A simple method for gene expression in endo- and ectodermal cells in mouse embryos before neural tube closure

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Abstract

The lack of a widely accessible method for expressing genes of interest in wild-type embryos is the fundamental obstacle in understanding genetic regulation during embryonic development. In particular, only a few methods are available for introducing gene expression vectors into cells prior to neural tube closure, a period for the drastic development of many tissues. In this study, we present a simple technique for transferring vectors into the endo- and ectodermal cells of mouse embryos at E7.0 or E8.0 via in utero injection, without any specialized equipment. Using this technique, introduction of retroviruses can facilitate the labeling of cells in various tissues, including the brain, spinal cord, epidermis, and digestive and respiratory organs. As such, this technique can aid in analyzing the roles of genes of interest during endo- and ectodermal development prior to neural tube closure.

Keywords: Development, gene transfer method, viral vector, electroporation

Introduction

To understand the molecular mechanisms underlying mammalian development, it is necessary to manipulate the expression of the genes of interest in mouse embryos. The most conventional method for such manipulation involves generating gene-targeted knock-in or knock-out mice and crossing them with specific Cre- or CreERT2-expressing mouse strains [1–3]. Although the advent of genome editing tools, such as the transcription activator-like effector nuclease and clustered regularly interspaced palindromic repeat (CRISPR)/CRISPR-associated protein 9 systems [4–7], has made it easier to develop transgenic mice, these tools are expensive, labor-intensive, and inaccessible to many biologists owing to the need for specialized equipment, such as micromanipulators or electroporators.

An alternative approach for gene manipulation is the direct delivery of vectors into wild-type embryos [8]. In the central nervous system, in utero injection of a DNA plasmid, followed by electroporation or introduction of a viral vector into the ventricle is a widely utilized method for embryos after embryonic day 10.5 (E10.5) [9–11]. In utero injections of viral vectors were also applied to the epithelium and nervous system after E9.5 [12–15]. For analyzing the role of genes of interest at E7 or E8, a period of rapid organ development, ultrasound-guided method is useful to introduce viral vectors into embryos. Although previous methods for E8.5 embryos resulted in low survival rates and abnormalities in many surviving embryos[16], a recent method for E7.5 embryos, called NEPTUNE, greatly improved the survival rate of embryos [17]. As the neural tube is still open at E7.5, a viral vector injected into the amniotic fluid can access the neuroepithelial cells in addition to the epithelial and endodermal tissues[17]. The high efficiency of gene transduction achieved by the NEPTUNE-mediated knockdown of *Olig2* or *Sptbn2* resulted in phenotypes similar to those of their knockout embryo counterparts [17]. However, this approach requires specialized and expensive equipment as well as an ultrasound microscopy system.

In this study, we developed a method to introduce retroviruses into the amniotic fluid at E7.0 and E8.0 for gene manipulation during organ morphogenesis without an ultrasound microscopy system. Most embryos survived and exhibited normal development. We confirmed ectopic gene expression in the endodermal and ectodermal tissues, similar to that observed with the NEPTUNE system. In addition, we demonstrated the possibility of introducing plasmids via electroporation, although this requires further

investigation. Therefore, our method is useful for analyzing the functions of genes of interest and beneficial to the broader scientific community.

Results

Development of a simple and efficient method to introduce a retrovirus in mouse embryos prior to neural tube closure

We hypothesized that it would be feasible to introduce a reagent into amniotic fluid without using an ultrasound microscopy system by regulating the position of the capillary tip in the uterus. To this end, we employed optical fibers to visualize the inner structure of the uterus, including the amnion and embryo (Figure 1a–c). Optical fiber-based visualization of the ventricles of embryos from E9.5, has been previously described for targeted DNA injection and electroporation following neural tube closure (Figure 1c)[18]. We found that lighting with this system was adequate to enable the injection of the dye inside the amnion when positioning the tip of the capillary at the center of the uterus (see Methods), although it was not possible to directly visualize the amnion within the uterus. To precisely control the developmental stage of embryos, mice were mated for only 2 h, and females with a confirmed vaginal plug were used for in utero injection. We first injected carboxyfluorescein succinimidyl ester (CFSE), a cell-permeable dye that becomes fluorescent following its reaction with intracellular proteins[19,20], into the uterus at E8.0. To minimize the potential damage caused by the injection, we inserted a sharpened capillary at the center of the uterus at an angle perpendicular to the uterine wall while it was positioned (Figure 1d). We injected a small volume (approximately 0.5 μL) of CFSE solution at a depth of 1–2 mm below the uterine wall, which resulted in reproducible labeling of the embryos. The duration of surgery is relatively short (approximately 20 min) because of the simplicity of the method, which may contribute to minimal damage to the embryos. After three days (at E11.0), fluorescence microscopy without sectioning revealed that 77% of the injected embryos were labeled with CFSE (Figure 1e–f). Approximately 80% of the embryos survived without gross developmental malformations at E11.0. We also injected CFSE into the amniotic fluid at E7.0, which yielded a high rate of embryo labeling (64%) and a high rate of survival without gross developmental malformations (89%) at E9.0 (Figure 1e–f).

