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Efficient generation of transgenic mice by direct intraovarian injection of plasmid DNA

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Abstract

We established a rapid procedure for obtaining transgenic mice by directly injecting an enhanced green fluorescent protein (EGFP)-expressing plasmid (pIRES-EGFP) into the ovaries of fertile mice. The frequency of transgenic mouse production was determined by pair-mating, and by polymerase chain reaction (PCR) and sequence analysis of DNA taken from the tails of the offspring. The mice that received the EGFP gene transmitted it to their offspring (F_1). Genetic and PCR analyses of F_1 progeny confirmed that the inserted EGFP was stably inherited. Of six female F_1 mice, all were able to pass the foreign DNA on to the next generation (F_2). *In situ* hybridization using paraffin-embedded sections of ovarian and testicular tissues from the F_1 and F_2 progeny showed that the introduced gene was expressed in the gonads of the animals. The chromosomal location of the injected DNA was determined by fluorescence *in situ* hybridization, and the frequency of multiple site versus single site insertions is 85.71% (18/21) analyzed by FISH. We anticipate great progress in murine genetic engineering using this technique.

Keywords: Direct intraovarian injection; Enhanced green fluorescent protein; Fluorescence *in situ* hybridization; Plasmid; Transgenic mice

Transgenic technology is an evolving biotechnological tool that permits the creation of genetically modified animals for scientific, pharmaceutical, and agricultural purposes. Pronuclear microinjection has become the most widely used method of germ line genetic transfer, despite the fact that it remains an intrinsically costly and laborious approach [1]. Other methods are also used to produce transgenic animals, including retroviral infection and the use of embryonic stem cells and spermatozoa as vectors; however, each of these methods is inefficient and costly. Thus, it is necessary to develop an improved method of transgenic animal production. Sperm-mediated gene transfer (SMGT) may be a practical alternative for producing transgenic animals. Lavitrano and colleagues [2–8] have shown that sperm cells can bind and internalize exogenous DNA and that they subsequently transmit that DNA to offspring.

We asked whether oocytes also have the ability to take up and transmit exogenous DNA. Direct injection of circular plasmid DNA with subsequent *in vivo* electroporation has been used for the efficient delivery of genes into ovarian cells, including mouse follicular cells and oocytes [9]. Our objective was to evaluate a novel transgenic technology involving direct intraovarian injection (DII) of plasmid DNA into mice. Our results demonstrate that DII can be used to successfully generate transgenic mice. The founder mice transmitted the enhanced green fluorescent protein (EGFP) gene to their offspring, and stable transmission to the F_2 generation was detected. Additionally, the reporter gene was not only integrated into the genome, but was

Abbreviations: EGFP, enhancing green fluorescent protein; TB, trypan blue; DII, direct intraovarian injection; ISH, *in situ* hybridization; FISH, fluorescence *in situ* hybridization; PCR, polymerase chain reaction.

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also transcribed, translated into a functional protein, and transferred to the next generation. Thus, oocytes are capable of efficiently taking up and integrating exogenous DNA *in vivo*. This method will be useful for studying the functions of novel genes and to create transgenic animal models for use in medicine and animal biotechnology.

Materials and methods

Animals. Five-week-old female Kunming mice were purchased from Peking Union Medical College, Tsinghua University. The mice were housed in a single room for more than 1 week after purchase under conditions of constant temperature (20–23 °C) and humidity (60–70%), with a 12-h light/dark cycle.

Intraovarian injection of the EGFP expression vector. Eleven adult female mice were used. After being narcotized, the ovaries of the recipient mice were surgically exposed, and a solution (2–5 μl of Tris/EDTA, pH 8.0) containing pIRES-EGFP (10 μg/μl, a vector in which EGFP expression is driven by the human cytomegalovirus promoter; Clontech, Mountain View, CA, USA) and 0.1% trypan blue (TB) was directly injected into the ovaries using a glass micropipette [9] (the establishment and optimization of the DII will be published by Jigui Wang in another paper). Mock injections were also performed using phosphate-buffered saline (PBS) plus 0.1% TB. After suturing and 2 days of rehabilitation, they were mated with male mice of the same line and were maintained in the same conditions.

