Producing Recombinant Human Milk Proteins in the Milk of Livestock Species

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Abstract Recombinant human proteins produced by the mammary glands of genetically modified transgenic livestock mammals represent a special aspect of milk bioactive components. For therapeutic applications, the often complex posttranslational modifications of human proteins should be recapitulated in the recombinant products. Compared to alternative production methods, mammary gland production is a viable option, underlined by a number of transgenic livestock animal models producing abundant biologically active foreign proteins in their milk. Recombinant proteins isolated from milk have reached different phases of clinical trials, with the first marketing approval for human therapeutic applications from the EMEA achieved in 2006.

Introduction

Recombinant DNA technology has revolutionized the production of therapeutic proteins. Even before the sequence of the Human genome became known, genes of a great number of human proteins have already been identified and cloned, including clotting factors VII (hfVII), VIII (hfVIII), and IX (hfIX), growth hormone (hGH), protein C (hPC), insulin (hI), insulin-like growth factor-1 (hIGF-1), interleukin-2 (hIL 2), antithrombin III (hAT-III), tissue plasminogen activator (htPA), α-1 antiprypsin ($h\alpha_1AT$), lactoferrin (hLF), extracellular superoxide dismutase (hEC-SOD), and erythropoietin (hEPO). Human proteins have been used in medicine for many years, but the supply was limited by the availability of the human tissue from which they were extracted (e.g., sourcing hGH from pituitary glands of human cadavers). The first attempts toward producing therapeutic proteins from cloned genes were made in microorganisms, such as yeast and bacteria. Unfortunately, for many

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proteins this is not a viable option, because microorganisms are not capable of reproducing the posttranslational modifications necessary for protein activity and stability (Swartz, 2001). Furthermore, lower eukaryotic systems such as yeast, filamentous fungi, and unicellular algae are often limited by their ability to duplicate human patterns of protein production and yield recombinant products that are immunogenic and lack activity (Dyck et al., 2003). Insect cell systems have unique glycosylation patterns and are usually restricted to use at the laboratory scale (Farrell et al., 1998). Although mammalian cell culture systems provide the required complex posttranslational modifications, they are expensive and technically demanding when used at a commercial scale. The use of transgenic animals as "bioreactors" overcomes these problems. The expression of human proteins in the mammary gland of livestock (e.g., rabbits, pigs, sheep, and goats) provides a practically unlimited source of correctly processed, active, and stable proteins for clinical use at lower costs than mammalian cell culture, though they still have many regulatory hurdles to cross.

Producing Transgenic Animals

The Targeted Tissue

The main objective in biopharming is the economical production of valuable complex human therapeutic proteins using transgenic livestock species. Ideally, protein production in an animal should allow collection of the product in significant amounts without killing the animal and be isolated such that practical and cheap purification methods can be applied. Harvesting proteins from body fluids (blood, milk, urine) rather than from solid tissue is desirable because the fluids are renewable and most of the biomedically important proteins are secreted into body fluids. Collecting the protein from the bloodstream is possible by targeting expression to the liver or kidney, or to the blood lymphocytes. The main drawback, however, is that high circulating levels of biologically active proteins may have an adverse effect on the health of the animals. Therefore, the most obvious and viable tissue to target the expression of foreign proteins is the mammary gland. Milk is readily collected in a repeatable manner and is available in large quantities. The presence of large amounts of active foreign proteins in milk usually does not interfere with the health of the lactating animals. In some cases adverse effects have been observed, e.g., with human erythropoietin in transgenic rabbits (Massoud et al., 1996) and with hGH in transgenic mice (Devinoy et al., 1994); however, in these animals high concentrations of the recombinant proteins were detected in the blood during lactation. Since it is very unlikely that lactogenic hormones enhance the ectopic expression of the transgenes, the most probable cause is "leaking" of the recombinant protein from the mammary epithelium into the blood.

Milk contains a relatively small number of major protein components, which are secreted exclusively by the mammary gland and belong to either the caseins (α_{S1}-, α_{S2}-, β-, and κ-CN) or the whey proteins (BLG, α-LA, and WAP). The milk protein genes of several species have been cloned and characterized (Mercier & Vilotte, 1993). These single-copy genes are transcribed at high levels specifically in the mammary gland during pregnancy and lactation. Using promoter sequences from different milk protein genes allows high expression of foreign proteins into the milk of transgenic animals to be achieved.

The Gene Construct

The first step toward creating a transgenic animal is to engineer a DNA construct that will target the expression of the candidate protein specifically to the mammary gland. The recombinant protein expression must be restricted to milk to avoid any deleterious side effects on the animal's health. Therefore, the construct usually consist of a milk protein–specific promoter linked to the coding sequence of the desired protein. The regulatory elements that control the temporal and spatial expression of a gene are usually located within a few kilobases of the 5'-end of the transcribed region of the gene. Heterologous gene expression can be targeted specifically to the lactating mammary gland by using promoters isolated from different milk-specific genes. Additional regulatory elements, e.g., insulators, may be added to the construct to ensure high-level and/or position-independent expression of the transgene (Fig. 1). Genomic sequences coding for the candidate proteins was found to be expressed at higher levels than cDNA sequences (Whitelaw et al., 1991; Brinster et al., 1988), although at least the goat β-casein (Ziomek, 1998) and the mouse whey acidic protein promoter (Velander et al., 1992) seem to be capable of directing high-level expression of some cDNA-based constructs.

Large DNA fragments, e.g., BAC or YAC, ensure integrated copy number–dependent tissue and developmental specific expression of the coded genes, which was the case for human and goat α-lactalbumin (Fujiwara et al., 1997; Stinnakre et al., 1999) and porcine whey acidic protein (Rival-Gervier et al., 2002) genes in transgenic mice. Those BAC or YAC vectors could also be used to express human genes at a high level in milk (Fujiwara et al., 1999; Soulier et al., 2003).

Milk protein promoters have been isolated and well characterized from several species, including mice, rats, guinea pigs, rabbits, goats, sheep, and cattle. These promoters usually work well across species. However, the most commonly used promoters in commercial transgenic pharmaceutical production are murine and rabbit whey acidic protein, bovine α_{S1}-casein, goat β-casein, and ovine β-lactoglobulin. Nevertheless, an ideal mammary gland–specific vector still remains to be designed.

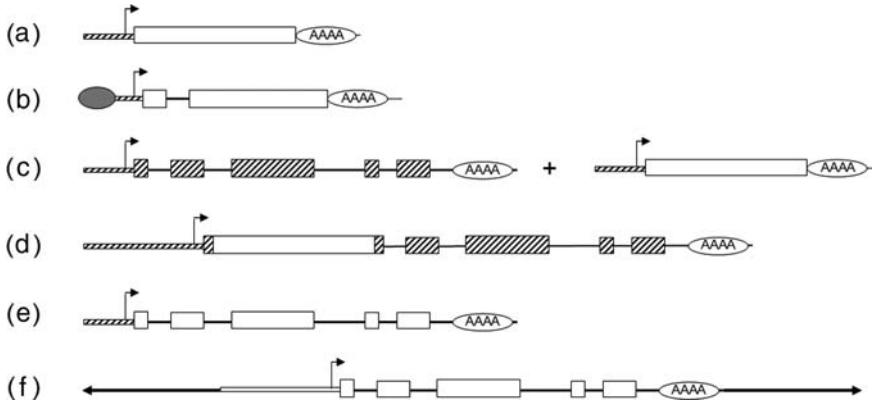

Fig. 1 The most typical types of transgene constructs. (a) cDNA-based gene constructs containing a homologue or heterologue promoter sequence attached to the cDNA sequence of the desired protein. (b) Different sequence elements can be added to the cDNA construct to enhance expression and/or tissue specificity, for, e.g., homologue or heterologue intron sequences, scaffold or matrix attachment elements [SAR/MAR], locus control regions [LCR], insulator elements. (c) Transgene expression can be "rescued" by co-injection with a high-expressing transgene, (d) or the cDNA can also be inserted into the genomic sequence of a highly expressed transgene. (e) Genomic gene constructs containing the whole coding region of the desired protein driven by a homologue or heterologue promoter sequence. (f) Gene constructs containing large genomic sequences with all the endogenous regulatory elements are mainly used with artificial chromosomes [BAC/YAC]

The Choice of Livestock Species

The next step in creating a transgenic animal is the process of introducing the transgene construct into the fertilized eggs of the species of interest. The key consideration for choosing a species for protein production is the quantity of protein product required and the timescale for production (Table 1). The feasibility and the costs of keeping and breeding the animals should also be considered.

The smallest and easiest to keep are rabbits, with about 1 L of milk per lactation, up to 8–10 lactations per year, and a minimum of 6 months to produce a lactating animal. Cattle with the longest timeline are the most costly. Up to 10,000 L of milk per cow can be collected, but a minimum of 2.5 years is necessary to produce a lactating cow (with an additional 2 years if the founder was a bull). Sheep, goats, and pigs are between these two extremes (Table 2). From sheep and goats, several hundred liters of milk can be obtained per lactation and about 1.5 years are needed to reach the first lactation. The generation of pigs can be established in about 15 months, and usually 100–150 L of milk can be collected per lactation (two lactations per year). Milk collection from ruminants is achieved with milking machines.

