# Gene Transfer to Sperm and Testis: Future Prospects of Gene Therapy for Male Infertility

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**Abstract:** Male infertility has been considered a major contributory factor to infertility. The causes of spermatogenetic failure found in most cases of male infertility remain largely idiopathic. Unfortunately, there is no effective treatment to improve spermatogenesis for idiopathic male infertility patients. Intracytoplasmic sperm injection (ICSI) is the current treatment of choice for severe male infertility and has brought the joy of childbearing to couples for whom it was previously impossible; however, several problems exist with this treatment. In addition, if there are no spermatozoa in the testis of these patients, they do not have paternity potential even if ICSI is conducted. Ultimately, fertilization is better *in vivo* than *in vitro*. Recently, on the other hand, gene transfer to sperm and testis has been developed to find more effective and simple methods to obtain transgenic animals. This technique has the potential to be the most useful approach for the future treatment of male infertility. In this review, we will give an overview of the recent advanced technique of gene transfer to sperm and testis, and discuss the future prospects of gene therapy for the treatment of male infertility. In conclusion, although more investigations on the mechanism of spermatogenesis and male infertility and the establishment of techniques for more efficient and safer gene transfer to the sperm and testis will be needed, gene therapy will enable a revolutionary advance for reproductive treatment and provide great benefit for patients with male infertility in the future.

**Keywords:** Gene transfer, sperm, testis, transgenic animal, gene therapy, male infertility, sertoli cell, leydig cell.

## INTRODUCTION

Infertility is defined as the inability to conceive after one year of unprotected intercourse; approximately 10–15% of couples have difficulty conceiving. The cause of infertility can be divided into four major categories, the male factor, the female factor, combined factors, and unexplained infertility. Although it is difficult to assign an exact percentage to each of these categories, it is generally reported that in approximately 30% of cases, infertility is mainly due to a male factor, and 70 to 90% of male infertility arises from impaired spermatogenesis with the clinical presentation of abnormal sperm production, such as oligozoospermia and azoospermia [1]. Although the presented cases of male infertility include varicocele, cryptorchidism, obstruction of spermatic ducts, and endocrine disorders, in most cases of male infertility, the underlying pathogenesis is not clear, and when no cause is known, it is described as idiopathic male infertility.

Spermatogenesis is a complicated process involving many interesting phenomena that are biologically important, such as stem cell proliferation and differentiation, meiosis, generation of haploid germ cells, and morphogenesis of the developing sperm [2]. All these phases of spermatogenesis are supported by and dependent on an intimate interaction between germ cells and Sertoli cells, which provide the microenvironment essential for functional spermatogenesis. Disruption of spermatogenesis, which results in male infertility, can therefore be caused by defects affecting the germ cells or Sertoli cells or sometimes Leydig cell dysfunction.

It is obvious that when few sperm are available, the possibility of sperm-egg interaction is reduced. To overcome this difficulty, *in vitro* fertilization (IVF) is a possible option. Recently, severe male infertility has been treated successfully by intracytoplasmic sperm injection (ICSI) [3,4]. Earlier techniques of oocyte micromanipulation, such as partial zona dissection and subzonal insertion, were supplemented by ICSI, in which one variable spermatozoa obtained by testicular sperm extraction (TESE) and microsurgical epididymal sperm aspiration (MESA) were needed for each oocyte retrieved. Fertilization rates were in the range of 60–90% and the delivery rate per retrieval was 38.5% in the first 560 couples [4]. ICSI is now the treatment of choice for severe male infertility and has brought the joy of childbearing to couples for whom it was previously impossible. However, without doubt, the ICSI procedure itself is more invasive [5]. Although assisted reproductive technologies, including ICSI, do not alter the mutation frequency or spectrum compared with natural reproduction in mice [6], a follow-up study involving 1,082 karyotypes of ICSI children demonstrated a slightly increased incidence of chromosomal abnormalities [7,8]. In addition, the long-term safety of ICSI is still insufficient in humans. Problems such as the collection of ova from healthy women remain. Moreover, there is concern over the possibility of transmitting normally non-transmissible gene abnormalities, such as those related to spermatogenic disorders or to other diseases, to the next generation. Therefore, the establishment of new therapy as a substitute for ICSI is necessary.

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The increased molecular understanding that has accompanied the genomic technological revolution has important implications for the improved understanding, diagnosis and treatment of human diseases. Gene transfer is a powerful tool for studying basic biological mechanisms and is an increasingly attractive approach for treating several diseases, including inherited or acquired genetic disorder and cancer [9-12]. Recently, there have been many reports about gene transfer to sperm and testis, the original purpose of which was to develop more effective and simple methods to obtain transgenic animals than the most widely used method, which is the direct microinjection of foreign DNA into pronuclei of fertilized eggs. In the future, however, this technique has the potential to be the most useful approach for the treatment of male infertility. The purpose of this review is to summarize the possible role of gene transfer to mammal sperm and testis and to discuss the future prospects of gene therapy for the treatment of male infertility.

## 1. GENE TRANSFER TO SPERM

### 1.1. Gene Transfer Vectors

#### *1.1.1. Non-Viral Vector*

***Naked DNA***

Sperm cells have the capacity to capture foreign DNA [13]. Ejaculated rabbit sperm (30–35%), exposed to SV40 DNA labeled by [$^3$H]-thymidine, was reported to contain radioactive material, mostly localized in the postacrosomal area [13]. Although sperm-mediated gene transfer has the potential to markedly simplify the generation of transgenic animals, its efficiency is controversial [12,14].

Several reports have demonstrated that sperm-mediated gene transfer, which it is based on the intrinsic ability of sperm cells to bind and internalize exogenous DNA and to transfer it into the egg at fertilization, has succeeded in generating transgenic animals [15-19]. In this technique, DNA is mixed with spermatozoa before used for *in vitro* fertilization, and in approximately 30% of the obtained offspring, the foreign DNA was integrated and the germ line transmitted [15]. Sperm-mediated gene transfer could also be used to generate multigene transgenic pigs that would be of benefit as large animal models for agricultural applications [20-22]. There is a recent report on transient transgene transmission to piglets by intrauterine insemination of spermatozoa incubated with DNA fragments (30% at 3 days old, but 0% at 70–100 days old) [23]. Treatment of spermatozoa with DMSO sometimes enables effective DNA incorporation into spermatozoa, and recently, the production of transgenic mice and rabbits by transfected spermatozoa with exogenous DNA/DMSO complex was reported [24].

On the other hand, several independent research groups have expressed scepticism about the fundamental basis of sperm-mediated gene transfer to generate transgenic animals **[**25,26**]**. They insist that sperm can associate with exogenous DNA but cannot convey the DNA into the oocyte.

To increase the efficiency of DNA uptake into sperm, two modifications, liposome and electroporation, have been described.

