INTRODUCTION

Autologous skeletal muscle stem cells (SMSC) are the source of cells very frequently studied in terms of their suitability for regenerating the infarcted myocardium (1). The primary aim of their application is the ability for the potential reduction of scar tissue in the infarcted heart by muscle stem cells (2), which are resistant to hypoxic conditions (3). Myoblasts were one of the first cells used in clinical trials because of the promising results of many preclinical attempts (4-5), the feasibility of their isolation (muscle oligobiopsy), expansion in in vitro culture and lack of in situ tumour formation risks due to their unipotential characteristics and structural similarity to cardiomyocytes. However, evidence suggests that myoblasts cause arrhythmias after transplantation, which may be caused by a lack of electromechanical coupling of myoblasts and cardiomyocytes or an inflammatory reaction caused by this procedure. This issue may be eliminated by modifying the cells with connexin 43. However, arrhythmias are not dangerous for the patient and do not affect the quality of the graft (3).

A variety of successful attempts with combined cell and gene therapy in animal models in relation to further improvements in myoblast delivery to the infarcted heart provides the basis of consistency for this type of approach. One of the primary goals of this type of cell engineering involves increased blood perfusion within the injured ischaemic area, termed therapeutic angiogenesis (6), which is applied to compensate for coronary artery occlusion and to provide better oxygen supply and nutrients, preventing further cardiomyocyte loss. Thus far in rodent heart infarction models, commonly tested factors such as VEGF (7-9), FGF-2 (10) and hepatocyte growth factor (HGF) (11) have been successfully used to improve myocardial vasculature and function. The application of angiogenic synergism of several factors was shown in different animal studies on hindlimb ischaemia (12, 13) when the sequential delivery of pro-angiogenic growth
factors has been used. Therefore, angiogenesis can be perceived as a complex phenomenon, and best possible outcomes are obtained when pro-angiogenic factors work cooperatively to promote the stability of newly formed blood vessels. Lately, the study by Yang et al. (14) showed that the administration of both VEGF and basic bFGF in alginate beads to the injured pig myocardium resulted in the facilitation of vascular growth.

Vascular endothelial growth factor is a potent pro-angiogenic factor that exerts its action through a reduction in apoptosis and the stimulation of proliferation and migration of endothelial cells (15). Fibroblast growth factors such as FGF-4 are also involved in the first stages of angiogenesis but may also stimulate arteriogenesis, thus playing a role in more mature blood vessel formation. Furthermore, FGF-4 has been shown to have a helpful role in the induction of VEGF synthesis (16).

In the present study, we examined the influence of the simultaneous overexpression of two potent pro-angiogenic factors, FGF-4 and VEGF, on human primary myoblast proliferation, cell cycle, resistance to hypoxic stress conditions and myogenic gene expression. We also evaluated the efficiency of the expression of both proteins provided by a bicistronic non-viral vector as well as their function in a in vitro capillary formation test.

MATERIALS AND METHODS

Cell preparation

Human primary myoblasts were isolated from a ~1 cm³ fragment of skeletal muscle tissue harvested from patients under anaesthesia during anterior cruciate ligament resection (ACL). For this purpose, approval from the Local Bioethical Committee (Medical University of Poznan) as well as written consent from patients were received. Briefly, the biological sample was stripped of fat, and after 3 digestions with 0.2% collagenase 1 solution (Sigma-Aldrich Corp. St. Louis, MO USA) in a water bath (37°C), the cell suspension was filtered through 70-µm mesh. Finally, the cells were centrifuged and seeded onto 25 cm² culture flasks (Beckton Dickinson, NJ, USA) coated with 0.1% gelatin (Sigma-Aldrich Corp. St. Louis, MO USA), and the modified "preplate" method was used to obtain the human primary myoblast cultures (18). To verify the purity of the isolated cells, cytometric analysis was performed. The cells were stained using an antibody against the CD56 marker because the presence of CD56 on the cell surface is typical for myoblasts. Further verification was also performed by staining desmin, a muscle cell marker. After the confirmation of myogenic properties, the cells were maintained in the standard Dulbecco’s modified Eagle’s medium with 4.5 g/l of glucose supplemented with 20% foetal bovine serum (Lonza Group Ltd, Basel, Switzerland), antibiotics (Lonza Group Ltd, Basel, Switzerland), 1% ultraglutamine (Lonza Group Ltd, Basel, Switzerland), and appropriate growth factors including bFGF (Sigma-Aldrich Corp. St. Louis, MO USA).

To investigate the pro-angiogenic properties of the transfected cells, we used human umbilical vein endothelial cells (HUVECs). For pro-angiogenic properties experiment HUVECs were purchased from Lonza Group Ltd, Basel, Switzerland and cultured in endothelial basal medium (EBM) supplemented with 2% fetal bovine serum 1 µg/ml hydrocortisone, 3 µg/ml bovine brain extract, 10 µg/ml epidermal growth factor and antibiotics. The cells were maintained at the standard in vitro culture conditions.

Table 1. Primer sequences used in real-time qPCR reactions.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF-4</td>
<td>Fgf_f</td>
<td>5'-TATGGCTCGCCCCTTCTTC-3'</td>
<td>142 bp</td>
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<tr>
<td></td>
<td>Fgf_r</td>
<td>5'-CTCCTCTCTTTGTC-3'</td>
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<tr>
<td>VEGF-A</td>
<td>Vegf-a_f</td>
<td>5'-AAGGAAGGGAGCAATCAT-3'</td>
<td>143 bp</td>
</tr>
<tr>
<td></td>
<td>Vegf-a_r</td>
<td>5'-CACACAGGATGCTTAAGA-3'</td>
<td></td>
</tr>
<tr>
<td>VEGF-B</td>
<td>Vegf-b_f</td>
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<td>123 bp</td>
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<td></td>
<td>Vegf-b_r</td>
<td>5'-TCAGGGAGAAGGGATGG-3'</td>
<td></td>
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<tr>
<td>VEGF-C</td>
<td>Vegf-c_f</td>
<td>5'-GGTTTGGAGAGGGCCTAGA-3'</td>
<td>114 bp</td>
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<td></td>
<td>Vegf-c_r</td>
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<td>VEGF-D</td>
<td>Vegf-d_f</td>
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<td>Act-β</td>
<td>ActB_f</td>
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<td></td>
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<td>Myf5_r</td>
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</tr>
<tr>
<td></td>
<td>MyoD_r</td>
<td>5'-CGACTCAGAAGGCACGT-3'</td>
<td></td>
</tr>
<tr>
<td>Myogenin</td>
<td>Myogenin_f</td>
<td>5'-GCTGATGACACTCCCCCTA-3'</td>
<td>226 bp</td>
</tr>
<tr>
<td></td>
<td>Myogenin_r</td>
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</tr>
<tr>
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<tr>
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<td>Mef2_f</td>
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<td>193 bp</td>
</tr>
<tr>
<td></td>
<td>Mef2_r</td>
<td>5'-ATCCTTCGAAAGTGCATG-3'</td>
<td></td>
</tr>
</tbody>
</table>