Subsequently, we used retroviral vectors for gene introduction using this method. We initially injected a concentrated suspension of a retrovirus encoding green fluorescent protein (GFP) under the control of the phosphoglycerate kinase (*PGK*) promoter, which is the same promoter utilized in the NEPTUNE system, into the uterus

102 at E8.0. Our results revealed that 89% of embryos survived without gross abnormalities,
103 and 74% of embryos expressed GFP at E11.0 (Figure 2a and c). Injection of the retrovirus
104 at E7.0, also induced GFP expression at E11.0, although the labeling rate was lower
105 (Figure 2b–c). Flow cytometric analysis of the cortical tissue obtained from E12.0
106 embryos that had been injected with the retrovirus encoding GFP at E8.0 showed that
107 approximately 70% of the cortical cells were positive for GFP in successful cases (Figure
108 2d). Immunohistochemical analysis of sagittal sections at E10.0 embryos that had been
109 injected with the retrovirus encoding GFP at E8.0 revealed that most tissues exposed to
110 amniotic fluid expressed GFP (Figure 2e–g). The labeled tissues included the central
111 nervous system (forebrain, midbrain, hindbrain, and spinal cord) as well as the epidermis
112 and endodermal tissues, such as digestive and respiratory organs. This suggests that our
113 method is suitable for gene introduction into ectodermal and endodermal tissues at the
114 early stages of mouse development.
115
116 **Possible application of plasmid introduction via electroporation**
117 Given that in utero electroporation of plasmids is generally more efficient and results in
118 higher expression levels than retroviral infection as a method of gene introduction, we
119 next employed our new method to inject a plasmid encoding mCherry and performed in
120 utero electroporation using electrodes (disc size, 2.5 by 4 mm) positioned on each side of
121 the uterus at E7.0 or E8.0. The proportions of mCherry-expressing embryos at E9.0 and
122 E10.0 were 19% and 40% among those electroporated at E7.0, and E8.0, respectively
123 (Figure 3a and b). The expression pattern of mCherry varied among embryos, likely
124 because of differences in the orientation of the electrode relative to the embryo during
125 electroporation. Most embryos expressed mCherry in the somites (Figure 3a), whereas
126 only one of them expressed mCherry in the midbrain and hindbrain (Figure 3c). To date,
127 no embryos have expressed mCherry in the forebrain. These results demonstrate that it is
128 possible to perform gene introduction by in utero electroporation in mouse embryos at
129 E7.0 or E8.0 using our method, although further improvements are necessary to allow the
130 targeting of a specific tissue.
131

Discussion

Manipulation of gene expression is necessary for the detailed investigation of molecular mechanisms underlying embryonic development. However, in the central nervous system, existing methods, such as the injection of viruses or plasmids for in utero electroporation into the ventricles, can only be applied after neural tube closure [9–11]. The gene introduction method developed in the present study has several advantages over other methods that can be used before neural tube closure, such as whole-embryo genomic manipulation (gene targeting or transgene introduction) and ultrasound-guided injection of viruses into the mouse nervous system. Genomic manipulation allows the precise expression of a gene of interest in a temporally and spatially regulated manner; however, it requires a specific Cre-expressing mouse strain or knowledge of specific distal elements to control expression, and a significant investment of time, cost, and space to establish and maintain a transgenic mouse strain. The NEPTUNE method, which uses the ultrasound-guided injection of viruses, addresses many weaknesses of genomic manipulation and allows for the analysis of a gene of interest in less time, space, and animals [17]. However, it still requires specific equipment, such as ultrasound microscopy.

