生物化学与生物物理进展 *Progress in Biochemistry and Biophysics*, 2006, 33(7): 685~690
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A Novel Method to Transfer Gene *In vivo* System*

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Abstract A new and effective method to produce transgenic animals was established. Without a surgical incision, the recombinant plasmid containing green fluorescence protein (GFP) cDNA was repeatedly injected into male mouse testis at multi-sites. After few weeks of the final injection, the injected male was mated with normal oestrus female to produce transgenic mice. The presence of the GFP cDNA in F_1 transgenic individuals were detected by polymerase chain reaction and Southern blot hybridization, which showed that the transgenic rate of mouse F_1 offspring was 41%. The transferred gene was integrated into the host genome and could be transmitted to its offspring. When the positive F_1 individuals were mated with the wild type ICR mice, the F_2 individuals had a transgenic rate of 37%. The results indicate that the high efficiency of gene transfer and the limited number of manipulations make the method suitable for creating a large number of transgenic animals, especially, for producing domestic animals.

Key words testis injection, gene transfer, *in vivo*, transgenic animal

The technique of producing transgenic animals by microinjecting DNA into the pronucleus of the fertilized egg or through transferring embryonic stem cells is well established[1,2]. During the last decade, the use of spermatozoa as a vector for transfer genes in mammals and other vertebrates has been studied in different laboratories[3~6]. Consequently, a number of different approaches for making transgenic spermatozoa have been developed.

One of the alternative ways is using spermatozoa as vectors for transferring genes. Some animals have been produced by incubating DNA with sperm cells and then using this sperm cells for *in vitro* fertilization (thus called sperm-mediated gene transfer: SMGT)[7~10].

Another approach using spermatozoa as vectors was to transfer foreign DNA to the progeny *via* the testicular spermatozoa *in vivo* (testis-mediated gene transfer:TMGT). The method was based on the microinjection of DNA constructs mixed with mediums into the animal seminiferous tubules[11~15].

These two techniques have the advantage of being able to create transgenic progeny simply by mating with wild-type females, which avoids the possibility of interference or damage as a result of assisted fertilization or the manipulation of embryos.

Along this line of study, we recently study that DNA was injected directly into testis without incision and microinjection. The results showed that exogenous DNA could be transferred into egg *via* sperm at fertilizations and produced transgenic mice F_1 individuals. We also verified these transgenic offspring by PCR and Southern blotting, and confirmed that foreign gene can be integrated into transgenic F_1 animal genome and transmitted to the F_2 descendant.

1 Materials and methods

1.1 Animals

Adult ICR mice of six-week old were purchased from The Animal Center of Academy of Military Medical Science (Beijing, China).

1.2 Preparation of plasmid pCX-GFP

The plasmid pCX-GFP was kindly provided by Professor Chen Yong-Fu, China University of

*This work was supported by a grant from The National Basic Research Program of China (G2000016107)
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Received: February 15, 2006 Accepted: April 29, 2006

Agriculture, which has a construct with the CMV-IE enhancer, chicken β-actin promoter, green fluorescence protein (GFP) cDNA and rabbit β-globin poly-A signal. This plasmid was used in production of transgenic mice.

The plasmid DNA was prepared by the standard extraction method, followed by purification on cesium chloride gradients (Cold Spring Laboratory Manual). Before gene transfer, the plasmid was linearized by digestion with *Bam*HⅠ and *Sal* Ⅰ restriction enzymes. The 3.18 kb DNA fragment containing GFP cDNA was purified for the injection.

1.3 Exogenous DNA- lipofectin mixture

The transfection reagent N-[1-(2,3-dioleoyloxy) propyl] - N, N, N- teimethyl- ammoniummethylsulfate [DOTAP](Roche), was used to deliver the GFP gene. Preperation of the DNA-lipofection mixture was achieved by mixing solution A (dilute 20 μl (1 g/L) DOTAP up to 30 μl with HBS (which contained 20 mmol/L Hepes, 150 mmol/L NaCl, pH 7.4)) with solution B (dilute 10 μg DNA up to 30 μl with HBS), the mixture was incubated for 20 min at room temperature. The transfection-mixture was for one male ICR mouse testis. Each male mouse received 60 μl of DOTAP-DNA mixture each time.

1.4 Injection of the DNA-lipofectin mixture into testis of mice

ICR mice were aged 6～8 weeks. The mature males were anesthetized and the solution containing 10 g/60 L DOTAP-DNA was directly injected from the scrotum into testis with a $1\frac{1}{2}$# needle attached to a 0.5 ml disposable syringe. After the injection, the needle was slowly removed. The mice received three injections of the foreign gene and the interval of each injection was 2～3 days. Few weeks after the final testis injection, the male ICR mouse was mated with normal oestrus female to produce offspring.

