Chapter

Possible Production of Genome-Edited Animals Using Gene-Engineered Sperm

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Abstract

CRISPR/Cas9 is widely used for genome editing in a variety of organisms, including mammals, fishes, and plants. In mammals, zygotes are considered an appropriate target for gene delivery of CRISPR/Cas9 components [Cas9 endonuclease and a single-guide (sgRNA)] via microinjection or *in vitro* electroporation. However, these approaches require *ex vivo* handling of zygotes, which is necessary for egg transfer to recipient females to allow the treated zygotes to develop full-term. These procedures are often laborious, time-consuming, and use numerous mice. In our previous experiments, the plasmid DNA encapsulated by liposomal reagent introduced into the internal portion of a testis can be transferred to the mature sperm present in the epididymal ducts, and is finally transferred to oocytes via fertilization. Although it was not integrated into their genome, this approach would be useful for creating genome-edited animals, since CRISPR/Cas9 can be performed by transient interaction of Cas9 and sgRNA, whereby chromosomal integration of the CRISPR components is not a prerequisite. Here, we will review past achievements concerning in vivo transfection of immature/mature sperm and present experimental proposals for possible genome editing via gene-engineered sperm based on recent findings.

Keywords: sperm, CRISPR/Cas9, guide RNA, testis-mediated gene transfer, *in vivo* transfection, genome editing, vas deferens, epididymis, artificial insemination, intratesticular injection

1. Introduction

Transgenesis is a method to induce genetic change in an organism by delivering exogenous DNA (also called transgenes) to early embryos (i.e., zygotes), and is now considered an important technique to examine gene function *in vivo* and for creating animal models of human disease [1, 2]. In 1980, Gordon et al. [3] first demonstrated that microinjection of purified DNA fragments into the pronuclei of zygotes led to the production of mice carrying the transgenes, which are generally referred to as transgenic (Tg) or genetically modified (GM) mice. When the injected transgenes are successfully integrated into the host chromosomes of the zygotes, they are transmitted to the next generation through mating with normal mice in a Mendelian ratio, and gene expression derived from the integrated transgenes will occur in the Tg offspring depending on the property of the promoter used. For the production of Tg animals through zygote microinjection, several steps

are required for the "*ex vivo* handling of embryos," including: collection of zygotes, DNA microinjection using an expensive micromanipulator, temporal incubation of the injected zygotes, and egg transfer (ET) to the oviducts of the pseudo-pregnant females to allow full-term development of the injected eggs [4, 5]. Furthermore, all of this requires highly specialized and skilled personnel for the preparation of pseudo-pregnant females and vasectomized males, which is time-consuming and tedious, and requires a large number of mice.

Since 1980, several methods for bypassing microinjection-based transgenesis have been provided, which include infection of zygotes with viral vectors like a retrovirus [6, 7], embryonic stem (ES) cell-based gene transfer [8–10], transgenesis via somatic cell nuclear transfer [11–13], and intracytoplasmic sperm injection (ICSI) using sperm associated with the transgene (TransICSI) [14, 15]. All of these methods deal with zygotes and require *ex vivo* handling of embryos, although a micromanipulator system is not used in almost the cases.

Genome-editing via Oviductal Nucleic Acids Delivery (GONAD) is a recently developed method for creating GM mice and rats without *ex vivo* handling of embryos [16–21]. It can be simply performed by injecting a solution containing nucleic acids into the oviductal lumen of pregnant females at zygote to two-cell stages and subsequent *in vivo* electroporation (EP) to enhance DNA uptake by early embryos *in situ*. In this case, there is no need for the large number of mice that is normally required for the traditional microinjection-based transgenesis: only four to five pregnant females are required for modifying an endogenous gene [18]. Thus, creation of GM animals is simplified with the development of GONAD, but an expensive apparatus electroporator is still required for the technique.

In 1989, Lavitrano et al. [22] reported the simplest, convenient, and cost-effective method to create GM animals, which was called sperm-mediated gene transfer (SMGT), where isolated sperm were incubated in the presence of naked plasmid DNA for a short period and these DNA-associated sperm were subjected to *in vitro* fertilization (IVF) with normal oocytes. The resulting progeny are later judged as those carrying the exogenous DNA in their genome. Since the report, there has been controversy over its reproducibility among researchers [23–25]. However, several recent improvements were made on this SMGT system by several researchers who employed reagents capable of enhancing gene delivery towards isolated sperm [26–28]. For example, Shen et al. [29] incubated mouse sperm in a solution containing 3% dimethyl sulfoxide (DMSO) and plasmid DNA for 10–15 min at 4°C prior to IVF. Embryos (42%; 25/60) obtained from this experiment showed bright enhanced green fluorescent protein (EGFP)-derived fluorescence. Furthermore, Kim et al. [30] reported that nanoparticles, such as magnetic nanoparticles, can be a vehicle for delivering exogenous DNA to sperm from various animals such as boar. They incubated boar sperm in the presence of 0.5% (v/v) of magnetic nanoparticles (MNPs) and plasmid DNA coding for green fluorescent protein (GFP) on the magnetic field for 90 min, and the magnetofected sperm were subjected to IVF with normal oocytes. As a result, they obtained fertilized eggs expressing GFP. Unfortunately, for further development of IVF-derived embryos, it still required ET towards recipient females, which is laborious and requires specialized skill. Notably, it is possible to perform artificial insemination (AI) using *in vitro*-transfected sperm, which can be simply done by injecting those sperm into the uterine horn or lumen of the oviducts of recipient females showing oocyte ovulation. This method, called "SMGT-based AI" (SMGT-AI), has already been performed by several laboratories and will be discussed in more detail in the last part of this paper.

Testis-mediated gene transfer (TMGT) is the *in vivo* version of SMGT, in which sperm is transfected *in vivo*. This technology was first developed by Sato et al. [31], who performed intratesticular injection of calcium phosphate-precipitated plasmid DNA using a glass micropipette in mice. The injected exogenous DNA is transferred

to epididymal sperm or spermatogenic cells within a seminiferous tubule (ST) of the testis, and those transfected sperm will transmit it to an oocyte through fertilization (**Figure 1**). They could detect the injected DNA in isolates of sperm from the epididymis and from the uteri of females mated with the injected males, but the DNA could not be detected in embryos [31]. In contrast with SMGT, TMGT does not require *ex vivo* handling of embryos such as collection of oocytes, IVF and ET. In this context, TMGT appears to be more convenient and simpler than SMGT for the purpose of GM animal production. Since the report of Sato et al. [31], several *in vivo* gene delivery approaches targeted to male reproductive systems have been reported: gene delivery to spermatogenic cells by intratubular injection of STs (ST-mediated gene transfer, STGT) (**Figure 2a**), to epididymal sperm present in the epididymal ducts (epididymis-mediated gene transfer, EpiGT) (**Figure 2b**), and to sperm present in the vas deferens (vas deferens-mediated gene transfer, VDGT) (**Figure 2c**).

Based on this background, the previous terminology TMGT appears to be now recognized as "direct *in vivo* gene delivery approach towards male reproductive system." In this context, it may be better to re-name TMGT as "intratesticular injection-based gene transfer" (IIGT), which involves direct injection of genetic materials into the interstitial space of a testis. Thus, IIGT, STGT, EpiGT and VDGT can be considered as TMGT-related experiments. In **Table 1**, a summary of previous studies on TMGT-related experiments is listed. Furthermore, there are several excellent papers reviewing the SMGT/TMGT-related studies [28, 90–93], which provide a helpful survey of this field.

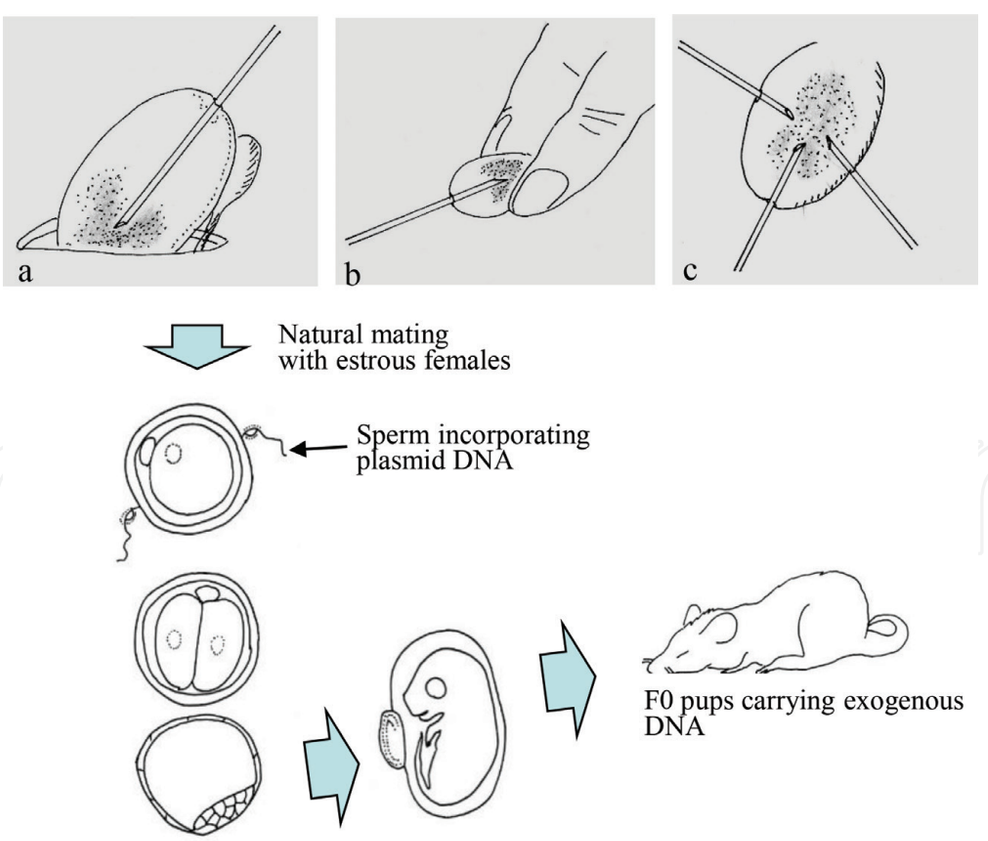

Figure 1.
IIGT in mice. To perform IIGT, at least three different ways to inject a DNA-containing solution into the testis have been employed. The first way (shown in a) is to perform IIGT towards a testis (that is exposed outside after surgery) under anesthesia [31, 32]. The second way (shown in b) is to perform IIGT through insertion of a needle via scrotum under anesthesia [33]. The third way (shown in c) is to insert a needle at three times to different sites [34]. In these latter two cases, no surgery is required. Three to five days after IIGT, the IIGT-treated males are subjected to mating with normal estrous females. The in vivo transfected sperm may fertilize ovulated oocytes, leading to creation of offspring carrying the introduced exogenous DNA.

