Transgenic animal technology, from DNA microinjection to gene targeting and cloning, has had a significant impact on human health, pharmaceutical discovery and the drug pipeline

Foundation Review:

Transgenic animals and their impact on the drug discovery industry

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The ability to direct genetic changes at the molecular level has resulted in a revolution in biology. Nowhere has this been more apparent than in the production of transgenic animals. Transgenic technology lies at the junction of several enabling techniques in such diverse fields as embryology, cell biology and molecular genetics. A host of techniques have been used to effect change in gene expression and develop new pharmaceutical and nutraceutical compounds cost-effectively. Scientific advances gained by transgenic capabilities enable further understanding of basic biological pathways and yield insights into how changes in fundamental processes can perturb programmed development or culminate in disease pathogenesis.

Transgenic animals serve as important models in translating findings in the basic sciences to potential clinical applications. Moreover, the use of transgenic animals as bioreactors in pharmaceutical manufacturing has far-reaching uses from protein production in various end-organs (e.g. milk, blood, urine and other tissues) to modification of tissues and organs for transplantation. Here, it will become apparent that the applications of transgenic technologies, although potentially of great significance, have yet to be fully realized.

History of transgenesis: foundational technologies

The term 'transgenic' was coined in 1981 by Gordon and Ruddle [1] to describe an animal in which an exogenous gene was introduced into its genome. In the late 1980s, the term transgenic was extended to gene-targeting experimentation and the production of chimeric or 'knockout' mice in which a gene (or genes) has been selectively removed from the host genome [2,3]. Today, a transgenic animal can be defined as one having any specific, targeted genetic modification. Transgenic animals are most commonly produced through: (i) germline modifications of gametes; (ii) microinjection of DNA or gene

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BOX 1

Embryological nomenclature

- **Blastocyst**: An embryo generally around the 64–128-cell stage, where the inner cell mass and trophoblast layers are differentiated and a blastocoel cavity is apparent.
- **Egg**: A female gamete, ovum or reproductive cell at any stage before fertilization and/or its derivatives after fertilization.
- **Embryo**: In animals, a fertilized ovum and its descendants, which eventually become the offspring, during their period of most rapid development.
- **Oocyte**: A developing egg cell at any stage before or after ovulation, but before fertilization.
- **Ova**: Plural of ovum.
- **Ovum**: A female gamete synonymous with egg. Ovum is also used to designate any early stage of a conceptus.
- **Zygote**: A fertilized ovum.

constructs into zygotes [unicellular embryos (Box 1)]; or (iii) incorporating modified cells, including embryonic stem (ES) cells, into later stage embryos. After gamete or embryo modifications, the resultant embryos are matured to term in a recipient female. Several scientific paths converged in the early 1980s to establish transgenic animal technologies. Through the 1990s, a host of new techniques and modeling systems further extended the scope, utility and commercial aspects associated with animal transgenesis.

Embryology and embryo culture

Embryo transfer was the first of the pieces of the transgenic puzzle to fall into place. In 1891, Heape [4] transferred Angora rabbit embryos into a gestating Belgian doe, which subsequently produced a litter containing young of both breeds. Methods for embryo transfer in other species were developed during the early- and mid-1900s. Embryo transfer in the mouse was first accomplished in 1942 [5]. Advances in understanding the endocrinology of ovulation with the subsequent development of superovulation protocols across several species facilitated the broad range of genetic engineering technologies in common use today [6].

In vitro manipulation of embryos requires a culture system that supports physiological and metabolic processes and ensures a high level of viability for embryos *ex utero*. In 1949, Hammond [7] gave an account of viable blastocyst stage embryos that had been cultured from the eight-cell stage. With time, culture conditions and media formulations improved considerably. One notable example is the exhaustive work conducted by Brinster [8–11] on optimizing ova culture media.

Microinjection

A seminal achievement that paved the way for the transgenic age occurred in 1966 with the description of embryo microinjection [12]. After fabricating an 'egg holding' pipette with a ~60–70 μm tip diameter, negative pressure was used to hold the embryo. Then, a fine glass microinjection pipette (1 mm tip diameter) was used to pierce the embryo and subsequently deliver a picoliter volume of bovine gamma globulin. Fertilized mouse zygotes at the pronuclear stage survived the microinjection and developed to term. It was nearly 15 years after this event before successful experiments were reported describing the production of transgenic mice [1,13–18]. Indeed, these efforts would not have been possible had it not been for recombinant DNA technologies, which were necessary to characterize genetic elements and the resultant offspring.

Recombinant DNA

The ability to insert DNA into a cloning vector from which myriad copies can be reproduced marked the beginning of a new age in biology. Several advances made DNA cloning possible, including the discovery and isolation of restriction endonucleases [19,20], development of plasmid manipulation and transformation protocols [21] and the discovery and isolation of DNA ligase [22]. The pioneering experiment in recombinant DNA technology that led the way for the generation of transgenic animals occurred in 1973, with the introduction of a DNA fragment into an exogenous plasmid [23].

