Techniques of Transgenic Animal Production: A Review

A Thesis Submitted

By

Dilip Dhali
Examination Roll No. M.S 070722
Session: 2006-2007

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August, 2008.

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DEDICATED TO MY VENERATED

PARENTS AND BELOVED SIBS

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ABSTRACT

The possibility that phenotype of cells and whole animals could be manipulated in selected and intended ways by introducing foreign DNA has enchanted scientists. Recent progress in molecular biology has made it possible to transfer genes of interest into cells cultured in vitro and even in target tissues of living animals in vivo, albeit at a low rate. This enables one to manipulate phenotype of cells and whole animals in selected and intended ways. The consequence of such gene transfer attempts has been used for the production of various types of transgenic animals. Transgenic technology is the process by which controlled genetic modifications of multicelluler eukaryotic species are produced. It has led to an explosion of research in developmental genetics. It attracts the interest of many investigators who were not engaged in animal research previously. Animal in which foreign DNA is integrated into the genome are called transgenic. The foreign genetic material will generally consists of a structural genes with regulatory sequences those are required for the transcription of the introduced genes. Within the mammalian species, the mouse has been extensively used to study gene regulation through the introduction of fusion genes. Fundamental information on gene expression and regulation is being generated from the transgenic mouse model. Transgenic rats, rabbits, sheep, cows, pigs, goats, chickens, birds, amphibians, reptiles and even fish have already been produced. Transgenic manipulation of livestock can facilitate precision agriculture, minimizing the use of antibiotics and pesticides, and can bring increased efficiency to the processing industry. Transgenesis offers a new paradigm in disease control and can assist in preserving genetic diversity. Outside agriculture, transgenic livestock have an important role in biopharmaceutical production. Biotechnology, including transgenic livestock, will play a key role in meeting the challenge of feeding the world population, while creating and maintaining sustainable agricultural systems. Transgenesis is not an end in itself; its only justification in agriculture is to benefit the quality of life of man, or animals, or to achieve better use of environmental resources. The main target of my review was to explore the methods of transgenic animal production and therapeutic applications of the transgenic animal as well as other possible implications of transgenesis.

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INTRODUCTION

CHAPTER ONE

Introduction

Transgenic animal is an animal in which there has been a deliberate modification of the genome - the material responsible for inherited characteristics - in contrast to spontaneous mutation[1]. Foreign DNA is introduced into the animal, using recombinant DNA technology, and then must be transmitted through the germ line so that every cell, including germ cells, of the animal contains the same modified genetic material.

An animal is "transgenic" once a scientist inserts DNA from another organism. This process allows scientists to transfer beneficial genes from a different animal, bacterium, or plant. Currently scientists are developing transgenic pigs, cattle, fish and poultry. Prior to the development of molecular genetics, the only way of studying the regulation and function of mammalian genes was through the observation of inherited characteristics or spontaneous mutations. Long before Mendel and any molecular genetic knowledge, selective breeding was a common practice among farmers for the enhancement of chosen traits, e.g., increased milk production.

During the 1970s, the first chimeric mice were produced[2]. The cells of two different embryos of different strains were combined together at an early stage of development (eight cells) to form a single embryo that subsequently developed into a chimeric adult, exhibiting characteristics of each strain. The process to achieve controlled genetic modifications of multicellular eukaryotic species by the insertion of genetic material is known as transgenic technology. It has led to an explosion of research in developmental genetics. It attracts the interest of many investigators who were not engaged in animal research previously. Transgenic is the production of animals whose genetic make-up has been changed in some way[6].

Transgenic is one of the fastest growing areas of research. Latest Home Office figures show that 511,607 experiments on transgenic animals were carried out in 1999. The first transgenic animal was created nearly 20 years ago, and was a giant mouse that had the gene for human growth hormone incorporated into its genome (genetic makeup). Since then transgenic rats, rabbits, sheep, cows, pigs, goats, chickens and even fish have all been 'designed'[7].Transgenic animals provide the investigator with an extremely powerful tool for the development of disease models, since the mechanisms of gene regulation will receive a greater understanding. In addition, the use of transgenic mouse models that more closely mimic the human disease can replace the need to use more sentient animals as models. The better specificity of models may in time also lead to a reduction in the number of animals used. Genetic modification of livestock may also be seen as a benefit to human health, in the economic and efficient production of important pharmaceutical proteins [8].

Transgenesis is not an end in itself; its only justification in agriculture is to benefit the quality of life

of man, or animals, or to achieve better use of environmental resources. Transgenic manipulation of livestock can facilitate precision agriculture, minimizing the use of antibiotics and pesticides, and can bring increased efficiency to the processing industry. Transgenesis otters a new paradigm in disease control and can assist in preserving genetic diversity. Outside agriculture, transgenic livestock have an important role in biopharmaceutical production. Biotechnology, including transgenic livestock, will play a key role in meeting the challenge of feeding the world population, while creating and maintaining sustainable agricultural systems.

The nucleus of all the cells in every living organism contains genes made up of DNA. These genes store information that regulates how our bodies form and function. Genes can be altered artificially, so that some characteristics of an animal are changed. For example, an embryo can have an extra, functioning gene from another source artificially introduced into it, or a gene introduced which can knock out the functioning of another particular gene in the embryo. Animals that have had their DNA manipulated in this way are known as transgenic animals. Transgenic rats, rabbits, pigs, sheep, cows and fish have been produced, although over 95% of all existing transgenic animals are mice.Science today is unable to grasp a complete understanding of the workings of genetics. The Central Dogma of Molecular Biology might offer us the capability to manipulate genes to obtain desired phenotypes but also undesired ones frequently. Current genetic engineering techniques do not consider much about gene expression and regulatory networks. For example, some genetically engineered fish with growth hormone genes are known to develop tumours and abnormal fins[10]. Such undesired traits cannot be detected until the transgenic species are nurtured. As no one can guarantee that none of these transgenic animals will escape into natural habitats, the problem of genetic pollution arises. In an article from the Green Peace International website[11], it is said that transgenic animals could never have evolved naturally and have a natural habitat. If allowed to inter-breed and reproduce with natural organisms, they may pose unacceptable risks to ecosystems, and threaten biodiversity, wildlife and sustainable forms of agriculture. Food chains may be disrupted and we risk the lost of the already diminishing natural habitats. We must also not neglect the possibility that various environmental problems may meet at crossroads to develop unimaginable disasters.

Hunger exists as a problem for many developing countries. Farming transgenic animals can increase food productivity as a solution to these countries. In conclusion, we witness the different perspectives of transgenic animal production. The underlying rationale behind individual arguments lie in the kinds of advantages and disadvantages such production can bring to mankind.

The objectives of my review are: (1) to elucidate the molecular basis/techniques of growth and development of transgenic animals production, (2) to study how various products e.g. pharmaceuticals, food, proteins, drugs and other products produced from transgenic animal are improved both in quality and quantity, (3) to study how they can be used in various sectors for human welfare.

CHAPTER TWO

METHODS OF CREATION OF TRANSGENIC ANIMALS

CHAPTER TWO

Techniques of Transgenesis

2. Methods of creation of transgenic animals:

The underlying principle in the production of transgenic animals is the introduction of a foreign gene or genes into an animal (the inserted genes are called transgenes). The foreign genes "must be transmitted through the germ line, so that every cell, including germ cells, of the animal contains the same modified genetic material."(Germ cells are cells whose function is to transmit genes to an organism's offspring.)

The first step is the identification and isolation of the relevant gene that will eventually be responsible for the production of protein or feature that you wish to manifest in the animal. The piece of DNA containing that gene is then inserted into a fertilized egg or embryo. This can be achieved in a number of ways.

The three principal methods used for the creation of transgenic animals are as follows:

1. DNA microinjection into the pronucleus
2. Embryonic stem cell-mediated gene transfer and
3. Retrovirus-mediated gene transfer.

The most successful of these is the microinjection technique.

2.1 DNA microinjection.

Microinjection of DNA is done by injecting liner DNA molecules into fertilized eggs in pronuclear stage using an inverted microscope, micromanipulation equipment and injection or holding devices. Highly purified DNA is essential for successful transgenic production. Particles within the DNA could easily clog the microinjection needle and/or interfere with integration of the DNA. Therefore, the fragment should be isolated by electrophoresis followed by electro-elution.

The first successful production of transgenic mice using pronuclear microinjection was reported in 1980[3]. However, the technique has also been extended to species like rats[27], rabbits[28], sheep[19], goats[29], cattle[30], and pigs[28]. Although the recombinant viral construct was proven to have integrated into the mouse genome, it was rearranged and did not express.

Subsequent reports[31] proved that integrated transgenes were capable of functional expression following pronuclear microinjection. The first visible phenotypic change in transgenic mice was described in 1982 for animals expressing the rat growth hormone sequence[32]. Currently, several hundred transgenic expression papers are published each year, the majority examining the effects of microinjected viral sequences on mammalian growth and pathology.

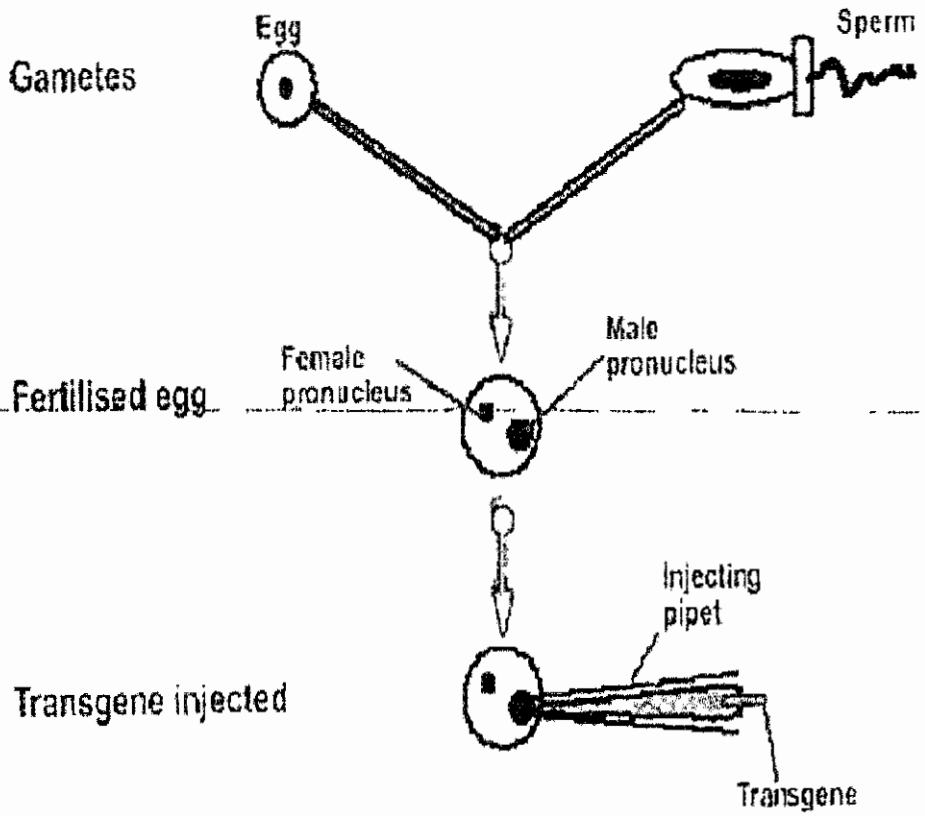


Fig 1: Microinjection process

The pronuclear microinjection method of producing a transgenic animal results in the introduction of a purified double-stranded DNA sequence into the chromosomes of the fertilized mammalian egg. If this transferred genetic material (i.e., transgene) is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information in every cell. The foreign DNA must integrate into the host genome prior to the doubling of genetic material that precedes the first cleavage or a mosaic animal may be produced in which many cells do not possess the new gene. For this reason, the transgene DNA is introduced into the zygote at the earliest possible stage, i.e., the pronuclear period immediately following fertilization. For several hours following the entry of the sperm into the oocyte, the male and female pronuclei are microscopically visible as individual structures. The transgene may be microinjected into either of these pronuclei with equivalent results. However, Xchromosome or Y-chromosome integration events do occur and obviously may be influenced by the choice of pronucleus. Usually, the male pronucleus may be distinguished because it is larger than the female nucleus and also because it is closer to the oocyte surface.

The animal that develops after receiving the transgene DNA is referred to as the founder (Fo) of a new transgenic lineage. If the germ cells of the founder (mosaic or not) transmit the transgene stably, then all descendants of this animal are members of a unique transgenic lineage. Integration of foreign DNA into the embryonic genome generally is a random event with respect to the chromosomal locus. Therefore the probability of identical integration events in two embryos receiving the same transgene is overwhelmingly unlikely. In addition, it is impossible to regulate exactly how many copies of the transgene will be introduced into the embryo and how many will join together to integrate (usually at a single site) as a single linear array called a concatamer [33]. Many studies have found dramatic differences in the expression of a specific transgene within individual sibling embryos simply due to different integration loci. The number of copies of the transgene that have joined the founder's genome is referred to as the copy number, and rarely appears to be correlated with the degree of transgene expression in the animal.

Because the locus of transgene integration is random, the transgene frequently inserts into functional genetic sequences. Interruption of the normal expression of an endogenous gene may be inconsequential or lethal. Alternatively, observable insertional mutagenesis might be apparent when the insertion interferes with the expression of an endogenous developmentally active gene. These mutations are distinguished from the true transgenic phenotype because only a single lineage exhibits the defect. The mutations can involve any system including the special senses, cardiovascular, neurological and reproductive systems, and severe morphogenetic abnormalities may be observed[34]. The identification of the locus of transgene insertion is of great value because it maps the locus of an important endogenous gene.

Because the new transgenic locus is present in only one member of a particular paired chromosome, the genotype of the founder is described as homozygous for the transgene rather than heterozygous. The mating of a pair of homozygous F_1 siblings may produce a homozygous genotype, in which a pair of transgene alleles is present. Obviously, mating a pair of animals with identical transgenes but from different founder lineages cannot result in a true homozygote in which independent segregation of the loci is predictable.

