DEVELOPMENT OF MURINE STEM CELLS WITH CONDITIONAL KNOCKOUT OF HUMANIZED *SNCA* GENE

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α-synuclein is one of the key molecular links in the pathogenesis of Parkinson's disease. The accumulated data indicate that pathogenic mutations in the *Snca* gene are associated with the development of neurodegenerative brain damage, indicating the relevance of studying the synuclein neurobiological role.

The aim of the study was to create a genetically modified clone of mouse stem cells with a conditional knockout of humanized α-synuclein, which can be used for the reinjection into mouse blastocysts, as well as for basic and applied *in vitro* research in the field of pathophysiology and neuropharmacology.

Materials and methods. To create mouse stem cells with a conditional knockout of the humanized *Snca* gene, a previously obtained clone with the first *Snca* exon flanked by LoxP sites, was used. The CRISPR/Cas9-mediated homologous recombination system with donor DNA oligonucleotides of the human sites of the corresponding gene sites was used to humanize the fourth and fifth exons. Cas9 nuclease, single guide RNA, and donor DNA were transfected into mouse cells.

Results. An approach to obtaining clones of mouse genetically modified stem cells expressing pathological humanized α-synuclein, has been proposed and implemented. The resulting clones were plated on Petri dishes for propagation and a further genetic analysis. Clone 126-2F4 was found out carrying the necessary genetic modifications. The results obtained are fundamentally important not only for understanding the development of the pathological process in α-synucleinopathies, but which is more important, for the development of new therapeutic approaches that will stop the extension of the human α-synuclein aggregation pathology throughout the nervous system, and the validation of these approaches in preclinical trials.

Conclusion. As a result of the study, a strategy for CRISPR/Cas9-assisted homologous recombination in the genome of mouse embryonic stem cells has been developed to create a fully humanized *Snca* gene encoding α-synuclein, and the clone genome of mouse embryonic stem cells has been edited using a CRISPR technology. The RNA and DNA oligonucleotides necessary for the creation of RNP complexes that carry out a directed homologous recombination in the *Snca* locus of the mouse genome have been synthesized. The developed cell clone can serve to create a line of genetically modified mice that serve as a test system for pathophysiological and neuropharmacological studies associated with synucleinopathies. Herewith, before the induction of the Cre-dependent recombination, this line is a representative model for studying a biological role of mutant *Snca*. At the same time, after a Cre-dependent knockout activation, it is possible to imitate the pharmacological inhibition of α-synuclein, which is of particular interest for applied research in neuropharmacology.

Keywords: α-synuclein; neurodegeneration; Parkinson's disease; conditional knockout; CRISPR/Cas9

Abbreviations: NDs – neurodegenerative diseases; sgRNA – single-guide RNA; NAC – non-amyloid-β component; RNP – ribonucleoprotein; PCR – polymerase chain reaction; PD – Parkinson's disease.

СОЗДАНИЕ КЛОНА ЭМБРИОНАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК МЫШЕЙ С КОНДИЦИОННЫМ НОКАУТОМ ГУМАНИЗИРОВАННОГО ГЕНА *SNCA*

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Одним из ключевых молекулярных звеньев в патогенезе болезни Паркинсона является белок α-синуклеин. Накопленные данные свидетельствуют о том, что патогенные мутации в гене *Snca* ассоциированы с развитием нейродегенеративного повреждения головного мозга, тем самым указывая на актуальность изучения нейробиологической роли α-синуклеина.

Цель. Создание генетически-модифицированного клона стволовых клеток мышей с кондиционным нокаутом гуманизированного α-синуклеина, который может быть использован для реинъекции в мышиные бластоцисты, а также для фундаментальных и прикладных *in vitro* исследований в области патофизиологии и нейрофармакологии.

Материалы и методы. Для создания мышиных стволовых клеток с кондиционным нокаутом гуманизированного гена *Snca* был использован прежде полученный клон с фланкированным LoxP-сайтами первым экзоном *Snca*. Для гуманизации IV и V экзона была использована система CRISPR/Cas9-опосредованной гомологичной рекомбинации с донорными ДНК олигонуклеотидами человеческих сайтов соответствующих генов. Нуклеаза Cas9, гидовые РНК и донорная ДНК были трансфецированы в клетки мыши.

Результаты. Нами был предложен и реализован подход к получению клонов мышиных генетически-модифицированных стволовых клеток, экспрессирующих патологический гуманизированный α-синуклеин. Полученные клоны были высеяны на чашки Петри для размножения и дальнейшего генетического анализа. Был обнаружен клон 126-2F4, несущий необходимые генетические модификации. Результаты проведенного исследования принципиально важны не только для понимания развития патологического процесса при α-синуклеинопатиях, но и, что ещё важнее, для разработки новых терапевтических подходов, которые позволят остановить распространение агрегационной патологии человеческого α-синуклеина по нервной системе и валидации этих подходов в доклинических испытаниях.