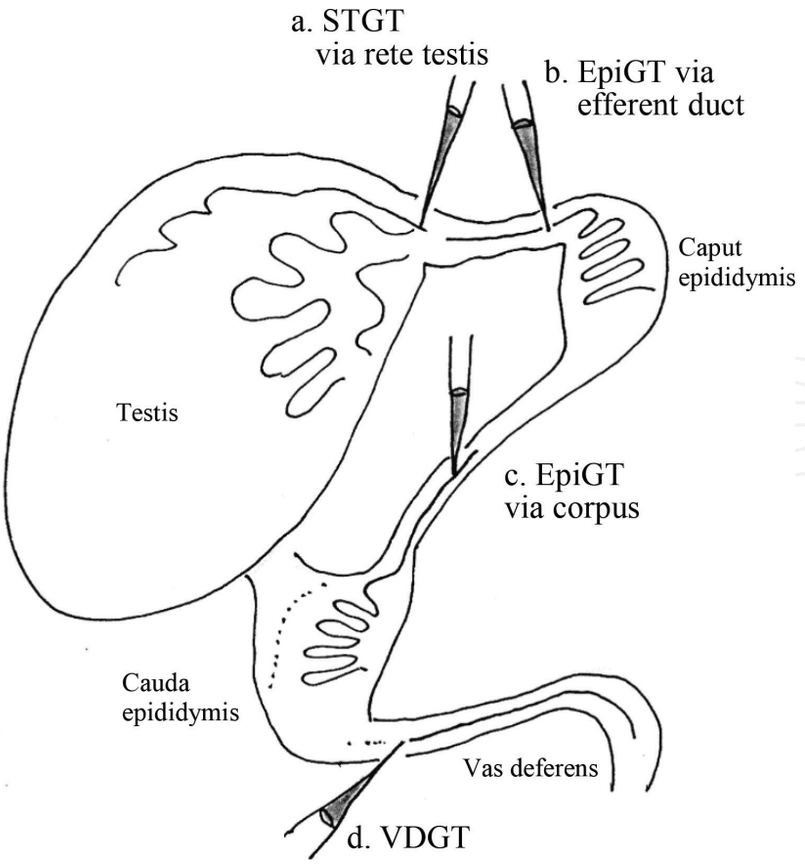

Figure 2.
There are several routes for introducing exogenous DNA into the male reproductive system. The routes for DNA injection are as follows: (a) STGT via rete testis; (b) EpiGT by inserting a glass pipette into the proximal region of caput epididymis; (c) EpiGT by inserting a glass pipette into the proximal region of cauda epididymis; and (d) VDGT by inserting a glass pipette into the proximal region of vas deferens.

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	Plasmid/calcium phosphate	The exogenous DNA was detectable in the sperm isolated from caput and cauda epididymides as early as 6 h after IIGT and in the ejaculated sperm from the female uteri, but not in 1-cell eggs	[31]
IIGT	Mice	Plasmid/liposome/IIGT 3 times at 4-day intervals	Eighty percent of blastocysts expressed lacZ from the exogenous DNA, suggesting successful transmission of the exogenous DNA to F0 offspring	[34]
IIGT	Rats	Adenovirus	Leydig cells expressed the transgene	[35]
IIGT	Mice	Plasmid/EP	Some spermatogenic-like cells expressed the transgene	[36]
IIGT	Mice	Plasmid/liposome/2–5 days after IIGT, the males were mated to females	Transmitted the foreign DNA to F0 offspring at frequencies of 4.3–92.3%; the copy number of the exogenous DNA was estimated to be less than 1 copy per diploid cell; no transgene expression in mid-gestational F0 fetuses	[32]

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	Plasmid/liposome/2 days after IIGT, the males were mated to females	Transmitted the foreign DNA to F0 offspring at frequencies of 50.0–84.6%; expression of lacZ in most (71.9%) blastocysts, but no expression of foreign DNA in the mid-gestational F0 fetuses; transmission of the exogenous DNA from F0 to F1 generation at frequencies from 16.1–23.1%, suggesting that the introduced DNA was chimeric in these F0 mouse testes	[33]
IIGT	Mice/rats	Plasmid/liposome/3–4 days after IIGT, the males were mated to females	Transmitted the foreign DNA to F0 offspring at frequencies of 18% in rats and 20% in mice; transgene expression in F0 offspring; transmission of the exogenous DNA up to F4 generation	[37]
IIGT	Mice/rats	Plasmid/liposome/4 days after IIGT, the males were mated to females	Detection of exogenous DNA in the head and tail of epididymal sperm; also, in the ejaculated sperm isolated from the female uteri; failure to detect the exogenous DNA in the DNase-treated sperm, suggesting no integration of the transgene in sperm DNA	[38]
IIGT	Mice	Plasmid/liposome/2 days after IIGT, the males were mated to females	Although seven commercially available transfection reagents were tested for possible improvement of IIGT to increase copy number of transgenes integrated and transgene expression, drastic improvement failed; transgene expression indeed occurred, but the degree of its expression diminished with development; choice of reagents used appears not to be so critical for IIGT	[39]
IIGT	Rats	Plasmid/liposome/4 days after IIGT, the males were mated to females	Detection of foreign DNA (encapsulated by DMRIE-C and SuperFect among eight liposomes tested) in sperm in the cauda epididymis isolated 1, 4, and 14 days after IIGT; more than 80% of morulae expressed EGFP; the ratio of animals carrying the foreign DNA decreased as they developed, suggesting high incidence of mosaicism	[40]
IIGT	Mice	Plasmid (lacZ/retroviral integrase gene)/EP	One month after IIGT, some spermatocyte-like cells in STs expressed the lacZ gene; co-transfection with integrase gene resulted in prolonged expression of lacZ, suggesting the usefulness of integrase gene co-transfection for stable transformation of spermatogenic cells *in vivo*	[41]

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	TB or Hoechst 33342/ plasmid/EP	TB was rapidly transported to the epididymal portion after IIGT; TB reached the corpus and cauda epididymis within 2–4 days after injection; sperm isolated from epididymal portion had the exogenous DNA even after DNase I treatment, suggesting incorporation of the DNA inside the sperm	[42]
IIGT	Mice	Adenovirus	Presence of the transgenes in sperm from 7 to 16 days after inoculation; transgenes are detected in the sperm heads in oocytes after IVF; the transgene product was mainly present in the interstitial tissue of a testis; it was also present in the STs and collected sperm; the transgene product was located in the head and the mid-piece of sperm	[43]
IIGT	Fishes (silver sea bream)	Plasmid/liposome	IIGT was performed 48 h before spawning. After mating these IIGT-treated males to females, between 59 and 76% of the hatched fry were found to be Tg; the efficiency of gene transfer was improved more than 80% by injecting multiple doses of the transgenes; Southern blot analysis showed that the transgene was integrated into the host genome	[44]
IIGT	Mice	Plasmid/EP	To study regulatory elements of genes specifically active in spermatocytes, IIGT-EP was performed; in the EP-based IIGT-treated testes, only small fraction of cells expressed the transgenes; this method can be useful for preliminary screening of constructs aimed to study in Tg mice	[45]
IIGT	Mice	Adenovirus/IIGT at 3 sites per testis	The transgene expression was found in Leydig cells, but no expression was noted in germ cells	[46]
IIGT	Mice	Plasmid/liposome/repeated injections (singly, 3 or 6 times 3 days apart)	Repeated injections of the exogenous DNA led to a high rate of gene transfer, but failed to introduce high numbers of copies (more than 1 copy per diploid cell); expression of transgene-derived mRNA was observed, although its strength appeared still to be very low	[47]
IIGT	Mice	Plasmid/liposome/3 times 3 days apart/7–21 days after IIGT, the males were mated to females	Detection of the presence of at least two types of the exogenous DNA, intact and deleted form of plasmid in F0 offspring, suggesting degradation of the exogenous DNA during the process of IIGT	[48]

