Targeted gene disruption in a marsupial, *Monodelphis domestica*, by CRISPR/Cas9 genome editing

Graphical abstract

**Highlights**

- The first successful demonstration of gene knockout in a marsupial

- Efficient generation of mutant opossums by zygote microinjection of CRISPR/Cas9

- Successful germline transmission of mutant alleles to the F1 generation

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**In brief**

Kiyonari et al. report the first case of a genetically engineered marsupial, *Monodelphis domestica*, the most commonly used experimental marsupial model. By refining reproductive biology parameters and zygote manipulation techniques, CRISPR/Cas9 genome editing was successfully applied to generate animals carrying targeted mutations of the *Tyr* gene.
Targeted gene disruption in a marsupial, *Monodelphis domestica*, by CRISPR/Cas9 genome editing

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SUMMARY

Marsupials represent one of three extant mammalian subclasses with very unique characteristics not shared by other mammals. Most notably, much of the development of neonates immaturity born after a relatively short gestation takes place in the external environment. Among marsupials, the gray short-tailed opossum (*Monodelphis domestica*; hereafter “the opossum”) is one of very few established laboratory models. Due to many biologically unique characteristics and experimentally advantageous features, the opossum is used as a prototype species for basic research on marsupial biology.1,2 However, *in vivo* studies of gene function in the opossum, and thus marsupials in general, lag far behind those of eutherian mammals due to the lack of reliable means to manipulate their genomes. In this study, we describe the successful generation of genome edited opossums by a combination of refined methodologies in reproductive biology and embryo manipulation. We took advantage of the opossum’s resemblance to popular rodent models, such as the mouse and rat, in body size and breeding characteristics. First, we established a tractable pipeline of reproductive technologies, from induction of ovulation, timed copulation, and zygote collection to embryo transfer to pseudopregnant females, that warrant an essential platform to manipulate opossum zygotes. Further, we successfully demonstrated the generation of gene knockout opossums at the Tyr locus by microinjection of pronuclear stage zygotes using CRISPR/Cas9 genome editing, along with germline transmission of the edited alleles to the F1 generation. This study provides a critical foundation for venues to expand mammalian reverse genetics into the metatherian subclass.

RESULTS

The opossum, *M. domestica*, as a potential genetic model

Among marsupials, the opossum is the most commonly used experimental model in laboratory settings,3 making it a reasonable choice to be the first marsupial species to have its whole genome sequenced.7 After only 14 days of gestation, at birth, opossum offspring correspond to embryonic day 13 to 15 in the mouse and rat or to 40-day human embryos.5 Hence, various developmental events, including organogenesis, that would otherwise proceed in the intrauterine environment in eutherian counterparts occur externally. In addition, female opossums do not have a marsupium (a pouch), making it feasible to continuously observe and analyze the development of the same offspring over time. Furthermore, the opossum provides a valuable marsupial model to study many other biological attributes, such as preimplantation development,6 structure of the urogenital system,7 X chromosome inactivation,8,9 and genomic imprinting.10,11 Despite such desirable features, *in vivo* molecular studies of the opossum have been largely limited to gene expression analyses in embryos and early offspring.12–16 This apparent limitation is, at least in part, attributable to the lack of established methodologies for *in vivo* genetic manipulation. To tackle this technical hurdle, we aimed to develop a reliable platform for reproductive biology and zygote micromanipulation in this species.

Induction of copulation and collection of zygotes in the opossum

For genetic engineering by zygote manipulation in mammals, it is necessary to coordinate preparation of zygote donor females and pseudopregnant recipients for embryo transfer. However, the opossum is an induced ovulator,17 making it difficult to predict appropriate timing of pairing for timed copulation. Male pheromones are thought to be the key to activate female reproduction.18 Baggott et al.19 described that mating occurred over a relatively large time frame after female-male pairing with a...
majority of pairs copulating between 6 and 14 days during an observation period of 28 days. Such tendencies in copulation timing are not desirable for the development of efficient production colonies suitable for zygote genetic manipulation in the opossum.