Methods for genetic modification

DNA microinjection

DNA microinjection generally involves the use of micromanipulators and a microinjection apparatus to inject a solution containing recombinant DNA into embryos. Virtually any cloned DNA fragment (construct) can be used, albeit some caveats do apply. Linearized (as opposed to closed, circular) DNA constructs are more readily integrated into the host genome. The presence of extraneous plasmid or vector sequences can adversely affect expression of the integrated transgene. Also, considerable care must be taken when handling large DNA constructs, such as those derived from bacterial or yeast artificial chromosomes, to avoid damage by shearing during the microinjection process.

Microinjected gene constructs generally integrate randomly into the genome of the embryo, but typically only in a single chromosomal location. This can be exploited to obtain functional linkage of independent transgenes by co-injection of one or more DNA constructs. In such cases, the introduced constructs tend to integrate randomly in the same site and resulting animals might exhibit coordinated expression of the transgenes. During the integration of transgenes, a single copy or multiple copies of a transgene are incorporated into the genomic DNA. When multiple copies are integrated, the transgenes concatenate before the integration event, and as many as a few hundred copies of the particular sequence can be incorporated into a single chromosomal location, predominantly as head-to-tail concatemers. Regulatory elements in the host DNA near the site of integration, and the general availability

of this region for transcription, appear to have major roles in affecting the level of transgene expression. This 'positional effect' is proposed as an explanation of why the levels of expression of the same transgene can vary dramatically between individual founder animals and the resulting transgenic lines.

Host DNA near the site of integration frequently undergoes various forms of sequence duplication, deletion or rearrangement as a result of transgene incorporation. Such alterations, if sufficiently drastic, can disrupt the function of endogenous genes at the integration site and constitute 'insertional mutagenesis', wherein an aberrant phenotype can result. Because DNA microinjection is usually accomplished in ova at the one-cell (zygote) stage, the transgene is incorporated in essentially every cell that contributes to the developing embryo. Incorporation of the transgene into germ cell progenitors makes heritability of the transgene by offspring of founder animals probable. In such cases, the transgene is said to be germline or the animals are referred to as 'germline-competent'. Occasionally, integration of the microinjected DNA construct into the genome of the host can be delayed. If individual blastomeres undergo mitosis before the transgene is integrated, some, but not all, of the cells of the embryo will contain the transgene. The founder animal will be classified as a mosaic, where only a proportion of the cellular composition (including gametes) could harbor the introduced transgene.

Advantages of the DNA microinjection method are: (i) a high frequency of generating transgenic animals from viable microinjected embryos transferred to recipient females (for mice: 20–25% of total pups obtained); (ii) a lack of constraint on the size or type of DNA constructs used; and (iii) the stability of the transgene as it is transmitted from generation to generation. By contrast, disadvantages of this method include: (i) the random and potentially significant influence that the site of integration can exert on transgene expression (positional effects); (ii) the potential for undesired insertional mutagenesis; and (iii) the time and expense required developing the necessary micromanipulation and microinjection skill sets [24].

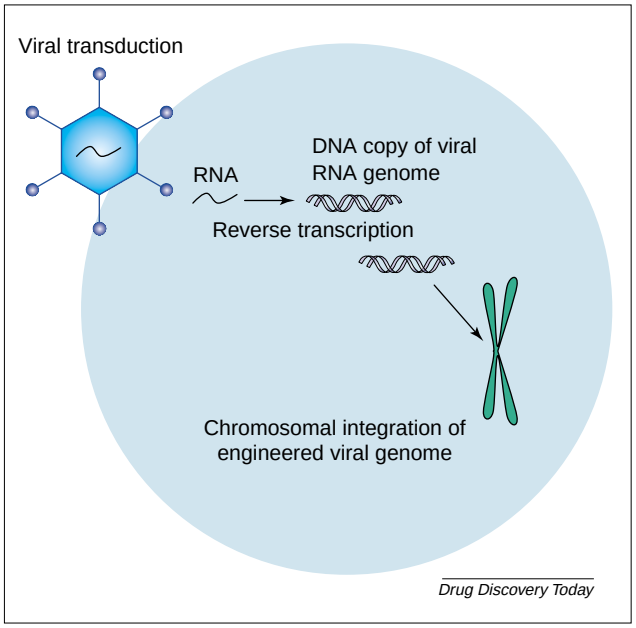

FIGURE 1

Retrovirus-mediated gene transfer. Modified viral genomes containing a transgene are packaged into virions. On transduction of the cells, the viral RNA genome is reverse-transcribed to a DNA copy of the viral RNA genome (cDNA) by reverse transcriptase. The cDNA is then integrated into the genome of the host cell using integrase. Reverse transcriptase and integrase are packaged into the virion and delivered into the cell with the RNA transgene.

