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TRANSGENIC ANIMALS

An Interactive Qualifying Project Report

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By

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ABSTRACT

The ability to create transgenic organisms has been present since the 1970's. Since then this power has increased greatly, and current uses of this technology include pharmaceutical production, organ transplants and disease research. Because this science involves creating new animals, various issues regarding the moral acceptability of this technology have arisen. Our recommendations include increasing public awareness about the benefits of the technology so that educated decisions can be made about its future.

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EXECUTIVE SUMMARY

Scientists have been able to create transgenic organisms since the 1970's. This ability came as a surprise to the public since technology such as this had been unheard of before then, and the possibilities and consequences are numerous. This technology was so new and unpublicized that there were no existing laws that dealt with this type of technology. Old patent laws had to be reviewed, and precedents had to be set regarding transgenic organisms. The applications of this technology are very broad with potentially a very large affect on a great many people. While this technology has great possibilities for good there are also dangers involved.

The public does not yet understand the medical and economic benefits of transgenic organisms. This lack of information greatly inhibits the public's ability to make knowledgeable and informed decisions. Instead, their opinions are founded on rumor and fear, not fact and experimentation. The public has quite a large say in what laws are passed and what kind of technology is allowed to be funded and developed. Due to this, a well-educated public is required. An uneducated public in Europe caused a moratorium to be put in place on transgenic technology. This greatly hindered discoveries that could have been made during that time. One such discovery included the production of human proteins in a transgenic animals' milk. Such transpharmed proteins could become a primary source of medication for human diseases and allow a greater area of distribution to third world countries. Transgenic animals could also become organ donors for human transplant patients. These animals could produce human organs that mimic the recipient's organ and prevent rejection of the transgenic organ. Transgenic animal disease

models allow for a disease to be researched more in depth as well as allowing for pre-clinical drug trials to be more useful and applicable to humans.

A well-educated public would be able to make fact-based conclusions and well developed informed decisions about the future of transgenic technology. These decisions would not be based on the fear of things that are not understood or are biologically impossible. Education is the key to further research and useful application of this technology. Well-developed studies and additional research into possible consequences of transgenic technology should be made and geared towards the general public for education purposes.

PROJECT OBJECTIVE

The purpose of this interactive qualifying project was to investigate and understand in-depth the new transgenic technology. We attempted to meet that goal by breaking the project down into easy to understand sections. Each section systematically and methodically described a different aspect of transgenics. First, we explored the science of how these animals are created. We next explored medical and economic applications, and ethical issues of this technology. Based on our research a major objective was to make informed recommendations and suggestions to the reader.

Although the project goal was to educate the readers, in writing it the authors gained a lot of knowledge. This knowledge has instilled in us a pro-transgenic point of view with some cautions. It is our hope that this paper may affect the readers in a similar way. It is also a goal of the authors to see transgenic research progress.

INTRODUCTION

In recent years enormous strides have been made in the biological sciences. The amount of knowledge that has been gained is incalculable and is constantly increasing. As this amount of knowledge grows, so does the technology that is used to study it. Of particular importance to this project is the creation of the transgenic animal.

In the past, scientists would often be forced to use the human body to study things such as diseases. This was difficult for two reasons. First, it meant that a subject with that affliction must be found and most likely moved to the proper setting for more thorough study, particularly since it would be inhumane to purposely infect another human being. Finding such patients was not always easy, especially in cases of more exotic diseases. Second, testing of any new treatment for the disease would have to be done on the person, and this could possibly make the situation worse.

After having practiced within these boundaries, scientists eventually moved onto using animals as models for these diseases. Rather than risking a human life in the study of the disease they could just infect an animal. This allowed a more thorough study of the disease as well as a pre-clinical stage to test new treatments. One major problem with this animal model approach was that although the animals did seem to be a more suitable form of study they did not always accurately duplicate the human pathological responses, especially to treatments. So although this did give a decent platform for study of the disease it was not the best way to study treatments.

The question of how to more accurately study these animals then arose. And thus the field of transgenics came into existence. By altering the genetic code of one type of

animal such that it contains attributes of another type, scientists have gained the ability to more completely study these diseases using animal models. This technology proved to be extremely useful once perfected. An excellent case in point was the development of the world's first Alzheimer's disease mouse model (in part here at WPI), which was used to make the first Alzheimer's vaccine. Although transgenics was originally used for the study of diseases, it has begun to show other uses as well, such as pharmaceutical, production and even organ transplants.

The uses of animal subjects that occur in transgenics have come under much public fire. As what occurs so often when the public is told about something that they do not entirely understand, they begin to fear it. As a result of this lack of information the public opinion turns against the new technology, especially in light of some well publicized misuses of the technology. This could affect the future of transgenics and can only be remedied after the public is properly educated and is then in a position where educated choices can be made. Scientists also need to be made aware of the public's concerns, and design experiments to minimize or eliminate animal suffering. Until then the negative public opinion that is felt toward transgenics is only counterproductive.

CHAPTER 1 - HISTORY AND BACKGROUND

1.1 What is a Transgenic Animal?

All living creatures are made of cells, the basic building blocks of life. Deep within these cells lie genes, the material from which everything that we are made of is encoded. These genes are furthermore comprised of deoxyribonucleic acid also know as DNA. By using it's specialized chemical makeup, DNA encodes hundreds upon thousands of different proteins that are essential for the body to function. Each organism has a highly specific set of genes that makes it unique from every other organism. These genes are passed from generation to generation to members of the same biological species through reproduction. A transgenic animal, however, is an animal that carries a foreign gene that has been purposely inserted into its genetic makeup. The foreign gene is created using recombinant DNA technology (discussed in chapter 2), and is inserted into the germ cells (sperm or egg), or young embryo of the specific organism. The offspring of this organism will then contain the modification within every cell of the transgenic organism. This change permanently modifies the animal's genome and is much quicker than the natural change that would occur as a result of mutation, evolution or traditional breeding.

1.2 History of Transgenics

The first transgenic organism on record was the bacterium *Escherichia coli* (*E. coli*). Although prior plant hybridization resulted in a plant with a combination of DNA, it was not truly transgenic because the two sets of DNA were allowed to combine

completely. This combination of complete chromosomes results in what is known as a "**hybrid polyploid**." The 'hybrid' refers to the combination of two different species, breeds, or varieties. The -ploid refers to a chromosome set of which there are usually two in plants, and the poly- meaning many.

The research on genetic alteration of *E. coli* began in the early 1970's. The act of altering a living organism's genetic makeup was a novel and poorly understood field. Due to a lack of knowledge, a major scientific outcry slowed down the research. They demanded an inquiry looking into the possible risk of creating, and then inadvertently releasing, a super strain of *E. coli* that was potentially lethal.

The federal government then imposed strict research restrictions that only allowed the use of a special kind of defective *E. coli* bacteria, one that had been bred to require a nutrient agar with certain amino acids in it. This specific agar, with its six extra amino acids, as well as the special breed of *E. coli*, was developed at the University of Alabama at Birmingham in their Microbiology lab in 1974. The researcher in charge of the lab at that time was professor J. C. Bennett, MD.

E. coli is an organism normally found in the mammalian intestinal track. Its appeal to the scientific community is that it has a reproductive rate of one division every twenty minutes. This degree of replication is difficult to grasp for many; a few examples are illustrated in the following. If a single *E. coli* started dividing and was provided with all the space and nutrients it needed, at the end of 24 hours then there would be 2^71 *E. coli*, 2,361,183,241,434,822,606,848, or 2.36 septillion for short. This many cells would occupy a volume roughly equal to the volume of the Earth. If the same *E. coli* were to

reproduce for two days, this would result in 2^143 *E. coli* or 11,150,372,599,265,311,570,767,859,136,324,180,752,990,208.

The most promising implications of the work with *E. coli* have been the production of uniform strength recombinant insulin. The insulin that was previously used to treat diabetics was extracted from pigs because of their pre-disposition to make a large quantity of the drug. The problem with this insulin is that since it is extracted from different animals, the strength is not constant. This makes it difficult and dangerous for diabetics trying to regulate their blood sugar. The recombinant insulin (termed humulin), produced when human DNA containing the gene for insulin is combined with *E. coli*, is of a uniform strength. This is due to the fact that it is all from the same human based gene. These transgenic *E. coli* are placed in agar vats where they are allowed to split, divide, and produce insulin. If the marketing demands for insulin rise, you simply place a culture in another agar vat and start a new colony.

The first transgenic animal was a mouse. It is not agreed upon as to who created this mouse ("Short History Of", 1995). It is however agreed upon that the first human gene to be successfully transplanted into a mouse was the growth hormone gene. This occurred in 1982, one to two years after the first documentation of transgenic mice. The following time line gives a rough idea of the events leading up to, and following the success in 1982 ("Short History Of", 1995).

1.2.a. Genetic and Transgenic Timeline:

-1866: Czech monk Gregor Mendel, studying peas through many generations, postulates an explanation for the inheritance of biological characteristics. His ideas, however, were not widely recognized for approximately four decades.

-1891: The first successful embryo transfer was accomplished in in-vitro cultures.

-1903: American biologist William Sutton proposes that genes are located on chromosomes, which had been identified through a microscope.

-1911: Danish biologist William Johanssen devises the term "gene" and distinguishes genotypes (genetic composition) from phenotypes (how the organism appears), which is open to influence from the environment.

-1911: Biologist Charles B. Davenport, head of the U.S. Eugenics Record Office in New York, publishes a book advising eugenic practices, based on "evidence" that undesirable characteristics such as "pauperism" and "shiftlessness" are inherited. This eugenics movement becomes quite popular in the U.S. and Northern Europe in the next three decades, until Nazism forces people to understand its implications.

-1922: American geneticist Thomas Hunt Morgan and colleagues devised gene mapping techniques and prepared a gene map of fruit fly chromosomes.

-1944: Oswald Avery and colleagues at the Rockefeller Institute, New York, demonstrate that genes are composed of deoxyribonucleic acid (DNA).

-1944: Erwin Schrodinger publishes the classic *What Is Life?* pondering the complexities of biology and suggesting that chemical reactions don't tell the whole story of life.

