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Review Article

Direct gene delivery to murine testis as a possible means of transfection of mature sperm and epithelial cells lining epididymal ducts

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The use of a sperm cell to introduce exogenous DNA into an oocyte at the time of fertilization is of interest for the simple production of transgenic mice, and is now called 'sperm-mediated gene transfer (SMGT)'. *In vivo* transfection of sperm cells has been developed as an alternative method for SMGT and can be carried out by direct gene delivery into an interstitial space in a testis (now called 'testis-mediated gene transfer [TMGT]'), into the vas deferens, or into seminiferous tubules. This review summarizes what has been achieved in the field of *in vivo* gene transfer using sperm cells. (Reprod Med Biol 2006; **5**: 1–7)

Key words: epididymis, gene transfer, mice, testis, transgenic.

INTRODUCTION

TRANSGENIC ANIMALS HAVE become valuable tools for both research and application. The most widely used method for creating transgenic animals is to microinject foreign DNA into the pronucleus of a fertilized egg.[1] However, this pronuclear microinjection method has had only limited success in producing transgenic animals in larger species. It also requires great technical skill and is labor intensive.

An alternative possibility is to use a sperm cell as a vehicle for the introduction of exogenous DNA into an oocyte at the time of fertilization. Basically, this involves incubating murine sperm cells with transgene molecules *in vitro* and then using these sperm cells for *in vitro* fertilization (IVF).[2] This method, now called 'sperm-mediated gene transfer (SMGT)', is very simple and convenient compared with the more established pronuclear microinjection method. This SMGT protocol has been tested in several species (chicken, cow and fish), but the experiments were poorly replicated.[3–5] The possible uses and limitations of SMGT technology have been fully described in a number of reviews.[6–12]

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In vivo transfection of sperm cells is also an alternative method for the simple and rapid production of transgenic animals. Transfection can be carried out by the direct introduction of gene constructs into an interstitial space of a testis (called 'testis-mediated gene transfer [TMGT]')[13–25] or into seminiferous tubules.[26–32] In this review, I focus on recent progress in techniques for *in vivo* gene delivery to testicular cells and epithelial cells lining epididymal ducts.

GENE TRANSFER TO MALE GERM CELLS BY DIRECT INTRODUCTION OF DNA INTO SEMINIFEROUS TUBULES

GENE TRANSFER INTO male stem cells, such as spermatogonia, by the direct introduction of foreign DNA into seminiferous tubules appears to be a promising approach to the production of transgenic animals. Spermatogonia, which are actively proliferating and concomitantly providing mature sperm, may be easily transfected with exogenous DNA. Muramatsu *et al*.[26] first demonstrated that testicular cells within seminiferous tubules can be transfected by the introduction of plasmid DNA and subsequent *in vivo* electroporation (EP). Yamazaki *et al*.[29,30] showed, using the method of Muramatsu *et al*.,[26] that almost all spermatogenic cells within seminiferous tubules could be transfected by DNA; however, males undergoing DNA introduction failed to produce transgenic mice despite mating with normal females. Kim *et al*.[27] injected

