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Testis-Mediated Gene Transfer: an Alternative Method for Efficient Production of Transgenic Animals

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Introduction and Historical Background

The ability to introduce functional genes into organisms provides a powerful tool for dissecting complex biological processes. Gene transfer is especially valuable in diploid organisms with long life cycles where classical genetic approaches are impractical. A variety of methods for introducing foreign DNA into both somatic and germ cells of mammals have been developed over the last 27 years. Of fundamental importance for germ-line transformation was the contribution of mammalian embryologists, who developed techniques for removing embryos, culturing them briefly in vitro, and returning them to foster mothers where normal embryogenesis could proceed[1]. These techniques opened the way for combining embryonic cells from one animal with those of another to produce chimeric animals. They also provided a means of introducing cultured teratocarcinoma cells, which are derived from germ cells or early embryos, into developing embryos[2]. At the same time, geneticists and virologists were developing methods for introducing selectable genes into tissue culture cells. These advances presented the possibility of transferring genes into teratocarcinoma cells and then introducing those cells into the blastocyst of developing embryos to produce mosaic animals[3]. As an alternative to cell transfer, replacing the pronuclei of fertilized 1-cell eggs with nuclei of genetically modified teratocarcinoma cells was suggested because of the success of nuclear transplantation in amphibians[4].

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The ability to manipulate embryos also presented a way for infecting early embryos with intact viruses or viral DNAs. Experiments performed in the mid-1970s showed that infection of preimplantation embryos with murine leukemia virus (MuLV) resulted in mice with the retroviral DNA integrated into both somatic and germ cells[5]. Although each of these techniques has considerable potential for introducing foreign genes into the germ line, none of them has developed into a routine procedure with wide application. In contrast, a method that has been used extensively involves direct microinjection of DNA into the pronucleus. Techniques for injecting mRNA and then cloned genes, as they became available, were rapidly developed for the large amphibian eggs[6]. Meanwhile, microinjection of viral or cellular genes into tissue culture cells and means of detecting their expression were mastered. During the late 1970s, these methods were adapted for microinjection of mRNA, and then DNA, into mouse eggs[7,8]. In late 1980, the first report describing transgenic mice that developed from microinjected eggs was published[9], and within the next few months four other groups reported similar success in stably integrating foreign DNA into the genome of the mouse[10-13]. Moreover, evidence suggested that at least some of the foreign genes could be expressed[10,12,13] and that the foreign genes were not only incorporated into somatic tissues but also into the germ line[11,14-16]. Furthermore, offspring of transgenic founder mice often continued to express the foreign genes[15]. These facts, along with the observation that it was possible to produce significant amounts of biologically active gene products that would affect the phisiology of the mouse[17].

Sperm-Mediated Gene Transfer

By microinjection technique a maximum 25% of transgenic mice have been obtained in which functional genes of different origins were stably integrated and, in most cases, transcriptionally active[18]. Although in some laboratories this is a routine technique, the generation of transgenic mice is a procedure of certain complexity. Furthermore, for domestic animals the microinjection is possible only after additional treatments that are time consuming and that might influence the efficiency; e.g., pig eggs must first be centrifuged to make the pronucleus visible. Virus adsorption to the surface of mouse and fish sperm[19,20] indicates that sperm can be vectors for egg infection, a notion supported by the report that sperm can deliver foreign genetic information to rabbit eggs[21]. Therefore, the idea of using sperm as a vector to transfer exogenous DNA into eggs is very attractive, and a publication reported a successful attempt at this procedure[22]. However, this result could not be reproduced, although more than 1,300 mice were generated by several groups following the published protocol[23]. In addition,

