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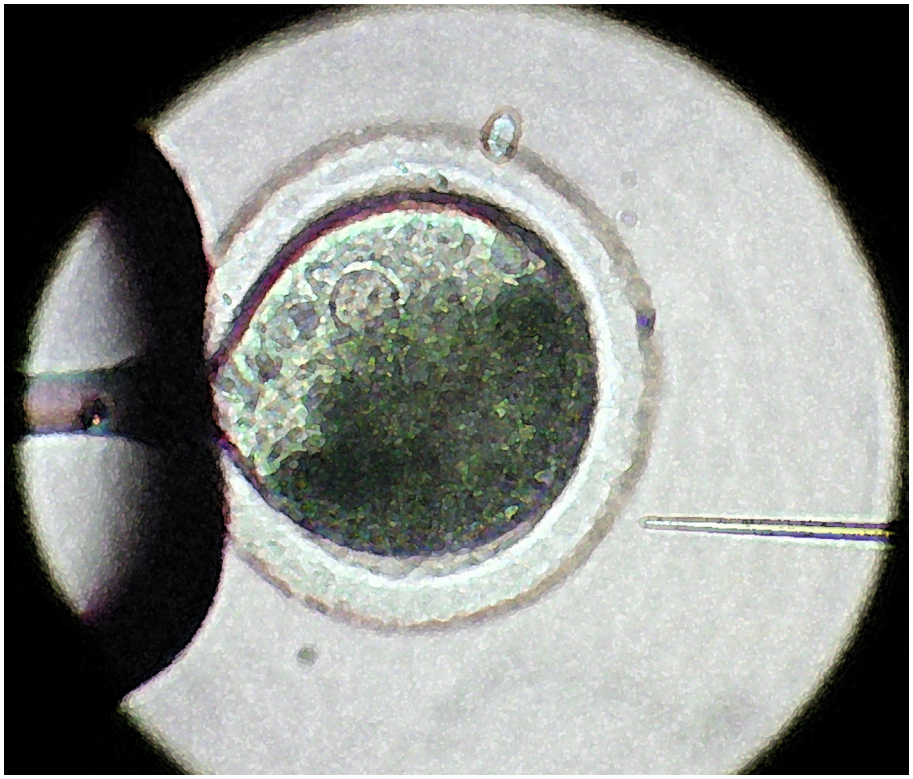
The Role of Transgenic Livestock in the Treatment of Human Disease

Animal Agriculture's Future through Biotechnology, Part 6

Abstract

Transgenic livestock have the potential to play a critical role in the production of new medications for the treatment of human disease. This role may consist of actual production of recombinant proteins (including biotherapeutic proteins and antibodies) for treatment of human diseases. Or, it may involve the development of new animal models that can be used in studies relating to human diseases. Both approaches can provide significant advances in the development of new treatments. Two techniques discussed in this paper—pronuclear microinjection and somatic cell nuclear transfer (SCNT)—are the predominant methods used to produce transgenic livestock. An in-depth description of the SCNT methodology is provided in an appendix. The paper describes two improvements that have been made in SCNT-based gene transfer technologies that enhance the potential for its application: gene targeting and transchromosomic technology. In addition, economic and regulatory issues are addressed, as are societal issues. The authors suggest that education regarding the advantages and challenges associated with this new technology is the key to public understanding.



Colorized image of a bovine pronuclear oocyte and DNA microinjection needle. (Photo courtesy of C. Keefer)

Introduction

Transgenic livestock have the potential to play a critical role in the treatment of human disease through the production of biopharmaceutical proteins and the development of disease models. After the development of *recombinant deoxyribonucleic acid (DNA) technologies*[1] in the early 1970s, scientists began to envision using *transgenic animals* not only as medical models of human and animal diseases but also for production of biopharmaceutical products. This exciting prospect was deemed feasible because of the ability to select, copy, and alter identified genes of interest. Using specialized techniques and appropriate experimental conditions, scientists could transfer these small pieces of genetic material into another plant or animal, resulting in an altered or novel trait such as faster growth or better disease resistance.

Since the time these uses were first projected, some transgenic animals (mice, pigs) have been developed as medical models for human diseases and for animal diseases such as bovine mastitis and bovine spongiform encephalopathy (BSE). Other transgenic animals (cattle, chickens, goats, pigs, rabbits, and sheep) have been developed for biopharmaceutical production. As scientists continue to perfect DNA technologies in the near future, more applications of transgenic animals for the treatment of human diseases will become available.

In this Issue Paper, the authors describe the potential for transgenic livestock to advance the development of new medications and treatments of human disease and offer a brief overview of current production methods and challenges. They also discuss the

[1] Italicized terms (except species names) are defined in the Glossary.

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economic, regulatory, and societal factors that impact the commercialization of products and treatments derived from transgenic animals. An in-depth description of the production of transgenic animals by somatic cell nuclear transfer is given in an appendix.

Methods of Transgenic Animal Production

An animal is considered transgenic if it has a copy of foreign DNA stably integrated into its genome. The foreign DNA (called a transgene) usually consists of a gene coding for the protein of interest and genetic regulatory sequences that protect or enhance *gene expression*. The protein coded by the transgene is known as a *recombinant protein* because it results from the recombination of the desired gene with the controlling elements. This transgene can be derived from the DNA code of another animal of the same species, an animal of a different species, or even from bacteria or plants.

The production of transgenic mice has become a common research tool to study gene function and to create disease models. In many instances, however, transgenic livestock (pigs, sheep, goats, and cows) may provide a better model than mice for studying human diseases because of physiological similarities. Furthermore, domestic livestock, which have been bred for high production traits, provide an effective means of producing biopharmaceuticals (*therapeutic proteins*) for the treatment of human disease.

