



New personalized genetic mouse model of Lesch-Nyhan syndrome for pharmacology and gene therapy

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Abstract

Introduction: Lesch-Nyhan syndrome is a clinical and laboratory disorder caused by X-linked disruption of the purine metabolism. The deletion in the HPRT1 gene leads to the disappearance of valine in the eighth position of the protein amino acid sequence. The disease occurs in males and is accompanied by an excess of uric acid, urate nephropathy and neurologic impairment.

Objective of the Study: Generation of the new personalized genetic mouse model of Lesch-Nyhan syndrome for pre-clinical study of new approaches to the pharmacological and gene therapy

Materials and Methods: For genomic editing, the sequence was synthesized the sequence of the matrix GACCG-GTCCCGTCATGCCGACACGCAGTCCCAGCGTGGTGAGCCAAGGGGACTCCAGCAGAGCCCCACAG was synthesized. For the cultivation of viable mouse embryos after microinjection, KSOM media was used. Amplification and sequencing was performed by the standard methods.

Results: A boy with not previously described hemizygous variant in the HPRT1 gene, was observed in our clinic. The mutation was the deletion of 8Val in the first exon of the HPRT1 gene. To introduce this mutation, we used the CRISPR-Cas9 genomic editing system. The genetic construct for microinjections included a mixture of the vector for the expression of Cas9 and sgRNA (px330), as well as the matrix for homologous recombination (ssODN), in a ratio of 1 part Cas9 to 3 parts of the ssODN matrix. Four of the 12 obtained animals were mosaic transgenes. One of 4 mice mated with a male from the hybrid strain CBA x C57BL/6, and descendants of F₂ have already been received from this mating.

Discussion: During the creation of HPRT1 genetically modified mice, we encountered certain difficulties. First, from 615 transplanted embryos, only 12 were able to complete full embryonic development. 9 recipients we observed abortions in the later stages. These data may indicate possible violations of embryonic development in animals carrying a mutant copy of the HPRT1 gene.

Conclusion: In the current study, we present the results of the generation of a genetically modified mouse strain carrying a deletion in the HPRT1 gene. These mice can be effectively used for the preclinical testing of new drugs aimed at the treatment of Lesch-Nyhan syndrome.

Keywords

transgenic mice, Lesch-Nyhan syndrome, CRISPR-Cas9, personalized medicine, orphan diseases, CBAx57BL/6

Introduction

The problem of orphan (rare) diseases, despite the low prevalence in the population, is very important for medicine. It is now believed that most of these diseases are incurable. However, hereditary metabolic diseases, as global medical practice shows, are considered one of the most promising directions in the development of therapies. Lesch-Nyhan syndrome (ICD-10: E79.1; OMIM 300322; ORPHA: 510) is an X-linked inborn error of purine metabolism that is caused by mutations in the HPRT1 gene encoding the purine recycling enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT), which catalyzes the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate in the presence of phosphoribosylpyrophosphate (Harris 2018, Nguyen and Nyhan 2016, Vargiami et al. 2016). The disease was first described in 1964 by Nyhan W.L. and Lesch M. The prevalence of Lesch-Nyhan syndrome is 1-9/1 000 000, and the estimated prevalence at birth is between 1/380 000 and 1/235 000 live births. Males are generally affected, and heterozygous females are carriers (usually asymptomatic) (García and Torres 2010). Uric acid overproduction and urate nephropathy in this disease can be combined with neurologic impairment (Campolo González et al. 2018), which leads to three overlapping clinical phenotypes: (1) the classical form of Lesch-Nyhan disease, characterized by cognitive impairment, dystonia, choreoathetosis, spasticity, self-injurious behavior, and urate nephropathy; (2) an intermediate phenotype with hyperuricemia and variable neurological manifestations with no self-injurious behavior; and (3) hyperuricemia alone (Harris 2018). The basic principles of therapy include the prevention and treatment of urolithiasis and gout (in adults) and the correction of neurological manifestations. Hyperuricemia and uric acid nephropathy respond well to treatment with allopurinol, but this treatment has no effect on behavioral and neurological symptoms. However, for patients with the classic variant of Lesch-Nyhan syndrome, the effective therapy of neurological disturbances remains a task to be solved.

