

# Detection of Transgene in Progeny at Different Developmental Stages Following Testis-Mediated Gene Transfer

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**ABSTRACT** We recently reported that exogenous DNA injected into testis as a liposome complex can be transferred into the egg via sperm by natural mating and integrated in the genome (testis-mediated gene transfer: TMGT). Here, we studied the efficiency of each of the several liposomes in associating foreign DNA with sperm, the expression of an introduced gene in early embryos, and the presence of the DNA in fetuses and pups at different ages. The CMV/ $\beta$ -actin/EGFP fusion gene, encapsulated with different liposomes, was injected into rat testis, and spermatozoa in the cauda epididymis were obtained 1, 4, and 14 days after injection. We tested each of the 8 liposomes, and found that only 2, DMR1E-C and SuperFect, led to the detection of foreign DNA on all of the days examined, with relatively higher ratios of rats having positive sperm. By means of TMGT using either of those two liposomes, more than 80% of morula-stage embryos expressed EGFP, as observed by fluorescence microscopy. Then we detected introduced DNA in the progeny by PCR and Southern dot blot, and found that the ratio of animals carrying the foreign DNA decreased as they developed, and that only a part of postpartum progeny were foreign-DNA-positive with high incidence of mosaicism. These results suggest that, although, the success rate is still limited, foreign DNA could be integrated into the genome of the progeny by TMGT at least under specific experimental conditions, the efficiency of which depends largely on the characteristics of the liposome. The results also suggest that TMGT could be applicable to fetal gene therapy as well as to the generation of transgenic animals. *Mol. Reprod. Dev.* 60: 196–201, 2001. © 2001 Wiley-Liss, Inc.

**Key Words:** gene transfer; sperm vector; testis; liposome; EGFP

DNA into pronuclei of fertilized 1-cell stage eggs. One of the alternative ways is to use spermatozoa as vectors for gene transfer (Brackett et al., 1971). Transgenic mice and pigs have been produced by simply incubating naked DNA with sperm cells and then using these sperm cells for in vitro fertilization of oocytes (thus called sperm-mediated gene transfer: SMGT) (Arezzo, 1989; Lavitrano et al., 1989; Atkinson et al., 1991; Lauria and Gandolfi, 1993; Zani et al., 1995). Although, the capacity of the spermatozoa to capture foreign DNA during in vitro incubation has been intensively studied (Huguet and Esponda, 1999; Sciamanna et al., 1999), the use of SMGT to produce transgenic animals has had unstable, or even not reproducible, results (Brinster et al., 1989; Maione et al., 1998).

Another attempted approach to utilizing spermatozoa as vectors was to transfer foreign DNA to the progeny via the testicular spermatozoa in vivo (testis-mediated gene transfer: TMGT) (Anderson, 1984; Sato et al., 1994; Ogawa et al., 1995). By this approach, DNA was injected directly into the testis with the expectation that testicular spermatozoa at various maturation steps may be more susceptible to DNA transfection than fully matured epididymal spermatozoa (Kim et al., 1997). Along this line of study, we recently reported that liposome-encapsulated DNA injected into the testis could be transferred into the egg via sperm at fertilization, and expressed in and transmitted to the descendants (Chang et al., 1999a). We have also shown that, in our method of TMGT, exogenous DNA injected with liposomes is not integrated into the genome of the sperm and that the integration occurs after fertilization (Chang et al., 1999b). Thus, the validity of sperm as vectors for TMGT appears to depend on a method suitable for loading spermatozoa with foreign DNA.

## INTRODUCTION

Transgenic animals are recognized as a powerful tool for analyzing the biological function of cloned genes in vivo (Brinster et al., 1985; Hammer et al., 1985; Palmiter and Brinster, 1986; Biery et al., 1988). Most of these animals have been produced by microinjection of

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In recent years, cationic liposomes for transferring DNA into cells have been much improved, though large differences in efficiency have been reported among various types of liposomes (Ochiya et al., 1999). In the present study, we therefore first examined the efficiency of each of the several liposomes in associating foreign DNA with epididymal spermatozoa. Then, to further establish optimum conditions for TMGT and to find a reliable application of this method, we investigated the expression of the introduced gene in early embryos, as well as the existence of the DNA in fetuses and pups at different ages. For these investigations, we used the enhanced green fluorescence protein (EGFP) gene, a recently developed reporter gene (Cormack et al., 1996), inserted in the strong expression vector (Okabe et al., 1997).

