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Transgenic Animal Production and Applications

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Abstract
A transgenic animal is an animal in which foreign DNA has been incorporated into its original DNA. The generation of transgenic animals is one of the most complex aspects of genetic engineering, both in terms of technical difficulty and in the ethical problems that arise. To establish a transgenic animal, foreign DNA constructs need to be introduced into the animal's genome, using recombinant DNA technology, so that the construct is stably maintained, expressed and passed on to subsequent generations. The first successful transgenic animal was a super mouse since that time the development of transgenic animals and the exploration of their applications has been a steady process, although at a slower rate than what was initially expected. DNA microinjection, Somatic cell nuclear transfer, Embryonic stem (ES) cells mediated gene transfer and Retrovirus-mediated gene transfer are the commonly used methods to transfer foreign gene from one species to another. Use of transgenic animals will provide solutions for disease model, drug research, food production and improved disease resistance. This review summarizes recent research based on the transgenic animal production and their potential applications.
Keywords: Application; Production; Transgenesis ; Transgenic animals

INTRODUCTION

Transgenesis is a procedure in which a gene or part of a gene from one individual is incorporated in the genome of another one. A transgenic animal is an animal that carries a specific and deliberate modification of its genome – analogous to a transgenic plant. The first transgenic experiments in mammals were performed in mice (Gordon *et al*., 1980) afterward rabbits, pigs, sheep and cattle (Hammer *et al*., 1985, Pursel *et al*., 1987, Rexroad *et al*., 1989, Roschlau *et al*., 1989). When considered on a global scale, the potential for exploitation of transgenic animals would appear to be almost unlimited. Achieving that potential is likely to be a long and difficult process in many cases, but the rewards are such that a considerable amount of money and effort has already been invested in this area.

Transgenic farm animals are important in human medicine as sources of biologically active proteins, as donors in xenotransplantation (Xenografts from transgenic pig) and for research in cell and gene therapy. Typical agricultural applications include: improved carcass composition, lactational performance, wool production as well as enhanced disease resistance and reduced environmental impact (Niemann *et al*., 2005). Commercial applications include the preparation of recombinant proteins, protection of animals against disease, and introduction of new genetic traits into herds.

Recent developments in animal gene transfer technique includes DNA microinjection, Somatic cell nuclear transfer, Embryonic stem (ES) cells mediated gene transfer and Retrovirus-mediated gene transfer method to improve efficiency and gene targeting to improve accuracy (Miao, 2012). But each of techniques has their own limitations and most of those techniques are uncertain and have long term effects on transgenic animal production. Therefore, the objective of this paper is: to highlight the techniques that are currently applied to produce transgenic animals and method of applications and its implications.

TRANSGENIC ANIMALS

A transgenic animal has been defined as an animal that is altered by the introduction of recombinant DNA through human intervention. To establish a transgenic animal, foreign DNA constructs need to be introduced into the animal's genome, using recombinant DNA technology, so that the construct is stably maintained, expressed and passed on to subsequent generations. The first transgenic mice were produced in the early 1980s, but it is only within the past seven years that transgenic livestock have been produced on a routine basis (Wall, 1996). Transgenic animals can be created for a variety of different purposes: to gain knowledge of gene function and further decipher the genetic code, study gene control in complex organisms, build genetic disease models, improve animal production traits, and produce new animal products (Melo *et al*., 2007).

Methods for the production of transgenic animals

Foreign gene to be used in creating the transgenic animal is constructed through a process known as recombinant DNA methodology. In this process, desired genes or a piece of DNA to be inserted from another species are cut with a restriction enzyme. Isolate plasmids from cloning vector and by using the same enzyme cut plasmids and then insert desired DNA into plasmid and then join the two DNA with DNA Ligase (Kinsey and Cooey, 2000).

