ATOH1 FACTOR EXPRESSION INDUCES RAPID DIFFERENTIATION OF IPSCS INTO NEURONS

Stepanov AI[1,2], Putlyaeva LV[1,2], Didych DA[2], Galiakberova AA[3,4], Gurskaya NG[1,3] ✉, Lukyanov KA[2]

[1] Skolkovo Institute of Science and Technology, Center for Molecular and Cellular Biology, Moscow, Russia
[2] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia
[3] Pirogov Russian National Research Medical University, Moscow, Russia
[4] Lomonosov Moscow State University, Moscow, Russia

The study of human induced pluripotent stem cells (iPSCs) and developing the technology for their practical use is one of the most knowledge-intensive areas of modern biomedical research. Despite the potential of using iPSCs in personalized medicine and to build cell-based models for disorders of various etiology, iPSC utilization remains challenging. Thus, the iPSC intercellular heterogeneity and the lack of effective identity determination and assessment methods considerably hamper reproducibility of such studies. The study was aimed to generate an iPSC line carrying the gene encoding the ATOH1 transcription factor controlled by the Tet-One expression induction system, along with TagBFP2 fluorescent protein and the puromycin resistance gene for cell selection. Molecular cloning, lentiviral transduction, cell culturing, immunofluorescence staining, and fluorescence microscopy were used during the study. The created cell model will allow analyzing the state of single cells and, therefore, has great practical potential for both laboratory and medical research.

Keywords: IPSC, ATOH1, lentivirus, neural differentiation

Funding: the study was supported through the grant by RSF (№ 22-14-00141).

Author contribution: Stepanov AI — experimental procedure; Putlyaeva LV — experiment planning, manuscript writing; Didych DA — design and molecular cloning of the leniviral plasmid components, processing of figures; Galiakberova AA — cell culture; Gurskaya NG — concept and design of iPSC study; Lukyanov KA — general management.

✉ **Correspondence should be addressed:** Nadya G. Gurskaya
Ostrovitianova, 1, str. 1, Moscow, 117997, Russia; ngurskaya@mail.ru

Received: 01.08.2023 **Accepted:** 09.09.2023 **Published online:** 22.09.2023

DOI: 10.24075/brsmu.2023.036

ЭКСПРЕССИЯ ФАКТОРА ATOH1 ИНДУЦИРУЕТ БЫСТРУЮ ДИФФЕРЕНЦИРОВКУ ИПСК В НЕЙРОННОМ НАПРАВЛЕНИИ

А. И. Степанов[1,2], Л. В. Путляева[1,2], Д. А. Дидыч[2], А. А. Галиакберова[3,4], Н. Г. Гурская[1,3] ✉, К. А. Лукьянов[2]

[1] Сколковский институт науки и технологии, Центр молекулярной и клеточной биологии, Москва, Россия
[2] Институт биоорганической химии имени М. М. Шемякина и Ю. А. Овчинникова, Москва, Россия
[3] Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва, Россия
[4] Московский государственный университет имени М. В. Ломоносова, Москва, Россия

Изучение индуцированных плюрипотентных стволовых клеток человека (иПСК) и создание технологий их практического использования — одно из самых наукоемких направлений современных биомедицинских исследований. Несмотря на потенциал применения иПСК в персонализированной медицине и в создании клеточных моделей заболеваний различной этиологии, использование иПСК остается крайне сложным. Так, межклеточная гетерогенность иПСК при отсутствии эффективных способов определения идентичности и оценки существенно затрудняет воспроизводимость подобных исследований. Целью работы было создать линию иПСК, несущую ген транскрипционного фактора ATOH1 под контролем системы индукции экспрессии Tet-One, ген флуоресцентного белка TagBFP и ген устойчивости к пуромицину для селекции клеток. В работе использовали методы молекулярного клонирования, лентивирусную трансдукцию, культивирование клеток, иммунофлуоресцентное окрашивание и флуоресцентную микроскопию. Созданная клеточная модель позволит анализировать состояние единичных клеток и, следовательно, имеет большой практический потенциал как для лабораторных, так и для медицинских исследований.

Ключевые слова: иПСК, ATOH1, лентивирус, нейронная дифференцировка

Финансирование: работа была поддержана грантом РНФ № 22-14-00141.

