Generation of a Transgenic Mouse Model for Investigating Mitochondria in Sperm

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Abstract

Mitochondria play an important role in the development of sperm, yet the mechanisms regulating their function in sperm remain poorly understood. The development of a method to regulate the expression of a target gene in the mitochondria of the sperm is an essential step in this line of research. In this study, we attempted to generate a system to express the transgene in the mitochondria of the sperm. As a proof of concept, we generated the transgenic mice expressing green fluorescent protein (GFP) fused with a mitochondrial localization signal (MLS) driven by PGK2 promoter which expresses the transgene in the sperm. Although the PGK2 promoter has been previously demonstrated to drive gene expression in spermatocytes and spermatids, the novelty of our method is the combination of the PGK2-driven MLS-GFP expression to study the mitochondria in vivo. We established two founder lines of transgenic mice by pronuclear microinjection, and MLS-GFP expression was confirmed in the mitochondria of the sperm cells by fluorescence microscopy and flow cytometry. Therefore, we provide a novel platform for studying mitochondrial function in sperm where the GFP can be replaced with other genes of interest to study their effect on mitochondria. These results may also help in understanding mitochondrial inheritance, and mitochondrial dysfunction in vivo.

Introduction

Mitochondria play a crucial role in the metabolism of spermatozoa, and they are implicated in energy production, cell signaling, as well as apoptotic pathways, all of which are necessary for the proper function of sperm (Aitken et al., 2001; Aitken et al., 2012; Amaral et al., 2013; Boguenet et al., 2021). The mitochondria in spermatozoa regulates important functions relating to human fertility and infertility (Spinelli et al., 2018; Fu et al., 2021; Tesarik et al., 2023). The genetic modification of mitochondria and its role in sperm development and in spermatogenesis has gained significant attention in recent years. The understanding of the function of mitochondria in sperm cells may provide critical insights into the molecular basis of male infertility (Park et al., 2021; Zini et al., 1993; Freitas et al., 2017). Recent reports suggested that male infertility is associated with a loss of mitochondrial proteins in spermatozoa, which induces low sperm motility, reduces OXPHOS activity, and results in male infertility (Park et al., 2021).

The dynamics of mitochondria in sperm, including their distribution, fusion, and fission, are important for sperm function and viability. During the development of sperm, mitochondria undergo significant structural and functional changes. The role of mitochondrial fusion and fission in sperm motility is increasingly understood, as proper mitochondrial dynamics are necessary for the motility and structural integrity of the sperm tail (Green et al., 2022; Raad M et al., 2024). Therefore, any defect in these processes leads to sperm dysfunction and infertility. However, studying the mitochondria in sperm has been challenging due to the difficulty of tracking these dynamics in live sperm cells.

The PGK2 promoter has been used as a testis-specific promoter for studying spermatogenesis (Zhang et al., 1999). The PGK2 promoter drives expression of transgene in spermatocytes and spermatids in transgenic models to study gene function during sperm development (Kramer et al., 1981; Danshina et al., 2010; McCarrey et al., 1992; Yoshioka et al., 2007). Green fluorescent protein (GFP) is often used as a marker to visualize the expression and localization of specific proteins in live cells. Moreover, the combination of GFP with a mitochondrial localization signal (MLS) allows for study of mitochondria within sperm (Otera et al., 2013; Wakai et al., 2014; Sandoval et al., 2014; Bader et al., 2020). This strategy will help to visualize the mitochondria of the sperm in vivo.

Although PGK2 has been used in a previous study related to spermatogenesis, it isnot used for studying mitochondria of the sperm. In this study, we used the PGK2 promoter to regulate the expression of GFP-tagged to a mitochondrial localization signal (PGK2-MLS-GFP) in transgenic mice.

Material and methods

Animals. Mice (FVB/J), were obtained from the small animal facility of National Institute of Immunology, were used for the present study. All animals were kept at 24 ± 2°C under 14 h light and 10 h dark cycle and used as per the National Guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA). Protocols for the experiments were approved by the Institutional Animal Ethics Committee. Animals were kept in a hygienic air condition with suitable humidity and handled by trained personnel. The animals were killed individually in a separate room by

86 carbon dioxide inhalation in an in-house built carbon-dioxide device and by cervical
87 dislocation (Russell and Burch;1959).