The success of the microinjection technique relies upon the careful collection of a relatively large group of accurately timed embryos from a reproductively synchronized group of female embryo donors. In addition, the techniques of microinjection and embryo transfer to a suitable recipient female must be mastered. Of course, the combined success of all of these manipulative skills ultimately depends upon the fastidious construction and preparation of the transgene DNA fragments to be injected.

General Equipments:

The equipments required to perform microinjection can cost between $50,000 and $80,000 and includes:

1. C02 incubator to maintain manipulated embryos at 37-38° C in an atmosphere of 5-6 percent C02.

2. Inverted microscope with a fixed stage.

3. Phase contrast, Nomarski differential interference, or Hoffman modulated contrast optical systems to visualize pronuclei. With lOx or 15x eyepieces, a 20x or 40 x objectives is required.

4. A pair of micromanipulators to control the DNA injection pipette and the embryo-holding pipette.

5. A pair of micro-volume syringes and associated tubing to regulate the fluid dynamics in the injection and holding pipettes. (Expensive automatic microinjection systems are available in lieu of the injection syringe.)

6. Pipette-pulling apparatus.

7. Vibration-free pneumatic table (optional).

8. Micro forge apparatus to heat-polish and bend pipettes.

9. Pipette beveling apparatus (optional).

10. Supply of clean capillary pipettes for the manufacture of holding and injection pipettes.

11. Fluorine solution (optional) to provide optimal fluid dynamics in the pipettes.

12. Micro photographic equipment (optional) including 35mm camera and/or video recording apparatus

Steps involved in microinjection process:

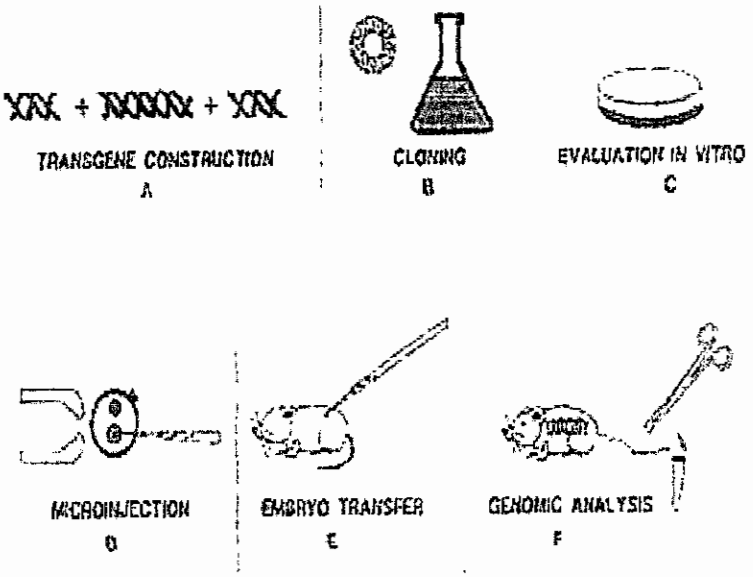


Fig 2: Sequence of events in the generation of a transgenic animal by pronuclear microinjection.

A. The double-stranded DNA components of the transgene are combined enzymatically to yield a transgene expression cassette.

B. Transgene cassettes are inserted into plasmid vectors and cloned.

C. Transgene-bearing plasmids are transfected into cultured eukaryotic cells to evaluate expression of the transgene.

D. Plasmid-free transgene fragments are introduced directly into embryonic pronuclei.

E. Manipulated embryos are placed in the reproductive tract of a pseudopregnant recipient.

F. The genomic DNA of live-born pups is analyzed for the presence of the transgene DNA sequence.

Transgene DNA Preparation:

The transgene DNA is engineered in the molecular laboratory to achieve fairly predictable expression in the animal. Using restriction enzymes and ligase, different functional regions of genes from different species may be recombined in the test tube. All components of endogenous genes may be isolated and recombined to form a transgene expression cassette or construct (Fig 3). The ends of the completed construct may be modified by the addition of polylinker sequences containing several different restriction enzyme recognition sites. The polylinker permits the construct to be inserted into a variety of vectors for testing and cloning. The following review of endogenous gene components will clarify these strategies:

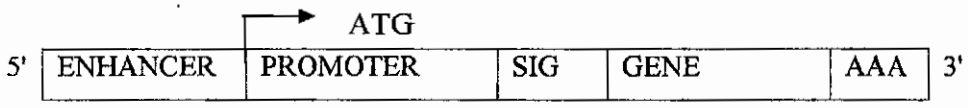


Fig 3: Schematic diagram of the double-stranded DNA regions of the transgene expression cassette.

{Restriction enzyme recognition sites are clustered at either end of the cassette (i.e., upstream and downstream). "ATG" indicates the beginning of the transcriptional reading frame. "SIG indicates the signal sequence"AAA" indicates the poly-A tail}

The endogenous gene contains exons that code for specific portions of the final protein and introns that appear necessary for optimal expression of the gene (Fig 4). The endogenous gene is flanked by non-coding DNA sequences that regulate gene expression. (Regulatory elements may also reside within intragenic intron sequences.) Sequences located at the 5' end of the gene are known as upstream elements, while downstream elements are found past the 3' end of the gene sequence[50]. Regulatory elements called promoters are usually found immediately upstream of the gene, and have critical roles in the temporal and tissue-specific regulation of gene expression. Other regulatory

elements called enhancers function to enhance gene expression, independent of their location and orientation with respect to the gene. Enhancer regions appear to correlate with DNase hypersensitive sites and may be several kilobases (kb) distant from the gene. Signal sequences are short sequences that target protein synthesis into specific intracellular pathways and frequently direct secretion of the protein from the cell. Secretory signals usually are found directly adjacent to the 5' end of the gene, and organelle targeting sequences usually are found within the 3' end of the gene or immediately downstream of the 3' end. These signal sequences are within the reading frame or transcriptional region of the gene and therefore encode mRNA and short polypeptide products[51]. The 3' end of the reading frame also must contain a poly-A nucleotide sequence to ensure proper mRNA transcription and translation.

GENOMIC	INTRON	EXON-1	INTRON	EXON-2	INTRON	EXON-3

cDNA	EXON-1	EXON-2	EXON-3

Fig 4: Comparison of the two forms of transgenes that may be introduced into embryonic pronuclei.

{The genomic form includes all naturally-occurring intron elements that are involved in mRNA splicing and expression, whereas the shorter cDNA form is a synthetic sequence representing only the protein-encoding exon elements of the gene. }

Any transgene fragments used in pronuclear microinjection represent several months or years of intensive efforts by a team of molecular biologists. The transgene cassette is inserted into a plasmid vector and cloned (copied) within E. coli. The transformed bacteria containing the plasmids are identified by survival selection culture techniques using the antibiotics to which the plasmids are resistant. Surviving bacteria are grown in nutrient medium overnight, and the transgene/plasmid is copied each time the bacteria divide. Several million copies of the transgene-bearing plasmid are then extracted from the bacteria, and the transgene fragments are excised from the plasmids using restriction enzymes[52]. The transgene fragments may be purified by electroelution from electrophoretic gels or by collecting them from a sucrose gradient. The transgene fragments are dissolved in a specific microinjection buffer (i.e., a Tris EDTA solution), and the DNA concentration is calculated. The working concentration of DNA for microinjection is usually between 1 and 5 μg/ml. Before introduction into mouse embryos, the transgene construct is tested for expression in cultured cells, usually following a DNA introduction technique known as transfection.

The presence of vector DNA sequences may inhibit expression of the integrated transgene, so this plasmid DNA usually is snipped off with restriction enzymes before purification of the fragments. Also, linear fragments have been observed to integrate at a higher frequency than circular or supercoiled molecules[35]. It is apparent from several reports [36] that genomic forms of transgenes containing introns or mRNA splice sites can be expected to exhibit greater expression levels than their cDNA versions (Fig 4), which merely contain the exon sequences of the gene. The introns may be involved in critical splicing activities and/or regulatory functions.

Embryo Collection:

The choice of the donor parental strains for production of the pronuclear embryos is a point of extreme proprietary concern to most laboratories. Many factors are cited including the response to super ovulation, frequencies of embryo survival following microinjection, size of pronuclei and the incidence of specific pathologies inherent in various strains. The relative merit of inbred versus outbred backgrounds may be important for the evaluation of a specific transgene expression. Other factors may involve coat color, the availability of a certain strain, or simply anecdotal rationales. Certain hybrids (e.g., C57BL/6 x SJL/N) and out breeds (e.g., CD-1) are reported to yield large numbers of viable pronuclear embryos following super ovulation. The FVB/N inbred strain is reputed to survive microinjection procedures better than many other strains and has been shown to possess pronuclei of relatively larger volumes.

Whichever strain is chosen to provide embryos, fewer animals will be needed and less variability encountered if exogenous gonadotropins are used to super ovulate the donor females. Successful super ovulation protocols must consider the strain, age and weight of the animals. Breeding should be monogamous, and the light cycle in the breeding room must be strictly regulated. The super ovulation and synchronization of rats, rabbits and larger mammals present additional technical challenges.

After following breeding, oviducts are removed from euthanized donors, and clumps of pronuclear embryos are collected from the oviducts by flushing or by dissection into a microdrop of sterilized buffered medium. The embryos are clumped together with sticky follicular cumulus cells that must be removed by brief treatment in a series of microdrops. The first drop, a solution of the enzyme hyaluronidase, is followed by two or more wash drops. Using heat-pulled tapered micropipettes controlled by mouth suction (a new pipette for each drop), the embryos are transferred from drop to drop until they are free of cumulus cells, debris and enzyme. Finally, the embryos are transferred into a pool of medium in a petridish that will be placed under the microscope[49]. A layer of sterile-filtered, autoclaved mineral oil to prevent contamination by microorganisms and debris and to prohibit evaporation and the resultant pH changes that would kill the embryos covers the embryo-containing pool. All collection and manipulation media contain a buffering system (i.e., bicarbonate or HEPES) and protein source (e.g., bovine serum albumin) to prevent embryos from adhering to

the dishes and pipes. In addition, media may contain antibiotics (e.g., penicillin and/or streptomycin) and a heavy-metal chelating agent (e.g., EDTA).

Embryo Transfer:

The manipulated embryos must be transferred into a suitable reproductive tract in order to have an opportunity to become live-born transgenic mice. The recipient female optimally should be somewhat earlier in her reproductive cycle than the embryo donor because manipulated and cultured embryos exhibit slightly retarded development when compared to embryos that developed in vivo. Recipients for embryo transfer are prepared by mating with vasectomized males at the same time that the superovulated donor females are mated with fertile males. It is advisable to use vasectomized males and recipient females with a coat color dominant to the embryo donor so that resources are not wasted testing embryos generated by insidiously fertile vasectomized males. Recipient females are anesthetized, the skin and peritoneum are incised, and the ovarian fat pad and bursa are exteriorized and draped over the midline[55]. The bursa is opened, avoiding any prominent vessels, and the infundibulum is located. An embryo transfer pipette with an internal diameter of less than 150 μm is loaded in the following sequence: one small air bubble, approximately 10 μl of medium, a second air bubble, 2-15 embryos in less than 25 μm of medium, and a third air bubble. The pipette tip is inserted into the infundibulum of the oviduct, and the contents are gently transferred into the oviduct by mouth pressure until the middle air bubble is expelled. The reproductive tract is gently replaced and the incision is closed. Pregnancy should be visible about two weeks after the embryo transfer (post-ET), and the litter should be delivered about three weeks post-ET. Animals may be analyzed for the presence of the transgene in their genomes after weaning at six weeks post-ET. It should be noted that certain transgene sequences may be activated in utero and may affect embryo survival or gestation length. Also, transgenic females in subsequent generations should be observed for abnormal gestation lengths.

In a short brief, we can say that this method involves the direct microinjection of a chosen gene construct (a single gene or a combination of genes) from another member of the same species or from a different species, into the pronucleus of a fertilized ovum. It is one of the first methods that proved to be effective in mammals [37]. The introduced DNA may lead to the over- or under-expression of certain genes or to the expression of genes entirely new to the animal species.

The insertion of DNA is, however, a random process, and there is a high probability that the introduced gene will not insert itself into a site on the host DNA that will permit its expression. The manipulated fertilized ovum is transferred into the oviduct of a recipient female or foster mother that has been induced to act as a recipient by mating with a vasectomized male.

A major advantage of this method is its applicability to a wide variety of species.

Currently the technique of DNA microinjection is the most successful and most widely used method of producing transgenic animals. Using this technique, transgene sequences up to about 50 kilobases

(kb) in length from viral, prokaryotic, plant, invertebrate or vertebrate sources may be introduced into the mammalian genome, where they may be expressed in both somatic cells and germ cells. Microinjection of DNA constructs into pronuclei of zygotes has been the method of choice for the generation of transgenic livestock. However, this procedure is characterized by low efficiency (1-4% transgenic offspring), random integration and variable expression of the transgene as well as a considerable proportion of mosaicism. Furthermore, it is extremely time consuming and costly. As a consequence, commercial application has focused on the production of recombinant proteins in the mammary gland of transgenic animals and xenotransplantation, e.g. the use of porcine organs in human organ transplantation[56].

Time-line for the production of transgenic animal by microinjection technique

The following graphic provides a representation of the expected timeline from inception of service request to transfer of potential founder animals to investigator care.

