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Cryopreservation for Broader Production of Transgenic Mice by DNA Injection into Zygotes

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Abstract: Mutant mice are indispensable to biological and medical research, and transgenic mouse production by DNA injection into zygotes has been an important method for producing these mice which are used to examine the over-expression of genes and to analyze gene transcriptional regulatory sequences. Recently, cryopreservation of zygotes by a simple vitrification method has become popular, saving the labor of isolating zygotes following each injection. However, the DNA injection technique requires training in the use of special equipment, and the injection cannot be accomplished routinely in every laboratory. The exchange of live mice also risks the propagation of common murine pathogens and possible escape of the animals; therefore the transfer of mice in frozen zygotes or embryos is recommended. Here we propose injecting DNA into frozen and thawed zygotes, refreezing them before transportation to any destination, where they can be thawed and developed into pups. The rate of transgenic mouse production using this method does not decrease significantly, even if zygotes are frozen and thawed before and after their DNA injection, and will make transgenic studies more popular on a worldwide scale.

Key words: cryopreservation, DNA injection, mouse, transgenic, vitrification

Introduction

Mutant mice are one of the essential tools currently used in biological and medical research. The quality and efficacy of research is greatly dependent on how easily mutant mice can be generated, propagated, and housed. Transgenic mouse production by DNA injection into pronuclei of zygotes has been one of the important methods of mutant mice generation for examining the functions of genes in their over-expression and analyzing gene transcriptional regulatory sequences [1]. Increased use of mutant mouse technology has spurred the need for the development of cryopreservation technology. Initial success was achieved by the slow freezing method [15], and the technology has markedly advanced, recently, with more simple vitrification methods [2, 7, 9, 13], which frees researchers from the labor of collecting zygotes for each injection. The number of eggs obtained by superovulation is variable each time, but transgenic mice can be constantly generated if frozen embryos are utilized [4, 8, 14]. The DNA injection technique, however, requires training with special equip-

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ment, and the injection cannot be accomplished routinely in every laboratory. Many academic researchers have difficulty in establishing transgenic mice, since not everyone can find a collaborator who can conduct the production. Commercially it is expensive, and non-commercial organizations that support the production are limited in number.

Only common pieces of equipment are required to obtain pseudo-pregnant females and a stereo microscope and a CO_2 incubator for the transfer of the zygotes injected with DNA into the oviduct [6]. In contrast to the DNA microinjection technique into zygotes, this technique can be easily mastered. The exchange of live mice inherently involves problems such as the propagation of common murine pathogens, the escape of animals and accidental animal death. The transfer of mice in frozen zygotes or embryos is therefore recommended and will undoubtedly become more widespread in the near future when each animal facility should have a technician in charge of developing frozen embryos into live mice. Transgenic studies would become more widespread on a worldwide, if zygotes injected with DNA can be successfully frozen and efficiently recovered. To demonstrate that this is indeed feasible, here we examined the efficiency of transgenic mouse production when DNA was injected into frozen and thawed zygotes and the DNA injected zygotes were then refrozen, thawed and developed in foster mothers.

Materials and Methods

Animals

C57BL/6N (B6N) is a standard strain in mouse genetics, and all experiments in this study were performed using this strain. Random bred CD1:ICR (CD1) is one of the cheapest mice available and was used for foster mothers. B6N and CD1 mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and Charles River Japan Inc. (Tokyo, Japan), respectively. Animal housing and experiments were performed according to the Riken Center for Developmental Biology Animal Experiment Guidelines. The housing rooms were in a restricted facility, and were kept at 21 ± 1°C with a humidity of 50 ± 10% under a lighting regimen of 12L:12D (light on from 08:00 to 20:00).