Act - β actin; Myf5 - myogenic factor 5; MyoD - myogenic differentiation factor 1; Myog - myogenin; Myf6 - myogenic factor 6; Mef2 - myocyte enhancer factor 2.
conditions (5% CO₂, 37°C, 95% humidity) and subcultured with 0.25% trypsin/EDTA solution (Sigma-Aldrich Corp. St. Louis, MO USA).

In addition, to assess the ability of cells to differentiate, we maintained the myoblasts in special differentiation medium containing 2% horse serum. After almost 100% confluency of cells, the standard medium was changed into medium with horse serum, which allowed myotubes to form from myoblast cells.

Plasmids

To investigate the effect of the transfection process on the transfected cells, a control plasmid, pTRUF-22, without sequences coding for pro-angiogenic genes was used to electroporate human control primary myoblast suspensions. To visualise and optimise the transfection efficiency, the pEGFP-C1 commercial plasmid was used (Clontech Laboratories Inc., USA) that contains the enhanced green fluorescence protein (eGFP).

The pTRUF-22-FGF4-ires-VEGF (called FGF-4/VEGF) bicistronic vector was used to overexpress human FGF-4 and human VEGF-A simultaneously as described in detail in our previous report (18).

Additionally, to evaluate the change in expression of the remaining VEGF isoforms, constructs carrying VEGF-B, VEGF-C and VEGF-D inserts were prepared. To obtain the sequences of the examined isoforms, polymerase chain reaction (PCR) with primers for particular genes was conducted using cDNA obtained from HUVECs. Next, the reaction products were purified by gel electrophoresis and appropriate columns (GeneJET Extraction Kit, Thermo Scientifics, Lafayette, USA). Subsequently, the obtained insert was cloned into a p-SC-A vector using the Strata Clone Kit (Agilent Technologies, Japan) and isolated from bacterial culture. The construct was designed to prepare the standard curve for particular genes to verify the expression profile by real-time qPCR obtained in test samples.

Transfection of human myoblasts

Prior to gene transfer, the cells were grown to approximately 70% confluency. The transfection of primary myoblasts was performed using electroporation in 0.2-cm gap electroporation chambers with the use of the Gene Pulser X-Cell Electroporation System (BioRad, USA) and optimised using different concentrations of the pEGFP-C1 plasmid, electroporation buffer (F-10 Ham medium, Sigma-Aldrich Corp. St. Louis, MO USA) and cell quantity. The optimal conditions were found to be the following: 5.2 pmol of plasmid, 200 µl of buffer, and 2–4×10⁶ cells electroporated with a single pulse of 150–175 V. After the pulse, the cells were incubated for 10 minutes at room temperature and resuspended in 1 ml of culture medium. The cells were then plated on 35 mm culture dishes with the media changed daily after the transfection. The green-fluorescent cells and dead cells (identified by using propidium iodide staining) were assessed at 48 hours after electroporation using the Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Fullerton, USA), and documentation was made under the inverted fluorescent microscope (Zeiss Axiovert 200, Germany). The transfection was performed for each planned experiment separately (its efficacy depended on the number of cells subjected to

<table>
<thead>
<tr>
<th>Experimental groups: WT (Mb) - wild type myoblasts, WT (Mc) - wild type myocytes, pTRUF-22 (Mb) - myoblasts transfected with empty control plasmid, pTRUF-22 (Mc) - myocytes containing control plasmid, FGF/VEGF-A (Mb) - myoblasts transfected with FGF/VEGF-A gene, FGF/VEGF-A (Mc) - myocytes with FGF/VEGF overexpression.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 2. Statistical differences between pro-angiogenic isoforms presented in Fig. 3.</strong></td>
</tr>
<tr>
<td>Bonferroni’s Multiple Comparison Test</td>
</tr>
<tr>
<td>WT (Mb) vs. pTRUF-22 (Mb)</td>
</tr>
<tr>
<td>WT (Mb) vs. FGF/VEGF-A (Mb)</td>
</tr>
<tr>
<td>WT (Mb) vs. HUVEC</td>
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<tr>
<td>WT (Mb) vs. WT (Mc)</td>
</tr>
<tr>
<td>WT (Mb) vs. pTRUF-22 (Mc)</td>
</tr>
<tr>
<td>WT (Mb) vs. FGF/VEGF-A (Mc)</td>
</tr>
<tr>
<td>pTRUF-22 (Mb) vs. FGF/VEGF-A (Mb)</td>
</tr>
<tr>
<td>pTRUF-22 (Mb) vs. HUVEC</td>
</tr>
<tr>
<td>pTRUF-22 (Mb) vs. WT (Mc)</td>
</tr>
<tr>
<td>pTRUF-22 (Mb) vs. pTRUF-22 (Mc)</td>
</tr>
<tr>
<td>pTRUF-22 (Mb) vs. FGF/VEGF-A (Mc)</td>
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<tr>
<td>FGF/VEGF-A (Mb) vs. HUVEC</td>
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<td>FGF/VEGF-A (Mb) vs. WT (Mc)</td>
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<td>FGF/VEGF-A (Mb) vs. pTRUF-22 (Mc)</td>
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<tr>
<td>FGF/VEGF-A (Mb) vs. FGF/VEGF-A (Mc)</td>
</tr>
<tr>
<td>HUVEC vs. WT (Mc)</td>
</tr>
<tr>
<td>HUVEC vs. pTRUF-22 (Mc)</td>
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<tr>
<td>HUVEC vs. FGF/VEGF-A (Mc)</td>
</tr>
<tr>
<td>WT (Mc) vs. pTRUF-22 (Mc)</td>
</tr>
<tr>
<td>WT (Mc) vs. FGF/VEGF-A (Mc)</td>
</tr>
<tr>
<td>pTRUF-22 (Mc) vs. FGF/VEGF-A (Mc)</td>
</tr>
<tr>
<td>pTRUF-22 (Mc) vs. HUVEC</td>
</tr>
</tbody>
</table>