***Liposome***

Cationic lipids interact with negatively charged nucleic acid molecules forming complexes in which the nucleic acid is coated by lipids [27]. The positive outer surface of the complex can associate with the negatively charged cell membrane, allowing the internalization of nucleic acid. In this technique, DNA is mixed with cationic lipid just before addition of this complex to the sperm suspension [28]. Although DNA transfer into sperm mediated by liposomes was very efficient and no obvious reduction in the fertilization frequency of oocytes could be detected, it was impossible to generate transgenic mice by this method [29]. Ball *et al.* [30] recently have reported that the incorporation of exogenous DNA by equine spermatozoa was enhanced by liposome-mediated transfection. Although the transgene was detected in a proportion of Day 7–10 embryos, there was no evidence of gene expression in these embryos. The effectiveness of sperm-mediated gene transfer may be significantly affected by the choice of the transfection reagent and by plasmid architecture [31]. We also investigated the effectiveness of liposome-mediated gene transfer into mouse sperm [32]. After incubation of the DNA-liposome complex and mouse sperm of epididymis in the medium, the gene expression of the fertilized ovum and fetus was investigated. In a few fetuses 12.5 days after embryo transfer, gene transfer was confirmed by PCR, but no gene expression was found and no transgenic mice were generated; however, we proved that sperm with a gene transferred by the liposome method could fertilize the ovum and transfer adventitious genes to ova.

***Electroporation***

Electroporation is a safe and efficient system to introduce genes into a wide variety of tissues [33]. Exposure of a cell to an adequate amplitude and duration of electric pulses leads to a temporary increase of cell membrane permeability. This allows exogeneous DNA to cross the membrane and enter the cell. Electroporation provides more sperm cells to increase DNA uptake than unelectroporated spermatozoa [34,35]. Gagne *et al.* [34] reported that the association of DNA with spermatozoa was increased several fold using electropration. There was a 5–10% increase in the amount of DNA bound by pig sperm when the samples were electroporated. [35]. Oocytes fertilized with electroporated DNA-treated spermatozoa developed beyond the 16-cell stage in proportions that were significantly lower than the control without DNA, although electroporation significantly increased the uptake of DNA [36].

#### *1.1.2. Viral Vector*

***Adenoviral Vector***

When *in vitro* fertilization was performed after sperm were exposed either to 10 or 100 plaque-forming units (PFU) per sperm cell, gene expression was not detected in embryos [37]. It is speculated that this is because sperm are not capable of infection by an adenovirus. Adenovirus-mediated gene transfer to sperm may be not suitable to generate transgenic animals.

### 1.2. Sperm-Mediated Transgenesis

The processes of making transgenic animals by microinjecting DNA into the pronucleus of a fertilized oocyte or

after the transfection of embryonic stem cells is now well established; however, some researchers have attempted to develop a simple and cost-effective method to generate transgenic animals. Several methods for gene transfer to sperm (sperm-mediated gene transfer) in animals have been reported in an attempt to generate transgenic animals and to clarify the molecular mechanism of spermatogenesis. These include directly incubating mature, isolated spermatozoa with DNA or pretreating mature, isolated spermatozoa before assisted fertilization [38,39]. As a potential tool for genetically manipulating animals, sperm-mediated gene transfer may have the advantage of simplicity and cost-effectiveness, in contrast with more established methods of transgenesis such as pronuclear microinjection [14]. Some researchers have succeeded in generating transgenic animals using sperm-mediated gene transfer. As described above, a non-viral vector may be more useful to generate transgenic animals than a viral vector; however, it has not yet been established as a reliable and stable method to generate transgenic animals.

### 1.3. Possibility of Clinical Application of Sperm-Mediated Gene Transfer for Male Infertility

Originally used in women with tubal damage, IVF is now acknowledged to be an effective treatment for long-term subfertility from other causes. [40]. The rate of embryo implantation associated with IVF is 9–12% [41-43]. A possible purpose of the clinical application of sperm-mediated gene transfer may be to improve the IVF rate; however, in the three methods described above, including naked DNA, liposome and electroporation, although the fertilization capacity of DNA-treated sperm was tested in *in vitro* fertilization experiments, the fertilization rate of the oocyte was depressed to varying degrees, depending on the concentration of DNA and liposomes and the electric conditions used during electroporation [28]. These techniques were designed to improve basic reproductive technology with little consideration of the possibility of cell injury caused by gene transfer. On the other hand, Ball *et al.* [30] reported that liposome-mediated gene transfer did not adversely affect sperm viability, acrosomal integrity or fertility. We also performed liposome-mediated gene transfer into sperm and evaluated its effects on sperm motility and the fertilization rate [32]. The results were DNA-concentration-dependent decreases in both sperm motility and the fertilization rate. Although no gene expression was observed in the embryos in our study, it is expected to pose serious problems if the method is applied to infertile men because there is a possibility that exogenous DNA will be transmitted to offspring and affect subsequent generations. Moreover, it is presently impossible to transfer genes to target sites in chromosomes, and further research is necessary. Although gene transfer to sperm is very easy compared with that to fertilized ova or in subsequent stages, the clinical application of sperm-mediated gene transfer to improve the fertilization rate may be impracticable.

Gene analysis of infertile men has revealed the deletion of *azoospermia factor* (*AZF*), a domain of the gene related to spermatogenesis in the long arm of the Y chromosome, in some azoospermic patients [44-46]. The deletion frequency of one or more of these regions on the Y chromosome in men with azoospermia or severe oligozoospermia is approximately 5–15%, depending on the phenotypic criteria of the studied population of infertile men [44,45,47]. To date, *RBM* and *DAZ* on the human Y chromosome have been cloned among genes related to spermatogenesis [44,45]. These reports suggest that idiopathic male infertility is a genetic disease in many patients and advances in assisted reproductive technology may result in the transmission of abnormalities on the Y chromosome of azoospermic men to male offspring with the possible occurrence of azoospermia in males of subsequent generations. For male infertility patients with *AZF* deletion, sperm-mediated gene transfer with the deletion gene may be useful to prevent azoospermia in males of subsequent generations; however, in conclusion, under the present technique and consensus, the clinical application of sperm-mediated gene transfer is not acceptable because of the possibility that uncontrollable transmission of exogenous DNA would severely affect subsequent generations.