The first steps in making a transgenic animal are *selection* of the gene of interest and determining where and when the gene should be expressed. A gene is said to be expressed when the DNA code of the gene is interpreted into messenger ribonucleic acid (mRNA) in a process called transcription, after which the mRNA is translated into the specific protein. Usually the expression of the protein is targeted to a particular tissue. For example, if the plan is to produce the biopharmaceutical protein in the milk of a cow, then genetic sequences that specify expression and secretion of the protein in the lactating cow's mammary gland will be needed, in addition to the gene coding for the biopharmaceutical protein itself.

Thus, a construct for the gene of interest would be designed to contain a *promoter* that specified the location and timing of expression and the gene of interest. The construct also could contain sequences that enhance or protect gene expression and marker genes that could be used to follow the incorporation of the gene construct into the genome of the animal. In addition to construct design, a method for transferring the transgene into an animal must be selected.

Three techniques—*pronuclear microinjection, somatic cell nuclear transfer* (SCNT), and *embryonic stem cells*—have been the predominant methods used to produce transgenic mice. But only two of those techniques—pronuclear microinjection and SCNT (also referred to as cloning)—have been used to produce transgenic livestock, because embryonic stem cells are not available in livestock.

Embryonic Stem Cells

Whereas embryonic stem cells can be established readily from certain strains of mice, derivation of embryonic stem cell lines from other strains of mice and from primates is more difficult, and no validated lines have been established from other mammals. The question of why embryonic stem cells are so difficult to establish from livestock species is an ongoing topic of research (Keefer et al. 2007).

Pronuclear Microinjection

In pronuclear microinjection, many copies of the gene construct are injected directly into one or both of the pronuclei of a recently fertilized *oocyte*. If the transgene is incorporated at this pronuclear stage, then all cells of the resulting animal (the founder animal for a transgenic line) will contain the transgene. In reality, however, the transgene does not always incorporate at this stage but incorporates one or more cellular divisions later. The result can be a mosaic animal in which some cells contain the transgene and others do not. Furthermore, there is no guarantee that the founder animal or its offspring will express the gene of interest because of the random integration of the transgene into the genome.

Despite these challenges, pronuclear microinjection has been used successfully to generate livestock, including sheep and goats, that produce valuable biopharmaceutical proteins (Clark et al. 1989; Ebert et al. 1991). In fact, the first biopharmaceutical product produced by a transgenic animal to receive European Medicines Agency (EMEA) approval was ATryn—a recombinant form of human antithrombin. This product was produced in transgenic goats as a result of pronuclear microinjection (Zhou et al. 2005).

Somatic Cell Nuclear Transfer

In the meantime, SCNT, using pre-

selected transgenic donor cells, is rapidly superseding pronuclear microinjection as the method of choice. The SCNT technique (Figure 1) overcomes many of the inefficiencies found in pronuclear microinjection and may help lower the costs of transgenic animal production by decreasing the number of animals used during production (Baldassarre et al. 2002). A more in-depth description of the methodology involved in SCNT is given in Appendix 1.

Recent Developments in SCNT-based Gene Transfer Technologies

Two improvements have been made in SCNT-based gene transfer technologies that significantly enhance the potential for its application: *gene targeting* (Kuroiwa et al. 2004) and *transchromosomic technology* (Kuroiwa et al. 2002).

Gene Targeting

The first improvement, gene targeting, allows the foreign DNA to be targeted to a precise location in the host genome. Gene targeting relies on a process known as *homologous recombination*. This process can occur between two identical DNA sequences where an *exogenous sequence* can actually replace an identical *endogenous sequence*. The intent of gene targeting is to replace a length of endogenous DNA with a different length of foreign DNA. This action can be accomplished by hiding the new piece of DNA between two long flanking pieces of foreign DNA that are identical to a stretch of endogenous DNA. The specific site of integration is the site of identical endogenous DNA. The site of integration can be chosen simply to ensure that no endogenous gene is disrupted, or a gene could be targeted if the goal is to alter its function.

Unlike random integration, gene targeting ensures that the site of integration is known and that the same modification can be made in the same genotype, in different genotypes, in males and females, or in different alleles to produce offspring that are homozygous for the modification. Gene targeting has been successful in sheep, pigs, and cattle (Denning and Priddle 2003; McCreath et al. 2000).