there have been other reports of sperm-mediated gene transfer. In the case[24], sea urchin sperm were shown to carry pSV-CAT and pRSV-CAT genes into eggs upon fertilization that gave rise to CAT enzyme activity in pools of swimming blastulae. However, no evidence of chromosomal integration of foreign DNA was presented in the report. After that, in many study concerning sperm-mediated gene transfer, the ability of spermatozoa to bind DNA strongly has been described in mice[25-27], porcine[28,29], chicken[26,30], cattle[31,32], insect[31,33]. Recently, Lavitrano's group tried a large, collaborative study of sperm-mediated gene transfer to mouse eggs[34]. Sperm were incubated with plasmid DNA during the capacitation period and then added to freshly ovulated mouse oocytes for fertilization *in vitro*. From a total of 75 experiments, 13 produced 130 transgenic offspring, amounting to 7.4% of total fetuses. In five experiments, more than 85% of offspring were transgenic. But the factors leading to this high success rate were not descovered. Discovering the factors important to success would not only allow this simplified approach to become an important tool in the generation of transgenic mice, but could also lead to important insights into natural protective mechanisms against sperm-mediated transfer of foreign DNA. In addition, this high binding ability appears to be mediated by the complex structure of the MHC class II molecules, localized at the posterior region of mouse sperm head[35]. Such binding resist multiple washing steps, gradient centrifugations and prolonged dialysis in porcine spermatozoa[29]. Considering the high affinity of spermatozoa to DNA, suggests that binding of exogenous DNA to spermatozoa may be in part mediated by simple binding of DNA to the cells.

In the advanced approach, liposomes have been used for introducing foreign DNA into spermatozoa. Cationic liposomes interact with the negatively charged nucleic acid molecules forming comlexes in which the nucleic acid is coated by the lipids. The positive outer surface of the complex can then associate with the negatively charged cell membrane, allowing the internalization of the nucleic acid. This new type of liposomes turned out to be very efficient in the transfer of DNA into cells[36,37] or tissues[38-43].

Bachiller *et al.* have firstly reported evidence that liposomes can efficiently transfer DNA into mouse spermatozoa[44]. About 80% of the liposome-treated spermatozoa showed a signal specific for the exogenous DNA inside the sperm head as shown by laser confocal microscopy. After fertilization *in vitro* with the DNA-introduced spermatozoa, resulting 2-cell embryos were transferred and subsequent offspring were born. However, the introduced DNA were unable to detect for a signal in offspring by Southern blot analysis. There is no obvious reason for this failure. This results suggests it might be that the majority of the sperm with low motility become labeled and that the more active sperm, which fertilized the oocytes, are for unknown reasons not susceptible to liposomes.

A promising approach for producing transgenic animals by using male stem cells was recently reported by Brinster and Zimmermann[45] and by Brinster and Avarbock[46]. However, in order to apply this technique to producing transgenic animals, some difficulties have to be overcome. These include a satisfactory method for short-term *in vitro* culture for drug selection after transfection with exogenous DNA, and methods for use of livestock such as pigs. Kim *et al.* developed a new method for transferring foreign DNA into male germ cells[47]. Mice and pigs were treated with busulfan, an alkylating agent, to destroy the developing male germ cells, and liposome/lacZ gene complexes were introduced into each seminiferous tubule by using a microinjection needles[47]. In mice, 8.0-14.8% of seminiferous tubule expressed the introduced lacZ gene, and 7-13% of epididymal spermatozoa were confirmed as having foreign DNA by polymerase chain reaction (PCR). The liposome-injected testes were all negative for X-gal staining. These results indicate that some spermatozoa were successfully transformed in their early stages by liposome/DNA complexes. In pigs, foreign DNA was also incorporated efficiently into male germ cells, and 15.3-25.1% of the seminiferous tubules containing germ cells expressed the lacZ gene. However, no evidence suggestes that foreign DNA expressed in transgenic animals and their offspring.

