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Technology Report

Establishment of embryo transfer in the musk shrew (*Suncus murinus*)

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Abstract. The present study established techniques to induce pseudopregnancy, *in vitro* oocyte cultures from pronuclear to 2- to 4-cell stages, and embryo transfer in musk shrews, a reflex ovulator. Offspring were subsequently obtained by transferring *in vivo*-developed or *in vitro*-cultured embryos. Female musk shrews received human chronic gonadotropin (hCG), with or without mating stimuli, from vasectomized males to produce pseudopregnant recipients. Embryos at the 2- to 4-cell stage were collected 44–48 h after mating. Another set of embryos was collected 26–27 h after mating and then cultured for 20 h from the pronuclear to 2- to 4-cell stages. Subsequently, embryos were transferred into the oviducts of pseudopregnant recipients 24 or 48 h after the induction of pseudopregnancy. Offsprings were successfully obtained from recipients that received hCG 24 h before embryo transfer, regardless of mating stimuli. These techniques may be valuable for producing transgenic musk shrews.
Key words: *Insectivora,* Peudopregnancy, Reflex ovulation

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The musk shrew (*Suncus murinus*), a reflex ovulator, is a small experimental animal that belongs to the order *Soricomorpha* (the former order *Insectivora*) and the family *Soricidae*. The musk shrew has a 29–31 day gestation period and delivers approximately 3–4 pups per litter. Puberty occurs at approximately 20–30 days of age in both male and female musk shrews [1, 2]. Musk shrews have been used as animal models for human conditions, given that musk shrews, unlike mice and rats, exhibit a vomiting reflex and diphyodont dentition. In addition, they are among the most primitive placental animals, and some, such as *Gnrh2* and *MLN* [3, 4], are commonly expressed in musk shrews and humans but not in mice and rats. Thus, studies using musk shrews could improve our knowledge of various human biological phenomena, which might be impossible to clarify using mice and rats. Furthermore, *in vitro* studies using musk shrew embryos or transgenic musk shrews would afford a powerful strategy to enhance our existing understanding of these phenomena. On the other hand, to the best of our knowledge, no report has described essential techniques for generating transgenic animals, such as induction of pseudopregnancy, *in vitro* embryo culture, and embryo transfer in musk shrews. Therefore, in the present study, we aimed to establish these techniques.

To generate pseudopregnant recipients, six female musk shrews were intraperitoneally administered human chronic gonadotropin (hCG, 10 IU/head), with two subsequently mated with vasectomized males. Nine days after hCG administration, the formation of corpora lutea (CL) was detected in the ovaries of all six animals, regardless of mating stimuli (hCG, n = 4; hCG + mating stimuli, n = 2) (Fig. 1); no CL was detected in control females without hCG injection (n = 2). These results suggest that a single hCG administration can successfully induce CL formation and pseudopregnancy and that mating stimuli are not essential to induce pseudopregnancy in the musk shrew. This result also suggests that mating stimuli are required only to trigger the gonadotropin surge, which, in turn, induces ovulation and the consequent CL formation in the musk shrew. This finding is largely consistent with the induction of pseudopregnancy in rabbits, another reflex ovulator in which a single hCG injection induces CL formation and pseudopregnancy [5]. Interestingly, unlike musk shrews, mating stimuli are required to induce pseudopregnancy in rats and mice, which are non-reflex ovulators. Indeed, mating stimuli or vaginal mechanical stimuli induce daily prolactin surges, which in turn maintain progesterone production in the CL of rats and mice [6, 7].

The experimental protocols for embryo transfer are shown in Figs. 2A and 2 B. Sixty-six embryos were collected from donor X musk shrew (n = 16), which had been mated with a stud male 44–48 h before embryo collection (Fig. 2A, see Supplementary Table 1 for details). Overall, 59 out of 66 embryos developed to the 2- to 4-cell-stage *in vivo,* and 50 were used for embryo transfer (Table 1 and Supplementary Table 1). The embryos were collected from donor females without hormone treatment for superovulation, given that the number of fertilized embryos obtained from the donors without hormone treatment was higher than that obtained from superovulated females (see Supplementary Table 2 for details). Pseudopregnant recipients were prepared using three different methods to undergo treatments 1–3 (Fig. 2A): treatment 1, females were injected with hCG and then mated with a vasectomized male 48 h before embryo transfer; treatment 2, females were injected with hCG and mated with a vasectomized male 24 h before embryo transfer; treatment 3, females were injected with hCG without mating 24 h before embryo transfer. Fifty embryos at the 2- to 4-cell stages, developed *in vivo* in donor X, were immediately transferred (2–8 embryos/head, Table 1) to the oviductal ampullae of pseudopregnant recipients receiving treatment 1, 2, or 3. Another set of five embryos, collected from donor Y musk shrews (n = 2), which had been mated with a stud male 26–27 h before embryo collection, was cultured *in vitro* from the pronuclear stage to the 2- to 4-cell-stage for 20 h (Fig. 2B, see Supplementary Table 3 for details). The five *in vitro*-cultured embryos were then transferred into the oviductal ampullae of a pseudopregnant recipient who received treatment 2 (Fig. 2B) to confirm implantation