-1953: James Watson and Francis Crick, working at the Molecular Biology Laboratory in Cambridge, England, explained the double-helix structure of DNA.

-1961: Mouse embryo aggregation to produce chimeras.

-1966: First report of microinjection of mouse embryos.

-1971: Stanley Cohen of Stanford University and Herbert Boyer of the University of California, San Francisco, developed initial techniques for recombinant-DNA technology.

-1973: Foreign genes function after cell transfection in *E. coli* models.

-1974: Patent applied for transgenic oil digesting bacteria, granted in 1981.

-1974: Development of teratocarcinoma cell transfer.

-1976: Recombinant insulin produced and marketed by Genentech. Inc.

-1977: mRNA and DNA transferred to xenopus (a type of toad) eggs

-1980: mRNA transferred into mammalian ova

-1980: 1980-81 transgenic mice first documented

-1980: U.S. Supreme Court rules that recombinant microorganisms can be patented, in the Diamond vs. Chakrabarty case, involving a bacterium that is engineered to break down components of oil. The microorganism is never used to clean up oil spills, however, because of concern over its uncontrollable release into the environment. On the strength of the patent approval, commercial biotechnology investments take off. The first Genentech public stock offering sets a Wall Street trading record.

-1981: Transfer of embryonic stem (ES) cells derived from mouse embryos.

-1981: First monoclonal antibody diagnostic kits approved for sale in the U.S.

-1981: First automatic gene synthesizer marketed.

-1982: First rDNA pharmaceutical insulin approved for use in the U.S.

-1982: The first successful cross-species transfer of a gene, a human growth gene inserted into a mouse.

-1983: Tissue specific gene expression in transgenic mice accomplished.

-1985: First transgenic domestic animal produced, a rabbit.

-1985: The first environmental release of genetically engineered microorganisms ("ice-minus" bacteria, intended to protect crops from frost) is approved in the U.S. in the face of controversy.

-1985: U.S. declares genetically engineered plants patentable.

-1985: Microinjection for transgenic pigs, sheep, rabbits, fish first successful.

-1987: Retrovirus mediated transgenic chicken created.

-1988: Oncomouse, a mouse that was engineered to develop breast cancer by scientists

at Harvard University with funding from DuPont, obtains a U.S. patent but at the time is not patented in Europe. Many other types of transgenic mice are soon created.

-1988: Human genome mapping project started.

-1988: German courts stop the Hoechst pharmaceutical company from producing genetically engineered insulin after public protests.

-1989: Targeted DNA integration & germline microinjection for transgenic cattle (Russia).

-1992: Under much controversy, the Oncomouse is patented in Europe.

-1993: Germline chimeric mice produced using embryo co-culture.

-1996: ES cells used for nuclear transfer in sheep.

-1997: Somatic cells from adult sheep used for cloning by nuclear transfer.

CHAPTER 2 - TRANSGENIC METHODS

2.1 Preparation of DNA

The first step in making a transgenic animal is to prepare the DNA that will be inserted into the target animal. The preparation of the DNA can be broken down into 3 steps, choosing the DNA, cloning it, and DNA purification.

2.1.1 Choice of gene and cloning

Humans contain hundreds of thousands of genes that code for an equal number of proteins. The selection of which gene is worthy of expressing in another animal requires great consideration, including what medical benefit to us will be created, and will the final product be financially feasible. Once a gene, or specific series of nucleotides of particular interest have been chosen in an organism they must be cloned. In order to do this **restriction enzymes** are added to the DNA. These enzymes are used to cleave the DNA duplex at a specific short sequence known as a **restriction site**. Restriction sites are often 4 or 6 nucleotides long, and each restriction enzyme has a unique restriction site. Restriction enzymes are 'symmetrical' in that the restriction sequence that they recognize is the same on both strands of the DNA duplex. For example a common restriction enzyme, known as *Eco*RI recognizes the site 5'-GAATTC-3' which is identical on the opposite strand and reads as 3'-CTTAAG-5', since the nucleotides Adenine (A), and Thymine (T) pair together, as do Cytosine (C) and Guanine (G). This type of symmetry is known as a **palindrome** (Hartl, D., 1998).

Once the enzyme finds its restriction sites it will then cleave the DNA at these sites. Two types of cleavage are done by restriction enzymes. In the first case the enzyme cuts the restriction sequence symmetrically down the center. This leaves the DNA fragments with ends that are of an even length, known as **blunt ends**. The second type of cleavage occurs when the cuts in the restriction site are not made directly across from each other on the DNA duplex. This results in a DNA fragment that had uneven ends, known as **sticky ends** (Hartl, D., 1998).

Once the DNA fragment representing the transgene of interest has been cleaved from the donor genome, certain base sequences are then added to it enzymatically. Sequence elements added toward the 5' end of the strand, known as upstream, can include enhancers, promoters and signal sequences. **Enhancers** do exactly what their name states, they enhance expression of the transgene and can be located several kilo-bases from the gene in question. **Promoters** play a vital part in the temporal and tissue-specific regulation of gene expression and are often found directly upstream of genes. Thus they are used to control which tissues the transgene is made in. Between the promoter region and the gene is the **signal sequence**. These are short sequences that target the synthesis of proteins into specific metabolic pathways of the cell. Signal sequences are mostly found on the 5' end of the gene, while some **organelle targeting sequences** are found on the 3' end. There is also a poly-A nucleotide sequence that is added to the 3' end (downstream of any signal sequences that may be residing there) that is used to ensure the correct mRNA transcription and translation. The gene that has had all of these elements added to it is then called a transgene expression cassette. ("Transgenic Animal Science", 1991)

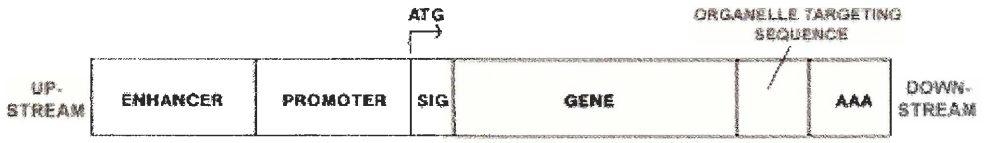

Figure 2.1 Transgenic Expression Cassette
The setup of a generic expression cassette

Once a cassette is completed, it must then be inserted into a **vector**. A vector is a circular DNA molecule into which an expression cassette can be added and then reproduced by a suitable organism. Depending on the size of the cassette that needs to be cloned different types of vectors can be used. Many different types of vectors can be produced and are available commercially, four of the most common vectors are: Plasmid vectors for cloning sequences of approx. 5kb (kilo-bases), Bacteriophage λ vector for sequences of approx. 15kb, Cosmid vectors for sequences of approx. 40kb and P1 phage vectors for sequences of approx. 85kb. Often times, the most common vector used is an *E. coli* plasmid (Hartl, D., 1998).

Once a vector type is chosen, the DNA fragment must be inserted into it. In order to do this a vector is chosen that has a single restriction site for the same restriction enzyme that was used to digest the original DNA strand. Because the sticky ends are complementary to each other, this allows the vectors to bind with the ends of the expression cassette that have had the restriction site added. The new recombinant plasmid is then ligased in order to seal the restriction sites back together to produce a circular DNA strand.

Next, the recombinant vectors are introduced into the host cells through a process called **transformation**. By exposing the cells to a solution of Calcium Chloride ($CaCl_2$) the cells become "competent", or gain the ability to take up free DNA. Another way to

introduce the vector into the DNA is through a process known as **electroporation**. By exposing the cells to an electrical current, pores on the surface of cells open more than they normally would, allowing some of the smaller recombinant vectors to diffuse into the cell.

Finally, once the vectors have been incorporated into the host cells they must be replicated. By nature, plasmids and other vector types, can exist inside the host cell as a separate entity and not be incorporated into the cell's genome. This allows for the vector to multiply inside the host cell without losing its integrity. It does stand to reason however that not all the host cells that are exposed to the recombinant vectors will successfully uptake them. For this reason an antibiotic resistance gene is often included in the DNA of the original vector. As a result the host cells are often grown on a media that includes that antibiotic. That way those cells that have incorporated the recombinant vector express the resistance to the antibiotic while those that didn't uptake the vector die.

2.1.2 DNA purification

Once it is established which cell cultures have taken up the vector and which haven't, those that have taken up the vector are grown until a desired number is established. Once there are enough cells (and enough copies of the gene) the cells are lysed, broken apart, and their plasmids removed. The original inserted gene is then removed by another round of restriction enzyme digestion in order to remove as much vector DNA as possible from the insertion sequence. Then by running the solution on an agarose electrophoretic gel or sucrose gradient the transgene can be specifically isolated.

Furthermore, by treatment of chloroform ($CHCl_3$) and Isoamyl Alcohol, followed by Sodium Acetate (NaOAc) and Ethanol, the transgene is precipitated out of solution and then dissolved into a microinjection buffer. The specific concentration of DNA is then calculated by spectrophotometry. The average concentration for microinjection is between 1 and 5 μg/ml ("Transgenic Mouse Facility", 1999).

2.2 Gamete maturation and extraction

In the production of transgenic animals, eggs from a female donor mouse must be harvested and then evaluated for implantation. Most often the eggs are fertilized inside the donor, but sometimes in vitro fertilization is used.

Hybrid donors are generally preferred because inbred mice demonstrate poor reproduction performance and lowered production of fertilized eggs. (However, if a specific genetic make-up is required, then an inbred mouse donor is used). The donor is administered hormones that superovulate her producing up to 20-30 eggs, while naturally ovulating mice produce only 6-10 eggs depending on the strain (Hogan, 1994). Because of the decreased number of donor mice needed and decreased collection time, superovulation is preferred over natural ovulation for embryo donors.

Since mice ovulate according to the light dark cycle it is vital that the donor and recipient mouse conditions be properly monitored and both mice be on the same light dark schedule. Females can be housed up to 5 in a cage, while male breeders are housed in separate cages and away from females. The males should be celibate for a minimum 72 hours prior to breeding, but not longer than several weeks. They are also separated from other males for 1 week prior to mating so as to assert their territory and to increase

testosterone production, since if housed together the dominant male can inhibit testosterone production in the other mice ("Embryology and Cryopreservation", 1998). Breeding is monogamous and restricted to 18-22 hours for the most successful results.