## 2. GENE TRANSFER TO TESTIS *IN VIVO* AND *EX VIVO*

### 2.1. Target Cells of Gene Transfer in the Testis

The testis has two primary functions: the production of sperm (spermatogenesis), which ensures the survival of our species, and the secretion of steroid hormones (steroidogenesis), which is necessary for male sexual differentiation, spermatogenesis and male secondary sexual functions, including behavior. Three main cells, germ cells (spermatogenic cells), Sertoli cells and Leydig cells play a significant role in maintaining testicular function. Germ cells in the testis are composed of spermatogonia, spermatocyte and spermatid. Spermatogenesis can be divided into several processes, including the proliferation and differentiation of spermatogonia, which are stem cells, meiosis of spermatocytes, and their morphological changes to spermatids, but complicated interactions among cells, including supporting cells such as Sertoli cells and Leydig cells, are involved in these processes. Within the seminiferous epithelium of seminiferous tubules, spermatogenesis occurs on the surface of Sertoli cells. In other words, Sertoli cells directly support spermatogenesis. They are involved in metabolic exchange with germ cells, as well as in their nutrition and the secretion of tubular fluid into the luminal compartment and the exclusive targets of follicle-stimulating hormone (FSH), androgens and other hormones within the testis [48]. On the other hand, Leydig cells are the main source of the androgen hormone, testosterone, which is located in the interstitial space, outside the seminiferous tubules. This function is regulated by luteinzing hormone (LH), paracrine factors secreted by cells within the seminiferous tubules and autoregulatory factors.

Germ cells are appropriate target cells in the testis for generating transgenic animals, while Sertoli cells and Leydig cells may be appropriate target cells for clinical application.

### 2.2. Methods of Gene Transfer to Testis

#### *2.2.1. In Vivo Gene Transfer*

As described above, germ cells and Sertoli cells are located in the seminiferous tubule, while Leydig cells are located in the interstitial space, which is outside the seminifer-

ous tubule. Two methods, intratubular injection and intratesticular injection, can be used to perform gene transfer to the testis depending on the target cells. If target cells are germ cells or Sertoli cells, intratubular injection may be better, whereas if they are Leydig cells, intratesticular injection may be better (Fig. **1**).

For intratubular injection, two injection techniques are available, antegrade injection and retrograde injection. The testis is pulled out and exposed under a dissecting microscope in both techniques. For the antegrade injection, a small incision is made in the tunica, and then DNA solution is directly injected into the seminiferous tubules using an injection glass micropipette. Injection is usually made at several sites in the testis [49]. For the retrograde injection, the injection glass micropipette is inserted into the rete testis via the efferent duct to allow direct injection into the seminiferous tubules. [50]. This technique enables the more thorough spread of exogenous DNA throughout the entire seminiferous tubule. We usually inject DNA containing Trypan blue dye to monitor the accuracy of the testis injection.

On the other hand, the intratesticular injection is a simpler method. DNA solution is injected directly into the testis through the scrotal skin using a 30-gauge needle and a 1.0-ml disposable syringe. This technique allows the spread of DNA throughout the entire interstitial space of the testis.

### *2.2.2. Ex Vivo Gene Transfer*

The testicular cell and tissue transplantation system with gene transfer is sometimes a useful procedure for gene transfer to the testis to produce transgenic animals. In this system, transduced donor testicular cells populations are transplanted by efferent duct injection into recipient animals [51,52] or transduced donor testicular tissues are transplanted directly into the testis.

## 2.3. Gene Transfer Vectors

Several researchers have attempted gene transfer into animal testis using several vectors to produce transgenic animals. Since these vectors have their own characteristics, they should be clarified before using them for testis-mediated gene transfer *in vivo* (Table **1**).

### *2.3.1. Non-Viral Vector*

#### ***Naked DNA***

Huguet E *et al.* [53] analyzed the uptake of exogenous DNA by mouse and rat spermatozoa using *in vivo* methods. DNA was injected into the vas deferens, and spermatozoa were recovered 6 hr later. Uptake of exogenous DNA occurred in 60–70% of spermatozoa after *in vivo* DNA injection into the proximal region of the vas deferens [53]. These male mice injected with exogenous DNA encoding green

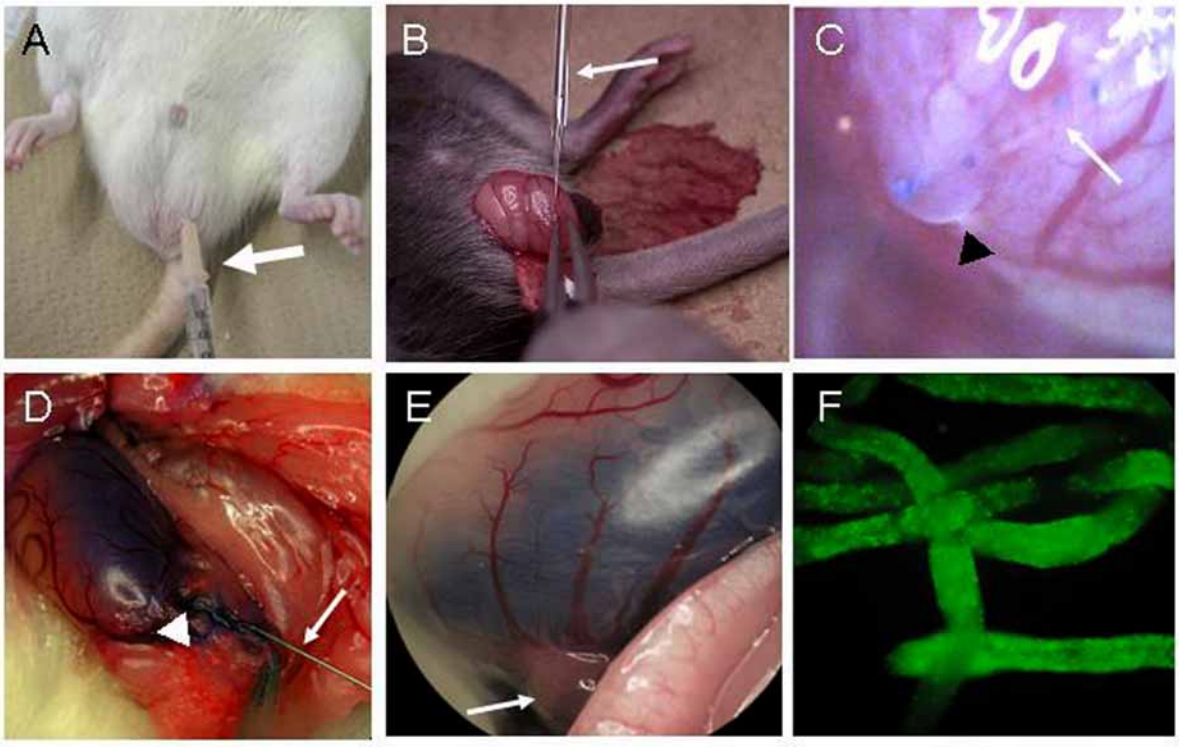


**Fig. (1).** Methods of gene transfer to animal testis and expression of exogenous gene. (A) Intratesticular injection. DNA solution is injected directly into the testis through scrotal skin using a 30-gauge needle and a 1.0-ml disposable syringe (B)(C) Antegrade intratubular injection. A small incision is made in the tunica, and then DNA solution is directly injected into the seminiferous tubules using an injection glass micropipette. (D)(E) Retrograde intratubular injection. An injection glass micropipette is inserted into the rete testis via the efferent duct to allow direct injection into seminiferous tubules. The injection of DNA solution containing Trypan blue dye is useful to confirm the accuracy of the injection and the spread of exogenous DNA into seminiferous tubules. (F) Expression of transgene (GFP) in the seminiferous tubles after adenovirus-mediated gene transfer using retrograde intratubular injection. Large arrows: 30-gauge needle and 1.0-ml disposable syringe Arrows: glass micropipette, black arrowhead: seminiferous tubules, white arrowhead: rete testis

