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Treatment with small molecules is an important milestone towards the induction of pluripotency in neural stem cells derived from human cord blood

Ilona Szablowska-Gadomska[1], Joanna Sypecka[1], Valery Zayat[1], Martyna Podobinska[1], Anna Pastwinska[2], Barbara Pienkowska-Grela[2], and Leonora Buzanska[1]*

[1]NeuroRepair Department, Mossakowski Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland, *Email: buzanska@imdik.pan.pl; [2]Memorial Cancer Centre and Institute, Warsaw, Poland

Standardization of methods for obtaining iPS cells from the human somatic cells and then their successful differentiation are important in the context of their possible application in personalized cell therapy and the development of toxicological and pharmacological tests. In the present study, the influence of the small molecules representing epigenetic modulators (histone deacetylase inhibitor Trichostatin A and DNA methyltransferase inhibitor RG-108) on the process of reverting neural progenitors from HUCB-NSC (Human Umbilical Cord Blood Neural Stem Cell) line to the pluripotent state was tested. The experiments were conducted in low oxygen tension, in three different experimental layouts: (1) in the presence of reprogramming/recombinant polyarginine–tailed proteins; (2) with recombinant proteins and small molecules; (3) only in the presence of small molecules. We wanted to find out, whether it will be possible to induce pluripotent state of neural stem cells only by epigenetic modulators. Our results revealed that the inhibitors of DNA methylation and histone deacetylation used along with 5% oxygen tension can only transiently induce or elevate some pluripotency genes in neural progenitors with different pattern, but were not sufficient for stable reprogramming. The iPS cells from neural progenitor cells of HUCB-NSC were obtained only when TSA, RG-108 and reprogramming proteins have been applied simultaneously. These cells were tested for the expression of the selected pluripotency genes and in functional assays to prove their pluripotency stage. The obtained data show that the small molecules in conjunction with reprogramming factors are the potent tools in cell reprogramming.

Key words: stem cells, pluripotency, reprogramming, epigenetic, DNMT, HDAC

INTRODUCTION

Induced pluripotent stem cells (iPSC) are central to developing new strategies for personalized regenerative medicine without the use of embryonic material. This become evident since Yamanaka's group for the first time reprogrammed mice and human fibroblasts (Takahashi and Yamanaka 2006, Takahashi et al. 2007) to the pluripotent stage. Since that time iPS cells have been generated from most types of human somatic cells by transgenic expression of Oct4 (Pou5f1), Sox2, Klf4 and Myc transcription factors usually delivered to the cells by viral vectors (Maherali and Hochedlinger 2008, Braun et al. 2012). To reduce potential risks of tumor induction or genomic instability, research has focused on "viral free" delivery systems of reprogramming factors (Stadtfeld et al. 2008). The safest, though not most efficient, is the recombinant protein-based approach. Specially designed charged polyarginine domains can bind to plasma membrane and facilitate protein transmembrane penetration (Kim et al. 2009, Zhou et al. 2009). In this report, for reprogramming purpose, stable HEK 293 (Human Embryonic Kidney 293 cells) cell lines producing OCT4, KLF4 and SOX2 recombinant proteins tagged with polyarginine residues have been established according to the published protocol (Kim et al. 2009).

Correspondence should be addressed to L. Buzanska, Email: buzanska@imdik.pan.pl

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During the past decade human umbilical cord blood has emerged as a novel valuable source for stem cells (Domanska-Janik and Habich 2011) which possess several unique characteristics making them suitable for other than hematopoietic clinical applications (Sanberg et al. 2005, Park et al. 2010). The CNS (central nervous system) repair is one of such possible applications. This is due to the ability of cord blood stem cells to attain neural features (Buzanska et al. 2002, 2006, Jablonska et al. 2010) and, under appropriate conditions, to differentiate into functional neurons and oligodendrocytes (Jurga et al. 2009, Sypecka et al. 2009). Although mononuclear fraction of cord blood may harbor limited number of stem cells with pluripotent characteristics (McGuckin et al. 2005, Habich et al. 2006, Kucia et al. 2007), iPS cells generation (Yamanaka 2007) is required to obtain homogenous population of pluripotent stem cells. This was already achieved for human cord blood in a number of laboratories (Haase et al. 2009, Giorgetti et al. 2010, Zaehres et al. 2010). Moreover, mononuclear cells derived from human cord blood have the advantage over age-matched fibroblasts for iPS derivation as they display unique gene signatures that are closer to iPS cells and human embryonic stem cells (ESCs) and are more efficient (up to 100 times in iPS generation) when DNA integration-free methods are used (Chou et al. 2011, Hu et al. 2011). Experimental proofs of cord blood suitability for iPS cells generation lead to further increase of possible therapeutic applicability of this cell source and induced increased CB (Cord Blood) banking (Broxmeyer 2010).

Epigenetic modifications of chromatin play an important role in maintaining the pluripotency of stem cells. Withdrawal of the cell from the pluripotency stage is accompanied by methylation of CpG islands in the promoter region of *Oct4, Sox2* and *Nanog* genes, as well as histone H3 and H4 tail modifications (Ho and Crabtree 2010). Additional epigenetic control over stem cell reprogramming and fate decisions may be exerted by manipulation of oxygen levels (Yoshida et al. 2009) and the application of small chemical molecules (Lin et al. 2009) as well as microRNAs. It was documented that low oxygen tension increases the expression of pluripotency genes and this is regulated by the hypoxia inducible factors (HIFs), a family of transcription factors some of which (HIF1) are responsible for cell adaptation to hypoxic conditions (Yoshida et al. 2009, Szablowska-Gadomska et al. 2011). In this study we apply 5% oxygen in order to support neural progenitor reprogramming by possible up-regulation of pluripotency genes expression. Since small chemical molecules inhibiting DNA methylation and increasing histone acetylation were shown to promote iPS cell derivation (Feng et al. 2009, Yu et al. 2011), it was also crucial in this report to investigate of the role of the epigenetic modulators on the process of neural progenitors reprogramming. Partial, non-transgene induced reprogramming of cord blood-derived cells to a pluripotent state was shown to be correlated with epigenetic chromatin changes (Wong et al. 2010).

The aim of the present study was therefore to test the influence of epigenetic modulators (histone deacetylase inhibitor Trichostatin A and DNA methyltransferase inhibitor RG-108) on the process of reverting neural progenitors from HUCB-NSC back to their more pluripotent state without the use of reprogramming factors or in the presence of recombinant poly-arginine – tailed proteins in low oxygen tension.

METHODS

Cell culture

HUCB-NSC is a non-transformed neural stem cell line derived from human cord blood (Buzanska et al. 2006). The starting population of progenitor cells were cultured in atmospheric oxygen, then the progenitor cells from spontaneously differentiation of HUCB-NSC were cultured in an environment of 5% oxygen, and in two different medium conditions. Low Serum (LS) medium containing: DMEM/F12, 1% ITS, 2% FBS, 1% AAS (antibiotic-antimycotic solution) and Serum Free (SF) medium containing: DMEM/ F12, 1% AAS EGF (20 ng/ml), B27 (1:50) were used.

