21 days), a part of cells died with disruption of the cell monolayer.

Under pulsatile flow conditions, we determined the following optimal mode of culturing cell-based vascular graft: 2 days of preliminary culturing in static conditions, 5 days of subsequent culturing in the pulsatile flow unit at the following pulsatile flow parameters: ejection volume 0.7 ml; ejection frequency 20 beats/min; shear stress 2.85 dyn/cm^2. The shear stress was gradually increased to the specified value after pre-culturing the cells in static conditions for two days.

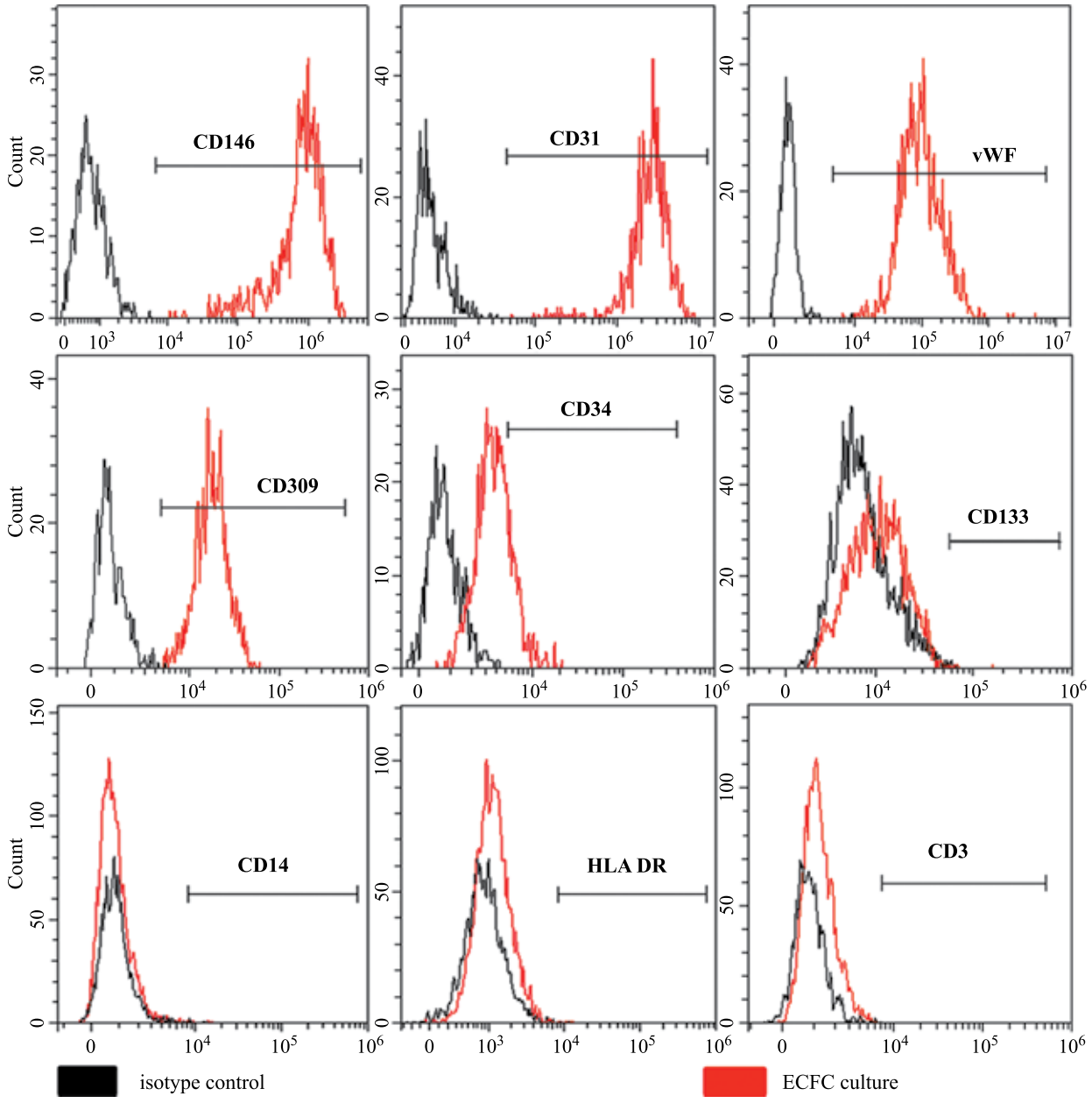

Fig. 1. ECFC culture phenotype. Flow cytometry [16]

