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Reproductive technologies for the generation and maintenance of valuable animal strains

Takehito KANEKO[1–3]

[1)]*Division of Science and Engineering, Graduate School of Arts and Science, Iwate University, Iwate 020-8551, Japan*
[2)]*Department of Chemistry and Biological Sciences, Faculty of Science and Engineering, Iwate University, Iwate 020-8551, Japan*
[3)]*Soft-Path Science and Engineering Research Center (SPERC), Iwate University, Iwate 020-8551, Japan*

Abstract. Many types of mutant and genetically engineered strains have been produced in various animal species. Their numbers have dramatically increased in recent years, with new strains being rapidly produced using genome editing techniques. In the rat, it has been difficult to produce knockout and knock-in strains because the establishment of stem cells has been insufficient. However, a large number of knockout and knock-in strains can currently be produced using genome editing techniques, including zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system. Microinjection technique has also contributed widely to the production of various kinds of genome edited animal strains. A novel electroporation method, the "Technique for Animal Knockout system by Electroporation (TAKE)" method, is a simple and highly efficient tool that has accelerated the production of new strains. Gamete preservation is extremely useful for maintaining large numbers of these valuable strains as genetic resources in the long term. These reproductive technologies, including microinjection, TAKE method, and gamete preservation, strongly support biomedical research and the bio-resource banking of animal models. In this review, we introduce the latest reproductive technologies used for the production of genetically engineered animals, especially rats, using genome editing techniques and the efficient maintenance of valuable strains as genetic resources. These technologies can also be applied to other laboratory animals, including mice, and domestic and wild animal species.
Key words: Electroporation, Gamete preservation, Genome editing, Rat, Sperm freeze-drying
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The rat is an important animal for understanding the mechanisms of human diseases [1, 2]. Spontaneous mutant and transgenic strains have been used as models of human diseases in various biomedical research fields [3, 4]. Although knockout and knock-in rat strains are also required as animal models, it has been extremely difficult to produce these strains because no high-quality rat embryonic stem (ES) cells [5, 6] or induced pluripotent stem (iPS) cells [7, 8] have been established. Transposon-mediated mutagenesis [9, 10] and N-ethyl-N-nitrosourea (ENU) mutagenesis [11, 12] have been used as alternative protocols for the random production of knockout strains.

Developing genome editing techniques overcame this serious problem. Genetically engineered strains can be rapidly produced by the direct introduction of engineered endonucleases into embryos with a requirement for neither ES cells nor iPS cells. Genome editing techniques, including zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system, are powerful tools for the generation of genetically engineered rats [13–16]. At present, a large number of knockout and knock-in rat strains produced by genome editing techniques are used worldwide [17].

The use of the genome editing techniques to produce genetically engineered strains has triggered an explosive increase in the numbers of animal populations available for biomedical research. Although breeding by natural mating is the optimal method for the maintenance of these strains, genetic contamination by mispaired mating and infection by pathogenic microorganisms can cause the extinction of valuable strains. Furthermore, the lack of breeding space as a result of the increased number of strains and the decline in fertility caused by inbreeding inhibit the reproduction of subsequent generations. Reproductive technologies, including gamete preservation and artificial fertilization methods using preserved gametes, are important tools in regulating animal breeding conditions. Although several reproductive technologies have been established and are used routinely on rats, these methods must be developed further to accommodate the rapid advances in genome editing techniques. In this review, we introduce the latest reproductive technologies for the production of genetically engineered rats based on genome editing techniques and the efficient maintenance of valuable strains. These technologies can also be applied to other laboratory animals, including mice, and domestic and wild animal species.

Production of Genome Edited Rats Using the Conventional Microinjection Method

In general, genetically engineered rats, such as transgenic strains, are produced by the microinjection of endonucleases into pronuclear-stage embryos. Fortunately, the same microinjection method can be used to produce genome edited rats. In brief, engineered ZFN, TALEN, or CRISPR-Cas9 systems, which encode the target genes, are introduced into the pronuclei or cytoplasm of embryos with a thin glass pipette installed in the holder of a micromanipulator [18–20]. The first genome edited rats were generated by microinjecting self-transcribing mRNAs of ZFN that targeted the immunoglobulin M (*IgM*) and Rab38 genes in the pronuclei of embryos [13]. The rat strain in which the interleukin-2 receptor subunit gamma chain (*Il2rg*) gene was knocked out (X-SCID) [21], and the strain in which the *Il2rg* and *Prkdc* genes are both knocked out, were then both generated [22], and these strains have since been widely used in biomedical research [23, 24].

