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INFLUENCE OF HYPOXIA PREVAILING IN POST-INFARCTION HEART ON PROANGIOGENIC GENE EXPRESSION AND BIOLOGICAL FEATURES OF HUMAN MYOBLAST CELLS APPLIED AS A PRO-REGENERATIVE THERAPEUTIC TOOL

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Cardiovascular diseases along with MI (myocardial infarction) lead to regional ischaemia and hypoxic conditions, which prevail after infarction. Diminished O_2 saturation which is related to elevated level of hypoxia inducible factor 1 (HIF-1) transcription factor, may switch the expression of many genes. To maximize effect of therapies proposed by regenerative medicine, it is essential to verify (within different time points after MI) the expression of proangiogenic genes and their receptors that are regulated, along with the expression of HIF-1α. We demonstrated a connection between the expression of *Hif-1α* (in murine post infarcted heart model) and the proangiogenic genes *Vegf-a* and *Plgf* and their receptors during myocardial hypoxia. The innovative part of the study required establishment of the most accurate *in vitro* O_2 level corresponding to the hypoxia level prevailing in myocardium after MI. We determined the influence of hypoxia on the biology of human myoblasts in *in vitro* oxygen conditions (3%), corresponding to those prevailing in the heart after an infarction using a murine model. We also tested myoblasts that were genetically modified with *VEGF-A/FGF-4* and *PlGF* under hypoxic conditions and compared their characteristics with cells cultured under normoxia and hyperoxia (standard *in vitro* conditions) with respect to myogenic gene expression, cell proliferation, fusion potential and proangiogenic function. The examination of genetically modified myoblasts under optimized *in vitro* hypoxia conditions led to the conclusion that hypoxia did not negatively influence the biological functions of the myoblasts, such as cell proliferation and/or proangiogenic characteristics. These results support the expected increased proregenerative effects of such genetically modified human myoblasts.

Key words: *myocardial infarction, hypoxia, regenerative medicine, proangiogenic factors, human skeletal myoblasts, growth factor, cell proliferation*

INTRODUCTION

Circulatory system supplies oxygen and nutrients to the cells, tissues and organs in the entire human body. Therefore, blood vessels, including veins, arteries and the heart itself, are responsible for maintaining of cardiovascular homeostasis. When part of the cardiovascular system fails, the balance becomes affected. This failure may be due to regional ischaemia and hypoxic conditions, which prevail in myocardial infarction. Hypoxia can be defined as the state in which O_2 levels decrease below physiological levels (characteristic for a particular tissue- state called normoxia) (1). For example, arterial blood demonstrates normoxia of pO_2 at a level of 14%, myocardium at 10%, and skeletal muscle at 5%. In contrast, the natural pO_2 levels of bone marrow, the thymus, and cartilage are 1% (2, 3). Thus, when the O_2 homeostasis is impaired, cells must adapt themselves to low oxygen concentration. In eukaryotic cells, the main response to hypoxia is regulated by hypoxia-inducible factor-1 (HIF-1). This regulation occurs through hypoxia response elements (HRE) that are located within the genes that are transcriptionally activated by HIF-1 (4). It has been proven that hypoxia particularly regulates the genes that are involved in angiogenesis, such as *VEGF*, *PlGF*, *PDGFB*, *ANGPT1*, and *ANGPT2* (5, 6). Increasing knowledge regarding the influence of HIF-1 on proangiogenic genes allows for its application in the treatment of ischaemic diseases.

There are two basic strategies to apply HIF-1 as a therapeutic tool: delivery of HIF-1α/2α subunits to the site or inducing HIF-1 expression by the modification/administration/inhibition of molecules associated with HIF activity.

Induction of HIF-1α and HIF-2α expression in mouse heart and/or rabbit ischaemic limbs induced the process of capillary formation, enhanced the amount and density of capillaries and was able to attenuate the cardiac remodelling. Described processes stimulated recovery of the muscles and improved tissue energy metabolism (7, 8). Another study applied an AdCA5- adenovirus encoding a constitutively active form of the HIF-1α subunit due to a deletion and a point mutation in the region responsible for O_2-dependent degradation. Therapy with AdCA5 improved the recovery of blood flow by stimulating both angiogenesis and arteriogenesis (9) and caused the upregulation of several proangiogenic genes/proteins that are targets for therapeutic angiogenesis in hindlimb and cardiac ischaemic models, including

fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), monocyte chemoattractant protein 1 (MCP-1), platelet derived growth factor subunit B (PDGF-B), PlGF, placental growth factor (PlGF), stromal cell-derived factor 1 (SDF-1), and vascular endothelial growth factor (VEGF) (10). Currently, a rising trend has been noted in combining therapies using proangiogenic factors with stem cells as the tissue vector.

In the case of myocardial ischaemia, regenerative medicine has proposed autologous skeletal muscle stem cells (SkMC) as a potential therapeutic tool (11). These cells are considered to be good candidates due to the relative simplicity of obtaining high numbers of cells in primary *in vitro* culture and their great resistance to hypoxia (12, 13). Despite the fact that grafted myoblasts tolerate the poor graft microenvironment, a large number of cells transplanted into myocardium do not survive due to inadequate blood supply in post infarcted tissue. To enhance proregenerative effects, the genetic modifications of SkMCs seem to be very effective at influencing delivery of oxygen and nutrients to the damaged tissue, which could be beneficial in prevention of massive apoptosis of engrafted cells. Grafted myoblasts can be modified with cytoprotective and/or proangiogenic factors (11, 14) thus enhancing proregenerative virtues. The greatest potential has been attributed to VEGF, which acts directly in neoangiogenesis (15, 16). Most importantly, *VEGF* gene can be triggered during angiogenesis in many different pathways. Moreover *VEGF* gene is activated by ageing and impaired cardiomyocytes not only during ischaemia therefore it may regulate and stabilize myocardium when maintained at steady levels (17). Many reports have emphasized PlGF as a proangiogenic factor with the abilities to enhance capillary formation (18). Additionally, fibroblast growth factors, such as FGF-4, are involved in the first stages of angiogenesis but may also stimulate arteriogenesis, thus playing a role in the maturation of blood vessels (19). Many factors are upregulated during hypoxia, although some only temporarily. Correlations among the ability of stem cells, proangiogenic factors and hypoxia may be interesting topics for the field of regenerative medicine.

In our study, we focused on human myoblast cells modified with the proangiogenic genes *FGF-4/VEGF-A,* and *PlGF*. Our first step was to verify how murine infarcted hearts regulate the proangiogenic genes and their receptor expressions, along with the expression of *Hif-1α*, at different time points after myocardial infarction. Because *in vitro* studies with genetically modified myoblasts were scheduled to be implemented next in preclinical studies, we also wanted to establish the most likely *in vitro* oxygen conditions corresponding to those prevailing in the heart after infarction. For this purpose, we evaluated the expression of *HIF-1α* in human myoblasts grafted into murine failing hearts compared to the expression of *Hif-1α* in myoblasts that were subjected to diminished oxygen levels in *in vitro* conditions. Thus, we were able to test and compare human myoblasts during hypoxia, normoxia and standard *in vitro* culture conditions (hyperoxia). We evaluated the influence of oxygen concentrations on the biological functions of genetically modified cells to define how these conditions may impact selected gene/protein expressions to induce proangiogenic and myogenic effects.

MATERIALS AND METHODS

Animal studies

All experiments involving animals were approved by the Local Ethical Committee for Animal Research in Poznan.

Twelve-week-old SCID mice purchased from The Jackson Laboratory USA were used in experiments. Myocardial infarction was performed following left coronary artery ligation. The SCID mice were used in two different experiments. The initial step of our study involved the characterization of proangiogenic gene expression in mouse post-infarcted hearts without cell therapy. Our interest was focused on proangiogenic genes (*Vegf* isoforms *A*, *B*, *C*, *D*; *Plgf*) and genes coding for their respective receptors (*Flt-1*; *Kdr*). To evaluate the influence of hypoxia in the hearts of mice, the expression of *Hif-1α* was examined. Mice were sacrificed at 24 h, 7 days and 28 days after coronary artery ligation. The RNA samples were isolated from the collected material, and the expression of the mentioned genes was evaluated by qPCR. As a control, the physiological heart samples were applied.

A second experimental series involving animals was included to evaluate the expression of human *Hif-1α* gene in human myoblasts transplanted into post-infarcted hearts (concomitantly with MI induction) to verify how myocardial hypoxia impacts *HIF-1α* expression in the grafted cells. For this purpose, upon coronary artery ligation, human myoblast cells were transplanted into the mouse hearts (3×10^5 cells per intervention in mice). Next, the animals were sacrificed at 24 hours, 7 days, 28 days after infarction/transplantation, and the hearts with the grafted human myoblasts were collected. After collection of the heart tissue samples, RNA was isolated from the left ventricle (the area into which the cells were injected), and the expression of human *HIF-1α* was evaluated using the qPCR with primers that do not interfere with the murine template. In both experiments, the myocardial infarction was verified by echocardiographic examination before heart sampling. The data were used to standardize the hypoxia *in vitro*, that is, to obtain culture conditions corresponding to those prevailing in the post infarction heart. The controls were physiological hearts (normoxia) injected with myoblasts.

Hypoxia optimization

The conditions for hypoxic *in vitro* cultures of the myoblast cells were designed by plotting the oxygen concentration curve to compare hypoxia levels in myoblast cells transplanted into post-infarcted hearts of SCID mice. The cells were further maintained for 24 h and 1 week at the following different oxygen concentrations: 1%, 2%, 3%, 5%, 7%, 10% and 15%. Next, the expression of hypoxia marker (*HIF-1α*) was evaluated to establish at which oxygen concentration it exhibited the highest expression in human myoblasts. To establish hypoxic *in vitro* conditions comparable to those prevailing in the heart, the *in vitro* expression of *HIF-1α* extrapolated using the oxygen curve was compared with the data obtained from animal studies in which the *HIF-1α* expression in human myoblast cells grafted into post-infarcted hearts was determined.

Cell in vitro culture

Human myoblasts were isolated from tissue fragments derived from skeletal muscle biopsies obtained from three patients. For this purpose, approval from the Bioethical Local Committee (Medical University of Poznan) and written consents from the patients were obtained. Isolation of muscle stem cells from oligobiopsies were conducted according to modified 'preplate' technique. Before further studies, the cells were characterized by flow cytometry (antibody against the CD56 surface marker) and desmin immunostaining. Cells were cultured in standard Dulbecco's modified Eagle's medium with 4.5 g/l glucose, supplemented with 20% foetal bovine serum (Lonza Group, Base, Switzerland), 1% antibiotics (Lonza Group, Base, Switzerland), 1% ultraglutamine (Lonza Group, Base, Switzerland), and other routine supplements. Additionally, the bFGF (Sigma-Aldrich, Saint Louis, USA) was added to the medium. Cells were cultured in standard *in vitro* conditions (95% humidity and 5% CO_2, at 37°C) and, in further experiments, oxygen levels were changed from standard *in vitro*

hyperoxic conditions (21%) to skeletal muscle normoxia (5%) and hypoxia (3%) by regulation using N_2.

