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Transgenic animals: The melding of molecular biology and animal reproduction

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Abstract

Biotechnology applied to livestock encompasses various reproductive techniques supported by molecular biology. Technologies for the transfer of gene constructs involve microinjection into the pronucleus of fertilized oocytes or DNA mass transfer. The last one can be made through the use of sperm, which carry the incorporated gene construct into the ovum at fertilization, or through the use of retroviral vectors in cell lines. One of the prerequisites to establishing transgenic lines is the presence of the foreign DNA in the gametes or one-cell embryos to ensure that the conceptus develops into a transgenic animal. To reach this objective, foreign genes can be transferred using different methods and strategies depending up on the species of domestic animal used for this venture and their biological potential. Transgenic animals are now commonly used worldwide as models for human disease and the commercial availability of transgenic protein products for therapeutic use is thought to be nearing realization. Advanced research is being conducted in areas such as organ development for human transplantation and improved animal production. Transgenic animals provide a true in vivo environment for evaluating the mechanisms by which gene expression is modulated during development and in adults. "Animal pharming", the process of using transgenic animals to produce pharmaceutical proteins for human use, is staking its claim in a lucrative world market since the inserted gene, enables an animal to generate the targeted pharmaceutical protein in its milk, urine, blood, sperm, or eggs, or to grow rejection-resistant organs for transplant. This paper is a brief review of the most recent events in the area of domestic animal trangenesis.

Keywords: transgenic animal; genes; foreign DNA; vectors; bioreactor.

Introduction

The optimization of the animal production efficiency depends on the success of advanced reproductive techniques (Deschamps *et al*., 2000). Transgenesis is one of these techniques which depend on the fusion of knowledge-base in genetics, molecular biology, and animal reproduction. Functional genomic analyses in vertebrate model systems, including fish, frogs, and mice, have greatly contributed to the understanding of embryonic development and human disease processes. However, new molecular tools and strategies are needed to meet the increasing demands for information on gene function (Ivics and Izsvak, 2004).

In 1982, a gene construct containing the mouse metallothionein promoter (*mMT*) and the rat growth hormone gene (*rGH*) were introduced by microinjection into mouse zygotes (Palmiter *et al*,. 1982). This was not the first, but the main paper published in the area, being considered as the initial mark on animal transgenesis. Sequencing projects have supplied molecular geneticists with raw material which, along with the advent of bioinformatics and information on gene expression obtained from *in silico*, are expected to allow transgenesis in animal models to reach its full potential (Carter, 2004). In fact, with the advances in molecular biology techniques applied to animal reproduction, new methods directed to the introduction of specific genes into the genome of farm animals, started to be used. The stable incorporation of these genes into the germ line has been a major technological advance in agriculture (Wheeler, 2003). The production of animals with large transgenes is a valuable tool for biotechnology and for genetic studies, including the characterization and manipulation of large single gene traits and polygenic traits (Moreira *et al*., 2004).

Transgenesis includes the introduction of foreign DNA sequences in the genome of multicellular organisms, and ensuring that the sequences are transmitted to the progeny of the manipulated species (Houdebine, 2003). On the other hand, Brink *et al*., (2000) define transgenesis as the alteration of the genetic information with the intention of modifying a physical characteristic of an animal. However, the latter concept does not encompass introduction of the gene to obtain new functions such as the production of proteins of pharmacological interest. Transgenesis differs from gene therapy since in the former, the inserted gene is expected to be transmitted to the next generations. Further more, the term "transgenic" has wider implications since it could comprise animals which had addition, or deletion (knock out) of genes, from the genome.

Transgenic technology is a fast method for introducing "new" genes in cattle, swine, sheep, goats, chicken and fish. It is a more extreme methodology, but

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does not differ in its essence from the long-term results obtained by classic genetics (Wheeler, 2003). In this way, the techniques to generate transgenic models represent one of the most promising biotechnologies for commercial use, as well as for different areas of basic research.

Transgenic animal production has various applications, including generation of animals with better or improved performance (Maclean *et al.,* 2002; Karatzas, 2003), animals as models to study human diseases (Duverger *et al*., 1996; Carter, 2004), animals for the production of proteins of pharmacological interest (Brem *et al.*, 1994; Houdebine, 1994; Limonta *et al*., 1995; Wall, 1999; Hwang *et al.* 2004), animals for the production of organs for transplant (xenotransplants) (Houdebine, 2000; Niemann, 2001), and animals for gene expression and regulation - promoters and coding sequences - (Montoliu, 2002; Giraldo *et al.*, 2003). Current applications of gene transfer in farm animals include the improvement of product quality and quantity, disease resistance, production of valuable proteins in the mammary gland or other organs, the genetic modification of pigs for the production of xenotransplants and the generation of new animal models where rodent models are not useful or practical for studying the problem under evaluation (Wolf et al., 2000).

The developmental costs and the inefficiency of the technique to produce transgenic animals, particularly large animals, together with the fact that the majority of interest characteristics are complexes and controlled by more than one gene, have restricted the use of transgenesis in animal production (Clark and Whitelaw, 2003).

The present review will focus on the currently used techniques to generate transgenic animals, the principal events in gene manipulation, and the main applications of this biotechnology.

Methods to generate transgenic animals

During the past few decades, various methods have been developed to generate transgenic animals. With the advent of gene sequencing, many sequences have been determined, bringing the knowledge of promoters and genes of interest, for various species. The advent of genomics, proteomics, and the new generation of reproductive biotechnologies hold the promise of successful application of transgenesis to domestic animals.

The techniques and methodologies to be implemented in the generation of a transgenic animal depend on the targeted use of the animal. Many transgenic animal models have been created to study gene function, to serve as bioreactors and as models for new approaches in animal breeding (Houdebine, 2002a, c; Maclean *et al.*, 2002; Montoliu, 2002; Dyck *et al.*, 2003; Houdebine, 2003; Niemann and Kues, 2003; Baldassarre *et al.*, 2004; Hwang *et al.*, 2004; Keefer, 2004;). The objective of the research will determine the costs and the tools necessary for the approach. A summary of the main techniques used to generate transgenic animals is presented in Fig. 1. These techniques comprise basically three forms of foreign DNA transfer: DNA microinjection into the pronuclei, mass transfer of genes through gametes, and somatic cell nuclear transfer (SCNT). Techniques using gene transfer mediated by retro-transposons and retrovirus are also presented (Fig.1).

