Chapter 8

Intraoviductal Instillation of a Solution as an Effective Route for Manipulating Preimplantation Mammalian Embryos *in vivo*

Masahiro Sato, Masato Ohtsuka and Shingo Nakamura

Additional information is available at the end of the chapter

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Abstract

Preimplantation embryos of mammals are enclosed by a translucent layer called zona pellucida (ZP), which is composed of glycoproteins. ZP is important for protecting against infection by virus and bacteria, and to prevent attachment of embryos to the oviductal epithelia. Due to the presence of ZP, it has been difficult to transfect preimplantation embryos existing within the oviductal lumen, with exogenous nucleic acids, such as DNA and mRNA. However, intraoviductal instillation of nucleic acids, and subsequent *in vivo* electroporation in pregnant females, enables transfection of these embryos, leading to the production of gene-modified animals. This new method for production of genetically modified animals does not require any *ex vivo* handling of embryos, which has been essential for traditional transgenesis. In this article, we describe recent advances in the *in vivo* transfection of preimplantation mammalian embryos, and also the possibility of simple transfection of these embryos through intraoviductal instillation of a solution, alone.

Keywords: genome editing, GONAD, *in vivo* gene transfer, oviduct, preimplantation embryos

1. Introduction

Ex vivo handling of early embryos, which includes embryo collection from pregnant animals, introduction of genetic material or genetically modified embryonic stem (ES) cells into the isolated embryos, and egg transfer (ET) to a recipient female reproductive tract, has been considered essential for production of transgenic and knock out (KO) animals [1, 2]. The recently

developed genome editing technology, as exemplified by CRISPR/Cas9 system, requires *ex vivo* handling of embryos, namely, microinjection of the genome editing component into the zygote, or *in vitro* electroporation (EP) in the presence of those materials, embryo culture, and subsequent ET [3–7]. Unfortunately, such *ex vivo* handling of early embryos requires an expensive manipulator, special technicians to handle this machine and perform ET, and preparation of recipient females for ET, all of which are sometimes time-consuming and laborious. If this *ex vivo* handling of embryos is omitted, embryo manipulation, including production of genome-edited animals, would become more convenient.

Preimplantation embryos present within the oviductal lumen would be the most favorable targets for genetic manipulation, since the oviducts themselves can be easily exposed outside the individual, under anesthesia, and manipulation within this organ would be possible under observation, using a dissecting microscope. Genetic material can be introduced into zygotes or cleaving embryos, floating in the oviductal lumen, by inserting a glass pipette through the oviductal wall, and introducing genetic material successfully into these embryos by penetrating the zona pellucida (ZP). If this event occurs, *in situ* production of genetically modified embryos (animals) would be possible.

The ZP is a multilayered porous matrix of glycoproteins that envelopes mammalian oocytes and preimplantation embryos, protecting them from environmental insults, including viral infection, and injury by chemical or physical substances [8]. Therefore, it has been difficult to transfect mammalian oocytes and preimplantation embryos, with the usual transfection methods that have proven useful for somatic cells. Early attempts at gene delivery to preimplantation embryos involved the transfection of ZP-free embryos. However, such embryos are vulnerable, adhesive, and easily damaged [9]. The most commonly method used for penetration of ZP for gene delivery is the pronuclear microinjection using purified DNA [10], or the microinjection of viral elements into the perivitelline space, between ZP and the zygotes [11]. Furthermore, it is possible to transfect mouse fertilized eggs with lentiviral vectors, *via* laser perforation of ZP [12]. *In vitro* EP was found to be effective for incorporation of plasmid DNA and morpholino, into mouse fertilized eggs, after ZP was weakened by a brief treatment with acidic Tyrode's solution [13–15]. This treatment allowed the enhanced uptake of exogenous DNA, and protected the embryos from electroporation damage. Notably, previous reports clearly indicate the need for penetrating the ZP for manipulation of early embryos, and also the *ex vivo* handling of embryos prior to ET. Thus, it may be desirable to perform gene delivery to preimplantation embryos, without handling the embryos *ex vivo,* and if possible, to employ substances that will allow direct gene delivery through penetration of ZP.

In the present article, we first discuss studies that have reported successful delivery of substances to ZP-enclosed embryos *in vitro,* followed by reports on *in vivo* transfer of substances into ZP-enclosed preimplantation embryos, after intraoviductal instillation. Lastly, we describe the recent advances in *in situ* genome editing of embryos, present in the oviductal lumen.

2. Transfer of exogenous substances into ZP intact embryos *via* simple incubation

As mentioned previously, it may be possible to perform gene delivery to embryos if a substance capable of penetrating ZP is used in combination with nucleic acids. For example, Ivanova et al.

[16] demonstrated that when ZP intact preimplantation embryos from mice and rabbits were incubated in a medium containing DNA-carrying constructs with insulin as an internalizable ligand, (insulin-polylysine)-DNA and (insulin polylysine)-DNA-(streptavidin-polylysine)-(biotinylated adenovirus), the constructs penetrated the ZP and accumulated within each blastomere. Southern blot hybridization revealed chromosomal integration of transgenes in mid-gestational fetuses and in a newborn. The ligand-mediated gene delivery to early embryos can be explained by the following mechanism. Insulin provides delivery inside the cell, while adenoviruses ensure release from the endosomes. They called this type of gene delivery "receptor-mediated gene transfer". According to the authors, the construct containing DNA and insulin, penetrates inside, to accumulate in the peri-nuclear space of the embryos. Munk et al. [17] demonstrated that multiwall carbon nanotubes (MWNTs) could cross the ZP to help the delivery of plasmid DNA into bovine embryos *in vitro*. Exposure of embryos to MWNTs did not affect their viability and gene expression. Interestingly, Joo et al. [18] recently developed a hydrophilic, Cy5.5-labeled organic compound called "VisuFect". When VisuFect is conjugated with poly(A) oligo, this complex successfully penetrates the ZP of the fertilized eggs of various species, including zebrafish, mice, and pigs, suggesting that VisuFect can be used to deliver genome-editing nucleic acids to ZP intact embryos to generate genetically modified animals. Unfortunately, VisuFect can deliver oligonucleotides to embryos, but not larger molecules, such as the plasmid DNA. Probably, genome editing in embryos derived from Cas9-expressing transgenic (Tg) mice [19–25] may be possible when VisuFect is used in combination with a smaller molecule such as guide (g) RNA.

