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Design of iPSC-based cell model to study the functions of the *UBE2A* gene

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ABSTRACT

BACKGROUND: The UBE2A protein belongs to the E2 family of ubiquitin-binding enzymes involved in the ubiquitination of substrate proteins. *UBE2A* mutations lead to congenital X-linked mental retardation syndrome-type Nascimento. How *UBE2A* participates in the central nervous system development is still unknown.

AIM: To establish a cell model based on induced pluripotent stem cells (iPSCs) to study the molecular and cellular functions of *UBE2A* in neurogenesis.

METHODS: Using genomic CRISPR-Cas9 editing and lentiviral transduction, a cell model based on iPSCs from two healthy donors was designed. This cell model includes isogenic iPSCs with knockout and inducible hyperexpression of *UBE2A*. In addition, iPSCs were obtained by reprogramming peripheral blood mononuclear cells of a patient diagnosed with X-linked mental retardation of Nascimento type, which has a deletion spanning the whole *UBE2A* locus.

RESULTS: The obtained iPSCs demonstrate an ESC-like morphology. They express pluripotent cell markers OCT4, SOX2, SSEA-4, and TRA-1-81 and have normal karyotypes. iPSCs with *UBE2A* knockout or hyperexpression had significantly increased nuclei size compared with the isogenic control.

CONCLUSION: The developed iPSC-based cell model can be used for fundamental studies of the functions of *UBE2A* in neurogenesis.

Keywords: iPSC; ubiquitin-conjugating enzyme E2A (RAD6 homolog), human; X-linked mental retardation; gene knockout.

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Создание клеточной модели на основе индуцированных плюрипотентных стволовых клеток для изучения функций гена *UBE2A*

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АННОТАЦИЯ

Обоснование. Белок UBE2A относится к семейству E2 убиквитин-связывающих ферментов, которые участвуют в процессе убиквитинирования белков-субстратов. Известно, что мутации гена *UBE2A* связаны с синдромом врождённой Х-сцепленной умственной отсталости типа Насименто. До сих пор остаётся неизвестным, каким образом дисфункция гена *UBE2A* приводит к нарушению развития центральной нервной системы.

Цель исследования — создание клеточной модели на основе индуцированных плюрипотентных стволовых клеток (ИПСК) для изучения молекулярных и клеточных функций гена *UBE2A* в нейрогенезе.

Методы. Используя геномное CRISPR-Cas9-редактирование и лентивирусную трансдукцию, мы создали клеточную модель на основе ИПСК двух здоровых доноров, включающую изогенные ИПСК с нокаутом и индуцибельной гиперэкспрессией гена *UBE2A*. Дополнительно к изогенным системам мы получили линию ИПСК путём репрограммирования мононуклеаров периферической крови пациента, которому поставлен диагноз Х-сцепленной умственной отсталости типа Насименто и у которого выявлена делеция, целиком захватывающая локус гена *UBE2A*.

Результаты. Полученные ИПСК демонстрируют морфологию, подобную эмбриональным стволовым клеткам. Они экспрессируют маркёры плюрипотентных клеток OCT4, SOX2, SSEA-4 и TRA-1-81 и имеют нормальный кариотип. Обнаружено, что у ИПСК с нокаутом или гиперэкспрессией гена *UBE2A* происходит статистически значимое увеличение размера клеточного ядра по сравнению с изогенным контролем.

Заключение. Созданная клеточная модель на основе ИПСК может быть использована для фундаментальных исследований функций гена *UBE2A* в нейрогенезе.

Ключевые слова: ИПСК; убиквитин-конъюгирующий белок E2A (гомолог Rad6) человека; Х-сцепленная умственная отсталость; нокаут гена.

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INTRODUCTION

The protein UBE2A, encoded by the X-linked gene by the same name, belongs to the family of E2 ubiquitin-binding enzymes and participates in ubiquitination — the attachment of a short polypeptide of ubiquitin to protein substrates. During the transfer of ubiquitin to protein substrates, UBE2A interacts with several E3 ubiquitin ligases, each of which is involved in various cellular processes. For example, UBE2A interacts with PARKIN ubiquitin ligase during mitophagy [1], with RNF20/RNF40 ubiquitin ligase during transcriptional regulation by histone H2B monoubiquitination [2], and with RAD18 ubiquitin ligase during DNA damage repair by tranlesion DNA synthesis [3]. UBE2A also interacts with the neuro-specific ubiquitin ligase UBR4 [4].

The protein UBE2A is highly conserved, with a 152-amino-acid sequence that is identical in humans, mice, and clawed frogs. Yeast also has a very close homolog, the protein Rad6. The fact that the protein UBE2A is highly conserved indirectly indicates its functional importance. However, its absence is not lethal. Deletions and mutations in the gene *UBE2A* have been associated with the development of the Nascimento syndrome, a form of X-linked mental retardation that manifests as mental retardation and intellectual disability in carriers [5, 6]. This disease is exceedingly rare; no more than three dozen cases of mental retardation associated with mutations in this gene have been reported [7]. In mothers of Nascimento syndrome patients with pathogenic mutations in the *UBE2A* gene, there is a skew from random inactivation of X chromosomes to completely nonrandom inactivation of a specific X chromosome in somatic cells [6, 8].

Interestingly, there are also duplications of the locus encompassing the *UBE2A* gene [7]. Some of the duplications detected in patients are associated with mental retardation, which suggests that a high dose of the gene *UBE2A* may have a pathogenic effect.

The precise manner by which the UBE2A protein participates in the formation of the central nervous system remains unknown. The complexity of studying the role of this protein in neurogenesis lies, in particular, in the multiplicity of its partners among E3 ubiquitin ligases and the diversity of cellular processes in which it participates. Additionally, the study of the UBE2A protein is complicated by the fact that in mammalian cells there is a homolog of the gene *UBE2A*, the gene *UBE2B*, which is located on chromosome 5. The amino acid sequences of proteins encoded by the genes *UBE2A* and *UBE2B* are 96% identical in humans [9]. The high degree of sequence similarity raises questions about the functional activity of these proteins. However, the amount of the products of these genes in various organs and tissues is not the same. Additionally, mutations in these genes lead to different phenotypic manifestations [10].

Cell models based on induced pluripotent stem cells (iPSCs) are used to study the molecular mechanisms of inherited diseases *in vitro* in many modern studies. One of the advantages of iPSCs is that they can be derived from cells of patients with a precisely established diagnosis [11]. Additionally, thanks to genomic editing technology (e.g., CRISPR-Cas9), it is possible to create isogenic iPSC lines differing only by a certain mutation, which allows exclusion of the influence of genetic background. iPSCs can differentiate into various cell types, such as neural cells. They can also be used to obtain three-dimensional cell structures, commonly referred to as organoids, such as brain organoids [12]. Consequently, iPSCs can be used to study developmental pathologies at both cellular and tissue levels [13]. Particularly, iPSCs derived from patients with congenital brain developmental disorders can be differentiated into a neural cell type of interest (e.g., forebrain neurons) for further investigation of the molecular pathogenetic mechanisms underlying these disorders [14].

