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Gene transfer technologies leading to transgenic animals

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Abstract

Gene that is introduced into the cell from an external source is called transgene. A fertile animal that carries an introduced gene (s) in its germ line is called transgenic animal. The introduction of gene (s) into animl cells that leads to the transmission of the input transgene to successive generation is called transgenesis. This article discuses and summarizes important work in the literature regarding the gene transfer technologies leading to transgenic animals. The main techniques focused in this article are DNA transfer to anmals by microinjection, biolistics and electroporation. Much stress has been given to embryonic stem cell technology, sperm-mediated gene transfer, intracytoplasmic sperm injection (ICSI), liposome/DNA delivery method, gene transfer by retrovirus and nuclear transfer method. Models for transgenic animals were also focussed. The author also reviewed the transgenic animals as bioreactors, pharmaceutical proteins derived from these animals and transgenic expression of immunoglobulin. This article will be a valuable source of information for the researcher who are starting their career in transgenic animals.

1. Introduction

Gene is a segment of DNA containing biological information and hence coding an RNA and/or polypeptide molecule. A transgene is a gene that is introduced into a cell or animal from an external source. Recombinant DNA technology has brought about complete revolution in the way living organisms are exploited. By transferring new DNA sequences into animals or by removing or altering DNA sequences in the endogeneous genome, completely new strains or varieties can be created to form specific tasks. An organism whose genetic composition has been altered by the addition of exogenous DNA is said to be transgenic.

Transgenic animals are produced by introduction of 'foreign' deoxyribonucleic acid (DNA) into preimplantation embryos. The foreign DNA is inserted into the genetic material and may be expressed in tissues of the resulting individual. The author reviewed a number of gene transfer technologies like DNA transfer by microinjection, biolistics and electroporation. The author gave much emphasis to embryonic stem cell technology, sperm-mediated gene transfer, intracytoplasmic sperm injection (ICSI), liposome/DNA delivery method, gene transfer by retrovirus and nuclear transfer method. He also reviewed the different models for transgenic animals. In this article various applications of transgenic animals were discussed. The author also reviewed transgenic animals as bioreactors and studied pharmaceutical proteins derived from these animals and also transgenic expression of immunoglobulin.

These technique are of great importance to many aspects of biomedical sciences including gene regulation, the immune system, cancer research, developmental biology, biomedicine and agriculture. The production of transgenic animals is one of a number of new and developing technologies that will have a profound impact on the genetic improvement of livestock. The reason why transgenic animal is made is listed in the Table 1.

Table 1. Reasons for producing transgenic animals

S. No	Reasons
1.	Gain new knowledge.
2.	Decipher the genetic code.
3.	Study the genetic control of physiological systems.
4.	Build genetic disease models.
5.	Improve animal production traits.
6.	Produce new animal products.

This article will be beneficial for those readers who are going to start their career in the field of gene transfer technologies in animals leading to transgenic.

2. Methods for genetic modification

It was Gorden and Ruddle who in 1981 coined the term 'transgenic' [1] to explain an animal in which an exogenous gene was introduced into its genome. In the late 1980s, the term transgenic was extended to gene-targeting experimentation and the production of chimeric or 'knockout' mice in which a gene (or genes) has been selectively removed from the host genome [2]-[3]. Today, a transgenic animal can be defined as one having any specific, targeted genetic modification. There are a number of methodologies which can be utilized for the production of transgenic animals and are listed in the Table 2.

Table 2. Methodologies that can be used for the production of transgenic animals.

S. No	Technologies that produce transgenic animals
1.	DNA microinjection.
2.	DNA transfer by retroviruses.
3.	Injection of embryonic stem (ES) cells and/or embryonic germ (EG) cells, previously exposed to foreign DNA, into the cavity of blastocysts.
4.	Microinjection of genes into pronuclei of fertilized ova.
5.	Sperm-mediated exogenous DNA transfer during *in vitro* fertilization.
6.	Liposome-mediated DNA transfer into cells and embryos.
7.	Electroporation of DNA into sperm, ova or embryos.
8.	Biolistics.
9.	Nuclear transfer (NT) with somatic cells.