As mentioned previously, EP enables the delivery of exogenous substances to somatic cells and ZP-enclosed embryos. Grabarek et al. [13] was the first to demonstrate that nucleic acids can be efficiently delivered to isolated embryos (oocytes and zygotes) by EP. Recently, Kaneko et al. [26] first demonstrated successful induction of genome editing of the target locus in rat embryos, when they were electroporated *in vitro* in the presence of CRISPR/Cas9-related components (Cas9 mRNA + gRNA). In this case, there was no need to weaken ZP, as Grabarek et al. [13] demonstrated. Since this report, similar success in the production of genome-edited animals has been reported in mice and pigs [27–29].

3. Intraoviductal instillation of a solution

Delivery of liposomally encapsulated DNA directly into the oviductal lumen was first reported by Esponda's group [30, 31]. The purpose of their study was to transfect epithelial cells lining the oviductal lumen, not to deliver genetic materials to preimplantation embryos floating in the oviductal lumen. They found that ~6% of oviductal epithelial cells were successfully transfected. Sato [32] injected a solution containing plasmid DNA [conferring enhanced green fluorescent protein (EGFP) expression] into the oviductal lumen of pregnant female mice on Day 0.4 (~11:00 h; corresponding to early zygotes; the day when the vaginal plugs are detected is designated as Day 0 of pregnancy), and then performed *in vivo* EP of an entire oviduct, in an attempt to transfect zygotes with the exogenous DNA. Unfortunately, the attempt to transfect zygotes failed; only a cellular remnant, probably derived from a part of the zygotes, was found to be fluorescent. In contrast, a maximum of 43% of oviductal epithelial cells facing the oviductal lumen, were fluorescent. It was speculated that the failure of gene delivery to the zygotes may be because the cumulus cells surrounding the zygotes function as a barrier [32].

Figure 1 represents a schematic illustration of the structure of murine oviduct and ovary, on different days of pregnancy; Days 0.4 (**Figure 1a**), 0.7 (~16:00 h; corresponding to late zygotes; **Figure 1b**) and 1.4 (~1d, 11:00 h; corresponding to 2-cell embryos; **Figure 1c**), are illustrated. After mating with males, ovulated oocytes, tightly surrounded by cumulus cells, are transferred to the specific site of the oviduct - the ampulla- following which fertilization occurs at

