# GENETIC ENGINEERING, ANIMALS

## 1. Introduction

Most cells within an animal contain a complete copy of genetic information. The genetic information within cells is encoded by deoxyribonucleic acid (DNA). The sequence of nucleotides within DNA is paired with its complementary

base within the double-stranded DNA molecule. A complete sequence of DNA is known as a genome and the sequence of DNA dictates an animal's genotype. For humans and most mammals, a single cell contains about six billion base pairs of DNA. The six billion base pairs are composed of two copies of the genome, one from each parent, of approximately three billion base pairs each. Genes are nested within the six billion base pairs of DNA. The exact number of genes in a genome is still debated but estimates range from 30,000 to 100,000 for humans, laboratory animals, and farm animals. Surprisingly, most of the genome consists of DNA that does not encode specific genes. The function of the DNA that lies outside known genes is poorly understood. Genes consist of regulatory regions (DNA that controls gene transcription) as well as transcribed regions [DNA that is transcribed into ribonucleic acid (RNA)]. Transcribed regions of DNA consist of introns and exons. Exons contain the DNA that encodes the specific protein for the gene. Introns are regions of DNA between exons whose function is poorly understood. Transcription is the synthesis of RNA from DNA. The RNA is chemically modified after synthesis and introns are spliced out of the RNA molecule. The processed RNA (termed messenger RNA or mRNA) encodes the sequence of amino acids for cellular proteins. The amount of specific proteins within a cell determines its function within the organism. All proteins have some function within a cell and certain proteins are directly involved in growth and disease. Genetic engineering is the process of modifying genes within an animal so that the amount or type of cellular protein is changed. Changing cellular proteins through genetic engineering can have a variety of applications in animal agriculture and human medicine (see below).

## 2. Classical Methods of Genetic Engineering to Improve Animal Genetics

Humans have been practicing genetic engineering for a long time. For example, farm animals have been selected for superior traits for several hundred years. The variation in productivity of farm animals is partially dependent on the activity and function of genes within their DNA. Selection for superiority in a certain trait is equivalent to selecting for superior DNA sequence within the animal. In the case of a dairy cow, the trait would be milk production. Cows with the greatest milk production are kept on the farm and their offspring are also kept for their superior milk production. Beef cattle and pigs are selected for growth rate and muscling. We assume that cows that produce the most milk and cattle and pigs that have the fastest growth are equipped with superior genetics that enables the performance that we observe. A technology that accelerates genetic progress is artificial insemination. Semen is collected from superior males, diluted, frozen in liquid nitrogen, and shipped to farms. The frozen semen is thawed on the farm and used for artificial insemination of females. By taking advantage of diluted and frozen semen from superior males, farmers can breed an average female on their farm to a superior male to produce above average progeny. Artificial insemination is widely used in the dairy industry. Very few dairy bulls are used to breed nearly all of the dairy cows in the United States. Although superior male genetics can be exploited through artificial insemination,

it is more difficult to exploit superior female genetics. Females produce very few ova, or eggs, relative to the number of sperm cells that a male can produce. Female cattle can be treated with stimulatory drugs that cause the release of numerous ova (superovulation) and these treatments can be used to maximize the production of embryos from a single female (perhaps 100 ova annually). Nonetheless, it is impossible for female animals to achieve the gamete production of male animals (billions of spermatozoa per week). Therefore, methods to produce identical copies of a single embryo or a single adult cell (cloning) were initially developed to capitalize on the genetics of superior females.

**2.1. Embryo Splitting.** Perhaps the oldest method for creating multiple copies of a single individual is embryo splitting. The procedure for embryo splitting is not complicated but requires delicate instrumentation. By using microdissection tools (either a knife or needle), embryos are separated into two to four pieces (1) (Fig. 1). The split embryos are then transferred into the uteri of foster mothers for development. Animals that are produced from the same original

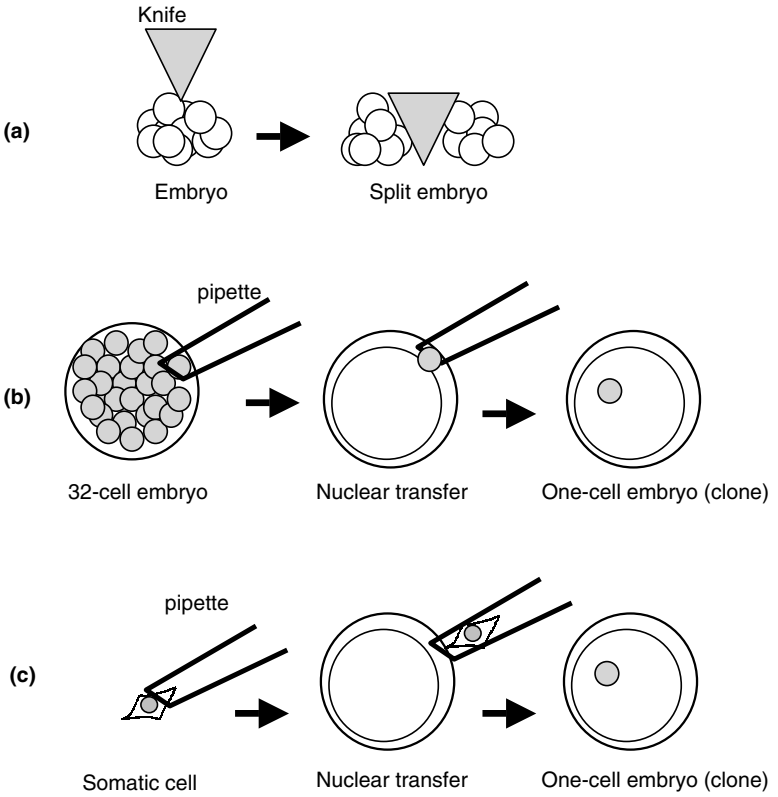


**Fig. 1.** Methods for the production of identical animals. (**a**) Embryo splitting. A microdissection knife is used to cut a morula stage embryo into two identical halves. (**b**) Embryo cloning by nuclear transfer from blastomeres. A cell from a morula stage embryo is removed and fused with an oocyte that has had its nucleus removed. The newly produced one-cell embryo is identical to the original morula. (**c**) Embryo cloning by nuclear transfer from somatic cells. A somatic cell is inserted next to an enucleated oocyte by using a specialized pipette. The somatic cell and the oocyte membranes are then fused. The newly produced one-cell embryo is identical to the original somatic cell.

embryo are identical twins and should be genetically equivalent. The highest success rate for the production of identical offspring occurs when embryos are split into halves resulting in identical twins. Splitting embryos into four pieces can yield identical quadruplets, but the probability of pregnancy is much lower because the quarter-sized embryos are less likely to develop within the uterus. Although the technology showed initial promise, embryo splitting is generally not practiced commercially because it requires additional time and technical expertise. Conception rates after split embryo transfer may also be slightly lower than conception rates after whole embryo transfer.

**2.2. Embryo Cloning by Nuclear Transfer from Blastomeres.** A principal limitation to embryo splitting is that an embryo can be split only a few times before the pieces are too small to continue development. In contrast, embryo cloning can be used theoretically to make an unlimited number of copies of the same embryo (2). Cloned embryos are not limited by size because cells housing a complete copy of the embryonic genome are transplanted back into an oocyte that has had its nucleus removed. Therefore, the transplanted cell becomes part of a new one-cell embryo. The nucleus and surrounding cytoplasm (karyoplast) from a single cell of a 32-cell embryo is removed using a wide-bore micropipette. Oocytes are isolated from ovaries collected from slaughtered animals and are used as recipients for the karyoplast from the 32-cell embryo. Prior to the nuclear transfer, the oocyte nucleus is removed using a micropipette. The cell from the 32-cell embryo is inserted next to the oocyte and the cytoplasmic membranes of the oocyte and the karyoplast are fused using a mild pulse of electricity. When the karyoplast and the oocyte are joined, a single-cell embryo is formed that proceeds with normal development. If all of the cells from the original 32-cell embryo are used, then 32 genetically identical embryos can be produced. The embryos can be cultured *in vitro* and transferred to foster mothers or allowed to develop and used to make more embryo clones. Therefore, a large number of identical cloned animals can theoretically be produced from a single embryo. Embryo cloning by nuclear transfer from blastomeres is not routinely practiced because embryo cloning by nuclear transfer from somatic cells is a superior technology that can be applied to adult animals whose phenotype is already known.