Karyotyping

Cytogenetic analysis was performed according standard procedures with G-banding method. Cells were incubated for 1 hour in the presence of Colcemide (concentration 0.1 μg/ml). Next step was hypotonic shock with 0.075 M potassium chloride for 45 minutes. Then cells were fixed in Carnoy's fixative (methanol: glacial acetic acid mixture v/v 3:1). Metaphases were banded with Wright's stain. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (Shaffer 2009).

Derivation of stable HEK 293 lines producing reprogramming proteins

Plasmid amplification

The pCMV cDNA-9R-myc plasmids containing the *myc* (*c-myc*) epitope and coding for one of the selected factors: OCT4 (NP_002692), SOX2 (NP_003097), or KLF4 (NP_004226) were received from Harvard Stem Cell Institute (courtesy of Dr. KS Kim). The plasmids were amplified in *E.coli* and purified using the Qiagen EndoFree Plasmid Medi Kit and the quality of the plasmids was assessed by electrophoresis on the 1.5% agarose gel (Fig. 1A).

Generation of stable cell lines expressing reprogramming factors

The cells of HEK 293 (Human Embryonic Kidney) line (ATTC #CRL15-73) have been transfected by non-viral, HiFect method (LONZA) with the pCMV cDNA-9R-myc plasmid (Fig. 1), coding one of the selected factors: OCT4, SOX2 and KLF4. The 2 μg of plasmid DNA was gently mixed with 10 μl of HiFect reagent and incubated for 5 min in order to form complexes then added to 4×10^5 cells for 24 hours. HEK 293 cells (both control and transfected) were maintained in DMEM (Gibco) medium supplemented with 10% fetal bovine serum, containing penicillin (100

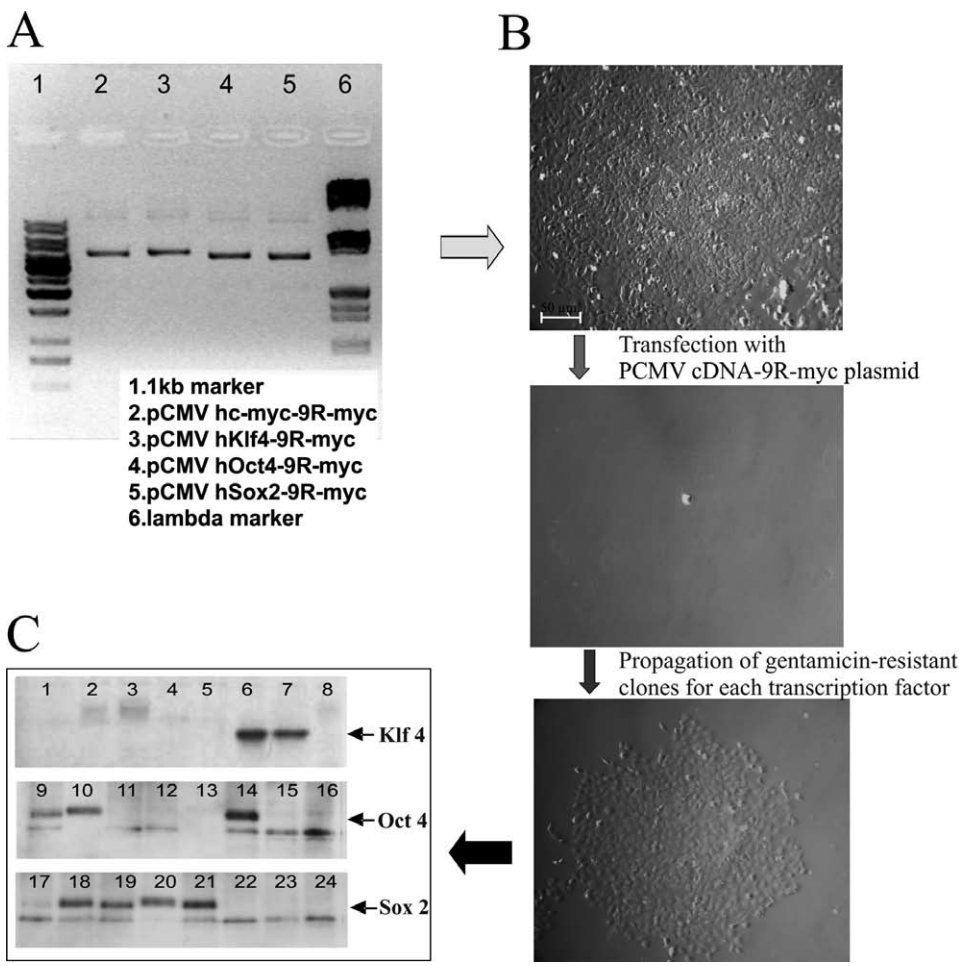

Fig. 1. Derivation of stable HEK 293 line clones with OCT4, SOX2 and KLF4 overexpression. (A) assessment of the quality of reprogramming factors by electrophoresis; (B) derivation of stable cell lines overexpressing reprogramming proteins connected to 9R and myc residues; (C) assessment of protein overexpression by Western blotting using anti-myc antibodies, numbers 1–24 represent different HEK clones. After transfection with PCMV cDNA -9R-myc plasmids 8 gentamicin-resistant clones for each tested transcription factor has been derived: (1–8 for Klf4, 9–16 for Oct4, 17–24 for Sox2). The clones overexpressing pluripotency markers were chosen: 6 and 7 –Klf4; 9, 10 and 14 – Oct4; 18 to 21 – Sox2. Scale bar is 50 μm.

units/ml) and streptomycin (100 μg/ml), in a moist atmosphere of air with 5% CO_2 at 37°C. After transfection, cells were cultured at low density for 2–3 weeks in the presence of 500 μg/ml Gentamicin (G4180) to select the resistant (i.e. transfected) colonies. The individual colonies were used to establish stable cell lines expressing reprogramming factors (i.e. OCT4, KLF4 and SOX2) (Fig. 1B).

Western blot analysis

The expression of reprogramming factors in gentamicin-resistant stable clones was determined by Western blot analysis (Fig. 1C). The cell extracts were mixed with Laemmli buffer and boiled for 5 minutes. Proteins were separated for 1.5 hours in 12% SDS-PAGE (Bio-Rad). Subsequently, proteins were transferred to nitrocellulose membranes (Amersham) using semidry transfer buffer (25 mM Tris, 150 mM glycin, 10% (v/v) methanol) and 3 mA/cm2 current for 45–50 minutes. Nitrocellulose membranes were blocked with 3% milk in TBS-T (0.1% Tween-20) for 1 hour at room temperature. The anti-myc antibody (Invitrogen) in blocking buffer was applied overnight at 4°C. After washing with PBS, the membranes were exposed to the horseradish peroxidase-conjugated anti-mouse IgG as a secondary antibody for 1 hour, and then detected by ECL technique (Amersham).

