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TRANSGENIC ANIMALS

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ABSTRACT

Through advancements in biotechnology, scientists now have the ability to genetically manipulate living organisms to possess genes from other species. Such techniques have since resulted in transgenic animals being created for multiple applications including the observation of disease pathways and testing treatments, drug production, food production, creation of organs for transplantation, and observation of biological processes. Unfortunately, the act of altering an animal's genome and then using the animal for testing raises many ethical and legal issues, including animal welfare, environmental safety, and public safety. The debates also focus on whether these altered animals can and/or should be patented, and whether such patenting helps or hinders medical research. Overall, this project reviews these topics, and the author concludes that despite negative connotations, these animals can be extremely beneficial to society in many aspects.

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PROJECT OBJECTIVES

The objective of this project was to discuss transgenic animals and their effects on society. Chapter-1 focuses on the specifics of techniques used to create transgenic animals and how to screen the animals for the presence of the transgene. The purpose of Chapter-2 was to explain the different applications that transgenic animals have been used for, and introduce examples of each category. Chapters -3 and -4 concern the ethical issues and legalities, respectively, of transgenesis. The latter two chapters finish with the author's opinion on whether certain applications should be discontinued, and if it is acceptable to patent animals.

CHAPTER-1:
TRANSGENIC ANIMAL TECHNOLOGY

Before the advent of transgenic technology, to study genetic changes in animals, scientists were forced to induce random mutations in their genome to alter the animal's or their offspring's characteristics. But with key advancements in the field of biology in the 1970s, scientists were able to genetically modify living organisms purposefully, to give them new properties to help society. The purpose of this chapter is to briefly describe the technology for creating transgenic animals.

Cloning the Transgene

By definition, a transgenic animal is an animal that possesses a foreign gene purposely incorporated into its genome (Transgenic Animals, 2011). The first step in creating a transgenic animal is to clone the gene that will be inserted in the animal, termed the *transgene*. The breakthrough experiments that allowed manipulating genes included the ability to cut DNA at specific sites and the ability to clone it into vectors. DNA can be cut at specific sequence sites using restriction nucleases. These enzymes are naturally used by bacteria to cleave invading viral DNA to inactivate it. Scientists use restriction enzymes to cut DNA at specific sequence sites creating a DNA fragment containing a gene of interest. Alternatively, scientists can amplify specific short segments of DNA using polymerase chain reaction (PCR) (discussed later in this chapter). This process was discovered in 1986 by Kary Mullis, who in 1993 earned a Nobel Prize in chemistry (Mullis et al., 1986). In PCR, short primers designed to flank the gene of interest are hybridized to the DNA, and a special type of DNA polymerase is used to synthesize DNA from the primers. Another breakthrough technology of the 1970's was the ability to ligate DNA fragments into cloning vectors, such as plasmid DNAs or viruses. These vectors are used

to amplify the DNA, making thousands or millions of copies of it for later insertion into the animal's genome (Charles River Laboratories, 2005).

The world's first transgenic *organism* was an *E. coli* bacterium transformed with plasmid DNA (Cohen et al., 1973). This technology was extended into animals shortly after in 1974, with the first transgenic *animal*, a mouse containing SV-40 viral DNA fragments (Jaenisch and Mintz, 1974). However, the SV-40 transgenes in this case were not actually expressed in the animal. The first transgenic animal *expressing* its transgene was a mouse containing a cloned growth hormone gene under the control of a metallothionein promoter (Palmiter et al., 1982). Later, the transgenic techniques evolved into manipulating embryonic stem cells, creating new species of transgenic animals other than mice, and infecting embryos with retroviruses.

Pronuclear Microinjection

Once the transgene has been cloned, it has to be inserted in the animal's DNA. Currently, there are two main ways for creating a transgenic animal: pronuclear microinjection and embryonic stem cell manipulation (Charles River Laboratories, 2005). Depending on the type of research being done, one technique may better suit the investigator's needs.

With pronuclear microinjection, female egg donors are given injections of pregnant mare serum gonadotropin and/or human chorionic gonadotropin that cause her to release three to four times as many eggs (super-ovulate). The eggs are either fertilized *in vitro*, or *in vivo* by males (LATG, 2010). When fertilization occurs *in vivo*, the fertilized eggs can be taken by euthanizing the female and removing her oviducts (Charles River Laboratories, 2005). Eggs taken early in development will contain the male and female pro-nuclei that have not yet fused. The cloned transgene solution is microinjected into either one or both of the pronuclei using an extremely fine glass pipette (LATG, 2010). The male pro-nucleus is usually chosen for microinjection due

to its slightly larger size and its close proximity to the periphery of the egg. After the pro-nuclei fuse into one nucleus, producing the zygote, the injected embryos are implanted into a pseudo-pregnant foster mother (Transgenic Animals, 2011). The pseudo-pregnant foster mother (recipient female or surrogate mother) is made pseudo-pregnant by either injecting her with hormones, or by mating her with vasectomized males which causes a false pregnancy and allows the uterus to receive the egg. The process stimulates the reproductive system, preparing the females' bodies for implantation of the embryos. Pseudo-pregnant females prepared by mating are identified by the presence of a copulatory plug.

When making a transgenic animal by pronuclear microinjection, the injected DNA is randomly incorporated into the animal's genome, which can harm the animal if the insertion location inactivates a necessary gene, or if the insertion activates a cancer-causing oncogene (LATG, 2010). In addition, the transgene can insert in an inactive area of the chromosome and not be expressed, or can insert multiple times in an active area of the chromosome and become highly expressed, so founder animals vary widely in their properties. The inserted sequences can vary in length, and may be expressed in either somatic cells, germ cells, or both, but chimeric animals are produced less frequently than when manipulating embryonic stem cells. For these reasons, transgenic pups are screened for presence and expression of the transgene (discussed below). Figure-1 shows an approximate timeline for the creation of a transgenic mouse line by pro-nuclear microinjection. Overall, it takes around twenty-three weeks to begin a colony of the transgenic founder animals.

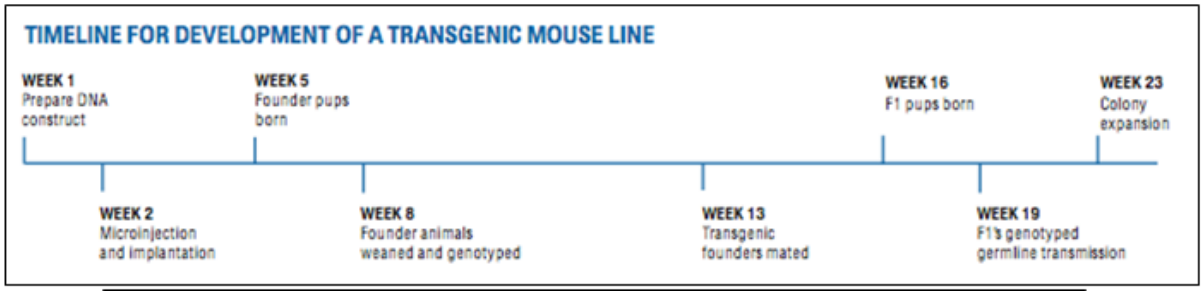

Figure-1: Approximate Timeline for the Development of a Transgenic Mouse Line Using Pronuclear Microinjection. The entire process takes about 23 weeks, beginning with the cloning of the transgene in week-1, and the microinjection of the cloned DNA into the pro-nucleus in week-2. (Charles River Laboratories, 2005)

Embryonic Stem Cell Manipulation

The second main method for creating transgenic animals is by the manipulation of embryonic stem cells. The advantage of using this technique is it allows the transgene to be targeted to a specific location by replacing a host gene or host DNA domain with the transgene (LATG, 2010). Embryonic stem cell manipulation, like pronuclear microinjection, begins by creating the desired DNA construct, but in this case the construct can contain sequences homologous to the host chromosome to facilitate crossing over. Figure-2 shows an example of a vector used for homologous recombination. The vector includes segments of the target chromosome (blue in the diagram), the transgene (red), a gene encoding neomycin resistance (neo^r, green), and a gene encoding thymidine kinase (*tk*, purple). Note that the regions of homology with the animal's chromosome flank the transgene and the neo^r gene, while the *tk* gene lies outside of the homologous region. Neo^r inactivates the antibiotic neomycin and its analogs like G418. Thymidine kinase is an enzyme responsible for adding phosphate groups to

the drug gancyclovir, thus activating it. These genes facilitate selection of cells that have correctly incorporated the transgene (Transgenic Animals, 2011).

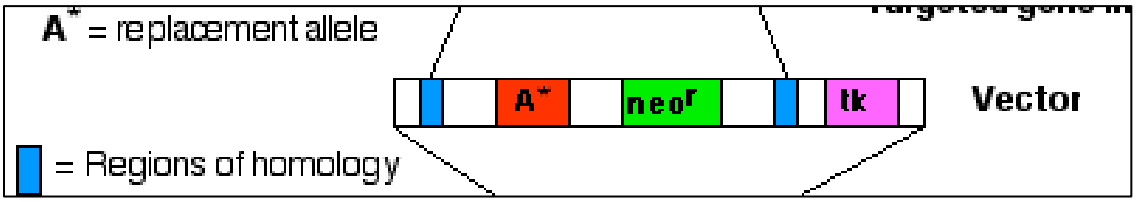

Figure-2: Diagram of Vector Created for Embryonic Stem Cell Manipulation by Homologous Recombination. Note that the vector contains regions of DNA homologous to the host chromosome (blue), the cloned transgene (red), a neomycin-resistance positive selection marker (green), and a thymidine kinase negative selection marker (purple). (Transgenic Animals, 2011)

The embryonic stem (ES) cells used for this process are taken from the inner cell mass (ICM) of a three-day old blastocyst embryo. The blastocyst is created by *in vitro* fertilization (IVF), and then grown 3-5 days to make a hollow ball of cells containing ES cells (LATG, 2010). At this stage of life, ES cells are called pluripotent. Pluripotent cells have the ability to differentiate into any type cell. The ES cells are isolated and grown into an ES cell line, then the transgene DNA is introduced by transfection or electroporation (Charles River Laboratories, 2005). Transfection uses chemical agents to allow the movement of the DNA into the ES cells eliminating the problem of negatively charged DNA particles trying to pass through a negatively charged or hydrophobic membrane (Promega.com). Alternatively, electroporation involves "shocking" the cells with an electric pulse, thus disturbing the membrane of the cell and allowing the DNA to pass inside (Purves et al., 2001).

