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Transgenesis: Embryo modification to sperm mediated gene transfer

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The generation of transgenic animals by conventional transgenic protocols is cumbersome and not very efficient. To improve the efficiency of obtaining transgenic animals, different groups have attempted to genetically modify spermatozoa and these technologies are collectively referred to as sperm mediated gene transfer (SMGT). SMGT technologies involve the modification of either spermatozoa or spermatogonial stem cells that give rise to spermatozoa, followed by either *in vitro* fertilization or mating with a wild type female to generate transgenic progeny. In addition to the generation of transgenic mice, the use of SMGT technologies has resulted in multiple insights into male reproductive biology. SMGT has bypassed most of the problems associated with the traditional methods of transgenesis and has considerably improved the efficiency of obtaining transgenic animals. Various techniques have been developed by which SMGT can be achieved and this review provides an overview of the evolution of SMGT technology and will indicate how these might be used to further our understanding of mammalian growth and development.

Keywords: ICSI, SMGT, spermatozoa, SSC, transgenic animals, wild type

Introduction

Except for the recent past, the study of mammalian biology and its impact on disease progression was restricted to observations made on patient material and *in vitro* studies in cell lines. While much has been learnt from these efforts, the results of these studies could not be directly extended to human beings, as these studies did not address the inherent physiological complexity of mammalian systems. In order to study the contribution of various cellular pathways to human physiology, it became necessary to generate models for human disease conditions in small animals, which led to the advent of transgenic technology.

Transgenic animals are generated by introducing an expression construct (the transgene) stably into the genome of the animal. To stably modify the genome of an organism, it is necessary that the modification is performed at the early stages of embryogenesis so that most of the cells of embryo will contain the transgene. Pioneering work from the Brinster laboratory led to the technology that permitted the culture of the ovum and early stage embryos[1]. Once this technical hurdle was crossed, multiple laboratories (including the Brinster laboratory) demonstrated that cultured embryos could be aggregated with teratoma cells to generate progeny, which expressed teratoma specific genes[2,3]. The next step in the generation of transgenic animals involves the injection of purified plasmid DNA into the male pronucleus of a 0.5 dpc mouse embryo. The microinjected embryos are then implanted into the pseudopregnant female mouse to allow the development of embryos. This technique resulted in the generation of mice, in which the plasmid DNA has integrated into the host genome[4]. This technique has been used to generate transgenic mice, which expressed high levels of Herpes Simplex Virus (HSV) thymidine kinase[5]. Other methods for generating transgenic mice, such as, retroviral infection of embryos and introduction of modified embryonic stem cells into the blastocoel cavity of embryo, have since been developed[6-8]. While this process has been used in many laboratories leading to significant advances in knowledge of mammalian biology, the technology is not used as widely as it could be because of the following issues. The pronuclei often burst post injection and integration of the transgene is often inefficient and does not improve with an increase in DNA concentration. Further, many of the embryos failed to implant or the implanted embryos fail to survive[9]. In addition, the efficiency of obtaining transgenic mice was variable, the foreign DNA is often not inherited in the germ line and these procedures took a long period of time and placed the animals

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under severe physiological stress. Thus, generating transgenic animals by the standard procedures described above is a laborious, expensive and time consuming process.

Spermatogenesis

To overcome the above-mentioned problems and yet to ensure that plasmid DNA is incorporated into the genome of the embryo, different groups attempted to modify spermatozoa. The reason behind choosing spermatozoa is their ability to bind foreign DNA, whereas unfertilized ova do not bind to foreign DNA[10,11]. A brief description of the process of spermatogenesis is required to understand the principle behind the methods that use sperm as a vehicle for transgene delivery. Spermatogenesis is the process by which spermatogonial stem cells (SSC) divide and differentiate to give rise to mature sperm (Fig. 1). The process of spermatogenesis is divided into three steps, *i.e.*, spermatogonial renewal, meiosis and spermiogenesis[12]. In spermatogonial renewal, a type A spermatogonium undergoes a series of division to give rise to a type B spermaotgonium (for details refer[13]). Type B spermatogonia differentiate into primary spermatocytes, which enter the meiotic programme. After meiosis I, primary spermatocytes give rise to secondary spermatocytes, which undergo meiosis II and differentiate into round spermatids with a haploid DNA content. During the process of spermiogenesis, haploid round spermatids undergo a series of changes, such as, nuclear condensation, acrosome and flagella formation and the removal of large portion of cytoplasm resulting in the formation of elongated spermatids[12]. Elongated spermatids leave the seminiferous tubules and migrate to the epididymis, where they mature to give rise to spermatozoa (Fig. 1). Therefore, each SSC gives rise to multiple spermatozoa and spermatozoa are regularly produced as opposed to ova. Hence, it is easier to generate larger numbers of transgenic animals by modifying spermatozoa and using them as a vehicle for the delivery of the transgene. Multiple techniques have been used to generate transgenic mice by modifying spermatozoa or SSCs (as indicated in Fig. 1) and these have been broadly grouped under the term "Sperm Mediated Gene Transfer" (SMGT). The different techniques by which SMGT is achieved are listed below.

