Chapter 3

# Transgenic Animals and its Applications

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## Introduction

The discovery of genetic code about 40 years ago suggested that the gene isolation and transfer in to living organism would become major tool for biologist. The first gene transfer into mouse using isolated DNA revealed that the generation of animals stably harboring foreign DNA (Gordon *et al.,* 1980) with modified phenotypic characters (Palmiter *et al.,* 1982) was made. These pioneer and fascinating experiment also revealed some of the limits of transgenesis, the generation of transgenic animals by gene microinjection appeared laborious. It is now well established that transgenesis is one of the major tool of the biologist to study gene expression and function. Transgenesis is still going to be used more extensively and systematically with the identification of all human genes. Transgenesis also play an essential role for application in the medical and agronomic fields. The study of human diseases is greatly facilitated by the generation of transgenic animals mimicking health disorders of allowing the evaluation of the new pharmaceuticals interest, one being prepared in the milk as transgenic animals. A few lines or farm animals having improved breeding properties have been generated and are understudy for human consumption. Despite the

impressive and growing success, transgenesis still suffers from many imperfections.

## Transgenic Construction and Design

The original definition described a transgenic animal as one carrying recombinant DNA molecules that was introduced by intentional human intervention (Gordon and Ruddle, 1981). Transgenesis defined as the introduction of foreign DNA sequence in the genome of multicellular organism and ensuring that the sequences are transmitted to the progeny of the manipulated species (Houdebine, 2003). On the other hand, Brink *et al.,* (2000) defined as alteration of the genetic information with the intention as modifying a physical charactertics of animals. Transgenesis differs from the gene therapy since the farmer; the inserted gene is exported to be transmitted to the next generation but in gene therapy, the gene will not be transmitted to next generation. Furthermore, the term transgenic has wider implications since it could compromise animals which had additional (knock in) or deletion (knock out) of genes from the genome.

A transgene is a recombinant DNA molecule that includes two parts regulatory element and structural element. The regulatory element confers tissue specificity and controls when the gene expression during development and modulates the amount of gene expression. The structural element is composed of DNA sequence that encodes the genetic information needed to synthesis the gene product (Wall*et al.,* 1997). The regulatory element includes promoter, enhancer, silencer, insulator element locus control region (LCR), introns and poly - A sequence. The selection of transgene, which should be based on the economic, scientific and social realities. The transgene which codes for the protein of interest can be derived from another animal of the same species, from a different species, even from the bacteria or plants. The regulatory element should be chosen such a way that enhance or enable control of gene expression (Wiqmann and Kues, 2003).

### Methods to generate transgenic animals

During the past decades various methods have been developed to generate the transgenic animals. With the advent of gene sequencing

many sequences have been determined bringing the knowledge of promoters and gene of interest for various species. The advent of genomics, proteomics and the new generation of reproductive biotechnologies hold the promise of successful application as transgenesis to domestic animals (Collores *et al.,* 2005).

The techniques and methodologies to be implemented in the generation of the transgenic animal depend upon the targeted use of the animals.

Transgenic animals have been produced by a number of techniques

- Pronuclear microinjection
- Nuclear transfer
- Retroviral mediated gene transfer
- Lentiviral mediated gene transfer
- Sperm mediated gene transfer
- Testis mediated gene transfer
- Transposon mediated gene transfer
- Artificial chromosome mediated gene transfer

## Applications of Transgenesis

### Livestock production

Enhanced prolificacy and reproductive performance increased feed utilization and growth rate, improved carcass consumption, improved milk production or composition and increased disease resistance are practical application of transgenesis.

### Modification of milk

Advances in recombinant DNA technology have provided the opportunity to change the composition of milk, increase in milk volume and to produce entirely novel proteins in milk. These changes may add value, as well as increase the partial uses of milk. Clearly altering the charactertics of one of the components to enhance a particular processing feature may make milk unsuitable for other uses. Many of proposed changes in milk structures are listed in table 1.