Immediately after the successful production of knockout rat strains with ZFN in 2009, a new genome editing tool, called TALEN, was reported [25, 26]. TALEN was immediately used as an alternative tool for genome editing in rats and other species [14]. We successfully produced knockout rats that targeted the albino (*Tyr*) gene by microinjecting TALEN mRNA into the pronuclei of embryos, although the initial TALEN only had a low activity in the embryos [27]. However, their activity was significantly increased by the co-injection of exonuclease 1 (*Exo1*) with the TALEN mRNA [27]. Sakuma et al. [28] also constructed TALEN with periodically patterned repeat variants harboring non-repeat-variable di-residue (non-RVD) variations (Platinum TALEN). Platinum TALEN showed a higher activity than conventional TALEN after its introduction into embryos, and all offspring obtained from these microinjected embryos showed the mutation of the targeted *Il2rg* gene [28].

After the ZFN and TALEN technologies became standard methods for producing knockout rat strains, another technology, the CRISPR-Cas9 system, was developed [29, 30]. The successful production of genome editing rat strains using CRISPR-Cas9 was immediately reported [15, 16]. We also successfully produced a knockout strain that targeted the *Tyr* gene by microinjecting both Cas9 mRNA and guide RNA (gRNA) into the pronuclei of embryos [31]. Targeted knock-in strains could also be generated by introducing single-stranded oligodeoxynucleotides (ssODN) together with the Cas9 mRNA and gRNA into embryos [32]. CRISPR-Cas9 is now the most popular genome editing tool for the production of knockout and knock-in rats and other animal strains, because the Cas9 endonuclease can be used regardless of the targeted gene, and gRNA is a customized construct that can be designed using online web applications. Importantly, the CRISPR-Cas9 system shows high target specificity in the embryos [31].

The F344/Stm rat strain is recommended as a suitable animal for the production of genome edited rat strains, because it has been optimized to collect a sufficient number of pronuclear-stage embryos, although it is an inbred strain [33]. Furthermore, the whole genome sequence [34] and the bacterial artificial chromosome end sequences [35] of this strain have been analyzed. Fortunately, all endonucleases, including Cas9 mRNA, Cas9 nuclease protein, and custom-designed gRNA, can be purchased commercially and are highly active in embryos [36, 37]. This ease of preparation for the production of genome editing animals strongly promotes their use in biomedical research. Although microinjection is now the gold standard method routinely used for the production of genome edited animals, it requires a micromanipulator and sophisticated technical skills to prevent cell damage. Furthermore, microinjection is not convenient when many cells must be assessed simultaneously, because the endonucleases must be injected into the embryos one by one. For easy preparation, it is important to develop a fully automatic micromanipulator and another system for introducing endonucleases into embryos.

Electroporation Method for the Introduction of Endonucleases into Intact Embryos

The electroporation method can introduce nucleases into living cultured cells. However, this method cannot be used to introduce nucleases into animal embryos because the strong electric pulses of conventional electroporation protocols damage the embryos. Weakening the zona pellucida by treatment with Tyrode's acid solution before electroporation increases the chance of introduction of endonucleases [38, 39]. However, this may affect subsequent embryonic development because its function is important in *in vivo* development [40, 41].

We developed a new electroporation device, NEPA21 (Nepa Gene, Chiba, Japan), that reduces the damage to embryos by using a three-step electrical pulse system (Fig. 1a) [42]. In brief, pronuclear-stage embryos are placed in a line between metal plates in a glass chamber, filled with phosphate-buffered saline (PBS) or Opti-MEM (Thermo Fisher Scientific Inc., MA, USA), that contains the endonucleases (Fig. 1b and c). Three-step electrical pulses are then discharged into the embryos. The first pulse, the poring pulse, make micro-holes in the zona pellucida and oolemma of the embryos. The second pulse, the transfer pulse, transfers the endonucleases into the cytoplasm of the embryos. The third pulse, the polarity-changed transfer pulse, increases the opportunity of introducing the endonucleases into the embryos [43]. It should be noted that intact embryos with no weakening of the zona pellucida can be used for electroporation.