First, in an attempt to increase the copulation efficiency and to narrow the time frame of mating, we tested whether hormone stimulation can promote copulation. Previous studies used hormone stimulation for estrus induction in several marsupial species, such as brushtail possums and tammar wallabies. Copulation was monitored by video recording because, unlike mice and rats, vaginal plugs and detection of spermatozoa in vaginal smears are not reliable in opossums. All copulation events among untreated females (67.5%) were confirmed only between day 4 and day 7; no females copulated on or before day 3 (Figure 1A; Table S1A). In contrast, females injected with pregnant mare’s serum gonadotropin (PMSG) 72 h before pairing copulated throughout the period from day 0 to 7 with the majority having copulated by day 3 even at a low dose (1 IU), in contrast to the untreated group (Figure 1A; Table S1A). Additional injection of 1 IU of human chorionic gonadotropin (hCG) 72 h after 1 IU of PMSG resulted in more than 70% of females copulating within 4 nights of pairing, drastically narrowing the overall range of copulation timing (Figure 1B; Table S1A).

Furthermore, more than 50% of females injected with a higher dose (5 IU) of hCG copulated on the day or the next day of pairing (Figure 1B; Table S1A). Although higher doses (30 IU and 5 IU) of PMSG apparently shortened the window of copulation timing, neither of these conditions yielded viable zygotes (Figure 1C; Table S1B). On the other hand, a low dose (1 IU) of PMSG was sufficient only to yield apparently healthy zygotes in more than 80% of females. Contrary to our expectation, however, additional injection of hCG (1 or 5 IU) appeared to lower the rates of zygote collection at either dose (33.3% or 61.9%, respectively) (Figure 1C; Table S1B). The average number of zygotes recovered from females stimulated with hormones showed no notable increase compared to untreated donors (Table S1C). These results indicated that hormone stimulation can aid in shortening of the time window for copulation. However, excessive doses of hormones may be counteractive to zygote formation. Taking these characteristics into account, in the subsequent studies we took an interim approach to collect zygotes from copulated females, regardless of whether hormones were administered or not, that suited the timing of microinjection sessions.

In the opossum, it has been suggested that fertilization occurs around 22 h after copulation, and that pronuclear stage zygotes can rapidly reach the uterus as early as 24 h postcoitus. Since copulation predominantly takes place during the night, we presumed that collection of zygotes from the uterus in the 2nd morning after copulation (estimated as 30–34 h after copulation; Figure 2A) would be suitable in a routine experimental schedule. Under the initial light-dark setting (light, 8:00 a.m. to 9:00 p.m.; dark, 9:00 p.m. to 8:00 a.m.), a large majority of females copulated shortly after the entry into the dark cycle (Figure 2B), and thus most of collected zygotes would be over 34 h after copulation by the time of routine zygote collection. These zygotes might not have been optimal for microinjection because not only had they formed a shell layer too hard for a needle to penetrate, but also their pronuclei often had already become invisible. Noting this tendency of copulation timing, delaying the onset of
the dark period (0:00 a.m. to 10:00 a.m.) allowed the collection of slightly younger zygotes more suitable for microinjection (Figure 2C).

Preparation of pseudopregnant opossums and embryo transfer
The next important task was preparing pseudopregnant females for embryo transfer. The estrus induction by hormones has also been practiced in livestock and rabbit. In rodents, the estrus is easily identified by the appearance of the vulva (mice and hamsters) or by measuring impedance in the vagina (rats). Embryo transfer is typically performed in pseudopregnant females prepared by copulation with vasectomized males. However, in the case of the opossum, which is an induced ovulator, there has been no effective method established to obtain timed pseudopregnant females. To alleviate this technical issue, we attempted to produce pseudopregnant recipients by letting females copulate with vasectomized males upon estrus induction by pairing per se or by hormone administration. Copulation was confirmed by video recording, and pseudopregnancy was further verified by uterine swelling at the time of embryo transfer (Figure S1A). This gross anatomical assessment of pseudopregnancy was complemented by histological comparisons between non-pregnant, pregnant, and pseudopregnant females, which clearly indicated the degrees of proliferation of uterine glands and transformation of the uterine luminal epithelium (Figure S1A). About a third of untreated females (33.3%) copulated between day 2 and day 7, whereas 27.4% of hormone-injected females copulated during the first 3 nights (days 0–2) upon pairing (Figures 1A and 1B; Table S1A). Next, freshly collected zygotes were transferred into the uterus of same-day (synchronous) pseudopregnant females. Although no pups were obtained from two females that were hormone-induced, seven pups were obtained and thrived from the other female whose estrus was pairing-induced (experimental rounds [exp.] 1–3 in Table S2). These results indicated that induction of estrus and subsequent copulation were successful to produce a pseudopregnant recipient by pairing with a vasectomized male.

