Translational Clinical Biology

eISSN 2330-2135

Journal home page at www.VedicJournals.com

Review

DOI: http://dx.doi.org/10.142598/tcb.v2i2.137

The Role of Recombinant Sperm Technology in Enhancing Mammalian Transgenesis

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Article Info: Received: *May 7th, 2014*; **Revised:** *May 25th, 2014*; **Accepted:** *May 28th, 2014*

Abstract

In the field of animal transgenesis, many attempts have been made recently to simplify facilitate, and reduce the cost and labor required to do such tasks. Although several transgenesis techniques (such as DNA microinjection and somatic cell nuclear transfer) have been applied successfully to produce transgenic animals, these traditional techniques are so tedious and have several disadvantages. Retroviral mediated gene transfer has solved some of these usual problems but has, however, inevitable disadvantages represented most prominently by its biological hazard. Many researchers found that the simplest and the most non-cost effective way to produce transgenic animals are to focus on the natural ability of the sperm to "carry" the foreign DNA and to "fertilize" the oocyte. The most important breakthrough obtained in this aspect is the accumulated information that demonstrated the ability of foreign DNA to be internalized into the sperm head after simple incubation step. Accordingly, the only manipulation step is restricted into the head of the sperm. Then, nature will be allowed to fulfill its scheduled task of reproduction. This method is known as sperm mediated gene transfer or SMGT. However, simple incubation of naked DNA with sperm head is not efficient enough to integrate the foreign DNA into the genome of the sperm. Thus, this review aims to pave the way for every effort to enable the researchers to undergo the transgenesis experiments in the routine laboratories. This, potentially, can be done by testing the validity of the most modern enhancement approaches suggested on the original SMGT.

Keywords: *Transgenesis, Recombinant, Sperm, Mammals, Technology, DNA, Genome*

Introduction

Transgenesis is a dramatic line of technology for altering the characteristics of animals by directly modifying the genetic material. In general, it is as a procedure by which a gene or part of a gene from one individual is incorporated in the genome of the other one [1]. It can be identified as a mere transfer of an exogenous gene into a host genome [2]. However, whatever the technique used to generate the transgenic animal, the general goal of transgenesis remains the same, which is "to add foreign genetic information to a genome" [3].

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The generation of transgenic animals is a cumbersome process and remains problematic both in its methodology and impact [4]. However, there are several methods and modern approaches of inserting a transgene in the mammals have been reviewed in [2,5,6], The most currently used techniques include pronuclear microinjection, retroviral mediated gene transfer, somatic cell nuclear transfer, and ovary mediated gene transfer but a special emphasis of the present review is sperm mediated gene transfer (SMGT), which is considered the simplest and the most cost effective technique

The original idea of SMGT was conceived from the observation that simple incubation of ejaculated sperm cells with the exogenous DNA was sufficient to transfect these cells, then all the subsequent steps are mimicry of nature.

ISSN 2330-2135 (Online); Translational Clinical Biology, VRI Press, Vedic Research, Inc. USA

Thus, this method is the simplest one in that manipulation steps are restricted to the transfection of sperm cells, and then natural physiological processes would fulfill subsequent events [7,8].

Practically, SMGT can be simplified by the incubation of either frozen or freshly collected sperm cells with DNA, for however short period of time, the exogenous DNA suspension at 37 to 39°C in a suitable fertilization medium. During this time, the exogenous DNA may penetrate the sperm cells (Figure 1). The resultant transfected spermatozoa are introduced into oocytes either *in vivo* or *in vitro* [9].

The process of exogenous DNA integration into the sperm head is very crucial step [10]. Traditional SMGT experiments are potentially characterized by lack of reproducibility [11]. However, the SMGT technique in mammalian systems is still controversial, since the viability of sperm outside the reproductive tract is not long enough to allow manipulation for efficient transfection [12].

Since seminal fluid contains many inhibitors of exogenous DNA, the removal of sperm cells natural protection medium introduces many variable factors which may contribute in the efficiency of this technique [13]. Such factors and species variability have led researchers develop methods to modify some conditions before conducting SMGT experiment.

Several enhancements have been made in the original SMGT method (Figure 2). These enhancements vary in complexity from simple chemical reaction to the sophisticated steps that require special skills and devices such as intracytoplasmic sperm injection (ICSI) and artificial chromosomes [14-17]. But the latter are associated with high cost of transgenesis technique since they utilize micromanipulators and, may only be applicable in laboratories with specialized equipment [8]. In this review, the following seven SMGT approaches are highlighted as most significant enhancements (Figure 2).

Testis Mediated Gene Transfer (TMGT)

Some scientists describe TMGT as an alternative and independent technique from SMGT [13], but others consider it as just a modification or simple variation of it [8,18], because, in both cases, the purpose is gene transfer into sperm cells. TMGT is still under development but the process can be simplified by direct injection of the transgene into