Заключение. В результате проведенного исследования разработана стратегия CRISPR/Cas9-ассистированной гомологической рекомбинации в геноме эмбриональных стволовых клеток мыши для создания полностью гуманизированного гена *Snca*, кодирующего α-синуклеин. Выполнено редактирование генома клона эмбриональных стволовых клеток мыши с использованием CRISPR технологии. Синтезированы РНК и ДНК олигонуклеотиды, необходимые для создания рибонуклеопротеиновых комплексов, осуществляющих направленную гомологическую рекомбинацию в *Snca* локусе генома мыши. Разработанный клон клеток может служить для создания линии генетически-модифицированных мышей, служащих тест-системой для патофизиологических и нейрофармакологических исследований, связанных с синуклеинопатиями. При этом до индукции Cre-зависимой рекомбинации данная линия является репрезентативной моделью для исследования биологической роли мутантного *Snca*. В то же время, после Cre-зависимая активация нокаута позволяет имитировать фармакологическое ингибирование α-синуклеина, что представляет особый интерес для прикладных исследований в нейрофармакологии.

Ключевые слова: α-синуклеин; нейродегенерация; болезнь Паркинсона; кондиционный нокаут; CRISPR/Cas9

Список сокращений: НЗ – нейродегенеративные заболевания; огРНК – одиночная гидовая рибонуклеиновая кислота; РНП – рибонуклеопротеины; ПЦР – полимеразная цепная реакция; NAC – неамилоидный компонент; БП – болезнь Паркинсона.

INTRODUCTION

A heterogeneous group of pathologies, united by the concept of neurodegenerative diseases (NDs), continues to acquire an increasing medical and social significance. In view of the increase in life expectancy, the burden of NDs, classically associated with an old age, is becoming one of the most relevant biomedical problems [1]. Herewith, despite high rates of the neurobiology development, many aspects of NDs pathogenesis remain disclosed only fragmentarily. One of these aspects is the role of α-synuclein in the main processes associated with the degenerative death of neurons.

As the main component of protein aggregates, α-synuclein has been found out in a number of NDs, which are combined into a group of synucleinopathy, including Parkinson's disease, dementia with Lewy

bodies, a Rapid Eye Movement sleep behavior disorder, and a pure autonomic failure [2].

α-synuclein is a product of the *Snca* gene located on chromosome 4 at position q22.1 [3], and is a small protein (140 amino acids) expressed mainly in neurons, as well as in some tumor cells [4]. Its structure is represented by three main domains. They are: an N-terminal domain (1–60) containing a conserved motif of several repeating amino acid sequences (a consensus sequence of XKTKEGVXXXX); the central domain (61–95), known as the non-amyloid-β component (NAC), which is highly hydrophobic and is involved in the aggregation of α-synuclein during the formation of the β-sheet structure; C-terminal domain (96–140) enriched in negatively charged residues and a proline which provides polypeptide flexibility [5].

Although an α-synuclein physiological function remains understudied, its localization in presynaptic terminals [6], the association with the synaptic vesicle reserve pool [7], and observed deficiencies in the synaptic transmission in response to the gene knockdown or overexpression, suggest that α-synuclein participates in the regulation of the neurotransmitters release, as well as in neuroplasticity [8].

A possible role of α-synuclein in the regulation of synaptic homeostasis is associated not only with its direct interaction with synaptic vesicles: it interacts with synaptic proteins that control vesicle exocytosis, such as phospholipase D and the Rab family of small guanosine triphosphatase [9]. The accumulated evidence suggests that α-synuclein can act as a chaperone, control the degradation, and influence the assembly, maintenance, and distribution of the presynaptic SNARE protein complex, which is involved in the release of neurotransmitters, including dopamine [10]. Taken together, these observations indicate that α-synuclein plays an important role in the movement and exocytosis of vesicles [8].

In this paper, a procedure for creating a clone of mouse embryonic stem cells with CRISPR/Cas9-mediated humanization of the *Snca* gene with the first exon flanked by LoxP sites, have been described.

THE AIM of the study was to create a genetically modified clone of mouse stem cells with a conditional knockout of humanized α-synuclein, which can be used for the reinjection into mouse blastocysts, as well as for basic and applied *in vitro* research in the field of pathophysiology and neuropharmacology.

MATERIALS AND METHODS

Ethics review of study

The experiments were carried out at the Research Institute of Pharmacology of Living Systems (Belgorod State National Research University) in compliance with the ethical standards regulated by the ARRIVE management. The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (protocol No. 08/21 dated February 8, 2021).

Obtaining cell clone with flanked first exon of *Snca* gene

A mouse clone of embryonic stem cells carrying identically oriented LoxP sites flanking the first coding exon of the *Snca* gene (Clone 126) had been obtained in the previous laboratory studies and was used to generate mice with a conditional knockout of this gene. The obtained line of mice and the evidence for the depletion of α-synuclein encoded by the *Snca* gene in the nervous system after the induction of the LoxP/Cre recombination have been described in the published articles [11–13]. The clone was used for a further genomic editing in order to obtain stem cells with the humanized *Snca* gene.

The surfaces of all plastic Petri dishes, flasks and plates used for the cultivation of mouse embryonic stem cells, had been preliminarily coated with a layer of gelatin: a 0.1% gelatin solution (Merk, Germany) was layered on the working surface of the plastic and aspirated after 15–30 min of incubation at room temperature. Immediately afterwards, the surface was covered with a layer of a culture medium.

