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i-GONAD: A method for generating genome-edited animals without ex vivo handling of embryos

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Abstract

The recent development of genome editing technologies has enabled the creation of genome-edited animals, with alterations at the desired target locus. The clustered regularly interspaced short palindromic repeats (CRISPR) system is widely used for this purpose because it is simpler and more efficient than other genome editing technologies. The conventional methods for creation of genome-edited animals involve ex vivo handling of embryos (zygotes) for microinjection or in vitro electroporation. However, this process is laborious and time-consuming, and relatively large numbers of animals are used. Furthermore, these methods require specialized skills for handling embryos. In 2015, we reported a novel method for the creation of genome-edited animals without ex vivo handling of embryos. The technology known as Genome-editing via Oviductal Nucleic Acids Delivery (GONAD) involved intraoviductal instillation of genome editing components into a pregnant female and subsequent in vivo electroporation of an entire oviduct. The genome editing components present in the oviductal lumen are transferred to preimplantation embryos in situ for introducing insertion or deletion (indel) mutations at the desired loci. This technology was further improved by optimizing several parameters to develop improved GONAD (*i*-GONAD) for the efficient generation of mutant or knock-in animals. In this review, we discuss the historical background, potential applications, advantages, and future challenges of GONAD/*i*-GONAD technology.

KEYWORDS

CRISPR, gene delivery, genome editing, *i*-GONAD, in vivo electroporation

1 | HISTORICAL BACKGROUND

1.1 | Genome editing technology

Gene modification based on recently developed techniques, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas), is recognized as a revolutionizing genetic engineering tool in vitro and in vivo (Fernández, Josa, & Montoliu, 2017; Kim & Kim, 2014). Among these techniques, CRISPR/Cas system has gained the most popularity because of its ease of application; CRISPR/Cas requires only two components, the Cas nuclease and a guide RNA (gRNA), which is a short RNA sequence that guides the Cas protein to the target site. CRISPR/Cas has an ability to elicit a double-strand DNA break (DSB) at a target genomic locus (Komor, Badran, & Liu, 2017). Subsequently, in the absence of homologous template to repair, non-homologous end joining (NHEJ) causes small insertions or deletions (indels). However, if a homologous template is present, homology-directed repair (HDR) occurs. The process of NHEJ introduces indels occur at a higher frequency than HDR-mediated knock-in of

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the gene-of-interest (GOI) (Mao, Bozzella, Seluanov, & Gorbunova, 2008; Ren, Xu, Segal, & Zhang, 2019).

Clustered regularly interspersed short palindromic repeats/Cas has been used to generate genome-edited cell lines, animals and plants for various research fields including preclinical drug testing, disease modeling and potential therapeutic purposes (Fellmann, Gowen, Lin, Doudna, & Corn, 2017; Puchta, 2017; Ren et al., 2019). In earlier studies of CRISPR/Cas-related genome editing, the use of DNA-based vector for expression of Cas9 and gRNA or in vitro synthesized gRNA and Cas9 mRNA was frequent (Harms et al., 2014). However, to achieve a high rate of genome editing using the CRISPR/Cas system, recombinant Cas9 protein mixed with synthetic gRNA (e.g., a complex between CRISPR RNA [crRNA] and trans-activating crRNA [tracrRNA] called CRISPR ribonucleoprotein [RNP]) is now frequently used as a cloning-free, direct delivery of CRISPR components (Aida et al., 2015; Quadros et al., 2017).

1.2 | Conventional gene delivery into zygotes

To produce genome-edited animals, such as mice, rats, rabbits, and monkeys, direct zygotic microinjection of CRISPR/Cas9 components has been used as a major tool at the initial stage of genome editing experiments (Figure 1) (Fujii, Kawasaki, Sugiura, & Naito, 2013; Harms et al., 2014; Ma et al., 2014; Niu et al., 2014; Shao et al., 2014; Yang et al., 2014). This microinjection-based approach requires ex vivo handling of embryos such as microinjection of genome editing components into the zygote using an expensive manipulator, tentative cultivation of injected embryos, and transfer of embryos (called embryo transfer [ET]) to the oviducts of pseudo-pregnant recipient females for further development of the embryos (Table 1). Furthermore, this requires skilled technicians to handle the manipulator and perform ET, and preparation of vasectomized males and pseudo-pregnant recipient females for ET, which is time-consuming and laborious.