## MATERIALS AND METHODS

### Preparation of CMV/ $\beta$ -Actin/EGFP and Cationic Liposomes

The plasmid (pCX-EGFP) of the EGFP gene that contains CMV enhancer, chicken  $\beta$ -actin promoter, and the rabbit  $\beta$ -globin 3' flanking sequence was generously donated by Dr. M. Okabe, Genome Information Research Center, Osaka University (Okabe et al., 1997). The plasmid DNA was purified by the Plasmid Purification System (Qiagen, Hilden, Germany). The plasmid was linearized using Sall, purified by phenol-chloroform extraction, and ethanol-precipitated twice.

The cationic liposomes used in this experiment were as follows. Lipofectin: *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA)/dioleoyl phosphatidylethanolamine (DOPE); Lipofect AMIN: 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/DOPE; CellFECTIN: *N,N,N,N*-tetramethyl-*N,N,N,N*-tetrapalmitylspermine/DOPE, and DMRIEC: 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE)/cholesterol were all obtained from Gibco BRL (Palo Alto, CA). Gene transfer: *N*-( $\alpha$ -trimethylalammonioacetyl)-didodecyl-D-glutamate chloride (TMAG)/DOPE was obtained from Wako (Osaka, Japan). SuperFect: *tris*(2-aminoethyl)-

amine (TAEA) core polymidoamine (PAMAM) dendrimers were obtained from Qiagen (Hilden, Germany). DOTAP: *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfaten (DOTAP) was obtained from Boehringer Mannheim (Mannheim, Germany). TransIT in Vivo (composition not announced) was obtained from Mirus (Madison, WI).

### Injection of Liposome-DNA Complexes Into the Testis

Male rats (Wistar-Imamichi strain) 7–8 weeks of age were purchased from Animal Reproduction Laboratory (Omiya, Japan). The liposome-DNA complexes for the TMGT method were prepared as follows. First, 10  $\mu$ g linearized plasmid DNA dissolved in HEPES buffered saline (HBS) was mixed with each liposome to make 100  $\mu$ l solution. The weight ratios of cationic liposomes to DNA were determined by the respective manufacturers' recommendations (Table 1). The mixed 100  $\mu$ l solution without visible aggregates was incubated at room temperature for 5–10 min. The entire solution was drawn into a sterilized Hamilton microsyringe with a 30 G needle, and injected into bilateral testes (100  $\mu$ l/side). Prior to being injected, the animals were lightly anesthetized by ether, the scrotum was disinfected, and the skin was cut to make the testes visible through the tunica muscularis. The tip of the needle was first placed near the rete testis through the rim of the testis, and then withdrawn slowly while liposome-DNA complexes were injected. The injection speed was  $\sim$ 100  $\mu$ l/50 sec. After injection, the skin was sutured.

### Observation of EGFP Expression in Early Embryos

Each male rat was mated with a proestrous female 4 days after an injection of liposome-DNA complex into the testis. Four days after mating, each females' uterus was flushed with saline to collect the embryos. The embryos, in a microdrop of saline, were observed by a fluorescence microscope (E800, Nikon) under a GFP excitation light (490 nm).