Our present method, which is based on the same principle as the NEPTUNE method, introduces a chemical and virus into embryos by injection into the amniotic fluid before neural tube closure; however, it is possible to introduce it without ultrasound microscopy. We also propose the possibility of introducing plasmids by electroporation, although further improvements, such as electroporation at multiple electrode orientations to a single embryo, are necessary. The gene transfection efficiency of the retrovirus using our method was lower than that using the NEPTUNE method (approximately 70% in our best case and >99% in NEPTUNE) [17]. This discrepancy may be attributed to the relatively imprecise targeting of the injection site in our method, which did not utilize ultrasound guidance as well as differences in the types and preparation methods of the viruses used. While Mangold et al. reported that an injection of more than 0.25 μL of lentivirus solution decreased the survival rate, our method allows for the injection of approximately 0.5 μL of retrovirus solution[17]. The simplified procedure of our method, with a shorter surgery time (approximately 20 min) and no requirement for pregnancy and stage verification due to the limited mating time, may contribute to a higher survival rate of embryos. However, transfected tissues were comparable between the two methods. As such, our method is a

useful tool for testing and screening various genes for their specific effects on development and will be beneficial to the broader scientific community.

167 **Materials and Methods**

168

169 **Mouse maintenance**

170 All animal studies were conducted in accordance with the protocols approved by the
171 Animal Care and Use Committee of the University of Tokyo. Mice were housed in a
172 temperature- and humidity-controlled environment (23 ± 3 °C and 50 ± 15%,
173 respectively) under a 12-h light/dark cycle. Animals were housed in sterile cages
174 (Innocage, Innovive) containing bedding chips (PALSOFT, Oriental Yeast), with two–six
175 mice per cage, and provided irradiated food (CE-2, CLEA Japan) and filtered water ad
176 libitum.

177

178 **Plasmid construction**

179 cDNA encoding mCherry was amplified via polymerase chain reaction, and its coding
180 sequence was confirmed via DNA sequencing. The cDNA was then subcloned into the
181 pCAGEN vector, kindly provided by C. L. Cepko, to generate the pCAGEN-mCherry
182 plasmid.

183

184 **Retrovirus production and concentration**

185 PLAT-GP cells, a gift from T. Kitamura, were harvested on a 10-cm diameter dish (55
186 cm^2 surface area) at 80–90% confluency in Dulbecco's Modified Eagle Medium (Sigma)
187 containing 10% fetal bovine serum and 1% penicillin–streptomycin (Thermo Fisher
188 Scientific). The cells were transfected with polyethyleneimine (PEI; Polysciences). A
189 mixture of 12 μg of the validated retroviral vectors pSIREN_EGFP-siLuc (Addgene
190 #122287) [21] expressing GFP under the control of *PGK* promoter and 9 μg of a VSVG
191 plasmid, kindly provided by H. Song, were mixed in a 1.5-mL tube with 500 μL of OPTI-
192 MEM (Thermo Fisher Scientific). Then, 63 μL of 1 mg/mL PEI in water in another 1.5-
193 mL tube with 50 μL of OPTI-MEM was shaken vigorously and incubated at room
194 temperature (20–25 °C) for 5 min. The plasmid and PEI solution were mixed, shaken
195 vigorously, and incubated at room temperature for 15 min. The mixture was then added
196 to the PLAT-GP cells.

197 The medium containing the transfection reagents was replaced with 10 mL of
198 fresh medium without penicillin–streptomycin, and the conditioned medium collected 72
199 h after the onset of transfection was cleared of debris via centrifugation at 2,500 × *g* for