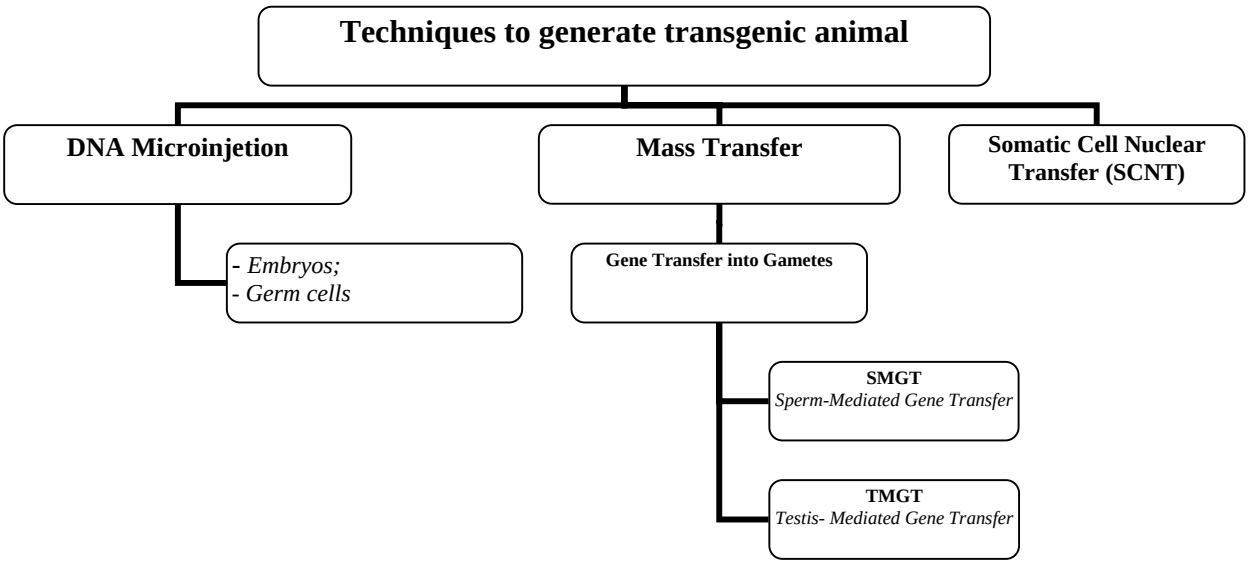

Figure 1. Main techniques used to generate transgenic animals.

Pronuclear Microinjection

Various methods can be used to produce transgenic animals. However, the main method used to-date is the microinjection of genes into the pronuclei of zygotes (Wheeler, 2003). Two decades ago, the microinjection of foreign genes into the pronuclei of newly fertilized embryos was the most efficient technique to generate the first transgenic mice (Gordon *et al*., 1980). In the 80's, this method was used on rabbits, pigs, and sheeps, and later on, on goats and cows. However, the efficiency of this method in domestic animals is still low (Wolf *et al*., 2000).

Production of transgenic livestock by pronuclear microinjection of DNA into fertilized zygotes is impaired by the low embryo survival and the low rate of integration of the injected DNA into the genome (Maga *et al*., 2003; Auerbach, 2004). The main obstacle with this method is that some copies of the foreign gene is integrated randomly into the host genome and upset the expression of the transgene, as well as the host genes. Generally, this method generates a mosaic transgenic animal. Because of these limitations, a large number of embryos in the pronucleus stage, need to be used in the experiment (Houdebine, 2002c). Thus, with the 500 to 5000 copies of the foreign DNA injected into the pronuclei, the mean progeny obtained ranges from 1 to 4 %. This means that less than 1 to 4 transgenic animals are obtained from a hundred injected cells. The lowest rate of success is obtained in cattle. In pigs, the pronuclear DNA microinjection has long been the most reliable method; however, even in this species, the efficiency of transgenic offspring production is low, with only 1% of the DNA-injected embryos resulting in transgenic animals (Nagashima *et al*., 2003).

The results obtained with this method vary greatly depending up on the species. However, there is also within-species variation in the success rate (Reviewed by Pinkert, 2002). The reasons for this difference are still not known, but they are probably related to the inherent difference in the DNA repair mechanism or intrinsic DNA integration process into host genome. Furthermore, the purity of the exogenous DNA, the strategy used for the construction of the artificial molecule (promoters and coding regions), and other factors involving cellular machinery, could lead to the low efficiency of transgenesis in domestic animals (Clark *et al*., 1994; Houdebine, 2002b).

The presence of lipids renders embryos of pigs and ruminants opaque, making the manipulation difficult and decreasing the efficiency of pronuclei microinjection. Centrifuging embryos before micromanipulation promotes the migration of the lipids to one side of the cell, facilitating the visualization of the pronuclei.

The pronuclear DNA microinjection method is routinely used to generate transgenic mice and some species of fish, despite the peculiar characteristics of the latter. In fish, egg microinjection poses some difficulties, since fish egg has a thick membrane. This membrane impairs the visualization of the nuclei during egg fertilization and penetration of the glass micropipettes (Kang *et al*., 1999; Lu et al., 2002).

According to Baldassarre *et al*. (2004), the production of transgenic goats through the traditional method of DNA microinjection also presents low efficiency, which discourages their use in advanced breeding programs. New alternatives proposed by this research group, using laparoscopic ovum pick-up (LOPU-IVF) and oocyte maturation *in vitro* prior to DNA microinjection, have shown interesting results. These efforts routinely result in the birth of transgenic offspring, showing that the established LOPU-IVF technology combined with pronuclear microinjection can be successfully used to produce transgenic goats (Wang et al., 2002; Baldassarre et al., 2004).

Although pronuclear microinjection has succeeded in the generating many transgenic cows, the success rates of transgenesis is low in this species (Hodges and Stice, 2003). The costs to produce a transgenic cow through pronuclear injection are of the order of U$ 300.000,00 (Whitelaw, 2004). Hence, a more efficient system of gene transfection that works in large animals is necessary. These inefficiencies are one of the major obstacles to the large-scale use of pronuclear microinjection techniques in livestock (Maga *et al*., 2003). Another method that has demonstrated success recently, is the nuclear transfer or "cloning" (Wheeler, 2003).

Somatic Cell Nuclear Transfer (SCNT)

The first important results with SCNT were obtained in 1986 by Willadsen, with the production of lambs cloned from nuclei of embryos at stage of 8 to 16 cells. This result stimulated the interest in the use of nuclear transfer to multiply embryos derived from animals with high agricultural value (Campbell *et al*., 1996). This laborious method also offered new and attractive possibilities to animal transgenesis.

Montoliu, 2002 opines that animals obtained by nuclear transfer could be considered as a group of transgenic animals when the nuclei used in the embryo reconstruction originates from a cell that carries some genetic modification (addition, substitution or alteration of some gene). In this sense, those embryos and animals generated by nuclear transfer of cells genetically modified will also be, by definition, transgenic, since they carry the initial modifications present in the nuclei of the donor cell from which the animal originated.

Exogenous genes of interest can be transfected into somatic cells and later on transferred by to pluripotent cells (cells of morulae or blastocysts). The resulting chimera can transfer the exogenous gene to the offspring, which will be transgenic (Wolf *et al*., 2000; Houdebine, 2002b). In this way, cultivated cells can be

transfected, and the insertion and expression of the transgene can be verified before using these cells for producing cloned animals genetically modified (Bordignon *et al*., 2003).