Testis-Mediated Gene Transfer

The process of spermatogenesis in the mouse is basically similar to that in any other mammal. One cycle of the seminiferous epithelium takes 207 ± 6 hours, and four such cycles occur between spermatogonium and mature spermatozoon[48]. The production of mature spermatozoa from the original spermatogonial cell takes about five weeks in the mouse (and about twice as long in human)[48]. DNA synthesis occurs only in spermatogonia and primary spermatocytes, since the succeeding stages do not involve mitotic divisions. The average life span of the spermatogonia is 27 to 30.5 hours, and DNA synthesis takes in spermatogonia. The nuclei of spermatids show a noticeable change in DNA pattern during the metamorphosis into spermatozoa. The DNA eventually becomes evenly distributed, and then resistant to desoxyribonuclease, so that the chromosomes undergo an orderly rearrangement. One can easily consider the possibility that certain proportion of spermatozoa, but not all, can be transfected by direct injection of DNA into testis, because actively proliferating cell can be more easily transfected than less proliferative one[49]. If the spermatogonia are successfully transfected with DNA, its descendants will transmit the DNA into oocytes, and result in the generation of transgenic mice. Furthermore, it is expected that testicular spermatozoa at various maturation

steps may be more susceptible to transfection by the exogenous DNA than matured epididymal spermatozoa.

Sato *et al.* firstly reported successfully direct injection of calcium phosphate precipitation of foreign DNA into testes of adult mice[50]. Circular plasmid pSV2-CAT was co-precipitated with calcium phosphate and directly injected *via* a needle into the mouse testis. Southern blot analysis indicated that the introduced DNA was present as a circular form at least up to 7 days after injection, but was undetectable upon autopsy at 28 days after injection. This suggests that testicular sperm cells may not be stably transfected by the DNA. PCR analysis revealed that the introduced DNA could be detectable in the freshly isolated spermatozoa from caput and cauda epididymides as early as 6 hours after the injection. The DNA was also detectable even in the ejaculated spermatozoa isolated from the uteri, which had been successfully mated to the male mice injected with the DNA. However, 1-cell eggs that were fertilized by these spermatozoa showed no evidence for transmission of introduced DNA. It has been reported that foreign DNA could be detected at least up to blastocysts by using a PCR [25], when spermatozoa were allowed to fertilize oocyte *in vitro* in the presence of the DNA. The remnants of the exogenous DNA could be histologically visualized in the surface of zona pellucida of oocytes when bovine epididymal spermatozoa were electroporated with DNA and then allowed to fertilize[51]. Considering these results, it can be speculated that in this system the number of ejaculated spermatozoa which carried the DNA seems to be very small, or if they possess, the amount of DNA may be scarce to be left onto the zona pellucidae of host oocytes which can be detected by PCR. Furthermore, in advanced study, liposome-encapsulated DNA was injected into mature mouse testes and 8% of blastocysts derived from eggs fertilized by spermatozoa of male mice injected with the DNA exhibited expression under the presence of injected DNA[52]. In this case, most of the blastocysts derived from eggs of the 2 females mated with 2 males injected with plasmid pMT-neo/MT-β-gal DNA three times into testis were positive for β-gal activity. Positive reactions were mainly localized to the inner cell mass of blastocysts. These findings suggest that the plasmid DNA exogenously introduced into the mouse testis is tranferred to eggs *via* spermatozoa. More than 20 blastocysts were analyzed for β-gal gene by PCR, of which 70% were positive. This report show for the first time that repeated direct injection of expression plasmid: cationic liposome complexes into matured mouse testes results in transfection of virtually all testicular spermatozoa, leading to very high rates of efficiency of production of transgenic blastocysts after mating of these injected males with normal females, although Bachiller *et al.* demonstrated that liposome-encapsulated DNA can be incorporated into mouse sperm head but these spermatozoa failed to generate transgenics[44] and similar results are occured by using direct injection of Ca-phosphate-