The clone cells stored in liquid nitrogen with a flanked first exon of α-synuclein were thawed, washed with ESGRO Complete Basal Medium (Sigma-Aldrich, USA), resuspended in 4 ml of the medium with GSK3 by the ESGRO Complete Plus Clonal Grade Medium (Sigma-Aldrich, USA) inhibitor and plated on plastic Petri dishes 6 cm in diameter (Nunc, Denmark). After 16 h of the incubation at 37°C in the atmosphere of 5% CO_2, the medium was changed for the fresh ESGRO Complete Plus Clonal Grade Medium, pre-washing the dishes with the ESGRO Complete Basal Medium. After 48 h, the cells concentration in the Goryaev chamber was calculated, and 200,000 cells were seeded on each of the prepared plastic Petri dishes using ESGRO Complete Accutase (Merk, Germany).

Preparation of cells for nucleofection

48 hours after the passage, the cells were treated with an Accutase solution as described above. 2 aliquots of 200 000 cells were taken, centrifuged; the supernatant was carefully removed, and each pellet was resuspended in 20 μl of Complete P3 buffer prepared immediately before use, by mixing 34.2 μl of the Nucleofector TM Solution and 7.6 μl of P3 Primary Cell 4D-Nucleofector® X kit S (Lonza, Switzerland).

The recombinant Cas9 protein, single-guide

ribonucleic acid (sgRNA), as well as single-stranded DNA oligonucleotides for the homologous recombination carrying nucleotide substitutions corresponding to the sequence of the human *Snca* gene, were used to introduce directed breaks into the edited regions of the *Snca* gene.

Ribonucleoprotein (RNP) complexes were formed by mixing 1 µl of 100 µM sgRNA5 solution, 1 µl of 100 µM sgRNA5 solution, and 1 µl of 10 mg/ml Cas9 solution. Incubated for 10 min at 20°C, 0.4 µl of a freshly prepared mixture of donor DNA solutions (ssODN4 and ssODN5) was added at the concentration of 250 µM for each of the oligonucleotides, and 20 µl of the cells resuspended in Complete P3 buffer, were immediately added as described above.

The CRISPR/Cas9-assisted homologous recombination strategy in the mouse embryonic stem cell genome to generate a fully humanized *Snca* gene expressing a human α-synuclein variant with an increased propensity for the aggregation associated with the development of a hereditary form of Parkinson's disease is shown in Fig. 1 and 5.

Delivery of RNP complexes into cells by nucleofection

The cell suspension was transferred into Nucleocuvette™ (Lonza, Switzerland) and a nucleofection was performed in a 4D-Nucleofector™ device (Amaxa, Ukraine) using a CA-120 program. At the end of the cells were transferred into 5 ml of ESGRO Complete Plus Clonal Grade Medium, resuspended to obtain a monocellular suspension. The concentration of survived cells in the Goryaev chamber was counted, and 200,

400, 600, 800, and 1000 cells were seeded for each of five prepared Petri dishes with a diameter of 10 cm in 10 ml of the same medium, an aliquot for the isolation of genomic DNA being previously taken.

The cells were grown until the appearance of separate colonies originating from one cell, separated by accutase in the well of a 96-well plate, incubated for 3 min at room temperature, 0.2 ml of ESGRO Complete Plus Clonal Grade Medium was added, resuspended, and the cells were grown until reaching a 30–50-percent monolayer. At this stage, the cells were subcultured into wells of 4-well plates in triplets. The last of the three parallel dishes was used to isolate the genomic DNA.

Genomic DNA isolation and exon editing analysis of *Snca* gene

After the medium removal, the cells were lysed directly on the wells surface and DNA was isolated using a Wizard Mammalian Cell DNA Extraction kit (Promega, USA) according to the manufacturer's instructions. DNA was used for the PCR amplification of DNA fragments containing mouse *Snca* exon sequences using a GenPak PCR Core kit (Isogen, Russia), according to the manufacturer's instructions.

The presence of homologous recombination with donor DNA was assessed using the allele-specific PCR and restriction analysis. The reaction mixture was incubated with restriction endonucleases specific to the mutant sequence and electrophoretically separated to assess the presence of the homologous recombination with donor DNA. The reaction products were analyzed in a 1.5% agarose gel.

Table 1 – Components of mixture transfected into clone 126 of embryonic stem cells for humanization of mouse *Snca* gene

Component	Sequence	Molecule type
sgRNA4 (Alt® CRISPR-Cas9 sgRNA for humanization of *Snca* gene exon IV)	5'- mG*mU*mC*CUUCUUGACAAAGCCAGGUUUUAG AGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC mU*mU*mU*U -3'	RNA
sgRNA5 (Alt® CRISPR-Cas9 sgRNA for humanization of *Snca* gene exon V)	5'- mG*mG*mG*UGAGGAGGGGUACCCACGUUUUAGAGCUArGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC mU*mU*mU*U -3'	RNA
ssODN4 (Alt-RTM HDR Donor single-stranded oligonucleotide for humanization of *Snca* gene exon IV)	5'- /Alt-R-HDR1/G*T*T ATT ACT GAG CAT AAA ACA GGC AGC CAT ACC TTG CCC AAC TGG TCC TTC TTG ACA AAG CCA GTT GCA GCA GCT ATG CTC CCA GCT CCC TCC ACT GTC TTC TGA GCG ACA GCT GTC* A*C/Alt-R-HDR2/ -3'	DNA
ssODN5 (Alt-RTM HDR Donor single-stranded oligonucleotide for humanization of *Snca* gene exon V)	5'- /Alt-R-HDR1/A*A*A ACA CTC TCT TAT TGT GCT TTC TCT TCC CTC TCT GTA GAA TGA GGA GGG GGC CCC ACA AGA AGG AAT CCT GGA AGA CAT GCC TGT GGA TCC TGA CAA TGA GGC TTA TGA AAT GCC TTC AGA GGT AAA TGC CTG TA*T* A/Alt-R-HDR2/ -3'	DNA
Cas9 nuclease	–	Protein