In situ hybridization (ISH) of paraffin-embedded sections. The injected ovaries were fixed in 4% paraformaldehyde in PBS at 4 °C for 1 day before being embedded in paraffin, sectioned, and subjected to ISH [10]. Four hundred and fifty-four nucleotides of EGFP from pIRES-EGFP were digoxigenin (DIG)-labeled (Roche, Basel, Switzerland) for use as a probe by polymerase chain reaction (PCR) with the following primers: 5′-ACTAGTGGATCTTTGTTCTAACC-3′ (forward) and 5′-GGAATTC TAGATCCTTGGCTAAG-3′ (reverse) (TaKaRa, Tokyo, Japan). To better observe the morphology of the ovaries, the sections were counterstained for 5–10 min using nuclear fast red (Sigma, St. Louis, MO, USA). The tissues were microscopically photographed under bright-field illumination. To detect the expression of EGFP in the F_1 generation, ISH was performed with the gonads of the transgenic animals using the same procedure.

Superovulation and embryo culture. Two days after injection, the mice were superovulated [11]. The females were immediately placed with the males for mating, and they were examined the following morning (day 0) for the presence of vaginal plugs. On the morning of day 0, the females were killed by cervical dislocation, and their oviducts were removed. The ampullae of the oviducts were opened using dissecting needles, and the zygotes with their cumulus cells were placed in a drop of M2 medium (modified Krebs–Ringer solution with partial substitution of Hepes for the bicarbonate) [12] and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. The embryos were examined under fluorescence microscopy for GFP expression.

PCR analysis and sequencing in the F_1 and F_2 generations. The transgenic mice were identified by PCR using DNA from the tail [2]. Screening of the genomic DNA was performed as described previously [13]. The coding and gene regulatory regions were screened using the following set of primers (TaKaRa): 5′-GGAGAAGAACTTTTCACTGGAGTTGT CC-3′ (forward) and 5′-TTATTTGTATAGTTCATCCATGCC-3′ (reverse). All of the reactions were performed at least in triplicate. An animal was considered to be transgenic only when the sequence of the PCR-amplified products was identical to that of the EGFP gene.

Chromosome preparation and fluorescence in situ hybridization (FISH). For FISH analysis, 3- to 4-week-old transgenic mice were injected intraperitoneally with 2.5% colchicines. Preparation of the chromosomes and FISH were performed as described by Matsuda et al. [14]. The pIRES-EGFP was digested using XhoI and BamHI. The 1.1-kb EGFP probe was labeled with DIG using the Random Priming Reaction Kit (Roche) according to the manufacturer's protocol. The hybridization signal was detected using the Fluorescent Antibody Enhancer Set for Dig Detection (Roche) according to instructions.

Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under Leica filter set I3 (BP 450-490, LP 515; Leica, Solms, Germany), which allowed the simultaneous visualization of fluorescein-labeled hybrid (yellow) and chromosomal (red) DNA.

Results

Ovarian distribution of pIRES-EGFP

GFP was clearly observed in the follicular cells and oocytes as dark brown deposits (Fig. 1A and B); in fact, 24 h after DII, the plasmids were dispersed throughout the ovary and had been absorbed by various ovarian cell types, including interstitial cells, follicular cells, granular cells, and oocytes. Assuming that the exogenous DNA had successfully integrated into the nuclei of the oocytes, we expected it to be transmitted to developing embryos upon fertilization.

Production of transgenic embryos

We cultured the embryos obtained from mice subjected to *in vitro* DII and analyzed their expression of GFP at the blastula stage under fluorescence microscopy. GFP-positive cells were easily distinguished from GFP-negative cells in the embryos (Fig. 2). Embryos with at least one blastomere showing GFP expression were considered to be GFP positive. Of 23 embryos, 17 (73%) were non-mosaic because GFP expression was detected in all of the blastomeres.

Production of transgenic mice

Four treated mice died due to anesthetic overdose. The remaining seven mice were subjected to the following experiments. Combined, the seven treated mice produced 19 litters of F_1 offspring with an average litter size of nine pups, for a total of 171 F_1 mice (Table 1). The transgenic individuals were identified based on the amplification of a 454-bp product Fig. 3, which was shown to be EGFP by sequencing (data not shown). PCR analysis indicated that 64.91% of the F_1 offspring were transgenic. The number of transgenics produced in the first litter was lower than that in the second and third litters. However, there was no significant difference among the frequencies of transgenic mice for different litters of the same generation. Six F_1 mice gave birth to 121 F_2 mice, 81 of which were transgenic (66.94%). The male to female ratio of the transgenic progeny was approximately 1:1 for both the F_1 and F_2 generations, and the transgenic mice were generally healthy. We are now in the process of confirming the transmission of the EGFP gene to the F_3 generation. The reproductive capability was decreased after the fourth and fifth litters. Mice were generally infertile 1 year. Therefore, we were

