Isolation of Insulin-Producing Cells from Different Populations of Multipotent Stromal Cells of the Umbilical Cord and Human Adipose Tissue

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Stromal cells of adipose tissue and human umbilical cord were isolated by the original method from general populations of multipotent subpopulation of multipotent stromal cells exhibiting perivascular phenotype ($CD146^+$, $CD31^-$). Effective directed differentiation of these cells into insulin-producing cells by transient transfection of the gene *Pdx1* was demonstrated. Transfection multipotent stromal cells $CD146^-$, $CD31^-$ derived from adipose tissue and umbilical cord and isolated by the standard method, did not result in activation of insulin gene transcription. It was shown that the expression of nestin was not necessary for effective pancreatic cell differentiation.

Key Words: *multipotent stromal cells of adipose tissue and umbilical cord; diabetes; insulin-producing cells; Pdx1*

Diabetes mellitus is one of the most prevalent diseases in the world. The number of diabetic patients increases almost twice every year. More than 5 million people (of them about 400,000 children) worldwide suffer from type 1 diabetes mellitus (DM1). Shortcomings of modern methods of DM1 treatment (insulin therapy and transplantation of pancreatic islets) necessitate the search for alternative sources of β-cells.

In light of this, new methods of regenerative cell therapy providing selection and *ex vivo* pre-differentiation of embryonic and adult stem cells are now actively developed and explored. Some studies showed directed *in vitro* differentiation of embryonic stem cells (ESC) into functionally active insulin-producing cells by culturing in specific media or retroviral transfection of genes encoding several transcription factors [3,6,11]. Many studies showed that effective directed differentiation of ESC into insulin-producing cells requires the stage of selection of cells expressing nestin. It is known that β-cells of the pancreas originate from the endoderm and express nestin at the early stages of embryonic development. Injection of cells obtained by differentiation of nestin-expressing ESC to DM1 mice reduced glycemia in these animals [5]. Most insulin-producing cells obtained from nestin-expressing cells did not synthesize insulin *de novo*, and in some cases they concentrated exogenous insulin from the culture medium, and then it was excreted by apoptotic cells [9]. Moreover, the obtained cells producing insulin had neural characteristics. It remains unclear whether the stage of the generation of nestin-expressing cells is necessary for obtaining insulin-producing cells.

Adipose tissue and umbilical cord are promising sources of mesenchymal stem cells (MSC). The main advantages of MSC from the adipose tissue and umbilical cord are their availability and the possibility of using these cells for autologous transplantation. MSC from these sources can differentiate into chondroblasts, osteoblasts, adipocytes, and myoblasts [14,17].

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Experimental data indicate that MSC *in vivo* are presented by perivascular cells (pericytes) that are identified by the expression of CD146 marker [1,4]. We showed that cells with this phenotype can be isolated from the adipose tissue and then propagated [1].

Transcription factor *Pdx1* (pancreatic duodenal homeobox 1) plays a key role in the differentiation of pancreatic β-cells [10,12]. Stable transfection of *Pdx1* gene induces the differentiation of ESC and MSC BM into insulin-producing cells [7,9]. Stable transfection is caused by incorporation of expressing cassette into the nuclear genome of the cell, which can result in oncotransformation. Thus, the obtained insulin-producing cells cannot be applied in clinical practice. That is why the greatest interest is the possibility of induction of pancreatic differentiation by transient transfection, *i.e.* without changing the structure of the nuclear genome.

The aim of the work was to receive insulin-producing cells from different populations of MSC adipose tissue and umbilical cord by adenoviral delivery of *Pdx1* gene.

MATERIAL AND METHODS

Obtaining cell cultures. Stromal-vascular fraction of the adipose tissue was obtained from the lipoaspirate of healthy donors. The adipose tissue was disaggregated with 1 mg/ml collagenase type 1 (PanEco) and 200 mg/ml dispase (PanEco) for 1 h at 37°C under conditions of constant shaking.