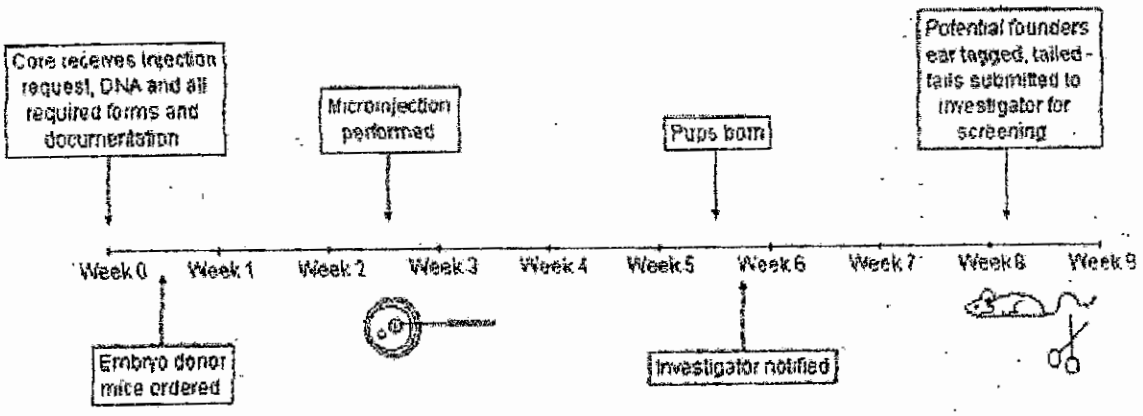


Fig 5: Time-line for transgenic animal production[39]

Limitation of Microinjection Method:

1. A highly trained practitioner can expect
2. Only 5% of the inoculated eggs to develop into live transgenic animal
3. A large number of microinjected fertilized eggs must be used
4. The injected DNA integrates at random sites within the genome
5. Sometimes multiple copies of the injected DNA are incorporated at one site.

2.2 Embryonic Stem Cell-Mediated Gene Transfer:

This method involves prior insertion of the desired DNA sequence by homologous recombination into an in vitro culture of embryonic stem (ES) cells. Stem cells are undifferentiated cells that have the potential to differentiate into any type of cell (somatic and germ cells) and therefore to give rise to a complete organism. These cells are then incorporated into an embryo at the blastocyst stage of development[57]. The result is a chimeric animal. ES cell-mediated gene transfer is the method of choice for gene inactivation, the so-called knockout method.

In short this method can be descrived as [5]:

1. Isolation of totipotent stem cells (stem cells that can develop into any type of specialized cell) from embryos
2. The desired gene is inserted into these cells
3. Cells containing the desired DNA are incorporated into the host's embryo, resulting in a chimeric animal

Unlike the other two methods, which require live transgenic offspring to test for the presence of the desired transgene, this method allows testing for transgenes at the cell stage.

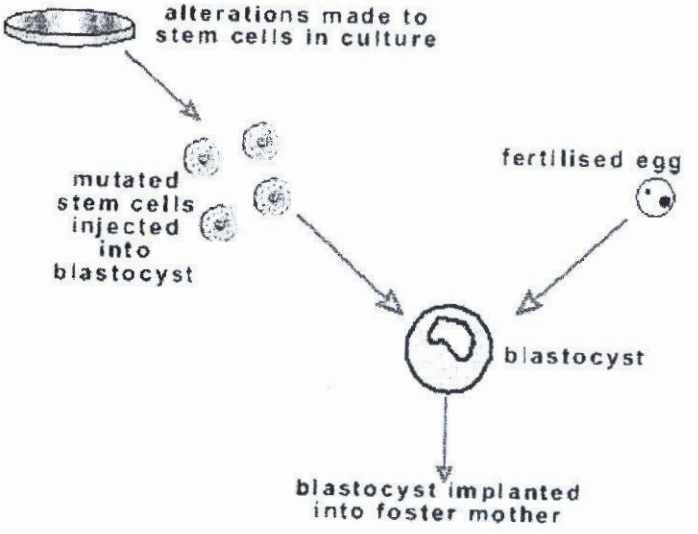


Fig 6: Embryonic stem cell-mediated gene transfer process

Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all

the cells of the mature animal, including its gametes.

Embryonic stem cells are derived from the inner cell masses of normal blastocysts (early embryos, mostly mouse embryos). These cells are pluripotent, which means they can develop into any type of tissue. Therefore, removing ES cells from the culture and placing them back into an early embryo (blastocyst) allows them to divide and become part of the embryo. Transfection allows foreign genetic material to be inserted in vitro into these cells. The target of this process is homologous recombination with the chromosome of the cell, i.e. introduction or exchange of DNA at one location with homologous DNA sequences. Following transfection, the descendants of one individual cell (clone) are analyzed using microbiological methods to ascertain whether any mutation is present. Cells of the identified clones which showed the desired reactions are multiplied *in vitro* and injected into blastocysts [40].These injected blastocysts are then implanted into the uterus of pseudo-pregnant females.

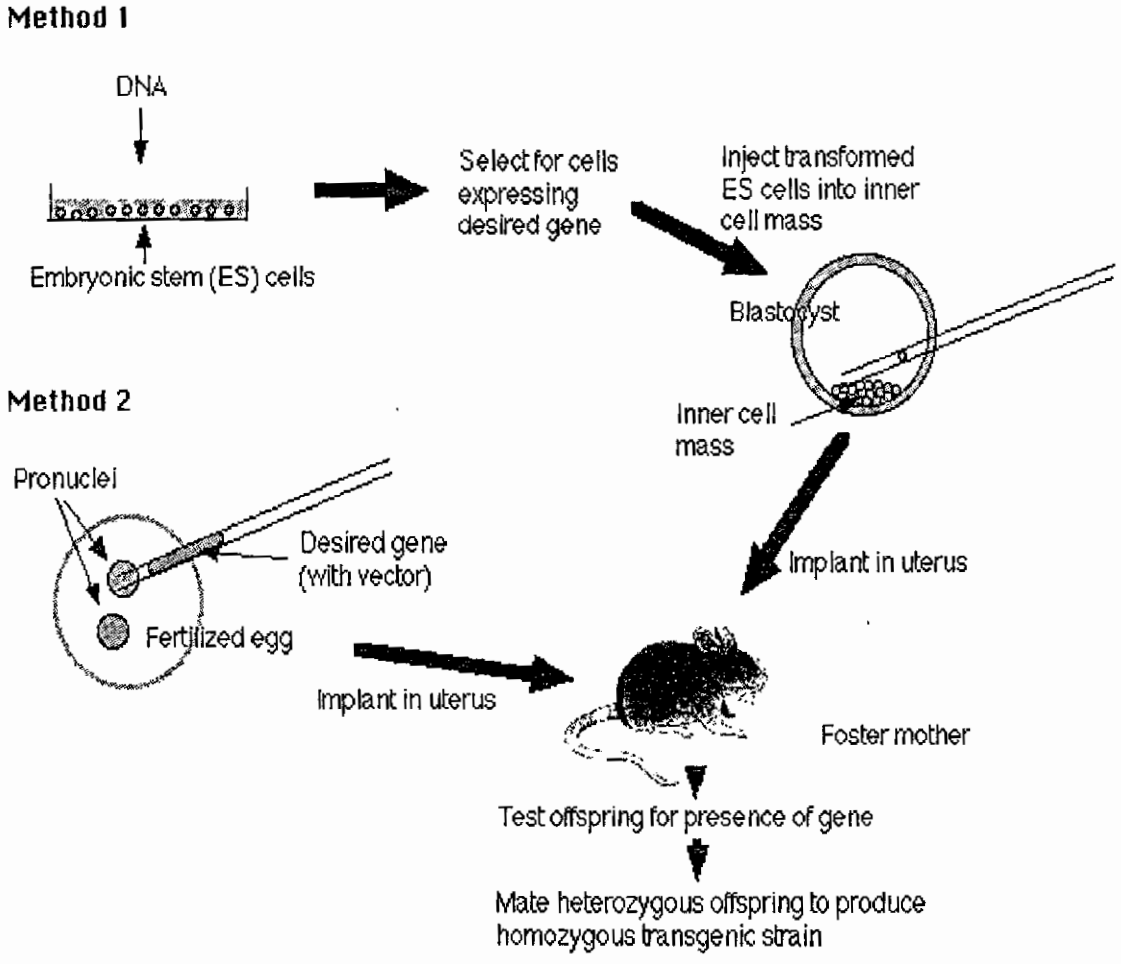


Fig 7: Transgenic animal production by ES cell mediated gene transfer[41].

Procedure:

Transgene DNA Preparation

Using recombinant DNA methods, build molecules of DNA containing -

- The structural gene you desire (e.g., the insulin gene)
- Vector DNA to enable the molecules to be inserted into host DNA molecules
- Promoter and enhancer sequences to enable the gene to be expressed by host cells
- Transform ES cells in culture
- Expose the cultured cells to the DNA so that some will incorporate it.
- Select for successfully transformed cells.
- Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

Embryo transfer

Prepare a pseudopregnant mouse (by mating a female mouse with a vasectomised male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive. Transfer the embryos into her uterus. Hope that they implant successfully and develop into healthy pups (no more than one-third will)[58].

Test her offspring

Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10-20% will have it, and they will be heterozygous for the gene.

Establish a transgenic strain

Mate two heterozygous mice and screen their offspring for the 1:4 that will be homozygous for the transgene.

Mating these will found the transgenic strain. This technique is of particular importance for the study of the genetic control of developmental processes. This technique works particularly well in mice. It has the advantage of allowing precise targeting of defined mutations in the gene via homologous recombination[59].

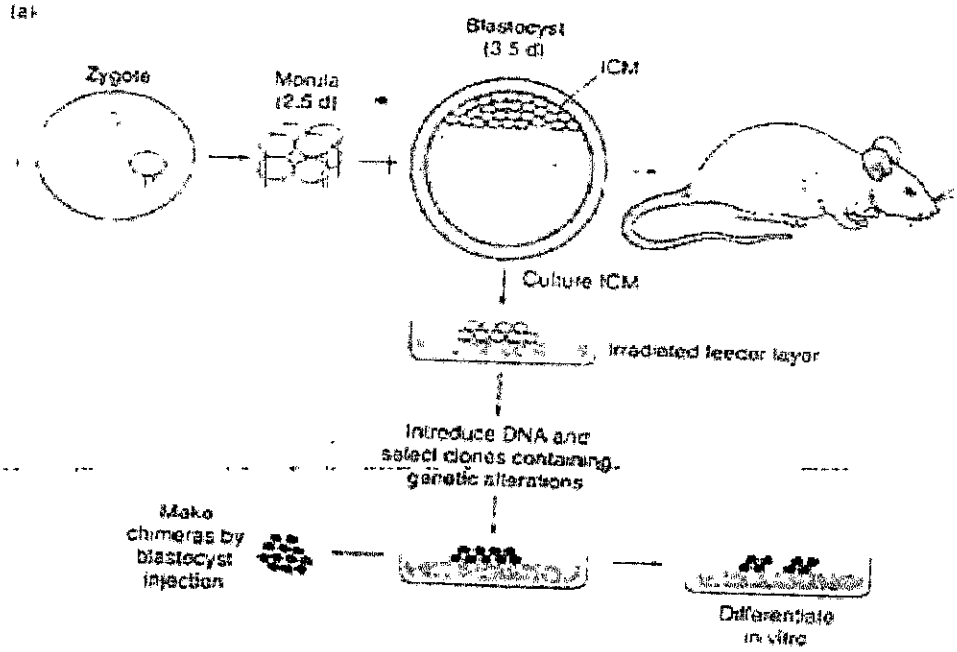


Fig 8: The production of ES cell lines and ES chimeric mice.

(a).The process of establishing ES cell lines from cultures of the inner cell mass off developing mouse embryos.

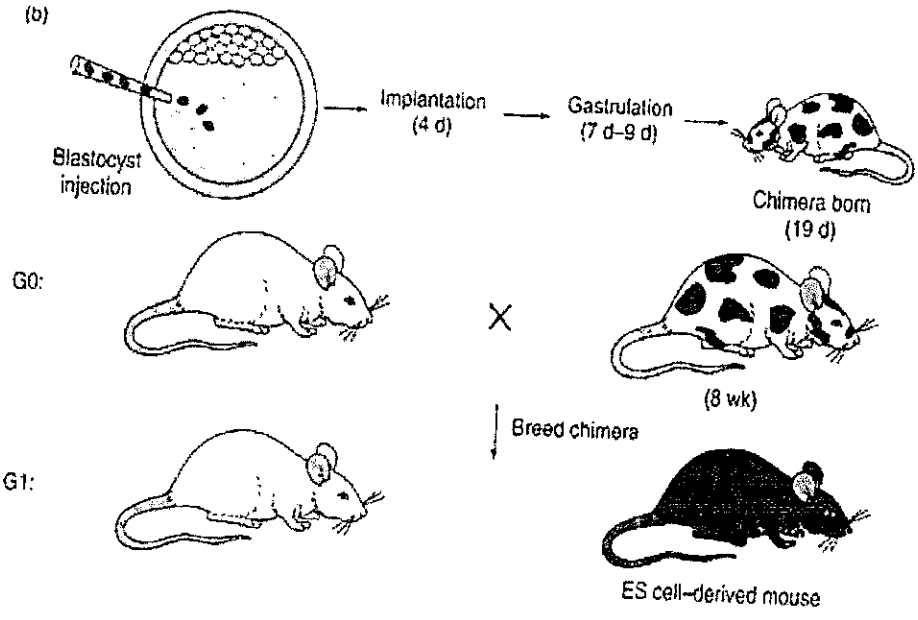
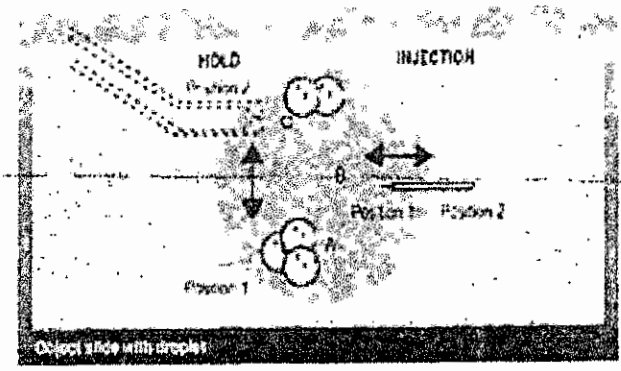


Fig 9: The production of ES cell lines and ES chimeric mice.