Retrovirus-mediated gene transfer

Retroviral methodology has also been used to transfer genes of interest into animal genomes [25]. Although embryos can be used up to midgestation, four- to 16-cell stage embryos are primarily used for infection with one or more recombinant retroviruses (effectively transducing only mitotically active cells). Immediately following infection, the retrovirus produces a DNA copy of its RNA genome using the viral enzyme reverse transcriptase (Figure 1). The DNA copy of the viral genome, or provirus, integrates randomly into the host cell genome, usually without deletions or rearrangements. High rates of gene transfer, approaching 100% efficiency, are achieved with the use of retroviruses. However, as is the case for gene transfer by microinjection, integration events are random.

For safety purposes, retroviruses are frequently modified by removing structural genes, such as *gag*, *pol* and *env*, which support viral particle formation. However, most retroviral lines used in transgenic animal experiments are ecotropic, meaning that they infect only the model systems (e.g. mice or rats); hence rodent cell lines, rather than humans, could be at risk of contamination if correct precautions are not established.

In the aggregate, disadvantages of retrovirus-mediated gene transfer include: (i) low copy number integration; (ii) the additional steps required to produce retroviruses in comparison to microinjection or ES cell-based techniques; (iii) a general limitation on the size of the foreign DNA insert (usually <15 kb in length); (iv) a potential for undesired genetic recombination that might alter the replicative characteristics of the retrovirus; (v) a high frequency of mosaicism; and (vi) possible interference of retroviral sequences on transgene expression.

Embryonic stem cell technology

Gene transfer has been used to produce random or targeted insertions, or ablation of discrete DNA fragments in the mouse genome. For targeted insertions, where the integration of foreign genes is based on homologous recombination, the efficiency of DNA microinjection is extremely low [26]. By contrast, taking advantage of cell culture and homologous recombination, the use of ES cell transfer into mouse embryos has been quite effective in enabling the targeting of a specific genetic modification at a precise

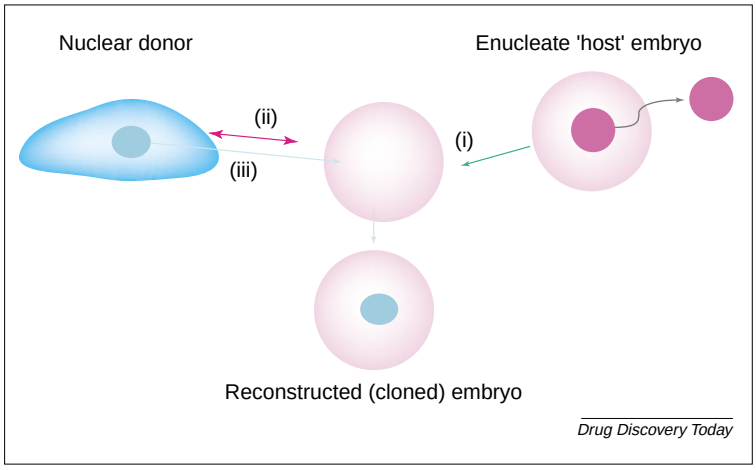

FIGURE 2

Nuclear transfer. **(i)** The host oocyte is enucleated. Either the nuclear donor cell is fused with the enucleated oocyte **(ii)** or the nucleus of the nuclear donor cell is dissected and transferred into the enucleated oocyte **(iii)**. Nuclei from many cell types, including terminally differentiated cells, can be transferred to enucleated oocytes. When transferred to a surrogate mother, the resulting animal will possess a nuclear genotype that is identical to that of the animal from which the nuclear donor cell was obtained.

chromosomal location. This preselection of specific clones in cell culture before ES cell transfer has led to the production of mice that: (i) incorporated a novel foreign gene into their genome; (ii) carried a modified endogenous gene (knockin models); or (iii) lacked a specific endogenous gene following gene deletion or 'knockout' procedures.

Pluripotent ES cells are derived from early preimplantation embryos and are maintained in culture for a sufficient period to perform various *in vitro* manipulations by introducing foreign DNA sequences using techniques such as electroporation, microinjection, precipitation reactions, transfection or retroviral insertion. One significant advantage of performing genetic manipulations in ES cells is that verification of the targeted modification can be performed *in vitro* before any animal work is initiated. ES cells are either injected directly into the blastocoel of a host blastocyst or incubated in association with earlier stage mouse embryos, followed by transfer of the embryos into intermediate hosts or surrogate females for continuing development. After birth, the coat color of the mice can be used as an early indicator of the level of chimerism. Because the coat color of the parental ES cell strain is different from that of the host embryo strain, mice in which both strains contribute to the development will display a mix of two coat colors. This phenomenon is termed chimerism and animals displaying this trait are referred to as chimeras.

The difficulties associated with the production, characterization and maintenance of pluripotent ES cell lines were perhaps the greatest obstacle for generating ES-cell derived transgenic animals. With the exception of some mouse strains, this difficulty reflected a general inability to maintain undifferentiated and pluripotent ES cells *in vitro*. Following the identification of ES cells, and subsequently gene transfer procedures, it became necessary to identify, accurately and efficiently, those ES cells that had integrated foreign DNA. In this regard, the use of marker-assisted selection schemes has significantly simplified mouse experimentation.