In 2014, Kaneko et al. first demonstrated that efficient genome editing can be achieved when rat zygotes are subjected to in vitro

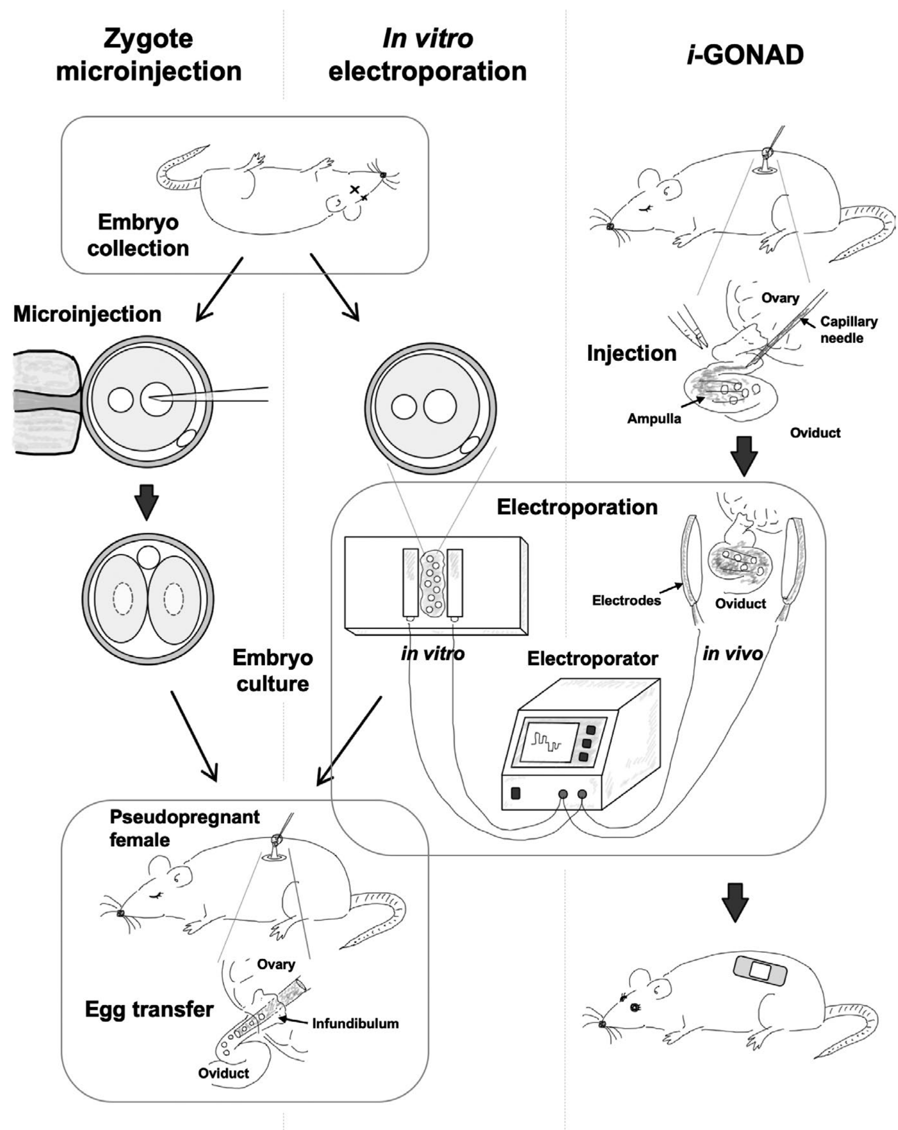

FIGURE 1 Schematic representation of three genome editing methodologies. Steps involved in all three methods (zygote microinjection, in vitro electroporation, and *i*-GONAD) used for generating genome-edited animals are shown

TABLE 1 Comparison of three methods, zygote microinjection, in vitro electroporation, and improved genome-editing via oviductal nucleic acids delivery (*i*-GONAD), used for generating genome-edited animals

	Zygote microinjection	*In vitro* electroporation	*i*-GONAD
Experimental procedures			
Ex vivo embryo culture	Req.	Req.	Not req.
Preparation of pseudo-pregnant female (maintenance of vasectomized male)	Req.	Req.	Not req.
Sacrificing pregnant females	Req.	Req.	Not req.
ET to pseudo-pregnant females	Req.	Req.	Not req.
Apparatus			
Micromanipulator	Req.	Not req.	Not req.
Electroporator	Not req.	Req.	Req.
Micropipette puller	Req.	Not req.	Req./not req.
Approximate number of mice required to obtain 20 pups[a]			
Pregnant female mice	8	3	5
Male mice for mating or Male mice for IVF	8 (mating) or 1 (IVF)	3 (mating) or 1 (IVF)	5
Vasectomized males	6	4	0
Pseudo-pregnant mice	6	4	0
Approximate amount of genome editing components required to obtain 20 pups[b]			
Amount of solution prepared (μl)	20	6–20	17
Cas9 protein (μg)	1	0.6–13	17 (can be reduced to 1/10)
gRNA (μg)	0.4	1.2–2.7	17
ssODN (μg)	0.2	2.4–10	34
DNA delivery capability for KI			
Plasmid DNA	Suitable	Not suitable	Not suitable
ssODN	Suitable	Suitable	Suitable
Approximate time required for experiment to obtain 20 pups (hours)			
Zygote collection or Egg collection (for IVF)	2 or 3 (IVF)	1 or 3 (IVF)	0
Delivery of CRISPR components	2	0.3	1
ET	1.5	1	0
Features	Highly developed skill is required for microinjection. CRISPR components can be delivered directly into the nucleus by pronuclear injection.	Many zygotes (~50) can be treated simultaneously. Microinjection is not required.	Free from all three steps required for conventional methods (zygote collection, microinjection and ET).