The intensive development of animal genetic engineering methods allows for a wider consideration of the concept of personalized medicine. Experiments on animal models are becoming an indispensable stage in the preclinical testing of therapeutic strategies. Thus, a personalized model of a genetic disease makes it possible to evaluate the contribution of the detected mutation to the phenotype to evaluate the potential efficacy of gene therapy.

Despite the fact that Hprt1-deficient mice models do not have a clinical complex characteristic of patients with Lesch-Nyhan syndrome, these models should be used in studies of brain metabolism (Bell et al. 2016) and precli-

nical studies of the effectiveness of new treatments for this disease, particularly of gene therapy.

At present, the genome editing technology CRISPR-Cas9 (Doetschman and Georgieva 2017, Yang et al. 2017) allows the creation of knockout mouse models with the introduction of mutations in the HPRT1 gene of certain patients (Kato and Takada 2017, Wu et al. 2013). We consider that such targeted models may be useful for the development of gene therapy for Lesch-Nyhan syndrome.

Materials and methods

The animals and the conditions of their care have been described previously (Silaeva et al. 2013). Protocols for the induction of superovulation, obtaining fertilized eggs at the pronucleus stage in mice, microinjection, transplantation of eggs into the oviduct of the pseudopregnant recipient, and for preparing vasectomized males and recipient animals have been published previously (Silaeva et al. 2013, Silaeva et al. 2018, Zvezdova et al. 2010).

We used the px330 vector (Cong et al. 2013) according to the protocol provided by Addgene (<https://www.addgene.org/crispr/zhang/>).

The matrix was synthesized by the Evrogen Company. The sequence of the matrix was GACCGTCCCGT-CATGCCGACACGCAGTCCCAGCGTGGTGAGC-CAAGGGGACTCCAGCAGAGCCCCACAG

Mouse embryo culture. For the cultivation of viable mouse embryos after microinjection, KSOM media was used. The cultivation of the embryos to the blastocyst stage was carried out in mineral oil (Merck, USA) under conditions of absolute humidity at 37°C in 5% CO₂.

The DNA isolation from blastocysts was performed according to a previously published protocol (Dimitrieva et al. 2016).

The DNA isolation from tissue samples was performed according to a standard protocol (Dimitrieva et al. 2016).

Sample preparation for Sanger sequencing included the amplification of the gene of interest (fragment size 544 b.p.) using the primers presented in Table 1. Amplification was performed under the following conditions: 95°C - 5 min, (95°C - 30 sec, 58°C - 25 sec) - 30 cycles, 72°C - 40 sec, 72°C - 10 min, and 4°C - storage. Then, the fragment was visualized on a 2% agarose gel. Further sample preparation was carried out according to the protocol of the Evrogen Company for performing sample sequencing.

The restriction analysis of the F₀ and F₁ generations was performed based on the presence of the BstX1 restriction site in the wild-type HPRT1 matrix and its absence in the mutant gene (Figures 1, 2).