88 **Transgene constructs.**

89 A total of three constructs were used in this study, and the schematic diagram were
90 provided in **Fig 1A**. The $EGFPN_2$ vector was used, and in this vector, mitochondrial
91 localization signal of *COXVIII* gene
92 (TCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTCGGCCCGGCGGCTC
93 CCAGTGCCGCGCGCCAAGA) (MLS) was added just before the coding sequence of
94 *EGFP* transgene. The CMV promoter from this vector was later replaced by PGK2
95 promoter of mice (1kb upstream region from the start codon of PGK2). The PGK2
96 promoter, a testis-specific promoter, was used to drive the expression of a green
97 fluorescent protein (GFP) tagged to a mitochondrial localization signal (MLS). Cloning was
98 performed using the standard method as described previously (Pradhan et al., 2016). The
99 sequences of the construct were provided in the Supplementary data.

100 **Validation of the mitochondrial localization signal**

101 HEK293 cells were transfected with the CMV-GFP-MLS plasmid using Lipofectamine
102 2000 (Invitrogen) following the manufacturer's recommendation. After the transfection,
103 cells were incubated for 24 hours and then analyzed for GFP expression and
104 mitochondrial localization as previously described (Pradhan et al, 2016). The
105 mitochondrial-specific dye, MitoTracker Red, was used to stain the mitochondria, and the
106 colocalization was evaluated for the GFP signal to determine the mitochondrial targeting
107 of the MLS.

108 **Pronuclear microinjection of transgene cassette and oviductal embryo transfer.**
109 This method was performed as described previously by us (Sarkar et al, 2020). Transgenic
110 mice were generated via pronuclear microinjection (Gordon et al., 1980; Costantini et al.,
111 1981; Auerbach et al., 2004). For pronuclear microinjection, female mice were super-
112 ovulated using PMSG and hCG followed by cohabitation with male mice. Oviducts were
113 collected from donor mice (euthanized by cervical dislocation) and placed into Brinster's
114 Modified Oocyte Culture Media (BMOC) containing hyaluronidase (1 mg/mL). Embryos

115 were collected and transferred to the 60 mm dish contain 100 µL drops of BMOC overlaid
116 with mineral oil. Linearized transgene cassette (PGK2-MLS-EGFP) was prepared at a
117 final concentration of 4ng/µl for microinjection. DNA was microinjected into the male
118 pronucleus of fertilized eggs using Narishighe micromanipulator. Each manipulated
119 embryo was then transferred into the pre-incubated BMOC containing dish and
120 maintained at 37°C in a 5% CO_2 incubator. Embryos were incubated till 2-cell stage.
121 Pseudo-pregnant female mice were generated by mating with vasectomized male mice.
122 2-cell stage embryos were transferred into the ampulla of the oviduct of pseudo-pregnant
123 recipient mice. Approximately 20 microinjected embryos were transferred in the oviductal
124 ampulla. After the gestation period (21 ± 2 days), recipient pups were born. Pups born
125 were analyzed for transgene integration by PCR.

126 **Establishment of Transgenic Lines**

127 Two founder mice expressing PGK2-GFP-MLS were screened through PCR (Pradhan et
128 al., 2016; Usmani et al., 2013). The founder mice were mated with wild-type females to
129 establish the separate transgenic lines, and the transgene integration in the progeny was
130 evaluated by PCR.

131 **Fluorescence Microscopy**

132 Fluorescence microscopy was employed to confirm GFP expression in the mitochondria
133 of sperm cells. Sperm from transgenic mice were isolated by standard methods and
134 immediately imaged using a confocal microscope (Pradhan et al., 2020).

135 **Flow Cytometry Analysis**

136 The sperm were isolated from the cauda epididymis from the wild-type mice and
137 transgenic mice. Samples were analyzed by a flow cytometer (FACS caliber, BD
138 Biosciences). Data were collected and analyzed using FlowJo software (TreeStar, Inc.) to
139 assess the percentage of GFP-positive sperm (Pradhan et al., 2016).

140 **Results and Discussion**

141 **Validation of MLS Functionality in HEK Cells**

142 The MLS sequences for mitochondrial targeting were used previously (Wakai et al., 2014;
143 Bader et al., 2020*).* The mitochondrial targeting ability of the MLS of *COXVIII* gene was
144 validated by expressing the CMV-MLS-GFP construct in HEK 293 cells. We observed a
145 colocalization of GFP with mitochondrial markers suggesting that the MLS was functional
146 in the mammalian system (**Fig 1B**). As a control, we used the CMV-GFP construct without
147 MLS.