Table 1 Demand of Some Human Proteins for Clinical Use in the United States and the Estimated Number of Livestock Animals Needed for Production Calculated with an Average Recombinant Protein Expression of 1 g/L of Milk

	Factor VIII(hfVIII)	Factor IX(hFIX)	Protein C(hPC)	Antithrombin III(hAT-III)	Fibrinogen(hFib)	Albumin(hAlb)	Annual Milk *Yield (L)*
Amount needed (kg/year)	*0.3*	*4*	*10*	*21*	*150*	*315,000*	
Rabbit	60	800	2,000	4,200	30,000	63,000,000	*5*
Pig	1	14	34	70	500	1,050,000	*300*
Sheep	1	8	20	42	300	630,000	*500*
Goat	1	5	13	27	188	393,750	*800*
Cattle	1	1	2	3	19	39,375	*8,000*

Table 2 Parameters to Be Considered When Choosing Animal Species for Transgenic Milk Expression

Species	Milk Yield per Lactation(L)	Gestation(Months)	Maturation(Months)	Elapsed Time from Microinjection to First Lactation (Months)
Rabbit	1–1.5	1	4–6	6–8
Pig	100–300	4	7–8	15–16
Sheep	400–600	5	6–8	16–18
Goat	800–1,000	5	6–8	16–18
Cattle	Up to 10,000	9	12–15	30–33

Methods to Create Transgenic Animals

Several methods are available for the generation of transgenic mice; however, they differ in their usefulness when working with livestock species. We describe some of these methods here.

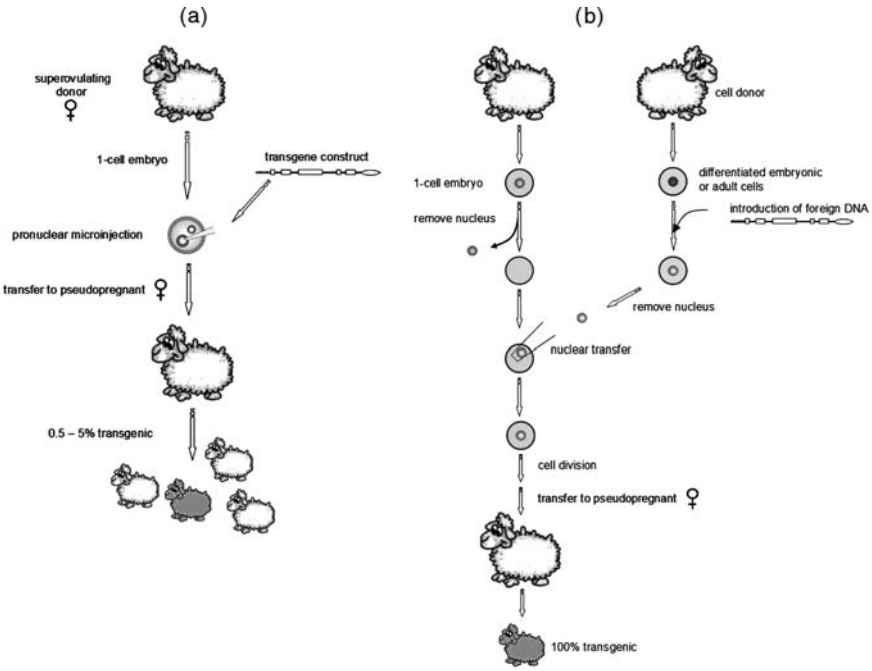

Fig. 2 Methods for producing transgenic livestock animals. (a) Pronuclear microinjection and (b) nuclear transfer. (*See* color plate 4)

Pronuclear microinjection (Fig. 2a) was considered the first successful mammalian transgenic technique (Gordon et al., 1980). It involves the direct injection of a foreign DNA sequence into the pronucleus of a fertilized egg, followed by a surgical implantation into the reproductive tract of a hormonally primed recipient foster mother. Successful gene transfers by this route have been described for all of the major livestock species, including rabbits (Bühler et al., 1990), pigs (Hammer et al., 1985), sheep (Simons et al., 1988), goats (Ebert et al., 1991), and cattle (Krimpenfort et al., 1991). The main assets of this method are as follows: It is well described, relatively simple to carry out for a skilled individual, relatively cost-effective, and DNA sequences of up to several hundred kilobases can be integrated. Further considerations include the low efficiency of generating transgenic founders (usually less than 10% of the animals carry the transgene) and the random integration of the transgene. When a transgene is integrated in a silent region of a chromosome, its product will be poorly or not expressed at all unless otherwise protected by regulatory elements. This phenomenon is called the "position effect." Pronuclear microinjection, however, is very labor-intensive, requiring special skills to carry out the micromanipulations. Nevertheless, despite its limitations, pronuclear microinjection remains the most straightforward and consistently successful means of gene transfer into the mammalian germline.

Retroviral transfection was first used in 1985 to create transgenic animals (Huszar et al., 1985; Jahner et al., 1985; van der Putten et al., 1985). The genetic material of the retroviruses is capable of stable integration into the chromosomes of the infected cells. The replication-defective viral vector construct to be used must contain not only the transgene but also regulating sequences for viral integration and packaging. Also necessary is the use of a "packaging" helper cell line, which allows assembly of the transgene-containing virus. After infection, the few days old embryos are implanted into recipient foster mothers. No micromanipulation is needed. The advantages of this method are its efficiency (nearly 100%) and the one-copy integration (Soriano et al., 1986), but since the viral infection does not occur at the one-cell zygote stage, the resulting animals will always be mosaic (not all cells carry the transgene). Only DNA sequences smaller than 8 kb can be integrated; therefore, in general, only cDNA constructs can be used. There is also the theoretical risk of recombination events leading to the development of new retroviruses. Combined with microinjection, this method has been adapted for cattle (Chan et al., 1998). Because of the above-mentioned limitations, the use of this method is restricted. More recently, the ability of lentivirus vectors to efficiently introduce transgenes has rekindled some interest in viral transgenesis (Clark & Whitelaw, 2003; Whitelaw, 2004).

Direct in vivo transfection of the mammary gland has been proposed as a faster and cheaper alternative to target the expression of a heterologous gene to the secretory mammary epithelial cells. Targeting of transgene carrying replication-defective retroviruses directly to the mammary secretory epithelia cells in lactating goats to produce foreign proteins in the milk has been demonstrated (Archer et al., 1994). Following trials through several ways of transducing the

mammary epithelium, recent publications point to a special advantage of the direct instillation of a recombinant adenoviral vector. This method allowed efficient secretion of human growth hormone (Sanchez et al., 2004) and human erythropoietin (Toledo et al., 2006) at levels of up to 2 g/L in the milk of mice and goats. Direct transduction of mammary epithelial cells by means of a recombinant adenovirus could be a suitable alternative to transgenic technology, especially for the production of potentially toxic proteins in milk, at levels high enough for their purification and biological characterization.

Sperm-mediated gene transfer has a history of increased transgenesis efficiency claims. Following controversial results, lactoferrin-producing transgenic rabbits were created through dimethylsulfoxide-treated sperm transfection (Li et al., 2006). The expression of the human lactoferrin (LF) gene was controlled by the goat β-casein gene 5' flanking sequence. Eighty-nine rabbit offspring were produced, with 46 of these being transgenic. Nevertheless, stable transmission of the transgene and expression levels in the consecutive generations has not been reported yet.

Embryonic stem (ES) cells are widely used at present to manipulate the mouse genome. These cells are isolated from the inner cell mass (ICM) of mouse blastocysts. They are undifferentiated cells, which, in the presence of the necessary growth factors, can be cultured without losing their pluripotency. Genetic modification of ES cells can be performed *in vitro,* making targeted transgene integration through homologous recombination possible. When injected back to a host blastocyst or using aggregation with normal diploid embryos (Nagy et al., 1990), their descendants contribute to the tissues of the resulting chimera including the germline. The main drawbacks of the ES technology include that it needs cell culture capabilities, it is very labor-intensive, a good ES cell line is needed, and only the second generation gives germline transgenic animals. Its main assets are that, in theory, construct expression can be tested prior to generating the animal and some advantages do result from site-specific recombination. Unfortunately, despite intensive efforts, no validated ES cells have been described for rabbits, pigs, sheep, goats, or cattle yet.