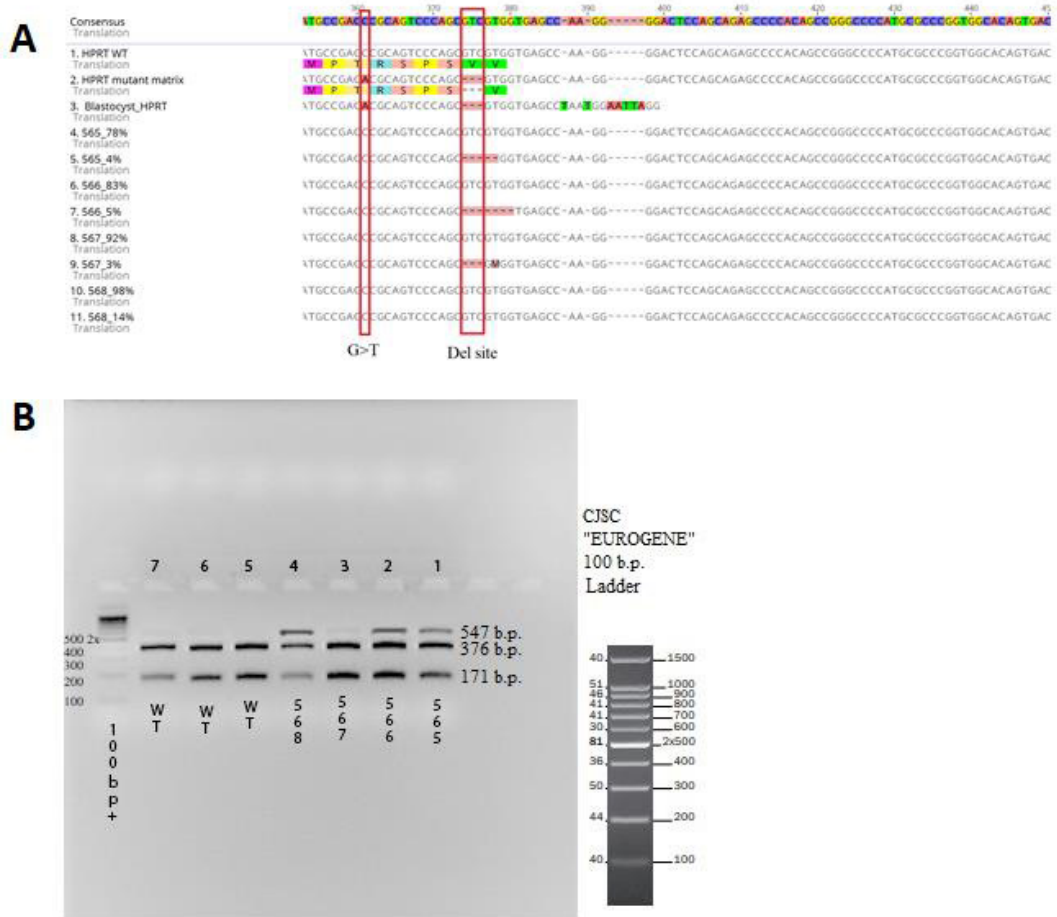


Figure 1. A) – Sequencing results for F₀ transgenes; B) – Restriction analysis results for F₀ transgenes