1.5 Observation of the green fluorescence protein (GFP)

GFP expression was analyzed at different time points with fluorescence microscopy. After one day of mating, fertilized eggs were collected from the oviducts of female and placed into microdrops of SOM medium. The embryos were observed by a fluorescence microscope under a GFP excitation light (490 nm). In addition, GFP expression of 2-week-old F_2 mice was determined in whole-mount tissue from freshly excised muscles.

1.6 Identification of transgenic individuals by PCR amplification

1.6.1 Extraction of genomic DNA. Genomic DNA was isolated from 1-week-old offspring by dissolving the tissue (tail) in 0.5 ml of 10 mmol/L EDTA, 0.5% SDS and 50 μl of 10 g/L proteinase K and the mixture was incubated at 40℃ overnight with gentle shaking. After the undigested materials were removed by a brief centrifugation, the mixture was extracted with phenol /chloroform and ethanol precipitation, the DNA was dissolved in TE buffer (10 mmoL/L Tris-HCl, pH 7.5 and 1 mmol/L EDTA). RNA was degraded by incubating the samples with 1 U of RNase A for 30 min at 37℃.

1.6.2 PCR analysis and hybridization. The primers used were 5′ TTGAATTCGCCACCATGGTGAGE 3′ (anti-sense) and 5′ TTGAATTCTTACTTGTACAGC-TCGTCC 3′ (sense) for GFP cDNA. The 20 μl PCR system contained 1 μg genomic DNA, 200 μmol/L dNTPs, 2 mmol/L $MgCl_2$, 1.2 U of Taq DNA polymerase (Promega) and 10 pmol of each primer. The PCR started with a denaturing at 94℃ for 5 min, then running 30 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 50 s, and an elongating at 72℃ for 5 min. The anticipated size of the amplified fragment was 750 bp for GFP cDNA. The PCR products were electrophoresed on a 1% agarose gel and then transferred to a nylon membrane for hybridization with radiolabeled GFP cDNA fragment.

1.7 Detection of transgenic integration

Southern hybridization of genomic DNA was performed to detect transgenic integration. Genomic DNA 20 μg, isolated from F_1 transgenic individuals, were digested to completion with *Bam*HⅠ-*Sal* Ⅰ (Promega) restriction enzymes, resolved on 0.6% agarose gel, and transferred to nylon membrane. Nylon membrane was placed at 80℃ for 50 min in order to fix DNA and prehybridized at 65℃ for 1 h in a prehybridization solution, then hybridized with α-^{32}P dCTP- labeled GFP cDNA at 65℃ for 18 h (according to Cold Spring manual). The hybridization signal was visualized by autoradiography on X-ray film.

1.8 Exogenous gene transmission from the transgenic individual (F_1) to the successor generation (F_2)

In order to investigate the regulation of the foreign gene transmission from F_1 to F_2, the positive individuals were mated with the wild type ICR mice. The presence of GFP gene in F_2 transgenic individuals

was determined by PCR analysis. GFP expression of 2-week-old F_2 mice was determined in whole-mount tissue from freshly excised muscles by fluorescence microscopy.

2 Results and discussion

2.1 Production of transgenic mice by testis-injection gene transfer method

During the past few years, the testis-mediated gene transfer (TMGT) was performed using surgical incision to expose some tubules from the testis. Foreign DNA was injected into the animal seminiferous tubules using microinjection[16,17]. In our present study, the foreign DNA was injected repeatedly at multi-sites into male mouse testis without any incision. Not only can it decrease injure to the animal but also improve the integrated efficiency of foreign gene.

In our first experiment, the F_1 offspring birth rate was very low, which was due to the fact that female estrus was induced by gonadotropic hormone. So in the next experiment, spontaneous oestrus female mice were used. The survival rates of F_1 generation were improved significantly (data shown in Table 1).

The presence of GFP gene in F_1 transgenic individuals was detected by PCR analysis and about 41% of the surviving mice carried the GFP transgene (Figure 1). Representative results of the assay were presented in Figure 2 and Table 1.