TABLE 1. Conditions of DNA-Liposome Complexes

Liposome	Applied condition		
	DNA:Lipid ratio <sup>a</sup>	DNA solution <sup>b</sup>	Lipid solution <sup>b</sup>
SuperFect	1 $\mu$ g: 5 $\mu$ l	10 $\mu$ g/50 $\mu$ l	150 $\mu$ g/50 $\mu$ l
DMRIE-C	1 $\mu$ g: 5 $\mu$ g	10 $\mu$ g/50 $\mu$ l	50 $\mu$ g/25 $\mu$ l+25 $\mu$ l HBS
LipofectAMIN	1 $\mu$ g: 5 $\mu$ g	10 $\mu$ g/50 $\mu$ l	50 $\mu$ g/25 $\mu$ l+25 $\mu$ l HBS
Cellfectin	1 $\mu$ g: 5 $\mu$ g	10 $\mu$ g/50 $\mu$ l	50 $\mu$ g/50 $\mu$ l
TransIT in Vivo	1 $\mu$ g: 1 $\mu$ l	10 $\mu$ g/10 $\mu$ l	17 $\mu$ g/10 $\mu$ l+80 $\mu$ l HBS
DOTAP	1 $\mu$ g: 5 $\mu$ g	10 $\mu$ g/40 $\mu$ l	60 $\mu$ g/60 $\mu$ l
Lipofectin	1 $\mu$ g: 5 $\mu$ g	10 $\mu$ g/50 $\mu$ l	50 $\mu$ g/50 $\mu$ l
Gene Transfer	1 $\mu$ g: 50 nmol	10 $\mu$ g	0.5 $\mu$ mol/100 $\mu$ l HBS

<sup>a</sup>The weight ratios of DNA to cationic liposomes were determined by the manufacturers' recommendations.

<sup>b</sup>DNA solution and Lipid solution were mixed and injected to each testis.

### PCR Analysis of the Sperm, Fetus, and Offspring

Detection of injected DNA in the spermatozoa of the cauda epididymis and the progeny was done by PCR analysis. DNA was extracted from the spermatozoa at 1, 4, and 14 days after the injection of the liposome DNA complexes, from whole fetus at day 14 post-coitus (p.c.), and from tails or fingers of pups at 1–7 days, 2–3 weeks, and 1 month old. The tissues were resolved in 500 µl of lysis buffer (30 mM Tris-Cl, 15 mM EDTA, 0.3% SDS) at 37°C overnight. The DNA was purified by phenol-chloroform extraction and then ethanol-precipitated.

The PCR primers designed to amplify a 548-bp fragment the promotor sequence of the pCX-EGFP construct were as follows: 5'-CTG CTA ACC ATG TTC ATG CC-3' (5' primer), 5'-CGT TGT GGC TGT TGT AGT TG-3' (3' primer). For a reaction mixture for use in PCR, 25 µl of reaction bufer containing 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin (Bio-Rad, Hercules, CA), 200 µM of each of dATP, dTTP, dGTP, and dCTP, 50 pM of each of two PCR primers and 0.1 µl of Ampli Taq Gold polymerase at 5 U/µl (Perkin-Elmer, Branchburg, NJ) were mixed with 0.25 µg of the extracted DNA. Respectively, 30 and 35 cycles of PCR amplification for the spermatozoa and for other tissues were performed with cycle times of 1 min at 94°C, 1 min at 60°C, and 1 min at 75°C. The reaction mixture was then analyzed on a 1.5% agarose gel. The gels were stained with ethidium bromide, and amplified DNA bands were visualized by ultraviolet transillumination.

### PCR and Southern Dot Blot Analyses of the Liver and Tail of Neonates

In the separate experiment, pCX-EGFP was encapsulated with SuperFect and injected into the testis of males, which were then mated with normal females. The neonates thus obtained were subjected to the analysis of foreign DNA in the liver and tail by PCR followed by Southern dot blot. DNA samples were isolated from the liver and tail of neonates of 1–7 days old. First, DNA was analyzed by PCR as mentioned

above. Second, the positive samples by PCR were further analyzed by Southern dot blot. Twenty micrograms of DNA from each sample was blotted onto nitrocellulose filters. An EGFP probe of 732 bp was cut out using *Eco* RI and purified according to Kato et al. (1999), and were labeled by [ $\alpha$ -<sup>32</sup>P] dCTP using a random priming labeling kit (Amersham, United Kingdom). Hybridization with the probe was performed in hybridization mix (5 × SSC, 2.5 × Denhardt's reagent, 5 mM EDTA, 0.1% SDS, 10% dextran sulfate, 100 mg/ml salmon sperm DNA) for overnight at 65°C. The filters were washed in washing mix (2 × SSC, 0.1% SDS) for 20 min and were exposed to the Imaging Plate. The signals were detected with FLA system (Fuji Photo Film, Japan).