The addition of ES cell–embryo co-culture techniques involving tetraploid host embryos (eight-cell stage to morulae) has resulted in first generation founders that are derived completely from co-cultured ES cells [27,28]. Hence, founders are no longer chimeras, because all cells are derived from the same progenitor cells and the founder animals will faithfully transmit the genetic modification. However, the genetic background of host and donor strains of mice has a considerable influence on the germline transmission of transferred genes in ES cell-derived mice. Whereas ES cell lines have been identified for species other than the mouse, the production of germline-competent, ES cell-derived chimeric animals has not been reported.

Nuclear transfer

Nuclear transfer (commonly referred to as 'cloning') involves the introduction of donor nuclei obtained from either stem cells or differentiated adult cells into enucleated oocytes, thereby reprogramming future development (Figure 2). Reconstructed oocytes are then transferred to a surrogate dam for the remainder of gestation. In the early 1980s, the first mammalian nuclear transfer experiments were reported in mice amid some controversy [29,30]. The successful cloning of a sheep by nuclear transfer was reported in the mid-1990s, overcoming the technological difficulties encountered in the early mouse studies and rekindling the imagination of researchers struggling with ES cell-based technologies in several species [31]. This landmark paper, followed by several technological feats, rapidly led to the production of transgenic animals using a variety of cloning techniques [32–34]. It should be noted that nuclear transfer was particularly important for gene targeting experiments in several mammalian species because pluripotent germline-competent ES cells were neither validated nor available beyond those characterized for a limited number of mouse strains. The advent of nuclear transfer enabled gene knockout experimentation in several biologically relevant species. Unfortunately, efficiencies of nuclear transfer experiments still pale in comparison with other techniques [35–37]. Cellular senescence as a consequence of telomere shortening is another potential drawback to nuclear transfer [38]. However, although nuclear transfer might be considered flawed in its current form, major strides in enhancing experimental protocols are envisioned.

Gene knockdown and RNA interference

Gain-of-function and loss-of-function modeling efforts have, for the most part, concentrated on introducing specific mutations into the nuclear genome. The emerging technology of RNA interference (RNAi) has broadened the

possibilities for the creation of loss-of-function models. Short interfering RNA (siRNA) exists in a double-stranded state and inhibits endogenous genes (and/or exogenous sequences, as in viral genes) as the result of complementary sequence homology [39,40].

From an evolutionary standpoint, RNAi appears to protect the cell against foreign (e.g. viral) RNA invasion. The mechanism of RNAi is thought to involve a double-stranded (sometimes hairpin) RNA molecule that is cleaved into small fragments of ~22 nucleotides in length and assembled into a ribonucleoprotein complex referred to as the RNA-inducing silencing complex (RISC). The RISC then binds to homologous mRNA and performs its endonucleolytic cleavage [41]. Recently, it has been shown that variations in the complementary oligonucleotide (i.e. length and nucleotide composition) can have a significant affect on both the degree and the duration of gene 'silencing' [42].

The short oligonucleotides that silence gene expression (i.e. protein synthesis) are referred to as siRNAs. siRNA has been shown to be a potent inhibitor of gene function *in vivo* [39]. Mouse and rat models harboring small hairpin RNA transgenes, following shRNA transcription, produced lower levels of the homologous protein when compared with controls [43]. Gene silencing and knockdown technology has potential medical and agricultural applications, including the inhibition of viral gene transcription and inhibition of endogenous genes coding for deleterious gene products [44].

In the mouse, RNAi has several advantages over homologous recombination and ES cell-mediated gene knockout methodologies. RNAi can be synthesized directly, thus avoiding laborious cloning steps. Most notably, this methodology is currently the most significant advance since nuclear transfer in effecting efficient loss-of-function modeling in mammalian species (particularly for non-murine species where ES cell transgenesis has not been successful).

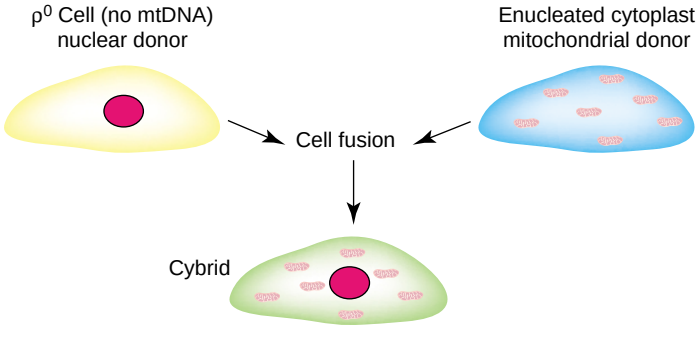

FIGURE 3

Mitochondrial transfer. Cytoplast or karyoplast transfer can be used to modify the mitochondrial populations within cells. Here, enucleated mitochondrial donor cells can be fused with P^0 cells (cells depleted of mitochondrial DNA) to create cells that are hybrids between the mitochondrial donors and the recipient host cells.