148 **Generation of Transgenic Mice**

149 To determine the in vivo implications of MLS sequence, we generated two transgenic
150 mouse lines using pronuclear microinjection. For this we used the PGK2-MLS-GFP
151 construct. The tissue-specific activity of the PGK2 promoter was previously demonstrated
152 to drive post-meiotic gene expression in the male germline (Zhang et al., 1999). We are
153 providing a diagram of the generation of the transgenic line (**Fig 2**). The transgene
154 integration was confirmed by PCR (**Fig 3**).

155 **Localization of GFP in the mitochondria of sperm**

156 We performed the fluorescence microscopy to determine the GFP fluorescence in the
157 sperm. We observed that GFP signal was localized to the midpiece of sperm, where
158 mitochondria are concentrated (**Fig 4**). Previous reports suggested that GFP expression
159 in the mitochondria of the sperm was nontoxic (Wang *et al., 2023*). Our study was in
160 agreement with the previous findings demonstrating the functional importance of
161 mitochondrial localization sequences in directing proteins to mitochondria (Wakai et al.,
162 2014; Bader et al., 2020). Mitochondria in the midpieces of the sperm are critical for
163 energy production and the motility and previous studies suggest a link between
164 mitochondrial dysfunction and male infertility (Park et al., 2021).

165 **Analysis of GFP Expression by Flow Cytometry (FACS)**

166 We performed the FACS analysis of sperm samples to validate the microscopy data. We
167 observed that GFP-positive sperm in various transgenic mice, suggesting that the
168 efficiency of the PGK2 promoter in directing mitochondrial GFP expression in vivo (**Fig 5**).
169 Our data also validated the microscopy data suggesting that the method is reproducible.
170 This study may help in studies of mitochondrial health and dynamics in large populations

171 of sperm cells (Park et al., 2021). Our study provides a platform to investigate
172 mitochondrial biology during spermatogenesis. The specificity of the PGK2 promoter
173 ensures minimal ectopic expression, which is critical for accurate functional analyses
174 (Zhang et al., 1999). Such advancements address limitations of ubiquitous promoters in
175 transgenic models. This work is a significant advancement for studying mitochondrial
176 dynamics in sperm cells. Mitochondria are vital for sperm function, playing key roles in
177 ATP production and motility, and defects in mitochondrial function are a major contributor
178 to male infertility (Tesarik et al., 2023).

179 The integration of a tissue-specific promoter with an MLS sequence enables precise
180 visualization of mitochondrial architecture and bioenergetics in vivo. This approach
181 addresses a critical gap in male reproductive biology, providing insights into mitochondrial
182 dynamics in sperm development, motility, and fertilization (Matilainen et al., 2017; Tesarik
183 et al., 2023). This approach can be used to study the effect of various mutations caused
184 by environmental stress on mitochondrial function, mitochondrial dynamics using
185 advanced imaging techniques during spermatogenesis and the role of mitochondria in
186 assisted reproduction technologies. These findings may have broad implications for
187 mitochondrial research, particularly in male infertility and sperm development.

188 **Data availability**

189 All the data were provided in the manuscript, and the reagents used in the manuscript
190 will be made available upon considerable request.

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292 **Conflicts of Interest:** The authors declare no conflict of interest.

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303 **Author Contributions**

304 Experiments were conceived and designed by B.S.P., H.S., and S.S.M. Experiments
305 were performed by H.S., S.B., N.W. and B.SP. Data of the manuscript was analyzed by
306 B.S.P, H.S. Manuscript was written and reviewed by B.S.P. and H.S. All authors have
307 read and agreed to the published version of the manuscript.

308 **Figure Legend**

309 **Figure 1**. Validation of MLS sequence in Hek293 cells. (A) A schematic diagram of the
310 constructs used in this study. (B) Hek 293 cells were transfected with CMV-GFP control
311 and CMV-MLS-GFP; and costained with mitotracker red dye. The images were analyzed
312 24h after transfection. Scale bar: 10μm.

313 **Figure 2**. A schematic diagram of generation of PGK2-MLS-GFP transgenic mice after
314 embryo transfer.

315 **Figure 3**. Generation of PGK2-MLS-GFP transgenic mice. Detection of transgene by
316 PCR. PCR screening of progeny generated through pronuclear microinjection. A band
317 size of 258 bp represented the transgene positive mice. b = blank containing only PCR
318 mastermix, numerical indicated the progeny numbers in the gel. Ppia was used as a
319 loading control.