Bonferroni’s Multiple Comparison Test VEGF A VEGF B VEGF C VEGF D
WT (Mb) vs. pTRUF-22 (Mb) ns ns ns ns
WT (Mb) vs. FGF/VEGF-A (Mb) *** ns ns ns
WT (Mb) vs. HUVEC ns ns ns ns
WT (Mb) vs. WT (Mc) ns *** ns ***
WT (Mb) vs. pTRUF-22 (Mc) ns ns ns ***
WT (Mb) vs. FGF/VEGF-A (Mc) *** *** *** ***
pTRUF-22 (Mb) vs. FGF/VEGF-A (Mb) *** ns ns ns
pTRUF-22 (Mb) vs. HUVEC ns ** ns ns
pTRUF-22 (Mb) vs. WT (Mc) ns *** ns ***
pTRUF-22 (Mb) vs. pTRUF-22 (Mc) ns ns ns ***
pTRUF-22 (Mb) vs. FGF/VEGF-A (Mc) *** *** *** ***
FGF/VEGF-A (Mb) vs. HUVEC *** ns * ns
FGF/VEGF-A (Mb) vs. WT (Mc) *** *** ns ***
FGF/VEGF-A (Mb) vs. pTRUF-22 (Mc) *** ns ns ns
FGF/VEGF-A (Mb) vs. FGF/VEGF-A (Mc) ns *** *** ***
HUVEC vs. WT (Mc) ns *** ns ***
HUVEC vs. pTRUF-22 (Mc) ns ns ns ***
HUVEC vs. FGF/VEGF-A (Mc) *** *** *** ***
WT (Mc) vs. pTRUF-22 (Mc) ns *** ns ns
WT (Mc) vs. FGF/VEGF-A (Mc) *** * *** ns
pTRUF-22 (Mc) vs. FGF/VEGF-A (Mc) *** *** *** ***
HUC vs. WT (Mc) ns *** ns ***
HUC vs. pTRUF-22 (Mc) ns ns ns ***
HUC vs. FGF/VEGF-A (Mc) *** *** *** ***
pTRUF-22 (Mc) vs. WT (Mc) ns *** ns ns
pTRUF-22 (Mc) vs. pTRUF-22 (Mc) ns ns ns ***
pTRUF-22 (Mc) vs. FGF/VEGF-A (Mc) *** *** *** ***
pTRUF-22 (Mc) vs. HUVEC ns ns ns ***
electroporation), and the transfection of the GFP plasmid was conducted in parallel with FGF-4/VEGF gene electroporation to reciprocally validate the efficacy of each procedure.

Fibroblast growth factor-4 and vascular endothelial growth factor gene expression

The bicistronic plasmid enabled obtaining the expression of two genes simultaneously. In our experiment, we wished to receive the overexpression of two genes FGF-4 and VEGF, and the cells were transfected using electroporation. Both genes were introduced into the bicistronic plasmid. Subsequently, the total RNA was isolated, and to obtain the mRNA fraction, Oligo(dT)25-Dynabeads (Invitrogen, USA) were applied. Next, using reverse transcription, the cDNA was prepared. To evaluate the expression of the introduced genes, real-time qPCR was performed. The primers were complementary to the FGF-4 and VEGF sequences located in the pTRUF-22 vector. The entire procedure is described below in the real-time qPCR section.

Fibroblast growth factor-4 and vascular endothelial growth factor protein levels

Human FGF-4 and human VEGF-A concentrations were measured using the ELISA quantitative assay (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions.

Cell proliferation

The intensity of cell proliferation was determined by the measurement of formazan salt formation in the MTT assay. The experiments were performed according to the manufacturer’s instructions (Roche, Germany).

Cell cycle stage determination by fluorescence activated cell sorting (FACS)

Cell cycle analysis was evaluated using nuclear isolation and staining solution, NIM-DAPI (Beckman Coulter, Fullerton, USA), according to the manufacturer’s protocol. For each experimental setting, at least 10^6 of 80% confluent cells were examined. The FACS experiments were performed using the Cell Lab Quanta™ SC MPL flow cytometer (Beckman Coulter, Fullerton, USA).

Angiogenic ‘sprouting’ test

The sprouting test was performed to measure the total length of the capillaries produced by the HUVEC aggregates. The experiments were performed according to the procedure previously described (18). Briefly, HUVEC aggregates (approx. 750 cells) were incubated for 24 hours in methylcellulose working solution (0.3% in HUVEC standard medium) in U-shaped plates (Greiner, Germany). For each spheroid culture, 48 aggregates were harvested and centrifuged (3 minutes, 500 rpm). The supernatant was discarded, and the HUVEC spheres were gently mixed with solution containing 30% FBS and 1.2% stock solution of methylcellulose. Afterwards, a culture medium was mixed with acidic collagen extract from rat tails, placed on ice, neutralised with 0.2 M NaOH, mixed with HUVEC aggregates and seeded onto preheated 24-well plates. After 30 minutes of incubation at 37°C, conditioned media from the transfected myoblasts were added to each spheroid culture. After 48 hours, the total length of the capillaries, which were produced by HUVEC aggregates, was assessed, and every spheroid culture was photographed. The capillaries sprouting from one HUVEC aggregate were measured and summed. This procedure was repeated for each spheroid in the examined variants. Next, the mean value for every particular variant under study was assessed. This value was counted for every group of investigated supernatants: standard medium-DMEM, FGF-4/VEGF, and pTRUF-22 conditioned media, and control conditioned medium- wild type were compared.