| S.No. | Changes | Consequences |
|---|---|---|
| 1. | Increase $\alpha$ and $\beta$- CN | Enhanced curd firmness for cheese making, improved thermal stability and increased calcium content |
| 2. | Increase phosphorylation site in caseins | Increased calcium content and improved emulsification |
| 3. | Introduce proteolytic sites in caseins | Increased rate of texture development (improved cheese ripening) |
| 4. | Increases $\alpha$ CN concentration | Enhanced stability of casein aggregates, decreased micelle size and gelation and coagulation |
| 5. | Eliminates $\beta$- LG | Decreased high temperature gelation, improved digestibility, decreased allergenic response and decreased primary source of cystein in milk. |
| 6. | Decrease $\alpha$ - LA | Decreased lactose, increased market potential of fluid milk, decreased ice crystal formation and compromised osmotic regulation of mammary glands |
| 7. | Add human lactoferrin | Enhanced iron absorption and protected against gut infection |
| 8. | Add proteolytic sites to $\alpha$-CN | Decrease rate of cheese ripening |
| 9. | Decrease expression of acetyl carcaboxylase | Decreased fat content, improved nutritional quality and reduced milk production cost |
| 10 | Express Ig genes | Protected against pathogens such as salmonella and listeria |
| 11 | Replace bovine milk proteins gene with human equivalents | Mimicked human breast milk |

Transgenic pigs containing the bovine alpha lactalbumin (α-LA) gene, which improve milk production and composition in swine. Alpha lactalbumin is a component of lactose synthesis complex and is involved in the synthesis of lactose in milk (Bleck *et al.,* 1998). Furthermore, higher concentration of α - LA early in the lactation boost milk output and piglet growth. In fact, piglet growth rate is higher when the piglet are sucking sow contain the a-LA transgenic. It has been suggested that bioactive substances (IGF, TGF- α and lactoferrin) in the milk possess important function in the neonate. These proteins influence growth as well as development and maturation of the gut, the immuno system and the endocrine organs (Grossvenor *et al.,* 1993). The over expression of a number of these proteins in milk through the use of the transgenic animals may improve the growth, development, health and survivability of the developing offspring.

## Modification of growth and carcass composition

Introduction of porcine growth hormone (PGH) genes into swine genome increases the growth rate, without increased arthritis and abnormal skeletal growth (Vise *et al.,* 1988). An alternative approach was performed hypertrophy of numerous muscles while reducing body fat by introduction of the chicken ski mutant oncogene, which was previously shown to cause (Sutrave *et al.,* 1990). This strategy, however, has resulted in limited success although muscle hypertrophy has been observed in some transgenic pig and transgenic cattle (Bowen *et al.,* 1994). The myostatin (growth differentiation factor-8, GDF-8) gene normally present in mouse skeletal muscle. Myostatin serves as autocrine prenatal inhibitor of myoblast differentiation and growth, when it knocks out result in an increase in lean muscle mass (McPherron *et al.,* 1997). Such genes also present in livestock hence same approaches can be used made in livestock to improve their lean muscle production.

## Modification of disease resistance

Identification of single gene in the major histocompatibility complex (MCH), which influences the immune responses, was instrumental in the reorganization up genetic basis of disease resistance or susceptibility. It has only been realized recently that there are many aspects of disease

resistance or susceptibility in livestock that are genetically determined (Lewin, 1989).manipulation of the MCH in farm animal through ES cell or NT transgenesis could have a major beneficial effect on disease resistance for livestock producers.

Mice and mouse fibroblast cell line that contain the Mx protein were shown to be resistant to infection with influenza virus (Haller *et al.,* 1981). The Mx cDNA has been introduced into porcine fertilized ova, producing pigs that are resistant to influenza infection (Muller *et al.,* 1992).

Prion is infectious agent that causes spongiform encephalopathies in human and animals. $PrP^c$ or the normal protein is expressed in outer surface of neurons. A switch causes the prion disease from the normal $PrP^c$ to a modified form of PrP. Bueler et al. (1993) demonstrated the inactivation of gene by homologous recombination in mice and this mouse is resistant to spongiform encephalopathy. The same knocking out can be done in sheep and cattle to improve the disease resistance to scrapie and bovine spongiform encephalitis. The antibacterial proteins such as lysostaphin can be used to confer resistance to bovine mammary gland infection. This protein has potent antistaphylococcal activity and its secretion into milk conferred substantial resistance to infection in three lines of transgenic mice (Kerr and Wellnitz, 2003).

## Modification of wool production

Alteration of the protein composition of wool fiber via transgenesis with sheep wool keratin and keratin associated protein (KAP) gene 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 to alteration in their micro and macro structure due to a high level of cortical specific expression of a wool type II intermediate filament (f) keratin gene.