Transchromosomic Technology

A second improvement that has been made in SCNT-based gene trans-

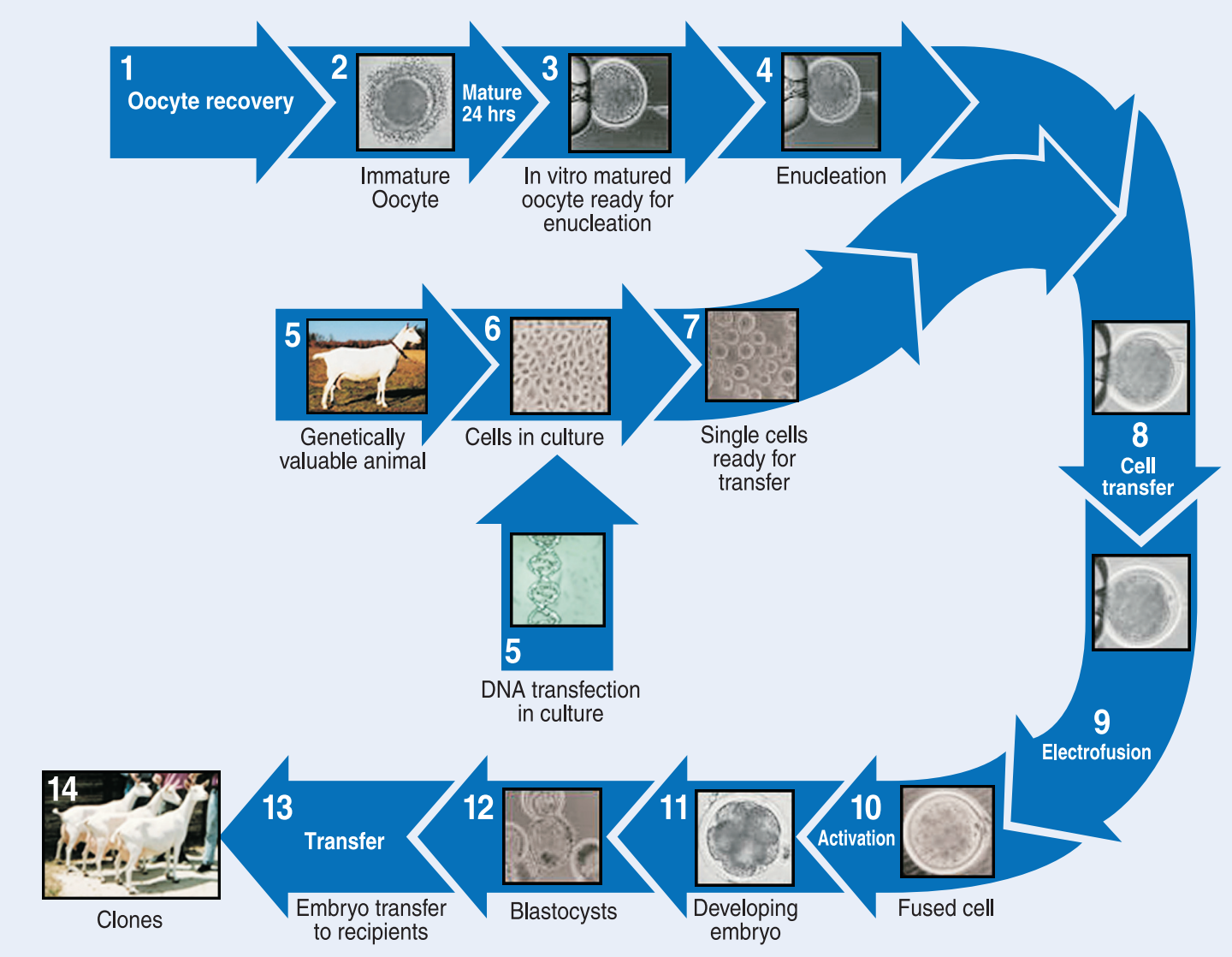

Figure 1. Steps in the Somatic Cell Nuclear Transfer process. (Courtesy of H. Baldassarre.)

fer technology is a system for transferring large pieces of DNA into an animal in the form of a *microchromosome*. A microchromosome contains all the elements of a normal chromosome, including *telomeres* at the ends of the chromosome, a *centromere*, and multiple origins of DNA replication. Unlike random or targeted gene transfer, however, a microchromosome does not integrate into the host DNA but is carried along through cell division as an independent chromosome. Furthermore, the length of DNA that can be transferred is substantially greater with a microchromosome compared with DNA that must integrate into an endogenous chromosome. The typical size for integrated DNA is 10,000 to 30,000 nucleotides in length, whereas a microchromosome can be 10 million or more of these DNA bases. Consequently, microchromosomes are capable of carrying either very long and complex genes or many genes.

Recently, the microchromosome approach was used to insert the entire unrearranged human heavy- and light-chain immunoglobulin loci into cattle (Kuroiwa et al. 2002). Incorporation of the entire immunoglobulin loci will allow the cattle to respond to an antigen challenge by production of a functional human antibody, which is composed of both heavy and light chains.

Transgenic Animals as Disease Models for the Development of New Treatments

Transgenic mice have been used to study how genes are controlled, how specific genes function, and what their role is in disease processes (Thyagarajan et al. 2003). The availability of embryonic stem cells in mice allows for sophisticated gene manipulation involving the disruption of normal gene function (*knock-out*); replacement with altered, mutant genes (*knock-in*); and site-time specific expression (*conditional expression*). Using these genetic manipulations, researchers have been able to develop mouse models for a variety of human diseases including Huntington's, Alzheimer's, cystic fibrosis, emphysema, diabetes, inflammatory arthritis, and cancer.

In some instances, these models can provide valuable information that can aid in the treatment of disease; in other instances, results obtained with mutant models are unexpected or significantly different from symptoms observed in affected humans. For example, cardiovascular physiology differs in the mouse model from that of the human; therefore, research could benefit from a larger animal model (Hoit 2004).

Pigs—in particular, minipigs—have been proposed to be better models than mice for studies relating to heart disease, organ transplantation, immunotherapy, and obesity. Transgenic models using livestock rarely have been developed, however, because of the difficulties and costs associated with production. The exceptions in which transgenic livestock have been the preferred model include transgenic pigs for *xenotransplantation* research (see CAST 2004) and transgenic pigs carrying a *mutated rhodopsin gene* as a model for *retinitis pigmentosa* (Petters et al. 1997).

With continued improvements in efficiencies, the advantages of SCNT, which allows selection of the transgenic donor cell before production of the transgenic animal, should increase interest in the production of transgenic livestock for studying both human and animal diseases. In fact, research projects are ongoing on several topics: transgenic cattle lacking the *prion receptor* (inhibits its susceptibility to BSE), transgenic cattle with resistance to staphylococcus infection (mastitis resistance), transgenic pigs as models for Alzheimer's disease, and xenotransplantation (Denning and Priddle 2003; Ramsoondar et al. 2003; Richt et al. 2007; Wall et al. 2005).

Transgenic Livestock as Producers of New Medications

Perhaps one of the biggest incentives for the production of transgenic livestock is their capacity to manufacture biopharmaceutical proteins. Livestock have been selected throughout agricultural domestication for superior production traits. With moderate alterations in production practices, it is possible to take advantage of the tremendous protein-producing capabilities of domestic livestock, such as dairy animals or poultry, through the production of valuable biopharmaceutical proteins in milk or eggs, respectively.

