*Review*

# Transgenic mammalian species, generated by somatic cell cloning, in biomedicine, biopharmaceutical industry and human nutrition/dietetics – recent achievements

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## Abstract

Somatic cell cloning technology in mammals promotes the multiplication of productively-valuable genetically engineered individuals, and consequently allows also for standardization of transgenic farm animal-derived products, which, in the context of market requirements, will have growing significance. Gene farming is one of the most promising areas in modern biotechnology. The use of live bioreactors for the expression of human genes in the lactating mammary gland of transgenic animals seems to be the most cost-effective method for the production/processing of valuable recombinant therapeutic proteins. Among the transgenic farm livestock species used so far, cattle, goats, sheep, pigs and rabbits are useful candidates for the expression of tens to hundreds of grams of genetically-engineered proteins or xenogeneic biopreparations in the milk. At the beginning of the new millennium, a revolution in the treatment of disease is taking shape due to the emergence of new therapies based on recombinant human proteins. The ever-growing demand for such pharmaceutical or nutriceutical proteins is an important driving force for the development of safe and large-scale production platforms. The aim of this paper is to present an overall survey of the state of the art in investigations which provide the current knowledge for deciphering the possibilities of practical application of the transgenic mammalian species generated by somatic cell cloning in biomedicine, the biopharmaceutical industry, human nutrition/dietetics and agriculture.

**Key words**: somatic cell nuclear transfer, transgenesis, animal bioreactor, mammary gland, human therapeutic protein, medical pharmacology

## Introduction

Effective mammalian somatic cell cloning, avoiding the sexual reproduction pathway, creates a possibility of not only providing numerous monogenetic offspring derived from existing transgenic individuals, but also generating monosexual genetically-transformed adult (post-pubertal) animals of high genetic merit with the use of *in vitro*-transfected nuclear donor cells. It is commonly believed that somatic cell

cloning could accelerate the rate of genetic progress, producing in a short time many identical animals with the most desirable, accurately defined genotypes. Somatic cell nuclear transfer (SCNT), in spite of many spectacular achievements, brings more and more questions, which remain as yet unanswered. It is beyond any doubt that the technical possibilities which enabled the production of cloned transgenic animals exceeded the understanding of the associated biological conditions, in particular the molecular and epigenetic aspects of the technology. While tremendous progress in the field of SCNT has been achieved during the past almost one and a half decades with the birth of numerous genetically-modified offspring of different mammal species worldwide, the overall efficiency remains low. The current high incidence of pre- and/or post-implantation embryonic, fetal as well as perinatal abnormalities limits the practical applications of somatic cell cloning and contributes to the negative perception of this assisted reproductive technology (ART) to society. The aims are to understand the mechanisms involved in donor cell nuclear reprogramming, which can lead to pathologic syndromes. At present the work involved in solving the specific problems of SCNT is challenging. The majority of these problems, however, are likely to be solved in the near future. This will make cloning technology safe, i.e., cases of individuals born with developmental abnormalities, as a result of its use, will not be more frequent than in natural reproduction (Samiec 2005a,b, Samiec and Skrzyszowska 2005).

The propagation of mammalian individuals by SCNT has important economical implications in biotechnology and biomedicine as so far has been shown by generation of cloned transgenic animals with the ability to produce valuable recombinant human proteins (Skrzyszowska et al. 2006, Kind and Schnieke 2008).

## Advantages of the use of somatic cell cloning in generating genetically-transformed animals

*In vitro* transfection of cultured differentiated cells combined with somatic cell nuclear transfer is currently the most effective procedure to produce transgenic mammals. Improvements in technologies to produce transgenic farm animals are highly desirable because the economic savings would benefit both biotechnology and basic research. The main barrier for transgenic animal production remains the identification of more efficient systems of transgene delivery and better mechanisms to optimize regulation of transgene expression levels. Although pronuclear microinjection has been used for more than two decades to produce genetically modified mice, rabbits, pigs, sheep, goats and cattle, variable transgene expression patterns and uncertain transmission through the germ line preclude widespread application of this technology (Chen et al.

2002a, Thomson et al. 2003, Niemann and Kues 2007). In turn, the successful production of cloned offspring originating from embryos reconstructed with transfected cell nuclei has important implications for various biomedical, agricultural and research purposes such as generation and/or multiplication of transgenic high genetic merit bioreactors providing human recombinant proteins (biopharmaceuticals) (Samiec et al. 2003, Wang and Zhou 2003, Skrzyszowska et al. 2006).