**2.3. Embryo Cloning by Nuclear Transfer from Somatic Cells.** The entire field of mammalian embryology changed when the cloned sheep named "Dolly" was born (3). Dolly demonstrated that mammals could be cloned from adult somatic cells. Dolly was created by fusing an adult mammary cell with an enucleated oocyte. Somatic cell nuclear transfer is similar to blastomere nuclear transfer because a karyoplast is fused with an enucleated oocyte. At the time of this writing a variety of somatic cell types have been used for cloning in a number of species (4) (Table 1). Cloning by somatic cell nuclear transfer enables the production of an unlimited number of cloned animals from a single adult animal.

The widespread application of embryo cloning for agricultural purposes (creation of genetically identical and superior animals) has not occurred. Cloning of agricultural animals for applications in human medicine, however, is becoming common (see below). The limitation to embryo cloning is the technical difficulty of the procedure. Very few laboratories worldwide are capable of

Table 1. **Species and Cell Types Used for Somatic Cell Nuclear Transfer (Cloning) in Animals**[a]

| Species | Cell types |
|---|---|
| sheep | embryonic cells |
| cattle | fetal fibroblasts |
| goat | mammary gland cells |
| pig | cumulus cells (ovary) |
| mouse | oviductal cells |
| cat | leukocytes |
| rabbit | granulosa cells (ovary) |
| | skin fibroblast (newborn and adult) |
| | newborn liver |
| | tail tip cells |
| | fetal germ cells |
| | fetal ovary |
| | fetal testicle |

[a] See Ref. (4).

successfully performing cell and embryo culture, embryo manipulation, embryo transfer, and management of the surrogate mother. The cloning procedure is inefficient, even in the best laboratories. To date, only a small percentage of cloned embryos from farm animals complete development and yield live offspring (5). Most embryos are lost because of inherent inefficiencies in each step of the cloning procedure. The cloned offspring that are born have a high rate of neonatal death caused by a variety of complications during pregnancy and the early postnatal period. Both inefficiency of production and genetic/developmental problems need to be solved before cloning will be broadly applied for agricultural purposes.

## 3. Genetic Modification of Animals by Transgenesis

Genetic selection is the process of manipulating the genome by selecting superior individuals with the best complement of genes in the genome. Animals are selected based on their phenotype (outward expression of the trait of interest). In most cases, the genes that we are selecting for are completely unknown. Transgenesis is the process through which the genome of an animal is modified. A transgenic animal may have a stable modification, a stable deletion, or a stable insertion of a foreign gene into its genome (6). The foreign gene is called a transgene. Transgenic animals are produced that either over- or underexpress specific proteins within certain cells. The changes in protein expression lead to animals with unique characteristics. Transgenic animals can be classified as traditional transgenics where a gene is inserted at a random location in the genome (7) or gene-targeted transgenics (knock-outs, knock-ins, and conditional knock-outs) where a specific gene within the genome is modified (8). The traditional transgenic technology (random insertion of a gene into the genome) will be discussed first.

**3.1. Utility of Transgenic Animals.** Genes may be randomly inserted into laboratory animal genomes for the purpose of studying basic biological questions or for the purpose of developing models for human diseases (9). Genes may be randomly inserted into the farm animal genome for the purpose of improving milk production, growth rate, or disease resistance (desirable traits for farmed animals) (10) or for producing recombinant proteins in milk (11). Transgenes may be a modified gene from the same species (eg, a porcine growth hormone gene engineered to increase blood growth hormone concentrations within a transgenic pig) (12) or may be a gene from an entirely different species (eg, the spider silk protein expressed mammalian cells) (13). Transgenic animals are produced by using a combination of molecular biology techniques (used to synthesize the transgene) and embryo manipulation/embryo culture (used to insert the transgene into the embryo). The most common methods for the production of transgenic animals with a random insertion include pronuclear microinjection of one cell embryos and retroviral infection of oocytes, zygotes, or early cleavage-stage embryos. Transgenes can also be introduced into animals by fusing transgenic embryonic stem cells with early cleavage stage embryos or by nuclear transfer from transgenic embryonic stem cells or transgenic somatic cells into enucleated oocytes.

**3.2. Creating Transgenic Animals with a Random Gene Insertion.** Transfer of foreign genes into animals is done at an early stage of embryonic development (one cell to blastocyst stage) prior to implantation or placentation. Embryos at this stage of development can be grown outside the uterus of the mother (*in vitro* embryo culture) in specialized medium containing nutrients that support their growth. For best results, micromanipulation and gene transfer are performed on one-cell embryos because integration of the transgene into the embryonic DNA theoretically assures that all of the adult cells carry the foreign gene. Original lines of transgenic animals had transgenes that were highly expressed in a wide variety of tissues (ie, constitutive, nonspecific, or ubiquitous activity of the transgene) (7). The goal for modern transgenesis is to create transgenic animals that have controllable transgene expression. The transgene expression may be tissue-specific, developmental-specific, or responsive to specific internal or external signals.

## 4. Construction of a Transgene for Random Insertion

Transgenes are assembled by splicing together DNA from selected genes (7,14). Transgene assemblies contain two important parts: a promoter or regulatory region and a protein-coding region. The promoter is a DNA sequence that dictates the activity as well as the tissue specificity for the expression of the protein-coding region of the transgene. A vast array of promoters is available for use in the construction of transgenes. Selection of the appropriate promoter depends on the goals of the project. Promoters may be homologous (similar to the endogenous promoter for the expressed gene) or heterologous (different from the endogenous promoter for the expressed gene). A DNA sequence that encodes mRNA for a protein is spliced next to the promoter. The DNA within this region generally includes exons and introns as well as a polyadenylation signals

for the mature mRNA. The DNA is transcribed when the promoter region is activated within certain cells. Activation of the transgene results in the synthesis of heterogeneous nuclear RNA (hnRNA), which is processed into mature mRNA for the production of a specific protein within the transgenic animal. In some cases, the protein from the transgene may have the same biological activity and structure as the naturally occurring protein. However, the protein from the transgene may be expressed in a greater amount or synthesized within a different tissue. The adjoining promoter within the transgene determines the amount and pattern of protein expression. Examples of promoters include the whey acidic protein promoter (active in mammary tissue and used to express recombinant proteins in milk), the mouse metallothionine promoter (activated by heavy metals and used in early studies of transgenesis), the β-globin promoter (ubiquitous expression) and the EIIa promoter (active in early stage embryos).