Preparation of protein extracts

For cell extract preparation, the cells were washed in PBS and lysed in with non-denaturating CellLytic buffer (Sigma) supplemented with protease inhibitor cocktail and stored at −80°C for future use. The lysates of non-transfected HEK 293 cells were prepared to serve as negative controls for the planned experiments.

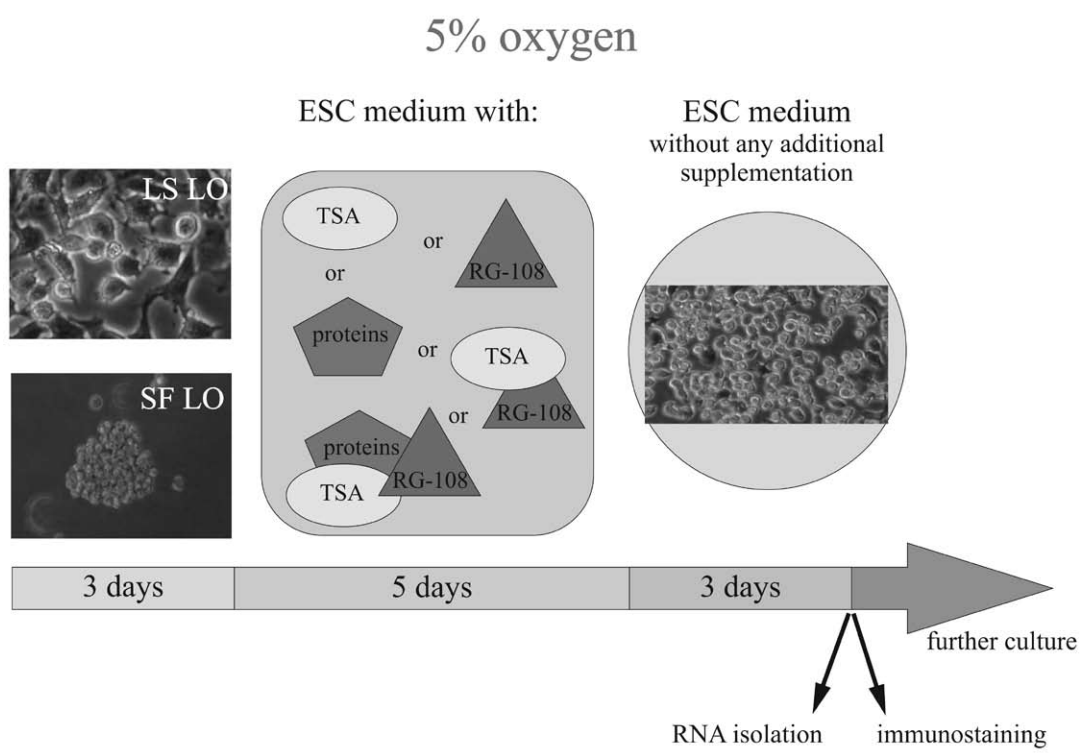

Fig. 2. Diagram of the experimental procedure for reprogramming of the NSC progenitors from umbilical cord blood using recombinant proteins as well as small molecules RG-108 and TSA. The cells grown under low oxygen tension (5%) and cultured in standard medium conditions (SF or LS) were subjected to various combinations of reprogramming factor cocktails including small molecules and TSA with or without the addition of reprogramming proteins in ESCM for 5 days. For the next 3 days, the cells were incubated in ESCM without any supplemental molecules. Then the cells were either harvested by trypsinization and RNA was isolated with TRIzol Reagent for gene expression analysis or cultured over prolonged time periods on Matrigel in ESCM and analyzed by immunocytochemistry.

Generation of iPS cells from human umbilical cord blood

Induction of pluripotency by reprogramming proteins and epigenetic stimulation

The progenitor cells from HUCB-NSCs were plated at density of $1 \times 10^4/cm^2$ and incubated in 5% oxygen in LS or SF medium (Fig. 2). After 3 days *in vitro*, the medium was changed to ESCM (Embryonic Stem Cell Medium) containing: KnockOut SR XenoFree 15%, KnockOut DMEM 1x, Glutamax 2 mM, 2-mercaptoethanol 0.1 mM, bFGF 8 ng/ml, NEAA 0.1 mM, AAS 1% supplemented with TSA (200 nM) and/or RG-108 (3 μM) separately or in combination with recombinant polyarginine–tailed protein lysate (whole cell lysate from HEK 293 lines producing reprogramming proteins OCT4, SOX2, KLF4 or OCT4 and KLF4 about 200 μg/culture flask) for 5 days. For the next 3 days, the cells were incubated in ESCM without any supplemental molecules. Then the cells were either harvested by trypsinization and RNA was isolated with TRIzol Reagent (Invitrogen) for gene expression analysis or cultured over prolonged time periods on Matrigel (BD Biosciences) in ESCM and analyzed by immunocytochemistry.

After one cycle of reprogramming procedure, the cells were maintained at 5% oxygen condition in ESCM. ES-like colonies were selected and transferred to the new Matrigel-coated 24-well plates. This step was repeated several times followed by continuous incubation and passaging by trypsinization at 1:3 ratio. The restricted culture conditions were applied to avoid too high or too low density of the cells as well as to minimize the colony size (about 150–200 cells in colony) to prevent cell differentiation and aging. The medium was changed 3–4 times per week. Most of procedures were performed in accordance with Human Embryonic Stem Cell Culture Protocols (Manual for Essential Human Embryonic Stem Cell Culture Methods Course 2007; Stem Cell Institute; University of Minnesota).

Identification of the pluripotency state by the expression of the ESC markers RT-PCR and real-time RT-PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen). The RNA samples were treated with the recombinant DNase I using the Ambion DNA-Free Kit (Life Technologies) and converted to cDNA by using High Capacity RNA-to-cDNA Kit (Applied Biosystems). PCR reactions were performed using the following reagents: Taq PCR Core KIT (Qiagen) in the presence of specific primers for pluripotency-associated genes including OCT3/4 F: CTCCTGGAGGGCCAGGAATC, R: CCA CATCGGCCTGTGTATAT 381bp; OCT 3/4 F: GACAG GGGGAGGGGAGGAGCTAGG, R: CTTCCCTCCA ACCAGTTGCCCCAAAC 144 bp (Real-time RT-PCR); SOX2 F: ACACCAATCCCATCCACACT, R: GCAAACT TCCTGCAAAGCTC 224bp**;** REX1 F: GCTGCCC TGAGAAAGCATCT, R: GCGTTAGGATGTGGGC TTTC 289 bp; NANOG (RT-PCR) F: TCCAGGA TTTTAACGTTCTGCT, R: TTCTTGCATCTGCTG GAGGC 580 bp; NANOG (Real-time RT-PCR) F: AATACCTCAGCCTCCAGCAGATG, R: TGCGTCAC ACCATTGCTATTCTTC 148 bp; hTERT F: AGCTAT GCCCGGACCTCCAT, R: GCCTGCAGCAGGA GGATCTT 185bp; Beta actin (ACTB) F: GCCAAC CGCGAGAAGATGA, R: CATCACGATGCCAGTGGTA 120 bp and differentiation genes (Takahashi et al. 2007). Amplification products were separated by electrophoresis on 1.5% agarose gel. Real-time RT-PCR was performed using cDNA and Syber Green Master Mix (Applied Biosystems) using 7500 AB Real-Time PCR System. The following conditions were applied: 50°C 2 min, 95°C 10 min, 95°C 15 s, and 60°C 1 min for 40 cycles. Standard curves were established and the results were analyzed using ΔΔCt method. Each sample was tested in triplicate. Genetic material from human ESC used as a calibrating and control factor was obtained from Masaryk University in Brno, Czech Republic (courtesy of Dr. A. Hampl).