2.1. DNA microinjection

Pronuclear injection is one of the methods to incorporate foreign genes of interest into animals. The most successful and widely used method for producing transgenic mice is the microinjection of cloned DNA into the pronucleus of a fertilized ovum. [4]-[8].

The technique of DNA microinjection generally demands the use of micromanipulators and a microinjection apparatus to inject into embryos a solution containing recombinant DNA. Virtually any cloned DNA fragment can be used, albeit some caveats do apply. Linearized DNA constructs are more promptly incorporated into the host genome. The presence of foreign plasmid or vector sequences can adversely affect expression of the integrated transgene. Much precaution must be taken when dealing large DNA constructs, such as those derived from bacterial or yeast artificial chromosomes, to avoid shearing during the process of microinjection. Gene constructs when microinjected, generally integrate randomly into the genome of the embryo, but typically only in a single chromosomal location [9]. After the gene transfer, pregnant recipients will follow normal gestation and deliver young at term. Advantges and disadvantages of DNA microinjection are listed in the Table 3.

Table 3. Advantages and disadvantages of the DNA microinjection

S. No	Advantages of DNA microinjection
1.	A high frequency of generating transgenic animals from viable microinjected embryos transferred to recipient females.
2.	A lack of constraint on the size or type of DNA constructs used.
3.	The stability of the transgene as it is transmitted from generation to generation.
	Disadvantages of DNA microinjection
1.	The random and potentially significant influence that the site of integration can exert on transgene expression (positional effects).
2.	The potential for undesired insertional mutagenesis.
3.	The time required in developing the necessary micromanipulation and microinjection skill sets.
4.	Process is inexpensive.

2.2. Retrovirus-mediated gene transfer

Retroviral methodology is also an efficient method to integrate genes of interest into animal genomes [10]. The retrovirus behaves as a naturally occurring delivery system to incorporate DNA into a number of mammalian cells [11]. Preimplantation embryos or oocytes [12]-[13] can be exposed *in vitro* to concentrated virus solutions or incubated over a single layer of virus-producing cells. Although embryos can be used up to midgestation, four- to 16-cell stage embryos are primarily used for infection with one or more recombinant retroviruses. After infection, the retrovirus produces a DNA copy of its RNA genome using the viral enzyme reverse transcriptase. The DNA copy of the viral genome, or provirus, integrates randomly into the host cell genome. Embryos infected with viruses are then

transferred back to recipient females to complete gestation. The use of retroviruses produces high rates of gene transfer, approaching 100% efficiency.

The virus contains the structural gene such as *gag*, *pol* and *env* which support viral particle formation. For safety purposes, retroviruses are frequently modified by removing these structural genes. However, a number of retroviral lines used in transgenic animal experiments are ecotropic, meaning that they infect only the model systems (e.g. mice or rats); hence rodent cell lines, rather than humans, could be at risk of contamination if correct precautions are not taken [9]. Various advantages and disadvantages of using viral vectors in genetic engineering of agricultural animals were also reported [14]. Important disadvantages of retrovirus mediated gene transfer are listed in the Table 4.

Table 4. Disadvantages of retrovirus-mediated gene transfer

S. No	Disadvantages
1.	Low copy number integration.
2.	The additional steps required to produce retroviruses in comparison to microinjection or ES cell-based techniques.
3.	General limitation on the size of the foreign DNA insert.
4.	A potential for undesired genetic recombination that might alter the replicative characteristics of the retrovirus.
5.	Possible interference of retroviral sequences on transgene expression.
6.	A high frequency of mosaicism.