Human umbilical cord vein cells (HUVEC) were required to evaluate the proangiogenic properties of protein secreted from genetically modified cells. Cells were routinely maintained in Medium 200 supplemented with Large Vessel Endothelial Supplement (Gibco) for *in vitro* culture in standard *in vitro* conditions at 95% humidity and 5% CO_2 at 37°C.

The medium was changed every two days, and the cells were passaged using 0.25% trypsin with EDTA (Sigma, St. Louis, USA). The experimental procedures were performed when the cell quantity reached approximately 70 – 80% of monolayer confluency, which was estimated by microscopic observation.

Plasmids

This study involved genetic modifications of myogenic stem cells with different proangiogenic genes (*PlGF* and *FGF4/VEGF-A*). The *PlGF* coding sequence was amplified from cDNA templates obtained from HUVEC cells. The polymerase chain reaction (PCR) was carried out using primers designed for the first isoform of *PlGF,* and the coding DNA seguence (CDS) was derived from BLAST database. Then, the PCR product was cloned into pSC-A vectors. To achieve *PlGF* expression in mammalian cells, the insert from the primary plasmid was subcloned using restriction enzymes XhoI and XbaI into pCi-neo vectors. The pTRUF-22-FGF4-ires-VEGF (called *FGF-4/VEGF-A*) bicistronic vector (which was a gift obtained from Prof. Dulak from Jagiellonian University, Cracow) was used to simultaneously overexpress human *FGF-4* and human *VEGF-A*.

Transfection of human myoblasts

Human myoblasts were transfected by electroporation (Gene Pulser X-Cell Electroporation System Biorad, USA). Cells were modified using the following conditions: one pulse 15 ms, wave tension 160 V, and cuvettes with 2 mm gaps. To reach the expected number of transfected cells, we used 3×10^6 myoblasts resuspended in a final volume of 200 µl F10 medium, and plasmid concentration was established according to its length. Medium was changed after 24 h of electroporation. After 48 h, when the transgene was activated, the cells were transferred for 24 h to different *in vitro* culture conditions (21%, 5%, and 3% oxygen levels) to test and compare their properties in different

Table 1. Sequences of primers used in the study. *Abbreviations*: *ACT-β,* beta actin; *FGF-4,* fibroblast growth factor-4; *HIF-1α*, hypoxia inducible factor 1 alpha; *Flt-1*, FMS-like tyrosine kinase receptor; *Kdr*, kinase insert domain receptor; *PlGF/Plgf*, placental growth factor; *TBP*, TATA-binding protein; *VEGF/Vegf-A*, vascular endothelial growth factor isoforms *A*.

	Gene		Sequence	Product bp
H. sapiens	*ACTβ*	F	5'-CTTCCTGGGCATGGAGTCC-3'	192bp
		R	5'-ATCTTGATCTTCATTGTGCTG-3'	
H. sapiens	*FGF-4*	F	5'-TATGGCTCGCCCTTCTTCAC-3'	142 bp
		R	5'-CTCGGTTCCCCTTCTTGGTC-3'	
M. musculus	*Hif-1α*	F	5'-GCTGAAGACACAGAGGCAAAG-3'	164 bp
		R	5'-GTGCTCATACTTGGAGGGCTT-3'	
H. sapiens	*HIF-1α*	F	5'-TTGGCAGCAACGACACAGAA-3'	82 bp
		R	5'-TTGGGTGAGGGGAGCATTACA-3'	
M. musculus	*Flt-1*	F	5'-GCTTTCACCGAACTCCACCT-3'	162 bp
		R	5'-AGTCCCGCCTCCTTGCTTT-3'	
M. musculus	*Kdr*	F	5'-TCTGGACTCTCCCTGCCTAC-3'	128 bp
		R	5'-CGGCTCTTTCGCTTACTGTTC-3'	
H. sapiens	*PlGF-orf*	F	5'-CTCGAGCTGAGAAGATGCCGGTCATGAGGCTGTT-3'	513 bp
		R	5'-TCTAGAAGCCGGGTGCGGGGTCTCTCTCCTCCAAG-3'	
H. sapiens	*PlGF*	F	5'-GGCTCGTCAGAGGTGGAAGT-3'	145 bp
		R	5'-GCAGCAGGGAGACACAGGAT-3'	
M. musculus	*Plgf*	F	5'- GTGTCCTTCTGAGTCGCTGT-3'	197bp
		R	5'- CCTTTCTGCCTTTGTCGTCT-3'	
H. sapiens	*TBP*	F	5'-CGGGCACCACTCCACTGTATC-3'	177 bp
		R	5'-ATTCGGCGTTTCGGGCA-3'	
H. sapiens	*VEGF-A*	F	5'- AAGGAGGAGGGCAGAATCAT-3'	143 bp
		R	5'-CACACAGGATGGCTTGAAGA- 3'	
M. musculus	*Vegf-a*	F	5'-TCCTGTGTGCCGCTGATG-3'	121 bp
		R	5'-GCTGGCTTTGGTGAGGTTTG-3'	
M. musculus	*Vegf-b*	F	5'-GTCGCCTGCTGCTTGTTG-3'	88 bp
		R	5'-ACTTTCTTCTGGTGGCTGGG-3'	
M. musculus	*Vegf-c*	F	5'-AGATGTGGGGAAGGAGTTTG-3'	193 bp
		R	5'-ACTGATTGTGACTGGTTTGGG-3'	
M. musculus	*Vegf-d*	F	5'-CTGGGGAAGACAACCAACAC-3'	172 bp
		R	5'-CAGGCACTAACTCGGGCA-3'	

oxygen concentrations. All experiments conducted on human myoblasts were performed 72 hours after electroporation.

Gene expression profile in myoblast cells

Cells were harvested after 24 h of culture in different oxygen concentrations (21%, 5%, and 3%). The RNA was isolated, and qPCR was performed. First, the expression of hypoxia marker *HIF-1α* and the overexpressions of introduced genes (*FGF-4*/*VEGF-A*; *PlGF*) were determined. Next, to check whether the culture *in vitro* conditions impact myogenic gene expression, early myogenesis genes, *MYOD* and *MYF 5*, and late myogenesis genes, *MYOG* and *MRF 4*, were examined (the respective primers are listed in *Table 1*).

Protein expression

Supernatants containing secreted proteins were harvested from the cells cultured for 24 hours in different oxygen concentrations. To determine the levels of secreted FGF-4, VEGF-A and PlGF protein, an ELISA quantitative assay was performed (R&D System, Abingdon, UK) according to the manufacturer's instructions.

Proangiogenic in vitro test

To determine the proangiogenic properties of overexpressed protein, a HUVEC angiogenesis test was performed. HUVECs were cultured (no further than the 4th passage) to reach approximately 80 – 90% confluency using Medium 200 supplemented with Large Vessel Endothelial Cells Supplement (LVES) (Gibco, Carlsbad, USA - Thermo Fisher Scientific). Then, the cells were trypsinized, counted and seeded at a density of approximately 25,000 cells per cm^2 on 24-well plates coated with Geltrex® Matrix. The supernatants collected from the myoblasts under study were transferred to prepared HUVEC cells to evaluate the functional properties of the secreted proteins. As a negative control, Dulbecco's modified Eagle's medium (DMEM) and a fresh myoblast medium were used, and, as a positive control, medium 200 (supplemented with LVES) was used. The HUVEC cells were further incubated with supernatants

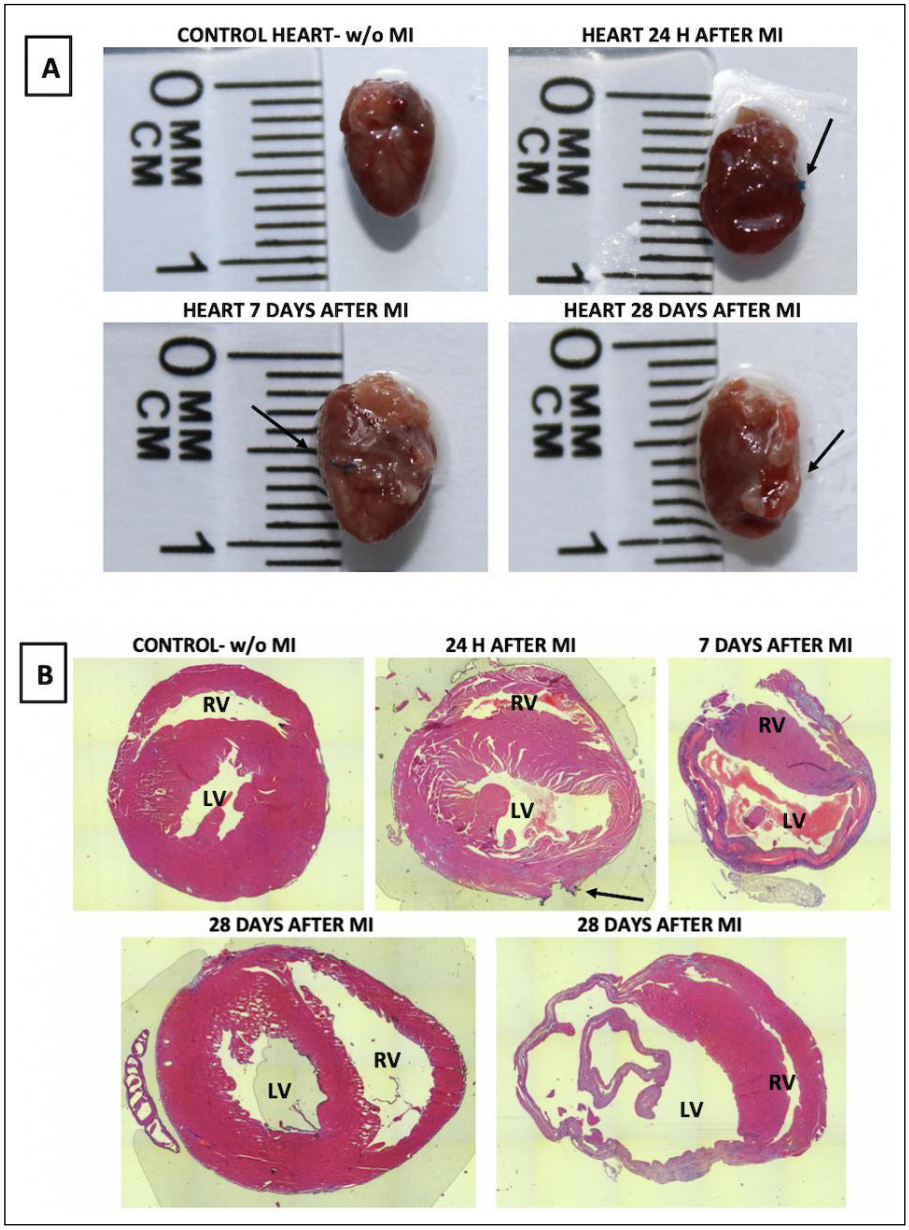

Fig. 1. Dynamics of post-infarction heart remodelling. (*A*): Photographs of heart remodelling in different post-infarction time points: 24 hours after MI, 7 days after MI and 28 days after MI compared to control physiological heart. (*B*): Histological analysis of hearts at 24 hours, 7 days and 28 days after MI, and control heart w/o MI. Haematoxylin and eosin staining. LV, left ventricle, RV, right ventricle. Arrows point out the coronary artery ligation regions.

harvested from myoblasts cultured under different oxygen concentrations for 14 – 18 hours. After this time, the newly formed capillaries were stained with 2 μg/mL Calcein, AM (Invitrogen, Carlsbad, USA, Thermo Fisher Scientific).