[a]The rates of development to two-cell stage are assumed to be 70% (for zygote microinjection) and 90% (for in vitro EP), and those of development to full term after transplantation 20% (for zygote microinjection) and 40% (for in vitro EP).
[b]These amounts were determined based on our experiences for zygote microinjection and *i*-GONAD, and on the data from other groups for in vitro EP (Hashimoto et al., 2016; Tröder et al., 2018).

electroporation (EP) in the presence of genome editing components (Kaneko, Sakuma, Yamamoto, & Mashimo, 2015). The success of this technology was subsequently reported by Kaneko et al. and other researchers using mice (Chen, Lee, Lee, Modzelewski, & He, 2016; Hashimoto, Yamashita, & Takemoto, 2016; Kaneko & Mashimo, 2015; Qin et al., 2015; Teixeira et al., 2018; Tröder et al., 2018). Although this approach simplified the process of gene delivery into embryos, it still requires ex vivo handling of embryos, such as embryo collection, short periods of embryo culture, and subsequent ET (Figure 1; Table 1).

1.3 | In vivo gene transfer into zygotes

The most desirable approach for obtaining genome-edited animals would be in situ transfection of zygotes (present within the oviductal lumen) with genome editing components that have been already

delivered to the oviductal lumen. This approach will help avoid the need for ex vivo handling of embryos during the production of genome-edited animals. Esponda and his colleagues first performed intraoviductal injection of liposomally encapsulated plasmid DNA in an attempt to transfect epithelial cells lining the oviductal lumen (Relloso, 2000; Relloso & Esponda, 1998). The authors succeeded to transfect approximately 6% of the oviductal epithelial cells using this approach. The objective of the authors was to demonstrate the transfection of murine oviductal epithelium by exogenous DNA and to investigate the biological function of an oviduct using this technology. We predict that this approach would also be useful for delivering genetic material to preimplantation embryos floating in the oviductal lumen, if the transfection efficiency is improved, and the genetic material could permeate through the zona pellucida.

In vivo EP is a powerful technology to deliver nucleic acids into organs or parts of the animal body and has been used since the 1990s (Aihara & Miyazaki, 1998; Muramatsu, Mizutani, Ohmori, & Okumura, 1997; Titomirov, Sukharev, & Kistanova, 1991). EP has been applied in various fields of study, particularly in the field of developmental biology, as reviewed by Nakamura et al. (Nakamura & Funahashi, 2013). This physical delivery method increases the permeability of cell membrane when exposed to an electric field. We hypothesize that in vivo gene delivery into zygotes is possible if DNA solution is introduced into the oviduct, followed by EP, which would induce the formation of microscopic holes in zona pellucida. Sato injected a solution (approximately 2 μl) containing naked circular plasmid DNA conferring enhanced green fluorescent protein (eGFP) expression into the oviductal lumen of pregnant female mice on Day 0.6 (between 14:00 and 15:00 on the day when vaginal plugs were detected) using a glass micropipette attached to the mouthpiece (Sato, 2005). Immediately after instillation, the entire oviduct was subjected to in vivo EP using tweezer-type electrodes with a square-wave pulse generator, T-820 electroporator (BTX), to enhance the delivery of DNA into zona pellucida encapsulated zygotes (at early one-cell stage) and oviductal epithelia. This attempt, however, failed to transfect zygotes, evident from the absence of eGFP fluorescence in morulae isolated from the treated oviducts. However, 36% of the oviductal epithelial cells facing the oviductal lumen were fluorescent. We speculate that the inability to transfect zygotes with exogenous plasmid may be ascribed to the presence of cumulus cells surrounding the zygote that protected the zygote from the introduction of foreign DNA. These experiments show that EP is a powerful tool for in vivo gene delivery toward into tissues/organs. Furthermore, if preimplantation embryos are free of cumulus cells, the transfection into zygotes can be possible.

