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REVIEW

Sperm and testis mediated DNA transfer as a means of gene therapy

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Assisted reproductive technologies (ART) have revolutionized the treatment of infertility. However, many types of infertility may still not be addressable by ART. With recent successes in identifying many of the genetic factors responsible for male infertility and the future prospect of whole individual human genome sequencing to identify disease causing genes, the possible use of gene therapy for treating infertility deserves serious consideration. Gene therapy in the sperm and testis offers both opportunities and obstacles. The opportunities stem from the fact that numerous different approaches have been developed for introducing transgenes into the sperm and testis, mainly because of the interest in using sperm mediated gene transfer and testis mediated gene transfer as ways to generate transgenic animals. The obstacles arise from the fact that it may be very difficult to carry out gene therapy of the testis and sperm without also affecting the germline. Here we consider new developments in both sperm and testis mediated gene transfer, including the use of viral vectors, as well as the technical and ethical challenges facing those who would seek to use these approaches for gene therapy as a way to treat male infertility.

Keywords gene therapy, sperm, testis, transgene, viral vector

Abbreviations ART: assisted reproductive technologies; SMGT: sperm mediated gene transfer; TMGT: testis mediated gene transfer; ES: embryonic stem; MHC: major histocompatibility complex; REMI: restriction enzyme-mediated integration; ICSI: intracytoplasmic sperm injection; YACs: yeast artificial chromosomes; BACs: bacterial artificial chromosomes; GFP: green fluorescent protein; EGFP: enhanced green fluorescent protein.

Introduction

Assisted reproductive technologies (ART) have revolutionized the treatment of infertility [Tesarik and Mendoza 2007]. However, cases of infertility where a man fails to produce sperm because of some defect in spermatogenesis [Krausz and Giachini 2007] or where some important factor necessary for a process such as egg activation is absent [Nasr-Esfahan et al. 2009], mean that some types of male infertility may still not be addressable by ART. One factor undermining efforts to treat such types of male infertility is that many cases of testicular failure are idiopathic, that is, we do not know their underlying cause.

However, with recent studies identifying some of the genetic factors responsible for infertility [Krausz and Giachini 2007; O'Flynn O'Brien et al. 2010], with whole individual human genomes recently being sequenced to identify disease causing genes [Lupski et al. 2010; Roach et al. 2010], and with sequencing costs falling to such an extent that it may soon be economical to sequence the genomes of every individual in the developed world, it may shortly be possible to link particular diseases or conditions, including male infertility, with specific gene defects [Snyder et al. 2010], with much greater ease. In these circumstances development of treatments for disease using the methods of gene therapy may assume much greater importance.

Currently, the term 'gene therapy' tends to mean the introduction into diseased cells of a gene construct encoding a specific protein that is missing or mutated in the diseased individual. However, with the discovery of RNA interference [Liu and Paroo 2010] there is much interest in using this as a form of gene therapy for suppressing expression of unwanted genes, particularly for cancer treatment [Ashihara et al. 2010], but potentially for treatment of any disease with a dominant phenotype.

Gene therapy in the sperm and testis offers both opportunities and obstacles. The opportunities stem from the fact that numerous different approaches have been developed for introducing transgenes into the sperm and testis, mainly because of the interest in using sperm mediated gene transfer and testis mediated gene transfer as ways to generate

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transgenic animals [Coward et al. 2007; Kojima et al. 2008a; Niu and Liang 2008]. The obstacles arise from the fact that it may be very difficult to carry out gene therapy of the testis and sperm without also affecting the germline. Currently, for both biosafety and ethical reasons, germline gene therapy is illegal [Smith 2003; Kimmelman 2008]. In this review we will consider both new developments in sperm mediated gene transfer (SMGT) and testis mediated gene transfer (TMGT) as well as the technical and ethical challenges facing those who would seek to use these approaches for gene therapy as a way to treat male infertility.