Once the DNA has entered the ES cells, homologous recombination recombines the transgene with the target location in the host chromosome. During homologous recombination, identical regions in the cloned construct find the corresponding sequences in the host's genome

and the DNA is replaced (Figure-3). Culturing the cells in a medium containing the drugs G418 and gancyclovir will reveal which cells incorporated the DNA. The incorporated *neo*^{r} gene acts as a "positive selection marker". Cells correctly taking up the transgene will contain *neo*^{r} and will be resistant to G418, while cells lacking the transgene and *neo*^{r} are killed by G418 (Charles River Laboratories, 2005). Thymidine kinase acts like a "negative selection marker". TK will be present in cells not undergoing homologous recombination, but will be absent in cells correctly incorporating the transgene by homologous recombination. So cells correctly containing the transgene by homologous recombination will be resistant to both G418 and gancyclovir (diagram upper), while cells containing the transgene and TK randomly will be resistant to G418 but killed by gancyclovir (diagram lower) (Transgenic Animals, 2011).

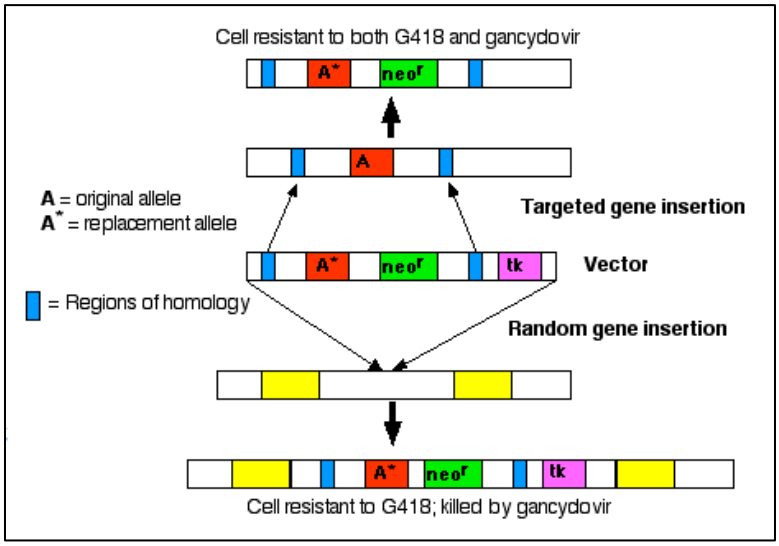

Figure-3: Diagram of the Difference Between Homologous Recombination and Random Insertion. Insertion of the transgene by homologous recombination (diagram upper) inserts the neo^r gene, but not thymidine kinase. Random insertion of the DNA (diagram lower) brings both neo^r and TK. (Transgenic Animals, 2011)

Once ES cells have been identified as correctly containing the transgene, the ES cells are injected into the inner cell mass of a blastocyst (Transgenic Animals, 2011), and the embryo implanted as described for pronuclear manipulation (LATG, 2010). Because only the injected ES cells contain the transgene and the remainder of the inner cell mass is normal, only some cells of the offspring are transgenic. This creates *chimeric* animals in which only some tissues are transgenic, so these animals need to be further bred with other transgenics to produce fully transgenic offspring. Figure-4 shows a timeline for developing a transgenic mouse by manipulating ES cells. This process usually takes around 27 weeks, which is slightly longer than pronuclear microinjection due to the time taken to screen the ES cells prior to injection into a blastocyst.

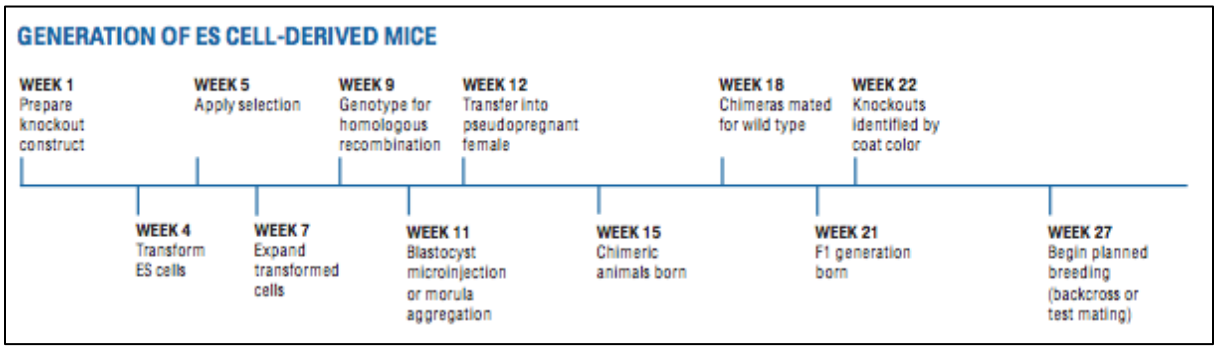

Figure-4: Timeline for the Development of a Transgenic Mouse by Manipulating Embryonic Stem Cells. The process takes approximately 27 weeks, including the time taken to screen the ES cells for the presence of the transgene. (Charles River Lab, 2005)

Both pronuclear microinjection and the manipulation of ES cells have contributed a large amount to the efficiency and reliability of creating transgenic animal lines. The evolution of this technology has created many opportunities to study the biological universe and continue research from medicine to agriculture to industry (Margawati, 2003). Disease models, food sources, and

transpharmers are just a few types of transgenic animals currently being used to further advance the field of biotechnology (LATG, 2010).

Identifying Transgenic Positives

The process of making transgenic animals is not efficient, and most pups born are not transgenic. To identify which pups have taken up the transgene in their genomes, they are usually screened by polymerase chain reaction (PCR) or Southern blots. Usually a very short section of tail section is cut off or an ear punch taken, and the DNA is isolated by phenol extraction. Crude DNA samples can also be prepared by alkaline lysis, but this process is less accurate (LATG, 2010). Other DNA may also be extracted from the animal's blood, saliva, or hair bulbs. In cases where polymerase chain reaction is used to analyze the DNA only a minimal amount of DNA is needed, such as DNA from ear punches. When Southern blots are used to genotype the animal, more DNA is required like with a tail clip (IACUC, 2009).

The process of PCR (Figure-5) copies DNA strands *in vitro*. The first step of the process is to separate the two strands of the DNA's double helix, leaving two halves of DNA to serve as templates for the copies (diagram, left side). Every piece of DNA consists of nucleotide bases containing adenine, cytosine, guanine, and thymine. Adenine is always paired with thymine, and cytosine is always paired with guanine. After separating the original strands of DNA, the primers anneal to regions flanking the transgene. The primers are designed to have sequences complementary to domains on each side of the transgene. The primers allow a special DNA polymerase to attach to the DNA and synthesize DNA from nucleotide precursors in the reaction tube (diagram, second column). When the DNA strand is complete, the process can be repeated to create more copies of the synthesized DNA (diagram, third column). As the number of cycles of DNA denaturation, primer annealing, and stand synthesis continues, the DNA is amplified

exponentially. For example, by the fourth round there are sixteen copies of the DNA region of interest (PCR, 1992). When screening potential transgenic positives, DNA samples amplified by the PCR reaction into visible "amplicon" bands on an electrophoresis gel are scored as positives. Screening DNA by PCR is sensitive and fast, but is prone to contamination, so when sufficient DNA is present Southern blots are sometimes used.

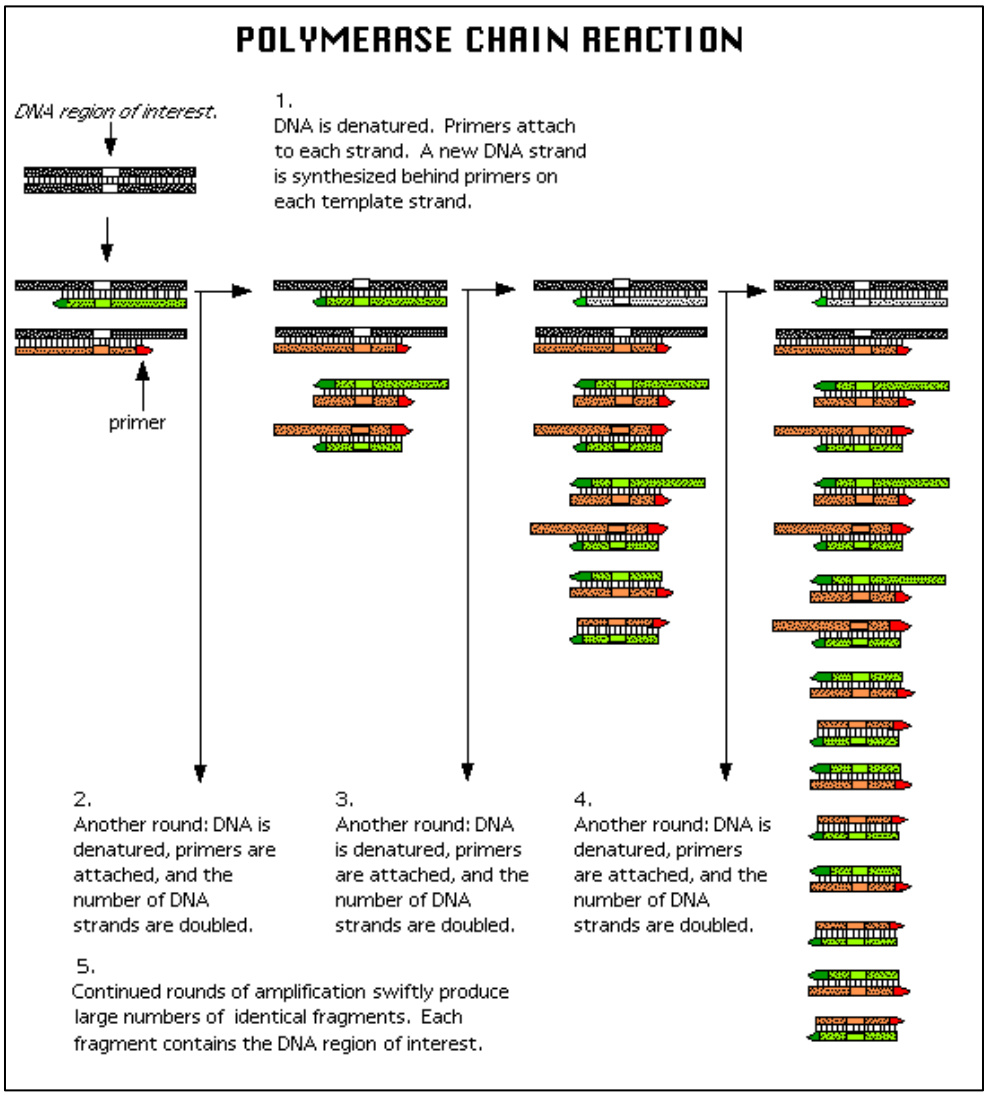

Figure-5: Diagram of Polymerase Chain Reaction. This process is used to amplify a segment of DNA containing the transgene. It can be used to initially clone the transgene, or to screen potential transgenic pups. (PCR, 1992)

When a larger amount of DNA is available, Southern blots can be used to genotype the animal (Figure-6). The technique begins by cutting the DNA into fragments with restriction endonuclease enzymes and separating them by size using an agarose gel and electrophoresis (diagram, upper left). During electrophoresis, a charge is place across a gel, and the DNA added to the end nearest the negative cathode. Because DNA is negatively charged (due to the presence of phosphate residues), it moves towards the positive anode. Smaller fragments move faster than larger fragments, so this separates the fragments by size (Cold Spring Harbor Lab).