(b).The ES cell injection process that leads to incorporation of the mutated Es cells into the germ line and soma of a recipient embryo, producing ES chimeric mice.

Setup of the workstation:

As for the injection of DNA, two TransferMan devices are used for controlling the holding and transfer capillaries. The actual holding of the blastocysts is performed by using CellTram Air. CellTram Oil is used for transferring the ES cells. As aforementioned, one inverted microscope, in combination with Hoffinann modulation contrast optics or differential interference contrast optics, makes the ES cell transfer quick and simple. The setting of the positions is carried out as shown in Fig 13



Figl0: TransferMan for Transgenic Animals Transfer of ES cells

Right-hand side - injection position:

The area where the transfer capillary is kept for injection (focus level, magnification x20 or x40 times) is defined as Position 1. Position 2 is its "parking" position. This can be outside and above the droplet (in the overlay medium). This capillary holder is connected to CellTram Oil.

Left-hand side - holding function:

The area where the uninfected blastocysts are placed is set to Position 1. Here one blastocyst can be easily taken by using CellTram Air and VacuTip. This blastocyst is moved via the joystick to a central position to inject the ES cells.

After ES cells have been injected, the capillary is moved to a "parking" position (can be outside the view). This position is defined as Position 2. Pressing the joystick button moves the capillary back to Position 1. The procedure then restarts.

Time-line for generation of Embryonic Stem Cell-Derived mice:

The following graphic provides a representation of the expected timeline for the generation of Embryonic Stem Cell-Derived mice[43].

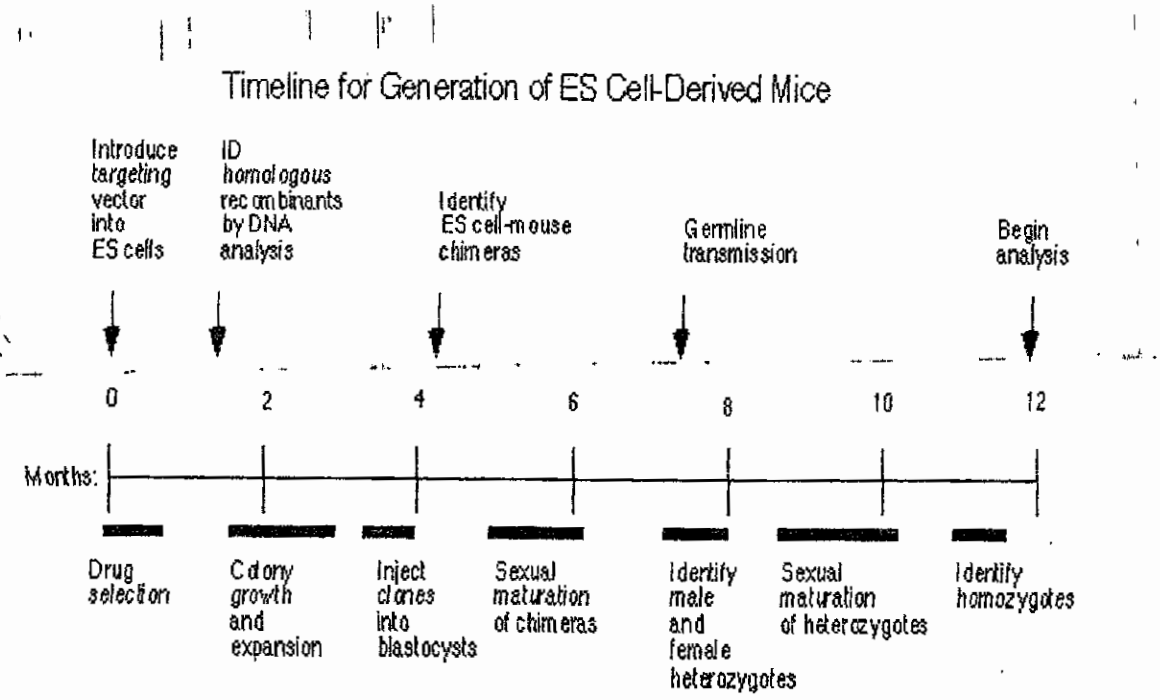


Fig11: Time-line for generation of embryonic stem cell-derived mice.

Limitation of ES cell method:

1. The genetically engineered cells can not be selected easilly.
2. In blastocyst stage it is difficult to control of a developing mouse embryo for its proliferatiating property.
3. For the randomness of integration the use of ES cell method is limited.

2.3 Retrovirus-Mediated Gene Transfer.

The viral information was successfully transferred into the genome of the recipient animal, and the technique of utilizing retroviruses as vectors for specific foreign DNA sequences was soon developed[44]. Retrovirus-mediated transgenesis produces a high degree of mosaicism; the size of the transgene sequence is limited, and the viral sequences may interfere with expression of the transgene. However, the integration of single copies of the transgene flanked by the viral DNA can be advantageous if it is desired to clone the locus of integration.

To increase the probability of expression, gene transfer is mediated by means of a carrier or vector, generally a virus or a plasmid. Retroviruses are commonly used as vectors to transfer genetic material into the cell, taking advantage of their ability to infect host cells in this way. Offspring derived from

this method are chimeric, i.e., not all cells carry the retrovirus. Transmission of the transgene is possible only if the retrovirus integrates into some of the germ cells.

A retrovirus is a virus that carries its genetic material in the form of RNA rather than DNA. This method involves [45]:

1. Retroviruses used as vectors to transfer genetic material into the host cell, resulting in a chimera, an organism consisting of tissues or parts of diverse genetic constitution.
2. Chimeras are inbred for as many as 20 generations until homozygous (carrying the desired transgene in every cell) transgenic offspring are born.

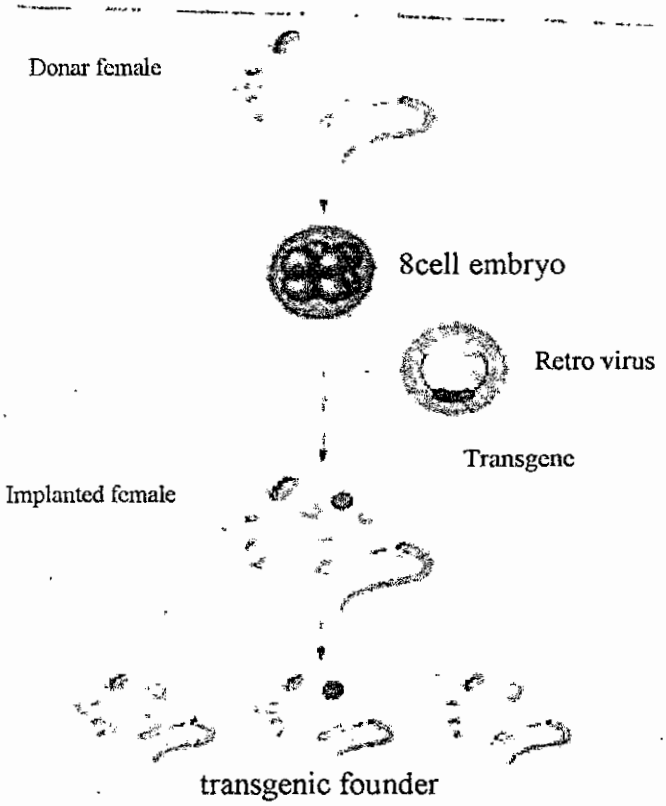


Fig12: Production of transgenic animal by Retrovirus-mediated gene transfer.

This method was successfully used in 1974 when a simian virus was inserted into mice embryos, resulting in mice carrying this DNA46. For any of these techniques the success rate in terms of live birth of animals containing the transgene is extremely low.

Providing that the genetic manipulation does not lead to abortion, the result is a first generation (F 1) of animals that need to be tested for the expression of the transgene.

Depending on the technique used, the Fl generation may result in chimeras. When the transgene has integrated into the germ cells, the so-called germ line chimeras are then inbred for 10 to 20 generations until homozygous transgenic animals are obtained and the transgene is present in every cell. At this stage embryos carrying the transgene can be frozen and stored for subsequent implantation.

This method has some limitations i.e.

i. Packages only 8 kb

ii. Requires a life helper phage

iii. The genome of retroviral strain (helper virus) that is needed to create large quantities of the vector DNA can be integrated into the same nucleus as the transgene.

iv. Retroviral vectors are rarely used for transgenic animal production.

Recent Development of Techniques of Transgenesis:

2.4 Transfection Technique of Transgenic Animal Production

While the technique of cloning has changed dramatically over the years, the basic principle remains the same [81]. Take a blood clotting factor protein we would like to clone in a cow as an example. Essentially, the gene for the factor would be linked to a promoter specific for a milk protein. This promoter is responsible for ensuring that the gene is only expressed in the milk of the animal. Many copies of this promoter-gene combo are then introduced into a cow's egg, which is allowed to develop in a surrogate animal (see Figure 1). A technique called 'Pronuclear injection' is used to do this and involves injecting the DNA directly into fertilized eggs using a very fine glass needle (transfection).

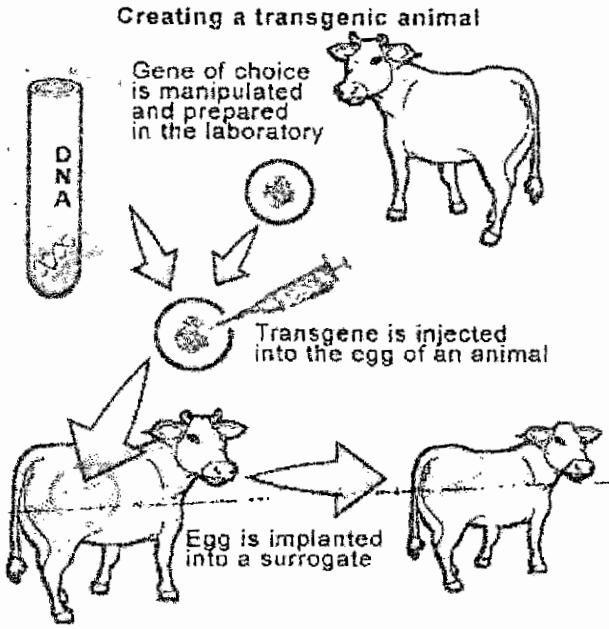


Figure13: Creating a Transgenic Animal. To create a transgenic animal, in brief, the gene of choice is manipulated and prepared in the laboratory. The transgene is then injected into the egg of an animal, which is implanted into the surrogate.

However, pronuclear injection is very unreliable. This is due to DNA uptake and integration into the genome of the egg cell being a very rare event, typically resulting in 1 to 5 transgenic animals out of every 100 being born. Dolly the sheep, the first animal cloned by pronuclear injection, was the result of one success out of 277 eggs[82]. Another Biotech company, PPL Therapeutics, took several years just to produce a flock of 600 transgenic sheep.

It was obvious that if pronuclear injection were the only animal cloning technique available, pharming would not be a feasible option. This problem has been solved by a new technique called 'nuclear transfer', which solves the low percentage of transgenic offspring by selecting for eggs that have cloned genes integrated into the egg's genome before it is implanted into the surrogate[83] (see Figure14) Essentially, cells are transfected as before, with the addition of a gene that makes the egg resistant to an antibiotic if it is expressed properly. This allows for the selection of only those cells that properly express the antibiotic resistance gene, and in conjunction, the gene of interest. The nucleus of these cells are then removed and transferred to an egg that has had its nucleus removed, followed by treating and culturing the egg to allow fusion with the nucleus. Since all the eggs contain the transgene, virtually 100% of the offspring will be transgenic animals.

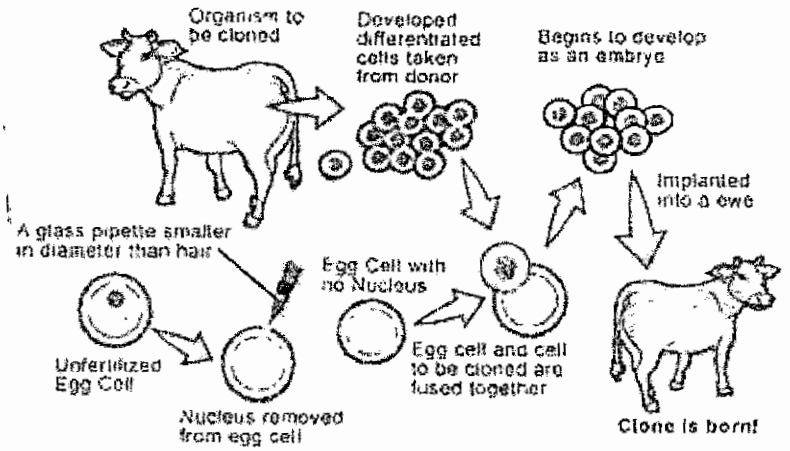


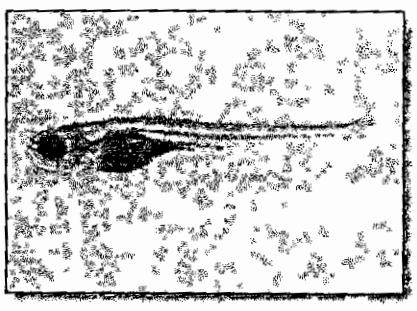
Figure14: Nuclear Transfer Technology. One method of cloning animals, nuclear transfer involves removing the nucleus from a donor animal and planting it into an egg cell that has had its nucleus previously removed.

2.5 Sperm-mediated gene transfer

BETHESDA, Md., Jan. 26, 2004 - A Japanese-U.S. team today reported the successful creation of transgenic animals using sperm genetically modified and grown in a laboratory dish, an achievement with implications for a wide range of research from developmental biology to gene therapy.