DNA Uptake Mediated Gene Transfer

Brackett *et al*[11] demonstrated that DNA bound non-specifically to spermatozoa. Lavitrano *et al*[14] showed that DNA bound to a receptor present on sperm and that this receptor could be blocked by Inhibition factor 1 (IF1), which was present in seminal fluid. Therefore, mature spermatozoa needed to be washed to remove IF1 to ensure that DNA could bind to spermatozoa. Binding of DNA to spermatozoa led to the internalization of the DNA bound receptor followed by release of DNA into sperm nuclei. In addition to the receptor, MHC II and CD4 molecules played an important role in DNA uptake and internalization by spermatozoa[15]. Even though MHC II was not detected in mature sperm, spermatozoa from MHC II knockout mice did not bind DNA, whereas spermatozoa from CD4 knockout mice could bind to DNA but failed to internalize the bound DNA[15]. Moreover, blocking CD4 receptors by treating them with neutralizing antibodies prevented DNA internalization in wild type spermatozoa[15]. Lavitrano *et al*[10] developed a technique in which circular plasmid DNA containing a cDNA encoding bacterial chloramphenicol acetyl transferase (CAT) was incubated with a suspension of spermatozoa (washed spermatozoa). Then this

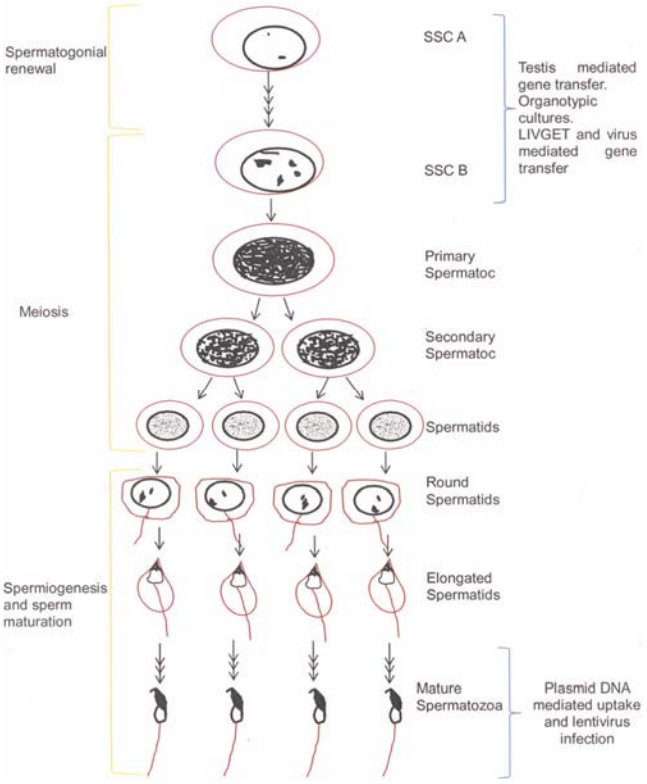

Fig. 1—The process of spermatogenesis is divided into three steps: Spermatogonial renewal, meiosis and spermiogenesis and sperm maturation. The figure also indicates which cell types are modified using the different SMGT techniques.

suspension was used in *in vitro* fertilization experiments. Progeny obtained from this method was found to be positive for the presence of plasmid DNA but did not express the CAT gene due to the rearrangement of the transgene[10]. Several groups have tried using this technique to obtain transgenic animals but not all of them were successful (reviewed in[16]). The major problems with this technique were: DNA uptake efficiency was highly variable in different species and in some species, *e.g.*, marine fish *Paralichthysolivaceus*, naked DNA damaged spermatozoa[17], the transgene did not integrate into the host genome and, most important, high occurrence of rearrangement of the transgene upon integration. The efficiency of obtaining transgenic animals using this method could be improved by using Intra-Cytoplasmic Sperm Injection (ICSI), where the sperm was injected into an ovum, instead of *in vitro* fertilization[18].

