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Research Article

Transgenic mice Cre-dependently expressing mutant polymerase-gamma: novel test-system for pharmacological study of mitoprotective drugs

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Abstract

Introduction: PolG-alpha is a nuclear-encoded enzyme which provides replication and repair of mitochondrial DNA. D257A mutation of PolG-alpha leads to change in the N-terminal ”proofreading” domain, which deprives the enzyme of 3′-5′ exonuclease activity, resulting in accumulation of mutations in the mitochondrial genome.

Materials and methods: Murine zygotes were microinjected with transgene construction carrying mutant murine *Polg* coding sequence and *GFP* coding sequence by a loxP-flanked STOP-cassette. Two Cre-activator strains, CMV-Cre (systemic activation) and Tie2-Cre (endothelial activation), were used for activation of the transgene. To confirm the insertion and Cre-dependent activation of the transgene, genotyping and qPCR copy number measurement of mutant *Polg* were performed, and GFP fluorescence was assessed.

Results: Two primary transgenic animals were used as the founders for two lines with copy numbers of transgene ~7 and ~5. After systemic activation, the number of the transgene copies decreases to ~1.0 while endothelial specific activation does not affect the number of transgene copies in tail tissue.

Discussion: A murine model with spatial control of mutant *Polg* expression has been developed. To our knowledge, this is the first transgenic model of tissue-specific mitochondrial dysfunction.

Conclusion: Transgenic mice Cre-dependent expressing mutant polymerase-gamma are a novel test-system for studying mitochondrial biology and efficacy of mitoprotective drugs.

Keywords

Cre-LoxP system, genome editing, IVF, mitochondrial polymerase-gamma, transgene copy number determination, transgenic mice

Introduction

Mitochondrial dysfunction (MD) represents an inadequate number of mitochondria, or an inability to provide necessary substrates to mitochondria, or a dysfunction in their electron transport and ATP-synthesis machinery (Nicolson 2014). Besides a large group of monogenic mitochondrial diseases (MD) is a key link in the pathobiology of a wide spectrum of disorders, including cardiovascular (Zakirov et al. 2020), neurodegenerative (Angelova et al. 2021) and metabolic diseases (Prasun 2020).

The mitochondrial genome is characterized by a high density of nucleotide substitutions and significant instability (10–17 times higher than the mutation rate of nuclear genes) (Wallace and Chalkia 2013; Aryaman et al. 2019). In addition, the mitochondrial genome is characterized by heteroplasmy, that is, copies can differ from each other in length, a set of genes, and the presence of mutations (Su et al. 2018). These features are due to rapid mutation processes (Fontana and Gahlon 2020), lack of recombination (El-Hattab et al. 2017), and cytoplasmic gene inheritance (Wei and Chinnery 2020) in the mitochondrial genome. The main reason for such mitochondrial genome instability lies in the absence of histones, the peculiarity of repair mechanisms, and the presence of free oxygen radicals, which are by-products of aerobic respiration (Woo et al. 2012; Nissanka and Moraes 2018). Many pathologies are associated with mitochondrial mutations, such as Leber hereditary optic neuropathy, chronic fatigue syndrome, encephalomyopathy, etc. (Orekhov et al. 2015; Hsu et al. 2016).

It is obvious that the improvement of approaches to treating mitochondrial pathologies requires adequate test systems. Herein, we describe an approach for creating a murine model that accumulates mutations in the mitochondrial genome of somatic and germ cells due to the presence of *Polg* gene mutants.

PolG-alpha is an enzyme that mediates mitochondrial DNA replication and repair (Copeland and Longley 2003). PolG-alpha is encoded by the nuclear genome and its mutant form PolG-alpha D257A leads to a change in the N-terminal "proofreading" domain, which deprives the enzyme of 3′-5′ exonuclease activity. The mutant form of the protein is unable to correct polymerization errors, which leads to the accumulation of mutations in the mitochondrial genome. Previously, transgenic animals with constitutive ubiquitous mutant *Polg* expression have been created, but their viability and fertility are critically reduced, which greatly complicates the experimental work (Trifunovic et al. 2004).