The major bottleneck in producing transgenic livestock is the low efficiency of generating transgenic founders. A radical improvement would be to carry out the required genetic manipulations not on the zygotes, but in conventionally cultured cells that could then be used to generate animals. Wilmut and co-workers (1997) introduced a new breakthrough in transgenic technology. *Cloning via nuclear transfer* enables viable animals to be created when nuclei from differentiated embryonic or somatic cells kept in *in vitro* culture are transferred into enucleated oocytes. Contradicting scientific dogma, the genetic material of the adult cell is capable of directing the growth and development of the oocyte into a healthy animal (Wilmut et al., 1997). This method is important because of the cloning technique, and it enables the creation of transgenic animals through the genetic manipulation of the donor cells in culture (Fig. 2b). The first transgenic sheep created using this method expressed human factor IX in milk

(Schnieke et al., 1997). Since then transgenic goats and cattle producing recombinant human proteins in their milk have been created by nuclear transfer (Parker et al., 2004). This method also allows gene targeting and the knocking in of transgenes to specified genomic loci, first demonstrated by the production of human α1-antitrypsin in sheep milk (McCreath et al., 2000). More recently, goat fetal fibroblasts have been gene-targeted by inserting the exogenous htPA cDNA into the β-casein locus through homologous recombination (Shen et al., 2006). The targeted insertion of the coding region of recombinant proteins into target loci will ensure more predictable expression levels in the future.

The advantages of cloning via nuclear transfer are that all animals are transgenic, the creation of transgenic animals can be shortened by one generation, the cultured cells can be stored almost indefinitely, and site-specific integration can be accomplished (McCreath et al., 2000). Though problems remain to be resolved, such as low efficiency and high mortality after birth, somatic cloning is already the preferred choice for producing transgenic ruminants. Notably, at the end of 2006, the U.S. FDA announced that food from cloned animals is safe to enter the food chain, although the debate about labeling continues.

Posttranslational Modifications

Many proteins require so-called posttranslational modifications, including signal peptide removal, forming of disulfide bonds, amino acid modifications, proteolytic processing, and subunit assembly. Bacteria, yeast, insect cell systems, or transgenic plants cannot provide all of the necessary modifications, which results in a lack of activity or immunogenicity of products. Mammalian cells and transgenic animals are the choice for recombinant protein production when complex posttranslational modifications are needed for the biologically active protein. Some of the amino acid modifications (e.g., glycosylation, carboxylation) are essential for the biological activity and/or stability of the proteins and are a key point in producing biologically active pharmaceuticals by recombinant organisms. It is by no means certain that the mammary gland is always capable of performing these modifications correctly. Since the recombinant proteins isolated from milk do not always have the same structure as their native counterparts, the possible differences and their effects must be evaluated case by case.

Glycosylation is the process (or result) of the addition of a glycosyl group to asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein. The process is one of the principal co-translational and posttranslational modification steps in the synthesis of membrane and secreted proteins, and the majority of proteins synthesized in the rough endoplasmic reticulum undergo glycosylation. It is an enzyme-directed, site-specific process with a very important role in affecting the folding, solubility, stability, biological

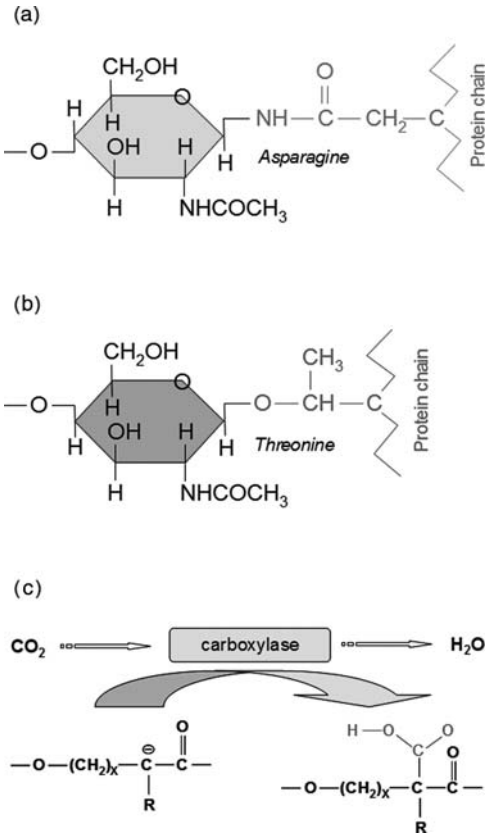

Fig. 3 Representative types of posttranslational modifications. Basic types of glycosylation: (a) N-linked glycosylation to the amide nitrogen of asparagine side chains; (b) O-linked glycosylation to the hydroxy oxygen of serine and threonine side chains. (c) Carboxylation. (*See* color plate 5)

activity, and immunogenicity of proteins. Two types of glycosylation exist: N-linked glycosylation to the amide nitrogen of asparagine side chains and O-linked glycosylation to the hydroxy oxygen of serine and threonine side chains (Figs 3a and b). Glycosylation is undoubtedly one of the most important posttranslational events for therapeutic proteins. It is essential for the stability of many proteins in blood circulation, required for the biological activity of gonadotropins, to some extent for antibodies, and often necessary for correct protein folding, conformation, intracellular transport, or tissue targeting.

The mammary gland naturally secrets N- or O-glycosylated proteins; although the mammary cells are capable of carrying out these modifications, the recombinant proteins isolated from milk are not always glycosylated in the appropriate manner. If this does not adversely affect the activity or stability, and does not cause ill side effects, it is of little consequence for the utilization of the recombinant protein. Detailed characterization of the recombinant human C1 inhibitor produced in the milk of transgenic rabbits showed an overall similarity of N-glycan structures with only the degree of

sialylation and core fucosylation being lower (Koles et al., 2004a). The first crystal structure of a recombinant protein produced in milk confirmed that the slightly modified glycosylation pattern of the recombinant human lactoferrin did not alter the protein's structural integrity (Thomassen et al., 2005). Systematic studies on the glycosylation capabilities of the mammary glands of the different species, including the effects of the stage of lactation and individual variations, are still to be performed.

Carboxylation is the introduction of a carboxyl group or carbon dioxide into a compound with formation of a carboxylic acid (Fig. 3c), e.g., the vitamin K–dependent blood clotting factors and regulatory proteins (e.g., hFVII, hFIX, hFX, hPC) require the conversion of glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla). The γ-carboxylated amino acid residues bind calcium, which is essential for their activity. Species-specific differences were observed in the ability of mammary epithelial cells to carboxylate heterologous recombinant proteins. Usually, carboxylation of a protein present at a low level is not adversely affected, while in some species high-level expression of the protein leads to the reduction in the amount of fully γ-carboxylated, biologically active components. This may result from the saturation of the cellular γ-carboxylase machinery. The species-specific differences may reflect differences in enzyme levels and/or substrate specificity.

Proteolytic processing is also of great importance. The first step in protein maturation is the removal of the signal peptide. Signal peptides are short peptide sequences (usually 13–30 amino acids) at the N-terminal part of proteins that direct the posttranslational transport of the proteins (which are synthesized in the cytosol) to certain organelles for further processing. In case of certain proteins (e.g., vitamin K–dependent plasma proteins) that are first synthesized as inactive preproteins, the removal or cleavage of some other parts of the precursor is essential for the development of the final structure and activity of the mature protein. The significance of the presence of the pro-protein processing enzyme furin has been confirmed experimentally in CHO cells and in transgenic mice. In both cases the expression of furin led to an increased level of mature recombinant human coagulation factor IX (rhFIX) (Lubon & Paleyanda, 1997). Significant species-specific differences were observed regarding the proteolytic processing capacity of the mammary gland.

It has been proven that the mammary gland is able to associate protein subunits in a correct fashion in a number of different cases (e.g., collagen type I, fibrinogen, and superoxide dismutase). Therefore, subunit assembly does not seem to be rate-limiting in recombinant protein production. The most impressive example in this regard is the recombinant fibrinogen produced at a high level in sheep's milk (Garner & Colman, 1998): Fibrinogen comprises six polypeptide chains of dimeric α, β, and γ chains. The recombinant fibrinogen isolated from sheep's milk was functional in clotting assay and was produced at a 1000-fold greater level than that achieved in cell culture.

Purification of Recombinant Proteins

The purification of recombinant proteins from milk for laboratory testing is not particularly problematic, although the purification procedure has to be adapted for each expressed recombinant protein individually. Milk has only a few main protein components, and simple procedures for removal of caseins, the major milk proteins, have been established. However, milk is a complex biological fluid, and eliminating some of its components requires more complex methods; chromatography can produce a high purity of the protein (Wright & Colman, 1997). Furthermore, if the recombinant protein has a high degree of similarity to an abundant milk protein, e.g., human serum albumin (hSA), separation from the equivalent host protein can be difficult.

For commercial products, since the recombinant proteins produced in transgenic livestock will be administered to humans, the therapeutic products must be purified to a very high degree and free of viral and prion proteins. The purification procedures usually involve the combination of several steps and methods like skimming, filtration, precipitation (e.g., selective precipitation by polyethylene glycol, enrichment by barium/citrate precipitation), viral inactivation, and, if necessary, chromatography (usually based on ion exchange, hydrophobic interaction, or immunoaffinity).