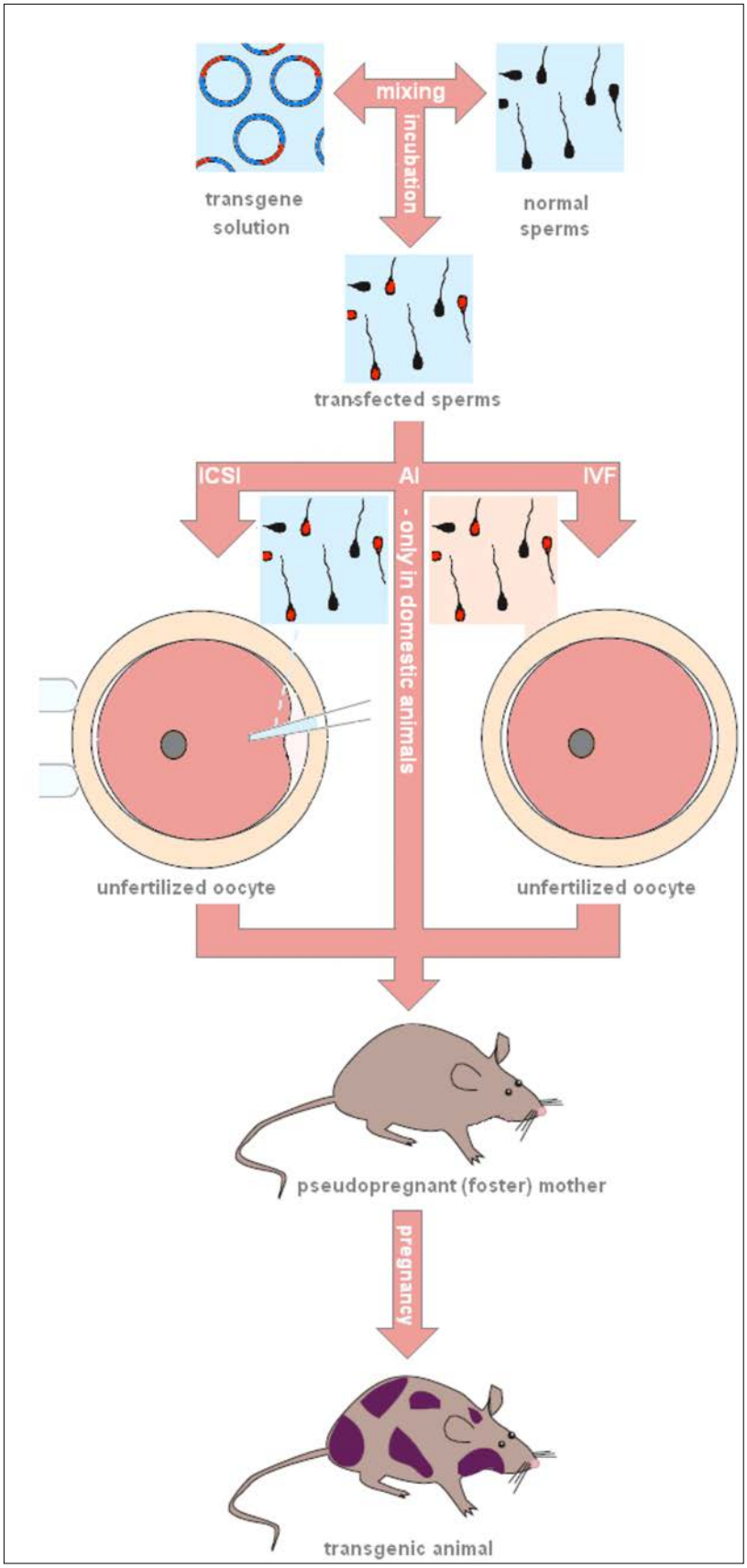

Figure 1: Illustration of sperm mediated gene transfer in transgenic animals. Simple incubation of sperm cells with exogenous DNA may permit the DNA to be inserted into the sperm cell. Then, this transfected sperm is used to fertilize oocytes either in vitro, such as in IVF, ICSI, or in vivo, such as in AI.