Umbilical cords were obtained from healthy women (all patients signed informed consent for participation in the study). Blood vessels isolated from umbilical cord were minced with scissors and then disaggregated with a mixture of enzymes 200 mg/ml dispase (PanEco) and 1 mg/ml collagenase (PanEco) over 4 h at 37°C in the Thermo-Shaker (1:2 v/v tissue—enzyme solution ratio).

The suspensions were centrifuged for 10 min at 1100 rpm and the cell pellet was resuspended in a small volume of DMEM/F12. The cells were cultured according to standard protocol [14] in DMEM/F12 (1:1; PanEco) supplemented with 10% FCS (PanEco), 2 mM L-glutamine (PanEco), 10 ng/ml recombinant basic FGF-2 (Prospec), 8 U/ml heparin (BRAUN), and 100 mg/liter amikacin (Krasfarma). The cells were seeded on 90-mm plastic Petri dishes (Corning) and grown to confluence in a CO_2 incubator (37°C, 5% CO_2).

Umbilical cords and adipose tissue were also used for isolation of MSC expressing perivascular phenotype ($CD146^+$). To this end, some cells were cultured according to previously published protocol for isolation of $CD146^+$-cell populations [1]. The cells were seeded in DMEM/F12 (1:1) with 10% FCS, 2 mM L-glutamine, 10 ng/ml FGF-2, 8 U/ml heparin, and 100 mg/liter amikacin; recombinant human insulindissolved in 1 M hydrochloric acid was added to a final concentration of 5 ng/ml. After 3-day incubation, nonadherent cells were transferred to new dishes and growth medium was changed.

Method of immunofluorescent flow separation of cells. Cell population (MSC $CD146^-$) from umbilical cord and adipose tissue obtained by the standard method and by the previously developed protocol (MSC $CD146^+$) were characterized by the presence of antigens CD29, CD44, CD49a, CD73, CD90, CD166, CD146, CD31 with Flow Cytofluorometer FACS Calibur (BD Biosciences). Mouse monoclonal antibodies (PharMingen and Chemicon) were used. Nonspecific mouse or rabbit antibodies of the same manufacturers served as the negative control. The results were processed using WINMDI 2.8 program.

TABLE 1. Primers Used in the Study

Gene	Primer		Annealing temperature	Amplicon length, b.p.
	5' (forward)	3' (reverse)		
Insulin	CCGCAGCCTTTGTGAACC	CGGGTCTTGGGTGTGTAGAA	59	100
NGN3	CCCTCTACTCCCCAGTCTCC	CCTTACCCTTAGCACCCACA	62	176
NeuroD	ACAGCTCCCATGTCTTCCAC	AAGATTGATCCGTGGCTTTG	59	250
MafA	CTTCAGCAAGGAGGAGGTCA	TTGTACAGGTCCCGCTCTTT	59	195
Pdx1	GAGCTGGCTGTCATGTTGAA	TTGTCCTCCTCCTTTTTCCA	59	88
ActB	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATAC	60	144
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	60	87
Nestin	GCCCTGACCACTCCAGTTTA	GGATCCTGGATTTCCTTCC	59	497

Adenovirus construction. Synthesis of *Pdx1* gene with flanking sequences containing restriction sites was carried out by Evrogen Company. The gene was cloned under human cytomegalovirus promoter and introduced into human adenovirus serotype 5 by homologous recombination in *E. coli*. Adenoviral particles were obtained using HEK-293T cells transfected with a plasmid with the full genome of the recombinant adenovirus. Monolayer HEK-293T cells were washed with PBS (PanEco) and then added to medium DMEM/F12 (1:1; PanEco) with 1% FCS (PanEco), 2 mM L-glutamine (PanEco), 100 mg/liter amikacin (Krasfarma), and adenovirus. The cells were incubated for 2-3 days for the penetration of adenovirus, after which the cells with the medium were frozen at -70°C, thawed at 37°C, and then carefully pipetted and centrifuged at 3000 rpm for 20 min. Supernatants containing adenovirus particles were collected.