Вклад авторов: А. И. Степанов — проведение эксперимента; Л. В. Путляева — планирование эксперимента, написание статьи; Д. А. Дидыч — дизайн и молекулярное клонирование элементов лентивирусной плазмиды, обработка рисунков; А. А. Галиакберова — культивирование клеток; Н. Г. Гурская — концепция и дизайн работы с ИПСК; К. А. Лукьянов — общее руководство исследованием.

✉ **Для корреспонденции:** Надежда Георгиевна Гурская
ул. Островитянова, д. 1, стр. 1, г. Москва, 117997, Россия; ngurskaya@mail.ru

Статья получена: 01.08.2023 **Статья принята к печати:** 09.09.2023 **Опубликована онлайн:** 22.09.2023

DOI: 10.24075/vrgmu.2023.036

Human induced pluripotent stem cells (iPSCs) were first obtained in 2007 [1]. iPSCs, that are similar to embryonic stem cells, can differentiate into various cell types. Since iPSCs can be obtained from the easily accessible patients' somatic cells (for example, dermal fibroblasts or peripheral blood mononuclear cells), the use of iPSCs solves important methodological and ethical problems, such as the problem of the neuronal cells' accessibility. iPSCs represent an extremely interesting model for fundamental studies of the human cells' differentiation and dedifferentiation, these cells are also of considerable practical relevance for medicine. Human iPSCs can be used to generate cells of various types and potentially for further transplantation in patients [2]. Many problems on the way to this (such as incomplete differentiation of human iPSCs and the development of teratomas) remain to be resolved and require further research. Testing of drugs on human cells, including in order to select the treatment regimen for individual patient (personalized medicine), is an equally important area of using iPSCs [3, 4]. Researchers can use iPSCs of patients generated from somatic cells to obtain isogenic cell lines, in which the

disease patterns are reproduced, through differentiation and use these cell lines for drug screening. Thus, iPSCs of patients after their differentiation into neurons or glial cells are used to study neurodegenerative diseases [5, 6].

Differentiation of iPSCs into neurons can be accomplished by using methods of two groups: 1) chemical induction; 2) genetically mediated induction [7]. In the first case, the mixture of small-molecule inhibitors and peptides suppressing the expression of pro-neuronal growth factors, as well as molecular pathways inducing neuronal induction are used to trigger differentiation. Dual SMAD inhibition is a well-known example [8]. The genetically mediated induction is aimed to introduce cDNAs encoding the gene of the transcription factor specific for the neuronal differentiation program (such as neurogenin-2) or regulatory sequences, such as promoters or enhancers, into the cell [9]. However, both strategies make it possible to ensure iPSC differentiation into neurons in at least two weeks.

In 2021, a groundbreaking report was published, where the authors proposed a new method for directed differentiation of iPSCs into neurons using the ATOH1 transcription factor overexpression [10]. The new approach is superior to earlier applied methods in terms of speed and simplicity, it allows one to ensure differentiation (up to 99%) in the standard medium within just a few days.

The study was aimed to generate an iPSC line carrying the gene encoding the ATOH1 transcription factor controlled by the Tet-One expression induction system and the TagBFP2 fluorescent protein as a selective marker. One of the objectives was to obtain a stable iPSC line allowing one to induce differentiation into neurons by adding doxycycline to the growth medium. This cell line can be used in various studies, including for analysis of the iPSC intercellular heterogeneity, development of the genetically encoded fluorescent sensors, testing of new drugs on human cells, and selection of treatment regimens for individual patients (development of personalized medicine protocols).

METHODS

Molecular cloning

The *ATOH1* transcription factor nucleotide sequence was amplified from the Addgene pTet-O-ATOH1-T2A-PuroR plasmid (Addgene #162342) using the ATOH1 for ATATGAAGACTTGAT CATGTCCCGCCTGCTGCATGCAGAAGAG and ATOH1 rev AT ATGAAGACAAACCTCTAGAACTTGCCTCATCCGAGTCACT GTAATGGGAATG primers. Then *ATOH1* and *TagBFP2* were inserted in the pRRLSIN.cPPT.EF1 lentiviral vectors at the BamH1 and EcoR1 (Thermo Scientific, Waltham, MA; USA) restriction sites. The following primers were used for *TagBFP2* amplification: TagBFP2 for ATATGAAGACGGAGGTGTGAGCG AGCTGATTAAGGAGAACATGC and TagBFP2 rev GCATGAAG ACATTCGATCATCACTTGTGCCCCAGTTTGCTAGGGAGGTC GCAGTATCTGGCC. The ATOH1 and TagBFP2 fusion was by using the T2A GSGEGRGSLLTCGDVEENPGP proteolytically cleaved peptide. The pRRLSIN.cPPT.EF1 lentiviral vector was kindly provided by Dr. D. Trono (Lausanne; Switzerland). Then the ATOH1-t2a-TagBFP2 DNA was transferred to the pLVX-TetOne-Puro vector (Clontech, #631847; USA) at the BamH1 and Age1 restriction sites (Thermo Scientific, Waltham, MA; PanEco; Russia). The T4 DNA ligase (Evrogen; Russia) was used for sticky end ligation.