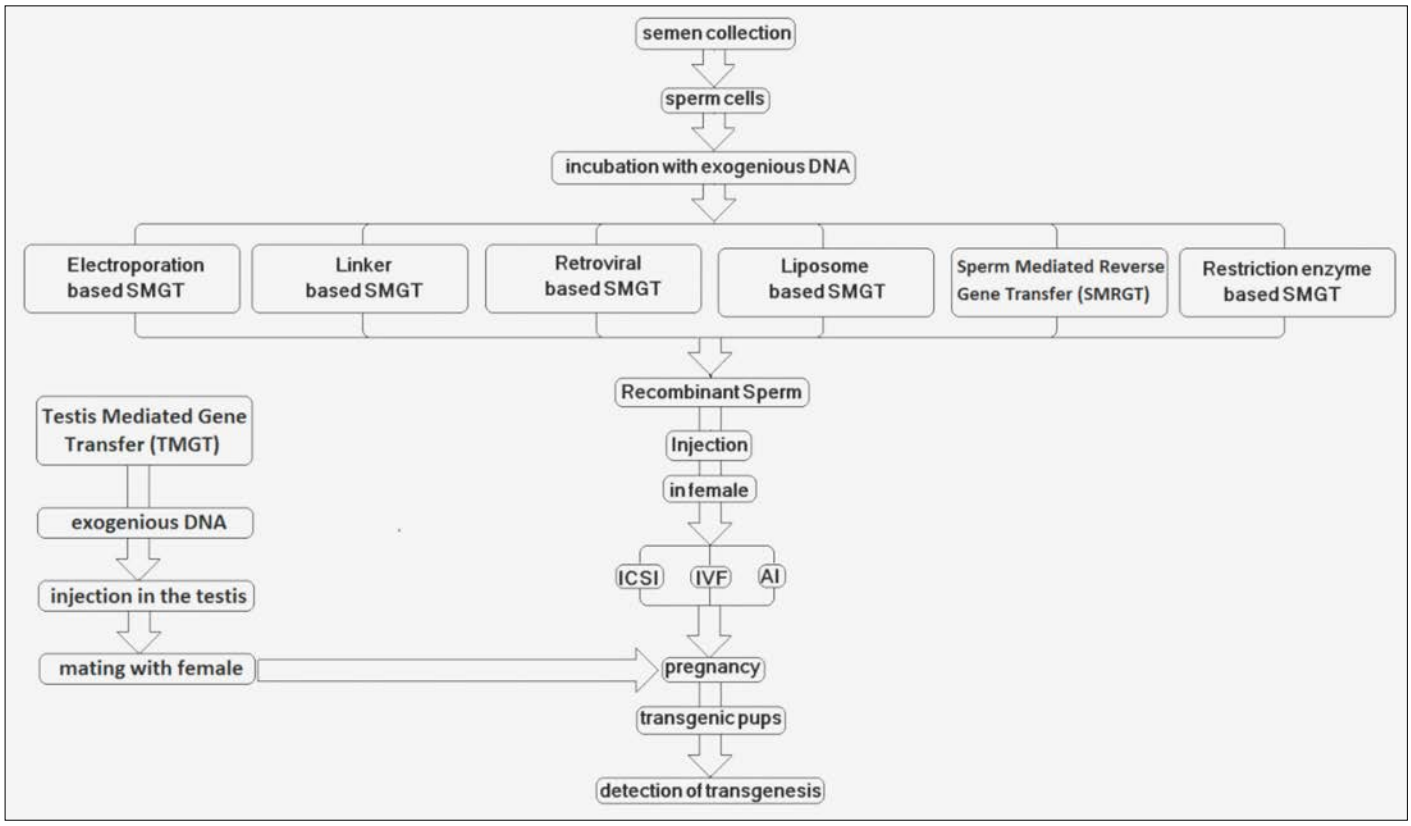

Figure 2: shows the details of the most notable types of SMGT which they increased the efficiency of gene transfer through sperm vector to a significant extent. The variable routes of transgenesis that be taken place are *per se* differ in their efficiencies.

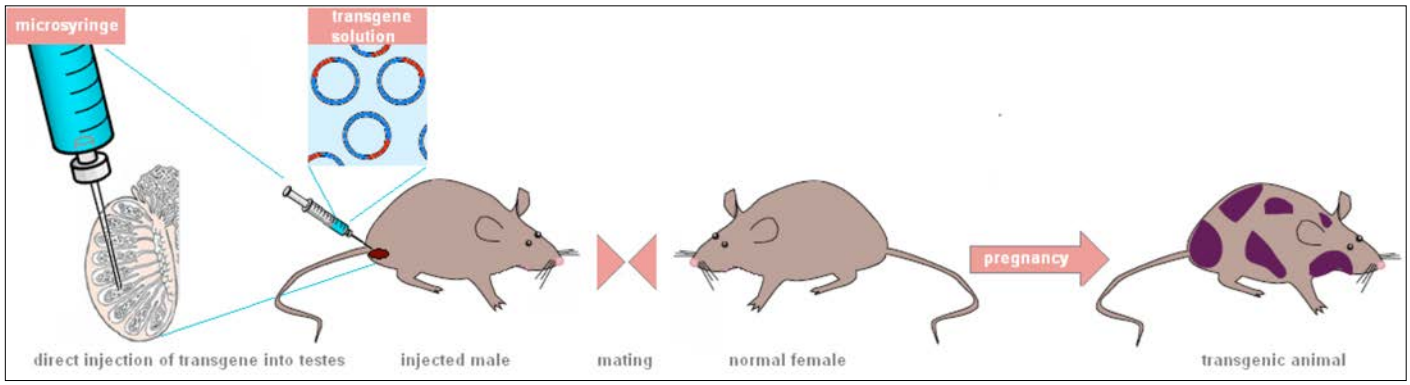

Figure 3: Illustration of testis mediated gene transfer (TMGT) technique. The injection of a transgene is done on the corner of testes near the caput epididymis to a depth 5-6 mm. Then, the male mice that have the recombinant gene are mated with normal female in order to transport the transgene from the testes of male to the oocyte of female. After natural mating followed by pregnancy period, the potentially expected transgenic offspring are generated.

testes (Figure 3).

TMGT not cost effective, low in technical demand, not require special techniques and equipments, easy to be understood since everything is natural except the recombinant testes that have the directly injected transgene [13,19]. Consequently, it become obvious that TMGT, as a derivative of the original SMGT, provides extreme simplicity compared with other SMGT derivatives represented by the absence of need for any manipulations either in sperm or oocytes [10].

The success of many groups in producing transgenic transgenic offsprings by TMGT doesn't mean that this method has an explicit ability of integrating the transgene into the genome. Rather, [20] discovered the episomal state of the transgenic mice generated by this method. This suggests that the plasmid they injected was diluted out along with cell proliferation [21]. Although the integration ability of the transgene into the host genome was confirmed in TMGT, a high incidence of mosaicism was observed [22].

Electroporation based SMGT

Electroporation is a technique by which a series of short electric pulses are conducted by gene pulser device to generate transient pores in the cell membrane to allow the transgenes to enter the cells (Figure 4). These electrically induced pores have the ability to be resealed spontaneously to get the transfected cell back into its normal state [23]. Thus, the purpose of introducing electroporation in SMGT is, *per se*, to enhance the rate of DNA uptake by sperm cells [8,24].

There are several major benefits of this method, which can be considered as such as the method is fast, less costly than microinjection and somatic cell nuclear transfer (SCNT), large number of cells can be treated, and high percentage of transfected sperm cells can be produced [23]. Several papers demonstrated the ability of electroporation to increase DNA integration ratio into the DNA of spermatozoa [7] and the technique is advocated to have a promising future [7,10,25-27].

This method, which it also named "electrogene therapy", is a safe method because it does not require viral vehicles, consequently, there is a high and promising ratio to apply this method on gene therapy [27]. At the same time, it was noted that this method of gene transfer may avoid several limitations and low transfection efficiency noticed in other methods [26].