Immunostaining

Cells were fixed in 4% PFA in PBS, blocked with 10% NGS (Normal Goat Serum) or 1% BSA and 10% DNS (Donkey Normal Serum) with either 0.25% Triton X100 for intracellular antigens or without Triton X100 for surface antigens. Primary antibodies against human proteins OCT4 (1:150 mouse IgG2b, Santa Cruz), SOX2 (1:200 rabbit IgG, Santa Cruz), NANOG (rabbit1:200 IgG, Abcam), KLF4 (1:500 rabbit IgG, Millipore), SSEA4 (1:100 mouse IgG3, Millipore), TRA 1-60 (1:100 mouse IgM, Millipore), HNF-3β/FoxA2 (1:20 goat IgG, R&D Systems), αSMA (1:400 mouse IgG2a α Smooth Muscle Actin, Sigma- Aldrich) were applied overnight at 4°C, followed by one hour incubation with AlexaFluor 488 or AlexaFluor 546

secondary antibodies. Verification of the specificity of the antibodies used have been done by omitting primary antibody for each performed staining. The cells nuclei are stained with Hoechst 33258.

Identification of generated iPS was performed with Stem Cell KIT (Human Pluripotent Stem Cell Functional Identification Kit R&D Systems) according to the manufacturer's instructions.

Verification of the pluripotency by testing differentiation potential of the reprogrammed cells

Two separate methods for evaluation of differentiation potential were applied: embryonic body formation and direct differentiation.

(1) Identification by embryonic body (EB) formation and differentiation. Cells from ES-like clone 1 were collected by trypsinization, centrifuged and then, for propagation/proliferation purposes cultured in suspension on plastic (without Matrigel) in modified ESC medium without bFGF. After about 8–20 days the cells were transferred to 24-well plates coated with Matrigel, gelatin or poly-L-lysine and further incubated in the same conditions for about 7–10 days before immunostaining and RNA isolation.

(2) Identification through differentiation into three germ layers of iPS cells from clone 1 was done according to the manufacturer's instructions using Stem Cell KIT (Human Pluripotent Stem Cell Functional Identification Kit R&D Systems). Culture media were used according to the manual and cells differentiating into ecto- and endoderm were fixed after 72 h, while cells differentiating into mesoderm were fixed after 36 h. In addition to the identification by immunostaining (SOX17 – endoderm, Otx2 – ectoderm and Brachyury – mesoderm – antibodies from Stem Cell KIT), RNA was isolated and gene expression was analyzed: FOX2, Sox17, AFP – for endoderm; PAX6, MAP2, GFAP – for ectoderm; and MSX1, Brachyury – for mesoderm markers (primers were designed according to Takahashi et al. 2007).

RESULTS

Derivation of stable HEK 293 lines producing reprogramming proteins

The quality of the plasmids overexpressing reprogramming factors connected to 9R residues has been assessed by electrophoresis (Fig. 1A). Isolation and propagation of individual cells from gentamicin-resistant colonies (Fig. 1B) allowed us to obtain about

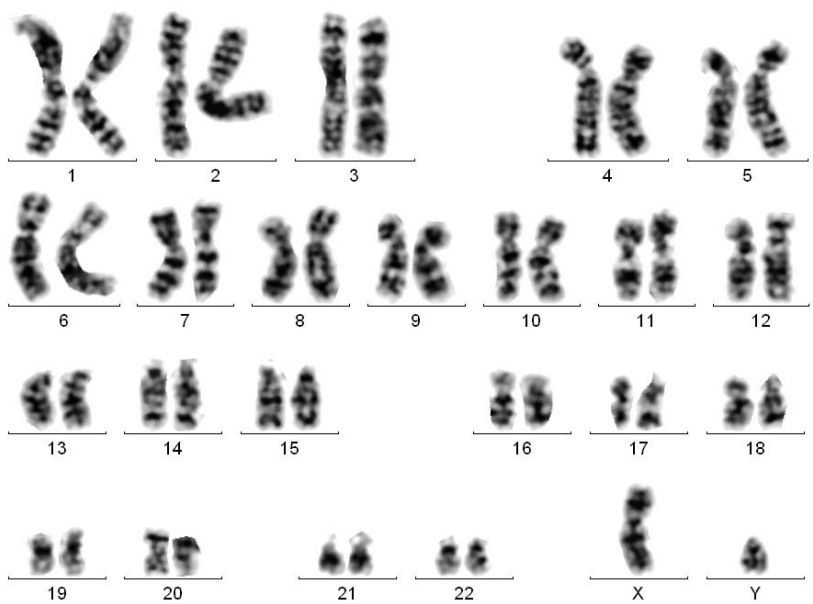

Fig. 3. Karyotype of neural progenitors starting population (HUCB-NSC growing in LS conditions). Cytogenetic analysis revealed no chromosomal aberrations, proving that the cells were characterized by the normal karyotype.

22–30 clones expressing each of the transcription factors, which have been subsequently analyzed by the Western blotting technique using anti-myc antibodies, due to the presence of c-myc epitope in reprogramming vectors (Fig. 1C). The 6–7 stable lines expressing each of the transcription factors have been selected for further *in vitro* culturing with the purpose of continuous generation of whole cell lysates expressing Oct4, Sox2 or Klf4 proteins tailed with 9R residues. Whole cell lysates overexpressing each of the reprogramming factors have been prepared and stored at −80°C for the future reprogramming experiments.

Generation of iPS cells from human umbilical cord blood

Before introducing the reprogramming procedure, the cells from starting population of neural progenitors were subjected to cytogenetic analysis. The detailed examination of metaphases revealed no chromosomal aberrations, proving that all the cells were characterized by the normal karyotype (Fig. 3) and could be used for the planned induction of the pluripotency state.