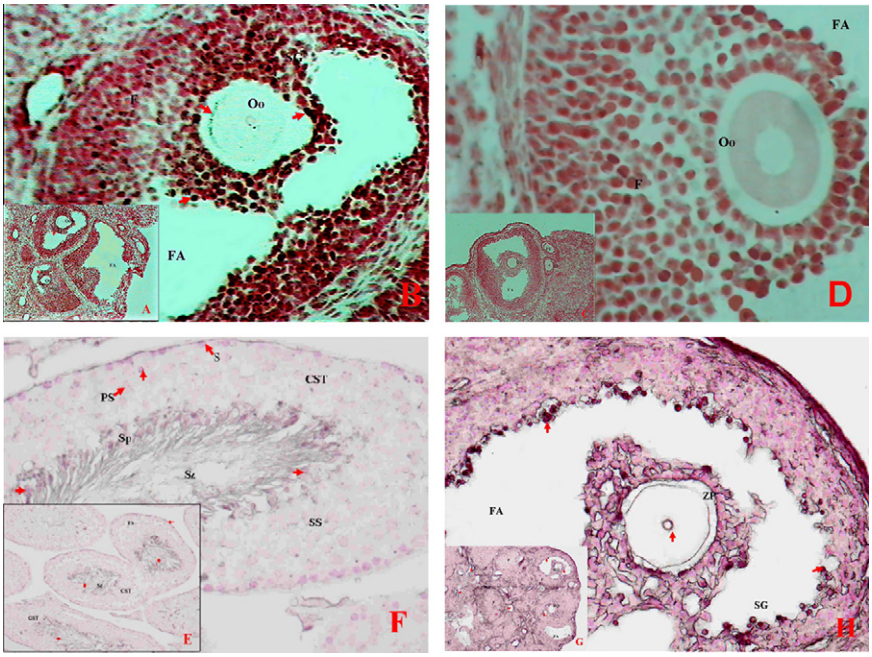

Fig. 1. *In situ* hybridization of mouse ovarian and testis tissues in founder mice and the F_1 generation. (A) Ovary injected with pIRES-EGFP (100×). (B) Ovary injected with pIRES-EGFP (250×). (C) Ovary mock-injected with PBS (−) (100×). (D) Ovary mock-injected with PBS (−) (250×). (E) GFP probe-hybridized section from a sexually mature female transgenic mouse (100×). (F) Enlargement of the section in G showing oocytes at different stages (250×). (G) GFP probe-hybridized section from a sexually mature transgenic male mouse (100×). (H) Enlargement of the section in C showing male germ cells at different stages (250×). SC, spermatocytes; ST, spermatids; S, spermatozoa; Oo, oocyte; F, follicular cells; FA, follicular antra; PrF, primordial follicles; PF, primary follicle. Some follicles and cells exhibited distinct staining for DIG activity (arrows).

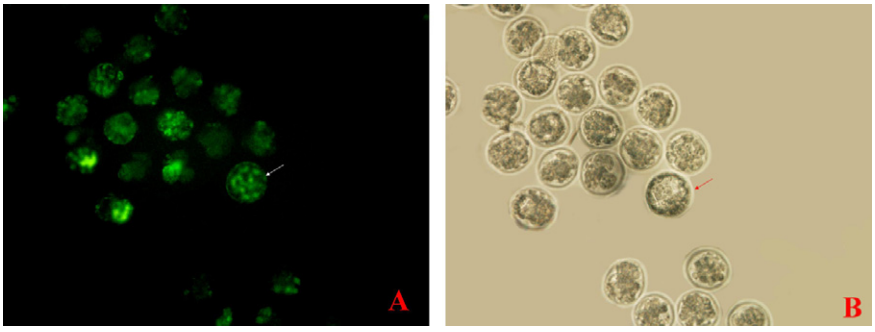

Fig. 2. Primary culture of embryos. (A) Fluorescence microscopic image. (B) Light microscopic image. Embryos harvested from mice that had received intraovarian injections were incubated in M2 medium to allow blastula formation.

Table 1
Analysis of the generation ratios of transgenic mice

F_0 lines			F_1 offspring			F_2 offspring			
No. treated	No. survival	No. litters	No. offspring	No. per litter	No. transgenic (%)	No. offspring	No. litters	No. per litter	No. transgenic (%)
11	7	19	171	9	111 (64.91)	121	12	10	81 (66.94)

unable to determine whether the production of transgenic mice would decline after 1 year.