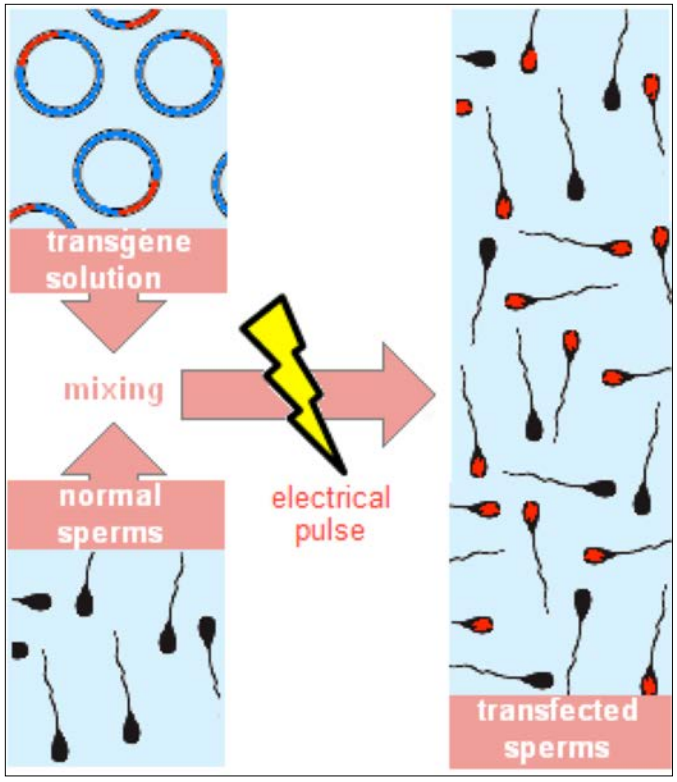

Figure 4: Illustration of electroporation based SMGT technique. Sperm cells are infected with the transgene solution with the aid of electrical pulse applied from an electroporator device.

Despite the ability of this technique in increasing the uptake of exogenous DNA to spermatozoa and its increased efficiency in SMGT [28], but, the increased electrical field strength had a deleterious effect on cell motility, causing clumping of spermatozoa at high voltages, so, this method require a careful optimization [29]. Additionally, high embryo lethality – despite its high transfection efficiency – is also associated with this technique [30].

Commercially available electroporators are not readily available due to their high cost (more than $40 000); hence high cost makes these devices unavailable at any routine labs.

Linker Based SMGT (LB-SMGT)

In this approach, researchers use special molecules that are recognized by cellular receptors, such as antibodies, peptides, and proteins. They are connected with exogenous DNA to form complexes able to penetrate cellular membrane through receptor mediated endocytosis [31].

There are several manufactured peptides which have potential ability to play crucial role in this approach [32]. The most popular peptides are cationic peptides; the peptides rich in positively charged amino acids such as lysine and argentine since they counteract the negative charge of DNA molecules. This neutralization of the DNA charge abolishes the repulsion forces in DNA and packs it closely [23].

Another extremely interesting utilization of LB-SMGT came from the work done by [33]; they used positively charged monoclonal antibody and bound it with DNA through ionic interactions (Figure 5). The antibody used by this group in mice and pigs was recognized by sperm cells and receptors of other mammalian species in precise manner [34].

Chang and his group firmly demonstrated that linker-based SMGT can be used to generate transgenic animals efficiently in many different species, especially in the farm livestock [33]. While others supported this results considering it as an effective way to improve the efficiency of SMGT [34].

However, to date, there are only few publications on this approach, so, it is still not clear how far this technique is applicable keeping in mind the applicability of using "a common ligand" which has the ability to recognize the variable receptors in variable species.

Retroviral based SMGT

One of the most promising areas used in the enhancement of the original SMGT is the retroviral-based method. In this method, the most important derivative of retrovirus, i.e., lenti virus is used as a high efficiency vehicle to facilitate the delivery the exogenous DNA into the head of the sperm cells (Figure 6). Recently, some reviewers shed light on several useful retroviral