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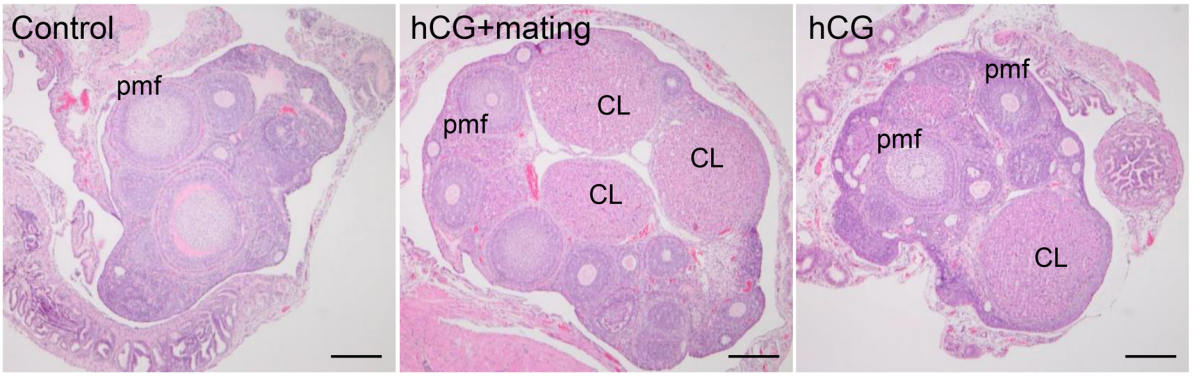

Fig. 1. Effects of human chronic gonadotropin (hCG) administration on the formation of corpora lutea (CL) in the musk shrew. Representative photomicrographs of hematoxylin-eosin-stained ovarian sections of the musk shrew that were untreated (control), hCG-injected and mated with a vasectomized male (hCG + mating), and hCG-injected (hCG). hCG-injected animals exhibit CL formation in the ovaries, regardless of mating stimuli. Scale bars, 200 μm; pmf, premature follicle.

and pregnancy outcomes. Figs. 2C and 2D present photomicrographs of representative uncultured and 20 h-cultured embryos, respectively. No apparent morphological differences were detected between *in vivo*-developed and *in vitro*-cultured 2- to 4-cell-stage embryos. Embryo fragments (arrowheads) were found in both *in vivo*-developed and *in vitro*-cultured embryos (Figs. 2C and 2D).

Two offspring (derived from *in vivo*-developed embryos) were obtained from a recipient that received an hCG injection 24 h before embryo transfer without mating stimuli from a vasectomized male (treatment 3) (Table 1, recipient #R8). This suggests that the mating stimulus is dispensable for embryo implantation and successful pregnancy in musk shrews. In addition, two offspring (derived from *in vitro*-developed embryos) were obtained when embryos, which were cultured for 20 h from the pronuclear stage to 2- to 4-cell stages, were transferred into a recipient that received both an hCG injection and mating stimuli 24 h before embryo transfer (treatment 2) (Table 1, recipient #R9). Typically, to produce transgenic mice and rats, pronuclear zygotes are used for DNA injection, cultured until the 2-cell stage, and then transferred to recipients [8, 9]. Therefore, we established a basic procedure for handling cultured pronuclear zygotes, which would be employed for generating transgenic musk shrews.