The donor mouse is between 3 and 6 weeks old, and a pathogen-free virgin ("Embryology and Development", 1998). She is given pregnant mare serum gonadotropin (PMSG) 0.1 ml **subcutaneous**, under the skin, or **intraperitoneally**, directly injected into the peritoneal cavity (Bowtell, 1998). PMSG mimics follicle stimulating hormone (FSH). Cumulus cells in the mouse are attached to the oocyte by gap junctions, and when the follicles are stimulated, these gap junctions are broken, and the follicle cells secrete proteoglycans, hyaluronan, and tissue Plasminogen activator (tPA). The proteoglycans and hyaluronan molecules cross-bridge to form an elastic matrix around the oocyte that helps protect the oocyte during ovulation (Salustri et al., 1998). PMSG should be administered 6 hours prior to the onset of the dark period, and 46-48 hours later 0.1 ml of human chorionic gonadotropin (hCG) is administered intraperitoneally (Bowtell, 1998). The hormone hCG mimics luteinizing hormone and induces ovulation 10-13 hours after injection. At ovulation the oocyte and the surrounding cells detach from the follicle wall and are released into the oviducts for fertilization. After being injected with hCG, the donor mouse is put in the genetically selected male breeder's cage and mating is expected to occur that evening. The following morning, 0.5 ED (embryonic days), the presence of copulation plugs is checked. A copulation plug consists of coagulated proteins from the male seminal fluid and indicates that the mice have actually mated. Upon the presence of plugs, 10-12 hours after

fertilization the donor is euthanized and placed on her back in a petri dish for easy movement (Hogan, 1994).

Once in the petri, dish a lateral incision is made at the midline, and the skin is pulled back so the peritoneum, the membrane that lines the abdominal cavity, is exposed. The peritoneum is then cut and pulled back. The intestines are then pushed up and out of the way and the uterus, ovaries, and oviducts are located. At this time in development, the embryos are still present in the oviducts, so it is necessary to remove both oviducts. The oviduct is cut just below where it connects to the uterus and between it and the ovary. Next the oviducts are moved to a petri dish with medium. The oviduct is then torn and the eggs released. In some circumstances the ducts must be flushed with a small volume of medium to remove all of the embryos (Hogan, 1994).

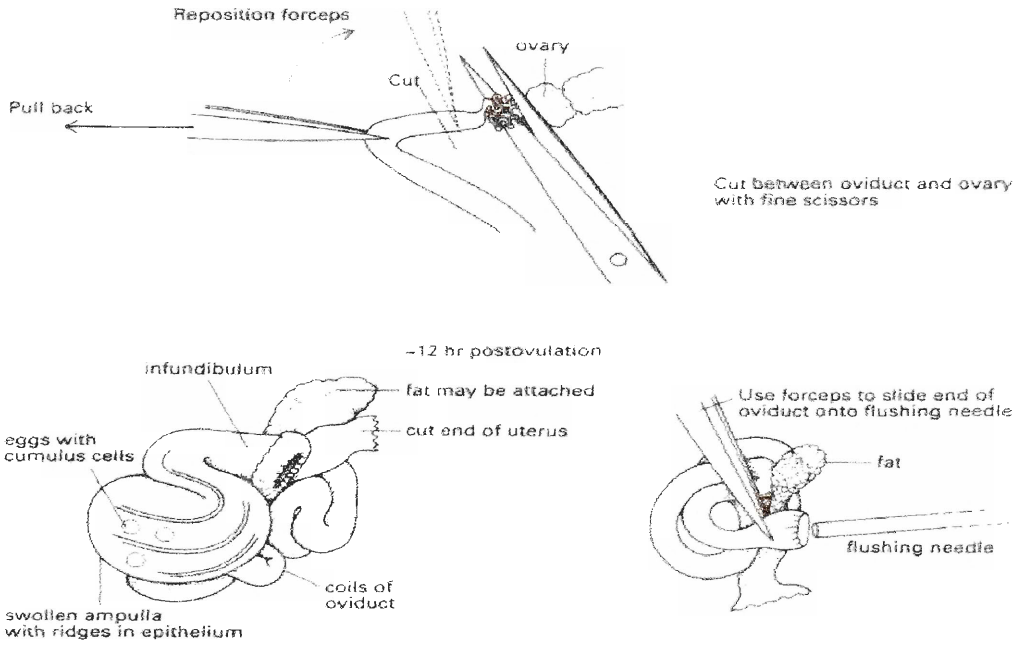

Figure 2.2 – Embryo Extraction This figure shows the procedure for collecting embryos from mouse oviducts. The oviducts are cut at the edge of the ovary, and between the oviducts and uterus. (Top) Then the oviducts are torn and flushed with medium (Bottom Left and Right). Figure is taken from (Hogan, 1994).

The removed pronuclear embryos exist clumped together with sticky cumulus cells. In order to separate them, the embryos are transferred to a medium with the enzyme hyaluronidase in it. The hyaluronidase dissolves the sticky matrix (containing hyaluronan) and cumulus cells, and allows for the isolation of the embryos (Salustri, 1998). After allowing the enzyme to work for a few minutes, the embryos are then carefully separated from debris and cumulus cells, and transferred to a petri dish. Then they are covered with mineral oil to prevent contamination, evaporation and pH changes, and can be stored at 37° C in culture until needed. The medium usually contains a buffering system and a protein source, and sometimes antibodies and a chelating agent ("Transgenic Animal Science", 1991). The optimal time for pronuclear microinjection of DNA into the embryos is about 12-18 hours post-fertilization.

If blastocysts are needed instead of pronuclear embryos, then the embryos are allowed to mature inside the mouse until 20-60 hours ED. At this time in development the embryos have progressed through the oviducts and into the uterus. The procedure is much the same as pronuclear embryo removal, except the uterus is cut just above the cervix and just below the junction with the oviducts. Each uterine horn is then flushed with medium and the embryos collected. The embryos will have since lost all of the cumulus cells attached so treatment with enzyme is not needed. A variety of 2-8 cell embryos, and 8-cell compacted morulae will be present.

2.3 Incorporation of transgene into host genome

There are three main methods commonly used to incorporate transgenic DNA into a zygote, Pronuclear microinjection, Embryonic Stem (ES) cell culture, and Retroviral vector delivery. Each method is described below.

2.3.1 Pronuclear Microinjection

For the transgenic material to be incorporated into every cell of the new organism it must be inserted when the embryo is at a very early developmental stage. In this case the transgene is introduced into the zygote at the **pronuclear** period immediately after fertilization has occurred. In this stage, the male and the female pronuclei are visible as separate structures, and only stay this way for a few hours after fertilization. Often it is easy to distinguish which pronuclei came from the male and which from the female because the male pronucleus is larger. The transgene may enter either of the pronuclei with the same probability regardless of the gene sequence. If by chance the transgene enters the zygote after the initial division of cells, a **mosaic animal** could develop, where the inserted DNA is only expressed in a percentage of the cells rather than all of them.

In order to inject the material special pipets must be used. These pipets are specially made out of thin walled glass capillary tubing. Two separate types of pipets are used in the process. The first is the holding pipet. It has a perfectly flat tip with an internal diameter between 15 to 25 μm in order to hold an embryo with an average diameter of about 85 μm. If the tip of the holding pipet is not perfectly flat there is a risk that the embryo may slide off and become damaged or destroyed in the process. The second pipet is known as the injection pipet. Much smaller than the holding pipet, the

injection pipet has a diameter of 1μm or less and is tapered at the end. During the procedure both pipets are held in place by an instrument holder and controlled by a micromanipulator, giving three-dimensional control of the pipets. The process must also be done in a perfectly clean, sterile room so that no contaminants are allowed to clog the pipets or potentially harm the cells if accidentally injected.

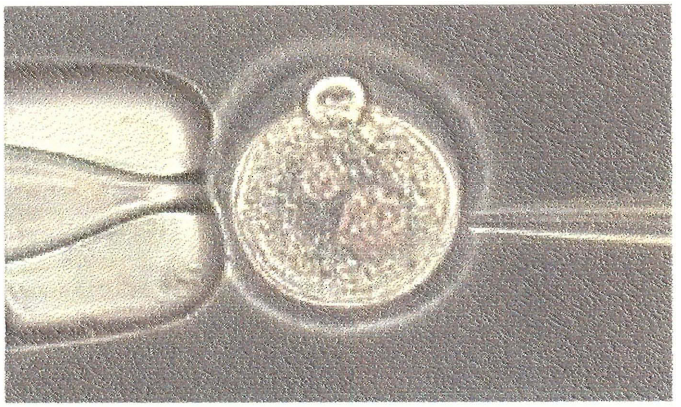

Figure 2.3 – Pronuclear Injection A photograph showing the pronuclear injection method. To the left can be seen the holding pipet and on the right the injection pipet. In the center is the cell, its two pronuclei are colored slightly pink. Figure is from ("Transgenic Animals", 1999).

The process in which the transgenes are injected into the cells can vary from facility to facility depending on what works best in each specific case. In general however the following procedure is followed; a petri dish containing the embryos in solution is placed under a low magnification, and any defective or degenerated embryos removed from the viable ones. The holding pipet is then slowly lowered into the solution, and the first embryo is slowly sucked onto the pipet using a very low amount of negative pressure suction. The tip of the injection pipet is then lowered into the solution, aligned with the embryo and a small amount of the microinjection solution, containing the DNA, is ejected to ensure that the pipet is not clogged. Then in one smooth step the injection

needle is injected through the cell membrane and cytoplasm and into the nucleus. Often it is hard to visually determine if the nuclear membrane has been pierced. The sure way to tell is by observing the small amount of swelling that occurs when the membrane is pierced. The microinjection solution is then injected into the nucleus and the needle quickly removed. Once all of the embryos are injected they are transferred into another medium for incubation and visual evaluation after a few hours, after which all viable embryos are implanted into host mothers (discussed in section 2.4).