Cells transfection and differentiation. After the second passage, the cell monolayer was incubated in DMEM/F12 (1:1; PanEco) with 1% FCS (PanEco), 2 mM L-glutamine (PanEco), 100 mg/liter amikacin (Krasfarma) and equal volume of freshly adenovirus for 2, 6, 24 or 30 h, removed with trypsin (PanEco), and placed in 6-well plates in differentiation medium consisting of CMRL-1066 (Invitrogen), 10 mmol/liter GLP-1 (Prospec), 1% BSA (PanEco), and 100 mg/liter amikacin (Krasfarma).

Analysis of changes in gene transcription. RNA was extracted using commercial reagents RNeasy Mini Kit (50) (QIAGEN). Synthesis of the first cDNA chains was performed according to standard protocol of Interlabservis with the use of a kit for reverse transcription. The reaction was conducted in the thermostat at 37°C for 30 min. For the analysis of gene transcription, real-time PCR was carried out with Sybr Green intercalating dye (Synthol) in a thermocycler BioRad iQ cycler. Unique pairs of primers for the analyzed genes were used (Table). The reaction mixture was collected in accordance with standard protocol of Synthol. The reaction protocol was as follows: initial denaturation (5 min at 95°C); denaturation (20 sec at 95°C); annealing of primers (20 sec at 56-62°C); 40

