Exp. Anim. 58(4), 415–419, 2009

—Note—

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

Yosuke KAWASE[1)], Takanori TACHIBE[1)], Toshio HANI[1)], Hiromi TATEISHI[1)], Kou-ichi JISHAGE[1)], and Hiroshi SUZUKI[2)]

[1)]*Chugai Research Institute for Medical Science, 1–135 Komakado, Gotemba, Shizuoka 412-8513 and* [2)]*Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2–13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

Abstract: Zona incision using a piezo-micromanipulator (ZIP) has 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 rates for oocytes compared with ZIP (42–75%) were significantly higher than that of nontreated oocytes (0–50%). Using only 90 oocytes for IVF with ZIP, 5 breeding pairs were produced 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 the maintenance of 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 which is strain commonly used in transgenic and mutagenesis studies is extremely poor [4, 10, 15]. 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 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, Inc. (Tokyo, Japan). These transgenic mice did not con-

(Received 21 January 2009 / Accepted 17 March 2009)

Address corresponding: H. Suzuki, Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, 2–13 Inada-cho, Obihiro, Hokkaido 080-8555, Japan

tain 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 an intraperitoneal injection of 5 IU equine chorionic gonadotrophin (eCG; Serotrophin, Teikokuzoki Co., Tokyo, Japan) followed 48 h later by an injection 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 [16] 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) which was then 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 [17] 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 pseudopregnant ICR recipients (CLEA Japan, Tokyo) on 0.5 days post-coitum (dpc) as described previously [12]. The recipients were euthanized at 19.5 dpc to determine the number of implantation sites and of term fetuses.

For the ZIP procedure, a holding pipette was prepared from a glass capillary tube (G1, Narishige, Tokyo, Japan), which was heated and pulled using an automatic electromagnetic pipette puller (P-197, Sutter Instruments, Novato, CA). 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 a 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 the Fluorinert (F77, Sumitomo 3M Co., Tokyo, Japan)-filled syringe system of the piezo electric actuator (Model PMM 150 FU; Prime Tech, Ibaraki, Japan) which was 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 [17] 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 statisti-

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).

cally 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 in ZIP/IVF (42–75%) than in 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, 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 an embryo was defined as fertilized at the 2-cell stage 24 h after insemination in this study. 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

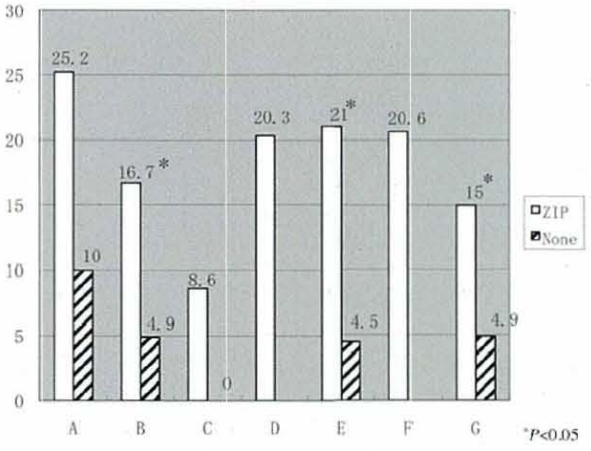

Fig. 1. The overall efficiency of mouse production by means of ZIP/IVF and conventional IVF. Efficiency rates were 8.6–25.2% using ZIP/IVF. In lines B, E, and G, the overall efficiency was 16.7, 21.0, and 15.0%, respectively, with ZIP/IVF, higher than values for conventional IVF (4.9, 4.5, and 4.9%, 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.

maintained through the freezing process [13]. 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 trans-

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)

genic 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 spermatozoa from the 14 lines developed to term. The average fertilization rate was 41% (1,839/4,474) and the percentage of live fetuses from embryo transfer was 27% (452/1,689). 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 among the C57BL/6J mice because of the variations of spermatozoa among the mice [13].