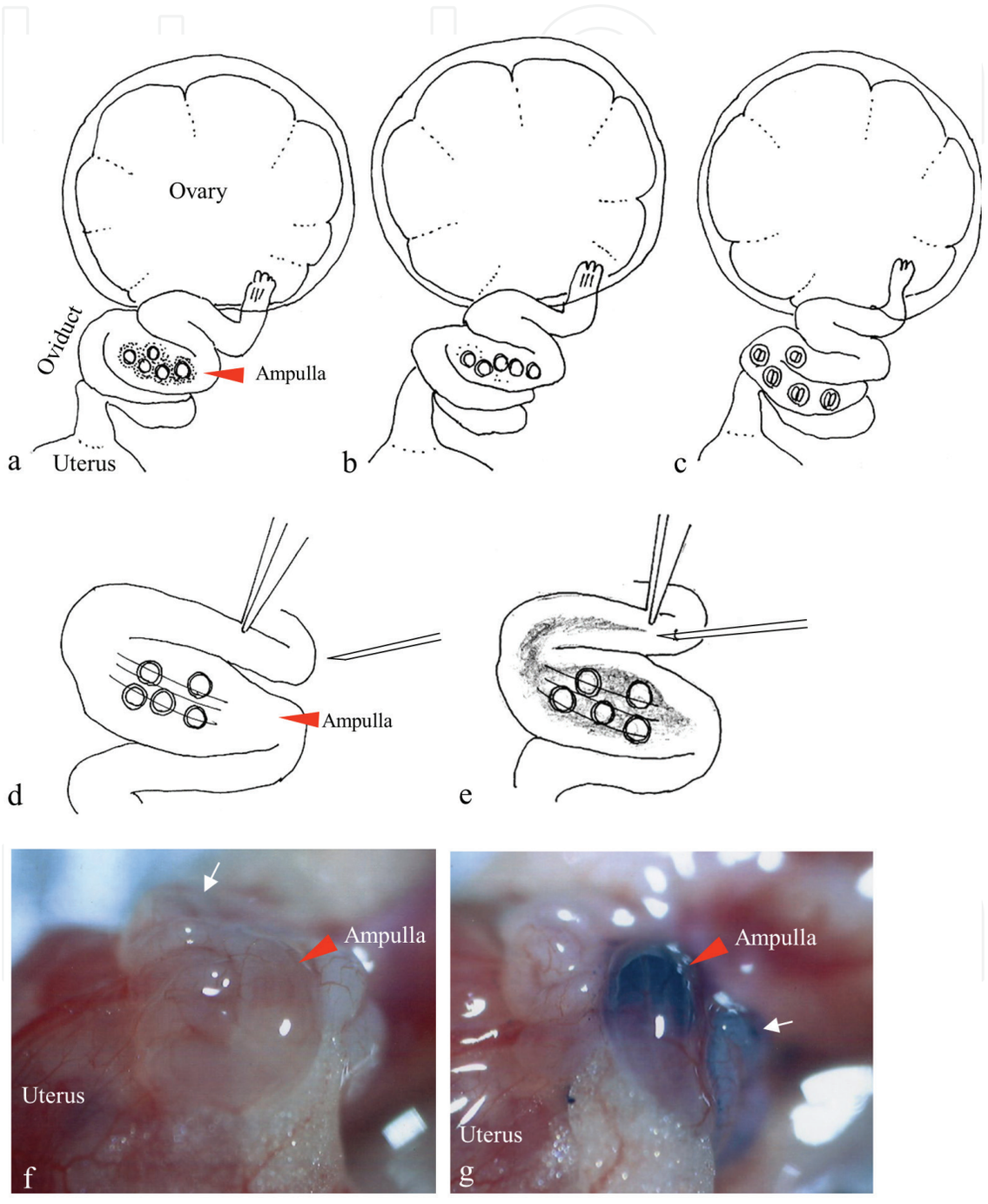

Figure 1. Procedure for the intraoviductal instillation of a solution. a-c: schematic illustration of ovary/oviduct/uterus of females at days 0.4 (a), 0.7 (b) and 1.4 (c) of pregnancy. d, e: schematic illustration of the moment before (d) and during (e) the intraoviductal instillation of a solution through oviductal wall. f, g: Oviduct at day 0.4 before (f) and after (g) intraoviductal instillation of trypan blue-containing solution. Arrows indicate the site through which a glass micropipette was inserted into the oviductal lumen.

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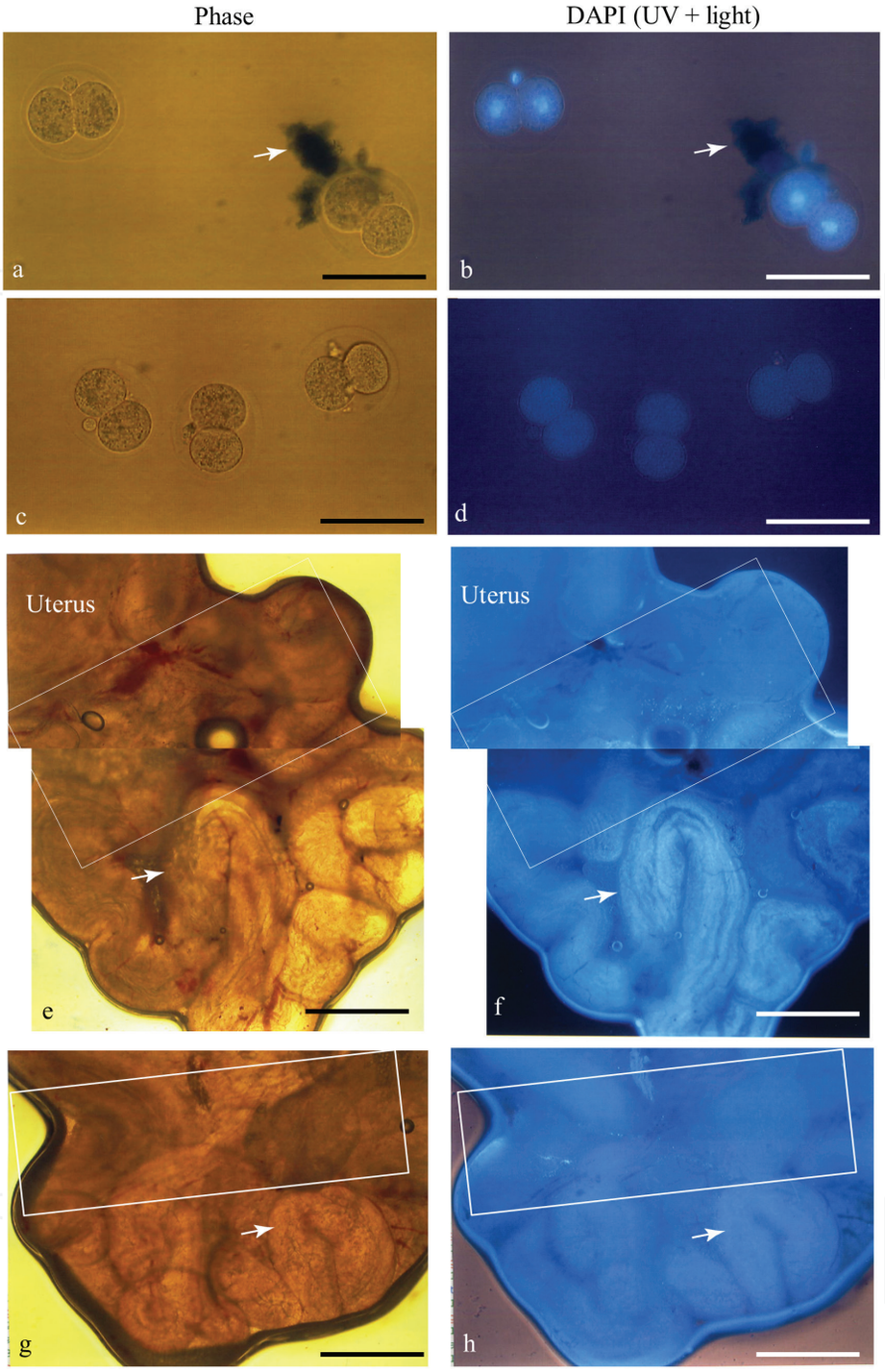

Figure 2. Intraoviductal instillation of a solution containing DAPI and trypan blue. a, b, two-cell embryos isolated from a female 1 day after the intraoviductal instillation of a solution containing DAPI and trypan blue on day 0.4 of pregnancy. Note the presence of bright DAPI-derived fluorescence in the nuclei of 2-cell embryos. Arrow indicates aggregates of trypan blue, which might have formed after introduction into the oviductal lumen. Bar = 100 μm. c, d, two-cell embryos isolated from a female one day after the intraoviductal instillation of a solution containing trypan blue alone, on day 0.4 of pregnancy. No fluorescence is detected in the nuclei. Bar = 100 μm. e, f, DAPI-derived fluorescence in the squashed oviduct from which 2-cell embryos [shown in (a) and (b)] were isolated. Note bright fluorescence in ampulla (arrows), and its neighboring segment of the oviduct. Box indicates the oviductal segment near isthmus. Bar = 250 μm. g, h: DAPI-derived fluorescence in the squashed oviduct from which the 2-cell embryos [shown in (c) and (d)] were isolated. No fluorescence is detected throughout the oviduct, including ampulla (arrows). Box indicates the oviductal segment near isthmus. Bar = 250 μm. a, c, e, g: Photographed under light; b, d, f, h: Photographed under UV and light.