In their study, to be published in the online edition of the Proceedings 'of the National Academy of Sciences during the week of Jan. 26-30, researchers from Fukui Prefectural University in Obama, Japan, and the National Human Genome Research Institute (NHGRI), which is part of the National Institutes of Health (NIH), describe the innovative techniques they used to produce genetically modified zebrafish using sperm cells grown under laboratory, or "in vitro," conditions.

An investigator in NHGRI's Genome Technology Branch and a co-author of the study said, "To our knowledge, this is the first time that sperm cells have been cultured entirely in vitro and used to produce a transgenic animal. It was a unique challenge that required creative solutions"[47].



A 21-day old zebrafish

Fig15: A 21 day old Zebra fish

Although further refinement and testing is needed, he said the new techniques have the potential to speed the production of many different types of transgenic animal models that will shed new light on human development and disease. The sperm culturing system will allow researchers to further explore the basic biology of sperm production in vertebrates. The findings also may prove helpful to researchers exploring pre-fertilization strategies for human gene therapy, thus allowing preventive treatment for certain genetic disorders.

NHGRI's scientific director and director of the Division of Intramural Research, said, "This is an outstanding example of our efforts to build upon the foundation laid by the Human Genome Project. Development of such novel technologies and methods will be essential for translating our rapidly growing knowledge of the genetic basis of disease into better diagnostic approaches and therapeutic options."

Past efforts to genetically modify sperm cells in animals prior to fertilization have been stymied by the failure of these cells to mature under in vitro growth conditions. Drawing upon the cell culture expertise of Fukui Prefectural University, the Japanese US. team developed a system that enables immature sperm cells, or spermatagonia, taken from male zebrafish to survive long enough in vitro that they can receive foreign genes inserted by a retrovirus. Those cells go on to develop into mature, functional sperm. The genetically modified, cultured sperm are then used to fertilize zebrafish eggs in a laboratory dish - a process known as in vitro fertilization - resulting in the production of transgenic embryos and, ultimately, transgenic zebrafish. One of the study's senior author and a reproductive biologist said, "The secret to our success was the idea of placing a layer of special 'feeder cells' under the spermatagonia in the laboratory dish. These feeder cells, derived from zebrafish testicular cancer cell lines, promote the growth of spermatagonia and stimulate them to mature into functional sperm".

One of the biggest advantages of the cultured sperm approach is that transgenic zebrafish created in this way carry the inserted, foreign gene in every cell of their bodies, including their germ cells. This means the fish will transmit the foreign gene along to their offspring in a pattern identical to their natural genes.

"Conventional techniques of producing transgenic animals, such as microinjection of genes into eggs and the retroviral transduction of genes into embryos, often produce many animals that are mosaic, which means they do not contain the foreign gene in all their cells. In the case of zebrafish, less than 20 percent of fish produced by injecting DNA into fertilized eggs will transmit the foreign gene to the next generation. So, we have to sort through hundreds of fish and breed them for two generations to produce a stable, transgenic line," said co-author of this study. "With the new approach, the transgenic fish are not mosaic, so screening for transgenics is reduced and an entire generation of breeding is skipped. That saves significant time and money."

The zebrafish, Danio rerio, is a small, transparent aquarium fish used as a model system for studying the biology of vertebrates. The fish, which share many of the same genes as humans, are ideal for genetic studies because of their rapid rate of reproduction and because their genes can be readily

mutated. However, researchers have been frustrated by the lack of methods to target mutations to specific genes in the zebrafish.

In their current system, the researchers can grow zebrafish sperm cells in culture for about 12 days. The team is now working to extend the period that sperm cells can be cultured, so there is more time to select cells that have the foreign gene inserted in specific areas of the genome in what is commonly called a "targeted gene knockout". Currently, such gene targeting is not possible in zebrafish or any vertebrate animal model system other than mice, for which such targeting involves the use of embryonic stem cells[48].

CHAPTER THREE

PRODUCTION OF VARIOUS TRANSGENIC ANIMALS

CHAPTER THREE

3. Production of Various Transgenic Animals:

For the generation of transgenic animals the transgene DNA is randomly integrated as several tandem copies at one site into the genome and is transmitted to the offspring of the founder animal derived from the injected embryo, thereby creating a stable transgenic line,

3.1 Transgenic Mice or Rat

A transgenic mouse is simply an organism that has had DNA introduced into one or more of its cells artificially. This is commonly done in one of two ways. DNA can be integrated in a random fashion by injecting it into the pronucleus of a fertilized ovum. In thegrate in a head-to-tail fashion. Targeted insertion, the other common method of producing transgenic animals, is accomplished by introducing the DNA into embryonic stem (ES) cells and selecting for cells in which the DNA has undergone homologous recomis case, the DNA can integrate anywhere in the genome, and multiple copies often intbination with matching genomic sequences. One of the most common uses of targeted insertion of DNA is to make "knockout mice". It is important to note that production of transgenic mice by pronuclear injection can also occasionally result in a mosaic animal. This happens when integration of the transgene is delayed until after the first cell division

Production of Transgenic Mice or Rat:

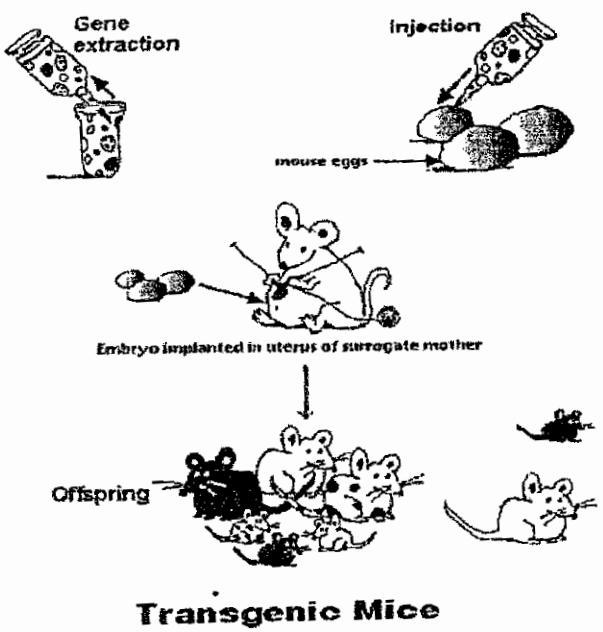


Fig 16: Production of transgenic mice.

3.1.1 Introduction.

The technique to generate transgenic animal models by the integration of foreign DNA sequences into the genome of mammals was established more than 20 yr ago. Most of the experiments since

then have been-performed in the mouse; however, the technology has been extended to other species such as the rat. Published values range from 3% to 5% transgene-positive offspring (founder) per injected zygote for mice and from 0.2% to 2% for rats[35].This technology includes the following steps:

- Design and generation of the transgene construct (Figure 17),
- Superovulation of donor animals, isolation of fertilized oocytes, microinjection of a small volume of solution containing the transgene construct into one pronucleus of the zygote (Figure 18),
- Transfer of injected embryos into a foster mother (Figure 19), and
- Identification of transgenic animals in the offspring (Figure 20).

Fig 17: Typical transgene DNA construct.

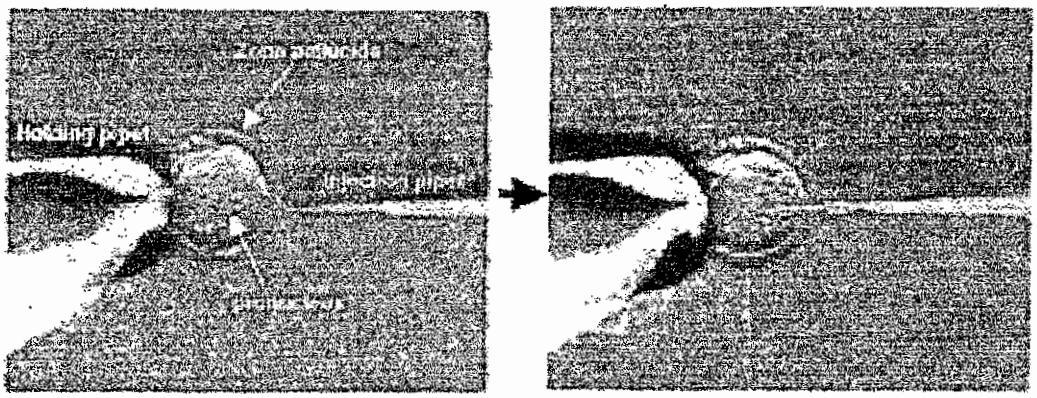


Fig 18: Microinjected of DNA construct into one pronucleus of a zygote.

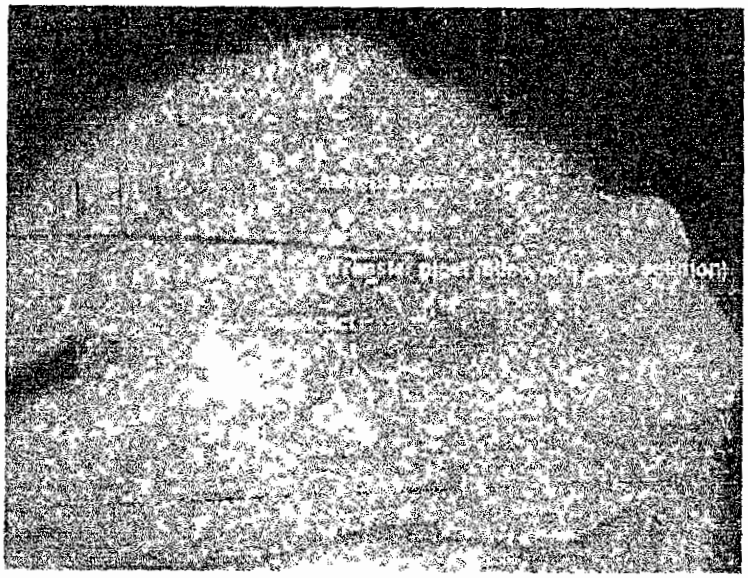


Fig 19: Transfer of microinjected zygotes into the oviduct of a foster mother.

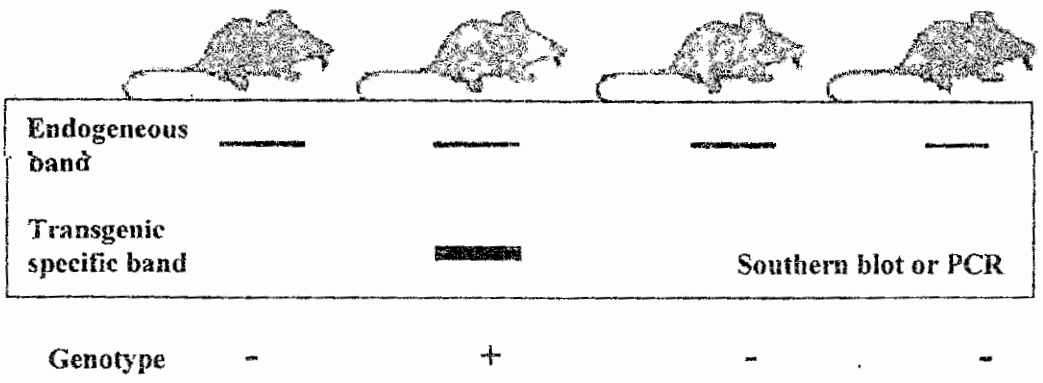


Fig 20: Identification of transgene positive founder animals in the offspring by detecting the integration of the transgene into the genomic DNA with the use of a trangene specific PCR or Southern blot.

3.1.2 Selection of Strain

A number of factors will play a role in the selection of an appropriate strain of females who will contribute the eggs to be used as experimental material. First, in all cases, it is important that the eggs are hardy enough to resist damage from the manipulations that they will undergo. Second, the particular experimental protocol may impose a need for eggs that have special genetically determined qualities. Third, in those cases where very large numbers of eggs are required, it will be important that the strain is one that responds well to super ovulation

3.1.3 Killing Mice and Rats

The recommended way of killing mice and young rats is cervical dislocation. This is quick and causes a minimum of distress for the animal. Other suitable methods, especially for older rats, include decapitation and ether overdosing.

3.1.4 Superovulation of Donor Animals (Mice and Rats)

Although one can recover on the order of 6 to 10 eggs directly from individual naturally mated inbred or F1 females, it is possible to obtain much larger numbers up to 60 eggs per animal by inducing a state of superovulation. For many experiments, it is important to begin with a large number of embryos; with superovulation, one can drastically reduce the number of females required to produce this large number. Superovulation is induced by administering two precisely-timed intraperitoneal injections of commercially-available gonadotropin reagents which mimic natural mouse hormones and initiate the maturation of an aberrantly large number of egg follicles. Superovulation, like normal ovulation, causes both a stimulation of male interest in mating as well as female receptivity to interested males.

Natural mating between mature animals can be used to supply the eggs for microinjection, but the yield is quite small, i.e., 10-15 ova per female depending on the strain used. Therefore, superovulation is conducted in immature animals in order to provide a large number of ova for the microinjection. Generally, superovulation is induced by the injection of gonadotropic hormones such as pregnant mare's serum gonadotrophin (PMSG) or follicle-stimulating hormone (FSH) followed by the injection of human chorionic gonadotropin (hCG). It has been reported that the efficiency of superovulation is dependent on the dose of the hormones, the route of administration, and the age and weight of the animals [84]. The most common method of superovulation in mice is intraperitoneal (ip) injection of five IIJ PMSG followed by ip injection of five IU hCG with 48-50 h of interval between the hormones.

3.2 Gene transfer Programme in Pigs

Gene transfer is the transfer of in vitro recombined gene constructs into animals. When a gene construct is integrated into the genome of the animal it is described as a transgene. The coded protein produced by this transgene is the transgenic product. Animals that contain transgenes are transgenic and, if the transgene is passed on to the offspring, transgenic lines or populations will be created. The target of the technology is the trait that is to be influenced by the transgenic product.