The DNA fragments in the gel are then transferred to a membrane (diagram, upper center and right), which allows hybridization of a labeled DNA probe complementary to the transgene to the membrane. DNA fragments containing the transgene hybridize to the labeled probe, and these fragments are visualized using x-ray film or by chemiluminescence (diagram, left, shown as a green band (Southern blot, 2009). DNA samples hybridizing to the labeled transgene probe are scored as transgenic positives.

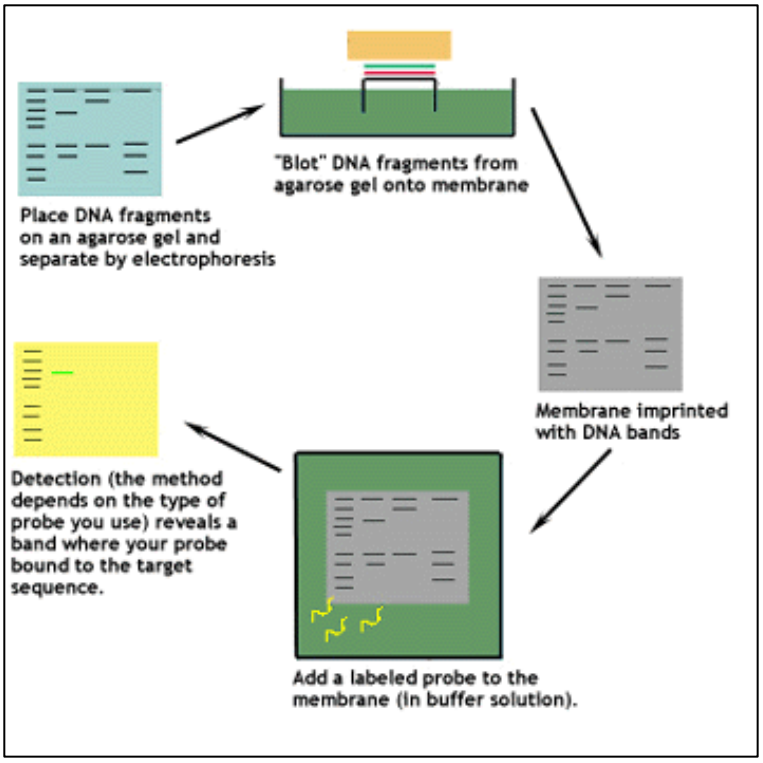

Figure-6: Diagram of a Southern Blot. DNA is cut into fragments using restriction nucleases and separated by size using electrophoresis (upper left). The DNA is then blotted to a membrane (diagram right) and hybridized to a labeled probe complementary to the transgene (lower and left) to identify bands containing the transgene. (Southern Blot, 2009).

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CHAPTER-2:
TRANSGENIC APPLICATIONS

Since the discovery of transgenesis, knockin animals, knockout animals, and animals created by random mutation have evolved to assist researchers in many different applications. The type of transgene inserted, the type of mutation it may contain, the type of gene promoter used to drive expression, and the animal species used depends highly on the scientific concept being explored. It is believed that all mammals have approximately 30,000 genes; however, some species' genomes are easier to manipulate. Mice, for instance, have become a "premier model," due to the ease of genetically modifying them (LATG, 2010). The start of engineering organisms began in the 1970s with *in vitro* manipulation of a bacterium (Cohen et al., 1973), and has advanced to make a wide variety of animals, ranging in size and classification from mammals, to reptiles and fish). In addition to serving as models of many biological conditions, transgenic animals are used for the production of pharmaceuticals in an animal's milk, transplant organs, and increasing food production. This chapter discusses the applications for transgenic technology, and provides examples for each application.

Disease Models

The first use of transgenic animals that will be discussed in this chapter is the disease model. Studies performed to observe disease pathways or to screen potential therapeutics are typically done by creating transgenic animals and/or gene targeted animals. Developed models include, but are not limited to, Parkinson's disease, cancer, Alzheimer's, AIDS, and sickle cell anemia. For the purpose of this review, AIDS mice, cancer mice, and Alzheimer's disease mice will be discussed.

AIDS Mice

Human immunodeficiency virus (HIV-1; the AIDS causing virus) was discovered in 1983 (Barré-Sinoussi et al., 1983), and is a retrovirus that enters human cells with the assistance of CD4 receptors (a glycoprotein) and a CCR5 co-receptor on the cell surface of T lymphocytes (a type of white blood cell) and deteriorates human immune systems. Early experiments with AIDS patients focused on assaying viral plasma load, CD4+ cell counts (and its deterioration), and human leukocyte antigens (HLA) to determine key characteristics contributing to disease progression (Saah et al., 1998). But in nature, only humans and primates can be infected with HIV, and only humans get AIDS. So scientists needed to develop a better experimental model.

To begin the studies, investigators were forced to find a way to overcome the fact that mice lack HIV CD4 and CCR5 receptors on the surfaces of their T cells. The original experiment, performed in 1988 at the Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases in Bethesda, involved injecting fertilized mouse eggs with the entire genetic proviral sequence of HIV (Leonard et al., 1988). This resulted in thirteen founder transgenic mice, none of which developed symptoms of AIDS. However, mating them with normal laboratory mice produced offspring that eventually acquired several AIDS-like symptoms, including enlarged lymph glands, enlarged spleens, and pneumonia, before they all died at twenty-five to twenty-eight days of age. Another transgenic litter consisted of all runts that developed a psoriasis-like skin disease. Mice that were sick tested positive for HIV DNA while healthy mice did not. Unfortunately, a laboratory accident resulted in all but three of the mice dying, so the strain was lost (Weiss, 1988).

In the same year, Joseph McCune at Stanford University School of Medicine developed a mouse line containing a human immune system (Namikawa et al., 1988). These mice are now termed SCID-hu (humanized severe combined immune deficient mice) or Thy/Liv (thymus-liver, pertaining to the human tissue grafted into the mice) (McCune, 1997; Weiss, 1988). To produce these mice, they were given severe combined immunodeficiency disease (SCID; a disease in which the animal has no B or T lymphocytes) by knocking out the gamma-C (γC) T-cell receptor that is required for immune system function. The SCID mice were then implanted with human fetal liver, thymus, and lymph node tissue to reconstitute a "human" immune system in the mice. It was hoped that the depletion of the mice's immune systems would prevent the foreign human tissue from inducing a foreign body immune response, and the engrafted tissue would produce human T-cells capable of being infected with HIV (McCune, 1997). The data indicated that the humanized graft survived, and it could be infected by HIV. Although the mice did not develop AIDS symptoms, the model allowed scientists to study HIV cell infection processes. These mice remain as one of the few models to study HIV-1 pathogenesis in the human thymus and T-cells *in vivo*. They also allow investigations into the mechanisms of "T cell turnover" during infection. Evaluating potential antiviral compounds in large groups of mice contributes to the development of preclinical drugs for HIV-1, and the development of gene therapy procedures against the disease.

More recent studies have been performed with rats, because rat cells contain host proteins that remain fully functional during HIV infection. Robert Gallo's lab developed a transgenic rat containing the full HIV proviral sequence that expresses a variety of functional viral proteins and shows multiple types of AIDS-like symptoms (Reid et al., 2001). These rodents allow an analysis of the interaction of viral proteins with host cell factors. Other HIV infection models are

created to express CD4 and CCR5 chemokine co-receptors, and are important for studying the pathway of the disease (McCune, 1997).

Alzheimer's Mice

Alzheimer's disease (AD) is a neurodegenerative disorder first characterized by German neurologist Alois Alzheimer almost 100 years ago, and is the fourth leading cause of death in the developed world (Alzheimer's Association, 2012). Five FDA-approved drugs are currently available, but they only temporarily treat the symptoms, and are not a cure. Both sporadic and genetic versions of AD exist. For the genetic versions, mutated genes responsible for early onset AD include the β-amyloid precursor protein (β-APP) gene, presenilin-1 (PS1), and presenilin-2 (PS2) (Duff et al., 1996). The β-APP gene is located on chromosome 21, the PS1 gene is located on chromosome 14, and PS2 gene is located on chromosome 12 (Janus et al., 2000). The cause of AD is unknown, but appears to involve the production of extracellular deposits of the peptide β-amyloid (β-A4, now known as Aβ) that accumulates and forms senile plaques in the cortex and hippocampus areas of the brain, which are involved in learning and memory. Aβ is produced from APP by an abnormal cleavage process catalyzed by beta and gamma-secretase enzymes. Other neuro-pathologic features of the disease include the deposits of intracellular neurofibrillary tangles (NFTs) comprised of tau protein, decreased synaptic density, and loss of neurons located in the basal forebrain (Janus et al., 2000). Humans and higher primates get AD, but neither is a good experimental model, so scientists sought to develop mice that mimic the disease.

The first functional AD model was created in 1995 in Worcester, Massachusetts, at WPI in collaboration with the former Transgenic Sciences Incorporated (TSI) (Games et al., 1995). The transgene inserted into this mouse strain was human APP mutated to mimic an early-onset

AD mutation found in an Indiana pedigree (Murrell et al., 1991). The human APP transgene was placed under the control of a PDGF promoter to drive APP expression in the cortex and hippocampus, the same regions affected in AD. The data showed a successful production of human APP and Aβ in the correct brain areas, and the initiation of neurodegeneration (Games et al., 1995). The mice were later shown to have learning and behavioral deficits similar to AD patients (Hsiao et al., 1996).