Lavitrano *et al*[10] also performed experiments in which they used a linear DNA fragment of a plasmid expressing CAT to generate transgenic spermatozoa in a protocol similar to the one described above. They found that transgenic animals, obtained from spermatozoa pre-incubated with linearized plasmid DNA, expressed the CAT gene and that the efficiency of obtaining transgenic progeny with this protocol was much higher compared to that observed with circular plasmid DNA. Using this technology, the Lavitrano laboratory had successfully generated transgenic pigs expressing human decay accelerating factor with an efficiency of 57%[19] and transgenic pigs expressing multiple transgenes, *i.e.*, enhanced blue fluorescent protein (BFP), enhanced green fluorescent protein (GFP) and DsRed2[20]. Kroll *et al*[21] modified the above-described protocol by incubating purified *Xenopus* sperm nuclei for a short period of time (10 min) with restriction enzymes in the presence of linearized plasmid DNA containing cDNA sequence of GFP. As a result, an improvement was observed in the efficiency of integration of linearized plasmid DNA into the genome and this method was named Restriction Enzyme Mediated Insertion (REMI) SMGT. Then these modified sperm nuclei were injected into the unfertilized ovum to generate transgenic embryos[21]. Another method used to improve the efficiency of DNA uptake by spermatozoa was to disrupt the cell membrane of spermatozoa by freeze thaw cycles and inject the membrane-disrupted spermatozoa along with a plasmid DNA fragment containing a GFP expression cassette into oocytes[22]. This change in protocol resulted in an increase in the efficiency of generating transgenic animals by 20%. The most critical factors associated with this method were the choice of enzyme, which is used to linearize the plasmid DNA, and the processing of sperm nuclei.

Since DNA uptake by spermatozoa have not been very efficient, some groups tried using transfection reagent lipofectamine to introduce exogenous DNA into spermatozoa[23-26] but lipofectamine complexed with linearized DNA (lipofection combined with REMI) gave efficient transgenesis compared to lipofectamine complexed with circularized DNA[24,26]. Some groups have also used nanoparticles, like magnetic nanoparticles, mesoporous silica nanoparticles and halloysite clay nanotubes, to deliver exogenous DNA to sperm[27-30]. Briefly, magnetic nanoparticles mixed with linearized DNA fragments were incubated with sperm and subjected to magnetic field for 90 min. Unbound DNA magnetic nanoparticle complex was removed and oocytes were fertilized with magnetofacted sperm to obtain GFP positive embryos[27]. The use of nanoparticles for SMGT was termed as NanoSMGT[30].

Transplantation of Modified Spermatogonial Stem Cells into Testis

Spermatogonial stem cells (SSCs) are progenitor cells, which undergo meiosis followed by differentiation to form spermatozoa as described above. These cells are mostly quiescent; however, they can be cultured and easily modified *in vitro*. Brinster *et al*[31] demonstrated that transplanting SSCs from a fertile animal to an infertile animal resulted in the restoration of male fertility and suggested that modified SSCs could be used to generate transgenic animals. This technique did not involve *in vitro* fertilization or ICSI. It was also shown that SSC transplantation in a fertile mouse resulted in the generation of some pups derived from spermatozoa from the donor and some from spermatozoa derived from the recipient[32]. Though Brinster *et al* did not use modified SSCs one could generate transgenic animals using this method. Briefly, SSCs can be isolated, cultured and transfected with a plasmid DNA. The SSCs can then be screened for the presence of the transgene and transgene expression. These stably transfected SSCs can then be transplanted into recipient testis and the recipient male mice upon mating with wild type female mice will lead to the birth of transgenic pups. These experiments demonstrate that even a few implanted SSCs are