this site. On Day 0.4, zygotes (fertilized eggs) are still enclosed by cumulus cells and exist at the ampulla. On Day 0.7, zygotes are still present at the ampulla, but the ampulla itself, exhibits shrinkage and detachment of cumulus cells from the zygote. On Day 1.4, zygotes cleave to form 2-cell embryos, and are present at the ampulla or the oviductal segment between the ampulla and the isthmus.

In Figure a schematic illustration of the moments before (**Figure 1d**) and during (**Figure 1e**) intraoviductal instillation of a solution is provided. Sato et al. [33] injected a solution containing Hoechst33342 dye, frequently used for vital nuclear staining, into the oviductal lumen of pregnant females at Day 0.4. One day after the surgery, 2-cell embryos were collected from the oviducts for checking the incorporation of the dye in their nuclei. They observed that all the collected embryos had fluorescent nuclei. This means that the dye injected into the oviductal lumen penetrates the ZP, enters the zygotes, and binds to their nuclear DNA. Here, we examined whether another dye 4′, 6-diamidino-2-phenylindole (DAPI), also used for staining of nuclei, can also bind to nuclear DNA of zygotes *in situ*. About 2 μL of a solution containing 1 μg/μL of DAPI and 0.05% trypan blue (used for monitoring successful injection) was injected into the oviductal lumen by piercing the oviductal wall, between the ampulla and the infundibulum (arrows in **Figure 1e** and **f**) of pregnant B6C3F1 (hybrid between C57BL/6 and C3H/He) females on Day 0.4. Successful instillation of a solution can be clearly visualized by the presence of blue dye inside the oviduct (arrowhead in **Figure 1g**). As control, trypan blue alone was similarly injected. When 2-cell embryos are collected from the treated females for inspection of fluorescence, all the collected embryos (6/6 tested) from DAPI-injected females had fluorescent nuclei (**Figure 2a** and **b**); in contrast, the control embryos did not have fluorescent nuclei (**Figure 2c,d**). Furthermore, inspection of DAPI-derived fluorescence in the squashed oviduct revealed the presence of fluorescence in epithelial cells of ampulla and its neighboring oviductal segment (**Figure 2e** and **f**), but no evident fluorescence in the distal segment of the oviduct, near the isthmus (boxed areas in **Figure 2e** and **f**), suggesting that DAPI introduced into the ampulla, might not have been transferred to the oviductal segment near the uterus in a day, after the instillation. The control oviduct never fluoresced (**Figure 2g** and **h**). Thus, DAPI introduced intraoviductally can penetrate ZP-enclosed zygotes and oviductal epithelia facing oviductal lumen.

4. Gene delivery into preimplantation embryos *in vivo*

To our knowledge, successful gene delivery into preimplantation embryos *in vivo* was first reported by Sato et al. [33], who demonstrated the intraoviductal instillation of a solution containing plasmid DNA (conferring EGFP expression) and trypan blue using B6C3F1 females on Day 1.4 of pregnancy (corresponding to 2-cell stage). Subsequently, *in vivo* EP into an entire oviduct was performed using a square-wave pulse generator, T-820 electroporator (BTX Genetronics Inc., San Diego, CA, USA), under the conditions of eight square-wave pulses, with a pulse duration of 50 ms, and an electric field intensity of 50 V. One day after the surgery, 8-cell embryos were collected for checking EGFP-derived fluorescence. About 33% of embryos exhibited fluorescence, although its intensity varied among embryos. However,

when the same procedure was later applied to 2-cell stage embryos using a different electroporator (NEPA21; NEPA GENE Co., Chiba, Japan), all the collected embryos failed to fluoresce (Sato et al., unpublished results). In this case, the ZP appears to act as a critical barrier, preventing substance penetration. In other words, ZP may allow penetration of smaller molecules such as gRNA, but not of large molecules like plasmid DNA. In this context, careful monitoring of EP conditions may be required when larger sized molecules are intended to be introduced into early embryos *in vivo*.

Takahashi et al. [34] employed the method of Sato et al. [33] to induce genome editing in the target gene (*EGFP* cDNA) in EGFP-expressing Tg 2-cell embryos *in situ*, through intraoviductal injection of a solution containing CRISPR/Cas9-related components (Cas9 mRNA + gRNA targeted to *EGFP* cDNA). Inspection of fluorescence under a fluorescence microscope demonstrated that 33% (2/6) of the resultant mid-gestational fetuses exhibited complete loss of EGFP-derived fluorescence, and 33% (2/6) exhibited reduced fluorescence. Sequencing analysis of genomic DNA isolated from these fetuses revealed that no mosaic mutations were detectable in the samples showing complete loss of fluorescence. Mosaic mutations were also detectable in the samples showing reduced fluorescence. Based on these findings, it was found that intraoviductal instillation of a solution containing CRISPR/Cas9-related components, and subsequent *in vivo* EP, enables genome editing in a whole embryo. Thus, Takahashi et al. [34] named this technology "Genome editing via Oviductal Nucleic Acids Delivery (GONAD)".