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], has been 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] techniques have also been reported; both have 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 its 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; however, 500 oocytes can be treated in 1 h without any damage [2]. ZIP, in contrast, 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 is mainly used for injection of certain materials into oocytes or embryos, and manipulating 100 oocytes for ZIP requires approximately 1 h [4]. Both ZIP oocytes and laser-assisted zona drilling oocytes can be transferred into oviducts [2, 3, 4]. Recently, there have been reports of new methods for IVF using frozen-thawed mouse spermatozoa involving the addition of solutions, for example, adding methyl-beta-cyclodextrin to the preincubation medium [14] or adding monothioglycerol to the cryoprotective medium [11]. With these methods, 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. The average fertilization rates and percentages of live fetuses are shown in Table 2. Five breeding pairs were produced from only 90 ZIP oocytes using spermatozoa cryopreserved for the maintenance of transgenic mouse colonies. Thus, it is easy to provide breeding pairs of mice from gene resource stocks using this method, indicating that ZIP/IVF is a useful tool for ART. ZIP/IVF offers significant advantages in the production of genetically modified mouse strains and the maintenance of transgenic mouse colonies.

Acknowledgment(s)

We thank Ms. S. Uchida and Ms. Y. Nakajima for technical assistance and Ms. F. Ford for proofreading the manuscript.

References

1. Abramczuk, J., Solter, D., and Koprowski, H. 1977. *Dev. Biol.* 61: 378–383.
2. Anzai, M., Nishiwaki, M., Yanagi, M., Nakashima, T., Kaneko, T., Taguchi, Y., Tokoro, M., Shin, S., Mitani, T., Kato, H., Matsumoto, K., Nakagata, N., and Iritani, A. 2006. *J. Reprod. Dev.* 52: 601–606.
3. Kaneko, T., Yanagi, M., Nakashima, T., and Nakagata, N. 2006. *Reprod. Med. Biol.* 5: 249–253.
4. Kawase, Y., Aoki, Y., Kamada, N., Jishage, K., and Suzuki, H. 2004. *Contemp. Topics* 43: 21–25.
5. Kawase, Y., Iwata, T., Ueda, O., Kamada, N., Tachibe, T., Aoki, Y., Jishage, K., and Suzuki, H. 2002. *Biol. Reprod.* 66: 381–385.
6. Kawase, Y., Kamada, N., and Suzuki, H. 2002. *J. Mamm. Ova. Res.* 19: 26–31.
7. Kimura, Y. and Yanagimachi, R. 1995. *Biol. Reprod.* 52: 709–720.
8. Knight, J. and Abbott, A. 2002. *Nature* 417: 785–786.
9. Nakagata, N., Okamoto, M., Ueda, O., and Suzuki, H. 1997. *Biol. Reprod.* 57: 1050–1055.
10. Nakagata, N. and Takeshima, T. 1993. *Jikken Dobutsu* 42: 317–320.
11. Ostermeier, G.C., Wiles, M.V., Farley, J.S., and Taft, R.A. 2008. *PLoS ONE* 3: e2792.
12. Suzuki, H., Ueda, O., Kamada, N., Jishage, K., Katoh, M., and Shino, M. 1994. *J. Mamm. Ova. Res.* 11: 49–53.
13. Sztein, J.M., Farley, J.S., and Mobraaten, L.E. 2000. *Biol. Reprod.* 63: 1774–1780.
14. Takeo, T., Hoshi, T., Kondo, Y., Toyodome, H., Arima, H., Yamamura, K., Irie, T., and Nakagata, N. 2008. *Biol. Reprod.* 78: 546–551.
15. Thornton, C.E., Brown, S.D.M., and Glenister, P.H. 1999. *Mamm. Genome* 10: 987–992.
16. Toyoda, Y., Yokoyama, M., and Hosi, T. 1971. *Jpn. J. Anim. Reprod.* 16: 147–151.
17. Whitten, W.K. 1971. *Adv. Biosci.* 6: 129–141.