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COLLECTION OF PLURIPOTENT STEM CELL LINES

Passport of the pluripotent stem cell line

Creation of Induced Pluripotent Stem Cells RCMGi014-A Using Reprogramming of Urine Cells of a Patient with Fibrodysplasia Ossificans Progressiva Associated with Heterozygous Mutation in the *ACVR1* Gene

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Abstract—Fibrodysplasia ossificans progressiva (FOP) is a severe genetic disease caused by mutations in the *ACVR1* gene for which there is currently no effective therapy. Therefore, obtaining iPSCs for further modeling and development of treatment methods, including gene therapy, can be very promising. The authors herein describe the generation of the induced pluripotent stem cell (iPSCs) line RCMGi014-A from urinary sediment cells of a patient with clinically expressed and genetically proven (*ACVR1*:c.6176G>A) FOP. These iPSCs proliferate in dense monolayer cell colonies, have a normal karyotype (46,XY), express pluripotency markers (OCT4, SOX2, TRA-1-60, SSEA-4), and show the ability to differentiate into three germ layers, which confirms their pluripotent status.

Keywords: fibrodysplasia ossificans progressiva, induced pluripotent stem cells, *ACVR1*, reprogramming

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INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP) is a rare genetic disease with an autosomal dominant type of inheritance; it occurs worldwide in one case per two million people. It is characterized by congenital malformations of the big toes and progressive heterotopic endochondrial ossification, in which soft connective tissues, including aponeuroses, fascia, ligaments, tendons, and skeletal muscles, transform into mature heterotopic bone, most often in response to injury (Pignolo, 2013). Most people diagnosed with FOP become wheelchair-bound by the third decade and need lifelong assistance in performing daily activities. The median life expectancy is approximately 45 years, and death often occurs as a result of respiratory failure due to limitations of chest mobility (Kaplan, 2008).

Heterozygous mutations with enhanced function in the *ACVR1* gene located on chromosome 2 (2q23-24) were found in all patients with FOP registered to date. *ACVR1* encodes a type 1 bone morphogenetic protein (BMP) receptor, also known as the activin A type 1 receptor. In most cases, patients with FOP show frequent heterozygous recurrent nucleotide substitution (c.617G>A), which leads to the replacement of arginine by histidine (R206H) in the intracellular glycine-serine (GS) domain of *ACVR1*, which causes aberrant signaling through the receptor and overactivation of the downstream SMAD 1/5/9(8) signaling pathway. Canonical BMP proteins bind to the mutated *ACVR1* receptor and cause overactivation of intracellular signaling pathways. It is the intensification of the BMP signaling pathway that is the cause of ectopic chondrogenesis, osteogenesis, and joint fusion observed in FOP (Pignolo, 2013).

Currently, there are no treatments for FOP, all developed therapy is symptomatic and includes the use of glucocorticoids and nonsteroidal anti-inflammatory drugs; however, these treatments are not highly effective and do not mitigate the progressive nature of this disease (Wentworth, 2019). Therefore, obtaining iPSCs for further modeling and development of treatment methods may be very promising.

Table 1. Antibodies for immunocytochemistry analysis and flow cytometry

Purpose	Antibody	Dilution	Manufacturer, cat. no.
Markers of pluripotency	Antibody against SSEA4 (MC813-70), mouse	1 : 100	Invitrogen, 41-4000
	Antibody against OCT 4, rabbit	1 : 200	Abcam, ab18976
	Antibody against SOX2 [9-9-3], mouse	1 : 200	Abcam, ab79351
	Antibody against TRA-1-60, mouse	1 : 50	Invitrogen, 41-1000
Markers of germ layers	Antibody against vimentin [EPR3776], rabbit	1 : 200	Abcam, ab92547
	Antibody against FOXA2 [EPR4465], rabbit	1 : 450	Abcam, ab108396
	Antibody against beta-tubulin [1E1-E8-H4], mouse	1 : 750	Abcam, ab78078
Secondary antibodies	Anti-mouse Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	1 : 150	Invitrogen, A-11032
	Anti-rabbit Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	1 : 150	Abcam, A21206

The most common way to obtain iPSCs is to reprogram fibroblasts; however, surgical intervention is necessary for isolation of these cells, which is risky for people with FOP since a skin biopsy can provoke an injury and the appearance of new ossifications. Therefore, an alternative and optimal option for obtaining iPSCs for these patients is to use urinary sediment cells (USCs) as a source. Within the framework of normal human physiology, approximately 2000–7000 cells of the tubular system and lower urinary tract are detached and excreted in urine every day. Their use is advantageous due to the low cost and noninvasiveness of obtainment; due to less exposure to UV radiation and, consequently, fewer genetic changes compared to skin cells (Gore, 2011); and also because of the higher efficiency of reprogramming compared to fibroblasts (Zhou, 2011), which is probably due to the fact that these cells do not require a mesenchymal-epithelial transition during reprogramming, unlike fibroblasts (Li, 2010).