Targeted disruption of the Tyr locus by CRISPR/Cas9 genome editing
With the successful establishment of zygote collection and embryo transfer schemes, we explored the feasibility of genome editing in the opossum to disrupt the Tyrosinase (Tyr) gene with the CRISPR/Cas9 system in pronuclear stage zygotes. The Tyr gene has been commonly used as a target to test the efficiency of CRISPR/Cas9-mediated genome editing in various organisms. The opossum Tyr gene on chromosome 4 consists of five exons, and two gRNAs were designed to target exon 4 (Figure 3A), which corresponds to exon 4 in the mouse previously used for highly efficient Tyr gene knockout. We employed the ribonucleoprotein complex of crRNA, tracrRNA, and Cas9 (ctRNP), which significantly improved genome editing efficiency compared to the use of sgRNA and Cas9 mRNA in mice in our hands.

At the time of collection around 34 h after copulation, opossum zygotes were already coated with thick mucoid and hard shell layers (Figure 2A), which were nearly impenetrable with the injection needle under our conventional microinjection setting for mice and rats (Video S1A). To overcome this, we employed a piezoelectric actuator, which can improve the penetration of injection needles into zygotes. We injected the CRISPR ctRNP into the pronucleus, or into the cytoplasm when pronuclei could not be clearly identified (Figures 3B and 3C; Videos S1B and S1C). Injected zygotes were transferred into the uterus of synchronized pseudopregnant females. Alternatively, when
synchronized pseudopregnant females could not be obtained, injected zygotes were cultured overnight to the 4-cell stage, and then transferred to pseudopregnant females copulated 1 day later. In the initial experiments where only gRNA1 (Figure 3A) was used, fourteen offspring without detectable changes in coat color were obtained (exp. n1, n2, and h1 in Table S2). PCR and sequence analyses revealed that no offspring showed evidence of insertions or deletions (indels) at the target site (Figure 3E; Table S3). The subsequent experiments where both gRNA1 and 2 were combined (Figure 3A) yielded a total of 19 offspring (Table 1; exp. n3–n5 and h2–h8 in Table S2). Seven of these offspring exhibited readily detectable changes in coat color ranging from complete white (albino) to mosaic loss of pigmentation (Figure 3D; Tables 1 and S2). PCR and sequence analyses confirmed that 15 out of 19 offspring (78.9%) exhibited evidence of indel alleles (Figures 3E–3G; Table S3). In total, we obtained 5 albinos, 2 coat color mosaics, and 8 with wild-type coat color carrying genome edited alleles. Subsequently, a pair of mosaic female (exp.h4-2) and albino male (exp.h4-1) were crossed to confirm germline transmission. All 9 F1 offspring were albinos, indicating that both mutant alleles have been passed on to the next generation (Figure 3H). These results clearly demonstrated successful generation of Tyr gene knockout opossums by CRISPR/Cas9-mediated genome editing.

**DISCUSSION**

In recent years, the emergence of CRISPR/Cas9-mediated genome editing technologies has accelerated mammalian targeted gene modification, not only in rodents, but also in livestock and non-human primates in which embryonic stem
cells and conventional gene targeting approaches are not available. In this study, we have successfully applied zygote microinjection of CRISPR/Cas9 to generate genome-edited opossums and demonstrated germline transmission of identified mutant alleles to the F1 generation. To our knowledge, this is the first report of genetically engineered marsupials. However, no previous attempts of embryo transfer in the literature have been incubated overnight could be transferred, leading to successful delivery of offspring with an overall birth rate apparently unaffected.

