**ADVANCEMENTS IN THE PRODUCTION AND APPLICATIONS OF TRANSGENIC ANIMALS: A MODERN REVIEW**

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**ABSTRACT:**

In genetic engineering, a transgenic animal possesses foreign DNA that has been intentionally introduced into its genome. We discuss cloning vectors which are used to genetically modify animals by introducing a short DNA fragment containing foreign desired DNA, which duplicates itself and then transferred into the other organism. Various types of vectors are plasmid vectors, cosmid vectors, bacteriophage, yeast artificial chromosomes, bacterial artificial chromosomes, and human artificial chromosomes. Different gene insertion techniques to insert the vector carrying the desired gene into the host cell including heat shock, electroporation, gene gun, microinjection, and liposomes have been discussed. We have compiled a list of numerous transgenic technologies and methodologies which includes transgenesis through the gonads such as sperm-mediated gene transfers and fertilised egg that is done by DNA microinjection, retrovirus-mediated gene transfers and more advanced techniques like cre-lox technique, viral vector, cytoplasmic injection,primordial germ cells, Spermatogonia manipulation**.** The general role of transgenic animals like rats, fish, cattle, and monkeys has also been mentioned. These genetically fabricated animals have an immense impact on human health and recombinant therapeutic application. This review focuses on various methods of transgenesis and their applications such as recombinant therapeutic protein, medical application, xenotransplantation, and human gene therapy.

Key words: genetic engineering, transgenic animals, restriction enzymes, vectors.

1. **INTRODUCTION**

               Transgenesis is the phenomenon of inserting the genetic material into a living cell for the species to exhibit new properties and then be able to transmit to its progeny. The novel gene of interest is separated, introduced into the receiver individual's cells, incorporated into the chromosomal DNA, and then transferred to the progeny. The added DNA is referred to as a transgene. Several methods are used to create transgenic animals. The microinjection approach is widely utilised, although it has numerous limitations, including poor efficiency and variable expression patterns. As a result, other methods such as sperm-mediated DNA transfer, fertilised eggs or embryos and somatic cell nuclear transfer (SCNT), intracytoplasmic injection of gametes containing foreign DNA manipulation, injection or infection in oocyte and/or blastocysts with different monoclonal antibodies, RNA interference techniques, including the use of nuclear transfer are being utilised. [1]

Transgenic technology has been used to develop various varieties of transgenic animals, for example, goats, sheep, fish, cattle, marine invertebrates, monoclonal antibodies, pigs, cows, rabbits, mice, chickens and eggs. Transgenesis has also been classified as a medical condition. In mammal developmental genetics, transgenic animals and technologies are widely used for biotic and abiotic stress tolerance to research disease processes by introducing the human genome into other creatures. The study of gene regulation in microbiology, pharmaceutical proteins manufacture, production of various pharmaceuticals products as well as their efficacy assessment has now become the modern tools for many respective pharmaceutical companies. Creating animals specifically for xenografting, in which the antigen of animals is altered so that their tissues and organs can be utilized in transfusions and transplanting. The research on the role of genes in the progression of a disease, the effects of gene regulation on human physiology and its development are also being studied thoroughly. It's also a component of the biological production of various vaccines and in their safety testing. [2] Transgenic animals expressing Green Fluorescent Protein (GFP) are widely used to study a variety of physiological processes, including organ development and cell migration. Several in vivo studies, however, claimed that GFP could harm the health of transgenic animals. Glomerulosclerosis was observed in transgenic mice and rabbits expressing a ubiquitous reporter protein. GFP expression in the heart caused dilated cardiomyopathy and altered cardiac function in transgenic mouse and zebrafish lines, respectively. Furthermore, GFP and yellow fluorescent protein (YFP) transgenic mice showed growth retardation and increased axon swelling. Transgenic livestock species have been studied as an alternative platform for recombinant protein production, primarily through milk secretion; the strategy has produced large amounts of biologically active proteins. Porcine cloning technology can be used to produce progenies genetically identical to the donor cells from high-quality breeding pigs. In addition, genetically modified pigs have been produced by somatic cell nuclear transfer using genetically modified porcine fetal fibroblasts. The method of preparing genetically modified pigs is critical for establishing pig models for human diseases, and for generating donor animals for future xenotransplantation.

