Table 1. Follicular loss of canine ovary after cryopreservation and subsequent xenotransplantation at 4 weeks after transplantation

Breed and age	Exp. Group	Mean number of follicles (/0.64 mm^2)					
		Pri mordial	Early primary	Primary	Transi-tional	Pre antral	Antral
Mixed	Fresh	38.4	2.0	0.2	0	0	0
6M	Cryo*	11.6	1.3	0.3	0	0.1	0
	Tp**	0.8	0.2	0.1	0.1	0.1	0.1
Mixed	Fresh	8.2	1.0	2.4	1.7	1.2	0
5M	Cryo	15.5	1.4	1.5	0.7	0.4	0
	Tp	0	0	0	0	0	0
Toy	Fresh	15.3	2.1	3.0	2.0	2.0	0.1
Poodle	Cryo	47.5	2.6	2.9	1.6	1.4	0
11M	Tp	1.5	0.1	0.1	0.1	0.7	0
Miniature	Fresh	14.8	3.3	4.1	3.6	0.8	0
Dachshund	Cryo	13.4	2.3	2.4	0.9	0.4	0
4M	Tp	0.3	0.3	0.1	0	0	0

*: Crypreserved ovarian tissues. **: Transplanted ovarian tissues.

Figure legends

Fig. 1. Percentages of surviving follicles in cryopreserved and subsequently transplanted canine ovaries. The survival rates of follicles were calculated as number of follicles in cryopreserved ovarian tissues / number of follicles in fresh ovarian tissue samples x 100, and number of follicles in transplanted ovarian tissues / number of follicles in cryopreserved ovarian tissue samples x 100.

Fig. 2 Hematoxylin and eosin staining of ovarian tissues from a 5-month-old mixed breed. Canine ovaries were cryopreserved by vitrification. The fresh (A) and cryopreserved ovarian tissues (B) are morphologically equivalent. Panel C shows an ovarian tissue recovered from NOD-SCID mice 4 weeks after transplantation of the vitrified-warmed canine ovarian tissues into the bursa. Note the much deeper stain seen in the mouse ovarian tissue (lower) compared to the canine ovary (upper). Canine ovarian grafts (pale stain) successfully adhered to the mouse ovary. Many follicles are seen in canine fresh (A) and cryopreserved (B) ovarian tissues but not in transplanted tissue (C). Note the much deeper stain seen in the mouse ovarian tissue compared with the canine ovary (C). white bar=100 μm.