In this study, we describe two independent isogenic iPSC systems, including iPSCs with knockout and inducible hyperexpression of the gene *UBE2A*. These cell lines were created from a healthy donor's iPSCs by genomic CRISPR-Cas9-editing and lentiviral transduction, respectively. Apart from the isogenic cell systems, we obtained a line of iPSCs from peripheral blood lymphocytes of a patient with the Nascimento syndrome, in whom the long arm of the X chromosome has a deletion of 167 Kbp, spanning five genes, including the UBE2A gene [8].

The aim of the study was to create a cell model based on iPSCs to investigate the molecular and cellular functions of the gene *UBE2A* in neurogenesis.

MATERIALS AND METHODS

Cell culture conditions

The iPSCs were cultured in mTeSR1 medium (STEMCELL Technologies, Canada) with 50 units/mL penicillin-streptomycin (PanEco, Russia) on Matrigel-coated (Corning, USA) Petri dishes at 37 °C in a CO_2 incubator in an atmosphere of 5% CO_2 and 80% humidity. Cells were passaged using 0.05% trypsin-EDTA (Gibco, USA) and 10 μM ROCK inhibitor Y27632 (Miltenyi Biotec, Germany). Spontaneous differentiation of iPSCs was performed through the stage of embryoid cell formation as described in [15].

HEK 293 and HEK 293 Phoenix cells were cultured in DMEM medium (Gibco) enriched with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 2 mM glutamine (PanEco), 50 units/mL penicillin-streptomycin at 37 °C and 5% CO_2. The medium was changed every 72 h. Cells were passaged using 0.05% trypsin-EDTA.

The absence of mycoplasma contamination of cell cultures was confirmed by PCR as described in [16].

Genomic CRISPR-Cas9-editing of induced pluripotent stem cells

The online resource CRISPOR [17] was used to design a guide RNA (gRNA) that was complementary to the second exon of the gene *UBE2A*. Oligonucleotides encoding gRNA (Table 1) were cloned into plasmid PX458 (Addgene, No. 48138), which carries genes encoding the Cas9 nuclease and green fluorescent protein (GFP). Transfection was conducted using the TransIT-LT1 reagent (Mirus Bio, USA) in accordance with the manufacturer's instructions. GFP-positive cells were sorted using FACS Melody (BD, USA) two days after transfection and seeded at low density in mTeSR1 medium with 10 μM ROCK inhibitor Y27632 and CloneR (STEMCELL Technologies). After 10 days, the iPSCs were seeded into individual wells of a 48-well plate (Corning).

DNA was extracted from selected iPSC clones using the "M-sorb" kit (Syntol, Russia) in accordance with the manufacturer's instructions. Sanger sequencing was conducted to assess the outcomes of genomic editing. Potential off-target sites of CRISPR-Cas9 genome editing were identified using the Off-Spotter online resource [18]. Subsequently, PCR was conducted with primers (see Table 1) targeting potential non-target editing sites,

Table 1. Oligonucleotides used in the study

Role	Gene/Locus	Product size, bp	Direct/reverse primer (5′-3′)
Reference gene (RT-PCR)	*GAPDH*	118	GAAGGTGAAGGTCGGAGTCA/GTTGAGGTCAATGAAGGGGTC
Markers of pluripotency	*SOX2*	278	AACCAGCGCATGGACAGTTAGA/CTTGACCACCGAACCCAT
	SALL4	303	TGGCGGAGAGGGCAAATAACAT/GCTGAAGAACTCCGCACA
	OCT4	887	CCTTCGCAAGCCCTCATTTCGCA/AGTTTGAGCATCCCTCGC
	DPPA5	331	AAGATGGGAACTCTCTCCCCGG/CGCAAGTTTGAGCATCCCTCGC
	LIN28	92	TTCGGCTTCCTGTCCATGAC/CCTTCCATGTGCAGCTTACTC
	SALL4	183	GTGCTCTTCCAGAGCCCTTT/AACCTTGACATAGGTCGGCG
	TDGF1	98	GCTGCTTTCCTCAGGCATT/ACGTGCAGACGGTGGTAGTT
Mycoplasma detection	16S рРНК	705–708	ACTCCTACGGGAGGCAGCAGTA/TGCACCATCTGTCACTCTGTTAACCTC
For the synthesis of guide RNA	*UBE2A*	—	CACCGTCCTCCAGCCGGAGTCA/GCGAAACCGCTGACTCCGGCTGGAGGAC
For Sanger sequencing	*UBE2A*	523	CGACCCTCGACTTCGGAGAAAC/ATTTTCCCCTACCCGCT
For CDS transfer of the *UBE2A* gene	CDS *UBE2A*	452–459	ATGTCCACCCCGGCTCGG/TCAACAATCACGCCAGCTTTGTTCTACTA
For the analysis of quantitative RT-PCR	мРНК *UBE2A*	100	GACTTCAAGAGGTTGCAGG/CTTCAGGCCCGAAAATGA
For transactivator integration analysis	FUdelta-rtTA	133	AACGCACTGTACGCTCTGTC/CCGCTTTCGCACTTTAGCTG
For the integration analysis of episomal transgenes	pCE-OCT4	106	CCCTGTCTCTGTCACCACTC/ CACACCAGCCACCACCTTC
	pCE-KLF4	131	ATGCGACCGAGCATTTTCC/ CACACCAGCCACCACCTTC
	pCE-SOX2	107	CATGTCCCAGCACTACCAGAG/ TTTGTTTGACAGGAGCGACAAT
	pCE-LIN28	113	AGAAATCCACAGCCCTACCC/ CACACCAGCCACCACCTTC
	pCE-L-MYC	122	GGCTGAGAAGAGGATGGCTAC/ TTTGTTTGACAGGAGCGACAAT
	pCE-mp53DD	236	CGTAAACGCTTCGAGATGTTCC/ CACACCAGCCACCACCTTC
	EBNA1	122	CGGGGTAGAGGACGTGAAAG/ GGAGACCCGGATGATGATGAC
	VPS4A	98	GGCAACCACTGCTAATCTACTT/ GCCACAAAGGACCACCTATT
Verification of the deletion	*HEXB*	136	CCGGGCACAATAGTTGAAGT/ TCCTCCAATCTTGTCCATAGC
	UBE2A	92	CAAAGCTGGCGTGATTGTTG/ GGAGTAGGGAGGTGACAAACA
	SEPTIN6	112	GACACCCTGTTCAACACCAAA/ GCTTTAGCCTCACGTTGCTC
Potential off-target effects of editing	*SNX11*	114	TGGAAAGTCCCACTCTCCCA/CATGATCTCCTCCCACAGCC

Note: RT-PCR — reverse transcription polymerase chain reaction, CDS — coding sequence.

and the resulting amplicons were sequenced by Sanger sequencing.

Lentiviral transduction of induced pluripotent stem cells

A virus with a tet-ON transactivator (Addgene, cat. No. 43915), a virus with the coding sequence (CDS) of the gene *UBE2A*, and a virus with GFP LeGO-G2 (Addgene, cat. No. 25917) were used to obtain lentiviruses. The virus with CDS of the gene *UBE2A* was obtained by transferring the CDS of the *UBE2A* gene from plasmid pDEST17-Ube2A (Addgene, cat. No. 15780) to plasmid pFU-tet-o-hSox2 (Addgene, cat. No. 19779). HEK 293 Phoenix cells were transfected using Unifectin-56 (Unifect group, Russia) with helper plasmids containing the viral genes *Rev*, *RRE*, and *VSV-G*, and the target plasmid at a ratio of 19:37:7:37%, respectively, according to the manufacturer's protocol. The supernatant containing viral particles was collected after 48 and 72 h. The supernatant was filtered through a 0.45 μm filter. To determine the titer of lentiviruses, cells were infected with the supernatant containing virus carrying the *GFP* gene at various dilutions (1:1, 1:5, 1:25, 1:125, 1:625). The titer was calculated by determining the percentage of GFP-positive cells.