Clinical Trials

Before administering new therapeutic products to human patients, the safety and effectiveness must be proven. A series of preclinical and clinical trials must be completed. During preclinical trials, biochemical, toxicity, and pharmacokinetic properties will be tested and detailed information will be collected on the source and means of production. The clinical trials consist of three phases. Phase I is conducted on a small number of healthy volunteers to test if there are any adverse effects. Phase II is carried out on patient and control groups to further evaluate safety and efficiency. In phase III, the product is evaluated in much bigger patient groups and controls to set the proposed use and dosage. Only new products that pass all three trials are licensed to be marketed commercially. The aim of phase IV—following the permission for human therapeutic application—is to evaluate the effect of long-term application on patients. The first transgenic product ATryn® (antithrombin III) approved for human therapeutic use is produced in transgenic goats; marketing approval was granted in 2006 to GTC Biotherapeutics (http://www.gtc-bio.com).

Livestock Species as Bioreactors

Rabbits

Among the transgenic livestock species, rabbits are the smallest and easiest to obtain and maintain. Because of their short generation time and large litter size, they are an attractive alternative to large dairy animals, where the major drawback is the time required to generate the transgenic animals and to deliver a product to the market. The rabbit has well-described laboratory breeds, it is easy to superovulate, and the manipulation of the embryos is quite simple. It is ideal as a model animal but can also be used as a bioreactor if the amount of expressed protein does not need to be more than a few kilograms, as in case of the blood clotting factors (Brem et al., 1998). Other advantages are that it can be kept pathogen-free, it can be easily milked, and its milk composition is well described (Dayal et al., 1982; Baranyi et al., 1995), containing about four times as much protein as cow's milk. Although the efficiency of generating transgenic rabbits by microinjection is lower than that in mice (about 1–2%), it still is at least equivalent to other livestock species (Brem et al., 1998). The ratio of mosaics among the founders is usually quite high, around 30%, resulting in a reduced rate of transgene transmission to the offspring (Castro et al., 1999). This may result from the fact that the embryonic development of rabbits is significantly faster than that of mice, with transgene integration often occurring after the first round of cellular division. Recombinant proteins and peptides that have been produced in the milk of transgenic rabbits are described in Table 3.

Pigs

Although pigs are not conventional dairy animals, they have a distinct advantage over ruminant animal models: Sows have a relatively short generation time of one year and produce two litters and about 20 offspring per year. Transgenic pigs can be created either by microinjection or by somatic cloning. The efficiency of transgenesis is influenced by the fact that pigs differ from many other types of livestock, because unless there are at least four viable fetuses in the womb, pregnancies fail to go to term. In pigs, the mammary gland has no cisternae, with stored milk ejected by an active process and up to 300 L of milk obtained annually from a sow. Table 4 summarizes the recombinant proteins produced in the milk of transgenic pigs. The mammary gland's capacity for performing posttranslational modifications has been compared in transgenic pigs producing rhPC and rhFIX (Van Cott et al., 1999). The γ-carboxylation of the two recombinant proteins was rate-limiting and showed

Table 3 Expression of Recombinant Human Proteins in the Milk of Transgenic Rabbits

Protein	Promoter	Expression Level	Status	Company	Reference
hIL-2	Rabbit β-casein	0.43 μg/mL			Bühler et al. (1990)
htPA	Bovine α_{S1}-casein	50 μg/mL			Riego et al. (1993)
hIGF-1	Bovine α_{S1}-casein	1 mg/mL			Brem et al. (1994)
hGH	Mouse WAP	50 μg/mL			Limonta et al. (1995)
hα_1AT	Goat β-casein	4.0 mg/mL	In development	GTC Biotherapeutics	Genzyme Transgenics (1996)
hPC	Ovine-BLG	0.7 mg/mL		GTC Biotherapeutics	Genzyme Transgenics (1996)
hEPO	Rabbit-WAP	50 μg/mL			Massoud et al. (1996)
hEC-SOD	Murine WAP	2.9 mg/mL			Stromqvist et al. (1997)
hEPO	Bovine BLG	0.5 mg/mL			Korhonen et al. (1997)
sCT*	Ovine β-LG	1–2.1 mg/mL		PPL	McKee et al. (1998)
hαGLU	Bovine α_{S1}-casein	8 mg/mL	Phase II finished		Bijvoet et al. (1999)
hNGF-β	Bovine α_{S1}-casein	250 μg/mL			Coulibaly et al. (1999)
hFVIII	Murine WAP	0.083 IU/mL			Hiripi et al. (2003)
hGH	Rat WAP(6xHisTyr)	Cleavage by trombin to activate			Lipinski et al. (2003)
hTNAP	Rabbit WAP	826 IU/mL			Bodrogi et al. (2006)
hLF	Goat β-casein	153 μg/mL			Li et al. (2006)
hFVII			In development	GTC Biotherapeutics/LFB Biotechnologies	http://www.transgenics.com/products/prod.html
hC1INH	Bovine α_{S1}-casein	12 mg/mL	Phase III	Pharming	Pharming literature online

*sCT salmon calcitonin: The human calcitonin aggregates; therefore, the piscine equivalent was produced.

Table 4 Expression of Recombinant Human Proteins in the Milk of Transgenic Pigs

Protein	Promoter	Expression Level	Company	Reference
hPC	Mouse WAP	1 mg/mL	GTC Biotherapeutics	Velander et al. (1992)
hPC	Ovine BLG	0.75 mg/mL	PPL Therapeutics	PPL literature
hFVIII	Mouse WAP	3 μg/mL		Paleyanda et al. (1997)
hEPO	Mouse WAP	878 IU/mL		Park et al. (2006)

differences between them, resulting in varying degrees of posttranslational modifications.

Sheep and Goats

The length of time to milk production is obviously the major factor in the choice of species; however, the disease status of animals, litter size, and volume of milk should also be taken into consideration. For proteins produced in sheep's milk, a special concern is related to animal health due to the issue of prions responsible for scrapie. Therefore, if sheep is the animal of choice for recombinant protein production, only animals from countries such as New Zealand should be used, because of their scrapie-free status. It was stated a decade ago that high-level (35–45-g/L) production of α1-antitrypsin in the milk of transgenic sheep does not seem to be at the expense of the production of other milk proteins (Colman, 1996).

The prototype of a transgenic goat producing recombinant protein was published in 1991 (Ebert et al., 1991). Ten years later a special dwarf breed of goat (BELE: breed early lactate early) was adapted to produce transgenic goats with nuclear cloning. The early sexual maturity of BELE goats shortens the generation time for producing recombinant proteins (Keefer et al., 2001). The type and expression levels of recombinant proteins produced in the milk of transgenic sheep and goats are described in Tables 5 and 6, respectively. More recently extension of the gene therapy techniques resulted in high-level expression of recombinant proteins in the milk through direct transduction of the mammary epithelium of goats (Sanchez et al., 2004; Toledo et al., 2006). In the future, the direct introduction of a foreign gene into a mammary gland could dramatically reduce the time of production of pharmaceuticals in milk from years to weeks.

Cattle

Transgenic cattle are the most economic choice if the aim is to produce huge amounts of recombinant protein. This species is attractive as a bioreactor

Table 5 Expression of Recombinant Human Proteins in the Milk of Transgenic Sheep

Protein	Promoter	Expression Level	Company	Reference
hα_1AT	Ovine BLG	35 mg/mL		Wright et al. (1991)
hFVII	Ovine BLG	2 mg/mL	PPL Therapeutics	PPL literature
hFVIII	Ovine BLG	6 ng/mL		Niemann et al. (1999)
hFIX	Ovine BLG	25 ng/mL		Simons et al. (1988)
hFIX	Ovine BLG	5 ng/mL		Clark et al. (1989)
hFIX	Ovine BLG	1.0 mg/mL	PPL Therapeutics	Schnieke et al. (1997)
hFIB	Ovine BLG	5.0 mg/mL	PPL Therapeutics	Garner and Colman (1998)
hFIB	Ovine BLG	5 mg/mL		Butler et al. (1997)
hPC	Ovine BLG	0.3 mg/mL	PPL Therapeutics	Garner and Colman (1998)

given the development of many established embryological techniques for cattle. Due to the high value of dairy cattle, "slaughterhouse-derived" oocytes are used for microinjection following *in vitro* oocyte maturation and fertilization. Alternatively, high-quality oocytes can be obtained via ovum pickup. Embryos are individually cultured, and a multiplex PCR analysis can be performed on biopsies to identify the males and the transgenics; since the aim is to produce foreign protein in the milk, preferably only the female transgenic embryos are selected for transfer into synchronized recipient heifers. Pregnancy initiation can be confirmed by ultrasound detection of a fetal heartbeat at ~28 days.