Results of cell seeding of PHBV/PCL/fibrin tubular scaffolds

The viability of endothelial colony-forming cells cultured on the inner surface of PHBV/PCL/fibrin biodegradable scaffolds was fully preserved regardless of the culture conditions. There were also no statistically significant differences in the density of cell populations per unit area (Fig. 3). Results obtained confirm that the chosen culturing regimen had no damaging effect on the endothelial lining, and the fibrin feeder layer provided sufficient adhesive surface characteristics for ECFCs.

Based on the results of staining with specific antibodies, we analyzed the effect of pulsatile flow intensity of 2.85 dyne/cm^2 on the phenotype of endothelial colony-forming cells, intercellular contacts, functional activity, cytoskeleton organization, and cell surface adhesion. It was found that ECFCs, which constituted a monolayer on the inner surface of PHBV/PCL/fibrin tubular scaffolds, regardless of the culturing conditions, were characterized by a high level of expression of specific endothelial markers: CD31, CD309, CD144, vWF (Fig. 4). However, under dynamic culturing conditions, these markers were higher, except for CD144 (VE-cadherin). Meanwhile, response to pulsatile flow was reflected in cytoskeletal rearrangements. The average fluorescence intensity of the structural protein F-actin was significantly higher under dynamic culture conditions, ($p < 0.01$). Under the influence of shear stress, chaotic orientation of F-actin microfilaments changed to a preferential orientation of cells in the flow direction, reflecting cell adaptation to shear stress effects. Also adaptive to flow was an increase in expression of the point adhesion protein Talin, ($p < 0.05$). Talin binds integrin receptors and actin cytoskeleton, is a mechanosensitive protein and transmits the mechanical signal of the environment to the cell, allowing it to adapt to the altered conditions.

A change in the morphology of endothelial cells cultured under pulsatile flow conditions was revealed. Under this operating regimen, the cells were stretched along the fluid flow, in close contact with neighboring cells, which was not observed under static conditions.