## Modification of digestion

Phytase is an enzyme that cleaves inorganic phosphorous form to organic form which increases the phosphorous availability to the animals. This enzyme normally presents in ruminates but absent in monogastric

animal. By additional supply of this enzyme along with feed make better utilization of feed (Umadevi, 2006). Golovan et al. (2001) produced the transgenic pig which expresses the phytase enzyme in their saliva, which enhance the digestion and reduce the feed cost in pig production.

## Application as bioreactor

The production of therapeutic proteins represents the first application of recombinant DNA technology, by the 2003; the European Union had approved 88 products. However none of this approved product was obtained in transgenic system. Despite this, domestic animals represent on efficient production system for large and complex (and biologically active) recombinant protein, which would be used to treat or prevent human disease. The production of these pharmaceutical proteins in the mammary gland of livestock originated the term biopharming (Keefer, 2004) or gene pharming (Wall, 1999). Various investigators have produced transgenic rabbit, sheep, goat, pig and cattle express heterologous protein successfully. The production of biopharmaceuticals present the most varied purposes: for treating such disease as multiple sclerosis, hepatitis, cystic fibrosis, blood disorders, some type of cancers, haemophilia, 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 bioreactors focused on the use of mammary glands as target but today blood, bladder, eggs and male accessory glands have all have been considered as bioreactors for pharmaceutical proteins.

## Transgenic mammary glands

1. Advantages of pharmaceutical proteins expressed in milk include
2. The mammary gland is a prodigious production system that is cable of generating between 23 gm (dairy cattle) and 205 gm (rat) of protein / kg of body weight during peak lactation (Oftedal, 1984).
3. Milk is clearly the least complicated body fluid to collect especially from ruminants.

4. Another advantage of producing biologically active products using the mammary gland is the isolation of the mammary gland from circulatory system. It is argued that bioreactor animals would be protected from the potentially untoward effect of biologically active compound because those compounds would be sequestered in the mammary gland and there fore would be unavoidable to the circulatory system.
5. Proteins which are expressed through mammary gland are able to post translation modification and also biologically active.

Human milk lysozyme is an important protein for innate immunity, but human breast milk is a fairly poor source of commercial production of this enzyme. Lysozyme is one of the most important antibacterial factors in milk, because it hydrolyzes the glycoside β linkage between N-acetylmuramic acid and N acetylglucosamine acid of the peptidoglycan polymer in the bacterial cell wall (Imoto *et al.,* 1972). Milk lysozyme has three times more lytic activity than that of egg white lysozyme because it possesses a greater positive charge than latter (Parry *et al.,* 1969). Lysozyme, which helps to increase the level of beneficial microorganism in the infants and strength of their disease resistance. Li *et al.,* 2006 successfully expressed the human lysozyme in the milk of transgenic mice and also it is biologically active. It can be express in cows milk that with helpful to create disease resistance in many orphan child.

Limonta et al. (1995) produced the transgenic rabbit for production of human growth hormone using whey acid protein gene promotor. The growth hormone enhances the growth retarded children. Recombinant pig growth hormone causes a massive in muscle mass (Evock *et al.,* 1988) hence it also used for increase the body weight in pig.

Lactoferrin present in human milk is an iron binding glycoprotein, which has bacteriostatic and bactericidal effect on the gram positive and gram negative bacteria. This helps to prevent bacterial infections that cause digestive problem that harm or kill millions of newborns around the world. Now transgenic bull was developed for human lactoferrin gene and it was named as Herman. Herman has now fathered for many

calves (Pursel, 1995). Herman is produced by gene pharming Europe, B.V. Company (Netherlands).

Breathed air contains many living organism where it enters the lungs. The lungs contain large numbers of neutrophils. These neutrophils secrete elastase and clear the organism. The walls of the alveoli in the lungs contain elastin, which maintains the elasticity of the lungs and this can be also be broken down by elastase and cause emphysema of the lung. To prevent this from happening there is secreted into the blood serum an enzyme called $\alpha$ 1- protease inhibitor (previously called $\alpha$1-antitrypsin) or $\alpha$ PI. Now this 1- antitrypsin successfully expressed in transgenic sheep and it named as Tracy grazes by PPL therapeutic company (UK). That can be purified and used for treatment of emphysema of lung (Pursel, 1995).

Several companies are developing protein therapeutics produced in milk. GTC biotherapeutics, C1 inhibitor for treatment of hereditary angio-oedema, produced in transgenic rabbit milk by pharming group N.V.