Herman, the world's first transgenic bull, was created in 1991 through microinjection of transgene αs1-casein promoter and the cDNA sequence for human lactoferrin (Krimpenfort et al., 1991). Today transgenic cattle can be generated far more efficiently via nuclear transfer (Table 7). Due to the use of female totipotent cells for genetic manipulation and the subsequent selection of transgenic cells before nuclear transfer, all calves born will be female and transgenic. Since calving-induced lactation will not occur until the animal is ~2 years old, to speed up the selection process, hormonal induction of lactation can be used when the animal is between 2–6 months of age. In the most optimal case, the elapsed time to obtain a lactating female for recombinant protein production is 48–56 months depending on the sex of the founder created by microinjection, which could be reduced to 33 months if nuclear transfer was applied.

Table 6 Expression of Recombinant Human Proteins in the Milk of Transgenic Goats

Protein	Promoter	Expression Level	Status	Company	Reference
htPA	Murine WAP	3 μg/mL 610,000 IU/mg			Ebert et al. (1991), Denman et al. (1991)
htPA	Goat β-casein	3 mg/mL (6 mg/mL?)			Ebert et al. (1994)
hAT-III	Goat β-casein	20 mg/mL	ATryn® EU: approved US: prelaunch	GTC Biotherapeutics/LEO Pharma	Genzyme Transgenics (1996), GTC literature Edmunds et al. (1998)
hAT-III	Goat β-casein	5.8 mg/mL			Baguisi et al. (1999)
hGH	Retrovirus	60 ng/mL			Archer et al. (1994)
hGH	Adenovirus	0.3 mg/mL			Sanchez et al. (2004)
hα_1AT	Goat β-casein	14 mg/mL		GTC Biotherapeutics	Genzyme Transgenics (1996), GTC literature
hαFP	Goat β-casein		Phase II (2004)	GTC Biotherapeutics/Merrimack Pharmaceuticals	Parker et al. (2004), http://www.transgenics.com/products/novel.html, http://www.clinicaltrials.gov/ct/show/NCT00147329?order=1
hEPO	Adenovirus	2 mg/mL			Toledo et al. (2006)
hLF	Adenovirus	2.6 mg/mL			Han et al. (2007)

Table 7. Expression of Recombinant Human Proteins in the Milk of Transgenic Cattle

Protein	Promoter	Expression Level	Method	Status	Company	Reference
hα-LA		2.4 mg/mL				Krimpenfort et al. (1991)
hα-LA	Human α-LA	2.4 μg/mL?	Microinjection		PPL Therapeutics	PPL literature
hCOL				Preclinical	Pharming	
hFIB	Bovine α_{S1}-casein	3 mg/mL	Nuclear transfer	Preclinical	Pharming	
hGH	Bovine α_{S1}-casein	5 mg/mL	Nuclear transfer		Bio Sidus SA, Buenos Aires, Argentina	Salamone et al. (2006)
hLF	Bovine α_{S1}-casein		Microinjection		PPL Therapeutics	McKee et al. (1998)
hLF	Bovine α_{S1}-casein	2.8 mg/mL	Microinjection	Phase I completed	Pharming	van Berkel et al. (2002)
hSA				In development	GTC Biotherapeutics/TransOva Genetics	GTC literature

Human Recombinant Proteins Produced in Transgenic Livestock

Blood Clotting Factors (hFVII, hFVIII, hFIX)

The blood clotting or coagulation factors are key players in blood coagulation. They are generally serine proteases, with some exceptions; for example, FVIII and FV are glycoproteins and factor XIII is a transglutaminase. Serine proteases act by cleaving other proteins at specific sites. Both plasma-derived and recombinant products are currently used in treating hemophilia. Clotting factors have been used for many years in medicine; initially isolated from human plasma, transgenic animals now provide a promising alternative to these limited recourses. Several blood clotting factors are currently produced in transgenic livestock.

Factor VIIa (also known as proconvertin, serum prothrombin conversion accelerator, or cothromboplastin) is an extrinsic endopeptidase with Gla residues that activates factors IX and X in the blood coagulation cascade. GTC Biotherapeutics, in collaboration with LFB Biotechnologies (http://www.lfb.fr), has developed a transgenically produced recombinant form of human factor VIIa (rhFVIIa) expressed in the milk of transgenic rabbits.

Factor VIII (also known as antihemophilic factor A or antihemophilic globulin) is an intrinsic protein co-factor of factor IX, with which it forms the tenase complex (the tenase complex is formed on a phospholipid surface in the presence of calcium and is responsible for the activation of factor X). FVIII is synthesized predominantly in hepatocytes as a single-chain macromolecule. Congenital X-linked deficiency of hFVIII (hemophilia A) is the most common human bleeding disorder and affects approximately 1 of every 5,000 males. Recombinant hFVIII is currently produced in cell culture system for replacement therapy of hemophilia A patients. The restrictive costs associated with cell culture–produced rFVIII have provided the incentive to develop an alternative production system. At first, transgenic pigs using the regulatory sequences of the mouse whey acidic protein gene and the human FVIII cDNA were created (Paleyanda et al., 1997), with the identity of processed heterodimeric rFVIII confirmed using specific antibodies, by thrombin digestion, and by activity assays. The secretion of 2.7 μg/mL of rFVIII in milk was detected. With an hFVIII cDNA/murine metallothionein I hybrid gene construct containing the ovine β-lactoglobulin promoter, transgenic sheep have been created by microinjection that produce up to 6 ng/mL of hFVIII in their milk (Niemann et al., 1999). The same hFVIII cDNA/murine metallothionein I hybrid gene coupled with the murine whey acidic protein promoter gave low expression levels in transgenic rabbits (Hiripi et al., 2003).

Factor IX (also known as Christmas factor, antihemophilic factor B, or plasma thromboplastin component) is an intrinsic endopeptidase. Through forming the tenase complex with factor VIII, it activates factor X. Its deficiency results in hemophilia B, which can be treated with FIX. Using the ovine-β-

lactoglibulin promoter, several groups have created transgenic sheep expressing hFIX in their mammary gland (Schnieke et al., 1997; Clark et al., 1989; Simons et al., 1988). Usually, expression levels were in the ng/mL range.

Protein C (hPC)

Human protein C (hPC) is a regulator of hemostasis, a zymogen of a serine protease that is activated by thrombin. hPC has a complex structure, containing nine γ-carboxylated glutamic acid residues that bind calcium at about 1 to 3 mM. Gamma-carboxylation is a vitamin K–dependent posttranslational modification. Transgenic pigs were generated that produced human protein C in their milk at up to 1 g/L. The gene construct consisted of the cDNA for human protein C inserted into the first exon of the mouse whey acidic protein gene (Velander et al., 1992). A monoclonal antibody that binds an epitope in the glutamic acid domain of hPC in the absence of calcium was used to study the conformational behavior of immunopurified rhPC. Immunopurified rhPC from higher-expressing pigs gave a less calcium-dependent response, suggesting that a rate limitation in γ-carboxylation by the mammary gland occurs at expression levels about >500 μg/mL in pigs (Subramanian et al., 1996). These studies provide evidence that γ-carboxylation can occur at high levels in the mammary gland of a pig.

The effects of rhPC expression levels on endogenous immunoglobulin and transferrin content of the milk of different lineages of transgenic pigs were studied. The levels of rhPC in the milk ranged from 40 to 1,200 μg/mL. Transgenic pigs with rhPC expression levels lower than 500 μg/mL had no significant differences in milk protein composition with respect to nontransgenic pigs. A line of transgenic pigs having rhPC expression levels of 960–1,200 μg/mL had two- to threefold higher IgG, IgM, and secretory IgA concentrations compared to other transgenic and nontransgenic pig groups. Since IgG, IgM, secretory IgA, and transferrin are transported into the milk by transcytosis, higher levels of these proteins indicate that transcyctosis in the mammary epithelial cell was likely upregulated in pigs having high rhPC expression levels (Van Cott et al., 2001).

Growth Hormone (hGH)

Human growth hormone (hGH) is not only a valuable recombinant therapeutic protein for hormone deficiency indications, but also is an extensively characterized molecule both from recombinant bacterial systems and as circulating in human blood. Treatment of growth hormone (GH) deficiency via parenteral administration of recombinant hGH has greatly benefited from recombinant DNA technology allowing the production of practically unlimited amounts of the pure hormone. An unwanted side effect of using recombinant human growth hormone, in combination with other products (e.g., androgens, erythropoietin), is for doping in sports. Although its effectiveness in enhancing

physical performance is still unproved, the compound is likely used for its potential anabolic effect on muscle growth.

There have been several attempts in the last 15 years to produce recombinant hGH in the milk of transgenic livestock animals as an alternative to production in recombinant *Escherichia coli*. One of the early models was a direct transfer of the *hGH* gene into the mammary gland by using replication-defective retrovirus vector in goats (Archer et al., 1994). Since then transgenic rabbits and transgenic cows producing high levels of rhGH in milk have been generated (Limonta et al., 1995; Salamone et al., 2006; Lipinski et al., 2003). Transgenic rabbits were created through microinjection with a chimeric gene comprising 5' sequences from mouse whey acidic protein gene linked to the *hGH* gene. The foreign protein was detected in the milk and serum of these animals at levels of up to 50 μg/mL and 0.6 ng/mL, respectively. In the milk of cloned transgenic cows, up to 5 g/L of rhGH were detected. The hormone is identical to that currently produced by expression in *Escherichia coli*. In addition, the hematological and somatometric parameters of the cloned transgenic cattle are within the normal range for the breed and display normal fertility, being capable of producing normal offspring. In the future, transgenic cattle could be used as a cost-effective alternative for the production of rhGH.