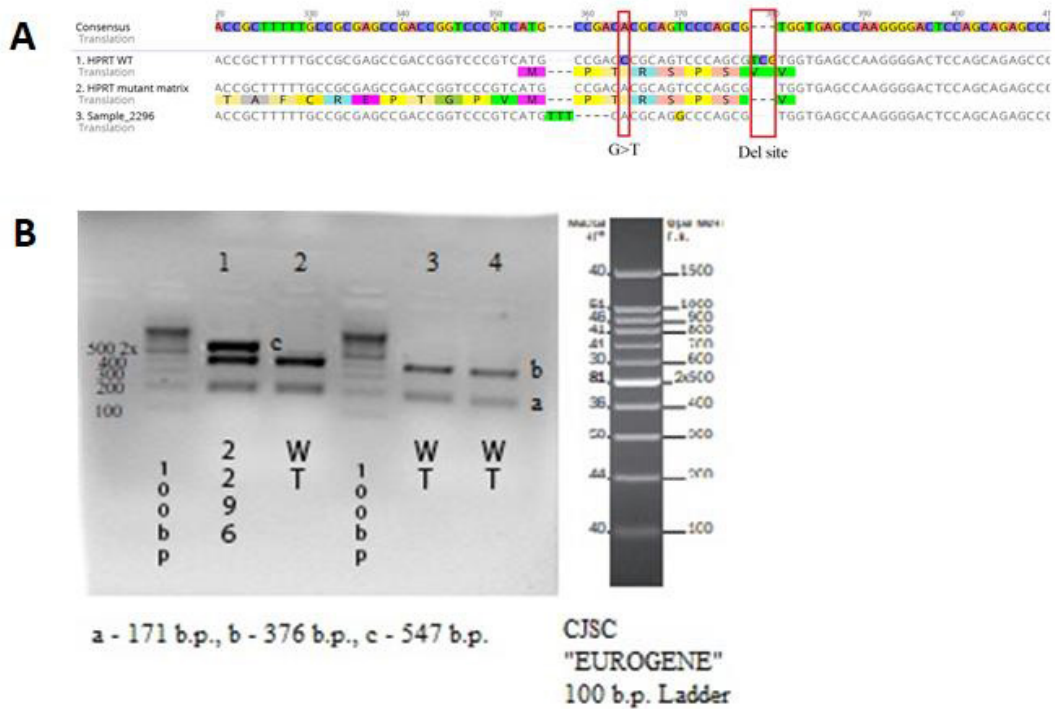


Figure 2. A) – Sequencing results for F₁ transgenes; B) – Restriction analysis results for F₁ transgene

Table 1. Primers used to amplify a fragment of the HPRT1 gene of 544 b.p. in size.

Primer	Sequence
Forward	TGATTATCTGGGAATCCTCTGG
Reverse	CATCTAGCCAGACTCCAGGAA

Software. Analysis of the sequencing results was performed using the program Peak Scanner (Thermo Fisher Scientific). Then, to identify matches with the mutant matrix and the wild-type matrix, the program Geneious (Biomatters Limited) was used. The protein models were visualized and aligned in PyMol (Schrödinger, Portland, OR). The analysis of the transgenic animal generation results was performed in Microsoft Excel (USA).

Results

Genetic analysis of the HPRT1 gene in our patient

Clinical case. A boy with a classic variant of Lesch-Nyhan syndrome was observed in our clinic. The age of the patient at the time of diagnosis was 9 years. Genetic analysis of the HPRT1 gene was performed by direct automatic sequencing of the entire coding region of the gene, including exon-intron boundaries. The patient had a hemizygous variant in the HPRT1 gene, which has not been previously described (Table 2). The boy showed neurological dysfunction characterized by moderate cognitive impairment, generalized action dystonia, choreoathetosis, mild pyramidal signs, and occasional ballistic movements. Self-injurious behavior included biting of hands, fingers and lips. The patient could not sit or walk without support. The neurological symptoms in this patient were combined with uric acid hyperproduction with hyperuricemia, an increased urinary urate-to-creatinine ratio and signs of urate nephropathy according to ultrasound data.

Table 2. Results of direct automatic sequencing of the entire coding region of the HPRT1 gene, including exon-intron boundaries.

Nucleotide	Effect	Amino acid change
c.23_25delTCG	Small deletion (exon 1)	p.Val8del

Structural modeling of HPRT1 del8Val loss of function mutation by bioinformatic algorithms

The BLAST algorithm (NCBI) was used for the comparison of human and murine HPRT1 gene sequences. The alignment shows 98.6% nucleotide sequence homology. The primary structure of the HPRT1 protein and mutation locus was similar enough to be studied in *Mus musculus*.

Structural models of the HPRT1 protein and its fragments (PDB) were used as templates for the M4T RaptorX structure prediction algorithm to obtain structural models of the mutation site and whole mutated protein with altered conformation (Kalmykov et al. 2018).

Models were visualized and aligned in PyMol (Schrödinger, Portland, OR). The cooperativity of HPRT1 homodimer subunits was suggested to be disrupted by the conformational changes observed in the predicted structure of HPRT1. The deleted valine was found to be located in the HPRT1 monomer surface close to the homodimer junction.