In the current study, embryo transfer was achieved using embryos collected from donors mated 48 h before transfer. Interestingly, offspring were obtained from recipients that received an hCG injection 24 h before the transfer (treatments 2 or 3, Table 1), whereas offspring were not obtained from recipients that received the hCG injection 48 h before transfer (treatment 1, Table 1). We speculate that musk shrews administered hCG 24 h before embryo transfer are suitable for successful implantation and pregnancy. This notion is consistent with embryo transfer experiments in mice and rats, showing that pseudopregnancy, induced by mating with a vasectomized male the day before the transfer, is reportedly suitable for developing 2-cell-stage embryos [10]. Importantly, the offspring obtained in the present study were healthy and grew normally (Fig. 2E).

To the best of our knowledge, this is the first report presenting methods for the induction of pseudopregnancy, *in vitro* embryo culture, and embryo transfer in musk shrews. Furthermore, we demonstrated that the examined techniques could successfully generate healthy offspring from both *in vivo* and *in vitro*-cultured embryos in the musk shrew. These results may contribute to improvements in future techniques, such as *in vitro* embryo studies and the production of transgenic musk shrews.

Methods

Animals

Male and female KAT- and BK-line musk shrews (*S. murinus*) were used at 2–12 months of age. KAT line musk shrews were captured in Katmandu, Nepal, and bred at Nagoya University [11]. BK-line musk shrews, a hybrid of KAT line animals, were captured in Bangladesh and bred at Nagoya University [11]. Animals were housed in a temperature-controlled (25 ± 2°C) room with 14 h-light and 10 h-dark cycles (lights on at 0600 h). Vasectomized male shrews were used for mating at least 2 weeks after the vasectomy procedure. Food (commercial trout pellets; Nippon Formula Feed Manufacturing Co., Ltd., Yokohama, Japan) and tap water were provided *ad libitum*. All animal experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences at Nagoya University.

Induction of pseudopregnancy

We examined three protocols to induce pseudopregnancy based on methods used to induce pseudopregnancy in rodents and rabbits: Briefly, in mice and rats, pseudopregnancy is generally induced by mating an estrous female with a vasectomized male [8]; in rabbits, a reflex ovulator, pseudopregnancy is induced by a single hCG injection without a mating stimulus [12]. Six musk shrews, weighing approximately 40 to 65 g, received intraperitoneal hCG (10 IU/0.1 ml/head, Aska Pharmaceutical Co., Ltd., Tokyo, Japan) to induce ovulation, based on a previous report on musk shrews [13]. Two of the six animals were immediately housed with vasectomized male musk shrews to provide mating stimuli, and the remaining four were housed without males. To verify the induction of pseudopregnancy, ovaries were collected nine days after hCG administration by observing CL formation. The ovaries of intact musk shrews, who did not undergo hCG administration or mating, were used as controls (n = 2). Ovaries were collected from the musk shrews immediately after cervical dislocation, fixed in 4% paraformaldehyde, and embedded in paraffin. The presence of CL was evaluated by histological analysis using hematoxylin and eosin staining.

Embryo collection and culture

For *in vivo*-developed embryo transfer, embryos (n = 66) were collected from the oviduct of donor X musk shrews (n = 16, Supplementary Table 1) 44–48 h after mating with a stud male, and