Cell line cultivation

The HEK293T cells were cultured at 37 °C (5% CO_2) in the DMEM medium (PanEco; Russia) supplemented with 10% fetal bovine serum (BioSera, Nuaille; France), 100 U/mL of penicillin and 100 mg/mL of streptomycin (PanEco; Russia).

The iPS-KYOU iPSC cell line was purchased at the ATCC cell bank (KYOU-DXR0109B, ATCC® ACS-1023™). iPSCs were cultured in the mTeSR medium (StemCell Technologies; USA) at 37 °C (5% CO_2) changed daily to ensure optimal growth and Matrigel (Corning; USA) as a matrix for the surface cover. Accutase (StemCell Technologies; USA) was used to detach cells from the flask surface.

Generating a stable cell line

The TetOne-ATOH1-t2a-TagBFP2 iPSC cell line was generated by lentiviral transduction. A total of 1.5×10^6 HEK293T cells were seeded on a cell culture dish with a diameter of 60 mm 24 h before transfection. In total, 2 μg of the pR8.91 plasmid, 0.6 μg of the pMD.G plasmid, and 6 μg of the TetOne-ATOH1-t2a-TagBFP2 plasmid were used for transfection. The Transfectin reagent (IBCh RAS; Moscow, Russia) in the ratio 2.5 μL of the reagent to 1μg of the plasmid was used for transient transfection of the HEK293T cells. The mixture of DNA and Transfectin was incubated for 20 min at room temperature and then added dropwise. After 4 h the medium was replaced with 2 mL of fresh DMEM. On the next day the medium containing the lentiviruses acquired was filtered (filter with the pore size of 0.45 μm) and concentrated by ultracentrifugation at 100,000 g (Beckman; USA) for 3 h at 4 °C. Precipitate was resuspended in 500 μL of mTeSR (StemCell Technologies; USA) and used for transduction of iPSCs. Lentiviral particles were added to 1×10^5 iPSCs to generate stable cell lines. Then the transduced cells were selected by adding the puromycin antibiotic (Thermo Fisher Scientific, Waltham, MA; USA) to the growth medium to a final concentration of 5 μg/mL.

Immunostaining of fixed cells

Cells were seeded and grown as described above, fixed in 4% formaldehyde solution in PBS for 15 min at room temperature, triple washed with PBS, permeabilized for 20 min in 0.1% Triton X-100 (Helicon; USA) in PBS, and incubated for 1 h with 1% BSA (Sigma; USA) in PBS to ensure blocking. Incubation with primary antibody was performed for 1 h, incubation with secondary antibody was performed for 1 h at room temperature. Cells were washed with PBS and imaged in the imaging medium using the BZ-9000 microscope (Keyence, Osaka; Japan). The rabbit anti-TUBB3 and goat anti-rabbit IgG Alexa Fluor 568 antibodies (ThermoFisher, Waltham, MA; USA) were used at a dilution of 1 : 500 and 1 : 1000, respectively.

Fluorescence microscopy of living cells

When conducting experiments on living cell imaging, cells were grown in confocal dishes with glass bottom (SPL Life Sciences; Korea). The mTESR medium was replaced with the MEM imaging medium (PanEco; Russia) supplemented with 10% fetal bovine serum (BioSera, Cholet; France) and 20 mM HEPES (Corning, NY; USA) immediately before microscopy.

The Keyence Biorevo BZ-9000 fluorescence microscope (Keyence; Japan) was used for in vivo fluorescence microscopy. Cells were imaged at 60× magnification using the CFI Plan Apo λ60xH/NA1.40 lens. Imaging was performed in the blue channel using the DAPI filter cube (excitation wavelength 360/40 nm, emission wavelength 460/50 nm) for detection of the TagBFP2 fluorescence.

