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Review

Recent Advances in the Production of Genome-Edited Animals Using *i*-GONAD, a Novel *in vivo* Genome Editing System, and Its Possible Use for the Study of Female Reproductive Systems

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Abstract

Gene-engineered animals created using gene-targeting technology have long been recognized as beneficial, valid, and valuable tools for exploring the function of a gene of interest, at least in early 2013. This approach, however, suffers from laborious and time-consuming tasks, such as the production of successfully targeted embryonic stem (ES) cells, their characterization, production of chimeric blastocysts carrying these gene-modified ES cells, and transplantation of those manipulated blastocysts to the recipient (pseudopregnant) females to deliver chimeric mice. Since the appearance of genome editing technology, which is now exemplified by the CRISPR/*Cas9* system, in late 2013, significant advances have been made in the generation of genome-edited animals through pronuclear microinjection (MI) of genome-editing components into fertilized eggs (zygotes) or electroporation (EP) of zygotes in the presence of these reagents. However, these procedures require the transfer of genome-edited embryos into the reproductive tracts of recipient females for further development. Genome editing via oviductal nucleic acids delivery (GONAD) and its modified version, called "improved GONAD (*i*-GONAD)," were developed as an alternative to the MI- or EP-based genome-edited animal production and now recognized to be very convenient and straightforward as genome editing can only be performed *in vivo* (within the oviductal lumen where fertilized embryos exist). This system also enables the simultaneous transfection of epithelial cells *lining the oviductal lumen*. In this review, we summarize the recent advances in GONAD/*i*-GONAD and their derivatives and discuss the potential of these technologies to study various biological systems related to female reproduction.

Keywords

Genome editing; CRISPR/*Cas9*; gene-engineered animals; GONAD; *i*-GONAD; oviductal epithelial cells; electroporation; zygotes; female reproductive system

1. Introduction

Gene-targeting technology is a technique to manipulate the function of an endogenous target gene through gene disruption (knockout (KO)) or insertion of a DNA fragment into a target locus ("knockin (KI)") in embryonic stem (ES) cells [1]. Engineered ES cells are then subjected to blastocyst injection to produce chimeric blastocysts. Then, the injected ES cells are mixed with the host embryonic cells (inner cell mass cells) for participation in their developmental process. The resulting chimeric blastocysts are then transferred to the uterine horns of recipient females to obtain viable chimeric offspring. In chimeric mice, ES cell-derived germ cells (sperm or oocytes) and host blastocyst cell-derived germ cells are thought to exist. By mating chimeric mice with normal fertile mice, the specific trait (KO or KI) present in ES cells can be transmitted to the next generation as heterozygous KO or KI mice. Unfortunately, producing these KO or KI mice is time-consuming and labor-intensive, as it usually takes over two years to obtain homozygous KO or KI mice.

Genome editing, as exemplified by the CRISPR/*Cas9* system, is a recently developed technology now widely recognized as a powerful tool for producing KO or KI animals [2, 3]. For example, the CRISPR/*Cas9* system requires only two components, *Cas9* endonuclease and guide RNA (gRNA), to induce mutations in a target locus. In the absence of donor DNA, a ribonucleoprotein (RNP) complex (consisting of the *Cas9* protein and gRNA) cleaves the double-stranded sequence(s) 3-4 bp upstream of the proto-spacer adjacent motif (PAM) in the target gene. Once cleaved, the cleaved portion is immediately repaired using a natural repair system called non-homologous end-joining (NHEJ). During NHEJ, insertions or deletions in DNA sequences, called insertion-deletion mutations (indels), occur frequently. In contrast, in the presence of donor DNA, homology-directed repair (HDR) often occurs at the cleavage site, leading to KI of the donor sequence. Notably, the efficiency of HDR-mediated genome editing is generally lower than that of NHEJ-mediated editing. Furthermore, HDR preferentially occurs in dividing cells, whereas NHEJ occurs in both dividing and non-dividing cells [4].

The emergence of genome editing technology in early 2013 has caused a paradigm shift in the production of genetically modified (GM) animals. Since late 2013, many genome-edited animals (including mice and rats) have been generated using this technology [2, 3]. In the early stages of genome-edited animal production, the primary platform was the pronuclear microinjection (MI) of genome-editing components. This was performed under a light microscope using a micromanipulator by a professional with specific skills. It takes approximately 2 h to complete MI for over 100 zygotes per session. The zygotes used are *in vitro*-fertilized (IVF) or those freshly isolated from the oviducts of pregnant females. In 2014, Kaneko et al. [5] reported the electroporation (EP)-based production of genome-edited rat zygotes. This was performed in an Opti-MEM-based drop containing 30-50 zygotes and genome editing components. EP was performed using a square pulse generator (NEPA21; NEPA GENE Co., Ltd., Chiba, Japan, or CUY21EDIT II; BEX Co., Ltd., Tokyo, Japan). EP is generally completed within 10 min, and several genome-edited zygotes can be collected simultaneously. In this context, EP appears more convenient than MI regarding cost performance. Furthermore, it does not require the specific skills needed for MI. However, MI- or EP-treated zygotes must be transferred into the oviducts of pseudopregnant females for further development. This procedure is called “egg transfer (ET)” and requires specific skill and vasectomized males (for inducing pseudopregnancy in females).

In 2015, a novel method enabling *in vivo* genome editing targeting two-cell mouse embryos was first reported by Takahashi et al. [6]. This technology is called Genome-editing via Oviductal Nucleic Acids Delivery system (GONAD) (Figure 1), which relies on intraductal instillation of 1-1.5 µL of solution containing genome editing components (*Cas9* mRNA + single guide RNA (sgRNA)) in a pregnant female (corresponding to the two-cell stage) using a mouthpiece-controlled glass micropipette and subsequent EP towards an entire oviduct. When the developing fetuses were assessed for possible indels in the target locus, 33% (2/6) were mosaic (comprising edited and non-edited cells), 33% (2/6) were edited fetuses, and 33% (2/6) were unedited fetuses.

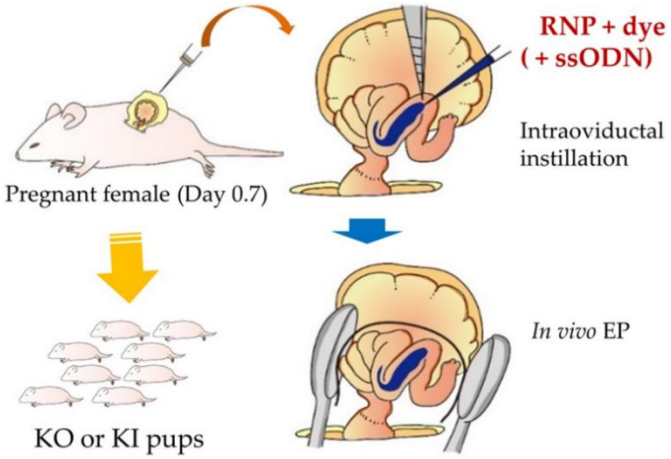

Figure 1 Schematic of genome-edited mouse production using the *i*-GONAD procedure. EP, electroporation; KI, knock-in; KO, knockout; RNP, ribonucleoprotein; ssODN, single-stranded oligodeoxynucleotide.

In 2018, the same group (Takahashi et al. [6]) improved GONAD and re-named it "improved GONAD (*i*-GONAD)" [7]. The significant improvements are that late zygotes are used for genome editing to avoid possible mosaicism, as has been frequently observed with GONAD. RNP was employed to induce genome editing more rapidly than when using *Cas9* mRNA [7]. First, late zygotes are almost free from cumulus cells, whereas early zygotes are surrounded by a thick layer of cumulus cells. The cumulus cell layer hampers transfection with exogenous nucleic acids, even in environments where EP enables forced gene delivery [6-8]. Second, *Cas9* protein is superior to *Cas9* mRNA because there is no need for protein synthesis when protein is used [9]. This is particularly beneficial for avoiding mosaicism since it enables rapid induction of genome editing in the injected zygotes. On the other hand, in the case of *Cas9* mRNA introduction (where time lag for protein synthesis occurs), during the cleavage from zygote to the two-cell stage, it is highly likely that one blastomere is genome-edited, but the other is not, leading to increased mosaicism. According to Ohtsuka et al. [7], the forkhead box protein E3 (*Foxe3*) locus could be successfully disrupted using RNP (comprised of *Cas9* protein and crRNA/tracrRNA) in the pregnant randomly bred females at Day 0.7 of pregnancy. In this case, the detection of a *vaginal plug* at noon was *designated* as Day 0.5 of *pregnancy*. After intraductal instillation of RNP-containing solution, *in vivo,* EP was performed using the NEPA21 apparatus, which yielded 97% of embryos with indels at the target locus. On the other hand, in the case of the *i*-GONAD-mediated KI experiment, a solution containing RNP and single-stranded oligodeoxynucleotide (ssODN) (as DNA donor) or long single-stranded deoxyribonucleic acid (ssDNA) (with ~1 kb in size) generated through a novel method, called Easi-CRISPR, a highly efficient CRISPR-based KI technique [10, 11], was intraoviductally injected. Subsequent *in vivo* EP resulted in the production of embryos with KI alleles in their genomes, with efficiencies of ~50% (for ssODN) and ~15% (for longer ssDNA). Furthermore, a large deletion of the retroviral fragment (~50 kb) inserted into the tyrosinase (*Tyr*) locus of C57BL/6 (starting now defined as B6) mice was possible using *i*-GONAD. Notably, *i*-GONAD does not require *in vitro* manipulation of embryos (such as zygote isolation, MI, EP, and embryo cultivation) or subsequent ET in recipient females. Furthermore, it does not require skill in manipulator-aided microinjection, as is required for MI. In this context, *i*-GONAD is more convenient and straightforward than the previous MI- or EP-based methods.

Since the report of GONAD/*i*-GONAD, many researchers have reproduced this technology using mice, rats, and hamsters, and some modifications have been made to these technologies [12]. For examples, these include production of KO or KI mice using ssODN as a donor [6, 7, 13-39], large deletion (LD) of a target site in chromosomes [7, 22, 37], maintenance of lethal mutant mice using an inversion balancer identified from the C3H/HeJJcl strain [38], production of rodents with floxed alleles [20, 22, 31] or long DNA fragments at a target locus [7], epitope-tagging at a target gene [32] or *in situ* somatic gene replacement in oviductal epithelium [39]. Furthermore, these techniques have recently been applied to carcinogenic studies, where oviduct-targeted gene delivery of oncogenes can cause the generation of malignant tumors of oviduct origin [40, 41]. Furthermore, *in situ,* gene modification of preimplantation mouse embryos (present in an oviductal lumen) using recombinant adeno-associated viral (AAV) vectors [15], which is called "AAV-based GONAD" [42], was reported. The AAV-based GONAD does not require *in vivo* EP after the intraductal instillation of rAAV [42].

More than 30 genes have been successfully modified by GONAD/*i*-GONAD [12]. GONAD/*i*-GONAD also enables the simultaneous transfection of epithelial cells lining the oviductal lumen [7, 8, 17]. Since recent advance in GONAD/*i*-GONAD-based production of genome-edited animals was already summarized in our previous review paper [12], we will show the recent *i*-GONAD-related works (reported on 2022 to 2023) in Table 1 and also focus on the following subjects: 1) present and future modification of GONAD/*i*-GONAD, 2) AAV-based GONAD, 3) possible application of GONAD/*i*-GONAD to manipulate the genome of animals other than mice, 4) the utility of these technologies to examine female reproductive systems, such as oviductal function related to the shape formation (coil or non coil) of the oviduct, embryo transfer and embryo survival, and 5) the utility of these technologies to generate ovarian tumors and exploration of the mechanism underlying carcinogenesis.

Table 1 Summary of gene-modified animals produced using GONAD/*i*-GONAD between 2022 and 2023.