The transgeneis inserted into a vector (which allows it to be amplified to high copy numbers). The vector also contains a promoter which allows the inserted foreign DNA to be expressed by the cells of the host animals (Anderson and Dowdy, 2005). In generating a transgenic animal, it is desirable that all the cells in the organism receive the transgene. The presence of the transgene in the germ cells of the organism will enable the gene to be passed on to succeeding generations, and this is essential if the organism is to be useful in the long term. Thus, introduction of genes has to be carried out at a very early stage of development, ideally at the single-cell zygote stage. The techniques that are currently applied to produce transgenic animals are listed below.

Microinjection Method

The direct DNA microinjection into the pronuclei of embryos was the first technique which led to regular and relatively easy success in mammals. DNA microinjection was successfully used for the first time in 1980 (Gordon *et al*., 1980), mouse was the first animal to undergo successful gene transfer. DNA microinjection method is based on the injection of a foreign DNA construct into a fertilized oocyte (Figure 1). The construct integrates randomly into the host oocyte genome, subsequently the zygote continues embryonic development, the embryo is transferred to a foster mother and eventually develops to a transgenic animal. This method has many advantages like, exogenous genes are expressed in an efficient manner, the size of the inserted DNA molecule has no clear limit and moreover this technique is simple, inexpensive and can be applied to a wide variety of species. However, as microinjection has several significant shortcomings including low efficiency, random integration and variable expression patterns related to the site of integration – research has focused on alternative methodologies for improving efficiency of generating transgenic livestock (Robl *et al*., 2007).