短報

凍結融解イヌ卵巣の異種移植後の卵胞減少について

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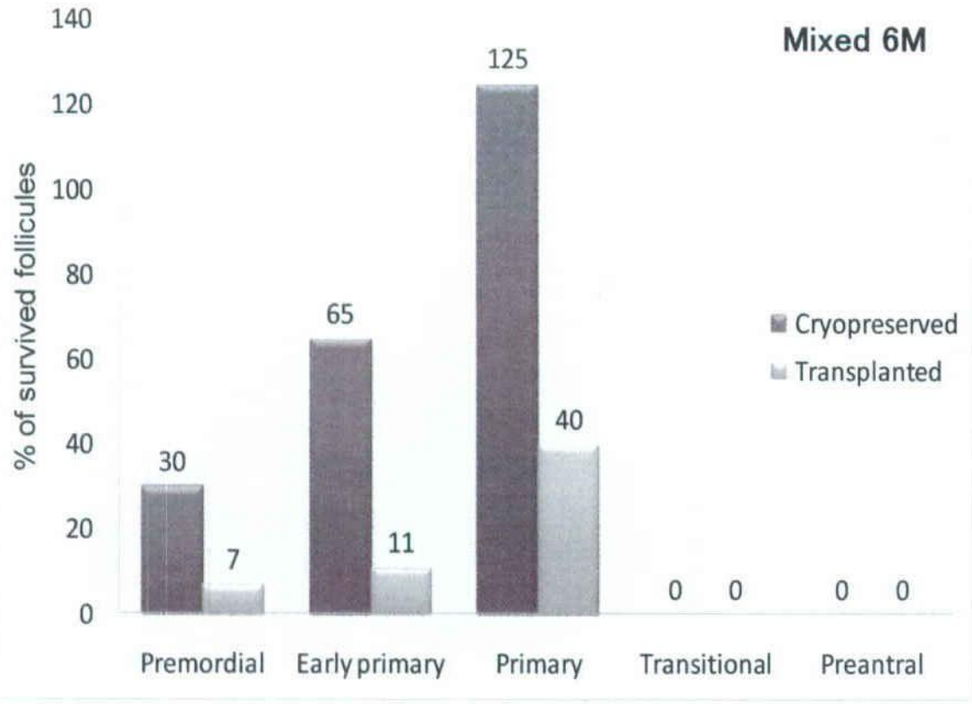
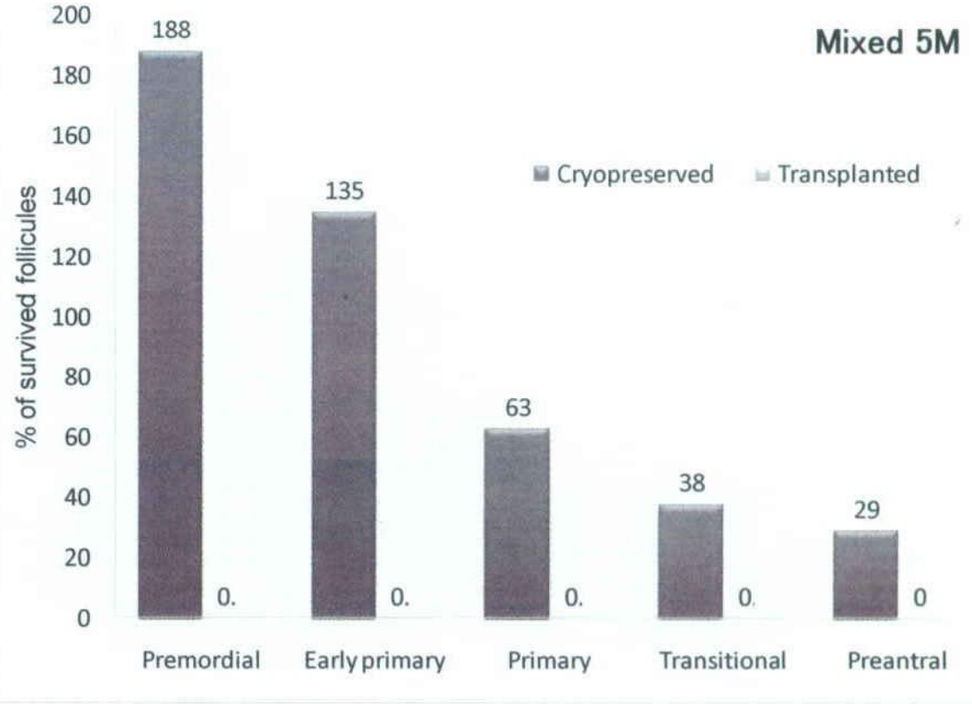
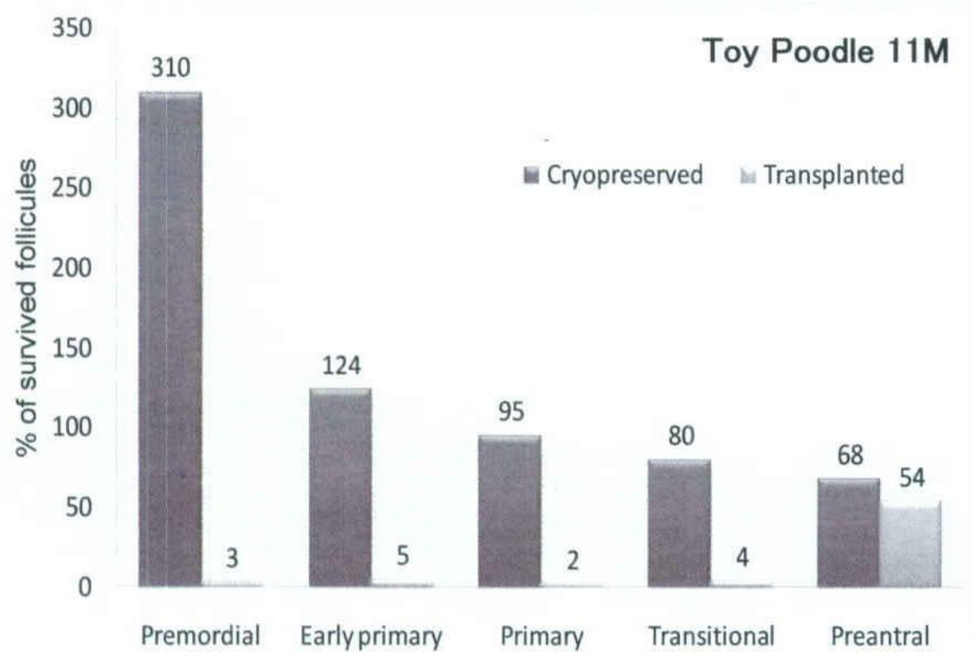
凍結保存および異種移植がイヌ卵巣の卵胞数減少におよぼす影響について検討した。イヌ卵巣をガラス化保存した後にNOD-SCIDマウスの卵巣のう内に移植し、移植後4週目に回収して組織学的に観察した。凍結保存後に、原始卵胞および初期一次卵胞は、それぞれ、30%以上および65%が残存していた。しかし、移植後4週目では、原始卵胞は0−7%、初期一次卵胞は0−15%が残存しているのみであった。これらの成績は、イヌ卵巣の異種移植によって、多数の卵胞が失われることを示しており、移植後の卵胞を保護するための検討が必要であると考えられる。