Materials and methods

Design and manufacture of genetic construct

The genetic construct was created based on the pKB1 vector and the mouse *Polg* gene ORF. The pKB1 vector was designed for Cre-dependent expression of the genes of interest. It contained an ampicillin resistance gene, as well as insulators and terminators to protect the transgene from the position effect (Bruter et al. 2021), a transcriptional unit under the control of the CAG promoter, a STOP-cassette flanked by LoxP sites (Deykin et al. 2019), multiple cloning site for transgene cloning, an encephalomyocarditis virus IRES element in the one reading frame with GFP gene and an SV40polyA signal. It is assumed that transgene expression in pKB1 is activated only after site-specific recombination at LoxP sites and subsequent STOP-cassette excision by Cre recombinase (Fig. 1).

Total RNA for *Polg* cloning was extracted from mouse liver biopsy. The tissue was homogenized using the Precellys 24 homogenizer, and RNA was extracted using a QIAGEN RNeasy Mini Kit. cDNA was synthesized using RevertAid Reverse Transcriptase (Thermo Scientific) and specific primer P1 (all primer sequences are presented in Table 1) for mouse *Polg* gene 3′-UTR. *Polg* gene (3653 bp) was amplified with Platinum SuperFi polymerase (Invitrogen), using primers P2 and P3. The amplified

Table 1. Primer sequences used in the study

Code	Sequence 5′→3′
P1	ATGGCTGCCTTTTGCAAAAAAGC
P2	ATTAACGCGTATGAGCCGCCTGCTCTGGAA
P3	TAATACGCGTCTAGGGTCCAGGCTGGCTTCG
P4	CTCGAGCCCATATCAGGGAACAG
P5	CAAAGGAAACATTGTGCCCCAC
P6	GTTAGATCTGCTGCCACCGT
P7	AGGTGGCAAGTGGTATTCCG
P8	GCGGTCTGGCAGTAAAAACTATC
P9	GTGAAACAGCATTGCTGTCACTT
P10	CTAGGCCACAGAATTGAAAGATCT
P11	GTAGGTGGAAATTCTAGCATCATCC
P12	GTGCATGTTTGCCTATAAGCTGG
P13	GGTAAATATCCAGTGCTTCACCCT

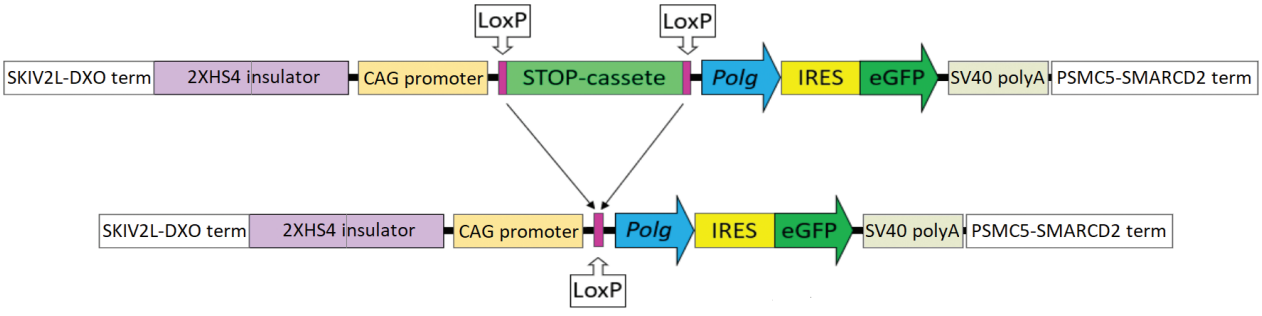

Figure 1. Linearized pKB1-*Polg* genetic construct design.

fragment was subjected to site-directed mutagenesis after cloning into the intermediate T-vector pTZ57R/T (Addgene). Site-directed mutagenesis was carried out using primers P4 and P5, due to which it was possible to introduce the D257A mutation into the native mouse *Polg* gene ORF, which deprives the enzyme of 3′-5′-exonuclease "proofreading" activity. This mutated *Polg* gene was sequenced, amplified using primers P2 and P3, treated with the restriction enzyme MluI (Thermo Fisher Scientific) with subsequent insertion into the pKB1 vector, enzyme treatment and dephosphorylation FastAP, Thermo Fisher Scientific).