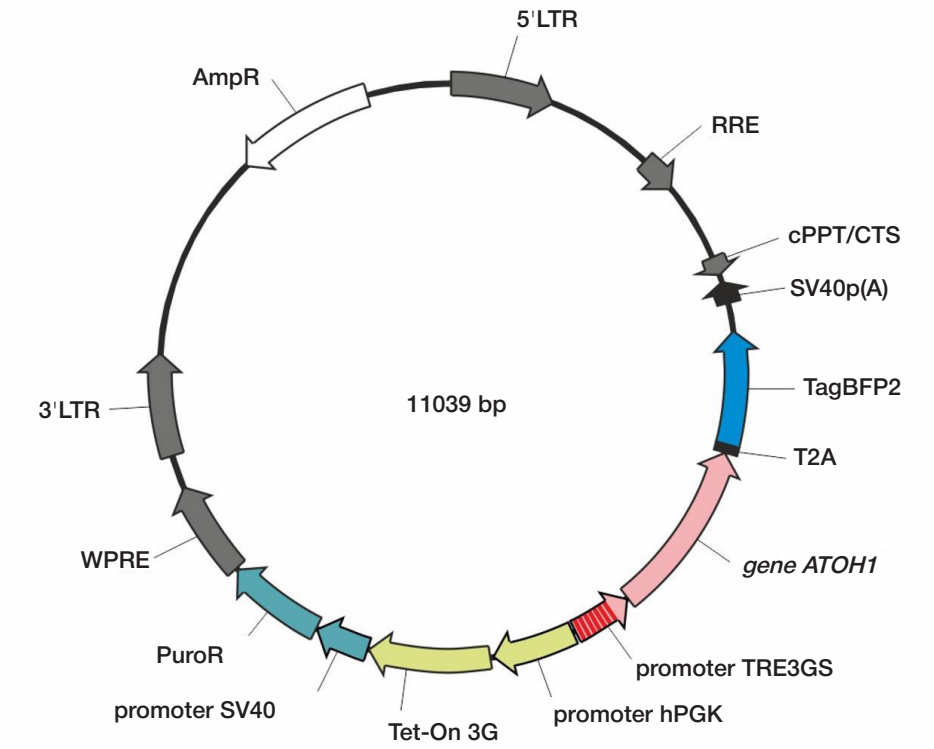

Fig. 1. Scheme of the *TetOne-ATOH1-t2a-TagBFP2* lentiviral plasmid used to generate a stable iPSC line with induced expression of the *ATOH1-T2A-TagBFP2* fusion gene. The *ATOH1-T2A-TagBFP2* gene is controlled by the TRE3GS inducible doxycycline-dependent promoter containing seven repeats of the tetO operator sequence. The plasmid also comprises the *Tet-On 3G* (TRE3GS promoter transactivator) and *PuroR* (puromycin resistance gene) genes controlled by the hPGK (promoter of human gene *PGK1*) and SV40 constitutive promoters, respectively. Standard components of lentiviral plasmids that are essential for correct and effective assembly of functional viral particles in the packaging cells and that ensure high expression of transgenes (5'LTR/3'LTR — long terminal repeats, RRE — Rev viral protein binding site (Rev response element); cPPT/CTS — central polypurine tract/central termination sequence; WPRE — Woodchuck hepatitis virus post–transcriptional regulatory element; SV40p(A) — SV40 transcription terminator with a poly(A) signal; AmpR — ampicillin resistance gene) are highlighted in gray

RESULTS

When constructing the most effective model of inducing iPSC differentiation into neurons, we used the approach reported by the group of G. Church in 2021 [10]. In this study a large-scale screening of three human iPSC lines was performed; it was found that the ATOH1 transcription factor was the most effective driver of the neuronal differentiation induction. In contrast to other pathways of iPSC differentiation into neurons, the ATOH1-induced differentiation requires no specific media or extra factors. Furthermore, this process takes little time (4 days). To create the most biologically relevant model of the ATOH1-induced neuronal differentiation, we decided to construct a stable iPSC line carrying the *ATOH1* gene controlled by under the control of the TRE3Gs inducible promoter. For that we made a lentiviral plasmid with induced ATOH1 expression (Fig. 1). This plasmid comprised three independent expression cassettes:

TRE3G promoter-ATOH1-t2a-TagBFP2 — the *ATOH1* gene controlled by the tetracycline promoter and fused with the expression marker, the fluorescent protein TagBFP2, through the T2A peptide. Induction of *ATOH1-t2a-TagBFP2* expression was achieved by adding doxycycline to the growth medium.

hPGK promoter-TetOn3G — *TetOn3G*, the gene encoding the TetOn tetracycline promoter activator, was controlled by the hPGK promoter;

SV40 promoter-PuroR — ensured puromycin expression that was essential for selection of iPSCs carrying a target construct only.