Type of method (a component of CRISPR/*Cas9* reagents)	Genome editing tool (mode for gene modification) EP apparatus	Animal (species strain)	Outcome	Target gene or transgene	References
i-GONAD *In vitro* EP (*Cas9* protein)	CRISPR/*Cas9* (indels) NEPA21	Mouse (B6D2F1 CD1 P3D2F1 NSG)	Pairs of guide RNAs were designed to generate controlled deletions that led to the absence of protein; *in vitro* EP or *i*-GONAD resulted in more than 90% and 30% genome-edited newborn animals, respectively, for each locus.	*Agxt1* *Pklr* *Rasa1*	Sanchez-Baltasar et al. [43]
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (KI) NEPA21	Mouse	Showing successful tagging of flag/DYKDDDDK epitope at the N-terminus of endogenous CaMKIIα and β proteins; showing differential activity of each promoter within a brain.	*CaMKIIα* *CaMKIIβ*	Aoto et al. [44]
i-GONAD (protein)	CRISPR/*Cas9* (indels) CUY21EDITII	Mouse	Showing successful indel induction in embryos after *i*-GONAD at early zygote stage; it requires waiting for 3 min before *in vivo* EP after intraductal instillation of RNP	*Tyr*	Takabayashi et al. [45]
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (indels) CUY21SC	Mouse	Showing gender difference in hair length when *i*-GONAD was performed for zygotes from long-haired mice with dysfunctional FGF5 mutations	*Fgf5*	Takahashi et al. [46]
AAV-based GONAD	-	Mouse (B6C3F1)	Intraductal instillation of rAAV6 carrying *EGFP* cDNA at Day 0.7 of pregnancy resulted in the highest levels of fluorescence when morulae were inspected for fluorescence; concomitantly, the oviductal epithelium was distinctly fluorescent; the fluorescence in embryos peaked at the morula stage, suggesting that rAAV6 is a valuable vector enabling	*EGFP*	Sato et al. [47]

			transduction of zona pellucida-enclosed preimplantation embryos *in situ*		
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (KI) NEPA21	Mouse (wild strains)	*In vitro* fertilization was extremely low in the case of using oocytes from wild strains, which hampered the efficient production of genome-edited animals; in contrast, *i*-GONAD (which does not require *ex vivo* handling of fertilized eggs) helped obtain genome-edited wild strains with an efficiency of 78% (7/9 strains tested)	*Adcyap1* *PACAP*	Imai et al. [48]
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (KI) NEPA21	Mouse (B6, ICR) Rat (SD, LE, LH)	Showing successful KI of a large fragment (~3 kb) corresponding to *ROSA26* locus using *i*-GONAD using rAAV carrying donor DNA and RNP resulted in the generation of rat pups with an efficiency of 10%	*Rosa26* *Erc1* *Erc2* *Thy1* *mCherry*	Abe et al. [49]
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (KI) CUY21EDITII	Mouse (B6, ICR, FVB/NJ, UBC-GFP)	Natural mating of C57BL/6 females that have been administrated with low-dose hormone failed to increase their low fertility rate; instead, diet enrichment had a positive effect on pregnancy success; furthermore, co-housing *i*-GONAD-treated pregnant B6 females with synchronized pregnant FVB/NJ companion was beneficial for increased generation of genome-edited mice; a two-step serial *LoxP* insertion, in which each *LoxP* sequence was inserted individually in different *i*-GONAD procedures, was effective for generating floxed mice	*GFP* *Ifna1,* *Ifna5,* *Ifna7*	Melo-Silva et al. [50]
i-GONAD (*Cas9* protein)	CRISPR/*Cas9* (KI) unknown	Mouse (CD1)	Showing successful KI of a fragment spanning 0.5~1 kb (containing fluorescent reporter or affinity tag) into transcription factor (TF) locus using *i*-GONAD using long ssDNA and RNP or ssODN and RNP, which led to the generation of new mouse lines related to neurodevelopmental and associated diseases	*Bcl11a* *Bcl11b*	Wiegreffe et al. [51]

AAV-based GONAD (TIGER)	CRISPR/*Cas9* (indels, KI) -	Mouse (African striped mouse)	Showing successful KO and large KI in a wild-derived rodent with high efficiency when intraductal injection of rAAVs (as the only vehicle to deliver all genome-editing reagents into the oviducts of pregnant females) was performed	*Tyr* *Sfrp2* *Alx3* *Rosa26* *EGFP* *mCherry*	Li et al. [52]

Abbreviations: AAV, adeno-associated virus; Adcyap1, adenylate cyclase activating polypeptide 1; *Agouti,* Agouti-signaling protein (ASIP); *Agxt1*alanine-glyoxylate aminotransferase; *Alx3,* aristaless-like homeobox 3; *Bcl11a,* BCL11 transcription factor A; *Bcl11b,* BCL11 transcription factor B; B6, C57BL/6 mice; B6C3F1, a cross between female C57BL/6 and male C3H/He mice; B6D2F1, a cross between female C57BL/6 and male DBA/2 mice; BN, Brown Norway rat; *CaMKIIα*, calcium/calmodulin dependent protein kinase IIα; *CaMKIIβ,* calcium/calmodulin dependent protein kinase IIβ; CD-1, *ICR* mice; CRISPR/*Cas9*, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9; DA, Dark Agouti rat; *EGFP,* enhanced green fluorescent protein; EP, electroporation; *Erc1*, ELKS/RAB6-interacting/CAST family member 1; *Erc2*, ELKS/RAB6-interacting/CAST family member 2; *Fgf5,* fibroblast growth factor 5; *GFP,* green fluorescent protein; *GONAD,* genome-editing via oviductal nucleic acids delivery; Ifna1 *Interferon alpha-1;* Ifna5, *Interferon alpha-5;* Ifna7, *Interferon alpha-7; i*-GONAD, improved GONAD; indels, insertions, deletions, or substitutions of nucleotides; KI, Knock-in; KO, Knock out; LD, large deletion; LE, Long-Evans (LE); LEW, Lewis rat; LH, Lister Hooded (LH); *mCherry,* monomeric derivative of DsRed fluorescent protein; *Mecp2,* methyl-CpG binding protein 2; *NSG,* NOD scid gamma mice; P3D2F1 mice , a cross between female Pep3 and DBA/2; PACAP, *pituitary adenylate cyclase activating polypeptide; Pklr,* pyruvate kinase L/R; rAAV6, recombinant AAV with serotype 6; *Rasa1, RAS p21 protein activator 1;* RNP, ribonucleoprotein; *ROSA26*, Gt(ROSA)26Sor; SD, Sprague-Dawley rat; *si*-GONAD, sequential *i*-GONAD; *Sfrp2*, secreted frizzled-related protein 2; sp*Cas9*, *Streptococcus pyogenes*-derived *Cas9*; ssDNA, single-stranded deoxyribonucleic acid; ssODN, single-stranded oligodeoxynucleotide; TIGER, targeted *in vivo* genome editing in rodents; TF, transcription factor; Tg, transgenics; *Thy1*, Thy-1 cell surface antigen; WKY, Wistar Kyoto rat; UBC-GFP, a GFP transgene under the ubiquitin C promoter.

Notably, since the first report by Takahashi et al. [6] in 2015, several protocols for the *i*-GONAD-mediated generation of genome-edited rats [53] and mice [54-56] have also been provided.

2. Further Modification of *i*-GONAD

Following the publication of the first report of *i*-GONAD in 2018, many reports have emerged describing modifications of this technique. The following sections discuss several topics related to *i*-GONAD.

2.1 Sequential i-*GONAD (*si-*GONAD)*

The targeted embryonic stages for GONAD and *i*-GONAD are the two-cell and late zygote stages, respectively. This indicates that a two-step *i*-GONAD, involving the first *i*-GONAD at the late zygote stage and the second *i*-GONAD at the two-cell stage, is theoretically feasible. Sato et al. [20] explored this by inducing indels at two closely situated sites (44 bp apart). They initiated the first *i*-GONAD using a solution containing RNP targeted the upper portion of exon 4 of the α-1,3-galactosyltransferase gene (*GGTA1*), which encoded the protein essential for synthesizing the cell-surface α-Gal epitope [57]. The following day, a 2nd *i*-GONAD was performed using a solution containing RNP targeted the lower portion of exon 4. One day after the final surgery, morulae were isolated for single-embryo-based analysis to identify possible indels at the target sites. The efficiency of the successful generation of morulae with indels at both sites was 18%. Based on these findings, Sato et al. [20] named this approach "*si*-GONAD" (Figure 2).

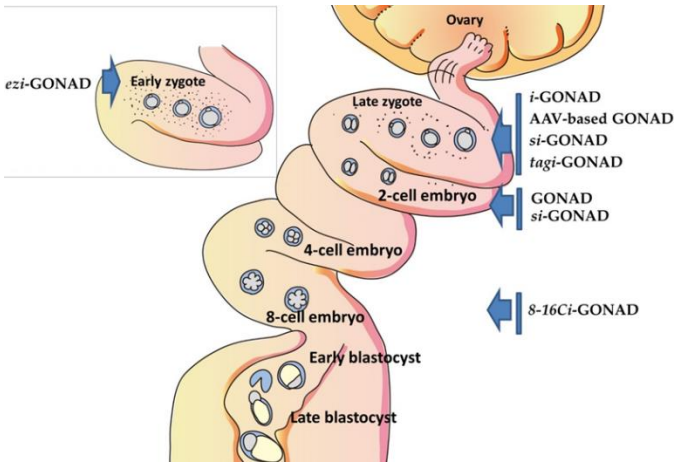

Figure 2 Schematic of mice's pre-implantation (Days 0.4-4.5) and post-implantation (Day 5.5) development. Embryos at early zygote (at Day 0.4; see box), late zygote (Day 0.7), two-cell embryo (Day 1.5), 8-16-cell embryo (Day 2.5), early blastocyst (Day 3.5), and late blastocyst (Day 4.5) stages are present within the oviductal lumen or uterine horn. Embryos at Days 0.4 to 3.5 are surrounded by zona pellucida (ZP). Still, embryos at Day 4.5 escape from ZP, called "ZP hatching," and are ready to attach to the uterine luminal epithelium for "implantation." At the early zygote stage (see box), zygotes are surrounded by compact layers of specialized granulosa cells called "cumulus cells," but at the late zygote stage, these cells begin to detach from the embryo. This figure was drawn in-house and reproduced with permission from Sato et al. [42], published by MDPI in 2020.

si-GONAD can also be used to create Cre/*loxP*-based floxed mice, also called "conditional KO mice," wherein two *loxP* sites flanking an exon of the target gene have been inserted. The Cre/*loxP* system utilizes Cre recombinase, an enzyme that can manipulate *loxP*-floxed chromosomally integrated genes of interest (GOI). The spatial and temporal control of Cre gene expression in *loxP*-floxed mice is beneficial for evaluating the *in vivo* functions of GOI. For the CRISPR-based generation of floxed mice, the simultaneous introduction of *Cas9*, two pairs of gRNAs, and two ssODNs containing *lox* sequences into mouse zygotes has been conducted in earlier studies [58, 59]. However, this approach is often challenging as it frequently causes LD at the target site [60]. To address this, Horii et al. [60] proposed the concept of "sequential KI," which involves two steps of CRISPR-based introduction of mutated *lox* sites into the target site with a one-day interval. To test the feasibility of creating floxed mice using this approach, Sato et al. [19] performed *i*-GONAD (*si*-GONAD) using a solution containing RNP (targeting both sides of the introns interposing exon 3 of the methyl CpG-binding protein 2 gene (*Mecp2*)) and ssODNs (as donor DNA containing mutated *lox* sites). However, this attempt failed, and only a morula with one floxed site in the 5′ site of *Mecp2* was successfully generated.