In this study, we obtained genome-edited opossums at the Tyr locus with a variety of allelic combinations in one generation under the injection conditions similar to those in mouse zygotes with CRISPR/Cas9. In particular, the generation of biallelic mutants would allow for direct analyses of recessive phenotypes under the injection conditions similar to those in mouse zygotes and demonstrated germline transmission of identified mutant alleles to the F1 generation. To our knowledge, this is the first report of genetically engineered marsupials.47–51 The current study effectively broke this technical barrier in marsupial reproductive biology. We observed that both hormone-treated and untreated females successfully copulated with vasectomized males. Presently, however, no offspring have been obtained from hormone-treated surrogate females, even though characteristic uterine swelling was confirmed to be similar to non-hormone-treated pseudopregnant females that subsequently carried successful pregnancies with transferred embryos. In contrast, mosaic and monoallelic mutants allow establishment of pedigrees for further genetic manipulation.

Another important factor was to promote copulation in a timely and predictable manner closely aligned with the schedule of zygote injection. In this study, we demonstrated that hormone treatment can be applied to gain better control over copulation timing. Based on previous reports, 20,21,56 female opossums treated with PMSG and then with hCG 72 h later exhibited an apparent tendency to copulate within shorter periods of time. In some other marsupial species and rabbits, estrous cycles can be adjusted by multiple hormone injections.46 In order to further improve the efficiency of copulation and zygote yield, it may be necessary to examine the optimal frequency, interval, and doses of hormone administration as well as the age of females used.

Successful production of genetically engineered opossums also required precise coordination of the production of pseudopregnant females for transfer of manipulated embryos. However, no previous attempts of embryo transfer in the literature led to the production of offspring in several species of marsupials.47–49 The current study effectively broke this technical barrier in marsupial reproductive biology. We observed that both hormone-treated and untreated females successfully copulated with vasectomized males. Presently, however, no offspring have been obtained from hormone-treated surrogate females, even though characteristic uterine swelling was confirmed to be similar to non-hormone-treated pseudopregnant females that subsequently carried successful pregnancies with transferred embryos. This phenomenon in hormone-treated pseudopregnant females seemed peculiar because similarly hormone-treated females were successfully impregnated by intact males and yielded offspring (Figure S1B). The underlying causes of these differences would be worth pursuing in future studies. Another caveat was that the copulation rate by vasectomized males was noticeably lower than that by intact males regardless of hormone administration. This result appears to be consistent with the previous report showing that the induction rate of estrus in females by vasectomized males was generally lower than that by intact males.

One valuable finding was that, when pseudopregnant females could be obtained 1 day later, 4-cell-stage embryos that had been incubated overnight could be transferred, leading to successful delivery of offspring with an overall birth rate apparently unaffected.

In this study, we obtained genome-edited opossums at the Tyr locus with a variety of allelic combinations in one generation under the injection conditions similar to those in mouse zygotes with CRISPR/Cas9. In particular, the generation of biallelic mutants would allow for direct analyses of recessive phenotypes in F0 offspring, which is extremely beneficial in species like the opossum whose sexual maturity takes considerably longer than in mice and rats. In order to increase the mutation efficiency, optimization of injection cocktail concentrations, injection timing suitable for a given species, use of multi-gRNA approaches such as the triple-target CRISPR method,53 and injection into both pronuclei40 may be effective. In contrast, mosaic and monoallelic mutants allow establishment of pedigrees for further genetic manipulation.