Nucleotide sequence of exon IV and flanking regions of mouse *Snca* gene
Green background: exon IV
Blue background: direct primer
Yellow background: reverse primer
Bold type: CRISPR-Cas9 crRNA and PAM sequence (underlined)
Red font type: nucleotides to be changed for exon IV humanization

Nucleide sequence homologous to ssODN4 (Alt-R™ HDR Donor a single-stranded oligonucleotide for exonIV humanization of *Snca* gene
Italic font type: "mismatch" primer
Red lowercase type: Nucleotide substitutions leading to IV exon humanization or creating new restriction endonuclease recognition sites

Nucleotide sequence of exon V and flanking regions of mouse *Snca* gene
Green background: exon V
Blue background: direct primer
Yellow background: reverse primer
Bold type: CRISPR-Cas9 crRNA and PAM sequence (underlined)
Red font type: nucleotides to be changed for exon V humanization

Nucleide sequence homologous to ssODN4 (Alt-R™ HDR Donor a single-stranded oligonucleotide for exon V humanization of *Snca* gene
Italic font type: "mismatch" primer
Red lowercase type: Nucleotide substitutions leading to V exon humanization or creating new restriction endonuclease recognition sites

Figure 1 – CRISPR/Cas9-assisted homologous recombination strategy in mouse embryonic stem cell genome to generate fully humanized *Snca* gene
Note: for some primers and CRISPR-Cas9 crRNA, their positions on the given DNA strand are shown.
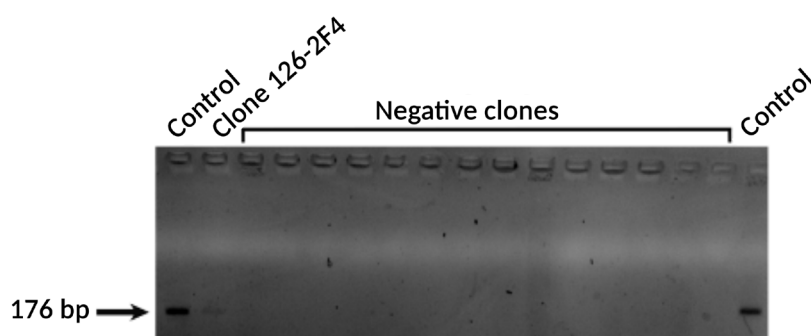The actual sequences can be found in the text.

**Figure 2 – DNA amplification analysis of PCR products of analyzed clones after cells nucleofection
of clone 126 of mouse embryonic stem cells with RNP complexes**
Note: Amplification of 176-nucleotide fragment using primers mexVfor-out and exV-rev-mism indicates successful homologous recombination
and, as a result, humanization of exon V in the genome of subclone 126-2-F4 cells. As a positive control,
amplification with the same template primers of a synthetic fragment corresponding to the humanized mouse V exon
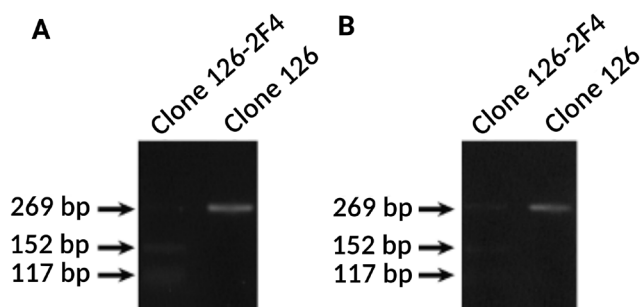with flanking sequences was used.

**Figure 3 – Analysis of two clones in which homologous recombination was detected in exon V
of *Snca* gene during primary screening by treating a 269-nucleotide PCR amplification product
with restriction endonuclease ApaI**
Note: complete cleavage of this fragment into fragments of 152 and 117 bps in size indicates that clone 126-2-F4 is homozygous
for the humanization of exon V (A), and only partial cleavage in the case of clone 126-3-B6 indicates that that only one
of the two allelic copies (B) of the *Snca* gene was humanized in this clone.
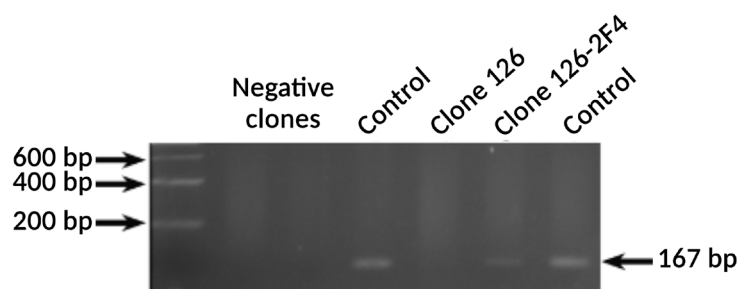
**Figure 4 – Analysis of exon IV humanization in genome of clone 126-2-F4 cells. DNA of maternal clone 126,
clone 126-2-F4**
Note: Amplification with the same primers from the template of the synthetic fragment corresponding to the
humanized mouse exon IV with flanking sequences was used as a positive control. The detection of a 167-nucleotide fragment
in the DNA analysis of clone 126-2-F4 indicates that exon IV of the *Snca* gene was humanized in the cell genome of this clone.