Biopharming, the production of biopharmaceuticals using domestic livestock, can have significant advantages compared with other production methods in terms of safety, biological activity, and production costs. For those biopharmaceuticals that are still harvested from human tissues, biopharming represents a safer procedure in regard to prevention of transmissible human diseases such as human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) or Creutzfeldt-Jakob disease (CJD).

In certain instances, biopharming can be used to produce products (e.g., human *polyclonal antibodies*, described later) that would not be feasible otherwise. Because recombinant proteins can be produced in transgenic animals that are the same as those produced by humans, the proteins produced should be less likely to cause allergic response than the corresponding nonhuman products harvested from nonengineered animal tissues. Many therapeutic proteins that previously were harvested from animal tissues (e.g., insulin, growth hormone, hemophilic factors) now are being produced as recombinant human proteins in mammalian, yeast, or bacterial fermentation systems. In these instances, biopharming may provide a more cost-effective production system (Van Cott et al. 2004).

Acceptable levels of recombinant protein production have been demonstrated in the milk of goats, sheep, and cattle (Clark et al. 1989; Parker et al. 2004). Moreover, the bioactivity of these proteins has been verified (Cerasoli et al. 2005; Levy et al. 2001). Obtaining acceptable levels in chicken eggs has proved more problematic; thus, further technological development is needed to compete with dairy animal production methods, although recent advances have been reported (Walsh 2006). Although further studies are required to establish the safety and efficacy of biopharmaceuticals produced using transgenic animals, a few biopharmaceuticals have progressed through preclinical and clinical trials and should establish a track record soon (Table 1) (Keefer 2004; Walsh 2006).

Whereas safety and effectiveness

Table 1. North American and European groups producing bioproducts or biomedical models in transgenic livestock[a]

System	Company/Group, Country	Company/Group Website	Products or Models	Status
Goats	GTC Biotherapeutics, United States	www.transgenics.com	Antithrombin III (ATryn) Monoclonal antibodies Malaria vaccine	ATryn received EU approval and is in clinical trials in the United States. Other products in preclinical
	Pharmathene, United States/Canada	www.pharmathene.com	Butyrylcholinesterase	Research
Cattle	Hematech, United States	www.hematech.com	Polyclonal antibodies	Research
	GTC Biotherapeutics, United States	www.transgenics.com	Human serum albumin	Research
Pigs	Revivicor, United States	www.revivicor.com	Xenotransplantation (cartilage implants) Polyclonal antibodies	Research
	Progenetics	http://www.progtx.com/	Factor-IX	Research
	Foulum Research Center, Denmark	http://www.agrsci.org/ny_navigation/forskning/centre/forskningscenter_foulum	Alzheimer's model	Research
	North Carolina State University (R.M. Petters), United States	http://www.ncsu.edu	Retinal pigmentosa model	Research
	University of Missouri (R. Prather), United States	http://www.missouri.edu	Xenotransplantation	Research
Rabbits	Pharming, The Netherlands	www.pharming.com	C1-inhibitor	Phase II clinical trials
	BioProtein Technologies, France	www.bioprotein.com	Recombinant proteins	Research
	Therapeutic Human Proteins, United States	www.polyclonals.com	Humanized polyclonal antibodies	Research
Chickens	Avigenics, United States	http://www.avigenics.com/	Interferon	Clinical trials
	Origen Therapeutics, United States	www.origentherapeutics.com	Recombinant proteins	Research
	Viragen, United States	www.viragen.com	Interferon alpha and single chain antibody	Research
	Vivalis, France	www.vivalis.com	Recombinant proteins using cell-based system	Research

[a]Company products and status are estimations due to limited types of available information (e.g., press releases, articles in popular press, etc.).

are of paramount importance, it also is critical to establish economic feasibility. Therefore, in addition to producing a transgenic line that secretes acceptable amounts of the targeted protein in the milk without affecting the physiology of the animal, issues involving the purification of the protein also are important. These issues will affect the final economics and commercialization of the product. Purification of the recombinant protein in commercially viable amounts is a critical but complicated step because of the complexities of milk composition (Goldman 2003). Furthermore, these processes must be completed in a highly controlled, documented manner to meet the regulatory demands of governmental agencies (Gavin 2001).

Bioproducts from Milk

Bioproducts produced from milk by transgenic techniques fall into two general categories: (1) proteins for treatment or prevention of human disease and (2) biomaterials (e.g., collagen or *spi-*

der silk proteins) (Karatzas and Turner 1997; Keefer 2004; Powell 2003; Zuelke 1998). The economic incentives are significant because pharmaceutical proteins are products with much higher profit margins than those found in traditional agricultural products.

Human pharmaceutical proteins can be either isolated from human fluids (e.g., blood-clotting factors) or produced as recombinant proteins in fermentation systems. The first method involves the risk of contamination (e.g., HIV/AIDS, CJD). The second method, production of recombinant proteins through mammalian cell culture and bacterial fermentation systems, can be expensive and requires dedicated production facilities (Datar, Cartwright, and Rosen 1992; Powell 2003). Therefore, production of these proteins in transgenic dairy animals provides significant advantages in terms of health risk and production costs. But stringent health surveillance for potential health hazards in the production herd must be factored into the economic equation.

Although the feasibility of producing human proteins in the milk of dairy animals has been well established, none has yet completed all the regulatory hurdles and demonstrated market acceptance and economic viability (Keefer 2004). One product, however, called ATryn (GTC Biotherapeutics, Inc)—an antithrombin for use as an anticoagulant and anti-inflammatory agent—recently received EMEA approval and currently is in phase III clinical trials in the United States (Schmidt 2006).