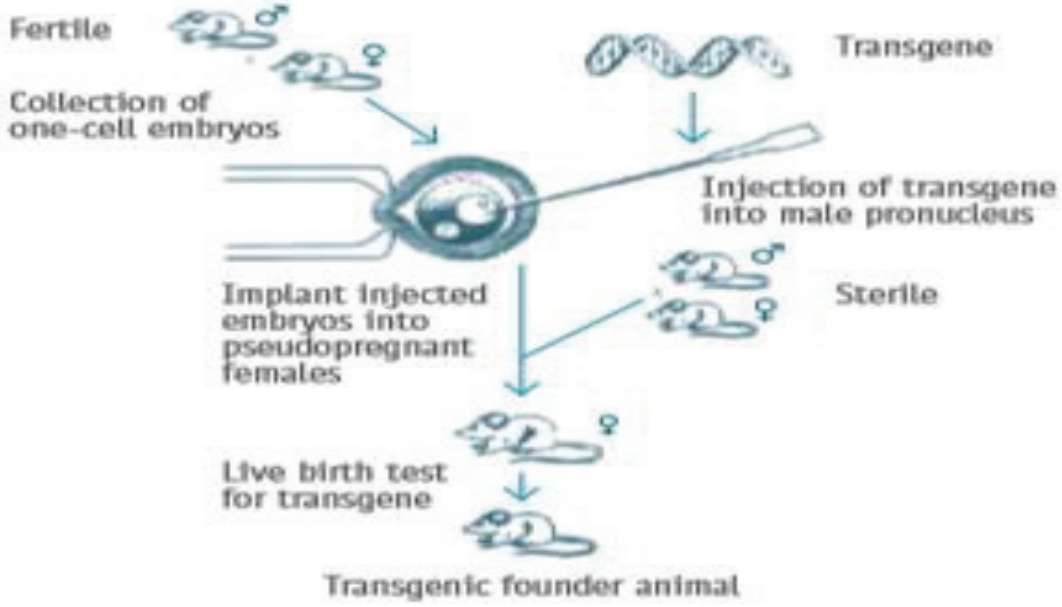

Figure 1. DNA micro-injection

Somatic cell nuclear transfer

The method of choice nowadays for the production of transgenic animals is somatic cell nuclear transfer (SCNT). In genetics and developmental biology, somatic cell nuclear transfer (SCNT) is a laboratory technique for creating a viable embryo from a body cell and an egg cell. The entire method is based on the following protocol: oocytes from a donor animal are enucleated, i.e. their nucleus containing the genome is removed. Subsequently a donor nucleus is injected into the enucleated oocyte, and the cells are fused by electrofusion. Following fusion, the oocyte is activated by chemical or mechanical means to initiate embryonic development, and the resulting embryo is transferred to a foster mother (Hodges and Stice, 2003). The donor nuclei can be derived from either somatic cells or ES cells that have been subjected to targeted genetic manipulation prior to injection into the oocyst. Somatic cell transfer may be used to generate multiple copies of genetically elite farm animals, to produce transgenic animals for pharmaceutical protein production or xeno-transplantation (Wilmut *et al*., 1997; Polejaeva *et al*., 2000) or to preserve endangered species. Dolly the Sheep, famous for being the first successfully cloned mammal was created using this process (Li *et al*., 2009).

Embryonic stem (ES) cells mediated gene transfer

Embryonic stem cells, as the name suggests, are derived from embryos at a very early stage (the blastula), and have achieved major consideration in recent years in the field of medicine, agriculture and biomedical research due to their unique property of pluripotency. Pluripotency is the ability of these cells to differentiate to any of the

cell types and tissues found in the adult organism. ES cells can be grown in culture for many passages and can be subjected to transformation with transgene constructs, resulting in modifications of their genome. The constructs used not only permit the selection of successfully transformed cells, but also allow gene targeting to be accomplished. Thus, genes can be specifically introduced, replaced or deleted (so-called knock-ins and knock-outs). Transformed ES cells are re-introduced into the blastocoel cavity of an embryo, where they integrate and produce a mosaic (chimaeric) animal, i.e. an animal that is made up of transformed and non-transformed cells. Possibly, the chimaeric animal carries the transgene in the germ line; in this case, it is possible to obtain completely homozygous transgenic animals through selective breeding (Figure 2). This technique, mainly through the feature of gene targeting, allows a broad variety of genetic modifications to be introduced. For many years, several laboratories worldwide have tried to produce ES cells from farm animals, and although some success has been claimed, no robust and reproducible method has been published. Indeed, even in mice the production of ES cells is a costly and labour-intensive technology (Melo *et al*., 2007).

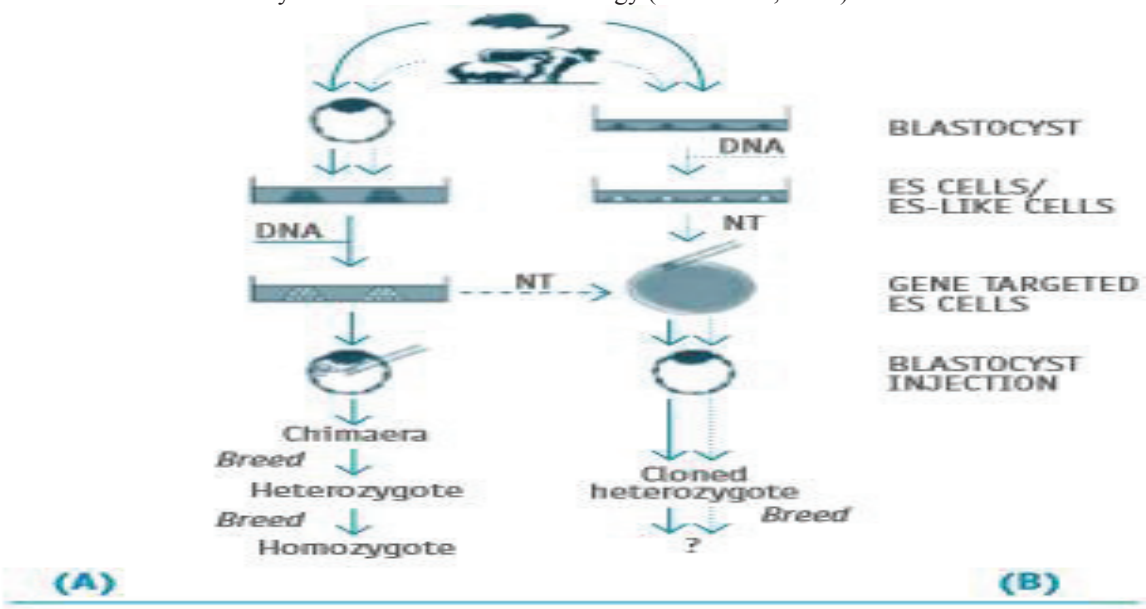

Figure 2. Embryonic stem (ES) cells mediated gene transfer (A) and Somatic cell nuclear transfer (B).