Sperm as vectors

In contrast to embryo manipulation, a completely different strategy was taken with the advent of sperm-related transfer procedures. Sperm-mediated gene transfer was reported in 1989, but many researchers hotly disputed the validity of this methodology because they were unable to duplicate the published procedures [45,46]. However, by 1994, spermatogonial cell transplantation procedures were established as a feasible alternative for *in vivo* gene transfer [47]. Genetically modified spermatogonia are transferred into testes lacking germ cells. These cells take up residence in the testis, divide and produce fully mature and fertile spermatozoa. Pluripotent spermatogonial stem cells have been isolated and grown under conditions allowing long-term maintenance and proliferation [48]. With embryo- and sperm-related procedures in further development, many existing procedures will become more practicable and efficient. However, whole animal and somatic-cell techniques, coupled with novel vectors and vector design, will continue to evolve and thus enhance the technologies and resources available for the effective genetic engineering of animals [28,35].

From the nucleus to the mitochondrial genome

In contrast to various methods targeting the nuclear genome, until recently, the importance of mitochondrial genetics and the mitochondrial genome in animal production had received only modest attention. This is, in part, related to the difficulty associated with *in vivo* mitochondrial gene transfer. The ability to manipulate the mitochondrial genome and to regulate mitochondrial gene function would provide an additional target for the modification of mammalian development. Studies revolving around mitochondrial transfer and techniques to produce animals harboring foreign mitochondrial genomes have been initiated (Figure 3) [49–52]. The creation of transmitochondrial animals represents a new model system that will provide a greater understanding of mitochondrial dynamics, leading to the development of genetically engineered production animals, as well as therapeutic strategies for human metabolic diseases affected by mitochondrial mutation or function.

Clinical applications

Models for human disease

The biomedical sciences rely heavily on animal models as tools for the discovery and development of therapeutic interventions. Of the many human conditions that medicine seeks to address, few naturally occur in animals. Instead, transgenic modifications, particularly in mice, are commonly used to model the human condition and studies in these animals have provided fertile ground for drug discovery across a continuum of human developmental and pathological conditions [53]. Some examples include:

(i) Gene therapy. Models for obesity and immunological, neurological, reproductive and hematological disorders,

among many others, have been developed [54–61]. It is now possible to circumvent and correct genetic disorders using a variety of transgenic technologies in animal models, providing future hope for a variety of human therapeutic interventions.

(ii) Genetic basis of human and animal disease and the design and testing of strategies for therapy. To date, many human diseases either do not exist in animals or are only developed by 'higher' mammals, making models scarce and expensive. Spontaneously (serendipitously) developed animal models do not exist [53].

(iii) Disease resistance in humans and animals. From a clinical and basic research perspective, it is imperative that models for enhancing characteristic pathways of human disease and disease susceptibility that target therapeutic applications are developed.

(iv) Drug and product testing and/or screening. Toxicological screening protocols using transgenic animal systems are already in trials [e.g. the list produced by the US National Institute of Environmental Health Sciences detailing characterized transgenic screening and reporter models(http://dir.niehs.nih.gov/dirlep/genmice2/open_me.htm)]. For preclinical drug development, a whole-animal model for screening is essential to the understanding of disease etiology, drug pharmacokinetics and evaluating therapeutic efficacy and safety.

(v) Novel product development through molecular pharming. Biologically active proteins have been developed in transgenic animals, which are used as bioreactors instead of traditional bacterial and cell culture-based systems. The use of animal transgenesis is an important contribution to pharmaceutical development, particularly for complex molecules where knowledge of tertiary protein conformation is crucial. Using genetically modified animal models, specific organs and body fluids have been targeted, with superior production efficiencies (Table 1).

A compelling example illustrative of the power of transgenic technology in dissecting human disease pathogenesis is the work on Alzheimer's disease (AD) [54–56]. Early experimentation with gain-of-function models considerably advanced knowledge of the etiology and progression of AD. Transgenic mice overexpressing amyloid precursor protein (APP) [54,55] have shed light on the manner in which β-amyloid plaques are deposited in the brains of Alzheimer's patients. Mice overexpressing both wild-type and mutant forms of the tau protein have advanced understanding of specific aspects of AD pathogenesis, particularly the formation of neurofibrillary tangles.

Transgenic models of human disease are also used extensively to assess the validity of therapeutic applications before clinical trials. Many of the transgenic mouse models created to study the pathogenesis of AD have been used to investigate therapeutic strategies. Studies aiming to decrease rates of β-amyloid production have made use of mice overexpressing APP [54,55]. Moreover, this and similar mouse models were used to test immunization strategies in which a host immune response was elicited to prevent β-amyloid plaque formation. At present, use of transgenic animals for preclinical research is the only way in which they directly impact clinical medicine.

Production of pharmaceuticals in transgenic animals

The impact of transgenic animals on pharmaceutical development could soon expand as recombinant proteins expressed and secreted by transgenic animals move toward regulatory approval and production (Table 1). The production of therapeutic proteins from transgenic animals usually involves their expression from mammary-gland-specific promoters to drive secretion of the transgene into milk [62]. An alternative is the use of kidney- or bladder-specific promoters that direct transgene expression to the urine [62–64]: these models enable the collection of recombinant protein from animals of both sexes during their complete lifetime.