This genetic construct referred to as *TetOne-ATOH1-t2a-TagBFP2* was used to generate lentiviral particles and infect the iPS-KYOU cell line (Fig. 2A). After that the cells were subjected to selection in the puromycin-containing medium, then doxycycline was added for the tetracycline promoter activation. Within 24 h after adding doxycycline we recorded weak fluorescence of the TagBFP2 blue protein, indicating the earliest stage of the ATOH1 factor expression. On day two the signal was strong and well-detectable for the fluorescence microscope, the neuron-like cell morphology changes were reported (Fig. 2B; *above*).

Differentiation efficiency was assessed by immunofluorescence staining aimed at detecting the neuronal stem cell marker (class III β-tubulin, TUBB3) expression (Fig. 3A). We showed that the cell culture obtained after differentiation was heterogeneous in terms of TUBB3 expression: some cells showing TagBFP2 fluorescence did not express TUBB3 and had no morphological features specific for neurons (Fig. 3B).

The findings suggest that the TetOne-ATOH1-t2a-TagBFP2 human iPSC line shows stable ATOH1 transcription factor expression controlled by the TetOne expression induction system. The use of this cell line in laboratory studies can provide unique information about the chromatin state changes during iPSC differentiation into neurons. This biological model can potentially be used to address diverse biological and biomedical challenges.

DISCUSSION

The generated TetOne-ATOH1-t2a-TagBFP2 cell line showed successful inducible expression activation within 1–2 days after the start of the experiment. However, the expression of blue protein reflecting the ATOH1 protein levels in the cell was heterogeneous (Fig. 2B, *below*; Fig. 3B). Perhaps, this phenomenon was due to heterogeneity of original iPSC line and no clonal selection among transduced cells in this experiment. We also assume that methylation of the tetracycline promoter