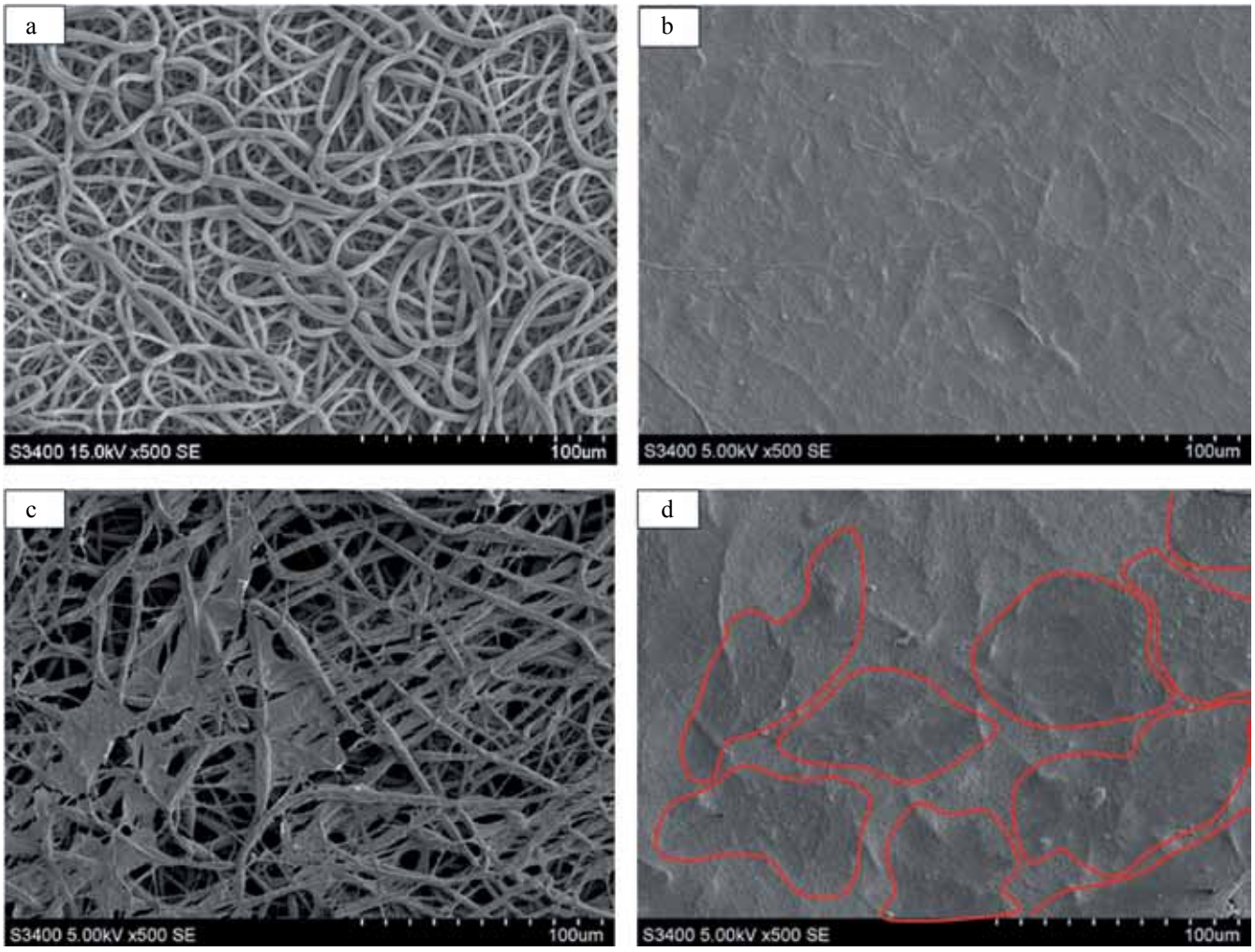

Fig. 2. Scanning electron microscopy of the inner surface of biodegradable vascular prostheses: a – PHBV/PCL; b – PHBV/PCL/fibrin, c – ECFC-populated PHBV/PCL; d – ECFC-populated PHBV/PCL/fibrin. The red line outlines the cell contour. 500× magnification

Endothelial homeostasis, maintained by the correct gene expression profile, affects the long-term patency of the vascular graft. Whole transcriptome sequencing (RNA-Seq) was performed to determine whether shear stress preconditioning has an effect on ECFC gene expression profile.

Sequencing of DNA libraries resulted in paired-end reads of 125 nucleotides long, with total reads ranging from 1–5 billion base pairs and coverage of 9.5–42.7 million reads. After filtering the reads by quality and length, as well as removing adapters, their number remained virtually unchanged. Mapping the library reads to the human genome showed that at least 98.2% of the reads in all samples corresponded to the human genome. Most of the reads (82.2–90.4) were for exons, i.e., the protein-coding part of genes.

Differences between ECFC transcriptomes cultured on the inner surface of PHBV/PCL/fibrin under static and pulsatile flow conditions were found. A total of 185 significant DEGs were identified in ECFC culture. DEGs were annotated using terms suggested in the Gene Ontology database to further disclose their molecular characteristics. DEGs were divided into 125 categories, which