Several advantages make transgenic production of recombinant proteins attractive. Many human proteins require posttranslational modification for biological activity. In many cases, comprehensive posttranslational modification can only be accomplished in mammalian expression systems and not in *Escherichia coli*-based bioreactors. Proteins for the treatment of blood clotting disorders are either isolated from human blood or produced *in vitro*. There are several factors that contribute to the high cost involved in isolating these proteins: (i) obtaining the starting material (e.g. blood); (ii) rigorous testing for bloodborne pathogens; and (iii) the difficulty and relative inefficiency (compared with the isolation of secreted products in milk) associated with cell culture systems. Isolating these same proteins from the milk of a specific pathogen-free transgenic dairy herd eliminates the possibility of viral contamination and enables flexible and scalable production quantities. These factors combine to reduce the cost and increase the efficiency of production for such therapeutics.

In January 2004, GTC Biotherapeutics submitted a Market Authorization Application to the European Medicines Agency for ATryn®, a recombinant form of human antithrombin produced in the milk of transgenic goats (www.gtc-bio.com/pressreleases/pr012604.html). This was the first product derived from a transgenic animal to be submitted for formal regulatory approval in Europe or the USA. Approval for ATryn® in Europe is expected to be granted in 2005 or 2006 and an application for FDA approval is planned. GTC has three other transgenic-derived proteins in its internal development pipeline, and is in collaboration with other companies to implement transgenic production of their therapeutic proteins.

Pharming Group NV has focused on developing a core of four transgenic products. The lead product in their transgenic-based portfolio is recombinant human C1 inhibitor for the treatment of hereditary angioedema. C1

inhibitor is currently undergoing Phase III clinical trials and the company expects to apply for market authorization in Europe in 2005.

Whereas GTC and Pharming are focused on developing pharmaceuticals derived from the milk of transgenic animals, Avigenics aims to express therapeutic proteins in transgenic chickens. Using egg-white-specific gene promoters, recombinant drugs are secreted in the eggs of the transgenic flock. Avigenics' lead product, α-interferon, will shortly begin clinical trials.

Transgenic expression of immunoglobulins

The transgenic production of immunoglobulins (Igs) dates back to the beginning of transgenic animals. The first Ig transgene expressed in a transgenic mouse encoded a murine κ isotype light chain [65]. Transgene expression was targeted to the spleen and the expressed protein was detected in the serum of transgenic animals. In 1984, a functionally rearranged gene encoding murine serum μ Ig was cloned and used to produce transgenic mice [66]. Not only was the transgene expressed in B and T cells of transgenic mice, it also combined with the endogenous light chain of the host to produce functional IgM. This research demonstrated that transgene encoded Ig heavy or light chains can combine with an appropriate endogenous counterpart to produce a fully functional Ig.

Working towards a transgenic animal producing a disease specific Ig, chimeric Igs were created by ligating the variable region exon of a previously characterized Ig to the constant region exon [67]. This strategy would facilitate the expression of an antigen-specific Ig with a unique constant region. Chimeric antibodies would have a reduced risk of allergic reactions because they are specific to the immune system of a particular organism. This technology also provides the potential to target an antigen-specific variable region to a location (i.e. tissue), which is not possible with the original antibody. For instance, a variable region derived from an IgG specific to an enterotoxigenic bacterial antigen could be combined with an IgA constant region, thus providing secretory capabilities to the chimeric antibody. In an attempt to develop a clinical application, such a model was created to demonstrate the application of transgenic technology in producing animals resistant to a targeted pathogen [68]; this study was intended to demonstrate the application of transgenic technology to produce animals resistant to a specific antigen. Although potentially important in particular disease models, the issue of producing human antibodies in a transgenic animal model was not addressed.

A similar approach to the chimeric antibody is the generation of humanized antibodies, in which only the complementary determining region (CDR) of the antibody is mouse derived [69]. CDRs represent the minimum amount of the variable portion of an antibody required to recognize a specific antigen. This 'humanization' of the antibody further reduces its potential to trigger an immune reaction. Similarly, transgenic mice have been produced in which murine Ig loci were knocked-out and replaced by human Ig loci. Resulting mice efficiently produced fully human antibodies [70]. There are several marketed chimeric and humanized antibodies, with others currently in development (Table 1).

The importance of having the capability to produce a wide diversity of human antibodies using an animal bioreactor, which would facilitate generating human antibodies to specific antigens for a wide variety of applications, was recognized early on in transgenic animal production. This approach would enable immunization in an animal model to produce clinically relevant human-specific antibodies efficiently. This technology required the microinjection of large segments of DNA ('translocus') containing the human Ig locus [71,72]. Transgenic mice containing human Ig gene loci express a wide variety of functional human Igs, and challenging these mice with specific antigens could potentially generate antigen-specific human Igs *in vivo*. The promise of such technology was demonstrated in an elegant study in which transgenic mice containing a human Ig-μ transgene produced a variety of receptor-binding variants of influenza virus hemagglutinin on exposure to the antigen [73].