The neural progenitors grown in normal atmospheric conditions (21% of oxygen) in LS medium – (master

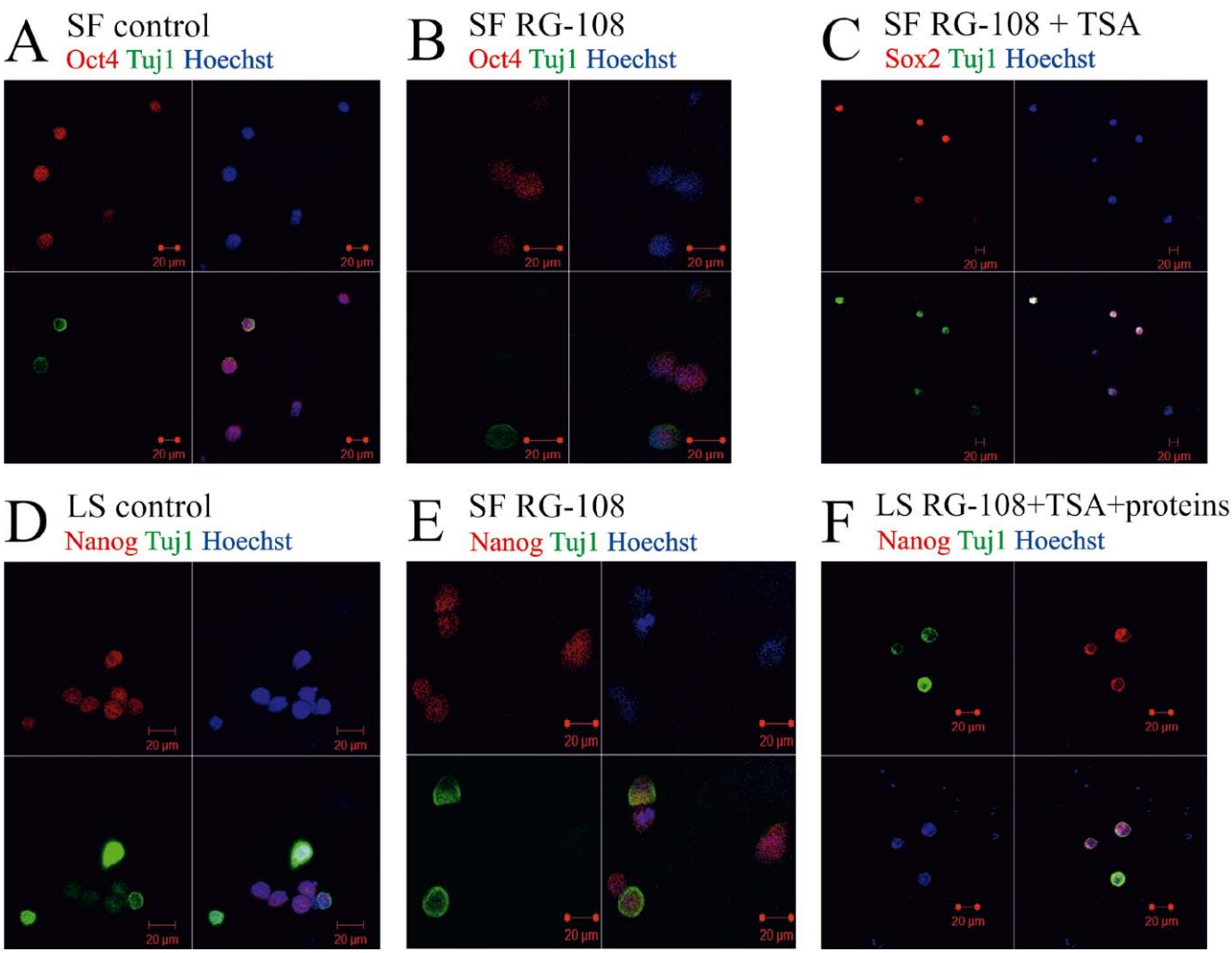

Fig. 4. Immunocytochemical analysis of protein expression in neural progenitors from HUCB-NSC, assessed 3 days after the reprogramming cycle as indicated on the diagram in Fig 2. The neural progenitors from HUCB-NSC line have been tested for the expression of proteins − OCT4, SOX2, NANOG (markers of pluripotency). (A, B) Cells from SF control (on Matrigel in ESCM and in 5% oxygen) and SF/RG culture conditions expressing β-tubulin3 (positive for Tuj1 antibody – green) and Oct4 (red); (C) SF/RG/TSA expressing β-tubulin3 (positive for Tuj1 antibody – green) and Sox2 (red), and (D, E, F) LS control (on Matrigel in ESCM and in 5% oxygen), SF/RG and LS/TSA/RG/protein expressing β-tubulin3 (positive for Tuj1 antibody – green), and Nanog (red). Cell nuclei are stained with Hoechst 33252.

control in Table I) did not express tested pluripotency markers. The designed reprogramming procedures were applied to cells derived from two different developmental stages of HUBCB-NSC (grown under SF and LS conditions, in 5% oxygen tension) (Fig. 2). The samples of each experimental variant were harvested and checked for the expression of undifferentiated markers Oct4, Sox2, Rex1 and Nanog on the mRNA (Table I) and proteins levels (Fig. 4).

The control cells grown in SF and LS medium under low oxygen tension, when tested three days after the reprogramming procedure (Fig. 2), did not express all tested pluripotency markers simultaneously. However low oxygen condition stimulated expression of Nanog in LS control and Oct4 and Nanog in SF control (Table I). The same mode in the expression of pluripotency markers in HUCB-NSC was observed when cells were preincubated for more than two weeks in 5% oxygen