Another example of a recombinant protein progressing through the research and development process is Protexia (Pharmathene, Inc.), a recombinant human butryl-cholinesterase produced in the milk of dairy goats. Protexia, which is targeted for use as a medical countermeasure against nerve toxins, has advanced to the preclinical research stage (Cerasoli et al. 2005; Schmidt 2006). Supplementation of milk with recombinant proteins that can provide antimicrobial or nutritive benefits is another strategy covered by ongoing research (Maga et al. 2006; Wall et al. 2005; Wheeler 2003).

Bioproducts from Serum

The use of animals for developing serum biopharmaceutical products is not new. Since the 1920s, insulin derived from pigs has been used for the treatment of diabetes. In 1982, insulin was the first recombinant protein approved for therapeutic treatment, and most insulin in use today is produced in bacteria or yeast (Walsh 2006).

Agricultural animals (horses and sheep) also have been used to develop polyclonal antibodies products for human medical treatments as antivenoms (Clark et al. 2002; Theakston and Smith 1995). Animal antibodies usually must be processed to remove the heavy-chain portion of the antibody to decrease unwanted anaphylactic reactions in patients. These products are developed for immediate, short-term uses and cannot be used for long periods of treatment because of these anaphylactic responses in patients. Even though animal polyclonal antibodies have limitations, these products have been approved by the U.S. Food and Drug Administration regulatory agencies and currently are being used for human medical treatments.

Donated human blood is the current production system for blood products such as red blood cells, plasma, clotting factors, immunoglobins, and serum albumin, all of which are used in the medical field today. Unfortunately, the human production system has many limitations, such as risks of human diseases (HIV/AIDS, CJD), lack of qualified donors, the need for a large number of donors, regulatory concerns, and ethical issues (e.g., humans are not *hyperimmunized* to boost their immune system for specific diseases).

Because of the increased application of advanced medical technologies, an increased global demand has developed for human-derived blood products; this demand has caused shortages and increased costs. Development of genetically engineered animals as a production system for human biopharmaceutical and blood products has great potential for biomedical applications with regard to these limitations, shortages, and increased costs.

Currently, genetically engineered animals such as cattle are being developed to produce human polyclonal antibodies in their blood (Kuroiwa et al. 2002). These transgenic animals are genetically engineered to have their own antibody-producing systems inactivated (knocked out) and artificial human microchromosomes inserted into the animal genome to produce full humanized polyclonal antibodies. This transgenic animal production system has a number of potential biomedical applications. Polyclonal antibodies can be used in the treatment of infections (e.g., staph, which is notorious for being resistant to antibiotics), cancer, organ transplant rejections, and autoimmune diseases; as antitoxins; and in biodefense.

Cattle are used as a human polyclonal production system because of their larger size (larger blood draws), the availability of cow oocytes from slaughterhouses to create production animals, and sufficient scientific research for understanding genetic manipulations. The resources used to raise cattle, such as facilities, feeds, animal management, disease control, and veterinary care, also are well understood and widely available.

To make these human polyclonal antibodies, the desired antigens from bacteria, viruses, toxins, and cancer cells are made into formulated vaccines and immunized into transgenic cattle. The animals go through a schedule of hyperimmunization to maximize the production of antibodies in the plasma. The plasma is collected from the cow jugular vein by *plasmapheresis*, which is similar to the process used for obtaining human plasma and differs only in the preferred vein for collection. Collected plasma is purified to remove unwanted bovine proteins (such as serum albumin, hormones, and enzymes), resulting in purified human polyclonal antibodies. The purification process also can remove unwanted bacteria, toxins, viruses, and prions. These purified, efficacious, and safe antibodies products are then ready to be used to treat patients.

Economic and Regulatory Issues

Perhaps the biggest challenge in the application of these technologies is their establishment as a viable commercial entity. The production of some of these products has been halted because of business decisions based on economic feasibility concerns and/or lack of sufficient funding. These are business issues caused not necessarily by technical challenges but by unknown factors that arise as new technologies develop without

Table 2. Websites providing information on animal biotechnology

Website	Information
NIH guidelines for Institutional Biosafety Committees http://www4.od.nih.gov/oba/IBC/IBCnihguidelines.htm	NIH guidelines detail safety practices and containment procedures for basic and clinical research involving recombinant DNA.
U.S. Regulatory Agencies Unified Biotechnology Website http://usbiotechreg.nbii.gov/index.asp	Description of regulatory oversight provided by federal agencies regarding genetically engineered agricultural products
U.S. Regulatory Agencies Unified Biotechnology Website: U.S. Laws and Regulations http://usbiotechreg.nbii.gov/lawsregsguidance.asp	U.S. laws and regulations used to regulate genetically engineered agricultural products
Office of Science and Technology Policy http://usbiotechreg.nbii.gov/Coordinated_Framework_1986_Federal_Register.html	Coordinated Framework for Regulation of Biotechnology
FDA Center for Veterinary Medicine http://www.fda.gov/cvm/bio_drugs.html	Animal drugs and genetically engineered animals 21CFR511.1(b)
Pew Initiative on Food and Biotechnology http://pewagbiotech.org/agtopics/index.php?TopicID=1	Animal biotechnology
Purdue Agricultural Biotechnology http://www.agriculture.purdue.edu/agbiotech/	Agricultural biotechnology including regulatory and ethical issues with links to other university and agency sites

an established track record or sufficient guidelines for completing the necessary regulatory steps. In fact, regulatory guidelines are being developed concurrently with the establishment of the new technology, creating uncertainty within the business community as to the costs and timelines associated with recombinant protein production.

One regulatory concern is altered immunogenicity. Although the transgene may code for a human protein, modifications may be made to the protein during its production in the transgenic animal. These changes are called posttranslational modifications. These modifications include glycosylation (addition of saccharides) of particular amino acids within the protein. Altered glycosylation patterns can affect the amount of time before the protein is cleared from a patient's system, which can affect treatment protocols. Furthermore, posttranslational modifications may alter the protein's immunogenicity. The EMEA's concern about these two issues—clearance time and immunogenicity—slowed the approval process for ATryn (Schmidt 2006).