**Table 1. Characteristics of Several Vectors in Testicular Gene Transfer System *In Vivo*.**

| **Non Viral Vector** | | | | | |
|---|---|---|---|---|---|
| | **Naked DNA** | **Liposome** | **Electroporation** | **Polyethylemine** | **HVJ-liposome** |
| **Transgene expression cell** | | | | | |
| Sertoli cell | NA | - | + | - | - |
| Leydig cell | NA | - | + | NA | + |
| Germ cell | NA | - | + | + | - |
| Sperm | + | + | + | NA | NA |
| Expression periods | NA | <1 week | 1-2 months | >1 month | >1 week |
| Production of transgenic animal by natural mating | + | - | - | NA | NA |
| References | Huguet *et al.* [53] | Ogawa *et al.* [56]<br>Yonezawa *et al.* [55]<br>Sato *et al.* [58]<br>Kojima *et al.* [71] | Murmatsu *et al.* [60]<br>Yamazaki *et al.* [61]<br>Umemoto *et al.* [64]<br>Kojima *et al.* [71] | Lu *et al.* [80] | Nakimura *et al.* [84] |
| **Viral vector** | | | | | |
| | **Adenovirus** | **AAV** | **Retrovirus** | **Lentivirus** | **Baculovirus** |
| Transgene expression cell | | | | | |
| Sertoli cell | + | - | - | + | + |
| Leydig cell | + | NA | NA | NA | NA |
| Germ cell | - | - | + | - | - |
| Sperm | - | NA | NA | - | - |
| Expression periods | >2 months | NA | NA | >6 months | NA |
| Production of transgenic animal by natural mating | - | NA | + | NA | NA |
| References | Blanchard *et al.* [91]<br>Kojima *et al.* [49] | Ikawa *et al.* [103] | Kanatsu-Shinohara *et al.* [109] | Ikawa *et al.* [103] | Tani *et al.* [117] |

fluorescent protein (GFP) were mated with normal estrus females, and 7.5% newborns were found positive by PCR for exogenous DNA. In these positive animals, some tissues showed GFP expression [54].

### ***Liposome***

Several researchers have reported testis-mediated gene transfer using the liposome method. As early as 3–4 days after microinjection of the exogenous gene mixed with cationic lipids into the seminiferous tubules, gene expression was observed within both immature and differentiated germ cells. By 40 days post-injection, the gene expression was restricted to the most immature germ cells in the basal portion of the seminiferous tubules [38]. Although transgene was transmitted to morula, blastocyst and mid-gestational fetuses, the ratio of animals carrying the exogenous gene decreased as they developed. Yonezawa *et al.* [55] reported that more than 80% of morula-stage embryos expressed the exogenous gene, but only some of the postpartum progeny were foreign-DNA-positive with a high incidence of mosaicism. Ogawa *et al.* [56] reported that repeated injection with linearized plasmid DNA containing the *β-galactosidase* gene encapsulated with cationic liposome into testis of adult mouse via the scrotum resulted in transmission of the exogenous DNA sequences to blastocysts through fertilization. Overall, 80% of blastocysts derived from mating with males receiving testis-mediated gene transfer were positively stained with X-gal staining for *β-galactosidase* gene expression. Sato *et al.* [57,58] also attempted to transfect testicular spermatozoa with plasmid DNA by direct injection into testes to obtain transgenic animals. When injected males were mated with superovulated females 2 and 3 days after injection, more than 50% gene transmission was achieved in the mid-gestational fetuses. The copy number of exogenous DNA in the fetuses was estimated to be less than 1 copy per diploid cell, and overt gene expression was not found in these fetuses. These findings suggested that plasmid DNA introduced into a testis is rapidly transported to the epididymis and then incorporated by epididymal spermatozoa; however, to generate transgenic offspring with stability, several modifications will be needed.

### ***Electroporation***

Non-viral gene transfer is markedly enhanced by the application of *in vivo* electroporation, since this system delivers exogenous DNA into any type of tissue cell and has mark-

edly higher transfer efficiency than other nonviral transfer systems. As a possible alternative and more effective method for generating transgenic mice, several researchers attempted testis-mediated gene transfer using an *in vivo* electroporation method. After injection of exogenous DNA into the testis, the testis are held between a tweezer-type electrode, and square electric pulses are applied several times at 20–50 voltage [59-64]. The expression activity by applying *in vivo* electroporation gene transfer to mouse testis was approximately 40-fold higher than with the *in vivo* liposome method [59]. Expression of the exogenous gene was clearly observed in spermatogonium- , spermatocyte- and spermatid-like cells of seminiferous tubules at 48 hours after transfecting the mouse testis by *in vivo* electroporation [60]. An exogenous gene was successfully expressed in epididymal sperm when analyzed 40 days after gene transfer and was localized in the head and midpiece regions [65]. Stable gene expression in spermatogenic cells using electroporation would be facilitated by *retroviral integrase* gene co-transfection [66]. We also directly injected DNA, which was constructed as a cytomegalovirus enhancer/chicken β-actin promoter connected with the *β-galactosidase* gene [67], into mouse testes using a square-wave electroporator, and investigated the efficiency of gene transfer. The β-galactosidase activity was detected in germ cells and Sertoli cells in seminiferous tubules and in Leydig cells for 4 weeks [63,64]. More recently, Hibitt *et al.* [68] also reported the gene expression in the germ cells of hamster testis and epididymal sperm by intratubular injection technique using a similar promoter/enhancer system and the same electroporator. However, although electroporation is a simple and convenient technique for gene transfer to germ cells in the testis, the gene expression was deemed transient and uncontrollable [64]. Generally, the gene is likely to be universally expressed in a mixed cell population of a variety of tissue *in vivo* unless tissue-specific promoter genes are used. If spermatogenic cell-specific gene expression is desired, suitable promoters are recommended [60]. The reporter gene driven by the *protamine 1* enhancer region enabled germ cell-specific expression [61]. Wildlak *et al.* [69] performed a functional study of spermatocyte-specific heat shock testis-specific *hst70* gene promoter and compared *in vivo* electroporation gene transfer to testis with a transgenic mice model. Although electroporated testes retained some spermatocyte-specific features, they included the basal activity of constructs which are not transcribed in the testes of transgenic mice, and low overall transfection efficiency [69]. Natural mating with normal adult females revealed that 65% of transfected males maintained fertilizable ability and could generate offspring normally, but no transgenic offspring were detected [62]. ICSI using spermatozoa with a transfected gene could produce transgenic mice [70].