One day prior to transduction, iPSCs were seeded at a density of 40,000 cells per cm^2 on a 12-well plate (Corning). Cells were transduced with two lentiviruses: a transactivator under the control of the tet-ON system and a vector encoding the CDS of the gene *UBE2A*. The multiplicity of infection (MOI) of both viruses was 10. After 48 h, cells were seeded at a low density. After 10 days, iPSC clones were selected. The presence of the target plasmids in the genome was confirmed by PCR using two primer pairs: one on the CDS fragment of the gene *UBE2A* and one on the fragment of the tet-ON system cassette (see Table 1).

Lymphocyte reprogramming

After obtaining informed consent of the patient's representative with the Nascimento syndrome, his blood was drawn. Reprogramming of peripheral blood lymphocytes was performed in accordance with the methodology described in [19, 20]. A total of five nonintegrative episomes (Addgene, No. 41813-41814, No. 41855-41857) were introduced into 500,000 peripheral blood mononuclear cells by electroporation using the Neon Transfection System (Thermo Fisher Scientific), yielding a total of 6 μg. In the process of reprogramming and obtaining iPSC clones, cells were cultured in the DMEM/F12 medium containing 20% Knockout serum substitute, 1% GlutaMAX-I, 1% substituted amino acids, 1% penicillin-streptomycin, 0.1 mM β-mercaptoethanol, and 10 ng/mL recombinant bFGF protein (all reagents from Thermo Fisher Scientific) on a feeder cell layer. Selection of the obtained iPSC clones was conducted manually. At the second to fifth passages, iPSCs were transferred to feeder-free conditions: in mTESR1 medium on Matrigel-coated dishes. The integration of episomal vectors into the genome of iPSCs was confirmed at the 12^{th} to 18^{th} passages using quantitative PCR with primers for all introduced transgenes. To confirm deletion, real-time PCR was used, using primers for the genes *UBE2A* and *SEPT6* localized in the deletion region, in addition to control primers for the gene *HEXB* (see Table 1).

Flow cytometry

The iPSC suspension was prepared using a 0.05% trypsin-EDTA solution. To stain surface markers SSEA-4 and TRA-1-81, the collected cells were incubated with primary antibodies for 15 min at room temperature. To stain the OCT4 nuclear marker, cells were fixed in 4% paraformaldehyde solution (Sigma-Aldrich, USA) for 15 min on ice, before they were treated with 80% cold ethanol for 30 min on ice, and finally incubated with primary labeled antibodies (Table 2) in phosphate-buffered saline (PBS) (PanEco) supplemented with 2% FBS at 4 °C for 60 min. To stain surface markers SSEA-4 and TRA-1-81, samples were incubated with secondary antibodies (Table 3) at 4 °C for 30 min in the dark. Analysis was performed using a NovoCyte flow cytometer (Agilent, USA).

Immunocytochemical staining

The cells were fixed in a 4% paraformaldehyde solution diluted in PBS for 10 min, before permeabilization in 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. They were then incubated with a blocking solution consisting of PBS, 0.1% Tween 20 (Thermo Fisher Scientific), 2.5% FBS, and 2.5% goat serum (Thermo Fisher Scientific) for 30 min. All procedures were conducted at room temperature. The primary antibodies were incubated with the cells overnight at 4 °C, whereas the secondary fluorescently labeled antibodies were incubated for 30–45 min at room temperature. The nuclei were stained with DAPI (Sigma-Aldrich). The preparations were analyzed on an Eclipse Ni-E fluorescence microscope (Nikon, Japan) equipped with a Qi2 camera (Nikon), and microphotometry was performed using BR NIS-Elements software (Nikon).

Western blotting

Protein lysates were obtained by resuspending cell sediment in a RIPA buffer (50 mM Tris HCl; 150 mM NaCl; 0.5% Sodium deoxylcholate; 0.1% SDS) and incubating it for 1 h at 4 °C. The lysate was then stored at –70 °C. Protein lysates were combined with twofold Lammli buffer (Bio-Rad, USA), incubated for 5 min at 95 °C, and 10 μg of total protein was applied per well of the gel. A 10% polyacrylamide separating gel and a 5% polyacrylamide concentrating gel were prepared and poured under traction. Protein electrophoresis was conducted in a vertical chamber (Bio-Rad) at 100 V in Tris-glycine buffer with SDS. The proteins were transferred from the gel to the PVDF membrane via a Trans-Blot Turbo device (Bio-Rad) for 30 min at 25 V. Subsequently, nonspecific antibody binding was blocked by incubation in 5% skim milk (Bio-Rad) for 16 h at 4 °C (see Table 2). The following day, the membrane was washed with PBST (PBS + 0.1% Tween 20), incubated with horseradish

Table 2. Primary antibodies used in the study

Antibody	Manufacturer, cat. No.	RRID
Rabbit IgG anti-OCT4	Abcam, ab18976	RRID:AB_444714
Mouse Alexa Fluor 488 anti-Oct4 (Oct3)	Sony Biotechnology, 3868525	RRID:AB_2940931
Rabbit IgG anti-SOX2	Abcam, ab97959	RRID:AB_2341193
Mouse IgG3 anti-SSEA-4	DSHB, MC-813-70	RRID:AB_528477
Mouse IgM anti-TRA-1-81	Cell Signaling Technology, 4745	RRID:AB_2119060
Mouse IgG2b, kappa anti-Tubulin β 3 (TUBB3)/ Clone: AMC0115	ABclonal, A18132	RRID:AB_2861923
Rabbit IgG anti-human HNF3β/FOXA2	ABclonal, A19053	RRID:AB_2862546
Rabbit IgG anti-human Vimentin/Clone: SP20	Thermo Fisher, RM-9120-S0	RRID:AB_722371
Mouse IgG1 anti-human cytokeratin PAN (CK PAN)	"PrajmBioMed", 10-310046	RRID:AB_2940932
Rabbit IgG anti-UBE2A	Thermo Fisher Scientific, PA5-112140	RRID:AB_2866876
Rabbit anti-human TATA binding protein TBP	Abcam, ab63766	RRID:AB_1281140

Table 3. Secondary antibodies used in the study

Antibody	Manufacturer, cat. No.	RRID
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Thermo Fisher Scientific, A21422	RRID:AB_2535844
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific, A11008	RRID:AB_143165
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific, A21236	RRID:AB_2535805
Rabbit anti-Mouse IgG (whole molecule) Peroxidase antibody	Sigma-Aldrich, A9044	RRID:AB_258431
Goat anti-rabbit IgG (whole molecule) Peroxidase antibody	Sigma-Aldrich, A9169	RRID:AB_258434

peroxidase-conjugated secondary antibodies for 1 h, before it was washed with PBST solution. Visualization was performed on a ChemiDoc gel-documentation system (Bio-Rad) using ECL Femto-Sensitivity reagents (Invitrogen, USA).