To examine the optimal electrical pulse conditions to introduce endonucleases into the embryos with this new system, we firstly introduced 3-kDa of tetramethylrhodamine-labeled dextran into intact rat pronuclear-stage embryos, because it can be easily and rapidly visualized and is nontoxic to embryos. The poring pulse was set to the following: voltage, 225 V; pulse width, 0.5, 1.5, or 2.5 msec; pulse interval, 50 msec; and number of pulses, + 4. The transfer pulse was set to the following: voltage, 20 V; pulse width, 50 msec; pulse interval, 50 msec; and number of pulses, ± 5. In this study, most embryos survived after electroporation, and dextran was introduced into the whole cytoplasms of all embryos at all pulse width settings for the poring pulse [42]. The results of this study are revolutionary, in that the new three-step electrical pulse system, NEPA21, can efficiently introduce high level of materials into intact embryos without any treatments that weaken the zona pellucida. Furthermore, the damage to the embryos by the electrical pulses is extremely low, ensuring a high survival rate among the embryos after electroporation. This new electroporation method was designated
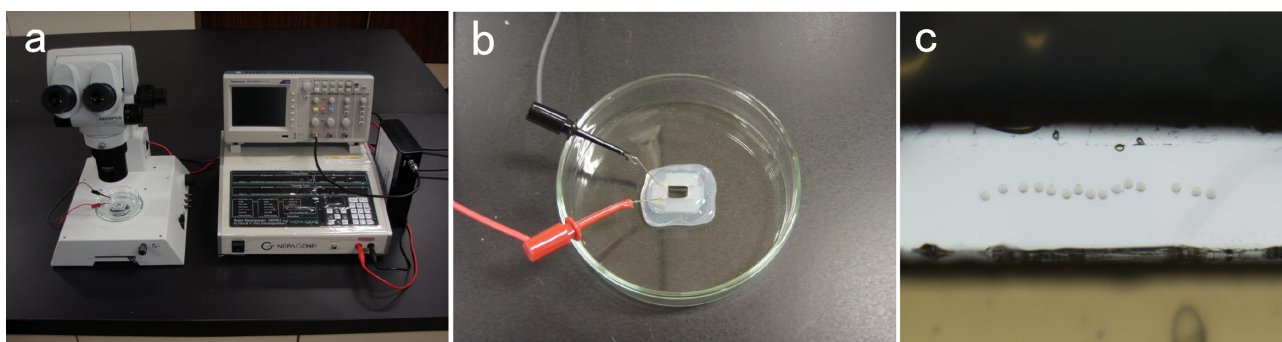
Fig. 1. (a) Super electroporator NEPA21. (b) Petri dish with platinum plate electrodes. (c) Pronuclear-stage embryos were placed in a line between metal plates in a glass chamber filled with a buffer that conducted the endonucleases.

Table 1. Development of rat embryos introduced to ZFN and TALEN mRNA by microinjection or electroporation

mRNA	Methods	Pulse width (ms)	No. of embryos examined	No. (%) of 2-cell embryos	No. (%) of offspring	No. (%) of offspring with mutation
ZFN	Microinjection	–	93	41 (44)	9 (10)	3 (33)
	Electroporation	0.5	61	58 (95)	19 (31)	7 (37)
		1.5	63	57 (91)	15 (24)	11 (73)
		2.5	66	16 (24)	4 (6)	3 (75)
TALEN	Microinjection	–	52	20 (39)	6 (12)	6 (100)
	Electroporation	1.5	57	55 (97)	25 (44)	1 (4)
		2.5	57	56 (98)	17 (30)	3 (18)

Targeted gene: *Il2rg* gene [42, 43].

the "Technique for Animal Knockout system by Electroporation (TAKE)" method [42].

Introduction of ZFN and TALEN mRNAs into Intact Embryos using the TAKE Method

We next introduced ZFN and TALEN mRNAs into intact rat embryos using the TAKE method. In this experiment, the mRNAs were self-transcribed from plasmid vector encoding ZFN and TALEN that targeted the *Il2rg* gene. The ZFN and TALEN mRNAs were suspended in PBS at 40 μg/ml. Up to 50 pronuclear-stage embryos were placed in a line between the metal plates in a glass chamber that was filled with PBS containing the mRNAs. The embryos were then electroporated with the same electrical conditions used to introduce tetramethylrhodamine-labeled dextran into embryos. The electroporated embryos that developed to the two-cell stage *in vitro* were transferred into the oviducts of pseudopregnant female rats.

