Viability of Transgenic Rat Embryos after Freezing and Thawing

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Abstract: *In-vivo* viability of frozen-thawed embryos derived from transgenic rats, as well as the transmission and the expression of transgenes in the resultant newborn rats, was investigated. Three strains of transgenic rats, carrying human growth hormone gene connected downstream to the promoter region of the bovine α -lactalbumin gene (α LA/hGH), bovine β -casein gene (β CN/hGH) or bovine α -S1 casein gene (α S1CN/hGH), were used. Two-cell stage embryos (non-transgenic Wistar female $\times$ heterozygous transgenic male) were placed in 10% (v/v) dimethylsulfoxide (DMSO) solution and cooled from -7 to -30°C at $-0.5^{\circ}\text{C}/\text{min}$ before being plunged into liquid nitrogen. After 2 to 4 years storage, the embryos were thawed by rapid warming. The intact embryos were transferred into the oviducts of Day 1 pseudopregnant recipients. The postthaw survival rate of frozen embryos was high in all 3 transgenic strains (88 to 92%), which was similar to that of control (non-transgenic) frozen embryos (95%). Development to newborn rats following transfer of embryos derived from the 3 strains (64 to 68%) was also similar to that of control embryos (60%). These transgenes (α LA/hGH, β CN/hGH and α S1CN/hGH) were detected in the DNA extracts from tail tissue of the newborn rats, but the transmission rates (41, 23 and 32%, respectively) were lower than 50% which is expected in the Mendelian fashion. In a transgenic line carrying α S1CN/hGH, hGH levels of secretion into the milk of transgenic newborn rats derived from frozen-thawed embryos and her transgenic offspring were the same mg/ml-level as that of their founder rat. Two-step freezing of embryos derived from transgenic rats was therefore an effective method for the long-term cryopreservation of transgene.

Key words: embryo freezing, gene expression, gene transmission, transgenic rat

Introduction

Recent development of recombinant DNA technology has made it possible to constitute new individuals by introducing new functions of foreign genes [1]. This

transgenic technology can be applied to not only small experimental rodents [2–4] but also large domestic animal species [5–9]. Since the maintenance of transgenic strains even in small animals is laborious and costly, cryopreservation of spermatozoa and embryos derived

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from transgenic animals has been explored [10, 11], but the stability of transgenes after freezing and thawing is not fully understood. There was only one report showing that rates of transmission of transgenes into newborn mice ranged between 33 and 46% when the mice were derived from frozen-thawed embryos of non-transgenic $\times$ heterozygous transgenic mice [11].

We previously developed a 2-step freezing technique appropriate for embryos from mouse and rat including spontaneous animal model of human diseases [12]. By this method, frozen-thawed embryos from model animals of human diseases could develop to newborn rats without losing their hereditary characteristics.

In the present study, the 2-cell stage embryos derived from 3 strains of transgenic rats were frozen-thawed, and the morphological survival and *in-vivo* developmental competence of postthaw embryos, as well as the transmission and the expression of transgenes in the resultant newborn rats, were investigated.

Materials and Methods

Source of Embryos: Three strains of transgenic rats (Wistar origin) [4], carrying human growth hormone gene connected downstream to the promoter region of the bovine α -lactalbumin gene (α LA/hGH), bovine β -casein gene (β CN/hGH) and bovine α -S1 casein gene (α S1CN/hGH) were produced by pronuclear microinjection. The α LA/hGH, β CN/hGH, and α S1CN/hGH strains composed from 3, 4, and 3 lines, respectively. Non-transgenic female Wistar rats (8 to 13 weeks old; Charles River Japan, Inc.) were superovulated by intraperitoneal injections of 150 IU/kg eCG (PMS-Zenyaku; Nippon Zenyaku Co., Ltd.) and 75 IU/kg hCG (Puberogen; Sankyo Co., Ltd.). On the day of hCG injection, the rats were coupled overnight with G1-generation transgenic male rats carrying the transgene heterozygously. Two-cell stage embryos were collected by oviduct flushing with HER medium [13] supplemented with 18% fetal bovine serum (HERs) between 44 and 50 hr after hCG injection. The embryos were washed 3 times with HERs medium. Non-transgenic 2-cell stage embryos (Wistar $\times$ Wistar) were served for frozen control and nonfrozen fresh control.