1. Scheme of a gene transfer programme in pigs

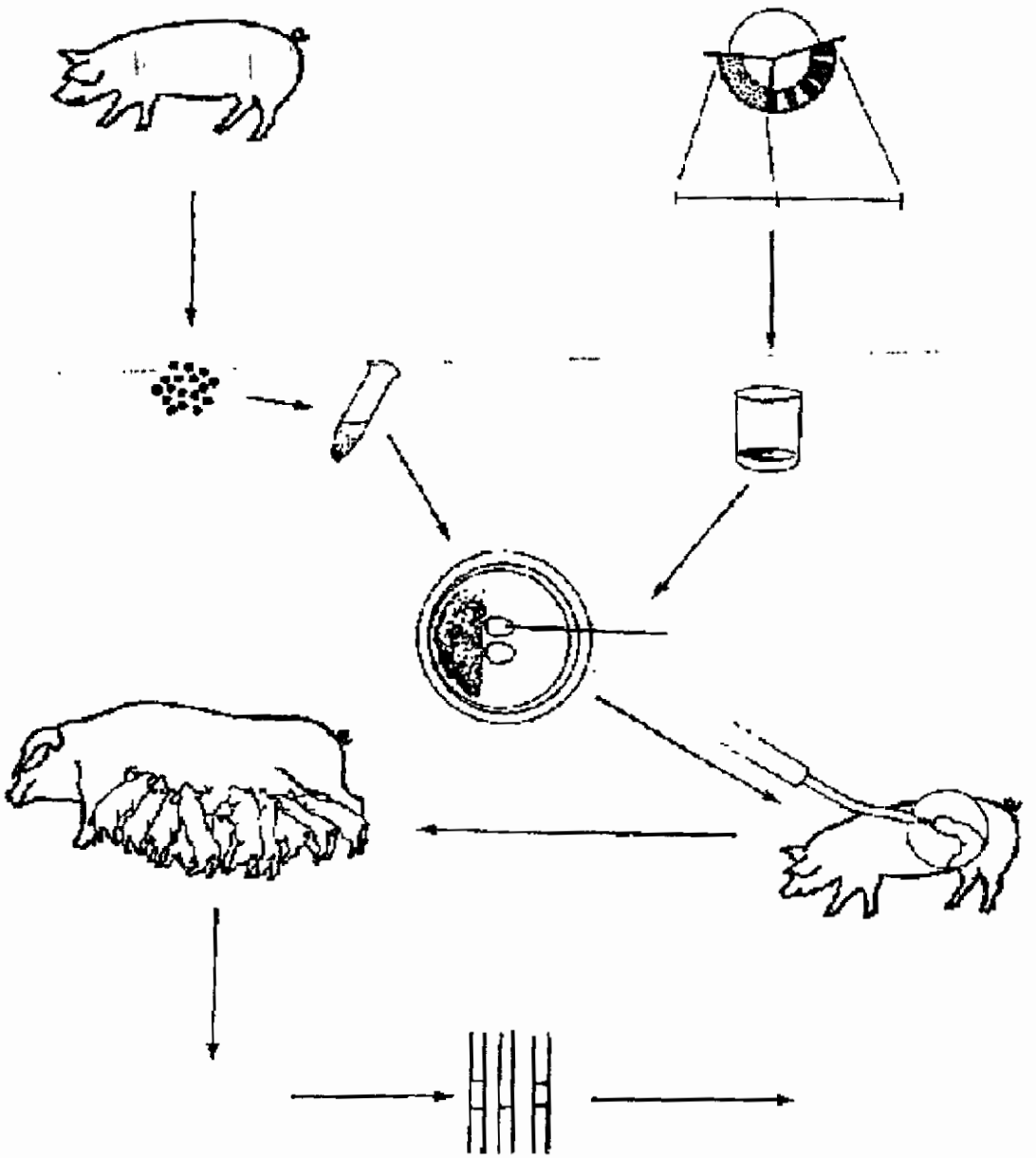


Fig21: Gene transfer programme in pigs.

2. Equipment for DNA microinjection

For gene transfer in mammals, three different techniques are possible:

· microinjection of DNA into the pronuclei of zygotes;

· DNA transfer using retroviral vectors;

· production of transgenic chimeras by injecting genetically transformed totipotent stem cells into embryos.

Gene transfer through direct DNA microinjection

Until now the microinjection of DNA into the pronuclei of zygotes is the only method used for gene transfer in domestic animals. The procedure consists of the following phases:

· cloning and recombination of a suitable gene construct;

· preparation of donors;

· recovery of zygotes (fertilized oocytes);

· visualization of pronuclei (necessary in some domestic animal species);

· preparation of the DNA solution to be injected;

· microinjection of DNA solution into the pronuclei of the zygotes;

· transfer of injected zygotes into the oviducts of synchronized recipients;

· investigation of newly born animals to ascertain whether they have integrated the gene construct (Dot - Southern - Blot).

Figure 21 shows the procedure of a gene transfer programme with a pig as an example. The donor animals are superovulated by means of gonadotropin injections according to the programme established for each species and mated or inseminated twice. The recovery of the zygotes is performed 12-24 hours after fertilization through surgical flushing of the oviducts. Persisting cumulus cells are removed using hyaluronidase treatment. In almost all domestic species no nucleus structures are visible because of dark lipid-containing granule. Centrifuging is a useful method of making pronuclei visible.

Gene constructions needed for gene injection are recombined in plasmids or cosmids and then cloned. After splitting the recombined vectors with restriction endonuclease the gene construct is extracted, precipitated, washed and placed in an injection buffer. In order to avoid problems during injection, all solutions utilized during the preparation of the injection fluid must be sterile-filtered. The gene solution must be free of contamination and particles. The DNA solution is diluted so that one picolitre contains about 1 000 copies of the gene construct.

The equipment needed for microinjection includes an invert microscope, two micromanipulators and injection equipment. Additional necessary items are an injection chamber and holding and injection pipettes. The injection pipette, with an outer diameter of 1-2 m m, is filled with DNA solution. During injection, the zygote is held with the holding pipette. The injection pipette is introduced into the pronucleus, passing through the zone pellucida, the cell membrane and the nucleus membrane. About one to two picolitres of the DNA solution are injected into the pronucleus, increasing its volume.

These injected zygotes are transferred, after brief in vitro culture, into the oviducts of synchronized recipient animals. Recipient animals are synchronized with the donors by means of hormonal treatment. After the birth of offspring from gene injection, high molecular DNA is isolated from tissue (blood or cells can be conveniently taken from the tail), to confirm successful integration. The integration of the injected DNA and the number of integrated copies can be determined after further processing with Southern Blot or Dot Blot hybridization. Integration sites in the chromosomes can be proved through hybridization of metaphase chromosomes, using the injected gene as a probe. Transgenic animals are raised and mated. Offspring from these matings are tested to discover whether the transgene has been passed on. In mating hemizygous-transgenic F_1 inter se attempts are made to produce homozygous transgenic animals.

3.3 Transgenic Cattle Production:

The generation of transgenic cattle is presently time-consuming and expensive, because of the inefficiency of the classical DNA-microinjection technique. Here, the process (use of lentiviruses) is used for the efficient generation of transgenic cattle. Initial attempts to generate transgenic cattle by lentiviral infection of preimplantation embryos were not successful. In contrast, infection of bovine oocytes with lentiviral vectors carrying an eGFP expression cassette followed by in vitro fertilization resulted in the birth of transgenic calves. Furthermore, all of the calves generated by infection of oocytes were transgenic and 100% of these animals expressed eGFP as detected by in vivo imaging and Western blotting. In addition, a transgenic calf was produced by infection of fetal fibroblasts followed by nuclear transfer into enucleated oocytes. Taken together, after adjusting lentiviral transgenesis to cattle, unprecedented high transgenesis and expression rates were achieved.

One of the obstacles in the production and rearing of transgenic cattle is the low efficiency of the introduction of new genes in the genome. Now, scientists working at the University of Wisconsin and at Gala Design, a private biotechnology company, have developed a remarkably more efficient method. They claim that by using their method, transgenic livestock can be produced on a routine basis. The research is published in the latest issue of the Proceedings of the National Academy of

Sciences. The PNAS article describes a new method of gene introduction, the transgametic method. It inserts a gene into the unfertilized oocyte or egg, which stably incorporates the gene into the maternal genniine. Once the egg is fertilized, all cells of the resulting embryo carry the new gene, and the calf is born with the capability to secrete a new protein in milk. Subsequent generations, offspring of each founder animal, will also carry the desired gene. Older, less efficient production methods made transgenic livestock very costly. Cloning and pronuclear microinjection typically lead to only 1 percent of animals born carrying the new gene. When DNA is microinjected into a fertilized embryo, the DNA is moreover often not taken up until cell division has occurred. As a result, only some cell lineages carry the new gene. If the germ or sex cells don't carry the new gene, then the gene isn't reliably transferred to offspring. The efficiency of the new technology could even make cloning and pronuclear microinjection economically obsolete for many biopharmaceutical purposes. It may also cover the way to applications of biotechnology in agricultural livestock, similar to those that have changed crop agriculture in recent years. The technology may also have applications in other mammals.

3.3.1 Cell Lines are Isolated and Cultured

High quality Friesian dairy cows are superovulated and artificially inseminated with proven semen. The resulting embryos are recovered and transferred to surrogate mothers for up to 90 days gestation. The embryos are then killed and fibroblast cells are isolated. Primary cell lines are continuously reproduced to test the lifespan of the cells and the stability of the genetic material. Similarly, cell lines are derived from adult cattle by collecting skin or follicle samples from the live animals. The sex of the cell lines is determined before genetic modification so 75% of the animals are female. The remaining 25 % must be male for breeding purposes.

3.3.2 DNA Is Isolated To Make a Library

DNA is extracted from the cell lines & used to prepare a genetic sequence library, a list of all the genes in the cattle's DNA. Existing cattle DNA clones are compared to this library to identify and isolate genetic sequences that will provide suitable fragments for further cloning.

3.3.3 Genes Are Modified (GM)

One of the three genetic modifications is introduced into the DNA of cultured cell lines using E. coil bacteria as a vector. Antibiotic resistance is integrated into the cell line so cells with the altered gene can be separated from those without the altered gene. If cells are exposed to the antibiotic neomycin those without the altered gene will die; the remaining live cells must contain the altered gene.

3.3.4 Genetically Modified (GM) Embryos Are Implanted

Cells identified as transgenic are injected into enucleated cattle eggs and the two components fused using nuclear transfer (a similar method was used to create "Dolly the Sheep"). The embryos are cultured for 7 days in vitro and then transferred to surrogate cows housed within the containment facility. Pregnancy is monitored regularly by ultrasound (on days 28, 35 and 50). The surrogate cows clearly identified as not pregnant are reused for another round of embryo transfer or, after 3 failures,

removed from containment and sold. In the last three months of pregnancy the cows have regular blood analyses to monitor their health. The calves are delivered by caesarian section within one week of full term.

3.3.5 Lactation Is Induced

Transgenic calves are induced into lactation (using the hormones oestrogen & progesterone) between 6 and 9 months of age (compared with 2 years normally). These cows are hand-milked, recovering 20 - 50 mL of milk over each of several days. Electrophoresis determines the levels of transgenic protein in their milk. Lines that express high levels of the protein are bred to produce small herds (up to 30 animals) to evaluate the expression of the gene in their milk. Eggs from these cows will be extracted, matured in vitro, fertilized with bull semen and cultured. When the embryos have divided to the 32-cell stage they will be biopsied and a diagnostic PCR test will be used to identify embryos that are female and carry the transgene. These embryos will then be transferred into a surrogate mother.

3.4 Production of transgenic goat

Generally transgenic goats are produced by nuclear transfer of fetal somatic cells. Donor karyoplasts were obtained from a primary fetal somatic cell line derived from a 40-day transgenic female fetus produced by artificial insemination of a nontransgenic adult female with semen from a transgenic male. Live offspring were produced with two nuclear transfer procedures. In one protocol, oocytes at the arrested metaphase II stage were enucleated, electrofused with donor somatic cells, and simultaneously activated. In the second protocol, activated in vivo oocytes were enucleated at the telophase II stage, electrofused with donor somatic cells, and simultaneously activated a second time to induce genome reactivation. Three healthy identical female offspring were born. Genotypic analyses confirmed that all cloned offspring were derived from the donor cell line. Analysis of the milk of one of the transgenic cloned animals showed high-level production of human antithrombin III, similar to the parental transgenic line.A transgenic goat carries about 55,000 goat genes and one human gene. They can produce milk at 12 months instead of 18. They are not allowed contact with other animals to prevent the passing of any diseases. One of the toughest tasks in establishing the farm, which was pulled together in four months, was finding a location where no other animals had been kept.

CHAPTER FOUR

APPLICATIONS OF TRANSGENIC ANIMAL PRODUCTION

CHAPTER FOUR

Applications of transgenic animal production:

4.1 Therapeutic Application

4.1.1 Targeted production of pharmaceutical proteins.

Another use for transgenic animals involves the biological production of valuable human protein enzymes, hormones and growth factors. These products may be recombinant or mutated, and collection of the functional protein from the animal employs tissue-specific regulatory DNA sequences, a strategy described below. Current techniques in the biotechnology industry use large-scale cell cultures to generate products in biological systems. Eukaryotic cell s or bacteria which have taken up genetic expression sequences (or constructs) are cultured in nutrient medium which is continually replaced and from which the bioengineered product is refined. This medium must be correctly buffered and must be temperature-regulated and maintained pathogen-free. The use of transgenic animals, particularly larger mammals, as bioreactors ("pharmaceutical pharming") is a cost-effective alternative to cell culture methods. Animals automatically supplement their bodily fluids with fresh nutrients, remove waste products, reliably regulate their internal temperature and pH and resist pathogens. By directing (or targeting) the expression of the transgene product so that it is produced by the secretory cells of the liver, lactating mammary gland or kidney, "pharmers" may collect and process bodily fluids with minimal effort. The mammary gland probably is the most promising target tissue because it produces large amounts of protein in a temperature-regulated fluid that may be collected daily in a non-invasive fashion. Transgenic animals are not only costeffective bioreactors but, with the complex secretory cell types and organs of the mammalian organism, can perform much more complicated protein modifications than simply cultured cells.