Our results showed that 10% or 12% of the embryos microinjected with 10 μg/ml ZFN or TALEN mRNAs as a control developed into offspring, and that 33% or 100% of these offspring had an edited *Il2rg* locus, respectively. In contrast, 24% of the embryos that were electroporated with ZFN mRNA with a pulse width of 1.5 ms developed into offspring, and 73% of these offspring had an edited *Il2rg* locus. In embryos that were electroporated with the TALEN mRNA at a pulse width of 2.5 msec, 30% developed into offspring and 18% of these had an edited *Il2rg* locus (Table 1). Surprisingly,

more than 90% of embryos survived after electroporation. These results demonstrated that our electrical settings minimized the damage to the embryos. The germline transmission of the edited *Il2rg* gene was also confirmed in the next generation [42, 43]. Thus, the TAKE method was established as an easy and efficient method of introducing endonucleases into animal embryos.

Introduction of Cas9 mRNA/nuclease Protein, gRNA, and ssODN into Intact Embryos with the TAKE Method

The development of the CRISPR-Cas9 system has had a great impact on our research into the production of genome edited animals. A method that could be used to quickly and completely produce genome edited animals, including rats, using this system was required. Although conventional microinjection is a reliable method that provides adequate results, the equipment preparation and requirement of sophisticated technical skills have hindered the progress of research. The successful production of genome edited rat strains with the TAKE method using the CRISPR-Cas9 system has become an urgent task.

At present, the CRISPR-Cas9 system has become most popular genome editing tool. It has a high target specificity, and allows the simpler and more rapid production of genome edited animals than ZFN and TALEN. In our first trial, a plasmid expressing human Cas9 (hCas9; ID #41815, Addgene, MA, USA) was modified by the addition of the T7 promoter and DNA encoding SV40 nuclear localiza-

Table 2.   Development of rat embryos introduced to Cas9 mRNA, gRNA and ssODN by electroporation

Cas9 mRNA (μg/ml)	gRNA (μg/ml)	ssODN (μg/ml)	No. of embryos examined	No. (%) of embryos developed to 2-cells	No. (%) of males offspring	No. (%) of knockout offspring	No. (%) of knock-in offspring
400	600	300	60	45 (75)	24 (53)	21 (88)	8 (33)
200	200	200	50	49 (98)	19 (39)	7 (37)	1 (5)
100	100	100	89	88 (99)	41 (47)	16 (39)	1 (2)

Targeted gene: *Il2rg* gene [43, 44].

tion signals at the N-terminal of hCas9 to increase its activity after introduction into the embryo. The mRNA was then self-transcribed from the modified hCas9 plasmid. The same electrical pulse settings (poring pulse: voltage, 225 V; pulse width, 2.5 msec; pulse interval, 50 msec; number of pulses, + 4; transfer pulse: voltage, 20 V; pulse width, 50 msec; pulse interval, 50 msec; number of pulses, ± 5) that were used to introduce the ZFN and TALEN mRNAs into the embryos were used to introduce the Cas9 mRNA and gRNA that targeted the *Il2rg* gene in the intact rat pronuclear-stage embryos. Of the embryos electroporated with 400 μg/ml Cas9 mRNA and 600 μg/ml gRNA, 53% developed into offspring, and 88% of these offspring had an edited *Il2rg* locus. The production of knock-in rats (33%) by co-introducing 300 μg/ml ssODN with Cas9 mRNA and gRNA was also successful (Table 2). The germline transmission of the edited *Il2rg* gene was also confirmed in the next generation [43, 44].

The TAKE method was further improved as a widely used method for producing genome edited rats with various engineered endonucleases, including ZFNs, TALENs, and CRISPR-Cas9 systems. Similar electroporation methods have been reported based on the protocol of the TAKE method [45, 46], and the TAKE method is now used as a highly reproducible method worldwide [47–49]. Cas9 mRNA, Cas9 nuclease protein, and custom-designed gRNAs can now be purchased commercially. All these endonucleases can be electroporated into embryos, and display high levels of activity (Tables 1–3) [42–44]. Therefore, the rapid and complete production of genome editing animals is now possible without molecular biological preparations, such as the self-transcription of mRNA from a plasmid vector.

TAKE is a revolutionary method that can introduce endonucleases into 100 intact embryos within 5 min without sophisticated technical skills such as conventional microinjection, and shows a high mutation efficiency in the generated offspring. As a further advantage, the same electrical pulse settings as we used in rat embryos can be applied to genome editing in mouse embryos [43, 44]. It is expected that the TAKE method will promote biomedical sciences by generating various genomically altered animal species.