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	Plasmid/EP	LacZ activity was detected in spermatogenic cells up to 4 weeks after IIGT	[49]
IIGT	Mice	Adenovirus/EP	EP might be effective for transfecting germ cells or somatic cells	[50]
IIGT	Mice	Plasmid/PEI	Transferred and expressed in germ cells (especially in primary spermatocytes); transfection into Sertoli cells was not observed; protein showed dynamic shifts in spermatogenic cells at different stages during spermatogenesis	[51]
IIGT	Mice/ rabbits	Plasmid/DMSO	F0 offspring (mice) expressed EGFP with an efficiency of 28.6%; also 56.3% of rabbits born were identified to be Tg	[29]
IIGT	Shellfishes (Japanese abalone)	Naked plasmid (linearized)	The gene-transfer efficiency of G_0 in larvae (9 h after fertilization), juveniles (3 weeks after fertilization), and 1-year-old adults was 90%, 92.5%, and 60%, respectively; genomic Southern blot analysis showed that the transgene was integrated in the genome of the Tg abalone	[52]
IIGT	Mice	Linearized plasmid/liposome (DOTAP)/IIGT at multi-sites/ after few weeks, the males were mated	41% of F0 offspring exhibited the presence of the transgenes when PCR and Southern blot hybridization were employed; 37% of F1 offspring obtained after mating of F0 offspring with wild-type mice were Tg	[53]
IIGT	Mice	Linearized plasmid/EP	Successful gene delivery to undifferentiated spermatogonia within the STs; about 94% of females after mating with the IIGT-treated males successfully sired Tg pups	[54]
IIGT	Chickens	Plasmid/cationic polymer	The percentages of gene expression reached the summit and became stable from day 70 to 160, being 12.7%, 12.8%, 15.9% and 19.1%, respectively; Southern blot showed that the transgene was inserted in their genomic DNAs	[55]
IIGT	Chickens	Plasmid for EGFP-lacZ dual reporter expression/liposome (Lipofectamine 2000)	Ten days post-IIGT, fluorescent sperms were not observed on semen slides; however, sperms positive for lacZ were detected; specific amplicons of EGFP and lacZ detectable in four of the six sequentially collected semen samples; staining with monoclonal antibodies demonstrated positive staining for subsets of testicle cells	[56]

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	Plasmid/DMSO, DMA or liposome (Lipofectin)/1 day after IIGT, each male was mated	The presence of transgene in the progeny (80% for PCR positive when repeated injection was done; 50% for RT-PCR positive) in the case of liposome was used; 55.5% for PCR positive, but 22.3% for RT-PCR positive in the case of DMSO was used; RT-PCR analysis of PCR positive animals showed EGFP expression in blood cells; repeated (4 times) injections of DNA complexes can affect spermatogenesis	[57]
IIGT	Mice	Plasmid/liposome (Lipofectamine 2000)/6 weeks after IIGT, each male was mated	38.46% of F0 positive for transgenes in the case of PCR; 30.77% by Southern blotting; 36.36% of F1 were positive for the transgene; expression of EGFP is recognized	[58]
IIGT	Mice	Plasmid/liposome (ExGen500)/at different angles into the testes of 7-day-old males	Transgene efficiencies were 11.76% (2/17), 14.29% (3/21), and 11.11% (2/18), respectively; semi-quantitative RT-PCR analysis further showed that the introduced GFP gene was expressed in 3/9 integration mice; GFP expression was observed in sperm from the F0 fetuses and F1 pups	[59]
IIGT	Mice	Linearized plasmid/EP	Electroporated testis expressed EGFP even 80 days after IIGT; EGFP expressing germ cells were discernible in the STs; after mating with the EP-based IIGT-treated males with normal females, the resultant Tg pups showed tissue-specific expression of transgene	[60]
IIGT	Mice	Plasmid/liposome (PEI)	Twenty days after IIGT, the transgene-derived fluorescence was detected in the testis and sperm; foreign DNA was successfully expressed in the treated mice: 4.0% for G0 and 30.23% for F1	[61]
IIGT	Mice	Plasmid/liposome (DOTAP)	The Tg positive rate in mouse F1 offspring was 39.69%; gene transmission beyond F2 generation; the transgene was expressed in the ovaries	[62]
IIGT (*in vitro*)	goats	Plasmid/EP	Cultivation (organ culture) of STs isolated from the IIGT-EP-treated testis led to expression of GFP 24 h after EP; green fluorescence was observed at best 23 days after EP-based IIGT	[63]
IIGT	Rats	GFP-expressing plasmid/EP	Possible integration of transgene into the genome of the spermatogonial cells; a transgenic disease model displaying alpha thalassemia was successfully generated with EP-based IIGT	[64]

Method	Species	DNA/transfection method	Outcome (note)	References
IIGT	Mice	Linearized Plasmid/hypotonic Tris-HCl solution	Successful internalization of the transgene in spermatogonia within STs; such IIGT-treated males generated Tg progeny by natural mating	[65]
IIGT	Mice	Plasmid/BMPs-PEI	DNA complexed with BMPs-PEI successfully reached the cytoplasm and the nucleus of spermatogenesis cell; the transgene was expressed in the testes of Tg F0 mice; the ratio of Tg F0 offspring was 88%	[66]
IIGT	Goats	Plasmid/EP	Successful transfer of the transgene into STs and testicular cells; chromosomal integration of the transgene and its expression in sperm; natural mating of a pre-founder buck produced a Tg baby goat	[67]
STGT	Mice/pigs	Plasmid/liposome (obtained from Gibco)	In mice, 8.0–14.8% of STs expressed the introduced LacZ gene, and 7–13% of epididymal sperm had the foreign DNA; in pigs, foreign DNA was also incorporated into male germ cells, and 15.3–25.1% of the STs containing germ cells expressed the LacZ gene	[68]
STGT	Rats	Adenovirus	Expression of the transgene in Sertoli cells and persisted for at least 10 days	[35]
STGT	Mice	Plasmid/EP	Specific lacZ expression only in haploid spermatid cells; spermatogenic differentiating cells maintained the transfected lacZ expression after more than 2 months of transfection, suggesting that spermatogenic stem cells and/or spermatogonia could also incorporate foreign DNA	[69]
STGT	Mice	Plasmid/EP	Transient expression of GFP in the innermost region of the testis uniformly, but confined to spermatogenic cells and Sertoli cells within the STs; GFP was detected in the spermatogenic cells even 2 months after EP; no Tg offspring were obtained	[70]
STGT	Mice	Plasmid/EP	After EP-based STGT, fluorescent sperm were collected from the STGT-treated STs; these fluorescent sperm were found to have the ability to produce Tg offspring, when ICSI was performed	[71]
STGT	Mice	adeno-, adeno-associated-, retro-, and lentiviral vectors	Transduction with either adeno- or lentiviral vectors led to reporter gene expression for more than 2 months after STGT; lentiviral vectors used to express the c-kit ligand in Sl/Sl(d) Sertoli cells restored spermatogenesis; lentiviral vectors failed to infect spermatogenic cells	[72]

Method	Species	DNA/transfection method	Outcome (note)	References
STGT	Mice	Plasmid/EP	By electrotransformation of a complete cDNA in Sertoli cells, defective spermatogenesis was rescued in infertile Sl(17H)/Sl(17H) mutant mice	[73]
STGT	Mice	Plasmid/liposome (noncommercial cationic lipids)	As early as 48–96 h post-injection, lacZ expression was observed within both immature and differentiated germ cells; by 40 days post-injection, it was restricted to the most immature germ cells; after mating with females, the transgene was transmitted to the offspring, but remained episomal	[74]
STGT	Mice	Adenovirus	Strong expression in Sertoli cells after STGT, but no expression in germ cells	[46]
STGT	Mice	Retrovirus	Transduction of spermatogonial stem cells with an average efficiency of 2.8%; the transgene was transmitted stably and expressed in the next generation	[75]
STGT	Mice	Plasmid/EP	STGT was performed to examine testis-specific gene promoter activity; successful *in vivo* transient transfection to living mouse testis was achieved	[76]
STGT	Mice	Plasmid/EP	LacZ activity was detected in spermatogenic cells up to 8 weeks after EP-based SMGT	[49]
STGT	Hamsters	Plasmid/EP	Sixty days following gene transfer, expression of the transgene can be detected in epididymal sperm	[77]
STGT	Mice	Plasmid/EP	The transgene products were found on the head and mid-piece regions of mature epididymal sperm	[78]
STGT	Mice	Plasmid/EP	A fluorescent reporter protein expressed in male germ cells	[79]
STGT	Mice	Plasmid RNAi targeting EGFP/EP	Sertoli cells were the main transfected cells	[80]
STGT	Mice	Plasmid/EP	STGT is useful for testing the tissue-specific promoter activity included in the construct *in vivo*	[81]
STGT	Mice	Adenovirus	Sertoli cell-specific expression of GFP	[82]
STGT	Mice	Lentivirus	All male pre-founder mice produced Tg pups with an overall success rate of over 60%	[83]
STGT	Mice	Lentivirus	14.3% of the lentivirus-injected mice successfully produced Tg pups; eight Tg founders were obtained from the total 336 pups; the Tg efficiency was around 2.4%	[84]
EpiGT	Rats	Plasmid/EP	After 72 h, the initial segments had intense fluorescence in the cytoplasm of the epithelial cells	[85]

Method	Species	DNA/transfection method	Outcome (note)	References
EpiGT	Mice	Plasmid/lipid (FuGENE 6)	Transfection was observed in 39.70% of epithelial cells after 2 days and in 31.77% after 7 days; the presence of the transgene in the DNA isolated from the treated epididymides (by PCR); GFP gene expression appeared in large areas of the cauda epididymis even after 2 weeks post-EpiGT	[86]
VDGT	Mice/rats	Plasmid	Uptake of exogenous DNA occurred in 60–70% of the spermatozoa after *in vitro* or *in vivo* treatments; positive signal was detected in the sperm nucleus and was not affected by DNase treatment	[87]
VDGT	Mice	Mixture of linearized and circular plasmids	From 53 newborns, four were found positive by PCR for the GFP gene; some tissues showed expression for GFP	[88]
VDGT	Mice	Plasmid/liposome (Lipofectamine)	Maximum of 6.8% in the epithelial cells of the vas (for lacZ staining); 13.3% after employing the GFP gene construction; expression of the GFP gene appeared from 1 week up to 3 months following injection	[89]

[1]TMGT can be defined as a method for in vivo gene delivery towards male reproductive systems (testis, epididymis, and vas deferens) and includes intratesticular injection-based gene transfer (IIGT), seminiferous tubule-mediated gene transfer (STGT), epididymis-mediated gene transfer (EpiGT), and vas deferens-mediated gene transfer (VDGT).
Abbreviations: BMPs, bacterial magnetic particles; DMSO, dimethyl sulfoxide; DOTAP, N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methyl sulfate; DMA, N,N-dimethylacetamide; EP, electroporation; GFP, green fluorescent protein; IVF, in vitro fertilization; PEI, polyethylenimine; STs, seminiferous tubules; Tg, transgenic; TB, trypan blue.