* 1. **Animal transgenesis technology:**

 **1.1.1. Transgenic vectors -** A cloning vector is a short fragment of DNA molecule that can transport foreign DNA and then duplicates itself for transferring or replicating inside the host organism. Gene expression seems to be more likely to take place when vectors are used. [3]

**1.1.2. Plasmid vectors-** Extrachromosomal DNA molecules are naturally occurring, self-replicating, circular, relatively small, double-stranded DNA molecules extracted from bacterial cells. Plasmids can be found in 10–700 copies per cell. The most often used plasmid vector is pUC18 (plasmid University of California), which can only receive DNA with a length of less than 5000 base pairs. Examples of plasmids also include pBR322 (plasmid "BOLIVAR" and "RODRIGUEZ" number 322), pUC19 etc. [4]

**1.1.3. Especially developed phagemid -** They also have unique potential as each of their DNA is inactive, making them ideal targets for gene transfer. E. coli is infected with Bacteriophage Lambda, which can integrate up to 15–16 kilobases of DNA. An example includes M13, F1M13 etc. [5]

**1.1.4. Cosmid (cos + plasmid) vector -** They are combined with 2 words COS + MID. COS means the site of lambda phage and MID is taken from the plasmid. They're a special combination of plasmids and bacteriophage chromosomes. They can incorporate DNA fragments of less than 50 kilobases and replicate them as plasmids. [6]

**1.1.5. Yeast artificial chromosome (YAC) -** It's utilized for cloning DNA strands using a linear yeast chromosome that is fewer than one million base pairs long.

**1.1.6. Bacterial artificial chromosome** (**BAC**)

It's a DNA regulation on a basis of functional fertility plasmid (or F-plasmid) which is employed in bacteria, most often E. coli, for conversion & duplicating. It can be used to clone 200-300 kb of DNA. [7]

**1.1.7. The human artificial chromosome (HAC)-** It's a vector which provides information potential characteristics for transferring the full human genes of different sizes into the host cell without preventing insertional mutagenesis and genomic instability by integrating genes into the host genome. [8]

* 1. **Foreign gene preparation method-** For the technology of foreign gene transfer, the first step is to prepare the transgene. It is accomplished by cutting and splicing together sections of DNA that have developed in rDNA. DNA ligase joins these segments of DNA ligase [9-10]
		1. **Isolation of the gene of interest** - It is accomplished by the use of restriction enzymes and recombinant DNA technologies. [11] for example, Eco RI identifies the GAA TTC sequence and cleaves it between letters G and A.
		2. **Restriction Enzyme –** these are enzymes that are also known as molecular scissors. It cuts the DNA at a specific site of nucleotide sequences known as restriction sites.

**Table 1- List of restriction enzymes, their, source, recognition site.**

|  |  |  |
| --- | --- | --- |
| **Restriction enzyme**  | **Source**  | **Recognition site** |
| Alu Ι | Arthrobacter luteus  | 5’-A-G-C-T-3’‘3-T-C-G-A-5' |
| Bam H Ι | Bacillus Amyloliquefaceiens H | 5’-G-G-A-T-C-C-3’3’-C-C-T-A-G-G-5’ |
| Eco R Ι | Escherichia coli RY13 | 5’-G-A-A-T-T-C-3’3’-C-T-T-A-A-G-5’ |
| Hae ΙΙΙ | Haemophilus aegyptius | 5’-G-G-C-C-3’3’-C-C-G-G-5’ |
| Sal Ι | Streptomyces albus | 5’G-T-C-G-A-C-3’3’-C-A-G-C-T-G-5’ |