Maintenance of Rat Strains as Genetic Resources Using Reproductive Technologies

Genome editing techniques have dramatically increased the number of new rat strains. Breeding by natural mating is ideal for maintaining the populations of these strains. However, the lack of breeding space that has arisen with the increased number of strains and the decline in fertility caused by inbreeding inhibit the reproduction of subsequent generations. Therefore, reproductive technologies, such as gamete preservation and artificial fertilization techniques, have

Table 3.   Development of rat embryos co-introduced to Cas9 nuclease protein and gRNA by microinjection or electroporation

Methods	No. of embryos examined	No. (%) of 2-cell embryos	No. (%) of offspring	No. (%) of knockout offspring
Microinjection	40	19 (48)	13 (68)	10 (77)
Electroporation	25	25 (100)	17 (68)	17 (100)

Targeted gene: *Tyr* gene. Cas9 protein and gRNA (Integrated DNA Technologies, IA, USA) were used [43].

been developed to overcome these problems.

Gamete preservation is a useful tool for reducing breeding space and preventing the genetic contamination of resources by mispaired mating. Rat embryos have mainly been frozen using a slow freezing or two-step freezing methods [50–52], and a vitrification method has been also investigated [53–56]. We now vitrify rat embryos using a solution containing 10% propylene glycol, 30% ethylene glycol, 20% Percoll, and 0.3 M sucrose, and the frozen embryos are rapidly thawed in 0.3 M sucrose warmed to 37°C [33, 57]. The vitrification of embryos has a high recovery efficiency after thawing in both the two-cell and more developed embryo stages [58]. However, the survival rates of unfertilized oocytes and pronuclear-stage embryos vitrified with this method are low. The rate of subsequent developmental to offspring was significantly increased when the vitrified pronuclear-stage embryos were transferred to pseudopregnant females after development to the two-cell stage *in vitro* [33]. Frozen unfertilized oocytes and pronuclear-stage embryos are useful entities for producing genome editing strains.

Sperm preservation is another gamete preservation technique, which allow the simple preparation of males and the use of smaller breeding spaces. Furthermore, the genetic traits of genome editing strains can be transmitted by only preserving sperm. Sperm preservation is a simple, space-saving, and cost-effective method for the maintenance of genetically modified strains, including genome edited strains. Offspring were obtained from sperm that were frozen in a solution containing 8% lactose, 0.7% Equex STM, and 23% egg yolk [59]. However, rat sperm are known to be extremely sensitive to physical damage, and the tolerance of sperm to freezing differs greatly between rat strains [60]. Therefore, freezing rat sperm has been studied to develop a routine protocol. Effective fertilization protocols using frozen sperm have been developed in various animal species [61]. It is anticipated that sperm freezing and fertilization techniques using frozen rat sperm will be improved to accommodate these strain variations.

Intracytoplasmic Sperm Injection (ICSI)

In vitro fertilization is a useful method for simply generating large numbers of embryos. However, it is essential that sperm with good motility are used to fertilize oocytes *in vitro*. It is often impossible to collect sperm with good motility from all rat strains and individuals. Intracytoplasmic sperm injection (ICSI) has been used as a powerful fertilization tool in various animals, including humans [62]. This technique involves direct injection into an oocyte of a spermatozoon that has been drawn into a thin glass pipette installed in the holder of a micromanipulator.

The ICSI technique has dramatically increased the fertility potential of sperm *in vitro*. Oocytes can be fertilized by ICSI even when the sperm are immotile [63] or immature [64]. Uehara and Yanagimachi [65, 66] first reported successful ICSI in mammals that demonstrated the formation of normal pronuclei in oocytes after the microinjection of hamster sperm. Since their study was published, the offspring of various mammals have been produced with ICSI [67, 68]. Interestingly, the successful results for mouse ICSI were reported by Kimura and Yanagimachi in 1995 [69] after the publication of human ICSI in 1992 [70], because the oolemma of the mouse oocyte is easily broken during sperm injection with the conventional sharp glass pipette used for human ICSI. The piezo pulse-driven micromanipulator unit overcame the vulnerability of the mouse oocyte to damage from physical stress, and significantly improved the survival of oocytes after the injection of sperm [69].

Although the piezo pulse-driven micromanipulator was also used for rat ICSI, another technical problem arose. The rat sperm head has a unique structure [61], and oocytes are extremely sensitive to damage during sperm injection with a glass pipette [71]. No oocytes survived after sperm injection using a glass pipette with a diameter sufficiently wide to allow the complete aspiration of the sperm head. Survival is significantly increased by hanging a single sperm head on the tip of a narrow diameter glass pipette [72]. Offspring were also successfully generated with frozen immature [73] and mature sperm [74] using this improved rat ICSI technique.