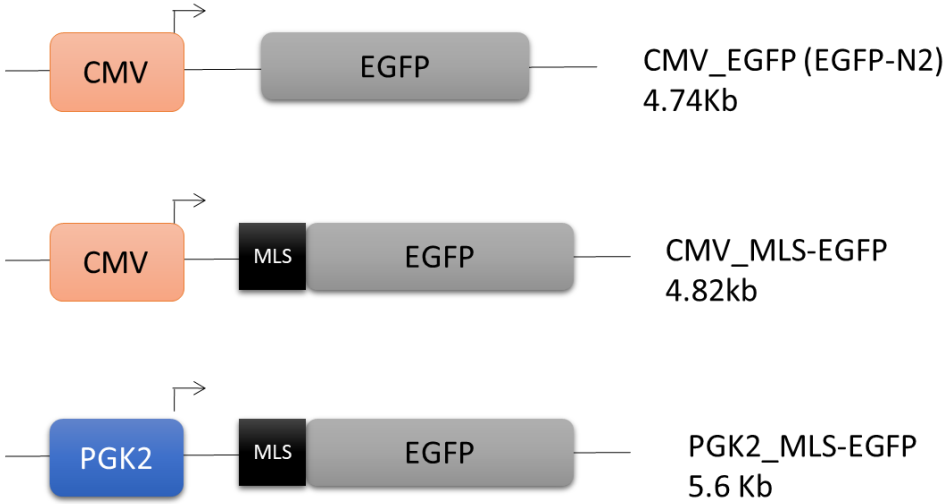
320 **Figure 4**. Detection of EGFP-positive sperm in the epididymis of the transgenic mice by
321 microscopy. The EGFP positive sperm was detected by microscopy in the live sperm of
322 the wild-type mice (A) and transgenic mice (B). Scale bar: 20μm.

323 **Figure 5.** Detection of EGFP-positive sperm in epididymis of the transgenic mice by flow
324 cytometry. (A) Scatter plot of one of the representative sperm samples and the
325 corresponding histogram of wild-type mice. (B) Scatter plot of one of the representative
326 sperm samples and the corresponding histogram of transgenic mice.

327

Figure 1

(A)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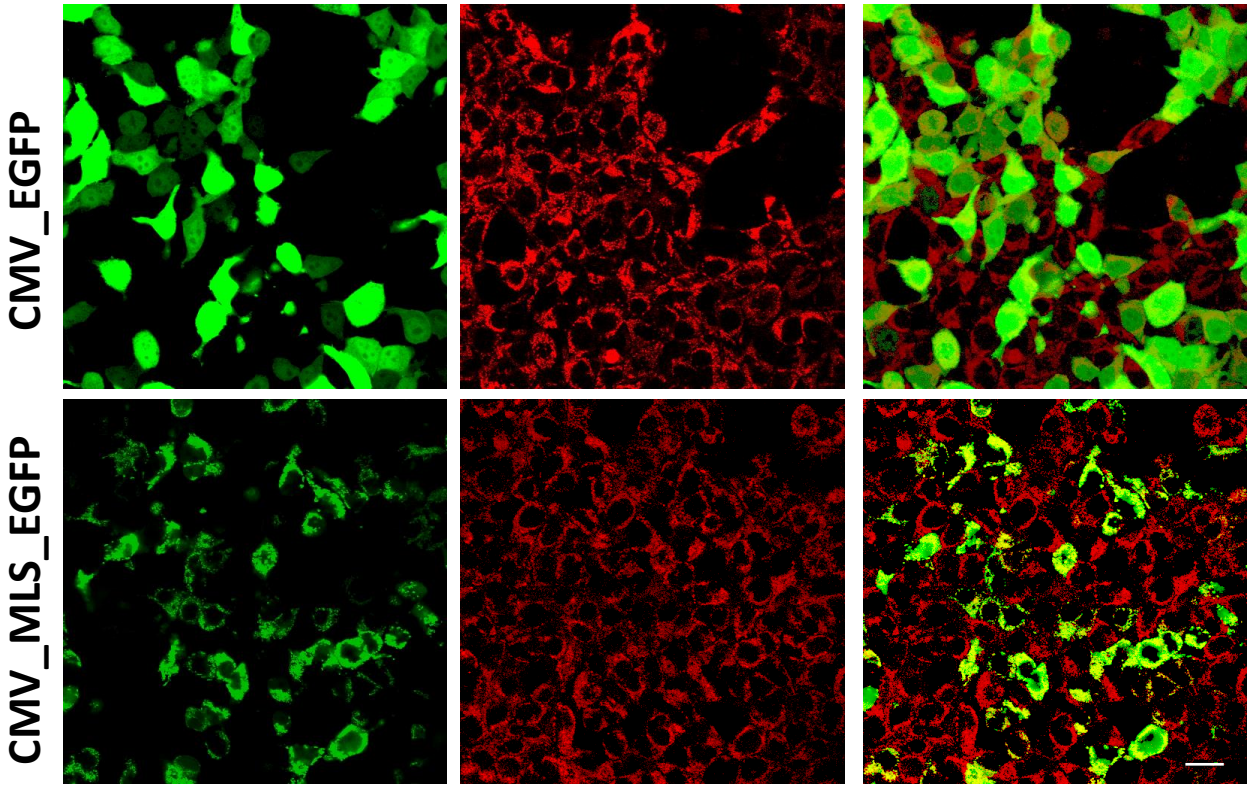
EGFP
CMV_EGFP (EGFP-N2)
4.74Kb
CMV
MLS
EGFP
CMV_MLS-EGFP
4.82kb
PGK2
MLS
EGFP
PGK2_MLS-EGFP
5.6 Kb