Shang et al. [31] demonstrated that one-step *i*-GONAD was sufficient to create mouse conditional KO alleles using two short ssODNs (uniquely designed as asymmetric *loxP*-ssODN) as HDR donors for *loxP* insertion, as described by Richardson et al. [61]. According to Shang et al. [31], each ssODN is 161 nucleotides (nt) long, comprised of 91 nt of the 5′ homology arm from the PAM-proximal side, 34 nt of the *loxP* sequence, and 36 nt of the 3′ homology arm from the PAM-distal side. When *i*-GONAD using a solution containing RNP (targeting the intron 2 and 3′ region of Fos-like antigen 1 gene (*Fosl1*)) and two short ssODNs was performed, one mouse out of 20 F0 mice obtained had the simultaneous 5′- and 3′-*loxP* insertions and six had either 5′- or 3′-*loxP* integrations. The critical factor for this success may be to employ asymmetric ssODN as HDR donors for targeted KI. Notably, Melo-Silva et al. [50] recently demonstrated that a two-step serial *loxP* insertion, in which each *loxP* sequence is inserted individually in different *i*-GONAD procedures, like in *si*-GONAD [20], is an efficient method for generating floxed mice.

2.2 i-GONAD Targeting Early Zygotes

As mentioned previously, it was difficult to induce genome editing in early zygotes (which are tightly surrounded by the cumulus cell layer) by *i*-GONAD because the cumulus cell layer blocks the efficient uptake of exogenous nucleic acids even in environments where EP-based forced gene delivery was applied [6-8]. It is well known that hyaluronidase (HA), an enzyme frequently used in IVF experiments, can disperse cumulus cells from zygotes [62]. Thus, it is conceivable that pretreatment with HA facilitates genome editing at the early zygote stage.

Kaneko and Tanaka [23] first tested the possibility through intraductal injection of a solution containing 0.1% hemagglutinin (HA) into the ampulla of a pregnant female mouse on Day 0.4 (10:00-11:00 a.m.). Several minutes after injection, *i*-GONAD was performed using a solution containing RNP (targeting the fibroblast growth factor 10 gene (*Fgf10*)). Inspection of fetal offspring revealed that the HA-treated group exhibited 2.5-fold higher genome editing (indels) efficiency than the control HA-untreated group (68% *vs*. 27%), indicating that *in vivo* HA-mediated removal of cumulus cells on Day 0.4 was possible and beneficial for increasing *i*-GONAD-mediated genome editing efficiency at the early zygote stage.

Subsequently, Takabayashi et al. [45] reported that electroporation (EP) performed 3 min after intraoviductal instillation of genome-editing reagents in pregnant females at Day 0.4 resulted in higher genome editing (indels) efficiency than EP immediately after intraoviductal instillation on Day 0.5 (70% *vs*. 18%). Furthermore, HA addition slightly enhanced the efficiency of genome editing in the early zygotes. These findings indicate that a 3-minute interval before EP is optimal for achieving *i*-GONAD-mediated genome editing at the early zygote stage.

Takabayashi et al. [45] reported that the introduced dye was not uniformly distributed immediately after introduction, as indicated by the circles in Figures 3b, 3e, and h. However, gradual dye dispersion occurred throughout the oviductal lumen during the 3-minute interval (Figures 3c, 3f, and 3i), indicating rapid exogenous infiltration of the solution into zygotes through the loose intercellular space of cumulus cells. In the studies by Kaneko and Tanaka [23] and Takabayashi et al. [45], no formal name was assigned to *i*-GONAD at the early zygote stage. Here, we refer to this approach as "*ezi*-GONAD (early zygote-targeted *i*-GONAD)" (Figure 2). Takabayashi et al. [45] demonstrated that a modified protocol (*in vivo* EP, 3 min after intraductal instillation of a solution containing HA, RNP, and dye on Day 0.4) was more effective than *i*-GONAD on Day 0.7 (85% *vs*. 57%). In this context, *i*-GONAD on Day 0.4 is a preferable approach for obtaining genome-edited animals with high efficiency.

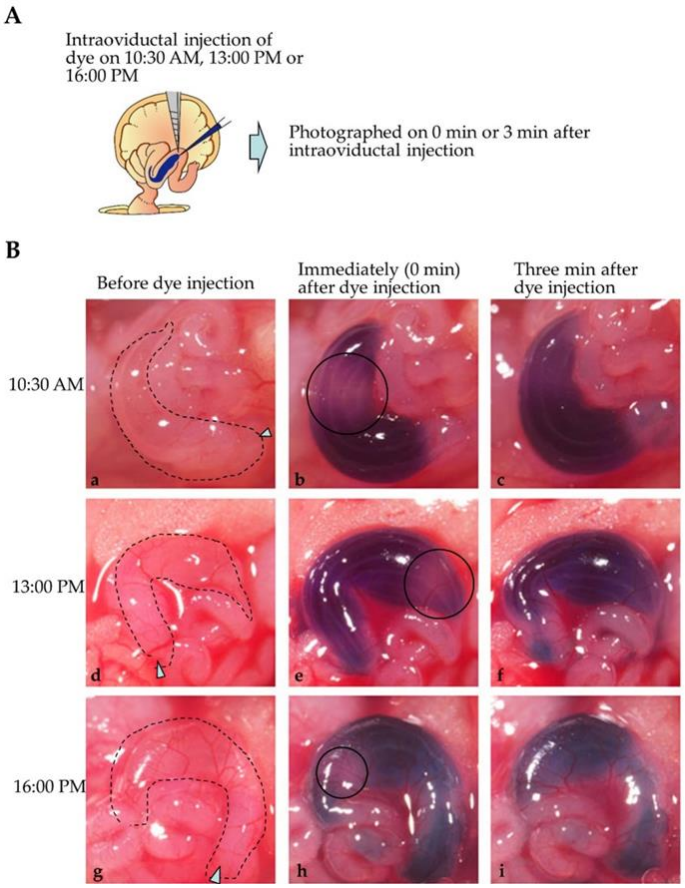

Figure 3 Monitoring of dye distribution (Fast Green FCF)-containing solution injected intraoviductally. (A). Schematic representation of intraductal injection of dye at various times (10:30 a.m., 1:00 p.m., and 4:00 p.m.) on the day the vaginal plug was first found. The mode of dye distribution in the ampulla before dye injection and immediately (0 min) or 3 min after injection was checked. (B). Photographs were taken before (a, d, and

g) and 0 min (b, e, and h) or 3 min (c, f, and i) after the dye injection. The ampulla portion is indicated by dotted lines in (a, d, and g). Arrowheads in (a, d, and g) show the injection sites. Circles in (b, e, and h) indicate the portions where the introduced dye is scarcely distributed and appears pale. (a-c) Injection at 10:30 a.m., (d-f) injection at 1:00 p.m., and (g-i) injection at 4:00 p.m. This figure was obtained from a paper by Takabayashi et al. [45], published by MDPI in 2022.

2.3 i-GONAD Targeted Two-Cell Embryos

Gu et al. [63] first reported a novel CRISPR/*Cas9*-based method, termed two-cell homologous recombination CRISPR, as a highly efficient gene-editing method involving the introduction of *CRISPR/Cas9* reagents into mouse 2-cell embryos. When 2-cell seeds were subjected to cytoplasmic microinjection (MI) with CRISPR reagents containing fluorescent template DNA and engineered *Cas9* protein (making the donor fragment more accessible to the target sequence *via* biotin-streptavidin complexing), up to 95% KI efficiency was achieved. Gu et al. [63] reported that two-cell embryos have a more extended phase than zygotes, which facilitates HDR-mediated KI of ssDNA donors into a target locus. In other words, the time (developmental stage of early embryos) required for genome editing is essential for increasing KI efficiency [64]. Consequently, this improvement resulted in a >10-fold increase in KI efficiency compared to existing methods. Based on a report by Gu et al. [63], it is theoretically conceivable that *i*-GONAD at the two-cell stage may result in the efficient production of embryos with successful KI. Takabayashi and colleagues recently obtained beneficial results from *i*-GONAD experiments at the two-cell stage (manuscript under preparation). Based on these findings, we call this technology "*2ci*-GONAD (two-cell embryo-targeted *i*-GONAD)" (Figure 2).

As for an attempt to increase KI efficiency, Aoshima et al. [33] employed several reagents [i.e., RAD51-stimulatory compound 1 (RS-1), L755,507, SCR7 and Alt-R® HDR Enhancer (HDR enhancer)], all of which are thought to be effective for increasing KI efficiency using cultured cells, for the *i*-GONAD experiment. Still, their effect on the improvement of KI efficiency was only marginal. Notably, the combined use of purified RAD51 protein (RAD51 recombinase) and CRISPR-*Cas9* reagents has recently been shown to significantly increase the efficiency of homozygous KI in mouse embryos [65]. Park et al. [66] reported similar observations. Notably, Ma et al. [67] recently developed mi*Cas9* by fusing a minimal motif consisting of 36 amino acids (encoded by *BReast CAncer gene 1* exon 27) to *Streptococcus pyogenes Cas9* (*SpCas9*). Mi*Cas9* binds to RAD51 through this fusion motif, stabilizes RAD51/ssDNA nucleoprotein filaments, and enriches RAD51 at the target locus. Compared to Sp*Cas9*, mi*Cas9* enhanced KI rates and reduced off-target indel events. Therefore, it would be worthwhile to investigate whether the use of RAD51 contributes to KI improvement in the *2ci*-GONAD system.

2.4 Gene Tagging Using i-GONAD

Specific antibodies are frequently used to assess the tissue localization of proteins expressed in GOI. However, the preparation of these antibodies is often time-consuming and effort-intensive. Furthermore, the resulting antibodies have low tissue specificity and nonspecific or high background in some cases. Epitope tagging an endogenous protein at an appropriate position is an excellent way to overcome this problem. Several anti-tag antibodies with high specificity and low background are now commercially available. However, these techniques depend on designing and creating specific

DNA donor templates with appropriate homology regions. In other words, this template generally contains homology arms of 500-1,500 bp homology arms on each side of the desired sequence specific to the targeted endogenous locus. Lackner et al. [68] first developed CRISPR/*Cas9*-based gene tagging to avoid this costly and laborious task. This alleviates the need for homology templates and enables the tagging of endogenous loci using a single generic donor plasmid. Since then, another CRISPR/*Cas9*-based gene tagging, which is called "Homology independent gene Tagging (HiTag)" [69], has been provided.

Nakano et al. [32] were the first to report the successful production of HA-tagged KI mice using *i*-GONAD. The target gene selected was the gene encoding activating transcription factor 5 (ATF5), a stress-responsive transcription factor that belongs to the cAMP response element-binding protein/ATF family. ATF5 is required to differentiate and survive sensory neurons in murine olfactory organs. Nakano et al. [32] inserted an HA tag sequence into the C-terminus of the *ATF5* coding sequence. Consequently, they observed ATF5-HA fusion proteins in the immature and mature olfactory and vomeronasal sensory neurons in the central olfactory epithelium and vomeronasal organs, respectively, which reflected the localization patterns of endogenous ATF5 proteins.

Aoto et al. [44] recently investigated epitope tag position using AlphaFold2 protein structure prediction and created developed Flag/DYKDDDDK tag KI calcium/calmodulin-dependent protein kinase type II subunit α (CaMKIIα) and β (CaMKIIβ) mice using *i*-GONAD (Figure 4) to determine the localization of endogenous CaMKIIα and β proteins in the mouse brain. In the genomes of these mice, a small fragment of up to 200 bp was successfully inserted into the target gene, enabling the tagging of a small epitope. Consequently, specific detection of endogenous CaMKIIα and β proteins was possible using the commercially available anti-Flag antibodies. Immunohistochemical analyses revealed that localization patterns of each tagged CaMKIIα and β in the GM mice were similar to the published expression patterns of CaMKIIα and β. Aoto et al. [44] concluded that the *i*-GONAD-mediated tag KI approach is valuable, especially when specific antibodies are unavailable. We therefore named this type of *i*-GONAD "*tagging*-GONAD (gene tagging based *i*-GONAD)" (Figure 2).