**Figure 5 – Strategy for creating clone of mouse embryonic stem cells with conditional knockout
of humanized *Snca* gene**

Note: I – to create a clone of mouse embryonic stem cells with a conditional knockout of the humanized *Snca* gene,
the cells with the first *Snca* exon flanked by LoxP sites, were taken; II – by transfection of CRISPR/Cas9, guide RNAs and fragments
of exons IV and V of human *Snca* for homologous repair, the mouse *Snca* gene was humanized; III – after the modification
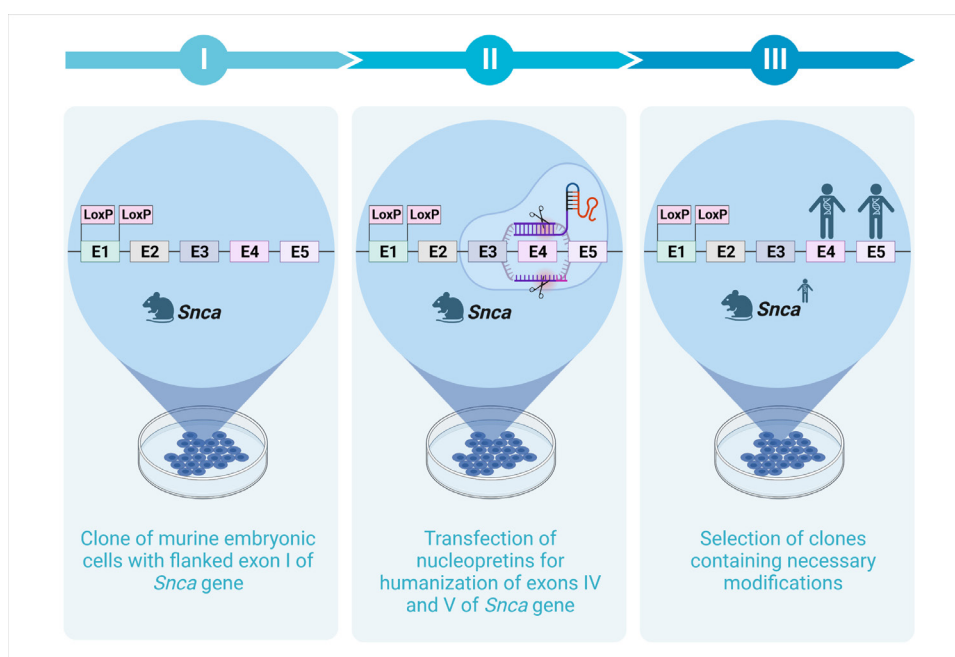by the allele-specific PCR and restriction analysis, the selection of clones carrying the necessary modification, was carried out.

The list of the primers used for the PCR analysis of the homologous recombination at the *Snca* locus of the mouse embryonic stem cell of clone 124 is as follows:

mexIV-for:	5'-GTCTCTGTCACACCATCATC-3'
mexIV-rev:	5'-AGTGTGCATCATGTGCATGC-3'
exIV-rev-mism:	5'-CAGTaGCAGCAGCTATgc-3'
mexVfor-out:	5'-CCAGTGGTTTGGTACACTTAG-3'
mexVfor-ins:	5'-CTGATAACACTTCGTGCAGC-3'
mexVrev-ins:	5'-TAGTGGCAGGGTTTTGATGG-3'
mexVrev-out:	5'-CTATGCCAACCATAATGTGAG-3'
exV-rev-mism:	5'-tTGTGGGgcCCCCTCCTCAtt-3'

RESULTS

Primary screening of clones
for humanized exon V

162 clones were selected for the presence of a 176-bp PCR amplification product analysis using mexVfor-out and exV-rev-mism primers after nucleofection. The two 3'-terminal nucleotides in exV-rev-mism corresponded to the nucleotides present in exon V of the human *Snca* gene, while the mouse gene contains 2 other nucleotides at these positions. In addition to those indicated, in the 5'-terminal part of this primer, there are 3 more nucleotides that are characteristic for only a human gene. Thus, an amplification product with this primer is formed only when the template is human DNA, or mouse DNA humanized for this gene. When the template is native mouse DNA, no amplification products are formed. An example of such an analysis

of PCR amplification products of DNA isolated from the cells 15 selected clones is shown in Fig. 2.

As Fig. 2 shows, 1 out of 15 clones tested for the presence of a 176-nucleotide PCR amplification product gave a positive result, i.e. a homologous recombination occurred in exon V in the DNA cells of this clone. That was the evidence that in the clone designated 126-2-F4, in accordance with its position in the well of one of the initial 96-well plates, this exon turned out to be humanized.