* 1. **DNA LIGASE-** DNA ligase is an enzyme that facilitates the binding of Okazaki fragments by DNA end-joining processes. DNA ligase is required for the completion of eukaryotic nuclear DNA replication. [12]
	2. **DNA POLYMERASE-** It's the primary enzyme catalyse the formation of DNA strands from nucleoside triphosphates, that are DNA's precursors. DNA replication frequently involves the use of these enzymes as well as frequently work in groups to create two identical DNA copies from a single original DNA molecule. Existing DNA strands are "interpreted" by DNA polymerase, which creates two new strands. which are identical to those currently available. DNA polymerases are categorized under 3 types based upon sequence similarities: type A, type B, and type C, which have similarities to polA (pol I), polB (pol II), and polC (pol III) from Escherichia coli, respectively. [13]
	3. **DNA TOPOISOMERSASE -** DNA topoisomerases are potent enzymes that preserve the cell nucleus’s structural equilibrium throughout DNA transaction events. The DNA topoisomerases I and II found in cells work by scissoring the DNA backbone on 1 or 2 strands, respectively, accompanied by helical stress release and replacement of the broken DNA backbone. [14]
		1. **Cloning of the desired gene -** It is a technique for inserting a foreign genetic material into a carrier vector (plasmid). The chimaera is a vector that contains a cloned gene and is delivered mostly to the host cell to enable it to exist differently. A transformed cell is a host cell that has received the vector. The sticky ends of the vector and particular DNA molecules is being generated by the similar protein for the cut DNA molecule. As a result, the sticky ends of vector and every particular DNA molecule are generated by the same protein, which joins the cut DNA molecule to the vector and to the ligase molecule that connects the sugar- phosphate bond of bases together.
		2. **Method of cloned gene Incorporation into a host cell** Various approaches should be utilized to attach an exogenous DNA molecule to a host cell.
			1. **Heat shock** Membrane permeability of the host bacterium is increased by quickly heating for 2–5 minutes at 42°C in a solution containing cold calcium chloride, chimaera plasmids, and normal host bacteria, allowing plasmid chimaeras to be integrated into the host cell.
			2. **Electroporation**The host cell membranes are temporally disrupted by a high voltage pulse, allowing the vector to enter the cell**.**  [15]
			3. **Viruses** The capacity of viruses to infiltrate and proliferate in prone cells made the insertion of desired DNA molecule sequences into target host cells a possibility. [16]
			4. **Genetic gun** It operates by bombarding golden particles containing foreign DNA sequences into the nucleolus of the host cell, or the nucleolus of a plant or animal cell. [17]
			5. **Microinjection** Foreign DNA is injected directly into the nucleus by using a fine needle while a cell is held in place with a pipette under magnification.
			6. **Liposome** Liposomes are tiny membrane-bound vesicles that can be encapsulated in a vector and transfer foreign DNA by fusing with a cell or nuclear membrane of the host cell. [18]
1. **Methods of transgenesis**

Animals that have been genetically altered may be completely or partly transformed through various useful ways like transferring genes by gonads, microinjection, stem cells, sperm vectors, somatic cells, and retroviral techniques are useful ways.

* 1. **Transgenesis through the Gonads-**Transgenes is infused into seminiferous tubules or germ cell precursors which are transferred in vitro and transplanted into the host testicle to transfer spermatids in situ. Testis-derived cells populate the testicles of infertile men, which produce sperm and offspring.
	2. **Sperm-mediated gene transfer-** Sperm cells are naturally able to internalize and bind foreign genomic DNA, allowing it to be transported into the egg during fertilization, which can be used as a substitute for microinjection. Moreover, it reduces the costs, increases productivity, and is more convenient than other procedures, as well as eliminating embryo handling or the use of advanced technology, are some of the benefits over other procedures, however, the results in different species of animals are quite diverse.
1. **Other transgenesis techniques**
	1. **DNA microinjection**

It's based on gamete cells' natural ability to bind and internalize foreign genomic DNA before carrying them further into the egg during fertilization, and it might be utilized instead of microinjection. The gene construct may be injected into a single embryo in quantities ranging from 200 to 500 copies. Before being placed into a “pseudopregnant” surrogate mother, transgene-positive embryos should be developed in vitro for 24 hours. [19]

* 1. **Stem cell-mediated gene transfer**

Embryonic stem cells containing the desired gene are inserted into totipotent stem cells and transformed into chimeric animals (animal stem cells derived from two or more species). A transgene can be tested through the cell stage without the need to use a live transgenic animal Gene targeting is enabled by its ability to target endogenous genes. [20]