Table 1.
Summary of testis-mediated gene transfer (TMGT)[1]-related studies.

In the following sections, the TMGT-related experiments will be mentioned in more detail.

2. Historical background of TMGT-related experiments

2.1 IIGT-related experiments

Between 1994 and 2006, over 20 reports on IIGT-related experiments were reported using various animal models such as mice, rats, hamsters, rabbits, boar, goats, chicks, fishes, and shellfishes [29, 31–41, 43–53, 68]. The DNA used were mainly plasmid DNA that had been mixed with calcium phosphate, liposomes/lipids, polyethylenimine (PEI), or DMSO, all of which were intended to facilitate uptake of DNA by sperm or spermatogenic cells [29, 31–34, 37–40, 44, 47, 48, 51, 68]. The method to use *in vivo* EP towards the entire testis after IITG with naked plasmid DNA was also employed [36, 41, 45, 49]. Furthermore, adenoviral vectors were introduced by IIGT [35, 43, 46, 50].

Through these works, the following information became available:

i. **Transgene expression in a testis after IIGT:** IIGT using an adenoviral vector resulted in preferential transgene expression in Leydig cells [46]. Mogas et al. [43] reported that transgene-derived protein is mainly present in the interstitial tissue of a testis, and in STs 7–16 days after inoculation. Li et al. [51] demonstrated that plasmid DNA mixed with PEI can be transferred within STs after IIGT and expressed in primary spermatocytes, but not Sertoli cells.

ii. **Detection of transgenes and expressed products on the epididymal or ejaculated sperm:** According to Yonezawa et al. [40], foreign DNA encapsulated by DMRIE-C and SuperFect can be detected by PCR in the cauda epididymis-derived sperm isolated 1, 4, and 14 days after IIGT. The exogenous DNA was also detectable in the ejaculated sperm collected from the uterine horn of the females in the morning after mating with the IIGT-treated males [31, 38].

iii. **Transgene expression in the F0 offspring obtained after mating with the IIGT-treated males:** PCR analysis revealed that detection of the transgenes in the mid-gestational fetuses was found at frequencies ranging about 50–85% [39]. However, the copy number of the transgenes in those fetal DNA are estimated to be <1 copy per diploid cell [32]. PCR/slot blot analyses revealed that 41% of F0 offspring had the transgenes [53]. Ogawa et al. [34] reported that 80% of blastocysts exhibited the transgene-derived lacZ gene coding for β-galactosidase, one of the key enzymes consisting of lactose operon. Sato et al. [39] reported that lacZ expression was evident in most (72%) of the blastocysts, but neither expression of the LacZ gene nor its mRNA was found in the mid-gestational fetuses. More than 80% of morulae expressed EGFP, but the ratio of animals carrying the foreign DNA decreased as they developed, and only some of the progeny were foreign DNA-positive with high incidence of mosaicism [40].

iv. **Transgene transmission to the next generation:** Gene transmission to F1 offspring was at frequencies ranging about 16–23%, but when F2 offspring was obtained from mating normal female with F1 male offspring, about 94% of F2 offspring had the transgenes, suggesting that the introduced DNA was chimeric in these F0 mouse testes [38]. On the contrary, He et al. [53] demonstrated that the transgene transmission rate from F0 to F1 generation was 37%, suggesting that the transgene transmission rate was similar to the Mendelian law of inheritance.

In 2007 and onward, attempts to improve IIGT systems were made by several laboratories to enhance the gene delivery efficiency [54–67]. In the following sections, we will describe several examples [(v) to (x)] about the improvement of IIGT, *in vitro* assessment for gene expression after IIGT or possible mechanism underlying IIGT.

v. **IIGT at a young stage:** In mammalian testis, spermatogenesis occurs in the STs of a testis (**Figure 3d**). In the ST, there are spermatogenic cells called spermatogonia (spermatogonial cells) that can be further matured into spermatocytes and spermatids. Spermatogonia are largely divided into two types, type A and type B cells. The former undergoes active mitosis and divide to produce type B cells. The type B cells divide to give rise to spermatocytes and spermatids, which move towards the lumen of the ST as they mature. According to Hui-ming et al. [59], type A spermatogonia first appear between 3 and 7 days postnatally in mice and are the only immortalized diploid cells. They considered that if these type A spermatogonia are stably transfected with the exogenous DNA, the transfected cells would be able to produce mature Tg

sperm leading to production of Tg mice through natural fertilization. Based on this hypothesis, they performed IIGT using GFP-expressing plasmid DNA encapsulated by the ExGen500 transfection reagent on 7-day old male ICR mice. When these treated mice reached different stages of sexual maturity (6, 12, and 24 weeks of age), they were mated with normal females. The resulting pups were identified as Tg, with efficiencies of 11–14%. They observed GFP expression in sperm cells isolated from F0 and F1 pups. They referred to this technology as "type A spermatogonia-mediated gene transfer" (TASMGT).

vi. **EP-based IIGT:** Majumdar's group developed a method for generating Tg mice by directly injecting the desired gene into the testis followed by *in vivo* EP [54]. In this method, one of the testis was surgically exposed for DNA injection (**Figure 1a**) and the other contra-lateral testis was removed. An improved version of this EP-based IIGT was provided by Usmani et al. [60], who introduced the transgenes to both testes of mice directly from the outside (**Figure 3a**), prior to *in vivo* EP. They employed a two-step EP in which four 60 V electric pulses (50 ms each with an inter-pulse interval of 1 s) in one direction (forward direction) (**Figure 3b**) and four more pulses after changing the sides of the electrodes (reverse direction) (**Figure 3c**). The EP-based IIGT-treated testis expressed EGFP from the introduced transgenes even at 80 days of age. Furthermore, fluorescent germ cells were discernible in the STs of those mice (**Figure 3d**). F1 offspring that were generated after mating the IIGT-EP-treated males with normal females showed tissue-specific expression of transgenes. This improved procedure is based on non-surgical gene delivery using a two-step EP, which appears to be a user-friendly technique for a person who is less experienced in performing surgery.

vii. ***In vitro* EP-based IIGT:** It is difficult to apply IIGT in the testis of larger animals such as goats, because the size of the testis is much bigger than that of smaller animals such as mice and rats. Raina et al. [63] hypothesized that IIGT may be possible when *in vitro* gene delivery is performed towards the testis dissected from goats. They slowly injected GFP-expressing plasmid DNA into the interstitial space at eight different sites of the isolated testis using a 1-mL syringe (**Figure 4a**). Then, the testis was subjected to *in vitro* EP using a pair of tweezer-type electrodes (**Figure 4b**). After that, ST was partially dissociated and placed under *in vitro* cultivation for checking GFP expression at regular intervals (**Figure 4c, d**). A strong green fluorescence signal was observed 24 h after EP and its expression was continuously observed for as long as 23 days post-EP. The authors mention that the results of this study cannot be applied straightforwardly for *in vivo* studies, but the *in vitro* transfection of ST using EP will provide valuable baseline information, prior to IIGT *in vivo*.

viii. **IIGT using a simple hypotonic solution:** Majumdar's group have developed a method to generate Tg mice by directly injecting the desired gene in the testis followed by *in vivo* EP, as mentioned above [54, 60]. This technique is less complicated for small animal like mice but appears to not be feasible for transgenesis in large animals, because these animals have larger testes and greater scrotal thickness, to which it is difficult to standardize voltage parameters. To overcome this difficulty, they developed an alternative technique for making Tg mice by hypotonic shocking male germ cells for gene delivery. According to Usmani et al. [65], treatment with hypotonic Tris-HCl solution reduced osmolarity and led to hypotonic-swelling of germ cells. The hypotonic-swelling eventually killed the cells with increased hypotonicity, but led to the uptake of surrounding molecules

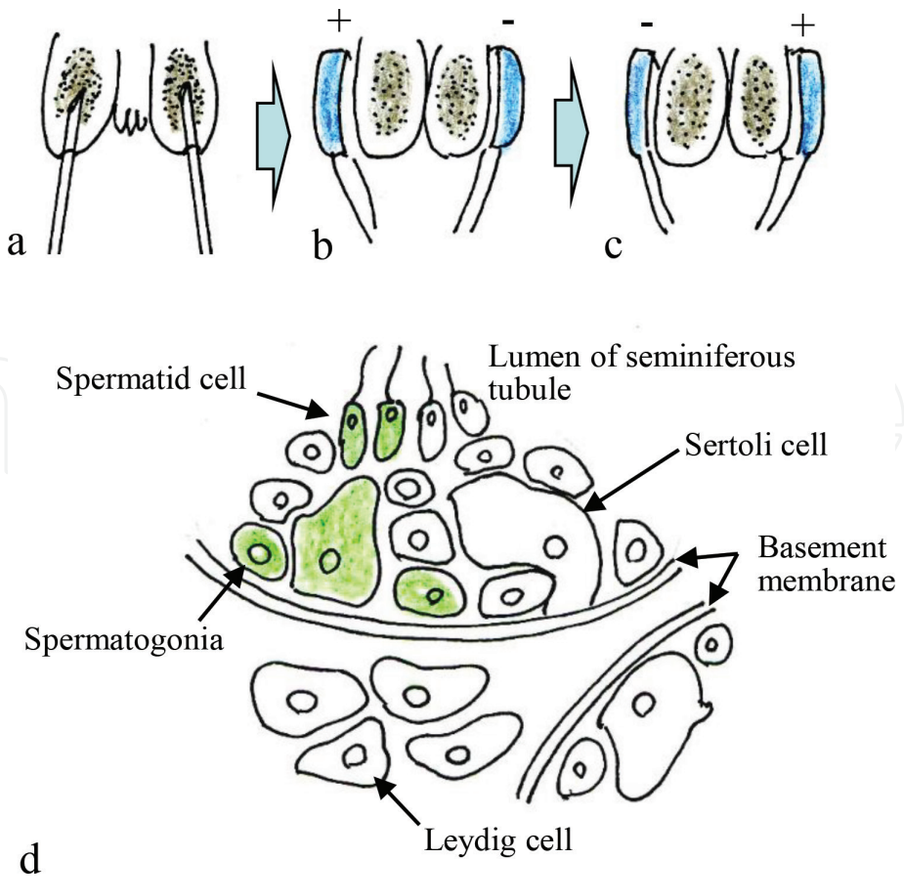

Figure 3.
EP-based IIGT in mice. a-c. Schematic illustration for EP-based IIGT. After IIGT towards both testes (a), they are held by a pair of tweezer-type electrodes and then subjected to the first in vivo EP (b). The second EP was next performed by changing the direction of electric pulse (c). (d) Structure of a ST. The colored spermatogenic cells and Sertoli cells indicate cells successfully transfected by the exogenous DNA that have been instilled within a lumen of ST.