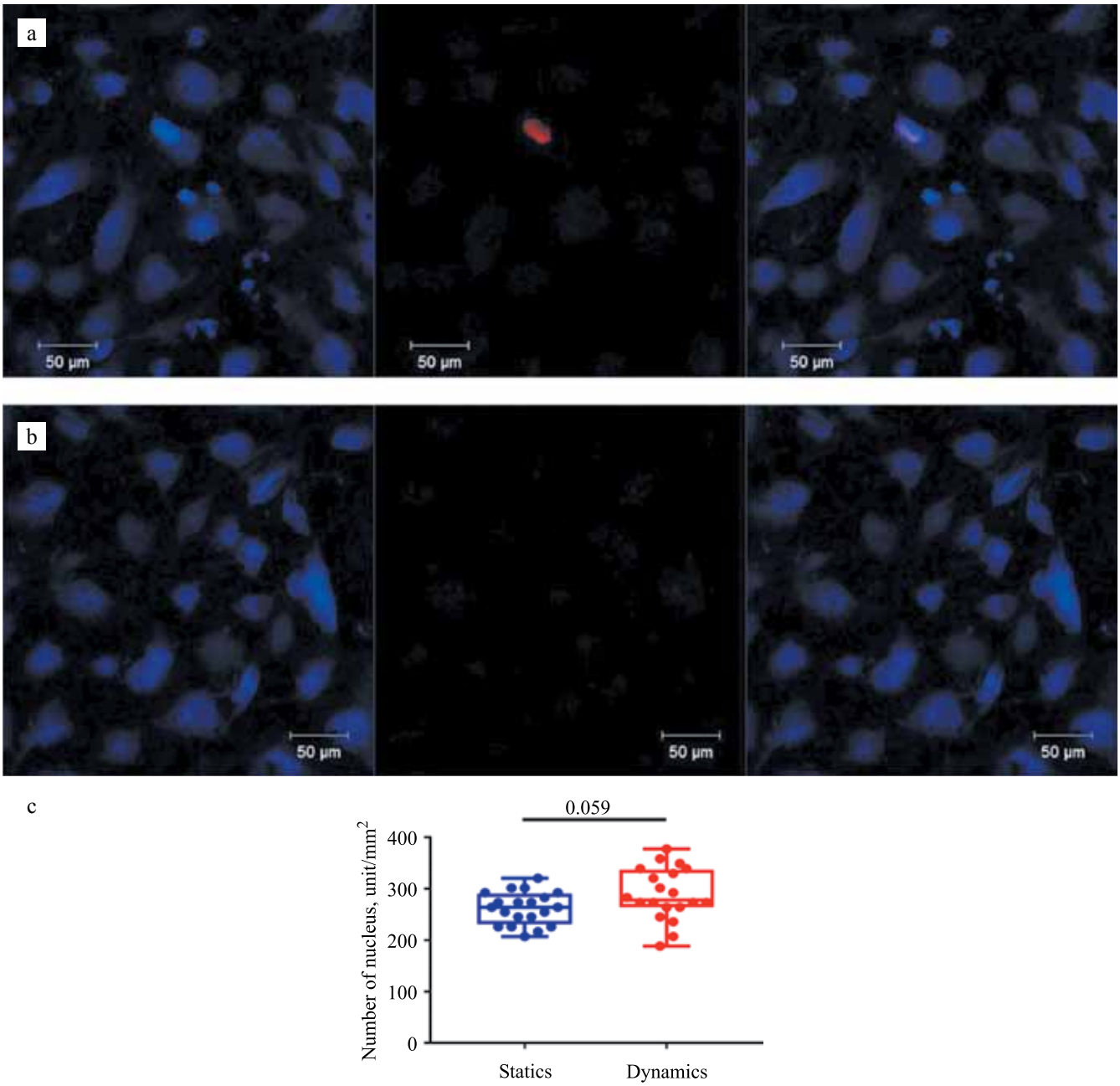

Fig. 3. Viability of human ECFC when cultured for 7 days on the inner surface of PHBV/PCL/fibrin biodegradable vascular prostheses: a – under static conditions; b – under pulsatile flow conditions; c – quantitative analysis of cell population density, $p < 0.05$. Combined staining with Hoechst 33342 fluorescent (blue) and ethidium bromide (red). Confocal laser microscopy, 200× magnification

can be roughly divided into 2 broad groups: endothelial metabolism (62) and processes involved in endothelial biology (63).

A comparative analysis of the differential expression systems associated with the endothelial gene phenotype showed that exposure to pulsatile flow activates metabolism in ECFCs. The Gene Ontology tool was used to detect these differences, which had an advantage when analyzing DEGs in the "endothelial metabolism" category, and determined that 23 DEGs were characterized by increased expression in statics, whereas 39 were characterized by increased expression in dynamics.