* 1. **Cre-lox technique**

Cre-Lox recombination implies a particular segment of DNA being targeted and spliced with the aid of an enzyme called Cre recombinase. A Cre-lox technique has become a genetic method of controlling site-specific recombination into genomic DNA. Researchers have used this technology to control gene expression and delete undesirable DNA sections, as well as manipulate chromosomal layout in many transgenic animals Cre has been a DNA recombinase that may catalyse DNA recombination across specified sites in a DNA molecule. These lox P sequences feature specialised Cre binding sites that surround a directed core sequence where recombination may occur. [21]

* 1. **Viral vectors**

The therapeutic gene can be delivered to the target cell either ex vivo or in vivo. The patient's target cells are initially extracted in ex vivo gene therapy. Once the correct gene has been inserted, the cells are returned to the patient. Although this method has shown potential, it is confined to a small number of cell types and diseases. [22]

* 1. **Cytoplasmic injection** The incorporation of the enhanced green fluorescent protein (EGFP) plasmid inside cattle fertilized ovum, is a promising technique for producing transgenic cattle. [23] When supercoiled covalently closed circular DNA (ccc plasmids) are injected into mice and cattle zygotes, a significant number of plasmid-expressing zygotes are created. At this phase, the plasmids remain episomally viable, as well as the promoter unique to the viral vector genome is intact. Injecting intracellular plasmids into mammalian germ cells is a straightforward and repeatable method for achieving early mammalian embryo reprogramming activities. In comparison to indirect pronuclear microinjection, direct pronuclear microinjection is more successful. [24]
	2. **Primordial germ cells** are extremely specialised cells that act as progenitors to gametes, which mature into haploid sperm and eggs during meiosis and produce a new organism when fertilized. They pass on genetic and epigenetic information from generation to generation, ensuring a species' survival. Through genetic and epigenetic control of genome function, germ cell development generates totipotency. Primordial germ cells (PGCs) are the earliest germ cell population to form throughout development and serve as the direct progenitors of both oocytes and spermatogonia. [25]
1. **Types of Transgenic Animals**

Genetically modified strains are taken into consideration for both research and commercial pharmaceutical production.

* 1. **Goats**

Many monoclonal antibodies are now being made in the mammary glands of genetically modified goats that make recombinant bi-specific antibodies in their blood. These goats reportedly generate cetuximab in their milk, which boosts the host body’s immune system.

* 1. **Sheep**

Dolly, a sheep, was the first animal discovered to be cloned from an adult cell and showed that DNA in a differentiated cell could be repurposed through nuclear transfer, opening up two new possibilities for milk including fibrinogen (major essentials with Factor XIII and thrombin). clotting Factor VII and IX activated protein C, and alpha-1-antitrypsin are all proteins that are potentiating pharmaceuticals for therapeutic purposes in treatments of [cystic fibrosis](https://en.wikipedia.org/wiki/Cystic_fibrosis) and [emphysema](https://en.wikipedia.org/wiki/Emphysema).

* 1. **Fish**

Zebrafish genetically engineered to have a deficient aortic valve development, allowed researchers to determine the involvement of an enzyme, UDP-glucose dehydrogenase, in the embryonic development of this valve. The present scientific discoveries and efforts related to transgenic fish are wide-ranging and, for the time being, unsurvey able. [26]

* 1. **Cattle**

Genetically modified animals generate milk that is identical to human milk and also devoid of the allergen BLG. Milk composition in dairy cattle has attracted significant study in attempts to enhance output, help human nutrition, and change different processing features made to fit the creation of certain food items. "Herman" was the first transgenic bovine. The first objective was to create humanised monoclonal C-1 antagonists. The primary purpose was to identify cattle that generate "human lactoferricin" in their milk, that's also subsequently utilised as a supplement in the newborn feeding formulation. [27]

* 1. **Cows**

 The first transgenic cow “Rosie” have an advanced human alpha-lactalbumin gene which contained about 2.4 g of human protein per litre of milk. It was established to be more dietetically balanced for infants. [28]

* 1. **Mice**

Products obtained from mice as transgenic animals include: synthesis of malaria proteins for vaccine research; Monoclonal antibodies, ATIII, beta interferon; cystic fibrosis transmembrane regulator; clotting Factor X, human albumin serum, Tissue plasminogen activator, myelin basic protein, prolactin and thrombin are all products derived from transgenic mice. They are used in genetic studies because they closely resemble human anatomical and physiological activities.