Biosteel is an extra ordinary new product that may soon be used in bullet proof vests and in suture silk for closing up of wounds. Fundamentally biosteel is spider web, which is among the strongest fiber on the earth. The gene of spider silk has been successfully transferred into goat by nexia biotechnologies (Canada) and those goat's milk contains the protein that make up spider silk (Thiemann *et al.,* 2004).

In 1997, Polly is the transgenic sheep that express the human blood clotting factor IX in their milk. It is produced by nuclear transfer technique. Human antithrombin – III (AT- III) is normally presents in human blood and it prevents clotting in the veins. Some people with an inherited deficiency (lack) of this protein are more prone to blood clots, which are dangerous when they break free lodge in lung or brain. To prevent this that patient need to take repeated injection at AT- III. Now Genzyme Corporation company and also produced AT – III producing goat farm successfully express it in goat milk.

A variety of human have been expressed in milk of several species of animal including insulin like growth factors I in rabbit, $\alpha$ - lactalbumin

in cows and protein C in pig (Rudolph, 1999). The enzyme $\alpha$-glucosidase from the milk of transgenic rabbit has been successfully used for by treatment of pompe's disease in infants (Hout, 2004).

The animal of choice for expression of foreign gene in milk is rabbit because of early age of maturity, short gestation period and multiparous than the other farm animals.

## Transgenic urinary bladder

Kerr *et al*. (1998) successfully expressed the human growth hormone in the transgenic mouse bladder epithelium using a promotor of mouse Uroplakin II gene.

There are some advantage in expressing in urine are

1. We can use both male and female animals
2. Non invasive collection of product
3. Ability to harvest product shortly after birth from both sexes

## Transgenic blood

Swanson et al. (1992) successfully expressed the human haemoglobin in transgenic swine blood. This hemoglobin could be purified from their blood and used to create a blood substitute to give to human patient because pigs are so easy to breed, they could provide an unlimited supply of inexpensive hemoglobin. This supply could help to solve the problem of blood shortages.

## Transgenic seminal vesicle

Dyck et al. (1999) expressed the human growth hormone in transgenic mice seminal vesicle using a mouse $P_{12}$ gene promotor. The concentration they obtained is 0.5 mg/ ml. it is prove to be an alternative to use of the mammary gland as a bioreactor. The animal of choice for this type of expression is boar, because their ejaculates 200-300 ml/ ejaculation with a total protein 30 mg/ml and can ejaculate 2-3 times per week, year round (Setchell, 1988). Therefore, a single transgenic pig expressing of foreign protein under the control of a similar promotor

specific to accessory sex gland at a rate of 1.0 mg/ml could produce 22.4gm of protein/ year.

## Transgenic eggs

Eggs are inexpensive source at high quality protein, but many people avoid them because they are rich in cholesterol. Altering the gene responsible for cholesterol production could result in healthier, lower cholesterol eggs. Low cholesterol eggs food that could help to produce many health problems in peoples (Thiemann *et al.,* 2004). Recently chicken anti-Prion single chain antibody expressed successfully in transgenic quail eggs using β actin promotor. They also checked their biological activity of that protein using western blotting analysis (Kawabe *et al.,* 2006).

## Application in xenotransplantation

The demand of organ transplantation is much greater than the supply of organ from human donors. It is estimated that every year, 10 -15 % patients in United States die while waiting for heart or liver donors (Logan, 2000). Pigs are primary interest for organ donation because their physiology and other parameters, including organ size, indicate that they are the among the most suitable non primate potential donor species. The major antigen that stimulate hyper acute rejection is the $\alpha_{1,3}$ – galactose ($\alpha_{1,3}$ Gal) epitope, which is found on the organ and secreted glycoprotein of all mammals except in humans, apes and old world monkeys (Sachs *et al.,* 2001). This epitope is synthesized by the enzyme $\alpha_{1,3}$ galactosyl transferase ($\alpha_{1,3}$ – GT), but the enzyme is inactive in humans. It has been shown that 1% of circulating B lymphocytes in humans produce anti $\alpha_{1,3}$ Gal antibodies as a reaction to gastrointestinal bacteria. The first target for the genetic engineering at pigs as organ donors has been to knock out the pig gene for $\alpha_{1,3}$ GT. This was achieved through gene targeting and nuclear transfer (Cooper, 2003). In xenotransplantation another potential problem is porcine endogenous retrovirus (PERV) to the recipient. To reduce the release of PERV by porcine transplants, a new approach, using synthetic short interfering RNAs (siRNAs) corresponding to the different part of the viral genes

gag, pol and env was applied by Karlas et al. (2004). This strategy was efficient in suppression of PERV replication.