Then the construction was prepared for microinjections: circular plasmid DNA was treated with the restriction enzyme PvuI (Thermo Fisher Scientific); the resulting fragments were separated by electrophoresis in agarose gel. A linear fragment 14832 bp, devoid of bacterial sequences, was extracted using a Monarch kit for isolation of DNA fragments from gels and reaction mixtures (New England Biolabs) and dissolved in TE buffer at a concentration of 1 ng/μl (Zvartsev et al. 2019).

Animals and keeping conditions

Several mice strains were used in the work. 6–8-week-old males of hybrid F1 (CBA X C57BL/6) (Stolbovaya Nursery) were used as breeders; immature females (12–13 g) CBA X C57BL/6 were used as donors; 8–10-week-old females CD1 were used as recipients. During the experimental work, the mice were kept in the vivarium of the Institute of Gene Biology of the Russian Academy of Sciences. The mice were kept in conditions of free access to water and food, a 12/12 light cycle, air temperature 23±1°C, and humidity of 42±5%.

Two activator mice of the Cre strain obtained from Jackson Laboratory were used to activate transgene expression, namely B6.C-Tg(CMV-cre)1Cgn/J Stock No: 006054|CMV-Cre (systemic activation) and B6.Cg-Tg(Tek-cre)1Ywa/J Stock No: 008863|Tie2-Cre (endothelial activation).

Solutions and media

The M2 medium (Sigma-Aldrich, USA) was used to wash out the oocytes, the "Droblenie" medium (Paneco, Russia) and mineral oil (Sigma, USA) were used for the oocytes cultivation. "Spermoprep" medium was used for sperm culture (Paneco, Russia), and IVF was carried out in the G-IVF PLUS medium (Vitrolife Sweden AB, Sweden).

Transgenic animals creation

Primary F0 transgenic animals were obtained through a microinjection of a gene construct into zygote pronucleus. The method for obtaining primary transgenic animals was described in detail earlier (Zvezdova et al. 2010; Silaeva et al. 2018).

In vitro fertilization

The method of intravital surgical sperm collection was used for IVF according to the protocol (Val and Robledano 2013) to obtain F1 offspring from the primary transgene #1683. Two-blastomeric embryos obtained after IVF were transferred to recipients.

Genotyping

Mouse DNA is derived from tail tissue. Tissue samples were lysed in alkaline lysis (25 mM NaOH, 0.2 mM EDTA) and purified by the phenol-chloroform method. The mice were genotyped using the PCR method and the following reagents: HS Taq-polymerase, Red buffer, and dNTP (Evrogen), and the STOP-cassette specific primers – P6 and P7 (292 bp fragment amplification, Fig. 2).

Genotyping of Cre mice was carried out in accordance with the Jackson Laboratory recommendations using primers P8, P9, P10, and P11.

Copy number determination

Transgene copy number was measured by real-time PCR as described earlier (Bruter et al. 2021). We used primers P12 and P13 to measure *Polg* transgene copy number.

Ethical approval

All the animal experiments were approved and controlled by the IGB RAS Bioethics Commission (the conclusion of the Bioethical Commission dated 30/04/2021). The animal experiments neither posed any biological hazards, nor required any biosafety facility.