Freezing and Thawing: The protocol for embryo freezing was the same way as that of Mizuno *et al.*

[12]. Briefly, embryos were suspended in the cryoprotective medium composed of 10% (v/v) dimethylsulfoxide (DMSO) in HERs medium at 22 to 24°C for 10 to 15 min. The embryos were then put into 0.25 ml plastic straws (FHK Co., Ltd.). The straws were placed in a 0°C methanol bath in a programmable freezer (ET-1; FHK Co., Ltd.), cooled to -7°C at $-1.0^{\circ}\text{C}/\text{min}$ and kept for 10 min. The straws were seeded at -7°C and then cooled to -30°C at $0.5^{\circ}\text{C}/\text{min}$ before being plunged into liquid nitrogen. They were stored in a liquid nitrogen tank for 2 to 4 years and thawed in a 37°C water bath. The contents of the straws were expelled into 0.8 ml of HERs medium and kept at 22 to 24°C for 10 min. The recovered embryos were washed 3 times with HERs medium.

Viability Assay of Frozen Embryos: The morphological survival of postthaw embryos was assessed by light microscopic observations at $\times 50$ magnification after washing. All of the morphologically normal embryos were transferred into the oviducts of the recipient rats (8 to 13 weeks old, Wistar strain) which had been previously mated with vasectomized male rats. The embryo transfer was performed on Day 1 of pseudopregnancy (the day when a vaginal plug was detected). Eleven to 21 embryos were transferred per recipient.

Transmission of Transgene: The presence or absence of the transgene in the resultant newborn rats was examined by the method of Ninomiya *et al.* [4]. Briefly, whole DNA extracts were prepared with proteinase K and pronase from the tail tissue of weaned rats. A part of the transgene was amplified by the PCR with a pair of primers, HGH003 (5^{OH} ACACCTACCAGGAGTTT-GTAAGCTC 3^{OH}) and HGH103 (5^{OH} CTGGAGACCA-GCTCCCATTTGTTACT 3^{OH}), or HGH004 (5^{OH} TCTC-AGAGTCTATTCCGACACCTC 3^{OH}) and HGH104 (5^{OH} AAGACACTCCTGAGAACTGCACGG 3^{OH}).

Expression of Transgene: In a transgenic line carrying α S1CN/hGH (#311-6), transgenic newborn female rats derived from frozen-thawed embryos (G2-generation) were allowed to mature and mate with non-transgenic rats. Ten days after delivery, their lactating milk was collected by hand. The amounts of human growth hormone (hGH) secreted into the milk were determined with the ELISA kit for hGH (GIBCO/BRL). Two G3-generation transgenic rats (heterozygously) were also examined for their abilities to express the transgene.

Statistical Analysis: The proportions of postthaw embryos with normal morphology in each replicate, the proportion of embryos developed to newborn rats per recipient, and the transmission rates of the transgene in each litter were arcsin-transformed before being subjected to one-way analysis of variance. Differences among means were compared by Fisher's protected least significant difference test (PLSD-test). A value of $P < 0.05$ was chosen as an indication of statistical significance.

Results

Morphological survival and *in-vivo* viability of postthaw 2-cell stage rat embryos after 2 to 4 years storage are shown in Table 1. Morphological survival rates of postthaw embryos derived from rats heterozygously carrying α LA/hGH, β CN/hGH and α S1CN/hGH were 92, 92 and 88%, respectively. No significant differences were found among strains and among lines in each strain. There was also no significant difference between each strain and the non-transgenic control (postthaw survival rate 95%). All the recipient rats became pregnant and delivered 4 to 16 newborn rats per litter. Proportions of embryos

developed to newborn rats were not significantly different among groups including the control (60 to 68%). These values were also similar to that for the nonfrozen fresh control (69%).

Detection of transgenes in the resultant newborn rats from frozen embryos is shown in Table 2. The transmission rates of these transgenes, (α LA/hGH, β CN/hGH and α S1CN/hGH, were 41, 23 and 32%, respectively, and were lower than the expected value, 50%, based on the Mendelian fashion. In contrast, transmission rates of transgenes via natural mating of transgenic and non-transgenic rats ranged between 40 and 56% (mean value 48%).

In a transgenic line carrying α S1CN/hGH (#311-6), the hGH secretion level into the milk of the newborn rat (G2-generation, $n=1$) was estimated to be 1.2 mg/ml. The hGH secretion levels in her transgenic offspring (G3-generation, $n=2$) were estimated to be 2.9 and 5.6 mg/ml. These levels of hGH secretion into the milk were similar to that of their founder female rat (G0-generation; 6.5 mg/ml).