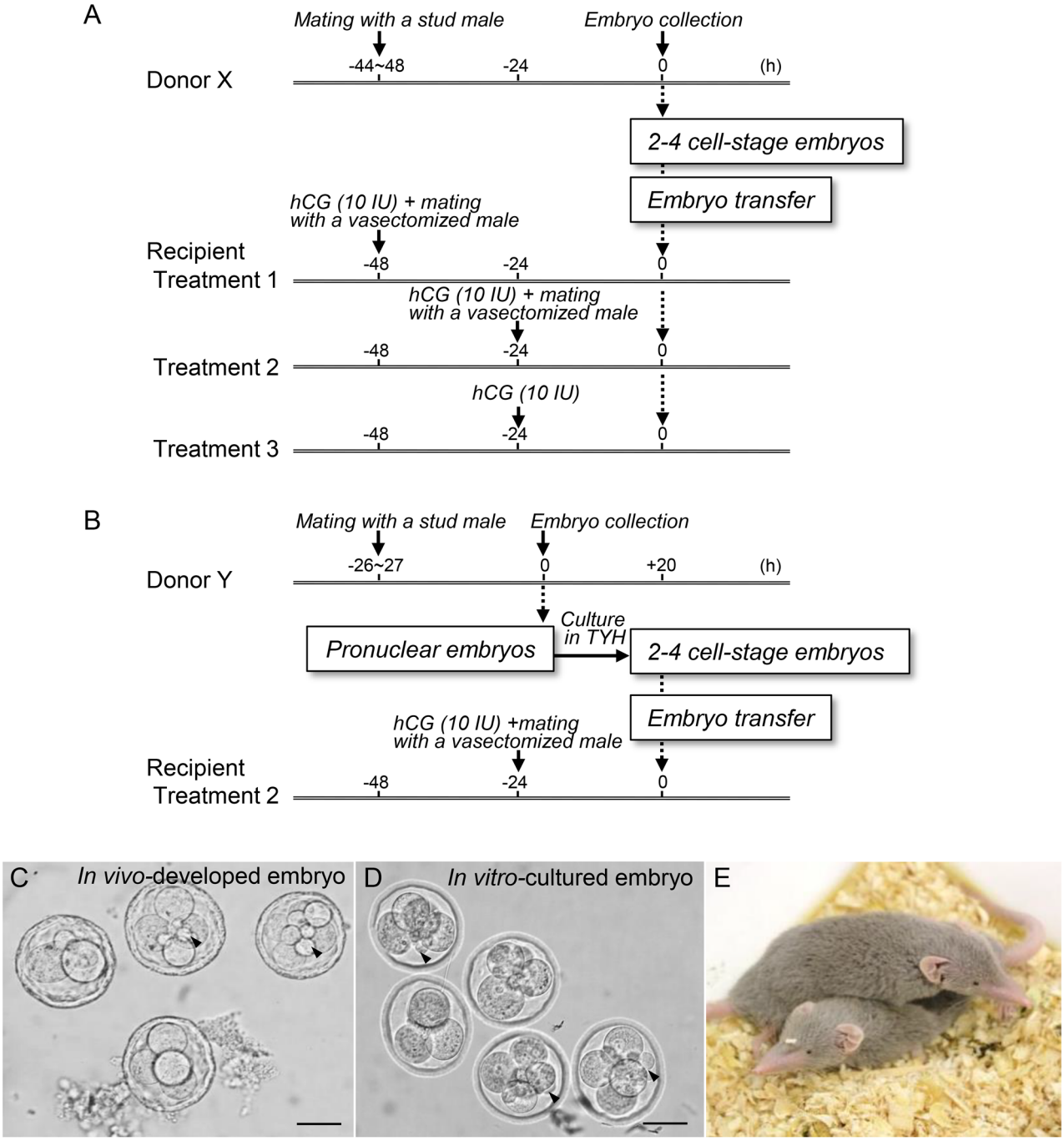

Fig. 2. Experimental protocols for embryo transfer, morphology assessment of *in vivo*-developed and *in vitro*-cultured 2- to 4-cell stage embryos, and offspring derived from transferred embryos in the musk shrew. (A) *In vivo*-developed 2- to 4-cell-stage embryos were collected from donor X musk shrews 44–48 h after mating with a stud male and immediately transferred to recipients. Pseudopregnant recipients were prepared using three different protocols: treatment 1, females were injected with hCG and then mated with a vasectomized male 48 h before embryo transfer; treatment 2, females were injected with hCG and mated with a vasectomized male 24 h before embryo transfer; treatment 3, females were injected with hCG without mating 24 h before embryo transfer. (B) Pronuclear zygotes were collected from donor Y musk shrews 26–27 h after mating with a stud male and then cultured for 20 h until the development of 2- to 4-cell-stage embryos. The *in vitro*-cultured embryos were transferred to the recipient receiving treatment 2. (C) Representative photomicrograph showing *in vivo*-developed 2 to 4-cell stage embryos, which were collected from the oviducts of donor X musk shrews. (D) Representative photomicrograph showing *in vitro*-cultured 2- to 4-cell-stage embryos for 20 h in TYH medium from the pronuclear stage, obtained from donor Y musk shrews. Scale bars, 50 µm. Arrowheads indicate embryo fragments. (E) Representative photograph shows the offspring (7 weeks of age), which were delivered from a foster mother musk shrew (recipient #R8) that had received *in vivo*-developed embryos.