200 5 min, passed through a 0.45-μm filter, and centrifuged again at 250,000 × *g* for 90 min
201 at 4 °C. After roughly removing the supernatant via decantation, the pellet was
202 resuspended with the remaining medium, transferred to a new 0.6-mL tube, and
203 centrifuged at 20,000 × *g* for more than 12 h at 4 °C. The supernatant was completely
204 removed via pipetting, and the pellet was resuspended in 10 μL of phosphate-buffered
205 saline. The 1,000-fold concentrated retrovirus was stained with 0.01% FastGreen
206 (Chroma Gesellschaft), rapidly frozen with liquid nitrogen, and stored at –80 °C. The
207 viral titer was not determined.
208
209 **Preparation of pregnant mice and capillary filled with virus, CFSE, or plasmid**
210 JCL:ICR (CLEA Japan) mice were used for in utero injection experiments. To ensure
211 precise control of the embryonic stage, mating was limited to a 2-h period, and the
212 presence of a vaginal plug was used to confirm the onset of gestation (E0.0).
213 Glass capillaries (GD-1; Narishige) were pulled using a PC-100 (Narishige)
214 puller, roughly cut with forceps, and shaped with an EG-45 (Narishige) grinder to achieve
215 a tip diameter of 70–80 μm and an angle of approximately 30°. These capillaries were
216 then backfilled with thawed viral, CFSE, or plasmid solution using a microloader
217 (Eppendorf) and attached to a FemtoJet (Eppendorf) injector. Any remaining air at the tip
218 was eliminated via flushing.
219
220 **In utero injection and electroporation before neural tube closure**
221 Pregnant mice were administered a sodium pentobarbital-based anesthetic, and the
222 uterine horn was exposed and pinched between a flexible fiber-optic cable and a finger [18]
223 (Figure 1c). Lighting from the fiber-optic cable helped in the visualization of the internal
224 uterine structure, but did not enable the identification of the embryo from the amnion.
225 CFSE (1 mM; Sigma), plasmid DNA (1 μg/μL), or retroviruses, each mixed with
226 FastGreen, were injected at an angle perpendicular to the center of the uterus using a
227 FemtoJet (Eppendorf) at 100–150 hPa pressure. An injection depth of 1–2 mm below the
228 uterine wall permitted consistent introduction of capillary contents into the amniotic fluid.
229 After visually confirming the presence of FastGreen at the center of the uterus, the
230 injection was halted, and the capillary was immediately removed. Generally,
231 approximately 0.5 μL of the reagent was injected, although this amount varies based on
232 the embryo. For electroporation, the uterus was held between tweezer-type electrodes

233 (CUY652P2.5x4; Nepa Gene) and electroporated with a NEPA21 system (Nepa Gene),
234 according to the following program: three poring pulses of 30 V with a 15-ms pulse width
235 and 450-ms pulse interval and 10% decay (+ pulse orientation), and two transfer pulses
236 of 10 V with a 50-ms pulse width and 450-ms pulse interval and 10% decay (± pulse
237 orientation). After injection and electroporation, the uterine horn was returned to its
238 original location. Pregnant mice were allowed to recover on a heating plate maintained at
239 38 °C until they regained consciousness following anesthesia. Following the designated
240 period of observation, the embryos were removed and examined using a fluorescence
241 stereo microscope equipped with a 2.0 × objective lens manufactured by Nikon (SMZ18).
242
243 **Flow cytometry**
244 Flow cytometry was performed using a previously established protocol [22]. Specifically,
245 the neocortices of the ICR embryos were dissected and subjected to enzymatic digestion
246 using Neuron Dissociation Solution (FUJIFILM Wako). To quantify the efficiency of
247 gene introduction, cells were analyzed using the FACSAria instrument (Becton
248 Dickinson) via direct flow cytometry.
249
250 **Immunohistofluorescence analysis**
251 Immunofluorescence staining was performed as described previously [23]. A 1:2,000
252 dilution of the anti-GFP antibody (Abcam; ab13970) and a dilution 1:000 of the anti-
253 DsRed antibody (Takara; 632496) for mCherry were used, and the nuclei were
254 counterstained with a 1:10,000 dilution of Hoechst 33342. Images were acquired using a
255 laser-scanning confocal microscope (TSC-SP5; Leica) and analyzed using the ImageJ
256 software (NIH).
257

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Author Contributions

Y.K. designed and supervised the study and wrote the manuscript. Y.G. supervised the study. Y.K., Y.M., and N.K. performed the experiments and analyzed the data.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Figure 1. In utero injection into mouse embryos before neural tube closure. (**a–b**) Exposure of the uterine horn of an anesthetized pregnant mouse at embryonic day 7 (E7.0) or E8.0. (**c–d**) Pinching of the uterus between an optical fiber and finger (**c**) for injection via a sharpened glass capillary of a solution colored with a dye (FastGreen), containing either carboxyfluorescein succinimidyl ester (CFSE), a retrovirus, or a plasmid (**d**). (**e**) Embryos at E7.0 (left) or E8.0 (right) were injected with CFSE, dissected out from the uterus at E9.0 or E11.0, respectively, and fixed for the visualization of CFSE fluorescence with a fluorescence stereo microscope. Scale bars, 0.5 mm (left) or 1 mm (right). (**f**) Survival and labeling rates for the in utero introduction of CFSE into the embryos of three pregnant mice in each condition as described in (**e**).