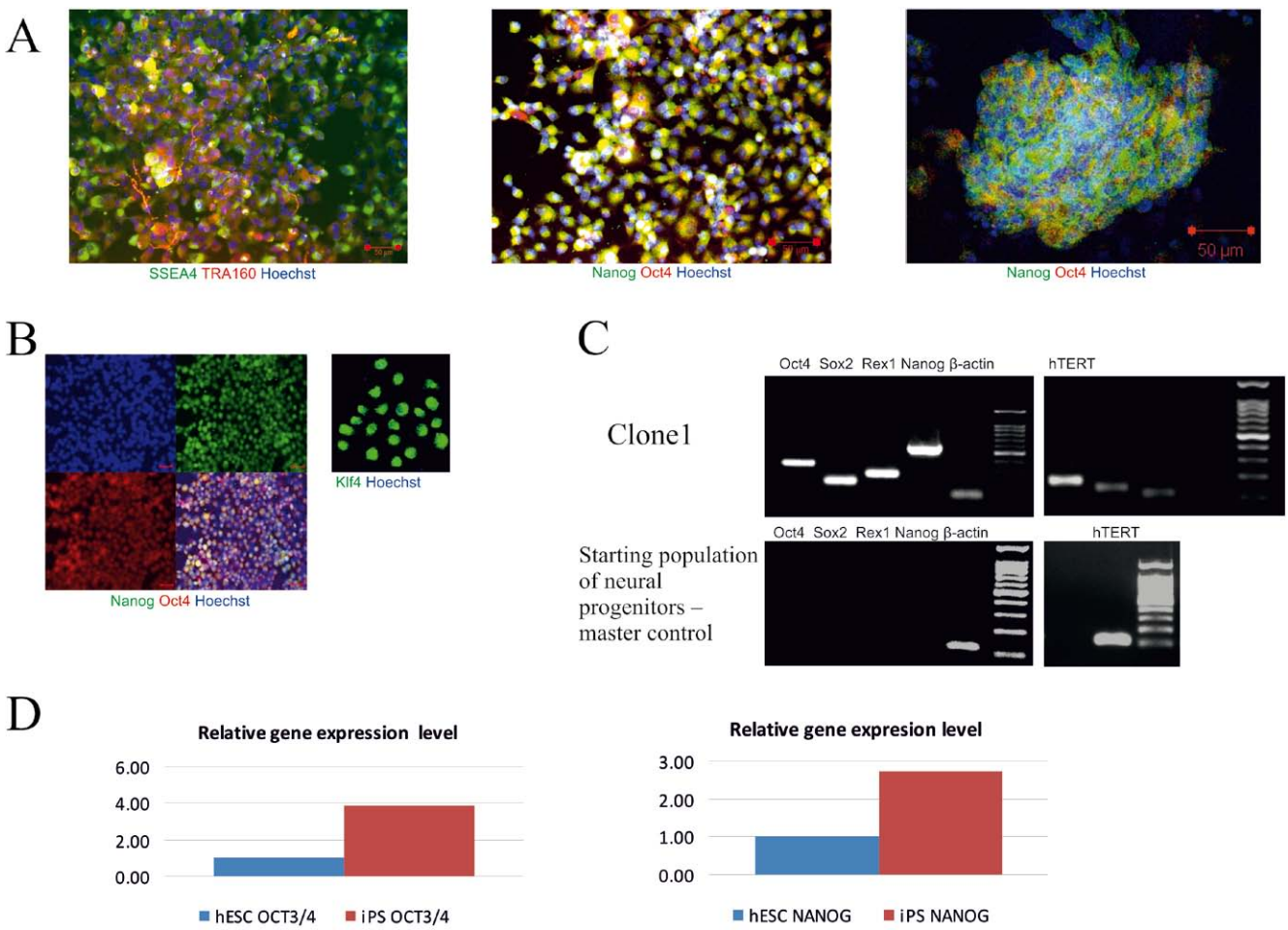

Fig. 5. Identification of the pluripotency state in HUCB-NSC-derived cells by the expression of ESC markers after derivation of iPS clone 1 with reprogramming proteins and small molecules under 5% oxygen tension, after 8 weeks of culture on Matrigel in ESCM medium. (A, B) The results of the protein expression analysis: OCT4, NANOG, KLF4 and surface markers TRA-1-60 and SSEA4 using immunocytochemistry. Cell nuclei were stained with Hoechst 33252; Scale bar is 50 µm. (C) The results of gene expression analysis in clone 1 derived iPS cells and the starting population of neural progenitor cells from atmospheric oxygen (master control in Table I): Oct4, Sox2, Rex1, Nanog and hTERT using RT-PCR; Control neural progenitor cells from atmospheric oxygen do not express pluripotency markers and do not express hTERT. (D) The comparison of gene expression of Oct4 and Nanog in HUCB-NSC-derived clone 1 of iPS cells and human ESCs. Expression analysis by Real-Time RT-PCR. ESCs were used as a calibrator and beta actin as a reference gene. The standard dev. in iPS cells for Oct4 dCt is 0.08963, for Nanog is 0.09899 dCt and for Oct 4 in hESC is 0.3704 dCt, for Nanog in hESC is 0.04. The starting population was not included in Real Time RT-PCR analysis since it was negative for tested pluripotency markers (as shown on C and in Table I).

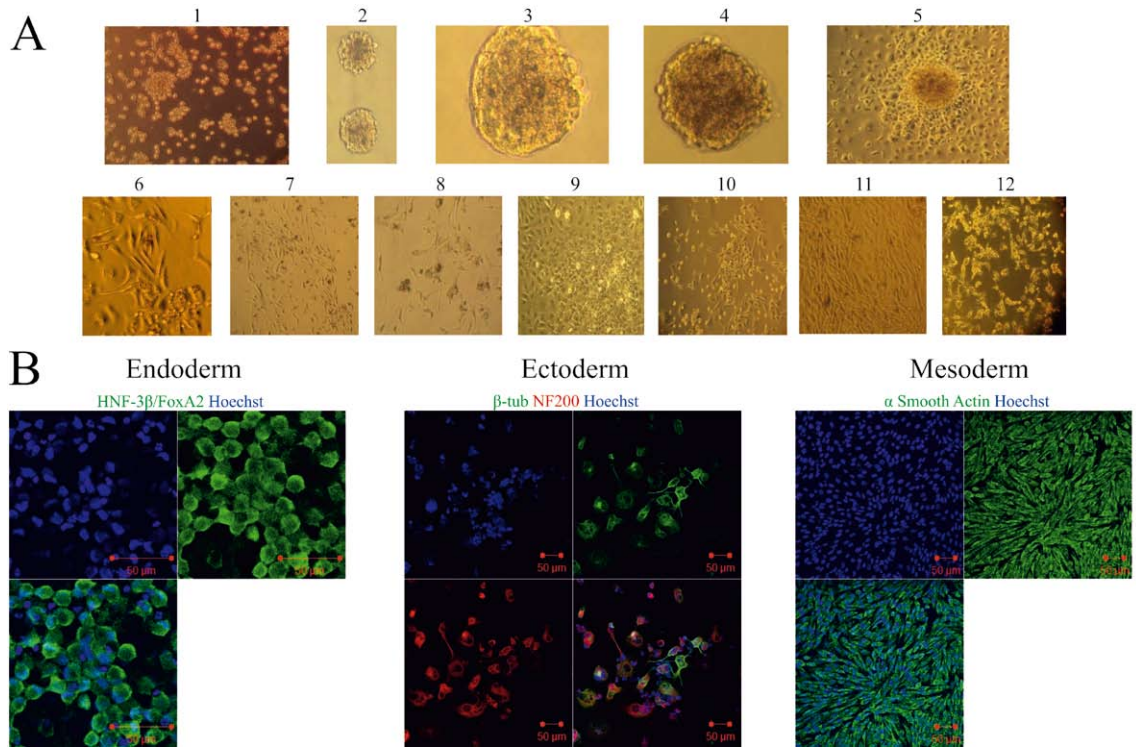

Fig. 6. Verification of the pluripotency of iPS cells by "EB formation method". Potential to differentiate of reprogrammed clone 1 cells into three germ layers was tested as described in Methods section. (A) Phase-contrast of spontaneously differentiating EB cells. (1–4) The stages of EB formation, (5) EB after attachment, (6–12) different morphology of cells representing the three germ layers. (B) Immunocytochemistry of spontaneously differentiating EB cells: HNF-3β/FoxA2, NF-200 with β-tubulin III and α Smooth Actin represent endoderm, ectoderm and mesoderm markers, respectively.