Mixed 6M
% of survived follicules
140
120
100
80
60
40
20
0
30
7
65
11
125
40
0
0
0
0
Cryopreserved
Transplanted
Premordial
Early primary
Primary
Transitional
Preantral


Mixed 5M
% of survived follicules
200
180
160
140
120
100
80
60
40
20
0
188
0.
135
0.
63
0.
38
0.
29
0
Cryopreserved
Transplanted
Premordial
Early primary
Primary
Transitional
Preantral


Toy Poodle 11M
% of survived follicules
350
300
250
200
150
100
50
0
310
3
124
5
95
2
80
4
68
54
Cryopreserved
Transplanted
Premordial
Early primary
Primary
Transitional
Preantral

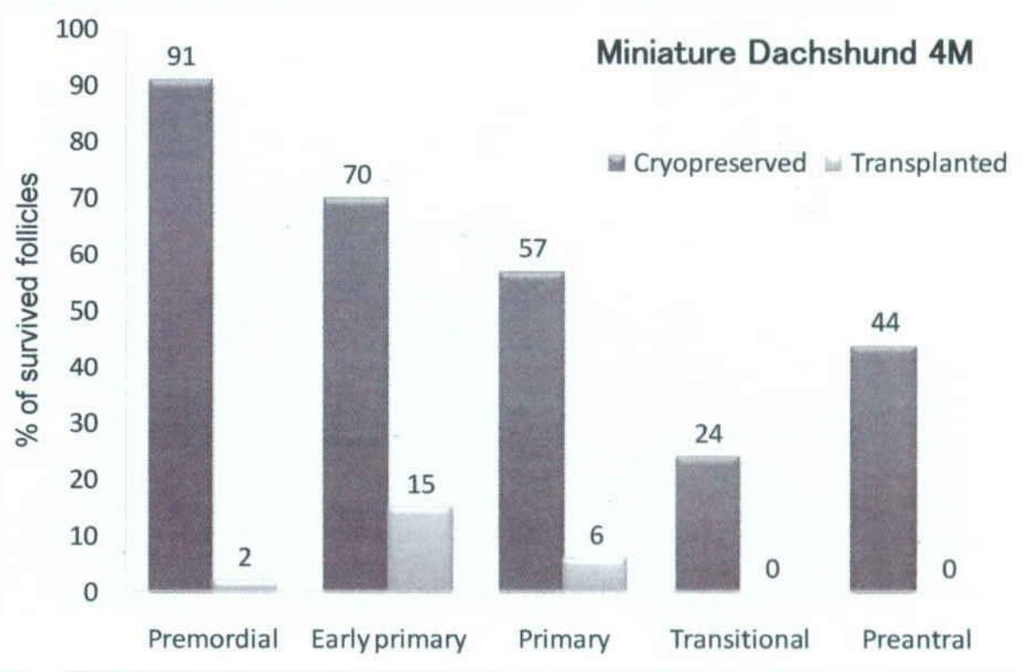

Miniature Dachshund 4M
% of survived follicles
100
90
80
70
60
50
40
30
20
10
0
91
2
70
15
57
6
24
0
44
0
Cryopreserved
Transplanted
Premordial
Early primary
Primary
Transitional
Preantral

Fig. 1

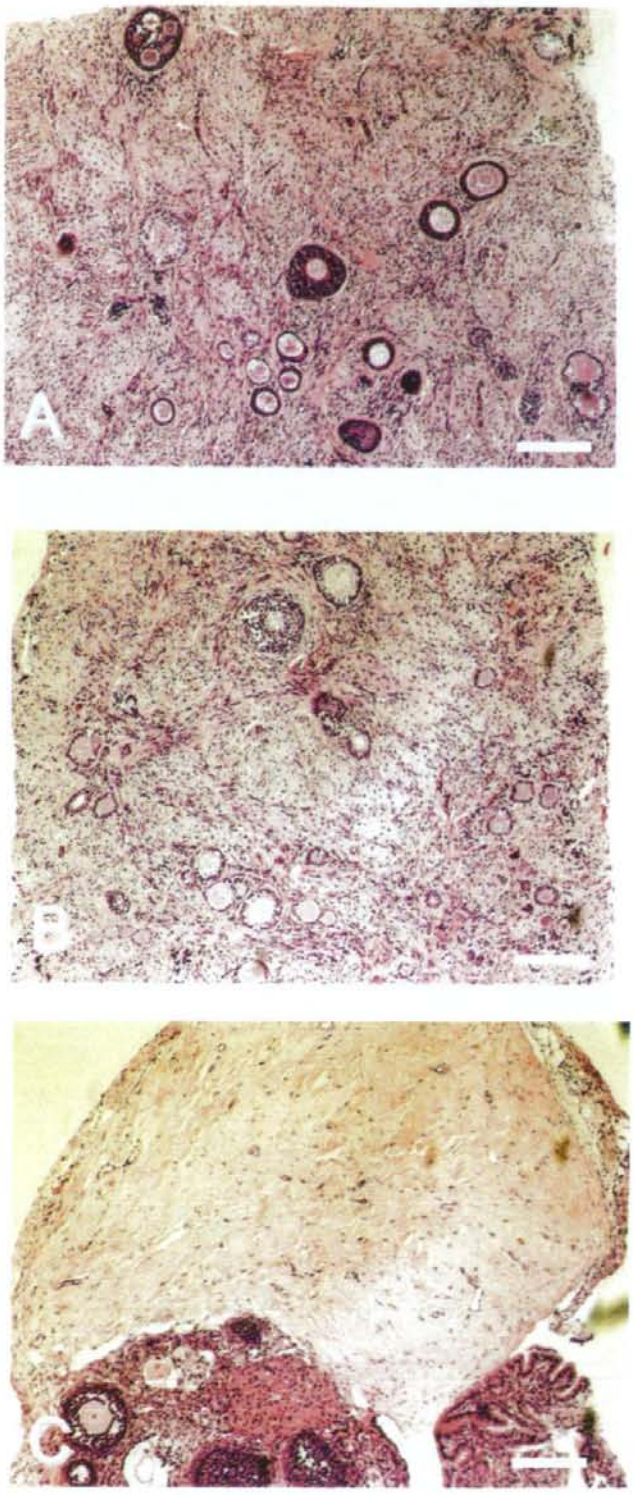


Fig. 2

Effect of Zona Incision by Piezo-micromanipulator (ZIP) on the *In Vitro* Fertilization in 21 Transgenic Mice Lines