Using this method, the DNA is randomly incorporated into the genome by selective pressure; however, the transgenic cells can be fully characterized (site of integration, number of integrated copies and integrity of the transgene) prior to use for nuclear transfer. As a result, although the developmental capacity of "reconstructed" nuclear transfer (NT) embryos is lower, the majority of animals born are transgenic, making this technology much more efficient than pronuclear microinjection. Somatic cell nuclear transfer has dramatically improved the efficiency rates of transgenesis (Baldassarre *et al.*, 2004). This approach would enable more efficient and sophisticated genetic modification of pigs (Nagashima *et al.,* 2003). Gene replacement by homologous recombination can be presently achieved only in somatic cells, used to generate genetically modified animals. Gene inactivation has been accomplished in sheep (McCreath *et al.*, 2000) and pigs (Lai and Prather, 2002). In pigs, the α-galactosyltransferase was knocked out in this way. The kidneys from homozygous pigs have become resistant to hyperacute rejection when grafted to experimental monkeys (Lai and Prather, 2002). Results obtained in cattle, sheep, goats and pigs demonstrate that the majority of animals cloned from transfected somatic cells express the transgene (Lai and Prather, 2002; Bordignon *et al.*, 2003; Nagashima *et al.,* 2003; Niemann and Kues, 2003; Baldassarre *et al.*, 2004).

Mass Transfer of DNA

Gene transfer into gametes

Sperm-mediated gene transfer (SMGT)

The microinjection technique results in high success rates in mice, but it is not an efficient method when applied to livestock (Lavitrano *et al*., 2003). A logical alternative strategy to generate transgenic animals theoretically consists of the introduction of foreign DNA into male gametes before the fertilization process (Spadafora, 2002).

Sperm cells are considered by some authors as metabolically inert cells, since they do not have most of the molecular and biochemical apparatus that exist in somatic cells engaged in such functions as DNA replication, gene transcription, and protein synthesis. This point of view has been corroborated, in some way, by their peculiar morphology, characterized by the extremely reduced cytoplasmic compartment and the nucleus which contains the genomic DNA compacted as condensed chromatin, connected to a long flagellum. These morphological observations lead to the conclusion that the only possible role of sperm cells is to act as vectors of their own genome during fertilization. The first evidence that mammalian sperm cells were capable of incorporating foreign DNA when incubated in solutions containing these macromolecules were described by Brackett *et al*. (1971).

In 1989, Lavitrano *et al*. demonstrated for the first time that (a) the epididymal sperm of the mouse can spontaneously incorporate plasmid DNA molecules; (b) genetically modified offspring can be generated by the approach using sperm cells containing plasmid, by *in vitro* fertilization procedures; (c) exogenous DNA sequences are expressed in the progenitors, and (d) that the sperm-carried exogenous DNA incorporated in the fertilized ovum, is transmitted from the parents to the F1 progeny. These characteristics are conserved in a variety of species and SMGT have been explored to generate genetically modified (transgenic) animals in a variety of species.

The SMGT technique in vertebrates has gone through many adaptations in the last 10 years, in different laboratories (Gandolfi, 2000). The incubation of sperm cells with foreign DNA, followed by *in vitro* or *in vivo* fertilization, has generated transgenic mice, rabbits, pigs, sheep, cows, chicken and fish. The definition and the establishment of work protocols for SMGT that could be effectively applied to different animal species would be of high value in biotechnology (Celebi et al., 2002). In addition, this procedure does not require any particular equipment or ability, and can be performed at field conditions. Another interesting aspect of the use of sperm as DNA vectors is referred to as mass transgenesis. Contrary to microinjection, which requires individual manipulation of the embryos, the genetic transformation of a great number of embryos can be obtained collectively, in one step, by SMGT. This can be of particular interest to transgenesis of aquatic animals including fish (Spadafora, 1998).

Wu *et al*. (1990) revealed that the main binding site of foreign DNA in mouse sperm is mediated by a complex structure of molecules from class II major histocompatibility complex, located in the posterior region of the sperm head. Associated DNA was also mainly located in the posterior area of the rabbit sperm head (Lavitrano *et al*., 1997; Wang *et al.*, 2003).

Attempts to elucidate the mechanism of DNA integration identified a complex net of factors, secreted by and linked to the sperm, which modulates this interaction. Carballada and Esponda (2001) identified two components in the mouse seminal plasma: a DNAse from the seminal vesicle, and diverse exogenous DNA binding proteins from the prostate. These components show inhibitory activity to exogenous DNA sequestration. These authors (Carballada and Esponda 2001) suggest that the mechanisms of control and uptake of exogenous DNA by mammalian sperm are highly regulated and specific. In fact, seminal fluid strongly antagonizes foreign DNA binding and, under normal con-

ditions, is a strong protection of sperm cells against foreign DNA (Celebi *et al*., 2003). A specific inhibitor of the DNA binding reaction factor (IF-1), was identified in the membrane surface of sea-urchin sperm (Arezzo, 1989). IF-1 is a glycoprotein and its inhibitory activity is linked to the polysaccharide component. In fact, the ability of IF-1 to inhibit DNA binding can be completely removed by pre-incubation with glycosidases. IF-1 binds to the subacrosomal segment of sperm head, which is the same area aimed by the foreign DNA, and can exert its inhibitory effect in heterologous as well as homologous sperm. Therefore, IF-1 has an important natural role, acting as a barrier and protecting epididymal sperm against the entry of undesirable exogenous molecules, which could compromise the sperm integrity and the genetic identity of the future progeny (Spadafora, 1998; Spadafora *et al*., 2002).

The ability of rabbit sperm to take up foreign DNA from the incubation media was tested by Wang *et al.* (2003), when spermatozoa were incubated with plasmid vector marked with tetramethylrodamine-6-dUTP. After incubation, spermatozoa were treated with DNAse I and evaluated by fluorescent microscopy. The results of this study demonstrated that rabbit sperm cells have the capacity to take up exogenous DNA from the media.

In domestic animals including cattle and pigs, SMGT is applied by the exploitation of the normal artificial insemination (AI) procedure used by the farmers. The fresh semen is collected from donor animals and repeatedly washed to remove seminal plasma by sequential centrifugations. Sperm cell suspensions are incubated with the foreign plasmid DNA (around 1 h at 18ºC), diluted in an appropriate media and used for AI (Shemesh *et al*., 2000).

Sasaki *et al*. (2000) demonstrated that significant loss of motility occurs in murine epididymal sperm incubated with complexes of DNA-liposomes, in keeping with the concentrations of the foreign DNA. Also, *in vitro* fertilization (IVF) rate decreases as the DNA concentration increases.