Further enrichment of endothelial phenotype-related gene systems by Gene Ontology category enlargement (especially by manual annotation of genes differentially expressed in ECFCs) showed that, in response to pulsatile flow, there is increased expression of gene encoding:

Table 1

Results of quantitative analysis of immunofluorescence staining of ECFCs cultured under static conditions ("Static") and under pulsatile flow conditions with 2.85 dyne/cm^2 shear stress ("Dynamic")

	Statics Me (25%; 75%)	Dynamics Me (25%; 75%)
Mean fluorescence intensity of CD31, AU	50.5 [40.8; 55.3]	56.0 [53.5; 62.3]
Dyed area of CD309, %	2.0 [1.8; 2.3]	3.1 [2.7; 4.3]
Mean fluorescence intensity of CD144, AU	34.5 [31.3; 37.6]	22.0 [19.6; 23.2]
Mean fluorescence intensity of vWF, AU	5.5 [4.0; 7.3]	17.5 [13.5; 23.3]
Dyed area of Talin, %	1.7 [1.6; 1.9]	2.7 [2.1; 3.3]
Mean fluorescence intensity of F-actin, AU	59.7 [52.2; 64.8]	66.5 [62.7; 71.6]

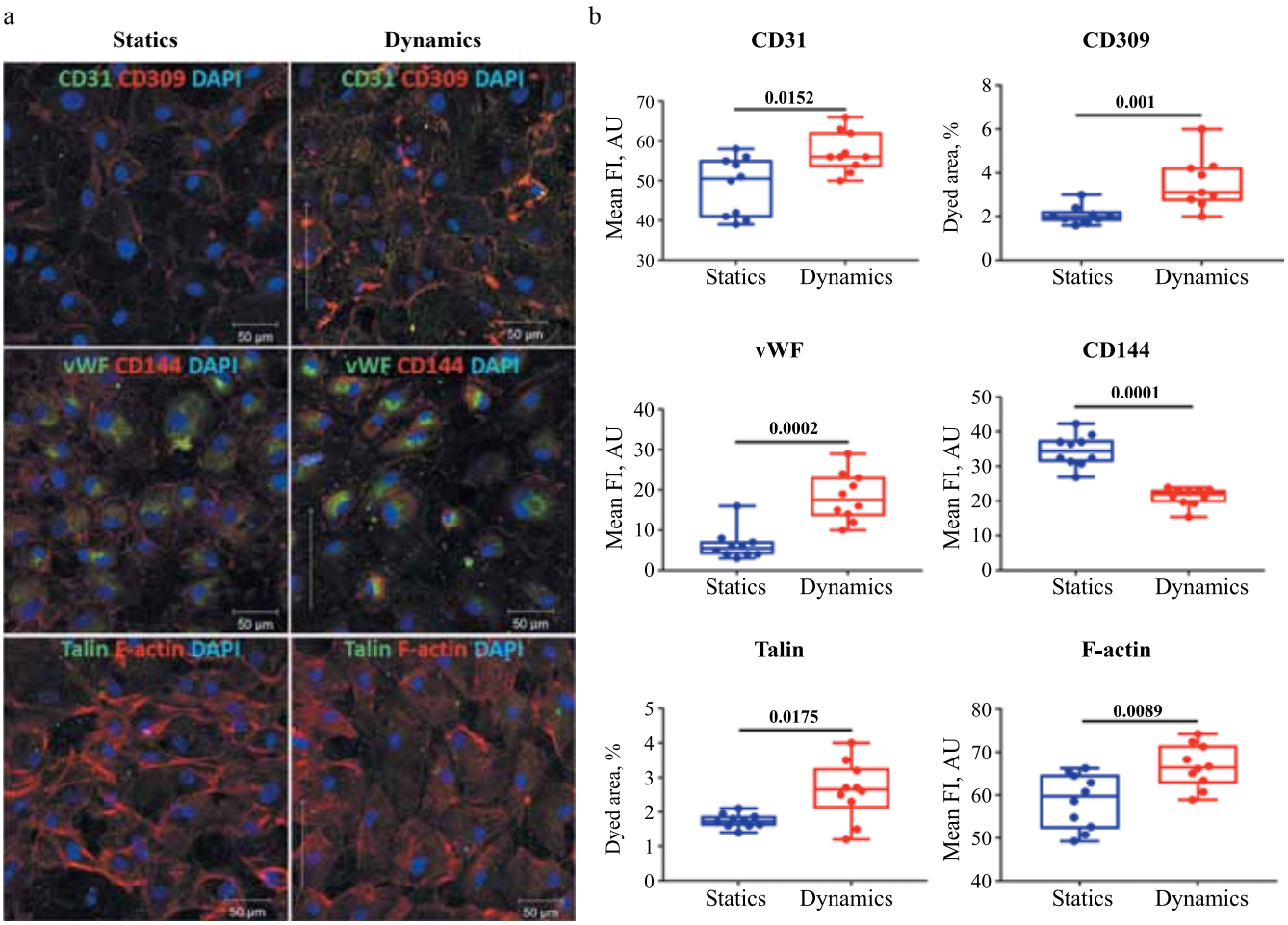

Fig. 4. Combined immunofluorescence staining of ECFCs cultured under static conditions ("Static") and under pulsatile flow conditions with 2.85 dyn/cm^2 shear stress ("Dynamic"): CD31/CD309/DAPI; CD144/vWF/DAPI; F-actin/Talin/DAPI. a – representative photographs, scanning confocal microscopy, 200× magnification; b – quantitative analysis. FI is fluorescence intensity

1) proteins responsible for blood vessel development (3 DEGs in pulsatile flow conditions (dynamic) versus 2 DEGs in static conditions);
2) proteins responsible for regulating endothelial integrity (9 DEGs in dynamic versus 6 DEGs in static conditions);
3) proteins stimulating angiogenesis (48 DEGs in dynamic versus 33 DEGs in static conditions);
4) proteins determining endothelial cell proliferation (17 DEGs in dynamic versus 12 DEGs in static conditions);
5) proteins promoting endothelial cell migration (21 DEGs in dynamic versus 15 DEGs in static conditions);
6) proteins responsible for inflammation regulation (20 DEGs in dynamic versus 16 DEGs in static conditions) (Fig. 5).

It was the manual annotation that allowed to establish the differences in genes differentially expressed in ECFCs in response to pulsatile flow in all the gene systems cited.

DISCUSSION

The native endothelial layer forms a continuous, selectively permeable, thrombosis-resistant barrier between the circulating blood and the vascular wall. It is the endothelium that is mostly subjected to shear stress exerted by blood flow. We have attempted to develop a