Retrovirus-mediated gene transfer

Transgenesis may also be accomplished by employing virus-derived vectors, namely vectors based on the retrovirus-class of lentiviruses (Whitelaw *et al*., 2008). Retrovirus is single stranded RNA virus which upon transfection gets converted to double strand DNA and integrates into the host genome (Eglitis *et al*., 1988). Genes that are essential for viral replication are deleted from the viral genome, maintaining only the capacity for integration of the viral genome into the host genome. Parts of the vector that were occupied by viral genes can then be replaced by the transgene of interest – an approach analogous to the modification of the A. tumefaciens Ti-plasmid. Viruses carrying the modified vector are then produced in vitro and subsequently injected into the perivitelline space of the zygote (or an unfertilized oocyte), resulting in infection of the zygote and integration of the viral genome into the host genome. Transgenesis rates reaching up to 100 percent of injected embryos have been described (Park, 2007). Major drawbacks of this method are a limited transgene size and random transgene integration. The maximal transgene size is 8 kb, which is rather low compared with other techniques. Random and possibly multiple transgene integration may lead to position effects, disturbance of the host genome and dose effects, as is the case with pronuclear injection. Solving these problems holds great promise for the further development and application of lentiviral vectors.

Application of transgenic animals

Transgenic animals for food production

Engineering transgenic animals with an application in food production focuses mainly on improved meat production, improved carcass quality and enhanced milk production. Milk is a complex biological fluid and has a high importance for contributing to the nutrition of many societies (Melo *et al*., 2007). The major goals for transgenic animal development concerning milk production are increased milk production, higher nutrient content or milk containing novel substances. Most milk proteins (circa 80 percent) belong to the caseins, and transgenic cattle were created that contain extra copies of casein genes. This resulted in elevated casein protein levels in milk (Brophy *et al*., 2003). Another milk application that is being investigated is the production of milk

with no lactose (milk sugar) present, since approximately 70 percent of the world population cannot metabolize lactose and thus cannot consume dairy products. Engineering milk with novel properties, e.g. milk containing the immune-stimulating human protein lactoferrin, is a further approach. Many other additives, e.g. different growth hormones or substances that stimulate health and development, have been proposed for overexpression in milk and thus possibly contribute to growth and health of developing offspring.

In pigs, the transfer of the bovine a-lactalbumin gene led to increased milk production, resulting in faster piglet growth and survival rate. One of the first reports with relevance for enhanced meat production was the article about the first transgenic mice, expressing rat growth hormone and showing increased body size and mass. However, transferring this approach to pigs initially did not yield promising results. Nevertheless, pigs showing increased muscle weight gain and feed efficiency by introducing porcine growth hormone or human insulin-like growth factor have been created (Niemann *et al*., 2005). Furthermore, pigs expressing the enzyme phytase in their salivary glands have been created: these animals can metabolise the phosphor present as phytic acid in corn and soy products, thus needing less phosphor as feed additives and releasing less phosphor with their manure, reducing the environmental impact of pig farming (Haefner *et al*., 2005). Experiments in cattle are focusing on the myostatin gene, a negative regulator of muscle mass, resulting in a high increase in muscle mass in animals with a myostatin mutation or deletion. Transgenesis is also employed for fish; injection of embryos with constructs containing either the bovine or Chinook salmon growth hormone has been reported, with the aim of improving fish growth in general and especially under adverse conditions, e.g. low water temperatures. This has resulted in an up to 5-11 fold increase in weight after one year of growth for transgenic salmon and 30-40 percent increased growth of transgenic catfish (Wheeler, 2007). All these studies demonstrate the fundamental feasibility of applying transgenesis to agricultural animals for improved food production, but so far no transgenic food producing animal has been released for commercial use. In addition to the research and development necessary for the establishment of a transgenic animal, there are several other factors that strongly influence the use of transgenic animals for food production. Among these are considerations concerning the economic practicability, social acceptance of transgenic food and, possibly most important, regulations concerning the approval of GMOs and derived products.