Figure 2. In utero gene introduction via retroviral infection in embryos before neural tube closure. (**a**) Embryos within the same uterus were administered with a retrovirus encoding green fluorescent protein (GFP) under the control of the phosphoglycerate kinase (*PGK*) promoter at E8.0. The embryos were subsequently dissected from the uterus at E11.0, fixed, and examined for GFP fluorescence using a fluorescence stereo microscope. The posterior half of the control embryo was excised at the yellow line for identification purposes. Arrows indicate the GFP-expressing regions. Scale bar, 1 mm. (**b**) An embryo at E7.0 was administered with a retrovirus as in (**a**) and examined for GFP fluorescence at E11.0. Arrows indicate the GFP-expressing regions. Scale bar, 1 mm. (**c**) Survival and labeling rates for embryos as in (**a**) and (**b**). (**d**) Flow cytometric analysis of the gene introduction efficiency. Neocortical cells dissociated from E12.0 embryos administered the retrovirus for GFP at E8.0 were subjected to flow cytometric analysis of GFP expression. This embryo exhibited the highest rate of GFP-positive cells in our experiments. (**e**) A sagittal section of an E10.0 embryo injected at E8.0 as in (**a**) was subjected to immunohistofluorescence staining with anti-GFP antibodies. Nuclei were counterstained with Hoechst 33342. Representative infected tissues are indicated. Scale bar, 1 mm. (**f–g**) High magnification images of the bronchus (**f**) and spinal cord (**g**) as shown in (**e**). Scale bar, 100 μm.

312 **Figure 3. In utero electroporation into mouse embryos before neural tube closure.**
313 (**a**) Embryos at E7.0 (left) or E8.0 (right) were injected with a plasmid encoding mCherry
314 and subjected to in utero electroporation. They were subsequently harvested from the
315 uterus at E9.0 or E10.0, respectively, fixed, and examined for mCherry fluorescence using
316 a fluorescence stereo microscope. Arrows indicate the mCherry-expressing regions. Scale
317 bars, 0.5 mm (left) or 1 mm (right). (**b**) Survival and labeling rates for the in utero
318 electroporation of embryos as described in (**a**). (**c**) A sagittal section of an E9.0 embryo
319 electroporated at E8.0 as in (**a**) was subjected to immunohistofluorescence staining with
320 anti-mCherry antibodies. Nuclei were counterstained with Hoechst 33342. Arrow and
321 arrowhead indicate the mCherry-expressing regions in third and fourth ventricles,
322 respectively. Scale bar, 1 mm.
323
324

325 **References**

326

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380

Fig. 1

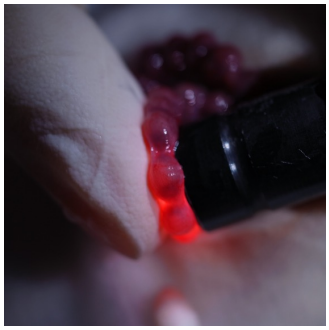
a

Anesthetized pregnant mouse (E7.0 or E8.0)