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Special field: Reproduction

Note

Running head: ZIP FOR IVF OF TRANSGENIC MICE

Abstract:

Zona incision using a piezo-micromanipulator (ZIP) had been demonstrated to be effective for *in vitro* fertilization (IVF) using cryopreserved C57BL/6 spermatozoa. In this study, ZIP oocytes inseminated with frozen-thawed genetically modified C57BL/6J or FVB mice spermatozoa (21 lines) showed fertilization rates of 22–75% and live fetus rates of 8–49%. In 6 of the lines, the fertilization rate for oocytes compared with ZIP (42–75%) was significantly higher than for nontreated oocytes (0–50%). From the use of only 90 ZIP oocytes for IVF with ZIP, 5 breeding pairs were possible from cryopreserved genetically modified mice spermatozoa. Our results indicate that application of the ZIP technique is effective for IVF using cryopreserved genetically modified mouse spermatozoa.

Key words: frozen-thawed spermatozoa / IVF / ZIP

The number of labs creating and studying transgenic and knockout mice has risen steadily since the technique was developed in the late 1980s. Around 3,000 knockout strains have become available and the number is growing exponentially [8]. Cryopreservation of mouse spermatozoa has been widely applied to maintain transgenic and knockout lines to cut down on breeding space, time, and cost [8]. However, the fertility of cryopreserved spermatozoa from some inbred strains such as the C57BL/6 strain commonly used in transgenic and mutagenesis studies is extremely poor [4, 10, 14]. There are reports that methods of zona-pellucida dissection, partial zona-pellucida dissection (PZD) [9], partial zona-pellucida incision by piezo-micromanipulator (ZIP) [5], and laser-assisted zona drilling [3] are effective in increasing fertility rates. Especially, ZIP in combination with *in vitro* fertilization (IVF) using cryopreserved C57BL/6J transgenic spermatozoa has dramatically improved fertilization rates and subsequent embryonic development [5]. IVF with ZIP (ZIP/IVF) is also an effective assisted reproductive technique (ART) for reproduction of infertile transgenic mice with low-motility spermatozoa [6]. We reported this technique 7 years ago and here we report the results of the application of ZIP/IVF in the breeding and conservation of 21 lines of transgenic mice at our laboratory over the past 7 years.

The mice (C57BL/6J, FVB, and ICR) used to make the transgenic mice were purchased from Clea Japan (Tokyo, Japan). These transgenic mice did not contain modified reproductive tissue-specific genes and were able to mate naturally. The mice were housed in polycarbonate cages and maintained under a specific pathogen-free environment in light-controlled (lights on from 05:00 to 19:00) and air-conditioned (temperature, 24 ± 1°C; humidity, 50% ± 10%) rooms. The mice had free access to standard laboratory chow (CE-2, Clea Japan). The Institutional Animal Care and Use Committee (IACUC) of Chugai Pharmaceutical reviewed the protocols and confirmed that the animals used in this study were

cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by IACUC.

Mature females were induced to superovulate by intraperitoneal injections of 5 IU equine chorionic gonadotrophin (eCG; Serotrophin, Teikokuzoki Co., Tokyo, Japan) and, after 48 h, given injections of 5 IU human chorionic gonadotrophin (hCG; Puberogen, Sankyo Co., Tokyo, Japan). Freshly ovulated oocytes were collected from the oviducts 15–16 h after the hCG injection. The oocytes for ZIP/IVF were treated with 0.1% hyaluronidase (280 U/mg; H-3506, Sigma Chemical Co., St. Louis, MO) in TYH medium [15] to remove cumulus cells. As a control, cumulus-intact (nontreated) oocytes were used in conventional IVF [9].