Karyotyping

Karyotyping was performed as described in [14].

RNA isolation and reverse transcription polymerase chain reaction

RNA was isolated using the RNAeasy Mini Kit (QIAGEN, USA). To synthesize complementary DNA (cDNA), degenerate d(N)10 primers (Evrogen, Russia) and the M-MLV reverse transcription kit (Evrogen) were used in accordance with the manufacturer's instructions. Quantitative RT-PCR (reverse transcription PCR) was performed using specific primers (see Table 1) on a CFX96 Touch amplifier (Bio-Rad). PCR was performed in a total volume of 20 µl, using a 5X qPCRmix-HS SYBR ready mix (Eurogen) and a primer concentration of 500 nM. Each reaction was performed in duplicate.

Ethical review

The study was approved by the Ethics Committee of Lopukhin Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency (Protocol No. 1 dated June 1, 2021). The patient's representative voluntarily signed the informed consent form approved by the Ethical Committee of the Research Institute of Medical Genetics of the Tomsk National Research Medical Center of the Russian Academy of Sciences (Protocol No. 3 dated May 17, 2018).

RESULTS

Creation and validation of a cell model for studying *UBE2A* gene functions

In this study, two isogenic iPSC systems were created, including iPSCs with gene *UBE2A* knockout and those with inducible hyperexpression of gene *UBE2A* (Fig. 1, *a*). This model was created based on two previously obtained iPSC lines reprogrammed from skin fibroblasts of healthy

donors. The IPSRG4S (RCPCMi009-A) line, which has a male karyotype [21], and the IPSFD5S line, which has a female karyotype [22], had also been obtained earlier. The IPSRG4S line was obtained as a knockout of the gene *UBE2A* [14].

An isogenic IPSFD5S iPSC line with a knockout (KO) of the gene *UBE2A* was obtained from IPSFD5S using genomic CRISPR-Cas9-editing. Genomic CRISPR-Cas9-editing of the IPSFD5S line was performed using gRNA complementary to the sequence of the second exon of the gene *UBE2A*. The editing resulted in a homozygous 7-bp deletion at the gRNA annealing site. This deletion consequently formed a premature stop codon in the matrix RNA (mRNA), which was then subjected to nonsense-mediated decay. Western blotting (Fig. 1, *c*) confirmed the absence of UBE2A protein expression in the selected knockout line. The resulting iPSC KO-*UBE2A* IPSFD5S line retained ESC-like morphology (ESC — embryonic stem cells) and normal karyotype (46, XX). Additionally, this line exhibited expression of pluripotency markers (OCT4, NANOG, TRA-1-81, SSEA-4) and the capacity to differentiate into derivatives of three germ layers (Fig. 2, *b–f*).

Given the pathogenic effect of a high dose of the gene *UBE2A*, it was decided to supplement the isogenic systems with lines with increased expression of the gene in question. Lentiviral transduction was used to obtain iPSCs with inducible hyperexpression of the gene *UBE2A*, for which two viruses were collected: one containing the tet-ON transactivator and another containing the CDS of the gene *UBE2A*. Lentiviral transduction of iPSCs IPSRG4S and IPSFD5S yielded corresponding isogenic iPSC lines with tet-ON-inducible hyperexpression of the gene *UBE2A*. Quantitative RT-PCR and Western blotting (Fig. 1, *b*; see Fig. 1, *c*) confirmed a significant increase in the expression of this gene in response to treatment of cells with doxycycline for 72 h. The lentiviral-transduced iPSCs exhibited a slight increase in *UBE2A* gene expression even in the absence of doxycycline induction (data not shown). This can be attributed to an imperfect genetic transgenic construct in which the transgene promoter is not completely "silent" in the absence of doxycycline induction. This phenomenon may become even more apparent through multiple transgene insertions during lentiviral transduction.

a

Parental IPSCs	**WT-*UBE2A* IPSFD5S [22]** Healthy donor, 46XX	**WT-*UBE2A* IPSRG4S [21]** Healthy donor, 46XY
***UBE2A* gene knockout**	**KO-*UBE2A* IPSFD5S** Homozygous 7-bp deletion hg38, chrX:119,574,961-119,574,967	**KO-*UBE2A* IPSRG4S** Homozygous 1-bp insertion (A) hg38, chrX:119,574,966-119,574,967
Inducible expression of the *UBE2A* gene	**Over-*UBE2A* IPSFD5S** Induction with doxycycline	**Over-*UBE2A* IPSRG4S** Induction with doxycycline

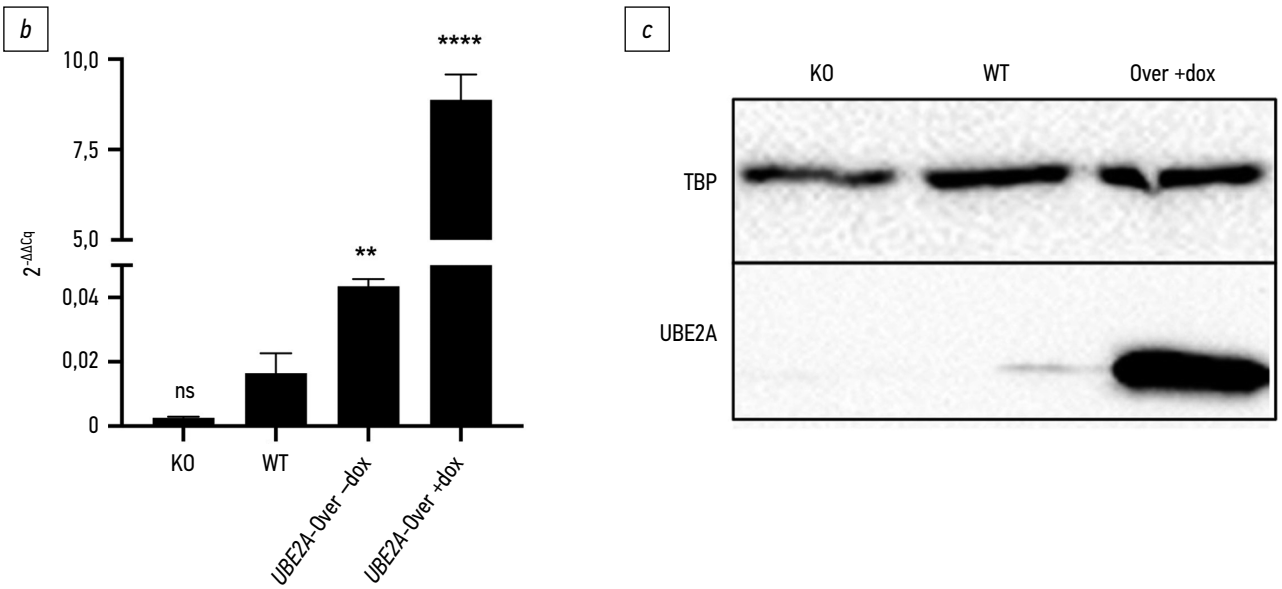

Fig. 1. Cell model based on iPSCs of healthy donors for studying *UBE2A* gene: *a* — iPSC lines of the cell model; *b* — validation of the iPSCs derived from IPSFD5S by analysis of *UBE2A* gene expression; *c* — validation of the iPSCs derived from IPSFD5S by western blotting with antibodies to UBE2A protein. Here: KO — KO-*UBE2A* IPSFD5S; WT — WT-*UBE2A* IPSFD5S; –dox — Over-UBE2A IPSFD5S without doxycycline induction; Over +dox — Over-UBE2A IPSFD5S after doxycycline induction; $2^{-\Delta\Delta Cq}$ — relative expression, calculated with delta-delta Cq algorithm; TBP — TATA binding protein; ** difference with WT is significant, p <0.01; **** difference with WT is significant, p <0.0001; ns — difference with WT in not significant.