Real-time qPCR

Real-time qPCR experiments evaluated the expression of myogenic (MyoD, Myogenin, Myf 6, Myf 2, Myf 5), pro-angiogenic genes and other isoforms of VEGF genes (VEGF-B, VEGF-C, VEGF-D) and connexin 43. In the case of VEGF gene isoforms and Cx43, we performed the expression analysis in undifferentiated myoblasts after transfection as well as in genetically modified myocytes. Briefly, RNA was isolated from 80% confluent cells at 48 hours post transfection using TRIzol Reagent (Invitrogen, USA). Oligo(dT)25-Dynabeads (Invitrogen, USA) were used to obtain a pure mRNA fraction from 10 µg of total RNA, which was then reversely transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen, USA). The standard curve method for the relative quantification of real-time qPCR was performed using the iCycler detecting system (BioRad, USA) and iQ SYBR Green Supermix Reagent (BioRad, USA). Each 25 µl test sample contained 2 µl of cDNA from the reverse transcription reaction mixture diluted to 4x, 400 nmol of each primer, and 50% (vol:vol) of iQ SYBR Green. Evaluation using SYBR Green may be less specific; however, in our studies, we assessed melting curves to confirm the accuracy of our primers, products, and the standard curve made by series dilution of the plasmid. The following conditions were used: 95°C for 1 minute, 56 cycles at 95°C for 20 second, 60°C for 20 second and 72°C for 20 second. The primer sequences are listed in Table 1. The relative gene expression levels were normalised using the β-actin housekeeping gene as a reference gene and were calculated according to the following pattern:

\[ \Delta \Delta C_{T} = C_{T_{n}} - C_{T_{b}} - (C_{T_{0a}} - C_{T_{0b}}) \]

Where:

- \( \Delta \Delta C_{T_{n}} \) – normalised, relative gene expression of sample n;
- \( C_{T_{n}} \) and \( C_{T_{b}} \) – value of threshold cycle for the examined and reference sample;
- \( C_{T_{0a}} \) and \( C_{T_{0b}} \) – value of threshold cycle for the examined and control sample.

Following this method, we were able to quantify the increase or decrease in the amount of obtained templates. In this case, we measured the fold change of the target gene expression level. For each gene, a standard curve was made to obtain the efficacy indispensable to calculate changes in particular gene expression.

Apoptosis/necrosis cells detection

The apoptosis of primary myoblasts was evaluated using the annexin V kit assay following the manufacturer’s instructions (Beckman Coulter, Fullerton, USA). Oxidative stress conditions were induced by the 24 hour incubation of cells with 500 µM H2O2, after which the cells were analysed by flow cytometer Cell Lab Quanta™ SC MPL (Beckman Coulter, Fullerton, USA). The percentages of necrotic and viable cells were determined using the propidium iodide assay.

Statistical analysis

Each experiment was performed at least in triplicate. For real-time qPCR analysis, the experimental samples were run at
least in duplicate. The data obtained were expressed as the means ± standard deviation. Statistical analysis was performed using variance assessment by single classification and then multiple group comparisons by the ANOVA test or χ² test.

RESULTS

Myoblast transfection efficiency and the overexpression of fibroblast growthfactor-4 and vascular endothelial growth factor-A genes

The purity of the primary cell suspension used for transfection purposes was verified based on myoblast markers (CD 56 and desmin) and was in the range of 90%. We optimised the myoblast electroporation efficiency using various concentrations of cells and plasmid DNA and by applying different electrical parameters. To optimise the transfection efficiency, a pEGFP-C1 plasmid was used. The mean percentage of green fluorescent cells observed at 48 hours after the transfection process was approximately 80%. In addition, variability was noticed between samples from different patients but oscillated at approximately 80%. Fig. 1 presents the representative cytometric profile of the GFP transfection.

The high efficacy of transfection resulted in the satisfactory overexpression of introduced genes. The bicistronic plasmid caused the high overexpression of FGF-4 and VEGF simultaneously (Fig. 2A and 2B). The levels of VEGF and FGF-4 transcripts markedly increased. The amount of VEGF transcript was almost 90-fold higher than in the control. We also detected greater expression of the FGF-4 gene in the transfected myoblasts than in the ‘native’ population; therefore, both outcomes were statistically significant (p<0.01 for VEGF expression and p<0.001 for FGF gene expression). We detected small amounts of VEGF and FGF-4 transcripts in cells transfected with control plasmid, which were also statistically significant compared to the wild type cell population. The gene overexpression resulted in the increased production and secretion of both proteins. The cells transiently transfected with the pTRUF-22-FGF4-ires-VEGF produced both human proteins: FGF-4 and VEGF, and the concentrations of these proteins have been shown in Fig. 2C. We did not detect FGF-4 production in the media collected from the non-transfected cells or the cells transfected with control plasmid (without pro-angiogenic genes coding sequences). The small amount of transcripts was revealed in both control cell populations; therefore, we can assume that not every FGF-4 transcript was translated into a stable and mature protein that is secreted from the cell. In contrast, VEGF protein was present in the media from both control cell populations (mock transfected and non-transfected), although at levels markedly lower than in the supernatants from FGF-4/VEGF-transfected cells (~1.4–1.8 ng/ml versus ~6 ng/ml, respectively).

Expression of vascular endothelial growth factor isoforms

We used HUVECs as a control population to evaluate VEGF isoform expression. In our study, we examined the expression of VEGF-A, B, C and D isoforms in transfected cells and both controls (mock-transfected and ‘native’ myoblasts) as illustrated in Fig. 3. Panel A illustrates the significant overexpression of VEGF-A in transfected cells compared to the control cell population. In panel B, a trend to increase the VEGF-B expression level in the examined cells is shown; however, the tendency is not statistically significant. The VEGF-C expression shown in panel C is elevated in comparison to the expression in HUVECs in every myoblast cell population under study. In addition, the expression of VEGF-C in FGF-4/VEGF-transfected myoblasts was statistically higher than in ‘native’ and pTRUF-22-transfected cells; however, in cells transfected with the control plasmid, the expression was the lowest. Comparing the FGF-4/VEGF-transfected myoblast population to the ‘native’ myoblasts, a
Fig. 2. Expression of examined genes evaluated by real-time qPCR. The relative expression of both genes was normalised according to the expression of a housekeeping gene. The data are presented as a relative mRNA fold change in comparison to wild type cells. Experimental groups: control – non-transfected cells, wild type; pTRUF-22 – transfected control with a coding sequence lacking plasmid; FGF-4/VEGF complete bicistronic plasmid. (A), expression of VEGF gene; the VEGF expression was found to be significantly higher between following cell populations: VEGF vs. control, and pTRUF-22 vs. control at p-value level p<0.01 (**). (B), expression of FGF-4 gene was found to be significantly higher in the following cell populations: FGF-4 vs. control and p-TRUF-22 vs. control at p value level, p<0.001 (**). These experiments were performed twice in triplicate for each gene, (C) the concentrations of human FGF-4 and VEGF proteins in media from human primary myoblast cultures measured at 48 hours after transfection in supernatants from in vitro cultures of ~1.5 ml cells. Data are presented as the mean ±S.D.; n=3 experiments. Experimental groups: control – non-transfected cells, wild type; pTRUF-22 – transfected control with a coding sequence lacking plasmid; FGF-4/VEGF complete bicistronic plasmid. The human myoblast spontaneous secretion levels for VEGF protein have been detected in the range of 1813 pg/ml. ND – non detectable.
visible, clear and significant induction of VEGF-D isoform expression level was observed in the transfected myoblasts. However, no change in VEGF-D expression was noted between the transfected myoblasts and HUVECs. Additionally, we compared the expression level of each isoform in myoblasts versus differentiated myocytes. Interestingly, the VEGF-A isoform was maintained at the same level in the FGF-4/VEGF-transfected population, even after differentiation procedure, which lasted approximately 7 days. Generally, we observed the upregulation of VEGF isoform expression in myocytes.