Cell in vitro proliferation

The proliferation of cells was examined by MTS tests using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA). The test was based on the ability to convert tetarazolium salt to formazan crystals by metabolically active cells, and the quantity of the formazan product measured by absorbance at 490 nm is directly proportional to the number of living cells.

Detection of apoptosis

Using an Annexin V-FITC Kit (Beckman Coulter, Fullerton, USA) assay, we were able to detect the percentage of apoptotic cells in the myoblast populations cultured in different oxygen conditions. The test was performed using a Cell Lab Quanta MPL flow cytometer.

Myotube formation

To estimate the genetically modified myoblast differentiation potential, *in vitro* cultures were maintained using the cell differentiation protocol. Photographs were taken using a standard light microscope.

RESULTS

Proangiogenic gene expression profile in post-infarcted mouse hearts

Progression of post-infarction remodelling is shown in *Fig. 1A*. There was visible scarring and progressive decrease in wall

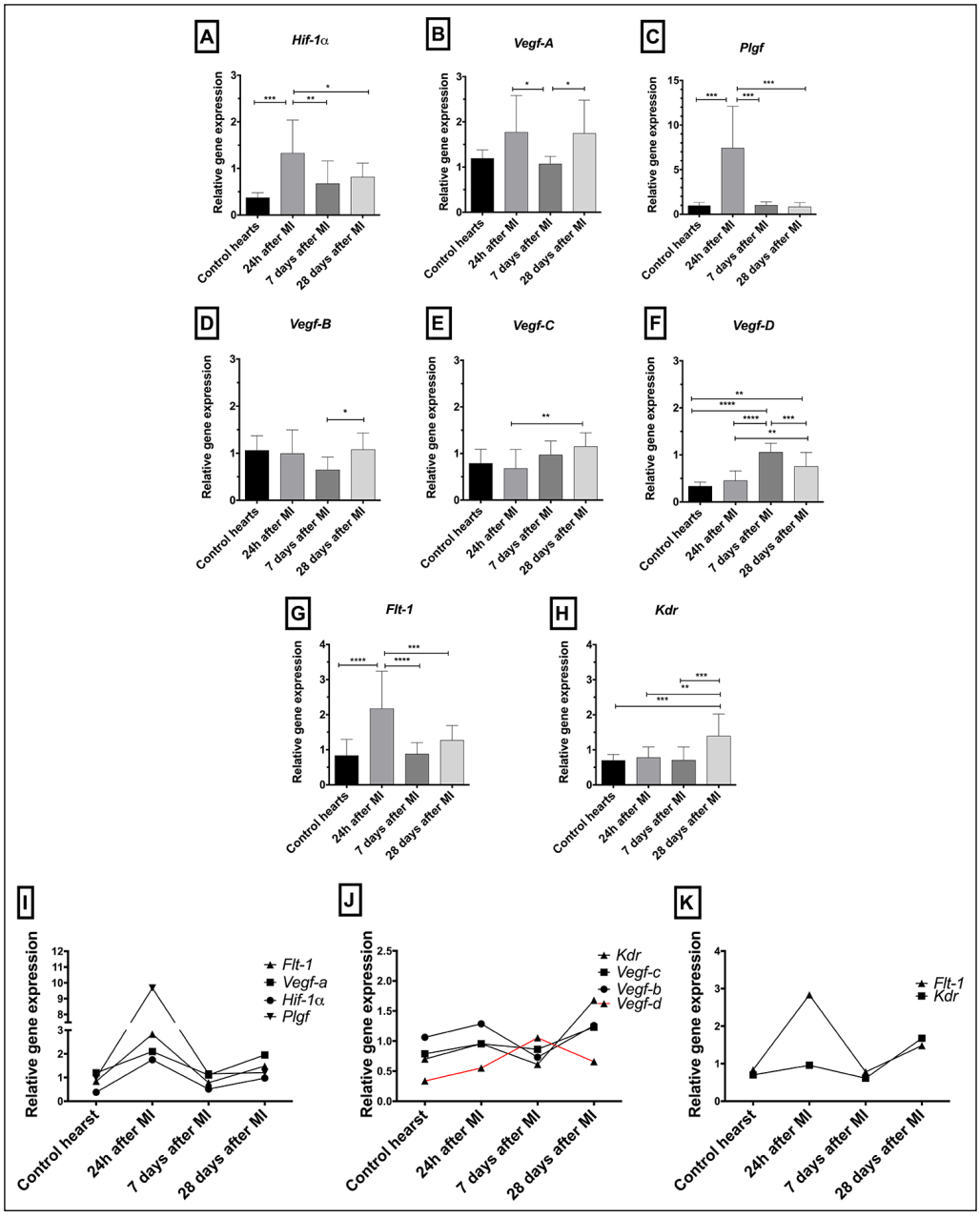

Fig. 2. (*A - H*): Expression of proangiogenic genes in murine hearts at 24 h after myocardial infarction (MI), n = 8; at 7 days after MI, n = 8; at 30 days after MI, n = 8; and in control hearts without MI, n = 6. (*I - K*): Summary of the time dependent proangiogenic gene expression in murine hearts. *Abbreviations: Hif-1α*, hypoxia inducible factor; *Vegf-A,B,C,D*, vascular endothelial factors isoforms A, B, C, D; *Plgf*, placental growth factor; *Flt-1*, FMS-like tyrosine kinase receptor; *Kdr*, kinase insert domain receptor gene.

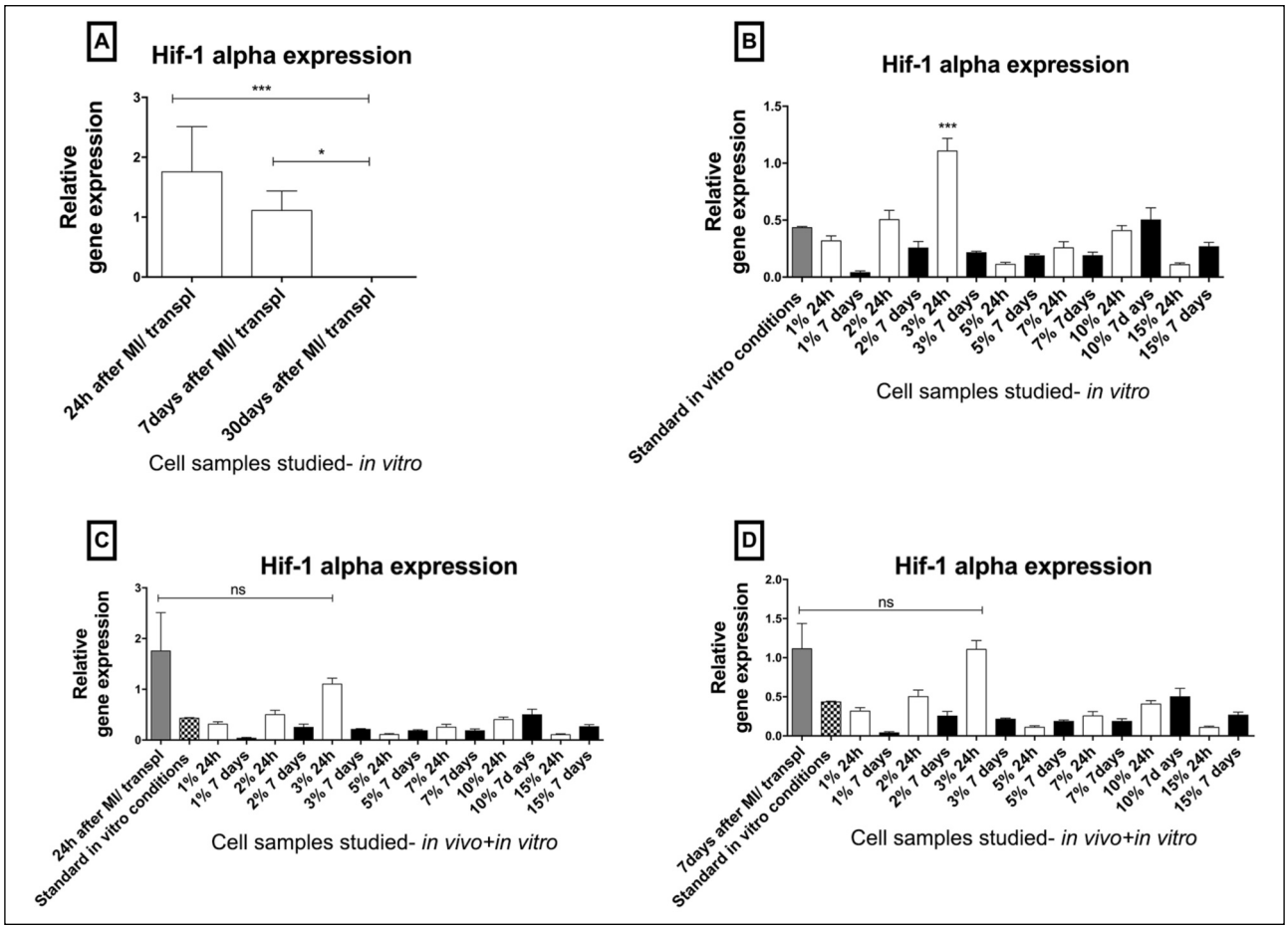

Fig. 3. Optimization of hypoxia. (*A*): An analysis of hypoxia inducible factor 1α (*HIF-1α*) expression in human myoblasts transplanted into mouse hearts during MI induction. Samples studied at: 24 hours after cell transplantation, n = 3; 7 days after cell transplantation, n = 4; and 30 days after cell transplantation, n = 3. The control group included murine heart samples with injected myoblasts without MI induction, n = 6 (2 mice for each time point) for which no *HIF-1α* expression was revealed (data not shown). (*B*): *HIF-1α* expression *in vitro*. Oxygen levels tested: 1%, 2%, 3%, 5%, 7%, 10%, 15% and standard *in vitro* conditions (21%); time points: 24 hours and 7 days. *HIF-1α* expression in 3% oxygen concentration reached the highest statistically significant value (complete statistics is given in *Table 2*). (*C*): Comparison of *HIF-1α* expression in myoblast cells in the *in vitro* experiments and in human myoblasts at 24-h after transplantation into post-infarcted hearts. (*D*): Comparison of *HIF-1α* expression in myoblast cells in *in vitro* experiments and in human myoblasts at 7 days after transplantation into post-infarction hearts. NS, not significant.