**4.1. Methods for Random Insertion of a Transgene into an Embryonic Genome.** *Microinjection of a Transgene.* Microinjection is performed on one-cell embryos that are surgically collected from the oviduct (14). Several thousand copies of the DNA construct are dissolved in a small quantity of buffered solution and loaded into a needle specifically designed for microinjection (Fig. 2). The needle is then inserted into the pronuclei where the DNA solution is injected. A piezoelectric microinjection system may be used to facilitate the process (15). The pronuclei of the mouse embryo are readily visible. The embryos of some farm animals (eg, pigs and cattle) are briefly centrifuged to polarize the opaque cytoplasm and reveal the pronuclei prior to injection. The process through which the foreign DNA integrates into the embryonic genome is poorly understood. A greater understanding of the factors that affect the process may improve the efficiency of transgenic animal production. The transgene hypothetically inserts where a break has occurred in a DNA strand. The likelihood of a strand break may be greater near sites of active gene transcription. Breaks in DNA strands can occur throughout the genome so that microinjected transgenes are randomly inserted into genomic DNA. The position of the transgene within the genome is believed to affect its activity (see below). Once the transgene is integrated into the DNA of the embryo, it is replicated and becomes a permanent part of the embryonic genomic DNA. Microinjected embryos are generally cultured *in vitro* for a short period of time and then transferred to the oviduct or uterus of a surrogate mother.

Following pregnancy and parturition, samples of DNA are extracted from tissues of newborn animals and analyzed for the transgene DNA. The methodology for DNA analysis varies according to the original DNA construct. In general, the DNA is either analyzed by a Southern blot (a sample of genomic DNA is transferred to a nitrocellulose membrane and the presence of the transgene is detected by using a radiolabeled probe) (16) or by a polymerase chain reaction (PCR; transgene detected by specific amplification of the transgene from a sample of genomic DNA) (17).

Estimates of out-of-pocket expenses for the production of transgenic animals are high and range from several hundred dollars for a transgenic mouse to several hundred-thousand dollars for the production of a transgenic cow (18). In practice, only a small percentage of microinjected embryos develop into transgenic animals (19). Most microinjected embryos fail to develop within the

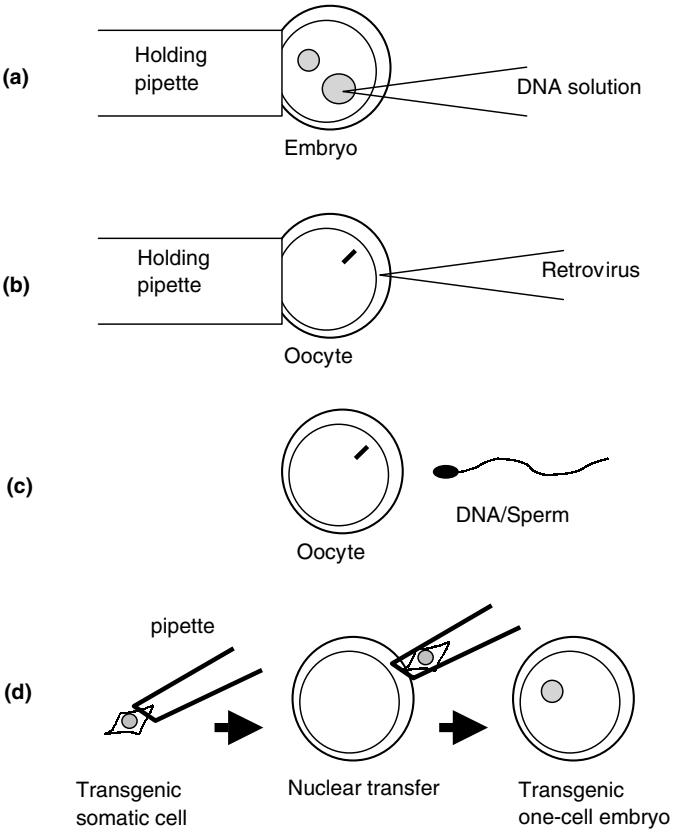


**Fig. 2.** Methods for the production of transgenic animals. (**a**) Microinjection. A DNA solution containing the foreign gene is injected into the pronucleus of a one-cell embryo. (**b**) Retroviral infection. A replication-defective retroviral vector is injected under the zona pellucida of an oocyte. (**c**) Sperm-mediated gene transfer. A sperm cell is incubated with the transgene DNA and used to carry the transgene into the oocyte during fertilization. (**d**) Gene transfer with somatic cells. A transgenic somatic cell is used to create a transgenic embryo by nuclear transfer.

uterus and die shortly after being transferred to the surrogate mother. Only a small percentage of the microinjected embryos that survive and develop normally will integrate the foreign DNA into their genomes. These inefficiencies do not preclude the production of transgenic mice. Mouse embryos can be collected, microinjected, and transferred in a short time. In addition, mice are conveniently housed within limited space, produce numerous embryos in response to superovulation, carry a large litter of potentially transgenic offspring, and have a short gestation period. The production of transgenic farm animals is a more tedious process. Compared to mice, eg, cattle require a lot of space, do not respond well to superovulation, can only carry one or two embryos, and have a long gestation interval. Pigs are somewhat more efficient than cattle for transgenesis because they require less space, have a high ovulation rate, can carry a large litter, and have a shorter gestation interval. The production of transgenic farm

animals is also less efficient than the production of transgenic mice because embryos from farm animals have a lower frequency of integration compared to mice and are less likely to survive after the microinjection procedure. The lower integration efficiency for farm animals may reflect the delayed onset of gene expression in farm animal embryos relative to mouse embryos. The aforementioned limitations combine to reduce the production efficiency for transgenic farm animals to <1% of the injected embryos. Although these factors increase the production costs for transgenic farm animals they do not preclude the production of transgenic farm animals for a variety of commercial applications (see below).

The transgene may not integrate into the embryonic DNA until several cleavages of the embryo have occurred. If integration is delayed then some of the embryonic cells integrate the transgene whereas other embryonic cells do not integrate the transgene. Delayed integration of the transgene results in an adult animal that is genetically mosaic; harboring mixtures of transgenic and nontransgenic cells. The cells within the adult may also have different sites of transgene integration because the insertional event occurred at a different location for each of the original cells in the early embryo. Gene transfer into later stage embryos (ie, two-cell, four-cell, or greater) can be done but it is less desirable because it exacerbates the problem of genetic mosaicism in the transgenic adult. Adult animal that are mosaic for the transgene can be used to produce nonmosaic offspring as long as their gonads harbor germ cells carrying the transgene. Mating transgenic founder animals to nontransgenic animals yields transgenic offspring that are "hemizygous" for the transgene (ie, harbor the transgene on a single chromosome). Hemizygous transgenics are used commonly when studying the effects of over-expressed genes. If a homozygous genetic line is desired (ie, the transgene is found on both homologous chromosomes of a diploid cell) then the hemizygous transgenics must be enrolled in breeding programs to produce homozygous offspring.

Several different lines of animals are generally created for a single transgene. The transgene is the same but the site of integration within the DNA will be different for each line. Animals carrying the same transgene but at a different genomic location can have vastly different levels of gene expression for the transgene (20). In some animals the transgene can be detected in the DNA but foreign protein is not detected in the body. Hence, the gene is nonfunctional; ie, not expressing mRNA and not producing protein. Other animals carry the transgene and express the foreign protein at extremely high levels. The extreme expression of the transgene can cause abnormal development and pathological conditions. The expression of the transgene should be under the control of the promoter region of the DNA construct. However, other factors, including the flanking DNA sequences at the site of transgene integration, can influence the level of expression of the foreign DNA. Producing transgenic animals by using artificial chromosomes (large fragments of DNA containing the gene of interest) may improve the consistency of gene expression by reducing the effects of flanking sequences (21). Negative effects of flanking sequences may also be decreased by coinjecting matrix attachment regions (MARs) or insulators with the transgene.