However, the problem of electroporation is that the electric pulses themselves could resulted in testicular damage. In our previous reports, the Johnsen scores were significantly decreased and apoptotic cells were significantly increased in the testis at several weeks after *in vivo* electroporation [63,64,71]. These results suggest that spermatogenic damage caused by electroporation itself could present big problems for clinical application.

Oatley *et al.* [72] evaluated the development of spermatogenesis in ectopically grafted neonatal bovine testicular tissue and investigated the utility of using electroporation to stably transfect spermatogonial stem cells within the graft with an exogenous gene. This technique could potentially be an alternative to testis-mediated gene transfer and pronuclear injection because of the high success rate of stable transgene chromosomal incorporation.

Yomogida *et al.* [73] examined the conditions for the introduction and stable expression of transgenes in Sertoli cells using electroporaltion and investigated the rescue of spermatogenesis in $Sl^{17H}/Sl^{17H}$ mutant mice, which are infertile because of an altered stem cell factor (SCF) cytoplasmic domain resulting from a splicing defect, by gene transfer using electroporation. The transgene was stably expressed in mature Sertoli cells for a long period, although the majority of transgene-positive germ cells soon disappeared. In addition, spermatogenesis was recovered only in seminierous tubules containing Sertoli cells transfected with the complete SCF cDNA, although no pups have been obained from these mice under normal mating conditions. They concluded that these findings support the future application of this method as gene therapy for patients with Sertoli cell dysfunction causing spermatogenic maturation arrest similar to *Sl* mutant mice. Another study showed that the transfer of *erythropoietin* to rat testes by *in vivo* electroporation might reduce the risk of germ cell loss caused by surgically-induced cryptorchidism [74]. To define the transcriptional regulatory elements of the *OAZt* gene promoter, *in vivo* DNA transfer to mouse testes was performed using electroporation [75]. RNA interference (RNAi) against endogenous *Dmc1*, which encodes a DNA recombinase that is expressed and functionally required in spermatocytes, was introduced into mouse testis by electroporation and led to the same phenotypes observed in null mutant mice [76]. Thus, an *in vivo* RNAi system using electroporation may provide a rapid means for assessing the physiological role of the spermatogenetic gene. Gene transfer to mouse testis may enable not only the production of transgenic animals but also detailed analysis of germ cell-specific gene regulation in animals.

### ***Polyethylenimine***

Polymer-based non-viral gene carriers have been used for their merits, including the avoidance of potential immunogenicity, the possibility of repeated administration, and the ease of establishing good manufacturing practice [77,78]. The linear 22kDa form of polyethylenimine (PEI) has been shown to function as an effective nonviral vector for gene transfer [79]. PEI-mediated gene transfer to the testis via seminiferous tubules showed the expression of the exogenous gene in germ cells, especially in primary spermatocytes, but not Sertoli cells, for more than 1 month after transfection [80]. However, since PEI is toxic, the development of new novel gene delivery carriers with high efficiency but less toxicity may be necessary [81].

### ***HVJ-Liposome Vector***

HVJ-liposome vector is a hybrid vector consisting of liposome and an inactivated Sendai virus (Hemagglutinating Virus of Japan [HVJ]), which has been reported to be less immunogenic and can also be administered repeatedly [82]. The HVJ-liposome was a highly efficient vehicle for introducing oligonucleotides into cells *in vivo* as well as for trans-

ferring genes <100 kbp without damaging cells [83]. In the HVJ-liposome-mediated gene transfer to testis, the gene expression was observed in interstitial cells (probably Leydig cells) by direct intratesticular injection, but there was no gene expression in germ cells and Sertoli cells by intratubular injection [84]. Further experiments are necessary before the production of transgenic mice and clinical application.

***Sleeping Beauty (SB) Transposon System***

The SB transposon system combines the advantages of viruses and naked DNA molecules for gene therapy purposes [85]. The SB transposon system directs the precise transfer of specific constructs from a donor plasmid into a mammalian chromosome. It has potential as a randam germline insertional mutagen useful for *in vivo* gene trapping in mice [86]. The activity of SB transposase has been demonstrated in cultured mammalian cells, mouse embryonic stem cells, mouse hepatocytes, the one-cell mouse embryo and the mouse germline [86-89]. Carlson *et al.* [86] demonstrated transposition in the male germline of mice and the transmission of novel inserted transposons in offspring; however, efficacious delivery of DNA molecules to animal tissues can still be problematic [85].

### *2.3.2. Viral Vector*

***Adenoviral Vector***

Adenovirus is an efficient vector for gene therapy due to its ability to infect post-mitotic cells, its high efficacy of cell transduction and its low pathogenicity. Numerous demonstrations of efficacious adenoviral vector-mediated delivery of a wide array of transgenes in several animal species and humans have been reported. Recently, recombinant adenoviruses have provided a versatile system for gene expression studies and therapeutic applications. Of late, there has been a remarkable increase in adenoviral vector-based clinical trials [90].

When the adenoviral vector was tested on Sertoli cell-germ cell cocultures *in vitro*, it efficiently mediated transgene expression in Sertoli cells but not germ cells [91]. Several researchers also attempted to perform adenovirus-mediated gene transfer to animal testis *in vivo* [49,71,92,93]. Intratesticular injection of adenoviral vector resulted in strong transgene expression in interstitial cells, including Leydig cells from seminiferous tubules [49]. By contrast, intratubular injection resulted in transgene expression along the seminiferous tubules, in which strong expression was observed in Sertoli cells. No transgene expression was detected in germ cells by either method [49,91]. Interestingly, transgene expression showed a stage-specific pattern of spermatogenesis, which was expressed predominantly in stage VI–VII of Sertoli cells [91].

Gene expression patterns by adenovirus-mediated gene transfer to testis are particularly interesting and important from the viewpoint of gene therapy for male infertility in the future. Since it is not impossible to introduce a foreign gene precisely into specific chromosomal loci, abnormal sperm could be formed if a foreign gene is introduced into germ cells in the testis. This would increase the rate of offspring with anomalies; however, when the adenovirus vector is used, such problems can be avoided because of its biological characteristic of not introducing genes into germ cells in the testis. Fetuses and offspring produced by mating with males on several days after adenovirus-mediated gene transfer to testis were normal and the number of neonates was not significantly different from that in the control group. None of the fetuses and offspring showed any abnormalities, and the transgene was not detected by RT-PCR and histochemical study [49,92]. These results demonstate that the risk of germline transmission following adenovirus-mediated gene transfer to the testis is extremely low [92].

There are two problems concerning transfection with the adenoviral vector, i.e., cytotoxicity as a general biological characteristic and the immune response [94-96]; however, although apoptosis was detected in some spermatocytes and a slight immune response was observed for a short period, it was temporary. The testis did not become highly atrophic, severe deterioration of the tubules was not detected, sperm motility was not lowered and sperm abnormalities were not observed [49]; therefore, transfection by adenoviral vector was not considered to have any adverse effect on spermatogenic function as a whole. Adenovirus-mediated gene transfer to the testis has several advantages and can be exploited for the treatment of male infertility in the future.