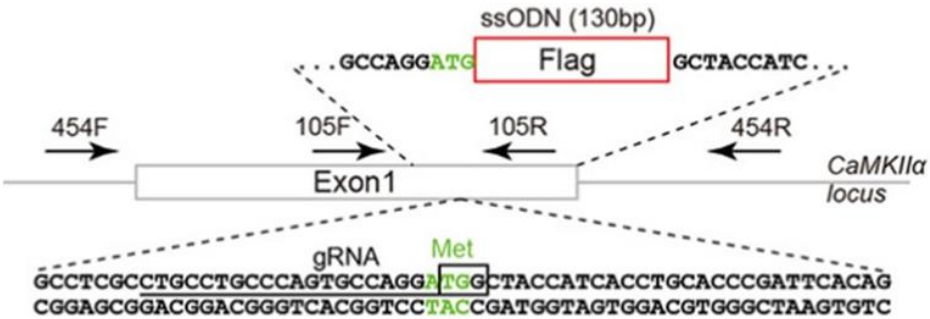

Figure 4 Generation of Flag KI mice for calcium/calmodulin-dependent protein kinase type II subunit α (CaMKIIα). gRNA target site (black underline) and single-stranded oligodeoxynucleotide (ssODN) are shown below or above exon 1 of mouse *CaMKIIα*, respectively. The square box indicates the protospacer-adjacent motif (PAM) sequence. The start codon encoding methionine (Met) is highlighted in green. This figure was reproduced with permission from Aoto et al. [44] and published in MDPI in 2022.

2.5 AAV-Based GONAD

Among the viral vectors, such as lentiviral, adenoviral, retroviral, and AAV, which have been widely used for therapeutic or experimental purposes [70], only AAV vectors can infect zona pellucida (ZP)-enclosed (or intact) early embryos by simple incubation in medium containing rAAV vector [15, 71]. There are over 10 serotypes of AAVs, each exhibiting different infectious abilities depending on the cell type [72].

Mizuno et al. [71] first explored which AAV serotype was adequate for the transduction of ZP-intact mouse two-cell embryos by co-incubation for 16 h with several types of rAAVs carrying an enhanced green fluorescent protein (EGFP) expression unit. When the morulae co-incubated with rAAVs were examined for fluorescence under a fluorescence microscope, rAAV serotype 6 (hereafter referred to as rAAV6) exhibited strong fluorescence. Yoon et al. [15] also reported similar results. Interestingly, rAAV6 was able to infect ZP-intact rat and bovine embryos *in vitro*, suggesting the utility of this vector for gene delivery to early origins beyond species. These observations indicate that this vector is functional even *in vivo*. Sato et al. [47] demonstrated that the injection of rAAV6 into the ampulla of a pregnant female on Day 0.7 resulted in the successful transduction of late zygotes. Notably, this was achieved without performing *in vivo* EP. The fluorescence intensity was transient and peaked at the morula stage. Notably, the injection of rAAV6 and subsequent *in vivo* EP failed to improve the fluorescence intensity of the resulting morulae, suggesting that using *in vivo* EP in this system is unnecessary.

These findings raise the possibility of genome editing in early embryos through transduction with rAAV6 carrying genome editing components. A two-step gene delivery approach was employed in *in vitro*-isolated zygotes to induce KI events in zygotes. For example, Mizuno et al. [71] first electroporated RNP targeting *Rosa26* locus into ZP-intact mouse zygotes, and then the treated embryos were incubated in a medium containing rAAV6 carrying a 1.8-kb GFP expression cassette flanked by two 100-bp *Rosa26* 5' and 3' homology arms. When the resulting newborn pups were analyzed, the KI efficiency at the *Rosa26* locus was 6%. Chen et al. [73] developed an approach called CRISPR-READI (CRISPR RNP electroporation and AAV donor infection), in which mouse zygotes were cultivated for 5 h in the presence of rAAV1 carrying HDR donor (containing a ~2 kb inducible Sox2-P2A-CreERT2 cassette flanked by two ~480 bp homology arms) and subsequently subjected to *in vitro* EP in the presence of the RNP-targeted *Sox2* locus. When the treated embryos were cultured and genotyped at the blastocyst stage, they exhibited correct targeting of the P2A-CreERT2 cassette, with a KI efficiency of 69%.

Yoon et al. [15] first demonstrated that *in vivo* genome editing of zygotes present within the oviductal lumen is possible through simple intraductal injection of a solution containing two types of rAAV6 (one carrying the *SpCas9* gene derived from *Streptococcus pyogenes* and the other holding the gRNA expression unit) on Day 0.5. Consequently, genome-edited newborn pups were obtained with an indel efficiency of 6%. These findings suggest AAV is useful for inducing genome editing in ZP-enclosed early embryos *in situ.* Abe et al. [49] recently demonstrated that a one-step approach was feasible for obtaining KI rats using a method similar to *i*-GONAD (Figure 5). They simultaneously introduced highly concentrated (2-3 × 10^{11} viral genome copy (VG)/mL) rAAV donors [rAAV1 vector carrying a 3.0-kb tetO-H2B tdTomato cassette comprised of a tet-responsive element (tetO) and a histone H2B-tdTomato fusion protein) flanked by two 0.5-kb homology arms targeting the rat *Rosa26* locus] and CRISPR RNPs into the oviductal lumen of a pregnant rat before *in vivo* EP. Viable

outbred Lister Hooded (LH) rats were obtained with a KI efficiency of 6.1%. Similarly, the KI efficiency was 10.0% when a closed-colony Sprague-Dawley (SD) rat strain was used. In this case, the EP of rAAV vectors in the absence of CRISPR RNPs failed to transduce a zygote, which is consistent with a previous report by Sato et al. [47]. In other words, the EP-mediated delivery of rAAV vectors to ZP-enclosed zygotes is possible only when rAAV vectors are mixed with CRISPR RNPs. It is also interesting to note that the GOI spanning ~3 kb can be knocked into a target locus because, in a previous *i*-GONAD-based KI experiment, a successful KI of a sequence of ~1 kb or less was shown [7]. In this context, a one-step (simultaneous) introduction of the rAAV vector + RNP may be more convenient than the two-step gene delivery approaches shown by Mizuno et al. [71] and Chen et al. [73] for creating KI animals with 3~4 kb GOI. Li et al. [52] recently developed a simple intraductal injection method similar to AAV-based GONAD using rAAV6 to deliver CRISPR reagents to pregnant female rodents. Using this technique, they successfully generated KO and KI (up to 3 kb) rodent lines, which were derived from non-traditional model species, such as the African striped mouse (*Rhabdomys pumilio*), with high efficiency (26-56% for indel efficiency). Li et al. [52] added a sgR26G1 hybridization sequence and a PAM site at the ends of the two homology arms as a double-cleaving rAAV vector to increase the KI frequency. They named this novel technique TIGER (targeted *in vivo* genome editing in rodents).

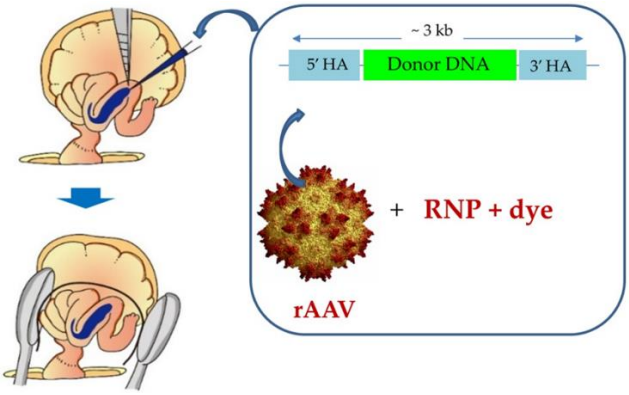

Figure 5 AAV-based GONAD uses ribonucleoprotein (RNP), dye, and recombinant adeno-associated virus (AAV) carrying large donor DNA in rats. This figure was based on the study by Abe et al. [49]. 5' HA, 5'-homologous arm; 3' HA, 3'-homologous arm; RNP, *Cas9* protein/sgRNA complex.

AAV-based GONAD does not require *in vivo* EP after intraductal injection of a solution, which is often harmful to embryo survival. In this sense, this system is more straightforward and convenient for obtaining genome-edited individuals than EP-dependent GONAD/*i*-GONAD-based genome editing systems. However, the construction and propagation of rAAV are laborious and time-consuming. Notably, unlike the oviducts of rodents, which have a helical structure, female pigs have a linear oviductal system 10-15 cm long [74]. In this case, a simple intraductal administration of rAAV6 may be sufficient to infect early porcine embryos to produce genome-edited piglets. However, large amounts of highly concentrated rAAV6 would be required for efficient infection.

2.6 i-GONAD as a Useful Tool for Assessing the Biological Function of Blastomeres in 8- to 16-Cell Embryos

Preimplantation embryos from the zygote to the morula stages exist in the oviductal lumen of a pregnant female rodent. They can serve as targets for gene delivery through GONAD/*i*-GONAD. Each blastomere is exposed to the external environment during the embryo cleavage stages (corresponding to the two-cell to early eight-cell stages). Thus, GONAD/*i*-GONAD-mediated genome editing at these stages may result in mosaic offspring of both edited and unedited cells. This mosaic nature is often undesirable for researchers aiming to produce biallelic KO animals to understand the function of a GOI. However, this is beneficial for exploring the roles of lethal embryonic genes. For example, mosaic fetuses or pups produced through the MI of genome editing reagents into one blastomere of two-cell embryos are viable and carry heritable lethal mutations [75].

Embryos at the 8-cell to morula stages (comprising 16 to 32 cells) are thought to be necessary for generating two types of cells, namely, trophectodermal (TE) cells and inner cell mass (ICM) cells, in a blastocyst [76]. More specifically, the blastomeres of 8-cell to 16-cell embryos facing the external environment tend to differentiate into TE cells, which are later involved in implantation and placenta formation. In contrast, blastomeres inside a source tend to become ICM cells, precursors of the embryonic properties generated after implantation. Thus, it is likely that GONAD/*i*-GONAD at the 8-cell stage will generate genome-edited cells that contribute to both the TE and ICM areas. In contrast, those at the morula stage (i.e., compacted 16-cell embryos) will develop genome-edited cells that preferentially contribute to TE cells. Therefore, GONAD/*i*-GONAD-mediated genome manipulation at these stages may be a novel tool for exploring the molecular mechanisms underlying the segregation of TE and ICM cells, together with those underlying implantation and placenta formation. To our knowledge, this approach has not yet been tested. We thus now named this technology as "*8-16Ci*-GONAD (8-cell to 16-cell embryo-targeted *i*-GONAD)" (Figure 2).

2.7 i-GONAD-Based Production of Wild Mouse Strains

Wild mouse strains (wild-derived inbred mouse strains) are valuable resources for biomedical research because they may possess many novel genetic traits that have not yet been examined [77]. Researchers at the National Institute of Genetics (NIG) in Japan have established nine wild mouse strains and attempted to apply them to reproductive biology experiments. Unfortunately, using genetic engineering technology on these wild strains is difficult because IVF was performed with similar efficiency as in the B6 strain only in two out of nine wild strains. IVF in the other seven wild strains was highly inefficient. Researchers at NIG applied CRISPR-based genome editing using the *i*-GONAD method. Using this method, they showed that it is possible to efficiently modify genes in most wild strains (seven out of the nine strains examined) [48]. These findings suggest that *i*-GONAD will contribute to the development of many wild GM strains in the future, which will also be helpful for future studies.

2.8 i-GONAD-Based Production in Rats

Rats (*Rattus norvegicus*) have been recognized as the most widely used model for biomedical research (especially toxicological, neurobehavioral, and cardiovascular studies) over the past four decades [78]. Since the first report 1997 by Guerts et al. [79] on the production of genome-edited

rats using ZFN technology, a total of 113 GM rats have been produced by MI and nine GM rats by *in vitro* EP [78].