To test this possibility, we next performed intraoviductal injection of a plasmid DNA-containing solution into pregnant female mice on Day 1.6 (corresponding to two-cell stage, when cumulus cells are detached) and subsequently applied in vivo EP to the entire oviduct. Analysis of the recovered 8-cell embryos showed eGFP fluorescence in 10% (19/192) of the embryos with varying intensity (Sato, Akasaka, Saitoh, Ohtsuka, & Watanabe, 2012). These findings suggest that preimplantation at the two-cell stage or more advanced stages increases in situ transfection efficiency when exogenous DNA is introduced within an oviductal lumen, followed by in vivo EP to enhance DNA uptake by the embryos.

1.4 | Development of GONAD

We recently demonstrated that genome editing is possible in murine two-cell embryos, when a solution containing CRISPR/Cas9-related components, including Cas9 mRNA and gRNA targeting *eGFP*, is injected into the oviductal lumen of a wild-type pregnant female, which was successfully mated with transgenic males homozygous for the *eGFP* transgene, followed by in vivo EP of the solution-injected oviducts (Takahashi et al., 2015). All resulting fetuses have mono-allelic *eGFP* sequence and its disruption by the CRISPR/Cas9-related components would result in the loss of eGFP fluorescence. When the developed mid-gestational and nearly full-term fetuses were examined for fluorescence, we obtained fetuses with complete loss of fluorescence or reduced fluorescence, indicating that the former fetuses have no mosaic mutations, while the latter fetuses harbor mosaic mutations because of the presence of a mixture comprised of genome-edited and non-edited cells, which was confirmed by sequencing analysis. Thus, intraoviductal instillation of a solution containing CRISPR/Cas9-related components and subsequent in vivo EP are effective for producing genome-edited individuals. We termed this novel technology as Genome-editing via Oviductal Nucleic Acids Delivery (GONAD).

2 | DEVELOPMENT OF *I*-GONAD AND ITS APPLICATIONS

In our initial attempt, as mentioned above, we performed the GONAD treatment on pregnant females on Day 1.5 (Takahashi et al., 2015). In this attempt, almost all zygotes were expected to be at the two-cell stage; thus all edits would result in mosaic offspring. However, there are possibilities of obtaining non-mosaic offspring if (a) one blastomere of the two-cell embryo dies, and the embryo develops only from the other blastomere, or (b) the same editing occurs in both blastomeres. To obtain non-mosaic offspring, it is desirable to perform GONAD at the zygote (one-cell) stage.

2.1 | GONAD on day 0.7

Pregnant females on the day of having copulation plug contain one-cell stage embryo in the ampulla (in which zygotes exist massively), the dilated area of the oviduct where the fertilization occurs. To assess the optimal time for GONAD, we injected *eGFP* mRNA into the oviductal lumen of pregnant females on Day 0.4 and Day 0.7, and performed in vivo EP (Ohtsuka et al., 2018). Observation of embryos from the two-cell to blastocyst stage showed eGFP fluorescence only in the embryos derived from females subjected to GONAD on Day 0.7 of pregnancy. By contrast, no fluorescence was detected in embryos subjected to GONAD treatment on Day 0.4 of gestation. These

results are probably explained by the presence of cumulus cells surrounding the zygotes at Day 0.4; however, by Day 0.7, connections of these cumulus cells become loose, which allows *eGFP* mRNA solution to reach close to zygotes. Based on this experiment, we conclude that Day 0.7 is the optimal time for GONAD treatment (Ohtsuka et al., 2018).

Next, we tried to create *forkhead box E3* (*Foxe3*) deficient mice by injecting *Cas9* mRNA and gRNA (targeting *Foxe3*) into the oviducts of pregnant females on Day 0.7 of gestation, followed by the application of electric pulses (Ohtsuka et al., 2018). The newborns delivered from these females harbored indel mutations at *Foxe3* with an efficiency of 31% (11/36). However, the frequency of mosaicism was high (82% [9/11]), and only two newborns were non-mosaic.

Performing GONAD on Day 0.7 of gestation is more advantageous than performing it on Day 1.5 because zygotes remain in a restricted area within the oviduct (ampulla) until Day 0.7, and therefore it is enough to elicit genome editing in embryos through injection of the genome editing solution into the ampulla. However, by Day 1.5, embryos are thought to move forward toward the uterus.

2.2 | Development of improved GONAD (*i*-GONAD) using CRISPR RNP

Genome editing efficiency was further improved using Cas9 protein together with crRNA/tracrRNA; we termed this RNP-based GONAD as *i*-GONAD (Figure 1, Table 1). Application of *i*-GONAD to the *Foxe3* locus resulted in 97% (35/36) genome-edited offspring compared with 31% edited using *Cas9* mRNA/gRNA (Ohtsuka et al., 2018). Interestingly, lower frequency of mosaic offspring (57% [20/35]) was obtained using *i*-GONAD compared with those obtained using *Cas9* mRNA-based GONAD (82%), although the difference was not statistically significant. Thus, RNP can lower the rate of mosaicism because it acts immediately after the delivery of CRISPR components to the zygotes.