Regulatory authorities need to consider three factors: Safety of the food product for human consumption; Environmental impact of the genetically modified animals and welfare of the animals. These factors need to be considered on a case-to-case approach for every new transgenic animal or product that has been obtained using GMOs. In principle, this safety investigation is identical to the safety regulations and procedures that apply for transgenic plants.

Transgenic animals for production of human therapeutics

One major application of animal transgenesis nowadays is the production of pharmaceutical products, also known as animal pharming. The costs for producing transgenic animals are high, but since the pharmaceutical industry is a billion-dollar market the input is likely to be a feasible and economically worthwhile investment (Sullivan *et al*., 2008). Since many human proteins cannot be produced in microorganisms and production in cell culture is often labour-intensive with low yields, the production of biopharmaceuticals in transgenic animal bioreactors is an attractive alternative (Kind and Schnieke, 2008). Furthermore, many human proteins cannot be produced in micro-organisms, since they lack post-translational modification mechanisms that are essential for the correct function of many human proteins.

Pharmaceutical proteins or other compounds can be produced in a variety of body fluids, including milk, urine, blood, saliva, chicken egg white and seminal fluid, depending on the use of tissue-specific promoters (Houdebine, 2009). Nevertheless, milk is the preferred medium due to its large production volume. Furthermore, it has been shown that the mammary glands can produce up to 2 g of recombinant protein per litre of milk; assuming average protein expression and purification levels, only relatively small herds of transgenic animals would be required to supply the world market with a specific recombinant protein (e.g. 100 transgenic goats for the production of 100 kg monoclonal antibodies required per year (Melo *et al*., 2007). In Table 1, biomolecules expressed in mammary glands and their anticipated applications are listed:

Table 1. Pharmaceuticals produced by transgenic animals

Pharmaceutical	Bioreactorspecies	Application/treatment	Company
Antithrombin III	goat	thrombosis, pulmonary embolism	GTC Biotherapeutics (USA)
tPA	goat	thrombosis	PPL Therapeutics (UK)
α-antitrypsin	sheep	emphysema and cirrhosis	PPL Therapeutics (UK)
Factor IX	sheep	hemophilia b	PPL Therapeutics (UK)
Factor VIII	sheep	hemophilia a	PPL Therapeutics (UK)
Polyclonal antibodies	cattle	vaccines	Hematech (USA)
Lactoferrin	cattle	bactericide	Pharming Group (NED)
C1 inhibitor	rabbit	hereditary angioedema	Pharming Group (NED)
Calcitonin	rabbit	osteoporosis and hypercalcemia	PPL Therapeutics (UK)

Adapted from: Melo *et al*., 2007.

Another advantage of biopharmaceutical production in transgenic animals is the reduced risk of transmitting diseases, compared with human-derived material. Several cases are known where hundreds of patients were infected with HIV, Hepatitis C or Creutzfeld-Jakob-disease following treatment with human-derived pharmaceuticals. Of course, animal-derived material needs to be subjected to a thorough purification procedure to exclude transmission of animal diseases (zoonoses) or contamination with animal DNA or protein that might induce an immune reaction. Nevertheless, the development of transgenic animals that secrete high contents of the desired product in their milk, and the subsequent development of an effective and high-yield purification protocol to get rid of contaminating proteins, requires a lot of knowledge and financial and intellectual input. So far, only GTC Biotherapeutics Antithrombin III has been approved for the united States market and is sold under the name of ATryn (FdA, 2009). Furthermore, many potential target proteins as well as the technologies to develop a transgenic animal are covered by patents and intellectual property rights, thus only a small number of proteins are being investigated by a small number of pharmaceutical companies at the moment (Kind and Schnieke, 2007).