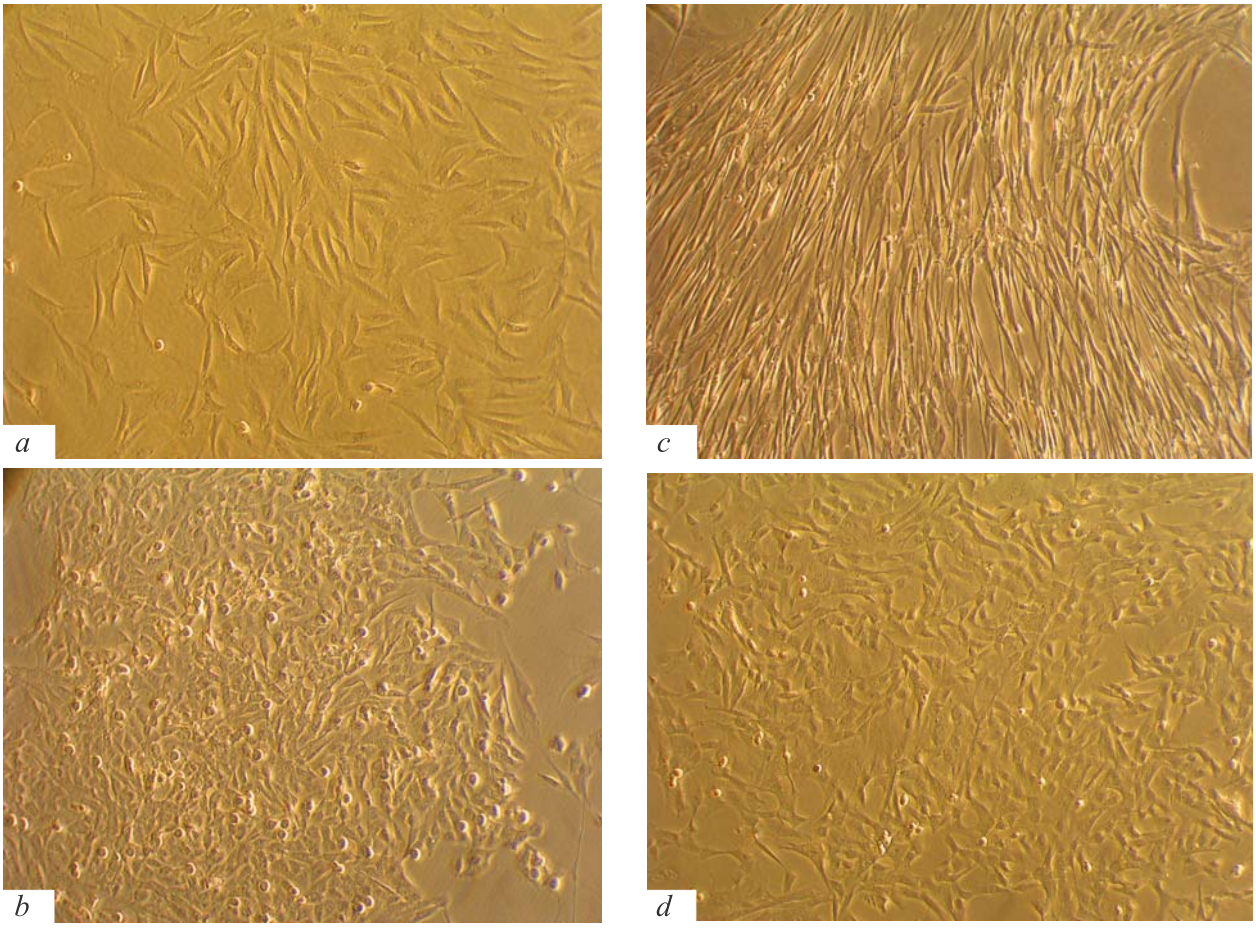

Fig. 1. Morphology of MSC cultures from the adipose tissue (*a, b*) and umbilical cord (*c, d*). *a*) culture of adipose tissue MSC obtained by the standard procedure, passage 2; *b*) isolated population of $CD146^+$ adipose tissue MSC; *c*) culture of umbilical cord MSC obtained by the standard procedure, passage 2; *d*) population of $CD146^+$ MSC from the umbilical cord. Relief phase contrast (×100).

cycles of elongation (20 sec at 72°C); melting of the amplification products.

The purity of reagents and work, control reaction with all the components except the matrix and electrophoresis of the reaction products in 1.5% agarose gel were carried out.

The content of mRNA of the analyzed genes was standardized by averaged amplification of two genes with unchanged expression levels (house keeping genes): *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *β-Actin*. The relative amount of mRNA was calculated using the ΔC (T) method.