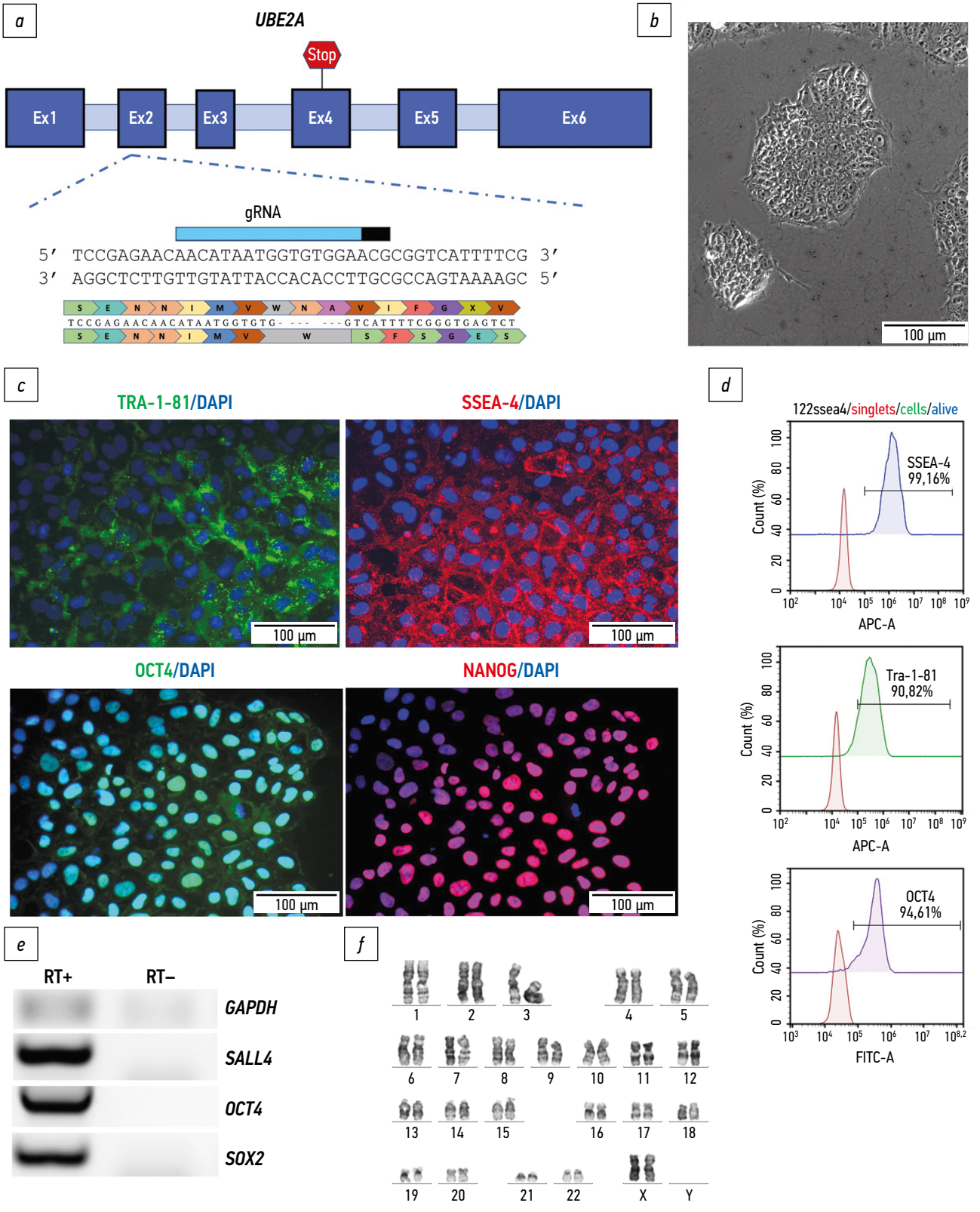

Fig. 2. Characterization of KO-*UBE2A* IPSFD5S line: *a* — localization of gRNA used for CRISPR-Cas9 genome editing and the obtained 7 bp deletion in the second exon of the *UBE2A* gene; *b* — ESC-like morphology of KO-*UBE2A* IPSFD5S; *c* — immunocytochemical staining for pluripotency markers OCT4, NANOG, SSEA-4 AND TRA-1-81, DAPI — blue color, corresponding marker — green or red; *d* — cytometry analysis for pluripotency markers OCT4, SSEA-4 and TRA-1-81; *e* — analysis of pluripotency state-specific gene expression in IPSFD5S IPSCs by quantitative RT-PCR; *f* — differential GTG staining of metaphase chromosomes revealed a normal 46, XX karyotype in KO-*UBE2A* IPSFD5S line. Ex — exon, RT+ — cDNA synthesis using reverse transcriptase; RT– — cDNA synthesis using no reverse transcriptase; APC-A — allophycocyanin fluorescence, area; FITC-A — fluorescein fluorescence, area. See text for other designations.

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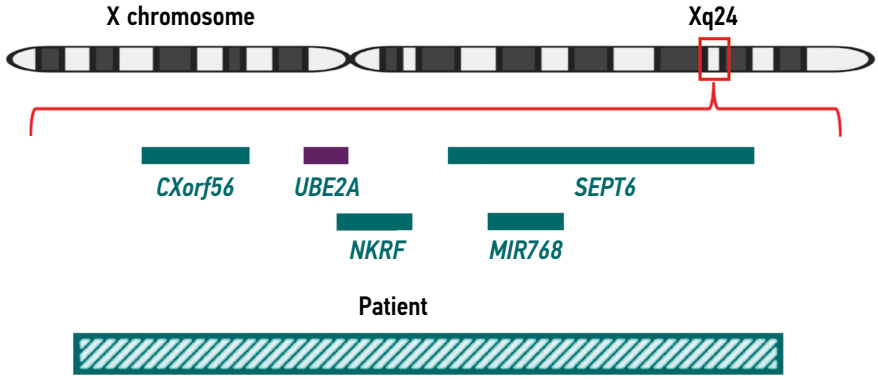
Generation and characterization of a cell line of induced pluripotent stem cells from a patient with *UBE2A* gene deletion

For more detailed study of the role of the gene *UBE2A* in the development of mental retardation, we created the IPSC line IPS67-7 by reprogramming peripheral blood lymphocytes from a patient with X-linked mental retardation of the Nascimento type. In this patient, a hemizygous deletion of 167.5 bp of the long arm of the X chromosome had been previously detected [8]. This deletion resulted in the complete absence of five genes in his genome, including the *UBE2A* gene (Fig. 3, *a*).