2.3 | Generation of mice carrying large deletions using *i*-GONAD

The *i*-GONAD technology was successfully adapted to induce large deletions in mice genome. Using two gRNAs with *i*-GONAD, we deleted a 16.2-kb genomic region containing retrotransposon sequence located in the intron 1 of the *Agouti* gene in C57BL/6J mice (Ohtsuka et al., 2018). The efficiency of deletion was estimated at 50% (3/6), based on the change in coat color from black to agouti and sequencing of PCR products.

2.4 | Single-stranded oligodeoxynucleotides (ssODN) knock-in using *i*-GONAD

The high frequency of indel mutation by *i*-GONAD encouraged us to apply this method for inducing small genetic changes by ssODN knock-in (Ohtsuka et al., 2018). The term ssODN refers to ssDNA up to 200 nt in length. The point mutation within the *Tyrosinase* (*Tyr*) gene of albino mice (e.g., MCH[ICR]) was chosen as a target because successful knock-in of ssODN containing wild-type sequence can be easily identified based on the change in eye pigmentation in fetuses and coat color in newborns. Offspring with pigmentation were obtained from the *i*-GONAD-treated females with a frequency of 49% (36/74). We referred to this experiment as the *Tyr* rescue experiment.

Similar ssODN knock-in experiments were successful in the C57BL/6N inbred mouse strain for targeting other loci including *Cdkn1a* and *Cdkn2a* with an efficiency of 31% (4/13) and 40% (4/10), respectively (Ohtsuka et al., 2018).

2.5 | Knock-in of longer sequences (up to 1 kb) using *i*-GONAD

Knock-in of a full-length gene coding sequence into the genome is one of the most demanding genetic changes in animal models. However, knock-in efficiency has been generally low even with CRISPR tools. Thus, the generation of knock-in mice has been challenging. We recently improved knock-in efficiency using long ssDNA as donor templates. This method referred to as *Easi*-CRISPR allows target insertion of approximately 2-kb sequence through zygote microinjection (Miura, Quadros, Gurumurthy, & Ohtsuka, 2018; Quadros et al., 2017). We successfully applied *Easi*-CRISPR with *i*-GONAD to generate reporter gene knock-in mouse models for *paired like homeodomain 3* (*Pitx3*) and *TPA inducible sequences 21* (*Tis21*) genes (Ohtsuka et al., 2018). The knock-in efficiency (15% [5/34] for *Pitx3* and 7% [1/14] for *Tis21*) appeared to be slightly lower than that obtained via zygote microinjection (25%–67%), although we have not compared these methods using the same loci in side-by-side experiments. Nonetheless, this experiment confirmed that *i*-GONAD could be applied for knock-in of DNA fragments up to 1 kb. The drawback of this strategy is that a large amount of ssDNA is needed. We used 0.85–1.4 μg/μl ssDNA for *i*-GONAD knock-in experiments. We used in vitro transcription and reverse transcription (*iv*TRT) (Miura, Gurumurthy, Sato, Sato, & Ohtsuka, 2015) to synthesize a relatively high amount of ssDNA by scaling up the reaction by 10-fold compared with the manufacturer's protocol. Therefore, easier and more cost-effective methods are needed to synthesize high amounts of long ssDNA. Additionally, the upper limit of the length of ssDNA that can be delivered into zygotes using *i*-GONAD needs to be determined.

2.6 | Cas12a application in *i*-GONAD

Cas12a, also known as Cpf1, is an RNA-guided DNA endonuclease that emerged as an alternative to Cas9 for genome editing (Swarts & Jinek, 2018). The CRISPR/Cas12a system is simple, as it requires only one RNA component (gRNA). Since we could design a gRNA for Cas12a within the region harboring the *Tyr* point mutation, we tried *Tyr* rescue experiment using a solution containing Cas12a protein/gRNA/ssODN components (Ohtsuka et al., 2018). Similar to the offspring obtained from females subjected to *i*-GONAD with Cas9/

gRNA/ssODN mixture, 58% (11/19) offspring exhibited pigmented phenotypes, indicating the success of genome editing with Cas12a. The precise correction of *Tyr* gene was confirmed by sequencing.

2.7 | Electroporators

In our first GONAD trial, we used the T820 electroporator (BTX) that generates square-wave pulses (Takahashi et al., 2015). However, this machine is no longer manufactured. During the development of *i*-GONAD, we used newer electroporators sold by BEX (CUY21EditII) and NepaGene (NEPA21) and optimized the conditions for EP (Ohtsuka et al., 2018). Both electroporators worked well in *i*-GONAD experiments, indicating that *i*-GONAD is reproducible and robust even when different electroporators are used. However, further optimization of EP conditions is needed for certain genetic strains and/or electroporators used. The EP conditions we are currently using are described in our protocol paper (Gurumurthy et al., 2019).