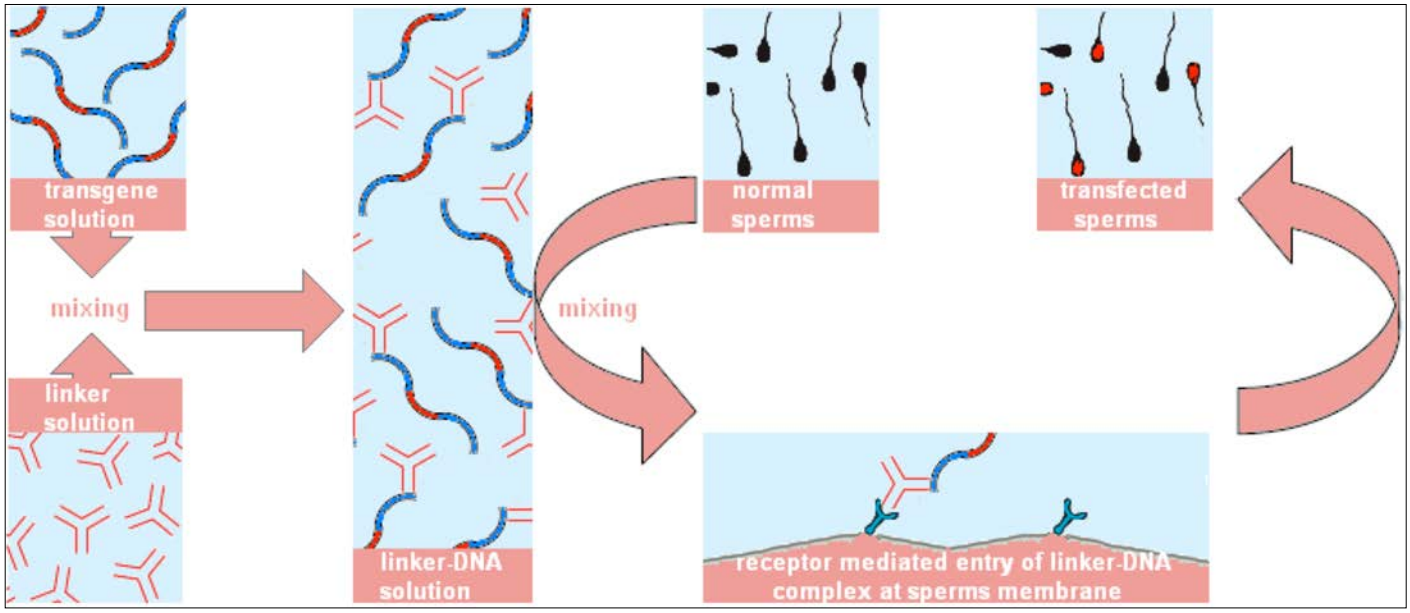

Figure 5: Postulated illustration of linker based SMGT; after the binding of linker, such as antibody, with transgene it is recognized and internalized by specific receptors found at the surface of sperm cells.

based approaches that have been applied on SMGT [13].

The main advantages of using RMGT arise from the stability of the integration of the viral genome into the host and to the technical feasibility of introducing a virus to embryos at several developmental stages [9,23]. These vectors are particularly characterized by their ability to be applied as suitable gene vehicles in that they infect a variety of cell types and introduce genes at high efficiency [35]. The ability of retroviruses to be integrated naturally into target cell genome provides a powerful tool for stable transfer of the gene of interest [36]. It makes gene transfer possible for species from which newly fertilized eggs cannot be readily obtained [12].

In contrary with pronuclear microinjection which is very inefficient in livestock, RMGT has two interesting advantages make it very appealing for use in livestock. The first one, only a fraction of the resources needed for conventional pro-nuclear injection would be required, while the second is the simplicity of delivery, abolishing the need for specialized equipment [37]. Furthermore, Molecular genetic analysis of transgenics produced by RMGT usually show integration of a single proviral copy into a given chromosomal site, and the rearrangements of the host genome are normally confined only to the short direct repeats at the site of integration [38], while in pronuclear microinjection the transgene may integrate in a more randomized manner [39]. In addition, the method is less invasive to the embryos, and technically less demanding. Delivering lentiviruses by co-incubation with denuded embryos obviates the need for micromanipulation and may be an easier option for many laboratories wanting to make transgenic animals. Furthermore, since the lentiviral delivery technique does not require visualization of the pronucleus, it has the potential to be extended to diverse mouse strains, as well as other animal species [38].

Despite many advantages that characterize RMGT, it is difficult to claim that this technique is "absolutely" the best one among other transgenesis technique [7] because of several disadvantages of RMGT.

These disadvantages include: 1) the size of DNA to be transferred is limited by size, 2) the inserted gene doesn't have the ability to express on itself in the second generation, which may, in turn, complicate the method. 3) Many transgenics are mosaic, with potentially multiple insertion sites [9], and (4) several safety concerns from using retroviruses [40].

The capacity of retroviral vectors to carry the transgene is not enough to provide the space required to transfer the DNA fragment wanted in transgenesis [41]. Size restriction imposed by the lent viral genome represents the most obstacles towards using this virus as vehicles for gene transfer. In such away, wild-type lent viruses have a genome of about 8 kb, and the genetic load of these viruses (comprising the internal promoter, transgene and enhancer elements) should therefore be less than this size [42].

The second potential disadvantage of RMGT is the complexity of the process as a consequence of the absence of transgene expression [43]. Though "introducing" viral particles to oocytes requires the least complicated embryo manipulation, but the packaging transgenes into virions takes many steps. For any gene transfer approach taken place through RMGT the transgene, both its structural and regulatory portions, must be built properly before proceeding to the next steps. Then the transgene must be introduced into the proviral genome by standard molecular cloning methodologies. The modified

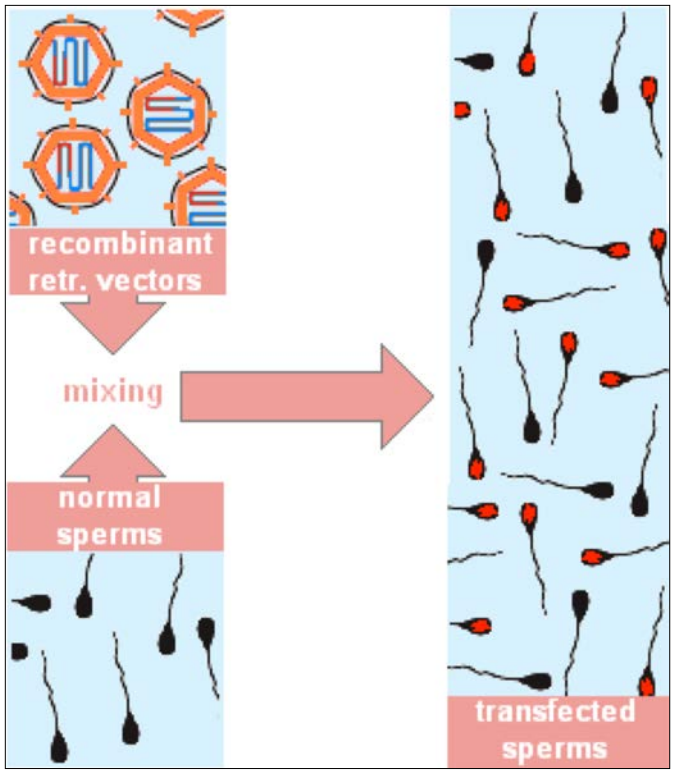

Figure 6: Retroviral based SMGT. This illustrated step is represented by infecting sperm cells with recombinant retroviral vectors.

proviral genome is then transfected into the packaging cells, and the packaging cells should be grown to produce the recombinant viruses [7]. Thus, the preparation of retroviral particles including the transgene of interest is a very laborious process, which may increase costs and requires more sophisticated technology [12].