(B)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CMV_MLS_EGFP

Figure 2

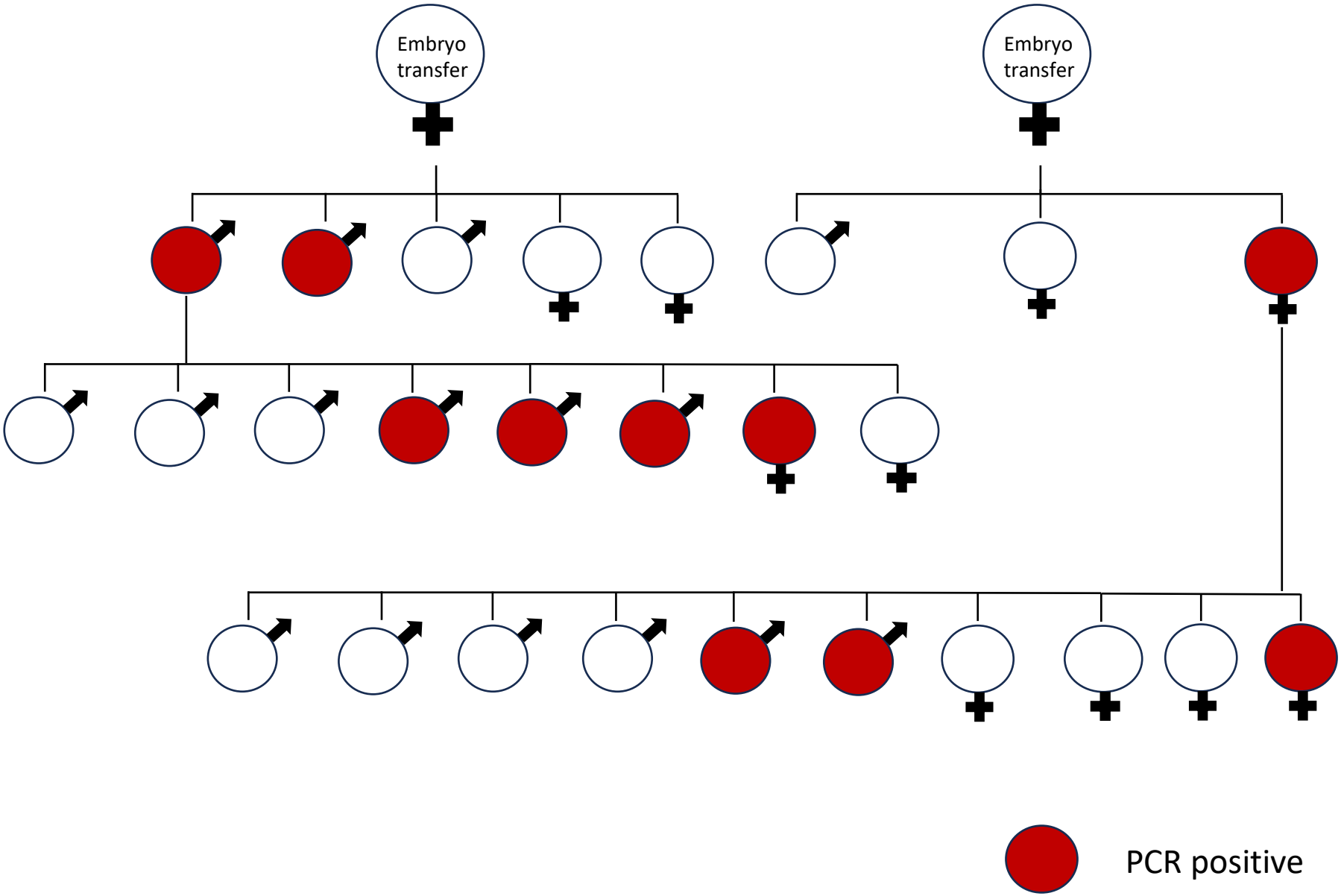
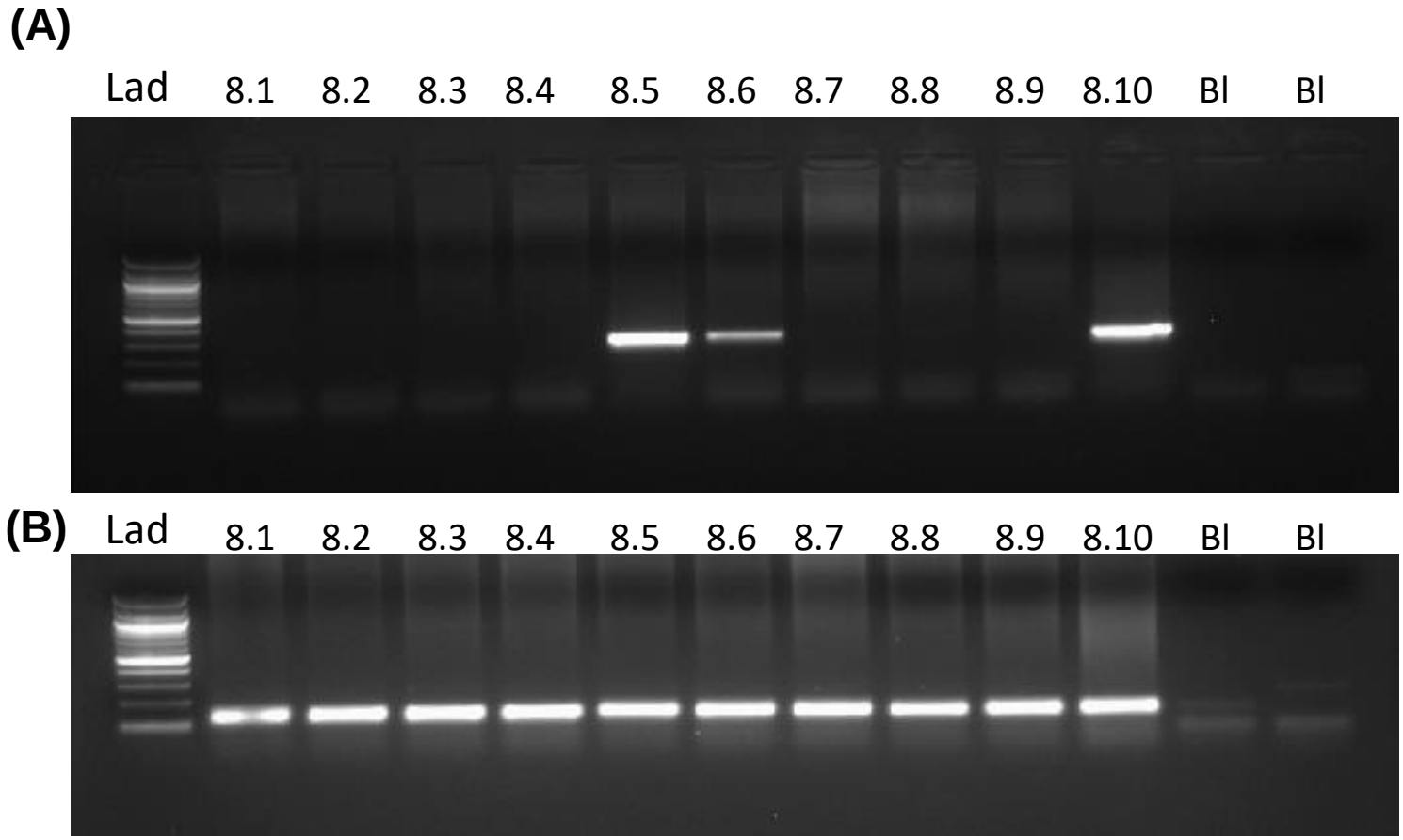