Expression of connexin 43 in human myoblast cells

We also evaluated the expression of Cnx43 in mature myoblast cells populations. Real-time qPCR analysis revealed the absence of connexin expression in the studied myoblast undifferentiated populations. However, the expression of connexin was elevated after differentiation, which was confirmed by the statistically significant increase in Cnx43 expression in myocytes compared to myoblasts (Fig. 4). Additionally, the genetic modification with the FGF/VEGF-A plasmid caused an increase in connexin expression in transfected differentiated myocytes in comparison to wild type myocytes.

Proliferation of transfected cells

We observed no statistically significant differences in the proliferation of the transfected primary myoblast cell populations measured by the MTT test (Fig. 5).

Cell cycle analysis

FACS analysis did not reveal any statistically significant changes in the proliferation rate (S+G2):(S+G0/G1+G2) of

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Fig. 3. The evaluation of the VEGF isoform expression levels in real-time qPCR analysis compared to the VEGF expression levels in HUVECs. Data are presented as the fold change in the relative mRNA level. Expression was normalised to the β-actin housekeeping gene. Experimental groups: WT (Mb) - wild type myoblasts, WT (Mc) - wild type myocytes, pTRUF-22 (Mb) - myoblasts transfected with control plasmid, pTRUF-22 (Mc) - myocytes containing control plasmid, FGF-4/VEGF (Mb) - myoblasts transfected with FGF-4/VEGF construct. FGF-4/VEGF (Mc) - myocytes with FGF-4/VEGF construct overexpression. (A) The expression of the VEGF-A gene in cell populations of myoblasts (Mb) and myocytes (Mc) (B) VEGF-B expression in myoblasts and myotube populations under study (C) the expression of the VEGF-C gene in cell populations of myoblast (Mb) and myocytes (Mc) (D) VEGF-D expression level in the examined cell populations of myoblasts (Mb) and myocytes (Mc). Statistical data are presented selectively in the figure. The complete statistical data are presented in Table 2. Data presented in the figure is in log scale.
myoblasts transiently transfected with the pTRUF-22-FGF4-ires-VEGF construct when compared to the control cell populations (Fig. 6). Most of the myoblast cells under study were in the G0/G1 phase, which is characteristic of cells after division. A very small percentage of the human myoblasts was in S or M phases, which is typical of cells before and during cell divisions. Comparing between transfected and unmodified cells, a decrease in the percentage of ‘native’ cells at G0/G1 phase was observed, which is reflected in the increased proliferation rate of wild type myoblasts.

Fig. 4. Connexin 43 expression evaluated by real-time qPCR analysis in myoblast cell populations and differentiated myocytes. The data are presented as the fold change in the relative mRNA level. The expression was normalised to the β-actin housekeeping gene. Experimental groups: WT (Mb)- wild type myoblasts, WT (Mc)- wild type myocytes, pTRUF-22 (Mb)- myoblasts transfected with control plasmid, pTRUF-22 (Mc)- myocytes containing control plasmid, FGF-4/VEGF (Mb)- myoblasts transfected with FGF-4/VEGF construct. FGF-4/VEGF (Mc)- myocytes with FGF-4/VEGF construct overexpression. Statistical significance was revealed between the following groups: WT (Mb) vs. FGF-4/VEGF (Mc) p<0.05 (*), and pTRUF-22 (Mb) vs. FGF-4/VEGF (Mc) p<0.01 (**) - these data are not presented in figure. Additionally, statistically significant differences are presented in FGF-4/VEGF (Mb) vs. FGF-4/VEGF (Mc) p<0.05 (*), and WT (Mc) vs. FGF-4/VEGF (Mc) p<0.05 (*).

Fig. 5. Cell proliferation evaluated using the formazan salt formation test (MTT). Data were presented as percentages normalised to non-transfected control cells. Experimental groups: control - non transfected cells, wild type; pTRUF-22- mock transfected cells; FGF-4/VEGF- cells transfected with complete bicistronic plasmid FGF-4/VEGF: 102±2; pTRUF-22: 102±2; control cells: 100±0; n=3 experiments. The differences between the cell populations under study were not statistically significant.

Fig. 6. Cell cycle analysis by the flow cytometry of human primary myoblast suspensions (n=3 experiments). Experimental groups: control- non transfected cells, wild type; pTRUF-22- mock transfected cells; FGF-4/VEGF- cells transfected with complete bicistronic plasmid. No statistically significant differences were observed between the cell populations under study.
Angiogenic ‘sprouting’ assay

The conditioned media with FGF-4 and VEGF transient overproduction increased the capillary formation of the HUVEC aggregate compared to the control media. The representative images of sprouts are shown in Fig. 7A-7D. Although some capillary formation was observed in all experimental settings (supernatants) due to both the foetal bovine serum content in the media used and spontaneous basic VEGF production by myoblasts, we observed a significantly higher rate of ‘sprouting’ when the HUVEC aggregates were incubated with media containing both pro-angiogenic proteins from FGF-4/VEGF-transfected stem cells of myogenic origin (Fig. 7E).