Some studies have been reported about the effect of gene transfers to an animal model testis on spermatogenesis using adenoviral vector *in vivo*. Transfer of the tumor suppressor protein *p53* gene to rat seminiferous tubules by adenoviral vector using the retrograde technique impaired spermatogenesis [97]. On the other hand, adenovirus-mediated *hepatocyte growth factor (HGF)* gene transfer into the testis in cryptorchidism rats inhibited germ cell apoptosis and restored spermatogenesis [98]. To explore the therapeutic usefulness of the *HST-1/FGF-4* gene, which could be an important factor for spermatogenesis, in impaired spermatogenesis, adenovirus carrying the *HST-1/FGF-4* gene was administered into the testis of mice after adriamycin treatment, and *HST-1/FGF-4* gene transfer had a protective effect on experimentally induced testicular toxicity [99].

To examine the feasibility of adenovirus-mediated gene transfer to spermatogonial stem cells, an adenovirus expressing the *Cre recombinase* gene was used to infect an enriched population of spermatogonial stem cells *in vitro*, and infected cells were transplanted into the seminiferous tubules of infertile animals. The infected spermatogonial stem cells could reinitiate spermatogenesis after transplantation into seminiferous tubules of infertile recipient testes [93]. Advanced molecular techniques may enable the realization of adenovirus-mediated gene transfer to germ cells in the testis and to generate transgenic animals.

***Adeno-Associated Viral (AAV) Vector***

The AAV has rapidly gained popularity in gene therapy [100,101]. With the recent discovery of novel AAV serotypes, there is one preferred serotype for nearly every organ or tissue to target. Thus, AAV-based vectors can successfully overcome the main gene therapy challenges such as transgene maintenance, safety and host immune response, and meeting the desirable vector system features of a high level of safety combined with clinical efficacy and versatility in terms of potential applications [102]; however, no gene

expression could be found in any cell of the testis after AAV-mediated gene transfer to the testis [103].

***Retroviral Vector***

Retroviral vectors based upon simple gammaretroviruses, complex lentiviruses, or potentially nonpathogenic spumaviruses represent relatively well-characterized tools that are widely used for stable gene transfer because of their efficiency and precision of integration [104,105]. In the testis injected with retroviral vector into seminiferous tubules via rete testes, no cells with gene expression could be found [103]; however, other researchers reported that retroviral vector was a powerful tool to transfer exogenous genes into the germ cell line and spermatogonia *in vitro* [106,107]. Retrovirus-mediated gene delivery into spermatogonial stem cells *in vitro* resulted in stable integration and expression of a transgene [52,108]. After transplantation of the transduced stem cells into the testes of infertile recipient mice, 4.5% of progeny from these males were transgenic, and the transgene was transmitted to and expressed in subsequent generations [51]. In addition, microinjection of a retroviral vector expressing the *β-galactosidase* gene into immature seminiferous tubules resulted in the direct transduction of spermatogonial stem cells, and the animals produced transgenic offspring after mating with females [109]. These experiments may have important implications for the improvement of transgenic animal production, but may be not useful for clinical application.

***Lentiviral Vector***

Lentiviruses, members of the retroviral family, have the ability to infect cells at both mitotic and post-mitotic stages of the cell cycle, thus opening up the possibility to target non-dividing target cells and tissues [110,111]. Testes transduced with lentiviral vector expressed the trangene without impairing spermatogenesis. Expression patterns were characteristics of Sertoli cells and gene expression was observed over a period of 6 months [103]. Although spermatogenesis can be resored in $Sl/Sl^d$ mutant mice by transducing Sertoli cells with lentiviral vectors generating a functional *c-kit* ligand, KL2, an insufficient number of spermatozoa for IVF were collected. Sperm collected from recipient testes were able to produce normal pups after intracytoplasmic sperm injection. None of the offspring carried the transgene, suggesting the inability of lentiviral vectors to infect spermatogenic cells *in vivo* [103].

However, when cultured spermatogonial stem cells with the lentiviral transgene were transferred to recipient male rat testis, about 60% of pups were derived from these cells and about 30% of pups carried the lentiviral transgene after mating [112]. Another group also reported that male germ line stem cells could be transduced *in vitro* by a lentiviral vector and generate complete spermatogenesis when transplanted into infertile host testes [113]. Lentiviral transduction of spermatogonial stem cells followed by transplantation may be an easy and effective method to produce transgenic animals [114]. Lentivirus-mediated gene transfer *in vivo* may be a good candidate for clinical application in the future because there is no germ cell transmission, at least *in vivo*; however, since the discrepancy between *in vivo* gene transfer and *ex vivo* gene transfer to the testis using lentiviral vector remains unknown, further study will be required before application for clinical use.

***Baculoviral Vector***

A relatively recent advance in the use of recombinant baculoviruses is their use for the delivery of genes and genetic elements into mammalian cells. Baculovirus-mediated gene transfer is simple to perform, reproducible, and demonstrates no overt cell toxicity, and is particularly useful for repetitive or moderately high-throughput procedures such as cell-based assays, or for situations where transfection procedures are inadequate [115,116]. Baculovirus-mediated gene transfer to the testis via intratubular injection resulted in the gene expression in Sertoli cells but not in spermatocytes or sperm [117]. This vector may also be a useful tool for clinical application for male infertility treatment although further investigations are needed.

### 2.4. Testis-Mediated Transgenesis

To generate transgenic mice more efficiently, some researchers have attempted to directly inject exogenous DNA into mouse testis instead of sperm-mediated gene transfer. This technique has the advantage of being able to produce transgenic mice more simply by mating with wild-type females, which avoids the possibility of interference or damage as a result of assisted fertilization or the manipulation of embryos. Germ cells are target cells for generating transgenic animals. Naked DNA has already been realized to generate transgenic offspring. Liposome, electroporation and polyethylenimine may have the possibility to generate transgenic animals in the near future. In addition, retroviral vector may be a more appropriate vector; however, further modification will be needed to establish a simpler and more efficient method to generate transgenic animals.

### 2.5. Possibility of Clinical Application of Testis-Mediated Gene Transfer for Male Infertility

If spermatozoa exist in the testis of infertile men, logically, there is a possibility of paternity using TESE-ICSI. On the other hand, if there are no spermatozoa in the testis in male infertility patients, as a result of such as pathological maturation arrest or Sertoli cells only, they do not have paternity potential even if TESE-ICSI is conducted. In addition, fertilization is better *in vivo* than *in vitro* with male infertility. Clinically, testicular gene therapy may be useful to treat male infertility in the future because it is possible to promote spermatogenesis in male infertility patients diagnosed with pathological hypospermatogenesis, maturation arrest or Sertoli cells only. Since testicular cells play a significant role in creating life and personality, it is very important to understand the safety issues and to control the expression of transferred genes [118]. Numerous difficult hurdles remain to be overcome in the clinical application of gene transfer to testis under the present conditions.