Once the transgene has been inserted into the nucleus there is no definitive way to determine where the integration will take place on the host genome or how many copies will be integrated. The integration is completely random, and controlling the process has not yet been successful. Even sibling animals can have a different number of integrated genes in many different locations on the genome. Many studies have shown a large difference in the expression of the transgene within siblings due to these different integration sites and the inserted copy number. Because of the random nature of the insertions, the transgene can often insert into functional gene sequences that are necessary for the animal to live. Interruption of these normal gene sequences is lethal or can lead to major deformities in the animal. The animals that do receive a functional transgene and appear to be functioning normally are termed **founder** (F_o). These animals are then mated in order to keep a functional lineage.

In 1980 the first successful transgenic mouse was created using the pronuclear injection method. Although it was shown that the transgene had integrated into the genome, it was not expressed because of a location problem. In 1982 the first expressive mouse was reported ("Transgenic Animal Science", 1991). Since then many leaps and

bounds have been made in the methodology and currently there are hundreds of different kinds of transgenic animals, and new breeds are constantly being produced.

The pronuclear method of transgene incorporation is widely used with tissue specific genes. By manipulating the promoter and enhancers that surround the gene, certain selectiveness can be used in choosing where the trans-protein will be targeted. Another use for this method is the study of over expression of certain proteins and their result on the organism.

2.3.2 Embryonic Stem Cell Method

The embryonic stem cell method of incorporating the transgene is somewhat different from that of the pronuclear injection method. In this method wild type animals are allowed to mate and the embryos are given a few days to develop and divide, these early embryos are known as **blastocysts** and are made up of cells known as a **embryonic stem (ES) cells**. These blastocysts are then harvested and the embryonic stem cells cultured. Embryonic stem cells are not especially hardy cells, and great care must be taken in culturing the cell so as not to kill them. This is often done by growing them on a layer of feeder cells (often irradiated fibroblasts) and changing the media they are in every 2 to 3 days ("What is a", 1998).

Once the cells have been cultured the next step is to incorporate the trangene. This can be done one of two ways. The trangene can be microinjected into the ES cell in a method very similar to that used in pronuclear injection, or the ES cells can be put through an elecroporation process. The transgene cassette that is added into the ES cells is flanked by homologous sections of the animal's own DNA sequence (2-7kb on each

end), this combination of homologous DNA and transgene cassette is known as a **construct**. Once the construct comes into contact with the genetic material of the cell, a process known as **homologous recombination** naturally occurs, where the construct replaces the genetic material of the original genome between the regions that correspond to those found on the flanking ends of the construct. The new transgene is then added to the genome and the cell discards the original sequence. It is very important that the genomic DNA used in creating the homologous ends of the construct is from the same strain of animal that the ES cell will be used on. If there are even small gaps between the strains, there can be a drastic loss of efficiency in the homologous recombination.

Running southern blot electrophoresis on the ES cells can reveal whether or not the DNA has been correctly incorporated because of the difference in the molecular weight of different parts of the genome. Once successful transgenic ES cells have been isolated, blastocysts are harvested from another nontransgenic host mother. Into each of these blastocysts 10 to 15 ES cells are injected, after which 8 to 10 blastocysts are reinjected into the host mother.

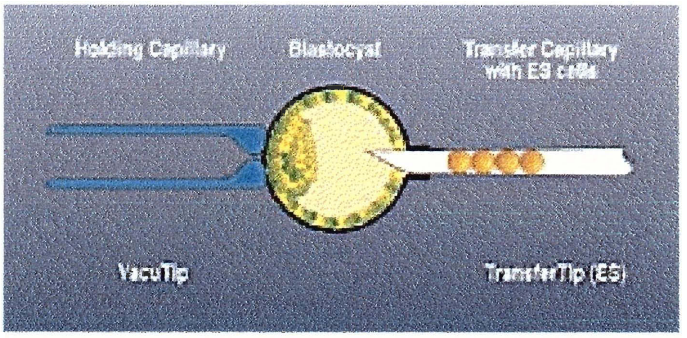

Figure 2.4 – ES Cell Injection A diagram showing the injection of the transgenic ES cells into the donor blastocyst. This figure is from ("Transgenic Animals", 1999).

Contained within each of these reinjected blastocysts is a mix of ES cells, some of which have the transgene and others that do not. Because ES cells occur at such an early

developmental stage they can develop into any type of tissue of the body. The resulting brood of animals then contains a mix of normal and mosaic (also know as **chimeric**) animals that have certain tissues in their body where the transgene is expressed. However, because of the randomness of the division of the ES cells, there is no definitive way in which to determine what cells will result in what tissues. As a result, a gene for a phenotypic characteristic is often inserted into the construct to aid in visualization, common insertions are a change in coat or eye color.

Once again, just because the subject shows expression in certain parts of its body does not mean that this is the same throughout the entire body. In order to propagate the strain an animal must be found where the transgene has been inserted into the germ cell line. Once a male and female with the germ line insertions have been found, these animals are then mated to create an animal that is completely transgenic expressing the new gene throughout its entire body. That is one of the major drawbacks of this technique, although it does allow a more targeted approach to the gene insertion than the pronuclear method, it does not guarantee the ability to procreate the strain. Often a large amount of offspring must be produced in order to find those few that can pass down the desired gene.

2.3.3 Retroviral insertion

The retroviral insertion technique is based on a virus that normally infects mammalian cells, called the retrovirus. Contained within the retrovirus is a single strand of genetic material known as RNA. The virus injects the RNA into the cell, infecting it. Then by means of an enzyme called **reverse transcriptase** the single stranded RNA

molecule creates a double stranded DNA molecule, which is then incorporated into the host's genome. In the case of transgenics, these retroviruses can be engineered to hold a RNA sequence that codes for the desired DNA sequence. The retroviruses are then allowed to infect the embryo at an early stage and insert the gene.

This method does have a few problems however. First, because of the physical size of the retrovirus capsid it can only contain extra sequences of up to 8kb. The second major problem with this technique is that the results can be very unpredictable and cause dangerous outcomes for the cell lines used. It is however, the most efficient way to introduce transgenes into a cell (Harlt, D., 1998).

2.4 Embryo Implantation

After the transgene has been incorporated into the blastocyst it is then necessary to transplant them into a foster mother so that they can be developed to term. At early stages of development oviduct transfer is used, while at later stages of development uterine transfer is used. Recipient mice are chosen for their large ampullae and good mothering techniques. Recipients are often between 20 to 30 grams, and 6 weeks or older. Females are allowed to ovulate naturally without hormone intervention and then mated with vasectomied males. The female body is then ready; she is pseudopregnant and she is ready to become the foster mother. The manipulated embryos show slower growth and development, so the recipient female needs to be earlier in her reproductive cycle than the donor to compensate for this ("Transgenic Animal Science", 1991). For embryos ready for injection at the 2-cell stage, they are injected into 0.5 embryonic day recipients via oviduct transfer. If the cells are allowed to culture to the blastocyst stage, they are

injected into 2.5 embryonic day pseudopregnant recipients via uterus injection. The earlier stages of transfer produce the highest success rates and are therefore the most commonly used (Hogan, 1994).

For oviduct transfer, the recipient female is anesthetized with Avertin anesthetic, laid on her belly, and the incision site cleaned. The incision is made 1 cm to the left of the spinal cord and level with the last rib. The incision is then moved until the ovary or fat pad can be seen through the peritoneum. The peritoneum is then cut, and the ovarian fat pad, ovary and oviducts are removed from the body and anchored to the mouse's back with a serrafine clip. Being careful to avoid any major blood vessels, the mouse is placed under a stereomicroscope, and a hole is made in the **bursa**, a thin membrane surrounding the oviduct and ovary. The infundibulum, the opening to the oviducts, is located partway between the ovary and the oviducts. The transfer pipet is inserted into the infundibulum and the embryos are transferred to the oviducts by mouth pressure (see figure 2.5). The uterus is then gently eased back into the body. If doing a transfer into the other oviduct, the procedure is then repeated with the other uterine horn. The incision site is then closed with wound clips, since mice chew at sutures.

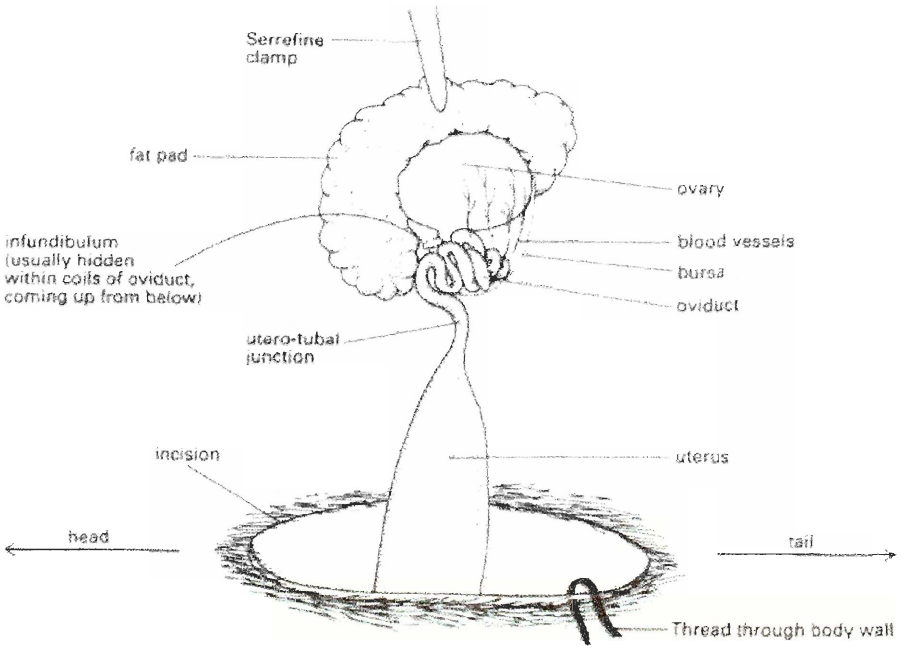

Figure 2.5 – Oviduct Transfer A diagram showing the method for oviduct transfer. The ovary and oviducts are extracted, a hole is made in the bursa, the infundibulum located, transfer pipet inserted, and embryos injected. Diagram is from (Hogan, 1994).