thickness within the observed time points. This was consistent with the histological analysis on *Fig. 1B*. The analysis of gene expression in mouse post-infarcted hearts revealed some interrelationship between studied angiogenic factors and their receptors. Initially, we investigated the expression pattern of *Hif-1α* in mouse hearts at different time points after MI (*Fig. 2A*). The greatest hypoxia in the heart was discovered 24 hours after coronary artery ligation. After 7 days, the level of *Hif-1α* expression significantly decreased, and further at the day 28th in the chronic phase of ischaemia, there was only a visible rising trend. According to the same scenario, we also evaluated the expression of *Vegf* isoforms (*Fig. 2B*). Seven days after MI, the level of *Vegf-A* significantly decreased to control levels, and after 28 days, *Vegf-A* expression again reached a similar level as that one observed at 24 h. *Vegf-B* expression was maintained almost at the same level as control, with some marked decrease observed after 7 days (*Fig. 2D*). *Vegf-C* exhibited an interesting increasing trend (*Fig. 2E*) along the observed time points that was roughly similar to *Vegf-D* (*Fig. 2F*). Interestingly, we found a high expression of *Plgf* in mouse hearts 24 h after coronary artery ligation (*Fig. 2C*). After analysing *Flt-1* and *Kdr* (*Fig. 2G*, *2H*) expression, we found that the *Flt-1* receptor is highly expressed 24 hours after MI, in contrast to the *Kdr* receptor, which was upregulated 28 days after MI. Upon comparing the obtained gene expression data, we were able to show that *Hif-1α, Vegf-A* and *Flt-1* receptor genes established similar expression pattern with time after MI (*Fig. 2I*). It is also worthy to note that the expressions of the B and *C Vegf* isoforms and *Kdr* also exhibited similar expression trends, unlike *Vegf-D* (*Fig. 2J*). The comparison of receptor genes expression strongly suggested that, after 24 hours, the *Flt-1* receptor gene was much more actively transcribed than *Kdr*, while after 28 days, both the *Flt-1* and *Kdr* genes exhibited similar pattern of expression (*Fig. 2K* and *2G*).

Optimization of hypoxia in vitro

The qPCR analysis of *HIF-1α* in the human myoblasts grafted into the mouse ischaemic hearts revealed the highest expression of *HIF-1α* occurring at 24 h after myoblast transplantation/MI induction and then at 7 days after the procedures. Unfortunately, we were unable to detect HIF-1α expression at 30 days after myoblast transplantation (*Fig. 3A*). In

Table 2. Statistical analysis of *HIF-1α* expression in different populations of myoblasts cultured under 1%, 2%, 3%, 5%, 7%, 10%, 15% of O_2 for 24 hours (h) and 7 days (d) (*Fig. 3B*).

	st. conditions	1% 24 h	1% 7 d	2% 24 h	2% 7 d	3% 24 h	3% 7 d	5% 24 h	5% 7 d	7% 24 h	7% 7 d	10% 24 h	10% 7 d	15% 24 h	15% 7 d
st. conditions			***		**	***	***	***	***	**				***	***
1% 24 hours			***	*		***		**					*	**	
1% 7 days	***	***		***	**	***	*			**					**
2% 24 hours		*	***		***	***	***	***	***	***	***			***	***
2% 7 days	**		**	***		***						*	***		
3% 24 hours	***	***	***	***	***		***	***	***	***	***	***	***	***	***
3% 7 days	***		*	***		***						**	***		
5% 24 hours	***	**		***		***						***	***		
5% 7 days	***			***		***						***	***		
7% 24 hours	**		**	***		***						***	***		
7% 7 days				***		***						***	***		
10% 24 hours					*	***	**	***	***	***	***			***	
10% 7 days		*			***	***	***	***	***	***	***			***	
15% 24 hours	***	**		***		***						***	***		*
15% 7 days	***		**	***		***								*	

parallel, we cultured *in vitro* human myoblast cells in different oxygen concentrations. As shown in *Fig. 3B*, the highest expression of *HIF-1α* was detected when the myoblasts were cultured *in vitro* for 24 h in 3% oxygen. It was interesting to note that *HIF-1α* expression was almost silenced in the myoblasts during longer periods of incubation (7 days) in diminished oxygen concentrations (*Fig. 3B*, statistical analysis in *Table 2*). Finally, when we obtained the data from the *in vitro* and *in vivo* experiments, we were able to compare the respective *HIF-1α* expression levels among all the samples performed. It is further shown in *Fig. 3C* and *3D* that hypoxia in the heart paralleled hypoxia occured in the *in vitro* cultured myoblasts at 3% oxygen concentration, which was shown by the lack of significant differences in expression only at this point (confirmed both for myoblasts after 24 h from transplantation and after 7 days from implantation).

Myoblast characteristics

The cytometric analysis against CD56 surface antigen revealed that around 90% of the cells in the isolated suspensions reached myogenic origin (*Fig. 4A-4C*). Additionally, immunostaining for desmin confirmed the myogenic features of the obtained cells (*Fig. 4D-4F*).

Expression of hypoxia inducible factor-1α and related transfected proangiogenic genes in genetically modified myoblasts cultured in different oxygen concentrations

Genetic modification of the myoblasts caused marked overexpression of the introduced genes – either *VEGF-A* (*Fig. 5C*), *FGF-4* (*Fig. 5D*) or *PlGF* (*Fig. 5E*) even when cultured in 21% O_2. The highest overexpression of the introduced genes *PlGF* (in *PlGF*-modified cells; *Fig. 5E*) and *VEGF-A* (in *FGF-4/VEGF-A*-modified cells; *Fig. 5C*) was observed in the 3% oxygen concentration, whereas *FGF-4* was most efficiently transcribed in the normoxic condition 5% O_2 (*Fig. 5D*).

Additionally, we found that, under hypoxic conditions, *PlGF* was also overexpressed in wild type myoblasts (*Fig. 5E*). Small amounts of *VEGF-A* transripts were determined in both controls (wild type and pTruf-22) cultured un hypoxic, normoxic and in standard *in vitro* conditions (*Fig. 5C*).

Protein overexpression

We detected the highest PlGF concentration in the supernatants of the myoblasts cultured in hypoxia and standard *in vitro* conditions. Interestingly, the amount of protein from the cultures grown in 5% oxygen was at a similar level for both control cell samples (wild type and those transfected with pCi-neo), cultured in 3% oxygen (*Fig. 6C*).

The opposite situation was observed in myoblasts transfected with the *FGF-4/VEGF-A* genes. Here, we observed the highest secretion of FGF protein in the cells cultured in 21% oxygen while, in case of VEGF-A protein, in cells cultured in 5% oxygen (*Fig. 6C*).

Proangiogenic properties

The supernatants harvested from *PlGF* and *FGF-4 VEGF-A*-transfected cells significantly enhanced the process of capillary formation in each of the applied oxygen concentrations (*Fig. 7*).

Myogenic gene expression

The analysis and comparison of myogenic gene expression for all the studied cell populations (either those transfected with *FGF-4*/*VEGF-A* or *PlGF* and their respective controls) proved that, in normoxia (5% oxygen), each of the examined genes exhibited substantial downregulation in expression while generally the expression of *MYOD* exceeded *myogenin* (*Fig. 8*). Additionally, in general, the highest myogenic gene expression was upregulated under standard *in vitro* conditions (hyperoxia) in genetically modified cells, either by FGF-4/VEGF-A or PlGF.

Cell proliferation

The *FGF-4*/*VEGF*-A-transfected cell controls revealed a similar trend and did not exhibit a change in their proliferation pattern for the studied oxygen concentrations (*Fig. 9A*) in opposite to its respective controls (*Fig. 9B*, *9C*). In general, myoblasts with forced overexpression of the *PlGF* gene and their respective controls exhibited the lowest proliferation rates in normoxia, whereas in hypoxia and standard *in vitro* conditions, they exhibited similar proliferation patterns (*Fig. 9D-9F*).

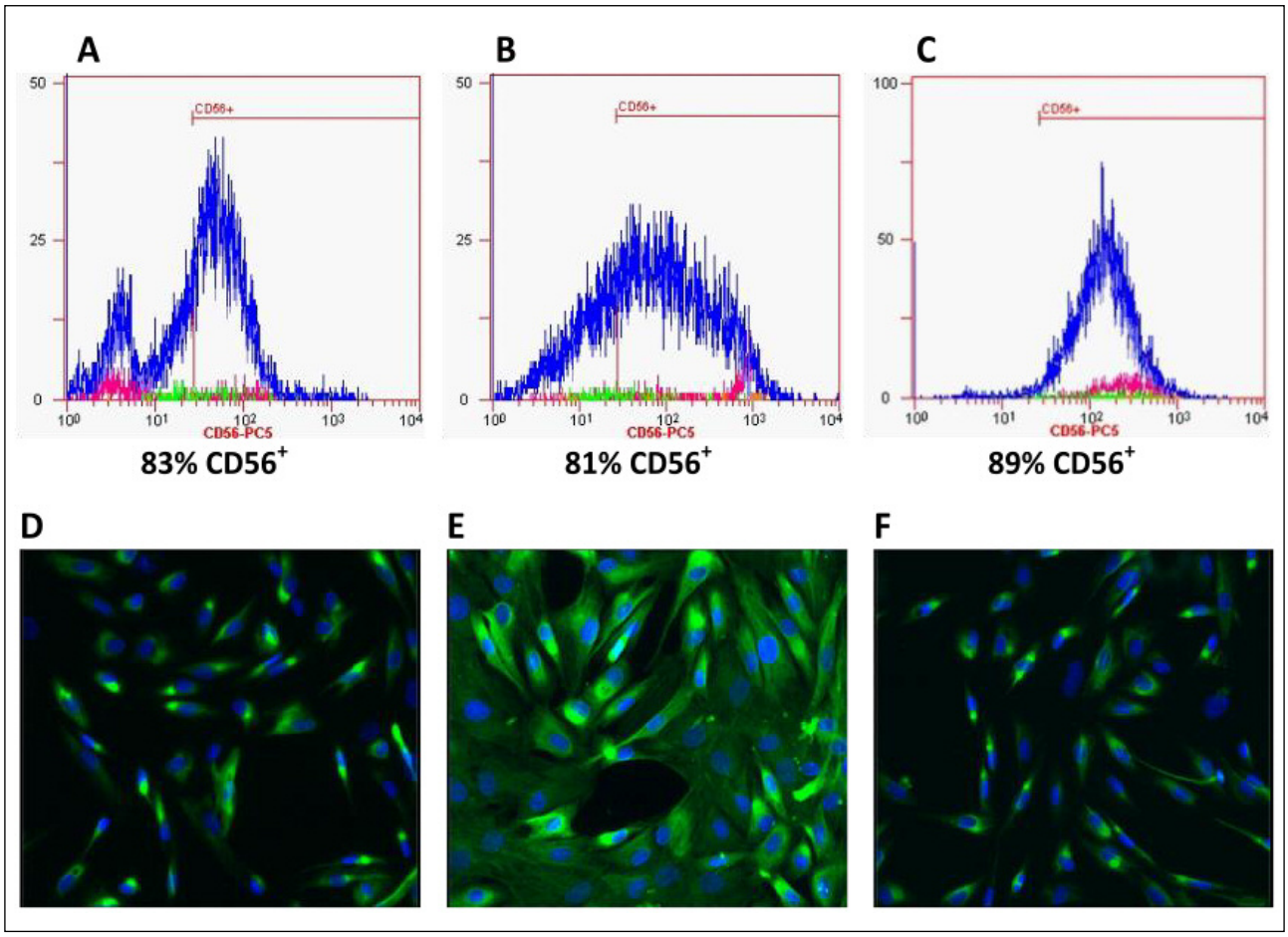

Fig. 4. Phenotypic characterization of human skeletal myoblasts. (*A*, *B*, *C*): Flow cytometry staining for the CD56 marker. (*D*, *E*, *F*): Desmin immunofluorescence staining.