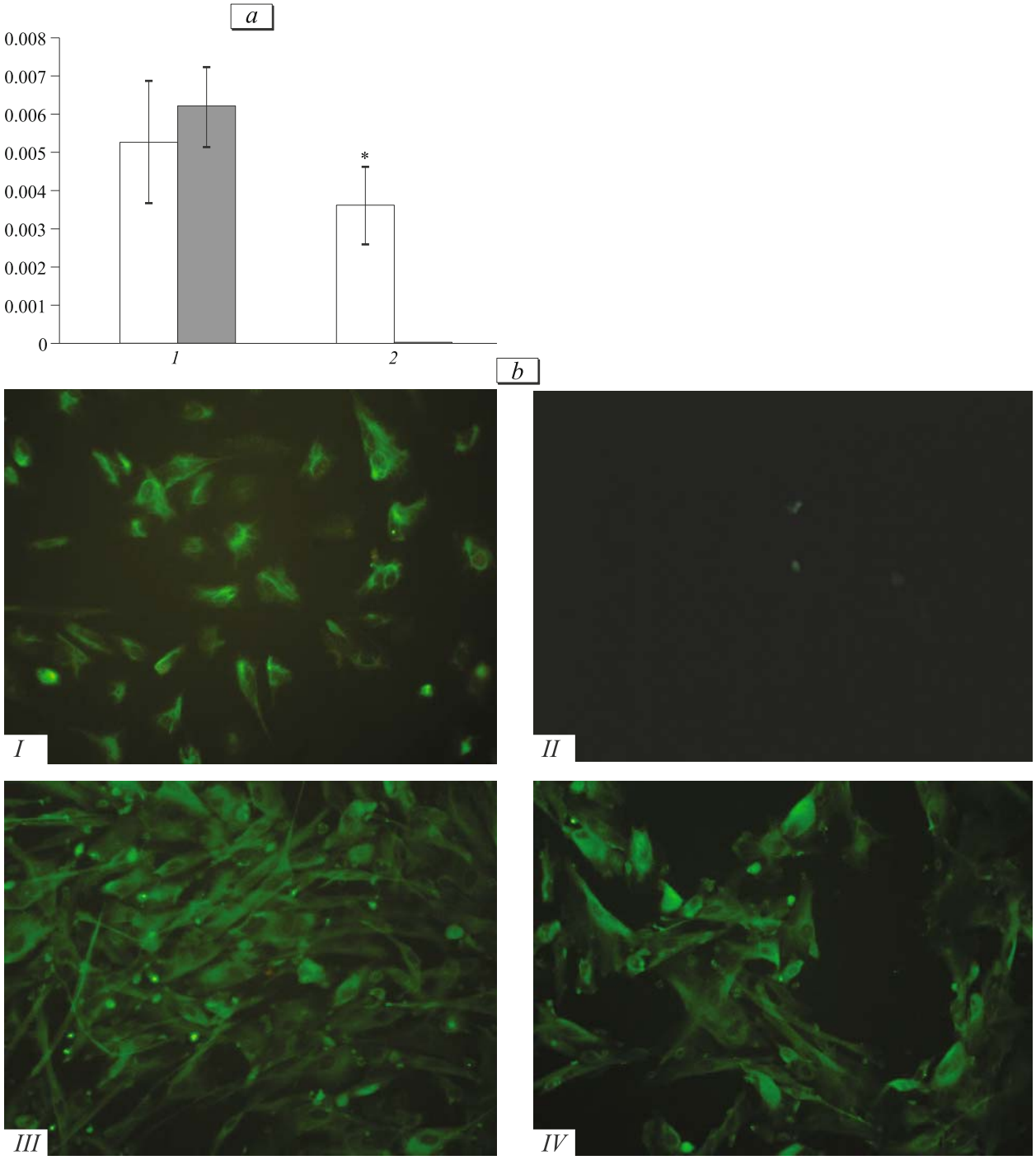

Fig. 2. Analysis of nestin expression in different MSC populations ($CD146^+$ and $CD146^-$) from the umbilical cord and adipose tissue. *a*) analysis of nestin transcription by real-time PCR: 1) umbilical cord MSC; 2) adipose tissue MSC. Ordinate: relative mRNA content; *b*) immunocytochemical staining for nestin: I, II: $CD146^-$ and $CD146^+$ MSC population from the adipose tissue, respectively; III, IV: $CD146^-$ and $CD146^+$ MSC population from the umbilical cord. Here and in Figs. 3 and 6: $^*p < 0.05$ compared to the control.

Immunocytochemical staining of cells. Cells were fixed with ice-cold acetone for 5 min, washed three times in PBS (PanEco) 3 times, then incubated in PBS with 0.25% Triton X-100 (Helicon) and 1% BSA (PanEco) for 30 min. After incubation, primary antibodies to nestin, insulin, or Pdx1 (Santa Cruz) in concentrations specified in the attached protocol in the same solution were added and incubated for 1 h at room temperature. The cells were washed 3 times with PBS. Secondary FITC-labeled antibodies to insulin and phycoerythrin-labeled antibodies to nestin and insulin (Abcam) were added and the cells were incubated for 1 h in the dark in PBS with 1% BSA. The cells were then washed 3 times with PBS and staining was visualized.

Test for glucose tolerance. Differentiated cells were washed 4 times with DMEM/F12 (1:1; PanEco) with 1% FCS (PanEco), 2 mM L-glutamine (PanEco), 100 mg/liter amikacin (Krasfarma) and seeded to 6-well plates (Corning) with 1 ml of DMEM/F12 (1:1) containing 5.56 and 25 mmol/liter glucose. After 24-h incubation, the medium was collected and frozen at -80°C. The content of insulin in the medium was determined using Ultrasensitive insulin ELISA commercial kit (Mercodia AB).

The data were processed by nonparametric Mann–Whitney test using Statistica 9 software. The differences were considered significant at $p<0.05$.

RESULTS

MSC were obtained from lipoaspirates of the adipose tissue (n=15) and from the umbilical cord (n=7) of healthy donors. MSC from two sources passaged by the standard method [11] (without adding insulin to the culture medium) were characterized by the phenotype $CD29^+$, $CD44^+$, $CD49a^+$, $CD73^+$, $CD90^+$, $CD166^+$, $CD146^-$, and $CD31^-$.