In 2018, two research groups in Japan generated genome-edited rat models using *i*-GONAD. Kobayashi et al. [13] employed *in vivo* EP using a NEPA21 electroporator and demonstrated that the endogenous tyrosinase (*Tyr*) gene, a gene encoding proteins essential for eye pigmentation, was disrupted in pigmented females with an efficiency of 42% when *i*-GONAD using RNP (targeted to *Tyr*) was performed. Based on these findings, Kobayashi et al. [13] named this rat-based *i*-GONAD as "rGONAD." Takabayashi et al. [14] performed experiments similar to those of Kobayashi et al. [13], demonstrating that 56% of fetuses produced had non-pigmented eyes when *i*-GONAD targeted the *Tyr* locus.

Since the reports of Kobayashi et al. [13] and Takabayashi et al. [14], several GM rats (including fetuses) with four genes (*COL4A3*, *COL4A4*, *COL4A5*, and *Tyr*) have been successfully generated [22, 28, 33].

2.9 i-GONAD-Based Hamster Production

The golden hamster (*Mesocricetus auratus*) is a small rodent extensively used in biomedical research. However, hamster embryos are highly vulnerable to damage when placed under *in vitro* conditions, which often hampers the efficient generation of GM hamsters [80]. In this context, *i*-GONAD provides an ideal experimental system by which hamster embryos can be manipulated without exposure to the external environment.

Hirose et al. [19] successfully produced KO hamsters using *i*-GONAD with the RNP-targeted acrosin gene (*Acr*), which is expressed in the sperm head and is thought to be essential for sperm penetration through the ZP. In the present study, six sgRNAs targeting several regions of *Acr* were simultaneously injected into the *Cas9* protein to disrupt *Acr* completely. Of the 15 pups obtained, eight survived beyond the weaning stage. Of these, five had mutant alleles. Notably, homozygous mutant males were sterile because the mutant spermatozoa were successfully bound to the ZP but failed to penetrate it. This finding indicates that acrosin in hamster spermatozoa is essential for regular fertilization in hamsters. Notably, the acrosin-KO mouse spermatozoa were fertile *in vivo* and *in vitro* [81]. Hirose et al. [19] suggested that the prevailing concept that acrosin is not essential for fertilization in mammalian species must be reconsidered.

2.10 Strain Differences in i*-GONAD-Mediated Genome Editing*

Ohtsuka et al. [7, 54, 55] were the first to demonstrate strain differences in *i*-GONAD-mediated genome editing. For example, when randomly bred mice (such as MCH(ICR) and B6C3F1, a hybrid between C3H/He and B6) were used for the *i*-GONAD experiment, *in vivo* EP under relatively stringent electrical conditions (40 V/100-200 Ω/~300 mA) was adequate. Still, zygotes from the inbred B6 strain frequently died under these conditions. On the other hand, *i*-GONAD using the B6 strain was successful under less stringent requirements (40 V/350-400 Ω/~100 mA). This principle was later observed for *i*-GONAD in rats. For example, *in vivo* EP under a current of >500 mA using the NEPA21 electroporator resulted in the successful production of genome-edited SD (albino) and LEW (albino) rats but not pigmented BN rats [14]. However, genome-edited BN rats were successfully obtained with 75-100% efficiencies when *i*-GONAD was performed at a current of 100-300 mA [22].

As described above, the NEPA21 electroporator employs a constant voltage and has been widely used for *i*-GONAD-mediated production of genome-edited animals. In contrast, other electroporators (as exemplified by the GEB15 (BEX Co., Ltd.)) employ a constant current. Kobayashi et al. [21] examined the optimal EP conditions generating 100-mA current, which is suitable for generating genome-edited B6 mice, using two electroporators, NEPA21 and GEB15. As a result, in a case where *i*-GONAD is performed using GEB15, EP under an average resistance of 367 Ω and an average voltage of 116 mA was the best. These findings suggest the importance of exploring optimal EP conditions when researchers intend to apply *i*-GONAD to animals other than mice and rats.

2.11 Advantages of Using a Highly Enriched Diet and a Highly Reproductive Female as a Foster Mother to Achieve Increased Performance in i-*GONAD*

Successful gene modification of inbred mouse strains of interest, such as B6, was reported by Ohtsuka et al. [7] and Kobayashi et al. [21] using EP conditions with relatively low stringency; however, this remains a problem owing to their low fertility and embryo fragility after *i*-GONAD. Melo-Silva et al. [50] explored the optimal conditions for efficiently producing genome-edited pups. They observed a reduction in litter size when *i*-GONAD was performed in superovulated pregnant females even though pregnancy rates remained unaffected. Furthermore, neither natural mating nor administering low doses of pregnant mare serum gonadotropin increased the low fertility rates observed in superovulated B6 females. In contrast, dietary enrichment had a positive effect on pregnancy success. Furthermore, cohousing *i*-GONAD-treated pregnant B6 females with synchronized expectant FVB/NJ companion mothers increased the survival of small litters. These results suggest the importance of using an enriched diet and sharing *i*-GONAD-treated pups with standard pups delivered from high-fertility females, such as FVB/NJ, to increase the productivity of *i*-GONAD-treated puppies.

3. Possible Application of *i*-GONAD to Assess Oviductal Function

The mammalian oviduct plays a supporting role in sperm locomotion toward the fertilization site (ampulla), uptake of ovulated oocytes at the infundibulum, fertilization of ovulated oocytes with sperm at the ampulla, oviductal transport of zygotes toward the uterus, and secretion of growth factors to support zygote development [82] (Figure 6). According to Li and Winuthayanon [82], during the early stages of preimplantation development, pyruvate and lactate provided by the oviductal fluid are the primary energy sources for oxidative metabolism. Glucose is also supplied by the oviductal epithelial cells, which convert glycogen into sugar through amylase. Notably, during the cleavage stage, the mitochondria in the embryos were immature and did not function. However, during the morula and blastocyst stages, the mitochondria mature and use oxygen and glucose to generate ATP through glycolysis. Notably, glutamic acid, one of the most abundant amino acids with the highest concentrations in female reproductive fluids, has recently been shown to act as a signaling molecule that exerts its effects by activating cell membrane receptors in preimplantation embryos [83]. In contrast, oviductal epithelial cells provide embryotrophic factors, such as epidermal growth factor, fibroblast growth factor, insulin-like growth factor, and transforming growth factor to promote cleavage and embryonic development.

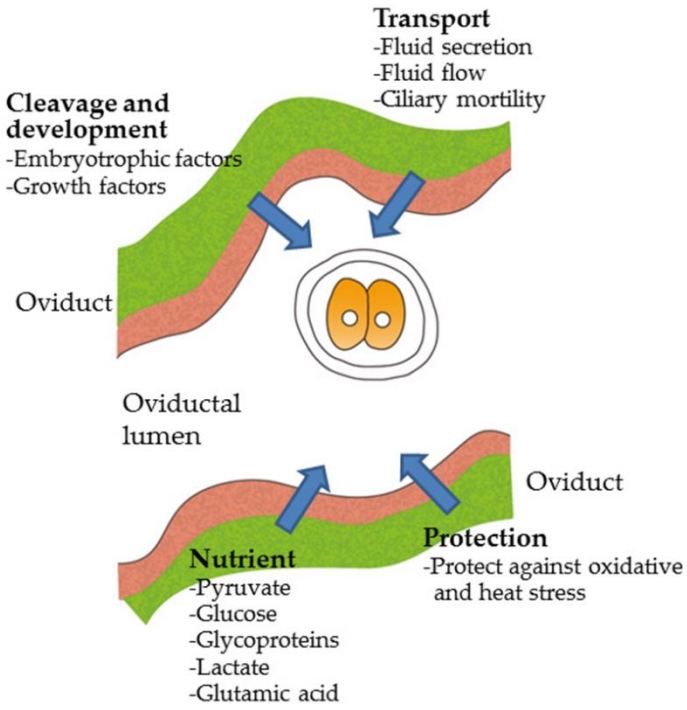

Figure 6 Schematic of the possible roles of the oviduct in assisting *in vivo* development of preimplantation embryos. Molecular interactions between the oviduct and the source can be divided into the following domains: protection, cleavage and development, transport, and nutrition. This figure was drawn in-house, according to Figure 5 in Li and Winuthayanon's paper [82].

In this context, *in situ* gene delivery to oviductal epithelial cells appears to be a promising choice for assessing the function of each oviduct. Unfortunately, little is known about effective *in vivo* transfection of these cells. The Biological Research Center group (Madrid, Spain) first succeeded in transfecting these cells through intraductal instillation of liposomal encapsulated plasmid DNA, but its efficiency was very low (less than 5%) [84]. Development of "gene transfer to the oviductal epithelium (GTOVE)" [8], a prototype of GONAD/*i*-GONAD, made it possible to assess the function of an oviduct directly because approximately 41% of oviductal epithelial cells (in the ampulla region) facing the oviductal lumen were successfully transfected with plasmid DNA (Figures 7a, 7b). Later, these oviductal epithelial cells were also efficiently infected by the intraoviductal administration of rAAV6 [47] (Figures 7c-7e). This suggests that GONAD/*i*-GONAD or AAV-based GONAD is helpful for gene manipulation in early embryos and oviductal epithelial cells facing the oviductal lumen.

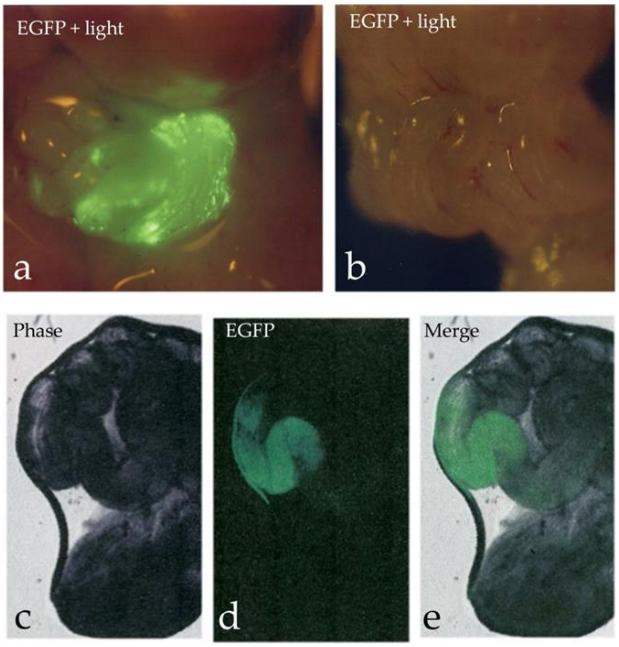

Figure 7 *In vivo* gene delivery to the oviductal epithelium after instillation of plasmid DNA and subsequent *in vivo* electroporation (EP) (a, b) or intraoviductal instillation of recombinant adeno-associated virus (rAAV) (c-e). Inspection of fluorescence in the oviducts after EGFP-expressing plasmid DNA delivery demonstrated that the transfected area exhibited bright fluorescence (a). In contrast, the oviduct of an untreated female was non-fluorescent (b). Inspection of fluorescence in the oviducts (c-e) after AAV-based GONAD demonstrated that the fluorescent oviductal area appeared to correspond to the ampulla, where the rAAV6 vector was directly injected. Photographs in (a, b) were taken under UV illumination and were reproduced in-house, based on the paper by Sato [8]. Pictures in (c, d) were captured under white light (phase) or blue light illumination (EGFP). A mixed image of white and blue light illumination is shown as "Merge." These are reproduced with permission from Sato et al. [47], published by MDPI in 2022.

Several genes thought to be important for oviductal function have been identified to date. These include oviduct-specific glycoprotein (OVGP1 or oviductin), which is a high-molecular-weight glycoprotein secreted from non-ciliated oviductal epithelial cells that can bind to the ZP and is thought to provide a positive effect on IVF [85]; oviduct-specific glycoprotein (OGP), a member of the chitinase protein family, which can directly associate with gametes or with the early embryo in the oviduct [86]; heat shock protein 70 (HSP70), which is expressed in the mammalian sperm and can stimulate sperm motility *in vitro* [87]; and sperm adhesion molecule 1 (SPAM1), which is a widely conserved sperm surface protein involved in ZP-sperm binding [88].