Figure 3


(A)
Lad
8.1
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(B)
Lad
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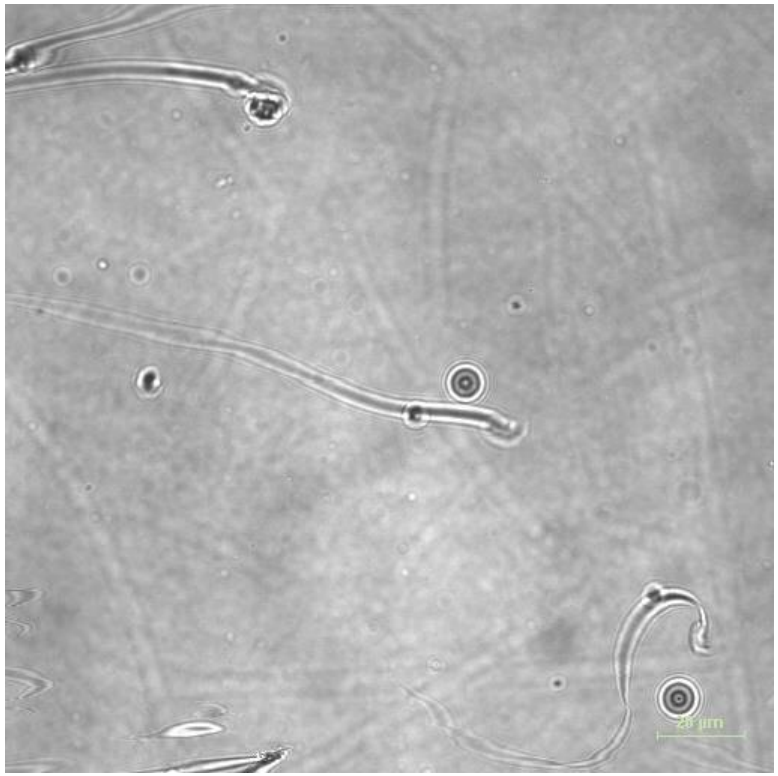
Figure 4