#### *2.5.1. Ethics of Future Gene Therapy for Male Infertility*

Generally, the protection of human subjects is the ethical and legal responsibility of scientists and clinicians conducting clinical trials [119]. There are several limitations, including ethical aspects, regarding research on genetic manipula-

tion in human subjects. Although biomedical science and engineering have made rapid advancements in the field of reproductive medicine over the past few decades, we can not overlook the ethical responsibility to the patient and society when we perform gene therapy for patients with infertility. The basic common consensus throughout the world is that somatic gene therapy can be applied to treat a wide range of disorders, including cancer and inherited disease [120]. The ethical issues in somatic gene therapy are primarily concerned with the risk of this procedure, such as vector toxicity and oncogenesis. On the other hand, although germline gene therapy is theoretically possible, it is rejected on the grounds that it would be possible to affect future generations without precise understanding about the mechanism and control of gene expression [118]. At present, we should not make exceptions to the standing ethical rules in future gene therapy for male infertility and consider appropriate gene therapy that avoids gene transfer to germ cells.

### *2.5.2. Appropriate Future Gene Therapy for Male Infertility*

As described above, there are two possible methods of gene transfer to the testis, *in vivo* and *ex vivo* gene transfer for clinical treatments (Fig. **2**).

*In vivo* gene transfer to the testis is considered to be an easier, more efficient and practical method for clinical application. Since somatic cells in the testis are Sertoli cells and Leydig cells, on the basis of ethical issues, they are the only appropriate target cells for gene therapy for male infertility. Viewed from this point, adenoviral- or baculoviral-mediated gene transfer may be effective for transfecting testicular somatic cells, Sertoli cells or Leydig cells, because they can not introduce exogenous genes into germ cells, and are applicable vectors for *in vivo* gene therapy for male infertility in the future [71,118]. Intratubular injection (antegrade injection or retrograde injection) is a useful technique to introduce genes into Sertoli cells, while intratesticular injection may be useful for Leydig cells.

Previously, we directly injected DNA into mouse testes *in vivo* using three methods, liposome, electroporation and adenovirus vector, to transfect testicular cells using the same exogenous DNA, and compared the three methods for transfection efficiency and effects of gene transfer on spermatogenesis to identify the most appreciate method for future clinical application [71]. Although liposome and electroporation methods were simple, they had low efficiency, short duration of the physiological effect and high spermatogenic damage in comparison with adenovirus-mediated gene transfer. The disadvantage of the liposome and electroporation methods for clinical application was that they showed potential for gene expression in germ cells and strong adverse effects on spermatogenesis. Our results suggest that adenovirus-mediated gene transfer may be a more effective and superior method for transfecting testicular cells among these three methods and may be more applicable for *in vivo* gene therapy for male infertility in the future [118].

*Ex vivo* gene transfer to the testis is another possible method of gene therapy for male infertility. The development of spermatogonial stem cell transplantation techniques provided access to this cell and the opportunity to manipulate the male germ line, because during the transfer of donor stem cells to the recipient testis, they can be genetically modified [51,121]. In particular, gene transfer to spermatogonial stem cells enables the generation of transgenic animals [51]. Using this technique, testicular cell autotransplantation after gene transfer may have great potential as an *ex vivo* system

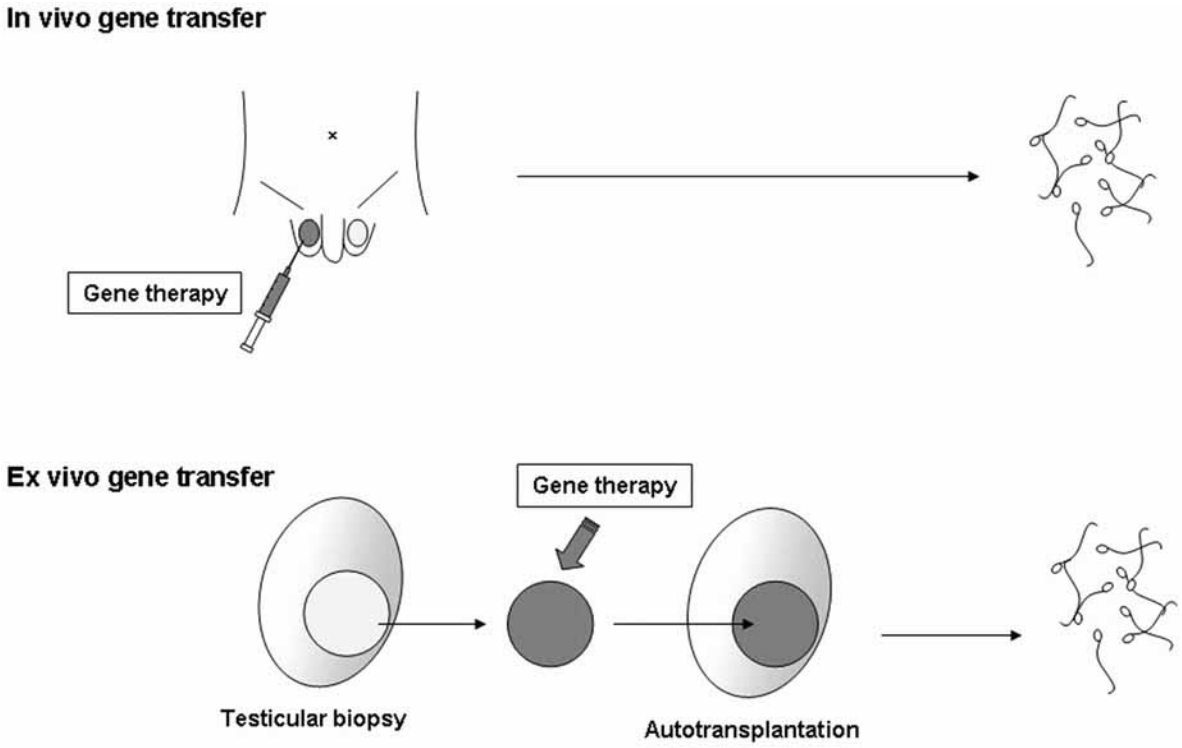


**Fig. (2).** Strategy of gene therapy for male infertility in the future

for the delivery of therapeutic proteins to the testis to treat male infertility (Fig. **2**). Establishment of an *ex vivo* gene transfer system using Sertoli cells and Leydig cells is desirable for clinical application.

### *2.5.3. Subjects Receiving Gene Therapy in the Future*

The causes of male infertility are multiple and varied. Based on the results of patient history, physical examination and laboratory testing, patients with male infertility are placed into etiologic categories [122]. These categories of male infertility include idiopathic, varicocele, cryptorchidism, obstruction of spermatic ducts and endocrine disorders. ICSI has emerged as the first-choice treatment for idiopathic male infertility, while several other treatments are usually effective in patients with varicocele, cryptorchidism and endocrine disorders.