Production of KO or KI animals derived from chimeric mice generated by chimeric formation between gene-targeted ES cells and blastocysts is necessary for examining the functions of these genes in oviducts. For example, Prunskaite-Hyyrylainen et al. [89] demonstrated that wingless-type integration family member 4 (*Wnt4*) signaling is required for the development of the female reproductive tract because *Wnt4* KO female mice exhibited a failure of *coiling* and *lack* of mucosal *folding* of the epithelial layer. Intraductal instillation of siRNA or microRNA for *Wnt4* or RNP targeting *Wnt4* into wild-type female mice and subsequent *in vivo* EP may result in the

manifestation of phenotypes similar to those observed in Wnt4 KO female mice, whereby the oviduct failed to coil.

Furthermore, Yuan et al. [90] recently reported that deleting two miRNA gene clusters (miR-34b/c and miR-449) in mice leads to female sterility. This female infertility phenotype is likely caused by a lack of motile cilia on the inner lining of the oviductal epithelium because *Wnt4* KO females exhibited average hormonal profiles and folliculogenesis. The cilia-less phenotype of *Wnt4* KO females can be recreated using *i*-GONAD, in which the endogenous expression of miR-34b/c and miR-449 in the oviductal epithelium can be partially blocked. Thus, *i*-GONAD-mediated disruption of oviduct-specific genes may help elucidate the physiological roles of GOI in the oviduct, which is more convenient and rapid than previous germline transgenesis-based approaches, such as the production of animal strains established from gene-targeted embryonic stem (ES) cells or embryo genome editing.

4. Oviduct-Derived Carcinogenesis Induced by *i*-GONAD

Proto-oncogenes (e.g., *src, ras, myc*, and *erbB/EGFR*) are healthy cellular genes found in the cell. Each produces a protein involved in cell growth, division, and other processes under normal conditions. However, if an error (mutation) occurs in these proto-oncogenes, the expression of the mutant gene causes normal cells to become tumorigenic cells with persistent proliferation ability [91].

Teng et al. [40] reported that high-grade serous ovarian carcinoma (HGSOC) is the *most common* form of ovarian cancer and has the lowest survival rate. Ford and Yamanaka [41] described an *in vivo* oviductal EP method similar to GTOVE that facilitates the delivery of multiple plasmids into oviductal epithelial cells. Teng et al. [40] employed this method to generate HGSOC models in mice through *in situ* induction of mutations in endogenous tumorigenesis-related genes (breast cancer susceptibility gene I [*Brca1*], transformation-related protein 53 [*Trp53*], neurofibromin 1 [*Nf1*], and phosphatase and tensin homolog [*Pten*]). When mutations were introduced into three of the four genes, *Trp53, Brca1,* and either *Nf1* or *Pten*, using the CRISPR/*Cas9* genome editing approach, the transfected sites exhibited the formation of tumors similar to those of human HGSOC and changes in chromosome number. Teng et al. [40] concluded that the *in vivo* oviductal EP method is helpful for the *in situ* generation of HGSOC models in mice, which facilitates the treatment of ovarian cancer.

5. Advantages and Challenges of GONAD/*i*-GONAD

In Table 2, we summarized a comparison of *i*-GONAD with other MI or EP-based genome editing methods. For example, based on its simplicity and cost-effectiveness, GONAD/*i*-GONAD is superior to pre-existing MI- or *in vitro* EP-based genome-editing technologies for genome manipulation of mammalian early embryos. It requires only four to five pregnant females per session. It does not require a micromanipulator, unique skills to operate it, *in vitro* cultivation of genome-eddied zygotes, ET, recipient pseudopregnant females, or vasectomized males for preparing those recipient females [54, 55]. Only an electroporator that generates a square pulse and tweezer-type electrodes are required. In some cases, intraductal injection into the ampulla of a pregnant female under a dissecting microscope with the aid of a mouthpiece-attached glass micropipette may be difficult for beginners. Still, compared to ET, its hurdle is not very high. Like MI- and *in vitro* EP-based genome editing, KO or KI at a target locus is possible using GONAD/*i*-GONAD. KI efficiency is comparable

between *i*-GONAD- and MI-based genome editing [7]. Ohtsuka et al. [7] also showed that *i*-GONAD-treated females could be reused for the next session of GONAD experiments because the *i*-GONAD-treated oviducts (which had been pierced by a glass micropipette and electroplated) were always intact. This is consistent with the concept of 3R because of animal welfare. Other approaches based on MI or *in vitro* EP always require the sacrifice of many females to acquire several zygotes. GONAD/*i*-GONAD was also applicable to animals sensitive to *in vitro* manipulation, such as hamsters and wild mice.

Table 2 Microinjection (MI), *in vitro* electroporation (EP), and improved genome editing via oviductal nucleic acid delivery (*i*-GONAD) have all been used to produce genome-edited mice[1].

	MI	EP	*i*-GONAD
Embryo culture	Req.	Req.	Not req.
Preparation of pseudopregnant female and vasectomized male	Req.	Req.	Not req.
Egg transfer (ET) to pseudopregnant female	Req.	Req.	Not req.
Micromanipulator	Req.	Not req.	Not req.
Electroporator	Not req.	Req.	Req.
Micropipette puller	Req.	Not req.	Req./not req.
The approximate number of mice required to obtain 20 pups			
Pregnant female mice	8	3	5
Vasectomized males	6	4	0
Pseudopregnant mice	6	4	0
Approximate amount of genome editing components required to obtain 20 pups			
Amount of solution prepared (μL)	20	6-20	17
Cas9 protein (μg)	1	0.6-13	17
gRNA (μg)	0.4	1.2-2.7	17
ssODN (μg)	0.2	2.4-10	34
Availability of donor DNA for knock-in (KI)			
Plasmid DNA	Suitable	Not suitable	Not suitable
ssODN (or ssDNA up tp ~1 kb)	Suitable	Suitable	Suitable
Approximate time required for obtaining 20 pups (hours)			
Zygote collection or egg collection (for in vitro fertilization (IVF))	2 or 3 (IVF)	1 or 3 (IVF)	0
Delivery of CRISPR components	2	0.3	1
ET to pseudopregnant female	1.5	1	0

Features	Required for manipulator system and highly developed skill	Many zygotes (~50) can be treated at once	*Ex vivo* handling of embryos (i.e., zygote collection, MI, and ET to pseudopregnant female) can be avoided

[1]Mainly based on the paper of Ohtsuka and Sato [55].

Plasmid DNA is frequently used for genome editing studies to introduce the *Cas9* gene and gRNA or as donor DNA for efficient KI at a target locus. For example, microhomology-mediated end-joining (MMEJ)-aided Precise Integration into Target Chromosome) (PITCh) [92], homology-independent targeted insertion (HITI) [93], two-hit by gRNA and two oligos with a targeting plasmid (2H2OP) [94], pCriMGET (plasmid of synthetic CRISPR coded RNA target sequence-equipped donor plasmid-mediated gene targeting)-based KI system [95], combination of NHEJ and HDR repair pathway (Combi-CRISPR) [96], and Targeted Knock-In with Two (TKIT) [97] have employed plasmid DNA. Unfortunately, introducing plasmid DNA into mouse zygotes is generally tricky when EP is used. Only a few reports have shown the successful introduction of plasmid DNA into early mouse embryos [98-100]. For this purpose, extensive exploration of optimal conditions for EP, such as strength of electric pulse, duration of vibration, and times of EP, is required. Hakim et al. [100] evaluated several *in vitro* EP parameters to determine the optimal conditions for delivering plasmid DNA into mouse follicles, oocytes, and early embryos. They employed *in vitro* EP with three square pulses of 30 V for 1 ms each at an interval of 10 s in 1-mm gap cuvettes. The advantage of using plasmid DNA is that >1 kb long inserts can be introduced into the target site of an endogenous gene. However, once plasmid DNA is used for EP-based gene introduction into zygotes, adjacent plasmid sequences can often be integrated into host chromosomes. Lackner et al. [68] demonstrated a strategy for scarless integration of a reporter gene into a plasmid at an endogenous target locus. In other words, the adjacent plasmid sequences were not integrated into this system. Lackner et al. [68] used a generic donor plasmid that contained a tag of interest flanked by two gRNA cleavage sites derived from a genomic locus in zebrafish (*tia1l*) that was absent in human cells. This donor plasmid contained a *U6* promoter that drove the expression of the *tia1l* gRNA. When cells were transfected with *Cas9*, the donor plasmid and a gene-specific gRNA, *tia1l* gRNA produced from the U6 promoter, cut *tia1l* into the donor plasmid, releasing the tag from the plasmid. The tag was then spontaneously integrated at the site specified by the gene-specific gRNA. In this context, the system developed by Lackner et al. [68] can be used if *i*-GONAD successfully incorporates plasmid DNA into zygotes.

GONAD/*i*-GONAD requires expensive electroporation procedures. Therefore, it is desirable to produce genome-edited animals without using an electroporator. In this context, AAV-based GONAD [15, 49] or TIGER [52], which do not require an electroporator, are very convenient for the one-step acquisition of GM animals. However, these approaches are always associated with laborious and time-consuming tasks such as viral vector preparation.

6. Conclusion

Since the reports on GONAD/*i*-GONAD in 2015 and 2018, several improvements have been made in these systems, including *i*-GONAD, applied at the early zygote stage ("*ezi*-GONAD") [45], two-step *i*-GONAD (*si*-GONAD) [20], and *i*-GONAD allowing tagging at a desired endogenous gene (*tagi*-GONAD) [32, 44]. These technologies now create pups with KO or KI phenotypes [12]. Thus,

GONAD/*i*-GONAD and their derivatives are now recognized as possible alternatives to pre-existing systems based on the *ex vivo* handling of zygotes because they have many advantages over the previous methods because of the reduced number of females used (therefore fitting the animal welfare 3R principle) and the absence of laborious tasks required for *ex vivo* handling of embryos (such as embryo isolation, MI or *in vitro* EP procedure, cultivation of sources, and ET to pseudopregnant recipient females).

GONAD/*i*-GONAD requires an expensive electroporator to enable the smooth delivery of nucleic acids into zygotes within the oviductal lumen. In this case, EP-based gene delivery applies only to rodent oviducts with tightly packed helical (coiled) structures but not linear (uncoiled) systems, such as porcine oviducts. However, whether GONAD/*i*-GONAD can be applied to oviducts with uncoiled facilities remains unclear. Besides gene delivery to zygotes *in situ*, GONAD/*i*-GONAD can edit the target genome of oviductal epithelial cells surrounding the oviductal lumen because gene correction in the oviductal epithelial cells of mice with mutated *EGFP* cDNA occurs after *i*-GONAD [39]. This implies that GONAD/*i*-GONAD is also beneficial for exploring oviductal function by manipulating genes coding for oviduct-specific factors or those related to intra-oviductal embryo transport. Furthermore, as shown by Teng et al. [40], GONAD/*i*-GONAD can help generate malignant tumors in the oviduct by overexpressing oncogenes in oviductal epithelial cells or genome editing of anti-oncogenes. These approaches provide an additional role for GONAD/*i*-GONAD in assessing the biological functions of the reproductive system in mammals.

The ZP surrounding a zygote is the most significant barrier to delivering nucleic acids into mammalian zygotes [17]. To date, an electroporator enables the delivery of small molecules such as mRNA and proteins; however, in the case of plasmid DNA delivery, extensive exploration is required for optimal EP conditions, as demonstrated by Peng et al. [99] and Hakim et al. [100]. Among the viral vectors tested, only the rAAV vector could penetrate the ZP after simple incubation with zygotes *in vitro* and *in vivo* [15, 47, 71]. However, the construction and preparation of rAAVs are laborious and time-consuming. Furthermore, the size of DNA inserts up to 4.3 kb is strictly limited owing to the capacity of the AAV cargo itself. To date, most known approaches are based on plasmid-based KI of large-sized DNA into a target locus, as exemplified by several unique names such as PITCh, HITI, 2H2OP, pCriMGET, Combi-CRISPR, and TKIT. Therefore, optimal EP conditions that enable plasmid DNA delivery into ZP-intact embryos are urgently required. Alternatively, developing new EP-free methods that are simpler than EP-based GONAD/*i*-GONAD may also be required. This is particularly important when large animals with linear oviductal systems are used for genome editing. The possible use of ZP-penetrating reagents [i.e., multi-wall carbon nanotubes (MWNTs) and VisuFect) will be highly desirable in this case, as shown in our previous paper [17]; however, there are no reports of successful genome editing of mammalian zygotes using these reagents.