b

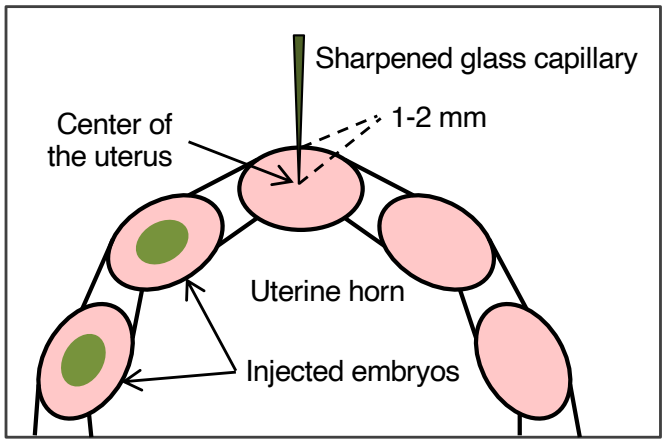
Exposed uterine horn

c



A uterus pinched between an optical fiber and finger

d



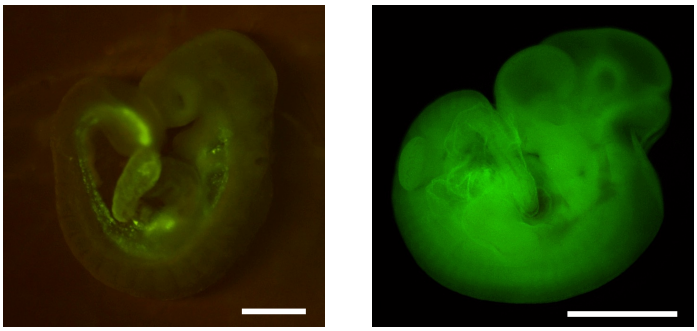
Injection of CFSE, retroviruses, or plasmids

e

E7.0 injection → E9.0

E8.0 injection → E11.0

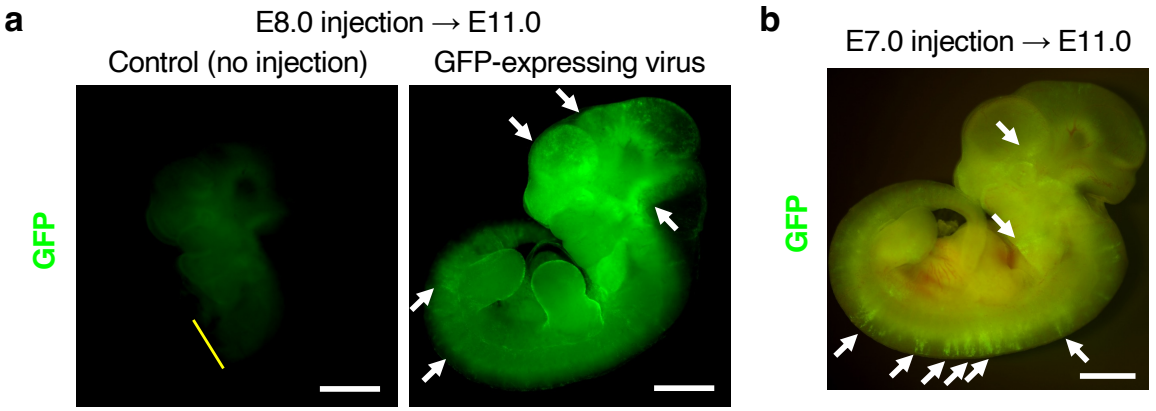
CFSE



f

	Injected embryos	Survived (% of injected)	CFSE-positive (% of injected)
E7.0 injection → E9.0	28	25 (89%)	18 (64%)
E8.0 injection → E10.0	30	24 (80%)	23 (77%)

Fig. 2



c

	Injected embryos	Survived (% of injected)	GFP-positive (% of injected)
E7.0 injection → E11.0	28	27 (96%)	2 (7%)
E8.0 injection → E11.0	19	17 (89%)	14 (74%)