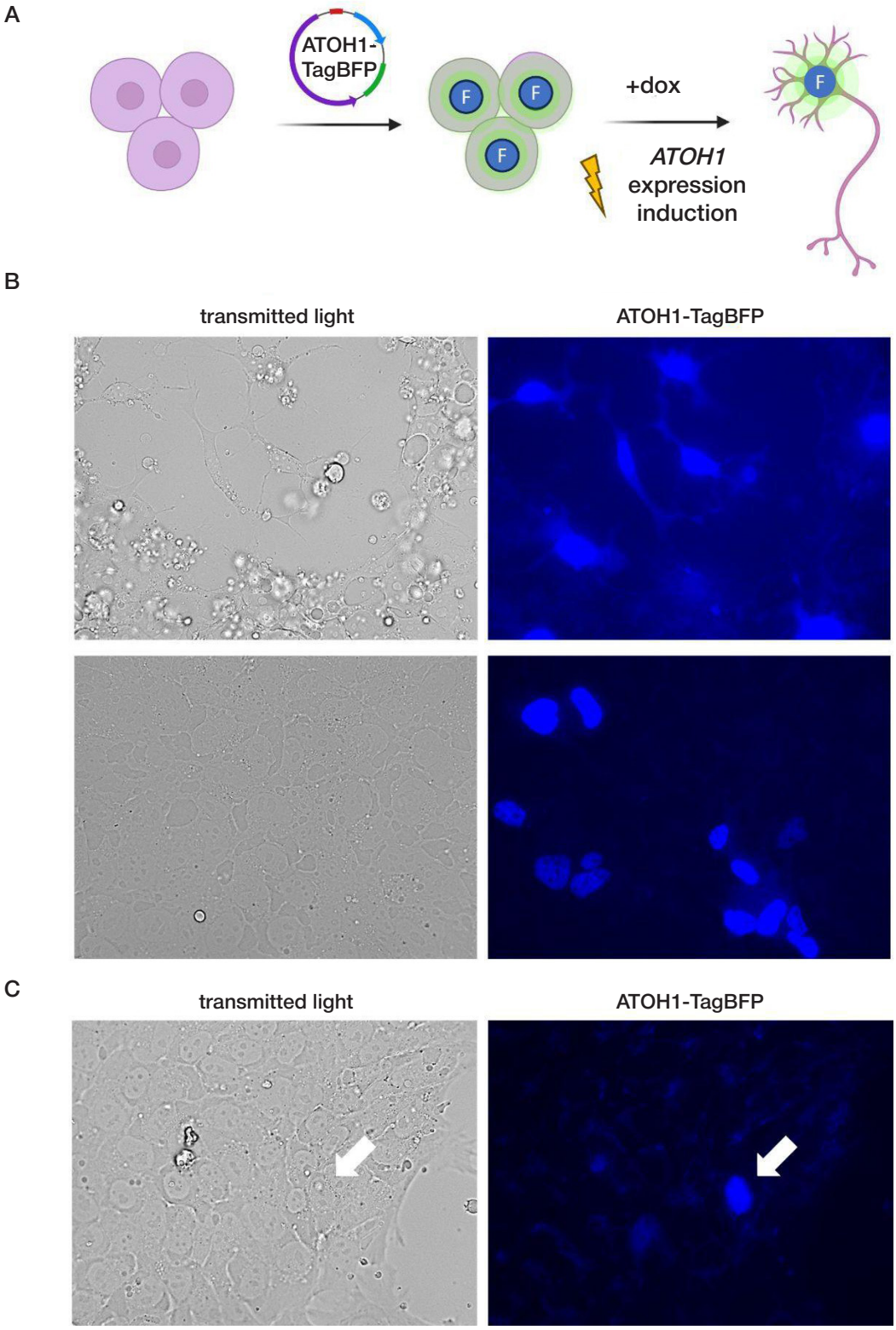

Fig. 2. Expression of the *TetOne-ATOH1-t2a-TagBFP2* construct in iPSCs. **A**. Scheme to generate a stable iPSC line carrying the *ATOH1* gene controlled by the TRE3G inducible promoter. F — TagBFP fluorescent protein. **B**. Cells 24 h after induction of expression by doxycycline. **C**. After freezing and thawing the majority of cells become incapable of doxycycline-dependent expression (the only cell in the field of view showing a bright TagBFP2 expression signal after adding doxycycline is marked with *arrow*)

and/or hPGK promoter can occur in iPSCs over time, resulting in the decrease in the tetracycline promoter activator protein levels. The ATOH1-t2a-TagBFP2 downregulation represents a cumulative effect of these processes. Indeed, we have found out that the number of cells showing the TagBFP2 expression has reduced after thawing a new aliquot of TetOne-ATOH1-t2a-TagBFP2 cells and repeating the experiment (Fig. 1C).

CONCLUSIONS

The use of the new method of directed iPSC differentiation into neurons makes it possible to quickly (in 4 days) generate the populations enriched with cells that differentiate into neurons. The cell line generated showing *ATOH1* expression controlled by the TRE3Gs inducible promoter is suitable for experiments