Alternative techniques to promote better incorporation of foreign DNA are being tested. To increase DNA uptake by the sperm cell, non-polar detergents, including Triton and Tween which promote destabilization of sperm membrane, could be used. Similar results have been obtained through sperm freezing and thawing. The chromatin cleavage by restriction enzymes in the sperm genome site, but not in the foreign DNA site, triggers repairing mechanisms and increases the possibilities of integration of the foreign DNA of interest. This method is known as restriction enzyme mediated integration (REMI). REMI utilizes a linear DNA derived from a plasmid by the cleavage with a restriction enzyme, which originates a cohesive end in one of the strips. The linear DNA with the cohesive end is then introduced, together with the restriction enzyme, into the sperm cells by lipofection or eletroporation. It is believed that the restriction enzyme cleaves the genomic DNA at the sites that allow the integration of the exogenous DNA by the pairing of the cohesive ends (Khoo *et al*., 1992; Khoo, 2000; Sparrow *et al*., 2000).

Another interesting alternative method is the direct injection of sperm treated and incubated with foreign DNA, into the oocyteby the method known as intra-cytoplasmic sperm injection (ICSI). ICSI was successfully used in mice to transfer long fragments of DNA, as in yeast, bacteria and other artificial chromosome constructs (YACs or , BACs and MACs) (Giraldo *et al.*, 1999; Giraldo and Montoliu, 2001**;** Moreira *et al*., 2004**).** The potential use of more recent approaches, such as REMI and ICSI are also being explored (Khoo, 2000).

The use of electroporation of sperm incubated in isosmotic solutions containing DNA, has been described in some species. Electroporation of sperm subjected to osmotic differential demonstrated an increase in foreign DNA uptake by fish sperm cells (Kang et al., 1999; Collares et al., 2004). However, the generation of transgenic animals by osmotic differential SMGT alone has not been described to date. Wang *et al.* (2003) demonstrated that 66% of rabbit spermatozoa incubated with lipofectin and marked foreign DNA carried the foreign DNA. Cationic detergents have been used with the intent of promoting sperm membrane solubility, thus allowing the entry of marked foreign DNA. Sin *et al.* (2000) showed that electroporated salmon sperm cells were more efficient and more reliable for picking up foreign DNA and subsequently transferring the DNA into salmon embryos, than untreated sperm. Indirect evidence suggests that some of the foreign DNA was internalized in the sperm nuclei and the incorporated DNA retained its integrity as demonstrated by PCR (Symonds *et al*., 1994).

Chang *et al*. (2002) present an extremely interesting strategy for generating transgenic animals, using incubation of sperm cells with marked foreign DNA and monoclonal antibody (mAb C). mAb C is a basic protein that binds to DNA through ionic interaction, allowing foreign DNA to be linked specifically to sperm. This linker protein is reactive to a surface antigen on sperm of all tested species, including pig, mouse, chicken, cow, goat, sheep, and human. It is important to note that foreign DNA uptake mediating mechanisms are integral parts of the biology of the species that have sexual reproduction.

Accordingly to Lavitrano *et al*. (2003), SMGT is highly efficient and relatively cheap, and can be used in species refractory to microinjection. The use of spermatozoa as noninvasive delivery vehicles to transfer foreign DNA into oocytes during *in vitro* fertilization has provided a new alternative to the approach in generation of transgenic animals (Lazzereschi *et al*., 2000; Spadafora, 2002).

Sperm-mediated "Reverse" Gene Transfer

Sciamanna *et al.* (2003) demonstrated the presence of an active reverse transcriptase (RT) in murine sperm. RT can reversely transcribe a foreign viral RNA into cDNA fragments that can be subsequently transferred to embryos during fertilization. The RNA vector was incorporated by sperm cells, reverse transcribed and transferred to in vitro-derived embryos which eventually will be passed on to their F1 progeny. These results suggests that the reverse transcribed cDNA molecules are maintained as extra-chromossomal structures replicating autonomously, while the integration into the host genome would rarely occur.

It has been shown that the sequenced human genome contains 223 bacterial genes (Lander *et al*., 2001). Probably, multiple independent gene transfers from different bacteria occurred during the evolution of the human genome. Some introduced genes appear to be involved in important physiological functions and have been fixed during evolution, because of the selective advantage they provide (Lander *et al*., 2001). Would a highly gene-mediated mechanism to ensure the genetic identity of sexually reproducing species exist? Do gametes have more extensive evolutionary functions?

Although strong natural barriers exist against sperm-mediated gene transfer, such barriers are unlikely to be absolutely inviolable (Smith, 2002). Sciamanna *et al.* (2003) demonstrated that sperm endogenous reverse transcriptase (RT) has the potential to reverse-transcribe exogenous RNA, generating transcriptional competent sequences that are transmitted to the progeny upon fertilization. This event, if proved to occur in nature, would reveal its profound implications to human health and to evolutionary processes.

This assumption is supported by the previous findings that extra-chromossomal structures are frequently hosted by eukaryotic nuclei. In deed, transgenic sequences can generate extra-chromossomal structures that are transmitted to the next generation, as documented in transgenic animals obtained by SMGT of mammals, birds, fish and insects (Giordano *et al.*, 2000; Sciamanna *et al.*, 2000; Spadafora, 2002).

Testis-mediated gene transfer (TMGT)

Other approaches have also been developed for making transgenic spermatozoa. One of these, is the testis mediated gene transfer approach which is considered as a simplified variation of SMGT, since it does not require IVF or embryo transfer (ET) procedures.

The testis is also considered an immune-privileged site. Transferring genes into specific cell types of the testis *in vivo* should provide a tool to study the regulation of spermatogenesis at the molecular level (Blanchard and Boekelheide, 1997). Liposome- based methods have successfully generated transgenic mice and fish by TMGT (Lu *et al.*, 2002; Celebi *et al.*, 2003; Zhao *et al.*, 2003).

The mechanism of gene transfer into epididymal spermatozoa by injection of a DNA-transfectant complex into the testis is under study. However, it is suggested that foreign DNA introduced into the testis is rapidly transported to epididymal ducts via the rete testis and efferent ducts, and then incorporated by epididymal epithelial cells and epididymal spermatozoa (Sato *et al*., 2002).

Round plasmid carrying the reporter gene lacZ mixed with lipossomal complexes were injected into mouse seminiferous tubules, prior to subjecting them to natural mating. The presence of the foreign gene was observed in the progeny, but in episome – like form (Celebi *et al.*, 2003). The efficiency of gene transfer was improved more than 80% by injecting multiple doses of the liposome-transgene mixture into the gonads of treated males (Lu *et al.*, 2002). More than 80% of morula-stage embryos generated by means of TMGT using liposomes, expressed EGFP, as revealed by fluorescence microscopy (Yonezawa *et al.*, 2001). High incidences of mosaicism, as well as a decrease in the rate of cells carrying foreign DNA during embryo development, have been noted with this technique, suggesting that TMGT efficiency is directly related to liposome characteristics (Yonezawa *et al.*, 2001).