More recently, transgenic cattle harboring intact unrearranged human Ig heavy- and λ light-chain loci were created [74]. These 'transchromosomic' cattle were shown to produce human Ig. It appears that transgenic animals can be engineered to produce a repertoire of human antibodies, which could provide passive immunity to humans, as well as be used in a variety of other applications.

Xenotransplantation

The extraordinary success of human-to-human (an example of intraspecies or allotransplantation) transplantation of vascularized organs (i.e. heart, kidney, liver, lung and pancreas) has saved many lives over the past 25 years, but it has also created a significant need for donor organs. Primate-to-human organ transplantation ushered the age of xenotransplantation, but it was not a long-term solution. Although the immunological barriers in a primate-to-human transplant were comparable with those of allotransplantation, the ethical and physical constraints (number of donor animals available) rendered the model unrealistic.

It was recognized early on that for physiological, anatomical, ethical and supply reasons the pig was the best choice as a donor animal for vascularized organs. However, serious immunological issues had to be overcome before the pig-to-human transplantation model could become a reality [75]. The hyperacute rejection mediated by preformed natural antibodies and complement provided the first significant immunologic barrier. The benefit of inhibiting complement was demonstrated when complement depletion before transplantation extended the duration of xenograft acceptance by the

TABLE 1

Pharmaceutically related products derived from transgenic animals

Product	Use	Product development stage	Organization
5G1.1	Rheumatoid arthritis Nephritis	Preclinical	Alexion–GTC Biotherapeutics
α-1 Antitrypsin	Hereditary emphysema Cystic fibrosis	I–T	Bayer–PPL ARC
α-Fetoprotein (rhAFP)	Myasthenia gravis Multiple sclerosis Rheumatoid arthritis	Phase II	Merrimack–GTC
ABX-EGF	Cancer	Phase II	Abgenix–Amgen
ABX-IL8	Rheumatoid arthritis	I–T	Abgenix–GTC
Antithrombin III (ATryn®)	Emboli Thromboses	Phase III Seeking marketing approval	GTC
Bile salt-stimulated lipase	Cystic fibrosis Fat malabsorbtion	I–T	AstraZeneca–PPL
Butyrylcholinesterase (Protexia™)	Biodefense	Preclinical	Nexia
C1 inhibitor	Hereditary angioedema	Phase III	Pharming
Calcitonin	Osteoporosis	I–T	PPL
CD137 agonist	Solid tumors	Preclinical	Mayo Clinic–GTC
CFTR	Ion transport Cystic fibrosis	I–T	GTC
Collagen	Rheumatoid arthritis	Preclinical	Pharming
CTLA4Ig	Rheumatoid arthritis	Preclinical	Bristol-Myers Squibb–GTC
D2E7	Rheumatoid arthritis	Preclinical	Abbott–GTC
Erythropoietin	Anemia	Preclinical	Avigenics
Extracellular superoxide dismutase	Ischemic reperfusion injury	I–T	Pharming
Factor VIII	Hemophilia A	Preclinical	ARC–Pharming
Factor IX	Blood coagulation Hemophilia	Preclinical	GTC PPL ARC–Pharming
Fibrinogen	Tissue sealant development	Preclinical	Pharming
G-CSF	Leukopenia	Preclinical	Avigenics
Glucagon-like peptide-1	Type 2 diabetes	I–T	PPL
Glucosidase	Glycogen storage disease	I–T	Pharming
Glutamic acid decarboxylase	Type 1 diabetes	I–T	GTC–BioSyn
Hemoglobin	Blood substitute development	I–T	Baxter
Human growth hormone	Growth failure Turner's syndrome Cachexia	I–T	GTC
Human serum albumin	Blood pressure Trauma Burn treatment	Preclinical	Fresenius–GTC
Humanized antibodies	Various indications	Phases I, II and III	Medarex
Humanized polyclonal antibodies	Various indications	Preclinical	Hematech Avigenics
huN901	Small-cell lung cancer	Preclinical	ImmunoGen–GTC
Insulin	Diabetes	I–T	GTC
Interferon	Antiviral	Preclinical	Avigenics
Lactoferrin	Immunomodulatory Anti-inflammatory	GRAS filing Phase I	Pharming
Lysozyme	Antimicrobial Immune modulator	I–T	UC-Davis
Merozoite surface protein 1	Malarial vaccine	Preclinical	GTC
PRO542	HIV/AIDS	Preclinical	Progenics–GTC

Continued on following page

TABLE 1 (continued)