Serum Albumin (hSA)

Human serum albumin (hSA), a protein currently derived from pooled human plasma, is used therapeutically to maintain osmotic pressure in the blood. Approximately 440 metric tons of plasma-derived albumin are used annually worldwide, with annual sales of approximately US$1.5 billion; 5,400 cows would be needed to produce 100,000 kg of hSA (Rudolph, 1999). The use of the recombinant form, produced in *Saccharomyces cerevisiae*, is limited to excipient applications. Animal bioreactor approaches have been established, with hSA produced in the milk of transgenic cows by GTC Biotherapeutics and Genzyme Biotech (http://www.genzyme.com); GTC Biotherapeutics has formed a joint venture with Fresenius (http://www.fresenius.de) to expand the commercial development opportunities of recombinant hSA. The aim is to manage the development of hSA for both the blood expander market and the use of hSA in the excipient market.

Lactoferrin (hLF)

Human lactoferrin (hLF) is a natural protein that helps to fight and prevent infections and excessive inflammation and strengthens the human defense system. The protein is present in significant amounts in numerous human biological fluids and mucus secretions, including tears and lung secretions,

and has been shown to fight bacteria that cause infections of the eye and lungs. In addition, hLF is present in substantial quantities in mother's milk and plays an important role in the defense system of infants as well as that of adults. Lactoferrin promotes the health of the gastrointestinal system by improving the intestinal microbial balance. Since the protein has the ability to bind iron, it is a natural antibacterial, antifungal, and antiviral agent. It is also an antioxidant and has immunomodulatory properties; large groups of people might benefit from orally administered hLF.

Pharming (http://www.pharming.com) has a patent on hLF from the Japanese Patent Office, which covers the production and purification of hLF with Pharming's technology as well as its use in sports and food formulations. In Japan, bovine lactoferrin is currently used as an additive in food products and as a nutritional supplement. Pharming is producing human lactoferrin for use as pharmaceuticals (for infection and inflammatory diseases) and as nutraceuticals, using the bovine α_{S1}-casein promoter to direct expression into the milk of transgenic cows; a method that fits functional food development very well as cow's milk is a common food source worldwide. Comparing the recombinant protein with its native counterpart, a slight difference in the molecular weights was identified due to differences in N-linked glycosylation (van Berkel et al., 2002). Natural hLF contains only complex-type glycans, while in recombinant hLF, oligomannose- and/or hybrid-type glycans were also found. The substitution of some of the galactose with N-acetylgalactosamine has also been observed in other transgenic systems, e.g., hAT-III produced in goat's milk (Edmunds et al., 1998). Importantly, the two most important functional activities of hLF, namely iron binding and release and antibacterial activity, were not influenced by these differences. Pharming has filed a GRAS (Generally Recognized As Safe) notification for its recombinant hLF in the United States and has completed clinical trials phase I.

Human lactoferrin has also been produced in goats. Directly transfecting the lactating mammary glands with a replication-defective adenovirus vector containing the human lactoferrin cDNA resulted in high-level expression of up to 2.6 mg/mL of the recombinant protein (Han et al., 2007).

Alpha-1-Antitrypsin ($h\alpha_1AT$)

Alpha-1-antitrypsin ($h\alpha_1AT$), also known as α-1-proteinase inhibitor, is an enzyme produced by the liver and released into the bloodstream. One of the primary roles of $h\alpha_1AT$ is to protect the lungs from neutrophil elastase, an enzyme released by white blood cells. Neutrophil elastase can attack healthy lung tissue if not controlled by $h\alpha_1AT$.

Proteinase-antiproteinase imbalances are recognized in several diseases, including the two most common lethal hereditary disorders of white populations, $h\alpha_1AT$ deficiency and cystic fibrosis (CF). In $h\alpha_1AT$ deficiency, the type

Z variant of hα_1AT forms polymers in the endoplasmic reticulum of hepatocytes, resulting in childhood liver disease. In CF, chronic bacterial lung infections due to impaired mucociliary clearance lead to a vigorous influx of neutrophils in the airways. Serine proteinases released from the neutrophils, particularly elastase, exceed the antiproteinase capacity of endogenous serine proteinase inhibitors in the airways. Strategies to augment the antiproteinase defenses in the airways of patients with severe hα_1AT deficiency or CF include the intravenous or aerosol administration of serine proteinase inhibitors. Studies in both patient groups using plasma-derived or transgenic recombinant secretory leukoprotease inhibitors or synthetic elastase inhibitors show promising results concerning drug safety and efficacy.

Wright et al. (1991) reported the generation of five sheep transgenic for a fusion of the ovine β-lactoglobulin gene promoter to the hα_1AT genomic sequence as one of the earliest successes in using transgenic farm animals as bioreactors. Analysis of the expression of hα_1AT in the milk of three of these females showed that all expressed the human protein at levels greater than 1 g/L. hα_1AT purified from the milk of these animals appeared to be fully N-glycosylated and had a biological activity indistinguishable from human plasma-derived material.

Transgenic hα_1AT has gone through phase II clinical trials in the cystic fibrosis patient, delivering it in aerosol form to assess its safety and efficacy. Trends toward a reduction in neutrophil elastase activity were observed in patients treated with 500 mg and 250 mg of recombinant hα_1AT compared to placebo. Although significant differences between recombinant hα_1AT and placebo for neutrophil elastase activity were not observed, some improvements were found for secondary efficacy variables. Results show that nebulized recombinant hα_1AT is safe and well tolerated but has a limited effect on neutrophil elastase activity and other markers of inflammation (Martin et al., 2006).

GTC Biotherapeutics also has established founder transgenic animals that express rhα_1AT. Using the goat β-casein promoter, they achieved a 4-mg/mL recombinant protein expression in rabbits, while that in goats was 14 mg/mL. GTC believes that rhα_1AT may also be developed as an effective treatment for other diseases, potentially including cystic fibrosis, chronic obstructive pulmonary disease, acute respiratory syndrome, and severe asthma.

Extracellular Superoxide Dismutase (hEC-SOD)

EC-SOD is the major SOD isoenzyme in plasma, lymph, and synovial fluids. Studies with SOD molecules have indicated a number of interesting therapeutic actions, including acute pancreatitis, cardiovascular disease, and renal transplantation. hEC-SOD has been produced at up to 3 mg/mL in rabbit's milk (Stromqvist et al., 1997). The milk-derived hEC-SOD was purified and

compared to the native and CHO cell-produced proteins. All proteins were glycosylated, tetrameric metalloproteins. Since each homotetramer contains one copper ion per monomer, production of hEC-SOD at a high level in milk means that the mammary gland will need to collect large amounts of copper, presumably from the blood.

Erythropoietin (hEPO)

Erythropoietin (hEPO) is a glycoprotein hormone that is a cytokine for erythrocyte precursors in the bone marrow. Also called hematopoietin or hemopoietin, hEPO is mainly produced by the adult kidney and circulates in blood plasma, a small portion is synthesized by the liver, and possibly by macrophages in the bone marrow, and it is the hormone that regulates red blood cell production. At present, hEPO is available as a therapeutic agent only through production by recombinant DNA technology in mammalian cell culture (Jacobs et al., 1985; Krantz, 1991; Kim et al., 2005). It is used in treating anemia resulting from chronic renal failure or from cancer chemotherapy. It is also effective as a blood doping agent that is believed to be common in endurance sports such as cycling, triathlons, and marathons.

Trangenic rabbit (Korhonen et al., 1997; Massoud et al., 1996), transgenic pig (Park et al., 2006), and nontransgenic goat (Toledo et al., 2006) animal systems have been developed for large-scale production of recombinant hEPO. In rabbits the rabbit WAP and bovine BLG promoters were used to direct expression into the mammary gland. High expression of biologically active hEPO into the mammary gland had adverse affects on the lactating female (Massoud et al., 1996); thus, only an expression level of 50 μg/mL could be achieved with the rabbit whey acidic protein promoter driving a genomic hEPO gene construct. Trying to compensate for unwanted side effects, a bovine β-lactoglobulin promoter driving a hEPO cDNA fusion protein with lower biological activity was expressed at a level of 500 μg/mL (Korhonen et al., 1997). The biological activity of the bovine β-lactoglobulin promoter linked to hEPO cDNA was less than 10–20% of that of the native hEPO due to different glycosylation. Upon digestion with IgA protease, the normal biological activity could be recovered (Korhonen et al., 1997). In spite of decreased biological activity, transgenic females expressing the fusion protein showed elevated hematocrit values (up to 80%) during lactation.

hEPO can be successfully produced in transgenic pigs (Park et al., 2006). hEPO-expressing pigs were created via microinjection and use of a mouse whey acidic protein-driven hEPO genomic construct. Expression level was up to 900 IU/mL (EPO levels in normal humans are between 10 and 30 IU/mL). The transgenic animals were generally healthy, except for a few examples of physiological problems (e.g., low sperm quality with erectile dysfunction in some males, and elevated reticulocyte counts and hematocrit levels in both sexes).