Another strategy for foreign gene introduction employs adenovirus vector solution injected into the interstitial space (intratesticular injection) or seminiferous tubules (intratubular injection) of the mouse testis. Although spermatogenesis is slightly impaired and the inflammatory response caused by these methods may present some problems, the results suggest that adenovirus mediated gene transfer may be effective for transfecting testicular somatic cells and that this approach may be applicable for in vivo gene therapy for male infertility in the future (Kojima *et al.*, 2003). In general, the results also suggest that TMGT could be applicable to fetal gene therapy, as well as to the generation of transgenic animals (Yonezawa *et al.*, 2001).

Retroviruses and Transposon -mediated gene transfer

The retrotransposons and retroviruses are vectors with highly efficient intrinsic capacity of integration into the genome (Linney *et al*., 1999; Houdebine, 2002b). Retroviral vectors are currently being used because of their ability to integrate the foreign gene into the host genome with high efficiency. Retroviruses and retrotransposomes belong to this category of natural gene delivery vehicles to mammalian cells (Houdebine, 2003). Vectors based on lentivirus have been shown to be an efficient transgene delivery system (Hofmann *et*

al., 2003; Whitelaw, 2004). Whitelaw *et al.* (2004) used a vector derived from equine infectious anaemia virus to carry a green fluorescent protein expressing transgene and showed that 31% of the injected/transferred eggs resulted in a transgenic founder animal and 95% of the founder animals displayed green fluorescence. This method is more efficient than the standard pronuclear microinjection, indicating that lentiviral transgene delivery may be a general tool to generate transgenic animals (Rottmann *et al.*, 1991; Hofmann *et al.*, 2003; Whitelaw *et al.*, 2004).

Simple structure and easy laboratory handling of transposome vectors are coupled with efficient and stable transgene integration and persistent, long-term transgene expression by transposome-mediated gene transfer (Ivics and Izsvak, 2004). Transposomes are DNA sequences which contain at least one gene coding for a transposase and motives located on both ends, to trigger integration. Transposome sequences are transcribed into RNA, which drives transposase synthesis. The RNA is retrotranscribed in DNA, which integrates in the multiple sites of the genome under the action of the transposase (Houdebine, 2002b). The transposome vectors must be transcomplemented with a plasmid capable of expressing the transposase gene required for the integration of the recombinant transposome. In practice, a circular plasmid containing a construct capable of expressing the transposase gene is injected with the recombinant vector. This allows the integration of the foreign gene with the vector whereas the assistant plasmid is rapidly degraded (Dupuy *et al.*, 2002; Houdebine, 2002b; Kawakami *et al.*, 2004).

Grabhera *et al.* (2003) tested the Sleeping Beauty (SB) transposable element for its ability to efficiently insert transgenes into the genome of medaka (Oryzias latipes), an important model system for vertebrate development. These investigators demonstrated that the SB transposome efficiently mediates integration of a reporter gene into the fish germ line with a transgenesis efficiency of 32% .The efficiency of transposome-mediated germline transformation is dependent on the mobility of transposomes in the host embryo, and on the detectability of the used transformation marker (Horn *et al*., 2000). These features contribute to the usefulness of transposable elements as tools for vertebrate functional genomics, as well as for animal biotechnology and human gene therapy (Ivics and Izsvak, 2004). These aspects will be of great interest to the field of evolutionary developmental biology and to modern pest management programs (Horn et al., 2000).

Genes of interest and detection of the transgene

Among the genes of direct interest for animal production application are the GH (growth hormone), the IGF-I and II (Insulin-like Growth Factors), and the hormones secreted by muscle, fat cells and stomach (leptin, adiponectin, myostatin, ghrelin), which regulate feed intake, energy metabolism, and body composition. Through genetic manipulation, there is the potential to exploit these genes in a range of livestock species.

Bovine GH over-expression in rabbits did not produce positive results on growth (Costa *et al*., 1998). The high level of expression was accompanied by the over-expression of IGF-I and, as consequence, resulted in the development of acromegaly and diabetes mellitus.

In contrast to the effects observed with the introduction of GH in large animals, the majority of GH introduced fish species showed a marked effect in growth (Hinits and Moav, 1999; Martinez *et al*., 1999; Rahman and Macclean, 1999; Morales et al., 2001). For example, 10% gain in food conversion and a 2.62 to 2.85 fold higher growth rates in transgenic than in non-transgenic salmon were obtained by Cook *et al*. (2000). Other investigators have presented positive results, among them the research of Du *et al*., (1992), with transgenic fish manifesting 2 to 6 fold higher growth than non-transgenic fish. Even higher results were obtained by Devlin *et al*., (1994) with coho salmon (*Oncorhynchus kisutch*), where the transgenic fish were 11 times faster in growth than control salmon.

The insulin-like growth factors (IGF-I e o IGF-II) produced in the liver, bones and other tissues, mediate some of GH functional effects (Strobl and Thomas, 1994). IGF-I has proved to be of more use as a growth reporter/selection marker in pigs, than as a viable treatment. However, a niche for this product could exist in the manipulation of neonatal growth, causing a life-long change in lean: fat ratio (Sillence, 2004).

Other genes of interest are related to food metabolism and disease resistance. For example, a reduction up to 75% in fecal phosphorus output was observed in transgenic pig expressing phytase gene in saliva, thus showing an effect on the digestion of dietary phosphorus (Golovan et al., 2001). Antibacterial proteins, such as lysostaphin, can be used to confer resistance to bovine mammary gland infection. This protein has potent anti-staphylococcal activity and its secretion into milk conferred substantial resistance to infection in three lines of transgenic mice (Kerr and Wellnitz, 2003).

Initially, the introduced foreign gene in a transgenic system was detected by PCR and Southern blot; however, now a day the detection system is built-in in the transgene so that its own expression can be evaluated. Among the detection systems built-in the construct are CAT, Luc, Lac-Z (Gibbs and Schmale, 2000; Maclean, *et al*., 2002), and more recently, GFP in swine (Whitelaw, 2004). The main methods used for transgene detection in transgenesis in animals, are presented in Table 1.

A rapid and simple method based on PCR was presented by Nam et al. (2003) for analysis of trans-

genic fish using small amounts of tissue. This method allows the screening of a large amount of larvae, but the cost of analysis is higher compared to the visual methods based on fluorescence. In spite of the problems GFP expression or other fluorescent protein could present, their use as reporter genes seems to be the best choice.

