Airway and lung organoids from human induced pluripotent stem cells can be used to assess CFTR conductance

Anna Demchenko[1], Ekaterina Kondrateva[1], Vyacheslav Tabakov[2], Anna Efremova[3], Diana Salikhova[3], Tatiana Bukharova[3], Dmitry Goldshtein[3], Maxim Balyasin[4], Natalia Bulatenko[3], Elena Amelina[5], Alexander Lavrov[1], Svetlana Smirnikhina[1]

[1]Research Centre for Medical Genetics, Laboratory of Genome Editing, Moscow, Russian Federation,
[2]Research Centre for Medical Genetics, Moscow Branch of the Biobank "All-Russian Collection of Biological Samples of Hereditary Diseases", Moscow, Russian Federation,
[3]Research Centre for Medical Genetics, Stem Cell Genetics Laboratory, Moscow, Russian Federation,
[4]Peoples' Friendship University of Russia, Scientific and educational resource center, Moscow, Russian Federation
[5]Research Institute of Pulmonology, Laboratory of Cystic Fibrosis, Moscow, Russian Federation

Corresponding author: Anna Demchenko, e-mail address: demchenkoanna@med-gen.ru

Additional methods

Derivation of intestinal organoids from human rectal biopsies

The protocols we employed were originally developed by the J.M. Beekman's team who used them for generation of intestinal organoids [29, Boj et al., 2017, Vonk et al., 2020]. The study was approved by the Ethics Committee of the Research Centre for Medical Genetics (Moscow, Russia) and conducted in accordance with the provisions of the Declaration of Helsinki (1975). A healthy donor signed an informed written consent form as anonymous participant of the study and donor of biological materials. The study was conducted on an individual with the wt/wt genotype.

The L-Wnt3A and Noggin-HEK293 cell lines were the courtesy of J.M. Beekman and were used to produce conditioned media with appropriate growth factors. Both cell lines were maintained in the DMEM + GlutaMax medium (Thermo Fisher Scientific, cat. no. 10566016) supplemented with 10% Fetal Bovine Serum (PAA Laboratories, cat. no. A15101) and penicillin/streptomycin. For selective propagation of transfected L cells and HEK293 cells, the medium was supplemented with zeocin (Thermo Fisher Scientific, cat. no. R25001) at a concentration of 125 μg/ml or G418 (Thermo Fisher Scientific, cat. no. 10131035) at a

concentration of 500 μg/ml, respectively. The conditioned media were obtained after 8–9 days of cultivation in the medium without selective antibiotics. The medium for culturing organoids contained 50% Wnt-3A- and 10% Noggin-conditioned media, 40% Advanced DMEM/F12 (Thermo Fisher Scientific, cat. no. 12634010), 50 ng/mL hEGF (Prospec, cat. no. CYT 217), 300 ng/ml hR-Spondin-3 (Peprotech, cat. no. 120-44), 2% B27, 1.25 mM N-acetylcysteine (Sigma-Aldrich, cat. no. A7250), 10 mM nicotinamide (Sigma-Aldrich, cat. no. N3376), 5 μM A83-01 (Tocris, cat. no. 29-391-0), 10 μM SB 202190, 100 μg/ml primocin [29, Boj et al., 2017, Vonk et al., 2020].

Crypt isolation from rectal biopsies was preceded by a series of washes with the Advanced DMEM/F12 medium and PBS solution. The biopsy samples were then incubated with a 10 mM EDTA solution (Thermo Fisher Scientific, cat. no. AM9260G) in PBS for 40 min with constant stirring at +4 °C. Upon completion of the incubation, the samples were resuspended to release individual crypts from the rectal biopsies. The resulting crypt suspension was precipitated by centrifugation for 5 min at 130 g and +4 °C. The crypt precipitate was mixed with Matrigel and then plated onto culture plates; these steps were performed on ice. The plates were placed for 30 min in a CO_2 incubator to allow Matrigel polymerization with the embedded crypts. Then the growth medium was added to each well. The organoids were re-plated once a week. Prior to that, the growth medium was removed from all wells, Matrigel droplets were mechanically destroyed, and organoids were mechanically dissociated into smaller fragments. The resulting suspension was precipitated for 5 min at 130 g and +4 °C. The organoid precipitate was mixed with Matrigel and plated. The medium for crypts and organoids was replaced every 2–3 days.

Immunostaining assay

Immunostaining of intestinal, airway and lung organoids was carried out according to the protocols of Dekkers et al. [20].