Hindered enzymatic soma assembly may be the reason for the biological function loss - the HPRT1 homotetramer appears in its native conformation as a dimer of dimers. The fully functional HPRT1 complex strongly depends on its intermolecular surface structure (Figures 3, 4, 5).

The alignment of three native monomers with the del8Val HPRT1 predicted model shows that the whole protein is slightly changed, with a rotational conformational distribution along the chain. Accordingly, dislocated charged residues are suggested to be the reason for the dimerization disruption.

Developing a system for gene editing

To introduce the 8Val deletion in the first exon of the HPRT1 gene, we used the CRISPR-Cas9 genomic editing system with the most successful sgRNA, the PAM site of which was removed at 13 bp from the place of the proposed deletion. For the selection of sgRNA, we used Chopchop (<http://chopchop.cbu.uib.no/>) (Chapman et al. 2017, Labun et al. 2018) web utilities and Algorithms (<http://www.e-crisp.org/>) (Heigwer et al. 2014). The scheme for introducing a deletion is shown in Figure 6. For homologous recombination, after making the incision, we used a synthetic matrix with a deletion length of 70 bp.

The genetic construct for microinjections included a mixture of the vector for the expression of Cas9 and sgRNA (px330), as well as the matrix for homologous recombination (ssODN), in a ratio of 1 part Cas9 to 3 parts of the ssODN matrix.

To verify the efficiency of the selected genomic editing system, microinjected embryos were cultured to the blastocyst stage, and then the DNA from these organisms was isolated and amplified. A fragment of the gene of interest was sequenced, as described above. As shown in Figure 6, the selected editing system leads to a deletion in the first exon of the HPRT1 gene at the 8Val position.

Generation and genetic analysis of primary genetically modified animals

The F₀ generation of a genetically modified animal model of Lesch-Nyhan syndrome was obtained by the microinjection of a solution of a genetic construct into the pronucleus of a fertilized egg cell and subsequent trans-

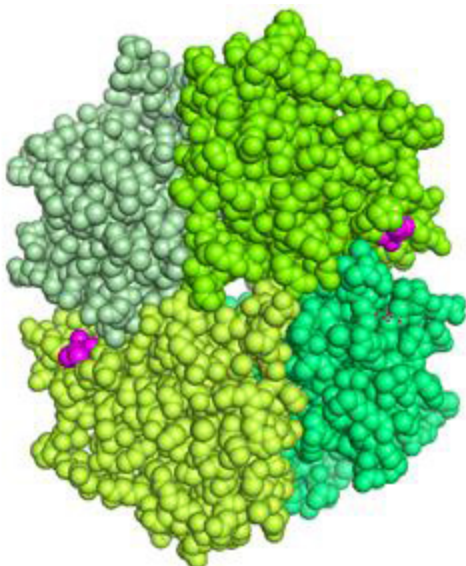


Figure 3. Dimer of dimers, HPRT1 homoheterotetramer (PDB ID 5w8v)

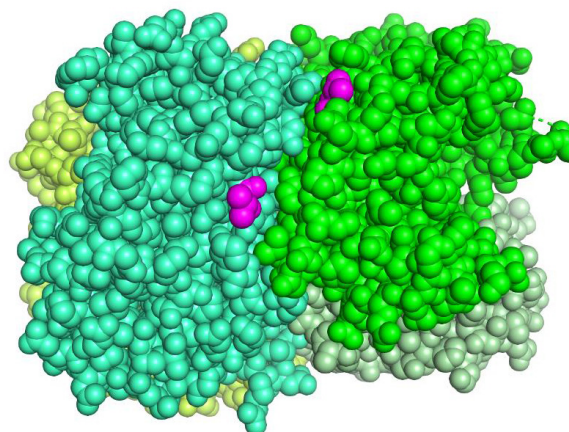


Figure 4. HPRT1 homodimer with 8Val highlighted in both monomers (magenta).

plantation into the oviduct of a pseudopregnant recipient (Ittner and Götz 2007, Liu et al. 2013, Raveux et al. 2017, Richa 2001). The results of this experiment are presented in Table 3.