2.3. Embryonic stem cell technology

Embryonic stem (ES) cell have the potential to form all of the cell types of the mature animal (muscle, nerve, skin etc) including the gamete [15]. Firstly the DNA of interest can be integrated into the cultured ES cells. The cells transfected with the gene of interest can be then selected. The recombinant selected ES ells are then incorporated into fresh blastocyst. In the blastocyst they are mixed with the cell of the inner cell mass. The blastocyst is then implanted into the uterus of pseudopregnant female and then pups were produced [16].

The level of chimerism can be known by seeing the coat color of mice after birth. Because the coat color of the parental ES cell strain is different from that of the host embryo strain, mice in which both strains contribute to the development will display a mix of two coat colors. This phenomenon is termed chimerism. The animals showing this trait are called as chimeras.

The advantage of performing genetic manipulations in ES cells is that verification of the targeted modification can be performed *in vitro* before any animal work is initiated. One of the major advantages of ES cell is that they are relatively efficient at homologous recombination in comparison to other animal cells. The greatest obstacle for generating ES-cell derived transgenic animals is due to the difficulties associated with the production, characterization and maintenance of pluripotent ES cell lines.

2.4. Nuclear transfer

Nuclear transfer (commonly referred to as 'cloning') involves the introduction of donor nuclei obtained from either stem cells or differentiated adult cells into enucleated oocytes, thereby reprogramming future development. Reconstructed oocytes are then transferred to a surrogate mother for continuation of gestation. The first mammalian nuclear transfer experiments were reported in mice in early 1980s amid some controversy [17]-[18]. The use of NT (cloning) techniques may have the potential to increase the number of offspring from a single female into the thousands and possibly the tens of thousands [19]. Since the famous cloned sheep "Dolly" was born [20], NT technology has become another methodology available for the production of transgenic animals.

2.5. Biolistics

Biolistics is one of the methods of DNA transfer. Biolistics or particle bombardment is a physical method to transfer gene of interest. This technique uses accelerated microprojectiles to transfer DNA or other molecules into intact tissues and cells [21]. Initially this method was developed to transfer genes into plants by Sanford [22]. The main advantage of biolistics, compared to other transfection methods, is its mechanical ability to cross biological membranes [23]. Biolistics do not depend upon the structure and characteristics of target cell membranes [24] nor on the interaction between DNA molecules and the membranes.

2.6. Sperm-mediated gene transfer

Sperm mediated gene transfer is also a method for introducing exogenous DNA into animals for producing transgenics. Sperms of many species have been shown to bind naked DNA [25-27] as well as DNA-liposome complexes [28]-[29]. Generally, sperm are collected at ejaculation or from the epididymis of the testis. It is then incubated in fertilization medium at 37-39 ◦C for varying lengths

of time. The transformed sperm may be used for *in vitro* fertilization systems [25] [27] [30] or artificial insemination [31] [32] [27]. Successful sperm-mediated gene transfer has been reported in the mouse [25] [33] [28] [30], rabbit [34]-[35], pig [36] [27] ,chicken, xenopus [37] and cattle [38] [27].

2.7. Liposome/DNA delivery methods

Liposome/DNA delivery method is also used for transfering cloned DNA into cells and embryos. Liposomes are small vesicles consisting of membrane like lipid layers that can actually protect foreign DNA from digestion of proteases and DNAses [39]. Cationic liposomes are capable of spontaneously interacting with DNA molecules, giving rise to lipid-DNA complexes [39]. Under appropriate conditions, exogenous DNA can be transferred into cells and a portion of this DNA becomes localized in the nucleus [39]. One-cell embryos may also be exposed to liposomes carrying cloned genes and potentially incorporate the DNA sequences into their genome.

2.8. Electroporation

Electroporation is anoher method to transfer cloned DNA into cells and embryos by using electric curent [40]-[45]. Briefly, the target cells or embryos are kept in a solution having the gene of interest. The solution and the cells are exposed to a very short duration of a high voltage electrical pulse. The pulse causes a temporary breakdown of the cell membrane. This technology has been utilized in attempts to produce transgenic animals by electroporation of DNA into sperm, which then carry the exogenous DNA to the egg at fertilization [43]. Electroporation of DNA into mouse ES cell lines [46] and their subsequent transfer into blastocysts by microinjection have resulted in transgenic mice [47]. This method has great potential either alone or in combination with others to efficiently transfer genes for the production of transgenic individuals.