MSC passaged in insulin-containing medium by the original method for separating $CD146^+$ populations had the phenotype $CD29^+$, $CD44^+$, $CD49a^+$, $CD73^+$, $CD90^+$, $CD166^+$, $CD146^+$, and $CD31^-$. Thus, the cell grown in the presence of insulin yielded subpopulation of MSC ($CD146^+$) carrying surface marker CD146, a glycoprotein expressed on endothelial and perivascular cells, as well as on some MSC. This population is characterized by high proliferative activity of cells, clonogenicity, and stability of the phenotype (Fig. 1).

Since nestin-positive cell populations are used in the majority of studies on generation of insulin-producing cells [13], we studied nestin transcription in the obtained MSC populations ($CD146^-$ and $CD146^+$) from the umbilical cord and adipose tissue.

Expression of nestin was low in the $CD146^-$ MSC population isolated by the standard method from the adipose tissue and was absent in $CD146^+$ cultures. In $CD146^-$ and $CD146^+$ cell population from the umbilical cord, high expression of nestin was observed (Fig. 2).

In our work, cell cultures isolated from umbilical cord and adipose tissue of the two phenotypes ($CD146^+$ and $CD146^-$) were transfected with recombinant adenovirus carrying *Pdx1,* the key gene of pancreatic differentiation. For each cell phenotype, the optimal time for adenovirus adsorption (control points 2, 6, 24 or 30 h) was determined.

Nontransfected cells served as the control. The cells were then removed with trypsin and cultured for 3-7 days until the formation of islets in serum-free medium CMRL 1066 containing glucagon-like peptide (GLP). Under these conditions, the cells lost their ability to adhere to the substrate and formed cell clusters

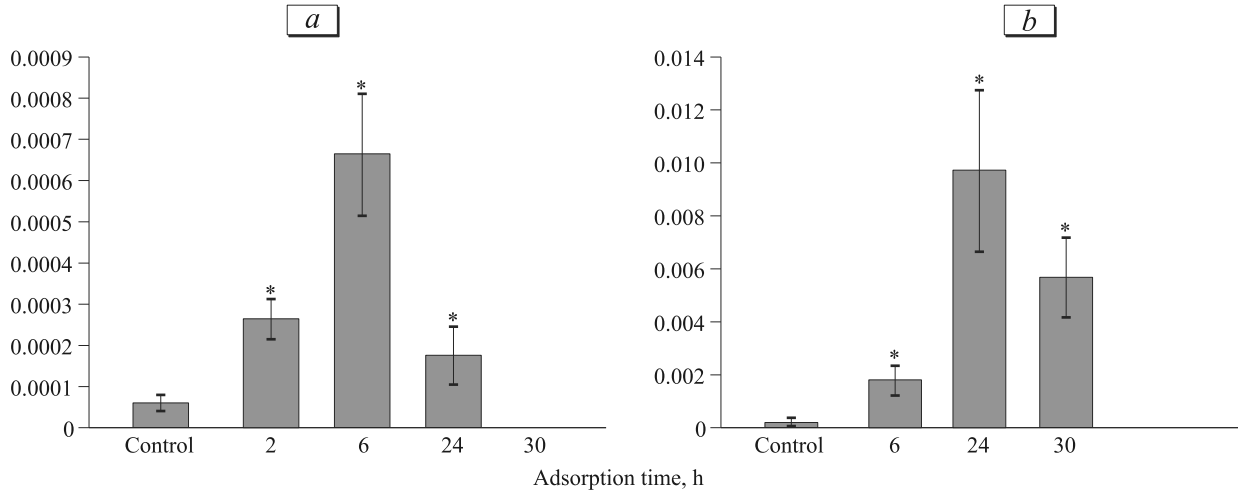

Fig. 3. Analysis of *Pdx1* gene transcription in transfected $CD146^+$ (*a*) and $CD146^-$ (*b*) MSC populations derived from the adipose tissue and umbilical cord.