Sperm mediated gene transfer

A primary impetus for the development of SMGT has been the search for new methods of creating transgenic animals. Transgenic animals can encompass those that have had foreign genes inserted into their genomes but also gene knockouts (where a gene is made inoperative) and knockins (where a gene is modified *in situ* in the genome) [Shastry 1998]. Transgenic and knockout/knockin mice have revolutionized the study of gene function in the whole organism and are usually generated by injection of DNA into the pronucleus of the fertilized egg [Wall 2001], or the injection of gene targeted embryonic stem (ES) cells into blastocysts to form chimeras [Capecchi 1989], respectively. However, despite the success of these two approaches, concerns still remain about their efficiency.

In addition, for the study of human physiology and diseases, mice may not always be the best model. Thus rats have been very important for the study of brain biology [Wells and Carter 2001], guinea pigs and rabbits for cardiac function [Hasenfuss 1998], while factors such as lifespan, size and similarities in physiology make pigs a potentially better model to study human diseases [Lunney 2007]. Finally, transgenic versions of large animals like sheep, pig, and cow offer immense possibilities for agriculture and also for the generation of therapeutic human proteins in their milk [Niemann and Kues 2007]. A major limiting factor is the lack of efficient methods for creating transgenic versions of such species. Thus pronuclear injection is highly inefficient with respect to transgene integration for cow, pig, and sheep, with only 1% of injections being successful [Wall 2002]. Moreover the failure to identify ES cells for mammalian species other than mice and humans has restricted possibilities for making knockout/knockin versions of such species. This has led to a search for other ways of generating transgenic mammals.

As a means to generate transgenic animals, SMGT is attractive since the sperm itself is a natural vector carrying genetic information into the oocyte. While Brackett et al. [1971] showed almost 40 years ago that rabbit sperm could take up exogenous DNA, it was not until approximately twenty years later that Lavitrano et al. [1989] demonstrated that mouse epididymal sperm incubated with plasmid DNA were able to transfer this DNA to the oocyte, resulting in transgenic offspring at an apparent impressive 30% efficiency. Although initial attempts to repeat these findings, for instance by Brinster et al. [1989], failed to find evidence for such gene transfer, subsequently SMGT has been demonstrated in chicken [Nakanishi and Iritani 1993], mouse [Maione et al. 1998], Xenopus [Jonak 2000], zebrafish [Khoo 2000], and pig [Lavitrano et al. 2002]. However, there is still controversy about the efficiency of this method, and also its mechanism of action.

According to a study by Camaioni et al. [1992], exogenous DNA can bind to the sperm head in the subacrosomal region and in the proximity of the equatorial segment. Francolini et al. [1993] showed that mature sperm could spontaneously take up exogenous DNA. Moreover, another study by Lavitrano et al. [1992] suggested that DNA binding and internalization was not a random event but was mediated by specific DNA binding proteins on the sperm surface, with further studies by the same group indicating that major histocompatibility complex (MHC) class II and CD4 proteins played important roles in this process of interaction [Lavitrano et al. 1997]. Finally, Zani et al. [1995] identified an inhibitory factor (IF-1) in mammalian seminal fluid that appeared to block binding of exogenous DNA to the binding proteins on the surface of the sperm. Another study also identified such endogenous inhibitory factors [Carballada and Esponda 2001]. These findings might explain why mammalian sperm are resistant under normal physiological conditions to the uptake of exogenous DNA (which could be disastrous from an evolutionary point of view) and could also account for the varying success of the different studies investigating SMGT.

As well as seeking to understand the physiological mechanisms underlying SMGT, many studies have focused on identifying ways to improve the efficiency of the process for the purpose of creating transgenics. One approach that has been pursued is the use of liposomes to enhance passage of exogenous DNA across the sperm cell membrane. Following initial studies by Bachiller et al. [1991] which showed that liposome treated sperm improved efficiency of DNA uptake without adversely affecting fertilization, subsequent studies have reported the generation of transgenic offspring in a variety of species including chicken and rabbit [Wang et al. 2001; Yonezawa et al. 2001; Yang et al. 2004], pig [Lai et al. 2001], and bull [Hoelker et al. 2007].

Another approach has been to use electroporation of sperm. However, although this method increased the uptake of DNA into the sperm, attempts to generate transgenic offspring by this method have only been successful in fish/shellfish [Patil and Khoo 1996; Tsai 2000] and cattle [Rieth et al. 2000]. A different approach has been to seek to enhance integration into the genome using restriction enzyme-mediated integration (REMI). In this case constructs were linearized prior to SMGT. Transgenic offspring were obtained using this approach in Xenopus [Kroll and Amaya 1996] and in cattle [Shemesh et al. 2000]. In the latter case REMI was combined with the use of liposomes to facilitate DNA uptake.