precipitated DNA into mouse testes[50]. Chang *et al.* also reported successful testis-mediated gene transfer using liposome-encapsulated cDNAs for human growth hormone receptor (hGHR), mouse leptin and genomic DNA for human growth hormone (hGH) under control of each promoter region of the mouse metallothionein-I (MT-I), mouse mammary tumor virus (MMTV) and mouse whey acidic protein gene (WAP), respectively[53]. The liposome/DNA complexes were injected into testes of a male rats and mice, and then these males were mated with females at 3 or 4 days after the injection, respectively. Southern blot analysis revealed the MT-I/hGHR fusion gene was introduced in 3 pups. In one of the 3 rats harbouring the transgene, hGHR expression was detectable in the liver by Northern blot analysis. This female transgenic rat was also confirmed the transmission of the transgene to offspring. In other transgene, germ-line transgenic rats or mice could be generated by this method. Moreover, Ogawa et al. also showed multiple direct injection of liposome/DNA complex into mature mouse testis can result in production of transgenic mice at very high rates (28~53%) of efficiency[54]. For injection of DNA into testes, linearized pMT-neo/MT-β-gal DNA was allowed to form liposome-DNA compex. This solution was directly injected into the mouse testis *via* a needle. Four days after the first injection, the DNA-injected males were again injected with liposome-encapsulated DNA into their testis, and two days after the second injection they were also mated to superovulated normal females. This procedure was repeated once. Analysis with PCR revealed that testis 2 days after injection once possessed the introduced DNA, and ejaculated spermatozoa, isolated from the uteri of pregnant females, were also positive for the presence of exogenous DNA even after DNAase treatment and subsequent extensive washing. The frequency of the embryos showing β-gal activity increased along with increased cycles of injection; approximately 80% of blastocysts, that were derived from eggs fertilized by spermatozoa of the males injected a total of three times, were found to be positive for β-gal activity. These results indicate that the exogenously introduced DNA into mouse testis can be transferred to eggs *via* spermatozoa. PCR analysis also confirmed the above results; the exogenous DNA was present in certain population of blastocysts in this experiment. Moreover, PCR analysis of genomic DNA isolated from the fetuses developed from eggs fertilized by spermatozoa of injectd males revealed that 28.6% of the fetuses had detectable levels of amplified products corresponding to the β-gal gene. Histochemical staining of the fetuses also revealed that 34% of fetuses were positive for β-gal activity. And also approximately 65% of F1 offspring from F0 transgenic mice, derived from eggs fertilized to spermatozoa of injected males, were detectable for the exogenous DNA by PCR analysis, suggesting that the transgene was transmitted to their offspring at very high rates of efficiency. In the subsequent experiment, Ogawa *et al.* attempted TMGT using liposome-encapsulated WAP/hGH gene construct and

then obtained F0 newborn mice from the injected males[54]. Southern blot analysis revealed the positive in 2 out of 28 DNA samples from F0 mice. Therefore, Ogawa *et al.* suggests that the foreign DNA could be introduced with high copy number into the host chromosomes by TMGT method[54].

Conclusions and Prospects

Testis-mediated gene transfer (TMGT) has a promising potential as an alternative method for production of transgenic animals because of its simplicity. However, in order to make this technique practically useful, several questions are remained[52,54]. If there is less than one copy of the foreign DNA per diploid cell, then why does it appear that the β-gal activity in their fetuses is so high and does not appear to be mosaic. It suggests that X-gal staining may be bringing up the possibility of background staining. The best reporter construct to be used would be a lacZ gene under the control of a region specific regulatory element. The use of a ubiquitously expressed promoter such as MT-I may be not sufficient for rulling out the possibility of nonspecific background activity. In addition, such low copy number of DNA per diploid cell in transgenic embryos and fetuses produced by TMGT method may be due to chimeric integration or non-integration such as transient expression of the introduced DNAs. Therefore, the presence of transgenes in TMGT-transgenic mice may be only detectable by PCR but not genomic Southern blot analysis because of their low copy number. However, Ogawa *et al.* could obtain F0 newborn mice harbouring a high copy number of the transgene, which was detectable using genomic Southern blot by TMGT method[54]. The resulted high copy number of transgene are difficult to explain at present. This suggests that it is possibility of producing transgenic mice with high copy number of transgenes by TMGT method. On the basis of the data obtained by the classical pronuclear injection technique, the embryos expressing the exogenous DNA (SV40-lacZ) showed various pattern, all showing a mosaic[55]. The DNA was expressed from the 4-cell stage until the blastocyst stage, although none of the embryos injected with the DNA showed a positive at the 2-cell stage. The staining frequency of 38% observed in morulae is extremely high in comparison with the frequency of production of transgenic mice (6-8%) using the SV40-lacZ gene[55]. Therefore, it is likely that some of the expression at morula stage is of a transient nature, originating from extrachromosomally remaining DNA, rather than being an indicator of successful gene integration. Stevens *et al.* also reported mosaicism in the expression of the MT-lacZ gene induced by zinc, although the frequency was low (38%)[56]. They explained that the mosaicism resulted from integration of the