For immunostaining of cells on days 3, 6 and 14 of differentiation, cells were fixed in a chilled 4% formalin solution for 15 min at +4 °C. Then cells were permeabilized in a solution of 0.1% Tween 20 in DPBS without Ca^{2+} and Mg^{2+} for 10 min at RT and blocked with a solution of 0.1% Tween 20 and 1% BSA in DPBS without Ca^{2+} and Mg^{2+} for 30 min at RT. After that, a solution of SOX2 primary antibodies at a final concentration of 5 μg/ml was added and the mixture was incubated for 1 hour at RT, then rinsed three times with DPBS without Ca^{2+} and Mg^{2+}. A solution of secondary antibody at a final concentration of 10 μg/ml was added and the mixture was incubated overnight at +4 °C, then rinsed three times with DPBS without Ca^{2+} and

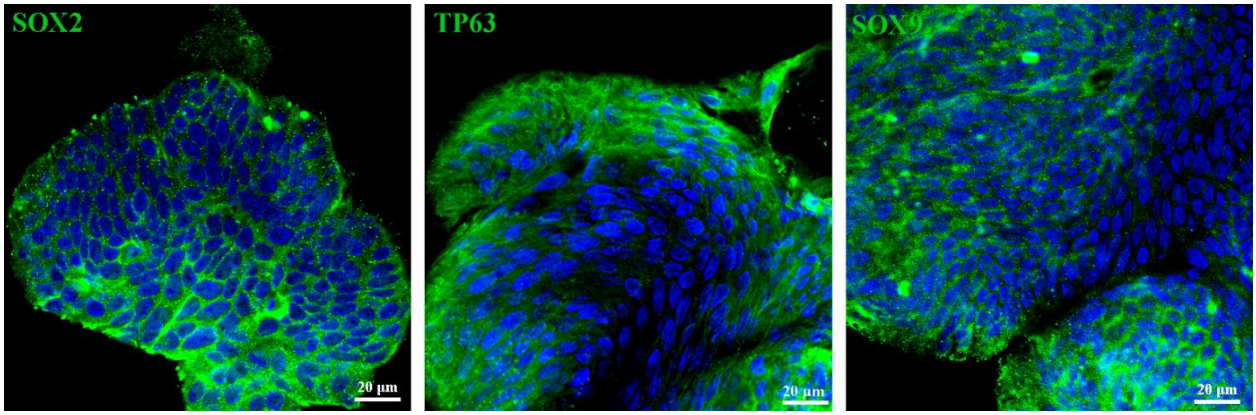
Mg^{2+}. DAPI was added and the mixture was incubated for 10 min at RT. Microscopy was performed on a TCS SP8 confocal laser scanning microscope (Leica Microsystems).

Immunostaining for the CFTR marker was carried out in airway and lung organoids derived from a healthy donor at day 22 of differentiation. Primary Anti-CFTR antibody (Abcam, cat. no. ab131553) was used at a final concentration of 6 µg/ml. Secondary Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam, cat. no. ab150077) was used at a final concentration of 20 µg/ml. For the flow cytometry screening of cellular composition of AOs and LOs, organoids after immunostaining were dissociated into single cells using 0.05% trypsin-EDTA for 10 min in an incubator. The cells were then centrifuged at 150 g for 5 min and the pellet was resuspended in DPBS without Ca^{2+} and Mg^{2+}. The analysis was performed on a CytoFLEX S flow cytometer. Before assessing expression of the markers, the cells were passed through a 50 µm filter. The number of analyzed events was 20,000. Stained cells were stored at +4 °C in the dark until analysis.

Additional results

Immunostaining of intestinal organoids

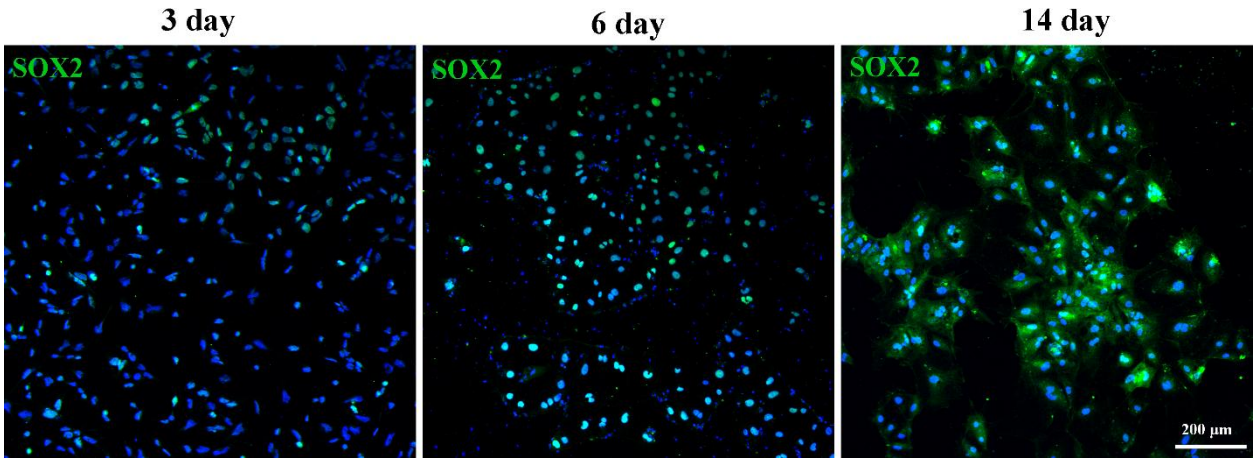
We analyzed localization of the SOX2, SOX9 and TP63 markers in intestinal organoids derived from human rectal biopsies. All of these markers, as well as airway and lung organoids, had a cytoplasmic localization (Additional Fig. 1).