3 | POTENTIAL APPLICATIONS OF *I*-GONAD

3.1 | *i*-GONAD in other species

Recently, two of our collaborators independently established *i*-GONAD in rats. The first report was published by Kobayashi and her colleagues (Kobayashi et al., 2018). In this study, Kobayashi et al. (2018) determined optimal EP conditions in rats (voltage for poring pulse: 50 V, number of transfer pulse: 6), and applied *i*-GONAD to disrupt the wild-type allele of *Tyr* locus in the DA strain and to repair the mutant *Tyr* locus by ssODN knock-in in the WKY strain. Gene disruption and mutation repair were accomplished at an efficiency of 58.7% (27/46) and 26.9% (7/26), respectively. Kobayashi et al. termed *i*-GONAD in rat as rGONAD. Subsequently, another report on *i*-GONAD in rats was published by Takabayashi et al. (Takabayashi et al., 2018). Takabayashi et al. (2018) successfully disrupted the wild-type allele of *Tyr* locus in the BN strain (62% [8/13]) and rescued the mutation in LEW (5% [1/22]) and SD (5% [2/40]) strains. The authors also performed *i*-GONAD in hybrid embryos (derived from a cross between SD and BN strains) and obtained embryos with mutation in the *paired box 6* (*Pax6*) locus (50% [4/8]). These two studies showed reproducibility and robustness of the *i*-GONAD method in rats.

Since the *i*-GONAD method allows delivering CRISPR components into the zygote within the oviduct, this method can also be used for other mammalian species such as cow and pig. However, the size and shape of oviduct vary among species. Therefore, optimization of parameters (e.g., volume of the solution injected, electric current, and shape of electrodes) of *i*-GONAD will be required for each species.

3.2 | Generation of mice with conditional knockout allele

Although conditional knockout mutants are one of the most highly demanded animals, generation of conditional knockout allele, containing a critical exon flanked by a *lox*P site on either side, is challenging. The *Easi*-CRISPR strategy, which was used to generate reporter knock-in mice, could also be used for generating conditional knockout mutant alleles with the *i*-GONAD method since the length of ssDNA required is generally less than 1 kb for conditional alleles (Miura et al., 2018; Quadros et al., 2017).

3.3 | mRNA delivery for other genes

In our first GONAD trial, we introduced *eGFP* mRNA into preimplantation embryos, showing that mRNA can be delivered by GONAD. It is believed that mRNAs of other genes, such as *Cre* and *FLP*, can be delivered using the same procedure. Cre and FLP recombinases have often been used to excise unwanted sequences, such as drug resistance genes, flanked by *lox*P sites (floxed cassette) or FRTs (FRTed cassette), respectively (Bunting, Bernstein, Greer, Capecchi, & Thomas, 1999). This can traditionally be accomplished by crossing “floxed” or “FRTed” mice with recombinase transgenic mice, or microinjection of recombinase expression plasmid or mRNA into zygotes recovered from these mice. The *i*-GONAD method serves as an alternative strategy to obtain mice without the unwanted sequence.

4 | ADVANTAGES OF *I*-GONAD

The *i*-GONAD method has various advantages over previous methods to generate genome edited animals. One specific feature of *i*-GONAD is that it does not require ex vivo handling of zygotes (Figure 1); this is advantageous for several reasons: (a) techniques for embryo collection and ET are not needed, which saves time (the entire surgical procedure of *i*-GONAD can be accomplished within 15 min per mouse); (b) preparation of pseudo-pregnant mice is not required, and thus there is no need to maintain vasectomized males; (c) genome editing can be performed in animals lacking a system of ex vivo culture for zygotes; and (d) females do not need to be sacrificed in *i*-GONAD and can be used for other purpose after the delivery of pups (Table **1**).

These features of *i*-GONAD allow us to use fewer animals for experimentation compared with those required for traditional technologies such as zygote microinjection. For example, repairing the *Tyr* mutation in the MCH(ICR) mouse strain requires 2.5 times less animals than with microinjection (Ohtsuka et al., 2018). Thus, *i*-GONAD promotes animal welfare, although the number of animals required for experimentation may change with the strain or species used.

5 | FUTURE CHALLENGES IN *I*-GONAD

Since *i*-GONAD is a recently developed technology, there is still room for improvement. For example, the application of *i*-GONAD in other species needs to be investigated, as described above. Below are other examples of future challenges.