*Sperm-Mediated Gene Transfer.* The most common method for the production of transgenic animals is DNA microinjection into the pronucleus. An

alternative approach is to use spermatozoa to transfer genes into the embryo (22). Spermatozoa are capable of penetrating the zona pellucida (outer egg covering) and entering the egg cytoplasm. The head of the spermatozoa will bind DNA. Gene transfer can occur through a sperm-mediated process if spermatozoa are incubated with a solution containing the DNA construct and then used. Sperm-mediated gene transfer is appealing because of its simplicity and because of its potential for creating transgenic animals from in vitro or in vivo fertilization. At this time, however, the results have been inconsistent and the technique needs to be optimized.

*Retroviral Infection of Oocytes and Embryos.* Infection of oocytes and embryos with genetically modified retroviruses is a strategy to improve the efficiency of gene transfer when compared to microinjection (19). Retroviruses are naturally equipped to perform gene transfer because they infect cells and integrate their genes into the DNA of the infected cell. Once inserted, the proviral DNA of the retrovirus may remain dormant in the host genome. However, when critical signals are received, the retroviral DNA is activated and new viral particles are produced that spread the infection. By slightly modifying the genes of a retrovirus, it is possible to infect oocytes or embryonic cells with a retrovirus that inserts its genes into the genome but cannot undergo subsequent replication. These "replication-defective" retroviral vectors are used to carry transgenes (promoter and protein coding sequences) into oocytes or embryos. Replication-defective retroviral vectors contain sequences for DNA integration (long terminal repeats) that flank the transgene. The retroviral DNA sequences needed for replication of the viral particle are deleted from the replication-defective retroviral vector. The defective retroviral vectors are propagated in a specially constructed cell line that expresses the proteins needed to make a mature retroviral particle. Therefore, the replication-defective retroviral vectors can only multiply within the specialized cell line. The oocyte or embryo is incubated with the replication-defective retroviral vector. The retroviral vector infects the cells and inserts the foreign DNA at a random location in the genome. The foreign viral DNA is not harmful to the embryo because the virus cannot replicate.

Once the embryo has been infected, it is transferred to a surrogate mother. The provirus is carried throughout development as a foreign element in the DNA. The presence of the provirus is confirmed by genetic testing of the newborn animal. Transgenic animals produced by retroviral infection are usually mosaic because the retroviral transduction can be delayed and occur in the multiple cell embryo. Founder animals that test positive for the transgene are used to produce offspring that are homozygous for the foreign genes. Mosaicism may be reduced if oocytes instead of embryos are transduced with replication-defective retroviral vectors (23).

*Gene Transfer with Embryonic Stem Cells and Somatic Cells.* The field of genetic engineering in animals has undergone rapid expansion with the widespread use of embryonic stem (ES) cells (mice) and somatic cells (mice and other species) for gene transfer and genetic modification (4). Embryonic stem cells are cells isolated from developing embryos and grown in culture (24). They are maintained in an undifferentiated state by either growing the cells on top of a mitotically inactive feeder cell layer or by growing the cells in the presence of

leukemia inhibitory factor (LIF). Embryonic stem cells are unique because when aggregated with an embryo they are capable of developing into somatic cells as well as germ cells of an adult animal. Therefore, ES cells are considered "pluripotent" (capable of directing the development of an animal to adulthood and contributing to the germline of the adult animal). Transgenes can be transfected or transduced (replication-defective retroviral vector) into the DNA of ES cells while the cells are grown *in vitro*. The ES cells can be screened for the presence of the transgene before an attempt is made to use them for the production of transgenic mice. Embryonic stem cells do not develop into an embryo if they are placed by themselves in the uterus. Therefore, cells from the ES cell line are either aggregated with developing morula stage embryos, injected under the zona pellucida of morula stage embryos or injected into the blastocoele of blastocyst stage embryos (most common method) (25). The ES cells and the cells of the embryo intermingle to form a chimeric embryo. Chimeric embryos are composed of a mixture of cells from different sources (the original embryo and the ES cells). Chimeric embryos survive within the uterus and give rise to adult animals whose cells are a mixture of the cells derived from the original embryo and the ES cells. Chimeric adults must be mated to produce a second generation of animals, some of which will be direct decedents of the ES cell line and carry the desired transgene. In practice, gene transfer using ES cells is not practiced in mice because microinjection is a faster and simpler procedure to produce mice with a random insertion of a transgene. Embryonic stem cells have been used for gene transfer in certain strains of mice that have low efficiency for the production of transgenic mice by microinjection (24). Embryonic stem cells are more commonly used for genetic modification by gene targeting (see below).

Despite intense efforts by a number of laboratories, mice are the only species where the conditions for creating ES cells have been developed successfully. Attempts to create ES cells from farm animals have yielded cells that have characteristics of stem cells. However, the farm animal stem cells did not contribute to the germ line of chimeric adult animals and therefore could not be classified as pluripotent (26). A different approach, however, can be used to create transgenic animals from cells in farm animals. "Dolly" (the original sheep cloned from an adult mammary cell) demonstrated that karyoplasts (nucleus with surrounding cytoplasm) from somatic cells could be transferred into enucleated oocytes and used to create adult animals ("clones" of the original individual that donated the somatic cell). The procedure for gene transfer using somatic cells differs slightly from the procedure used for ES cells (27). Embryonic stem cells are transfected or transduced with the transgene in culture and then the transgenic cells are combined with a morula or blastocyst stage embryo. Somatic cells used for gene transfer are also grown in culture and transfected or transduced with the transgene. Instead of aggregating the cells with an embryo, however, a single transgenic cell (karyoplast) is removed and fused with an enucleated oocyte. Thus the embryo is a "clone" of the original donor cell. The cloned embryo is transferred to a surrogate mother to create cloned offspring. The cloned offspring can be mated to create genetic lines that are homozygous for the transgene.

The use of ES cells or somatic cells for the production of transgenic animals has both advantages and disadvantages relative to pronuclear injection. For

mice, pronuclear injection remains the most popular method for gene transfer at a random location because it is relatively simple compared to the alternative approach that uses ES cells. For farm animals, however, the advantages of gene transfer by somatic cell nuclear transfer outweigh those of pronuclear microinjection. The methods for insertion of foreign DNA into cells in tissue culture are simpler and more efficient than the methods for inserting DNA into embryos. Cells grown in tissue culture can be tested for the transgene and selected before they are used for nuclear transfer. For example, porcine fetal fibroblasts were infected with a replication-defective retroviral vector carrying the neomycin resistance gene (28). The fibroblasts were cultured in G418 for 13 days to enrich for transduced cells. Cells surviving the G418 selection (assumed transgenic) were used for somatic cell nuclear transfer. The ability to select transgenic cells before nuclear transfer has a distinct advantage in farm animals because compared with mice, farm animals have long gestation intervals and are expensive to care for during pregnancy. These advantages make the use of somatic cells a possible alternative to either microinjection or retroviral infection for future production of transgenic farm animals. However, improved methods for establishment, *in vitro* culture, and genetic engineering of ES cells and somatic cells are needed. In addition, genetic factors that control the development of the embryo and the integration of transgenes within the embryo need to be more clearly understood.

**4.2. Genetic Modification of Animals by Gene Targeting.** Gene targeting is the process through which a transgene is used to modify or delete specific genes in the genome (8). It is distinctly different from traditional transgenic approaches (described above) that insert genes at a random location within the genome. In gene targeting, a specific gene within the genome is modified. The modification may involve an insertion, a deletion, or a point mutation in the gene sequence. Animals undergoing gene targeting for the purpose of deleting a gene or making a gene nonfunctional are called "gene knock-out" animals.