Frozen-thawed spermatozoa, collected from the cauda epididymis of mature males of the same strain, were prepared using the method described by Nakagata et al [9]. The spermatozoa were dispersed from two tails of the epididymides into 100 μl of cryopreservation solution. The sperm suspension was then divided into 10 aliquots, and 10 μl of each aliquot was placed in a plastic straw (volume, 0.25 ml; Fujihira Industry Co., Ltd., Tokyo, Japan) and the straws heat-sealed. The straws were then cooled by placing them into the neck (gas layer) of a liquid nitrogen container (volume, 2 L: Iuchi Seieido Co., Ltd., Osaka, Japan) for 10 min, then plunged into liquid nitrogen and stored before thawing.

To thaw the sperm, the frozen plastic straws were immersed in a water bath for 15 min at 30°C. Approximately 2 μl of thawed sperm suspension was added to 400 μl of TYH medium. After incubation of the frozen-thawed spermatozoa for 30 min at 37°C (for ZIP/IVF [5] and conventional IVF [9]) under 5% CO_2 in air, the ZIP oocytes and freshly collected oocytes with cumulus cells were separately introduced into TYH medium containing frozen-thawed spermatozoa. Fertilization was defined by the number of embryos that had developed to the 2-cell stage 24 h after insemination. All zygotes were cultured in Whitten's medium [16]

containing 100 μM EDTA [1] up to the 2-cell stage in 5% CO_2 in air at 37°C and subsequently transferred into the oviduct of ICR recipients (CLEA Japan, Tokyo) on 0.5 days post-coitum (dpc) of pseudopregnancy as described previously [12]. The recipients were euthanized on 19.5 dpc to determine the number of implantation sites and of term fetuses.

The holding pipette was prepared from a glass capillary tube (G1, Narishige, Tokyo, Japan), heated and pulled using an automatic electromagnetic pipette puller (P-197, Sutter Instruments, Novato, Calif.). The end of the pipette (about 80 μm in diameter) was polished using a microforge (De Fonbrune, Beaudouim, France).

The ZIP pipette was prepared from a glass capillary tube (Sutter Instruments) using the pipette puller (Sutter Instruments) and had a blunt end [7]. The outer and inner diameters of the tip of the ZIP pipette were approximately 7 and 5 μm, respectively. A small volume (about 0.5 μl) of mercury was introduced into the ZIP pipette from its proximal end. The pipette was connected to a Fluorinert (F77, Sumitomo 3M Co., Tokyo, Japan)-filled syringe system of the piezo electric actuator (Model PMM 150 FU; Prime Tech, Ibaraki, Japan) attached to a micromanipulator (Leica, Wetzlar, Germany).

The ZIP procedure was essentially the same as described previously [5]. The zona pellucida was incised by the micropipette through the application of piezo pulses (controller setting: speed 2, intensity 2) while the pipette was moved along the surface of zona pellucida. The length of the incision made in the zona pellucida using ZIP was approximately 26 μm, representing about 1/12 of the perimeter of the zona pellucida of a mature mouse oocyte (approximately 314 μm) [5]. After IVF as described, all zygotes were cultured until the 2-cell stage in Whitten's medium [16] containing 100 μM EDTA [1] in 5% CO_2 in air at 37.0°C. Fertilization was defined by the number of embryos that had developed to the 2-cell stage 24 h after insemination.

Data presented in this study were analyzed statistically by the chi-square test and Tukey's test for nonparametric multiple comparisons. In all statistical tests, the difference was considered significant when P was <0.05.