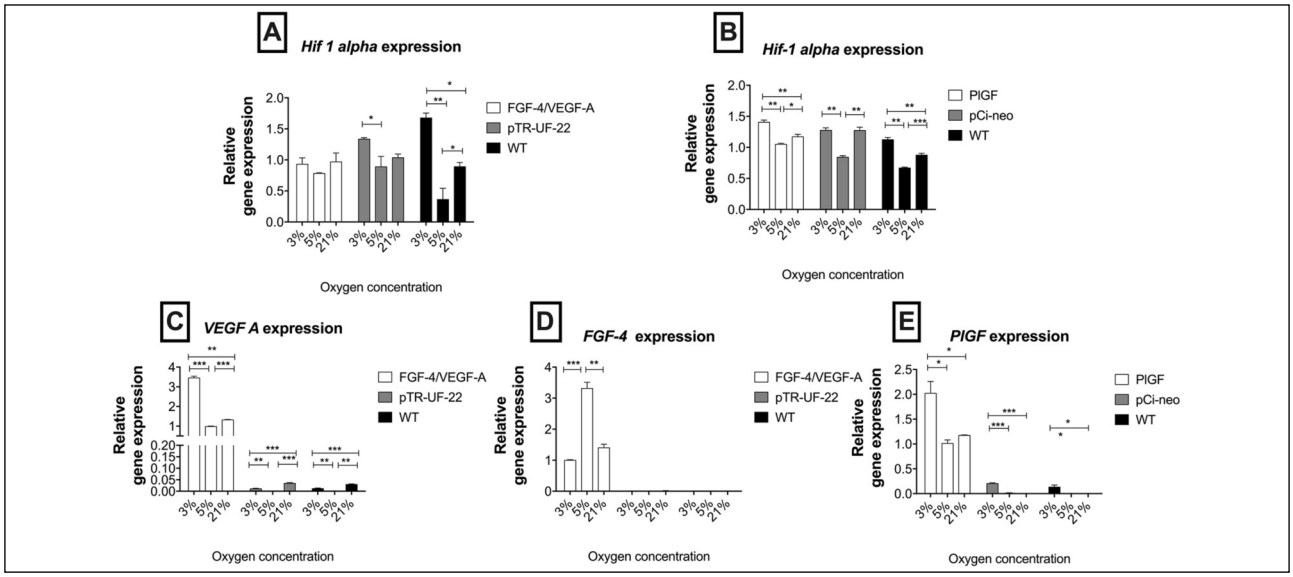

Fig. 5. Expression of proangiogenic genes in myoblasts transfected with *FGF-4*/*VEGF-A*, *PlGF*, pTruf-22, or pCi-neo control plasmids, and in wild type (WT) cells examined in *in vitro* cultures under different oxygen. concentrations: 3%, hypoxia; 5%, normoxia; and 21%, standard *in vitro* (hyperoxia) conditions. (*A*): Expression of *HIF-1α* in cells transfected with *FGF-4*/*VEGF-A* or the pTruf-22 control plasmid and in WT cells. (*B*): Expression of *HIF-1α* in cells transfected with *PlGF* or with the pCi-neo control plasmid and in WT cells. (*C*): Expression of *VEGF-A* in cells transfected with *FGF-4*/*VEGF-A* or with the pTruf-22 control plasmid and in WT cells. (*D*): Expression of *FGF-4* in cells transfected with *FGF-4*/*VEGF-A* or with the pTruf-22 control plasmid and in WT cells. (*E*): Expression of *PlGF* in cells transfected with *PlGF* or with the pCi-neo control plasmid and in WT cells. *Abbreviations*: *HIF-1α*, hypoxia inducible factor-1α; *VEGF-A*, vascular endothelial factors isoforms A; *PlGF*, placental growth factor; *FGF-4*, fibroblast growth factor 4.

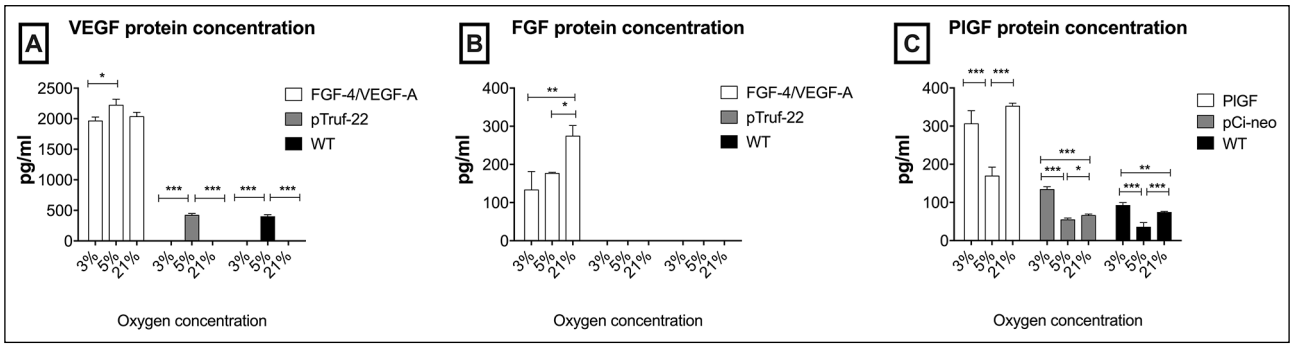

Fig. 6. Secreted proteins concentrations obtained under different oxygen concentrations: 3% - hypoxia; 5% - normoxia; and 21% - standard *in vitro* conditions (hyperoxia). (*A*): Concentration of VEGF-A protein in supernatants harvested from myoblasts transfected with the *FGF-4*/*VEGF-A* genes (FGF-4/VEGF-A) or the pTruf-22 control plasmid (pTruf-22) and WT cells. (*B*): Concentration of FGF-4 protein in supernatants harvested from myoblasts transfected with the *FGF-4*/*VEGF-A* genes (FGF-4/VEGF-A) or the pTruf-22 control plasmid (pTruf-22) and WT cells. (*C*): Concentration of PlGF protein in supernatants harvested from myoblasts transfected with the *PlGF* gene (PlGF) or the pCi-neo control plasmid (pCi-neo) and wild type (WT) cells.

Apoptosis

We detected slightly higher cell mortality in the genetically modified cell populations after transfection versus wild type cells. In population modified with *PlGF* and their related controls, cytometric analysis revealed the highest apoptotic rate in normoxia (*Fig. 10B*); however, we were not able to confirm this trend in cell populations modified with *FGF-4/VEGF-A* (*Fig. 10A*).

Myotubes formation

Hypoxia generated the highest percentage of unfused nuclei in the cell populations under study (*Fig. 11*).

DISCUSSION

It is well known that certain fish and amphibians retain a robust capacity for cardiac regeneration throughout life span, but the heart of adult mammals is not able to sufficiently regenerate after infarction. Although there is some evidence that heart of 1-day-old neonatal mice can regenerate after partial surgical resection, this capacity disappears by 7 days of age (20, 21). Poor ability of adult myocardium to self-renewal leads to a loss of cardiomyocytes which can be replaced by proliferating fibroblasts. The remodelling starts within the first twenty-four hours after infarction and lasts till 28th day when the scar tissue is formed and in general there are visible morphological anomalies of the heart (*Fig. 1A*). However, it is also important to note that during and after infarction there is ongoing inflammatory reaction within the heart. Dying cardiomyocytes during acute myocardial ischemia induce the pro-inflammatory response through the production of danger associated molecular patern (DAMPS), reactive oxygen species (ROS), and complement, which through the release of cytokines (such as IL-1β, IL-18, IL-1α, IL-6, CCL2, CCL5), mediate the accumulation of a variety of cells including neutrophils, monocytes, macrophages, B lymphocytes and $CD8^+$ T cells into the infarct zone. The subsequent anti-inflammatory reparative phase, mediates the resolution of the inflammatory response through the production of anti-inflammatory factors (such as IL-10, IL6, transforming growth factor-β), and changes in monocytes and macrophages, and the recruitment of Tregs, $CD4^+$ T cells and dendritic cells. As it was shown in *Fig.1B* either 24 hours or 7 days after infarction we were able to expose a massive inflammatory reaction using immunohistochemistry. Therefore, therapies involving stem cells transplantation are usually carried out when the scar tissue is formed and the inflammation gradually disappears – in the mouse model it is a period of about a month after the infarction. Inflammatory response serves to repair the heart, but excessive inflammation leads to adverse left ventricular remodeling and heart failure. It is also worth to point that recruitment of inflammatory cells plays a crucial role in capillary growth.