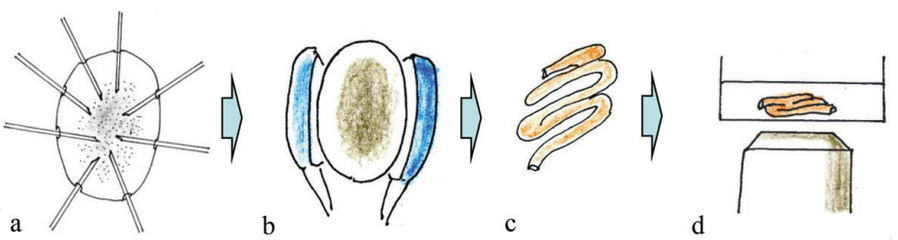

Figure 4.
In vitro EP-based IIGT in goats. The isolated goat testis is subjected to IIGT with a total of eight repeated injections from different directions (a). Then, the entire testis is hold by a pair of electrodes prior to EP (b). After that, the EP-treated STs are partially dissected and subjected to in vitro cultivation (c). By this, the transgene expression on the spermatogenic cells of STs can be monitored continuously (d).

such as nucleosides inside the cell. The authors hypothesized that a hypotonic Tris-HCl solution at a certain hypotonic concentration might allow the germ cells to internalize the surrounding solutes like DNA *in vivo* without being killed and the sperm produced from transfected germ cells may carry a desired DNA fragment (transgene) to generate Tg animals. Usmani et al. [65] suspended the linearized plasmid DNA (transgenes) in hypotonic Tris-HCl solution (pH 7.0) and simply performed IIGT to internalize the injected transgenes in the genome of spermatogonia residing at basal compartment of tubules. As a result, such males successfully generated Tg progeny by natural mating. This technique is easy and simple and does not require expensive apparatuses like electroporators. Usmani et al. [65] proposed that such a procedure enables researchers to generate their

own Tg animals, instead of outsourcing, and would drastically minimize the time required for studies on functional genomics.

ix. **IIGT using nanoparticles:** It has previously been reported that nanoparticles such as magnetic nanoparticles, mesoporous silica nanoparticles, and halloysite clay nanoparticle can be used as a vehicle for delivering exogenous DNA to sperm of various animals, such as boar and bovine [30, 94–96]. However, these early reports are confined to *in vitro* events. Wang et al. [97] applied this technology to IIGT using bacterial magnetic particles (BMPs)/PEI complex-conjugated foreign DNAs (BMP-PEI-DNA complex). According to Wang et al. [97], BMPs help to reduce the toxicity of the PEI, an efficient gene transfer agent, and assist gene delivery *in vivo*. After performing IIGT in mice, the testis was returned to the original position, and a neodymium-iron-boron magnet with an intensity of 600 milliteslas (mT) was placed onto the surface of the abdomen to facilitate gene delivery towards the spermatogenic cells. The authors report that this procedure is not harmful to the functioning of the testis. They reported that the clusters of BMP-PEI-DNA complex successfully reached the cytoplasm and the nucleus of spermatogenic cell and expressed in the testes of F0 mice. The resulting F0 mice could transmit the introduced transgene to the offspring with efficiencies of 88%.

x. **Possible mechanism for IIGT-mediated gene delivery to sperm:** When intratesticular injection of a DNA-containing solution into the interstitial space of a testis is performed, the fate of the injected solution is largely divided into two routes. This was first assessed by Sato et al. [42] who employed trypan blue (TB) as dye to visualize the transferring solution. One route is when a solution is transferred to the epididymal ducts, and the other route is when a solution is transferred to the lumen of the STs (**Figure 5**). After the intratesticular injection of 30 μL TB, the dye was rapidly transferred via rete testis to the proximal segment of caput epididymis [42]. One day after the injection, the dye was observed in the middle segment of caput epididymis. This was also confirmed by a previous observation using PCR analysis, which revealed the presence of the exogenous DNA in the spermatozoa isolated from caput epididymis [31]. Furthermore, the exogenous DNA can be detected in the ejaculated sperm collected from the uterine horn 1 day after mating with females [31]. Thus, it is reasonable to consider that the exogenous DNA injected into the interstitial space of a testis is rapidly transferred to the epididymal ducts and taken up by epididymal sperm *in situ*.

Only a minor portion of the solution injected into the interstitial space of a testis is transferred inside the STs. This may be elicited by mechanical shearing of STs upon insertion of a needle or glass capillary. To increase the transfection efficiency in the spermatogenic cells existing within STs, repeated needle insertions (over at least three times from different sites) have been employed by some research groups [34, 46, 53, 59, 63]. At present, it remains unknown how many STs are indeed transfected by this treatment. Usmani et al. [65] reported that spermatogenic cells, including spermatogonia present within STs, are transfected after IIGT and subsequent *in vivo* EP using fluorescent marker genes.

2.2 STGT-related experiments

ST is a tubular structure packed in a testis, which contains spermatogenic cells, such as spermatogonia, a precursor for mature sperm, and maturing sperm cells

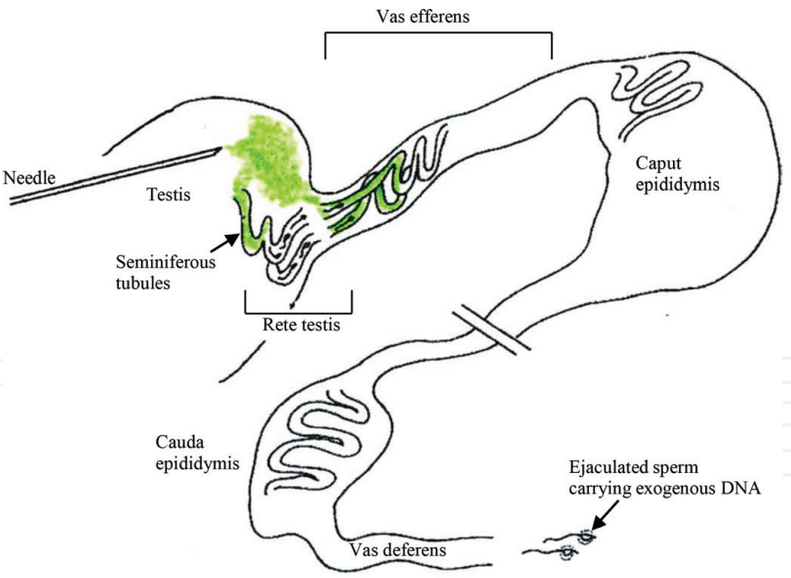

Figure 5.
Possible mechanism of how the exogenous DNA introduced into the interstitial space of a testis is transmitted to epididymal sperm or to spermatogenic cells within a ST.

(spermatocytes and spermatids), and Sertoli cells, which support the proliferation and differentiation of spermatogonia (**Figure 3d**). If the exogenous DNA is introduced within a lumen of the ST, it is possible to transfect those spermatogenic cells, from which transfected spermatozoa are transported via epididymal ducts and finally ejaculated upon mating with estrous females. As a result, the introduced exogenous DNA will be transmitted to oocytes via fertilization with the transfected sperm.

To our knowledge, STGT was first performed in 1997 by two groups: Blanchard and Boekelheide [35], with rats using adenoviral vector, and Kim et al. [68], with mouse and pigs using liposomally encapsulated plasmid DNA. The aim of the former group was to study transgene expression in the adult rat testis *in vivo*. They demonstrated that there was transgene expression in Sertoli cells and principal cells of the epididymis, and expression persisted for at least 10 days. The latter group demonstrated that in mice, 8.0–14.8% of STs expressed the introduced transgene, as evaluated by histochemical staining for the lacZ gene, and 7–13% of epididymal sperm had the exogenous DNA, as evaluated by PCR analysis. In pigs, 15–25% of the STs contained lacZ-positive germ cells. They suggested that STGT can be used as a powerful tool for producing Tg livestock.

In 1998, Yamazaki et al. [69] employed STGT for examining transcriptional regulatory elements of spermatogenic specific genes. After injecting DNA into STs, subsequent *in vivo* EP was used to enhance DNA uptake by spermatogenic cells. Based on these experiments, they suggested that spermatogenic stem cells and/or spermatogonia can incorporate foreign DNA, and that the transgene could be transmitted to the progenitor cells derived from a transfected proliferating germ cell. Later, the same group [70] examined the possibility to create Tg offspring using STGT coupled with *in vivo* EP (EP-based STGT). Although long-lasting transgene expression could be detected in the spermatogenic cells even 2 months after EP, no Tg offspring were obtained after natural mating with normal adult females.