a lacZ expression plasmid encapsulated by a liposome into the seminiferous tubules of mice that had been treated with busulfan, an alkylating reagent that destroys developing male germ cells, and demonstrated that 8–14.8% of seminiferous tubules expressed the foreign gene. Polymerase chain reaction (PCR) analysis revealed that 7–13% of epididymal spermatozoa possessed the DNA. However, no evidence was obtained to suggest that the foreign DNA had been transmitted to offspring after mating. Celebi *et al.*[32] carried out seminiferous tubule-targeted gene transfer using the method of Kim *et al.*[27] and demonstrated that murine spermatogenic cells were efficiently transfected, and that transfected sperm led to the transmission of a transgene in offspring. However, this transmission was transient and the transferred plasmid DNA had disappeared by the adult stage, probably because of its episomal state. Huang *et al.*[31] used a unique method for gene delivery to germ cells within seminiferous tubules. First they infused seminiferous tubules of young 2-week-old mice with a transgene and then used *in vivo* EP to encourage transfection. Two weeks later the testes were harvested. 'Transgenic testicular sperm' expressing yellow fluorescent protein (YFP) were selected under observation using a confocal microscope and used for intracytoplasmic injection of sperm (ICSI). Transgenic YFP-positive pups were produced. This result suggests that exogenous DNA can be incorporated into the genome of maturing sperm, probably into spermatocytes and spermatids. Importantly, Huang *et al.*[31] produced several lines of transgenic mice derived from ICSI-treated embryos. Thus, the introduced DNA may have been integrated into the chromosomes of maturing testicular sperm cells, thereby expressing YFP. If this integration event occurs frequently in these maturing sperm cells, it may be possible to carry out *in vivo* gene targeting in mammalian testis using appropriate gene targeting constructs. It is not known whether germ cells (spermatogonia) can be transfected using this seminiferous tubule-targeted gene transfer method. Almost all researchers suggest that it may be difficult to use, probably because Sertoli cells cover and shield stem cells from the inflow of the DNA present in the tubular lumen. This problem is easily solved if a plasmid construct carrying a spermatogonia-specific promoter and reporter gene could be introduced into the seminiferous tubule with several enzymes, such as trypsin and collagenase, believed to digest cell–cell junctions and intercellular matrix, and subsequently the entire testis were *in vivo* electroporated.

Attempts to infect testicular cells with viral vectors are of interest, but have met with little success. Blanchard and Boekelheide[28] introduced an adenoviral vector carrying a lacZ gene into seminiferous tubules by rete testis injection and found that lacZ gene expression was observed in Sertoli cells, but not in germ cells. Nagano *et al.*[33] introduced retroviral vectors carrying the lacZ gene and demonstrated that expression of a lacZ transgene was observed in male germ line stem cells and that differentiated germ cells persisted in the testis for more than 6 months. Nagano *et al.*[33] also demonstrated that the introduction of a retroviral vector into the seminiferous tubules of mice resulted in germ cells in 35% of the seminiferous tubules and in the production of transgenic mice with an efficiency of 35%.[34] The introduced DNA was found to have been integrated into the host chromosome when genomic Southern blot analysis was carried out in the offspring, and the gene transmission pattern from one generation to the next was Mendelian. This alternative route of transgenesis is now used in rats,[35,36] pigs[37] and goats.[38] The findings of Nagano *et al.*[33,34] appear to contradict previous findings based on the transfection of adenoviral vector by introducing it into seminiferous tubules[28] or by injection of plasmid DNA into seminiferous tubules and subsequent *in vivo* EP.[27,29,30,32] Germ cells may be more amenable to infection by retrovirus than by adenovirus.

GENE TRANSFER TO EPIDIDYMAL SPERMATOZOA BY DIRECT INTRODUCTION OF DNA INTO THE INTERSTITIAL SPACE OF MAMMALIAN TESTIS

DIRECT INTRODUCTION OF calcium–phosphate-precipitated circular plasmid DNA into the interstitial space of a testis of an adult mouse was first carried out by Sato *et al.*[13] The aim was to transfect testicular spermatozoa so that exogenous DNA could be transmitted to the offspring at fertilization. Polymerase chain reaction analysis revealed that the introduced DNA could be detected in epididymal spermatozoa freshly isolated from the caput and cauda epididymides as early as 6 h after injection and in ejaculated spermatozoa recovered from the uteri of females. These findings suggest that a DNA solution injected into a testis is transferred to the epididymal portion, where epididymal spermatozoa incorporate it.

Ogawa *et al.*[14] extended the study of Sato *et al.*[13] and demonstrated that 80.0% (16/20) of F0 blastocysts derived from mating with males whose testes had been exposed to liposome/lacZ expression plasmid complex exhibited lacZ activity. These findings clearly suggest that mature epididymal spermatozoa incorporate DNA

exogenously introduced into an interstitial space of a mouse testis, leading to the generation of transgenic blastocysts with relatively high efficiency. This technology has been termed 'testis-mediated gene transfer (TMGT)' as an *in vivo* alternative to SMGT.[14]

Sato *et al*.[15] showed that a single injection of circular plasmid DNA complexed with Lipofectin into mature mouse testes is sufficient for transfection of spermatozoa (epididymal spermatozoa), and for a relatively high efficiency (50–100%) of gene delivery to mid-gestational fetuses (F0) obtained by mating injected males with normal females. However, the introduced DNA appeared to be present in a mosaic pattern in the TMGT-derived fetal tissues because it was estimated to be present at less than one copy per diploid cell.[15] Sato *et al*.[16] also revealed that the exogenous DNA introduced into a testis was transmitted to at least the second generation.