Test for homozygosity of modification
in clone 126-2-F4

The DNA of clone 126-2-F4 and maternal clone 126 were amplified using primers mexVfor-ins and mexVrev-ins corresponding to the sequences flanking mouse V exon. As expected, the same amplification product, a 269-nucleotide fragment, was detected in both cases. The treatment of the reaction mixture with the restriction endonuclease ApaI did not lead to a cleavage of the parent clone 126 fragment amplified from the DNA template, since in the mouse genome in the analyzed region, there is no recognition site for this enzyme. However, the point substitutions used in the humanization of exon V resulted in the appearance of such a site. The fragment amplified from the DNA template of clone 126-2-F4, was cut with the restriction endonuclease ApaI into 152-nucleotide and 117-nucleotide fragments (Fig. 3A). It is important to notify that in this case, the original 269-nucleotide fragment completely disappeared, which

indicated that in the genome of clone 126-2-F4 cells, the homologous recombination and, consequently, the exon V humanization, occurred in both alleles of the *Snca* gene. In another clone, 126-3-B9, selected in the primary screening, only a partial cleavage of the 269-nucleotide fragment by the restriction endonuclease ApaI, was observed (Fig. 3B). It indicated that in this clone genome, the homologous recombination occurred in only one allelic copy gene or that this clone had originated not from one, but from two cells, in the genome of one of which the DNA sequences of exon V had not been edited.

Verification of humanized exon IV presence in genome of clone 126-2-F4 cells

The DNA of clones 126-2-F4, maternal clone 126 and two negative clones from the above screening were amplified using primers mexIVfor and exIV-rev-mism. As a positive control, amplification with the same template primers of a synthetic fragment corresponding to the humanized mouse exon IV with flanking sequences was used. The analysis result of the PCR amplification products is shown in Fig. 4.

A fragment of the expected size (167 bps) was detected only when the DNA of clone 126-2-F4 cells was used as a template, which indicated that this clone had also a homologous exon IV recombination in the mouse *Snca* gene. Testing for homozygosity of this exon modification was carried out according to the same scheme as had been used for exon V using primers mexIV-for and mexIV-rev and the amplification products treatment with the restriction endonuclease PvuII. It was found out that the 280-nucleotide fragment, the product of the DNA amplification of the clone 126-2-F4 cells, had been completely cut by this enzyme into fragments of 164 and 116 base pairs (Fig. 4). That indicates that in the genome of the clone 126-2-F4 cells, the homologous recombination and hence the exon IV humanization occurred in both alleles of the *Snca* gene. As expected, in the absence of the PvuII recognition site, in the studied fragment of the mouse genome, the 280-nucleotide PCR amplification product of maternal clone 126, the DNA cells were not cleaved by this enzyme. A partial cleavage was observed for the 280-bp PCR DNA amplification product of the clone 126-3-B9 cells, which supports the earlier assumption that this clone had originated from not one, but two cells.

DISCUSSION

Due to the progressive aging of the population, the incidence and prevalence of Parkinson's disease (PD) have increased significantly and will continue to grow - thus, this is a serious medical and social problem. The search for effective therapeutic approaches requires the use of optimal models for the development of the pathological process in sporadic (~90% of cases) PD. The models currently used, do not correspond to this task (not humanized - or incorrectly humanized, there is no possibility of regulation) and cannot be used in experimental neuropharmacology.

The most important role of α-synuclein in the degenerative cell death has been shown in the whole spectrum of neurodegenerative diseases. Moreover, mutations (A53T and A30P) were among the first discovered genetic correlates of the disease [14, 15]. This finding has intensified the molecular mechanisms study of α-synuclein-induced neuropathology. It is now known that under pathological conditions, α-synuclein tends to form the structures rich in β-sheets including oligomers, protofibrils, and insoluble fibrils, which are eventually accumulated to form Lewy bodies. Although the disease has traditionally been associated with insoluble forms of aggregated α-synuclein, it is the soluble intermediate oligomers that are characterized by neurotoxic effects. Oligomers have been found out to mediate aberrant calcium signaling, lipid peroxidation, oxidative stress, mitochondrial dysfunction, and neuronal death [16–18]. *In vivo* studies have shown that oligomer-prone and fibril-inhibiting forms of α-synuclein lead to the death of dopaminergic neurons. On the contrary, fibril-producing forms do not lead to the loss of these neurons [19].

In general, the molecular cascades associated with the aberrant function of α-synuclein, continue to be the most important topic of the study for modern neurobiology [20, 21].

In this regard, an approach to obtaining genetically modified mice expressing pathological humanized α-synuclein has been proposed and implemented. To obtain this line, the strategy of creating genetically modified animals through CRISPR/Cas9-mediated editing of embryonic stem cells has been used. The resulting clone of stem cells can be used for the reinjection into blastocysts, which will then be transplanted into recipient mice to carry genetically modified embryos.

The genetic model described in this work makes it possible to carry out the studies aimed at a precise assessment of the role of pathological α-synuclein in mice.

Thus, the exons IV and V humanization will make it possible to evaluate the phenotypic effects of pathogenic human α-synuclein on a representative test system. In addition, the presence of LoxP sites flanking the first exon allows spatial and temporal control of the humanized *Snca* expression due to the possibility of Cre-induced gene knockout. This feature makes it possible to precisely study the effects of a tissue-specific impairment of the protein expression, providing the information about its role in a specific cell population [22–25]. Moreover, the possibility of inducing knockout in adulthood eliminates the effect of the antenatal adaptation to the genetic modification.

A Cre-dependent knockout induction is intended to mean that crossing a line containing a gene region

flanked by LoxP sites with transgenic animals expressing Cre recombinase leads to the deletion of this region and the loss of this gene functional activity [18]. To date, the Cre-mice repertoire is characterized by a great diversity, and the variety between different strains lies in the tissue-specific recombinase expression. Moreover, there are lines in which the penetration of Cre-recombinase into the nucleus and, accordingly, its activity, depend on tamoxifen. In this type of mice, a site-specific recombination between the two LoxP sites occurs only after the treatment with tamoxifen, which makes the gene expression regulation over time possible [19].