Control



PGK2-MLS-GFP mice

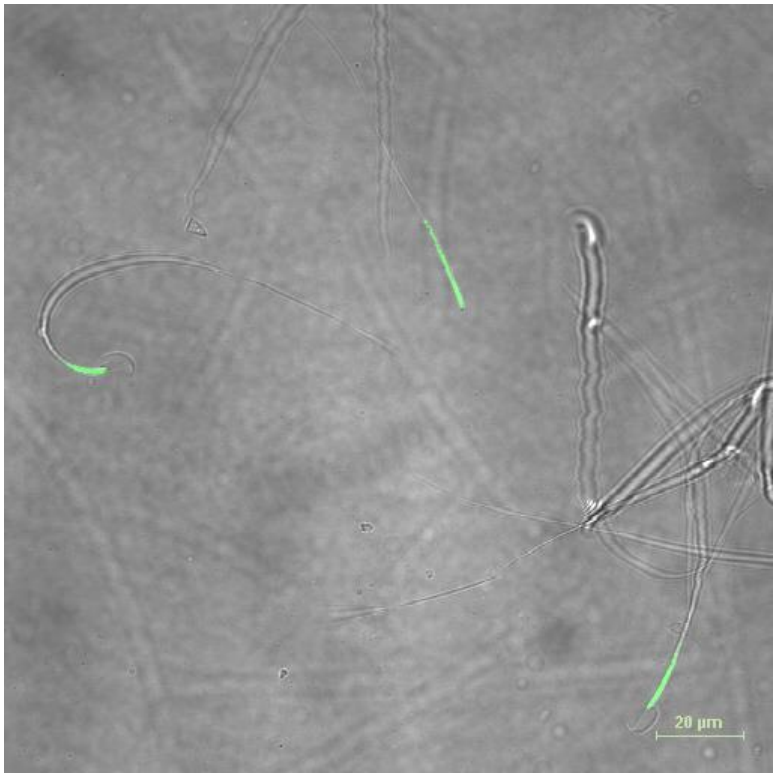
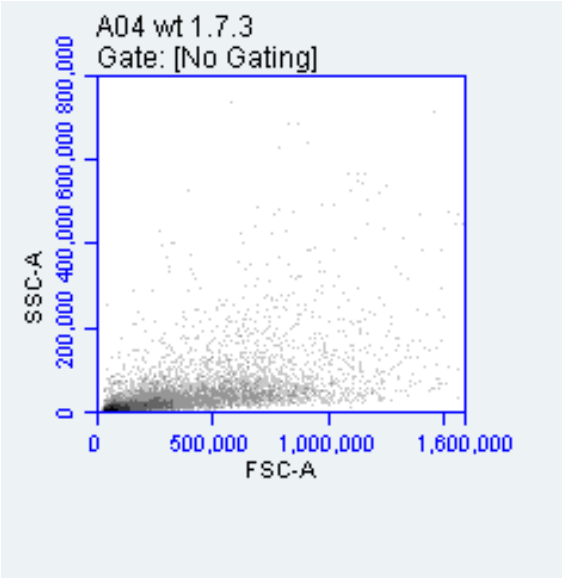
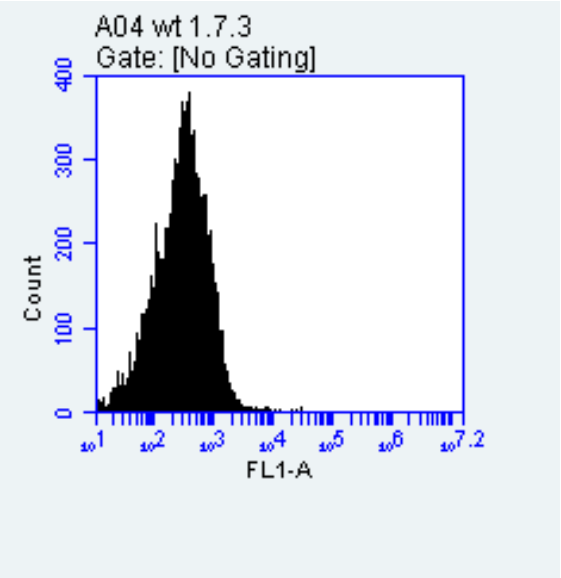
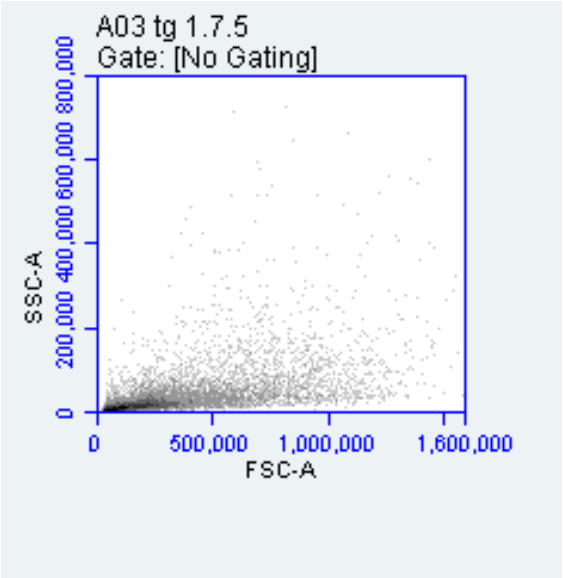
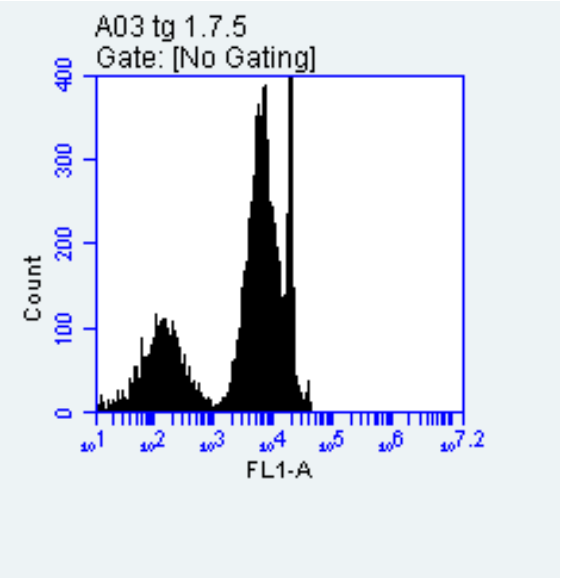


Figure 5

Control





PGK2-MLS-GFP mice