MATERIALS AND METHODS

Reprogramming the USCs

USCs were obtained from urine and cultured as previously described (Zhou, 2011). Reprogramming of 100 000 cells per well was performed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific), using MOI 5 : 5 : 4 for viruses KOS, c-Myc, and Klf4, respectively. On day 5–6, the appearance of colonies was observed. On day 8, cells were plated into culture plates precoated with Matrigel (Corning). A day later, the cells were replated to the TeSR™-E8™ (E8) medium (Stemcell Technologies), followed by its daily change. On day 14, several colonies were mechanically detached and manually plated into separate wells, these iPSC clones were then cultured in E8 medium at 37°C, 5% CO_2, replated every 4–7 days in a 1 : 6 ratio using the Versene solution and with the addition of the ROCK Y27632 inhibitor (Stemcell Technologies) in concentrations of 5 μM on the first day.

Immunocytochemical Staining and Flow Cytometry

Cells were fixed with a 4% formaldehyde solution, membranes were permeabilized, then staining with primary and secondary antibodies (Table 1), followed by staining with DAPI were performed. To assess an expression of pluripotency markers in iPSCs and germ layers' markers in embryoid bodies qualitatively, cell images were obtained using the Lionheart LX (BioTek) imaging system in transmitted light and fluorescent light through an appropriate filter. To quantify the expression of pluripotency markers in iPSCs, cells after staining were detached and analyzed using a Flomax flow cytometer (Partec) and FloMax® Software (Partec).

Genotyping USCs and iPSCs

To determine the patient's genotype and confirm the mutation in iPSCs obtained from the cells of this patient, we used the method of Sanger sequencing. DNAs from the samples were purified using the DNA Quick-gDNA MiniPrep genetic kit (Zymo Research). The *ACVR1* locus was amplified by PCR (protocol: 95°C 3 min; 35× (95°C 20 s, 58°C 20 s, 72°C for 25 s); 72°C 3 min); with DNA polymerase Taq (Eurogene) and specific primers (Table 2) using Eppendorf Mastercycler equipment (Eppendorf). Sanger Sequencing

Table 2. Primers for PCR

Purpose	Target	Size of product	Sequence
Sanger Sequencing	Locus c.6176G>A of gene *ACVR1*	394 bp	ACATGTACACTAACAGGCCA CAGGTGCTCCAACATTAGTCAT
Elimination of the Sendai virus	SeV	181 bp	GGATCACTAGGTGATATCGAGCACCAGACAAGAG TTTAAGAGATATGTATC
	KOS	528 bp	ATGCACCGCTACGACGTGAGCGCACCTTGACAAT CCTGATGTGG
	Klf4	410 bp	TTCCTGCATGCCAGAGGAGCCC AATGTATCGAAGGTGCTCAA
	c-Myc	532 bp	TAACTGACTAGCAGGCTTGTCG TCCACATACAGTCCTGGATGATGATG

(protocol: 95°C, 3 min; 25× (95°C, 15 s, 50°C, 15 s, 60°C, 2 min) from forward and reverse primers was performed in an ABI Prism 3130XL analyzer (Applied Biosystems).

Obtaining of Embryoid Bodies (Spontaneous Differentiation of iPSCs)

After the tenth passage, iPSCs were collected using Versene solution and transferred into low-adhesive plates (Corning) with E8 medium with Y27632. On day 3, half of the medium was carefully replaced; on day 6, the entire medium was replaced by EB medium consisting of Advanced DMEM/F12 (Gibco), 20% KO Serum replacement (Gibco), 2 mM L-glutamine (Gibco), 1% MEM NEAA, 50 μg/mL penicillin, 50 μg/mL streptomycin. Subsequently, the medium was replaced every 2–3 days, first by adding 1% fetal bovine serum (FBS), and then by gradually increasing its content from 1 to 10%. After approximately 3 weeks, the embryoid bodies were transferred to gelatin-coated plates in an EB medium with 10% FBS. After 7–14 days, the expression of the markers was analyzed by immunocytochemistry.