As mentioned above, GONAD on Day 1.5 results in production of individuals with highly frequent mosaic mutations. Genome editing at 2-cell embryo stage, appears to be the major cause of this problem. To circumvent the issue of mosaic mutations, performing GONAD on Day 0.7, a stage corresponding to late 1-cell embryos, from which cumulus cells begin to detach (see **Figure 1b**), was considered. Ohtsuka et al. [35] examined whether embryos at this stage are suitable for GONAD by injecting a solution containing *EGFP* mRNA (1 μg/μL) and trypan blue (0.05%) into the lumen of an oviduct of pregnant ICR females, since EGFP-derived fluorescence can be easily monitored under a fluorescence microscope. Two days after GONAD, 8-cell embryos were isolated from the oviducts of the GONAD-treated females, and checked for EGFP-derived fluorescence; several of the collected 2-cell embryos were found to be fluorescent. Notably, no or less biased fluorescence was evident in each blastomere among the fluorescent embryos, suggesting low mosaicism in the treated embryos.

5. Genome editing in preimplantation embryos after performing GONAD on day 0.7

Next, we tested whether GONAD can indeed induce specific mutations on the endogenous target locus in late 1-cell embryos. We chose α-1, 3-galactosyltransferase (α-GalT) gene (*GAAT1*) as a target gene to be knocked out. α-GalT is a key enzyme, capable of synthesizing the cell-surface glycoprotein, called α-Gal epitope that is expressed in all mammals, except humans and Old-Word monkeys [36, 37]. Complete KO of *GAAT1* leads to loss of α-Gal epitope expression, which can be easily monitored by staining with α-Gal epitope-specific

lectin called BS-I-B_4 (IB4) [38, 39]. Indeed, cloned porcine blastocysts, after reconstitution with *GAAT1* KO nuclei, lost the reactivity against fluorescence-labeled IB4. However, residual amounts of α-Gal epitope, which may have been derived from maternally accumulated products of α-GalT, were occasionally recognized by the α-Gal epitope-specific lectin in the cloned blastocysts [40].

We designed gRNA (5′-GAGAAAATAATGAATGTCAA-3′) for targeting exon 4 of murine *GAAT1*. A part of the gRNA was synthesized by Integrated DNA Technologies, Inc. (IDT; Coralville, Iowa, USA) as Alt-R™ CRISPR crRNA product. The crRNA and tracrRNA (purchased from IDT) were combined for annealing and then mixed with recombinant Cas9 protein (TaKaRa Shuzo Co. Ltd., Shiga, Japan) to form a complex called ribonucleoprotein (RNP). The final concentrations of the components in RNP were 30 μM (for crRNA/tracrRNA) and 1 mg/ml (for Cas9 protein). GONAD was performed in superovulated B6C3ICR (hybrids between B6C3F1 and ICR) females (10–16 weeks of age) using RNP. Two days later, morula were collected from the oviducts, fixed in 4% paraformaldehyde (PFA) for 1 day at 4°C, and then stained with Alexa Fluor 594 (AF594)-labeled IB4 lectin (hereafter referred to as AF594-IB4) for 1 day at 4°C. About 42% of morulae (5/12) tested, exhibited decreased levels of fluorescence after staining with AF594-IB4, as exemplified by embryo #4 (**Figure 3a** and **b**), suggesting successful genome editing at the target locus, *GAAT1*. Notably, the residual amounts of maternally accumulated α-Gal epitope, appeared to be still discernible in this embryo (#4 in **Figure 3a** and **b**). The remaining seven embryos (including the embryos #1 to #3 in **Figure 3a,b**)

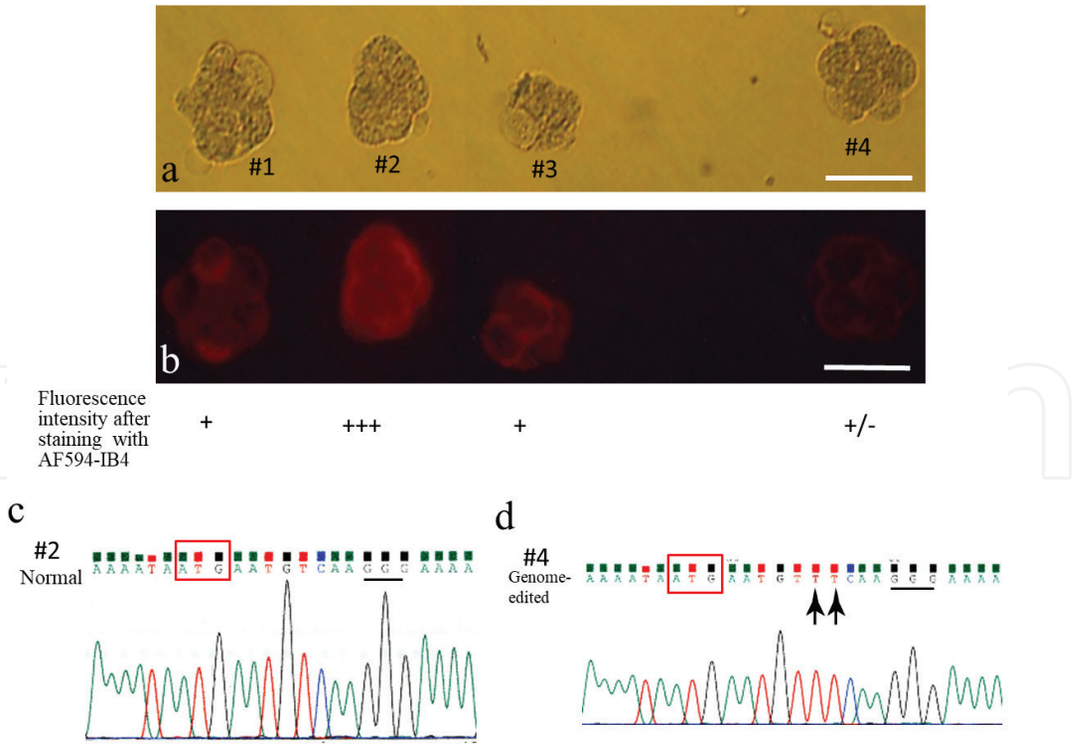

Figure 3. *i*-GONAD-mediated production of genome-edited morulae using RNP, targeted to mouse *GAAT1*. A, b, staining of *i*-GONAD-treated embryos with AF594-IB4. Of the four embryos stained, embryo #4 exhibited highly reduced expression of α-gal epitope. Bar = 100 μm. a: Photographed under light; b: Photographed under UV and light. c, d: direct sequencing of PCR products derived from the *i*-GONAD-treated single embryo, shown in (a) and (b), and corresponding to the embryos #2 and #4, respectively. Boxes indicate the translation initiation codon ATG; underlines indicate PAM.