The transfer pipet should have an internal diameter between 120-180 μm, but preferably less than 150 μm. There are multiple ways to load the transfer pipet. A common method is as follows; a volume of the buffered medium is taken up, then a small air bubble, a volume of medium, and then another air bubble. A minimum of 12 embryos (usually 20- 30) is taken up in the smallest possible volume of media, and then another small air bubble. When injected into the oviducts both the medium containing the embryos and the air bubbles are inserted. These air bubbles are inserted into the oviducts in order to prevent the embryos from immediately flowing back out, and to also help drive them forward into the ampulla region of the oviduct ("1-Cell Embryo Transfer" 1998). This increases the chances of implantation.

Any embryos allowed to mature to the blastocyst stage are transferred to the recipient mouse via uterine transfer. The uterine transfer procedure is the same as oviduct transfer described above, but instead of the embryos being transferred to the oviducts, a hole is made in the uterus a few millimeters down from the utero-tubal junction (see figure 2.6). The injection pipet is loaded with an air bubble, medium, another air bubble, and then up to 8 blastocysts in a small amount of medium. The injection pipet is then inserted 3mm into the hole in the uterus, and the pipet blown until the medium containing the blastocysts has been injected. This time none of the air bubbles are injected into the uterus because the air interferes with embryo implantation. No more than 8 blastocysts should be implanted into one uterine horn, and no more than 12 blastocysts total per recipient animal. The uterus, oviducts and ovary are all eased back in to the body and the incision site is closed with wound clips (Hogan, 1994).

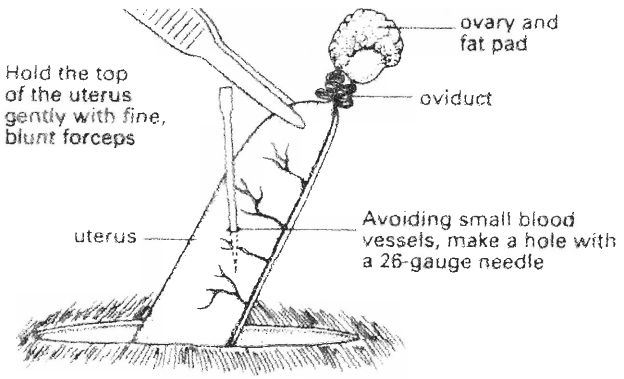

Figure 2.6 – Uterine Transfer A diagram showing the procedure for uterine transfer. Uterus, oviducts and ovary are extracted, a small whole is made in the uterus, the transfer pipet inserted and the blastocyst inserted. Figure is from (Hogan, 1994).

After surgery, the mouse is then placed into a clean sterile box with sawdust. To keep the mouse warm it is either wrapped or a heating pad is placed over the cage. Recipients are closely observed and the environment monitored. The temperature is

maintained at 21° ± 1° C, and light cycle should be constant at 12 hours dark and 12 hours light. Pregnancy should be visible in 2 weeks, and the pups delivered at 3 weeks.

CHAPTER 3 - TYPES OF TRANSGENIC ANIMALS

3.1 Pharmaceutical Producers

Ever since researchers discovered that human genes could be inserted into other organisms' genes, the gears have been turning in their heads as to what can be done with this ability. As the human genome project is progressing, and the specific coding sequences for individual proteins are being discovered, scientists can use this information to insert specific proteins where desired. This means that scientists can cut out the desired piece of DNA from the human genetic code and then insert it into another organism, and if done at the right stage of development of the other organism there is a chance that that organism will incorporate the foreign DNA into its genetic code. For practical purposes the new organism, genetically engineered and now called transgenic, will produce whatever protein the inserted DNA coded for.

This allows scientists to use animals as bioreactors to produce human proteins. The protein can be produced in almost any tissue or organ the researcher desires as long as they know the DNA sequence that controls its expression. The most common locations for protein production are the kidney, liver, blood, salivary, and mammary glands. When the protein is produced in the mammary glands, in a process called **transpharming**, the protein is secreted in the milk. The milk can then be easily collected and the human protein purified from the milk. This is non invasive and highly profitable since average dairy cows, for example, can produce more than 2,500 gallons of milk a year. Alternatively, when the human protein is produced in an animal's liver for example, the

tissue can then be used to fool a human body into thinking the foreign tissue is actually human and thus accepting it in transplants. This process is called **xenotransplantation** (trans species transplantation).

E. coli is capable of producing protein drugs such as insulin and growth hormone, however more sophisticated proteins (i.e. those that have complex structures or are glycoproteins) need higher level organisms to produce them. So in order to produce other human drugs, higher organisms are needed, such as mice, rabbits, goats, sheep, pigs, and cows.

The first such animal used to produce a therapeutic drug was the common lab mouse. The mouse was a logical choice as a subject due to researchers having a large amount of experience with it, its low cost, short gestation period and generation interval. The first mouse to produce a human therapeutic protein was in 1987 (Betsch, 1994). One group of these mice produced alpha 1-antitrypsin (AAT) (Sifers et al., 1987), and another group of these mice produced the protein tissue Plasminogen Activator, tPA (Betsch, 1994). AAT is used for treatment of mucous buildup in lungs of emphysema and cystic fibrosis patients, while tPA is used to dissolve blood clots and clear blocked arteries in heart attack patients. AAT was first produced in the plasma, liver, and kidneys of the transgenic mice. The next job of the researchers was to find a way to insert the foreign DNA in the correct spot so that the proteins were only manufactured in the desired tissues. Scientists were endeavoring to have the transgenic animals produce the protein entirely in the mammary glands due to the easy collection. After the initial transpharming hurdle was overcome, it was found that on a larger scale, such as a sheep, goat, or cow, the amount produced would be high enough for commercial success.

Soon after the successful production of a foreign protein in mice, researchers endeavored to broaden their animal base and they added sheep to their repertoire. In 1991 the first transgenic sheep was born ("Partners in Protein", PPL Therapeautics). Her name was Tracey and in her milk is the human protein AAT. Tracey, along with 3 other females, produces the protein in amounts greater than 1 gram per liter, and as much as 35 grams per liter. The ewes and the 1 male transgenic sheep also produce the protein in much lower levels in their plasma. The scientists' next step was to prove that the transpharmed protein was able to function just as normal human AAT would. In an analysis of the protein, it was found that the gross sugar content and the in vitro activity were undistinguishable from human plasma-derived proteins (Wright et al., 1991). Upon further investigation it was also discovered that the amino-terminal sequence and molecular weight determined by mass spectrometry were also identical to human plasma-derived protein (Carver et al., 1992).

Rabbits were initially not an obvious choice for transpharmers, but they have their own advantages. Rabbits reproduce, mature, and produce milk quickly, but do not lactate in large volumes compared to cows. This however is not a problem when only small volumes of protein are needed. A biotech company, known as Pharming, has begun using rabbits for these reasons and has made a facility for milking their transgenic rabbits. These rabbits produce in their milk the enzyme alpha-glucosidase ("Milking Rabbits for", 1996). This enzyme is being developed to treat Pompe's disease, also know as acid maltase deficiency, and glycogen storage disorder type II, a rare genetic disorder that prevents the conversion of glycogen to glucose in muscles. There is currently no treatment for this lethal disease. Pharming is now involved in Phase II clinical trials. This

means that they are assessing the safety of the drug and if the drug is producing the desired effect on actual patients with the disease.

Goats were chosen as transpharmers because of their optimal combination of milking ability and short gestation period. Researchers could find out much more quickly if the animal was transgenic, whereas in cows it would take a lot longer and thus cost the researchers more money to rear and care for non-transgenic animals. Transgenic goats owned by the Genzyme Corp., secrete a chemical variant of tPA. Genzyme developed this variant four years prior and believes that it will work longer than the original protein ("Genetically Modified Animals", 1991). Genzyme has also developed goats that secrete human antithrombin III (ATIII) ("Genzyme Transgenics", 1999). This protein is normally found in human blood plasma and it helps regulate the clotting of blood. The protein is being tested to see how well it functions for patients undergoing elective cardiac surgery with cardiopulmonary bypass.

By 1991 a wide variety of drugs and animals had been selected by researchers to experiment with, and pigs were not to be left out. Pigs also were a good choice for a drug producer because of the ease of growing, short generation interval, and the high number of offspring they produce. Pigs were an obvious choice for xenotransplantation due to the similarity of the anatomy and physiology of their organs to humans, while they are not easy to retrieve milk from they have also been used for pharmaceutical production in their milk (Bullock, 1996). Pigs have also been genetically engineered for another purpose, their blood as a substitute for human infusions ("Genetically Modified Animals", 1991).

Along with sheep, transgenic pigs now produce foreign proteins in their milk. In 1992 transgenic pigs were made that produced human protein C (Velander et al., 1992). This anti-coagulant had the same activity as human produced protein C from plasma (Velander et al., 1992).

In humans there is a complement system of proteins that initiates a cascade effect against foreign organisms or tissues. This cascade effect destroys the foreign material in a matter of minutes. Since pig tissue does not have the required proteins to be recognized as human, any pig organ or tissue transplants into humans would fall victim to the complement system. Researchers have now started to integrate human proteins into the tissue of pigs to prevent the human body from rejecting the foreign tissue or organ. These proteins are inserted into the pig's genome and are produced in the endothelial cells that line blood vessels, it is hoped that these proteins can fool the complement system and that the organ will not be destroyed. A different company has produced pigs that have the human shield protein, decay accelerating factor, DAF. When a transgenic pig heart expressing this shield protein was transplanted into a monkey, the heart showed an increase in time of survival (Wong, 1996). While this is promising, the organ still would be required to survive other later attacks from the immune system. It is hoped that these researchers can develop transgenic pigs that express proteins on the surface of their organs that will shield the organ from the host's immune system in its entirety.

The use of different species organs brings up the question of what types of viruses or diseases swine could pass to humans. Some possible viruses that pigs could infect us with could be retroviruses, cytomegalovirus and circovirus ("Xenotransplantation", 1997). While the large differences between humans and pigs are expected to lower the

chance of disease or pathogen transfer, it has not been fully researched to be able tell for certain if they will transfer. Another concern is the relative lifespan of the pig. Since it is so much shorter than a human's would the transplanted organ survive the required amount of time?