Expression of introduced DNA was first evident only in F0 early blastocysts, and almost absent in F0 mid-gestational fetuses and the organs of adult F0 mice.[14,16] Sato *et al*.[16] failed to detect any gene expression at Northern blot level in these F0 fetuses and succeeded in detecting it only in a limited number of samples when nested reverse transcription polymerase chain reaction (RT–PCR) was carried out. Furthermore, Sato *et al*.[16] tested several commercially available reagents that are used for *in vitro* gene transfer to determine which one was the most effective in introducing high numbers of exogenous DNA copies into the fetal mouse genome using TMGT.[19] Unfortunately they were unable to find any candidate reagents for this purpose.

Another research group[17] demonstrated with the use of a TMGT method essentially the same, that is, that plasmid DNA singly injected into testes was transmitted via fertilization to F0 offspring with efficiencies of 5.6–17.6% for rats and 9.7% for mice. Notably, Chang *et al*.[17] demonstrated using genomic Southern blot hybridization that these positive samples had more than one copy of the DNA per diploid cell, in contrast to the findings of Sato *et al*.[16]. Furthermore, they successfully detected gene expression in the F0 and F1 offspring of mice born at Northern blot level.[17] This is in marked contrast to the findings of Sato *et al*.[16] who failed to detect expression of exogenous DNA in F0 offspring (including fetuses and adult organs). Recently, the same group tested the efficiency of eight commercially available liposomes in associating exogenous DNA with sperm and found that only two liposomes, DMRIE-C and SuperFect, led to the detection of exogenous DNA on rat epididymal spermatozoa.[20] When TMGT was carried out on rat testes using enhanced green fluorescent protein (EGFP) expression plasmid/DMRIE-C (or SuperFect) complex, the resulting F0 neonates carried the foreign DNA at greater than one copy per diploid cell.[20] In addition, expression of EGFP was present in more than 80% of morula-stage embryos examined when DMRIE-C or SuperFect were used. Kojima *et al*.[22] injected adenoviral DNA into mouse testes using the TMGT method and found that Leydig cells, but not Sertoli or spermatogonic cells, were efficiently infected. They claimed that gene transfer of adenovirus into a testis using TMGT might be effective for *in vivo* gene therapy for male infertility resulting from Leydig cell dysfunction.

MECHANISM OF GENE DELIVERY INTO SPERM CELLS IN A TMGT SYSTEM

IT HAS BEEN suggested that with the use of the TMGT method exogenous DNA introduced into a testis can be transmitted to mature sperm within 4–7 days after DNA injection. Chang *et al*.[18] first examined the mechanism of TMGT using confocal microscopy of frozen sections of epididymis prepared 4 days after testis injection with fluorescence-labeled DNA and found that the exogenous DNA bound to the surface of spermatozoa in the cauda epididymis. This finding suggests that the exogenous DNA introduced into a testis is transferred to the epididymis within 4 days and binds to epididymal spermatozoa.

Sato *et al*.[21] recently assessed the mechanisms of TMGT in greater detail by injecting trypan blue (TB), a dye generally used for staining dead cells in cell culture systems, and Hoechst 33342, a fluorescent dye generally used for staining cell nuclei, into adult murine testes. It was observed that the solution introduced into the testis was transported to the ducts of the caput epididymis via the rete testis and efferent ducts immediately after injection and reached the corpus and cauda epididymis within 3–4 days. These findings suggest that exogenous DNA introduced directly into testis is mainly taken up by epididymal spermatozoa, which subsequently transfer the DNA to oocytes through fertilization. It remains unclear how the exogenous solution (TB) present between the seminiferous tubules and the testicular capsule enters the rete testis. Sato *et al*.[21] speculated that there are two 'gates' leading to the rete testis through which testicular sperm cells and fluid are transported: one (first gate) through which testicular spermatozoa are transferred from the seminiferous tubules and the other (second gate) through which interstitial fluid present between the seminiferous tubules and testicular