Discussion

The need for transgenic animals has increased world-

Table 1. *In vivo* survival of transgenic rat embryos after freezing and thawing

Transgene	Line	No. (%) of embryos		
		Frozen-thawed	Morphologically surviving	Developed to pups
α LA/hGH	#222-9	55	51 (93)	30 (59)
	#223-3	48	47 (98)	35 (75)
	#226-4	25	20 (80)	11 (55)
	total	128	118 (92)	76 (64)
β CN/hGH	#211-2	42	41 (98)	29 (71)
	#213-2	60	52 (87)	35 (67)
	#213-3	50	47 (94)	32 (68)
	#219-2	43	39 (91)	25 (64)
	total	195	179 (92)	121 (68)
α S1CN/hGH	#305-1	30	26 (87)	18 (69)
	#308-5	27	26 (96)	15 (58)
	#311-6	62	53 (86)	38 (72)
	total	119	105 (88)	71 (68)
Control	Frozen-thawed	112	106 (95)	64 (60)
	Nonfrozen	—	107 (100)	74 (69)

Table 2. Transmission of the transgene to newborn pups derived from frozen-thawed transgenic rat embryos

Transgene	Line	No. (%) of newborn pups derived from			
		Frozen embryos		Natural mating	
		Examined	With transgene	Examined	With transgene
α LA/hGH	#222-9	30	14 (47)	9	6 (67)
	#223-3	35	14 (40)	—	—
	#226-4	11	3 (27)	16	8 (50)
	sub total	76	31 (41) ^a	25	14 (56) ^a
β CN/hGH	#211-2	29	11 (38)	—	—
	#213-2	35	4 (11)	—	—
	#213-3	32	8 (25)	10	4 (40)
	#219-2	25	5 (20)	—	—
	sub total	121	28 (23) ^a	10	4 (40) ^a
α S1CN/hGH	#305-1	18	7 (39)	27	12 (44)
	#308-5	15	7 (47)	28	15 (54)
	#311-6	38	9 (24)	23	8 (35)
	sub total	71	23 (32) ^a	78	35 (45) ^a
total		268	82 (31) ^a	113	53 (47) ^b

a, b: Different superscripts within a row denote significant differences ($P < 0.05$).

wide, resulting in their commercial use or free-service for research co-operation. But it has become necessary to control the management of transgenic animals under the guidelines for experiments on recombinant DNA, and it is laborious and costly even in the case of small facilities. Cryopreservation of embryos as a tool for maintaining transgenic animals can overcome such disadvantages, and has another merit in protecting animals from microbial infections and spontaneous genetic changes induced throughout the passages. It has been reported by Anzai *et al.* [11] that transgenic mice could be derived from two-cell stage embryos following ultrarapid freezing procedures. The same authors have also reported that transgenic rats could be derived from frozen-thawed pronuclear stage embryos [14], but the *in vivo* viability of transgenic rat embryos was lower than that of transgenic mice. In the present study, two-cell stage embryos derived from transgenic rats were found to remain viable after cryopreservation for as long as 2 to 4 years, without an appreciable loss of viability. These results suggest that the 2-step freezing procedure employed here [12] is useful not only for spontaneous animal models for human diseases but also for transgenic rats.

The mean transmission rate of 3 transgenes in the newborn rats derived from frozen embryos (31%, 82/268) was lower than the expected value of 50% based on the Mendelian fashion, and the mean transmission rate of the transgenes via either natural mating of transgenic and non-transgenic rats or transfer of the embryos without freezing and thawing process was 47% (53/113). Selden *et al.* [15] have reported that a transgenic founder mouse carrying 1 copy of hGH gene transmitted the transgene to the G1-progeny at the rate of approximately 50%. Anzai *et al.* [11] have reported that a transgene, anti-sense sequence against rat growth hormone, was transmitted at the rate of 33% (5/15) into the newborn rats derived from frozen-thawed, non-transgenic $\times$ transgenic embryos. These results suggest that freezing and thawing procedures for transgenic rat embryos may affect the stable transmission of transgenes. From the viewpoint of developmental kinetics, selective loss of transgenic embryos is unlikely to occur under the oviduct/uterine environment of recipient rats, because no differences between experimental and frozen control groups in the proportions of newborn rats following embryo transfer were found. But if the transgenic embryos developed more

slowly than non-transgenic embryos and the uterine capacity to support embryonic development (destined number of newborn rats per recipient) was prearranged, the final proportion of transgenic rats in all examined newborn rats would be less than 50%. Further investigations with transgenic rats carrying other transgenes are necessary.

Expression of a transgene, α S1CN/hGH, was confirmed by ELISA analysis for hGH in the milk of G0-founder, G2- and G3-transgenic rats. Levels of hGH secretion into the milk were all at the same mg/ml level, indicating that once the cryopreserved transgene was revived in individuals, the level of expression of the transgene could be kept stable through generations as in the case of other genetic characteristics.

In conclusion, the 2-step freezing procedure, characterized by slow cooling to -30°C in 10% DMSO solution, for transgenic rat embryos is an effective method for the long term cryopreservation of transgenes.

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