A high level (2 mg/mL) of hEPO expression in the milk of nontransgenic goats has been achieved by direct transduction of the lactating mammary gland without causing any harm to the animals (Toledo et al., 2006). A replication-defective adenovirus vector containing the hEPO cDNA was used. The recombinant hEPO had low *in vivo* hematopoietic activity due to underglycosylation.

Tissue Plasminogen Activator (htPA)

Tissue plasminogen activator (htPA) is a serine protease that converts plasminogen to plasmin and can trigger the degradation of extracellular matrix proteins. The glycosylation variant of htPA designated longer-acting tissue-type plasminogen activator (LAtPA) has been produced in the milk of transgenic goats and rabbits (Ebert et al., 1991, 1994). Recombinant htPA was extensively purified from the milk of a transgenic goat by a combination of acid fractionation, hydrophobic interaction chromatography, and immunoaffinity chromatography. Although the early availability of this product had been predicted, recent indications suggest that predicted recombinant htPA, produced by GTC Biotherapeutics for the treatment of coronary clots, is not close to market.

Tissue-Nonspecific Alkaline Phosphatase (hTNAP)

Alkaline phosphatase is a promising therapeutic agent in the Gram-negative bacterial lipopolysaccharide-mediated acute and chronic diseases. Contrary to other alkaline phosphatase isozymes, purified tissue-nonspecific alkaline phosphatase (hTNAP) is not available in large quantities from tissue sources that would enable us to analyze its efficacy in animal sepsis models. Two transgenic rabbit lines were created by pronuclear microinjection with the whey acidic protein promoter-hTNAP minigene (Bodrogi et al., 2006). Alkaline phosphatase enzymatic activity was two orders of magnitude higher compared to normal human serum levels. As indicated by fractionation of milk samples, the recombinant hTNAP was associated with the membrane of milk fat globules.

The production of cystic fibrosis transmembrane conductance regulator in the milk fat globules of transgenic mice was the first report on the expression of a membrane-bound protein, but its biological activity was not examined (DiTullio et al., 1992). Therefore, the milk of transgenic rabbits could be a source of membrane receptors to define their structure after crystallization. This approach may be essential to define synthetic molecules acting on the receptors.

Acid α-Glucosidase (hαGLU)

The clinical spectrum of glycogen storage disease type II/Pompe disease comprises infants, children, and adults. All patients characteristically have acid α-glycosidase deficiency and suffer from progressive skeletal muscle weakness. Affected infants die of cardiorespiratory failure within the first two years of life. Cell culture and transgenic animal technology were explored to produce recombinant human acid α-glucosidase (hαGLU) on a large scale (Van Hove et al., 1996). Transgenic rabbits expressing the human acid α-glucosidase gene under the bovine αs1-casein promoter were constructed, resulting in a selected transgenic line producing up to 8 g/L of recombinant protein in milk. The therapeutic efficacy of the product purified from rabbit milk has been demonstrated in clinical trials. The enzyme is transported to lysosomes and lowers the glycogen concentration in the tissues. Phase II clinical trials in patients with classical infantile Pompe disease revealed an overall improvement in cardiac function, skeletal muscle function, and histological appearance of skeletal muscle (Klinge et al., 2005). Long-term intravenous treatment with recombinant hαGLU from milk encourages enzyme replacement therapy for several forms of Pompe disease and underlines that safe and effective medicine can be produced in the milk of mammals (Van den Hout et al., 2004).

Fibrinogen (hFIB)

Fibrinogen (hFIB) is a soluble plasma glycoprotein synthesized by the liver and a key component in blood clotting. In its natural form, fibrinogen is useful in forming bridges between platelets, by binding to their GpIIb/IIIa surface membrane proteins, though the major use of hFIB is as a precursor to fibrin. Processes in the coagulation cascade activate the zymogen prothrombin, producing the serine protease thrombin, which is responsible for converting hFIB into fibrin. Fibrin is then cross-linked by factor XIII to form a clot that serves as an *in vivo* hemostatic plug that prevents further blood loss. hFIB is a hexamer containing two sets of three different chains (α, β, and γ), linked to each other by disulfide bonds. The N-terminal sections of these three chains are evolutionarily related and contain the cysteines that participate in the cross-linking of the chains. Because of this complexity to the protein, expression in bacterial or yeast expression systems has not been feasible. Expression in mammalian cell culture systems has been demonstrated, but this approach is likely to be too expensive for the production of the large amounts of hFIB needed. Since the mammary gland appears to be able to secrete fully assembled recombinant hFIB, the only way to obtain sufficiently large amounts of human fibrinogen safely and cost-effectively is the transgenic production in the milk of large animals. Transgenic livestock—sheep and cattle—have been created for this purpose.

Transgenic sheep were produced (Garner & Colman, 1998; Butler et al., 1997) using the ovine β-lactoglobulin promoter to direct transgene expression to the mammary gland with hFIB expression levels up to 5 mg/mL, while Pharming has used the bovine α_{S1}-casein promoter and nuclear transfer technology to create transgenic cattle producing hFIB at a concentration of 3 mg/mL.

α-Fetoprotein (hαFP)

Alpha-fetoprotein (hαFP) is a serum glycoprotein expressed at high concentrations in the fetal liver, but its concentration drops dramatically after birth. Potential indications for the use of recombinant hαFP include autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, myasthenia gravis (a chronic autoimmune neuromuscular disease), and psoriasis. Since hαFP is produced normally during pregnancy, it is not commercially available from fractionation of the human blood supply. Using the goat β-casein promoter to direct transgene expression, GTC Biotherapeutics has developed transgenic goats that express hαFP in their milk. Since neither glycosylation of hαFP nor any bound ligands are necessary for activity (Semeniuk et al., 1995), to avoid unwanted glycosylation patterns, the single N-linked glycosylation site of the protein was removed (by mutagenesis) from the transgene construct. Characterization of the hαFP in the milk of transgenic goats shows that the structure was indeed not affected by removal of the glycosylation site. Furthermore, the cell binding and pharmacokinetic properties of the recombinant protein were identical to the native protein (Parker et al., 2004). Through cooperation between GTC Biotherapeutics and Merrimack Pharmaceuticals (http://www.merrimackpharma.com), recombinant hαFP purified from goat's milk entered phase II clinical trials in 2004.

Interleukin-2 (hIL-2)

Interleukin-2 (hIL-2), formerly referred to as T cell growth factor, is an immunoregulatory lymphokine that is produced by lectin- or antigen-activated T cells. It is produced not only by mature T lymphocytes on stimulation but also constitutively by certain T cell lymphoma cell lines. It is useful in the study of the molecular nature of T cell differentiation and, like interferons, augments natural killer cell activity. hIL-2 can act as a growth hormone for both B and T lymphocytes. Since hIL-2 and interleukin-2 receptor act as required for the proliferation of T cells, defects in either the ligand or the receptor would be expected to cause severe combined immunodeficiency.

At present, a recombinant form of hIL-2 is manufactured by the Chiron Corporation with the brand name Proleukin (http://www.proleukin.com). It is

produced by recombinant DNA technology using genetically engineered *Escherichia coli* containing a modified hIL-2 gene. Transgenic technology has also been used to produce hIL-2. Microinjection and a gene construct containing the rabbit β-casein promoter and the hIL-2 genomic sequence were used to create transgenic rabbits expressing hIL-2 in their milk (Bühler et al., 1990). The recombinant protein, produced at a concentration of up to 430 ng/mL, was stable and biologically active. But to be able to compete with the present method to produce hIL-2, the transgene constructs need to be significantly improved to direct protein production at considerably higher levels.

Insulin-Like Growth Factor-1 (hIGF-1)

The insulin-like growth factors (hIGFs) are polypeptides with high sequence similarity to insulin. They are part of a complex system that cells use to communicate with their physiological environment. This complex system (often referred to as the IGF "axis" or the growth hormone/IGF-1 axis) consists of two cell-surface receptors (IGF-1R and IGF-2R), two ligands (IGF-1 or somatomedin C and IGF-2 or somatomedin A), a family of six high-affinity IGF binding proteins (IGFBP 1-6), as well as associated IGFBP degrading enzymes. hIGF-1 is mainly secreted by the liver as a result of stimulation by hGH. It is important for both the regulation of normal physiology as well as a number of pathological states, including cancer.