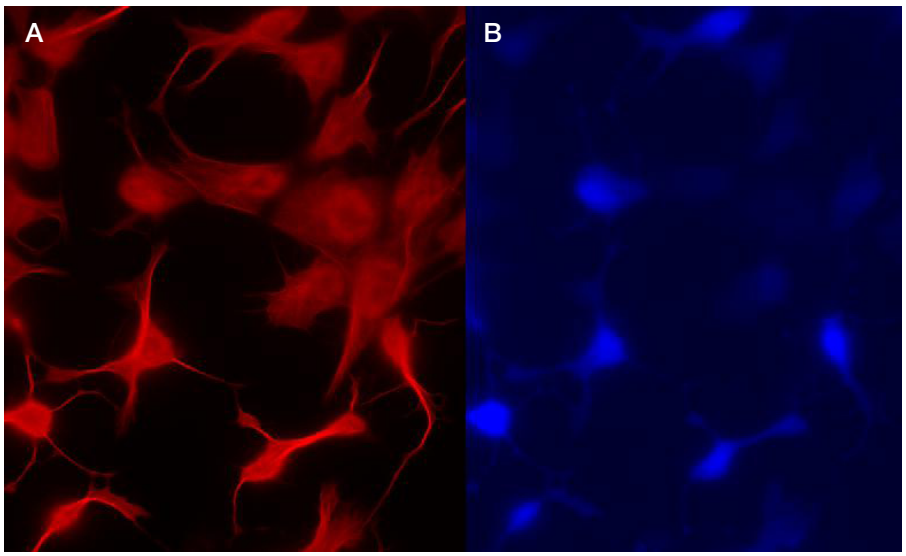

Fig. 3. Immunofluorescence analysis of simultaneous class III β-tubulin (TUBB3) and TagBFP2 expression in the cells on day 4 of differentiation after induction with *TetOne-ATOH1-t2a-TagBFP2*. **A**. *Red signal* — rabbit anti-TUBB3 (Affinity) antibody was used along with the goat anti-rabbit IgG Alexa Fluor 568 (ThermoFisher) secondary antibody. **B**. Blue signal — TagBFP2 fluorescence

requiring rapid iPSC differentiation, however, the experiment has to be carried out continuously (with no freezing/thawing cycles), which represents a limitation of this method when used to acquire experimental data.

References

1. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861–72.
2. Chang C-Y, Ting H-C, Su H-L, Jeng J-R. Combining Induced Pluripotent Stem Cells and Genome Editing Technologies for Clinical Applications. Cell Transplant. 2018; 27: 379–92.
3. Kleiman RJ, Engle SJ. Human inducible pluripotent stem cells: Realization of initial promise in drug discovery. Cell Stem Cell. 2021; 28: 1507–15.
4. Madrid M, Sumen C, Aivio S, Saklayen N. Autologous Induced Pluripotent Stem Cell-Based Cell Therapies: Promise, Progress, and Challenges. Curr Protoc. 2021; 1: e88.
5. Zhang N, Bailus BJ, Ring KL, Ellerby LM. iPSC-based drug screening for Huntington's disease. Brain Res. 2016; 1638: 42–56.
6. Chang C-Y, Ting H-C, Liu C-A, Su H-L, Chiou T-W, Lin S-Z, et al. Induced Pluripotent Stem Cell (iPSC)-Based Neurodegenerative Disease Models for Phenotype Recapitulation and Drug Screening. Molecules. 2020; 25. DOI: 10.3390/molecules25082000.
7. Telias M. Neural differentiation protocols: how to choose the correct approach. Neural Regeneration Res. 2023; 18: 1273–4.
8. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol. 2009; 27: 275–80.
9. Zhang Y, Pak C, Han Y, Ahlenius H, Zhang Z, Chanda S, et al. Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron. 2013; 78: 785–98.
10. Ng AHM, Khoshakhlagh P, Rojo Arias JE, Pasquini G, Wang K, Swiersy A, et al. A comprehensive library of human transcription factors for cell fate engineering. Nat Biotechnol. 2021; 39: 510–9.

Литература

1. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861–72.
2. Chang C-Y, Ting H-C, Su H-L, Jeng J-R. Combining Induced Pluripotent Stem Cells and Genome Editing Technologies for Clinical Applications. Cell Transplant. 2018; 27: 379–92.
3. Kleiman RJ, Engle SJ. Human inducible pluripotent stem cells: Realization of initial promise in drug discovery. Cell Stem Cell. 2021; 28: 1507–15.
4. Madrid M, Sumen C, Aivio S, Saklayen N. Autologous Induced Pluripotent Stem Cell-Based Cell Therapies: Promise, Progress, and Challenges. Curr Protoc. 2021; 1: e88.
5. Zhang N, Bailus BJ, Ring KL, Ellerby LM. iPSC-based drug screening for Huntington's disease. Brain Res. 2016; 1638: 42–56.
6. Chang C-Y, Ting H-C, Liu C-A, Su H-L, Chiou T-W, Lin S-Z, et al. Induced Pluripotent Stem Cell (iPSC)-Based Neurodegenerative Disease Models for Phenotype Recapitulation and Drug Screening. Molecules. 2020; 25. DOI: 10.3390/molecules25082000.
7. Telias M. Neural differentiation protocols: how to choose the correct approach. Neural Regeneration Res. 2023; 18: 1273–4.
8. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotechnol. 2009; 27: 275–80.
9. Zhang Y, Pak C, Han Y, Ahlenius H, Zhang Z, Chanda S, et al. Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron. 2013; 78: 785–98.
10. Ng AHM, Khoshakhlagh P, Rojo Arias JE, Pasquini G, Wang K, Swiersy A, et al. A comprehensive library of human transcription factors for cell fate engineering. Nat Biotechnol. 2021; 39: 510–9.