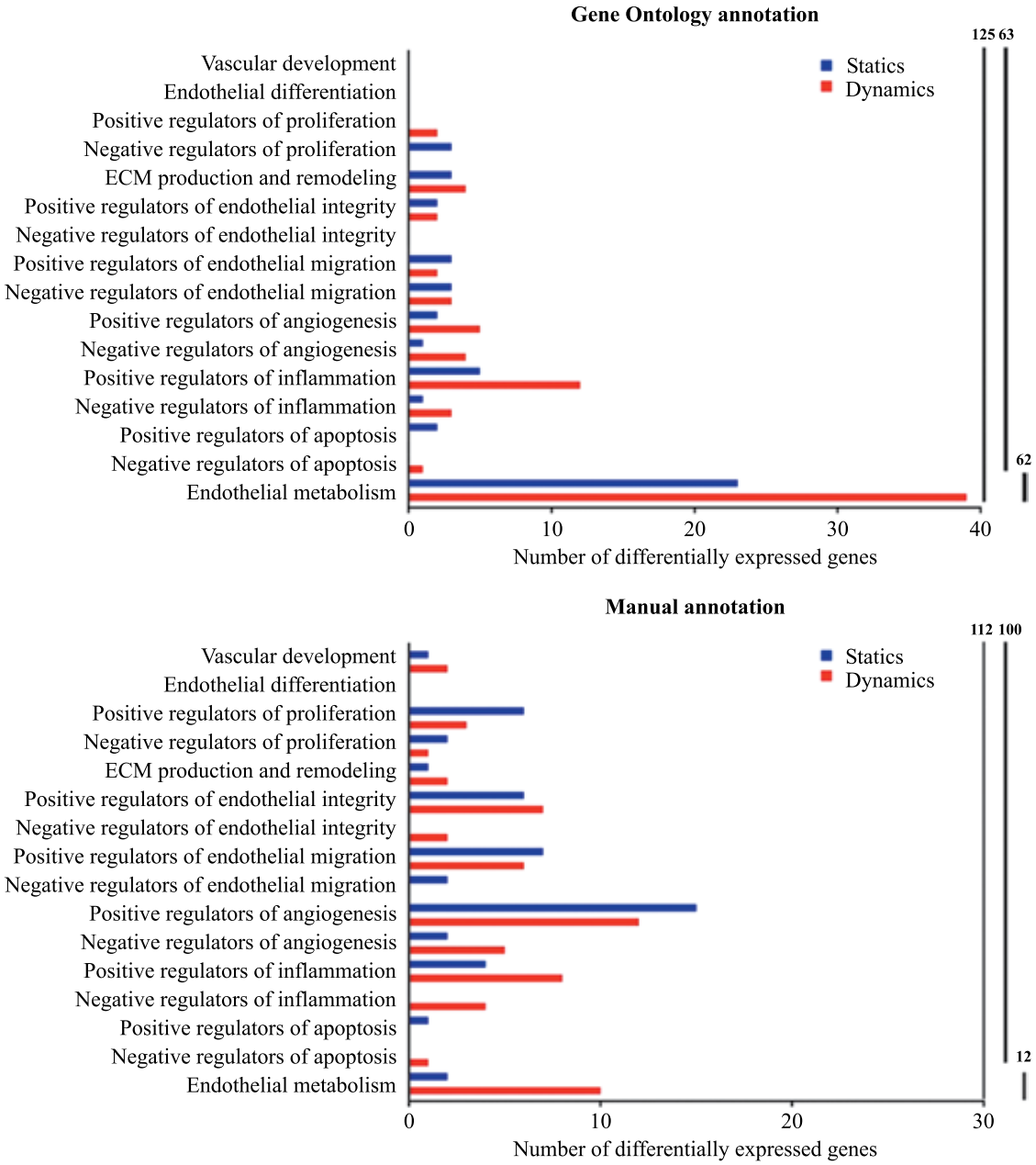

Fig. 5. Qualitative and quantitative comparison of the profile of DEGs in ECFC under static conditions and when exposed to pulsatile flow with 2.85 dyne/cm^2 shear stress using bioinformatic tool Gene Ontology and manual annotation. ECM – extracellular matrix

cell-based small-diameter vascular graft under pulsatile flow conditions. The main goal was to obtain a wash-resistant functional endothelial layer on the inner surface of the graft. The efficiency of the selected protocol was evaluated by the formation of endothelial monolayer, its integrity and viability, and the effect of shear stress on endothelial cell phenotype.

Under pulsatile flow, the cells were aligned relative to each other, formed a monolayer and had intercellular interactions. Some of these shear responses, such as cell alignment, were found to be mediated by PECAM-1, VE-cadherin and VEGFR2 receptors [19]. These receptors, which provide adhesion and form a monolayer, carry out mechanotransduction. Laminar shear stress promotes cell elongation and orientation along the vessel wall, activates mechanosensors and intracellular signaling pathways, and induces endothelial gene and protein expression [20]. Thus, shear stress plays a leading role in vascular homeostasis due to its atheroprotective, anticoagulant and anti-inflammatory functions [20–22].

Tondreau et al. obtained complete endothelialization of a decellularized tissue-engineered vascular scaffold produced by self-assembly of the extracellular matrix from fibroblasts. The endothelial monolayer was reconstituted using Human umbilical vein endothelial cell (HUVEC) culture and under flow conditions at a rate of 40 ml/min and a shear stress of 0.65 dyne/m^2 for 1 week [23].