The fertilization rates were higher using ZIP/IVF (42–75%) compared to conventional IVF in 6 of 7 lines: lines A–F had a C57BL/6J genetic background and line G had a FVB genetic background (Table 1). However, the embryos produced by conventional IVF from line C (i. e. the line that did not show a higher fertilization rate using ZIP/IVF) failed to produce any live born pups after embryo transfer. In line C there might be abnormally fertilized oocytes including, for example, parthenogenetic oocytes. However, the abnormal fertilization could not be confirmed because, in this study, an embryo was defined as fertilized at the 2-cell stage 24 h after insemination. Overall, 20–49% and 27–40% of the 2-cell embryos developed to term following ZIP/IVF and conventional IVF, respectively. Lines C, D, and F produced live fetuses following ZIP/IVF but not from conventional IVF.

Figure 1 shows the overall efficiency of mouse production after ZIP/IVF and conventional IVF using frozen-thawed spermatozoa. In 3 lines (B, E from C57BL/6J and G from FVB) of transgenic mice, the overall efficiency of ZIP/IVF was significantly greater than conventional IVF ($P<0.05$). The FVB/N mouse strain has a low sperm concentration but high fertility, which is maintained through the freezing process [17]. In this study, the overall efficiency using cryopreserved FVB transgenic spermatozoa was improved by using ZIP/IVF. The number of live born pups from 100 ZIP oocytes determined the overall efficiency. Figure 1 clearly indicates that, with cryopreserved C57BL/6J and FVB transgenic spermatozoa, ZIP/IVF is more effective for ART mouse production than conventional IVF.

As shown in Table 2, the fertilization rates of ZIP oocytes from 14 transgenic mice spermatozoa lines (H–U) were 22–74%. When ZIP zygotes at the 2-cell stage were

transferred into oviducts, 8–44%of transferred embryos that had been fertilized using the 14 lines spermatozoa developed to term. The average fertilization rate was 41% (1839/4474) and the percentage of live fetuses from embryos transfer was 27% (452/1689). The fertilization rates varied among lines as a result of variations in spermatozoa. Fertility dose not correlate with sperm concentration or with total motility and progressive fraction counts. Large variations were especially seen the C57BL/6J mice because of the variations of spermatozoa among the mice [17].

Cryopreservation of mouse spermatozoa has become a commonly used tool for preserving transgenic mice in many laboratories. The ZIP method, which we previously developed and reported [5], is suggested to be a useful technique for the exploitation of stored mouse spermatozoa. In addition to ZIP [5], PZD [9] and laser-assisted zona drilling [3] have been reported and have both advantages and disadvantages. With PZD, the technique is difficult because zona pellucida need to be dissected manually by needle and the PZD zygotes must be cultured *in vitro* up to the morula or blastocyst stage prior to embryo transfer because blastomeres often escape from the slit in the zona pellucida during the early stages of embryonic development and attach to the epithelial cells of the oviduct [5]. However, PZD requires only a microscope and needle so the cost is very low. In laser-assisted zona drilling, the laser equipment used for the drilling is very expensive and, in some cases, a sucrose solution (0.5 M) for shrinking the oocytes is needed to avoid laser-induced cytoplasmic damage, but 500 oocytes can be treated in 1 h without any damage [2]. ZIP, however, is easy to perform with little or no damage and new expensive equipment is unnecessary because many reproductive laboratories already have piezo micro-manipulators. The piezo electric actuator has been used mainly for injection of certain materials into the oocytes or embryos. However, manipulating 100 oocytes requires approximately 1 h with ZIP [4]. Both ZIP

oocytes and laser-assisted zona drilling oocytes can be transferred into oviducts [2, 3, 4]. Recently, there have been reports on a new method for IVF using frozen-thawed mouse spermatozoa. One is the addition of solutions, for example, adding methyl-beta-cyclodextrin to the preincubation medium [13] or adding monothioglycerol to the cryoprotective medium [11]. With each method, the collection of motile thawed spermatozoa from preincubation medium is necessary and previously cryopreserved spermatozoa cannot be used. However, the ZIP method is stable and easy. Moreover, ZIP allows the use of the vast number of previously cryopreserved spermatozoa at the many laboratories and gene resource banks.