2.9. Intracytoplasmic sperm injection (ICSI)

Transgenic animals can be also generated by intracytoplasmic sperm injection (ICSI). A passive technique for the production of transgenic mice called intracytoplasmic sperm injection-mediated transgenesis (ICSI-Tr) , sometimes referred to as metaphase II transgenesis have been developed [48]. In this technique mouse spermatozoa are taken. They are then demembranated. This is either done by freeze-thawing or by treatment with a detergent such as TritonX-100 [49]. It is then incubated with linear, double stranded DNA that contains the transgene. The principle behind this method was that the exposed perinuclear theca of the sperm head would interact with the DNA and act as a carrier for the transgene. This Sperm-DNA complex is then injected into mature metaphase II-oocytes by ICSI, allowing the transgene to be transfered into the embryonic genome via the DNA repair mechanism [50]. One of the main advantages of ICSI-Tr is its higher efficiencies at inserting very large DNA fragments into the host genome, including inbred mice, as compared to pronuclear microinjection [51]-[53].

3. Models for Transgenic

Mice were used as a model to study transgenesis because they have well-characterized physiology, genetics, and short lifespan. This allows the mice for rapid analysis of the phenotypic changes associated with the transgene over their entire lifespan. A number of transgenic rodents are available that model human diseases such as sickle cell anemia [54], AIDS , amylotropic lateral sclerosis (ALS), also known as Lou Gehrigs disease [55], hepatitis B infection [55], ankylosingspondylitis [55] and cancer [56]. Most of these are produced by the highly inefficient but preferred pronuclear microinjection technique. The literature contains transgenic livestock models produced by pronuclear microinjection for sheep, pigs and rabbits [57], cows [58] and goats [59]. Inducible and conditional transgenic mouse models of schizophrenia were also reported [60]. Transgenic models of neurodegenerative disease were also studied [61].

4. Transgenic farm animals as bioreactors

Transgenic mammals are used as bioreactors for protein production. This is very important for the pharmaceutical industry, because it is less expensive as compared to cell culture. The impetus of using animals as bioreactors starts from the experiments where transgenic mice for growth hormone (GH) expression demonstrated remarkable growth [62]. Mammary gland transgene expression allows mass production of large amounts of correctly processed proteins in a temperature-regulated fluid that may be collected daily in a non-invasive fashion [63]. ICSI-mediated transgenesis with improvements offers an opportunity to insert large transgenes into livestock animals for the correct expression and processing of gene products. Production of large quantities of biological products in animal bioreactors may help to furnish the big demand for biomolecules that are presently synthesized by very expensive methods.

5. Pharmaceutical proteins derived from animals

Livestock animals have made an important contribution to the health of human and well-being throughout humankind's history. A substantial contribution of farm animals to human health are the longstanding use of bovine and porcine for yield of insulin, gelatin, as well as horse and sheep antibody against natural venoms, toxins, drugs and microbial peptides [64]. Gelatin being the biggest animal protein consumed in human health, follows with antibodies fragments. The chronic problem of animal-derived therapeutics, especially those of high molecular weight, is the immunogenicity induction in addition to their biosafety. However, the invertebrates and lower vertebrates donate the human being a several crucial emergency saving life small-peptides or their analogs such as Refludan, Prialt, Exendin. Not only, but the farm animals are enormously using as models for novel surgical strategies, testing of biodegradable implants and sources of tissue replacements, such as skin and heart valves. Recently, they are being harnessing as bioreactor for production of biopharmaceutical related products through gene farming with efficiency far greater than any conventional microbial or cell-culture production systems. The efficiency of the different animal systems to produce pharmaceutical proteins are described and compared to others including plants and micro-organisms [65]. Reports were also made regarding the expression of VP1 protein in the milk of transgenic mice [66]. Several pharmaceutically related products derived from transgenic animals are listed in the Table 5.