Source of embryos

Female B6N mice 8–10 weeks of age were superovulated by intraperitoneal injections of 5 units of PMSG (Serotrophin, Teikoku Hormone Mfg. Co., Tokyo, Japan), and hCG (Gonatrophin, Teikoku Hormone Mfg. Co., Tokyo, Japan) 48 h after PMSG injection. Ovulated oocytes were collected from the ampulla of the superovulated female mice 15–16 h after the hCG injection. Supermatozoa were collected from cauda epididymis tail of B6N males and suspended in HTF medium covered with mineral oil (M8410, Sigma Aldrich Co., St. Louis, MO, USA) [12]. A small volume of sperm suspension was added to the HTF medium containing the oocytes. Pronuclear stage oocytes were obtained 6 h after the *in vitro* fertilization (IVF) [6].

Cryopreservation and transgenic mice

Fresh and DNA-injected zygotes were cryopreserved by the simple vitrification method at 60–100 zygotes per tube as previously described [9]. The freshly isolated and vitrified embryos were stored in liquid nitrogen (LN2), and after thawing, morphologically normal ones were injected with DNA. The DNA-injected and vitrified embryos were stored in LN2 for more than one week, and after thawing, morphologically normal ones were developed to the 2-cell stage in KSOM medium overnight [3]. The 2-cell stage embryos were then transferred into the oviducts of pseudopregnant CD1 female mice to gestate transgenic mice by Cesarean section. The transgene used in this study was Venus [5] directed by the ubiquitous CAG promoter [10] (Fig. 1A). It was microinjected into male pronuclei of B6N mouse zygotes as previously described [6]. The transgenic mice were identified by PCR for the Venus gene using primers: p1, GGCTGCCATGAACAAAGG, and p2, AATTAACCCTCACTAAAGGG. The expression of the integrated transgene was deduced by green fluorescence (Fig. 1B) under UV light [11].

Results

Our proposal for routine transgenic mice production is to inject DNA into zygotes from frozen stocks, then refreeze them and transport them to any destination where they can be thawed and transferred into the ovi-

ducts of foster mothers to produce live mice. First, we examined the effects of repeated freezing and thawing on the developmental rate of zygotes (Table 1). About 60% of the zygotes freshly obtained for IVF developed into blastocysts in culture, and about half the embryos transferred into the oviducts of pseudopregnant CD1 foster mothers developed into live-born mice (Table 1-I). Zygotes frozen by the vitrification method were recovered at a rate of 94% after thawing; 60% of them developed into blastocysts in culture and about half of these developed into live-born mice (Table 1-II). The effects of refreezing and rethawing the zygotes were then tested for their rate of development (Table 1-III). Two hundreds sixty-nine zygotes were frozen and thawed twice, and 261 (97%) were recovered in a viable state after the second thawing, about half of which also developed into live-born mice. Therefore, repeated freezing by the vitrification method and thawing had no appreciable effect on the viability of the zygotes.

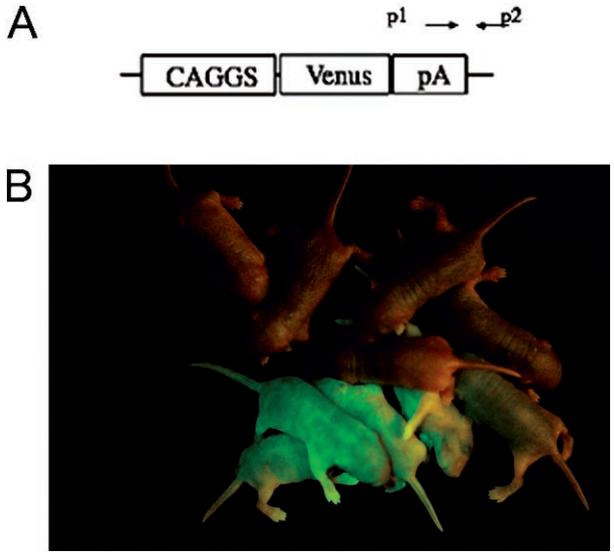

Fig. 1. A. The construct of the transgene. The length is about 3 kb. P1 and p2 indicate the position of primers for PCR genotyping. B. An example of a litter from a founder mouse with the Venus transgene. Under a UV light about half of the pups born fluoresced green.