**4.3. Production of Targeted Deletions.** Targeted deletion of a specific gene is done by using ES cells (mice) or somatic cells (farm animals). A DNA targeting vector is constructed that is designed to insert itself into the embryonic genome at a predetermined location (Fig. 3) (8). The targeting vector contains a long sequence of DNA that is homologous to the gene targeted for inactivation. The homologous DNA directs the targeting vector to a specific gene within the genome. The targeting vector is electroporated into ES cells or somatic cells. Once inside the cell, the DNA of the targeting vector exchanges with the DNA of the targeted gene through a process called "homologous recombination." Homologous recombination of the targeting vector with the genomic DNA results in either the deletion of the targeted gene or the insertion of the vector into the targeted gene. Either outcome theoretically inactivates gene function but targeting vectors that delete the target gene are preferred because the gene is removed from the genome. Homologous recombination only occurs in a small percentage of the targeted cells. Thus, a method is employed to identify cells undergoing the targeting event. A positive selectable marker gene as well as a negative selectable marker are carried in the targeting construct and used to enrich for cells undergoing successful gene targeting. For example, if the targeting vector contains a neomycin resistance gene then the targeted cells will grow in the

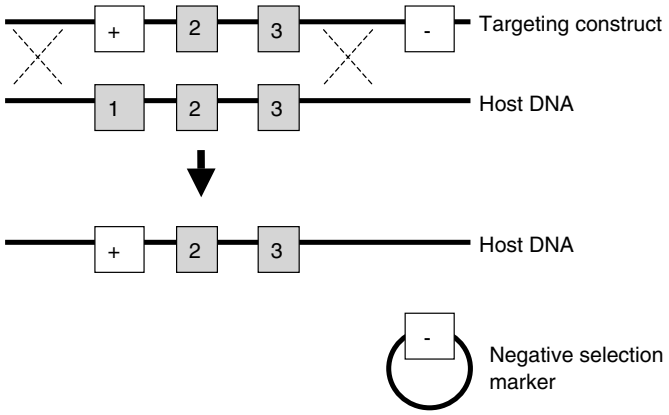


**Fig. 3.** Gene inactivation by homologous recombination (gene targeting or knock-out). The targeting vector contains homologous DNA, a positive selectable marker, and a negative selectable marker. In this example, the targeted gene in the host DNA contains three exons (protein coding region of the gene). The DNA of the targeting vector exchanges with the DNA of the targeted gene through a process called homologous recombination (dotted "X" in the diagram). Homologous recombination of the targeting vector with the genomic DNA results in deletion of exon 1 of the targeted gene (leads to gene inactivation). A positive selectable marker gene (+) as well as a negative selectable marker (−) are carried in the targeting construct and used to enrich for cells undergoing successful gene targeting. Cells undergoing successful targeting will retain the positive selectable marker and lose the negative selectable marker. The targeting is done in embryonic stem cells or somatic cells that are then used to create viable embryos.

presence of G418. Growing cells in culture with G418 enriches for transgenic cells by killing the cells that have not integrated the targeting vector. Negative selectable markers such as thymidine kinase (TK) can also be included in the targeting construct. The negative selectable marker is placed outside the region of homologous DNA. Insertion of the targeting vector into the correct location within the genome will excise the negative selectable marker and prevent its expression. If the targeting vector inserts randomly into the genome then the negative selection marker will be incorporated and expressed in the cell. Expression of the negative selection marker TK is lethal to the cell when used with the chemical gancyclovir. The use of a negative selectable marker enriches for cells undergoing the correct targeting event.

**4.4. Production of Conditional Targeted Deletions (Cre/*loxP* system).** Gene targeting in ES cells results in the deletion or inactivation of specific genes in the genome. The effect of gene targeting on the phenotype provides insight into the function of specific genes. Deletion of some genes results in what is known as an "embryonic lethal" where the embryo or fetus dies in utero (29). The function of genes whose knock-outs are lethal at the embryonic stage cannot be studied in adult animals. Other genes have knock-outs that are not embryonic lethal but for scientific reasons their function should be knocked out at specific times during development or within specific tissues of the adult animal. For example, knocking out a gene in the whole animal may create secondary effects where loss of function in one tissue prevents the study

of gene function in the primary tissue of interest. Animals whose genes are knocked out in a tissue- specific or developmentally specific manner are called "conditional knock-out" animals.

Conditional knock-outs are created by using a recombination system called Cre/*loxP* (30,31). A second recombination system known as Flp/*frt* will not be discussed here because Flp/frt is conceptually similar to Cre/*loxP* but less widely used. A traditional gene targeting approach is used to introduce short DNA sequences known as *loxP* sites around the targeted gene. The *loxP* sites are 34 base pairs in length and contain inverted repeat sequences separated by 8 base pair spacers. The gene is termed "floxed" (flanked by *loxP* sites) once the *loxP* sites are used to surround a portion of the gene (Fig. 4). The entire gene or perhaps only a protein coding exon from the gene may be floxed. The *loxP* sites do not affect gene expression. Therefore, the gene functions normally in the floxed state. Adult animals can be derived from mice carrying floxed genes that would otherwise be embryonic lethal if completely knocked out. Floxed alleles are deleted from the genome when a recombinase known as "Cre" is expressed. The recombinase excises the region of DNA between the two *loxP* sites and deletes the floxed gene.

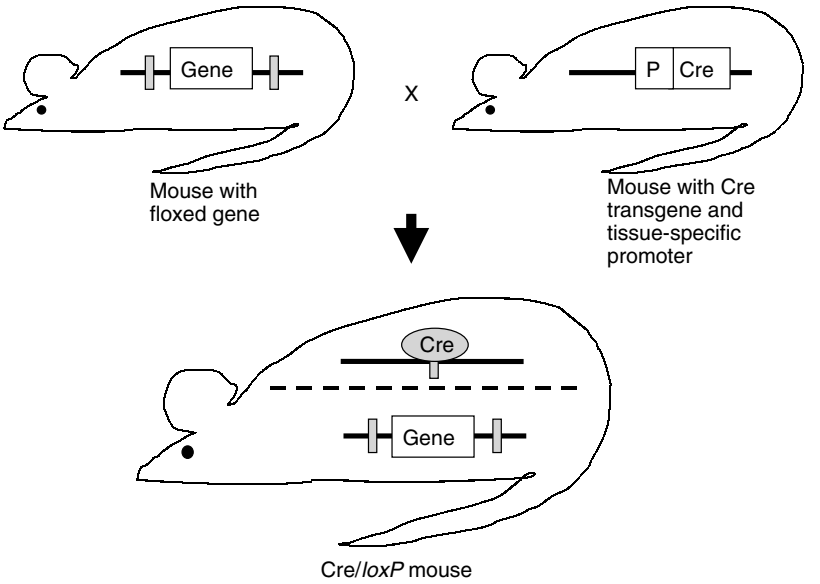


**Fig. 4.** Conditional gene inactivation by using the Cre/*loxP* system (conditional knock-out mouse). A traditional gene targeting approach is used to introduce *loxP* sites (shaded rectangles) around the targeted gene (floxed gene). A transgenic mouse line is created that has the Cre gene driven by a tissue-specific promoter (P). Mice with the floxed allele are mated to mice that carry the Cre transgene. The tissue-specific and (or) developmentally specific expression of Cre in the progeny deletes the floxed gene in target tissues (represented by DNA strand above the dotted line). Tissues that do not express Cre retain the floxed gene (represented by DNA strand below the dotted line). Thus, individual mice are chimeric for the floxed gene.

The Cre/*loxP* system depends on the cell, tissue and (or) developmental expression of Cre. A transgenic mouse line is created that expresses Cre in a manner that is consistent with the desired location or developmental window for the knock-out of the targeted gene (30,31). Creation of appropriate Cre expression depends on identifying a promoter that will initiate Cre expression at the appropriate time and in the appropriate tissue. For this conditional knockout approach, two distinct mice need to be generated: (*1*) floxed mice made by a gene targeting approach in ES cells; and (*2*) Cre mice made by traditional trangenesis via pronuclear injection. Mice with a floxed allele are mated to mice that carry the Cre transgene. The tissue-specific and (or) developmentally specific expression of Cre deletes the floxed gene in target tissues.