The world's first transgenic dairy calf, Bull Herman, was born in 1990 (Deforest, 1998). Due to the random nature of the microinjection process, the researcher cannot determine before injection if the embryo will be a male of a female. Unfortunately the calf was male and thus does not produce any milk, but he does carry the gene for the protein Lactoferin. Lactoferin is a natural antibiotic found in breast milk, it is also anti-microbial and active in the gastro-intestinal tract ("Pharming Produces World's", 1996). Lactoferin is aimed at use in patients with reduced immune systems (such as AIDS), cancer patients undergoing chemotherapy and premature infants. Herman's children have been proven to carry the gene and his female offspring produce Lactoferin in their milk. In tests, the bovine produced Lactoferin and human breast milk Lactoferin were undistinguishable ("Pharming Produces World's", 1996).

Another cow to produce a human protein in milk was created in 1997 ("First Human Protein", 1997). The company PPL Therapeutics, has 15 transgenic cattle producing two different proteins, alpha-lactalbumin and modified alpha-lactalbumin. Alpha-lactalbumin is also produced in breast milk and will be used to treat phenylketonuria.

3.2 Disease models

One of the two most important uses of transgenic animals is to create disease models. By specifically manipulating the genome of certain animals such as rats and mice, biotechnologists can produce a strain of animals that are afflicted by a disease, or a portion of the disease, that they would not normally get. There are many advantages of this type of genetic manipulation. First and foremost is that it can allow study of the way diseases function without having to risk human life. It gives an accurate model with which to study and test both the disease itself, as well as treatments for the disease. Because some treatments may have unforeseen side effects, 'pre-treatment' on animal subjects gives scientists a way of screening out things that may be harmful. If successful however, this pre-treatment can give researchers very accurate results on the effectiveness of the drug as well as information on how it works and any more negligible, nonfatal, side effects. Transgenic animals can also be used to study things such as gene expression, amount of protein made from a specific gene, and signal degradation, the degree to which the gene begins to become nonfunctional after a certain amount of time, without having to actually study the organism from which the genetic material came from. Some examples of transgenic disease models are the following:

3.2.1 Huntington's Chorea

Huntington's Chorea is one of a family of inherited neurodegenerative diseases, in which there is a repeat of a CAG (Cytosine, Adenine, Guanine) base sequence in human DNA causing an excess of the amino acid glutamine in the production of proteins, ("Choreographing Mice", 1996). Major symptoms of this disease include repetitive

involuntary movements, epileptic seizures, and increased appetite but a loss of weight. In November of 1996 Dr. Gillian P. Bates, Senior Lecturer in the Division of Medical and Molecular Genetics at Guy's Hospital in London, successfully created a transgenic mouse that contained these excess CAG sequences, ("Choreographing Mice", 1996). These mice have been shown to have all the symptoms of Huntington's disease and have been made available to researchers worldwide through Jackson Laboratories in Bar Harbor, Maine. Scientists can now study and address new questions about Huntington's disease as well as test new therapies for curing the disease.

3.2.2 Alzheimer's Disease

Alzheimer's disease is an affliction in which a person accumulates diffuse and neuritic plaques in the brain. These plaques in the brain induce neurodegeneration, apoptosis, astrogliosis, and eventually spongiosis, a condition where the brain becomes spongy and soft. Symptoms of the disease include mental and physical disability, loss of memory, and dementia. The constituent component of these neuritic plaques is the beta-amyloid peptide. This peptide is often found in victims of Alzheimer's disease and is thought to play a role in the development of the disease. In 1995 a team that included Worcester Polytechnic Institute scientist David Adams reported the creation of the first transgenic mouse to successfully mimic Alzheimer's disease (Games et al., 1995). By using a neuron-specific transcription promoter to express the beta-amyloid's genetic code, the transgenic mice accumulated the excess beta-amyloid in the same regions of the brain that Alzheimer's patients do. Studies have shown the accuracy of the Alzheimer's mice as a disease model. Many of the cellular features of the degeneration seen in

patients are also found in the animal model. The study also more clearly defined the link between increased levels of beta-amyloid and apoptosis of the neural tissue; in fact the study proved that amyloid deposition by itself was necessary and sufficient for initiating the disease. More recently, the summer of 1999 (Schenk et al., 1999) used the Adams model to create the world's first Alzheimer's vaccine. This vaccine was able to remove preformed plaques, or to prevent their formation. The study showed that removal of preformed plaques could reverse neurodegeneration in mice. This provides hope that doing so in humans would also reverse the progress of the disease.

3.2.3 Inflammatory (Rheumatoid) Arthritis

Arthritis is a general term used to describe any of a number of specific autoimmune diseases in which one's own immune system attacks their body. This happens primarily in the sanovial joints and causes loss of movement and decay of the joint and surrounding tissue. Because of the nature of this disease, two types of transgenic mice are often used for subjects in research. The first type, are mice that express human Tumor Necrosis Factor Alpha (TNF-a) and/or Phosopholipase A2 (PLA2) (DNX Transgenic Animal, 1995). Both TNF-a and PLA2 play a part in the pathogenesis of arthritis, and as a result these mice develop the disease without experimental manipulation. For the most part, these animals are used to test new drugs or other pharmaceutical treatments previous to human trials. Rather than having genes inserted into the genome, the second type of mice that are commonly used for arthritis research, are knockout mice. These mice have had certain genes removed from their genome such that they have a compromised immune system. Often times these mice must be kept in a

sterile environment and are used by researchers to find the causes of arthritis and other autoimmune diseases.

3.2.4 Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy is a type of heart disease that causes a thickening of the heart muscle and is mostly found in young athletes. The disease causes difficulty for the blood to get into the ventricles of the heart and ultimately a difficulty for the heart to pump blood into the arteries. Although the disease's symptoms include shortness of breath, dizziness and chest pain, death is often the first sign that one has been afflicted by the disease. The cause of the disease is a mutation in a small portion of one gene that produces the protein myosin. It is unknown however why this production of myosin causes death. It does however appear that in people with this mutation the structure of the heart muscle is not aligned correctly, which might be a clue to the mystery. By creating a transgenic mouse strain with this same mutation a team of researchers at the University of Colorado at Boulder led by Professor Leslie Leinwand ("Transgenic Mice Created", 1998) hope to study this disease and unravel it's secrets. The team has also created a second strain of mice with a mutant gene for troponin T, a protein that is also suspected to play a part in the disease.

3.2.5 Sickle Cell Anemia

1982 was not only the year that the first human gene was successfully placed into a mouse, but it was also a great year in disease research. A pathology professor at the University of North Carolina's school of medicine by the name of Dr. Oliver Smithies,

began to wonder if it was possible to cure human genetic disorders by removing the defective gene, and replacing it with a functional gene. Dr. Smithies' area of interest was sickle cell anemia. He began to use mice in his experiments in the mid 1980's and was able to isolate the defective gene (Kicklighter, 1998). The only problem was, that he could not do it reliably with odds on the order of one in one million.

In the early 1990's Dr. Smithies and his colleague Beverly Koller had advanced their technique to the point that they could reliably study different diseases by knocking out the gene in mice that shields them from a certain disease. One such disease where they have had much luck is cystic fibrosis. They are still also involved in research for sickle cell anemia as well as high blood pressure.

Gene knockout research is one of the most promising outlooks using transgenic animals. Historically, if a mouse with a certain trait for disease were needed, researchers would have to look at mice until one was found. Now all that has to be done is to make the mouse that exhibits the trait that is needed for the particular disease you are studying.

More and more transgenic animals are constantly being created for research. Most major biotech companies have separate divisions just to handle the constant need for these designer animals. Newer and more impressively engineered animals are always being produced, and it is only a matter of time before a cure for a variety of diseases will be found.

CHAPTER 4 - ETHICS

4.1 Transgenic Patenting Issues (Oncomouse)

As in so many industries in this country, no sooner had the field of transgenics gotten off its feet when corporate industry made its appearance known. In 1972 a research microbiologist, Ananda M. Chakrabarty, at General Electric Co. created a strain of transgenic bacteria (Walter, 1999). This bacterium was engineered to contain genetic material from different strains of bacteria, which were combined into one new strain. The new strain was then capable of breaking down the ingredients of crude oil into harmless components, allowing for the bacteria to be placed at the site of an oil spill. The bacteria would decompose the crude oil and then die out when all the oil had been decomposed.

Since Chakrabarty's industrial product would consist of a microbe, anyone would be able to reproduce the bacteria, thus Chakrabarty sought to protect his creation by applying for a patent. His patent claims included the method of producing the bacteria, the **inoculum** that contains the bacteria, and the bacteria itself (Chakrabarty, 1981). The U.S. Patent and Trademark Office (PTO) had no reservations against issuing a patent for the method to create the bacteria, or for the inoculum that contained it, but on the claim to patent an organism they rejected the application. The PTO stated that microorganisms were a product of nature and therefore unpatentable. They also stated that under the Plant Patent Act of 1930 living things other than plants could not be patented. Chakrabarty appealed this decision to the Board of Patent Appeals and they reversed the first count, saying that due to the new combination of genetic material, the bacteria could not be found in nature, but they upheld the second objection, saying that under U. S. Patent Law

35 U.S.C. § 101 it was not patentable. Code 35 U.S.C. states that "whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title." Chakrabarty once again appealed the decision, this time to the U.S. Court of Customs and Patent Appeals, and the previous decision against Chakrabarty's patent application was reversed. The PTO then appealed the reversal of the decision to the U.S. Supreme Court, but the Supreme Court also found in favor of Chakrabarty. The close 5 to 4 vote in favor of Chakrabarty allowed him to patent the bacteria, and a patent was issued on March 31, 1981. The court ruled that human-made microorganisms could be patented and that congress intended patentable material to include "anything under the sun that is made by man" (Hughes, 1996). They did however, invite Congress to change the patent law to exclude living organisms, but their offer was not taken (Diamond, 1980).