Economics and regulatory issues are linked closely because companies must determine whether the market for a product merits the costs of production and regulatory compliance. Because clinical trials are costly, the progress of some transgenic products has been halted after cost/benefit analysis. The protein must be clearly competitive with other products on the market and must have potential for profitable sales.

An example of a transgenic protein that failed this cost/benefit analysis is recombinant human alpha-1-antitrypsin, developed to treat a lung disorder. The project was shut down in 2003 by the former Scottish transgenic protein company PPL, after its partner, Bayer, concluded that the project would not be competitive enough to merit expending further funds on clinical trials and regulatory compliance (Vogel 2003).

Other regulatory issues have less to do with the therapeutic product and more to do with the production system. Regulations controlling the use of recombinant DNA technologies in agricultural species exist at many levels, including institutional, state, federal, and international (Table 2). These regulations can be quite explicit and rigorous, specifying the procedures and levels of containment required for manipulation of DNA (e.g., the National Institutes of Health [NIH] Guidelines for Biosafety). These regulations also set out restrictions on transgenic animals (e.g., Code of Federal Regulations: 21CFR511.1[b]).

Questions have been raised about the hazards associated with the transgenic animal production techniques themselves. These questions range from issues of food safety to environmental impact and animal welfare. These issues are complex and are outside the scope of this paper. For a more thorough coverage of these regulatory and welfare issues, the reader is directed to documents from the National Research Council (NRC 2002) and the Pew Initiative on Food and Biotechnology (Pew 2005).

Societal Issues

Societal issues also are linked closely to the regulation and economics of transgenic protein production. If a majority, or a vocal minority, of the population strongly objects to the use of transgenic production systems, companies may become reluctant to market the products, and governments may delay product approval. In most instances, the biopharmaceuticals produced by transgenic animals will carry the promise of significant medical and health benefits.

At the present time, transgenic products of all kinds have become a sensitive social issue. Therefore, it is apparent that unless a biopharmaceutical produced by transgenic animals offers clear and significant advantages compared with

more conventional products or procedures, it is unlikely to receive regulatory approval or attain commercial viability.

Established and widely accepted management practices similar to those for meat and milk production systems can be expected to facilitate acceptance of transgenic animal production systems by the majority of the population. Furthermore, the development of freely available information that clearly describes the regulatory controls on production methods and product testing would aid in allaying safety concerns. Currently, organizations such as CAST and the Pew Initiative provide information to the public on key issues regarding biotechnology. Other sources of information include websites maintained by the government and universities (see Table 2).

Groups that do not approve of the commercial exploitation of animals for food production, however, are unlikely to accept transgenic production systems, despite evidence of any benefits. Whereas government regulatory agencies should consider only the safety and efficacy of products, governmental legislatures will reflect the concerns of society in general and must consider the ethical issues and the societal cost/benefit balance of using animals to produce biopharmaceuticals.

Summary and Conclusions

Transgenic livestock have the potential to play a critical role in the production of new medications for the treatment of human disease. This role may consist of actual production of recombinant proteins (including biotherapeutic proteins and antibodies) for treatment of human diseases. Or, it may involve the development of new animal models that can be used in studies relating to human diseases. Both approaches—recombinant protein production and transgenic animals as disease models—can provide significant advances in the development of new treatments.

The development of therapeutic protein production systems and medical models is well under way (see Table 1). Not only has the feasibility of producing human proteins in domestic animals been well documented, but their effectiveness in treating human disease is being established through clinical trials. Although none of the recombinant therapeutic proteins has yet completed all the regulatory hurdles and demonstrated market acceptance and economic viability, one product—ATryn—has completed the first step of receiving EMEA approval (Schmidt 2006).

Because of the strong potential for these products to provide unique, vital therapeutic treatments, continued support of research by both government and commercial entities is needed such that additional promising biotherapeutics can be developed. Furthermore, open access to information concerning the production methods, products, and regulatory process should be available to the public. Education regarding the advantages and challenges associated with this new technology is the key to public understanding.

Appendix 1. Production of Transgenic Animals by Somatic Cell Nuclear Transfer

Production of transgenic animals by SCNT involves two general procedures. The first procedure includes introducing DNA into a somatic nuclear donor cell and selecting the properly modified transgenic cells. The second procedure entails the actual cloning process in which an embryo is produced using the transgenic nuclear donor cell (see Figure 1).

The typical nuclear donor cells used for genetic modification are *fibroblasts*, recovered after mincing and enzymatically digesting the skin and muscle tissue of a young fetus. Fibroblast cells are isolated easily because they readily attach to the bottom of a culture flask and generally outgrow any contaminating cells. Fibroblast cells, unlike the immortal mouse embryonic stem cells used in mouse gene transfer, only grow for a finite number of cell divisions in culture. The life span of the cells is related to the age of the animal from which they were derived. Cells obtained from an old animal have a shorter life span than cells obtained from a young fetus (Kasinathan et al. 2001). Therefore, young fetal cells are used for genetic modification to maximize the time that the cells can be grown and manipulated in culture. Within a couple of days of harvest from a 2-cm (40-day) bovine fetus, 50 million cells are available for *cryopreservation* and *banking*.

Introduction of DNA into the Donor Cell

Using a process called *transfection*, DNA is introduced into the fibroblast cells. There are several methods for transfecting cells, but one of the most reliable is *electroporation*, which involves applying an electrical charge across the membrane of the cell to induce formation of small pores in the membrane. The DNA, added to the electroporation medium, gains access to the inside of the cell through these pores. The DNA sequences then can associate with, and eventually become integrated into, the host DNA sequence.

