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Status of Sperm-mediated Delivery Methods for Gene Transfer

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The production of transgenic animals by sperm cell-mediated gene transfer continues to be a fascinating and intriguing possibility due to the simplicity and potential use of the technique for all animals. Some success in producing transgenic fish, lower vertebrates and invertebrates using this method has been reported by a number of laboratories. However, except for the group that first reported the production of transgenic mice, no one has produced transgenic birds or mammals by this method. Many examples of successful experiments where DNA has been 'bound' to sperm cells have been reported. Methods for transfer of DNA to sperm include simply incubating the sperm and DNA together, use of liposomes containing DNA or electroporation of sperm with the DNA. Treatment with DNase or extensive washing does not entirely remove this DNA. Recent work has suggested that some of the DNA is internalized into the sperm nucleus and becomes incorporated into sperm chromatin. The incorporation of DNA into the sperm genome may be a necessary prerequisite for producing transgenic animals, since unincorporated DNA may be degraded both within the sperm cell and after fertilization of the egg. An interesting methodology for the transfection of male germ cells by injection of liposome/DNA complexes into seminiferous tubules has been described which may overcome this difficulty. Other methods to increase the integration of the intact transgene in the sperm cell genome may allow stable expression and replication of the DNA in the fertilized egg.

Introduction

The potential use of sperm cells as vectors for gene transfer was suggested by Brackett *et al.* (1971) who demonstrated that SV40 virus binds to sperm. Interest in this area was renewed by reports that transgenic mice (Lavitrano *et al.*, 1989) and pigs (Gandolfi *et al.*, 1989) were produced after *in vitro*

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fertilization of oocytes with sperm that had been incubated with naked DNA. However, other laboratories were unable to repeat these experiments (Brinster *et al*., 1989; Gavora *et al*., 1991). Since then, work has continued on using sperm cells to transfer DNA, due to the simplicity and potential use of the technique for all animals. Work with pigs (Gandolfi *et al*., 1996), cattle (Schellander *et al*., 1995) and chickens (Martinez *et al*., 1992; Nakanishi and Iritani, 1993) has been largely unsuccessful in producing transgenics, although limited success has been claimed by some groups (Rottmann *et al*., 1992; Squires and Drake, 1994, 1997; Sperandio *et al*., 1996).

There have been many examples of successful gene transfer; with fish (Tsai *et al*., 1995; Patil and Khoo, 1996), Japanese abalone (Tsai *et al*., 1997), sea urchin (Arezzo, 1989) and *Xenopus laevis* (Habrova *et al*., 1996). Species differences in producing transgenics may be due to differences in the stability of the foreign DNA, both in the sperm and after transfer to the egg. In this regard, it is interesting that microinjection of foreign DNA into the cytoplasm of fertilized fish eggs can produce transgenics (Chourrout *et al*., 1986), while in other species the DNA is microinjected into the male pronucleus of the fertilized egg. This may reflect the presence of nucleases in the sperm or egg of higher vertebrates that degrade or rearrange the foreign DNA.

DNA Uptake by Sperm

Work using sperm cells as vectors for gene transfer has largely focused on methods to maximize the efficiency of transfer of DNA to sperm, while maintaining the viability of the sperm after transfer. Many examples of successful experiments where DNA has been 'bound' to sperm cells have been reported (Castro *et al*., 1990; Atkinson *et al*., 1991). Treatment with DNase or extensive washing does not remove all of this DNA. DNA binding apparently occurs with particular proteins on the sperm and is reported to be antagonized by glycoproteins present in seminal fluid (Zani *et al*., 1995). Others (Rottman *et al*., 1992) have reported that chicken sperm cells do not need to be washed before being treated with liposomes containing DNA. Recent work has suggested that 15–20% of the DNA is internalized into the sperm nucleus and becomes incorporated into sperm chromatin (Zoraqi and Spadafora, 1997). Although some DNA binding occurs after simply incubating the sperm and DNA together, the efficiency of binding can be dramatically increased by the use of liposomes containing DNA or electroporation of sperm with the DNA.

Use of liposomes

We have systematically investigated the use of various liposome preparations for efficacy in encapsulating DNA and transfer of DNA to chicken sperm cells

while still maintaining the fertility of the sperm (Squires and Drake, 1993). Liposomes containing DNA in Beltsville Poultry Semen Extender (Sexton and Fewlass, 1978) were prepared by reverse phase evaporation. The trapping efficiency of DNA into the liposomes was estimated by using ^{32}P-labelled DNA and determining the percentage of the total amount of radioactivity that was recovered in the liposome pellet after centrifugation. The liposomes containing the labelled DNA (0.1 ml) were mixed with sperm cells (0.1 ml) for 10 min at 40°C, and the liposomes were separated from the sperm cells by centrifugation through 0.25 M sucrose or silicone oil.