Generally, transgenic animals are mosaic and the transgenes are not always expressed in the second generation [4]. Infection of early embryos with retroviral vectors resulted in genetic mosaics represented by multiple insertion sites in different tissues [44]. Retroviruses sometimes integrate within genes, which become inactivated. Repeated inserted sequences also modify gene activity when they are in their vicinity or within those genes [3].

The safety problems associated with retroviral vectors would not be omitted with respect to the disadvantages of RMGT [40,45], since in many cases, cell culture systems used for production of replication-defective retroviral vectors may eventually produce replication-competent retroviruses after varying periods of incubation, because of the recombination of vector with helper viral sequences [46]. Several improvements of genetic manipulation of the lentivirus genome would ensure that the resultant vector would have a very high level of safety [47,48], but one would have to ask whether the our current understanding of retroviruses behavior is sufficiently qualified to wisely enhance vector design [49].

Recently, Klymiuk and his colleges developed new genetic engineering strategies to reduce the biohazard of these natural vehicles [50]. But the potential problem still exist in terms of the long terminal repeats (LTRs); the flanking sequences of the transgene of the recombinant retroviral genomes which have been reported to interfere with mammalian promoters, suppressing or misdirecting expression [6], or may lead to inactivation of tumor suppressor genes or activation of proto-oncogenes [51]. This, in turn, makes the transgenic animals more susceptible to develop tumor [52].

Some researchers are aware of the unwanted recombination event between the sequences of expression vector and a related sequence present in the same transgenic animal. This could lead to the dangerous oncogenic emergences [51]. As a result, some researchers are against the use of retroviral sequences in any experiments [43].

Sperm Mediated "Reverse" Gene Transfer (SMRGF)

The mode of communication that naturally exist between sperm and its corresponding oocyte is not a random phenomenon, rather, it's precisely regulated process involving several factors, one of these factors being endogenous reverse transcriptase [53].

The interaction of exogenous molecules triggers an endogenous reverse transcriptase activity in spermatozoa. This activity reverse transcribe's exogenous RNA molecules (specifically, the human poliovirus RNA genome) into cDNA copies, which are transferred to embryos following IVF ([54].

Thus this phenomenon is called "sperm-mediated reverse gene transfer" or SMRGT [55]. The discovery of functional RT in sperm cells provides the basis for SMRGT: in this process, the exogenous RNA is probably "captured" by the retro transposon-mediated mechanism active in sperm cells, reverse-transcribed, further propagated through the embryo as non-integrated structures in tissues of founder individuals and transmitted to F1 progeny. It is demonstrated that reverse-transcribed sequences behave as functional genes, being correctly expressed in tissues of F0 and F1 animals [56].

The role of this enzyme in SMGT is illustrated in model suggested by Smith and Spadafora [55] and updated by Spadafora [53].

SMRGT is one of the few interesting mechanisms in finding a way for the ex-gene to be internalized into the genome. This event, if proven to occur in nature, has wide implications for human health and evolutionary processes [18].

After the reverse transcription of exogenous RNA, the resulting cDNA molecules are located in and extra chromosomal location and while the ability of this segment to be integrated into the genome is a rare event [18]. Consistent with extra

chromosomal habits of these molecules are the negative results of various attempts to identify integration of the reverse transcribed cDNA copies [55]. The need for the use of expensive intracytoplasmic sperm injection equipment reduce the applicability of this technique and limited its readily availability in small budget labs.

Liposome Based (Lipofection) SMGT

Another interesting approach is represented by utilizing of liposomes in order to facilitate the entry of exogenous DNA in to the sperm head [15], see figure (7).

Currently available liposomes are spherical phospholipid vesicles, some of these structures have two faces hydrophilic head and hydrophobic tails, when the later moieties are used to associate with the hydrophobic moieties of the molecules to be transported, they tend to exclude water and encapsulate these molecules inside their structures [23]. Additionally, cationic liposomes use ionic interactions or electrostatic attractions. These cationic liposomes are much more capable of being interacted with DNA compared with the uncharged counterparts [57]. When the resulting complex is mixed with sperm cells in suitable solution (Figure 6), such vesicles can fuse with the cell membrane and deliver DNA directly into the cytoplasm [58].

Liposomes that are made up of cationic lipids can interact with the negatively charged nucleic acid molecules to form complexes forcing the nucleic acid to be associated with their structures [13]. The most commercially known cationic liposomes are lipofectin or lipofectamine, DOTAP, and DOTMA (Invitrogen, Boehringer-Manheim, Evrogen). They are commonly used as transfection reagents in many gene transfer protocols.

Liposomes enjoy many features made them used in many gene transfer protocols. They play very important role in the success of experiments such as their simplicity, easy of use, long term storage and stability, low toxicity, in addition to their ability to protect the passenger DNA from degradation [23].

