

—Mini Review—

Transgenesis Via Intracytoplasmic Sperm Injection (ICSI) in RodentsMasumi Hirabayashi^{1,2*}, Megumi Kato¹ and Shinichi Hochi³¹National Institute for Physiological Sciences, Okazaki 444-8787, Japan²The Graduate University for Advanced Studies, Okazaki 444-8787, Japan³Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan

Abstract: Intracytoplasmic sperm injection (ICSI) has been successfully achieved in mice and rats using a piezo-driven injection pipette. More than 30% of ICSI oocytes are capable of developing to full-term when the isolated sperm heads are microinjected. The ICSI technique has been applied not only to rescue infertile male strains, but also to produce transgenic rodents. ICSI-mediated DNA transfer, which mixes sperm heads and exogenous DNA solution and co-injects them into ooplasm, was as effective as conventional pronuclear DNA microinjection. The production efficiency of transgenic founders by ICSI-mediated DNA transfer was comparable between mice and rats, while the optimal DNA concentration for 1-min exposure was lower in rats than in mice. The production efficiency was improved when the membrane structure of sperm heads was partially disrupted by detergent or ultrasonic treatment before exposure to the exogenous DNA solution. Exogenous DNAs with various chain lengths have been stably integrated into rodent genomes of various genetic backgrounds using this method. ICSI-mediated DNA transfer in which preparation of pronuclear-stage fertilized zygotes is not required would be alternative to conventional pronuclear DNA microinjection.

Key words: DNA transfer, ICSI-tg, Transgenic mouse, Transgenic rat

animals, and the efficiency of producing transgenic mice and rats using this technique is generally 1 to 5% of the total injected zygotes or 10% of the newborn offspring. Sperm-mediated DNA transfer into mouse genomes was first reported by Lavitrano *et al.* [1]. They demonstrated that oocytes fertilized *in vitro* with exogenous DNA-bound sperm cells could develop into transgenic mice, with a maximum efficiency of 30% of 250 newborn offspring. This approach to producing transgenic mice appeared to be more effective and less laborious, but numerous numbers of experiments by other laboratories failed to reproduce their results [2]. Perry *et al.* [3] reported that transgenic mice were successfully produced by intracytoplasmic sperm injection (ICSI) of mouse oocytes using sperm heads co-incubated with exogenous DNA solution. ICSI-mediated DNA transfer has been found to be a reproducible technique in mice [4, 5] and rats [6, 7]. In addition to transgenesis via pronuclear microinjection, *in vitro* fertilization, and microinsemination, there is also testis-mediated DNA transfer, including direct delivery of exogenous DNA into the testis (see the review by Sato [8]). In the present manuscript, rodent transgenesis via ICSI using sperm cells as vectors of exogenous DNA is reviewed.

Introduction

Transgenic technology in mammals is increasingly important in the design and implementation of biological and physiological studies. Pronuclear microinjection of exogenous DNA is the most convenient and reproducible technique for production of transgenic

ICSI Protocols in Mice and Rats

ICSI has been used as a research tool for studying fundamental aspects of gamete interaction during fertilization and has been routinely applied to clinical treatment of male infertility in humans. Since 1988, normal live offspring following ICSI have been reported in several mammalian species, including small rodents, large domestic animals, and primates [9]. The first successful ICSI in rodents was achieved by Kimura and Yanagimachi (1995) [10] using a piezo-driven injection

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pipette 5 μm in diameter to dissociate the sperm head from the tail and to drill the zona pellucida and oolemma of mouse oocytes. When the mouse sperm head was aspirated into the injection pipette and microinjected into an oocyte, approximately 60% of the surviving oocytes were capable of developing into newborn mice.

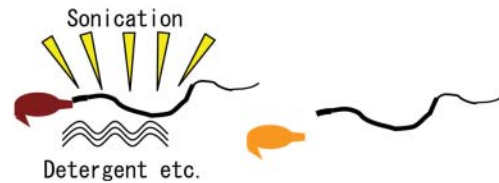
An earlier attempt at piezo-ICSI in rats [11] suggested that microinjection of larger rat sperm heads is extremely difficult even though the use of piezo-driven versus the conventional pipette (7 to 11 μm in diameter) significantly improved the post-injection survival of rat oocytes (71 vs. 24%). We reported in 2002 that technical improvement to minimize the amount of medium injected with sperm heads makes it possible to produce rat offspring from ICSI oocytes [12]. Rat sperm heads were isolated by ultrasonic treatment and cryopreserved until use. When the sperm heads were held on (rather than aspirated into) the tip of much finer injection pipettes (2 to 4 μm in diameter) and expelled into oocytes, more than 30% of the ICSI oocytes were capable of developing into newborn rats (from our latest results). The volume of polyvinylpyrrolidone (PVP) brought into the oocytes by the improved method is also obviously smaller than by the conventional method because the improved method only releases the sperm heads from the pipettes prior to zona drilling and they are then taken up again for ICSI. In cattle, reducing the PVP concentration in the sperm-surrounding medium has been reported to increase the rate of pronucleus formation following ICSI [13].