### Table 1. Knockout efficiencies by zygote injection with CRISPR/Cas9 in F0 offspring

| Exp. | gRNA | No. injected embryos (injected location) | No. transfer embryos (injected location) | Stage of transferred embryos | No. offspring (offspring rate) | Coat color | No. carrying indels (KO rate) |
|------|------|----------------------------------------|----------------------------------------|----------------------------|--------------------------------|-----------|-------------------------------|
| n1   | 1    | 12 (ND)                                | 12 (ND)                                | 1-cell                     | 6 (50.0%)                     | wild      | 0                             |
| n2   | 2    | 16 (PN14, Cyt2)                        | 11 (PN11)                              | 4-cell                     | 8 (72.7%)                     | wild      | 0                             |
| n5   | 1&2  | 16 (PN11, Cyt5)                        | 16 (PN11, Cyt5)                        | 4-cell                     | 3 (18.8%)                     | wild      | 3 (100%)                      |
| h2   | 1&2  | 25 (ND)                                | 25 (ND)                                | 1-cell                     | 5 (20.0%)                     | wild      | 1 (20.0%)                     |
| h4   | 1&2  | 15 (PN7, Cyt8)                         | 14 (PN6, Cyt8)                         | 4-cell                     | 2 (14.3%)                     | 1 white, 1 mosaic | 2 (100%)                      |
| h6   | 1&2  | 11 (PN10, Cyt11)                       | 11 (PN10, Cyt1)                        | 1-cell                     | 2 (18.2%)                     | white     | 2 (100%)                      |
| h7   | 1&2  | 12 (PN9, Cyt3)                         | 11 (PN9, Cyt2)                         | 1-cell                     | 2 (18.2%)                     | wild      | 2 (100%)                      |
| h8   | 1&2  | 18 (PN17, Cyt1)                        | 18 (PN17, Cyt1)                        | 1-cell                     | 5 (27.8%)                     | 2 white, 1 mosaic, 2 wild | 5 (100%)                      |

All injected zygotes in each experimental round were transferred to a single non-hormone-treated pseudopregnant female that was synchronized (1-cell) or copulated 1 day later (4-cell). An expanded set of data of all experimental rounds is shown in Table S2. Exp., experimental round; PN, pronuclei; Cyt, cytoplasm; ND, no data. See also Figure S1 and Table S2.

a The ratio of offspring to transferred embryos

b Percentage of offspring carrying any indels at the Tyr locus in each experiment
studies of early lethal mutations. Opossum zygotes tend to develop more rapidly to the 4-cell stage than mice, as early as the morning following injection. This may have contributed to the occurrence of allele variety if Cas9 activity persists during initial blastomere cleavages to generate a range of allele mosaicism.