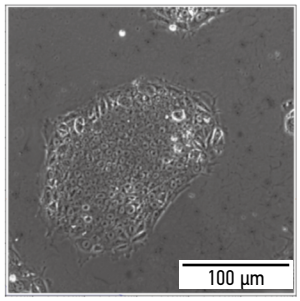
Transfection with non-integrating episomal plasmid vectors expressing reprogramming factors, including OCT4, MYC, LIN28, SOX2, and KLF4, was performed to obtain iPSCs from lymphocytes of a patient with X-linked mental retardation. Additionally, episomes were engineered to carry genes encoding auxiliary factors that enhance the efficiency of reprogramming, including the EBNA1 protein and the C-terminal fragment of the mouse p53 protein mp53DD [18].

The IPS67-7 iPSC line was characterized to ascertain its pluripotency status. The iPSC cell line exhibited the typical morphology of human pluripotent stem cells (Fig. 3, *b*) and displayed high proliferative activity. Flow cytofluorometry confirmed the high expression of pluripotency markers,

a



b



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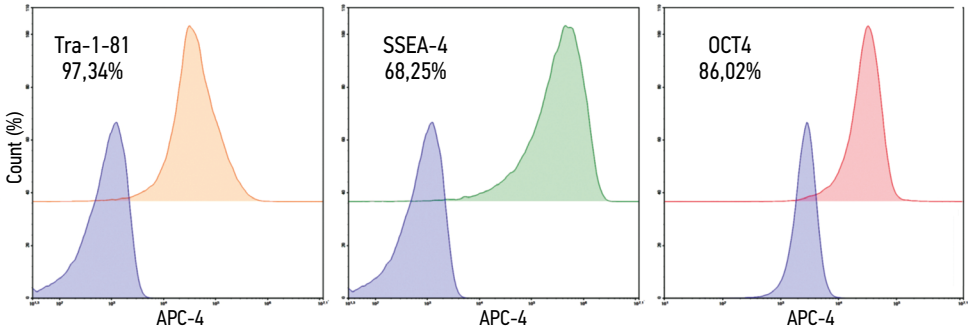
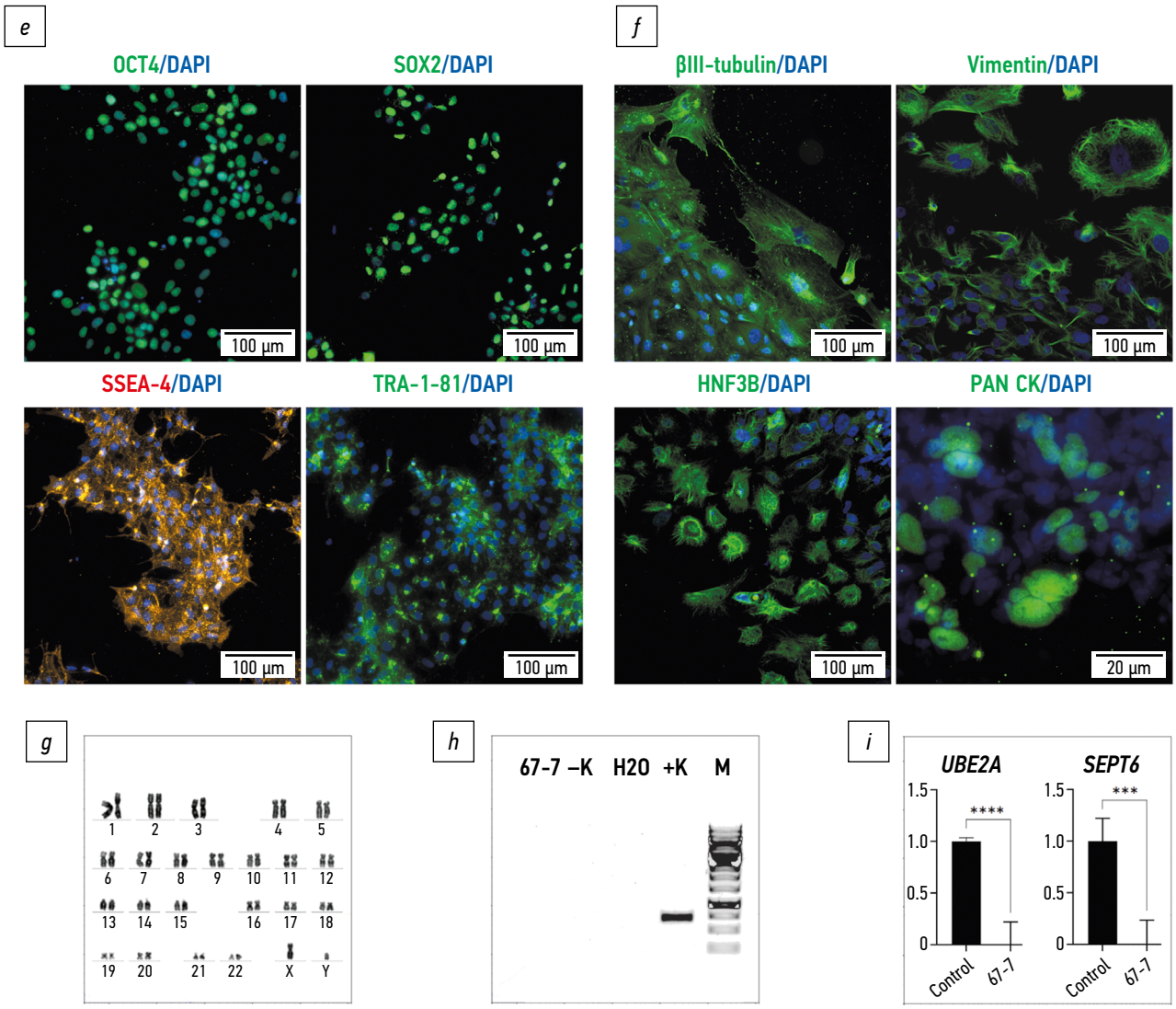


d

Genes	67-7	ESC Control	Fibroblasts
LIN28	+	+	–
SALL4	+	+	–
TDGF1	+	+	–
GAPDH	+	+	+

Fig. 3. Characterization of IPSC line IPS67-7 derived from lymphocytes of a patient with Nascimento syndrome: *a* — localization of 167.5 Kbp deletion with indication of genes captured by the deletion [8]; *b* — ESC-like morphology of IPS67-7 colony; *c* — flow cytometry analysis for pluripotency markers OCT4, SSEA-4 and TRA-1-81 in IPS67-7; *d* — RT-qPCR analysis for pluripotent gene expression , cDNA of HUES9 ESCs served as a positive control, cDNA of healthy donor fibroblasts was taken as a negative control; *e* — immunocytochemical staining analysis for pluripotency markers OCT4, SOX2, SSEA-4 AND TRA-1-81, DAPI — blue color, corresponding marker — green or red; The scale bar corresponds to 100 µm; *f* — immunocytochemical staining analysis of spontaneous differentiation derivatives of IPS67-7. Antibodies to βIII-tubulin (βIII-tubulin) and pancytokeratin (PAN CK) were used to detect ectodermal derivatives, antibodies to vimentin (vimentin) were used to detect mesodermal derivatives, antibodies to FOXA2 (HNF3B) were used to detect entodermal derivatives, DAPI — blue, corresponding marker — green; scale bar — 100 µm; *g* — differential GTG staining of metaphase chromosomes revealed a normal 46, XY karyotype in IPS67; *h* — PCR test did not reveal mycoplasma contamination of cells, 67-7 — DNA isolated from IPS67-7 cells, –K — negative control, H2O — PCR technical control, +K — positive control; *i* — verification of the deletion in IPS67-7 by quantitative PCR with primers for *UBE2A* and *SEPT6* genes. The HEXB gene was used as a reference gene; *** p <0.001; **** p <0.0001. See text for designations.