Huang et al. [71] used the EP-based STGT towards an entire mouse testis after intratubular injection of plasmid DNA that coded for fluorescent genes. To trace the fate of transfected spermatogenic cells, they obtained fluorescent sperm by fluorescence activated cell sorting (FACS), and performed ICSI to obtain their offspring. Almost all the individuals produced from fluorescent sperm were Tg. The authors

claim that this is the first report of gene transfer into germ cells and subsequent production of Tg offspring.

STGT appears to be the direct approach to transfect spermatogenic cells *in situ*, but most experiments [46, 49, 51, 72–84] have been confined to successful transfection of spermatogenic cells and Sertoli cells for rescuing damaged/inactive Sertoli cells, *in vivo* testing of efficiency of RNA interference (RNAi), or establishment of an *in vivo* assay system to evaluate the promoter activity of the gene of interest. There have been no trials to create Tg offspring through STGT. Only some groups have tried to test the possibility of creating Tg animals by STGT. For example, Celebi et al. [74] performed STGT using circular plasmid carrying the lacZ reporter gene mixed with noncommercial cationic lipids. These injected males were mated with wild-type females and the progeny were analyzed by PCR and Southern blot assay. They demonstrated that the transgenes were transmitted to the offspring, but remained episomal, since it was found in the tail of the young animals and was lost at adulthood. Therefore, the plasmid seemed to be lost during the numerous germ cell divisions. This plasmid stayed in some tissues, such as in the skeletal and cardiac muscles. No integrative forms have yet been found with the use of circular DNA. Kanatsu-Shinohara et al. [75] described a novel approach for producing Tg animals by transducing spermatogonial stem cells *in vivo* using a retroviral vector by STGT. When these injected males were mated with wild-type females, Tg offspring were obtained with an efficiency of 2.8%. The transgene was transmitted stably and expressed in the next generation. The authors, thus, concluded that this technique will be useful as an alternative to the pre-exiting microinjection-based transgenesis, as well as provide a means for analyzing the self-renewal and differentiation processes of spermatogonial stem cells *in vivo*. Sehgal et al. [83] described a technique for the generation of Tg mice by infection of spermatogonial stem cells with recombinant lentiviruses expressing EGFP with a high rate of success. When the infected males were mated to normal females, over 60% of the delivered pups were found to be Tg. Li et al. [84] employed methods similar to those of Kanatsu-Shinohara et al. [75] and Sehgal et al. [83] and reported that the Tg efficiency is around 2.4%, which is similar to the previous report of Kanatsu-Shinohara et al. [75].

2.3 EpiGT-related experiments

Epididymal sperm present on the ducts of caput and cauda epididymides and epididymal epithelial cells can be targeted for transfection by the exogenous DNA. Kirby et al. [85] performed intraluminal injections (2–5 μL) of plasmid DNA into the lumen of an initial segment tubule of caput epididymis (**Figure 2b**), and subsequent *in vivo* EP towards the injected portion to examine the function of epididymal epithelial cells, which are thought to play critical role in sperm maturation during transport through epididymides. They concluded that this procedure is useful for elucidating the activity of promoter elements included in the injected plasmid that may not be identified when traditional *in vitro* methods are used. Esponda and Carballada [86] injected plasmid DNA mixed with the lipid FuGENE6 into the lumen of mouse cauda epididymis (**Figure 2c**). Successful transfection was observed in about 40% of cells after 2 days and in about 32% after 7 days, and then diminished progressively over time. Gene expression continued up to 15 days after gene injection and occupied about 22% of the area of the tubules. They concluded that intraluminal injections of exogenous DNA are effective for the study of epididymal physiology or to change the fertilizing ability of sperm. These studies are not aimed to create Tg animals, but they hold a potential to transfect epididymal sperm as a useful Tg tool.

2.4 VDGT-related experiments

In 1998, the Esponda's group [87] first attempted to examine whether exogenous plasmid DNA introduced into the lumen of the proximal region of the vas deferens could be taken up by mouse and rat sperm (**Figure 2d**). They demonstrated that 60–70% of sperm recovered 6 h after DNA injection had positive signal for successful transfection in their sperm nucleus, which was not affected by DNase treatment. This was also confirmed by PCR and slot blot analyses. They concluded that sperm within the vas deferens had the ability to incorporate exogenous DNA, which can be transferred to their nuclei, and vas deferens secretions do not block these capacities. In 2000, the same group [88] showed that this VDGT is useful for production of Tg mice. They injected plasmid DNA encoding GFP into the lumen of mouse vas deferens. The night after injections, males were mated with normal estrous females, and the offspring were analyzed. About 8% (4/53) of the newborns delivered expressed the GFP gene. They concluded that VDGT is a simple alternative to the pre-existing microinjection-based production of Tg animals and can be used for species in which the microinjection procedure is not feasible. This technology was later found to also be useful for transfection of epithelial cells of the vas deferens using a direct injection of DNA-liposome complexes, which could modify vas fluid contents [89].

3. Historical background of SMGT-based AI

As mentioned previously, AI of transfected sperm with exogenous DNA through SMGT is a highly convenient route for producing Tg animals. To our knowledge, Sperandio et al. [98] was the first to demonstrate its usefulness in domestic animals, such as bovine and swine. They performed AI towards ten sows with boar sperm cells that had been preincubated with plasmid DNA and obtained 82 offspring. Southern blot analysis of the DNA extracted from the animal tails showed that five animals were Tg and contained sequences complementary to the exogenous plasmid DNA that appeared to be rearranged compared to the original plasmid. From this study, it was suggested that SMGT-AI can be successfully adapted for the generation of Tg livestock. Yonezawa et al. [99] tested whether liposome-peptide (derived from human protamine)-DNA complex (LPD), a new reagent known to stabilize transfection in cultured cells, was useful to increase the efficiency of SMGT. They performed AI using rat epididymal sperm that had been incubated in a solution containing GFP expressing plasmid DNA and LPD complex. Expression of GFP was detectable in the morulae isolated from the treated animals. Furthermore, the AI-treated animals produced pups carrying foreign DNA.

This SMGT-AI is applicable to avian species. Yang et al. [100] performed AI using freshly-ejaculated chicken sperm that had been incubated in the presence of plasmid DNA and liposome, and found that about 4% (2/53) newly hatched chicks was identified as Tg. Harel-Markowits et al. [101] employed restriction enzyme-mediated insertion (REMI) to increase the efficacy of the transfection towards the isolated chicken sperm. REMI was used to insert exogenous DNA linearized with a restriction enzyme that cuts the genomic DNA at sites that enable the exogenous DNA to integrate via its matching cohesive ends [102, 103]. Following insemination with sperm transfected with linearized DNA, restriction enzyme, and liposome, they obtained Tg offspring. Furthermore, when chicken sperm are incubated in a solution containing plasmid DNA and DMSO or N,N-dimethylacetamide (DMAc) and subsequently subjected to AI, the resultant newborn chicks have the transgene, with efficiencies of 38% (for the DMSO-treated group) and 19% (for the

DMAc-treated group) [104]. However, Chaparian et al. [105] recently reported that they were unable to create Tg chicks by SMGT-AI.

4. Exosomes as a possible carrier to deliver genetic materials to sperm

Exosomes, membrane-enclosed sub-cellular microvesicles shed from most cell types, are present in a wide variety of body fluids [106]. Recently, it was found that they can mediate various effects on the behavior of recipient cells, since they contain cytokines, growth factors, and membrane proteins [107]. Furthermore, they contain a substantial amount of small and functional RNA molecules, called microRNAs (<100 nucleotides in length) [108], which could potentially control gene expression of various endogenous genes. It has recently been shown that these exosomes are (1) found in human semen [109], (2) involved in sperm maturation process during the transit along the male epididymal tracts [110], (3) accumulated in mature spermatozoa nuclei [111], and (4) delivered to oocytes through fertilization [112].

Notably, there are some reports describing non-Mendelian germline-independent inheritance of phenotypes in the absence of any classically identifiable mutation or predisposing genetic lesion in the genome of individuals who develop the disease [113–115]. For example, Cossetti et al. [116] performed subcutaneous inoculation of EGFP-expressing human melanoma cells into an immunocompromised mouse, from which EGFP RNA was released from the grafted melanoma cells, delivered to the bloodstream, and finally brought to sperm. When epididymal sperm isolated from these tumor-bearing males were examined carefully, the EGFP RNA was found to be tightly associated with the extracellular fraction of these mature sperm. They termed this phenomenon "soma-to-germ line transmission of information," and thought that exosomes may be involved in this phenomenon as the carrier to deliver EGFP RNA. The findings of Cossetti et al. [116] appear to be well correlated with those obtained from the TMGT-related experiments done at earlier stages of IIGT development, which include (1) non-Mendelian transmission of the exogenous DNA in the offspring obtained [33], (2) extreme low copy number of the exogenous DNA (<1 copy per diploid cell) transmitted to these offspring [32], (3) mosaic expression of the exogenous DNA in the offspring (blastocysts) obtained [33, 40], and (4) reduction in the number of offspring carrying the exogenous DNA during development [40]. As mentioned in Section 2.1 (x), parts of a solution introduced into the interstitial space of a testis is transferred to the excurrent ducts of epididymides, and the exogenous DNA may be taken up by the extracellular fraction of epididymal sperm, possibly through exosomes. We detected the presence of exogenous DNA in the DNase I-treated epididymal sperm, which have been isolated from the IIGT-treated males [42]. This may be due to the fact that exosomes can protect its exogenous DNA against DNase I-mediated digestion.