capsule is transported. The TB introduced into the testis might have been transferred to the rete testis through the second gate. Notably, solution instilled in the rete testis never enters the interstitial space between the testicular capsule and seminiferous tubules.[39] These findings suggest that the latter gate would open when fluid flows from the interstitial space, but would close when fluid from flows from the rete testis and vas efferens. Another question of interest is the size of the molecules permitted passage through the second gate. My colleagues and I are currently attempting to answer this question by introducing fluorescence-labeled plastic beads of various sizes or epididymal spermatozoa isolated from enhanced green fluorescent protein (EGFP)-expressing transgenic males into a testis using the TMGT method.

Interestingly, *in vivo* EP toward the vas efferens and caput epididymis immediately after injection of EGFP-expression plasmid resulted in the generation of distinct fluorescence at the epithelium lining the ducts in these portions.[21] Instillation of liposome–plasmid DNA mixture also led to transfection of some epithelial cells lining the ducts of the caput epididymis.[21] These findings clearly indicate that gene delivery targeted to the male reproductive tract, such as the efferent ducts and epididymis, is possible. It is known that portions of the epididymis including caput, corpus and cauda epididymides play important roles in sperm transport, sperm storage and sperm maturation.[40] For example, the epididymis secretes several factors that protect spermatozoa from the harmful effects of toxic xenobiotics, chemicals and oxidative stress.[41] Thus, gene transfer targeted to epididymal epithelial cells would modify the properties of epididymal spermatozoa. To my knowledge, no such attempt to transfect epididymal epithelium has been made. In this sense, gene delivery of liposome/DNA mixture using TMGT or instillation of naked DNA by TMGT and subsequent *in vivo* EP appears to be useful for manipulating the physiological function of the epithelium lining the male reproductive tract.

STATE OF EXOGENOUS DNA TRANSMITTED TO OFFSPRING

IT IS OF interest to determine the state of the exogenous plasmid DNA in the mid-gestational F0 fetuses derived from superovulated females that had been mated to DNA-injected males. Polymerase chain reaction analyses of yolk sac DNA revealed that the majority (78–100%) of these fetuses possessed the exogenous DNA. However, genomic Southern blot analysis of these PCR-positive samples failed to detect any hybridizable band despite repeated trials.[16] In a preliminary test, Sato *et al.*[24] found that foreign DNA (Cre expression vector termed 'pCAG/NCre') was present intact in mouse offspring when PCR using several primer sets, which covered the entire region of pCAG/NCre DNA, was carried out. However, other samples had a deleted form of pCAG/NCre in which both end regions of the CAG/NCre inserted together with a pBluescript SK(–) vector backbone had been lost.[24] Based on these findings, my colleagues and I speculate that the exogenous DNA introduced into adult mouse testis may be present in the fetal tissues in a mosaic pattern. If this DNA is present in each cell, it may be located in close association with host chromosomes and/or integrated in part with the host chromosomes. Our previous findings demonstrated that the pattern of transmission of exogenous DNA to the next generation is not Mendelian[16]. Yonezawa *et al.*[20] obtained a finding similar to ours: greater than 80% of morula-stage rat embryos exhibited EGFP fluorescence, but the ratio of animals carrying the foreign DNA decreased as the animals developed. They also observed that only some postpartum progeny were foreign-DNA positive, with a high incidence of mosaicism. These findings suggest that there is extensive loss of the foreign DNA introduced via TMGT during transition from pre-implantation to postimplantation stages. In this sense, the gene delivery pattern in the TMGT system appears to differ from that observed in the previous system, which depended on pronuclear microinjection of DNA.[1]