Following the construction of the initial AD model, scientists later developed many other types of models, from those over-expressing other types of early-onset APP mutations (London, Sweden, Baltimore), over-expressing several types of PSEN mutations, or over-expressing beta and gamma-secretase mutations. Each of these models increases the formation of Aβ toxin in the brain. Most of the models focus on the formation of Aβ, since it is thought to be one of the key initiators of AD (Moran et al., 1995). For instance, a mutation that stimulates the β-secretase enzyme pathway results in over-production of the Aβ. In another case, targeting the γ-secretase pathway also causes an increase in the abnormal cleavage of APP to produce Aβ. A transgenic mouse line made by a different laboratory also enhances γ-secretase cleavage, but the mice contain all three splice variants of APP (695, 714, 751, and 770 amino acids long) in their genomes, similar to that done in the original 1995 AD model (Games et al., 1995). Allowing the production of all three APP isoforms by including introns six through eight and the platelet-derived growth factor β-chain (PDGF-β) is thought to be important to AD (Janus et al, 2000). The increased Aβ deposits can also result from AD mice containing mutations in the PS1 gene, which affect the gamma-secretase pathway (Duff et al., 1996).

Oncomice & Chemoprevention Models

There are two types of transgenic animals currently used in cancer studies: oncomice and chemoprevention models. Oncomice are genetically engineered to express cancer causing genes, or oncogenes, in order to study carcinogenesis (e.g. events leading to loss of cell cycle control and tumorigenesis) (Hanahan et al., 2007). On the other hand, chemoprevention models are created to study ways to reverse, suppress, and prevent cancer development (Alexander, 2000).

The world's first oncomouse was constructed in 1984 by Philip Leder at Harvard University, in collaboration with Dupont (Stewart et al., 1984). The transgene inserted into this mouse line was *c-Myc* oncogene under the control of a mouse mammary tumor viral promoter that expressed the oncogene only in mammary tissue. The mouse was patented in the U.S. in 1984, and became the world's first patented animal (discussed in Chapter-4) (Leder and Stewart, 1984). Based on previous attempts at constructs with the *Mt1* (mouse metallothionein-1) gene promoter incorporated with the *v-Src* oncogene, the scientists tested a *Mt1-Myc* complex and a *Eμ-Myc* complex, expecting to mimic the chromosomal translocation characteristic of human B-cell lymphomas. The *Mt1-Myc* consisted of the *Mt1* promoter gene and the *Myc* oncogene, and was predicted to cause broad expression of *Myc.* On the other hand, the *Eμ-Myc* consisted of the immunoglobulin enhancer (*Eμ*) with the *Myc* gene. *Mt1-Myc* transgenic mice did not end up developing any tumors, however, the *Eμ-Myc* mice showed development of pre-B-cell and mature B-cell lymphomas (Hanahan et al., 2007).

Mouse models used to study cancer pathways can also be used to study potential chemo-preventative agents. p53-KO mice have been developed in which the p53 tumor suppressor gene has been knocked out (Harvey et al., 1993). Protein p53 normally functions to help repair DNA mutations as they form. If it is knocked out, the mutations remain unrepaired, so cancer forms.

In one chemoprevention study, it was found that this particular type of knockout causes a type of tumorigenesis that is greatly affected by calorie restriction, and giving the mice dehydro-episandrosterone and 16-α-fluoro-5-androsten-17-one compounds caused reduced food intake and most likely delayed tumor development due to glucose-6-phosphate dehydrogenase inhibition and less DNA synthesis (Alexander, 2000).

Transpharmers

Animals termed "transpharmers" are transgenic animals manipulated to produce a certain pharmaceutical protein drug in large quantities in the milk, blood, or urine (Biotechnology, 1995). The aim of transpharmers is to not only provide a high level of product, but to also provide a cost effective and safe way to produce drugs. Early methods to isolate biologically active human proteins would either purify the protein from human cadavers, risking viral contamination, or produce the drug in mammalian cell culture system. Although mammalian cell cultures are safer, the amount of drug that can be produced is significantly lower (Brink et al., 2000).

Since the development of the first transpharmer mouse in 1987 that produced a human clot dissolver drug tPA in its milk (Gordon et al., 1987), milk has remained the most popular production fluid for transpharming. To express a protein in milk, the transgene must be under the control of a milk protein promoter, such as casein, beta-lactoglobulin, or whey acid protein. Drug development in milk has increased in popularity because transgenic animals can produce at least one gram of therapeutic protein per liter of milk, which in turn reduces the number of animals required (Brink et al., 2000). Producing in milk does not require euthanasia of the animal to access the drug. Drugs that have been expressed in "pharm" animals include tPA (tissue

plasminogen activator, a clot dissolver), human factors VIII and IX (blood clot treatments), lactoferrin (infant formula additive), CFTR (cystic fibrosis transmembrane conductance regulator), and hemoglobin (blood substitute for human transfusions) (Biotechnology, 1995). More recently, ATryn® (anti-thrombin blood thinner) has also been transpharmed, and in 2008 became the world's first FDA-approved drug for use in humans (discussed below).

Transpharmer Mice

The first transpharmer animal made was a mouse that expressed the human tissue plasminogen activator (tPA) protein in its milk (Gordon et al., 1987). Research performed in 1988, tested the levels of the protein transcripts present in the mammary gland of lactating mice. The mice were expected to express the gene and secrete the product in the milk. The construct in question, named WAP-tPA, hybridized tPA with the promoter region of whey acidic protein (WAP). The results showed that tPA was detected in the milk and was biologically active. A statistical analysis showed that significant differences in the degree of transgene expression occurred as a consequence of random insertion into the genome of various transgenic lines. It was also noted that because the concentrations of the protein increased in the mammary gland but did not increase in the sublingual gland, tongue, or kidney during lactation, the expression was limited to the mammary gland (Pittius et al., 1988).

Transpharmer Cows & Herman the Bull

Transpharming is not limited to mice. In 1989, a bull was created to produce lactoferrin in his milk (Hendolin et al., 1989). The world's first transgenic bull, named Herman, showed lactoferrin gene integration, and his female offspring transpharmed the drug in their milk. Since

then, other cows have been created by gene-targeted expression in the mammary gland, using promoters such as ovine β-lactoglobulin, caprine β-casein, murine whey acidic protein (WAP), and bovine αS1 casein. The transgene used to create Herman used the αS1 casein promoter and the cDNA encoding human lactoferrin. More recent studies have shown that the use of cDNA in contrast to using the entire gene (which includes exons and introns), is not as effective as using the full gene; this may explain why the lactoferrin levels in Herman's milk never exceeded 0.01 grams per liter of milk as his transgene was a cDNA (Brink et al., 1999).

Transpharmer Goats

Transpharmer goats manipulated to produce ATryn®, anti-thrombin blood thinner protein, were the first transpharmed product approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (ATryn®, 2008; Ledford, 2006). Before being approved, refinements needed to be done to increase the percentage of successfully transpharmed animals. One breakthrough study used the Gibbon ape leukemia virus (GaLV) retroviral vector to insert reporter genes into the mammary epithelial cells of goats. The experiment resulted in the secretion of human growth hormone (hGH) in the milk of the animals at high levels. Archer and colleagues (1994) observed that each of the goats had different levels of expression of the growth hormone, and the general trend decreased and eventually reached a plateau. The group concluded that the high level of protein on day-1 of lactation could have been due to a number of things including: hGH from the viral infusate still present from the injection on the previous day, mRNAs being more largely present on the first day, and/or long terminal repeat (LTR) stabilization. Finally, it was found that it is possible to introduce a drug to the mammary gland via retrovirus and have it be successfully expressed over a long period of time. Because of the

poor ratio of mammary cells to virus particles, it was proposed that improving the ratio by increasing the amount of virus, as well as altering the timeline and frequency of the viral treatments could lead to an improved protocol for creating transpharmer animals (Archer et al., 1994).

Xenotransplanters

Xenotransplantation is the procedure in which a nonhuman animal source is the donor of cells, tissues, or organs meant to be transplanted, implanted, or infused into a human. The concept is being explored as a potential alternative to help solve the organ shortage for patients in need of vital organs (U.S. FDA, 2010). Statistics in 1999 showed that only 2,300 out of the 40,000 people that needed hearts got one (Mooney et al., 1999). As of 2010, the numbers indicated that 10 patients die every day waiting for a transplant organ (U.S. FDA, 2010). Xenotransplanters are closely related to current methods of tissue regeneration that scientists are trying to perfect for "off-the-shelf" organs. Ideally, tissue engineers would like to create a scaffold of biodegradable polymers that can allow cell replication so when the polymer breaks down, the neo-organ (man-made organ) will remain as a natural product (Mooney et al., 1999).

A large problem with engineered tissue and organs from xenotransplanters is to overcome the human immune response. Since the first successful xenotransplantation in the 1960s, when chimpanzee kidneys were transplanted into 13 patients (Reemtsma et al., 1964), chimpanzee kidneys, hearts and a baboon heart have been tested in humans, and both eventually failed. The monkey donors were then abandoned, and scientists decided to try using pigs hoping that their organ size and function is similar enough to human anatomy to work. Currently, pig heart valves are routinely used worldwide. The ability to use porcine heart valves lies in the fact that they are

mostly connective tissue and do not cause immune rejection. Unfortunately for pig organs, a porcine sugar, α-galactose (α-gal), on the surface of pig cells causes rejection when antibodies in the human donor attack the sugar. Although some people believe that there is more than just the α-gal involved with rejections (e.g. the host attacks the porcine blood vessels as well), scientists set out to knock out the gene encoding the enzyme α-galactosyl-transferase that places the α-gal on the cell surface (Lai et al., 2002). January of 2002 marked an advance in preventing immune rejection; the pigs contained one knocked out allele of α-galactosyl-transferase. Although this is a good start, the pigs still contain one intact allele for the enzyme, so future experiments need to be performed to knock out the other allele (Couzin, 2002).