Expression profiling using microarrays enables to identify changes in global gene expression while linking them to the observed heart remodelling stages (22). We observed that *Hif-1α* reached its highest expression at 24 hours after MI in murine heart. We concluded that, at this point, acute hypoxia prevails in the heart (*Fig. 2A*). At the same time *Vegf-a, Plgf* and *Flt-1* were also upregulated (*Fig. 2I*). This tendency is not difficult to explain. HIF-1 regulates more than 2% of genes in vascular endothelial cells, either directly or indirectly (23). As a transcription factor, HIF-1 binds to HRE elements localized into different genes (*VEGF-A*, *PlGF* and *Flt-1*). In fact, *PlGF* expression under hypoxia seems to be cell-type specific. In human trophoblast (24), microvascular endothelial cells (25) and human hepatoma cells (hepG2) (26), hypoxia either reduced or played a neutral role with respect to *PlGF* expression. Nonetheless, in cardiac cells, hypoxia influences clearly *PlGF* expression. Moreover, in neonatal rat cardiomyocytes maintained longer than 6 h in less than 2% O_2, the expression of *PlGF* dramatically increased and reached the highest point after 24 hours. The activity of the *PlGF* gene was also shown in human hearts with advanced fibrosis, which is a long-term consequence of MI (27). We were able to prove that murine hearts exhibited the highest expression of *Plgf* at 24 h after MI, and this level decreased at further time points (*Fig. 2C*). *Vegf-a* significantly responded to hypoxia at 24-h and 30 days after MI. Its upregulated expression after 24 h was directly related to enhanced *Hif-1α* expression (*VEGF-A* is tightly regulated by hypoxia). Later, hypoxia interfered with *Vegf-a* expression, which dramatically increased in the chronic phase of ischaemia at 30 days after MI (*Fig. 2B*). This result may also indicate that, at this time point, the post-infarction scar is fully formed, and the surrounding tissues likely start to regenerate (after MI), initiating angiogenesis. Interestingly, the *Kdr* receptor gene also upregulated its expression 30 days after MI, whereas at earlier time points, its level was stable or slightly repressed (*Fig. 2H*). This suggests that there are differences in the regulation of two principal angiogenic receptors due to hypoxia (28) or that the

response could also be a cell-type specific process, as in case of *PlGF*. Waltenberger *et al*. suggested that hypoxia is able to stimulate the expression of *KDR* in HUVECs and transfected porcine aortic endothelial cells (29). On the other hand, Gerber *et al*. (28) concluded that hypoxia may indeed increase *FLT-1* expression but not *KDR* in HUVECs. In our case, increased expression of *Kdr* at 30 days after MI coincided with the elevated expression of *Vegf-a*. We might conclude, therefore, that the *Kdr* receptor gene can be indirectly regulated, for example, by upregulation of *Vegf-a* in an acute phase of hypoxia. Simultaneous expression of both receptors at the chronic phase of hypoxia (30 days after MI) may reflect the need for more

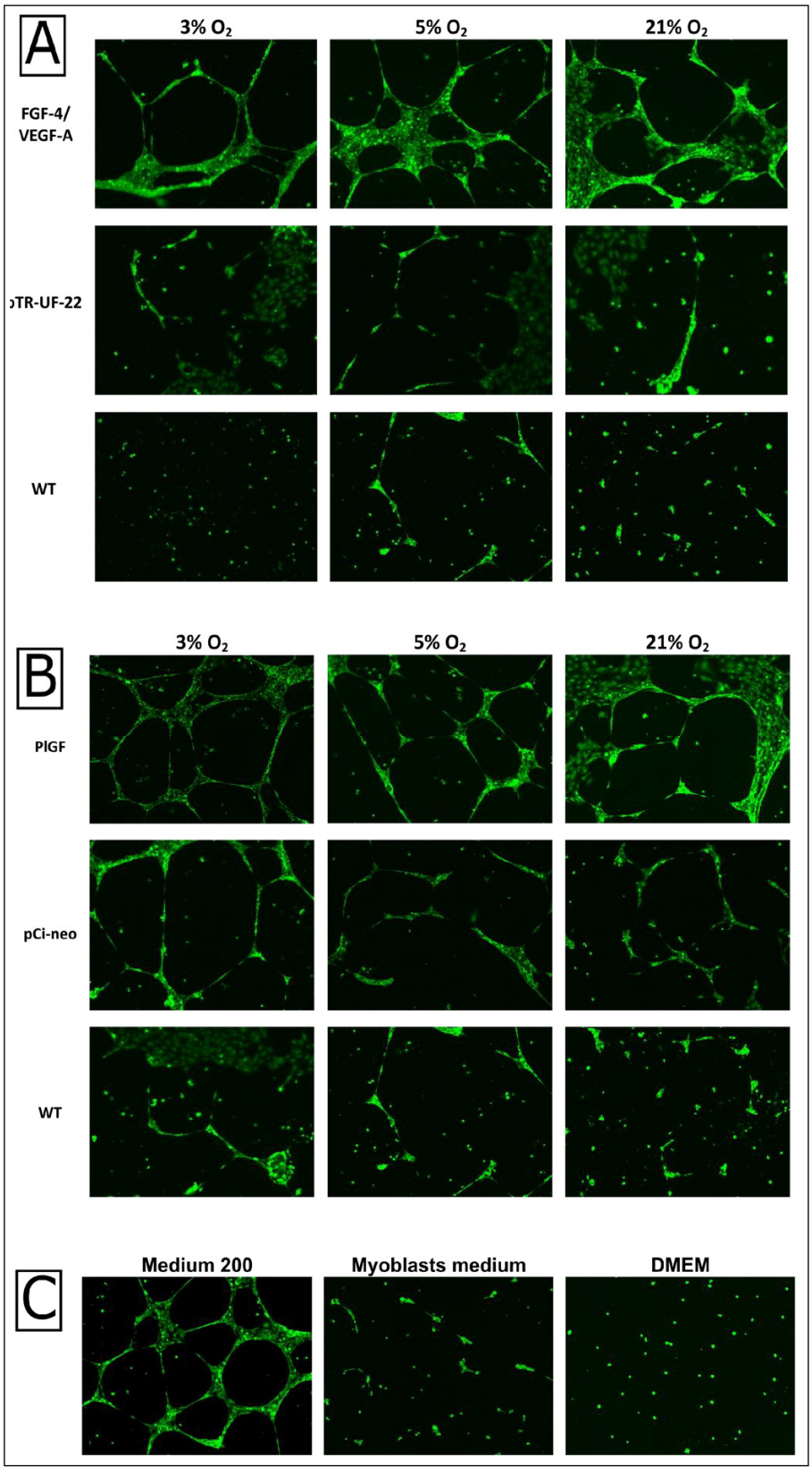

Fig. 7. Proangiogenic test. (*A*): Photographs of capillaries formed upon stimulation with proangiogenic proteins contained in the supernatants harvested from myoblasts transfected with *FGF-4*/*VEGF-A* or pTruf-22 control plasmids and wild type cells cultured for 24 hours in 3% O_2 (hypoxia), 5% O_2 (normoxia), and 21% O_2 (standard *in vitro* conditions). (*B*): Photographs of capillaries formed upon stimulation with proangiogenic proteins contained in the supernatants harvested from myoblasts transfected with the *PlGF* gene or the pCi-neo control plasmid and wild type cells cultured for 24 h in 3% O_2 (hypoxia), 5% O_2 (normoxia), and 21% O_2 (standard *in vitro* conditions). (*C*): Photographs of capillaries formed in Medium 200 supplemented with Large Vessel Endothelial Cells Supplement (positive control); capillaries formed in myoblast medium (negative control); capillaries formed in DMEM (negative control).

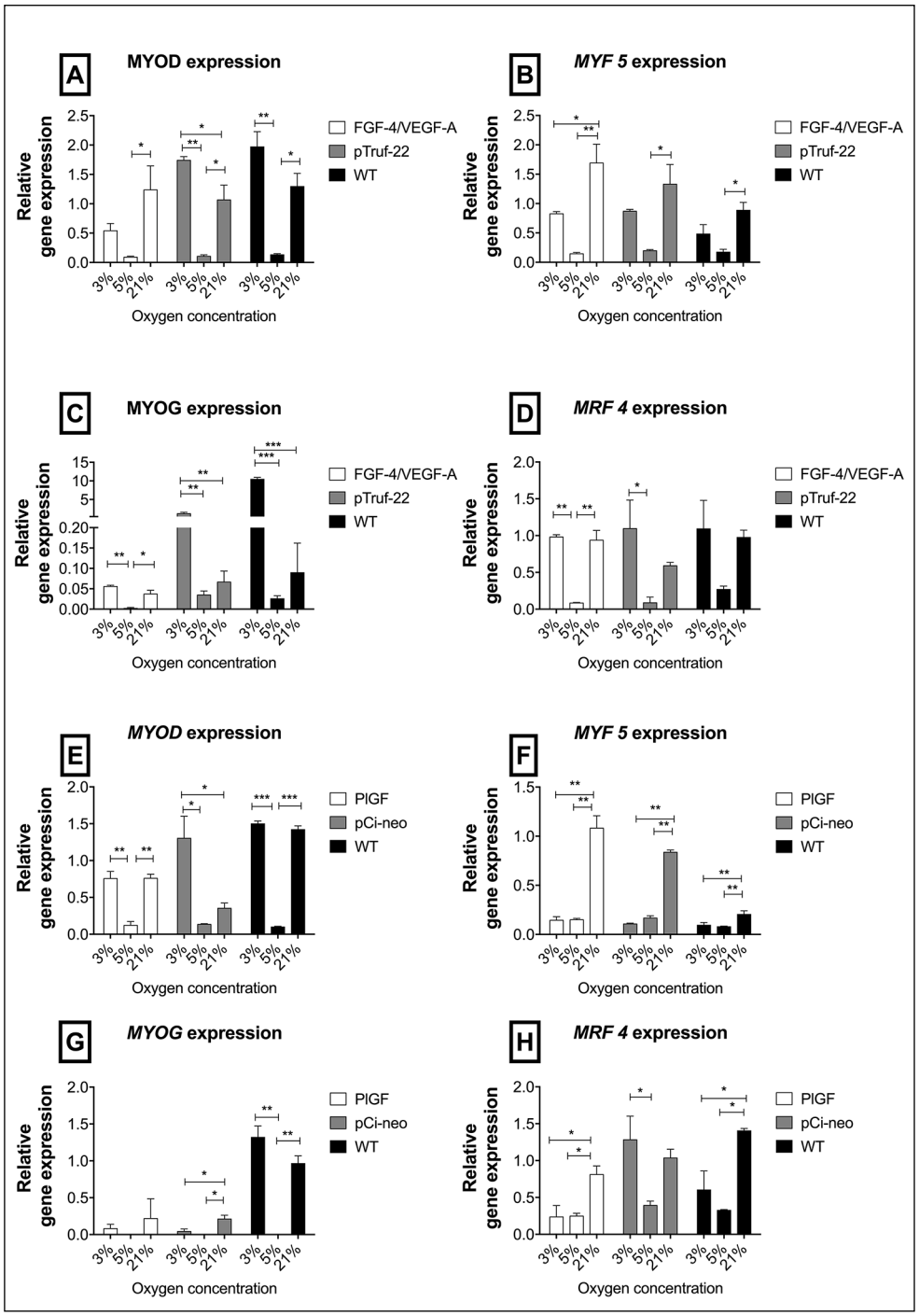

Fig. 8. Myogenic gene expression in myoblasts transfected with *FGF-4*/*VEGF-A*, *PlGF*, pTruf-22, or pCi-neo control plasmids, and WT cells examined in different oxygen concentrations: 3%, hypoxia; 5%, normoxia; and 21%, standard *in vitro* conditions. (*A*, *E*): *MYOD* expression; (*B*, *F*): *myogenic factor 5* expression (*MYF 5*); (*C*, *G*): *myogenin* expression (*MYOG*); (*D*, *H*): *myogenic factor 4* expression (*MRF4*).

complex regulation than by only *Flt-1*, being critically dependent on the integration of signals generated by ischaemia that are essential to activate angiogenesis in the post-infarcted region. Most importantly, we were able to prove that our genes of interest (*Vegf-a* and *Plgf*) were positively regulated by hypoxia *in vivo* in murine post-infarction heart (*Fig. 2B*, *2C*).