Next, we examined how refreezing and rethawing affects the rate of transgenic mouse production (Table 2). Although most of the zygotes freshly collected and injected with DNA were viable at the time of their transfer into oviducts of pseudopregnant females at the 2-cell stage, only about 30% of them developed into live-born pups (Table 2-I), in contrast to about 50% of uninjected zygotes (Table 1). Mechanical damage by the DNA injection would explain the loss. About 5% of the zygotes injected with DNA developed into transgenic mice, and about 30% of these developed into live-born mice of which about 20% of the pups were transgenic, expressing *Venus* (Table 2). A question is whether the damage by the DNA injection is enhanced by the freezing and thawing after the injection. However, 26% of the zygotes freshly collected, injected with DNA, frozen by the vitrification method, thawed and transferred into foster mothers developed into live-born mice, and the rate of transgenic mice was 7.6% of the zygotes injected with DNA (Table 2-II). Also, frozen and thawed zygotes might be more susceptible to mechanical damage than freshly collected zygotes. However, transgenic mice were obtained at a rate of 4.2% from the frozen zygotes. The DNA injection into frozen and thawed zygotes had

Table 1. Effects of freezing on development of zygotes

Treatment*	a: No. zygotes obtained by IVF	b: No. zygotes recovered after thawing (b/a)	c: No. zygotes developing into two cell stage (c/a)	*In vitro* culture		*In vivo* transplantation	
				f: No. embryos cultured	g: No. embryos developing into blastocysts (g/f)	h: No. embryos transplanted into foster mothers	i: No. pups born (i/h)
(I) Fresh	250	–	248 (99%)	48	30 (63%)	200	101 (51%)
(II) One freezing	330	311 (94%)	308 (93%)	68	41 (60%)	240	118 (49%)
(III) Two freezing	269	261 (97%)	240 (89%)	–	–	240	113 (47%)

*(I) 248 out of 250 zygotes obtained by IVF developed into two cell stage, of which 48 were further cultured into blastocysts and 200 were transplanted into foster mothers; (II) 330 zygotes obtained by IVF were frozen and thawed, yielding 311 viable zygotes of which 308 developed into two cells. 68 embryos were further cultured into blastocysts, while 240 were transplanted into foster mothers; (III) 269 zygotes obtained by IVF were frozen, thawed, refrozen, and rethawed, yielding 261 viable zygotes of which 240 developed into two cells and were transplanted into foster mothers. Each figure is the sum of three experiments.

Table 2. Effects of zygote freezing on rate of transgenic mouse production

Treatment*	a: No. zygotes collected or frozen	b: No zygotes recovered after thawing (b/a)	No. zygotes injected with DNA				g: No. pups born (g/a)	h: No. pups transgenic (h/a)	i: No. pups expressing *Venus* (i/a)
			c: No. zygotes subjected to injection (c/a)	d: No. zygotes surviving after injection (d/a)	e: No. zygotes recovered after injection and freezing (e/a)	f: No. embryos developing into two cells and transferred into foster mothers (f/a)			
(I) Injection	245	–	245	242 (99%)	–	239 (98%)	71 (29%)	13 (5.3%)	13 (5.3%)
(II) Injection-Freezing	237	–	237	234 (99%)	215 (91%)	212 (89%)	61 (26%)	18 (7.6%)	11 (4.6%)
(III) Freezing-Injection	260	245 (94%)	245 (94%)	237 (91%)	–	237 (91%)	57 (22%)	11 (4.2%)	10 (3.8%)
(IV) Freezing-Injection-Freezing	240	220 (91%)	220 (91%)	214 (89%)	200 (83%)	198 (83%)	51 (21%)	13 (5.4%)	12 (5.0%)