5.1 | Effect of strain on the success of GONAD

Because sensitivity of zygotes to electric pulses varies among mice strains, particularly in inbred strains such as C57BL/6, experimental conditions used for GONAD need to be optimized in each mouse strain. We have frequently used 150 mA of pulses for the MCH(ICR) outbred strain; however, this value is harmful for zygotes of the C57BL/6 inbred strain, which performs better with 100 mA pulses (Gurumurthy et al., 2019). The condition may need to be optimized in other strains such as BALB/c.

5.2 | Mosaicism

Presence of mosaicism in founder animals, especially mice, has been a common issue in many genome editing methods, including *i*-GONAD (Fellmann et al., 2017; Hsu, Lander, & Zhang, 2014; Singh, Schimenti, & Bolcun-Filas, 2015). In mice, the *i*-GONAD method is performed using embryonic Day 0.7 zygotes, which correspond to late one-cell stage. We anticipated that *i*-GONAD at this stage could reduce mosaicism in offspring compared with GONAD, which is performed at the two-cell stage (Day 1.5). The RNP-based delivery of CRIPSR components at Day 0.7 resulted in 36%–65% offspring with mosaicism (Ohtsuka et al., 2018). Although performing the procedure at an earlier stage (e.g., Day 0.4) could reduce the mosaicism further, the presence of cumulus cells around the zygotes hampers the successful delivery of CRIPSR components into zygotes. Although this problem could be avoided by forced dispersion of the solution throughout ampulla by gentle squeezing of ampulla after injection, this has not yet been tested (Gurumurthy et al., 2019).

5.3 | Concentration of CRISPR components

The *i*-GONAD method requires higher concentration of CRISPR components than the zygote microinjection method. For example, in our laboratory, we generally use 1 μg/μl and 50 ng/μl Cas9 protein for *i*-GONAD and microinjection, respectively. The concentration of Cas9 may be reduced to 100 ng/μl without enormous reduction of genome editing efficiency (Gurumurthy et al., 2019). The window of working concentrations of other components such as gRNAs needs to be determined.

5.4 | Delivery of plasmid DNA

Initially, we developed oviductal nucleic acids delivery method using plasmid DNA (Sato et al., 2012). Therefore, *i*-GONAD method could potentially be used for delivering plasmid DNA as well as protein, RNA, and ssDNA into preimplantation embryos. However, we have not tried plasmid delivery after the first study, and further optimization may be required to obtain reproducible results. Once the efficiency of plasmid DNA delivery by *i*-GONAD is improved, it can also be used for generating transgenic animals using transposon or recombinase/integrase-based target transgenesis system (e.g., pronuclear injection-based target transgenesis [PITT]) (Ohtsuka et al., 2010, 2015; Shinohara et al., 2007).

5.5 | Timing of GONAD

The *i*-GONAD method is usually performed at approximately 16:00 on the day of vaginal plug detection. However, this timing is often inconvenient for users, especially for technicians who perform it routinely. We recently showed that it is possible to shift the timing of ovulation, and subsequent fertilization in females at estrus by changing the timing of gonadotrophin administration (Sato, Ohtsuka, & Nakamura, 2018). For example, female mice are 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 after 48 hr. Then, these females are mated with males. The next morning (approximately 11:00; corresponding to Day 0.7 of pregnancy), ampulla is relatively shrunken, and zygotes are already detached from cumulus cells. For superovulation, administration of higher dose of gonadotrophins (>5 IU) is frequently adopted but often causes failure to deliver pups normally. We resolved this problem by administrating low-dose eCG (<5 IU) to facilitate the ovulation of a natural number of oocytes, leading to successful delivery of pups at least in females with B6C3ICR mixed genetic background. Another approach to shift the timing of *i*-GONAD would be the synchronization of estrous cycle by two daily injections of progesterone, as suggested by Hasegawa et al. (Hasegawa et al., 2016).

5.6 | Stable delivery of pups from *i*-GONAD treated females

In our initial trial, we used superovulation to prepare pregnant females. However, superovulation by eCG and hCG often reduces the number of pregnancies both in mice and rats, particularly when inbred strains, such as C57BL/6, are used. The use of low-dose eCG and/or synchronization of estrous cycle may help overcome this difficulty, as mentioned above. Further experiments are needed to test these strategies. Currently, we are using C57BL/6 females for *i*-GONAD experiments without superovulation. In this case, we usually mate 10 estrous C57BL/6 females (after examination for vaginal smears) with C57BL/6 males to obtain five plugged females, of which >90% tend to retain pregnancy after the *i*-GONAD procedure (Gurumurthy et al., 2019).

6 | HOW TO MASTER THE *I*-GONAD

Although the *i*-GONAD procedure is simple and easy to use, practice is required to master the technique, especially for beginners. Following experiments are recommended to quickly acquire the required skills for performing *i*-GONAD in mice (and rats) (Kobayashi et al., 2018) (Gurumurthy et al., 2019). Using strains with high reproducibility, such as MCH(ICR), is highly recommended. To perform *i*-GONAD, an electroporator, electrodes, a stereomicroscope, and a micropipette puller (optional: hand-made capillary made by flaming [Gurumurthy et al., 2019]) are needed.