Despite the success rates with the transfection of sperm head with several commercially available liposomes [59], it has not

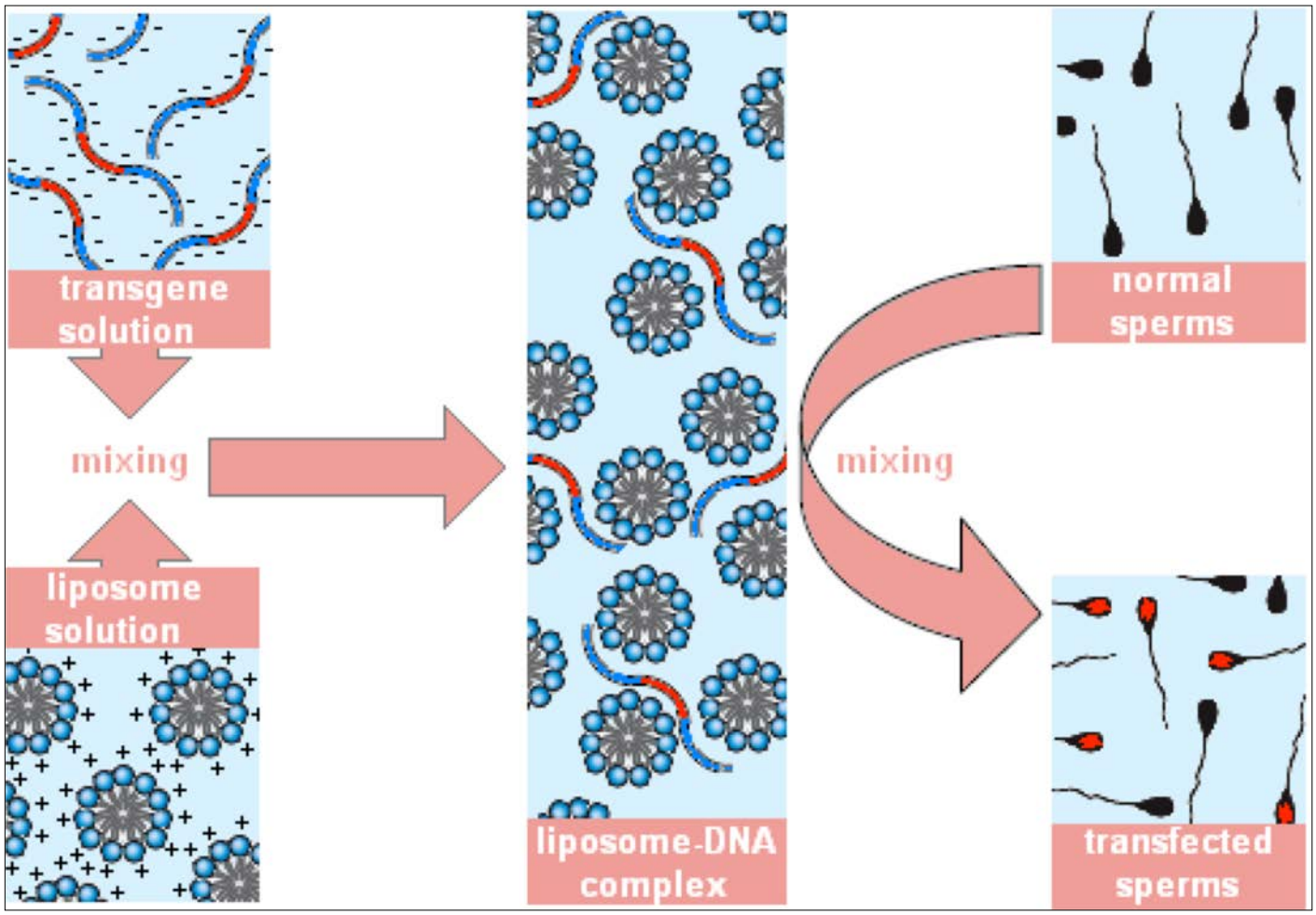

Figure 7: Illustration shows liposome based SMGT. After mixing cationic liposome with the transgene of interest a DNA-liposome complex is formed. Then, the resulting mixture is incubated with sperm cells. During this process, the fusogenic nature of sperm cells leads to the internalization of the complexes to form recombinant sperm.

been possible to generate transgenic animal by this technique [15,22,60].

Significant reductions in sperm motility is observed after treatment of murine sperm cells with liposome transfection reagent [61]. The most potentially important factor, which eliminates the wide applicability of liposomes, is represented by the high cost that is usually concomitant with the lipid drug delivery systems, as these systems are quite expensive to produce [62].

Restriction Enzyme Mediated Integration SMGT (REMI-SMGT)

One method that proved to be of interest in species for which there is a need for a more powerful technique to increase the success of transgenesis is restriction enzyme mediated integration (REMI).

REMI SMGT is a logical enhancement of the traditional SMGT but the combination of restriction enzymes made this technique very interesting with respect to the molecular mechanism by which the restriction enzyme enhance" the rate of integration.

This mechanism can be simplified by incubation of transgene located within a circular vector with its corresponding restriction enzyme. After digestion of circular DNA, its linear counterpart is produced (Figure 8). The linearized transgene and the same enzyme then are incubated with liposome. The role of liposome here is just to pass the transgene and its corresponding enzyme through the cell membrane of the sperm cells [30].

It is believed that once the exogenous DNA encounter the sperm genome, its corresponding restriction begins to digest its sensitive sites that are located on the hosting genome (Figure 8), meanwhile the exogenous DNA will seize the opportunity in order to integrate itself into the genome of the sperm cell by cellular DNA repair mechanism.