One advantage of using SCNT for making genetic modifications in animals is that a large number of nuclear donor cells can be manipulated to overcome the highly inefficient transfection procedures. Of the millions of cells that are electroporated and exposed to DNA, only a small portion survive, take up the DNA, and incorporate it into a host chromosome. The site of integration into the chromosome may be random or targeted to a specific site (homologous recombination) depending on the techniques used. Generally, out of approximately 5 million cells, only about 500 incorporate DNA and grow. Consequently, a method is needed to separate the transgenic cells from the nontransgenic cells. This separation process is called selection.

Selection of a Transgenic Donor Cell

A standard selection approach consists of including a *selection cassette* in the foreign DNA that is being inserted. The selection cassette is made up of a gene for a resistance protein and a strong, active promoter that drives expression of the resistance gene. Many resistance genes are available for a variety of cell toxins (typically antibiotics such as neomycin or puromycin). After transfection, cells are placed in a culture medium that contains the toxin. As a result, nearly all cells that survive and grow are cells that incorporate the foreign DNA and express the resistance gene. Cells generally are cultured at a low density; therefore, surviving cells

can be observed as colonies of cells, all derived from one original cell. Cells within a colony are considered to be clones of the single original cell.

After selection and growth for a few weeks, the cell colonies can be split and passed to separate culture vessels. Additional analysis can be done on one set of cells while the other set continues to grow. Typically, additional analysis consists of a *polymerase chain reaction* to *amplify*, or make multiple copies of, a DNA segment from the foreign DNA. If the segment is amplified, then the colony likely contains cells that incorporated the DNA. But sometimes nontransgenic cells can survive among the transgenic cells.

By the time selection, splitting, and additional analysis are done, the fibroblast cells are getting old. Some cell colonies may even show signs of senescence and slowed growth. Good colonies are selected for the second part of the process, which is SCNT with the transgenic donor cells.

Production of a Transgenic Embryo by SCNT

The SCNT procedure includes two types of cells and several procedures. The first cell type is the nuclear donor that contributes its DNA to the resulting SCNT embryo. Therefore, cells derived from a single, pure (all derived from one original cell) colony should all be clones. For transfection techniques that result in random integration, cells from a second colony (derived from the same original fetal cell line) will be genetically identical to the first colony, except for the site in which the foreign DNA integrated into the host chromosome and the number of copies that integrated. Because the site of integration and number of copies integrated are random, offspring derived from one colony likely will be clones of each other but not of offspring derived from a second colony. When homologous recombination techniques are used, clones are likely to be identical because the site of transgene integration was targeted to a particular genomic location.

The second cell type is a mature oocyte. The oocyte is a large cell, approximately 500 times larger than the donor fibroblast. The large size results from storage of components necessary for early embryo development. Some of these components are structural or metabolic, but the oocyte also contains factors that instruct the DNA so that appropriate genes can be inactivated or activated at the appropriate time to make a viable embryo. In the process of SCNT, the donor cell contributes its DNA and the oocyte contributes its cytoplasm to the resulting SCNT embryo.

Removal of the Oocyte DNA

The SCNT technique starts with the process of *enucleation* in which the oocyte DNA is removed by aspiration with a tiny micropipette mounted on a *micromanipulator*. This enucleation process is necessary because placement of the donor fibroblast DNA into the oocyte along with the oocyte DNA would result in too many chromosomes, and the SCNT embryo would not be viable. Therefore, the oocyte's own DNA must be removed. The oocyte's cytoplasm, however, must be retained because it contains materials needed to instruct and process the donor cell's DNA.

Nuclear Transfer

After enucleation, the donor cell DNA needs to be introduced into the enucleated oocyte or cytoplast. Although the nucleus of the donor cell can be injected directly into the cytoplast, a less disruptive method of introducing the DNA is fusing the entire donor cell to the cytoplast (Collas, Fissore, and Robl 1993). With the fusion approach, the donor cell is placed against the cytoplast cell membrane using a micropipette and micromanipulator. This couplet then is placed in an *electrofusion chamber*. Electrofusion is similar to electroporation in that a high voltage DC electrical pulse is applied to the cell membrane to induce formation of small pores. But with electrofusion, the cells must be aligned so that the two membranes to be fused (the donor cell and cytoplast membranes) are perpendicular to the flow of current.

Application of the electrical pulse results in close apposition of the membranes resulting from the charge across the membranes. Eventually, the charge causes formation of tiny pores between the two cells, which eventually coalesce and effectively join the two cells into a single entity. Although cell fusion seems to be a complex and disruptive procedure, it actually is quite efficient and has minimal effect on the health of the cells.

Oocyte Activation

Cell fusion results in joining the oocyte cytoplasm with the donor DNA. But another process is required to initiate cell division; just as the sperm normally contributes its DNA to the embryo, it also delivers a signal that activates embryo cell division. *Sperm-induced oocyte activation* has been the subject of considerable investigation during the past 15 years. Although much is known of its mechanism, much remains to be resolved on its involvement in regulating early embryo development. Based on information regarding sperm-induced oocyte activation, chemical methods of activating oocytes have been developed (Liu, Ju, and Yang 1998).

Essentially, these methods are designed to elevate intracellular calcium and suppress activity of an intracellular enzyme that arrests cell division in the oocyte. After activation, the SCNT embryo either can be transferred immediately to a recipient female or allowed to grow for a few days in culture before transfer. The efficiency in which the SCNT embryo and recipient establish a pregnancy can vary considerable between species; however, the general process of SCNT as shown in Figure 1 is similar whether the livestock animals being cloned are cattle, pigs, sheep, or goats.

Glossary

Amplify. To make multiple identical copies, as in "amplify a segment of DNA."

Banking. Storage of cells or embryos by cryopreservation for subsequent use.