Table 1. A summary of the principal techniques involving gene transfer for generation transgenic animal

Biological model	Technical	Detection	Reference
Rabbit	DNA Microinjection method and *in vitro* cultivation	RT-PCR	Bodo *et al*., 2004
	SMGT - Liposome	PCR; GE	Wang *et al.,* 2003
Mouse	SMGT	SB; GE	Lavitrano *et al.,* 1989
	SMGT	GE, SB, FISH	Chang *et al*., 2002
	SMGT	PCR, GE	Sciamanna *et al*., 2003 Celebi *et al*., 2003
	TMGT- Adenovirus	GE	Kojima *et al*., 2003
	TMGT – Liposome	---------	Yonezawa *et al.,* 2001
	TMGT - Liposome	PCR; SB	Zhao *et al.,* 2003
Cattle	SMGT - Eletroporation	PCR	Gagne *et al*., 1995
	Infection of bovine oocytes with lentiviral vectors		Hofmann *et al*., 2004
	SMGT - Eletroporation	PCR / HR	Rieth *et al*., 2000
	SMGT	PCR, GE	Shemesh *et al*., 2000
	SMGT	PCR	Sperandio *et al*., 1996
	SMGT	GE	Rottmann *et al.*, 1996
Pig	Lentiviral microinjection	PCR	Whitelaw *et al*., 2004
	SMGT	SB	Sperandio *et al*., 1996
	SMGT – monoclonal antibody	GE; SB, FISH	Chang *et al*., 2002
Goat	Pronuclear microinjection	PCR; SB	Baldassarre *et al*., 2003
	SMGT – monoclonal antibody	GE; SB, FISH	Chang *et al*., 2002
Fish	SMGT	DB; GE;	Jesuthasan and Subburaju., 2002
		PCR, SB	Khoo, 2000
	Transposon - mediated	GE	Kawakami *et al*., 2004
	Transposon - mediated	PCR, GE	Grabhera *et al*., 2003
	SMGT/Electroporation	PCR;	Sin, *et al.,* 2000
	SMGT/Electroporation/ Osmotic Differencial	PCR, GE	Kang *et al*., 1999 Collares *et al*., 2004
	SMGT TMGT - Lipossome	PCR,SB	Lu *et al.*, 2002

SB= Southern blot; PCR= polymerase chain reaction; FISH (fluorescent in situ hibrization). RT-PCR= real-time polymerase chain reaction; SMGT= Sperm-Mediated Gene transfer; TMGT = Testis-Mediated Gene Transfer. HR= Homologous Recombination; GE = Gene Expression.

Regulatory sequences and Artificial chromosomes (YAC, BAC, and MAC)

Genes in eukaryotic organisms have regulatory regions that participate in the control of their expression. Sequences of 150-200 nucleotides called promoters are part of these regulatory regions and are located near the transcription initiation sites (Houdebine, 2003; Hu *et al*., 2004). The promoters define the level and tissue specificity of genic expression. Thus, the transgene should have, besides the target gene, regulatory sequences in the upstream region and the poliadenilation signal in the downstream region of the construct. Other elements which participate in the genic expression control, are the enhancers, the insulators, the silencers and the locus control region (LCR), which contains different enhancers or insulator elements (Guglielmi *et al.*, 2003; Houdebine, 2003). The presence of introns in the gene constructions also can lead to a more efficient expression (Petitclerc *et al*., 1995; Rocha *et al*.,

2004) or to a less efficient expression if their sequences contain silencers (Lin *et al*., 2004).

The first promoters used in gene constructs were derived from human genes since there was a lack of knowledge of the target species sequence. Other promoters used include CMV (cytomegalovirus), β-actine genes, myosin light chain, WAP (Whey Acidic Protein), a protein expressed in salivary gland (Golovan et al., 2001), primordial cells (Yoshizaki et al. 2000), and gene P12 expressed in male accessory gland (Dyck *et al*., 1999). The CMV promoter present in various commercial vectors drives the expression predominantly to nervous tissue. The β-actin promoter has been fused to the growth hormone (GH) gene to direct the expression to muscular tissue. The light chain myosin promoter was used by Gong *et al.* (2003) to express different fluorescent proteins in zebrafish muscle. Extremely high levels of the target protein were observed in the transgenic products, demonstrating the potential use of fish muscle to synthesize proteins of interest. The WAP gene promoter was used by Limonta *et al.* (1995) to direct the hGH expression to transgenic rabbit mammary gland. Besides the WAP promoter, the ovine β-lactoglobulin, the goat β-casein, and the bovine S1-α-casein promoters drive the expression of milk secretion (Whitelaw et al., 1991; Brink et al. 2000, Parker *et al*., 2004). The promoters can also be used to mark cells, as was demonstrated by Yoshizaki *et al.* (2000). The authors used the promoter RtVLG to drive the expression of GFP (green fluorescent protein) to rainbow trout primordial cells. The promoter P12 was used by Dyck *et al.* (2003) to express human GH in transgenic mice seminal vesicle epithelium. GH was secreted in the seminal fluid ejaculated, with the seminal vesicle lumen contents containing GH concentrations of up to 0.5 mg/ml.

The transcription enhancers are sequences found upstream or downstream of the promoters and generally have multiple sites for transcription factors. The enhancers increase the transcription rate and direct the expression to a specific tissue. Glasser *et al.* (2005) demonstrated that an enhancer located in the proximal region of a 4.8KB SP-C is essential to the expression of pulmonary surfactant protein C. A distal and a proximal upstream element, as well as a downstream-located enhancer of pseudo-allelic versions of FoxD5 genes of *Xenopus laevis*, contribute to transcription (Schon *et al.*, 2004)**.** Besides, the downstream enhancer cooperates with the proximal upstream element and also contributes to the spatial expression.

The insulators or chromatin borders are DNA sequences that have the capacity of establishing genomic barriers, protecting DNA sequences from the neighbor heterochromatin expansion, and have the potential to interfere with the activity of enhancers distally located (Giraldo *et al*., 2003). A comparative analysis of the use of insulators in transgenic animals, produced from heterologous constructs, was presented by Giraldo et al. (2003). A functional analysis of suHw insulators was made by Majumder and Cai (2003) in *Drosofila* embryos. The suHw insulator is a sequence of 340-bp present in the *gypsy* retrotransposon. It was observed that the pairing of type suHw insulators or even suHw heterologous with other insulators could increase the enhancers blocking activity, suggesting that insulators can act independently or additively. In transgenesis, insulators are used to protect a transgene against chromatin position effects at their genomic integration sites, and they are able to maintain transgene expression for long periods of time (Recillas-Targa *et al.*, 2004). One application of the insulator type element in the transfection of animal cells was presented by Yao *et al*. (2003). The authors succeeded to block the silencer in transgenic mice using insulator elements to avoid the retrovirus blocking. Retroviral vector silencing is of interest to mark stem cells and for studies of gene manipulation, because it can compromise therapeutic gene expression during the application of gene therapy (Yao *et al.*, 2003).