Myogenic gene expression

The introduction of the bicistronic vector delivering the pro-angiogenic gene constructs FGF-4 and VEGF influenced the expression of Mef 2 and MyoD genes in human myoblast populations at the mRNA level as shown in Fig. 8. Both early myogenic genes showed an increase in their expression levels, which was statistically significant. The expression levels of the remaining genes were similar in all evaluated human myoblast populations. Moreover, we did not observe any changes between the studied cell populations in in vitro cultures, and the transfected cells further maintained the ability to differentiate into myotubes when transferred into differentiation media. To assess the ability of myotube formation, an in vitro differentiation test was performed each time; therefore, we confirmed that the myoblasts used in this study were able to form myotubes (Fig. 9).

Apoptotic/necrotic cell detection under oxidative stress conditions

The percentages of necrotic, apoptotic and viable cells in all myoblast populations under study without and after addition of 500 µM H₂O₂ are depicted in Fig. 10A. The statistics are
presented in Table 3. The oxidative stress conditions used in the study slightly influenced the apoptotic level in the FGF-4/VEGF-transfected human myoblast cells. The decrease in the viability was primarily due to developed necrosis; however, in all cell populations treated with H\textsubscript{2}O\textsubscript{2}, this phenomenon remained at a similar level. Overall, the quantity of viable cells appeared to decrease after oxidative stress in control and mock-transfected cells, but the percentage of viable cells remained primarily equal among cell populations. Nevertheless, we conclude that the viability of cells subjected to hydrogen peroxide remained satisfactory. In addition, in cell populations including viable cells, a statistically significant decrease in the amount of cells between FGF-4/VEGF-transfected cells and wild type cells not treated with hydrogen peroxide was revealed, and the same cell populations subjected to oxidative stress conditions did not reveal any statistically important changes.

Fig. 8. Myogenic gene expression in myoblast cell populations under study; n=3 experiments. Experimental groups: control- non transfected cells, wild type; pTRUF-22 - mock transfected cells; FGF-4/VEGF - cells transfected with complete bicistronic plasmid. Data are presented as fold change in relative mRNA level. The observed differences between the cell populations were statistically significant for MyoD and Mef2 gene expression. MyoD gene: pTRUF-22-transfected cells vs. control, \(p<0.05\) (*), Mef2 gene: FGF-4/VEGF-transfected cells vs. control \(p<0.001\) (***), and FGF-4/VEGF-transfected cells vs. pTRUF-22-transfected cells \(p<0.05\) (*). Experiments were performed twice in triplicate for each gene.

**DISCUSSION**

We optimised myoblast cell transfections using the electroporation method with various concentrations of DNA, buffer and cells. Using the optimised conditions, we obtained approximately 80% transfection efficiency at 48 hours after transfection (Fig. 1). Manipulations of growth factor expression is extensively studied area in case of myoblasts. So far genetic manipulation of myoblast cells was performed to obtain: a) appropriate gap junction formation (to prevent cardiac arrhythmias), b) reduction of cell death and increase of cell proliferation (by interference in myogenic gene expression) and c) modification of myoblast (mostly with VEGF-A and FGF-4) to prevent cell apoptosis and to induce the angiogenesis (19). FGF-4 has pleiotropic roles in various cell types and may act as a mitogenic, angiogenic and survival factor that is involved in conditions or after the addition of H\textsubscript{2}O\textsubscript{2} revealed only a slight decrease in the percentage of viable cells in FGF-4/VEGF-transfected cells and 'native' cells in normoxia. Fig. 10B presenting the FACS profile for populations under study.

**Fig. 9.** Cells maintained in differentiating medium containing horse serum and stained with Giemsa reagent. The observation was made using a light microscope with 100-fold magnification.
cell proliferation and differentiation (20). In contrast, the VEGF gene is primarily expressed by vascular endothelial stem cells and has a crucial role in capillary formation (15). Bicistronic plasmids enable the overexpression of both genes simultaneously in myoblast stem cells; however, this type of plasmid does not guarantee the equal expression of both factors.

Fig. 10. The percentages of necrotic, apoptotic and viable cells in all cell populations under study in standard medium for 24 hours or subjected to 500 µM H$_2$O$_2$ solution; n=3 experiments. Experimental groups: control- non transfected cells, wild type; pTRUF-22 - mock transfected cells; FGF-4/VEGF- cells transfected with complete bicistronic plasmid. Data are presented as the means (percentages) ± standard deviation. The statistical significance between cell populations is presented in Table 3. FACS analysis of cells incubated either in standard in vitro conditions or subjected to oxidative stress conditions is shown in panel B.
Table 3. Statistical differences between the myoblast populations studied in Fig. 10.

<table>
<thead>
<tr>
<th>cells</th>
<th>Population of cells transfected with FGF-4/VEGF gene, pTRUF-22 plasmid wild type cells (WT)</th>
<th>Statistical significance</th>
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<tr>
<td>Viable</td>
<td>FGF-4/VEGF vs. FGF-4/VEGF H2O2</td>
<td>p&lt;0.001 (***)</td>
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<tr>
<td></td>
<td>FGF-4/VEGF vs. pTRUF-22 H2O2</td>
<td>p&lt;0.01 (***)</td>
</tr>
<tr>
<td></td>
<td>FGF-4/VEGF vs. WT</td>
<td>p&lt;0.05 (*)</td>
</tr>
<tr>
<td></td>
<td>FGF-4/VEGF vs. WT H2O2</td>
<td>p&lt;0.01 (**)</td>
</tr>
<tr>
<td></td>
<td>FGF-4/VEGF H2O2 vs. pTRUF-22</td>
<td>p&lt;0.001 (****)</td>
</tr>
<tr>
<td></td>
<td>FGF-4/VEGF H2O2 vs. WT</td>
<td>p&lt;0.001 (****)</td>
</tr>
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<td></td>
<td>pTRUF-22 vs. pTRUF-22 H2O2</td>
<td>p&lt;0.001 (****)</td>
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<td></td>
<td>pTRUF-22 vs. WT H2O2</td>
<td>p&lt;0.001 (****)</td>
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<td></td>
<td>pTRUF-22 H2O2 vs. WT</td>
<td>p&lt;0.001 (****)</td>
</tr>
<tr>
<td></td>
<td>WT vs. WT H2O2</td>
<td>p&lt;0.001 (****)</td>
</tr>
<tr>
<td>Apoptotic</td>
<td>FGF-4/VEGF H2O2 vs. WT</td>
<td>p&lt;0.05 (*)</td>
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<tr>
<td>Necrotic</td>
<td>FGF-4/VEGF vs. FGF-4/VEGF H2O2</td>
<td>p&lt;0.001 (****)</td>
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<td></td>
<td>FGF-4/VEGF vs. pTRUF-22 H2O2</td>
<td>p&lt;0.01 (***)</td>
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<td>p&lt;0.001 (****)</td>
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<td></td>
<td>FGF-4/VEGF H2O2 vs. pTRUF-22</td>
<td>p&lt;0.001 (****)</td>
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<td>FGF-4/VEGF H2O2 vs. WT</td>
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<td>pTRUF-22 vs. pTRUF-22 H2O2</td>
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<td>WT vs. WT H2O2</td>
<td>p&lt;0.001 (****)</td>
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Experimental groups: WT - wild type; control, non-transfected cells, ; pTRUF-22 - transfected control with a coding sequence lacking plasmid; FGF-4/VEGF complete bicistronic plasmid.