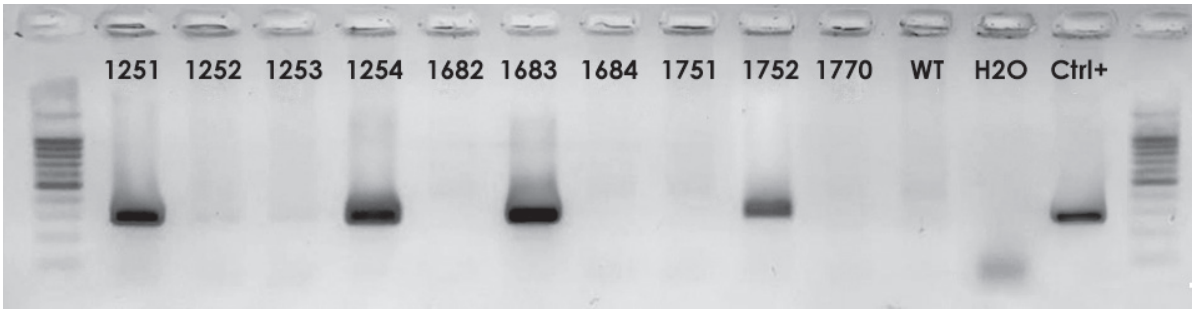

Figure 2. Polg F0 mice genotyping. Samples by numbers #1251, #1254, #1683 and #1752 are transgenes.

Results

Primary transgenic mice creation with mutant *Polg* gene inducible expression

In the process of creating the model, 1081 microinjected embryos were obtained; they were transplanted to 104 recipients. As a result, 29 mice were born from 17 recipients, of which 4 turned out to be the primary *Polg* transgenes. These were mice #1251♂, #1254♂, #1683♂, #1752♂. The results of genotyping are shown in Fig. 2.

Transgene copy number was determined for all primary transgenes. Primary transgenic mice often have a mosaic pattern of transgene construct insertion into the genome. The results of the copy number measurement are presented in Table 2. For mouse #1251, the copy number was 0.35 transgene copies per genome, for mouse #1752 – 0.1 transgene copies per genome, for mouse #1254 – 0.30 transgene copies per genome, and for mouse #1683, the copy number was ~7 transgene copies per genome.

Table 2. Polg transgene copy number in the strains of genetically modified mice

Line	Mouse number	Generation	Copy number
Polg	1251	F0	0.35
Polg	1254	F0	0.30
Polg	1683	F0	7.37
Polg	1752	F0	0.1
Polg	5479	F1	6.71
Polg	6981	F1	5.44
Polg	7513	F2	6.90
Polg	7515	F2	7.36
Polg	7538	F2	6.39
Polg	7539	F2	6.46
Polg*Tie2	893	F3	6.59
Polg*Tie2	898	F3	6.34
Polg*Tie2	903	F3	5.39
Polg*Tie2	904	F3	5.29
Polg*Tie2	905	F3	5.66
Polg*CMV	889	F3	1.77
Polg*CMV	1503	F3	1.61
Polg*CMV	8429	F2	1.20

Obtaining two independent animal lines with inducible expression of the mutant *Polg* gene

All primary transgenes were put into crosses to obtain F1 generation of transgenic animals. Mice #1251 and #1752 died, leaving no offspring. One F1 transgenic mouse (of 90 offspring) 5479♂, with a ~7 copy number, was obtained from transgenic mouse #1254 (Table 2). Transgenic animal #1683♂ with the highest copy number turned out to be viable, but not capable of independent reproduction. So, we performed a surgical collection of sperm from the epididymis and used the obtained material for IVF (the method is described above) to obtain F1 offspring. Two-blastomeric embryos resulting from IVF were transferred to the recipients. As a result, one transgenic mouse F1 was obtained with number 6981♀, with a ~5 copy number. Many F2 offspring with a ~7 copy number were obtained from F1 5479♂ (Table 2).

As a result, we obtained two independent lines of mice with a transgene ~7 and ~5 copy number for the inducible expression of the mutant form of *Polg*.