Transgenic Food Sources

Another application of transgenic animals resides in the agriculture and food industry. There are currently no transgenic animals commercially produced and sold for food purposes, although the methods for production are still being tested to eventually allow it. The improvements targeted in transgenic food animals include more efficient meat production and higher safety in areas such as disease and/or parasite resistance, increased growth rate and feed conversion efficiency, as well as increased nutrition (Harper et al., 2006). As of 2010, the FDA was close to approving a genetically modified Atlantic Salmon containing a growth hormone (explained below) (Marris, 2010). Transgenic pigs and other livestock are also being tested for their ability to produce food, however, those experiments are still under development (Harper et al., 2006).

Superfish

The first fish considered "commercially important" was mutated to express a human growth hormone transgene under the control of the mouse metallothionein promoter (Devlin et al., 1997). Created by injecting the DNA into the germinal disc of a goldfish embryo, the fish in general prove to be easily manipulated due to the large number of gametes they produce, and their eggs are fertilized outside the body so re-implantation is not necessary (Harper et al., 2006). The so-called Atlantic salmon ***superfish*** were further developed by Aquabounty Technologies, whose headquarters are located in Waltham, Massachusetts (Aquabounty, 2012), and are the closest animal to being approved by the FDA. The fish grow twice as fast as the non-transgenic salmon, and some strains now include the gene for Chinook salmon growth hormone and its regulatory sequences. Chinook salmon grow throughout the year, allowing the altered Atlantic salmon to do the same and grow to be twice as large as the normal Atlantic strain (Marris, 2010).

Superpig

The use of growth hormone transgenes in pigs does not tell as nice a story as the use of similar genes in fish. The first pigs given growth hormone genes, termed "superpigs" to describe their enlarged size, were created in 1988 using pronuclear microinjection (Vize et al., 1988). The transgene construct introduced to the fertilized eggs contained a porcine growth hormone (pGH) gene fused to the human metallothionein-IIA promoter (hMT-IIA) to ensure the transgene was expressed in a wide variety of tissues. Only one of the pigs successfully expressed the transgene and grew faster than the other pigs. Other littermates were found to contain the transgene in their genome, but did not express it, and two of the pigs passed the transgene to their offspring. It was

found that the pig that grew faster had the transgene integrated randomly and none of the resulting growth characteristics caused health problems (Vize et al., 1988).

A subsequent study done the following year in 1989 engineered pigs to contain either human or bovine growth hormone genes under the control of a mouse metallothionein-1 promoter (Miller et al., 1989), and although these initial superpigs did not appear to have any size benefits, they were found to have leg problems and heart problems. Leg problems were thought to result from the skeletal system not being strong enough to withstand the extra weight of the superpig. Similarly, the cardiovascular system must also be able to handle the weight of the pig, causing heart failure in early models (D'Silva, 1998).

Transgenic Biological Models

The world's first transgenic animal that *expressed* its transgene was not engineered to provide a direct benefit to mankind by making a disease model or by transpharming a life-saving drug. Instead, it was a mouse engineered to contain a growth hormone gene under the control of a metallothionein promoter to determine whether a biologically active product (growth hormone) could be produced by transgenesis (Palmiter et al., 1982). Different from food source transgenesis, where the animals size are meant to provide better food supply, supermouse was a *biological model* intended to aid our understanding of growth hormone fusion genes and how foreign genes are regulated *in vivo* (Palmiter et al, 1982).

Since the landmark 1982 transgenic animal experiment, other biological models have been created to aid our understanding of the consequences of knockout and knockin animals. As a recent example, the "Mouse Phenotyping Project" was creating in 2011 and will test over 5,000 different strains of knockout mice. The aim of the project is to create different transgenic mouse

lines missing a specific gene by stem cell manipulation, and then observe the effects on over 20,000 mRNAs and proteins in the mouse body by hybridization arrays. Researchers will also observe the visible phenotypes on anatomy, physiology, development, behavior, and disease traits in these mice to help understand the roles of each gene throughout the life cycle. It is hoped that the project will be complete by the end of 2016 (News of the Week, 2011).

Doogie the Smart Mouse

The original smart mouse, named "Doogie," was used in a study to determine whether a specific type of receptor in the brain acts as a switch for memory formation (Tang et al., 1999). The N-methyl-D-aspartate (NMDA) receptor binds glutamate in the brain, and is thought to help regulate learning and memory formation. The NMDA receptor is composed of two subunits; the NR1 subunit is required for function, while the NR2 subunit is a regulatory subunit and varies depending on the developmental stage of the animal. The NR2B subunit predominates when mice are young, and learn more easily, so the experiment was designed to determine whether over-expressing the NR2B subunit can facilitate learning and memory. The data indicated that over-expressing NR2B in the cortex and hippocampus caused an increased activation of the NMDA receptors, and increased learning in memory and behavioral tasks, compared to normal mice (Tang et al., 1999).

Youth Mouse

At the Weizmann Institute of Science in 1997, transgenic mice were created that lived longer than their wild type parents. The mice were designed to over-express urokinase-type plasminogen activator (uPA) in the brain. Results of the study showed that the mice consumed less food, grew less, gained less weight, and had longer lives. They also had lower blood sugar

levels, fewer pups, and less births. It is thought that the lifetime of the mice was extended because the uPA over-expression prevented atherosclerosis that is common in aged animals (Miskin et al., 1997).

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CHAPTER-3:
TRANSGENIC ETHICS

As is the case for experiments involving any animals, studies using transgenic animals pose problems from the public. Many ethical issues on animal research originate from the late 1960s, when pet dogs were abducted from people's homes and sold to research companies (Cowan, 2010). Although transgenic animal research has provided technologies that allow studying disease formation or testing potential cures, producing life-saving medication, providing organs for transplant, or producing food sources to aid a starving planet, the potential for abuse of these animals is apparent in each category. The purpose of this chapter is to discuss transgenic ethics and whether these animals *should* be created, by weighing the benefit of transgenic animals against animal welfare issues. Because the benefits and animal issues vary considerably, each category must be discussed separately.

Introduction to Animal Ethics

There are three ethical approaches that can be used to determine whether a particular animal experiment is cruel. The *utilitarian approach* is based on the opinion that animal welfare creates the standard to deem an experiment "right" or "wrong." Using only this standard, if human or animal welfare is enhanced, it might be justified regardless of the proposed experiment itself. However, it would eliminate an experiment that might produce a life-saving vaccine if it harmed a few mice. This creates the idea that animals matter morally completely equal to humans (Almond, 2000).

In 1991, Robin Attfield proposed an *ethical solution* where moral standing is different from moral significance (Attfield, 1991). Now called the *rights approach*, it is centered on the

idea that although animals matter, it can be supported to sacrifice them at the benefit of a superior being like humans (Attfield, 1991; Almond, 2000). The final concept, the *virtue approach*, states that objects and creatures were meant to "flourish" (i.e. meet their goal in life). So, if an animal was made for testing, then that is what shall happen to it (Almond, 2000).

In transgenesis, since the animals are not only tested, but also genetically altered, the ethics involved with them is more complex than with an animal solely used for testing. Brenda Almond's discussion in the *Health, Risk & Society* Journal focuses on the issue of how animals are treated individually, although newer issues involve the treatment of the species as a whole. One side of the argument is that humans have been genetically modifying animals since they found it possible to breed dogs and farm animals to better suite their owner. With transgenic animals, the problem is not just the genetic modifications, but the acceleration of creating them using genetic engineering, and their impact on the environment, economy, and health and safety of both animals and humans (Almond, 2000).

In terms of animal well being, genetically altered animals may be discussed ethically by two different aspects of the experiment: first, how they are created, and second by the amount of pain and distress the animal endures due to the application. As introduced in Chapter-1, there are two main techniques used to create transgenic animals: pronuclear microinjection, and the manipulation of embryonic stem (ES) cells. The main difference between the two is that pronuclear microinjection integrates the transgene randomly in the host genome, while ES cell manipulation allows gene targeted insertion. If the transgene insertion is random and the gene is incorporated in a spot that allows birth of the pup, then the mutation has a large range of possible effects on the living animal. If the transgene inserts into an inactive region of the chromosome, it might not induce any change in the animal's phenotype, while on the other side of the spectrum,

it could inactivate a required gene or activate an oncogene to cause illness or even death. Embryonic stem cell manipulation allows gene targeting, so the direct effect of the transgene is known prior to incorporation into the genome (LATG, 2010). The use of ES cells themselves, however, have developed an entirely individual ethical debate, since the cells are *pluripotent*, meaning one could potentially create any adult tissue once the cells begin differentiating (Charles River Laboratories, 2005). Because of the potentially surprising conditions that could arise with using either transgenic technique, it is up to the principal investigator (PI) leading the research, and the institutional staff to observe each animal's phenotypic behaviors and physical characteristics to ensure humane care of the animal. This way if necessary analgesics or euthanasia is required, it can be administered at an appropriate time (LATG, 2010; National Research Council, 2011).

One commentary, written by David Porter in 1992, proposes a scoring system similar to the system determined by IACUC committees to place a grade on how humane the study is. The categories considered involve the potential benefit of the experiment, the expected amount of pain, and the duration, number of animals, and quality of the experiment. Each of these features would be experiment specific, while others are more dependent on the application (Porter, 1992). For example, the "measurable" pain in which a transgenic disease model may undergo is far different than for a transpharmer animal. The remainder of this chapter will focus on the different ethical problems associated with specific transgenic examples.

Disease Model Ethics

Because disease models are created to study sickness and disease, they are a part of a large number of ethical debates. The main discussion involves the induced pain and/or distress in

the animals. Sources such as *Recognition and Alleviation of Pain in Laboratory Animals,* are helpful for determining and controlling pain and distress (NRC, 2009). It is up to the veterinarian to recognize when treatment is necessary. Treatments depend on the species, age, degree of pain, and length of the study. Often times, with disease models, the experimental endpoint has to be the same as the humane endpoint if there is vital information to be determined post death (National Research Council, 2011).

In this category, the health and safety of humans may also be of concern since investigators are in direct contact with animals that could potentially pass a disease on to humans (Almond, 2000). Of the diseases discussed in this project, the most risky model would be the AIDS mouse since the disease could, in theory, be transmitted by the animal's blood. In reality, the chance of human infection is low since HIV mice do not produce infectious HIV virions in large quantity, but scientists must always remain vigilant for potential transmission. Moreover, as long as people follow rules and wear the proper personal protective equipment (PPE), the likeliness of contracting any disease is very low. Environmental and economic impact is not usually of concern with disease models, given the animals are not freed into the wild, and although valuable, the personal investigators budget should be negligible in comparison to the affect of experiments involved with the U.S. Department of Agriculture (Almond, 2000). The rest of this section will focus on the three disease model applications discussed in Chapter-2 (AIDS mice, Alzheimer's mice, and Oncomice) in terms of pain and stress based on phenotypic traits versus the potential benefits of each model.