Detection of GFP by ISH in the gonads of F_1 animals

Prior to ISH, several testicular and ovarian sections were stained with hemalum and eosin, and germ cells at different stages were identified (data not shown). Hybridization signals were subsequently detected in the germ line cells at all stages (Fig. 1E–H); in fact, EGFP was strongly expressed in both adult transgenic mouse ovaries and testes.

FISH analysis

To determine the chromosomal localization of the EGFP gene, we performed ISH on chromosome spreads

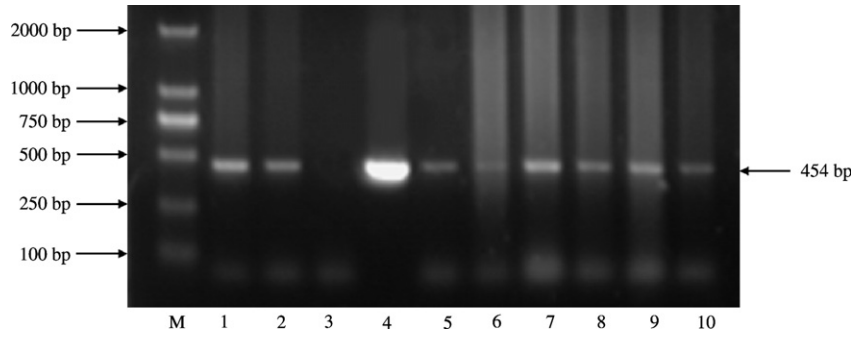

Fig. 3. Identification of transgenic mice by PCR using genomic DNA from the animals' tails. Lanes 1 and 2, detection of the transgene in the F_1 generation; lane 3, negative control (normal mouse); lane 4, positive control (pIRES-EGFP); lanes 5–10, detection of the transgene in the F_2 generation.

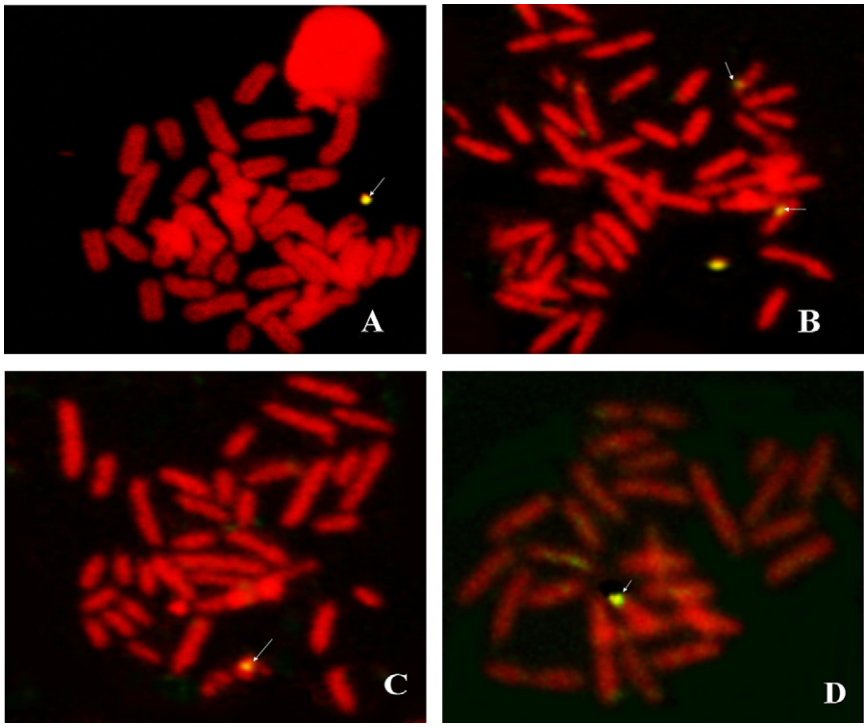

Fig. 4. Localization of EGFP DNA by FISH using metaphase chromosomes. (A) No. 113 transgenic mouse (B) No. 124 transgenic mouse (C) No. 153 transgenic mouse (D) No. 165 transgenic mouse. The chromosomes and one interphase nucleus from a transgenic mouse were stained (red) using propidium iodide. The digoxigenin-labeled EGFP probe (arrows) was visualized indirectly using avidin-FITC (yellow). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

prepared from 21 transgenic mice. FISH analysis using an EGFP probe confirmed that the transgenic arrays had integrated into the chromosomes of the mice. The transgenic locus is often located near the centromere in transgenic mice; however, our results indicate that the GFP locus array was located at variable positions and on different chromosomes in the transgenic animals (arrows, Fig. 4A–D). The frequency of multiple site versus single site insertions is 85.71% (18/21) analyzed by FISH.