sufficient for the generation of transgenic progeny. The principal behind this technique formed the basis for the evolution of SMGT techniques used today. Different groups used plasmid vectors, retroviral particles, lentiviral particles and transposons for modifying SSCs *in vitro* and then transplanting them into the testis of recipient mice to generate transgenic animals[33-35]. An organotypic culture technique, for culture of testicular cells of neonatal mouse, to obtain fertile spermatozoa *in vitro* has also been developed[36]. Using organotypic cultures from testicular cells of transgenic mice, transgene positive spermatozoa were obtained. These transgenic spermatozoa were fertile and were used for generating GFP positive transgenic mice[36]. Even though establishing organotypic cultures is not easy but once established, spermatogenesis can be maintained over two months[36]. It is tempting to speculate that recombinant spermatozoa generated *in vitro* in these organotypic cultures will result in the generation of compound transgenic animals carrying and expressing multiple transgenes in a single step rather than the multiple rounds of transgenesis, and mating that is the current norm.

Electroporation of Transgenic DNA into Testis

DNA can be introduced directly into target organs either in adult animals or pups and embryos using a number of procedures that are covered by the term LIVGET (localized *in vivo* gene transfer technique). LIVGET achieves the integration of plasmid DNA into the target organs by three distinct methods. The DNA is encapsulated either in a microparticle or a lipid particle that is injected into the target organ, or DNA is injected into the target organ followed by an electrical pulse across the organ. All these procedures result in the incorporation of the DNA construct into the cells of the target organ. Muramatsu *et al*[37] compared the efficiency of microparticle bombardment and electroporation for testis specific expression of the CAT gene. This was the first report in which cells in the testis were modified *in vivo*. They showed that, upon intratesticular DNA injection and electroporation, testicular cells showed CAT expression. Further, they also injected a plasmid expressing lacZ into the testes followed by the application of an electric current across the testis. Post 48 hours of electroporation, sections of electroporated testis were stained with X-gal, hematoxylin and eosin, and they observed lac Z expression in spermatogonium like cells, spermatocyte like cells and spermatid like cells. But they did not determine whether the plasmid DNA had integrated into the genome of these cells[37]. Yamazaki *et al*[38] demonstrated that injection of linearized plasmid DNA into seminiferous tubules, followed by the application of an electric current to the testis, resulted in long term expression of the lac-Z transgene and also in the modification of spermatogonial stem cells. In another study, by using plasmid DNA injection and electroporation of testis, transgene positive spermatozoa were obtained and used to fertilize ova using ICSI to generate transgenic progeny with high efficiency[39]. Some groups have also tried injecting calcium phosphate bound plasmid DNA or plasmid DNA encapsulated in liposomes into the testis to obtain transgenic animals; however, these methods were not always successful[40,41]. DNA injection into the seminiferous tubules or testis is termed as TMGT (testicular mediated gene transfer). Recently, the Majumdar laboratory has reported a method for DNA electroporation, in which linearized plasmid DNA was injected at multiple locations on mouse testis followed by electroporation. Post 35 days of injection, this pre-founder mouse was mated with wild type female mice to obtain pups and pups were screened for the presence of transgene. The results showed the efficiency of this method around 57-62%[42,43]. However, the pulse intensity and pulse interval will have to be standardized for different species such that the testicular cells will not be affected by electroporation leading to their cell death.

Virus Mediated Transgenesis

In 2000, Nagano *et al*[33] demonstrated that spermatogonial cells could be infected with retroviruses *in vitro* and these cells upon reintroduction into the testis differentiated into transgene containing spermatozoa. Briefly, spermatogonial cells were isolated, infected with retroviral particles carrying a lacZ transgene. Then transgene positive cells were implanted in the testes of a wild type mouse. These male mice were able to sire lacZ transgenic pups when mated with wild type female mice. The transgene positive pups obtained from the recipient male mouse were inbred and it was determined that the lacZ transgene was stably integrated as the pups obtained from inbreeding of transgene positive animals were also positive for lacZ expression[44]. The same group suggested that the use of lentiviruses would increase the efficiency of transgenesis due to their ability to infect quiescent germ cells[34]. Pfeifer *et al*[45] independently