Both factors separately greatly contribute to angiogenesis (even in small amounts) (15, 16) and in our study are highly but unequally overexpressed, which may lead to the enhancement of angiogenesis. Moreover, we detected the FGF-4 gene transcripts in pTRUF-22-transfected cells. After determining the protein concentration in supernatants, we assume that the lack of FGF-4 protein in the pTRUF-22 and wild type populations may indicate that not every transcript of the FGF gene became a stable protein that will be secreted from cells. The mature form of FGF-4 protein needs to be glycosylated before secretion because disrupting the glycosylation process (i.e., lack or mutations in signal amino acid residues) impairs the secretion of the protein (20). We observed several variations between primary myoblast cell suspensions isolated from different patients and because transfection is a random process, the concentrations of FGF-4 and VEGF secreted into the media slightly varied between biological replicates. Usually at 48 hours after transfection, we obtained up to 0.3 ng/ml and 6 ng/ml of secreted FGF-4 and VEGF, respectively. These levels diminished gradually with time because the transfection was also transient (data not shown). Control human primary myoblasts expressed VEGF protein spontaneously but at significantly lower levels (~4-fold lower). However, FGF-4 protein was not detected in any of the conditioned (non-transfected) cell media. Our previous findings from murine myoblast experiments (18) showed that FGF-4 is expressed at slightly lower concentrations than VEGF (2.0 ng/ml versus 2.8 ng/ml of FGF-4 and VEGF, respectively); however, in the case of human myoblasts, FGF-4 secreted levels were more than twenty-fold lower than VEGF protein (~0.3 ng/ml versus ~6 ng/ml; FGF-4 versus VEGF). Moreover, we found that the VEGF concentration was two-fold higher in human cells compared to mouse transfected C2C12 myoblasts (~6.0 ng/ml versus ~2.8 ng/ml). Based on previous reports from animal models (21, 22), we hypothesised that the high overexpression of VEGF may be beneficial at the time of transplantation of the cells into the infarcted myocardium, and this transient phenomenon may prevent the risk of haemangioma formation (23, 24). This set of experiments demonstrated highly efficient, non-viral angiogenic gene delivery followed by simultaneous FGF-4 and VEGF protein secretion by human primary myoblasts for the first time. We hypothesise that the co-expression of both pro-angiogenic factors results in the formation of more stable and mature blood vessels (14) because this process requires more than one factor to be effective. In fact, a previous study involving viral genetic modification of human myoblasts designed to overexpress two potent pro-angiogenic factors, VEGF and angiopoietin-1 (26), showed the benefits of this combinatorial approach because the transplantation of these cells into a pig infarcted myocardium resulted in stable and mature blood vessel formation. The previously mentioned adenoviral transduction study and our non-viral studies in both human and murine myoblasts (19) are the only studies that provide data from bicistronic vectors simultaneously carrying two growth factor genes for therapeutic angiogenesis. Our study is focused on finding the most efficient gene therapy for heart disease. For example aging process is inherently associated with increased risk of cardiovascular diseases, leading to heart failure and reduced tolerance to ischaemia. New vessel formation might be therefore reduced and delayed in aged individuals what may lead to impairment in revascularisation of ischaemic heart (26, 27). It has been proven that expression of VEGF and its receptor VEGF-R2 is downregulated in senescent rats. Due to this phenomenon the regeneration of damaged heart was insufficient. It is well known that VEGF is able to stimulate expression of VEGF-R2 receptor (27). In our view the solution for aging patients suffering from cardiac disease can be an administration.
of exogenous of VEGF within genetically modified myoblasts. This therapy requires adjustment of therapeutic gene/protein dose, for rat such efficient dose was calculated for 10 µg of recombinant protein (27). In our case, we shall calculate how many cells secreting proangiogenic factor must be grafted into the scar area to be sufficient for capillary formation. This could be done in prospective trial in porcine model. We were able to obtain high amounts of VEGF-A and FGF-4 proteins; therefore, we might consider their potential side-effects while applying cellular therapy with introduced two proangiogenic genes, while both of these factors participate in capillary formation. The question remains as to whether the administration of the provided pro-angiogenic factors is harmful for an organism. In our study, we used therefore the transient expression of both genes. The plasmid delivery gene system provides a short-term effect that prevents malignant capillary development. VEGF-A naturally occurs in blood plasma, and its gene expression is characteristic not only for endothelial cells, but several mRNAs have been detected in heart and skeletal muscles according to microarray experiments (28, 29). FGF-4 gene in several species is required for cardiac valve development during embryogenesis (30); however, in adult organisms, the expression of this gene is barely detectable. Nevertheless, FGF-4 participates in blood vessel maturation (16); therefore, we assume that the balanced expression of VEGF-A and FGF-4 gene provides the development of stable and mature capillaries.