DOI: https://doi.org/10.17816/gc623799



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including transcription factor OCT4 and surface markers SSEA-4 and TRA-1-81 (Fig. 3, *c*). Expression of the pluripotency genes *LIN28*, *SALL4*, and *TDGF1* was confirmed by quantitative RT-PCR using ESC line HUES9 as a positive control and healthy donor skin fibroblasts as a negative control (Fig. 3, *d*). Visualization of pluripotency biomarkers (transcription factors OCT4 and SOX2, and surface markers SSEA-4 and TRA-1-81) was performed by immunocytochemical staining (Fig. 3, *e*). The ability of IPS67-7 cells to form three germ layers (ento, meso- and ectoderm) was confirmed by spontaneous *in vitro* differentiation to the embryoid cell stage. The resulting differentiated cells expressed biomarkers of ectoderm (pancytokeratin and βIII-tubulin), mesoderm (vimentin) and entoderm (FOXA2) (Fig. 3, *f*). Karyotype analysis performed at 14th passage showed that the iPSC line obtained had a stable chromosomal composition and a normal karyotype (46, XY) (Fig. 3, *g*). Quantitative PCR using primers specific for the deleted region of the X chromosome confirmed the presence of the deletion in iPSCs from a patient with the Nascimento syndrome (Fig. 3, *i*). The resulting iPSCs at passage 18 were tested for the integration of episomes into the genome. This test showed that the genome of the iPSC line contained *OCT4*, *EBNA1*, and *mp53DD* transgenes, and the *mp53DD* transgene retained a strong expression in the iPSCs at passage 18 (data not shown).

Cell nucleus morphometry in induced pluripotent stem cell lines of the derived model

Immunocytochemical staining of the obtained iPSC lines indicated that the knockout of the *UBE2A* gene and its overexpression increased the size of cell nuclei (Fig. 4). Morphometric analysis of the obtained iPSC lines revealed a significant increase in the area of nuclei in the modified iPSCs. The most striking effect was observed in the iPSCs with inducible overexpression of the *UBE2A* gene (Table 4).

The increase in the size of nuclei in knockout iPSCs was observed only relative to the isogenic parental iPSCs. For instance, in iPSC IPSFD5S, the average area of nuclei was 101.4±0.9 μm^2, whereas in the isogenic knockout clone, it was 148.7±1.3 μm^2, which was lower than in normal

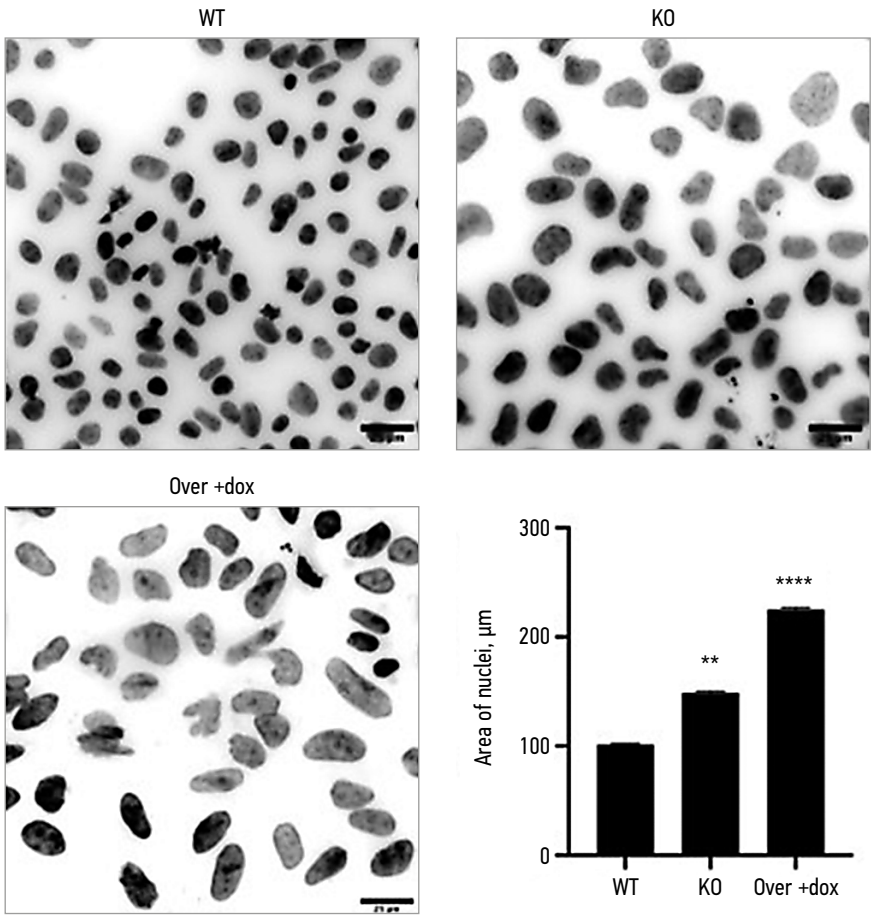

Fig. 4. Cell nuclei area in KO-*UBE2A* IPSFD5S p. 54 (KO) and Over-UBE2A IPSFD5S p. 52 (Over +dox) is significantly larger than in the isogenic normal IPSFD5S p. 46 (WT), ** difference from the normal IPSCs is significant, p <0.01; Student's t-test; **** difference from the normal IPSCs is significant, p <0.0001, Student's t-test. Scale bar — 25 µm, histogram whiskers indicate standard error of mean.

Table 4. Results of measurements of nuclei area in the induced pluripotent stem cells of cell model

Induced pluripotent stem cell line	*UBE2A* gene status	Number of nuclei analysed	Mean nuclei area ± SEM, µm²
IPSFD5S	Wild type	2127	101,4±0,9
KO-UBE2A IPSFD5S	Knockout	2130	148,7±1,3**
Over-UBE2A IPSFD5S	Overexpression	10225	220,6±0,8****
IPSRG4S	Wild type	1007	156,9±2,6
KO-UBE2A IPSRG4S	Knockout	1600	188,4±2,5**
Over-UBE2A IPSRG4S	Overexpression	2457	278,6±1,7****
IPS67-6	Deletion	282	136,7±2,8

** differences with isogenic normal induced pluripotent stem cells were statistically significant, p <0.01; Student's t-test; **** differences with isogenic normal induced pluripotent stem cells were statistically significant, p <0.0001; Student's t-test.

male iPSC IPSRG4S (See Table 4). However, in knockout derivatives of male IPSRG4S iPSCs, the average nuclei area was higher than in the parental normal iPSCs, with a value of 188.4±2.5 µm² (See Table 4). The mean area of the nuclei in iPSCs derived from the patient with a *UBE2A* gene deletion was similar to that of knockout clones of the female FD5S line, with a mean of 136.7±2.8 µm² (See Table 4).