Alongside the creation of a genetically modified clone of embryonic stem cells, the authors' team is also implementing a direct editing approach of mouse blastocytes. In other words, a mixture containing a DNA template for a homologous recombination, Cas9 mRNA, and guide RNAs, was microinjected into fertilized eggs of CBA×C57Bl6J mice. After a 24 h incubation, the survived embryos were transplanted into the oviducts of female recipients, who had served as surrogate mothers for the mutants. At present, the primary offspring of mutant mice has already been obtained in a similar way, which is undergoing a genetic analysis for the presence of the desired nucleotide substitutions.

The results obtained are fundamentally important not only for understanding the development of the pathological process in α-synucleinopathies, but what is more important, for the development of new therapeutic approaches that will stop the extension of human α-synuclein aggregation pathology throughout the nervous system, and the validation of these approaches in preclinical trials.

CONCLUSION

As a result of the study, a strategy for CRISPR/Cas9-assisted homologous recombination in the genome of mouse embryonic stem cells has been developed to create a fully humanized *Snca* gene encoding α-synuclein, and the clone genome of mouse embryonic stem cells has been edited using a CRISPR technology.

RNA and DNA oligonucleotides necessary for the creation of RNP complexes that carry out directed homologous recombination in the *Snca* locus of the mouse genome, have been synthesized.

Clones screening obtained by maternal clone 126 nucleofection of mouse embryonic stem cells with RNP complexes, made it possible to identify clone 126-2-F4 that meets the primary criteria for the successful humanization of both alleles of the endogenous *Snca* gene in the mouse embryonic stem cell genome.

Thus, the developed cell clone can serve to create a line of genetically modified mice that serve as a test system for pathophysiological and neuropharmacological studies associated with synucleinopathies. At the same time, before the induction of the Cre-dependent recombination, this line is a representative model for studying the biological role of mutant *Snca*. At the same time, after a Cre-dependent knockout activation, it is possible to imitate the pharmacological inhibition of α-synuclein, which is of particular interest for the applied research in neuropharmacology.

AUTHORS' CONTRIBUTION

Evgeniy A. Patrakhanov – DNA isolation, PCR analysis, restriction analysis, article writing; Vladimir M. Pokrovsky – article writing, cell cultivation and nucleofection; Anastasia Yu. Karagodina – list of references formalization, graphic materials preparation; Anastasia M. Krayushkina – list of references formalization, graphic materials preparation; Nikita S. Zhunusov – PCR analysis, article writing; Alexey V. Deykin – consultation on research methodology; Mikhail V. Korokin – consultation on research methodology, experiment design; Mikhail V. Pokrovsky – consultation on research methodology, experiment design; Oxana B. Altukhova - experiment design, article writing.

REFERENCES

1. Checkoway H, Lundin JI, Kelada SN. Neurodegenerative diseases. IARC Sci Publ. 2011;(163):407–19.
2. Bougea A. Synuclein in neurodegeneration. Adv Clin Chem. 2021;103:97–134. DOI: 10.1016/bs.acc.2020.08.007
3. Ozansoy M, Başak AN. The central theme of Parkinson's disease: α-synuclein. Mol Neurobiol. 2013 Apr;47(2):460–5. DOI: 10.1007/s12035-012-8369-3
4. George JM. The synucleins. Genome Biol. 2002;3(1):REVIEWS3002. DOI: 10.1186/gb-2001-3-1-reviews3002
5. Breydo L, Wu JW, Uversky VN. A-synuclein misfolding and Parkinson's disease. Biochim Biophys Acta. 2012 Feb;1822(2):261–85. DOI: 10.1016/j.bbadis.2011.10.002
6. Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, Saitoh T. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. Neuron. 1995 Feb;14(2):467–75. DOI: 10.1016/0896-6273(95)90302-x
7. Lee SJ, Jeon H, Kandror KV. Alpha-synuclein is localized in a subpopulation of rat brain synaptic vesicles. Acta Neurobiol Exp (Wars). 2008;68(4):509–15.
8. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α-synuclein: from structure and toxicity to

therapeutic target. Nat Rev Neurosci. 2013 Jan;14(1): 38–48. DOI: 10.1038/nrn3406

9. Dalfó E, Ferrer I. Alpha-synuclein binding to rab3a in multiple system atrophy. Neurosci Lett. 2005 May 20-27;380(1-2):170–5. DOI: 10.1016/j.neulet.2005.01.034

10. Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science. 2010 Sep 24;329(5999):1663–7. DOI: 10.1126/science.1195227

11. Ninkina N, Connor-Robson N, Ustyugov AA, Tarasova TV, Shelkovnikova TA, Buchman VL. A novel resource for studying function and dysfunction of α-synuclein: mouse lines for modulation of endogenous Snca gene expression. Sci Rep. 2015 Nov 13;5:16615. DOI: 10.1038/srep16615

12. Roman AY, Limorenko G, Ustyugov AA, Tarasova TV, Lysikova EA, Buchman VL, Ninkina N. Generation of mouse lines with conditionally or constitutively inactivated Snca gene and Rosa26-stop-lacZ reporter located in cis on the mouse chromosome 6. Transgenic Res. 2017 Apr;26(2):301–7. DOI: 10.1007/s11248-016-9995-8