## RESULTS

### Detection of Foreign DNA in Epididymal Spermatozoa

To evaluate the efficiency of liposomes for associating DNA with spermatozoa, we first examined the presence of foreign DNA by PCR in epididymal spermatozoa obtained 1, 4, and 14 days after intratesticular injection. As shown in Table 2, foreign DNA was already detected 1 day after the injection when the DNA was mixed with 6 out of 8 liposomes tested, though the ratio of animals exhibiting positive signals varied according to the liposome used. Similarly, 4 days after injection, foreign DNA injected with most of the liposomes was still detected in the sperm. On day 14, however, the DNA was present only when injected with DMRIE-C, SuperFect or Lipofectin. Without any liposome, foreign DNA was not detected in epididymal spermatozoa at any of the days examined. Thus, by using DMRIE-C and SuperFect, the DNA was detected at any of the days, with relatively higher ratios of animals having positive sperm. We therefore used DMRIE-C and SuperFect in the following experiments.

### EGFP Expression in Early Embryos

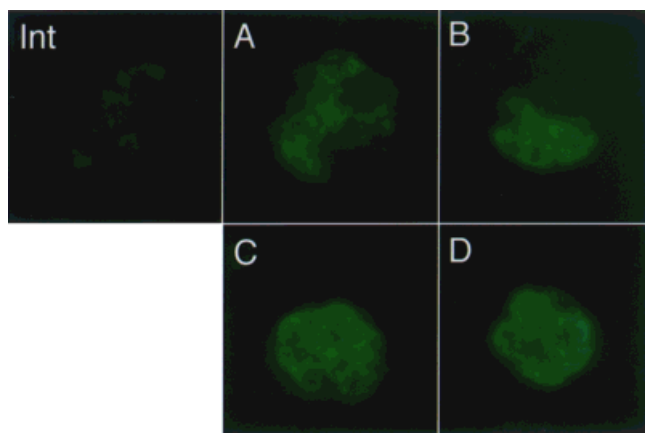
Four days after the injection of a liposome-DNA complex into the testis, each male was mated with a normal female. Then, EGFP expression in embryos of

TABLE 2. Detection of Foreign DNA in Epididymal Spermatozoa

Liposome	1 day <sup>a</sup> +/n <sup>b</sup>	4 days +/n	14 days +/n
DMRIE-C	2/2	3/3	1/3
SuperFect	2/2	2/3	3/3
LipofectAMIN	2/3	0/3	0/3
TransIT in Vivo	1/4	2/4	0/3
Cellfectin	1/3	1/3	0/3
DOTAP	1/4	1/4	0/3
Lipofectin	0/4	1/5	1/5
Gene Transfer	0/1	1/2	0/2
No liposome	0/2	0/2	0/1

<sup>a</sup>Days after intratesticular injection of DNA.

<sup>b</sup>Number of rats having PCR-positive spermatozoa/Number of rats examined.



**Fig. 1.** Representative microscopic figures of the morula-stage embryos subjected to the TMGT method. Each male rat was mated with a proestrous female rat 4 days after intratesticular injection of pCX-EGFP DNA. At day 4 Post-coitus (p.c.), the uterus was flushed to collect embryos. The embryos in a microdrop of saline were observed by a fluorescence microscope. Fluorescent images were taken under an EGFP excitation light (490 nm). Int, Intact embryo. (A, B) Embryos with mosaic fluorescent obtained by TMGT using DMRIE-C (A) and SuperFect (B). (C, D) Embryos with uniform fluorescent obtained by TMGT using DMRIE-C (C) and SuperFect (D).

day 4 p.c. was examined under a fluorescence microscope. As shown in Figure 1, morula-stage embryos with EGFP signal were observed. The ratios of EGFP-positive embryos to the total number of embryos examined were more than 80% when either SuperFect or DMRIE-C was used (Table 3). With either of them, however, some of the embryos showed mosaic expression. The ratios of mosaic embryos to total EGFP-positive ones were 38% (DMRIE-C) and 16% (SuperFect).