One of the biggest developments in SMGT has been its combination with intracytoplasmic sperm injection (ICSI). Perry et al. [1999] showed that such a combination was

more efficient when the sperm were pretreated with Triton-X or subjected to repeated freeze-thaw cycles prior to being incubated with exogenous DNA; such treatments are thought to cause disruption of the sperm membrane which could facilitate uptake and integration of exogenous DNA [Szczygiel et al. 2003]. Recently the efficiency of production of both transgenic mice and rats by SMGT/ICSI was shown to be improved when membrane structure of sperm heads was partially disrupted by detergent or ultrasonic treatment before exposure to the exogenous DNA solution [Hirabayashi and Hochi 2010]. In pigs, the integrity of the sperm plasma membrane has been shown to play a critical role in DNA interaction, and altered plasma membranes facilitate interactions between an injected exogenous DNA and the sperm chromatin. However, severe sperm treatments may damage the sperm nucleus, induce DNA fragmentation, and/or lead to chromosomal breakage with a detrimental effect on further embryonic development [Garcia-Vazquez et al. 2009].

Another recent study showed that pretreatment of mouse sperm with NaOH to remove the surface membranes allowed better uptake of DNA and subsequent high efficiency production of transgenic mice [Li et al. 2010]. The combined method of SMGT/ICSI was successfully used to create transgenic mice expressing expression constructs based on both yeast artificial chromosomes (YACs) [Moreira et al. 2004] and bacterial artificial chromosomes (BACs) [Osada et al. 2005]. The latter approach could be of great importance for creating transgenic animals expressing transgenes under the control of endogenous gene expression control elements, which can span many kilobases of DNA.

Other variations on the SMGT/ICSI approach involved use of single stranded DNA complexed with E.coli recombinase RecA [Kaneko et al. 2005]. Moreira et al. [2007] reported a further development of this approach by assessing the effect of varying transgene concentration, flanking matrix attachment regions, and degree of RecA coating. Another approach has made use of a hyperactive Tn5 transposase to facilitate integration of the transgene into the genome [Suganuma et al. 2005]. In addition, viral vectors have been investigated as a way to introduce transgenes into the sperm [Farre et al. 1999; Zi et al. 2009], although this approach still has to yield clear evidence that it can be used to create transgenic offspring.

In summary SMGT has proved to be a viable method for generating transgenic embryos and animals in a considerable number of species. However issues still remain to be addressed regarding its repeatability, efficiency between different species, and our understanding of the underlying molecular basis of its action. Such issues will be important to resolve if this approach is to be employed for the generation of transgenic animals on a routine basis. Some defining features of SMGT in comparison to TGMT are outlined in Table 1.

Testis mediated gene transfer

An alternative way of introducing transgenes into the sperm would be to genetically modify the spermatogenic cells in the testis. TMGT is also of interest as a way of studying the role of specific genes in testicular and sperm function [Coward et al. 2007; Kojima et al. 2008a; Niu and Liang 2008]. An initial study that injected calcium phosphate-precipitated plasmid DNA directly into the mouse testis showed that the injected DNA could remain in the testis for at least 7 days and was also detected in ejaculated sperm of treated animals [Sato et al. 1994].

Another study that injected plasmids encapsulated in liposomes into the mouse testis resulted in the transgene being detected in blastocysts following fertilization [Ogawa et al. 1995]. Subsequent studies that further developed this TMGT/lipofection approach reported detection of the transgene in fetuses generated by mating with treated males [Sato et al. 1999a], and in subsequent F2 offspring [Sato et al. 1999b]. However, other studies that used this approach demonstrated that TMGT/lipofection did not result in integration of transgenes into the sperm genome [Chang et al. 1999a; 1999b]. In general, the efficiency of transgenesis using this approach appears to be low [Coward et al. 2007].

A different method of TMGT has used *in vivo* electroporation to introduce transgenes into the testis. The first

Table 1. Characteristics of SMGT and TMGT.