demonstrated that lentiviruses could infect embryonic stem (ES) cells. They also showed that morula stage embryos could be infected with lentiviruses *in vitro* and the infected morula could be implanted into the surrogate mother to obtain transgene positive animals. Hamra *et al*[46] demonstrated that purified SSCs isolated from rat testis could be infected with lentiviruses *in vitro*. And these modified SSC's could give rise to transgenic progeny when implanted in the rat testis. While the techniques mentioned above resulted in the generation of transgenic animals, these were cumbersome for the following reasons. SSCs need to be isolated and cultured *in vitro* without them undergoing differentiation. Further, the transduced SSCs need to be propagated in culture, which is not a trivial protocol though it has become easier over time. In addition, spermatocytes need to be depleted from the recipient male mice and often the transplantation of the recombinant SSCs into recipient mice failed, probably due to immune reactions against the modified SSCs. This protocol is also difficult to use in larger animals where spermatocyte depletion and surgery might pose unforeseen problems. In 2004, Kanatsu-Shinohara *et al*[47] suggested that all the difficulties in obtaining and culturing SSCs can be bypassed by *in vivo* retroviral transduction of SSCs. By injecting retroviruses into the testis of sexually immature mice, they obtained transgenic pups but the efficiency of obtaining transgenic pups (22%) was little less compared to the *in vitro* transduction technique (33-38%).

In 2011, Sehgal *et al*[48] reported that, upon *in vivo* lentiviral transduction of pre-pubescent male mice, the EGFP-f transgene could integrate into the DNA of the SSCs and the transgene was expressed in all the cells in the testis. More than 60% of the pups generated by mating these pre-founder mice with wild type female mice were positive for the transgene. The transgene integration sites were mapped in the transgene positive pups and demonstrated that the transgene was stably inherited to next generation and that most of the animals contained one or two integrants[48]. This method is cost effective, simple and highly efficient for generation of transgenic animals as compared to the methods described above. But the site where integration is going to occur cannot be predicted and the transgene will be expressed by all the testicular cells, which are infected by lentiviruses. And if the transgene plays an important role in spermatogenesis or cell-cell adhesion then the pre-founder male mouse may become infertile[49]. However, these problems can extend to any SMGT technique that relies on the infection of SSC's. Few groups have also tried using adenoviruses for generating transgenic animals but adenoviral infection did not lead to integration of virus genome into the host cell. Hence, adenoviruses when used to generate transgenic animal by injection into seminiferous tubules did not show integration into the genome of the progenies but some of the pups expressed the transgene[50]. Recently, using GFP expressing pseudotyped lentivirus infected spermatozoa, GFP positive transgenic mice were obtained[51]. Breifly, cauda epididymis and distal end of vas deferens were placed in a dish, punctured with needle and incubated with concentrated virus soup for 30 min to 2 h. Then the spermatozoa were washed with buffer and used for IVF to generate transgenic mouse. The efficiency of obtaining transgenic animal using this technique was found greater than 42%[51]. The critical factor, which affected the efficiency of fertilization in this technique, was the time interval for which viruses were incubated with spermatozoa.

Site Specific Recombination and Targeted Knockouts

Until recently, the ability to generate knockout and knockin mice was limited to technology requiring the modification of embryonic stem cells *in vitro*, followed by embryo aggregation or blastocyst injection. These methods are difficult to develop and require facilities not available in most laboratories. Kanatsu-Shinohara *et al*[52] tried to generate knockout mice by infecting cultured SSCs with retroviruses containing neomycin resistant cassette. SSC colonies resistant to neomycin were sequenced to identify the genes flanking the retroviral genome and to find out if the neomycin resistanst cassette became the part of the ORF of the flanking gene sequences, suggesting disruption of that gene. Using this random mutagenesis approach, they found that one of the disrupted genes was *occludin*. The SSC colony with a disrupted *occludin* allele was transferred to the recipient testis to generate occludin knockout mice; though the efficiency to generate knockout mice with this method was very less (1.7%)[52]. However, the advent of the SMGT technologies allows the exploitation of the nucleases like TALENs (Transcription Activator-Like Effector Nucleases) and CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats; Cas-CRISPR associated protein) to achieve targeted insertions or deletions or modifications with higher efficiency.