The trapping efficiency of the DNA into the liposomes was increased with increasing concentration of lipid, but this reduced the subsequent transfer of DNA to the sperm. Increasing the positive charge by including stearylamine in the lipid mixture improved the trapping efficiency of the DNA into liposomes and the transfer of DNA to sperm cells. Including lysophosphatidylcholine in the lipid mixture promotes fusion of the liposomes with sperm cells and the combination of lysophosphatidylcholine and stearylamine gave the highest transfer efficiency to sperm. The fertility of the sperm cells was dramatically reduced by exposure to liposomes made from dimyristoylphosphatidyl choline, dilauroylphosphatidyl choline or lipids extracted from sperm cell membranes. Increasing sperm cell numbers reduced the total transfer of DNA from liposomes to sperm cells. However, the number of sperm cells should not be lowered below 5×10^7 sperm because of reduced fertility.

We found the optimum conditions for DNA transfer to chicken sperm cells while maintaining fertility are using liposomes comprised of 10 μmol ml^{-1} dipalmitoylphosphatidyl choline, 5 mol% stearylamine and 20 mol% lysophosphatidylcholine with 2.5×10^8 sperm. Other experiments with lipofectin reagent at 0.006 or 0.06 μmol ml^{-1} were also effective in transferring DNA to sperm, but lower numbers of sperm cells (5×10^7 sperm) must be used, which decreased fertility somewhat. Nakanishi and Iritani (1993) reported that 51.6% of sperm retained exogenous DNA after treatment with lipofectin, but the experimental conditions were not given. We have found that the optimum conditions for loading sperm with DNA varies with different species. In particular, fish sperm are quite different from other species since they are activated by water and do not have an acrosome. This requires that salmonid sperm be diluted in buffer (20 mM Tris, pH 9, containing 80 mM NaCl, 40 mM KCl and 1 mM CaCl) to prevent activation.

Use of electroporation

There have been reports of DNA transfer by electroporation to bovine (Gagne *et al*., 1991) and chicken (Nakanishi and Iritani, 1993) sperm as well as sperm from several fish species including zebrafish (Patil and Khoo,

1996) and loach (Tsai *et al*., 1995). Electroporation reduced the fertility of chicken sperm by causing premature breakdown of the acrosome (Nakanishi and Iritani, 1993). Similar reductions in the fertility of bovine sperm were seen after electroporation (Gagne *et al*., 1991), while the fertility of fish sperm was largely unaffected by electroporation (Tsai *et al*., 1995). In using electroporation, the electric field strength and DNA concentration must be optimized.

Fertilization Methods

A potential problem of intravaginal insemination is that 'sorting' of sperm could occur, preventing the sperm containing the DNA from fertilizing the egg. In some experiments (Squires and Drake, 1994), we compared the efficiency of intravaginal insemination versus insemination using intramagnal catheters (Lakshmanan *et al*., 1990). In our experience, we found little difference in the frequency of transgenesis by either method. Trefil *et al*. (1996) used intramagnal insemination of hens to improve the fertilizing ability of spermatozoa treated with lipofectin. The efficiency of sperm-mediated gene transfer could potentially be increased by sorting the sperm after transfection to select sperm containing the foreign DNA, perhaps using fluorescence-enhanced flow cytometry (Nakanishi and Iritani, 1993). In conditions where it is not possible to maintain sperm fertility while loading sperm cells with foreign DNA, the sperm cells could possibly be injected into the egg by intracytoplasmic sperm injection (ICSI).

Detection of the Transgene

A variety of methods have been used to determine if the foreign DNA was present in the developing animal. PCR or Southern blotting of genomic DNA can measure the presence of foreign DNA in the blastocyst, embryo or young animal. The expression of the transgene is usually estimated by measuring the enzyme activity coded by the transgene (CAT, chloramphenicol acetyl transferase, β-galactosidase, green fluorescent protein, etc.) or by Western analysis or enzyme-linked immunosorbent assay (ELISA) of the gene products.

Nakanishi and Iritani (1993) checked for the presence of DNA in freshly laid chicken eggs by PCR. They reported that the incidence of eggs containing foreign DNA was 67% after fertilization with sperm treated with lipofectin–DNA, 47% after fertilization with sperm treated with naked DNA and 23% after fertilization with sperm electroporated with DNA. However, there was no evidence of genomic integration of the foreign DNA. Similar results were previously reported by Rottman *et al.* (1992). They reported that, while naked DNA was not taken up by chicken sperm, liposome-encapsulated

DNA was. The foreign DNA was present in 26% of 11- to 13-day-old embryos but was not incorporated into the genomic DNA. Episomal replication of the foreign DNA had apparently occurred.