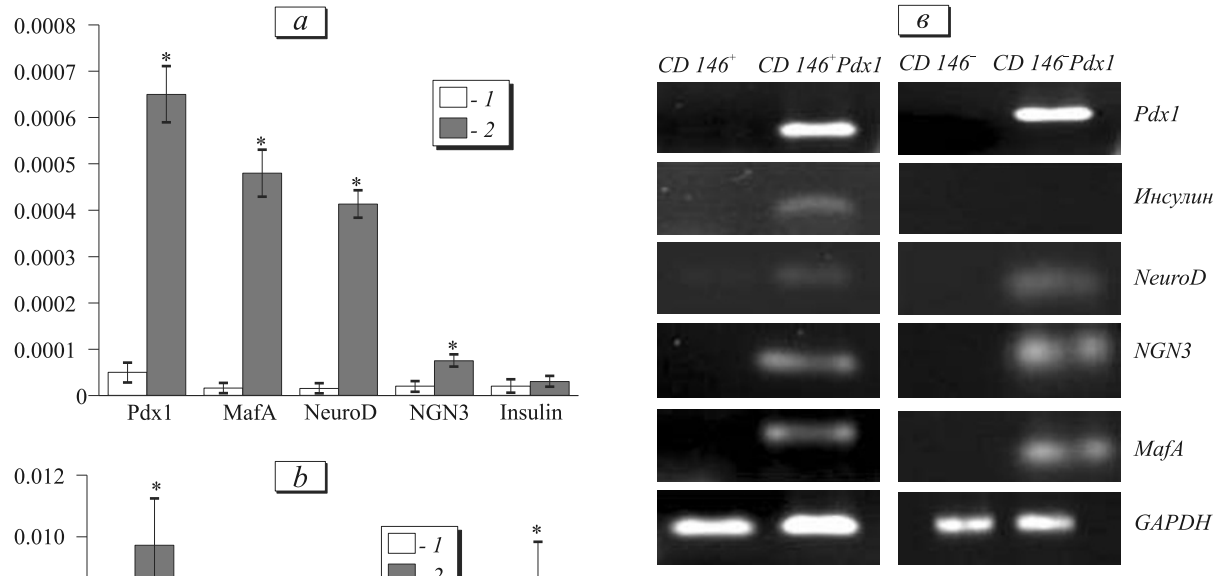

Fig. 4. Analysis of transcription of pancreatic differentiation genes in populations of CD146⁻ and CD146⁺ cells from the umbilical cord and adipose tissue. *a, b*: ordinate: relative mRNA content. a) analysis of transcription of pancreatic differentiation genes by real-time PCR in a population of CD146⁻ cells; *b*) analysis of transcription of pancreatic differentiation genes by real-time PCR in a population of CD146⁺ control (*1*) and transfected (*2*) cells; *c*) electrophoresis of PCR products in 1.5% agarose gel.