PROSPECTS FOR TMGT

IMPROVEMENT OF TMGT is still required before it can be used as an alternative method for transgenic animal production. For example, the introduction of DNA into the testis of a very young male, approximately 5–10 days old, is interesting because young testes are smaller than adult testes and have actively proliferating spermatogonial cells. Thus, it appears to be easy for exogenous DNA to contact sperm cells in a young testis. Repeated injections of DNA are also interesting. This strategy is aimed at increasing the frequency of DNA contact with testicular spermatozoa. However, our preliminary trial of repeated injections (up to six injections) of plasmid/liposome (FuGENE6) complex failed to improve the TMGT system.[23] The choice of reagents used for gene transfer may be important in improving TMGT: in particular, the use of DMRIE-C and TransFect appears promising.[20] If efficient methods for TMGT are developed in mice, I believe that this technology will provide a powerful tool for the production of domestic transgenic

animals, such as cows, pigs and horses, although it is considered difficult with the currently available microinjection techniques.[42]

TMGT IN FISHES

THE MOST SUCCESSFUL form of TMGT appears to be gene delivery to fishes. Lu *et al*.[25] presented interesting findings on the production of transgenic silver sea bream (*Sparus sarba*) using the TMGT method. They injected a liposome–transgene mixture into the gonads of male sea bream at least 48 h before spawning. The males were mated to reproductively active females and the fertilized eggs were collected for further incubation. The PCR analysis revealed that 59–76% of the hatched fly possessed the transgene. Southern blot analysis also confirmed that the transgene had been integrated into the host genome. Gene expression was also confirmed. Lu *et al*.[25] concluded that TMGT would be of great value in the modification of the phenotype of marine fishes.

GENE TRANSFER TO MATURE SPERMATOZOA USING DIRECT INTRODUCTION OF FOREIGN DNA INTO VAS DEFERENS

ANOTHER WAY TO transfect sperm cells with exogenous DNA *in vivo* is by the direct introduction of DNA into spermatozoa in the vas deferens. Huguet and Esponda[43,44] injected a plasmid DNA encoding a green fluorescent protein (GFP) into mouse vas deferens. The night after injection, males were mated with normal estrus females. When the resulting offspring were analyzed, four of 53 newborn mice were found to be positive for the presence of the GFP gene using PCR. Various tissues exhibited GFP expression in the positive animals. Unfortunately, they did not demonstrate that the transmitted constructs were still detectable in the offspring using genomic Southern blot analysis, nor did they determine what pattern gene transmission to the next generation took. Further studies will be needed before the vas deferens-mediated gene transfer system can become a simple and reliable alternative for the creation of transgenic animals. Huguet and Esponda[43,44] also found that epididymal and vas deferens secretions do not block binding of the DNA to spermatozoa. This is in agreement with our previous finding that DNA introduced into the interstitial space of a testis can bind to epididymal spermatozoa,[13,15,21] but not with the finding of Lavitrano *et al*. who found inhibitory factor-1 (IF-1), an inhibitor of binding of DNA to sperm, in seminal fluid.[45]

CONCLUSION

IT IS NOW clear that DNA molecules can associate with, and even penetrate, sperm cells when exogenous DNA is forced into contact with naked sperm.[46–50] However, in most cases the exogenous genes are completely or partially degraded and/or rearranged,[51] probably because of the presence of endonuclease in the sperm. Thus, it appears that exogenous DNA delivered using transformed fertilizing sperm into oocytes is rarely integrated.[15,16,20,23,24,32] In this context, the introduction of small DNA fragments such as oligonucleotides and double-stranded RNA into sperm using TMGT or SMGT may provide an excellent system for examining the regulation of expression of the endogenous target gene at early stages of development.

The conventional approach using pronuclear microinjection is still an effective way to produce transgenic animals; however, it is both costly and labor intensive and requires long periods of time to carry out. The use of either SMGT or TMGT would be more time and cost effective, and would allow genes to be delivered quickly and efficiently to target tissues. If an efficient method to introduce an intact form of exogenous DNA into the genome of immature sperm cells or matured spermatozoa is developed, these novel technologies would undoubtedly contribute to the use of transgenesis for research and development and to gene therapy for the treatment of human male infertility.

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