The new methodologies we have developed in this study for successful generation of genetically engineered opossum will contribute to the acceleration of in vivo studies of gene function in the marsupials that trail significantly behind those of eutherian mammals. It is conceivable that the techniques developed in this study can be readily applied to the production of transgenic opossums by random transgenesis. With the anticipation for a number of technical improvements that can potentially be implemented, our current study provides a fundamental platform toward the establishment of the opossum, M. domestica, as a unique and accessible genetic model system to explore comparative in vivo gene function studies in mammals.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2021.06.056.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.K. and M.K.; Methodology, H.K. and M.K.; Investigation, T.A., A.S., R.Y., K.I., H.K., and M.K.; Writing – Original Draft, H.K., T.A., and Y.F.; Writing – Review & Editing, H.K. and Y.F.; Funding Acquisition, H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| PMSG (SEROTROPIN) | ASKA Animal Health | N/A |
| hCG (GONATROPIN) | ASKA Animal Health | N/A |
| KSOM | ARK Resource | N/A |
| Isoflurane | AbbVie, UK | B506 |
| PFA(Paraformaldehyde) | nacalai tesque | 30525-89-4 |
| PBS(phosphate-buffered saline) | nacalai tesque | 14249-95 |
| Ethanol | nacalai tesque | 14713-95 |
| Paraffin | Leica | 39601006 |
| Xylene | nacalai tesque | 36612-93 |
| Mayer’s hematoxylin | FUJIFILM Wako Pure Chemical Corporation | 131-09665 |
| Eosin | MUTO PURE CHEMICALS | 32022 |
| RNase-free-water | Promega | P1193 |
| TrueCut Cas9 protein V2 | Thermo Fisher Scientific | A36497 |
| Proteinase K | Roche | 03 115 852 001 |
| Phenol, Saturated with TE Buffer | nacalai tesque | 25969-96 |
| Phenol:Chloroform:Isoamyl Alcohol | nacalai tesque | 25970-56 |
| Isopropanol | nacalai tesque | 29113-95 |
| GoTaq Green Master Mix, 2X | Promega | M712C |
| AMPure XP | BECKMAN COULTER | A63881 |
| ExTaq HS | TaKaRa | R300A |
| MiSeq reagent kit v2 | Illumina | MS-102-2003 |
| **Experimental models: Organisms/strains** | | |
| Gray short-tailed opossum (Monodelphis Domestica) | Texas Biomedical Research Institute | N/A |
| **Oligonucleotides** | | |
| crRNA1 (5'- AUU GUG UCC AAU GGG CGC AUg uuu uag acg uac gcu guu uug-3') | FASMAC | N/A |
| crRNA2 (5'-GGU AGG CGU AAU CAU AUC CCg uuu uag acg uac gcu guu uug-3') | FASMAC | N/A |
| tracrRNA (5'- AAA CAG CAU AGC AAG UUA AAA UAA GGC UAG FASMAC N/A UCC GGU AUC AAC UUG AAA AAG UGG CAC CGA GUC GGU GCU-3') | FASMAC | N/A |
| oTyr FW2: 5'-GTG GCT TCG AAG ACA TCG-3' | Thermo Fisher Scientific | N/A |
| oTyr REV: 5'-CCA ACA CTC ATG CTT GAG-3' | Thermo Fisher Scientific | N/A |
| oTyr REV3: 5'-GAG GAT TAT GTA GTC TGC-3' | Thermo Fisher Scientific | N/A |
| **Software and algorithms** | | |
| IINA | GitHub | [https://github.com/iina/iina](https://github.com/iina/iina) |
| Fiji 2.1.0 | [54](#) | [https://imagej.net/Fiji](https://imagej.net/Fiji) |
| CRISPRdirect | Database Center for Life Science | [https://crispr.dbcls.jp](https://crispr.dbcls.jp) |
| BLAST Search Genome | University of California Santa Cruz Genomics Institute | [http://genome.ucsc.edu/cgi-bin/hgBlat](http://genome.ucsc.edu/cgi-bin/hgBlat) |
| FLASH2 (v2.2.00) | [55](#) | [https://github.com/dstreett/FLASH2](https://github.com/dstreett/FLASH2) |
| CRISPy_v1-py3.py | [56](#) | [https://github.com/patrickc01/CRIS.py](https://github.com/patrickc01/CRIS.py) |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information should be directed to and will be fulfilled by the lead contact, Hiroshi Kiyonari (hiroshi.kiyonari@riken.jp).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Gray short-tailed opossums (Monodelphis domestica) used for all experiments were bred in-house, derived from founder animals from Texas Biomedical Research Institute (San Antonio, Texas). Housing cages measured 15 cm (W) x 30 cm (D) x 19 cm (H) for maintenance and 24 cm (W) x 41 cm (D) x 19 cm (H) for breeding supplemented with TAPVEI Aspen Bedding (Tapvei Estonia OÜ, Estonia) and Enviro-dri (Shepherd Specialty Papers, U.S.A.) for nesting. They were fed with ferret chow (DOUBUTSUMURA ferret food maintenance; Yeaster, Japan) and provided with water automatically or by bottle. The rooms were maintained at a temperature of 25°C and humidity of 50% under artificial lighting (14 h light (10:00 am to 0:00 am); 10 h dark (0:00 am to 10:00 am)). For breeding, females aged 8-20 months and males 8-25 months were paired for two weeks and then separated to observe signs of pregnancy and birth of offspring. To minimize as much as possible the decline in fertility due to inbreeding degeneration, we routinely selected females and males from separate parental breeders. In our hands, the gestation period was typically 14 days with an average litter size of 8.8 ± 3.0 and average weaning rate of 74.6% (n = 320). All animal experiments were conducted under the institutional guidelines for animal experimentation. The pertinent animal experimentation protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of RIKEN Kobe Branch.