In addition to improving gene delivery methods, a close examination of genome editing components that enable precise and off-target free genome editing at a target locus is also required. New CRISPR/*Cas9*-based genome editing systems known as “prime or base editing” systems have recently been reported [101-103]. These systems do not require DSBs and allow precise gene correction at the single-nucleotide level at a target locus. To our knowledge, these reagents have not been previously used in GONAD/*i*-GONAD-related studies. We believe that rapid advances in genetic engineering systems will help establish a more convenient and straightforward method for producing GM animals using modified GONAD/*i*-GONAD.

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Author Contributions

M.S. and S.N. designed and drafted the manuscript; K.M., M.O., S.T., E.I., I.S. and S.W. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

References

1. Clark JF, Dinsmore CJ, Soriano P. A most formidable arsenal: Genetic technologies for building a better mouse. Genes Dev. 2020; 34: 1256-1286.
2. Harrison MM, Jenkins BV, O'Connor Giles KM, Wildonger J. A CRISPR view of development. Genes Dev. 2014; 28: 1859-1872.
3. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-*Cas9* for genome engineering. Cell. 2014; 157: 1262-1278.
4. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-*Cas9* system. Nat Protoc. 2013; 8: 2281-2308.
5. Kaneko T, Sakuma T, Yamamoto T, Mashimo T. Simple knockout by electroporation of engineered endonucleases into intact rat embryos. Sci Rep. 2014; 4: 6382.
6. Takahashi G, Gurumurthy CB, Wada K, Miura H, Sato M, Ohtsuka M. GONAD: Genome-editing via oviductal nucleic acids delivery system: A novel microinjection independent genome engineering method in mice. Sci Rep. 2015; 5: 11406.
7. Ohtsuka M, Sato M, Miura H, Takabayashi S, Matsuyama M, Koyano T, et al. *i*-GONAD: A robust method for *in situ* germline genome engineering using CRISPR nucleases. Genome Biol. 2018; 19: 25.
8. Sato M. Intraoviductal introduction of plasmid DNA and subsequent electroporation for efficient *in vivo* gene transfer to murine oviductal epithelium. Mol Reprod Dev. 2005; 71: 321-330.
9. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified *Cas9* ribonucleoproteins. Genome Res. 2014; 24: 1012-1019.
10. Quadros RM, Miura H, Harms DW, Akatsuka H, Sato T, Aida T, et al. Easi-CRISPR: A robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol. 2017; 18: 92.
11. Miura H, Quadros RM, Gurumurthy CB, Ohtsuka M. Easi-CRISPR for creating knock-in and conditional knockout mouse models using long ssDNA donors. Nat Protoc. 2018; 13: 195-215.

12. Sato M, Ohtsuka M, Inada E, Nakamura S, Saitoh I, Takabayashi S. Recent advances in *in vivo* genome editing targeting mammalian preimplantation embryos. CRISPR Technology. Rijeka, Croatia: InTechOpen; 2023. doi: 10.5772/intechopen.106873.
13. Kobayashi T, Namba M, Koyano T, Fukushima M, Sato M, Ohtsuka M, et al. Successful production of genome-edited rats by the rGONAD method. BMC Biotechnol. 2018; 18: 19.
14. Takabayashi S, Aoshima T, Kabashima K, Aoto K, Ohtsuka M, Sato M. *i*-GONAD (improved genome-editing via oviductal nucleic acids delivery), a convenient *in vivo* tool to produce genome-edited rats. Sci Rep. 2018; 8: 12059.
15. Yoon Y, Wang D, Phillip WL, Tai PWL, Riley J, Gao G, et al. Streamlined *ex vivo* and *in vivo* genome editing in mouse embryos using recombinant adeno-associated viruses. Nat Commun. 2018; 9: 412.
16. Hirose M, Ogura A. The golden (Syrian) hamster as a model for the study of reproductive biology: Past, present, and future. Reprod Med Biol. 2018; 18: 34-39.
17. Sato M, Ohtsuka M, Nakamura S. Intraoviductal instillation of a solution as an effective route for manipulating preimplantation mammalian embryos *in vivo*. In: New Insights into Theriogenology. Rijeka, Croatia: InTechOpen; 2018. pp. 135-150.
18. Koyano T, Namba M, Kobayashi T, Nakakuni K, Nakano D, Fukushima M, et al. The p21 dependent G2 arrest of the cell cycle in epithelial tubular cells links to the early stage of renal fibrosis. Sci Rep. 2019; 9: 12059.
19. Hirose M, Honda A, Fulka H, Tamura Nakano M, Matoba S, Tomishima T, et al. Acrosin is essential for sperm penetration through the zona pellucida in hamsters. Proc Natl Acad Sci USA. 2020; 117: 2513-2518.
20. Sato M, Miyagasako R, Takabayashi S, Ohtsuka M, Hatada I, Horii T. Sequential *i*-GONAD: An improved *in vivo* technique for CRISPR/*Cas9*-based genetic manipulations in mice. Cells. 2020; 9: 546.
21. Kobayashi Y, Aoshima T, Ito R, Shinmura R, Ohtsuka M, Akasaka E, et al. Modification of *i*-GONAD suitable for production of genome-edited C57BL/6 inbred mouse strain. Cells. 2020; 9: 957.
22. Takabayashi S, Aoshima T, Kobayashi Y, Takagi H, Akasaka E, Sato M. Successful *i*-GONAD in Brown Norway rats by modification of *in vivo* electroporation conditions. OBM Genet. 2020; 4: 121.
23. Kaneko T, Tanaka S. Improvement of genome editing by electroporation using embryos artificially removed cumulus cells in the oviducts. Biochem Biophys Res Commun. 2020; 527: 1039-1042.
24. Ferez M, Knudson CJ, Lev A, Wong EB, Alves Peixoto P, Tang L, et al. Viral infection modulates Qa-1b in infected and bystander cells to properly direct NK cell killing. J Exp Med. 2021; 218: e20201782.
25. Umschweif G, Medrihan L, Guillén Samander A, Wang W, Sagi Y, Greengard P. Identification of Neurensin-2 as a novel modulator of emotional behavior. Mol Psychiatry. 2021; 26: 2872-2885.
26. Loubalova Z, Fulka H, Horvat F, Pasulka J, Malik R, Hirose M, et al. Formation of spermatogonia and fertile oocytes in golden hamsters requires piRNAs. Nat Cell Biol. 2021; 23: 992-1001.
27. Zhang H, Shang R, Bi P. Feedback regulation of notch signaling and myogenesis connected by MyoD-Dll1 axis. PLoS Genet. 2021; 17: e1009729.

28. Namba M, Kobayashi T, Kohno M, Koyano T, Hirose T, Fukushima M, et al. Creation of X-linked Alport syndrome rat model with Col4a5 deficiency. Sci Rep. 2021; 11: 20836.
29. Ho YT, Shimbo T, Wijaya E, Kitayama T, Takaki S, Ikegami K, et al. Longitudinal single-cell transcriptomics reveals a role for Serpina3n-mediated resolution of inflammation in a mouse colitis model. Cell Mol Gastroenterol Hepatol. 2021; 12: 547-566.
30. Yoshinaga S, Shin M, Kitazawa A, Ishii K, Tanuma M, Kasai A, et al. Comprehensive characterization of migration profiles of murine cerebral cortical neurons during development using FlashTag labeling. iScience. 2021; 24: 102277.
31. Shang R, Zhang H, Bi P. Generation of mouse conditional knockout alleles in one step using the *i*-GONAD method. Genome Res. 2021; 31: 121-130.
32. Nakano H, Kawai S, Ooki Y, Chiba T, Ishii C, Nozawa T, et al. Functional validation of epitope-tagged ATF5 knock-in mice generated by improved genome editing of oviductal nucleic acid delivery (*i*-GONAD). Cell Tissue Res. 2021; 385: 239-249.
33. Aoshima T, Kobayashi Y, Takagi H, Iijima K, Sato M, Takabayashi T. Modification of improved-genome editing via oviductal nucleic acids delivery (i-GONAD)-mediated knock-in in rats. BMC Biotechnol. 2021; 21: 63.
34. Hasegawa A, Mochida K, Nakamura A, Miyagasako R, Ohtsuka M, Hatakeyama M, et al. Use of anti-inhibin monoclonal antibody for increasing the litter size of mouse strains and its application to *i*-GONAD. Biol Reprod. 2022; 107: 605-618.
35. Takaki S, Shimbo T, Ikegami K, Kitayama T, Yamamoto Y, Yamazaki S, et al. Generation of a recessive dystrophic epidermolysis bullosa mouse model with patient-derived compound heterozygous mutations. Lab Invest. 2022; 102: 574-580.
36. Hiradate Y, Harima R, Yanai R, Hara K, Nagasawa K, Osada M, et al. Loss of *Axdnd1* causes sterility due to impaired spermatid differentiation in mice. Reprod Med Biol. 2022; 21: e12452.
37. Iwata S, Nakadai H, Fukushi D, Jose M, Nagahara M, Iwamoto T. Simple and large-scale chromosomal engineering of mouse zygotes via *in vitro* and *in vivo* electroporation. Sci Rep. 2019; 9: 14713.
38. Iwata S, Sasaki T, Nagahara M, Iwamoto T. An efficient *i*-GONAD method for creating and maintaining lethal mutant mice using an inversion balancer identified from the C3H/HeJJcl strain. G3. 2021; 11: jkab194.
39. Miura H, Imafuku J, Kurosaki A, Sato M, Ma Y, Zhang G, et al. Novel reporter mouse models useful for evaluating *in vivo* gene editing and for optimization of methods of delivering genome editing tools. Mol Ther Nucleic Acids. 2021; 24: 325-336.
40. Teng K, Ford MJ, Harwalkar K, Li Y, Pacis AS, Farnell D, et al. Modeling highgrade serous ovarian carcinoma using a combination of *in vivo* fallopian tube electroporation and CRISPRCas9–mediated genome editing. Cancer Res. 2021; 81: 5147-5160.
41. Ford MJ, Yamanaka Y. Reprogramming mouse oviduct epithelial cells using *in vivo* electroporation and CRISPR/Cas9-mediated genetic manipulation. Methods Mol Biol. 2022; 2429: 367-377.
42. Sato M, Takabayashi S, Akasaka E, Nakamura S. Recent advances and future perspectives of *in vivo* targeted delivery of genome-editing reagents to germ cells, embryos, and fetuses in mice. Cells. 2020; 9: 799.