13. Chaprov KD, Lysikova EA, Teterina EV, Buchman VL. Kinetics of alpha-synuclein depletion in three brain regions following conditional pan-neuronal inactivation of the encoding gene (Snca) by tamoxifen-induced Cre-recombination in adult mice. Transgenic Res. 2021 Dec;30(6):867–73. DOI: 10.1007/s11248-021-00286-3

14. Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kösel S, Przuntek H, Epplen JT, Schöls L, Riess O. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet. 1998 Feb;18(2):106–8. DOI: 10.1038/ng0298-106

15. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science. 1997 Jun 27;276(5321):2045–7. DOI: 10.1126/science.276.5321.2045

16. Angelova PR, Choi ML, Berezhnov AV, Horrocks MH, Hughes CD, De S, Rodrigues M, Yapom R, Little D, Dolt KS, Kunath T, Devine MJ, Gissen P, Shchepinov MS, Sylantyev S, Pavlov EV, Klenerman D, Abramov AY, Gandhi S. Alpha synuclein aggregation drives ferroptosis: an interplay of iron, calcium and lipid peroxidation. Cell Death Differ. 2020 Oct;27(10):2781–96. DOI: 10.1038/s41418-020-0542-z

17. Angelova PR, Horrocks MH, Klenerman D, Gandhi S, Abramov AY, Shchepinov MS. Lipid peroxidation is essential for α-synuclein-induced cell death. J Neurochem. 2015 May;133(4):582–9. DOI: 10.1111/jnc.13024

18. Choi ML, Chappard A, Singh BP, Maclachlan C, Rodrigues M, Fedotova EI, Berezhnov AV, De S, Peddie CJ, Athauda D, Virdi GS, Zhang W, Evans JR, Wernick AI, Zanjani ZS, Angelova PR, Esteras N, Vinokurov AY, Morris K, Jeacock K, Tosatto L, Little D, Gissen P, Clarke DJ, Kunath T, Collinson L, Klenerman D, Abramov AY, Horrocks MH, Gandhi S. Pathological structural conversion of α-synuclein at the mitochondria induces neuronal toxicity. Nat Neurosci. 2022 Sep;25(9):1134–48. DOI: 10.1038/s41593-022-01140-3. Epub 2022 Aug 30. Erratum in: Nat Neurosci. 2022 Nov;25(11):1582.

19. Choi ML, Gandhi S. Crucial role of protein oligomerization in the pathogenesis of Alzheimer's and Parkinson's diseases. FEBS J. 2018 Oct;285(19):3631–44. DOI: 10.1111/febs.14587

20. Srinivasan E, Chandrasekhar G, Chandrasekar P, Anbarasu K, Vickram AS, Karunakaran R, Rajasekaran R, Srikumar PS. Alpha-Synuclein Aggregation in Parkinson's Disease. Front Med (Lausanne). 2021 Oct 18;8:736978. DOI: 10.3389/fmed.2021.736978

21. Chaprov KD, Goloborshcheva VV, Tarasova TV, Teterina EV, Korokin MV, Soldatov VO, Pokrovskiy MV, Kucheryanu VG, Morozov SG, Ovchinnikov RK. Increased Expression of the Multimerin-1 Gene in α-Synuclein Knokout Mice. Dokl Biol Sci. 2020 Sep;494(1):260–3. DOI: 10.1134/S0012496620050014

22. Soldatov VO, Kubekina MV, Silaeva YuYu, Bruter AV, Deykin AV. On the way from SARS-CoV-sensitive mice to murine COVID-19 model. Research Results in Pharmacology. 2020;6(2):1–7. DOI: 10.3897/rrpharmacology.6.53633

23. Bruter AV, Korshunova DS, Kubekina MV, Sergiev PV, Kalinina AA, Ilchuk LA, Silaeva YY, Korshunov EN, Soldatov VO, Deykin AV. Novel transgenic mice with Cre-dependent co-expression of GFP and human ACE2: a safe tool for study of COVID-19 pathogenesis. Transgenic Res. 2021 Apr 14;30(3):289–301. DOI: 10.1007/s11248-021-00249-8. Epub ahead of print.

24. Kuzubova E.V., Radchenko A.I., Pokrovsky VM, Patrakhanov EA, Novikova AA, Stepenko YuV, Deikin AV. Pathological conditions associated with tau protein: mechanisms of development and possible biological targets for pharmacological correction of tau proteinopathy (review). Research Results in Biomedicine. 2022;8(4):474–94. DOI: 10.18413/2658-6533-2022-8-4-0-6. Russian

25. Dolskiy AA, Gudymo AS, Taranov OS, Grishchenko IV, Shitik EM, Prokopov DY, Soldatov VO, Sobolevskaya EV, Bodnev SA, Danilchenko NV, Moiseeva AA, Torzhkova PY, Bulanovich YA, Onhonova GS, Ivleva EK, Kubekina MV, Belykh AE, Tregubchak TV, Ryzhikov AB, Gavrilova EV, Maksyutov RA, Deykin AV, Yudkin DV. The Tissue Distribution of SARS-CoV-2 in Transgenic Mice With Inducible Ubiquitous Expression of hACE2. Front Mol Biosci. 2022 Jan 18;8:821506. DOI: 10.3389/fmolb.2021.821506

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