The most advantageous feature of REMI comes from the fact that the foreign endonuclease that is associated with foreign DNA has only one effect directed toward its genomic sensitive site rather than toward the linearized foreign DNA. This in

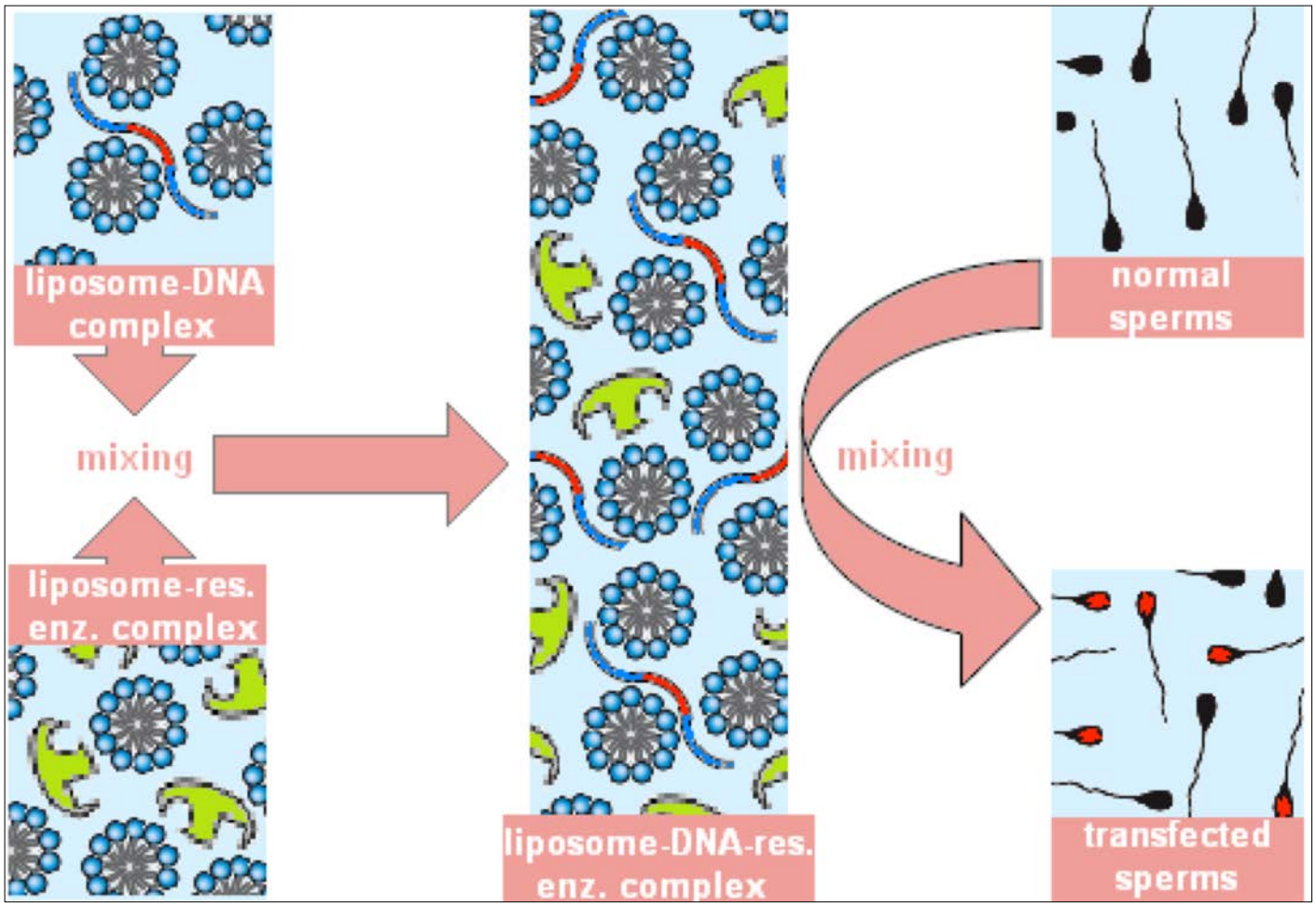

Figure 8: Suggested mechanism of restriction enzyme mediated integration sperm mediated gene transfer. In this mechanism, the corresponding restriction enzyme facilitates the integration of the transgene into the genome of the sperm, by mimicking a part of natural endogenous repair system.

turn will confuse the host genome by potentially speculated repair mechanism and then foreign DNA is integrated [18]. In this cellular repair mechanism, the host inserts the free cohesive ended foreign DNA within its original sequences.

Although Wall [7] referred to the absence of any significant disadvantages of REMI-SMGT, several experiments show high efficiency integration of DNA, there is a need to optimize this technique. Regrettably, the numbers of papers concerning REMI-SMGT is few; hence, further studies to elucidate more details on the validity of this particular approach are needed.

Conclusion

Several enhancements have been made to increase the efficiency of this promising method such as using electroporation, linkers, retroviral vectors, and liposomes. But, according to many data, the molecular mechanisms integrating the exogenous DNA by these approaches are little understood. Several researchers have further simplified SMGT by direct injection of foreign DNA into the testes of animals combined with electroporation or lipofection. Testis mediated gene transfer (TMGT), however, don't have significant differences compared with the original SMGT because each method relies upon sperm as a vehicles to carry the exogenous DNA. Thus, the problem of reduced integration still exists.

It has been reported that many enhancement approaches have increased the reproducibility of the original SMGT. Nevertheless, SMGT-induced integration of foreign DNA is still inefficient. The introduction of restriction enzyme mediated integration SMGT or REMI-SMGT by an Israeli group brought a new alternative feasible tool to achieve such task. However, this method is yet to be tested by other groups. We think it is very necessary to see how much these technique are capable on tricking the molecular repair mechanisms of sperm cell, since this tracking opens the door widely for more exploration of molecular manipulations of the sperm head for the sake of producing a transgenic animal with a minimum efforts and costs.

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