DISCUSSION

The UBE2A protein is highly conserved and its amino acid sequence is identical among vertebrates [5]. Therefore, it can be assumed that there is a high selective pressure against the emergence of new variants of the protein. Additionally, selection, at least at the somatic cell level, can explain the fact that 100% nonrandom inactivation of the X chromosome is observed in blood cells from mothers of patients with the Nascimento syndrome [6, 8]. However, the knockout of this gene is not lethal in mice. Only a homozygous knockout of the gene *UBE2A* leads to sterility in females [9]. This discrepancy suggests that mutations in the gene *UBE2A* is non-lethal only in a certain genetic context. The extreme rarity of the Nascimento syndrome is consistent with the assumption that mutations in the gene *UBE2A* may be non-lethal in

a certain genetic context. Furthermore, the effect of the gene *UBE2A* knockout on the cell nucleus area in iPSCs can only be detected by comparing isogenic cells. The model we have created, which includes two isogenic systems of iPSCs and iPSCs from a patient with the Nascimento syndrome, can be used to study the effect of gene *UBE2A* dysfunction without influence of genetic background. Moreover, it can be used to demonstrate how the dysfunction of this gene manifests in various genetic environments.

In our study, we reprogrammed blood lymphocytes from a patient with the Nascimento syndrome in a nonintegrative method using episomal transfection [18]. However, the iPSCs that we obtained integrated into the genome of three (*OCT4*, *EBNA1*, and *mp53DD*) out of the seven transgenes that were introduced during reprogramming. The transgene *OCT4* encodes the transcription factor OCT4, which is one of the four major reprogramming factors included in the Yamanaka cocktail [23]. The transgene *EBNA1* encodes an Epstein–Barr virus EBNA1 protein that promotes extrachromosomal replication by episomes and can also bind the p53 protein and prevent its activity as a transcription factor. The transgene *mp53DDD* encodes a C-terminal fragment of mouse p53 protein, which has a dominant-negative effect, preventing the formation of functional tetramers of protein p53 [18]. The transgenes *EBNA1* and *OCT4* do not exhibit expression in iPSCs at passage 18. This suggests that they underwent epigenetic silencing, which is common in exogenous factors upon completion of reprogramming. Residual activity of exogenous reprogramming factors impairs the ability of iPSCs to differentiate [24]. However, our findings demonstrate that the patient's iPSCs, obtained through our methodology, differentiated into derivatives of all three germ layers. These findings support the conclusion that the *OCT4* integrating transgene underwent reliable silencing.

In contrast to observations in *EBNA1* and *OCT4* transgenes, the anti-apoptotic transgene *mp53DD* continued expressing in the passaged 18 iPSCs in patient. The fact that human pluripotent stem cells *in vitro* are predisposed to upregulation of anti-apoptotic genes is well documented. A subsequent cytogenetic analysis of over 120 human ESC lines revealed that a significant proportion of these lines exhibited a recurrent duplication of the 20q11.21 region, which encompasses the anti-apoptotic gene *BCL2L1* [25]. The integration of the transgene *mp53DD* and the preservation of its expression even in advanced passages of iPSCs indicate that it is impossible to use the obtained iPSC line IPS67-7 in studies investigating apoptosis or cytotoxicity. Nevertheless, this iPSC line passed all tests for pluripotency, including the ability to differentiate into derivatives of three germ layers, and thus can be used as an addition to our isogenic system created on the basis of iPSCs from two healthy donors.

Quantitative RT-PCR indicated that the knockout of the gene *UBE2A* resulted in a minimal reduction in the quantity of *UBE2A* mRNA. The knockout in iPSCs was validated through Western blotting with antibodies directed against the UBE2A protein. The Human Proteome Atlas database [26] indicates that commercially available antibodies lack sufficient specificity for UBE2A protein due to cross-reactivity with its highly similar homolog, the UBE2B protein. This suggests that the successful validation of knockout in our work was possible due to the lower expression of the UBE2B protein than protein UBE2A in iPSCs. However, the cross-reactivity of antibodies precludes the application of western blotting or immunocytochemical staining in differentiated neural progenitor cells, where the used antibodies to UBE2A "recognized" a protein product even in patient cells in the complete absence of the *UBE2A* gene locus (data not shown). The cell model generated by our laboratory, which includes iPSCs with a deletion and overexpression of the gene *UBE2A*, can serve as a reliable validation platform for the development of antibodies that are specific to UBE2A and do not cross-react with UBE2B.

Our findings indicate that iPSCs with knockout or hyperexpression of the gene *UBE2A* exhibit a larger mean area of nuclei than the parental iPSC line. An abnormal increase in cell nuclei has been previously demonstrated for cells with overexpression of the gene *UBE2B*, which has high homology with the gene *UBE2A* [27]. The observed increase in the mean nucleus area in the cell population can be attributed to several factors, including an increase in the proportion of cells at the G2 stage, cell flattening due to increased cell adhesion, and alterations in the dynamics of nuclear-cytoplasmic transport.

CONCLUSIONS

In this study, we constructed a cell model based on iPSCs to investigate the role of the *UBE2A* gene in the pathogenesis of the Nascimento syndrome. This model comprised two isogenic cell systems, derived from two healthy donors, each consisting of iPSCs with knockout of the gene *UBE2A*, iPSCs with inducible overexpression of the gene *UBE2A*, and normal parental iPSCs. The model was supplemented with a line of iPSCs from a patient with the Nascimento syndrome caused by deletion of the X chromosome region containing the gene *UBE2A*. This model was used for the first time to demonstrate that both knockout and overexpression of the gene *UBE2A* result in a significantly higher mean area of nuclei in iPSCs than in the isogenic control. Additionally, our findings indicate that the morphological effect of *UBE2A* gene knockout in iPSCs can only be detected in an isogenic context. These findings suggest that genetic background plays a significant role in the manifestation of mutations in the gene *UBE2A*. The model can be used for fundamental studies of *UBE2A* gene functions, including its molecular and cellular functions in neurogenesis. Furthermore, the model can be used to generate antibodies that are specific to the UBE2A protein and not cross-reactive to the homologous UBE2B protein.

ADDITIONAL INFORMATION

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Competing interests. The authors declare that they have no competing interests.

Authors' contribution. E.A. Khomyakova and A.V. Fedorenko performed the cultivation and genetic modification of iPSCs and their characterization; M.A. Gridina and A.A. Khabarova obtained iPSCs from a patient with Nascimento syndrome; A.A. Kashevarova, D.A. Fedotov, and T.V. Limanskaya characterized the iPSCs of the patient; A.V. Surdina and L.D. Belikova analyzed the expression of pluripotency markers by immunocytochemical staining and flow cytometry, respectively; E.A. Volovikov and E.K. Sekretova performed western blotting; E.A. Zerkalenkova analyzed the karyotype of IPSC lines; M.A. Lagarkova, I.N. Lebedev, and A.N. Bogomazova developed the design of the experiment, analyzed the obtained data, and participated in writing the article.

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