Commercially available hIGF-1 has been manufactured recombinantly on a large scale using both yeast and *Escherichia coli*. Transgenic rabbits were also created to produce hIGF-1 (Brem et al., 1994). Brem and co-workers used microinjection to integrate a bovine α_{S1}-casein promoter driving the hIGF-1 cDNA construct into the genome of rabbits. The amount of recombinant protein in the milk of the transgenic rabbits was up to 1 mg/mL. Since the recombinant protein was associated with the casein micelles, purification included extraction with urea and dithioerythritol, gel filtration, and chromatographic enrichment. The recombinant protein was correctly processed and biologically active (Wolf et al., 1997). The local production of hIGF-1 in mammary tissue was found to be associated with increased secretion of IGFBP-2, which may prevent major biological effects by high levels of hIGF-1 on the mammary gland (Zinovieva et al., 1998).

Several companies have evaluated hIGF-1 in clinical trials for several indications, including growth failure, type 1 diabetes, type 2 diabetes, amyotrophic lateral sclerosis (Lou Gehrig's disease), severe burn injury, and myotonic muscular dystrophy. In August 2005, the FDA approved Tercica's (http://www.tercica.com) hIGF-1 drug, Increlex, as replacement therapy for severe primary IGF-1 deficiency. In December 2005, the FDA also approved IPLEX, Insmed's (http://www.insmed.com) IGF-1/IGFBP-3 complex. In the human body, 97 to 99% of hIGF-1 is always bound to one of six hIGF binding proteins, with

IGFBP-3 the most abundant binding protein, accounting for approximately 80% of all hIGF binding. Delivering the drug in a complex achieved the same efficacy as far as growth rates but with fewer side effects and less severe hypoglycemia. The drug is injected once a day versus Tercica's twice-a-day version.

Antithrombin III (hAT-III)

Antithrombin (hAT-III) is a plasma protein with anticoagulant and anti-inflammatory properties. It regulates thrombin, a blood protein that plays a key role in controlling clot formation. Patients with hereditary hAT-III deficiency can have either Type I or Type II deficiency. Type I is a quantitative deficiency characterized by low levels of hAT-III. Type II is a qualitative deficiency characterized by the presence of hAT-III variants that do not function properly. Individuals with Hereditary Antithrombin Deficiency are at risk for blood clots, organ damage, or even death. An acquired form of the disease is also known. It causes disseminated intravascular coagulation, a widespread formation of clots within blood vessels, which is most severe when it occurs in association with sepsis.

GTC Biotherapeutic's lead product, ATryn®, is a recombinant form of hAT-III. hAT-III is produced in the milk of transgenic goats. A goat β-casein promoter-driven hAT-III cDNA transgene was microinjected to create the transgenic animals, resulting in an expression of the transgene as high as 20 mg/mL. The specific activity of the recombinant hAT-III was found to be identical to human plasma-derived AT-III; however, its affinity for heparin was fourfold higher than plasma hAT-III. The recombinant protein was structurally identical to phAT-III except for differences in glycosylation. Oligomannose structures and some GalNAc for galactose substitutions were observed, along with a higher degree of fucosylation and lower degree of sialylation. It was concluded that the increase in affinity of the recombinant protein resulted from the presence of oligomannose-type structures on the Asn155 glycosylation site and differences in sialylation (Edmunds et al., 1998).

Transgenic goats to produce hAT-III have also been created via fetal somatic cell nuclear transfer (Baguisi et al., 1999). Somatic cell lines were generated from 35-day- to 40-day-old fetuses resulting from the mating of hAT-III–expressing transgenic goats (goat β-casein promoter-driven hAT-III cDNA). Analysis of the milk of the transgenic cloned animals showed high-level production of hAT-III (up to 5.8 mg/mL with an activity of 20.5 IU/mL), which was similar to the parental transgenic line.

In 2006, ATryn® was approved for human therapeutic application in the EU. It has completed phase III in the United States and is at the state of prelaunch indicated for Hereditary Antithrombin Deficiency; for other

indications (disseminated intravascular coagulation in sepsis), it is in phase II of clinical trials.

C1 Inhibitor (hC1INH)

C1-inhibitor is a serine protease inhibitor (serpin) protein, the main function of which is inhibition of the complement system. It circulates in blood at levels around 0.25–0.45 g/L. Human C1 inhibitor (hC1INH) is used for the treatment of hereditary angioedema (HAE). In the Western world, approximately 1 in 30,000 persons, or some 22,000 people, suffers from HAE, a life-threatening genetic disorder. The shortage of hC1INH results in recurrent attacks of edema, causing painful swelling in the body's soft tissues. The disease seriously affects the quality of life of patients and can even be lethal if attacks in the throat area lead to asphyxiation.

Pharming has developed a method for the easy, quick, and clean production of hC1INH in large quantities, highly suitable for pharmaceutical applications and treatment of HAE. Its recombinant hC1INH is purified from the milk of transgenic rabbits. The DNA construct used contains the bovine α_{S1}-casein promoter sequence functionally linked to the gene encoding hC1INH and directs the expression at levels of 12 mg/mL. The glycosylation pattern of the recombinant protein is essentially similar to the native protein, only the degree of sialylation and core fucosylation was lower (Koles et al., 2004a, b). Pharming is nearing the end of the development program, practically all safety tests in laboratory animals have been finalized, and the product is now in phase III of clinical testing in humans.

Nerve Growth Factor Beta (hNGF-β)

Nerve growth factor beta (hNGF-β), the founder member of the protein family termed neurotrophins, is a protein secreted by a neuron's target. hNGF-β is critical for the survival and maintenance of primary sensory neurons, sympathetic neurons, and cholinergic neurons of the basal forebrain. When hNGF-β is released from the target cells, it binds to and activates its high-affinity receptor (TrkA) and is internalized into the responsive neuron. The hNGF-β/TrkA complex is subsequently trafficked back to the cell body. This movement of hNGF-β from axon tip to soma is thought to be involved in the long-distance signaling of neurons. Secreted pro-hNGF-β has been demonstrated in a variety of neuronal and nonneuronal cell populations. It has been proposed that secreted pro hNGF-β can elicit neuron death in a variety of neurodegenerative conditions, including Alzheimer's disease, following the observation of an increase of pro-hNGF-β in the nucleus basalis of postmortem Alzheimer's brains. Recombinant hNGF-β may be used to treat neuronal dysfunction of

the central and peripheral nervous system as well as HIV-related peripheral neuropathy (clinical trials are in progress).

In the past 15 years, mass production of hNGF-β has been carried out by mammalian cell culture systems (now commercially available), and although great progress could be made to increase the yield, transgenic technology offers a more viable solution. Recombinant hNGF-β has been produced in transgenic rabbits (Coulibaly et al., 2002). Using a construct containing the pre-pro-hNGF-β cDNA under the control of bovine α_{S1}-casein promoter, an expression of up to 250 μg/mL of the recombinant protein was achieved. hNGF-β could be purified from the milk by a two-step chromatographic procedure. Biological activity of the purified protein and also of crude defatted milk from transgenic animals demonstrated full biological activity when compared to commercial recombinant hNGF-β.

Collagen Type I (hCOL)

Recombinant human collagen type I (hCOL) is being developed by Pharming and its partner Cohesion Technologies (http://www.cohesiontech.com) for the biomaterials market. hCOL accounts for 85% of the total collagen in humans and is found in almost all collagen-based products on the market. Collagen is the main protein in connective tissue in animals and the most abundant protein in mammals (about 25% of the total protein content). Collagen is partly responsible for skin strength and elasticity, it strengthens blood vessels, and during aging its degradation leads to wrinkles. In crystalline form it is also present in the cornea and lens of the eye. Collagen is a commonly used biomaterial in the medical and pharmaceutical industries based on its structural role and compatibility within the human body. Applications include hemostats, vascular sealants, tissue sealants, implant coatings (orthopedic and vascular), artificial skin, bone graft substitutes, "injectables" for incontinence treatment, dental implants, and (antibiotic) wound dressings. Many additional applications are currently under development, such as engineering of cartilage, bone, skin, artificial tendons, blood vessels, nerve regeneration, and several drug delivery applications.

Pharming has successfully produced recombinant hCOL at high expression levels in transgenic cattle. The recombinant hCOL is indicated as an intermediate for medical devices and aesthetic products. The purified protein is now in the preclinical phase of trials.

Conclusions

Less than 25 years ago the first transgenic livestock animals with altered milk composition were born. Since then a number of valuable animal models have been created and characterized, with improved transgene constructs. The methods of creating transgenic livestock species have also been developed and have become more efficient, with fewer side effects. Some of the recombinant

proteins are at advanced stages of clinical trials, and ATryn®, the flagship of pharmaceutical proteins produced in the milk of transgenic animals, was approved in 2006 by the EMEA for human therapeutic application. Notwithstanding issues of public acceptance and authorization hurdles that are specific to pharmaceutical proteins produced by genetically modified mammals, the scientific developments achieved so far make animal bioreactor production systems a viable option. This is especially so when considering large-scale production of human proteins that require complex posttranslational modifications.

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