Table 1 Percentage of inheritance of GFP gene in F_1 transgenic mice

Genetransfer male mice	Produced F_1 number	Survival number	Transgenic positive number	Rate of foreign gene positive/%
1	18	10	4	40
2	13	12	5	41
3	11	11	5	45
4	12	12	5	41
5	13	13	6	46
6	13	13	5	38
7	7	5	3	43
Total number	92	71	29	41

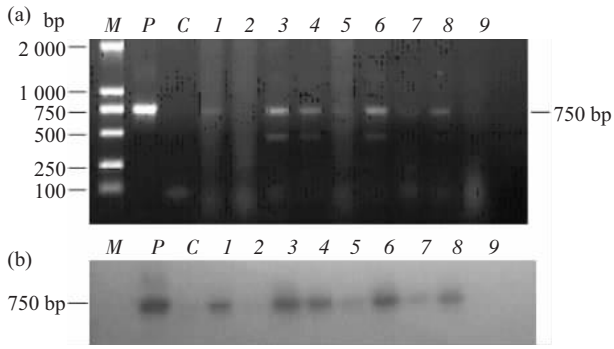

Fig. 1 PCR analysis for GFP cDNA in F_1 offspring

Genomic DNA was isolated from F_1 mice and the PCR products were confirmed by Southern blot hybridization. (a) Agarose gel photograph of the PCR products. (b) Photograph of Southern blot hybridization using ^{32}P-labeled GFP cDNA as probe. *M*: Molecular mass marker (DL2000); *P*: Positive control (GFP cDNA); *C*: Negative control; *1*～*9*: DNA samples from F_1 transgenic mice.

We injected a lipofectin- DNA mixture (the GFP reporter gene mixed with commercial cationic lipids) from the scrotum into the testis of anesthetized adult mice. After injection 2 days, 4 days, 6 days, 8 days, 10 days, 14 days, 16 days ..., the male mice were mated with wild female and the expression of GFP in F_1 pups were observed with fluorescent microscope. The data showed that after injection 2～6 days, no positive F_1 pup was observed. The time after 2～5 weeks post-injection was the suitable time for the males to be mated with wild-type females. It was demonstrated that the transferred gene could be transmitted to the offspring and be observed in the tail of the new born mice. The mechanism of TMGT has not been investigated extensively, Sato *et al.*[12] believed that TMGT was possible *in vivo* gene transfer system *via* epididymal sepermatozoa.

In the past few decades, some teams explored the TMGT, they thought a possible mechanism of TMGT: when foreign DNA was introduced directly into testis, it was rapidly transferred to epididymal spermatozoa, which will subsequently transfer the DNA to oocytes-through fertilization[15]. The foreign gene was inserted into sperm during the spermatogenic processes from spermatogonia to mature spermatozoa[16～18]. Coward *et al.*[19] found that foreign

gene was localized to the head and midpiece regions of sperm. Lu *et al*.[20] believed that the subcellular localization of the foreign gene showed dynamic shifts in spermatogenic cells at different stages during spermatogenesis. In mice one cycle of the spermatogenic processes is estimated about 2 ～5 weeks. So in our experiment, few weeks after the final injection, male mice was permitted to mate with spontaneous oestrus female mice.

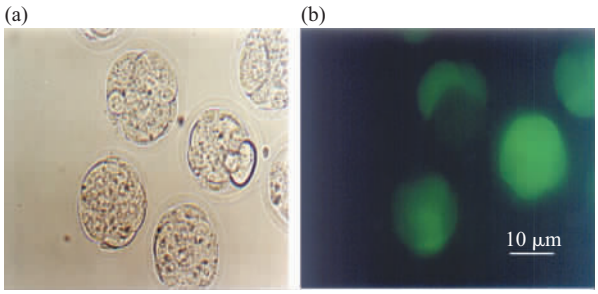

Fig. 2 Representative microscopic figures of the transgenic embryos subjected to the TMGT method

After mating 30 h, eggs were collected from the oviducts of female mated with male and placed into microdrops of SOM medium. Under a fluorescent microscope, FITC filter was suitable for the observation with B excitation (488 nm). (a) Visible light. (b) Exciting light (488 nm).

2.2 Integration, inheritance and expression of GFP gene

In previous study on TMGT offspring, there are two opinions about the existing forms of foreign gene. The foreign gene existed in extrachromosomal or integrated into F_1 genome[18,19].

To investigate whether or not the foreign gene (an intact GFP) was integrated into the genome of F_1 transgenic mice, Southern blot was performed. Genomic DNA was isolated from 4 PCR- positive F_1 transgenic individuals and then digested with *Bam*H Ⅰ and *Sal* Ⅰ restriction enzymes. The resulting DNA was hybridized to the radio-labeled GFP cDNA. As shown in Figure 3, a signal band of 3.2 kb was found. Since the transferred DNA contains a single restriction enzyme recognition site for *Bam*H Ⅰ and *Sal* Ⅰ, digestion of genomic DNA of F_1 transgenic with *Bam*H Ⅰ and *Sal* Ⅰ would yield a predicted 3.2 kb fragment. These results suggested that the transferred gene was stably transmitted to the F_1 generation and integrated into genome.