Transgenic mice with Cre expression under the control of inducible promoters have also been created (32). Tetracycline can be fed at the desired time ("Tet-ON" system) or taken out of the diet at the desired time ("Tet-OFF" system) to turn on Cre gene expression and delete the targeted gene. Transgenic mice carrying fusion proteins of Cre and steroid receptors have been used for the purpose of inducing Cre translocation to the nucleus in response to tamoxifen or RU486 (orally-active steroid antagonists that bind steroid receptor).

**4.5. Knock-In Mice.** Mice that have a deleted gene are knock-out mice. A knock-out mouse can either be a complete knock-out or a conditional knock-out mouse. Knock-in mice also undergo gene targeting. However, knock-in mice have a gene replacement instead of a gene deletion at the targeted locus (8,32). Foreign genes can be knocked in and expressed under endogenous regulatory elements. The replacement gene may be a completely foreign gene or may be the target gene with a mutated protein-coding region. Alternatively, the replacement gene may contain a mutated promoter region that can be used to study the effects of a promoter mutation on the expression of a specific gene. In some cases, only subtle mutations are introduced into the gene. Knock-in technology has been used to create mice with mutations that are similar to those found in humans. The mice with the mutated allele can then be used to study the analogous genetic disease in humans.

Marker genes are genes whose protein product can be easily assayed in the whole animal. Marker genes have been knocked into specific loci for the purpose of studying the tissue-specific and developmental-specific expression of genes (33). The marker is expressed in the same way as the targeted gene. A simple assay can be used to detect the marker. The *lacZ* gene encodes β-galactosidase, an enzyme that catalyzes the hydrolysis of X-gal to produce a blue color. When LacZ is knocked into a gene, the tissues that express the gene will stain blue when incubated with X-gal. Tissue-specific expression of a gene, therefore, can be rapidly assayed by examining the whole embryo or by performing histological sections of specific tissues. The green fluorescent protein can be knocked in so that tissues glow green when a gene is active. Knock-in mice have also been used to create transgenic animals that are transgenic for a variety of genes at the same locus (8,32). Creating transgenic animals whose transgene is knocked into the same locus eliminates problems associated with different integration sites in different lines of transgenic animals.

## 5. Applications of Transgenic and Gene-Targeted Animals

The production and use of transgenic and gene targeted animals represents an evolving technology of engineering animal species for specific roles in science and agriculture. Transgenic and gene targeted animals have become mainstays for scientific research. Although the bulk of the genetically modified animals used for scientific research are mice, farm animals are being created when mouse models do not recapitulate the physiology of human disease. Transgenic farm animals are quickly becoming essential for the low-cost production of recombinant proteins that cannot be produced by bacteria. The productivity of farmers may be improved through the use of transgenic farm animals in their herds. Pigs with deleted immune recognition genes may soon provide organs for transplantation into humans.

**5.1. Transgenic Farm Animals.** *Transgenic Animals with Growth Promoting Genes.* Agricultural productivity may be increased by using lines of transgenic farm animals that preferentially express selected genes in their tissues. The first transgenic livestock carried genes designed to increase growth, milk production, or muscling (6,10,19,21,27). The transgenes included growth hormone (GH), growth hormone releasing factor (GRF) and insulin-like growth factor-I (IGF-I). Growth hormone releasing factor causes the release of GH from the pituitary gland and GH causes the release of IGF-I from the liver (34). The original transgenes were chosen because increased concentrations of GH and IGF-I in the blood are associated with increased growth and milk production in farm animals. In addition, cattle or swine injected with GH have greater IGF-I in the blood and grow more efficiently. Dairy cows injected with GH produce more milk (35). A transgenic animal carrying an additional GRF, GH, or IGF-I gene, therefore, theoretically should grow faster and more efficiently, and produce more milk. The concept of using a GH transgene to increase growth was initially tested in mice. Transgenic mice were produced that carried a GH gene under the control of an inducible promoter. The mice had increased concentrations of GH in the blood and grew considerably larger than the nontransgenic mice (36). The experiment suggested great promise for transgenic technology in agricultural species; however, farm animals carrying the GH transgene have not shown similar responses. Transgenic pigs with high levels of GH have increased feed efficiency and rate of gain but suffer from a variety of illnesses including gastric ulcers, synovitis, dermatitis, joint abnormalities, and infertility. The pathologies observed in pigs with the GH transgenes were similar to those observed in humans with acromegaly (giantism; an endocrine disease of excess GH secretion). Thus, in the case of GH transgenesis, pigs were found to be different from mice. Overexpression of GH in mice led to faster growing and larger mice. Overexpression of GH in pigs led to faster growing pigs but the improved growth was associated with sickness and disease. Likewise, transgenic mice harboring additional copies of the c-*ski* gene (a protein that is involved in muscle fiber development) were healthy and had larger muscles. Transgenic swine carrying the c-*ski* gene also had larger muscles but their fore and hind legs were weak. The *GH* and c-*ski* transgene examples demonstrate that different species can respond differently to similar transgenes. The different responses reflect unique physiological differences between species.

Although the initial studies of GH transgenesis in farm animals were disappointing, there is at least one line of GH transgenic pigs that are healthy and have increased growth and muscling (12).

The illnesses and physical abnormalities in transgenic swine carrying the *GH* or c-*ski* genes made the animals useless for agricultural purposes. Transgene expression must be controlled so that transgenes are active during periods of growth and inactive during other periods. Reasonable control of transgene expression can be achieved through the selection of appropriate promoters. However, few promoters tightly control transgene expression. Identifying new promoters that elicit better control of gene expression will be necessary for the future success of transgenic technologies. In addition, the actual site of transgene insertion within genomic DNA may have to be controlled. Controlling the site of integration could prevent the complicating effects of flanking DNA sequences that can either increase or diminish the activity of the promoter within the DNA construct.

Perhaps the only meat producing species known to tolerate GH transgenesis is salmon (37). Transgenic salmon carrying a GH transgene grow rapidly and do not suffer from disease or illness. Farming of salmon with GH transgenes may occur in the near future. Ecological concerns are the primary impediments to the use of transgenic salmon (38). If transgenic salmon escaped from sea pens then they could breed with wild salmon populations. The effect of the transgene in wild salmon populations is not known. The potential for the rapid spread of the transgene to the detriment of wild populations, however, has raised concerns among ecologists and fishery biologists.

*Transgenic Animals with Innate Immunity.* One method to increase the productivity of farm animals is to increase their resistance to common diseases (39). Transgenic farm animals can be produced that carry genes for immunoglobulins directed against viruses or bacteria. In theory, animals carrying foreign immunoglobulin genes would not need to be vaccinated against certain diseases because the expression of the transgene would cause the production of immunoglobulins. Transgenic rabbits, sheep, and pigs that carry mouse immunoglobulins have been produced (40). The foreign immunoglobulins are correctly assembled and secreted into the blood. Immune function may also be improved in transgenic animals with greater cytokine or immune resistance gene expression. Knocking out intestinal receptors may reduce the incidence of systemic infections for pathogens entering through the gut wall (27).