exhibited reactivity to AF594-IB4, suggesting that they might be unedited or have mono-allelic mutations for *GAAT1* locus. These stained embryos were individually subjected to lysis for genomic DNA isolation, whole genome amplification (WGA), and PCR/nested PCR for direct sequencing, to examine the presence of mutations in the target gene, based on our previous procedure for detecting mutations in the porcine *GAAT1* gene [41, 42]. The primers used were 5′-GCAAATGTGGATGCTGGGAAC-3′ (sense primer)/5′-ACAGTTTTAATGGCCATCTGG-3′ (reverse primer) and nested primers were 5′-TGAATCGAGCAGGTGTTTCAT-3′ (sense primer)/5′- AGGAACACAGGAAGACTGGAC-3′ (reverse primer). The expected size of PCR products was 390 bp (for 1st PCR) and 344 bp (for nested PCR). Direct sequencing of the PCR products demonstrated that, in the embryo showing decreased expression of α-Gal epitope (#4 in **Figure 3a** and **b**), there was an insertion of two nucleotides (AA) (shown by arrows in #4 of **Figure 3c**) above the protospacer adjacent motif (PAM) on the target locus, *GAAT1*. Furthermore, the sequence around the insertion site had no additional peak in the electrophoretogram, suggesting that #4 is a homozygous bi-allelic KO embryo. The amino acid sequence deduced from the nucleotide sequence of sample #4 is Met N V S R E K Stop, which differs from that of the authentic sequence (Met N V K G K V I L L Met L I---) and had premature termination of protein synthesis at seven amino acids starting from Met. On the other hand, embryo #2, showing normal expression of α-Gal epitope, had no mutation on the target region (#2 of **Figure 3c**). Thus, GONAD, using RNP, is useful for inducing genome editing within the target locus in preimplantation embryos.

6. Improved (*i*)-GONAD in mice

As already mentioned in Section 4, Ohtsuka et al. [35] modified GONAD as follows: 1) RNP was employed instead of Cas9 mRNA and gRNA; and 2) *in situ* genome editing within embryos was carried out at Day 0.7 of pregnancy. As a result, they succeeded in obtaining mice with insertion/deletion mutations in their genome at relatively high frequencies (~100%), as well as mice with large deletions in a target gene, and with knock-in of a desired sequence, into a target locus. Furthermore, the mutations acquired through genome editing, in the target locus, were transmitted to the next generation. Therefore, they re-named this improved technology "improved GONAD (*i*-GONAD)". According to Ohtsuka et al. [35], *i*-GONAD is usually performed at ~16:00 from the day of the vaginal plug detection. However, this timing often causes inconvenience for users, such as researchers and technicians. If possible, experiments are done during the daytime, from 9:00 to 16:00. Since the timing for ovulation, and fertilization by sperm, can be shifted by changing the time of gonadotrophin administration, we first examined whether the time at which late 1-cell embryos are obtained may be shifted forward, by administering gonadotrophins, at an earlier time. Adult B6C3ICR females (10–16 weeks of age) were intraperitoneally (i.p.) injected with 5 IU of pregnant mare's serum gonadotrophin (PMSG; eCG) at 11:00, followed by human chorionic gonadotrophin (hCG) of the same dose, 48 h later. Thereafter, the females were allowed to mate with the males. The next morning (~11:00 h; corresponding to Day 0.7 of pregnancy), oviduct/ovary/uterus were dissected and the morphology of ampulla was observed under a dissecting microscope; the ampulla exhibited a tendency of shrinkage (arrow in **Figure 4a**). Flushing of the oviduct resulted in release of cumulus cell-free zygotes (**Figure 4b**). These findings were

indeed consistent with the previous findings of Ohtsuka et al. [35], who observed shrinkage of ampulla and cumulus cell-free zygotes when females, at Day 0.7 of pregnancy, were inspected at ~16:00. Thus, hormone-mediated shift in timing for acquiring late 1-cell embryos was found to be successful.

Administration of higher dose (in this case, more than 5 IU) of gonadotrophins can induce superovulation, but often causes failure to deliver pups [43–45]. Some reports state that the administration of low-dose gonadotrophins (less than 5 IU) facilitates the ovulation of a natural number of oocytes, and successful delivery of pups [46, 47]. This evoked us to suppose that the use of females who have achieved pregnancy after administration of low-dose gonadotrophins and subsequent mating with males would be preferable for *i*-GONAD experiments. To verify this strategy, three adult B6C3ICR female mice (10~16 weeks of age) were i.p. administrated with either 5, 2, or 0.5 IU of PMSG at 11:00, followed by 5 IU of hCG, 48 h later (**Figure 4c**). In this case, light/dark cycle (7:00/19:00) in a mouse room remained unchanged. Thereafter, the females were mated to males (with a ratio of one female: one male). In case of administration of 5 IU PMSG, two of three females had vaginal plug, but failed to deliver their pups (**Figure 4d**). When these females were inspected later, one female was found to