AIDS Mice

Any model involved with AIDS is extremely valuable because in nature only humans and chimpanzees spread HIV, and only humans get AIDS. SCID-hu mice (introduced in Chapter-2) benefit the study of HIV-1 because they are one of the few settings to evaluate the disease's effects on human thymopoiesis *in vivo*. Studies involved with differential HIV-1 pathology are not possible in humans, therefore, any model that provides key information on T-cell turnover and infection is important (McCune, 1997).

Despite their benefits, SCID-hu mice have a significant downfall. Since fetal human organs are implanted into the mice, the physiology may not be fully accurate in analyses meant for adults. The implanted human thymus tissue differentiates into the main cellular reservoir for HIV (T-cells), but HIV cannot infect the surrounding mouse tissues; so the model cannot be used to assay HIV spread. Also, a mouse environment housing human organs may result in insufficient functionality of the human cells. However, in some instances there is the reverse where the mouse cells lack functionality. Transgenic HIV mice are so dissimilar in comparison to humans that they are not expected to be used in vaccine studies, but are of general interest because of the ability to analyze infections with HIV (McCune, 1997).

Since AIDS is a disease that essentially destroys the immune system, it is possible that the animals used for testing are expected to endure pain and sickness at some point during the study. The main ethical question for this model is not only what is the expected degree of pain but can the animal's pain be relieved. Both of these questions are experiment specific, and can be determined only by further analysis.

Alzheimer's Mice

Alzheimer's disease (AD) models have not been fully established, therefore, they have many downfalls. AD is a very complex disease, and has many criteria that makes transgenic testing very difficult on a single model with a specific transgene (Janus et al., 2000). There are currently three different genes believed to contribute to AD, and techniques for making transgenic animals do not make it easy to make an animal with a triple mutation to study the effects simultaneously (Duff et al., 1996). This also contributes to the fact that studies testing familial Alzheimer's disease (FAD) may not provide help for the sporadic form of the disease. On top of this, the behavioral studies in mice must be analyzed to support their reproducibility (Janus et al., 2000). Positively speaking, any study currently being performed to observe AD pathway is bringing biotechnology one step closer to finding the ideal model to mimic symptoms and help develop preventative measures against the disease (Janus et al., 2000; Moran et al., 1995). In spite of the fact that no current AD model fully mimics the disease, the current partial models have at least provided a means for testing key theories in AD research (i.e. does the amyloid-beta neurotoxin (encoded by the AD mouse) really initiate AD, and can behavior improve after removing the amyloid-beta toxin). Two separate studies have proven that production of Aβ is sufficient for initiating AD (Games et al., 1995) and removal of brain Aβ can improve animal behavior (Morley et al., 2002).

In terms of pain, AD mice are not expected to experience physical pain due to illness, but they risk the possibility of having high stress and anxiety levels (Moran et al., 1995; Janus et al., 2000), although these symptoms are not present in all the models. Stress is indicated in mouse studies largely by either reduced activity and neophobia, or boredom shown with consistent circling. If the mice are going to experience pain it would be due to seizures that are often caused

by stress or aggressiveness which would cause pain if the mouse was fighting with its cage mates (Janus et al., 2000). However, these symptoms are not characteristic of all models, and can be reduced with correct husbandry and environmental conditions as with any laboratory animal (National Research Council, 2011).

Oncomice & Chemoprevention Models

According to the American Cancer Society, more than 1.6 million new people are expected to be diagnosed with cancer in 2012, and almost 12 million people will be diagnosed with cancer at some point in their life (American Cancer Society, 2012). This high statistic makes cancer models extremely valuable. Since there are so many different types of cancer, each model needs to be approached differently. Although there is not a singular model demonstrating all types of cancer, current models have evolved to more accurately mimic specific tumors in organs. Early cancer studies were done by transplanting tumors under the skin of immune-deficient mice. But these models could not fully imitate the disease. Lacking in the transplant models but important in cancer studies are: benign premalignant tumor progression, significant histological traits, prediction for therapeutic benefits, and shortcomings of previously tested drugs (Hanahan et al., 2007).

As concluded by Jan Alexander, every mutation being studied provides a little more information towards the cure (Alexander, 2000). For instance, mutations in the p53 tumor suppressor gene (targeted in the p53-KO mouse), have been found to be involved with over 50% of all known human tumor types. However, there are several forms of cancer that are not affected by loss of p53 function (Harvey et al., 1993). Generally, the ethical issues in tumor models are found with the discussion of physical pain that the animal has to endure. The tumor

may not only cause direct pain, but could cause physical impairment if the tumor is located next to a leg and grows to a significant size. Such problems must be closely reviewed by the IACUC committees to determine both the experimental endpoint and humane endpoint (National Research Council, 2011).

Transpharmer Ethics

The ultimate goal with transpharmer animals is to create animals not physiologically affected by the drugs they produce. Regardless, they must be first approved by the USA's Food and Drug Administration (FDA) (or the country's equivalent) prior to human use of the produced therapeutic. Due to the fact that the transpharming application must be adequate for consumer use, both animal and human safety must be considered. Specific drugs have already proven to be safe for both animals and humans. For example, in 2008, Genzyme Transgenics Corporation (GTC) received U.S. FDA approval for human use of its anti-thrombin blood thinner drug ATryn® transpharmed from goats' milk (ATryn®, 2008). Other proteins such as lactoferrin and tissue plasminogen activator (tPA) can be expressed in the mammary gland, with no observable effects on the animal, while remaining biologically active in humans. Production of pharmaceuticals in the milk has shown to be more humane towards the animals than production in blood, since the former does not interfere with the animal's health. On the other hand, proteins such as hemoglobin are better isolated from the animals' blood, requiring controlled bleeding or euthanasia to obtain the drug (Biotechnology Information Series, 1995). Purification from blood also risks viral contamination, endangering humans with the potential of infection with animal viruses (Brink et al., 2000; Almond, 2000). Also, since the drug is expelled from the animal's body there is a risk of allowing the drug to enter the environment by either consumer or producer

error (i.e. if the milk is dropped). This has the possibility of creating antibiotic-resistant bacteria, increasing human risk of sickness and environmental problems (Almond, 2000).

The main purpose of these transgenic farm animals is to provide a cheap alternative for large scale drug production (Pittius et al., 1988). Early cell culture systems are safer than purification from cadavers, however, the few number of animals needed to produce a large quantity of drugs provides a cost effective strategy (Brink et al., 2000). Ongoing problems include the low transgenic success rates with large animals due to the low number of embryos that can be transferred, and the long time periods involved with making large animals transgenic (Brink et al., 2000), which has proven to increase the cost in advancements (Biotechnology Information Series, 1995). Unfortunately for small farms, once the application is approved, large farm enterprises will be able to adopt the system, causing traditional farming to lose value (Mepham, 1994; Almond, 2000).

Xenotransplanter Ethics

Ethically speaking, xenotransplantation is frowned upon due to the bad image these animals have been given for this application. Some people believe that this type of application has turned animals into tools rather than living things (Mepham, 1994). According to Juan Correa, speaking in terms of religion and creation, the animals have their own value and they were brought to this earth to serve man. Looking at it from the human standpoint, however, religious issues state that taking organs from non-humans interfere with the order of creation (Correa, 2001). On the other hand, the concept of xenotransplantation has opened many doors to solving the organ shortage problem. Couzin presents the argument that even if long term survival

with a xenotransplant is not possible, creating organs that can be used will at least increase the amount of time the person has to find a permanent human organ (Couzin, 2002).

Since xenotransplanters are created for organs that can be put into a human body, the animal has to be designed so that the donated organ will not cause an immune response in the recipient human. Not only do humans have to concern themselves with the natural immune response of having an animal organ in their body, but they also must think about any diseases that may be passed between species. For example, all pigs have porcine endogenous retrovirus (PERV), but the problems the disease may cause in humans are not known. Problems like this most likely would occur with diseases that appear harmless to pigs. When the organ is implanted into a different species, if the disease has not been studied, there is the possibility of something being harmful to the recipient. Because of these risks, the FDA is extremely stringent on allowing clinical trials to test the transplants (Couzin, 2002). When the FDA finally approves a xenotransplantation model, large farms may begin threatening the economics of smaller farms (Mepham, 1994).

Food Source Ethics

The aim of transgenic food sources is to increase production and quality of food from animals. Like xenotransplanters and transpharmers, the issues largely depend on approval by the FDA since the animals would be consumed (Harper et al., 2006). People believe there are two main benefits to increasing growth hormones in farm animals. First, faster growth results in quicker slaughter of the animal, thus decreasing the amount of saturated fats in the meat which should be avoided in human diet. Also, if the animal is larger, the increased amount of meat will require less animals overall. This not only benefits animal welfare but proves to be

environmentally friendly. Utilizing the animal resources for food, can decrease the environmental impact of production systems and slaughter houses, while increasing economic risks for small farming (Mepham, 1994).

But the debate of killing and consuming animals for human consumption has become an ever-growing debate, because food production raises the issue of the treatment of such animals. With genetically engineered animals, however, the welfare of the animal could directly affect humans that consume the meat. Situations similar to the "Beltsville Pigs" (Pursel et al., 1997), are of highest concern when it comes to the welfare of both humans and animals (Mepham, 1994). The Beltsville Pigs were produced to increase their growth for food production, and the size ended up affecting every organ system in the animals. Their bodies could not handle the weight; as a result, they contracted diseases such as pneumonia, arthritis, and peptic ulcers (Pursel et al., 1997). As with non-genetically engineered animals, if an animal previously had an illness that could be passed to humans, it would pose a very dangerous risk when consumed. One may believe that this could be easily solved by giving the animal antibiotics, although, then scientists risk creating antibiotic-resistant bacteria (Mepham, 1994).