Discussion

We confirmed that DII results in a high rate of transgenic mouse production, with efficient and functional integration of the transgene into the genome and stable, Mendelian-like transmission to the progeny.

DII is very simple and inexpensive. It does not require skilled technicians or substantial embryo manipulation. If the treated mice are housed in optimal conditions, there is no risk during the course of DII. All mice survived the procedure and produced two to three litters each. It should be noted that the first litters contained fewer pups than the second and third litters. This may indicate that the reproductive capability was initially affected by DII, but that it recovered within 1 month. We did not observe any other negative effects of DII treatment.

Of 171 F_1 offspring, 111 were transgenic (64.9%), suggesting that the EGFP sequences detected by PCR were

integrated into the host genome (Table 1). The transmission of EGFP was also possible; 66.94% of the mice in the F_2 generation were transgenic (Table 1). Sato [9] indicated that integration of transgenes into the host genome by transfection into ovarian cells via intraovarian injection and subsequent *in vivo* electroporation is unlikely. Sato's [9] findings are in contrast to our results in that gene expression was observed *in vitro* in cultured mouse embryos obtained by intraovarian injection of pIRES-EGFP, with subsequent mating and zygote collection (Fig. 2A). In addition, healthy transgenic mice were obtained by DII, which suggests that it has no negative effects on embryonic development *in vivo*. These discrepancies may have occurred because we did not use *in vivo* electroporation.

Although various mechanisms have been suggested, the sequence of events underlying the integration of foreign DNA into the host genome following microinjection is unknown [15,16]. DNA may be integrated when chromatin is freed from its nuclear context such as at fertilization, oocyte activation, nuclear decondensation, or at the formation of the pronucleus. Some studies have suggested that integration may result from the activation of endogenous enzymatic machinery, as during DNA repair [17].

Wilkie [18] reported that about 30% of mice produced through pronuclear microinjection were mosaic in the germ line. This would be expected if DNA replication occurs prior to the integration of the foreign DNA; however, transgenesis occurs only within a fraction of the embryonic cells. Our EGFP expression data (Fig. 2A) revealed green fluorescence in nearly all of the blastomeres in the blastula-stage embryos. This suggests that the integration of the DNA occurs prior to fertilization, but it does not explain what takes place after the injection of pIRES-EGFP.

We infer that foreign DNA enters an oocyte by penetrating the ovarian follicle and crossing the zona pellucida of the oocyte within the follicle. Furthermore, we believe that the successful integration of the DNA into the oocyte genome occurs at a very low frequency; however, the factors controlling the uptake of foreign DNA and the molecular basis of this phenomenon have yet to be clarified. The fact that integration occurs via an unknown mechanism does not prevent it from being a powerful laboratory tool. We have developed a simple and efficient protocol that can be used to generate stable transgenic mice by DII. Our data show a high rate of success with stable integration of the transgene into the chromosomes of the transgenic mice.

In addition, FISH is a powerful technique that can be used to visualize transgenic integration sites and to gain a better understanding of transgene behavior. Studies using FISH to characterize transgene integration have focused primarily on metaphase chromosomes because the number and position of the integration sites are more easily determined at this stage, although gene (and transgene) expression occurs mainly during interphase. However, the unpredictability of integration sites and a lack of stable transgene expression are still limitations in transgenic technology.

The transfer of DNA into the germ line, which is central to the production of a transgenic animal, requires the transport of the construct across cellular and nuclear membranes with insertion of the construct into the genome of a multipotent cell. Several approaches have been used to mediate this transport, although the original and most common method is DNA microinjection into a newly fertilized zygote.

In conclusion, intraovarian injection is a useful technique with a high success rate for producing transgenic mammals via efficient and functional integration of the transgene into the genome and stable transmission of the transgene to the progeny. It has the advantages of simplicity and cost-effectiveness in contrast to the more established methods of transgenesis such as pronuclear microinjection. More importantly, transgenic animal production can be accomplished efficiently using DII. We anticipate that DII will be very useful in the field of gene targeting.

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