Next, we decided to test whether these factors could be similarly regulated under hypoxia *in vitro* in myoblast progenitor cells. However, this part of the study first required to the establishment of the most accurate *in vitro* O_2 level corresponding to the hypoxia level prevailing in myocardium after MI. To our knowledge, no study has previously reported such an experiment. It is interesting that *HIF-1α* in long-term *in vitro* cell cultures in diminished O_2 levels was slightly downregulated (*Fig. 3B*). We concluded that there could be a mechanism that may silence *HIF-1α* expression during long periods of hypoxia, likely to prevent cells from undergoing pathological changes. The mechanism leading to normoxic degradation of HIF-1α has been elucidated as previously described by Kaelin and Masson (30, 31). Thus, under prolonged hypoxia, the cells are able to downregulate the expression of *HIF-1α*, likely by PHD upregulation by HIF-1 itself. This autoregulation therefore works as a negative feedback loop (32).

The exposure of stem cells to a non-physiological hyperoxic state in general CO_2 incubators may lead to oxidative stress and induce DNA damage. Hyperoxia is known to be associated with excessive formation of ROS, especially after chronic exposure. ROS exert harmful action towards the subcellular structures.

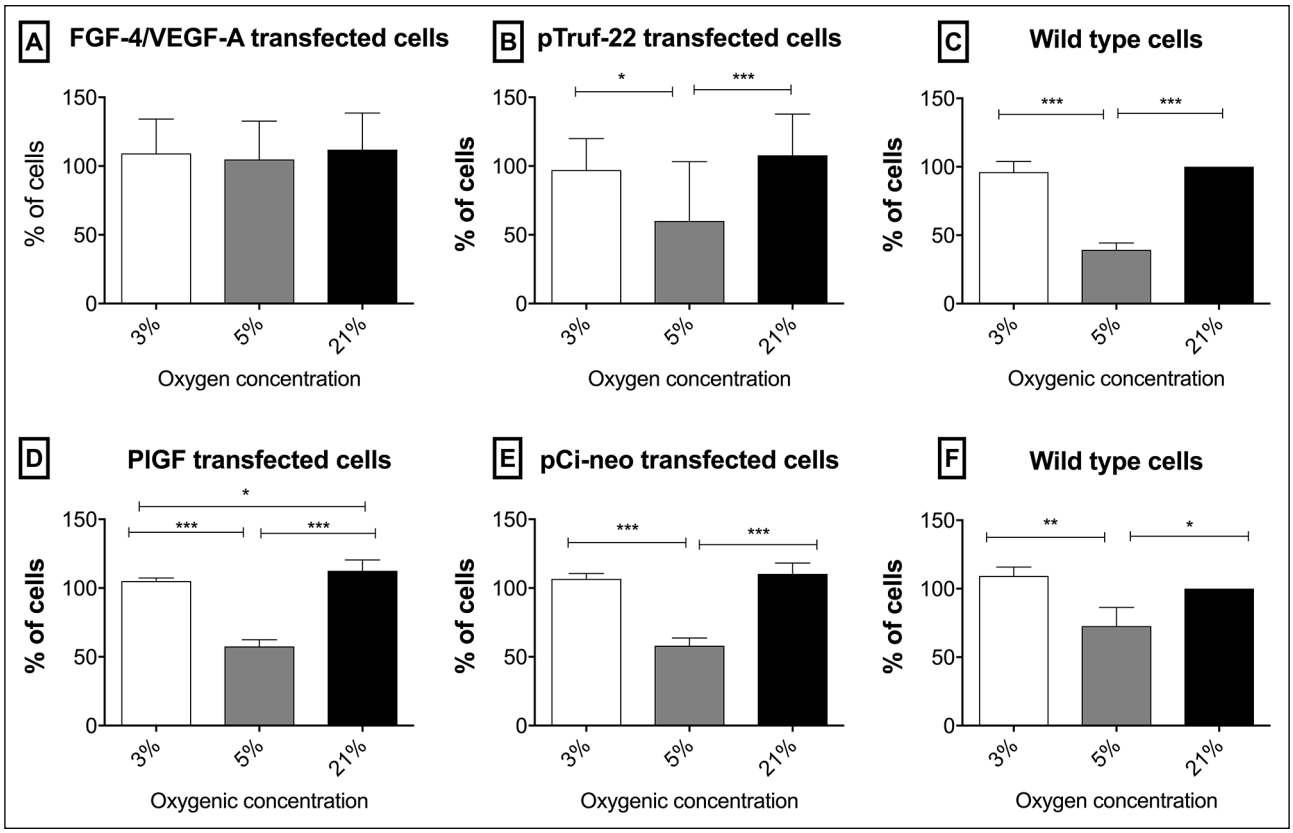

Fig. 9. Cell proliferation of cells transfected with *FGF-4*/*VEGF-A*, *PlGF*, pTruf-22, or pCi-neo control plasmids, and WT cells evaluated by the MTS test in different oxygen concentrations: 3%, hypoxia; 5%, normoxia; and 21%, standard *in vitro* conditions (hyperoxia). (*A*): Proliferation of cells transfected with *FGF-4*/*VEGF-A*. (*B*): proliferation of cells transfected with the pTruf-22 control plasmid. (*C*): proliferation of wild type cells. (*D*): proliferation of cells transfected with the *PlGF* gene. (*E*): proliferation of cells transfected with the pCi-neo control plasmid. (*F*): proliferation of wild type cells.

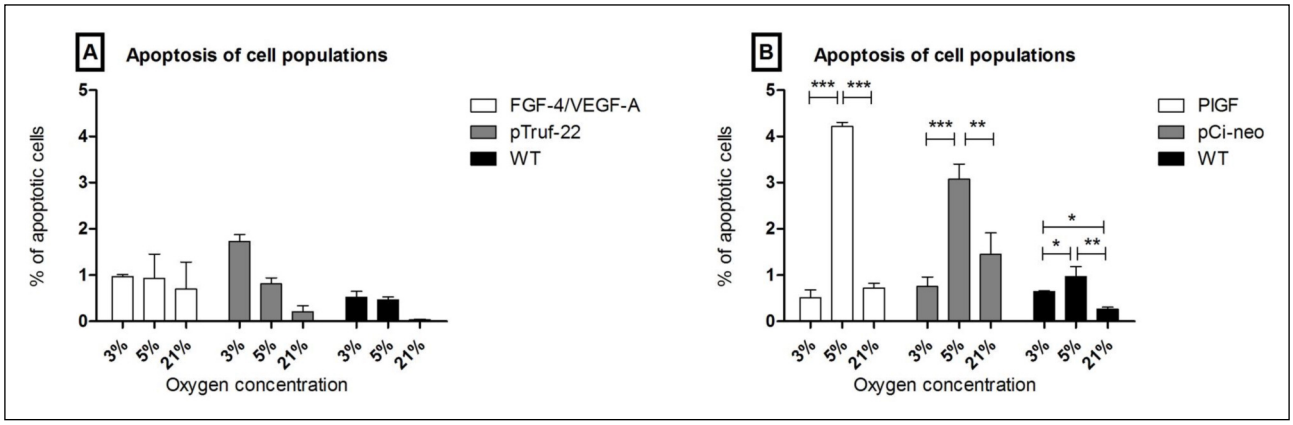

Fig. 10. Apoptosis of all the cell populations under study maintained in 3% O_2 (hypoxia), 5% O_2 (normoxia), and 21% O_2 (standard *in vitro* hyperoxic conditions). (*A*): apoptotic cells in myoblasts transfected with *FGF-4*/*VEGF-A* genes (FGF-4/VEGF-A) or the pTruf-22 control plasmid (pTruf-22) and wild type cells. (*B*): apoptotic cells in myoblasts transfected with the *PlGF* gene (PlGF) or the pCi-neo control plasmid (pCi-neo) and wild type cells.

However, in human microvascular endothelial cells, hyperbaric oxygen induces cytoprotective and angiogenic responses against oxidative insults, but the changes have been minimal following treatment with 100% O_2 in the absence of elevated pressure. Hyperoxia may also modify the response to different vasoactive stimuli by enhancing vasodilatation in certain circumstances. Moreover hyperoxic preconditioning significantly improved the post-ischaemic functional recovery of rat and mice hearts, reducing the infarct size, and necrotic damage (33). Thus in our study we examined the cells also in hyperoxic conditions, comparing their biological and therapeutic properties in 3 different oxygen concentrations.

For muscle precursor stem cells, normoxia is considered to be 5% O_2. This fact has been reported in previous studies (3), and we were also able to show that myogenic progenitors, among all the myoblast populations cultured for 24 hours,