*Production of Recombinant Proteins in Transgenic Farm Animals.* Transgenes engineered for mammary gland expression can direct the synthesis and secretion of recombinant proteins into the milk of sheep, goats, and cattle (11,18,41,42) . The gene coding for the protein is driven by a promoter that is active within the mammary gland. The recombinant protein is synthesized in mammary cells and secreted into the milk. The milk is collected and the protein is extracted and purified. Proteins secreted into milk are appropriately modified for mammalian systems and can have greater biological activity when compared to similar proteins produced in bacteria. A variety of pharmaceutical proteins can be produced by mammary cells (Table 2). A single cow can potentially produce enough milk to supply the worldwide needs of some specialized drugs (18). Goats have been genetically engineered to produce spider silk protein in

Table 2. **Pharmaceuticals Produced in Milk or Blood of Transgenic Farm Animals**[a]

| Protein | Treatment application |
|---|---|
| $\alpha_1$-antitrypsin | emphysema |
| α-lactalbumin | infection |
| antibodies | vaccines, cancer |
| antithrombin III | thrombosis |
| CFTR | cystic fibrosis |
| collagen I, II | tissue repair |
| factor VIII | hemophilia |
| factor IX | hemophilia |
| fibrinogen | wound healing |
| hemoglobin | synthetic blood |
| glutamic acid decarboxylase | type 1 diabetes |
| human serum albumin | trauma and burn treatment, blood volume |
| lactoferrin | GI tract infection, infant formula |
| protein C | thrombosis |
| tissue plasminogen activator | thrombosis |

[a] See Refs. (11,18,41,42).

milk. The spider silk protein is stronger and more flexible than steel and offers a lightweight alternative to carbon fiber (13).

*Improving the Amount or Nutritional Value of Milk.* Farm animals have a copious milk supply. Nonetheless, milk production of the mother can sometimes limit the growth of her offspring. In pigs, a large percentage of piglet growth is dependent on the volume and composition of the milk produced by the mother. Transgenic pigs that express bovine α-lactalbumin in their milk have a higher milk protein percentage (27). Piglets suckling sows expressing bovine α-lactalbumin in milk grew faster perhaps because of the higher milk protein content of the milk that they consumed. Newborn animals depend on milk for nutrients but they also depend on growth factors and immunoglobulins found in milk. In addition to increasing mammary production of milk components, therefore, it should be theoretically possible to increase the production of growth factors or immunoglobulins in milk (27). The increased concentration of nutrients, growth factors, and immunoglobulins could lead to faster growing, healthier newborn animals. Ultimately it may be possible to improve bovine milk for human consumption by altering the composition of bovine milk to more closely fit the nutrient requirements or the manufacturing needs of humans (18).

*Creating Environmentally Friendly Animals.* Farm animals require phosphorous in their diets. Most of the phosphorous in farm animal diets, however, is unavailable to the animal because it is in the form of phytate (natural molecule containing phosphorous in plants). Farmers add inorganic phosphorous to farm animal diets so that the phosphorous requirements of the animals are met. Unfortunately, much of the inorganic phosphorous ends up in the manure of the farm animals and is spread on the land surrounding the farm. The phosphorous from the manure either builds up on the land or runs off the land into streams, rivers, and lakes. Phosphorous build-up on land can become toxic to plants. Phosphorous run-off lowers water quality of surrounding waterways (43).

Transgenic pigs have been created that partially solve the phosphorous problem described above (44). Fungi and bacteria (but not animals) have the gene for phytase; the enzyme that breaks down phyate. A transgene was constructed containing a mouse promoter for a salivary protein and a phytase gene from a bacterium. The pigs carrying the transgene express phytase in their salivary glands. They can utilize the phosphorous in phytate because their salivary glands release the phytase enzyme when they consume feed. Thus, the total amount of phosphorous in the feed is reduced because a previously unavailable source of phosphorous (phytate) is now available to the pig. Less phosphorous in the diet results in less phosphorous in the feces and less phosphorous spread on to the land surrounding the farm. Transgenic pigs with the phytase gene are called "Enviropigs" because of their environmentally-friendly trait. The pigs may be commercially available in the near future.

*Gene Targeting in Farm Animals.* Gene targeting in farm animals is an important new area of research with exciting applications for human health. Methods for the maintenance of ES cells have not been developed for farm animals. However, somatic cells (usually fetal fibroblasts) can be used for gene targeting in farm animals. The first successful gene targeting was done in lambs by the same group that created "Dolly" (3). The gene for ovine α1 procollagen was replaced by the gene for human α1-antitrypsin (valuable pharmaceutical protein) (45). Shortly afterward, the same group knocked out the prion protein gene from sheep (46). The prion protein gene was an important target because the gene is associated with spongiform encephalopathies in cattle (BSE or "mad cow disease), sheep (scrapie), and humans (Creutzfeld-Jacob disease). Farm animals are believed to pass spongiform encephalopathy to humans through the prion protein. Removing the gene ensures that sheep cannot contract scrapie and cannot pass spongiform encephalopathy to humans.

Perhaps the most exciting new knock-outs animals are sheep and pigs that have the α-1,3-galactosyltransferase gene removed from their genome (46,47). The hope is that these new knock-outs will be used as a source of transplant organs for humans (xenotransplantation or cross-species organ transfer). The surface of pig cells (and the cells of most mammals) contains glycoproteins that carry terminal α-1,3-galactosyl residues. The surface of human cells (as well as the cells of old world monkeys) does not contain α-1,3-galactosyl residues because humans have lost the gene for α-1,3-galactosyltransferase (the enzyme that places α-1,3-galactosyl residues onto glycoproteins). Transplanted organs are destroyed when pig tissue is transplanted into humans because the human immune system has preformed antibodies against α-1,3-galactosyl residues. Immune system attack results in organ failure within a few minutes after transplantation (hyperacute rejection). Organs from sheep and pigs with an α-1,3-galactosyltransferase knock-out should not undergo hyperacute rejection because they do not express α-1,3-galactosyl residues on their cell surface. Farm animals with a α-1,3-galactosyltransferase knock-out are being tested in xenotransplantation experiments.

**5.2. Transgenic and Gene Targeted Mice for Biomedical Research.** Transgenic mice revolutionized the methods used to study the functions of specific genes. Gene targeting carried the revolution one step further. It could be argued that collectively, transgenic and gene targeted mice represent some of the most

important tools for biomedical research in the twenty-first century. To understand the function of a specific gene, a transgenic mouse is created that overexpresses the gene. The amount, location, and time of gene expression can be controlled somewhat by the promoter in the transgene. The physiology and morphology of the mouse in the context of excess gene expression is studied. A change in the normal physiology or morphology of the mouse provides an insight into the function of the gene. The mice can be used for basic research (simply understanding the function of an unknown gene) or they can be used for applied research questions. For example, transgenic mice can be created that mimic the physiology of known diseases; particularly if the disease is caused by the overexpression of a specific protein. Once the mouse model is created it can then be used to develop therapies that may someday be applied to humans. Large databases catalogue transgenic lines of mice and their corresponding phenotypes (TBASE; http://tbase.jax.org).

Transgenic mice are excellent models for the overexpression of certain genes and they have played a critical role in understanding gene function. Transgenic mice have also been used to create dominant negative genes that inactive intracellular pathways (48) and to create antisense mRNAs that cause gene inactivation (49). Gene targeting enabled the modification of genes by either knocking out the gene altogether or by knocking in a mutation into the gene. Gene targeting is a step beyond simple overexpression of genes because in gene targeting the function of a gene is accessed by evaluating the phenotype of the gene-deleted mouse. Targeted mice have been created to address nearly every important disease state known to be linked to a single gene defect in humans. The disease state can be mimicked by completely knocking out the gene or by introducing mutations than can cause subtle changes in gene expression or function. In some cases, the knock-out model in mice poorly recapitulates the symptoms of human disease. For example, mice lacking the cystic fibrosis transmembrane conductance regulator (CFTR) gene do not develop the lung disease found in human cystic fibrosis patients. In these specialized cases, transgenic farm animals may more closely resemble the human condition (9).