Biological Model Ethics

Unlike disease models and the rest of transgenic applications described in this review, there is no medical life-saving goal for biological models, except to increase our understanding of specific genes. Since biological models are not created to interact with or be consumed by humans, there are no FDA regulations involved. This means that ethics mainly revolve around the animal's welfare. As with any other knockin or knockout animal, when the gene is deactivated there will be a lack of its protein product in the animal, while gene over-expression

will increase the protein product. The aim of biological models is to see what happens as a result of over-expression or under-expression of the transgene. Unfortunately, until tested, the mutation could potentially be extremely harmful to the animal. Where, on the other hand, it could be harmless (LATG, 2010). For instance, youth mouse (described in Chapter-2) was essentially no different from a non-transgenic mouse, and had no health problems. They were found to be smaller and had less breeding potential, but overall were not sick or harmed by the over-expression of urokinase-type plasminogen activator (uPA). They just displayed increased learning and memory (Miskin et al., 1997). As with youth mouse, Doogie the Smart Mouse's welfare was unaffected by overexpression of the NR2B subunit of the N-methyl-D-aspartate (NMDA; Tang et al., 1999).

Author's Conclusion on Transgenic Ethics

Advancements in transgenesis have revealed many controversial opinions regarding human health, animal welfare, and the impact on the environment and economy. After evaluation, the author has concluded that in a majority of cases, the benefits of these animals outweigh the potential problems. Ongoing refinements of the technology, continue to help scientists minimize animal suffering while maximizing human benefits. Over the years, each improvement pushes science one step closer to achieving a significant lifesaving goal. For instance, the human genome project has resulted in significant improvements to the cost of accurately sequencing large segments of DNA, and this now makes it easier to accurately assay transgene insertion sites. Knowing the human genome has allowed scientists to target all 20,000-25,000 genes responsible for human bodily functions, ranging from growing and learning to suppressing tumors (Human Genome Program, 2012). This has led to the revolution of

understanding disease pathways and developing models and treatments for the diseases. Although public perception is that genetically modified animals are treated poorly, books (i.e. *The Guide*; National Resource Council, 2011), laws (i.e. The Animal Welfare Act; U.S. Congress, 1966), and organizations (i.e. IACUC; IACUC, 2005) (discussed in Chapter-4) have been developed to regulate animal use in experimentation and help ensure animals are treated humanely. Each transgenic application is widely different, though, causing IACUC committees to analyze each proposed study individually. Similarly, people who argue an opinion on the technology must do so in view of all the issues from an educated point of view.

With transgenic disease models, depending on the disease modeled and how accurate the model is, they may endure pain and stress. Despite this, when looking in terms of the future of the application, the decreased animal welfare is worthwhile. First, the animal is sick, but protocols require relief and treatment of sick animals when observed. As long as standard animal husbandry requirements are met, the animals' stress will be lessened (National Resource Council, 2011). Once the experiment is complete, findings will work towards the treatment, prevention, and/or cure to the disease in humans. The final step of this type of experiment seems to be often overlooked by the public: when the results are successful, it might not only benefit human welfare, but might benefit the animal's welfare too. The same treatment may one day be used to cure an animal with the same disease. Because of this cycle, in the long run, it is *not* humans being selfishly cruel to helpless creatures below them in the levels of creation, as it is frequently perceived. The animals have nearly the same probability of benefitting from the study as humans do.

In the opinion of the author, transpharmers, xenotransplanters, and food sources fall into the same category, ethically speaking. Agreeing with Mepham in his review, these applications

create animals as tools (Mepham, 1994). It is believed that each type of transgenic animal is created in good nature, though, and has the potential to be very helpful to the world. Approval by the FDA could lead to improved medical care, increased organs for transplant, and increased food for starving people in third world countries. Since each of these modifications could directly affect the humans involved and the environment, it is crucial that scientists aim to perfect the technology in a way that will not create pain for the animal as a result. Unfortunately, both xenotransplanters and food sources require the eventual death of the animal. However, the animal was bred to do so, and this debate is not specific to transgenic animals but also applies to the millions of animals sacrificed daily for human consumption. Some groups may believe this is selfish of humans to create animals to only benefit themselves. The author could argue that raising animals to be domesticated is also selfish when animals were put on this Earth to roam and be free in the wild. The fact that these transgenic animals are consumer products also contributes to a similar defense to that of disease models. Animals eat, have organs and need medicine to feel better when sick. As the technology grows, the findings can be applied to multiple species. Perhaps one day a pet dog's life can be sustained by the liver of a transgenic pig.

On an entirely different note, biological models are not as easy to defend. Thoughts retreat back to the old saying: "Don't fix what isn't broken." It is true that it would be nice to understand how each gene in the human body works, but the author believes it would be more beneficial to focus transgenesis on known diseases and conditions. The only benefit to such study lies in the futuristic opinion. If we study the knockin and knockout results of each gene, doctors could predict the conditions someone may develop throughout their lifetime, and thus give advice on how to prevent it from occurring. Because of this, scientific models should not be

abandoned, but delayed. It may be more widely accepted if investigators work to try and solve the health problems of today instead of jumping into entirely unknown territory.

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CHAPTER-4
TRANSGENIC LEGALITIES

Controversial technologies need laws regulating the use of those technologies. Transgenic technology versus conventional technology is one of five comparisons involved with analyzing "biolaw." In this discussion, law and biology are treated as though they are related topics, and it must be realized that any knowledge that pertains to one of them also applies to the other, and each is dependent on rules and procedures as well as the people involved with them. Since there are several different branches of transgenic technology, evaluation of the laws must also be looked at in reference to environmental health and human health (Chen, 2008).

To start understanding the laws needed to regulate transgenic animals, one must first distinguish the definition of the term "biotechnology." In one sense, it means all areas of an industry that create and market things through manipulation of life forms and/or the use of knowledge of living organisms. Other times, biotechnology is the sole use of *new* technologies to modify plants and animals. From here, regulations must decipher between the ownership and use of such technologies. In the U.S., granting *ownership* of biotechnology is a responsibility of the Patent and Trademark Office (PTO), while the Environmental Agency, Department of Agriculture and the Food and Drug Administration (FDA) control the *use* of biotechnology jointly (Chen, 2008). The larger issue among transgenic animals, however, is the question of whether or not life should be patented.

Animal Welfare Regulation

Although it took until 2009 before the FDA rules were finalized for the use of transgenic animals, animal welfare laws date back to 1966 when a law, later named "The Animal Welfare

Act," was passed to protect pets (Cowan, 2010). The original bill states that animals may not be used for research if they have been personal pets, and any animals used for research must be provided with humane care (US Congress, 1966). Currently, the act is used as the minimum standard accepted for animals used in research, exhibition, transport, and dealing (U.S. Dept. of Agriculture, 2012).

Since the formation of this law, multiple organizations have been created to ensure that all animal facilities treat animals humanely. One group, for instance, the Office of Laboratory Animal Welfare (OLAW), is a part of the National Institutes of Health (NIH). Aiming to provide help to institutes, OLAW is responsible for interpreting the Public Health Service's regulations on humane care and use of animals (OLAW, 2005). Also working with OLAW and NIH is the Institutional Animal Care and Use Committee (IACUC), which is established at each laboratory. These committees are in charge of overseeing and evaluating the institution's animal protocols (IACUC, 2005). The IACUC works with scientists, institutional officials, and the attending veterinarian to ensure humane animal use and regulatory compliance of all animal research performed at that institution. They also confirm and enforce policies, procedures, and the overall animal care program for the species present at that site. Guidelines are created to determine humane endpoints of studies, alternative studies using *in vitro* models or fewer animals, and appropriate veterinary care. A quick way IACUC's analyze different protocols is using "The Three R's": replace, refine, and reduce. It is the obligation of the institution to first make sure that the animal study is not currently being done elsewhere and has never been done before. Once that is determined, the model must be checked to ensure that no other *in vitro* experiment can be done to receive the same information (i.e. the proposed experiment cannot be "replaced"). Refine refers to modifying husbandry and experimental procedures to decrease pain and distress

in the animals. The final "R", reduce, is meant to help lessen the number of animals used for a study by obtaining more information from fewer animals while not increasing pain or stress (National Research Council, 2011). By doing all of this, it will be ensured that all of the animals' welfare are being considered.

Transgenic Regulations

After nearly a decade of review, in 2009, the U.S. Food and Drug Administration (FDA) released the guidance they plan to use to regulate genetically engineered animals. The basis of these rules considers animals as drugs under the New Animal Drug provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA), but are not legally binding (Wadman, 2008; FDA.gov, 2009). The section of the FFDCA concerning new animal drugs outlines laws saying that the secretary has responsibility for determining if the new animal drug can be deemed safe, and if not, he has the ability to propose levels that can be considered safe or prohibit use. Tolerance levels are based on similar criteria used for food safety. In this law, one can also find regulations on filing an application for the use and manufacturing of the new animal drug (U.S. Congress, 2006). The FDA guidance goes as far as addressing environmental safety by controlling shipping and labeling of the animals, as well as requiring authorization for edible products from genetically engineered animals to enter food supplies. The FDA must also have knowledge of the characterization of the animals and manufacturing processes used with the animals. Once approved, the institution is responsible for recordkeeping and ensuring compliance by submitting annual reports and supplements with any changes in the investigation. In addition to these regulations, institutional guidelines and National Institutes of Health (NIH) guidelines must also be considered (FDA.gov, 2009). NIH guidelines cover topics such as human health and safety,

environmental safety, confinement used at the laboratory, and the roles of people at each institution (National Institutes of Health, 2011).

Introduction to Patent Laws

The original patent laws date back to 1790. Currently, the patent law specifies how patents may be gained and any conditions involved with such. However, the US Constitution states that Congress has the ability to grant authors and inventors the right to secure writings and discoveries for a limited time period. By law, a patent can be issued to any person who creates a "new and useful process, machine, manufacture or composition of matter, or any new and useful improvement thereof…" (35 USC 101: 2007; U.S. Patent and Trademark Office, 2005). As a part of the FFDCA, approved patents for new animal drugs can be filed with the request application used for claiming a use of the drug and protecting the applicant from patent infringement (U.S. Congress, 2006). Patent infringement law states specifically that submitting such application covers *only* new animal drugs manufactured by recombinant DNA and other genetic manipulation techniques (U.S. Patent and Trademark Office, 2007).

In other countries, there are different outlooks on the definition of what is "patentable." Europe for instance, says that patented objects can be "inventions which are susceptible of industrial application, which are new, and which involve an inventive step." As a side note, the patent law has a section stating that patents *cannot* be awarded to inventions that are "offensive to public morality" or plant and animal varieties (European Patent Convention Art. 53(a), 1980; European Patent Convention Art. 53(b), 1980; Jozwiak, 1994). Japan's patent laws are very similar to Europe's. The invention must be novel and useful, but may not be considered publically immoral (Japan Patent Law, n.y.). Australia's Patents Act of 1990 also includes

inventions believed to be useful, novel, and inventive, however, humans are specifically not included under the law (Australia Patents Act, 1990).