Biopharming. Production of therapeutic proteins using plant or animal production systems.

Centromere. Specialized region of the chromosome to which spindle fibers attach during cell division.

Conditional expression. Expression of the protein is limited to certain situations; for example, the protein is expressed only in a lactating mammary gland.

Cryopreservation. Storage of cells at subfreezing temperatures.

Electrofusion chamber. Small chamber used to hold cells during electroporation process.

Electroporation. Process using an electrical shock to make cell membranes permeable to allow introduction of new DNA; commonly used in recombinant DNA technology. Also used to fuse two cells together or to fuse a donor cell and oocyte during somatic cell nuclear transfer.

Embryonic stem cells. Embryonic cells that can replicate indefinitely, transform into other types of cells, and serve as a continuous source of new cells.

Endogenous sequence. The DNA sequence found within a particular animal as opposed to the exogenous transgene obtained from another DNA source.

Enucleation. Removal of the nucleus (nuclear DNA) from an oocyte.

Exogenous sequence. A DNA sequence originating from a source outside of a particular animal as opposed to the animal's own DNA (endogenous sequences).

Fibroblasts. Type of cell found in connective tissues, e.g., skin. Fibroblasts can be cultured relatively easily.

Gene expression. Process by which a gene's coded information is transcribed into either messenger RNA (mRNA) and then translated into protein, or into RNA but not translated into protein (e.g., transfer and ribosomal RNAs).

Gene targeting. Insertion (or removal) of DNA at a specific site (gene) within the genome to alter expression of that gene. *See also* **Homologous recombination**

Homologous recombination. Swapping of DNA fragments between paired chromosomes or between a piece of DNA that can pair with a specific DNA site and the chromosome containing that specific site because of alignment of complementary (matching) DNA sequences. *See also* **Gene targeting**; **Knock-in**; **Knock-out**

Hyperimmunized. Strong immunological response resulting in the production of a high level of immunoglobulins (antibodies) after exposure to an antigen.

Knock-in. Incorporation of an exogenous sequence into a specific site that results in altered gene function. *See also* **Homologous recombination**

Knock-out. Incorporation of an exogenous sequence into a specific site that results in disruption of normal gene function. *See also* **Homologous recombination**

Microchromosome. Small, artificially produced chromosome.

Micromanipulator. Instruments (attached to a microscope) used to manipulate oocytes and embryos.

Mutated rhodopsin gene. Mutations (alterations) in the genetic code for rhodopsin, a component of the photoreceptor cells in the eye. Mutations can lead to the disease *retinitis pigmentosa*, which can result in degeneration of the photoreceptor cells and blindness.

Oocyte. Female germ cell, which after maturation into an ovum (egg) can be fertilized by the sperm (male germ cell).

Plasmapheresis. Process of separating cells and other components from plasma in the blood by a machine. This process can be used to remove antibodies from the blood.

Polyclonal antibodies. Mixture of immunoglobulin molecules secreted against a specific antigen, each recognizing a different site on the molecule.

Polymerase chain reaction. Method for amplifying (copying) a DNA base sequence using a heat-stable enzyme that copies DNA and two primer sequences, complementary to the strands of the DNA. The newly synthesized DNA strands subsequently can serve as additional templates for the same primer sequences. Successive rounds of DNA copying produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample.

Prion receptor. Membrane receptor that allows prions (abnormal proteins responsible for brain disease such as bovine spongiform encephalopathy [mad cow disease]) to enter a cell.

Promoter. In molecular biology, the term promoter refers to the binding site on DNA to which the enzyme that transcribes DNA into RNA can attach and initiate transcription.

Pronuclear microinjection. Process in which transgenes are injected directly into one or both pronuclei. A pronucleus is the nuclear structure formed by the male- or female-derived chromosomes in a recently fertilized oocyte. Each fertilized oocyte should have two pronuclei, one female derived and one male derived.

Recombinant DNA technologies. Procedures used to join together DNA segments (sequences). Under appropriate conditions, a recombinant DNA molecule can enter a cell and replicate there, either autonomously or after it has become integrated into the chromosome(s).

Recombinant protein. Protein produced using recombinant DNA technologies.

Retinitis pigmentosa. *See* **Mutated rhodopsin gene**

Selection. Process of selecting those cells that have incorporated the transgene into one or more of its chromosomes.

Selection cassette. The DNA sequence that contains the coded information for several genes, one being the sequence for the protein of interest and another being a protein that can be used to mark or select those cells that contain the cassette. In some instances, the gene used for selection will code for resistance to a toxin such that only those cells containing the transgene can survive exposure to the toxin.

Somatic cell nuclear transfer (SCNT). Process by which an oocyte's DNA is replaced with the DNA from a somatic cell (donor). In a process that is inefficient and poorly understood, the oocyte is able to reprogram (reset) the somatic cell DNA so that it can direct normal embryonic development.

Sperm-induced oocyte activation. An unfertilized oocyte is maintained in a state of arrested development until activated either by the sperm during fertilization or artificially using chemical or electrical stimulation. If the oocyte is not activated, it will degenerate.

Spider silk proteins. Proteins produced by a spider, which comprise the silk filaments found in spider webs.

Telomere. Specialized structure located on the end of a chromosome, which is involved in the replication and stability of the chromosome.

Therapeutic proteins. Proteins used in medical therapies to treat disease.

Transchromosomic technology. Method of producing a transgenic organism using small artificial chromosomes rather than incorporation of smaller DNA sequences into the organism's own chromosome(s).

Transfection. Introduction of foreign DNA into a host cell.

Transgenic animals. Experimentally produced animal in which exogenous DNA has been artificially introduced and incorporated into the animal's cells.

Xenotransplantation. Tissue or organs from an individual of one species transplanted into or grafted onto an organism of another species (e.g., the use of pig heart valves in humans).

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