Notably, in their review article, Jiang and Gao [117] demonstrated that exosomes can be used as naturally occurring cell-to-cell transporters or as novel biocarriers for gene and drug delivery. These exosomes are naturally secreted by the cells and pass through additional biological barriers. They are more biocompatible and biodegradable and can avoid immune response which is most likely due to the surface expression of the complement regulatory proteins, such as CD55 and CD59. For these natural characteristics, exosomes are being extensively explored as gene delivery vehicles. For example, in 2011, Alvarez-Erviti et al. [118] first demonstrated that exosomes are useful for delivering short interfering (si)RNA to the mouse brain. They engineered dendritic cells to express lysosome-associated membrane protein 2 (Lamp2) isoform (Lamp2b), an exosomal membrane protein fused to a neuron-specific peptide. Following that, exosomes were isolated from

the gene-engineered dendritic cells and loaded with siRNA using electroporation and were administered intravenously to mice. As a result, the targeting peptide was shown to be successfully delivered to the brain. The concomitantly delivered siRNA caused reduced expression of a target protein associated with the pathogenesis of Alzheimer's disease. Furthermore, Lin et al. [119] proposed that exosomes can be a good carrier to introduce various cargoes, including plasmid DNA, into a cell. They prepared a mixture composed of purified exosomes isolated from HEK293FT cell line, pEGFP-C1 plasmid DNA, and Lipofectamine 2000 liposomes *in vitro*. During the incubation at 37°C for 12 h, exomes and liposomes are fused together and the exogenous plasmid DNA becomes incorporated into exosome-liposome hybrid nanoparticles. Transfecting mesenchymal stem cells (MSCs), which cannot be transfected by the liposome alone, with this complex resulted in successful generation of fluorescent cells when evaluated by FACS. Now, an exosome-based transfection kit, possibly based on this principle, is commercially available: Exo-Fect Exosome Transfection Kit (System Biosciences). We confirmed the usefulness of this kit by *in vivo* transfecting oviductal epithelial cells through intraoviductal instillation of a solution prepared using this kit. Some oviductal epithelial cells were found to be fluorescent after transfection with a plasmid expressing GFP (unpublished results). Thus, it may be possible to transfect isolated sperm by incubating plasmid DNA and exosome/liposome hybrid vesicles provided from the Exo-Fect Exosome Transfection Kit, prior to AI as mentioned below.

5. Genome-editing sperm

Gene modification based on recently developed techniques such as zinc-finger nucleases (ZFNs), TAL effector nucleases (TALENs), and clustered regularly interspersed short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) are now recognized as a revolutionary genetic engineering tool *in vitro* and *in vivo* [120–124]. Three types of endonucleases from ZFNs, TALENs, and CRISPR/Cas9 have been developed to promote precise genome editing at a target gene. All these enzymes have a DNA-binding ability and an ability to elicit double-strand DNA break (DSB) at a target genomic locus. Subsequently, in the absence of a homologous template to repair, nonhomologous end joining (NHEJ) occurs and causes small insertions or deletions (termed "indels"). In the presence of a template donor DNA, site-specific recombination through homology-directed repair (HDR) occurs. Generally, the frequency of NHEJ is thought to be higher than that of HDR in most of the cell types [125]. **Table 2** shows comparison among these three technologies.

ZFNs are the first engineered endonucleases [127] that combine the DNA recognition ability of zinc-finger protein (called zinc-finger motifs) and restriction enzyme *Fok I* to introduce DSB [120–124]. In 2005, Urnov et al. [128] first demonstrated that ZFNs are effective as a genome editing system in the human cells.

TALENs are similar to ZFNs and require a string of TALEN motif (consisting of a series of 33–35 amino acid repeats) to bind to the specific sequence of a target gene and *Fok I* enzyme to introduce DSB [120–124]. TALENs provide more flexibility to the target sequences since ZFNs are known to be more active towards GC-rich region, whereas TALENs can be assembled to the target AT-rich regions [120–124].

Since both ZFNs and TALENs require assembling of an array to build each set, which is a complex and time-consuming process [124], CRISPR/Cas9 has become the favorite because of its easy application. CRISPR/Cas9 requires only two components,

	ZFN	TALEN	CRISPR/Cas9
Working mechanism	DNA/protein interaction	DNA/RNA/protein interaction	DNA/RNA/protein interaction
Core components	TALE and *Fok* I fusion protein	TALE and *Fok* I fusion protein	Cas9 protein and sgRNA
Design	Easy	Easy	Very easy
Construction	Easy	Easy	Very easy
Efficiency	High	High	High
Off-target rate	Low	High	High

[1]*Based on Chen and Gao [126].*

Table 2.
Comparison of ZFN, TALEN, and CRISPR/Cas9-mediated genome editing systems[1].

the Cas9 nuclease and a single-guide RNA (sgRNA), which is a short sequence to guide the Cas9 protein to a target site. More importantly, these events are performed by transient interaction of Cas9 and sgRNA, whereby chromosomal integration of the CRISPR components is not a prerequisite [129]. There is a concern of off-target cleavage activity from the endonuclease from CRISPR/Cas9 because the system requires recognition of only 20 bp target sequence and allows up to 5 bp mismatches for the formation of DSB [124]. Several strategies for minimizing the off-target cleavage have been employed including use of double nickase mutant form of Cas9, which induces a single-strand break instead of DSB [130]; use of Cas9-sgRNA ribonucleoprotein (RNP) complex, whose half-life is shorter than that the time in which plasmid or viral nucleic acid is transcribed [131]; or use of fusions of catalytically inactive Cas9 with *Fok* I nuclease domain (fCas9) to improve the DNA cleavage specificity [132].

In the case of producing GM animals using SMGT or TMGT, it is better for the exogenous DNA (transgenes) to be integrated into the chromosomes of sperm. This event appears to occur more frequently in immature sperm cells present in the ST of a testis than in the mature epididymal sperm because the chromosomal DNA in the latter cells is tightly packed in the head region of a sperm. In this context, STGT is a preferable system to create GM animals because it is targeted to transfection of spermatogenic cells present within the STs. However, it takes about 4 weeks for mature sperm to reach the epididymal portion for fertilization. If a researcher wants to generate GM animals within a short period of time, direct transfection of mature sperm present in epididymides or vas deferens is recommended. In this case, as mentioned above, the introduced exogenous DNA may be associated to the extracellular fraction of a sperm, as episomal DNA. Notably, CRISPR/Cas9-based genome editing does not always require chromosomal integration of its components; it can be performed by transient expression of their components after transfection [129]. In this sense, an attempt to transfect mature sperm would be a useful alternative for GM animal production.

6. Proposal of new experimental systems for simple creation of genome-edited animals using *in vivo* or *in vitro* transfected sperm

In the following section, we propose two experimental plans to create genome-edited animals using VDGT or AI-based systems, all of which are simpler and more convenient than the previously described systems.

6.1 VDGT-based genome editing

As previously described in Section 2.4, VDGT enables transfer of exogenous DNA to oocytes via fertilization by mature sperm transfected within vas deferens [87, 88]. Injecting a solution containing genome editing components (e.g., sgRNA + DNA/mRNA/protein for Cas9) into the lumen of vas deferens of anesthetized males and subsequent mating between the VDGT-treated males and normal estrous females the day (night) after the surgery may result in production of genome-edited offspring.

In **Figure 6a–c**, experiments obtained after TB injection into the lumen of mouse vas deferens is shown (unpublished results). Under anesthesia, cauda epididymis and vas deferens were pulled out and a small slit was made at the proximal region of vas deferens using micro scissors (**Figure 6a**). Then, a glass micropipette containing TB was inserted into the lumen of vas deferens under observation using a dissecting microscope and about 15 μL of the solution is slowly injected (**Figure 6b**). It is easily discernible that the injected TB still remains within the proximal portion of vas deferens immediately after the injection (arrows in **Figure 6c**). However, the injected TB moved to the distal portion of vas deferens the next day (arrow in **Figure 6d**). Thus, to produce genome-edited animals by VDGT, a solution containing CRISPR/Cas9 components (sgRNA + DNA/mRNA/protein for Cas9), gene delivery enhancing reagents (such as DMSO, liposomes, microparticles, etc.) and fluorescent marker expression plasmid DNA has to be prepared first (**Figure 6e**).