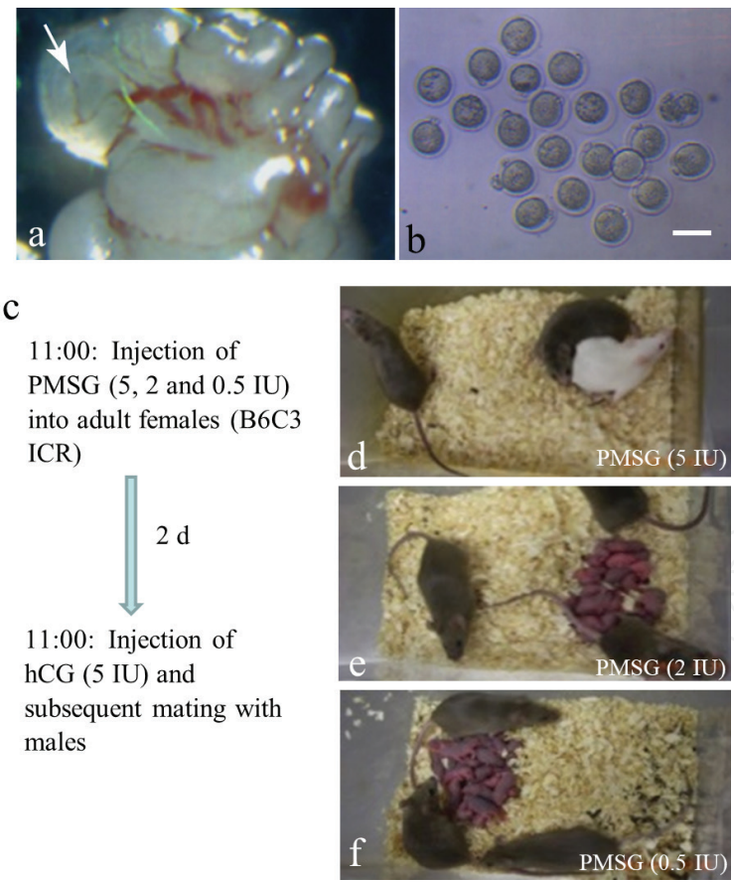

Figure 4. Late zygotic stage embryos can be obtained when superovulation schedule is shifted forward. a, oviduct dissected from a female one day after administration of superovulation-inducing hormones at 11:00. Ampulla (arrows) begins to show shrinkage. b, late zygotes collected from the oviduct shown in (a). Note that the isolated zygotes are already free from cumulus cells. Bar = 100 μm. c, a regime for performing *i*-GONAD towards late 1-cell embryos by administering gonadotrophins at an early time. d-f, birth of pups from females administered varying amounts [5 IU (d), 2 IU (e), or 0.5 IU (f)] of PMSG. Note that females treated with low-dose PMSG (2–0.5 IU) successfully delivered normal number of pups, but those treated with 5 IU PMSG failed to deliver their pups.