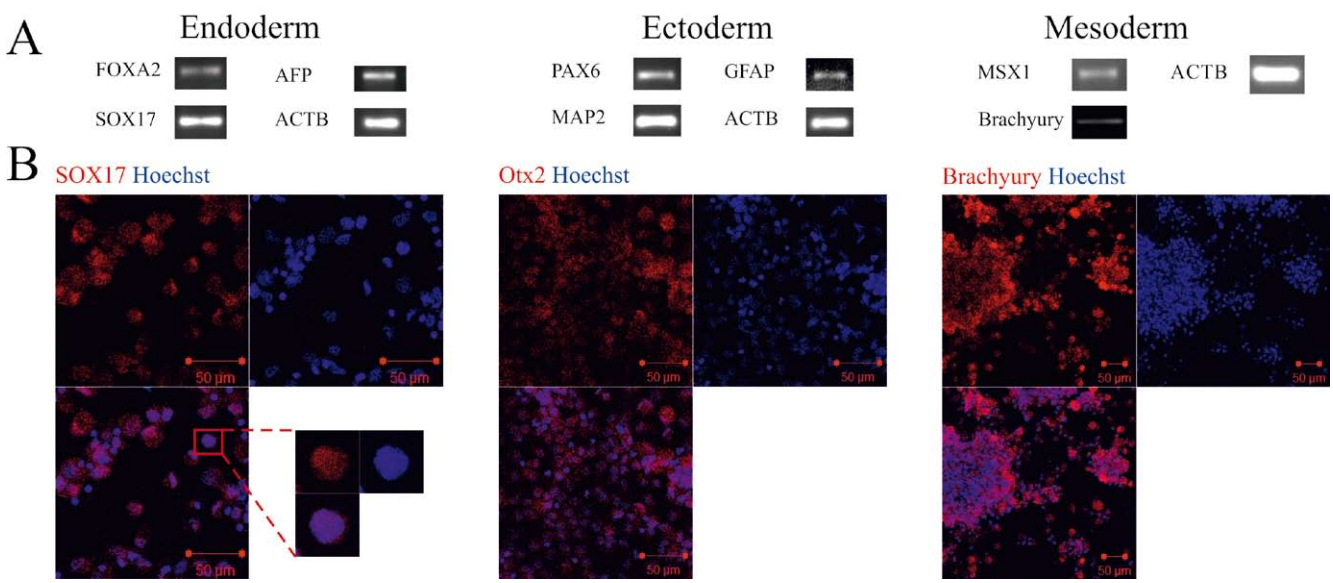

Fig. 7. Verification of the pluripotency of iPS cells by "direct differentiation method". Differentiation potential of the reprogrammed clone 1 cells into three germ layers was assessed using Stem Cell Kit as described in Methods section. (A) Gene expression analysis by RT-PCR confirmed the expression of: endoderm (FOX2, Sox17, AFP), ectoderm (PAX6, MAP2, GFAP) and mesoderm (MSX1, Brachyury) markers. Beta-Actin - loading control. (B) Protein expression revealed by immunocytochemistry: Sox17, Otx2 and Brachyury – endoderm, ectoderm and mesoderm cell marking, respectively

conditions and regardless of the used medium (LS,SF or ESCM) (unpublished observation).

In all experimental variants, the tested pluripotency markers were expressed but not simultaneously after 3 days of reprogramming procedure. Further incubation in 5% oxygen medium and ESCM conditions led to observation that only cells grown under SF conditions either treated with RG-108 and TSA or treated with combination of RG-108, TSA and recombinant proteins (OCT4- 9R and KLF4-9R) gained and maintained pluripotent cell morphology (round cells with large nuclei surrounded with small amount of cytoplasm). Out of this two variants, the only one with recombinant proteins expressed all pluripotency markers, including Oct4A and Rex1, when tested 8 weeks after the reprogramming procedure (Table I and Fig. 5C). The cells from the rest of experimental variants either undergone differentiation process or did not survive.

The generated ES-like colonies were selected and transferred to the new Matrigel-coated 24-well plates in ESCM under 5% oxygen tension. After 8 weeks, the cells were analyzed for the expression of pluripotency markers by using RT-PCR and immunostaining techniques (Fig. 5).

The results showed that the cells grown under SF conditions treated with RG-108, TSA and reprogramming proteins expressed continuously *Oct4*, *Sox2*, *Rex1*, *Nanog* and *telomerase* (*hTERT*) genes as well as proteins OCT4, NANOG, TRA 1-60, SSEA4, KLF4, thus revealing the features of induced pluripotent stem cells (Fig. 5A, B, C).

Quantitative Real-Time PCR analysis was performed to compare the expression of main pluripotency genes revealing higher levels of OCT4 and NANOG in derived pluripotent clone as compared to hESC (used as a calibrator probe) (Fig. 5D). The starting population of neural progenitors (spontaneously differentiating from HUCB-NSC) was not subjected to Real-Time PCR due to lack of the investigated pluripotency gene expression revealed as shown by RT-PCR (Fig. 5C and Table I).

Those cells exhibiting ES-like morphology were maintained in culture in undifferentiated state up to 42 passages.

Identification of the pluripotency state

The pluripotency of the received reprogrammed cells were verified through their ability to differentiate into cells representing endoderm, ectoderm and mesoderm germ layers using two different approaches: by embryonic body formation (Fig. 6) and by direct differentiation (Fig. 7). The iPS-like cells go through distinct stages of EB formation (Fig. 6A1–5) and differentiated into 3 germ layers as evidenced by the expression of HNF-3β/FoxA2 (endodermal marker), NF-200, β-tubulin III (ectodermal marker), α Smooth Actin (mesodermal marker) (Fig. 6B) and different morphologies of differentiated cells (Fig. 6A6–12). Similar results were obtained by culturing the iPS-like cells in differentiation media using commercially available Stem Cell Kit (R & D Systems). The cells expressed proteins typical for all three germ layers: the endodermal markers (FOX2, Sox17, and AFP), the ectodermal markers (PAX6, MAP2, and GFAP) as well as mesodermal markers (MSX1, Brachyury) (Fig. 7A). The immunostaining confirmed the presence of Sox17 (for endoderm), Otx2 (for ectoderm) as well as Brachyury (for mesoderm) (Fig. 7B).

DISSCUSSION

The reprogramming of the neural progenitor cells from HUCB-NSC was carried out by a dual approach. The first one was based on the selected transcription factors, known as potent inducers of pluripotency. These included Oct4, Sox2 and Klf4 (Kim et al. 2009). For this purpose, the stably transfected HEK293 cell lines producing each of the three human reprogramming factors fused with the myc tag and with polyarginine (9R) residues to facilitate intracellular trafficking have been generated. The obtained protein extracts were used in the attempts to induce pluripotency in the human neural stem cells, cultured in 5% oxygen. We have previously tested the influence of different oxygen conditions on the expression of pluripotency genes in progenitor cells from HUCB-NSCs and the results suggested that lowered oxygen (5%) environment is more conducive for to reprogramming processes and for maintaining pluripotent state than that of standard 21%. Low oxygen tension activates some key regulatory genes in HUCB-NSC, such as Nanog (data unpublished) involved in reprogramming. This confirmed previous observation (Forristal et al. 2010, Szablowska-Gadomska et al. 2011) that lowering the atmospheric oxygen tension helps to maintain cell in undifferentiated state. Thus, all reprogramming experiments were performed under low oxygen tension (5%).