	SMGT	TMGT
Cells to be transformed	Spermatozoa	Spermatogenic cells Spermatozoa
Technical complexity	Simple	Simple Surgical techniques
Equipment required	Simple	Simple
DNA preparation	Simple	Simple
Species of application	No limitations	No limitations
Possibility of using	Electroporation	Electroporation
complementary	Liposomes	Liposomes
methodologies	Virus	Virus
Mosaicism	Yes	Yes
Multitransgenesis	Possible	Possible
DNA integration	Random	Random
Efficiency	Low-moderate	Moderate
Repeatability	Low	Low-moderate
Cost	Low	Low

study using this approach was by Muramatsu et al. [1997] who used it to achieve expression in various types of testicular cells, with expression levels being related to the voltage and time of electroporation. A study by Yamazaki et al. [1998] achieved higher levels of expression by a modification of the injection technique; while a subsequent study by the same group found evidence for expression of a green fluorescent protein (GFP) transgene more than two months after treatment [Yamazaki et al. 2000], suggesting that the transgene might have become incorporated into spermatogonial stem cells. In another study, Huang et al. [2000] provided the first evidence that this approach could be used to generate transgenic offspring via ICSI using testicular sperm from treated animals.

In other developments of this technique, Kubota et al. [2005] showed that injection into the rete testis is the optimum injection route for efficient expression and minimal damage to the testis. Hibbitt et al. [2006] first systematically studied the effect of varying different parameters of the electroporation technique upon both transgene expression and testicular integrity and sperm motility and viability. This study showed that under optimum conditions, *in vivo* electroporation of the testis does not have any adverse effects upon testicular integrity and sperm quality. In addition, expression of a GFP variant was shown in the testis and in epididymal sperm in this study; however no attempt was made to demonstrate the transmission of the transgene to offspring. One negative feature of gene transfer into the testis by electroporation is that the proportion of cells expressing the transgene is still relatively low, limiting its current potential as an efficient transgenic approach.

Apart from transgenics, TMGT using electroporation has shown potential as a way of studying gene function in the testis and sperm. Thus, two studies have shown that electroporation can be used to introduce reporter constructs driven by germ cell-specific promoters into the testis as a way of analyzing the function on these promoters [Ike et al. 2004; Somboonthum et al. 2005]. In another use of this approach, Shoji et al. [2005] showed that it was possible to knockdown expression of a specific gene in mouse testis following delivery of small hairpin RNAs (shRNAs) using electroporation. Coward et al. [2006] showed that it was possible to express an EYFP tagged form of the sperm protein in mouse sperm following electroporation of constructs carrying this gene product into the mouse testis. This suggested that such an approach might be used to study the factors mediating localization of sperm proteins. From a gene therapy perspective, it is also interesting that *in vivo* gene transfer into the testis using electroporation has been used to express transgenes in Sertoli cells and thereby boost spermatogenesis [Yomogida et al. 2002; Dobashi et al. 2005].

Gene transfer by viral vectors

One important limitation about all the above methods of introducing transgenes into the sperm or testicular cells is that they are relatively non-specific. Viruses offer a potentially far more powerful tool for gene transfer since they have evolved specifically to enter host cells and deliver their genetic information to those cells [Thomas et al. 2003; Mancheno-Corvo and Martin-Duque 2006]. Current gene therapy strategies focus heavily on viral vectors for this reason. However viruses also pose significant problems, both in terms of general safety but also because they can have adverse effects upon the cells and tissues into which they are introduced. An important aspect of using viral vectors is selecting the best class of virus for transgene delivery. Current gene transfer strategies tend to focus on three classes of virus: adenovirus, retrovirus, and lentivirus.