Conditional knock-out mice may represent the final step in the evolution of genetic modification of animals. The original knock-out mice had the targeted gene deleted in embryonic cells. For some genes, loss of function was lethal to the embryo. It was impossible to study the function of embryonic lethal genes in adult animals because the embryo died in utero. Conditional knock-out mice (Cre/*loxP* system) were originally developed to circumvent the problem of embryonic lethality. The system has now evolved into a sophisticated methodology that can be used to remove genes within specific tissues at specific times during fetal, neonatal or adult life. The conditional knock-out system is a powerful technique that can be used to study a wide range of scientific questions.

## 6. Gene Therapy in Humans

Gene therapy is the genetic modification of humans for the purpose of correcting genetic diseases (50). The procedures for gene therapy in humans are related to those described here. However, gene therapy in humans is typically practiced on

somatic cells of children and adults and is intended to relieve the disease state of the treated individual. The genetic modifications are not passed to offspring because germ cells are not targeted. The techniques used for genetic modification of laboratory and farm animals could be applied to human embryos for the purpose of correcting genetic defects. If successful, the genetic defect would be corrected in both somatic and germ cells of the individual arising from the manipulated embryo. However, genetic modification of animals is still an imperfect technology with limitations that are poorly understood. There is a high rate of embryonic loss associated with embryo manipulation and embryo culture. In farm animals, the offspring created by cloning and gene targeting typically have poor health. Second generation animals, however, appear normal (26). The techniques for genetic modification in animals will need to be optimized before they can be considered safe for the purpose of correcting genetic defects in both somatic and germ cells of humans.

## BIBLIOGRAPHY

"Genetic Engineering" in *ECT* 3rd ed., Vol. 11, pp. 730–745, by A. M. Chakrabarty, University of Illinois at the Medical Center, Chicago; in Suppl. Vol., pp. 495–513, by E. Jaworski and D. Tiemeier, Monsanto Co.; in *ECT* 4th ed., Vol. 12, pp. 465–480, by M. Lucy, University of Missouri, Columbia, and R. Collier, Monsanto Company; "Genetic Engineering, Animals" in *ECT* (online), posting date: December 4, 2000, by Matthew Lucy, University of Missouri, Columbia, and Robert Collier, Monsanto Company.

## CITED PUBLICATIONS

1. J. M.E Robl and N. L. First, *J. Reprod. Fertil. Suppl.* **33**, 101 (1985).
2. R. S. Prather and N. L. First, *J. Reprod. Fertil. Suppl.* **40**, 227 (1990).
3. I. Wilmut and co-workers, *Nature (London)* **358**, 810 (1997).
4. G. Brem and B. Kühholzer, *Cloning and Stem Cells* **4**, 57 (2002).
5. A. Dinnyés and co-workers, *Cloning and Stem Cells* **4**, 81 (2002).
6. R. J. Wall, *Theriogenology* **57**, 189 (2002).
7. R. D. Palmiter and R. L. Brinster, *Cell* **41**, 343 (1985).
8. P. Hasty and co-workers, in A.L. Joyner, ed., *Gene Targeting A Practical Approach*, Vol. 1, Oxford University Press, Oxford, U.K., 2000.
9. R. M. Petters and J. R. Sommer, *Transgenic Res.* **9**, 347 (2000).
10. H. Niemann H and W. A. Kues, *Anim. Reprod. Sci.* **60–61**, 277 (2000).
11. L. M. Houdebine, *Transgenic Res.* **9**, 305 (2000).
12. M. B. Nottle, in J. D. Murray, G. B. Anderson, A. M. Oberbauer, and M. M. McGloughlin, eds., *Transgenic Animals in Agriculture CAB International*, Wallingford, U.K., 1999, pp. 145–156.
13. Lazaris and co-workers, *Science* **295**, 472 (2002).
14. A. Hammes and A. Schedl, in I. J. Jackson and C. M. Abbott, eds., *Mouse Genetics and Transgenics A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 217–245.
15. G. Tigyi and I. Parker, *J. Biochem. Biophys. Methods* **22**, 243 (1991).
16. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).

17. H. A. Erlich, D. Gelfand, and J. J. Sninsky, *Science* **252**, 1643 (1991).
18. R. J. Wall and co-workers, *J. Dairy Sci.* **80**, 2213 (1997).
19. R. J. Wall, *Theriogenology* **57**, 189 (2002).
20. R. E. Hammer and co-workers, *Nature (London)* **315**, 680 (1985).
21. L. Montoliu, *Cloning Stem Cells* **4**, 39 (2002).
22. F. Gandolfi, *Theriogenology* **53**, 127 (2000).
23. A. W. S. Chan and co-workers, *Proc. Natl. Acad. Sci.*, U.S.A. **95**, 14028 (1998).
24. B. Ledermann, *Exp. Phys.* **85.6**, 603 (2000).
25. V. Papaioannou and R. Johnson, in A.L. Joyner, ed., *Gene Targeting A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 133–175.
26. R. S. Prather and co-workers, *Theriogenology* **59**, 115 (2003).
27. M. B. Wheeler and E. M. Walters, *Theriogenology* **56**, 1345 (2001).
28. K.W. Park and co-workers, *Anim. Biotech.* **12**, 173 (2001).
29. S. Bultman and T. Magnuson, in A.L. Joyner, ed., *Gene Targeting A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 255–283.
30. S. M. Dymecki, in A.L. Joyner, ed., *Gene Targeting A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 37–99.
31. A. F. Kolb. *Cloning Stem Cells* **4**, 65 (2002).
32. A. Plagge and co-workers, in I. J. Jackson and C. M. Abbott, eds., *Mouse Genetics and Transgenics A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 246–284.
33. S. G. Nonchev and M. K. Maconochie, in I. J. Jackson and C. M. Abbott, eds., *Mouse Genetics and Transgenics A Practical Approach*, Oxford University Press, Oxford, U.K., 2000, pp. 61–86.
34. T. D. Etherton and D. E. Bauman, *Phys. Rev.* **78**, 745 (1998).
35. D. E. Bauman, *Domest. Anim. Endocrinol.* **17**, 101 (1999).
36. R. D. Palmiter and co-workers, *Nature (London)* **300**, 611 (1982).
37. S. J. Du and co-workers, *Biotechnology (N.Y.)* **10**, 176 (1992).
38. E. Check, *Nature (London)* **418**, 805 (2002).
39. M. Muller and G. Brem, *Reprod. Fertil. Dev.* **6**, 605 (1994).
40. U. H. Weidle, H. Lenz, and G. Brem, *Gene* **98**, 185 (1991).
41. R. J. Wall, *Livestock Prod. Sci.* **59**, 243 (1999).
42. C. A. Pinkert and J. D. Murray, in J. D. Murray, G. B. Anderson, A. M. Oberbauer, and M. M. McGloughlin, eds., *Transgenic Animals in Agriculture*, CAB International, Wallingford, U.K., 1999, pp. 1–18.
43. Boesch and co-workers, *J. Environ. Qual.* **30**, 303 (2001).
44. Golovan and co-workers, *Nat. Biotechnol.* **19**, 741 (2001).
45. K. J. McCreath and co-workers, *Nature (London)* **405**, 1066 (2000).
46. C. Denning and co-workers, *Nat. Biotechnol.* **19**, 559 (2001).
47. L. Lai and co-workers, *Science* **295**, 1089 (2002).
48. D. Rosenberg and co-workers, *Ann. N. Y. Acad. Sci.* **968**, 65 (2002).
49. R. P. Erickson, *Methods* **18**, 304 (1999).
50. G. Romano and co-workers, *Stem Cells* **17**, 191 (1999).

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