In conclusion, all 21 lines of the transgenic mice freeze-thawed spermatozoa produced live fetuses using ZIP/IVF. As shown by the average fertilization rate and percentage of live fetuses (Table 2), only 90 ZIP oocytes resulted in 5 breeding pairs from cryopreserved spermatozoa for the maintenance of transgenic mouse colonies. Thus, it is easy to provide breeding pair of mice from gene resource stock using this method, indicating that ZIP/IVF is a useful tool for ART. The most important advantage of ZIP/IVF is the production of genetically modified mouse strains and the maintenance of transgenic mouse colonies.

Figure legend

Fig. 1: The overall efficiency of mouse production by means of ZIP/IVF and conventional IVF. Efficiency rates were 8.6–25.2% using ZIP/IVF. In lines B, E, and G, the overall efficiency was 16.7%, 21.0%, and 15.0%, respectively, with ZIP/IVF, higher than values for conventional IVF (4.9, 4.5, and 4.9%, respectively). The overall efficiency of lines D and F using conventional IVF could not be calculated because of the small number or lack of oocytes fertilized and thus not transferred.

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Table 1. Effect of ZIP on the fertility of transgenic mouse freeze-thawed spermatozoa and subsequent embryo transfer

Line	Strain	Treatment	No. of fertilized / no. of inseminated oocytes (%)	No. of implantation sites / no. of embryos transferred (%)	No. of live fetuses / no. of embryos transferred (%)
A	C57BL/6J	ZIP	222/300 (74)*	123/205 (60)	70/205 (34)
		Non	5/20 (25)	3/5 (60)	2/5 (40)
B	C57BL/6J	ZIP	277/626 (44)*	172/277 (62)	104/277 (38)
		Non	15/85 (18)	7/15 (47)	4/15 (27)
C	C57BL/6J	ZIP	158/367 (43)	75/157 (48)*	31/157 (20)
		Non	15/30 (50)	0/15 (0)	0/15 (0)
D	C57BL/6J	ZIP	203/292 (70)*	120/202 (59)	58/202 (29)
		Non	1/34 (3)	Not done	Not done
E	C57BL/6J	ZIP	184/245 (75)*	93/184 (51)*	51/184 (28)
		Non	19/69 (28)	4/19 (21)	3/19 (16)
F	C57BL/6J	ZIP	184/440 (42)*	135/151 (89)	74/151 (49)
		Non	0/36 (0)	Not done	Not done
G	FVB	ZIP	131/279 (47)*	65/131 (50)	42/131 (32)
		Non	11/61 (18)	4/11 (36)	3/11 (27)

*$P<0.05$, compared to the control (non-treated).

Table 2. Effect of ZIP on the fertility of C57BL/6J transgenic mouse freeze-thawed spermatozoa and subsequent embryo transfer

Line	No. of fertilized / no. of inseminated oocytes (%)	No. of implantation sites / no. of embryos transferred (%)	No. of live fetuses / no. of embryos transferred (%)
H	81/148 (55)	32/81 (40)	20/81 (25)
I	77/138 (56)	31/77 (40)	14/77 (18)
J	38/133 (29)	5/38 (13)	3/38 (8)
K	95/200 (48)	34/95 (36)	20/95 (21)
L	85/200 (43)	40/85 (47)	16/85 (19)
M	174/517 (34)	80/174 (46)	53/174 (30)
N	102/460 (22)	55/102 (54)	27/102 (26)
O	256/750 (34)	140/256 (55)	77/256 (30)
P	183/434 (42)	135/168 (80)	74/168 (44)
Q	293/395 (74)	108/210 (51)	51/210 (24)
R	187/281 (67)	78/187 (42)	38/187 (20)
S	78/132 (59)	21/40 (53)	8/40 (20)
T	46/126 (39)	9/32 (28)	6/32 (19)
U	144/560 (26)	82/144 (57)	45/144 (31)
Total	1839/4474 (41)	850/1689 (50)	452/1689 (27)