Oncomouse Patents: United States, Canada, & Europe

In 1980, the first patented living organism in the U.S. was a microbe designed to eat oil slicks. However, the Patent and Trademark Office did not originally give Chakrabarty the patent. After denial, the Supreme Court took up the case for judicial review (Jozwiak, 1994). The outcome of the case, *Diamond v. Chakrabarty*, awarded the investigator the patent because it was not 'naturally occurring' to have microbes with such genetic make-up and Congress had intended for patent law to consist of everything made by man, and it was considered 'useful' thus satisfying the usefulness requirement (Jozwiak, 1994; *Diamond v. Chakrabarty,* 1980; Council for Responsible Genetics, 2000). Since that court case, the category of "non-naturally occurring, non-human multicellular living organisms" has become a standard for allowing patents in the U.S. (O'Connor, 1993). This 1980 case was a breakthrough for transgenic technology applied to higher organisms, such as animals.

In 1988, the U.S. PTO awarded first patented animal, the Havard-Dupont mouse, an oncomouse (Council for Responsible Genetics, 2000). This mouse has since been considered for patenting throughout the world. The United States currently has three patents awarded to the mouse created by Philip Leder and Timothy Stewart, researchers at Harvard Medical School. All three patents do not include humans to avoid moral and legal concerns regarding alteration of the human genome (WIPO Magazine, 2006). The first patent on the oncomouse (Leder et al., 1984) was specifically for: "a 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." The claims in the patent also include the endogenous coding region, the location of the transgene location (different from the site of the endogenous region), an inducible promoter sequence, a c-myc gene, various viral promoters and the fact that the animal is a mouse (Leder and Stewart, 1984). Years later, in 1992, Leder and Stewart filed another patent for the same oncomouse. This time the patent included the method for creating a cell culture from the somatic cells of the oncomouse (Leder and Stewart, 1992). Moving forward, the most recent U.S. patent for the oncomouse was given in 1999. It incorporated a method for testing suspected carcinogens and the method for testing treatment for any induced tumors (Leder and Stewart, 1999). Since transgenic technology was still fairly new at the time of the original 1984 oncomouse patent filing, questions arose about the patentability of animals. Mainly, should high-order mammals be patented, and how should moral implications be handled? As a result, there have been different responses to the subject in different countries (WIPO Magazine, 2006).

Although Europe approved the original oncomouse, the patent, once awarded, was limited to mice (WIPO Magazine, 2006). Like the first patented microbe, the oncomouse was not originally approved in Europe. After discussion, the Technical Board of Appeals of the European Patent Office (EPO) reversed their decision. They believed in the end that the detriments of the model were outweighed by the benefits it provided to the human species. Since then, the EPO has decided that animal patents shall be decided on a case-by-case basis (Jozwiak, 1994). Canada, on the other hand, concluded in 2002 that only the techniques that are used to create the mouse are patentable. The Canadian Supreme Court ruled that the transgenic mouse is not, by definition, a "new and useful art, process, machine, manufacture, or composition of matter" (Kondro, 2002). It had been interpreted that the term "manufacture" specifically meant a non-

living process, and a "composition of matter" referred to ingredients mixed together by a person. Since the original Canadian Patent Act of 1869 did not include mammals or higher life forms, it had been decided that further debate was necessary to address moral and social issues (WIPO Magazine, 2006).

Opponents to Patenting Higher Life Forms

Because higher life forms are not specifically targeted in many countries' patent laws, some people have developed negative, disapproving views towards animal patents. One group, Council for Responsible Genetics, has expressed an opposition to animal patents in general, whether they be naturally occurring or engineered. They state nobody should be able to claim ownership over another living organism. Primary reasons for this include accessibility, cost and secrecy issues. However, they also mention that allowing patents will promote a decrease in genetic diversity, creating agricultural policies that are difficult to enforce and support. Their final argument focused on the morality, questioning: if genetically altered DNA in an animal is patentable where do human reproductive cells lie in this scheme (Council for Responsible Genetics, 2000)? In addition to this, groups such as the American Anti-Vivisection Society argues that patenting animals conflicts with laws encouraging replacement experimental procedures (as described earlier in the chapter; Letterman, 2007).

Animal rights activists and small farmers are also among the people who are against the concept of patenting animals. The activists are concerned that patents will encourage inflicting disease upon the animals. Farmers, on the other hand, are afraid that the patents will hurt their business. They are under the impression that licensing fees will increase the cost in purchasing the transgenic animals from large biotechnology companies, while increased productivity will

cause the farmers to become dependent on purchasing the animals, thus hurting their budget (Jozwiak, 1994).

On a much larger scale than individual farmers or small organizations, Elisabeth Jozwiak mentions in her review the reasons entire countries deny patents. Often, the countries that do not allow patents are the lesser-developed countries (LCDs) of the world. Their argument is that by restricting patents, their own industries will be promoted which will ensure providing low cost products to their citizens. Also, similarly to Council for Responsible Genetics, LCDs believe that patents promote secrecy and any knowledge should be shared publically. In order to avoid patenting, LCDs require compulsory licensing that allows piracy and selling patented items at a much lower cost (Jozwiak, 1994).

Benefits to Patenting Transgenic Animals

Despite large opposition towards patenting higher life forms, there are several large benefits that are often overlooked. As noted in Jozwiak's article, the main concern lies with developing countries. Transgenic technology provides ways to increase food production, produce life-saving drugs, and increase knowledge of medical concepts. If these ideas are patented, then the patenting countries will have a higher likeliness of having that animal available mostly to them. Not only will people from developing countries be more likely to make their invention available to the LDC because they will not be afraid of piracy and fraud, but the cost of the patented items will decrease if they obey adequate patent laws (Jozwiak, 1994).

This could help solve the problems with starving and sick people on multiple levels. If pharmaceuticals are produced by the transgenic animals, developed countries can make a profit from selling these animals to lesser countries by investing. In the end, the pharmaceutical

industry will grow and increase drug availability in countries in need of it. Likewise, food production and medical research will also be promoted. Another benefit to patenting food production and disease models is the overall health of these lesser-developed countries will become better as a result, even if those countries do not hold the patent. This lies in the fact that transgenic food sources are created to increase nutritional value and prevent the animals from developing diseases. The disease models will increase understanding of said diseases, making it more likely for doctors to diagnose and properly treat sick people (Jozwiak, 1994).

Disadvantages of Patenting Animals

The large issues that arise with the debate of patenting higher-life forms, such as mammals, are ethical considerations, especially those issues that remain unchanged with or without a patent. For instance, religious people argue that transgenic animals disrespect God, reduce the value of life, and encourages people to put money before traditional values. Generally speaking, people arguing against animal patents also overlook that humans have been owners of animals as pets for centuries (Jozwiak, 1994). Patent protection is also called intellectual property, thus showing that patents should be no different that owning a pet (O'Connor, 1993).

Economic issues and the regulation of patented animals must also be considered. The problem here lies in the fact that the subject areas are too broad to predict any issues that could potentially arise. There is no single statute that regulates all uses of genetically altered animals. Economically speaking, commercial production is most likely to increase in the agricultural transgenic applications such as food production and transpharmers. Even among the food production industry, there is a wide range of economic relationships. Within the dairy market, federal price supports are very important, while poultry is more competitive. This shows that

intellectual property issues associated with animal patenting could cause different problems, even if inside one branch of the food market (O'Connor, 1993).

Author's Opinion on Animal Patents

Similar to many of the author's conclusions in the previous chapter, there are more benefits to patenting of animals than disadvantages. Although patenting has a negative connotation when including higher life forms, patenting animals is seen as no different from owning a pet. The only reason there is a difference is because most patents are associated with inanimate objects, and animal rights activists find that demeaning to the animal. As long as worldwide issues, such as piracy in lesser-developed countries, do not interfere, patenting has the potential to promote the use of transgenic animals. Knowledge and resources involved with the health and well being of people will then become more available to all people in need, thus improving health worldwide.

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PROJECT CONCLUSIONS

Transgenic technology has evolved to allow genetically manipulated animals to possess a specific gene (termed "transgene") not normally possessed by that particular species. The gene in question is typically incorporated into a cloning vector possessing regulatory sequences of DNA to help control which tissues the transgene is expressed. The transgene can be inserted to the animal's genome via two main techniques: pronuclear microinjection or introduction into embryonic stem (ES) cells. Pronuclear microinjection involves injecting a solution containing the transgene that will randomly integrate itself into the animal's genome. ES cell manipulation, on the other hand, allows the use of homologous recombination, so the transgene will insert into a known gene locus, replacing the endogenous gene entirely. However, even with gene targeted integration, there is still a possibility that the transgene can insert into spots that either inactivate the transgene, or create an animal with a mutation. Since both ways can result in an incorporation that could harm the animal, the ethical considerations and legalities must be looked at carefully and individually for each application. Currently, transgenic animals are created to aid our analysis of disease pathways and treatments for diseases, food production, drug production, organs that can be transplanted into other species, and observation of biological processes.

By creating a genetically altered animal to suit man, many different debates arise involving animal welfare, effects on the environment, public safety, and laws that regulate the ownership and use of the animal. Following performing the research for this project, the author's conclusion is that, overall, the advantages of transgenic technology outweigh the disadvantages, as long as there are proper committees and legislative oversights to regulate and enforce the rules for proper animal care. Animals should be humanely treated, and experiments should be altered to use as few animals as possible. Since the animals are bred in captivity they are not meant to

be released into the wild, and even if they were it would not be likely that they would survive. However, experiments should be designed to have minimal environmental effects.

In the long run, transgenic technology can benefit both humans and animals. Knowledge of disease pathways and biological processes has the ability to contribute to possible disease prevention and cures in humans and animals. Drug production can contribute to creating affordable medical care for people who need it. Likewise, food production can help the food shortage in places with starving people, and xenotransplanters can help solve the severe organ shortage problem. Because of these benefits, patenting the transgenic animals can be advantageous. Patents can increase the availability of knowledge and resources for the public's welfare by promoting the use of this technology by allowing the labs who created the animals in the first place to re-claim some of the money it took to make them. The knowledge gained through transgenesis can help improve people's health worldwide, and doctors will be better able to diagnose problems and will have access to drugs necessary for treating the diseases.