Pharmaceutically related products derived from transgenic animals

Product	Use	Product development stage	Organization
Prolactin	Enhancement of immunity	I–T	GTC
Protein C	Blood coagulation	I–T	ARC PPL
Remicade®	Crohn's disease Rheumatoid arthritis	Preclinical	Centocor–GTC
Rotavirus virus-like particles	Vaccine development	Preclinical	Bioprotein
Spider silk (Biosteel™)	Materials development	Preclinical	Nexia
Tissue plasminogen activator	Dissolution of blood clots Heart attacks	I–T	Genzyme
Tissues or organs	Engineered for xenotransplantation	Preclinical	Alexion Bresagen Novartis Mayo Clinic
Tysabri® (formerly Antegren®)	Neurological disorders	Preclinical	Elan–GTC
Xenomouse® (fully human antibodies)	Various indications	Various	Abgenix

Table adapted from [62].
Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; G-CSF, granulocyte-colony-stimulating factor; GRAS filing, generally regarded as safe (here for nutraceutical filing); I–T, transgenic product manufacture inactive or terminated.

recipient [76]. Because only closely related species (i.e. mouse-to-rat or primate-to-human) have the intrinsic ability to regulate complement in a xenograft, it became obvious that a pig genetically engineered to express human complement regulatory proteins would be required to abrogate this initial barrier. However, some reasoned that, even with such a transgenic model available, the role of pig-to-human xenotransplantation would be that of a temporary solution while a patient awaited an allotransplant [77]. In any case, overcoming the initial hyperacute rejection was the highest priority. The cloning of human complement regulatory proteins [78] provided the molecular tools necessary to produce the transgenic pig required to overcome this problem.

The first published transgenic pig-to-primate xenograft used a novel transgenic delivery system for human complement regulatory proteins [79,80]. Although this work demonstrated the advantages of a transgenic model for overcoming hyperacute rejection [81], it also highlighted the need to surmount additional rejection barriers, such as natural killer (NK) cell-mediated rejection similar to that observed in allotransplantation [82,83]. However, subsequent barriers will not require further transgenic work, with the possible exception of knocking out immunologically important carbohydrate epitopes. Early attempts to block these important epitopes relied on either transgenic production of α-galactosidase and α-1,2-fucosyltransferase [84] or extracorporal removal of specific antibodies [85]. Each of these strategies was aimed at the removal or lowering of the number of galactose (Gal) epitopes on the graft endothelium to prevent hyperacute rejection. Although these strategies led to a marked reduction in natural antibody deposition, it appears that complete antigen removal could be required for pig-to-human xenotransplantation to be clinically relevant.

As xenotransplantation technologies progressed, porcine stem cell and nuclear transfer (cloning) procedures came together in the production of α-1,3-galactosyltransferase knockout pigs [86,87]. Although these studies effectively removed a specific xenoepitope [Gal(α1,3)Gal], immunologic hurdles were not overcome, for example, lymphocyte-mediated cell death, which occurs in the absence of antibody deposition. Accordingly, although antibody deposition and the subsequent complement lysis were prevented in the knockout pig model, xenogeneic NK damage was still apparent [88].

Several groups have now performed pig-to-nonhuman primate xenotransplants with varying results. The first such trial involved the transplantation of hearts from transgenic pigs expressing human complement regulatory proteins into pigs [80]. Transgenic hearts exhibited markedly reduced vascular injury and were functional for longer when compared with controls. Moreover, a transgenic pig liver expressing a human complement regulatory protein exhibited normal metabolic function in a nonhuman primate for eight days, whereas controls were rejected in less than 12 h (on average) [89]. However, complications related to acute vascular injury ultimately led to the rejection of the liver and heart transplants, even though complement activities were largely abrogated. Pig-to-primate renal transplants have been described, where transgenic pig organs expressed human complement regulatory proteins in concert with human antithrombin III [90]. Transgenic pig kidneys showed a doubling of the survival rates of the organs compared with nontransgenic control transplants. These modest, but significant, improvements in organ survival demonstrate the potential for transgenic pig-to-human xenotransplantation.

While research on overcoming xenograft barriers and preclinical trials were underway in the USA and UK,

issues regarding zoonotic disease detection and transmission became a concern and transplantation was debated in the scientific literature, as well as within societal and legislative arenas [91,92]. Nonetheless, transgenic models for human xenotransplantation remain a viable option for overcoming severe donor organ shortages and research continues to address the biological barriers that are yet to be circumvented or surmounted [93,94].

Conclusion

In the mid-1980s, the advent of transgenic technologies generated great excitement in the scientific community and the pharmaceutical industry. The acknowledged consequences and potential were equal to the societal implications and translation of the outlined basic research technologies toward fruitful applications. At the time, inroads in transgenesis were considered the next wave in the maturation of the developing field of biotechnology. Twenty-five years later, we now find ourselves positioned to reap the benefits of advances that are still deemed to be, if not in their infancy, in their adolescence. Although there are various products poised for launch, market, ethical and product concerns have matured considerably as we have advanced into the 21st century. The challenges are daunting, the implications thought provoking, with results still appearing to loom just around the corner. As in the case of related technologies, including stem cell-based genetic therapies, it will be interesting to observe the acceptance of newly engineered products that hold the promise of having a tremendous impact on societal needs and human health.

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