A new platform has been created by the monumental research work of Dr. Wilmut that has led to the production of drugs through the milk of livestock. Nuclear transplantation in an enucleated ovum has led to the process of cloning. By a blend of cloning and recombinant DNA technology (genetic engineering) it is possible to make a herd of animals that produce the drug of choice especially human therapeutic proteins in the milk. Wilmut group with the funding from PPL Therapeutics UK has successfully produced Alpha- l-Antitrypsin (used in emphysema) in the milk of sheep. As a model peptide Calcitonin from Salmon has been produced. The method has expressed peptides as inactive fusion protein joined by a cleavable linker in the peptide terminal. Amidation can occur in VIVO by extending the peptide by a single Glycine residue which acts as a substrate for the endogenous amidating enzyme in the mammary gland.

This successful work has led to the concept of biofacotories dispensing the cumbersome highly expensive fermentation technology. The production is the same regardless of the length of the peptide whether it is Insulin or Erythropoietin or Clottong factors or immunoglobins.

Expression in the milk of cows or sheep not only produced high yields up to 15 grams per litre of milk but also exploits the specific production abilities of the organ enabling purification, fidelity of sequence and the most important post-transnational medifications. This occurs without any additional cost of enzymatic processing and solvent disposal. The cost to establish such a unit is much cheaper than a fermentation facility. Moreover the purification is easy from the whey fraction of the milk. Since the gene expression operates in a temporal and temporal system that is responsible for normal development when cloned genes are constructed with using promoter fragment of bovine lactog; obulin. This construction expresses the desired genes in the milk during lactation[79].The global market for human proteins alone has been estimated to be worth US $ 24 billion in 1999 and the market for solid organs cloned from pigs has been estimated to be US $ 5 billion. In view of the advantages from its current applications as well as tremendous potential of emerging uses, the use of transgenic animal's models is likely to rise considerably.

Table 1: Proteins with therapeutic and industrial value that have been produced (but not commercialized) in the milk of transgenic animals:

Protein	Animal	Use
Antithrombin III	Goat	Reduce the amount of blood needed in some surgeries
Factor VIII, Factor IX	Goat, Pig, Sheep	Treatment of hemophilia
CFTR	Sheep	Treatment of cystic fibrosis
Lactoferrin	Cow	Natural antibiotic and used in coronary surgery
Alpha-I antitrypsin	Sheep	Treatment of cystic fibrosis and emphysema
Lysostaphin	Cow	An anti-bacterial compound that prevents mastitis in cows
Spider silk protein	Goat	Production of ultra-strong, lightweight medical and industrial materials

4.1.2 Pharming:

"Pharming" is a term used to describe the production of human pharmaceuticals in animals. As an example, transgenic mice have been used to produce a human drug, tPA (tissue plasminogen activator to treat blood clots) since 1987. This type of approach for producing pharmaceuticals is particularly useful when the product is modified (glycosylated, etc.). and correct modification is not

feasible in an in vitro system. A popular method of "pharming" is to couple the DNA gene for the protein drug with a DNA signal directing production in the mammary gland. The foreign protein is expressed and secreted into the milk (of a goat or cow, for example) and doesn't interfere with the health of the animals. A goat expressing tPA in its milk is worth an estimated $75,000 per year. A pig expressing human protein C (another clotbusting drug) is worth about $1,000,000 per year. Pig-napping may become a more common crime, as the pharming develops.

Animals are genetically engineered to produce drugs or other pharmaceutically active compounds e.g. vaccines, in their milk. This is called pharming, a new word in the biotechnological industry that is a play on the word 'farming'. The 'pharmer' milks the medicines and vaccines when he milks his herd of transgenic animals. The animals are seen as living factories that offer a low-cost alternative for the large scale production of drugs and proteins. The first clinical trial of drugs produced in the milk of transgenic goats is underway in the UK. PPL Therapeutics, a biotechnology company based in Scotland, has produced a flock of transgenic sheep that produce significant amounts of alpha- 1-antitrypsin (AAT) (used in the treatment of cystic fibrosis), in their milk. AAT will be isolated from the milk and administered directly to the lungs via an aerosol.

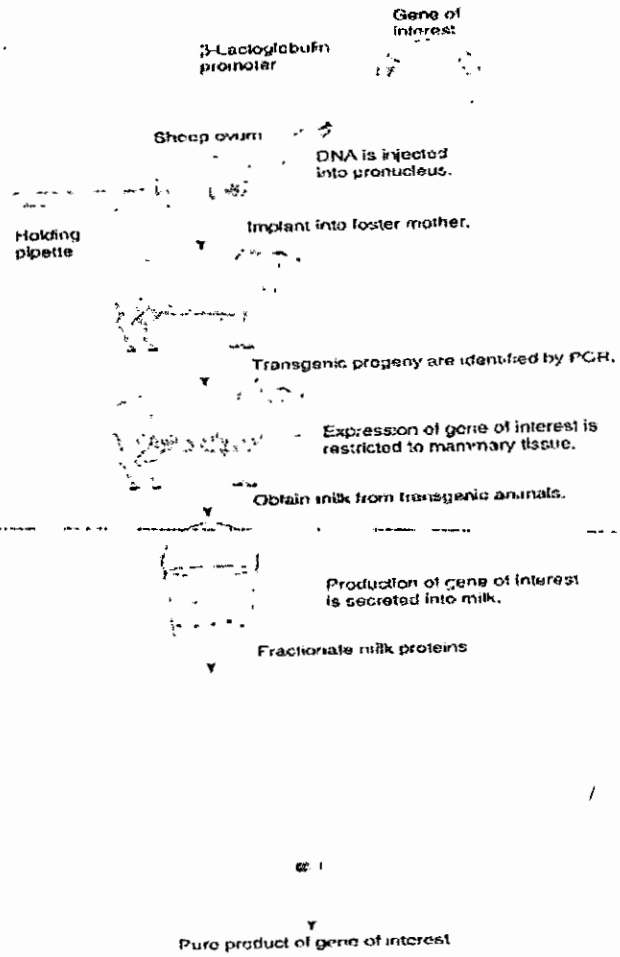


Fig 22: Protein Production of a pharmaceutically important protein in the milk of transgenic sheep.

The gene of interest encodes a protein that is of therapeutic importance, such as tissue plasminogen activator used to dissolve blood clots in humans. The gene is placed under control or the beta-lactoglobulin promotor, active only in the mammarty tissue, and is injected in sheep ova by microinjection of the expression vector in the nucleus. The injected ova are implanted into foster mothers, and progeny expressing the transgene are identified by PCR amplification from chromosomal DNA using primers specific Ibrom the sequence of the gene of interest. Transgenic sheep express this gene only in mammary tissue and secrete high levels of the gene's protein in the milk, from which it can be purified.

However, some people believe that such gene therapy will not cure cystic fibrosis (CF). CF is a disease that affects the pancreas, intestines, liver and reproductive system as well as the lungs. Even if the inhaled gene was incorporated into the patient's genetic make-up it could not reverse the damage already caused [85].

Another genetically modified animal is Babe, a rabbit that can produce a fish protein in her milk. The fish protein is similar to the human protein, calcitonin, that could be used to fight against osteoporosis. However, it is known that a diet that is rich in calcium and protein can help in cases of

osteoporosis. Huomen, a transgenic cow was designed to produce erythropoietin (stimulates the production of red blood cells by the bone marrow) in her milk. However after findings that the transgene caused unacceptable health problems in mice, research with

Huomen was terminated (Successful Farming, 1991; Transgenic Animals in the Production of Therapeutic Proteins, 1992; Transgenic Pharming Advances, 1992).

The plight of transgenic animals clearly underlines the need to regulate the use of transgenic technology in order to prevent the unacceptable suffering that many of these animals experience. Many eminent workers are urgently calling for an international committee to be set up to look into the welfare issues surrounding transgenic animal[7].

4.1.3 Improved human health:

As much demand as there is for the advancement of transgenic livestock in the agricultural industry, demand for recombinant proteins and vaccines is also on the rise, though many are still on clinical trials. These proteins come in forms as serum, sweat, urine or milk from livestock such as rabbit, sheep and cattle.

The potential usages of these therapeutic proteins produced via transgenic livestock as bioreactors are proven to be able to replace that of the conventional method such as mammalian cell cultures, because of their more genuine properties and economic reasons (Walsh, 2002). Various issues surrounding our main objective, the therapeutic milk protein-hFIX (human factor IX), inflict on further issues in human health concerns and animal welfare. Hemophilia B, a genetic defect that is present in individuals who have a deficiency of blood coagulation hFIX that leads to hemorrhage and other blood-borne complexities[86]. Recoveries from transgenic milk proteins obtained from livestock may provide Hemophilia patients a greater quantity and safety. Furthermore, with the advent of methods generating transgenic livestock such as nuclear transfer and novel research into the molecular level such as gene regulation, they will facilitate the process of regulating and marketing the efficient, safe and economical therapeutic proteins.

The use of livestock as bioreactors for therapeutic proteins can improve human health in three main ways:

1. By achieving genuine protein production with the correct pattern of post-translational modification: Recombinant proteins produced from transgenic livestock ensure the necessary modification after synthesis which will reduce antigenicity. Many complex proteins have been expressed with high efficiency and full bioactivity. Although cell culture may be an alternative method, it is a complex and expensive procedure that will result in relatively high operating costs. Since the post-translational modification is carried out naturally in the transgenic livestock without additional facilities such as fermentation, overall capital investment is much lower.
2. By using milk in as a vehicle to deliver the desired recombinant proteins in enough quantity: Although in principle proteins can be produced in various tissues of the body, by far the most

progress in the development of transgenic bioreactors has been made in targeting transgene expression to the epithelia] cells of the mammary gland, thus the production of recombinant proteins in milk. There are several good reasons for recovering recombinant proteins from milk. Transgenic livestock could tolerate only limited concentrations of blood products in their bloodstreams, whereas milk is effectively outside of the livestock[33]. Also, the harvesting of milk can be easily, regularly, and sterilely obtained from the livestock without causing too much stress on the livestock. In addition, large quantity of proteins can be expected from milk with high expression. Thirdly, milk is perceived by consumers to be benign.

3. By creating pure proteins without the potential infectious risks associated human blood: Hemophilia B is currently mainly treated with hFIX derived from human plasma or cell cultures. For many years proteins have been isolated from human sources such as blood and pituitary glands (growth hormone). Recipients of human products have contracted hepatitis, AIDS, and CJD (CreutzfeldJakob disease). Therefore, it is not certain that there will not be a new infective agent. On the other hand, animals may also present a different media for zoonotic disease (disease caused by an animal infectious agent crossing the species barrier and infecting humans). Stringent removal steps in downstream processing, thereby promising high degree of purity before distribution.

4.2 Others applications of transgenic animals to human welfare:

The benefits of these animals to human welfare can be grouped into areas:

1 Agriculture
2 Medicine
3 Industry

The examples below are not intended to be complete but only to provide a sampling of the benefits.

2.1.1 **Agricultural Applications**

a) Breeding:

Farmers have always used selective breeding to produce animals that exhibit desired traits (e.g., increased milk production, high growth rate) [65]. Traditional breeding is a time-consuming, difficult task. When technology using molecular biology was developed, it became possible to develop traits in animals in a shorter time and with more precision. In addition, it offers the farmer an easy way to increase yields.

b) Quality:

Transgenic cows exist that produce more milk or milk with less lactose or cholesterol[66], pigs and cattle that have more meat on them [67], and sheep that grow more wool [68]. In the past, farmers used

growth hormones to spur the development of animals but this technique was problematic, especially since residue of the hormones remained in the animal product.

c) Disease resistance:

Scientists are attempting to produce disease-resistant animals, such as influenzaresistant pigs, but a very limited number of genes are currently known to be responsible for resistance to diseases in farm animals [69]

4.2.2 Medical Applications

a) Xenotransplantation:

Patients die every year for lack of a replacement heart, liver, or kidney. For example, about 5,000 organs are needed each year in the United Kingdom alone, Transgenic pigs may provide the transplant organs needed to alleviate the shortfall [70]. Currently, xenotransplantation is hampered by a pig protein that can cause donor rejection but research is underway to remove the pig protein and replace it with a human protein [71]

b) Nutritional supplements and pharmaceuticals:

Products such as insulin, growth hormone, and blood anti-clotting factors may soon be or have already been obtained from the milk of transgenic cows, sheep, or goats [72]. Research is also underway to manufacture milk through transgenesis for treatment of debilitating diseases such as phenylketonuria (PKU), hereditary emphysema, and cystic fibrosis [72].

In 1997, the first transgenic cow, Rosie, produced human protein-enriched milk at 2.4 grams per liter. This transgenic milk is a more nutritionally balanced product than natural bovine milk and could be given to babies or the elderly with special nutritional or digestive needs. 4, 21, 23 Rosie's milk contains the human gene alpha-lactalbumin.

c) Human gene therapy:

Human gene therapy involves adding a normal copy of a gene (transgene) to the genome of a person carrying defective copies of the gene. The potential for treatments for the 5,000 named genetic diseases is huge and transgenic animals could play a role. For example, the A. 1. Virtanen Institute in Finland produced a calf with a gene that makes the substance that promotes the growth of red cells in humans .

4.2.3 Industrial Applications

In 2001, two scientists at Nexia Biotechnologies in Canada spliced spider genes into the cells of lactating goats. The goats began to manufacture silk along with their milk and secrete tiny silk strands from their body by the bucketful. By extracting polymer strands from the milk and weaving them into thread, the scientists can create a light, tough, flexible material that could be used in such applications as military uniforms, medical microsutures, and tennis racket strings [73].