Transgene copies in three *Polg* strain variants

As a result of *Polg* transgenic mice crossing of the strain with mice expressing Cre-recombinase systemically and

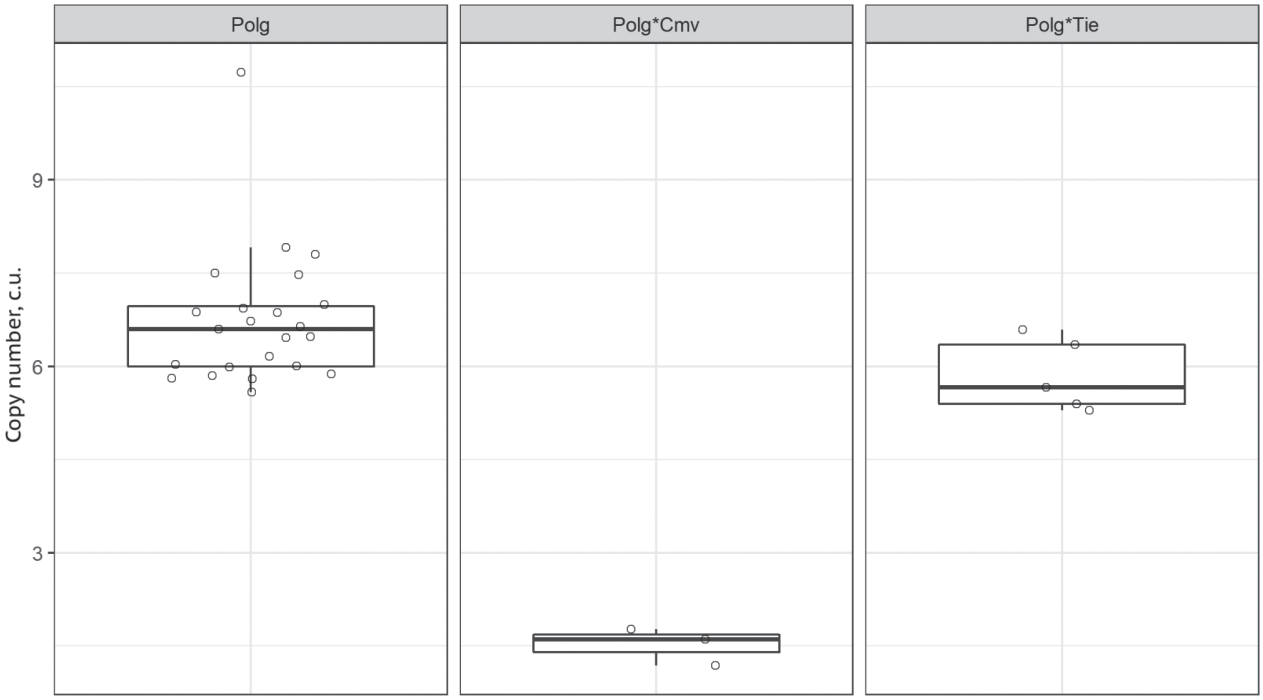

Figure 3. The results of copy number determination in different Polg strains. Transgene copy number of the Polg*CMV is significantly reduced compared to Polg and Polg*Tie2. Copy numbers of Polg and Polg*CMV significantly differ ($p < 0.01$). Copy numbers of Polg and Polg*Tie insignificantly differ ($p \sim 0.05$).

in the vascular endothelium, we obtained double transgenes *Polg**Tie2 and *Polg**CMV. The copy number was determined in all samples from double transgenic mice (Table 2, Fig. 3).

From the chart above, with systemic activation, the transgene copy number decreases to ~1, while with endothelial activation, the transgene copy number remains at the same level in the tail tissue. This is the expected result, since systemic activation presupposes widespread recombination of the STOP-cassette from the entire genomic DNA of the organism, leaving only one copy of the transgene per genome. This allows us to evaluate the efficiency of the Cre-LoxP system. In transgenic *Polg**Tie2 mice, similar recombination occurs only in vascular endothelial cells.

GFP fluorescence of *Polg**CMV pup

We detected GFP fluorescence in transgenic *Polg**CMV mouse skin via a UV lamp in just one day after birth (Fig. 4). This means that mutant PolG-alpha and GFP coexpression is activated during embryogenesis.