This led to the attempt to patent something more complex than bacteria. In 1984, Standish K. Allen and his co-inventors applied for a patent for pacific oysters. These oysters became **polyploid** after hydrostatic pressure had been applied to them. This patent application however was denied on the grounds that the oysters occur naturally and do not satisfy the non-obviousness requirement. Allen then appealed the decision to the Board of Patent Appeals and Interferences. His patent application was once again denied, but the board only denied it due to obviousness and not because oysters were naturally occurring. U. S. Patent Law states in code 35 U.S.C. § 103 that an invention must not be obvious in order to be patented. The PTO however made no comment on the actual patentability of oysters or other non-human organisms. The PTO then made a

public announcement on April 7, 1987 saying that they would now consider non-naturally occurring non-human multi-cellular living organisms, including animals, to be patentable subject matter. This announcement made it clear that genetically engineered non-human animals could now be patented (Walter, 1999).

In April of 1988 the first patent was then passed for such an animal. Created by Harvard University scientists, the Oncomouse is a transgenic mouse with an inserted human cancer gene, which made it very useful in cancer research. The process for making such a mouse was successfully patented, giving the rights to create such animals solely to Harvard University and E. I. Du Pont Nemours & Company were given exclusive licensure ("Who owns this", 1998).

The patent on Oncomouse is very broad however. The patent covered any "transgenic non-human eukaryotic animal whose germ cells and somatic cells contain an activated oncogene sequence introduced into the animal, or an ancestor of the animal, at an embryonic stage"(Leder et al., 1988). Thus the patent not only applies to the mouse and its offspring, but to any mammal that has had cancer genes inserted into its genome during the embryonic stage. As a result this monopolizes the entire subclass of animal rather than just one species of animal. This patent includes any animal that can be made with the more than forty known cancer-causing genes.

This was the first patent awarded on a multicellular organism, and caused an uproar with members of the scientific community. Up until then the ability to patent another living animal was unheard of and generally thought to be unethical. Because these animals were now patentable this gave companies, universities, and individuals the ability to completely monopolize entire species and/or subspecies. Having the first patent

on such an animal would be the equivalent to patenting the first light bulb or steam engine, and there is much money and power that could be made. The author Leon Kass says, "It is one thing to own a mule; it is another to own mule."

Once the patent was passed in the US, the European Patent Office (EPO) also passed the patent in 1992 under some pressure from members of the international scientific community, after initially rejecting it in 1989. After the patent was finally passed by the EPO there was some resistance from 17 European groups including animal rights campaigners, environmentalists and religious groups who thought that a patent of this caliber should not be granted because of a clause in the European Patent Convention which states "...a patent cannot be granted if it is contrary to morality". After hearing the arguments, the EPO advised the Harvard team to revise the claims of the patent. One of these redrafted claims turned out to be of significant importance to the group. Upon redrafting, the new claim states that the patent is only valid for rodents, whereas the earlier draft was applicable to any non-human animal. This was a somewhat major blow to the Harvard team, seeing as how now they could only license out their technique on rodents rather than for complete comprehensive use such as in the US patent ("Oncomouse trial ends", 1995).

A few more incidents of interest have occurred recently as well. After an outcry from many scientists regarding concerns about the rush to patent DNA segments, on Dec. 21, 1999, the PTO proposed a new set of guidelines ("Revised Interm Guidelines", 1999). The new proposed guidelines will affect the way in which examiners look at the function of the invention (DNA segment in this case) and whether or not it serves a particular practical purpose. Within these new guidelines the examiners will be forced to take a

closer look at "specific and substantial utility." The numerous companies that are submitting up to 6,000 applications for DNA sequences brought on this change.

There are many in the scientific community who are opposed to the idea of patenting these segments and believe that the genetic information should be freely available. Many are "theologians" who don't like the idea of patents in this field of study and believe that the new guidelines haven't gone far enough to prevent the patenting of these segments. By patenting these segments they believe that other researchers could be deprived of tools that could prove to be essential in the future of medical research (Slind-Flor, 2000). The PTO is accepting written comments on the new guidelines until March 22, 2000, after which it will make a final decision on whether they will pass.

Also in the news is the patent that has been granted to the researchers who cloned Dolly the sheep. This patent, given on January 20, 2000 to Geron Bio-Med, will hold for 17 years and covers the nuclear transfer technology, used as well as any animals that it produces ("Dolly cloning method", 2000). Although this is technically not a transgenic issue, it does show how yet another major bio-medical technique has been patented. Critics do argue that this patent in particular gives the American owned company too much power, a claim that Geron Bio-Med denies.

4.2 Ethics of Transgenics

Ethics is defined in Webster's dictionary as follows:
-The discipline dealing with what is good and bad and with moral duty and obligation
-The set of moral principles or values,
-A theory of moral values,
-The principles of conduct governing an individual or group.

There are two basic ethical questions that we must consider on the topic of whether or not it is right for transgenic research to be performed. The first question need

not apply to transgenic animals, it is simply the question of whether or not it is ethically all right to use an animal in a way that may cause it harm or suffering, possibly even death to better our quality of life. The second question applies to whether or not it is all right for human beings to tamper with the "natural order of things" by altering an organism's genetic make up.

A popular stance on the use of animals in research is that it is regretful to have to use animals and whenever possible it should be avoided. However when it is necessary the animals should be treated as humanely as possible. This opinion is one that is morally sound; the trouble lie's in deciding when it is necessary to use animals. It is clearly not acceptable to use them in an experiment where no new or useful information will be provided to a researcher. However such clear decisions are not always present. For example, if the use of animals in research will expedite the outcome of an experiment that can be done without animals should this type of use be condoned? Normally the answer would be no, and it would not be condoned, however what if the research were for a drug that could save the lives of people?

There are many sets of guidelines for the ethical treatment of lab animals, and when it is acceptable to use them in research. The trick however, is to find a set of standards that represents the median opinion of today's society. That is to say that in some cases many guidelines go too far and vastly hinder science while in others they do not go far enough resulting in the cruel treatment of lab animals. One of the better sets of guidelines we have found is published by the Canadian Council on Animal Care (CCAC), these guidelines (which follow) do not vastly hinder scientific advancement, nor do they

result in prolonged cruelty to animals. They also represent a fairly close approximation of the ethical median of society.

1.If animals must be used, they should be maintained in a manner that provides for their physical comfort and psychological well being, according to CCAC's policy statement on Social and Behavioral Requirements of Experimental Animals.

2.Animals must not be subjected to unnecessary pain or distress. The experimental design must offer them every practicable safeguard, whether in research, in teaching or in testing procedures; cost and convenience must not take precedence over the animal's physical and mental well-being.

3.Expert opinion must attest to the potential value of studies with animals. The following procedures, which are restricted, require independent, external evaluation to justify their use:

I. Burns, freezing injuries, fractures, and other types of trauma investigation in anesthetized animals, concomitant to which must be acceptable veterinary practices for the relief of pain, including adequate analgesia during the recovery period;

II. Staged encounters between predator and prey or between conspecifics where prolonged fighting and injury are probable.

4.If pain or distress is a necessary concomitant to the study, it must be minimized both in intensity and duration. Investigators, animal care committees, grant review committees and referees must be especially cautious in evaluating the proposed use of the following procedures:

a. Experiments involving withholding pre and post-operative pain-relieving medication;

b. paralyzing and immobilizing experiments where there is no reduction in the sensation of pain;

c. electric shock as negative reinforcement;

d. extreme environmental conditions such as low or high temperatures, high humidity, modified atmospheres, etc., or sudden changes therein;

e. experiments studying stress and pain;

f. experiments requiring withholding of food and water for periods incompatible with the species specific physiological needs; such experiments should have no detrimental effect on the health of the animal;

g. injection of Freund's Complete Adjuvant. This must be carried out in accordance with CCAC Guidelines on Acceptable Immunological Procedures.

5.An animal observed to be experiencing severe, un-relievable pain or discomfort should immediately be humanely killed, using a method providing initial rapid unconsciousness.

6.While non-recovery procedures involving anesthetized animals, and studies involving no pain or distress are considered acceptable, the following experimental procedures inflict excessive pain and are thus unacceptable

a. utilization of muscle relaxants or paralytics (curare and curare-like) alone, without anesthetics, during surgical procedures;

b. traumatizing procedures involving crushing, burning, striking or beating in un-anesthetized animal

7.Studies such as toxicological and biological testing, cancer research and infectious disease investigation may, in the past, have required continuation until the death of the animal. However, in the face of distinct signs that such processes are causing irreversible pain or distress, alternative endpoints should be sought to satisfy both the requirements of the study and the needs of the animal.

8.Physical restraint should only be used after alternative procedures have been fully considered and found inadequate. Animals so restrained must receive exceptional care and attention, in compliance with species specific and general requirements as set forth in the Guide.

9.Painful experiments or multiple invasive procedures on an individual animal, conducted solely for the instruction of students in the classroom, or for the demonstration of established scientific knowledge, cannot be justified. Audiovisual or other alternative techniques should be employed to convey such information.

There are currently many projects in existence that make adhering to these guidelines easier. Such projects include the "Virtual Frog" so that students can get an understanding of an animal without the need for an animal to have died. One of the best places to find such information is at a website furnished by The Norwegian Reference Center for Laboratory Science & Alternatives. Their website contains over 4300

alternatives to animals form working models to overhead projector sheets. http://oslovet.veths.no/NORINA/titles.html

The next question is whether or not it is ethically sound to genetically engineer organisms. This question seems easier to answer because there is no harm implied in genetic engineering. However, there are many questions concerning the morality of genetic engineering. One of the questions arises when organisms are altered to be susceptible to diseases they are normally immune to. This is answered reasonably well in the above CCAC guidelines, however the ethical question of whether or not it is acceptable to alter the "natural order of things" is omitted.

The basis for many people's morality is a result of their religions beliefs. Because of this it is no surprise that the opinion of the world's religious leaders plays a large part in shaping the ethical view of society. There are far too many religions in the world to explore all of their feelings on genetic engineering and transgenic organisms so discussion will be limited to two, Judaism and Christianity.

Judaism opposes transgenic organisms as they pertain to food. There are rules of cleanliness, which must be adhered to, in the orthodox Jewish faith. These rules state that you may only consume food that is kosher. Kosher food is clean food as specified by the torah, the scripture that is the basis for the Jewish faith. If DNA from a non-kosher source is placed into the genetic make up of a kosher food then that food becomes non-kosher. This ruling is generally agreed upon in orthodox circles, however most reformed Jewish sects look upon genetic alteration less severely. Reformed sects represent a larger percentage of the Jewish community in this country.