* 1. **Rabbits**

Transgenic rabbits are commonly employed as models for the study of cardiovascular disorders such as atherosclerosis, cardiomyopathy, and prolonged QT syndrome, as well as malignancies, AIDS, and other pathological conditions. Rabbit proteins include recombinant human C1 antagonist, human erythropoietin, and human alpha-fetoprotein. The ApoE gene, which is required to uptake chylomicrons and very-low-density lipoprotein particles, is mutated, resulting in a model with atherosclerotic lesions that are histologically comparable to those seen in humans.

**5. Human Health**

**5.1 Recombinant Therapeutic Proteins**

Some new therapeutic enzymes are produced from transgenic animals' mammary glands. Many approaches for producing therapeutic enzymes were employed. Transgenic cattle are used for a large amount of production of important proteins like Antithrombin III (AT III), tissue plasminogen activator (TPA), and -antitrypsin. [29]

**5.2 Medical Applications**

**5.2.1. Xenotransplantation**

Every year, patients die due to a lack of replacement heart, liver, or kidney. Transgenic pigs may be capable to provide the transplant organs that are needed to help with the loss. The creation of genetically modified pigs by transferring a gene sequence that would avoid acute resistance of organs transferred among pigs and humans was among the earliest genetic changes of larger animals. [30]

 **5.2.2. Nutritional supplements and pharmaceutical**

Insulin, growth hormone, and blood anti-clotting proteins may be acquired from the milk of mutant cows and goats shortly or are already acquired. Transgenic milk is being researched for the treatment of disorders such as phenylketonuria (PKU), genetic emphysema, and cystic fibrosis. Rosie's milk is a nutritious product that can be given to infants or the elderly who have unique dietary or gastric demands [31]

 **5.3 Disease Models Using Transgenic Animals for Drug Development**

An animal model is a functional, non-human mammal that is used to study and learn disease in humans to acquire a greater understanding of the issues without endangering humans during the drug development procedure. [32]

* + 1. **(HIV/AIDS) Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome. -** The very first genetic model for the research of HIV was the Tg26 HIVAN (human immunodeficiency virus-associated nephropathy) Mouse Model. These genetically modified animals can express HIV-1 molecules and acquire symptoms and immunological deficits that are similar to those seen in people with AIDS. AIDS Mice and Advanced Mice are two such models. [33]
		2. **Alzheimer’s Disease -** Studies have shown few transgenic animals are used for the treatment of Alzheimer’s. Vaccination with Amyloid precursor (A42) in pigs and transgenic mice shows that Alzheimer's disease vaccination has therapeutic possibilities. [34-35]
		3. **Diabetes Mellitus -** For researching the gene and their function in the production of peripheral insulin, genetically modified mice have been developed. Insulin secretion models, such as glucokinase and hepatic glucose production (type II diabetes), are developed. Insulin was expressed in this model. [36]
		4. **Cancer Diseases -** The "Oncomouse" was the only transgenic animal to be patented. Its germline and cell cultures include an active human oncogenic genome that was implanted inside the animal at an embryogenesis phase to make sure that the oncogene was expressed in most of the organism's cells. Various transgenic knockouts can enable tumour development and spread via E-cadherin and other adhesion molecules in mice.
		5. **Angiogenesis-** Angiogenesis, artery stenosis, atherosclerosis, thrombosis, thrombolysis, and haemorrhage models are used to study vascular development in mice. Angiogenesis prevention is one of the most popular methods for novel cancer therapies. To uncover antagonists of particular angiogenesis processes, genetically modified animal models of angiogenesis have been used. [37]
		6. **Blood Replacement** - Due to sickness concerns, a scarcity of qualified donors, and legal issues, the present blood product manufacturing process is dependent on various blood donors. Livestock with human antibody genes that can manufacture human polyclonal antibodies can give a continuous source of polyclonal antibodies for the treatment of diseases & health conditions like transplant rejection, cancer, and autoimmune illnesses. [38]
		7. **Industrial Applications -** Toxicology-sensitive specific genes are developed for toxicity studies and to produce a wide variety of proteins, which can then be used to create enzymes that accelerate industrial chemical reactions, pharmaceutical protein production, drug development, and product development in the pharmaceutical industry. [39]