Figure 4. Transgenic pup (left) skin in the UV chamber compared to two non-transgenic siblings (right).

We only see GFP fluorescence in one pup out of three, the other two are not transgenic, which is confirmed by genotyping results.

Discussion

Mutations of several genes involved in the maintenance of vital functions of mtDNA and in mitochondrial protein synthesis have been described. One of these genes is polymerase-gamma, which replicates and repairs mitochondrial DNA. PolG-alpha is an attractive target for the creation of model animals, since this enzyme is encoded by the nuclear genome, within which it is much easier to make genetic engineering modifications. Moreover, a decrease in PolG-alpha function leads to spontaneous mutations accumulation, resulting in heteroplasmic mutant mtDNA, which represents genuine MD caused by oxidative and nitrosative stress, inflammation, xenobiotics exposure, etc. (Payne and Chinnery 2015).

Trifunovic et al. (2004) carried out an experiment to create mice with a mutant form of this enzyme, namely, they deprived it of its 3′-5′-exonuclease activity. Thus, these mice did not repair the errors created by PolG-alpha in the mitochondrial DNA of all mouse organism cells. That study showed that an increase in mtDNA mutations in somatic cells is associated with a decrease in life expectancy and premature onset of aging. In the mice of this strain, the following was observed: weight loss, decreased subcutaneous fat content, hair loss, kyphosis, osteoporosis, anemia, decreased fertility, and enlarged heart (Trifunovic et al. 2004). This phenotype negatively affects the viability of transgenes and significantly complicates the maintenance of the animal strain. Our model is free from these drawbacks since it assumes inducible and tissue-specific expression of the mutant form of the protein.

To obtain a universal and viable model, we used a genetic construct based on the Cre-LoxP system, due to which the inducible tissue-specific expression of the mutant *Polg* gene is possible. Due to the presence of a STOP-cassette, the expression of the protein mutant form and the subsequent phenotypic manifestation are possible only after crossing the obtained model animals with mice with tissue-specific Cre-recombinase activation (Bruter et al. 2021).

For the first time, we were able to develop a model of tissue-specific rather than systemic mitochondrial dysfunction. We believe that mutant PolG-alpha tissue-specific expression will lead to an increase in the number of mitochondrial genome mutations in certain tissues (depending on the activator used), for example, in the vascular endothelium. These animals can be further used in basic research and preclinical trials of mitoprotective drugs.

Separately, it is worth mentioning that our murine model with spatial control of mutant *Polg* expression has been developed. This means that we can obtain mice with different degrees of heteroplasmy, and at the same time mitochondrial dysfunction. An increase in a degree of heteroplasmy is achieved by crossing *Polg* females of the same line in each generation with males of any lines. Thanks to this, we can assess the effect of a degree of heteroplasmy on the phenotype and assess a phenotypic effect of mitotherapeutic drugs.

This strain will make it possible to assess an increased mutational load effect of the mitochondrial genome of a certain tissue on the vital activity of the whole organism. The model of mitochondrial dysfunction proposed by us is more viable and adequate for studying this pathology in specific tissues. It provides wide opportunities for the study of mitochondrial dysfunction in neurons, immune, muscle, endothelial cells, which is of considerable scientific interest. In this work, we did not confirm the mitochondrial dysfunction phenotype in transgenic mice, which is the subject of further research.

Conclusion

Here we describe the creation of the first mouse strain with conditional expression of the mutant polymerase-gamma. As a result of microinjection and embryo transfer of genetically modified embryos, primary transgenic animals were born and bred. After breeding with Cre activators, the transgene expression was confirmed by GFP fluorescence and a decrease in the copy number of the mutant *Polg* transgene. Thus, transgenic mice Cre-dependently expressing mutant polymerase-gamma is a novel test-sys-

tem for studying mitochondrial biology and efficacy of mitoprotective drugs.

Conflict of interest

The authors have declared that no competing interests exist.

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