Karyotyping of iPSC

To confirm the absence of chromosomal rearrangements that may occur during reprogramming, we performed karyotyping of the iPSC line using GTG-differential chromosome staining with Giemsa staining. iPSCs were cultured to achieve 70–80% confluence. To stop mitosis, we treated cells with the solution of colchicine (PanEco) at a concentration of 10 μg/mL for 40 min. The cells were then treated with a hypotonic solution (0.075 M KCl) at 37°C for 13 min and fixed in a cooled solution consisting of three parts methanol and 1 part glacial acetic acid twice for 30 and 20 min. Samples were analyzed using an Axio Imager 2 (ZEISS) microscope. The distribution of chromosomes was evaluated in at least 15 metaphase plates in accordance with the International System for Human Cytogenomic Nomenclature ISCN-2016.

STR Analysis of USCs and iPSCs

To confirm the origin of the iPSC line from the initial USCs, we analyzed short tandem repeats (STR analysis) using a set of 18 microsatellite markers evaluating the genetic profile. DNAs from the samples were prepared using Quick-gDNA MiniPrep Kit (Zymo Research).

Confirmation of the Elimination of the Sendai Virus

After passage 15, the elimination of Sendai virus in the obtained iPSC lines was confirmed by PCR using specific primers (Table 2). cDNA obtained from RNA using the MMLV RT reverse transcription kit (Evrogen) was used as a template for PCR. RNA was obtained from cells using the Total RNA Purification Kit (Norgen Biotek).

Detection of iPSCs Mycoplasma Contamination

Mycoplasma contamination analysis was performed using the MycoReport kit (Evrogen); it is based on endpoint PCR with amplification of species-specific sections of 16S rRNA genes. The supernatant from cell cultures for this analysis was collected during at least 48 h of cultivation.

RESULTS

In a male patient aged 11 years with a clinical picture of FOP, a pathological variant *ACVR1:c.6176G>A* (p.Ary206His) was revealed by genotyping. The collected and cultured USCs of this patient were transformed into iPSCs using reprogramming vectors based on Sendai virus expressing four Yamanaka factors: OCT3/4, SOX2, KLF4, and c-MYC. A clone of iPSCs called P20L7 was isolated (Fig. 1a) and fully