**Table 2: pharmaceutically related products derived from transgenic animal**

|  |  |  |  |
| --- | --- | --- | --- |
| **S no.**  | **Cattle** | **Pharmaceutical product** | **Indication** |
| 1 | sheep | Alpha anti-trypsin | Emphysema is caused by a deficiency. |
| 2 | sheep | Cystic fibrosistransmembrane conductance regulator (CFTR) | Cystic fibrosis treatments |
| 3 | sheep  | Tissue plasminogen activator | Thrombosis medication |
| 4 | sheep | Factor VIII, IX | Haemophilia medication |
| 5 | sheep | Fibrinogen  | Thrombosis medication |
| 6 | Pig | Tissue plasminogen activator | Thrombosis medication |
| 7 | Pig | Factor VIII, IX | Haemophilia medication |
| 8 | Cow | Alpha - lactalbumin | Anti - infection |
| 9 | Cow | Factor VIII | Haemophilia medication |
| 10 | Cow | Fibrinogen | Treatment of wounds |
| 11 | Cow | Collagen I, Collagen | Rheumatoid arthritis treatments, tissue healing |
| 12 | Cow | Lactoferrin | Infant formulation additive |
| 13 | goat | Antithrombin III | Thrombosis, pulmonary embolism |
| 14 | goat | tPA | Thrombosis medication |
| 15 | sheep | α- antitrypsin | Treatments of Emphysema and cirrhosis  |
| 16 | sheep | Factor IX | Treatment of Hemophilia b |
| 17 | sheep | Factor VIII | Treatment of Hemophilia a |
| 18 | cattle | Polyclonal antibodies | Production of vaccine |
| 19 | rabbit | C 1 inhibitor | Treatment of Hereditary angioedema |
| 20 | rabbit | calcitonin | Treatment of Osteoporosis and hypercalcemia |
| 21 | Sheep | Cftr | Treatment of fibrosis |
| 22 | Goat | Glutamic acid decarboxylase | Treatment of type 1 diabetes |
| 23 | Goat | Pro 542  | Treatment of HIV |

1. **LIMITATIONS OF TRANSGENESIS:**

The development of transgenic animals may be a complex, time-consuming, and costly process. Generally, this causes breeding issues, mutagenesis, and functioning impairments. The transgenic technique has also low success and survival rates in transgenic animals. The efficiency of external genes at the specified spot is poor and unstable, and so the effect of the intrinsic gene causing problems in animals is uncertain; this system is still in its growing stage, suggesting that further research is needed.

* **Transgenic Technology and Ethics** There are risks involved if different methods and items fail to obtain customer acceptance owing to moral objections. Animal suffering is produced by the introduction of transgenes that generate tumours or neurological illnesses. Adverse effects may emerge from genetically modifying an animal's genes.  Though transgenic animals may benefit humans   foreign genes influence the host, creating several challenges to ecological balance and species diversity.
1. **CONCLUSION:**

Various steps in animal transgenesis technology were discussed in detail. The most successful approach for producing transgenic mammals is in vitro transfection of cultivated differentiated cells combined with somatic cell nuclear transfer. Cre-Lox is an effective tool for *in vivo* genetic engineering because it enables precise spatial and temporal regulation of gene expression. Advances in transgenic livestock technology are truly wanted since the cost savings would help both biotechnology and basic research. Among all the methods described above the pronuclear microinjection has been most commonly used to make genetically engineered mice, rabbits, pigs, sheep, goats, and cattle. [40]

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