Transgenesis Via ICSI

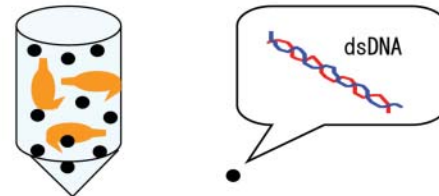
In 1999, Perry *et al.* [3] reported the first successful production of transgenic mice by ICSI using sperm heads co-incubated with exogenous EGFP DNA solution. ICSI-mediated DNA transfer provided similar efficiencies for production of transgenic rodents (3.8 to 4.5% of the transferred embryos for mice [3], 0.9 to 8.2% of the transferred embryos for rats [7]) when compared to the conventional pronuclear DNA microinjection. The standard protocol, as shown in Fig. 1, is as follows: (1) isolation of sperm heads and partial disruption of acrosomal/plasma membranes, (2) co-incubation of the sperm heads with exogenous DNA solution, (3) piezo-ICSI, followed by temporal *in vitro* culture and embryo transfer to foster mothers, and (4) identification of transgenes in the newborn offspring.

Disruption of the spermatozoal membrane would facilitate the binding of exogenous DNA molecules to sperm heads. Francolini *et al.* [14] reported that

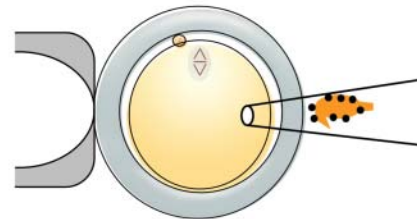
(1) Sperm head isolation / Membrane disruption



(2) Co-incubation with exogenous DNA



(3) ICSI using a piezo-micromanipulator



(4) Identification of transgenic offspring



Fig. 1. Protocol of ICSI-mediated DNA transfer in small rodents. (1) A sperm head is isolated from the midpiece and tail by piezo-pulses or ultrasonic treatment. One of several different procedures for partial disruption of spermatozoal membranes (*e.g.* Triton X-100, freeze-thawing, freeze-drying, or ultrasonic treatment) is routinely added. (2) Sperm heads are co-incubated with linearized dsDNA solution for a period of 1 min. (3) Denuded mature oocytes are microinseminated with the DNA-bound sperm heads using a piezo-manipulator, and the ICSI-derived embryos are transferred into recipients after a temporal culture for 1 to 3 days. (4) The resultant viable offspring are analyzed for the presence of the exogenous DNA by PCR, southern blotting, or another appropriate method.

exogenous DNA could reversibly bind to the subacrosomal segment of mouse spermatozoa. Different procedures have been used to induce

disruption; treatment with detergent (e.g. Triton X-100) [3], freeze-thawing [3, 15], and freeze-drying [3, 15] have been reported for mouse spermatozoa, while ultrasonic treatment combined with or without freeze-thawing [6, 7] has been applied for rat spermatozoa. Ultrasonic treatment has been considered to have an adverse effect on mouse spermatozoa and ICSI oocytes because Kuretake *et al.* [16] reported successful mouse ICSI at offspring rates of 24 to 26% in sonicated spermatozoa and 19% in sonicated and frozen-thawed spermatozoa, both of which were lower than the 50% offspring rate for whole spermatozoa. Ultrasonic treatment *per se* seemed not to be detrimental to the sperm chromosomes of the mouse [17]. In addition to our results for rat ICSI [6, 7, 12], rat offspring have been produced from ICSI using sonicated sperm heads [18].

Linearized exogenous DNA (dsDNA in common) was exposed to the isolated sperm heads at an ambient temperature for only 1 min. There was a considerable difference in the optimal DNA concentration for sperm treatment between mice and rats (in a model case that used EGFP DNA). The concentration of exogenous DNA solution used for exposure to mouse sperm heads was 5 $\mu\text{g/ml}$ [4] or 5 to 10 $\mu\text{g/ml}$ [3]. However, the optimal concentration of the EGFP DNA solution for exposure of rat sperm heads was 0.1 $\mu\text{g/ml}$ [7] or 0.5 $\mu\text{g/ml}$ [6], and ICSI after exposure to 5 $\mu\text{g/ml}$ DNA resulted in no development beyond the four-cell stage *in vitro*. Due to the larger surface area of the sperm heads in rats than mice, exposure to a higher concentration of exogenous DNA may lead to excess DNA association to the sperm heads and introduction into oocytes by ICSI, which may have a toxic effect on the development of the oocytes derived from ICSI-mediated DNA transfer.