Restricting transgene expression to specific cell types and maintaining long-term expression are major goals for gene therapy (Kim *et al*., 2004). Therefore, the development of systems to induce expression of transgene that could control time and tissue expression, and the development of methodologies to direct construction, are desirable for the control of gene expression, insertion efficiency, and loci incorporation into genomes (Rocha et al., 2004). Recent advances in transgenic technologies to generate temporally and spatially restricted targeted gene disruptions are promising for the understanding of epididymal genes involved in sperm maturation process (Lye and Hinton, 2004).

Although plasmid and viral gene delivery systems have been used successfully to introduce genes of interest into mammalian cell lines and transgenic animals, they are limited with regard to the amount of foreign DNA sequence that can be delivered (Lindenbaum *et al*., 2004). Potential problems of conventional transgenes include insertional disruption of the host genome and unpredictable, irreproducible expression of the transgene by random integration (Katoh *et al*., 2004).

Artificial chromosomes (engineered mini-chromosomes and other chromosome-based DNA constructs) are promising new vectors for use in gene therapy, protein production and transgenesis. Artificial chromosomes are able to carry extremely large DNA fragments of more than one megabase (Mb) (Oberle *et al*., 2004).

The use of YAC (yeast artificial chromosome) and BAC (bacterial artificial chromosome), constructs is usually associated with optimal performance in transgenic experiments. The size of their genomic inserts habitually ensures the inclusion of most regulatory elements that are relevant for the right expression of a given gene. Therefore, artificial chromosome-typetransgenes are normally expressed in spatially and

temporally correct manners (Giraldo *et al*., 1999; Giraldo and Montoliu, 2001; Montoliu, 2002; Oberle *et al.*, 2004).

The generation of artificial chromosomes, known as MACs (mammalian artificial chromosomes), are expected to incorporate all the benefits of the classical artificial chromosome-type vectors while maintaining the normal chromosomal status within the mammalian host cells (Montoliu, 2002). Compared to traditional methodologies, MACs offer significant advantages for cellular protein production, animal transgenesis and gene-based cell therapy applications on account of their capacity for carrying large constructs and ability to self replicate without relying on integration into the host genome. Despite the numerous advantages of MAC technology, systematic limitations have precluded its widespread implementation. These limitations include the requirement for *de novo* chromosome synthesis for each individual application, the inability to shuttle MACs easily across various cell types and the inability to precisely engineer gene targets onto the artificial chromosome. For broad applicability of MAC technology, all of these limitations must be addressed (Lindenbaum *et al*., 2004).

The intra-cytoplasmic sperm injection (ICSI) method for the stable incorporation and phenotypic expression of large yeast artificial chromosome (YAC) constructs has been able to produce founders exhibiting germ line transmission of an intact and functional transgene. Compared with the standard pronuclear microinjection method, the efficiency of the ICSI-mediated YAC transfer system by co-injecting spermatozoa and YACs into metaphase II oocytes has been significantly greater (Moreira *et al.*, 2004).

The benefits of artificial chromosomes in transgenesis will soon be exported to biotechnological applications, including the production of recombinant proteins of interest in the mammary gland of transgenic animals, with the hope that animal transgenesis will eventually become more reproducible, efficient, and predictable (Montoliu, 2002).

Applications of transgenesis

Livestock production

Enhanced prolificacy and reproductive performance, increased feed utilization and growth rate, improved carcass composition, improved milk production and/or composition, and increased disease resistance are practical applications of transgenesis in livestock production (Wheeler, 2003; Gerrits *et al*., 2005).

The first livestock targeting experiments have been directed at engineering animals either to render their organs immunologically compatible for use as human transplants, or for improving the commercial production of recombinant proteins in the transgenic mammary gland (Thomson *et al*., 2003).

Alpha-Lactalbumin plays a role in lactose synthesis and in the regulation of milk volume. Transgenic hemizygous sows over-expressing the milk protein, bovine alpha-lactalbumin produced as much as 0.9 g bovine alpha-lactalbumin per litre of milk obtained from the sow (Wheeler *et al*., 2001). A higher weight gain (days 7-21 after parturition) of piglets suckling alpha-lactalbumin gilts was also observed. Therefore, the over-expression of milk proteins in transgenic sows could contribute to a better lactation performance of pigs (Noble *et al.*, 2002).

Transgenic cows containing extra copies of the genes encoding bovine beta- and kappa-casein (CSN2 and CSN3, respectively) produced milk with an 8-20% increase in beta-casein and twofold increase in kappa-casein levels (Brophy *et al*., 2003). This work showed that it is feasible to substantially alter a major component of milk in high producing dairy cows by the transgenic approach to improve the functional properties of dairy milk.

Alteration of the protein composition of the wool fiber via transgenesis with sheep wool keratin and keratin associated protein (KAP) genes may lead to the production of fiber types with improved processing and wearing qualities (Bawden *et al*., 1998). These authors obtained wool fibers with higher luster and reduced crimp, as a result of alterations in their micro and macrostructure due to a higher level of cortical-specific expression of a wool type II intermediate filament (F) keratin gene.

Application as Bioreactors

The production of therapeutic proteins represents the first application of recombinant DNA technology (Walsh, 2003). By the 2003, the European Union had approved 88 products. However, none of these approved products were obtained in transgenic systems. Despite this, domestic animals represent an efficient production system for large and complex (and biologically active) recombinant proteins which could be used to treat or prevent human diseases. The production of these pharmaceutical proteins in the mammary gland of livestock originated the term biopharming (Keefer, 2004). Transgenic rabbits, sheep, goats, pigs and cattle express heterologous proteins have been have been produced successfully by various investigators (Lubon et al., 1996; Paleyanda et al., 1997; Houdebine, 2000; van Berkel et al., 2002, Fan and Watanabe**,** 2003). The production of biopharmaceuticals presents the most varied purposes (Rutovitz and Mayer, 2002): for treating such diseases as multiple sclerosis, hepatitis, cystic fibrosis, blood disorders, some types of cancers, hemophilia, thrombosis, growth disorders, Pompe's disease, osteoporosis, Paget's disease and anemia, and for improving infant's formula.

Initially the use of transgenic animals as biore actors focused on the use of mammary gland as target

organ (Whitelaw *et al*. 1991, Wright *et al.,* 1991; Van Cott *et al*. 1999, Houdebine, 2000; Wheeler *et al*., 2001, An *et al*., 2004). For example, human protein alpha-glycosidase is secreted in the milk of transgenic rabbit. It has been successfully used to treat patients who are genetically deficient in this enzyme (Fan and Watanabe, 2003). However, nowadays other systems are being evaluated, including the excretion of specific proteins in mouse urine (Ryoo *et al*., 2001) and in pig semen (Dyck *et al*., 2003).