Simple Gamete Preservation by Freeze-drying Sperm

It is difficult to store the sperm of all rat strains so that they retain their motility and can be used for artificial insemination and *in vitro* fertilization. However, sperm motility is no longer required when oocytes are fertilized by the ICSI technique. Offspring can be generated from oocytes that are fertilized by immotile sperm frozen without cryoprotectants [74–76]. The sperm of a large number of strains must be stored efficiently to save maintenance costs and space; sperm preservation by freeze-drying is an attractive and ultimate method for simple gamete preservation (Fig. 2) [77]. Unfortunately, freeze-dried sperm cannot penetrate the zona pellucida and oolemma of oocytes because their motility is lost during freeze-drying. However, sperm nuclei are strongly protected from damage during freeze-drying [78] by chelating agents, such as EDTA [79] or EGTA [76], in a slightly alkaline solution [80], and oocytes fertilized with these sperm by ICSI develop into normal offspring [74, 81].

The successful freeze-drying of sperm has already been reported in various animal species, including mice and endangered animals
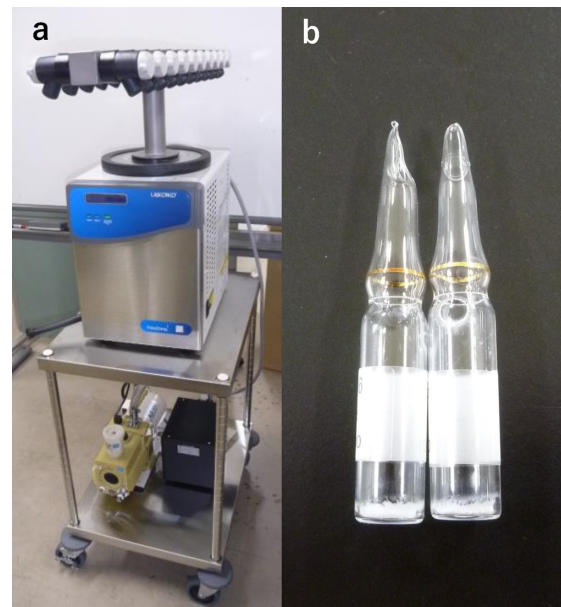
Fig. 2. (a) Freeze-drying machine and (b) freeze-dried sperm in glass ampoules.

Table 4. Development of rat oocytes fertilized with freeze-dried sperm stored at 4°C for various time periods

Storage term	No. of embryos transferred	No. (%) of embryos implanted	No. (%) of offspring
1–4 days	36	11 (31)	5 (14)
6 months	18	11 (61)	3 (17)
1 year	19	8 (42)	3 (16)
5 years	92	18 (20)	10 (11)

Rat: Crlj:WI [84, 87].

[82]. The advantage of freeze-drying sperm is that the sperm can be stored long-term in a simple solution (Tris and EDTA or EGTA) without cryoprotectants in a refrigerator (4°C) (Table 4) [83–85]. Furthermore, short-term storage [86] and worldwide transportation at ambient temperatures [87] are also possible. A similar simple sperm preservation method using evaporation has been investigated in the mouse [88]. Conventional gamete preservation requires a continuous supply of liquid nitrogen and the mechanical maintenance of equipment for long-term preservation. Unfortunately, valuable sperm samples that are stored in liquid nitrogen may be lost if the liquid nitrogen supply is compromised, especially during natural disasters, such as earthquakes and typhoons [89]. It is not realistic to fully prepare a facility in which samples can be safely stored in each laboratory. The freeze-drying and evaporation methods of sperm preservation are simple, safe, and cost-effective for the maintenance of valuable rat strains in the long term. At present, the freeze-drying of sperm has also been used to maintain the genetic diversity of endangered wild animal species [90].

Conclusions

The quality and quantity of rat research has been dramatically improved by the development of genome editing techniques. Genetically engineered rats with changes to uniquely targeted genes can already be rapidly produced. Advances in reproductive technologies have also been made in parallel with the development of gene targeting technologies. However, these require further development to become stable routine technologies. Few researchers are highly skilled in rat reproductive technologies, although we have held a technical workshop and released technical protocols [19, 20, 43, 87] to disseminate these animal reproductive techniques globally. However, these reproductive technologies are still inadequate for application to some experimental animals. We anticipated the development and popularization of reproductive technologies that can produce and maintain new valuable strains in various animal species.

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