Toxicity-sensitive transgenic animals have been produced for chemical safety testing. Microorganisms have been engineered to produce a wide variety of proteins, which in turn can produce enzymes that can speed up industrial chemical reactions.

4.3 The Value of Transgenic Animals:

Transgenic animal systems combine the virtues of cell culture and congenic breeding strategies while avoiding the negative aspects of each system. Using transgenic techniques, a characterized genetic sequence may be evaluated within the specific genomic background of the whole animal. Transgenic animals are used: in the basic biological study of regulatory gene elements; in medical research, to identify the functions of specific factors in complex homeostatic systems through over- or under-expression, as models of human disease; in toxicology as responsive test animals; in biotechnology as producers of specific proteins; and in agriculture and aquaculture to improve yields of meat and other animal products. This list is not inclusive; the use of transgenic animals is likely to expand in the future.Transgenic animals have been created for use by both the pharmaceutical and the agricultural industry. Therefore, transgenic animals may be utilized to study the regulation of a specific genetic sequence in a realistic fashion. Many uses have been developed and many more are forecast, particularly in three areas:

4.3.1 Models of human disease processes.

Hundreds of transgenic rodent lines have been produced by introducing into the genome genetic sequences such as viral transactivating genes and activated oncogenes implicated in specific pathologies. The phenotype and regulatory parameters of the gene then may be evaluated in an animal model with a relatively short generation time. Also, normal rodent genetics and physiology are highly characterized. The predictability of many transgenic phenotypes permits the innovative testing of diagnostic and therapeutic agents while using a reduced population of experimental animals. The generation of novel cell lines from transgenic organs also promises to reduce the number of research animals required to evaluate a therapeutic compound. In addition, transgenic genomes may be created in which more than one transgene may interact, or in which a transgene may interact with an endogenous normal or mutated gene. The use of transgenic disease models in biomedical research promises to accelerate dramatically the development of new human diagnostic and therapeutic treatments. transgenic rodent models have been characterized for several human diseases including cardio-vascular disease, cancer, autoimmune disease, AIDS, sickle cell anemia and neurological disease[19].

4.3.2 Modification of animal anatomy and physiology.

The most controversial aspect of transgenic animal usage involves the "selective improvement" of species by the modification of the genome. Most often, foreign genes are added to the host genome, but selective deletion of specific genes or regions has been attempted. It has become apparent that merely adding genes for growth factors or hormones to the genome is a simplistic approach to

altering the complex multigenic physiology of the mammal. The goals of this type of experiment may include decreased body fat, increased speed, novel disease resistance or higher yields of meat or milk. At present, these types of phenotypic alterations are more realistically achieved in plants and bacteria than in animals.Transgenic animals present unique possibilities for medical, agricultural and fundamental research, and as a means of producing valuable pharmaceutical and nutrient products. Their use has increased dramatically in recent years and is set to increase further in the near future. It has been claimed that transgenic animals might allow reduction and refinement in animal use, via more precise gene targeting in breeding programs. However, these objectives are threatened by transgenic procedures which may promote greater animal use, more varied applications, and the greater likelihood of animal suffering.

4.4 Improved food supply:

Transgenic livestock can affect food supply in two ways, either by increasing the food supplied or by changing the type of food that is supplied. However the vast utility of transgenic livestock is masked by numerous costs, risks and ethical concerns. The project would not only exceed 10 years but will also require a substantial amount of funding. Potential risks associated with transgenic cattle include possible allerginicity and hypersensitivity of human physiology to transgenically introduced proteins. In addition, ethical concerns regarding this issue have resulted in vast public unacceptance of transgenic cattle. However, with the promise of food safety and greater benefits, public acceptance would be easy to gain. Thus continuous research in the area despite the present lack of benefits, we believe would result in economical and social benefits that would surpass the cost of development with much ease[61].

Transgenic cattle could be used to increase food supply in three major ways:

1. By increasing the rate of growth of cattle: Regulating cattle development and their growth rate comes with great economical benefits. For instance, cattle can be produced with desirable alterations to growth rates or feed conversion efficiency to produce leaner or thicker meat faster. Such modifications have been successfully done in fish such as salmon, where transfection with exogenous genes has resulted in upto 250% increase in their mass. However inadequate advances in technology have till now deterred such genomic manipulations in cattle. If developed, these transgenic cattle could be an alternative to the current use of non-bovine growth hormones that produce allergic reactions in a large number of people. This would induce an increase in the demand for milk, thereby affecting the supply of milk itself.

2. By creating resistance against diseases and environmental conditions in cattle: Transgenics could also provide a means of producing animals resistant to many diseases, which would be of direct benefit to those animals and in addition increase our food supply. The use of such transgenics would be greatly beneficial in disease prone developing countries. Resistance to diseases such as scrapie and trypanosomiasis could be of great advantage in African cattle. Although such gene modifications have been done in mice, the production of such transgenic cattle still seems far away. One of the

major current attempts at producing disease-resistant cattle includes the production of BSE (mad cow disease) - resistant cattle. A valiant attempt by Texas A&M College researchers utilizes homologous recombination to replace the PrP gene in cattle embryonic fibroblasts with a BSE -resistant form. Successful attempts would not only decrease death rates of cattle but also decrease occurrences of, Creutzfeldt-Jakob disease, the human isoform of cattle BSE.

3. By increasing or altering the production of cows milk: Milk has been the target of many transgenic experiments. Potential applications include the production of low lactose milk through replacement of genes responsible for lactose secretion into milk. Another is the alteration of casein in cows milk to increase cheese production[87]. Two specific caseins, [S, and F1, each occurring at a concentration of around 1 Og/1 in normal bovine milk, are important for cheese production. By transfecting cattle embryos with additional copies of casein transgenes, researchers hope to increase the amount of cheese that could be churned from milk.

In addition, the elevated expression of growth hormones in cows milk can result in faster development of calves that feed on the milk. Bateson et al discusses that the expression of bovine lactalbumin in pigs milk has increased piglets growth. Such manipulations in bovine milk would extend the demand for milk to lactose-intolerant human populations.

4.5 Organ transplants:

Transgenic pigs have been produced that carry a human gene. Scientists hope that these pigs will be the answer to the current, chronic shortage of organs available for transplantation. When the tissues and organs from the transgenic pig are transplanted into humans, they will be recognised as human and will trick the immune system into not attacking it. There is however serious concerns those transplanting pigs' organs into humans may allow an animal virus to pass on into the human population causing a fatal epidemic. AIDS is now thought to have come from monkeys and somehow crossed the species barrier.

4.6 Commercialization Issues:

Success in creating a transgenic animal that can produce the drug is far from guaranteed. About 10 to 30 percent of mouse embryos produce transgenics, but less than 5 percent of goats, sheep, or cows do. Production of the drug is measured during lactation after the animal is raised to maturity and bred. Because of the long time periods involved and low success rates, developing transgenic animals is currently very expensive, as the dollar amounts in Table 2 indicate.

Table 2: Production of drug from transgenic animals.

Drug	Animal	Value /anima/ yearl
AAT	sheep	$15,000
tPA	goat	75,000

Factor VIII	sheep	37,000
Factor IX	sheep	20,000
Hemoglobin	pig	3,000
Lactoferrin	cow	20,000
CFTR	sheep, mouse	75,000
Human Protein C	pig	1,000,000

'Current market price of the drug and supply produced by one animal.

Table 3: Drug descriptions

AAT	alpha- l-antitrypsin, inherited deficiency leads to emphysema
tPA	tissue plasminogen activator, treatment for blood clots
Factors VIII, IX	blood clotting factors, treatment for hemophilia
Hemoglobin	blood substitute for human transfusion
Lactoferrin	infant formula additive
CFTR	cystic fibrosis transmembrane conductance regulator, treatment of CF
Human Protein C	anticoagulant, treatment for blood clots

(Source: Whole Animals for Wholesale Protein Production, 1992.)

Although most protein drugs are made in milk, a notable exception is human hemoglobin that is being made in swine blood to provide a blood substitute for human transfusions. Because hemoglobin is naturally a blood protein, it is likely to be one of few exceptions to the usual method of production in milk. Furthermore, the economics of blood production are less favorable, because to recover human hemoglobin, the animal producing it must be slaughtered.

Drugs currently made by or being developed in transgenic animals are listed in Table 1. Notice that pharming is expected to increase the value of animals dramatically. In general, animal pharming is considered to be 5 to 10 times more economical on a continuing basis and 2 to 3 times cheaper in startup costs than cell culture production methods.

4.7 Contribution of Transgenic Livestock:

A. Improved Production Traits

a. Somatotropin/Growth Hormone

b. Stimulation of muscle development: c-SKI gene

c. Wool Production: Improve Cysteine Utilization

B. Improve Animal Health

a. Incorporate disease resistance genes

b. Preformed antibodies

c. Interferon

C. Transgenic Milk

Milk is used in transgenesis because milk is readily collected and available in large quantities. Protein composition (Caseins and Whey Proteins) is simple and specific. Regulatory sequences well characterized, Mammary gland can carry out post-translational modifications that microbial systems cannot. Some other uses are

1. Production of medically important products in milk.

a. Blood Factors

b. Peptide Hormones and Growth Factors

c. Enzymes

2. Manipulation of components already in milk

a. Simulate human breast milk (Natural Formula)

b. Remove Lactose (Lactose Intolerance)

c. Alter casein for cheese production

D. Human organ transplant

1. Incorporate human genes into pig organs to use as transplants

a. Pig organs similar in size to humans

b. Incorporated human genes would lower rejection.

CHAPTER FIVE

DISCUSSION

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Discussion:

The process to achieve controlled genetic modifications of multicellular eukaryotic species by the insertion of genetic material is known as transgenic technology. These terms transgenic animals now include the use of living organisms or their parts to make or modify products, to change the characteristics of plants or animals, or to develop micro- organisms for specific uses. Genetic modification of livestock may also be seen as a benefit to human health, in the economic and efficient production of important pharmaceutical proteins. Biotechnology, including transgenic livestock, will play a key role in meeting the challenge of feeding the world population, while creating and maintaining sustainable agricultural systems[3].

We can produce the transgenic animals by three principal methods namely DNA microinjection into the pronucleus, Embryonic stem cell-mediated gene transfer and Retrovirus-mediated gene transfer. Among the three techniques the use of embryonic stem cell-mediated gene transfers and Retrovirus-mediated gene transfer techniques are limited because of its constraints. The successful rate of DNA microinjection technique is greater than the techniques of embryonic stem cell-mediated gene transfer and Retrovirus-mediated gene transfer. The technique of Retrovirus-vector mediated gene transfer is not available because the transgene size is very small (about 8 kb only) and it requires a life helper phage which may harmful to the animal body. One the other hand for the randomness of integration the use of ES cell method is limited. Although expertise are essential for performing the DNA microinjection technique, several hundred male pronuclei can be inoculated one a good day.

Another recent development of techniques for the production of transgenic animal are used which has cent percent success rate. A technique called 'Pronuclear injection' is used to do this and involves injecting the DNA directly into fertilized eggs using a very fine glass needle called transfection techniques. It was obvious that if pronuclear injection were the only animal cloning technique available, pharming would not be a feasible option. This problem has been solved by a new technique called 'nuclear transfer', which solves the low percentage of transgenic offspring by selecting for eggs that have cloned genes integrated into the egg's genome before it is implanted into the surrogate[83]. Essentially, cells are transfected as before, with the addition of a gene that makes the egg resistant to an antibiotic if it is expressed properly. This allows for the selection of only those cells that properly express the antibiotic resistance gene, and in conjunction, the gene of interest. The nucleus of these cells are then removed and transferred to an egg that has had its nucleus removed, followed by treating and culturing the egg to allow fusion with the nucleus. Since all the eggs contain the transgene, virtually 100% of the offspring will be transgenic animals[75].

There are various types of animals produced by Transgenisis. Cattle are being produce in USA, Canada, England and many other countries in the world. Dog are being in Japan, South corea and North-corea.
We can use the transgenic animals as a therapeutic purpose. Protein production of a pharmaceutically important protein in the milk of transgenic sheep. The gene of interest encodes a protein that is of therapeutic importance, such as tissue plasminogen activator used to dissolve blood clots in humans. The

gene is placed under control or the betalactoglobulin promotor, active only in the mamary tissue, and is injected in sheep ova by microinjection of the expression vector in the nucleus. The injected ova are implanted into foster mothers, and progeny expressing the transgene are identified by PCR amplification from chromosomal DNA using primers specific from the sequence of the gene of interest. Transgenic sheep express this gene only in mammary tissue and secrete high levels of the gene's protein in the milk, from which it can be purified.

There are various types of drugs produce from transgenic animals such as Antithrombin-III from goat which uses are reduce the amount of blood needed in some surgeries. Alpha-I antitripsin from sheep uses as treatment of cystic fibrosis and emphysema. Some drugs are also produced from transgenic animals such as AAT from sheep, hemoglobin from pigs etc.

The benefits of these animals to human welfare can be grouped into areas: Agriculture (breeding, increase in quality and quantity, disease resistance varieties), Medicine (xenotransplantation, nutritional supplements and pharmaceuticals, human gene therapy), Industry (proteins). Transgenic animals are used to improved human health by achieving genuine protein production with the correct pattern of post-translational modification, by using milk in as a vehicle to deliver the desired recombinant proteins in enough quantity, by creating pure proteins without the potential infectious risks associated human blood; to improve food supply by increasing the rate of development of cattle, by creating resistance against diseases and environmental conditions in cattle, by increasing or altering the production of cows milk and organ transplantation. The former are useful for rapid production of recombinant proteins, although the level of gene expression is generally low. In contrast, the latter are useful for a large-scale recombinant protein production especially in the mammary gland, and could also serve to supply xenotransplantation organs for human patients[84].In each case, however, a number of obstacles still exist to accomplish these therapeutic uses in practice. Possible research targets were suggested to surmount the problems encountered at present.

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