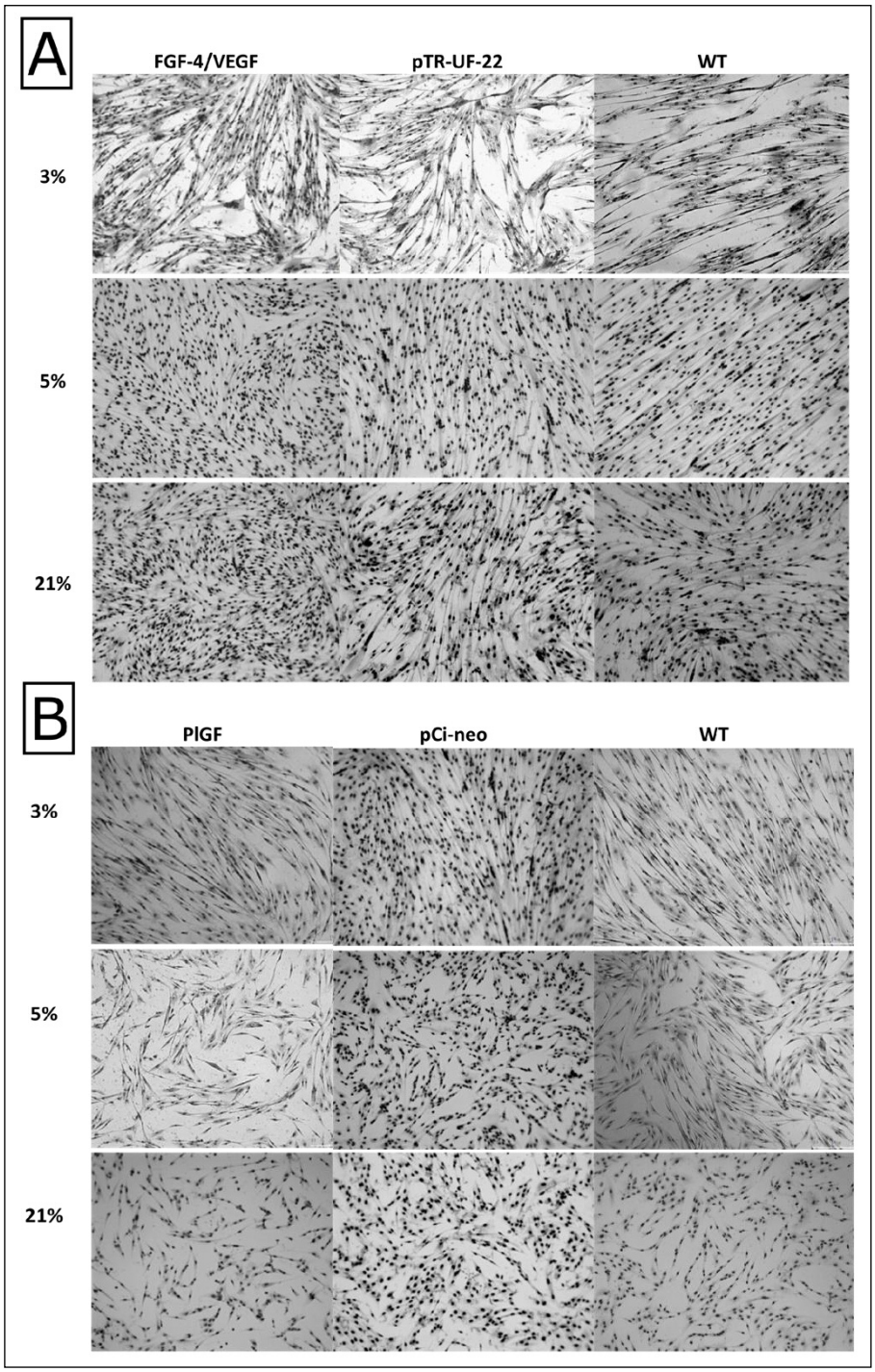

Fig. 11. Photographs of fused *FGF-4*/*VEGF-A*- and *PlGF*-transfected myoblast cells maintained in 3% O_2 (hypoxia), 5% O_2 (normoxia), and 21% O_2 (standard *in vitro* conditions). (*A*): Myoblasts transfected with *FGF-4*/*VEGF-A* genes (FGF-4/VEGF-A), pTruf-22 control plasmid (pTruf-22) and wild type cells. (*B*): myoblasts transfected with the *PlGF* gene (PlGF), myoblasts modified with the pCi-neo control plasmid (pTruf-22), wild type cells.

exhibited the lowest *HIF-1α* expression when incubated *in vitro* in 5% oxygen (*Fig. 3B*). After comparison of the *in vitro* and *in vivo* experiments, we established that only cells cultured for 24 hours in 3% oxygen exhibited *in vitro* similar *HIF-1α* levels with no statistically significant differences to *HIF-1α* expression in myoblasts transplanted into murine post-infarcted heart (*Fig. 3C* and *3D*). Therefore, 3% oxygen for *in vitro* hypoxic myoblast cell culture was considered equivalent to post-infarcted heart environment. Meanwhile, according to the literature hypoxia for myoblasts cells was previously established as 0.5 – 2% O_2. However, the 3% O_2 concentration mimicking the the post-infarctional hypoxia was able to induce the highest expression of HIF-1α expression in the cells of myogenic origin.

We genetically modified human myoblasts (transient transfection) with the proangiogenic genes *FGF-4*/*VEGF-A* or *PlGF* and compared the profiles of angiogenic gene expression (*Fig. 5*). In both transfections, we obtained high and significant expression of the corresponding transgenes. Moreover, in *PlGF* control cell populations (pCi-neo and WT) cultured under hypoxic conditions, we also detected visible *PlGF* gene expression (*Fig. 5C*) consistent with dramatic upregulation of *PlGF* in the heart at 24 h after MI (*Fig. 2C*). This analogy led to the conclusion that *PlGF* is upregulated in hypoxia, not only in cardiomyocytes, but possibly also in myoblast cells, and this is another striking similarity between these two types of cells that can be used as an advantage for application of myoblasts for

therapeutic treatment of post-infarcted heart. Most importantly, the overexpressed proteins were fully functional (*Fig. 7*). We detected many capillaries in the HUVECs cultured in supernatants from transfected myoblasts, which was comparable to the positive control (Medium 200). Despite the fact that 3% oxygen did not enhance proangiogenic protein secretion, we were able to confirm that hypoxic conditions did not disturb the functionality of proteins because the harvested supernatants from the transfected cells (maintained in 3% O_2) strongly stimulated capillary formation (*Fig. 7*). Taking into account these results and the fact that *in vitro* hypoxia (3% O_2) corresponded to the oxygen conditions prevailing in the post-infarcted myocardium, we may cautiously postulate that the grafted genetically modified myoblasts can well fulfil their proangiogenic role after transplantation into ischaemic hearts.

Myogenic gene expression revealed a very unexpected trend. Cells cultured for 24 hours in 5% oxygen, which is considered as normoxia for skeletal muscle, exhibited diminished expression of all the myogenic genes in each cell population studied (*Fig. 8*). A previous report indicated the opposite trend during *in vitro* culture (hyperoxia) where with increasing myoblast density, *MYOD* gene expression increased, followed by a delayed increase in *myogenin* (*MYOG*) gene expression (34). Our study indicates that myoblast cells, in general, exhibit downregulated myogenic gene expression after *in vitro* culture in 5% oxygen, which is then followed by a decrease in the proliferation of cells in normoxia- with one exception- FGF-4/VEGF-A modified cells (*Fig. 9*). Moreover, in the case of myogenic gene expression, it was previously reported that human myoblasts (35), primary rat cells (3), and bovine satellite cells (36) exhibited upregulated *MYF 5* and *MYOD* expressions. Because MYOD and MYF5 are transcription factors that take part in the early stage of myogenesis, this could be promising scenario for cell propagation, as opposed to cells that exhibit myogenin prevalence, which is activated at the point of cell differentiation. In turn, a C2C12 murine myoblast cell line exhibited transient downregulation of the *MyoD* gene (37). Both *FGF-4*/*VEGF-A*- and *PlGF*-transfected cells seemed to increase their *MYOD* expression inversely to *MYOG* expression (*Fig. 8A* versus *8C* and *8E* versus *8G*). We also observed upregulation of the *MYOG* gene in the wild type myoblast population (*Fig. 8C*, *8G*) cultured under 3% oxygen concentration. There were no marked differences, however, in *MYF5* gene expression, with the exception of its suppression during hypoxia, for both types of genetically modified cells (*Fig. 8B*, *8F*). This result may justify speculation that an increase in the expression of early myogenic genes may potentially enhance myoblast propagation *in vitro,* as an inverse relationship between *MYOD* and *MYOG* gene.

It has been proven that for the most cell types, hypoxia induced a decreased cell proliferation. It seems to be obvious since an increased number of cells increases O_2 demand. However, certain cell populations maintain cell proliferation during prolonged time of hypoxia. This is a common pathological feature of cancer cells, but can also serve as physiological in the maintenance of the stem cell population that reside in a hypoxic niche. It is worthy to point out that muscle tissue is rather resistant to hypoxia because during extended exercise the environment becomes anaerobic. Diminished oxygen supply is one of the physiological trigger that activate the satellite cells to proliferate, whereas normoxic conditions, may maintain the cells in quiescent state (*Fig. 9*). We could put the attention that low proliferation rate was consistent with the silenced myogenic gene expression within the examined myoblast populations (*Fig. 8*), which can lead to conclusion that in normoxia cells may remain quiescent.

According to the literature, apoptosis under hypoxia seems to be a controversial aspect. Some reports indicate that the molecular changes during hypoxia may shift the balance of pro and antiapoptotic genes. In our case, hypoxia that was established to mimic the oxygen conditions prevailing in the post-infarcted myocardium did not cause a massive apopototic death within the studied cell populations (*Fig. 10*). Moreover, genetically modified cells exhibited relatively low level of apoptosis. In case of VEGF there is some evidence that this factor possess some antiapoptotic features. Addition of VEGF prevented HUVEC apoptosis from the high glucose exposure. What is interesting, elevated ROS generation, calcium overload, Bax/Bcl-2 ratio, caspase-3 activation in HUVEC induced by high glucose were reversed by VEGF. This may represent a mechanism for the anti-apoptotic effect of VEGF (38). In case of PlGF there are some evidences on their role in induction of apoptosis but under hypoeroxic conditions (39). Our study revealed that in 3% O_2 genetic modification with *PlGF* gene generated lower apoptosis then in *FGF-4*/*VEGF-A* myoblasts (*Fig. 10*). This may indicate a protective role in respect to myoblasts cells which can be transplanted into hostile environment.

The presented results are focused on expanding our knowledge of hypoxia. A very important aspect of our study was to evaluate the changes in proangiogenic gene expression at different time points in post-infarcted heart. The time dependent analysis allowed us to understand changes in myocardium during hypoxia in terms of proangiogenic gene expression. We believe that, in post-infarcted heart, not only proangiogenic genes participate in regeneration, but this is orchestrated by a variety of other genes, including those taking part in inflammatory response and cell recruitment. Because human myoblasts have been considered as promising cell therapeutic candidates, we evaluated the influence of hypoxia on these cells and compared the results obtained with the other cell samples cultured under physiological oxygen conditions (5%) or standard *in vitro* conditions (hyperoxia). The most important fact was that, due to *in vitro* optimization of hypoxia, we obtained similar oxygen profiles *in vitro* that corresponded to murine conditions in post-infarcted hearts. To the best of our knowledge, we are the first group to adjust the *in vitro* hypoxic conditions of cells to their corresponding *in situ* concentrations to study their functions which can be relevant for future cellular therapies. The examination of myoblasts modified with either proangiogenic factors or 'native' cells cultured *in vitro* under hypoxia, normoxia and hyperoxia led us to the following conclusions: 1) There is no negative influence of hypoxia on the biological functions of genetically modified or wild type myogenic progenitor cells. Additionally, HIF-1 as a transcription factor enhances the expression of genes that are meant to stimulate angiogenesis. 2) Short duration of normoxia negatively influences myogenic gene expression and cell proliferation. However, myoblasts are able to fully differentiate under prolonged exposure to either hypoxic or normoxic conditions. 3) Standard *in vitro* conditions, sometimes termed hyperoxia, did not markedly influence the biology of the cells. However, in the absence of the active HIF-1 transcription factor, the cells may exhibit low expression of proangiogenic genes. The major conclusions of this study, however, are that myoblasts can be used for the regeneration of ischaemic heart and that the hypoxic conditions prevailing in the post-infarcted myocardium may provide an environment for the high expression of proangiogenic genes that can be translated into functional proteins. This in turn may potentially improve conditions within the area of the post-infarction scar by new blood vessel formation.

Abbreviations: ANGPT, angiopoietin; CDS, coding DNA seguence; DAMPS, danger associated molecular patern; DMEM, Dulbecco's modified Eagle's medium; FGF-4, fibroblast growth factor 4; FLT-1, Fms related tyrosine kinase 1; HIF-1, hypoxia

inducible factor 1; HRE, hypoxia response element; HUVEC, human umbilical cord vein cells; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MI, myocardial infarction; PCR, polymerase chain reaction; PDGFB, platelet derived growth factor subunit B; PlGF, placental growth factor; ROS, reactive oxygen species; SDF-1, stromal cell-derived factor 1; SkMC, skeletal muscle stem cells; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

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Conflict of interests: None declared.

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