Another group of animals with potential as organ donors is fish. A group of Canadian investigators has produced transgenic tilapia in which the islets of β cells in the Brockmann body synthesis human insulin. These transgenic fish could serve as donor of islets for xenotransplantation, even in the encapsulated from form (immuno-isolated), because they display higher hypoxia resistance than mammal (Pohajdak *et al.,* 2004). Advantages of this xenotransplantation is low cost and do not cause any zoonotic infection to recipient.

### Application of as animal model for human disease

The animal model used for studying human disease process is the mice. Disease studied using the transgenic mouse model includes sickle cell anemia, amyotrophic lateral sclerosis, chronic hypertension, renal degeneration, osteogenesis imperfeita, cystic fibrosis, mitochondrial cardiomyopathy, neurodegenerative disease, Werner syndrome, rhodopsin mutation, retinitis pigmentosa, melanoma, Alzheimer's disease, prostate cancer and atherosclerosis (Venkateswaran *et al.,* 2004) mutamouse and big blue are transgenic mouse strains currently used for mutagenicity tensing (Mepham *et al.,* 1998).

Transgenic rabbits used as an animal model for human cardiovascular disease and atherosclerosis (Bosze *et al.,* 2003). The transgenic pigs used as large animal model for human eye disease retinitis pigmentosa (Petters *et al.,* 1997).

## Current risks in Transgenesis

### Cost of production

The operational cost of making transgenic mouse is about Rs. 4,400 making an expressing transgenic livestock founder can cost approximately anywhere between 11,00,000 and Rs. 2,20, 00,000 (Wall *et al.,* 1992).

## Success rate

Still using different method of transgenesis, the success rate is less than 10% only due to multifactorial problem.

## Animal health

Insertion of a transgene may upset the expression of the genome of host animal (and the consequence functioning of the animal)

**Table :** Current Company producing transgenic products

| Species | Company | Website | Products | Status |
|---|---|---|---|---|
| Goats | GTC Biotherapeutics (USA) | http://www.transgenics.com | Antithrombin III, Monoclonal antibodies, Malaria vaccine | Completed European efficacy study (AT – III) |
| | Nexin Biotechnologies (Canada) | http://www.dexiabiotech.co | Spider silk Protein, Herman | Other products in preclinical Fiber development research |
| Cattle | Hematrech (USA) | http://www.hematech.com | Human polyclonal antibodies (vaccines) | Research |
| | GTC biotherapeutics (USA) | http://www.transgenics.com | Human serum albumin | Research |
| Sheep | PPL (UK) | http://www.ppl-therapeutics.com | Alpha 1 antitrypsin | Trials postponed Assets business being gold |
| Rabbit | Pharming (Netherlands) | http://www.pharming.com | C1 – inhibitor | Phase II clinical trials |
| | Bioprotein Technologies (France) | http://www.bioprotein.com | Recombinant protein | Research |
| Chicken | Vivalice (France) | http://www.vivalis.com | | |
| | Avigenics. Inc (USA) | http://www.avigenics.com | Recombinant Proteins | Research |
| | Transxenogen (USA) | http://www.tranxenogen.com | | |
| | Virage (USA) | http://www.virage.com | | |

## Virus transfer

This is particular concern in animal bred as tissue donor for xenotransplantation, but now a day it can be over come with help of siRNAs, shRNA technique.

## Dissemination

Normal reproduction may result in a transgene being released to the wild population.

## Conclusion

Currently many companies start producing gene farming for therapeutic protein (listed in table 3) but now USA is the first country approved products were obtained in transgenic systems in last week of December 2006. Currently anti-thrombin III proteins produced in transgenic animals just completed the regulatory process. Many biopharmaceuticals proteins are under clinical trails and some are awaiting for regulatory approvement. We expect many transgenic therapeutic protein reaches the commercial market with in a decade. If it happens, it going to good revolution in transgenic animal production and pharmaceuticals industry and that will helpful in economic uplifting of poor farmers. Using modern technique such as knocking out siRNAs, shRNA and ribozymes along with transgenesis. We can remove particular receptor (that makes the animal susceptible to disease) and there by we can make disease resistant herd and other hand we can stamp out zoonotic disease to human beings.

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