2- to 4-cell-stage embryos (n = 50) were used for embryo transfer. Embryos obtained from donors were manipulated in M2 medium before embryo transfer. For *in vitro*-developed embryo transfer, embryos (n = 8) were collected from the oviducts of donor Y musk shrews (n = 2, Supplementary Table 3) 26–27 h after mating with a stud male. The pronuclear zygotes (n = 5) were cultured in TYH medium

Table 1. Condition of recipients and embryos and results of embryo transfer in the musk shrew

Individual number of recipients	Recipients	Recipient treatment		Donors and embryo conditions	Number of transferred embryos	Number of offspring
		hCG injection (h) before the embryo transfer	Mating stimulus in the recipient			
#R1	Treatment 1	–48	+	*Donor X, *in vivo* developed embryos	7	0
#R2					8	0
#R3					7	0
#R4	Treatment 2	–24	+		2	0
#R5	Treatment 3	–24	–		4	0
#R6					6	0
#R7					8	0
#R8					8	2
#R9	Treatment 2	–24	+	**Donor Y, *in vitro* developed embryos	5	2

hCG, human chronic gonadotropin. * Embryos were collected from donor X (n = 16), mated with a stud male 44–48 h before embryo collection. Then, 2- to 4-cell-stage embryos, developed *in vivo*, were immediately transferred to recipients. ** Embryos were collected from donor Y (n = 2), mated with a stud male 26–27 h before the embryo collection, and then pronuclear zygotes were cultured for 20 h. The 2- to 4-cell-stage embryos, developed *in vitro*, were transferred to recipients.

for 20 h at 37°C in a 5% CO_2 atmosphere before embryo transfer. TYH medium was selected because our preliminary experiments revealed that 75% of pronuclear zygotes developed to 2- to 4-cell stages in TYH medium, but not in other media, such as KSOM, modified Krebs–Ringer bicarbonate medium (m-KRB), and modified porcine zygote medium (m-PZM) (Supplementary Table 4). The *in vivo* and *in vitro*-developed 2- to 4-cell-stage embryos were observed using a light microscope (Eclipse TE300, Nikon, Tokyo, Japan) with a digital camera unit (DP21, Olympus, Tokyo, Japan).

Embryo transfer to the recipient oviduct

Embryos were transferred into the oviduct of a pseudopregnant recipient using glass micropipettes (150 µm inner diameter, Drummond Scientific Company, Broomall, PA, USA) under anesthesia with pentobarbital sodium (0.035 mg/g body weight; Somnopentyl, Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan). Two to four embryos per oviduct were transferred (4-8 per individual). Owing to the small number of embryos, recipient No. 4 had two embryos transferred to only one side of the oviducts. Pseudopregnant recipients were generated according to the following treatment protocols: treatment 1, animals were injected with hCG (10 IU/0.1 ml/head) and mated with vasectomized males 48 h before embryo transfer; treatment 2, animals were injected with hCG and mated with vasectomized males 24 h before embryo transfer; treatment 3, animals injected with hCG 24 h before embryo transfer. Specifically, 2- to 4-cell-stage embryos developed *in vivo* were transferred to recipients receiving treatment 1, 2, or 3, whereas *in vitro* cultured 2- to 4-cell-stage embryos were transferred to recipients receiving treatment 2. The delivery and number of offspring were examined at 33 and 34 days after embryo transfer.

Evaluation of the development of embryos obtained from superovulated musk shrews

Female musk shrews (approximately 40–65 g) were intraperitoneally administered pregnant mare serum gonadotropin (PMSG; 5 IU [n = 7], 7.5 IU [n = 5], 10 IU [n = 5]/0.1 ml/head; Aska Pharmaceutical Co., Ltd.,) and hCG (5 IU [n = 7], 7.5 IU [n = 5], 10 IU [n = 5]/0.1 ml/head) at an interval of 72 h to induce superovulation, according to a previous report on musk shrews [13]. The animals were immediately housed with a vasectomized male musk shrew to provide mating stimuli. Thirty hours after hCG administration, embryos were collected from the oviducts of animals, and the embryo developmental stage was confirmed using a light microscope.

Evaluation of medium for in vitro culture of pronuclear zygotes

Pronuclear zygotes (n = 24) were collected from the oviducts of female musk shrews (n = 9) 26–27 h after mating with a stud male. The zygotes were cultured for 20 h in KSOM, TYH, m-KRB with 1 mM hypotaurine (Sigma-Aldrich Inc., MO, USA), or m-PZM at 37°C in a 5% CO_2 atmosphere. The developmental stages of embryos were confirmed under a light microscope.

Conflict of interests: The authors declare no competing interests.

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