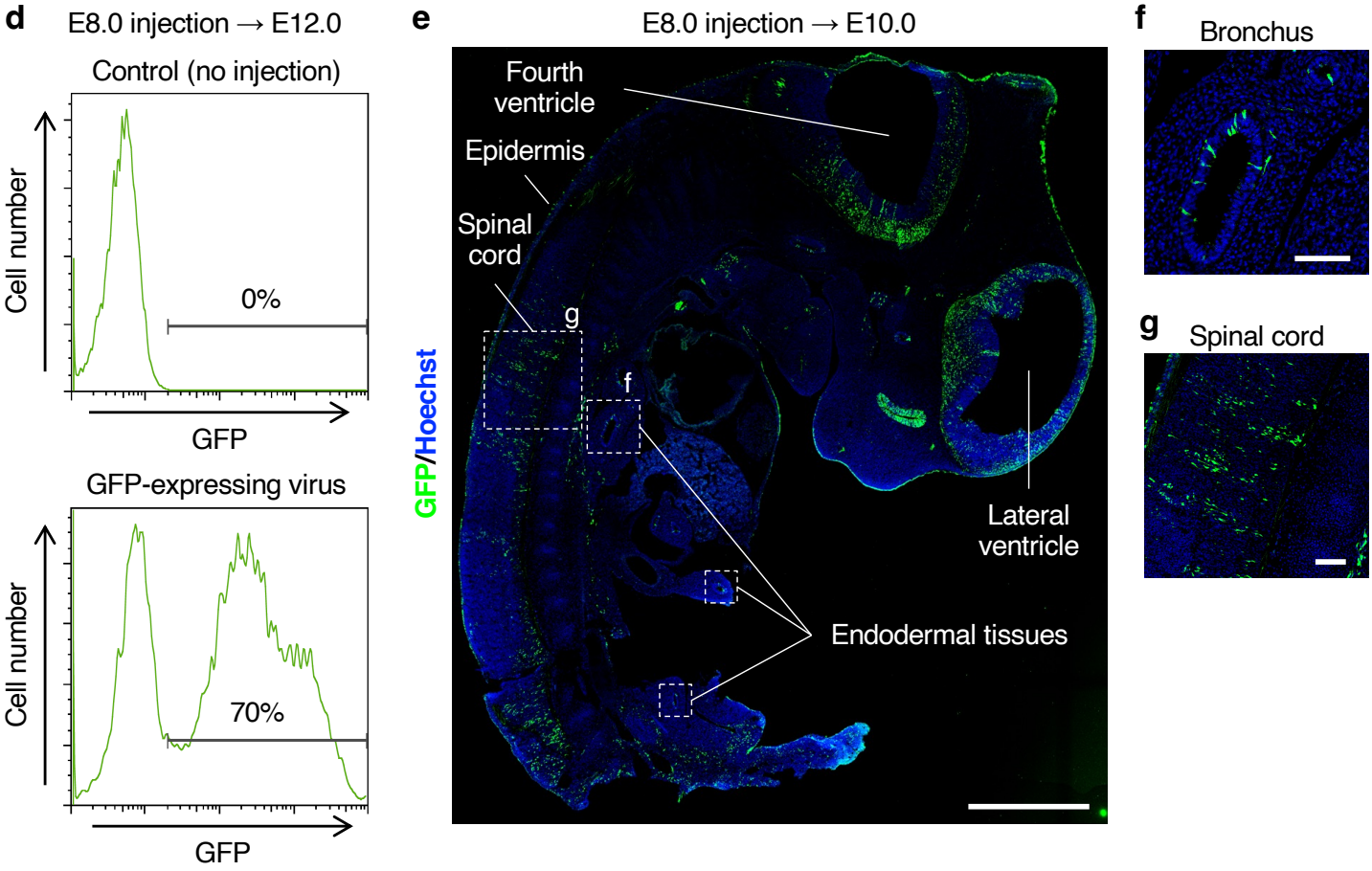
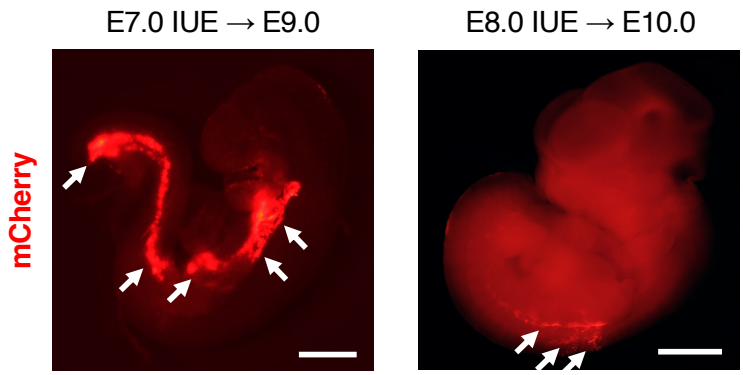
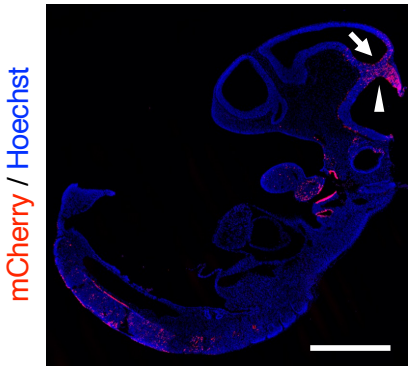


Fig. 3

a



b

	Injected embryos	Survived (% of injected)	mCherry-positive (% of injected)
E7.0 injection → E9.0	36	26 (72%)	7 (19%)
E8.0 injection → E10.0	20	11 (55%)	8 (40%)

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