In the Christian faith the dislike of genetically altered substances goes to a deeper level than rules of cleanliness. In the Christian scripture it is said that the role of Satan is to oppose or go against the beauty of God. It is believed by some members of the Christian faiths, that changing God's creations via genetic engineering, goes against god's beauty. When taken to extreme, this makes genetic engineering the work of Satan.

The view of the population however, is not generally as extreme as this. Rather, they are useful in understanding the thoughts and beliefs that help to fuel some of the criticism of transgenic research. Another element that plays a part in the view of the populous is fear, the general population does not always understand the benefit of what science is doing, or how it is doing it, and therefore is often afraid of it.

The pro stance on genetic engineering is founded in beliefs of societal benefit. There is no dominant religious sect in favor of genetic engineering, save for perhaps science. The belief that one can genetically alter a species and learn something to better humankind without putting ourselves in danger is the strongest moral argument in favor of genetic engineering.

Those in favor of genetic engineering are however also knowledgeable in the fact that a set of guidelines as to how to apply genetic knowledge are needed to prohibit abuse of the science. And similarly to before, we need to find a set of criteria which do not hinder the advancement of science, or prohibit unbeneficial use of genetic technology. Once again we found the best set of guidelines to be published by the CCAC to a sub committee The Animal Care Comity (ACC):

1. **Investigator and Animal Care Committee Responsibilities**
    a. **Education**

It is the responsibility of the ACC to ensure that all its members are informed about the ethical and technological aspects of transgenic animal use. A suggested reading list is attached. It is also recommended that researchers applying for ACC approval to create or use transgenic animals be conversant with ethical concerns surrounding the use of these animals, and be prepared to justify their work as being in the public interest.

b. **Proposals to create new transgenic strains**

i. Standard procedures for creating transgenic animals can be dealt with by ACC's according to their usual practices for surgical procedures.

ii. In reviewing applications for creation of novel transgenic animals, ACC's should determine that:

- The investigator has competent technical assistance and experience in the necessary record-keeping for breeding colony maintenance;
- Arrangements for surgical procedures, colony housing and maintenance, have been discussed with and approved by the local Animal Facility Management;
- the investigator and the technical staff involved in daily monitoring of the transgenic colony are familiar with signs of distress in the species of study;
- a frequent, reliable, thorough, and documented monitoring system is in place to detect behavioral, anatomical and physiological abnormalities indicative of animal distress; and
- endpoints for survival are clearly defined.

Standard operating procedures (SOPs) can be developed to deal with these concerns.

iii. Proposals to create or use transgenic animals should include information about expected phenotype (as indicated in the Appendix), to include information about anticipated pain or distress levels in the transgenic animal, measures which will be taken to alleviate such distress, and the required monitoring system.

iv. Proposals to create novel transgenics initially should be assigned CCAC category of invasiveness level "D". If approval is merited, it should be provisional, limited to a 12-month period, and subject to the requirement that the investigator report back to the ACC as soon as feasible on the animals' phenotype, noting particularly any evidence of pain or distress.

After receiving the report from the investigator, the ACC may confirm approval of the proposal and adjust the level of

invasiveness. However, if the animals are noted to be suffering unanticipated pain or distress, the ACC will ask the investigator to provide a revised protocol which will minimize and alleviate distress, and will reconsider its approval of the proposal.

c. **Proposals to utilize existing transgenic strains**
   i. A proposal on transgenic animals may have two parts: creation of the transgenic animals, and subsequent experimental manipulations of the animals. Except where subsequent manipulations are restricted to observation and euthanasia of the transgenic animal, creation and use proposals should be considered as separate proposals.
   ii. In reviewing use proposals, ACC's should consider whether procedures regarded as acceptable in non-transgenic animals, are still acceptable in transgenic animals where altered phenotype may impose additional stresses.
   iii. Proposals to use existing transgenic strains should also include the information requested in the Appendix.

d. **Accounting**
   i. Estimates of all animals to be used or generated in a transgenic study should be stated in proposals to the ACC, listed by use category (e.g., oocyte donors, pseudopregnant females, male "studs", successful transgenics, etc.)
   ii. When completing the *Animal Use Data Form* for reporting annual animal usage to CCAC, investigators should identify transgenic animals separately from non-transgenic animals in the "Species" column.
   iii. To reduce overall animal use, CCAC encourages, when appropriate, assignment of non-transgenic animals, bred in a transgenic creation procedure, to other ACC-approved protocols. Asymptomatic heterozygotes must be clearly identified and should only be used for breeding purposes when the investigator is aware of their altered genotype. Accounting procedures within animal facilities must prevent double counting of such transferred animals in annual use statistics.

e. **Containment**
   i. All proposals for creation or use of transgenic animals must assure the ACC that risks to human health and the environment are

minimized to an acceptable level. For transgenic animals created using microinjection or replication-defective viruses, the containment risks are limited to those associated with the escape of the animal and interbreeding with wild stocks. Proposals should include information about:

- containment and security procedures in animal facilities and, if applicable, during transportation when importing the animal;
- plans for recapture should a breach of containment occur; and
- the consequences to human health or wild populations should containment fail.

ii. For commonly used transgenic species, each animal facility should have SOPs for containment, which can be referenced by proposals.

iii. ACC's should discuss with the institutional Biohazard Committee any proposal, which raises biohazard containment concerns.

f. **Other regulations**

i. ACC approval of a proposal does not relieve the investigator of responsibility to satisfy the regulations of any other governmental agencies. For example, creation of any transgenic fish strain requires approval of the Department of Fisheries and Oceans. Biohazard approval may also be required for some proposals.

2. **Responsibilities of CCAC**

a. **Education**

To update at least every two years a reading list on ethical and technical aspects of transgenic animal use, which can be distributed to members of ACC's, this list to include articles appropriate for all members.

b. **Accounting and reporting**

To include in its annual animal usage statistics separate totals for transgenic strains of each species used in experiments.

It is the belief of this group that animal testing should be kept to a minimum, only done when no acceptable alternative exists. That no organism should be subject to genetic

alteration simply because science has the techniques at it's disposal. The ability to alter genetic make up is a powerful and dangers tool. One that humanity needs to wield with extreme caution but one that it cannot afford to simply disregard.

CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS

After much research, and the process of writing this paper, we the authors have come to the following conclusions.

Fist and foremost we believe that the public awareness of transgenic issues needs to be raised. We must increase the level of knowledge of society regarding transgenic research such that the public can make an educated decision about how the field should further progress if at all. When society comes across something that they don't understand they begin to fear it, which then leads to irrational conclusions. It is out belief that an educated public will have its fears assuaged, and desire the continuation of transgenic research.

Next, we believe that the uses of transgenics should be further explored as it relates to disease research. Disease models are already extensively used in medical research. Other uses for this technology do exist however; for example, the use of genetically altered bacteriophages as a means of specifically eliminating human bacterial pathogens. Although this type of usage is in its infancy the sheer prospect of such a thing existing is a major breakthrough.

Although numerous transgenic disease models are already in use we do believe that these models could be improved such that the traits of the animals more accurately emulate human characteristics. With a more accurate model fewer animals will be needed to test and the results of the tests would be more accurate and consequently speed up much of the research.

Finally, we must decide whether the ends justify the means. Is the advancement of human knowledge worth the suffering inflicted upon the subjects of the research? The answer that we have arrived at is that if the 'ends' significantly better the quality of life of the human race as a whole, then the suffering of the organisms is unfortunate but justified. These genetically engineered animals would not have existed in nature, but were created only for the purposes of research. As a society with the unique means of inflicting our will upon others we find ourselves using, with some regret, lesser organisms to better our own existence. This research does in fact justify their existence as well as their suffering. Perhaps as this science progresses, new ways will be found to maximize potential medical benefits while minimizing any animal suffering.

GLOSSARY

Blastocysts – Immature embryos that have had 2-3 days to develop and divide

Blunt ends – DNA fragments in which each strand of the helix is the same length at both termini

Bursa – Thin transparent membrane containing blood vessels that surround the oviducts and ovary

Construct – A transgene cassette that has been flanked by homologous sections of the recipients own DNA in preparation for embryonic stem cell injection.

Embryonic stem (ES) cells – One type of cell that makes up the blastocyst. These cells have the potential to make all the tissues in the body

Electroporation – Opening the pores on the surface of a cell by exposing it to an electric current in order to allow foreign material (i.e. DNA) into the cell

Enhancers – Gene sequences located upstream of a transgene in order to enhance it's expression

Founder – The animals that begin a new transgenic line

Homologous Recombination – The natural process within a cell, in which one series of genetic material shuffles into the genome and an equivalent portion is pushed out

Hybrid Polyploid - The result when two complete genomes from different organisms are allowed to merge together

Inoculum - Material used to introduce a pathogen (i.e. virus) or antigen into a living organism

Intraperitoneally – Injected directly into the peritoneal cavity

Mosaic Animal – An animal that does not have a single genome throughout its entire body.

Oncomouse – The first patented transgenic animal, which contained activated oncogenes that caused the mouse to be more susceptible to cancer.

Organelle Targeting Sequences – A genetic sequence that directs protein synthesis into specific metabolic pathways

Palindrome – A DNA fragment that reads the same in one direction as it does in the other

Polyploid – An organism containing more than one set of genetic material

Pronuclear – The period immediately after fertilization prior to fusion of the male and female nuclei

Promoters – Genetic sequences that direct mRNA synthesis to occur in specific tissues

Restriction enzymes – Enzymes that cleave DNA at a specific sequence of nucleic acids

Restriction site – The site at which a restriction enzyme cleave a DNA strand

Reverse transcriptase – An enzyme that creates a DNA strand out of an RNA template

Signal Sequences – A specific gene sequence that aids in directing protein synthesis into specific metabolic pathways

Sticky ends –DNA sequence where one side of the duplex is longer than the other, usually by 2 - 4 bases

Subcutaneous – An injection directly beneath the skin

Transformation – The process of entering DNA vectors into a host cell.

Transpharming – Genetically engineering another organism to produce a protein from another species in the milk

Vector - a circular DNA molecule into which an expression cassette can be added and then reproduced by a suitable organism

Xenotransplantation – Cross species transplantation

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