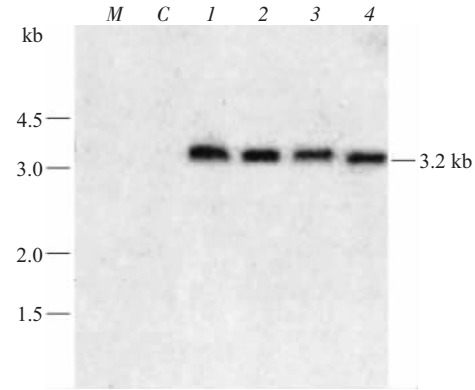

Fig. 3 Southern blot analysis of genomic DNA of F_1 transgenic mice

20 μg genomic DNA from PCR positive F_1 individuals were digested with *Bam*H Ⅰ and *Sal* Ⅰ, electrophoresed on 1% agarose gel, transferred to nylon membrane and then hybridized with ^{32}P-labeled GFP cDNA. *M*: Molecular mass marker; *C*: Genomic DNA isolated from PCR- negative F_1 transgenic individuals; *1* ～*4*: Genomic DNA isolated from PCR positive F_1 transgenic individuals.

To study the pattern of foreign gene transmission into the subsequent generation, F_1 transgenic positive individuals were mated with wild type mice. Approximately 37% of progeny were transgenic positive (Table 2). GFP expression of 2-week-old F_2 mice was determined in whole-mount tissue from freshly excised muscles (Figure 4). The EGFP gene had been integrated into genome and it can be inheritance to F_2 offspring. These numbers were substantially lower than the expected 50% of the progeny carrying the GFP gene, suggesting that some of the F_1 transgenic mice were chimera. Sato *et al*.[21] obtained the F_2 positive transgenic animals. However

Table 2 Percentage of inheritance of GFP transgene in F_2 offspring mice

Positive mouse number	Produced F_2 number	Survival number	Transgene positive number	Rate of foreign gene positive/%
1 (♂×2♀)	30	30	12	40
2 (♂×2♀)	26	26	10	37
3 (♀×♂)	15	15	6	33
4 (♀×♂)	13	13	4	31
Total number	84	84	31	37

other groups didn't found transgene positive in the F_2 generation[16, 22].

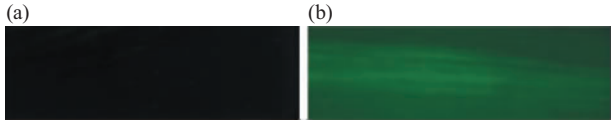

Fig. 4 GFP expression was determined in whole-mount tissue from freshly excised muscles of F_2 mice by flurescence microscopy

(a) Normal mouse muscle. (b) GFP gene positive of F_2 mouse muscle.

In summary, we have demonstrated that GFP gene can be introduced into ICR mice by testis injection method. This is the first report of producing transgenic mice by testis injection directly without a surgical incision. Not only can the exogenous gene be integrated in the F_1 transgenic mice, but also the foreign gene can be transmitted into F_2 generation. Using this method, we prepared transgenic rabbits, with a positive rate of 43% (not reported in this paper). So, the new method to transfer gene *in vivo* system can be used to produce different transgenic animals.

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体内系统转基因新方法 *

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摘要 建立了一种全新而高效的制备转基因动物新方法. 无需外科手术，将含有绿色荧光蛋白的重组质粒直接多次多点反复注射到雄性 ICR 小鼠的睾丸内. 几周后，上述雄性动物与自然发情的雌性交配，制备转基因动物. 经 PCR 检测、DNA 印迹，

实验结果表明：F_1 代小鼠转基因阳性率为 41%. 经 DNA 印迹证明：外源基因已经整合到子代转基因动物的基因组内并能遗传给后代. 将 F_1 代阳性鼠与正常 ICR 鼠交配，产生 F_2 代转基因鼠，F_2 代转基因阳性率 37%. 上述实验结果表明，建立的体内系统转基因方法简便、高效，适用于大规模制备转基因动物，特别适用于一些大型家畜.

关键词 睾丸注射，基因转移，体内，转基因动物
学科分类号 Q 812，Q953

*国家重点基础研究发展计划(973)资助项目(G2000016107).
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收稿日期：2006-02-15，接受日期：2006-04-29