Varicocele is the most common diagnosis in men attending fertility clinics, and surgery or percutaneous embolization are two possible treatment options [123]. Male infertility is considered the principal consequence of cryptorchidism, and orchiopexy is one of the most frequently used surgical procedure for boys with cryptorchidism, which has been shown to have a beneficial effect on future fertility [124, 125]. Hypogonadotropic hypogonadism is an uncommon cause of male infertility, and hormone therapy is an appropriate way to initiate testicular growth, including fertility and virilization [126]; however, these treatments do not always guarantee subsequent fertility and paternity [125]. Although ICSI is sometimes a treatment option for these patients [127,128], gene therapy may be good indication for patients with not only idiopathic male infertility but also varicocele, previous cryptorchid and endocrine disorders in the future [129] (Table **2**).

### *2.5.4. Candidate Genes of Future Gene Therapy for Male Infertility*

Spermatogenesis is a complex program of differentiation and takes place within the testicular seminiferous tubules, which are composed of germ cells and somatic Sertoli cells [48,130]. Two control mechanisms are involved in the regulation of spermatogenesis; hormone and genetics. Sertoli cells trigger germ cell development by not only mediating hormonal stimuli of FSH and androgen secreted by Leydig cells, but also numerous genes. Understanding the mechanisms by which genes and gene products are expressed or repressed in the testes enables the development of strategies to treat male infertility. At least 150 candidate male fertility-associated genes have been revealed from knockout mice phenotypes [131,132]. It is very important to perform detailed phenotypic analysis in order to extract the maximum amount of information from each model.

Several possible genes may be essential for Sertoli and Leydig cell function to support normal spermatogenesis. Testicular gene therapy for these cells as target cells may be useful to treat male infertility in the future, especially for those who have no spermatozoa in their testes at all, such as pathological maturation arrest or Sertoli cells only. From our viewpoint, Sertoli cells are more appropriate target cells, because they perform crucial functions that initiate, promote and maintain spermatogenesis directly in seminiferous tubules.

Disorders of testicular function may originate as a result of abnormal development or proliferation of Sertoli cells [133]. Idiopathic male infertility, including pathologically maturation arrest and Sertoli cell-only syndrome, may result in abnormal Sertoli cell function, which disrupts the normal progression of spermatogenesis [134]. Marker genes of maturity and immaturity of Sertoli cells include *anti-Müllerian hormone*, *vimentin*, *connexin 26*, *connexin 43*, *aromatase*, *cytokeratin-18*, *GATA1*, *laminin* $\alpha$*5*, *M2A antigen*, *p27*$^{kip1}$, *inhibin* $\alpha$, *sulphated glycoprotein 2*, *androgen receptor* and *WT-1* [48,133]. In addition, alterations of transcription regulation in the testis may cause idiopathic male infertility [134,135]. Transcription factors include *CREB* [136], *Sox3* [137], *Pem* (*Rhox5*) [138] and *DAX1* [134,139], which are essential for spermatogenesis and are expressed not in germ cells but in Sertoli cells of seminiferous tubules. Clarifying the role of these factors in spermatogenesis may advance the clinical application of gene therapy for patients with male infertility.

On the other hand, Leydig cells may also be target cells of gene therapy. Hypotheses about the underlying mechanism of the depletion of germ cells in Klinefelter syndrome males, which is the most common sex chromosome abnormality and the most frequent genetic cause of male infertility, include Leydig cell insufficiency and disturbed apoptosis regulation of Leydig cells [140]. There is also evidence of Leydig cell impairment in a proportion of men treated with cytotoxic chemotherapy for malignant desease [141]. Endocrine disruptors can disrupt not only spermatogenesis but

**Table 2. Future Prospects of Gene Therapy for Male Infertility**

| | **Present Treatment** | **Future Treatment** |
|---|---|---|
| Idiopathic male infertility | TESE-ICSI | Gene Therapy |
| Varicocele Cryptorchidism | Surgery<br>and/or<br>TESE-ICSI | Surgery<br>and/or<br>Gene therapy |
| Endocrine disorders | Hormone therapy<br>and/or<br>TESE-ICSI | Hormone therapy<br>and/or<br>Gene therapy |

also normal endocrine function of Leydig cells, acting directly on Leydig cells to diminish testosterone production by interfering with the expression of specific genes in the steroidogenic pathway [142]. Marker genes, including *P450 (CYP) steroid hydroxylase, 3β-HSD* [143], *Androgen receptor* [144], *LHR* [145], *Ad4BP/SF-1* [134,146,147] are expressed in Leydig cells, which are implicated in steroidogenesis or spermatogenesis. They may also be candidate genes for gene therapy in the future.

Genetic causes currently account for only 10–15% of severe male infertility, including chromosomal abnormality, Y chromosome deletions and single gene mutations [148]. There is increased interest in using genetic markers such as single nucleotide polymorphism to discover the genetic causes and risk factors of male infertility [149-151]. Microarray technology will offer a novel tool for the diagnosis and identification of therapeutic targets for male infertility [152]. Our limited understanding of the complex regulatory mechanisms underlying normal spermatogenesis makes it difficult to identify specific target genes for gene therapy. More genetic information about the molecular basis of spermatogenesis and male infertility will be needed to apply gene therapy for male infertility patients, especially for Sertoli or Leydig cell dysfunction in the future.

## CONCLUSION

With the progress of genetic engineering, hereditary diseases are being studied at the gene level. Not only is molecular technology being used for diagnosis and pathological studies, but attempts are also being made to use the technology in therapy. Actual trials of gene therapy for certain cancers and hereditary diseases have already been reported. New techniques are also being applied in the field of reproduction research using animal models, and gene therapy is expected be clinically applicable for treating male fertility caused by impaired spermatogenesis in the near future. Compared with other organs, gene transfer into the testis requires careful consideration, particularly for biological and ethical reasons. Although more investigations into the mechanism of spermatogenesis and male infertility, and the establishment of techniques for more efficient and safer gene transfer will be needed for clinical application, gene therapy will enable revolutionary advances in reproductive treatment and provide great benefit for patients with male infertility in the future.

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## ABBREVIATIONS

ICSI = Intracytoplasmic sperm injection

IVF = *In vitro* fertilization

TESE = Testicular sperm extraction

MESA = Microsurgical epididymal sperm aspiration

PFU = Plaque-forming units

AZF = Azoospermia factor

FSH = Follicle-stimulating hormone

LH = Luteinzing hormone

GFP = Green fluorescent protein

SCF = Stem cell factor

RNAi = RNA interference

PEI = Polyethylenimine

HVJ = Hemagglutinating Virus of Japan

SB = Sleeping Beauty

HGF = Hepatocyte growth factor

AAV = Adeno-associated viral

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