METHOD DETAILS

Detection of copulation
A custom video recording system was installed to monitor copulation because, unlike mice and rats, vaginal plugs and detection of spermatozoa in vaginal smears are not reliable in opossums.1,19 To record reproductive behaviors, an infrared video camera (TS-WPTCAM2, I-O DATA DEVICE, Japan) was placed directly above the cages, allowing three cages to be recorded per camera. In
this study, we prepared three cameras that covered up to nine cages at a time. Recorded images were played by IINA software (https://iina.io/) in a 4-32-time fast-forward mode, and reproductive behavior was manually inspected. Unique behaviors of the opossum prior to copulation, such as sniffing each other’s rumps and a male chasing in a loop to catch a female, were readily recognized, and copulation was confirmed upon brief cessation of their movements (1-2 min) and subsequent parting from each other in opposite directions.

**Zygote collection and culture**

Female opossums aged 8 to 18 months were used for zygote collection. Pregnant mare’s serum gonadotropin (PMSG) (SEROTROPIN, ASKA Animal Health) and human chorionic gonadotropin (hCG) (GONATROPIN, ASKA Pharmaceutical) were administrated by intraperitoneal injection. PMSG-only treated females were paired with males after 72 h of PMSG administration. When both PMSG and hCG were administered, the interval was 72 h, and animals were paired immediately following hCG injection. Copulated females were sedated with isoflurane and sacrificed by cervical dislocation. Zygotes were incubated in potassium simplex optimized medium (KSOM) (ARK Resource) from the uterus via midline incision. KSOM was also used as the culture medium, and the collected zygotes were incubated at 32.6°C, 5% CO2 as previously reported.1

**Production of pseudopregnant females**

Pseudopregnant female opossums were generated by copulation with vasectomized males with or without intraperitoneal hormone administration (PMSG, 1 IU; hCG, 5 IU). Copulation was confirmed by video recording as described above. Male opossums at weaning ages (around 8 weeks) were used for vasectomy. Each anesthetized male opossum was placed supine, the lower abdomen was incised, and the bladder was exteriorized. Two thin vas deferens near the ureters were pinched with tweezers and burned off with heated tweezers. The vas deferens was identified by confirmation of testicular movement when the vas deferens was pulled. Sexually mature vasectomized males were copulated at least twice with females to confirm their sterility prior to their use in the experiments.

**Embryo transfer**

Pseudopregnant female opossums anesthetized with isoflurane were placed in a face-down position. A surgical incision was made slightly on the head side from the base of a hind leg to exteriorize the uterus. After confirming the uterine swelling, a hole was made in the upper tip of the uterine horn with a 23G needle, and the embryos were transferred into the uterus with a glass capillary having an inner diameter of about 400 μm. Embryo transfer was performed unilaterally or bilaterally, depending on the number of embryos. After recovery from anesthesia, surrogate females were isolated for 14-15 days until due dates.

**Histological analysis**

Uterine tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline for overnight at 4°C. Fixed uterine tissues were rinsed and then dehydrated in a graded series of ethanol (70% for 30 min, 90% for 30 min x 2 and 100% for 30 min x 2). After replacing ethanol with xylene, the tissues were impregnated with paraffin (10 min x 3 times at 66°C) and embedded. The resulting paraffin blocks were mounted on a microtome (HM355S, MICRO EDGE INSTRUMENT) and cut into seven μm transverse sections. The sections affixed to slide glasses were deparaffinated with xylene and then rehydrated with a graded series of ethanol (100% for 10 min x 2, 90% for 10 min, 85% for 10 min, 70% for 10 min) for hematoxylin and eosin staining. Stained sections were observed under a compound light microscope (DMRA2, Leica).

**Guide RNA design and preparation**

For gRNA design, the gray short-tailed opossum (Monodelphis domestica) Tyr (tyrosinase) cDNA sequence (Gene ID: 100010166) was used for BLAT Search Genome (http://genome.ucsc.edu/cgi-bin/hgBlat) to obtain the sequence of the entire Tyr locus, and gRNA target sites in exon 4 were selected by CRISPRdirect (https://crispr.dbcls.jp).27 crRNA1 (5’- AUU GUG UCC AAU GGG CGC AGG uu uag acg uac gcu guu uug-3’), crRNA2 (5’-GGU AGG CGU AAU CAU AUC CCG uuu uag agc uac gcu guu uug-3’) and tracrRNA (5’-AAA CAG CAU AGC AAG UUA AAA UAA GGC UAG UCU GGU AUC AAC UUG AAA AAG UGG CCC GGC GCC GCC UC CUG JUC-3’ were purchased from FASMAC. crRNAs and tracrRNA were dissolved in RNase-free water (crRNA, 500ng/μl; tracrRNA, 1μg/μl), and stored at −80°C until use.