Adenoviruses are popular in somatic cell gene therapy because of their suitability for high titre production and ability to infect a wide range of cell types [Hitt et al. 1995]. Also, they do not tend to integrate into the host genome which can be important from a biosafety point of view. Retroviruses have been used widely in gene therapy as well. Although these viruses only infect dividing cells, thus potentially limiting their usefulness, they are very efficient at integrating transgenes into the genome. While this increases the likelihood that transgenes carried by retroviral vectors will be carried into the subsequent progeny of infected cells and thus increases their efficacy for gene therapy, it also poses potential problems of biosafety, since the integrating viral DNA may disrupt the expression of endogenous genes in the genome. Conversely, integrated transgenes carried by retroviruses may be silenced by protective mechanisms employed by the host genome, thus limiting their effectiveness. Finally, lentiviruses are a subclass of retroviruses which are of interest for gene therapy because, unlike other retroviruses, they can infect both dividing and non-dividing cells [Naldini et al. 1996; Park 2007], thus increasing their potential therapeutic range. In addition, although lentiviral vectors also integrate into the host genome, they are better able to avoid gene silencing and exhibit stable gene expression *in vivo* than other types of retrovirus [Hamaguchi et al. 2000; Ikawa et al. 2003].

In terms of viral delivery of transgenes to the testis, research findings have been mixed. Thus, a number of studies have suggested that the male germ cells appear to be resistant to infection by adenovirus. For instance, Blanchard and Boekelheide [1997] reported successful adenovirus-mediated gene transfer into Sertoli cells *in vitro*, as well as expression in both Sertoli and Leydig cells *in vivo* following injection of adenoviral vector into the rat testis. Kojima et al. [2003] also found that adenoviral vectors could infect and lead to gene expression in Sertoli and Leydig cells, but not male germ cells after injection into the mouse testis. Moreover, a subsequent study by this group showed that there was no evidence of transmission of the transgene either in epididymal sperm or in progeny derived from mice whose testes had been injected with adenoviral vectors in this manner [Kojima et al. 2008b]. While these findings would appear to limit the possibilities of adenovirus being used to introduce transgenes into the male germ cells, from the point of view of gene therapy in humans they are potentially very important. They suggest that it might be possible to correct defects in the Sertoli and Leydig cells without at the same time infecting the male

germ cells and thus running the risk of transmitting the transgene to the next generation. These findings also have relevance for somatic cell gene therapy as they suggest that use of adenoviruses to treat different somatic cell diseases is unlikely to lead to inadvertent genetic transformation of the germline.

In contrast to adenoviruses, retroviruses do appear to be able to infect the male germ cells. Nagano et al. [2000] showed that retroviral vectors could deliver transgenes into spermatogonial stem cells *in vitro*. This group also showed that transfer of such genetically modified stem cells into the testes of mice whose own spermatogonial stem cells had been depleted, led to repopulation of the testis with the donor cells and subsequent ability to transfer the transgene to progeny. In another study, De Miguel and Donovan [2003] characterized some of the factors affecting the ability of retroviral vectors to infect spermatogonial stem cells *in vitro*, such as proliferative status of the infected cell, type of viral envelope and retroviral long terminal repeat, and the mode of delivery of the virus. Importantly from the point of view of selective gene targeting, as opposed to delivering a transgene randomly into the genome, Kanatsu-Shinohara et al. [2006] showed that it was possible to use a retroviral vector to carry out such gene targeting to knockout a gene in spermatogonial stem cells *in vitro*. They subsequently showed that such cells could be transplanted into recipient animals to create knockout mice. While the previous studies all involved retroviral infection of spermatogonial stem cells *in vitro*, Kanatsu-Shinohara et al. [2004] also demonstrated direct transduction of mouse spermatogonial stem cells *in vivo* by injecting a retroviral vector into the seminiferous tubules. In addition, this study showed that transgenic offspring could be generated from mice treated in this manner and that transgene expression was observed in subsequent generations.

Lentiviruses have also been shown to be capable of infecting the male germ cells. Nagano et al. [2002] showed that lentiviral vectors can infect spermatogonial stem cells *in vitro* as well as retroviruses. In another study, Hamra et al. [2002] demonstrated the successful production of transgenic rats following infection of spermatogonial stem cells *in vitro* with lentiviral vectors and subsequent transfer of such cells to the depleted testes of recipient animals, with the transgene being transmitted to the F2 generation. The first study to pursue the potential of lentiviral vectors for gene transfer into the testis *in vivo* was by Ikawa et al. [2002], who showed that they could restore spermatogenesis in mice with absence of expression of the c-kit ligand on the surface of their Sertoli cells, by injecting the testis with a lentiviral vector carrying the c-kit gene. While this study found no evidence that lentiviral vectors could infect the male germ cells *in vivo*, Parrington et al. [2007] presented evidence that injection of a lentiviral vector carrying a GFP transgene into hamster testis led to strong expression of the transgene up to 72 h after infection in what appeared to be various types of male germ cells of different stages. A subsequent study by Kim et al. [2010] used a dual expression vector capable of expressing both firefly luciferase and enhanced green fluorescent protein (EGFP) and found that after injection into the mouse seminiferous tubules, expression of both the bioluminiscent reporter protein (visualized non-invasively) and the EGFP (detected via an anti-EGFP antibody) could be detected up to 3 months following infection. A variety of different male germ cells at different stages of spermatogenesis were shown to express the EGFP protein. In neither of these studies was any evidence provided that this approach could lead to germline transmission.