In total, 78 recipients were used, and 615 one- and two-cell embryos were transferred at 3-5 embryos per horn of the uterus. We significantly reduced the number of embryos transferred to an individual recipient compared to standard protocols (Kalmykov et al. 2018), as it was previously shown that with a decrease in the number of transferred embryos, the efficiency of transplantation increases (Silaeva et al. 2013).

9 recipients, we observed abortions in the later stages (genetic analysis of abortive material was not performed due to the unsuitability of the material). Of the 615 transferred embryos, 12 newborn mice were received. Seven to 14 days after birth, all mice were sampled, the DNA was isolated and sequenced, and restriction analysis was per-

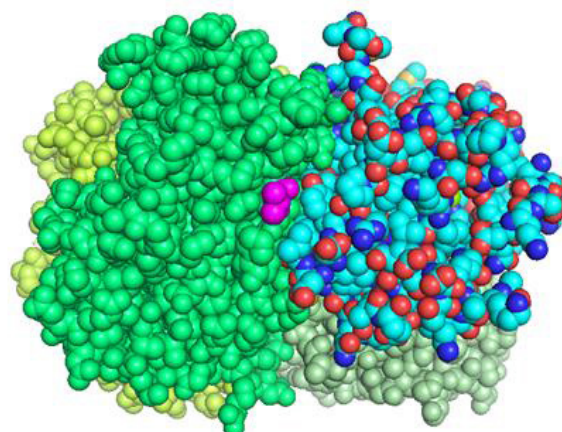


Figure 5. Native conformation of the HPRT1 monomer (left green colored) in contact with predicted mutated rotamer (cyan) built by the RaptorX algorithm.

Table 3. The results of the generation of primary genetically modified animals to obtain a model of Lesch-Nyhan syndrome.

Used Recipients	Amount of Transplanted embryos	Amount of recipients giving birth	The amount of newborn mice	Amount of genetically modified animals from newborns
78	615	9	12	4

Table 4. The results of experiments conducted to obtain F1 animals carrying the mutant HPRT1 gene.

Primary transgene, number	Descendants of F ₁ , amount	Transgenes from these animals
565	31	1
566	8	0
567	0	0
567	21	0