transgene into a single chromatid after DNA replication[55,56]. In the TMGT method, resulting transgenic embryos and fetuses indicates the DNA may be not integrated through developmental stage, although the introduced DNA is integrated at very early stage (1~2 cell stage) of preimplantation with a transient form in the pronuclear injection technique. However, Ogawa *et al.* indicates that the transgene transmission to offspring occurs at very high rates of efficiency[54]. These high rates of positive transmission or inconsistant transmission are difficult to explain for the transgene of this sort at present. It should be noted that the high rate of transmission might be overstimated due to the possibility of episomal replication. Thus, it might be necessary to introduce greater amount of exogenous DNA into the sperm cells for genomic integration of exogenous DNA by modified TMGT. Moreover, the activity of the liposomes might be inhibited for the incubation with the sperm and this step resulted in a decrease of the fertilization efficiency. And also the introduced DNA into spermatozoa may enter the nucleus but does not give any information on whether this DNA is still intact. When DNA is directly microinjected into the pronuclei of fertilized eggs, it becomes integrated into the host genome without gross alterations in most of the cases[18]. Therefore, the transfer of DNA through the cytoplasm of the sperm may be the site of intensive DNA digestion. In fact, Bachiller's report describe the faint signals of unexpected fragment size were observed in about 1% of born pups from DNA/liposome-treated spermatozoa by Southern blot analysis, suggesting less than one copy of truncated construct per cell[44]. The DNA rearrangement evident may be the result of such a protective mechanism. The evidence of the activation of endogenous nucleases in sperm interacting with plasmid DNAs supports this concept[57] and may provide at least a partial explanation of the highly variable nature of TMGT. It is tempting to speculate that the unique packaging of sperm DNA in spermidine and protamine is somehow involved in the ability to distinguish sperm DNA from DNA of other organisms or somatic cells. The inhibited frequency of integration into host genome in TMGT relative to pronuclear injection supports the existance of "foreign DNA" sensing mechanisms that operate during fertilization. Therefore, the developments for inducing exogenous DNA into male stem cells such as spermatogonia by TMGT will be needed.

In TMGT, the liposome/DNA complex will be injected into the interstitial space on testicular tube. Since a testis has sperm cells with several steps of differentiation pathway, what kinds of cells can be transferred by exogenous DNA must be dissolved in future. Probably, matured or maturing testicular spermatozoa may be transferred with exogenous DNA, because transmission of the introduced DNA could be observed in the blastocysts derived from the females that had been mated with the males 2 days after the first injection[54].

Cationic liposomes have been found to effectively bind nuclei acid molecules and fuse with cell

plasma membranes, thus providing for an even more pronounced transfection than pH-sensitive vesicles[58]. Particularly, the protocol using mixed cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA),and dioleoyl phosphotidylethanolamine (DOPE) liposomes ("Lipofectin") has been widely accepted as a reliable and effective method for targeting DNA into cells[36]. However, the method has various specific disadvantages and limitations. Particularly, the method based on calcium phosphate and polycation precipitation that are probably most widespread in laboratory practice are characterized by a relatively low transfection efficacy and strong cytotoxicity, appear ineffective for introduction of DNA in cells, and may be difficult to be used for genetic transfection *in vivo*. Therefore, despite a great variety of already existing method, it is nessessary to develop more effective transfection techniques of exogenous DNA into testes including the use of other liposome complexes, virus-transfection and the combination of electropolation.

Finally, we believe that TMGT can be used as an alternative for production of transgenic animals. This technique will be potentially useful for production of transgenic domestic animals, whose production has been thought difficult by the pronuclear injection, although it remains to be dessolved to clarify several problems.

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