In order for stable integration of the transgene to be demonstrated, it is necessary to show that the transgene is transferred to the offspring in a Mendelian fashion. In our work (Squires and Drake, 1994) we were able to demonstrate by PCR analysis of genomic DNA that the transgene was present in the offspring from a backcross to control birds, but the occurrence was less than the expected 50% of progeny. In the first generation backcross, this may have been due to mosaicism, as has been reported for transgenics produced by other methods (Overbeek *et al.*, 1991; Love *et al.*, 1994). However, similar results were also obtained in the next generation, suggesting that the transgene is partially eliminated during cell division. We were also unable to detect significant expression of the transgene in any of the birds that were positive by PCR analysis.

Problems with the Method

The main obstacles to the use of sperm cells as vectors for DNA transfer to the egg are the rearrangement/degradation of the foreign DNA and lack of incorporation into the genome. Tsai *et al.* (1995) reported success in producing transgenic loach using sperm electroporated with a growth hormone gene construct from chinook salmon. While the frequency of transgenesis was about 50%, a number of different size bands were seen on Southern blots of digests of genomic DNA, suggesting that rearrangement of the transgene had occurred before integration. In a later report (Tsai *et al.*, 1997) where they produced Japanese abalone transgenics with a CAT construct, the size of the transgene fragment on Southern blots was larger than expected. Recent work (Zoraqi and Spadafora, 1997) has shown that plasmid DNA internalized into sperm is associated with the nuclear scaffold and it has been suggested that topoisomerase II may play a role in the non-homologous recombination of foreign DNA into specific sites in the sperm genome. The plasmid DNA also becomes extensively rearranged. Preliminary reports suggest that nuclease activities are stimulated by the presence of high amounts of foreign DNA and this can be inhibited by pretreating the sperm with the apoptotic inhibitor, aurintricarboxylic acid (Spadafora *et al.*, 1997).

Problems with the integration efficiency and expression of transgenes are also found with other methods of gene transfer. The expression of transgenes can be affected by cotransfection with other actively transcribed genes (Thorey *et al.*, 1993). Improvements in efficiency may also occur if the DNA is targeted to the nucleus. In this regard, a nuclear DNA attachment element has been identified which confers integration site-independent expression of the lysozyme gene (Stief *et al.*, 1989). Another problem in common with gene transfer by microinjection is multiple copy insertion.

This can be reduced by treating the transgene with alkaline phosphatase to avoid ligation of linear DNA and the formation of head-to-tail arrays. In addition, mosaicism of expression in which the transgene is not expressed in all tissues has been reported (Tsai *et al*., 1995).

New Approaches

Studies by Brinster and Zimmerman (1994) and Brinster and Avarbock (1994) have shown that spermatagonial cells can be injected into the seminiferous tubules of sterile recipient mice. The testicular stem cells seeded the recipient seminiferous tubules and populated the recipient testes with spermatozoa that were capable of fertilizing eggs in female mice and producing normal live offspring. The use of this technique to produce transgenic animals requires that undifferentiated spermatogonia be cultured *in vitro* so they can be transfected with the transgene.

Recently, Kim *et al*. (1997) have further investigated this idea by transfecting male stem cells with DNA *in vivo*. They injected lipofectin–DNA complexes into seminiferous tubules of mice and at random sites in the testes of pigs. In all animals, the developing male germ cells were first destroyed with busulphan, an alkylating agent. After the remaining male stem cells differentiated, 7–13% of mouse spermatozoa contained the transgene and 15–25% of the pig seminiferous tubules contained sperm with the transgene. However, the busulphan treatment was extremely toxic, killing a large percentage of the animals and reducing the numbers of spermatozoa in the survivors. Earlier work by Sato *et al*. (1994) indicated that DNA injected into mouse testis appeared in the sperm up to 7 days later, but had disappeared by 28 days.

Kim *et al*. (1997) also conducted experiments in which epididymal spermatozoa were treated with lipofectin–DNA complexes. While the exogenous DNA was bound to the sperm and was resistant to DNase treatment, male pronuclei removed from oocytes fertilized by the transfected spermatozoa did not contain the exogenous DNA. However, exogenous DNA was occasionally detected in the cytoplasm. It is thus apparent that in order to transfer foreign DNA using sperm as a vector, the exogenous DNA must be incorporated into the sperm genome. The DNA in fully differentiated sperm cells is highly condensed into a small volume by protamine and it may be extremely difficult to integrate foreign DNA into the sperm genome at this stage.

Conclusions

The successful generation of transgenic animals using sperm cells as carriers for the foreign DNA is still an attractive possibility. DNA can be efficiently

loaded into the sperm cells using liposomes or by electroporation and successfully transferred to the egg. The main obstacles are the degradation and rearrangement of the transgene and the lack of integration of the transgene into the developing embryo. Methods that stabilize the foreign DNA and increase the efficiency of integration of DNA into the sperm chromatin would help to make possible the use of sperm cells for generating transgenic animals.

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