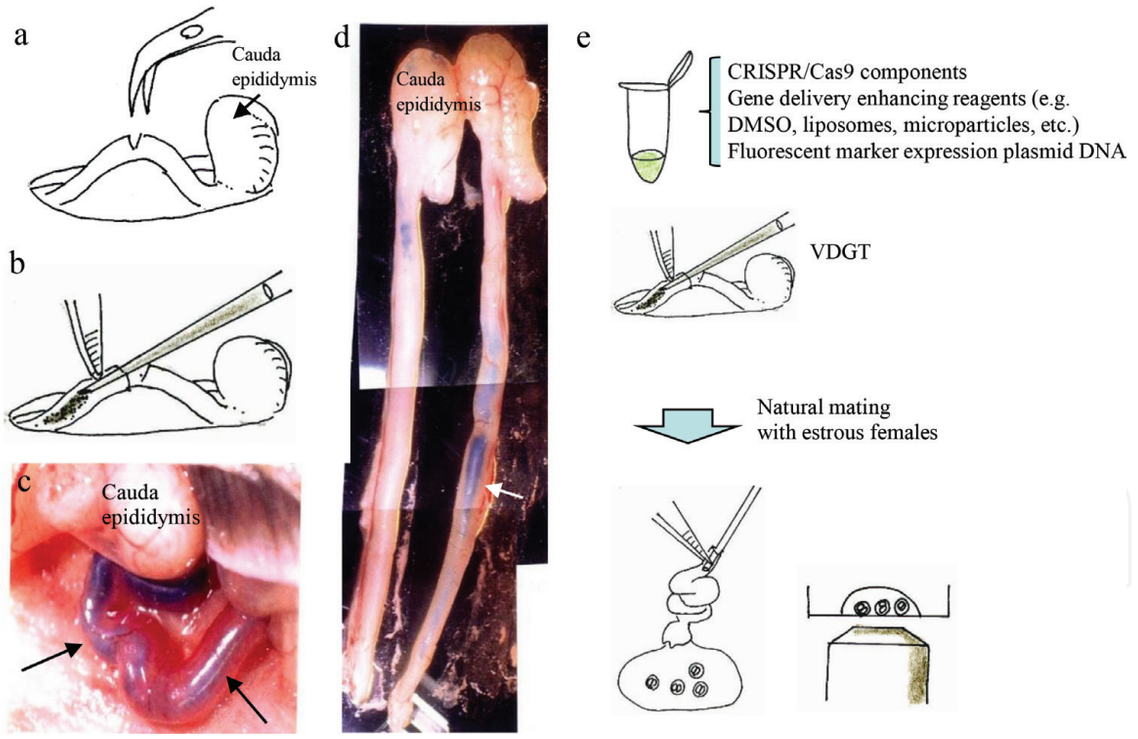

Figure 6.
Procedure for VDGT-based genome editing. (a–d) Experimental procedure of VDGT when TB as a visible marker is injected into the lumen of vas deferens. Under anesthesia, a small slit is made at the proximal region of vas deferens using micro scissors (a). Then, a glass micropipette is inserted into the lumen of vas deferens under observation using a dissecting microscope and the solution is slowly injected (b). After TB injection, the injected TB still remains within the proximal portion of vas deferens (arrows in c). One day after VDGT, the injected TB moves to the distal portion of vas deferens (arrow in d), showing the flow of the injected substance. e. Experimental procedure of VDGT when genome-editing components are injected into the lumen of vas deferens. First, a solution containing CRISPR/Cas9 components, gene delivery enhancing reagents (such as DMSO, liposomes, microparticles, etc.), and fluorescent marker expression plasmid DNA is prepared in a tube. Then, about 15 μL of this solution is immediately injected into the lumen of vas deferens. On the night following the VDGT or the next day, the VDGT-treated males are mated to normal estrous females. Later, cleavage stage embryos are collected to examine the presence/expression of the transgene (plasmid), as well as possible mutations in a target locus.

After short incubation period, this solution is injected into the lumen of vas deferens. Then, the VDGT-treated males are mated to normal estrous females on the day (night) or next day. Later, cleavage stage embryos were collected from the VDGT-treated females to examine the presence and expression of the transgene (plasmid) (**Figure 6e**, bottom) and occurrence of mutations in a target locus. In some cases, the SMGT-AI-treated females were allowed to deliver their pups to see whether genome editing is induced in their chromosomes.

6.2 SMGT-AI-based genome editing

AI is one of the assisted reproduction technologies that is based on the introduction of isolated sperm into the female reproductive tracts, such as uterine horn or oviductal lumen, to *in vivo* fertilize ovulated oocytes. As previously described in Section 3, isolated sperm are incubated in a solution containing exogenous DNA and gene delivery enhancing reagents such as DMSO, liposomes, and microparticles, for a short period (SMGT), and then the transfected sperm are subjected to AI, called "SMGT-based AI" (SMGT-AI). During this process, the exogenous DNA should be transmitted to oocytes via fertilization resulting in Tg embryos.

We previously reported that transfer of sperm into a space near the infundibulum between the ovary and ovarian bursa enables *in vivo* fertilization of ovulated oocytes in the ampulla region of the oviduct [133, 134]. In more detail, 2 μL of fresh epididymal B6C3F1 (F1 hybrid mice between C57BL/6 and C3H) sperm (containing 2 x 10^5 spermatozoa) were intrabursally injected 7 h after human chorionic gonadotropin (hCG) administration to B6C3F1 females that had been administrated with pregnant mare serum gonadotropin (PMSG) 48 h before. At 1.7 days after AI, normal cleaving embryos were recovered at rates of 40–100%. We called this AI technology "intrabursal transfer of sperm" (ITS) [133]. In **Figure 7a**, the ITS procedure is schematically illustrated. In **Figure 7b** and **c**, photographs before (b) and after (c) ITS are shown by using TB as a dye to visualize the process of AI. It is clear that the injected solution is present between the ovary and ovarian bursa (arrow in **Figure 7c**). In **Figure 7d**, an example for SGMT-AI-mediated genome editing in embryos is schematically shown. First, sperm isolated from the vas deferens are treated with CRISPR/Cas9 components (sgRNA + DNA/mRNA/protein for Cas9), gene delivery enhancing reagents (such as DMSO, liposomes, microparticles, etc.), and fluorescent marker expression plasmid DNA for a short period. Then, the solution containing the transfected sperm is subjected to AI towards females 7 h after hCG administration. The next day, 2-cell embryos are collected from the AI-treated females to examine the presence and expression of the transgene (plasmid) and occurrence of mutations in a target locus. In some cases, the SMGT-AI-treated females are allowed to deliver their pups to see whether genome editing is induced in their chromosomes. Notably, the selection of a successfully genome-edited sperm prior to AI may accelerate the production efficiency of genome edited offspring, although the practical approach for this remains unknown at present. Therefore, the success or failure of genome editing performed in this system may depend on the molecular analysis of the offspring (e.g. blastocysts or fetuses) generated after AI of the SMGT-treated sperm.

7. Conclusion

TMGT, based on direct *in vivo* gene delivery towards interstitial space of a testis, ST within a testis, or excurrent ducts of epididymides and vas deferens, is less labor

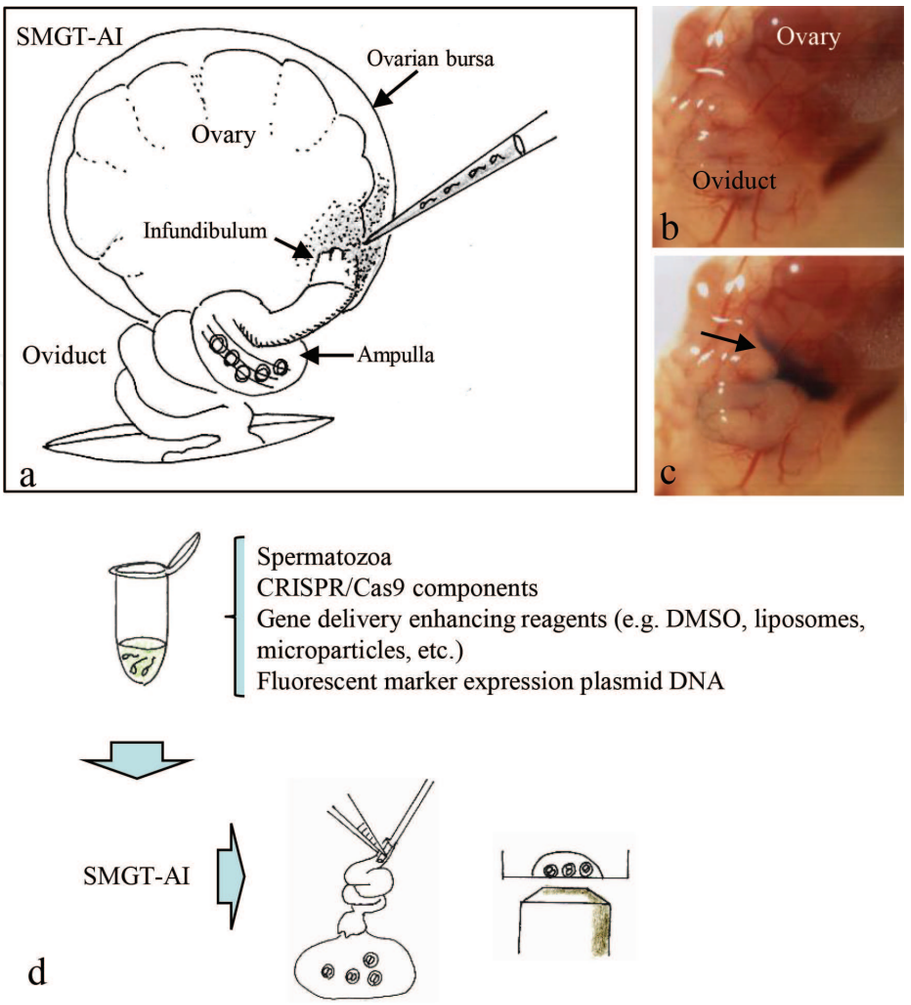

Figure 7.
Procedure for SMGT-AI-based genome editing. (a) ITS procedure schematic. (b, c) Photographs during before (b) and after (c) ITS, which is shown by intrabursal injection of TB. Note the presence of TB between the ovary and ovarian bursa (arrow in c). (d) Experimental procedure of SMGT-AI when genome-editing components are injected between the ovary and ovarian bursa. First, sperm isolated from the vas deferens are incubated in a solution containing CRISPR/Cas9 components, gene delivery enhancing reagents (such as DMSO, liposomes, microparticles, etc.) and fluorescent marker expression plasmid DNA for a short period. Then, the solution containing the transfected sperm is subjected to AI towards females 7 h after hCG administration. Later, cleavage stage embryos are collected to examine the presence/expression of the transgene (plasmid), as well as possible mutations in a target locus.

intensive and less time consuming for the production of GM animals. This testicular route is also ethically superior since fewer mice are required than existing alternative methods of transgenesis. The TMGT-treated males can be used to mate with estrous females, through which the exogenous genetic materials are transferred to oocytes at fertilization. During this process, there is no need for *ex vivo* handling of embryos, which is strictly required for zygote-based gene modification such as microinjection, EP, viral infection, and TransICSI. SMGT-AI, based on AI of sperm that have been transfected *in vitro* with the exogenous DNA, is also a convenient system for production of Tg animals, like TMGT. The CRISPR/Cas9 system, one of the recently developed genome editing technologies, is now recognized as a powerful and simple tool to create GM animals. More importantly, in this system, chromosomal integration of the genome editing components is not the prerequisite. Coupling this genome editing system with TMGT or SMGT-AI would accelerate creation of genome-edited animals in a more convenient manner. Furthermore, TMGT/SMGT-AI will be particularly useful for other animals that are difficult to manipulate as early embryos *in vitro*.

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Conflicts of interest

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

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