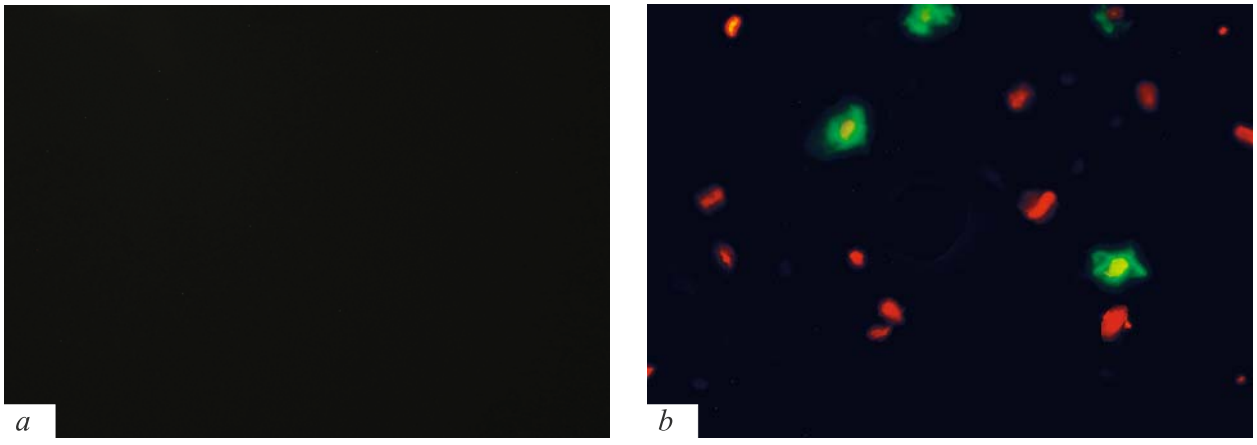

Fig. 5. Immunocytochemical staining of CD146⁺ MSC populations with antibodies to Pdx 1 and insulin: staining of CD146⁺ populations of MSC from the adipose tissue (result of a typical experiment). *a*) staining of non-transfected CD146⁺ MSC for Pdx1 and insulin; *b*) staining of transfected CD146⁺ MSC for Pdx1 (red fluorescence) and insulin (green fluorescence), ×200).

in suspension (islets). After that, mRNA was isolated from them and cDNA was synthesized.

The best time for adenovirus adsorption was determined by the level of transcription of *Pdx1* gene by real-time PCR. The experimental results indicated that the optimal time for adsorption of adenoviral vector varied in different populations. The maximal level of *Pdx1* transgene transcription in CD146⁻ and CD146⁺ MSC cultures was noted after 6 and 24 h, resectively (Fig. 3). It was found that transfection of Pdx1 in cell cultures of both types results in activation of transcription of *NeuroD, NGN3,* and *MafA* genes involved in the differentiation of pancreatic CK into β-cells. In the control, transcription of these genes was not detected. Transcription of the insulin gene in transfected CD146⁻ cultures was not found. Under similar conditions, transfection of the CD146⁺ cell population leads to activation of insulin gene transcription at a

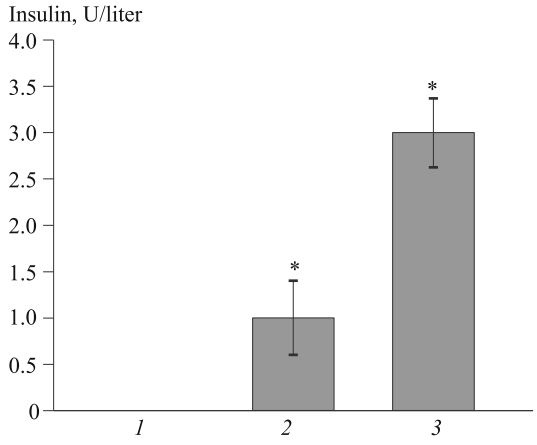

Fig. 6. Test for glucose tolerance. *1*) medium from non-transfected cells, *2*, *3*) medium from transfected cells with glucose content of 5.56 and 25 mmol/liter, respectively.

high level (Fig. 4, *a-c*; Fig. 5). This confirms the assumption that nestin expression is not required for effective directed differentiation into insulin-producing cells. $CD146^+$ MSC population from the adipose tissue not expressing nestin efficiently differentiated into functionally active insulin-producing cells. In turn, transfection of $CD146^-$ MSC populations from the umbilical cord characterized by intensive expression of nestin did not activate insulin transcription.

At the final stage of culturing, transfected cells were tested for glucose tolerance using Ultrasensitive insulin ELISA kit (Mercodia AB). It was shown that in transfected $CD146^+$ cells expression of insulin increases in response to high glucose concentrations in the culture medium (Fig. 6).

Thus, we demonstrated efficective differentiation of MSC subpopulations exhibiting perivascular phenotype ($CD146^+$), selected by the original method of adipose tissue and umbilical cord into insulin-producing cells by adenoviral delivery of the *Pdx1* gene. Glucose tolerance of the obtained insulin-producing cells was demonstrated. Transfection of MSC ($CD146^-$ cultures isolated by the standard method) did not activate insulin transcription. The results also show that nestin expression is not necessary for effective pancreatic cell differentiation.

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