Table 5. Pharmaceutically related products derived from transgenic animals

S. No	Product	Uses	S.No	Product	Uses
1.	ABX-EGF	Cancer	2.	Calcitonin	Osteoporosis
3.	ABX-IL8	Rheumatoid arthritis	4.	C1 inhibitor	Hereditary angio edema
5.	CD137	Against solid tumors	6.	CFTR	Ion transport Cystic fibrosis
7.	Collagen	Rheumatoid arthritis	8.	CTLA4Ig	Rheumatoid arthritis
9.	D2E7	Rheumatoid arthritis	10.	Erythropoietin	Anemia
11.	Extracellular superoxide dismutase	Ischemic reperfusion injury	12.	Factor VIII	Hemophilia A
13.	Factor IX	Blood coagulation Hemophilia	14.	Glutamic acid decarboxylase	Type 1 diabetes
15.	Fibrinogen	Tissue sealant development	16.	Glucosidase	Glycogen storage disease
17.	G-CSF	Leukopenia	18.	Hemoglobin	Blood substitute development
19.	Human growth hormone	Growth failure Turner's syndrome Cachexia	20.	huN901	Small-cell lung cancer
21.	Insulin	Diabetes	22.	Lactoferrin	Immunomodulatory Anti-inflammatory
23.	Interferon	Antiviral	24.	PRO542	HIV/AIDS
25.	Lysozyme	Antimicrobial Immune modulator	26.	Prolactin	Enhancement of immunity
27.	Interferon	Antiviral	28.	Protein C	Blood coagulation
29.	Remicade®	Crohn's disease Rheumatoid arthritis	30.	Rotavirus virus-like particles	Vaccine development

6. Transgenic expression of immune-globulins

Immunoglobulins are also produced using the transgenic models. The first Immunoglobulin (Ig) transgene expressed in a transgenic mouse encoded a murine $\varkappa$ isotype light chain [67]. Transgene expression was targeted to the spleen and the expressed protein was detected in the serum of transgenic animals. In 1984, a functionally rearranged gene encoding murine serum μ Ig was cloned and used to produce transgenic mice [68]. An elegant study was reported in which transgenic mice containing a human Ig-μ transgene produced a variety of receptor-binding variants of influenza virus hemagglutinin on exposure to the antigen [69]. More recently, transgenic cattle harboring intact un rearranged human Ig heavy- and λ light-chain loci were created [70].

7. Other applications and issues

Some emerging applications of livestock transgenesis in the field of pharmacology, meat and dairy industry, xenotransplantation, and human disease modeling were analyzed. Few bioethical and commercial concerns raised by the transgenesis applications are discussed [71]. Human lysozyme expressed in the mammary gland of transgenic dairy goats can inhibit the growth of bacteria that cause mastitis and the cold-spoilage of milk [72]. Recombinant porcine lactoferrin expressed in the milk of transgenic mice enhances offspring growth performance was also reported [73]. A method for rapid generation of transgenic animals to evaluate testis genes during sexual maturation was studied [74]. Endocrine effects of growth hormone over expression in transgenic coho salmon were also reported [75]. Cataracts in transgenic mice caused by a human papillomavirus type 18 E7 oncogene driven by KRT1-14 were also studied [76]. It was reported that valproate ameliorates the survival and the motor performance in a transgenic mouse model of Huntington's disease [77].

8. Conclusion

Transgenic technologies generated great excitement in the scientific community and the pharmaceutical industry. These technologies solved a number of biological problems. There are a number of new and developing technologies that will have a profound impact on the genetic improvement of livestock. The rate at which these technologies are incorporated into production schemes will determine the speed at which we will be able to achieve our goal of more efficiently producing livestock, which meets consumer and market demand. It is the requirement of the present time to have more advanced technologies for integrating the gene of interest without damaging its biological property so to produce the efficient transgenic animals with minimal error.

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