In addition, a gradual increase in the applied fluid current from 5 dyne/cm^2 to 10 dyne/cm^2 was found to promote cell adhesion at the level of 99.31 ± 4.97% after 32 hours of culturing with a nearly complete HUVEC monolayer [24].

Yazdani et al. succeeded in obtaining effective endothelialization at high shear stress. Scaffolds obtained from decellularized porcine carotid arteries (4–5 mm) were populated with autologous endothelial cells and preconditioned for 9 days. Three regimens were tested in the study: low steady shear stress LSS (1.7 dyn/cm^2), high steady shear stress HSS (13.2 dyn/cm^2), and cyclic high shear stress CSS (13.2 dyn/cm^2, 60 bpm). Preconditioning of grafts under HSS and CSS conditions resulted in monolayer formation and stable adhesion as well as cell orientation in the flow direction. In the arteriovenous shunting model, preconditioned grafts under HSS and SSS conditions remained intact, cell morphology was preserved, there was a statistically significant decrease in attachment of blood elements, especially thrombocytes, compared to other conditions. Western blotting demonstrated increased expression of eNOS protein and prostaglandin-I synthase for cells conditioned with cyclic high shear stress compared to cells conditioned with only high shear stress [25].

In a 2016 study by the Melchiorri AJ team, polyglycolic acid (PGA) polymer scaffolds populated with endothelial progenitor cells (EPCs) were subjected to a low shear stress of 0.6 dyne/cm^2, similar to the venous system. The researchers found increased proliferation, infiltration, and differentiation of EPCs under dynamic loading. In dynamically cultured grafts, there was increased expression of functional endothelial markers – vWF and VEGF – in comparison to statically cultured EPCs by PCR results [26].

Under normal physiological conditions, endothelial integrity maintains the dynamic balance between mechanical shear stress and biological responses [27]. Shear stresses resulting from various flow patterns initiate various signaling events in the endothelium, including mechanosensitivity, intracellular stress transmission, conversion of mechanical force into biochemical signals, and feedback mechanisms [28–29]. Focal adhesion proteins are a dynamic mechanosensitive multiprotein complex that binds integrin receptors of the extracellular matrix to intracellular actin. This complex is represented by many different proteins (vinculin, zyxin, talin, paxillin), which perform scaffold, adaptor, and regulatory functions [30–31]. The presence of focal adhesion proteins reflects the cell's response to the external environment and allows adhesion of the cellular matrix to adapt to the

Table 2

Characterization of DNA libraries prepared from ECFC RNA cultured under static and pulsatile flow conditions and their sequencing results

Sample ID	DNA-library average, nucleotides	Coverage, million reads	Percent of reads mapped to hg38	Percent of reads mapped to exons
	ECFC *Statics*			
S1	356	15.7	98.2	84.9
S2	354	14.5	98.4	84.5
S3	389	42.7	98.2	82.2
S4	360	9.5	98.2	84.1
	ECFC *Dynamics*			
D1	346	15.1	98.2	88.8
D2	351	15.5	98.3	89.8
D3	353	15.3	98.3	90.4

composition and mechanical properties of the extracellular matrix [32–34].

Culturing ECFCs on the surface of PHBV/PCL/fibrin scaffolds under pulsatile flow conditions changed the orientation of cells in the flow direction, resulted in increased expression of specific endothelial markers CD31, CD309 and vWF, and stimulated the expression of structural protein F-actin and focal adhesion protein Talin. In addition, endothelial cells exhibited increased expression of genes encoding proteins that:

– ensure blood vessel development;
– are responsible for the regulation of endothelial integrity;
– stimulate angiogenesis;
– determine endothelial cell proliferation;
– promote endothelial cell migration;
– responsible for the regulation of inflammation.

CONCLUSION

Personalized biodegradable, cell-based, small-diameter vascular graft can be created when autologous fibrin is used as a feeder layer, and an autologous ECFC culture is used for cell colonization. Endothelial lining cells formed on the internal surface of the graft under shear stress conditions possess increased synthetic activity, adhesion ability, and are oriented along the pulsatile flow path. Shear stress also affects cell culture transcriptome, enhancing endothelial ability to migrate, proliferate, and maintain integrity.

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