An interesting alternative for the production of therapeutic proteins is to use the initial developmental stage of embryos of some species of fish (Hsiao and Tsai, 2003; Hwang *et al.,* 2004; Morita *et al.,* 2004). Hwang *et al.,* (2004) demonstrated the production of factor VII in fertilized eggs of zebrafish, catfish, African catfish, and tilapia. However, the method used for introducing the transgene into the embryo was the micromanipulation, which is extremely laborious.

The search for other animal models, and other tissues for protein production, continues because of the cost involved in obtaining a large transgenic animal such as a cow. Even in goats which serve as a better model than cattle for transgenesis, there are some adverse effects on the mammary gland due to the production of certain proteins. Also, the necessary post-translational protein modifications are not invariably realized even in the mammary gland epithelium (Houdebine, 2002b). All these point to the fact that an efficient and inexpensive system of producing transgenic animals, is yet to be found in spite of the advances already achieved in this area.

Applications for organ donation

An organ transplant between discordant (non-related) species is defined as xenotransplants this procedure is usually associated with a hyperacute rejection response (HAR) that destroys the transplanted organ within minutes (Niemann, 2001). The HAR occurs as a result of pre-formation of antibodies and complement activation and it can cause irreversible vascular damage and cellular necrosis (Lazzerechi *et al*., 2000). Some authors consider the pig as the best organ donor because of various reasons: their organs have anatomical and physiological similarities to human organs, they have short reproductive cycle and large number of offspring at a time, they can be maintained with a high level of hygiene at relatively low cost, and they are a domesticated species (Lazzerechi *et al*., 2000; Niemann and Kues, 2003). Despite these advantages, it is still necessary to avoid the HAR that occurs in xenotransplants from pigs to humans. In the attempt to avoid this problem, some groups have developed transgenic pigs (hDAF) expressing species-specific complement activation system inhibitors (Lazzerechi *et al*., 2000) as well as HLA-DP and HLA-DQ pigs, which, being more similar in the HLA-II system leads to decrease in allotransplant rejection (Tu *et al.,* 2003; An *et al.,* 2004; Pohajdak *et al*., 2004). Other points to be considered include the differences in growth and life span between humans and pigs, and the potential for disease transmission from the xenotransplant to the recipient. Preventing the potential transfer of pathogenic microorganism, especially of porcine endogenous retrovirus (PERV) is a major prerequisite in the use of pig organs as xenotransplants (Niemann, 2001). Production of pigs under specified pathogen-free conditions is not totally effective in eliminating the risk of infection by PERVs. To reduce the release of PERVs by porcine transplants, a new approach, using synthetic short interfering RNAs (siRNAs) corresponding to different parts of the viral genes *gag*, *pol*, and *env*, was applied by Karlas *et al*. (2004). This strategy was efficient in the suppression of PERV replication. Moreover, the use of cells or organs from transgenic pigs producing short hairpin RNAs (shRNAs) should increase the safety of xenotransplants (Karlas *et al*. 2004).

Another group of animals with the potential as organ donors is fish. For example, a group of Canadian investigators has produced transgenic tilapia in which the islets of β cells in the Brockmann body synthesize human insulin. These transgenic fish could serve as donors of islets for xenotransplants, even in the encapsulated form (immunoisolated), because they display higher hypoxia resistance than mammals (Pohajdak *et al*., 2004). It should also be considered that the costs for producing SPF animals and the collection of the islets from tilapias would be much lower compared to swine. Furthermore, the potential for transmission of xeno-zoonotic infections is lower with transplanted fish cells because of the larger phylogenetic distance between teleosts and humans.

Applications as models for disease process

Analysis of disease processes and questions related to developmental biology require more elaborated models than those involving the expression or knock out, of one or more genes (Ryding *et al*., 2001). Nevertheless, genetically modified laboratory animals provide a powerful approach for studying gene expression and regulation, and allow the direct examination of structure-function and cause-effect relationships in pathophysiological processes and development (Fan and Watanabe, 2003; Kimura-Yoshida *et al*. 2004). However, it is necessary to direct the expression to a specific tissue and to control the levels of expression.

The use of DNA microinjection to produce transgenic animals to serve as human disease models is not practical or meaningful since, this method does not offer any control over the number of copies integrated and the sites in the genome where integration takes place (Petters and Sommer, 2000). On the other hand, Chen *et al*. (2004) demonstrated that foreign DNA could effectively be introduced into the cells of cornea

, retina and lens of birds through electroporation of the eggs. Electroporation offers a faster and easier way to manipulate gene expression during embryo development (Chen *et al*., 2004).

The animal commonly used as the model for studying human disease process is the mouse (Giraldo and Montoliu, 2001; Guglielmi *et al*., 2003). Diseases studied using the mouse model include sickle cell anemia, amyotrophic lateral sclerosis, chronic hypertension, retinal degeneration, osteogenesis imperfecta, cystic fibrosis, mitochondrial cardiomyopathy and neurodegenerative disease, Werner syndrome, rhodopsin mutations and retinitis pigmentosa, melanoma, Alzheimer's disease, prostate cancer and atherosclerosis (Shapiro *et al*., 1995; Petters and Sommer, 2000; Karnani and Kairemo, 2003; German and Eisch, 2004; Venkateswaran *et al.,* 2004). However, other organisms as rabbits, cows, pigs, and fish can potentially be used to model human diseases (Duverger *et al*. 1996; Bõsze *et al*., 2003; Fan and Watanabe, 2003). Transgenic rabbits expressing human genes have been used as models for arterioscleroses, cardiovascular disease, acquired immune diseases (AIDS), and cancer (Duverger *et al*., 1996; Fan and Watanabe**,** 2003).

The generation and analyses of transgenic animals carrying different constructs that lead to different phenotypes will be among the initial steps to the understanding of the relationship between different genes and the role of each one in the development of the organisms.

Perspectives

The search for new strategies that improve animal transgenesis could potentially promote significant advances in basic and applied biology. This possibility and the potential for economic benefit, have stimulated the development of a new industry. As a result, different methods to improve the efficiency of production of transgenic animals are constantly being tested.

Microinjection has made significant progress in transgenic research, and it will continue to be the method of choice until efficient mass gene transfer techniques (for example, SMGT, TMGT, and cell line transfection) become available. The application of methodologies that improve mass gene transfer techniques, such as lipofection and electroporation, in transgenic research is still in its developmental stage. Further tests of these methods using a wider range of organisms may provide more information on their suitability for use in gene transfer, routinely.

The use of transgenic animal models, together with the actual molecular biology tools, will help to identify the role of specific genes in molecular, biochemical, physiological and endocrine events in development and disease processes in animals and humans. Parallel advances in the localization and characterization of genes that control quantitative traits will contribute to the understanding of the variability of transgenic products generally encountered when these techniques are applied to livestock production. Significant improvements have been achieved in transgenic animal generation in the past few decades. However, for some species, a more efficient and low cost production system needs to be developed.

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