While using TALENs, the DNA or RNA sequences (transgene) can also be injected into the oocytes or embryos. Once inside the nucleus, TALEN binds to specific DNA sequences and introduces a double stranded break, which causes activation of the Non Homologous End Joining (NHEJ) pathway. During NHEJ, the exogenously provided template DNA or RNA is used to insert or delete a few bases at the site of strand breakage. Recently, knockin and knockout mice, and mice with a site-specific mutation were generated using TALENs[53-55]. Elimination of germline mitochondrial DNA mutations has also been achieved with the help of TALENs[56]. Instead of the embryo injections, the TALENs can be encoded in a lentiviral vector and used to infect cultured SSCs. Recently, TALENs complexed with lipofectamine were used to transfect SSCs to generate or correct mutations, even electroporation of SSCs has also been done to deliver TALENs without affecting the cell viability or proliferation of SSCs[57,58].

In the CRISPR-Cas system, a single guide RNA (sgRNA) (20 nucleotides) directs the Cas nuclease to its target. Once activated, Cas generates double stranded breaks, activates NHEJ and NHEJ activation leads to insertion or deletion of specific nucleotides. To generate transgenic mice using CRISPR-Cas system, Cas mRNA, sgRNA and a linearized vector coding for the transgene were injected into the pronucleus or cytoplasm of a two/four cell embryo[59]. Transgenic mice containing a conditional allele for Mecp2 was also generated by injecting mouse embryos with a mixture of Cas9 mRNA, sgRNA and loxPoligos[59]. These mice with conditional alleles can be mated with mice expressing tissue specific CRE recombinase to generate mice with a tissue specific knockout of Mecp2 gene. Conditional allele can also be generated by using a pair of Cas9 nucleases and a single DNA expressing guide RNA flanked by loxP sites[60]. Recently, using a single plasmid system for expressing sgRNA targeting the gene of interest and Cas9 nuclease, a tyrosinase knockout rabbit was generated[61]. CRISPR-Cas had been used in SSCs for genome correction with 100% efficiency, as in the case of mice carrying mutant *Crygc* gene (responsible for causing cataract), resulting into the generation of healthy progeny[62]. To make the activity of Cas highly specific and reduce off target effects, multiple sgRNA or a pair of sgRNAs can be used[63].

TALENs and CRISPR-Cas allowed selective modification of a genome as compared to the conventional random mutagenesis where integration of the exogenous DNA or viral genome could not be controlled. This uncontrolled integration gave rise to different phenotypes depending upon the gene, which was disrupted due to integration of exogenous DNA. Moreover, plasmid modification used for generating transgenic mice was cumbersome in conventional methods with requirement of selective promoters, intron or exon of a gene, poly A tail and other accessory DNA elements, and was often associated with recombination problems, whereas with CRISPR-Cas systems, a mixture of DNA or RNA molecule (complementary to the targeted genomic region) and mRNA encoding for the nuclease could be directly used to achieve transgenesis in embryo or SSCs. Use of CRISPR-Cas, TALENs and other nucleases like Zinc Finger Nucleases is changing the field of transgenesis but still the questions relating to their specificity, cytotoxicity and off target effects remains to be answered.

Future Prospects

The field of sperm mediated transgenesis has evolved a great deal since the first experiments in which spermatozoa were incubated with plasmid DNA. With the development of technology, the efficiency of transgenesis has improved from approx 2-10% to above 60%. SMGT techniques also allows the development of transgenic animals in other species, *e.g.*, the development of transgenic pigs expressing human decay accelerating factor. The tissues from this transgenic pig can be used for transplantation, as the peripheral blood mononuclear cells from this animal upon incubation with fresh human serum were able to resist antibody and complement mediated cell lysis[19]. Isolation and culture of SSCs for *in vitro* modification of SSCs led to the standardization of process of isolation, culture, maintenance and cryopreservation of human SSCs. This has given hope to the human patients suffering from infertility or cancer (reviewed in[64]). The techniques described here could be further modified, so that one day scientists will be able to generate single gene or multi gene transgenic animals with almost 100% efficiency. In addition, the transgenes will be integrated at specific sites in the genome and their expression regulated either temporally or in a tissue specific manner. These could lead to the generation of genome wide screens in small animal models. And the development of such a technology will result in the generation of animal models of

human disease that could be used to develop therapeutic strategies and to study the biology of different pathways involved in growth, development and disease pathogenesis. Finally, these technologies will allow scientists to extend their studies to larger animals, which might be better models for studying human disease.

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