#### Detection of Foreign DNA in Fetuses and Offspring

Foreign DNA in fetuses of day 14 p.c., and offspring of 1–7 days, 2–3 weeks, and 1 month old were examined by PCR. As shown in Figure 2, the PCR product, if any, was obtained as a clear single band at the predicted size in any of the experiments. Table 3 summarizes the

results of the PCR analysis. When SuperFect was used, more than 20% of fetuses of day 14 p.c. were shown to carry foreign DNA. After birth, at any of the ages examined, around 5% of the offspring still had the foreign DNA. On the other hand, in the case of DMRIE-C, only 2.4% of the fetuses showed a positive signal, and the signal disappeared after offspring reached 2–3 weeks of age.

In the separate experiment, DNA extracted from the liver and tail of neonates was analyzed by both PCR and Southern dot blot analyses. From the tail, foreign DNA was detected in 5 of the 50 neonates examined by PCR in this experiment (Table 4). The liver of one out of these 5 animals was shown to carry the DNA by PCR. Foreign DNA was detected in the liver and tail of only this particular animal by Southern dot blot analysis (Table 4, Fig. 3).

#### DISCUSSION

In the present study, we found that foreign DNA was already detected in the sperm of the cauda epididymis the day after intratesticular injection, when the DNA was mixed with most of the cationic liposomes tested. Since it takes more than 4 days for the sperm to descend from the caput to the cauda epididymis (Bedford, 1978), the present observation suggests that the DNA injected into the testis is carried to the epididymis by the rete testis fluid and then transferred through the epididymis unaccompanied by the sperm. Yet cationic liposomes seem mandatory for the processes, because foreign DNA was not detected without liposomes. The liposomes may facilitate the transport of DNA from the interstitial space to the lumen of the seminiferous tubules and/or the stability of DNA, and finally the association of DNA with spermatozoa in the cauda epididymis.

The foreign DNA remained in epididymal spermatozoa 4 days after intratesticular injection, but disappeared 14 days after injection in most cases. The DNA was still present there, however, when DMRIE-C, SuperFect or Lipofection had been injected. Especially, DMRIE-C and SuperFect appear to be more efficient than other liposomes in terms of associating DNA with sperm: when these liposomes were used, the ratio of

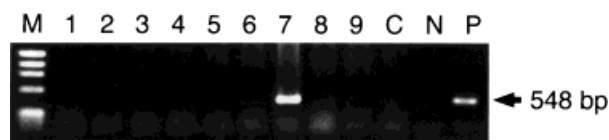
**TABLE 3. EGFP Expression or DNA Detection in the Progeny**

Ages	DMRIE-C		SuperFect	
	+/ <sup>a</sup> n <sup>a</sup>	(%)	+/ <sup>a</sup> n <sup>a</sup>	(%)
Day 4 p.c. <sup>b</sup>	18/21	(85.7)	19/23	(82.6)
Day 14 p.c. <sup>c</sup>	1/41	(2.4)	10/44	(22.7)
1–7 day old <sup>c</sup>	3/54	(5.6)	2/38	(5.3)
2–3 week old <sup>c</sup>	0/43	(0)	4/74	(5.4)
1 month old <sup>c</sup>	0/20	(0)	1/25	(4.0)

<sup>a</sup>Number of positive samples/number of samples examined.

<sup>b</sup>EGFP expression by the fluorescence observation.

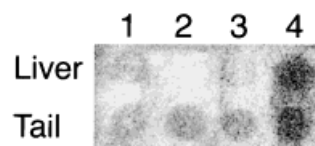
<sup>c</sup>Detection of pCX-EGFP DNA by PCR analysis.



**Fig. 2.** Representative PCR analysis of genomic DNA from littermate of 2-week-old pup littermates (Lanes 1–9) subjected to TMGT using SuperFect. Note the presence of amplified products of 548 bp derived from the pCX-EGFP DNA in lane 7. M, marker ( $\phi$  X174/*Hae*III digest); C, Nontransgenic control DNA; N, No DNA; P, Positive control of pCX-EGFP DNA.

animals having foreign-DNA-positive sperm was always relatively high. The ability of sperm cells to take up foreign DNA, which is at least partially mediated by specific DNA-binding protein on the sperm surface, has been well documented (Spadafora, 1998). But exactly how the liposomes are involved in this process remains to be fully elucidated. Unlike other liposomes, DMRIE-C and SuperFect do not contain DOPE, and are multilamellar (Ochiya et al., 1999) and dendrimer in shape, respectively. These characteristics in composition and/or structure might be favorable for associating foreign DNA with sperm.