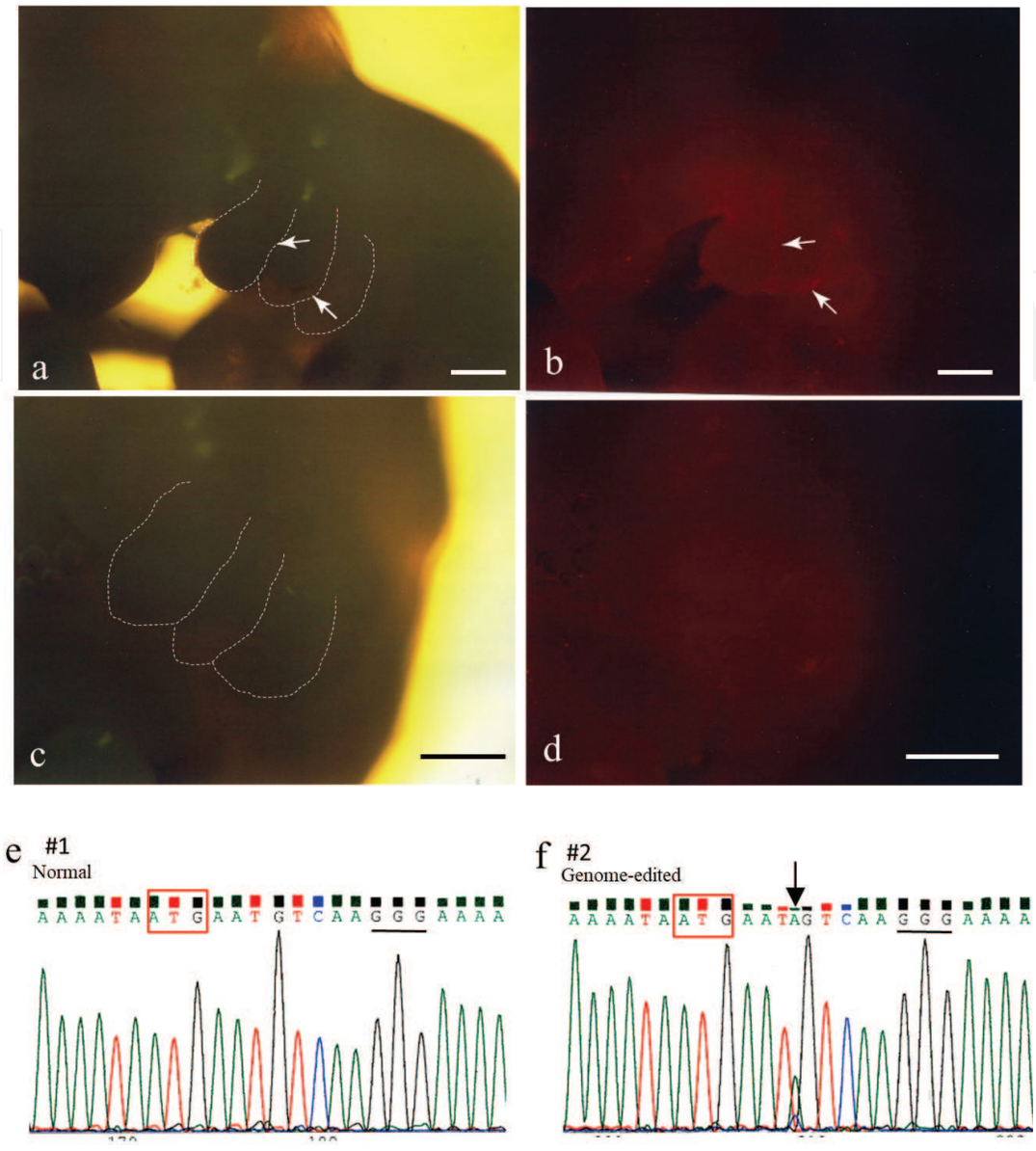

Figure 5. *i*-GONAD-mediated production of genome-edited mid-gestational fetuses, using RNP targeted to mouse *GAAT1*. a–d, staining of *i*-GONAD-treated fetuses with AF594-IB4. One fetus [numbered #1; shown in (a) and (b)] was strongly stained by lectin (indicated by arrows), while the other fetus [numbered #2; shown in (c) and (d)] exhibited highly reduced expression of α-gal epitope. Bar = 500 μm. a, c: Photographed under light; b, d: Photographed under UV and light. c, d: Direct sequencing of PCR products derived from the *i*-GONAD-treated single fetuses, shown in a-d. Boxes indicate translation initiation codon ATG; underlines indicate PAM.

have two dead fetuses in its uterus. In case of administration of 2 IU PMSG, all three females had vaginal plug, and then delivered a total of 23 pups (average: 8) (**Figure 4e**). In case of administration of 0.5 IU PMSG, also, all three females had vaginal plug, and subsequently delivered a total of 25 pups (average: 8) (**Figure 4f**). These findings suggest that administration of low-dose PMSG at 11:00 can lead to natural delivery of normal numbers of pups.

Next, we tested whether genome-edited live mid-gestational fetuses can be obtained by *i*-GONAD, using the same conditions mentioned in Section 4. Adult B6C3ICR females (10–16 weeks of age) were administrated with low-dose hormones according to the regime shown in **Figure 4c**. After administration of hCG, females were mated to males. Next morning,

i-GONAD, using RNP targeted to mouse *GAAT1*, was performed in vaginal plug-positive females at ~11:00. One of the four females had 14 normal and viable fetuses, when inspection was done 11 days after the surgery. All fetuses dissected were fixed in 4% PFA for 1 day at 4°C, and then stained with AF594-IB4 for 1 day at 4°C. Untreated Day 11.5 fetuses were similarly stained with lectin as control. Fluorescence on the surface of the stained fetuses was then inspected under a fluorescence microscope. Ten of the 14 fetuses showed positive and strong staining at the branchial arch (shown by arrows in **Figure 5a** and **b**), like the control fetus. However, the remaining four fetuses exhibited reduced staining for AF594-IB4, at the surface of the branchial arch (**Figure 5c** and **d**). Two of these four α-Gal epitope-negative fetuses, together with another α-Gal epitope-positive one, were subjected to genomic DNA isolation and subsequent PCR analysis, as described in Section 5. Direct sequencing of PCR products demonstrated that the α-Gal epitope-positive samples exhibited unedited sequence of *GAAT1* (**Figure 5e**), like an intact control fetus. In contrast, the samples showing reduced staining for AF594-IB4 (shown in **Figure 5c** and **d**) had one nucleotide (A) insertion above PAM (**Figure 5f**), creating an abnormal truncated protein with sequence MetN S Q G K S N P S Stop. Notably, the presence of a minor single nucleotide "C" overlapped with the inserted nucleotide "A", suggesting that this sample may comprise bi-allelic KO, with different mutated nucleotides. The other α-Gal epitope-negative sample also had mutations (insertion of AA) above PAM in the exon 4 of *GAAT1* (data not shown). From these results, *i*-GONAD performed in females at Day 0.7 of pregnancy, in which natural ovulation was induced after administration of low-dose PMSG, is proven useful for obtaining genome-edited animals (fetuses).

7. Conclusion

Since preimplantation embryos, including embryos at stages of fertilized eggs to cleaved eggs, are floating in the oviductal lumen, it is easy to manipulate them through introduction of substances (such as genome editing-related components, plasmid DNA, viral vectors and chemical reagents) into the oviductal lumen. Particularly, intraoviductal instillation of genome editing-related components and subsequent *in vivo* EP into an entire oviduct are now considered one of the most useful ways to create genome-edited animals (at least in mice and rats) in a convenient manner. Unfortunately, these approaches still require an expensive electroporator apparatus for gene delivery. Rather, in situ transfection of preimplantation embryos without using EP would be desirable. To achieve this, intraoviductal instillation of ZP-penetrating substances (as exemplified by reagents for receptor-mediated gene transfer, MWNTs and VisuFect), together with genome editing-related components, would be one of the promising approaches. If this attempt meets with success, the creation of gene-modified animals, including larger animals such as pigs and bovines, would be accelerated.

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Conflicts of interest

The founding sponsors had no role in the design of the study, collection, analyses, or interpretation of data, writing of the manuscript, and decision to publish the results.

Author contributions

Masahiro Sato designed the study and drafted the manuscript; Masato Ohtsuka provided information of *i*-GONAD-related study and revised the manuscript; Shingo Nakamura critically revised the manuscript.

Author details

Masahiro Sato[1], Masato Ohtsuka[2,3,4] and Shingo Nakamura[5]*

*Address all correspondence to: snaka@ndmc.ac.jp

1 Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima, Japan

2 Division of Basic Molecular Science and Molecular Medicine, School of Medicine, Tokai University, Kanagawa, Japan

3 Center for Matrix Biology and Medicine, Graduate School of Medicine, Tokai University, Kanagawa, Japan

4 The Institute of Medical Sciences, Tokai University, Kanagawa, Japan

5 Division of Biomedical Engineering, National Defense Medical College Research Institute, Saitama, Japan

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