**Microinjection**

Zygotes were microinjected in a drop of KSOM. Injection cocktails consisting of Cas9 protein (100 ng/μl) (Thermo Fisher Scientific), crRNA(s) (50 ng/μl each), tracrRNA (200 ng/μl) were injected into pronuclei or cytoplasm if pronuclei were not readily visible. Injection cocktails were mixed just before microinjection and kept on ice. The microinjection setup was composed of an inverted microscope (DMIRE2, Leica), micromanipulators (TransferMan 4r, Eppendorf), microinjectors (CellTram vario, Eppendorf) and a piezo micromanipulator controller (PiezoXpert, Eppendorf). The piezo conditions are as follows: for penetration of mucoid and shell layers, Int. 50, Speed 4 s, Pulse 18 (seamlessly); for penetration of zona pellucida, Int. 20, Speed 2, Pulse 25. Videos were recorded by XYClone system (HAMILTON THORNE). The recorded footage were edited by Fiji.54

**Genomic DNA analyses**

Tissues recovered from F0 opossum ears or tails were lysed with proteinase K, and genomic DNA was purified by sequential extraction by phenol and phenol-chloroform, followed by isopropanol precipitation. Genotyping of the Tyr locus was performed by PCR.
using primer pairs oTyr FW2: 5’-GTG GCT TCG AAG ACA TCG-3’ and oTyr REV: 5’-CCA ACA CTC ATG CTT GAG-3’ (465 bp) or oTyr FW2 and oTyr REV3: 5’-GAG GAT TAT GTA GTC TGC-3’ (291bp) using GoTaq Green Master Mixes (Promega). The PCR cycling condition was as follows: 96°C for 2 min, 35 cycles of 96°C for 30 s, 55°C for 30 s, 72°C for 30 s. PCR products were resolved on a microchip electrophoresis system using the 500 or 1000 reagent kit (MultiNa, Shimadzu). For next-generation sequencing, the primer pairs with Illumina adapters oTyr FW2: 5’-aca ctc ttt ccc tac acg acc ctc ttc cga tct GTG GCT TCG AAG ACA TCG-3’ and oTyr REV3: 5’-gtg act gga gtt cag acg tgt gct ctt ccg atc tGA GGA TTA TGT AGT CTG C-3’ (291bp) were used for the 1st PCR. Following AMPure XP beads purification (BECKMAN COULTER), the products were used for 2nd PCR reaction with Illumina index primers according to the manufacturer’s instructions. The 1st and 2nd PCR cycling conditions using ExTaq HS (TaKaRa) was; 94°C for 2 min, 20 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, followed by 72°C for 5min. For the 2nd PCR, 94°C for 2 min, 8 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, followed by 72°C for 5min. The PCR amplicons were sequenced by using the MiSeq reagent kit v2 (500 cycles) and the MiSeq sequencer (Illumina) at FASMAC (Kanagawa, Japan). The default setting of FLASH2 (v2.2.00) assembler was used to merge the overlapping paired-end fastq, and the merged fastq files were analyzed with Spyder 4.0.1 (Python 3.7.6) by using a Python-based CRISpy_v1-py3.py software according to the developer’s protocol (https://github.com/patrickc01/CRIS.py). As with the wild-type, the opossums with a total indel of 1% or less were counted as non indel carriers. The graphs of the resulting data were made by using Microsoft Excel software (ver. 16.49).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Average numbers of pups (Figure S1B) and zygotes (Table S1) from pregnant female were shown as means ± standard deviation (SD). The p-value of t test was calculated using the R software publicly available for statistical computing (https://www.r-project.org/). No statistically significant difference was defined as p > 0.05. The number of copulated females used were described as “n =” in Figures 1A, 1B, 2B, and 2C. The number of transferred zygotes in each experimental round is indicated as “n =” in Figure S1B.