Possibilities of gene therapy in the testis and sperm in humans

The findings discussed so far relating to studies of SMGT and TMGT in various animals raise the possibility of whether it would ever be possible to use such approaches for gene therapy of the testis and sperm in humans. As discussed at the beginning of this review, gene therapy in the future might encompass both introduction of genes that are absent or defective in a human disease, into a diseased human cell type or tissue, and shRNA hairpin constructs or dominant negative constructs that could suppress unwanted expression of a gene. In the context of the testis and sperm, this might mean that gene therapy could be used to treat both infertility and testicular cancer; in the latter case either shRNAs or tumor suppressor genes might be employed [Tanimoto et al. 2007]. However, a unique problem faced by those seeking to carry out gene therapy in the testis and sperm in humans is that such attempts could inadvertently lead to introduction of transgenes into the germline. Currently, germline gene therapy in humans is illegal because of biosafety and ethical concerns. One concern about using retroviruses for somatic human gene therapy is that such viral vectors integrate into the host genome. While this has the valued effect of ensuring transmission of the transgene to the progeny of treated cells and continued delivery to a diseased tissue, the use of retroviruses for gene therapy has recently raised concerns following the finding that some patients successfully treated for the single-gene immune disorder X-SCID, subsequently contract leukemia, apparently as a result of activation of endogenous proto-oncogenes by the viral vector [Nienhuis et al. 2006]. A sound reason for being concerned about the use of retroviruses for gene therapy in the human testis would be the possibility of such viral vectors integrating into the sperm genome and being transmitted to future offspring; if such an integration event carried with it a susceptibility towards cancer this would be catastrophic. In this context, apart from the fact that such concerns might again stress the potential importance of non-viral methods of TGMT such as those involving electroporation, it is worth noting that studies such as those mentioned already that have shown that male germ cells, but not Sertoli and Leydig cells, appear to be resistant to infection by adenoviral vectors [Kojima et al. 2003; 2008b], may mean that this type of viral vectors may have clinical potential for treatment of certain types of infertility in which there is a deficiency of some Sertoli or Leydig cell factor. For retroviruses, including lentiviruses, it is difficult at present to imagine an easy way to ensure that they would not be

passed to the germline if used for gene therapy in the testis, and if so, to ensure that side-effects caused by integration of the transgene would not create hazards in resulting offspring. However, the current interest in developing safer forms of retroviral vectors for somatic cell gene therapy means that such obstacles are not necessarily insurmountable.

There are also ethical reasons for opposition to germline gene therapy. Although manipulation of the germline might feasibly be used to prevent the transmission of disease traits, ethical problems would arise from the difficulties of distinguishing between gene therapy and genetic enhancement, and the specter of eugenics [Smith 2004]. Nevertheless, if it were ever deemed ethically acceptable to carry out germline gene therapy, one possible route to carry out a safe form of germline gene therapy might be through the development of targeted gene replacement. As noted already, Kanatsu-Shinohara et al. [2006] have shown that it is possible to make knockout mice by carrying out gene targeting by homologous recombination in spermatogonial stem cells *in vitro*. Could such a strategy be used to substitute a healthy gene for a disease-causing one in humans? If such an approach were ever deemed acceptable on safety grounds, it would remain to be demonstrated whether it was both ethically and socially desirable to unleash such a technology upon the world. Meanwhile, the continued search for new ways to create transgenic animals and study gene function in the testis and sperm mean that we can expect further exciting developments in this area of research in the future.

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