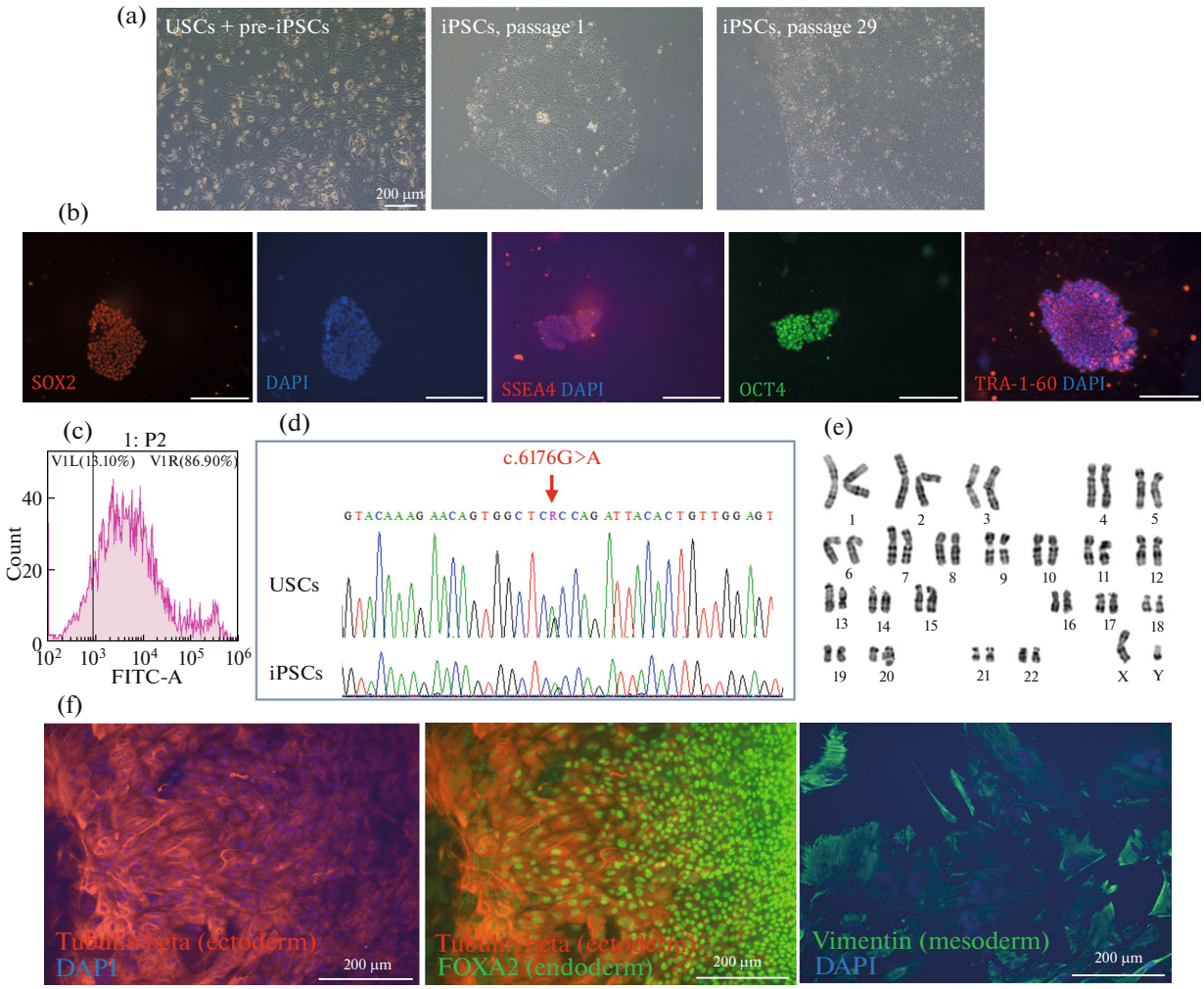

Fig. 1. Characteristics of the obtained iPSC line. (a) Morphology of urinary sediment cells (USCs) and pre-iPSCs on day 5 of reprogramming, iPSC colonies on passage 1 and 29. (b) Immunocytochemical staining of iPSCs for pluripotency markers for TRA-1-60, OCT4, SOX2, SSEA4. (c) Flow cytometry of iPSCs stained with OCT4. (d) Sequenograms of *ACVR1* locus with the c.6176G>A mutation obtained on DNAs from USCs and iPSCs. (e) Karyotype of the iPSC line. (f) Immunocytochemical staining of embryoid bodies obtained from iPSCs for markers of germ layers.

characterized. At passage 14, immunocytochemical analysis and flow cytometry showed that this line demonstrates a high level of expression of pluripotency markers SSEA4, OCT4, SOX2, and TRA-1-60 (Figs. 1b–1c). Sanger sequencing confirmed the presence of the *ACVR1:c.6176G>A* mutation in the heterozygous state in the USC and iPSC lines (Fig. 1d). Karyotyping of these cells on passage 15, showed a normal diploid karyotype 46,XY without any detectable deviations (Fig. 1e). The functional pluripotency of the obtained iPSCs, i.e., the ability to differentiate into three germ layers, was proved by spontaneous differentiation by the formation of embryoid bodies and their analysis using immunocytochemical staining, which was positive for FOXA2 (endodermal marker), beta-tubulin (ectodermal marker), and vimentin (mesodermal marker) (Fig. 1f). STR analysis of 18 microsatellite markers proved the identity of the DNA profiles of the original USCs and obtained iPSC clone P20L7. An elimination of Sendai virus genome and transgenes was confirmed at the 15th passage. In addition, cells were tested for mycoplasma contamination on the 18th passage, and the result was negative. The stable iPSC line P20L7 is deposited in the Network Shared Resources Center Russian Collection of Biological Samples of Hereditary Diseases on the basis of the Research Center for Medical Genetics. The line is registered in the hPSCreg stem cell registry under the name RCMGi014-A.

AUTHOR CONTRIBUTION

E.V. Kondrateva participated in the cultural and molecular genetic part, analysis of the obtained data, and writing of the article. O.V. Grigorieva performed the main part of cell culture work, reprogramming of USCs, and obtaining

individual iPSC clones. I.O. Panchuk participated in cell culture work. E.V. Kurshakova performed the main part of the molecular genetic work (immunocytochemistry, PCR). V.O. Pozhitnova and E.S. Voronina performed karyotyping of iPSCs. I.P. Nikishina, S.V. Arsenyeva, and V.G. Matkava carried out medical support of the patient and provision of urinary sediment cells. V.Yu. Tabakov obtained a culture of urinary sediment cells. A.V. Lavrov, S.A. Smirnikhina, and S.I. Kutsev performed experimental design and expert evaluation of the data and participated in the preparation of the publication.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of the Research Center for Medical Genetics, protocol no. 2015-5/3. The patient was provided with all information about this study and signed an informed consent and information sheet with his own hand.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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