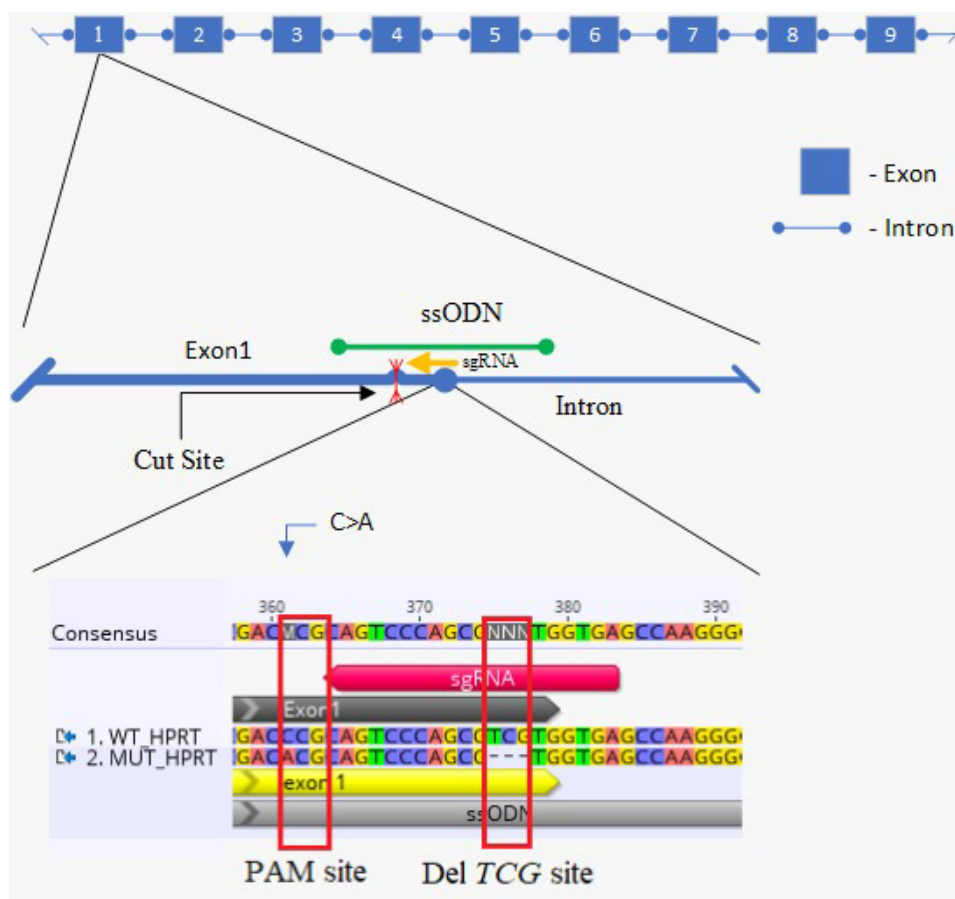


Figure 6. Scheme for introducing a deletion at the 8Val position in the first exon of the HPRT1 gene

formed on the obtained samples. Four of the 12 obtained animals were mosaic transgenes (Figure 1, a, b).

Generation and genetic analysis of F₁ genetically modified animals

The resulting primary transgenes were assigned numbers 565, 566, 567, and 568. Males 565, 567 and 568 were mated with females of the CBA x C57BL/6 hybrid strain, and female 566 was mated with a male of the CBA x C57BL/6 hybrid strain. A total of 31 descendants of F₁ were received from mouse 565, 8 descendants of F₁ were received from mouse 566, 21 descendants of F₁ were received from mouse 568, and no offspring were produced from mouse 567. All progeny obtained were analyzed using Sanger sequencing and restriction analysis, and one genetically modified F₁ animal, female 2296, was identified (Fig 2, a, b). The results of these experiments are presented in Table 4. Mouse 2296 was also mated with a male from the CBA x C57BL/6 hybrid strain, and descendants of F₂ have already been received from this mating.

Discussion

Mutations in the HPRT1 gene can cause severe neurological and metabolic disorders in humans (Fu et al. 2014,

Harris 2018). It has been shown that mice carrying a defective gene, as a rule, lack bright phenotypic manifestations (Bell et al. 2016). However, during the creation of genetically modified animals with a deletion of the nucleotide triplet, which causes the absence of valine in the eighth position of the amino acid sequence of the protein, we encountered certain difficulties. First, from 615 transplanted embryos, only 12 were able to complete full embryonic development. In addition, in 9 recipients (11.5% of the total), we observed abortions in the later stages. These data may indicate possible violations of embryonic development in animals carrying a mutant copy of the HPRT1 gene. In addition, all of the primary transgenes obtained were mosaics, with a mutant gene content of less than 20% in the genome. Only one of the obtained primary transgenes was capable of vertical transgene transmission, with a rather low probability. However, at this stage of the experiment, we have already obtained an F₂ generation of genetically modified animals with a mutated copy of the HPRT gene.

Conclusion

In this paper, we presented the results of the development and production of a new genetically modified mouse line model for Lesch-Nyhan syndrome.

We believe that the proposed model can be successfully used for basic research and preclinical testing of drugs for the treatment of Lesch–Nyhan syndrome.

Conflicts of interests

The authors state no conflict of interest concerning with the present submitted manuscript.

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