We have previously reported that EGFP-transgenic rats produced by ICSI-mediated DNA transfer transmitted the transgenes to their G1 progeny in a Mendelian fashion [7], suggesting that the transgenes were stably integrated into the rat chromosomes, as already reported for mice [3, 15]. A wide range of exogenous DNA sizes (chain lengths of 3.0 to 208 kbp) is applicable to the production of transgenic rats by ICSI-mediated DNA transfer [7], as also reported for the production of transgenic mice (range: 11.9 to 170 kbp) [15]. Long cassette DNA of more than hundreds kbp, such as BAC, YAC, and MAC, is often used to avoid the positional effect on expression of exogenous DNA. Since the ICSI-mediated DNA transfer procedure includes the use of an injection pipette larger than the pronuclear DNA microinjection procedure does, longer DNA may be favourably introduced without mechanical

damage by the ICSI-mediated procedure. More attention should be paid to the composition of the buffers in which sperm heads are prepared and exposure to the exogenous DNA solution because chelating divalent cations by EDTA/EGTA is effective in stabilizing paternal chromosomes during early embryogenesis [4].

The efficiency of producing transgenic rodents by pronuclear DNA microinjection using inbred strains (e.g. C57BL/6 and DBA/2 mice and, LEW and F344 rats) is generally low when compared to those using outbred (e.g. Wistar and SD rats) or hybrid strains (e.g. B6D2F₁ and B6C3F₁ mice). This is probably due to the low offspring rate from the transferred zygotes. This is also true for the efficiency of producing transgenic rats by ICSI-mediated DNA transfer, as the offspring rates per transferred zygote in the F344 (23%) and LEW strains (29%) were lower than that in the SD strain (47%) and the efficiency of producing transgenic rats in the LEW strain (0.9% of the transferred embryos) was lower than that in the SD strain (8.2% of the transferred embryos) [7]. In addition, gametes from inbred strains may be sensitive to exposure to exogenous DNA and/or *in vitro* circumstances. F₁ hybrid female mice are often used as oocyte donors for ICSI-mediated DNA transfer [3–5, 15]. Although use of F₁ hybrid rats (Donryu \times LEW) results in moderate efficiency of transgenic rats (3.3% of the transferred embryos) [7], the combination of two rat strains for hybrid design may be among the factors affecting such efficiency.

To improve Production Efficiency

Low integration of exogenous DNA is still an obstacle to the widening of the transgenic technology. It was reported in 2003 that recombinase-A protein (RecA)-coated ssDNA was more likely to be integrated into goat and pig genomes after pronuclear DNA microinjection (6- and 10-fold higher than controls, respectively) [19]. In mice, the production efficiency of transgenic founders was improved only if the exogenous DNA was previously coated with the RecA and then subjected to ICSI-mediated transgenesis [5]. ICSI-mediated DNA transfer using RecA-coated EGFP ssDNA (5 to 40 $\mu\text{g/ml}$) produced transgenic mice at efficiencies of 3.6 to 11.1% of the transferred embryos, while the same protocol using non-coated ssDNA resulted in no transgenic offspring. However, we found that the RecA-coating of the exogenous EGFP and OAMB DNAs contributed very little to the production of transgenic rats by both pronuclear DNA microinjection and ICSI-

mediated DNA transfer [20].

Very recently, Suganuma *et al.* [21] indicated that hyperactive Tn5 mutant transposase assists the integration of exogenous DNA into both the inbred C57BL/6 mouse genome (4.3% of the transferred embryos) and hybrid B6D2F₁ mouse genome (13.4% of the transferred embryos, vs. 1.1% in the transposase-free control group) when the transposase-DNA complexes are co-injected with spermatozoa. Since the positive effect of Tn5 transposase-mediated mouse transgenesis was exerted not only from ICSI but also from round spermatid injection (ROSI), such a pretreatment for exogenous DNA may be applicable for transgenesis using azoospermic animals.

Conclusion

ICSI-mediated DNA transfer is as effective as conventional pronuclear DNA microinjection in terms of the efficiency of producing transgenic rodents (up to several percent of the total number of treated oocytes), the stability of transgenes, and the applicable size of the exogenous DNA (up to a few hundred kbp). A possible advantage of applying ICSI for DNA transfer may have appeared in species (*e.g.* *mastomys*, *Praomys coucha* [22]) or strains of unique genetic characteristics (*e.g.* t^{w5}/t^{w32} mutant mouse [23]) from which recovery of pronuclear-stage fertilized zygotes after superovulation is difficult. In fact, practical application of ICSI-mediated DNA transfer using a growth hormone gene-deficient (Dwarf) rat strain reduced the cost of producing one founder to one-tenth when compared with that from pronuclear DNA microinjection. Since labor and space for animal care can also be significantly reduced, ICSI-mediated DNA transfer would become a possible alternative to the conventional pronuclear DNA microinjection under these circumstances.

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