An interesting topic appears to be an interaction between VEGF isoforms. We decided to examine the expression of remaining VEGF isoforms upon VEGF-A overexpression in human myoblasts cells. Additionally, we compared VEGF expression in undifferentiated myoblasts and myocytes. As we expected, VEGF-A expression achieved the highest level in transfected myoblasts. VEGF-A mRNA was already observed in the adult lung, kidney, heart, adrenal gland and in endothelial cells. Low but also detectable quantities of VEGF-A transcripts levels occur in the liver, spleen, and gastric mucosa (31). However, this factor is most prominent when it is activated during hypoxia or the disruption of blood vessels (32). After myoblast differentiation, VEGF-A expression was maintained at a similar level, which may indicate that even after the differentiation period, our myogenic cells exhibit a very high therapeutic dose of pro-angiogenic gene. VEGF-B has been found in the heart and skeletal muscles (31) and was shown to be induced by VEGF-A in our study (Fig. 3B). The upregulated expression of VEGF-B in transfected myoblasts may indicate the cooperation between both of these isoforms. As shown in Fig. 3, the elevated expression of VEGF-A may contribute to the upregulation of the expression of other VEGF isoforms. VEGF-C and VEGF-D are considered to be lymphangiogenic factors. In modified myocytes, the expression of VEGF-C is elevated; therefore, we assume that by applying this therapy, we will be able to obtain the pro-angiogenic effect and also lymphangiogenic effect induced by VEGF-C. Evidence also exists for the cooperation between VEGF-A and VEGF-D isoforms (especially in myocytes). Taking into account previous reports where these isoforms were able to induce arteriogenesis in rabbit hindlimb model (33), we presume that myoblasts modified with the FGF/VEGF-A genes will be able to interact with VEGF-D and cause similar effects.

Additionally, we examined the expression of the Ctnn gene. Genes from the connexin family ensure cell-cell communication through gap junction channels. In the heart, such communication is essential to maintain electrical coupling. The presence of Ctnn has been described in myoblasts of differentiating skeletal muscle (34). Real-time qPCR analysis revealed that in case of undifferentiated cells the expression of connexin 43 in modified myoblasts was preserved at least on the same level as in ‘native’ myoblasts cells (Fig. 4). Additionally, we performed a comprehensive study (34) where human skeletal myoblasts after differentiation into myocytes demonstrated significant increase in Ctnn expression level in comparison to undifferentiated cells and it is worth to point out that the most remarkable increase was noticed between FGF/VEGF-A transfected myoblasts and myocytes. Therefore, we can assume that in genetically modified myogenic cells, the amount of connexin transcripts increases, thus the cell-cell communication will be properly maintained.

Performing the transfection with pro-angiogenic factors did not influence human primary myoblast proliferation, which is contradictory to previous findings where experiments were conducted using separate FGF or VEGF overexpression models in rat primary skeletal muscle stem cells (35, 36). Although we reported similar observations confirming the pro-proliferative potential of FGF-4/VEGF overexpression in our previous experiments with murine myoblasts (18), we did not notice significant changes in the cell cycle for human primary myoblasts. This outcome may not necessarily be of negative meaning because in our future in vivo model hypoxia resistance and the survival rate of transplanted myoblasts in the ischaemic myocardial environment will be of more importance than an increase in cell proliferation, which may be difficult to control in in situ conditions. Taking into consideration possible future clinical applications of stem cells with pro-angiogenic properties, transplanting cells with limited proliferative capacity may be safer.

The natural expression of VEGF has been previously shown to be enhanced in ischaemic skeletal muscle and is thought to play a key role in the angiogenic response to ischaemia (37). Although human myoblasts naturally express low levels of VEGF, the formation of capillaries from HUVEC sprouts in media from control cell populations were also observed, and the simultaneous overexpression of FGF-4 and VEGF transfectants provided statistically significant, increased numbers of sprouts forming blood vessels in the in vitro assessment (Fig. 7B). VEGF is a potent inducer of capillary formation in vivo (39), and combined adenaloviral overexpression of this factor along with angiopietin-1 showed an improved pro-angiogenic ability (25); therefore, we assume that our model of non viral overexpression of VEGF with FGF-4 may also provide sufficient stimulus for angiogenesis in vivo and needs to be first tested in animal models.

Consistent with our cell proliferation assays that did not reflect any differences in the proliferation of our transfected cells versus control cell populations, the data from myogenic gene expression profiles (Fig. 8) showed commitment to different stages of the myogenic differentiation pathway (39, 40). The genes involved in early myogenesis such as Myf5, Myod and Mef2 primarily influence the proliferation of myoblasts, which are in equilibrium with the genes mostly responsible for the differentiation of the muscle stem cells, myogenin and Myf6. The Mef2 gene, which is involved in induction of muscle differentiation and may participate in the maintenance of muscle differentiated state, underwent an acceleration in expression level. Moreover, we also detected the increase in expression level of Myod but only in cells transfected with control plasmid. Nevertheless, the cells did not show any changes in the cell cycle and proliferation profile. The high levels of expression of all tested genes showed the commitment of our cells to the myogenic lineage when compared to our previous studies conducted with murine myoblasts in which the expression levels of the studied myogenic genes were significantly lower in cells overexpressing FGF-4/VEGF (18). As previously mentioned, these facts may also be advantageous for the use of human myoblasts in cell therapy of cardiac failures as their proliferative
potential would be more predictable. Additionally, myoblast unipotential character and equal ability to both proliferate and differentiate presumably allows both the sufficient overexpression of introduced angiogenic factors along with cell differentiation to myotubes, which potentially reinforces scar tissue formation.

Although different studies have shown a positive effect of VEGF overexpression in human myoblasts increasing their ability to survive in oxidative stress conditions (41, 42), our in situ study with murine skeletal myoblasts (18) as well as this study failed to reproduce this phenomenon. Perhaps our experimental design with the use of hydrogen peroxide was more demanding than the conditions influencing the cell death after in situ implantation; however, the aim of our assay was to assess cell survival in acute stress conditions. However, we noticed that the FGF-4/VEGF-transfected cells (in applied hydrogen peroxide conditions) showed almost equal viability to both control cell populations, proving that the sensitivity to the hostile environment did not increase. The viability of stem cells overexpressing the FGF-4/VEGF gene under stress conditions was comparable to the viability of 'native' stem cells treated with hydrogen peroxide. The same dependence was observed also in the case of apoptotic and necrotic cells. Subsequent in vivo studies will reveal the true behaviour of human myoblasts genetically modified with two pro-angiogenic factors (genes) when cells are transplanted into the mouse post-infarction heart.

Overall, we managed to present an efficient model of non-viral simultaneous overexpression of both FGF-4 and VEGF pro-angiogenic factors in human skeletal muscle primary cell suspensions, which are considered as good candidates for the treatment of the post-infarcted myocardium. Taking into consideration the involvement of different pro-angiogenic factors in the process of novel blood vessel formation and the ability of myoblasts to repopulate and to strengthen the fibrous scar formed after the heart infarction, this combined approach with the delivery of two potent pro-angiogenic factors and the concomitant use of stem cells as their vectors may prove to be beneficial in in vivo conditions.

* A. Zima and A. Janeczek equally contributed to the study.

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