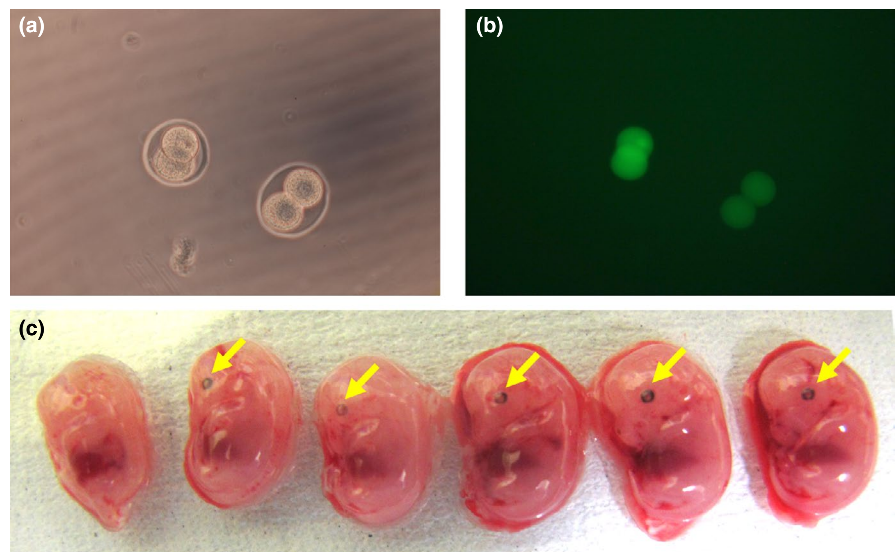

FIGURE 2 The trial of *i*-GONAD to master the method. (a,b) Two-day protocol using *eGFP* mRNA. Green fluorescence was observed in two-cell embryos isolated from females subjected to *i*-GONAD. (c) *Tyr* rescue experiment using the MCH(ICR) strain. All embryos, except for the embryo on the extreme left, exhibited pigmented eyes (yellow arrows). All animal experiments were performed in accordance with the institutional guidelines and were approved by *The Institutional Animal Care and Use Committee of Tokai University* (Permit Number: #165009, #171003 and #181037)

6.1 | Two-day protocol using rhodamine-dextran marker and *eGFP* mRNA

Skills for the steps involved in *i*-GONAD (e.g., preparation of pregnant females, surgical procedure, injection, and EP) can be checked by the two-day protocol. In this protocol, we use an injection solution containing fluorescent marker(s), such as tetramethylrhodamine-labeled dextran (3 kDa) and/or *eGFP* mRNA, instead of CRISPR components. Successful delivery of these materials can be confirmed one day after the in vivo EP procedure, based on the fluorescence in two-cell embryos recovered from *i*-GONAD-treated females (Figures 2a,b). Detection of fluorescence is an indicator of the acquisition of *i*-GONAD skill. This strategy was used to determine optimal EP condition in rats (Kobayashi et al., 2018).

6.2 | *Tyr* rescue experiment

Since pigmentation is one of the visually apparent characteristics in animals, it is easy to assess the outcome of genome editing through the manipulation of pigmentation-related genes, as described in section 3.22.4. For example, the presence or absence of a pigment can be easily assessed by checking the eyes of fetuses at the mid-gestational stage (e.g., embryonic Day 13.5 in MCH[ICR] strain) (Figure 2c). Unlike the 2-day protocol, this experiment can be used to determine the success of genome editing. Therefore, this system can also be used for optimizing experimental conditions, such electric pulse and concentration of CRIPSR components.

7 | SUMMARY

GONAD/*i*-GONAD, unlike conventional methods, does not require ex vivo handling of zygotes for the generation of genome-edited animals. Thus, it provides a simple strategy for researchers who do not possess specific skills required embryo handling and micromanipulation. In addition, from the gene delivery point of view, in vivo gene delivery into zygotes through oviduct is considered to be novel. This delivery method offers the possibility to manipulate germline in animal species in which ex vivo embryo culture procedure has been proven difficult or impossible under the conventional approaches. The oviductal gene delivery protocol will be further modified/improved in future. For example, Yoon et al. (2018) recently applied this delivery route to create genome-edited mice by intraoviductal injection of adeno-associated virus (AAV) (Yoon et al., 2018); in this method, no additional procedure such as EP is required. Furthermore, as we shown in gene repair experiments of *Tyr* locus by *i*-GONAD, the method can repair disease-causing mutations in vivo without sacrificing pregnant females, indicating the potential of this method for in vivo germline gene therapy.

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