Table I

Analysis of transcriptional expression of key pluripotency genes by RT-PCR in the progenitor cells from HUCB-NSC line assessed 3 days after reprogramming procedure (as indicated in Fig. 2), except starting population of neural progenitors from atmospheric oxygen and ESC positive control. In SF/LO RG-108 + TSA + proteins variant cells were additionally tested after 8 weeks for the expression of tested genes.

	Oct4A	Sox2	Rex1	Nanog	β-actin
Neural progenitors starting population (LS/atmospheric oxygen) – master control	–	–	–	–	+
LS/LO control*	–	–	–	+	+
SF/LO control*	+	–	–	+	+
LS/LO RG-108	–	–	–	+	+
SF/LO RG-108	+	+	–	+	+
LS/LO TSA	–	–	–	+	+
SF/LO TSA	–	–	+	+	+
LS/LO proteins	–	–	–	+	+
SF/LO proteins	–	+	–	+	+
LS/LO RG-108 + TSA	+	+	–	+	+
SF/LO RG-108 + TSA	–	+	–	+	+
LS/LO RG-108 + TSA + proteins	–	–	–	+	+
SF/LO RG-108 + TSA + proteins**	–/+	+/+	–/+	+/+	+
ESC positive control	+	+	+	+	+

* In these controls the cells have been preincubated in 5% oxygen conditions and regardless of the used medium (LS,SF or ESCM) no difference was noted in the expression of pluripotency markers (unpublished results). ** The cells which gained ES-like morphology, checked after 3 days of reprogramming procedure (as indicated in Fig. 2) didn't show expression of Oct4A and Rex1. However, prolonged incubation (up to 8 weeks in ESCM on Matrigel in 5% oxygen) allowed the stable induction of all tested pluripotency genes, and promoted derivation of stable iPS clones.

The performed study was based on the assumption that induction of pluripotency in neural stem cells could be regulated by the reversion of the epigenetic processes which occurred under differentiation and development. To induce changes in the epigenetic pattern of differentiated cells, TSA and RG-108, inhibitors of the deacetylation and DNA methylation processes, were chosen. Applying different combination of reprogramming factors, the prolonged incubation allowed only cells grown under SF conditions treated with RG-108 + TSA and grown under SF conditions after combined treatment with RG -108, TSA and recombinant proteins treatment to gain and to maintain the morphology similar to human ES and rhesus monkey ES (i.e. round cells with large nuclei surrounded with small amount of cytoplasm) (Thomson et al. 1998). Neural progenitor cells from HUCB-NSC line treated with reprogramming proteins, after prolonged incubation time in ESCM on Matrigel exhibited preserved ESC morphology and at the same time expressed pluripotency markers (Oct4, Nanog, hTERT, SSEA4, TRA1-60) (Fig. 5). However the cells from starting population treated with RG108 and TSA only, characterized by the activity of pluripotency genes 3 days after the reprogramming procedure, lost expression of these genes during prolonged culturing and passaging.

The effect of TSA on chromatin decondensation by inducing histone acetylation was previously described, and was shown to promote upregulation of pluripotency genes (Tóth et al. 2004, Kang and Roh 2010).

Ruau and collaborators have indicated that transient expression of pluripotency genes was induced by TSA /AzaC treatment. In neurosphere cells, induction of Oct4, Nanog, Klf4 activation as well as constitutive expression of Sox2 and c-Myc were insufficient to achieve stable pluripotent state (Ruau et al. 2008), which is in line with our observations. This underscores the importance of stoichiometry of reprogramming factors. Jaenisch and collaborators showed that only high expression of Oct4 and Klf4 together with low expression of Sox2 and c-Myc are the most conducive to generation of "good-quality" iPSCs (Maherali et al. 2007).

It is possible that inhibitors of DNA methylation and histone deacetylation are affecting the activation of pluripotency genes through the epigenetic changes, but this influence might be only temporary or the level of expression of those genes is insufficient to achieve the pluripotency level using only epigenetic factors. That is why it was important to use the reprogramming proteins as an additional booster factor. The efficiency gained by application of reprogramming proteins is low (about 0.001% of input cells), nonetheless this method has a great advantage over the most effective viral-based methods because of the safety issues (Kim et al. 2009).

High activity of human telomerase, characteristic for early embryonic development, is gradually down-regulated during differentiation process. The only known exceptions are lymphocytes, endothelial cells and adult stem cells (Marión and Blasco 2010, Saretzki 2011). The opposite situation takes place during reprogramming process (Marión and Blasco 2010) suggesting that changes in chromatin conformation during iPSC generation include telomeric chromatin. When pluripotency state is achieved, telomeres are re-elongated which usually correlates with telomerase activity (Marión et al. 2009). Similar to ES cells and cancer cells, the iPS cells are characterized by high level of telomerase activity (Saretzki 2011). The cells grown under SF conditions, treated with RG-108, TSA and reprogramming proteins were analyzed for hTERT expression and the obtained results confirmed that the cells treated with combination of small molecules and reprogramming proteins showed the same telomerase activity compared to that observed in ES cells.

The potential application of iPS cells for regenerative medicine relates to their ability to differentiate into any cell type of the body in autologous transplantation, therefore assessing their differential potential into three germ layers is very important. In present work the pluripotency of the obtained reprogrammed cells was verified through their ability to differentiate into cells representing endoderm, ectoderm and mesoderm germ layers in two ways: by direct differentiation as well as by embryonic body formation (Rungarunlert et al. 2009). In this work it was demonstrated that the influence of small chemicals on the expression of Oct4, Sox2, Rex1 and Nanog genes depended on developmental stages of progenitor cells from HUCB-NSC line. The cells treated with different combinations of small molecules and reprogramming factors did not express all of the pluripotency markers 3 days after the reprogramming treatment. It is possible however that the 3 days of incubation following the reprogramming procedures was not sufficient for induction of all tested pluripotency genes. This would be consistent with previous observations that at least 8 to 10 days are needed to efficiently reprogram differentiated cells to pluripotent state (Anokye-Danso et al. 2011). Nevertheless, the differences in expression pattern between tested treatment conditions were observed. The most important finding is that only the cells grown under SF culture conditions treated with a combination of inhibitors of histone deacetylases (Trichostatin A-TSA) and methyltransferases (RG-108) as well as recombinant proteins after prolonged incubation time in ESCM on Matrigel were able to gain typical iPSC morphology and showed continuous expression of undifferentiated state markers which are the evidence of achieved pluripotent state. It appears that all of the used reprogramming factors (RG-108, TSA, and reprogramming proteins) together with optimized oxygen conditions were important for reprogramming of neural progenitor stem cells derived from human umbilical cord blood into pluripotent state. In all tested conditions, none of mentioned above factors was alone sufficient to reprogram neural stem cells to a stable pluripotency state.

CONCLUSION

The present study demonstrated that small molecules such as TSA and RG-108 together with reprogramming proteins in lowered oxygen conditions can activate and maintain pluripotent state in neural pro-

genitor cells. In conclusion, it is evident that the developmental stage of the cells and epigenetic changes play an important role in the induction of pluripotency gene expression.

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