Four days after normal females were mated with males injected with a liposome-DNA complex into the testis, embryos were collected and EGFP expression was examined. More than 80% of the embryos were found to express EGFP by TMGT when either DMRIE-C or SuperFect had been injected, though some of the embryos (16–38%) exhibited mosaic expression. Kato et al. (1999) reported that ~40% of morula-stage embryos that had been subjected to pronuclear microinjection of the same gene construct used in the present study were EGFP-positive, among which 62% showed mosaic fluorescence. Thus, the efficiency in transferring foreign DNA into the egg is much higher by the TMGT method than by the pronuclear microinjection method. The reason for this difference may be that foreign DNA is introduced into the egg under much more physiological condition by the TMGT method, using the sperm as a vector, than in the case with the microinjection method. In addition, the difference in the timing of the introduction of foreign DNA into the egg between the TMGT method (at the time of fertilization) and the microinjection method (after formation of the pronucleus) could account for the difference in the ratio of mosaic embryos. The high and uniform expression of transgenes in early embryos following TMGT suggests that TMGT could be applic-



**Fig. 3.** Southern dot blot analysis of genomic DNA from the liver (upper) and tail (lower) of neonates having PCR-positive tail. Note the positive signal in lane 4.

able to fetal gene therapy for the rescue of embryonal dysfunction and early organ damage as a result of inherited genetic diseases or gene targeting.

At day 14 p.c., the ratio of fetuses having foreign DNA, as detected by PCR, dramatically decreased from the ratio at the earlier stages, indicating that most of the foreign DNA could be excluded in the course of embryonal development without integration into the innate chromosomes. Expression of EGFP in most of the early embryos might result from transient transcription from the DNA of extrachromosomal form. As well, transient expression of foreign genes has been commonly observed in liposome-mediated DNA transfection both in vitro (Bebok et al., 1996; Boulo et al., 1996; Carballada et al., 2000) and in vivo (Relloso and Esponda, 2000). Despite the precipitous decrease in PCR-positive fetuses in the case of DMRIE-C, in the case of SuperFect a considerable percentage of fetuses still carried foreign DNA. Since percentages of EGFP-positive embryos were not different between the two liposomes, it is difficult to interpret the large difference in PCR-positive fetuses on day 14 p.c. There might be difference in the amount and/or status of foreign DNA molecules in the cells between the two cases, which were not reflected in EGFP expression on day 4 p.c.

In the first week after birth, about 5% of neonates were shown to carry foreign DNA in the cases of DMRIE-C and SuperFect. Thereafter, in the case of SuperFect, but not in the case of DMRIE-C, the DNA was still detected in the progeny during the whole experimental period, up to one month of age. We also observed in the separate experiment that foreign DNA was detected by PCR in the tail of 10% of neonates when it was injected into paternal testis with SuperFect. It should be noted, however, that the gene was detected in the liver of only one fifth of the neonates with PCR-positive tail. In this particular animal, foreign DNA could be detected in both the tail and liver by Southern dot blot, suggesting that the animal was transgenic. Thus, although there seemed to be considerable numbers of mosaic animals after birth, foreign DNA could be integrated into the genome of the progeny by TMGT, at least under the specific experimental conditions. Taking the convenience of TMGT into account, this method could be promising for generating animals, in particular transgenic farm animals.

**TABLE 4. Detection of Foreign DNA in the Tail and Liver by PCR and Southern Dot Blot**

Tissue	PCR +/n <sup>a</sup>	Southern dot blot +/n <sup>a</sup>
Tail	5/50	1/5 <sup>b</sup>
Liver	1/50	1/5 <sup>b</sup>

<sup>a</sup>Number of positive samples/number of samples examined.

<sup>b</sup>Samples were obtained from rats with PCR-positive tails.

In summary, the present study demonstrated that the great majority of early embryos expressed the foreign gene by means of TMGT using specific liposomes. Although the ratio of animals carrying the foreign gene decreased as the animals developed, at least a part of them was suggested to integrate the gene into their own genome. The TMGT method could be applicable to fetal gene therapy as well as to the production of transgenic animals.

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