A particularly promising approach is the development of transgenic animals that express human polyclonal antibodies. Antibodies are the fastest growing set of new biopharmaceuticals, for therapeutic use in cancer, autoimmune diseases, infections, transplantations, biodefence and immune deficiencies. Currently all approved therapeutic antibodies are produced by cell culture techniques.

The possibilities for the production of polyclonal human antibodies in transgenic cattle are currently being investigated; such antibodies would mimic the natural human immune response to a pathogen. Cattle would be especially suited for this purpose, since the total amount of antibodies in an adult animal is approximately 1 kg. One approach towards this end is the use of artificial chromosomes to transfer the human antibody genes to the target animal (Kuroiwa *et al*., 2002). Concomitantly, the endogenous antibody genes of the animal are knocked out to prevent their expression and thus allow purification of human antibodies without contaminating bovine antibodies. To obtain human polyclonal antibody sera from the animal, the animal would need to be immunized with a vaccine containing the pathogen of interest, e.g. a bacterium or a virus. Subsequently, the animal would build up an immune response and express the human antibodies directed against that pathogen. These antibodies could subsequently be extracted and purified from the animal's blood plasma and used to treat humans suffering from an infection with that particular pathogen. This perspective for a quick availability of large amounts of human antibody sera targeted against a certain pathogen or disease agent has raised speculations about a transformation of medicine similar to the introduction of antibiotics in the 1940s and 50s (Kind and Schnieke, 2007). Similar approaches, based on the same methodology, are being pursued for the use of plants as bioreactors for the production of medically valuable proteins and small-molecule drugs (Twyman *et al*., 2005).

Transgenic animals for improved disease resistance

Resistance or susceptibility to diseases and the immune response typically depend on a variety of genes, but identification of some key genes has brought up the possibility of gene transfer to target important and specific aspects of the immune system (Niemann *et al*., 2005). Diseases that are under investigation, by either introducing resistance genes or removing susceptibility genes, include bovine spongiform encephalopathy (BSe), brucellosis, other viral or bacterial infections, parasitic organisms, and intrinsic genetic disorders.

one often-cited example is resistance against mastitis: mastitis is a bacterial infection of the bovine mammary gland, leading to decreased productivity and milk contamination. Transgenic cattle have been produced that secrete the small protein lyostaphin in their milk, which is a potent inhibitor of Staphylococcus

aureus (S. aureus), the bacterium responsible for the majority of mastitis cases. According to first trials, the transgenic cows are resistant to S. aureus – mediated mastitis (Donovan *et al*., 2005). Further approaches of animal transgenics target animal reproductive performance and prolificacy, development of organs for transplantations (xenotransplantation) that do not evoke a rejection response, or improvement of animal fibre and wool.

CONCLUSION

The establishment of stable transgenic animals implies that the foreign DNA is present in gametes or one-cell embryos to allow its transmission to progeny. The techniques that are currently applied to produce transgenic animals include DNA microinjection, Somatic cell nuclear transfer, Embryonic stem (ES) cells mediated gene transfer and Retrovirus-mediated gene transfer. In coming years transgenic animals will play a significant role for disease model, drug research, food production and improved disease resistance. The regulatory aspects and ethics should be given due consideration while using transgenic animals. As transgenic animals become more mainstream, a small yet growing portion of the animal production industry. It will shift its operations from farming livestock for food production, to transgenic animals for pharmaceutical production. The world market is growing for human pharmaceutical products. Producing transgenic animals is still relatively expensive. However, costs are trending down and transgenic animals have certain advantages over traditional laboratory methods for producing human proteins. More commercial use of transgenic animals in food production is also likely. Regulators will need to review existing policies and guidelines regarding transgenic animals. Regulatory authorities need to consider three factors: safety of the food product for human consumption, environmental impact of the genetically modified animals and welfare of the animals. Transgenic technologies will ensure that further research and analyses will be demanded by animal producers, regulators, environmentalists, and the general public.

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