Additional Fig. 1 Confocal images of SOX2, SOX9 and TP63 markers in intestinal organoids at day 7. Nuclei were stained with DAPI (blue). Scale bar, 20 µm

Immunostaining of cells during differentiation

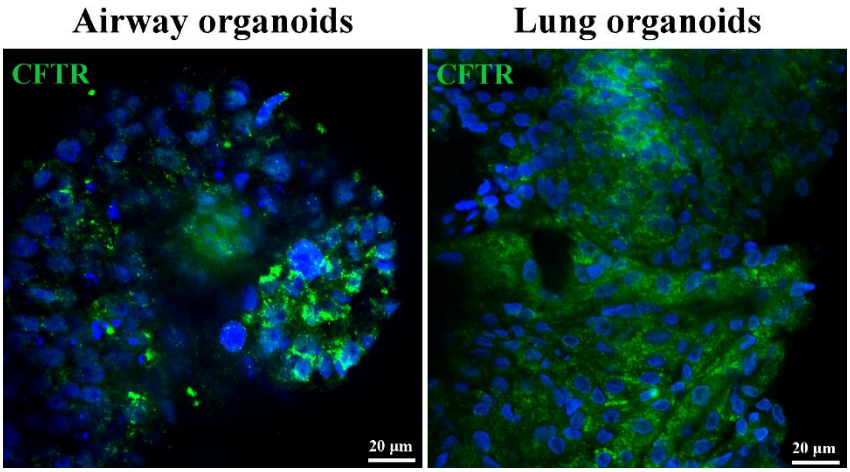
To analyze the localization of SOX2, we performed immunostaining of hiPSCs during differentiation into organoids. We chose three time points: day 3 (DE cells), day 6 (AFE cells) and day 14 (NKX2.1+ lung progenitor cells) from the start of differentiation. According to the results, the nuclear localization of SOX2 is maintained at the stages of obtaining DE and AFE cells, while NKX2.1+ lung progenitor cells have both cytoplasmic and nuclear localization of SOX2 (Additional Fig. 2).



Additional Fig. 2 Fluorescent images of SOX2 markers in organoids at days 3 (DE cells), 6 (AFE cells) and 14 (NKX2.1+ lung progenitor cells) of differentiation. Nuclei were stained with DAPI (blue). Scale bar, 200 µm

CFTR protein expression in airway and lung organoids

To confirm the presence of the CFTR channel in organoid cells, we performed a quantitative and qualitative assessment of the CFTR marker expression in airway and lung organoids derived from healthy hiPSCs at day 22 of differentiation. By immunostaining for the CFTR marker, we confirmed the presence of CFTR channels in airway and lung organoid cells (Additional Fig. 3). The quantification of CFTR+ cells in AOs and LOs by flow cytometry comprised 13.7% and 75.3%, respectively. This result may indicate that LOs contain a higher percentage of cells expressing the CFTR channel then AOs; as a result, LOs respond to forskolin more strongly. However, current evidence is limited and further research is required.



Additional Fig. 3 Confocal images of the CFTR marker in airway and lung organoids derived from a healthy donor at day 22 of differentiation. Nuclei were stained with DAPI (blue). Scale bar, 200 µm

Additional references

1. Boj SF, Vonk AM, Statia M, Su J, Vries RRG, Beekman JM, et al. Forskolin-induced Swelling in Intestinal Organoids: An In Vitro Assay for Assessing Drug Response in Cystic Fibrosis Patients. J Vis Exp 2017;2017. https://doi.org/10.3791/55159.
2. Vonk AM, van Mourik P, Ramalho AS, Silva IAL, Statia M, Kruisselbrink E, et al. Protocol for Application, Standardization and Validation of the Forskolin-Induced Swelling Assay in Cystic Fibrosis Human Colon Organoids. STAR Protoc 2020;1:100019. https://doi.org/10.1016/J.XPRO.2020.100019.