* (I) DNA was injected into zygotes freshly obtained by IVF; zygotes surviving after the injection and developing into the two cell stage were transferred into oviducts of foster mothers; (II) DNA was injected into zygotes obtained by IVF. The zygotes surviving after the injection were frozen, thawed, allowed to develop into two cell stage and transfered into oviducts of foster mothers; (III) zygotes obtained by IVF were frozen, thawed, injected with DNA, allowed to develop into two cell stage and transferred into oviducts of foster mothers; (IV) zygotes obtained by IVF were frozen, thawed, injected with DNA, refrozen, rethawed, allowed to develop into two cell stage and transferred into oviducts of foster mothers. Each figure is the sum of three experiments.

no adverse effect on the rate of transgenic pups (Table 2-III). Moreover, refreezing and rethawing together with the DNA injection into frozen and thawed embryos had no appreciable effect either on the birth rate of transgenic pups, 5.4% (Table 2-IV). These F_0 transgenic mice transmitted the transgene into their offspring yielding green mice at the Mendelian ratio (Fig. 1B). A few of the transgenic pups identified by PCR genotyping of tail DNA did not express *Venus*, significantly, most probably because of site of integration (Table 2).

Discussion

The rate of transgenic mouse production (about 5% of injected embryos) when zygotes were frozen before and after DNA injection was not different from that when DNA was injected into zygotes freshly isolated from pregnant females and immediately transferred into oviducts of foster mothers. Thus, DNA injection can be entrusted to a specialist and transgenic mice can be generated via receipt of frozen DNA-injected embryos, if the technology of thawing zygotes and transplanting them into foster mothers is locally available. Adoption of this method would make transgenic studies more accessible worldwide. The training of the techniques required for freezing and thawing of zygotes by the simple vitirification method, production of vasectomized males and the transfer of zygotes into oviducts can be relatively easily accomplished and requires no special equipment. There is nothing special in our freezing and thawing technique, but the technique is not standardized even among animal facilities in Japan. The exchange of mice as frozen embryos would minimize problems such as the propagation of common murine pathogens and the escape of animals. The transfer of mice as frozen embryos will undoubtedly become more widespread, and each animal facility should and soon have a technician in charge of developing frozen embryos into live mice. This must facilitate the standardization of the vitrification method to be shared commonly.

Currently, as the supplier of transgenic mice, after DNA injection, we undertake a series of processes, starting with the transfer of DNA-injected zygotes into foster mothers and housing of the foster mothers. At the time of weaning, tails are cut and sent to recipient researchers. DNA integration is a random event, and it is a long process to identify transgenic mice appropriate for a particular research project. Minimum characterization will be made at the F_1 generation of transgenic mice, but it is laborious for the supplier to breed transgenic mice for this characterization. It is practically impossible to freeze more than five uncharacterized transgenic lines at F_0 or F_1 to send to recipient researchers; therefore, we cannot support transgenic mice production unless the recipients are able to receive live F_0 transgenic mice. It is also laborious to send live mice, especially abroad, and this is further complicated by the potential for transmission of common murine pathogens, a problem that

is now serious for both suppliers and recipients. Sending DNA-injected zygotes would solve these problems. Moreover, cryopreserved mouse zygotes are now marketed and can be purchased. A specialist can support researchers by injecting DNA into zygotes even if he or she does not have an animal facility. Microinjected zygotes would then be cryopreserved again and sent to third party laboratories for embryo transfer procedures. This dramatically increases supplier's capacity to support researchers for the DNA injection into zygotes. On the recipient researcher side, receiving DNA-injected zygotes makes transgenic studies more direct, easy, and speedy. For example, in an enhancer analysis of a series of DNA fragments, a researcher can receive and store the zygotes injected with DNA in a frozen state and analyze them by thawing and transferring into foster mothers according to his/her schedule.

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