43. Sanchez-Baltasar R, Garcia-Torralba A, Nieto-Romero V, Page A, Molinos-Vicente A, López-Manzaneda S, et al. Efficient and fast generation of relevant disease mouse models by *in vitro* and *in vivo* gene editing of zygotes. CRISPR J. 2022; 5: 422-434.
44. Aoto K, Takabayashi S, Mutoh H, Saitsu H. Generation of flag/DYKDDDDK epitope tag knock-in mice using *i*-GONAD enables detection of endogenous CaMKIIα and β proteins. Int J Mol Sci. 2022; 23: 11915.
45. Takabayashi S, Iijima K, Tsujimura M, Aoshima T, Takagi H, Aoto K, et al. Successful *i*-GONAD in mice at early zygote stage through *in vivo* electroporation three min after intraoviductal instillation of CRISPR-ribonucleoprotein. Int J Mol Sci. 2022; 23: 10678.
46. Takahashi R, Takahashi G, Kameyama Y, Sato M, Ohtsuka M, Wada K. Gender-difference in hair length as revealed by Crispr-based production of long-haired mice with dysfunctional FGF5 mutations. Int J Mol Sci. 2022; 23: 11855.
47. Sato M, Sato-Yamamoto N, Wakita A, Haraguchi M, Shimonishi M, Okuno H. Direct injection of recombinant AAV-containing solution into the oviductal lumen of pregnant mice caused in situ infection of both preimplantation embryos and oviductal epithelium. Int J Mol Sci. 2022; 23: 4897.
48. Imai Y, Tanave A, Matsuyama M, Koide T. Efficient genome editing in wild strains of mice using the *i*-GONAD method. Sci Rep. 2022; 12: 13821.
49. Abe M, Nakatsukasa E, Natsume R, Hamada S, Sakimura K, Watabe AM, et al. A novel technique for large-fragment knock-in animal production without ex vivo handling of zygotes. Sci Rep. 2023; 13: 2245.
50. Melo-Silva CR, Knudson CJ, Tang L, Kafle S, Springer LE, Choi J, et al. Multiple and consecutive genome editing using *i*-GONAD and breeding enrichment facilitates the production of genetically modified mice. Cells. 2023; 12: 1343.
51. Wiegreffe C, Ehricke S, Schmid L, Andratschke J, Britsch S. Using *i*-GONAD for cell-type-specific and systematic analysis of developmental transcription factors *in vivo*. Biology. 2023; 12: 1236
52. Li S, Mereby SA, Rothstein M, Johnson MR, Brack BJ, Mallarino R. TIGER: Single-step *in vivo* genome editing in a non-traditional rodent. Cell Rep. 2023; 42: 112980.
53. Namba M, Kobayashi T, Koyano T, Kohno M, Ohtsuka M, Matsuyama M. GONAD: A new method for germline genome editing in mice and rats. Dev Growth Differ. 2021; 63: 439-447.
54. Gurumurthy CB, Sato M, Nakamura A, Inui M, Kawano N, Islam M, et al. Creation of CRISPR-based germlinegenome-engineered mice without ex vivo handling of zygotes by i-GONAD. Nat Protoc. 2019; 14: 2452-2482.
55. Ohtsuka M, Sato M. *i*-GONAD: A method for generating genome-edited animals without *ex vivo* handling of embryos. Dev Growth Differ. 2019; 61: 306-315.
56. Sato M, Nakamura A, Sekiguchi M, Matsuwaki T, Miura H, Gurumurthy CB, et al. Improved genome editing via oviductal nucleic acids delivery (*i*-GONAD): Protocol steps and additional notes. In: Transgenesis. Methods in Molecular Biology. New York, NY, US: Humana; 2023. pp. 325-340.
57. Galili U. The α-gal epitope (gal α1-3Gal β1-4GlcNAc-R) in xenotransplantation. Biochimie. 2001; 83: 557-563.
58. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/*Cas*-mediated genome engineering. Cell. 2013; 154: 1370-1379.

59. Ma X, Chen C, Veevers J, Zhou XM, Ross RS, Feng W, et al. CRISPR/*Cas9*-mediated gene manipulation to create single amino-acid-substituted and floxed mice with a cloning-free method. Sci Rep. 2017; 7: 42244.
60. Horii T, Morita S, Kimura M, Terawaki N, Shibutani M, Hatada I. Efficient generation of conditional knockout mice via sequential introduction of lox sites. Sci Rep. 2017; 7: 7891.
61. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-*Cas9* using asymmetric donor DNA. Nat Biotechnol. 2016; 34: 339-344.
62. Hogan B, Beddington R, Costantini F, Lacy L. Manipulating the mouse embryo. A laboratory manual. New York, NY, US: Cold Spring Harbor Laboratory Press; 1994.
63. Gu B, Posfai E, Rossant J. Efficient generation of targeted large insertions by microinjection into two-cell-stage mouse embryos. Nat Biotechnol. 2018; 36: 632-637.
64. Reyes AP, Lanner F. Time matters: Gene editing at the mouse 2-cell embryo stage boosts knockin efficiency. Cell Stem Cell. 2018; 23: 155-157.
65. Wilde JJ, Aida T, Del Rosario RCH, Kaiser T, Qi P, Wienisch M, et al. Efficient embryonic homozygous gene conversion via RAD51-enhanced interhomolog repair. Cell. 2021; 184: 3267-3280.
66. Park SJ, Yoon S, Choi EH, Hyeon H, Lee K, Kim KP. Elevated expression of exogenous RAD51 enhances the CRISPR/*Cas9*-mediated genome editing efficiency. BMB Rep. 2023; 56: 102-107.
67. Ma L, Ruan J, Song J, Wen L, Yang D, Zhao J, et al. Mi*Cas9* increases large size gene knock-in rates and reduces undesirable on-target and off-target indel edits. Nat Commun. 2020; 11: 6082.
68. Lackner DH, Carré A, Guzzardo PM, Banning C, Mangena R, Henley T, et al. A generic strategy for CRISPR-*Cas9*-mediated gene tagging. Nat Commun. 2015; 6: 10237.
69. Zeng F, Beck V, Schuierer S, Garnier I, Manneville C, Agarinis C, et al. A simple and efficient CRISPR technique for protein tagging. Cells. 2020; 9: 2618.
70. Sung YK, Kim SW. Recent advances in the development of gene delivery systems. Biomater Res. 2019; 23: 8.
71. Mizuno N, Mizutani E, Sato H, Kasai M, Ogawa A, Suchy F, et al. Intra-embryo gene cassette knockin by CRISPR/*Cas9*-mediated genome editing with adeno-associated viral vector. IScience. 2018; 9: 286-297.
72. Ellis BL, Hirsch ML, Barker JC, Connelly JP, Steininger RJ III, Porteus MH. A survey of *ex vivo*/*in vitro* transduction efficiency of mammalian primary cells and cell lines with nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. Virol J. 2013; 10: 74.
73. Chen S, Sun S, Moonen D, Lee C, Lee AY, Schaffer DV, et al. CRISPR-READI: Efficient generation of knockin mice by CRISPR RNP electroporation and AAV donor infection. Cell Rep. 2019; 27: 3780-3789.
74. Ishii K, Yanagisawa T. Structure of the female reproductive organ of pig ascaris. Jpn J Med Sci Biol. 1954; 7: 95-109.
75. Wu Y, Zhang J, Peng B, Tian D, Zhang D, Li Y, et al. Generating viable mice with heritable embryonically lethal mutations using the CRISPR-*Cas9* system in two-cell embryos. Nat Commun. 2019; 10: 2883.
76. Marikawa Y, Alarcón VB. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. Mol Reprod Dev. 2009; 76: 1019-1032.

77. Poltorak A, Apalko S, Sherbak S. Wild-derived mice: From genetic diversity to variation in immune responses. Mamm Genome. 2018; 29: 577-584.
78. Sato M, Nakamura S, Inada E, Takabayashi S. Recent advances in the production of genome-edited rats. Int J Mol Sci. 2022; 23: 2548.
79. Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, et al. Knockout rats via embryo microinjection of zincfinger nucleases. Science. 2009; 325: 433.
80. Schini SA, Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. Biol Reprod. 1988; 39: 1183-1192.
81. Baba T, Azuma S, Kashiwabara S, Toyoda Y. Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. J Biol Chem. 1994; 269: 31845-31849.
82. Li S, Winuthayanon W. Oviduct: Roles in fertilization and early embryo development. J Endocrinol. 2017; 232: R1-R26.
83. Špirková A, Kovaříková V, Šefčíková Z, Pisko J, Kšiňanová M, Koppel J, et al. Glutamate can act as a signaling molecule in mouse preimplantation embryos. Biol Reprod. 2022; 107: 916-927.
84. Relloso M, Esponda P. *In vivo* transfection of the female reproductive tract epithelium. Mol Hum Reprod. 2000; 6: 1099-1105.
85. Zhao Y, Vanderkooi S, Kan FWK. The role of oviduct-specific glycoprotein (OVGP1) in modulating biological functions of gametes and embryos. Histochem Cell Biol. 2022; 157: 371-388.
86. Malette B, Paquette Y, Merlen Y, Bleau G. Oviductin possess chitinase- and mucin-like domains: A lead in the search for the biological function of these oviduct-specific ZP-associating glycoproteins. Mol Reprod Dev. 1995; 41: 384-397.
87. Hiyama G, Matsuzaki M, Mizushima S, Dohra H, Ikegami K, Yoshimura T, et al. Sperm activation by heat shock protein 70 supports the migration of sperm released from sperm storage tubules in Japanese quail (Coturnix japonica). Reproduction. 2013; 147: 167-178.
88. Zhang H, Martin DeLeon PA. Mouse Spam1 (PH-20) is a multifunctional protein: Evidence for its expression in the female reproductive tract. Biol Reprod. 2003; 69: 446-454.
89. Prunskaite Hyyryläinen R, Skovorodkin I, Xu Q, Miinalainen I, Shan J, Vainio SJ. Wnt4 coordinates directional cell migration and extension of the Müllerian duct essential for ontogenesis of the female reproductive tract. Hum Mol Genet. 2016; 25: 1059-1073.
90. Yuan S, Wang Z, Peng H, Ward SM, Hennig GW, Zheng H, et al. Oviductal motile cilia are essential for oocyte pickup but dispensable for sperm and embryo transport. Proc Natl Acad Sci. 2021; 118: e2102940118.
91. Bister K. Discovery of oncogenes: The advent of molecular cancer research. Proc Natl Acad Sci. 2015; 112: 15259-15260.
92. Sakuma T, Nakade S, Sakane Y, Suzuki KT, Yamamoto T. MMEJ-assisted gene knock-in using TALENs and CRISPR-*Cas9* with the PITCh systems. Nat Protoc. 2016; 11: 118-133.
93. Suzuki K, Tsunekawa Y, Hernandez Benitez R, Wu J, Zhu J, Kim EJ, et al. *In vivo* genome editing via CRISPR/*Cas9* mediated homology-independent targeted integration. Nature. 2016; 540: 144-149.
94. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated knock-in with CRISPR-*Cas* for large genomic regions in zygotes. Nat Commun. 2016; 7: 10431.
95. Ishibashi R, Abe K, Ido N, Kitano S, Miyachi H, Toyoshima F. Genome editing with the donor plasmid equipped with synthetic crRNA-target sequence. Sci Rep. 2020; 10: 14120.

96. Yoshimi K, Oka Y, Miyasaka Y, Kotani Y, Yasumura M, Uno Y, et al. Combi-CRISPR: Combination of NHEJ and HDR provides efficient and precise plasmid-based knock-ins in mice and rats. Hum Genet. 2021; 140: 277-287.
97. Fang H, Bygrave AM, Roth RH, Johnson RC, Huganir RL. An optimized CRISPR/*Cas9* approach for precise genome editing in neurons. Elife. 2021; 10: e65202.
98. Sato M, Akasaka E, Saitoh I, Ohtsuka M, Watanabe S. *In vivo* gene transfer in mouse preimplantation embryos after intraoviductal injection of plasmid DNA and subsequent *in vivo* electroporation. Sys Biol Reprod Med. 2012; 58: 278-287.
99. Peng H, Wu Y, Zhang Y. Efficient delivery of DNA and morpholinos into mouse preimplantation embryos by electroporation. PLoS One. 2012; 7: e43748.
100. Hakim BA, Tyagi V, Agnihotri SK, Nath A, Agrawal AK, Jain A, et al. Electroporation of mouse follicles, oocytes and embryos without manipulating zona pellucida. J Dev Biol. 2021; 9: 13.
101. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature. 2017; 551: 464-471.
102. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019; 576: 149-157.
103. Kantor A, McClements ME, MacLaren RE. CRISPR-*Cas9* DNA base-editing and prime-editing. Int J Mol Sci. 2020; 21: 6240.