The generation of the first cloned transgenic sheep (Schnieke et al. 1997), exhibiting the expression of blood coagulation/clotting factor IX targeted at the mammary gland (udder), stimulated the interest of animal biotechnologists working with farm livestock species, since the potential of genetically-engineered individuals, especially those possessing high genetic merit and productive yield, as bioreactors for the synthesis and secretion or excretion of valuable biopharmaceuticals was immediately recognized. Relatively quickly, transgenic cloned sheep, cattle, goats, pigs as wells rabbits were produced. In contradistinction to the propagation of transgenic animals by zygote intrapronuclear injection which results, respectively, in only 0.5-3% and less than 10% of neonates carrying the transgene in relation to all the transferable microinjected embryos and all the offspring born, somatic cell cloning by nuclear transfer with the use of transfected and positively-selected transgenic donor cells provides a much more efficient system. *In vitro* cultured nuclear donor cells can be subjected, after transfection, to screening for transgene incorporation into the genomic DNA, gene construct copy number, and also for confirmation of chromosome integration sites prior to their use in the SCNT (Skrzyszowska et al. 2008). This selection of the transgenic donor cell ensures that nearly all the progeny produced will be transgenic. Moreover, the ability to transfect and select somatic cells prior to cloning procedure leads to generation of genetically-transformed specients of the desired gender and overcomes the problem of founder animals being mosaic. Somatic cell cloning can be used to propagate transgenic animals produced by standard DNA microinjection into the pronuclei of zygotes (Bondioli et al. 2001, Reggio et al. 2001, Lee et al. 2003, Ramsoondar et al. 2003).

## Genetically-engineered mammary gland as an animal bioreactor of recombinant human proteins or therapeutic proteins (biopharmaceuticals)

### The perspectives of somatic cell cloning and transgenesis of farm livestock species for biomedicine and pharmacy

Recent advances in the large scale production of human recombinant proteins have had a significant impact on the pharmaceutical industry. The mammary

Table 1. Expression of recombinant human proteins in the mammary gland of transgenic cloned livestock species.

| Animal species | Gene construct/ Method of nuclear donor somatic cell transfection | Therapeutic human protein | Translational activity rate/Level of secretion to milk[1] | Reference |
|---|---|---|---|---|
| Cattle | **Tg(pBSSLIII)** **Co-transfection** with backbone-free PGKneo transgene **Cationic lipid/liposome-mediated transfection**, advanced conjugation enhancement (ACE), using **GenePOR-TER 3000** (GP3K) transfection reagent (Gene Therapy Systems; Genlantis) | **Bile salt-stimulated lipase (BSSL)** | Unknown/not determined | Chen et al., 2002b |
| Cattle | **Tg(CSN2)** **Tg(CSN3)/Tg(CSN2/3)** **Co-transfection (unknown transgene carrier)** | **β-casein (β-CN)** **κ-casein (κ-CN)** | 15.8-20.9 mg/ml 8.4-14.1 mg/ml | Brophy et al., 2003 |
| Sheep | **Tg(pMIX1)** **Co-transfection** with PGKneo transgene **Lipofection** using **Lipofectamine 2000** (LF2K) (Gibco BRL; Invitrogen) | **Blood coagulation/ clotting factor IX (FIX)** | Unknown/not determined | Schnieke et al., 1997 |
| Sheep | **Tg(AATC2)** transgene *COLT-2* targeting vector Targeted *COL1A1 locus* **Lipofection** using **Lipofectamine 2000** (LF2K) (Gibco BRL; Invitrogen) | **α1-antitrypsin (AAT)** | 650 μg/ml | McCreath et al., 2000 |
| Goats | **Fibroblast cells derived from transgenic fetuses expressing caprine** β-*casein-hAT cDNA fusion gene* | **Antithrombin III-α (AT/AT III-α) – Atryn®** | 3.7-5.8 g/l | Baguisi et al., 1999 |
| Rabbits | **Fibroblast cells derived from ear dermal tissue of transgenic rabbit doe expressing Tg(Wap-GH1) fusion gene** | **Growth hormone/ somatotropin (GH)** | Unknown/not determined | Skrzyszowska et al., 2006 |
| Pigs | **Fibroblast cells derived from ear dermal tissue of transgenic sow expressing two transgenes: Tg(αLA-pLF)** and **Tg(αLA-hFIX)** | **Porcine lactoferrin (pLF) Human blood coagulation/ clotting factor IX (hFIX)** | Unknown/not determined | Lee et al., 2003 |

[1] Measured with concentration of the recombinant human protein in milk (quantity/content per volume unit).

gland (udder) of cloned species of both small ruminants (sheep, goats) and large ruminants (cattle) or lactiferous glands of cloned pigs are well suited for the targeted expression and secretion of xenogeneic therapeutic proteins. Their synthesis in the secretory epithelial cells of udder lactogenic vesicles can be induced through transfection of nuclear donor somatic cells with structure transgenes under the control of promoters, including the regulatory/intron sequences of either genes encoding protein variants/isoforms of casein ($\alpha_{S1}$-, $\alpha_{S2}$-, β- and κ-casein) or the genes encoding milk whey proteins (i.e., β-lactoglobulin, α-lactalbumin, whey acidic protein/WAP). Such biopharmaceuticals as human recombinant α-1-antitrypsin (McCreath et al. 2000), antithrombin III-α (Baguisi et al. 1999), human coagulation/clotting factors VIII and

IX (Schnieke et al. 1997, Chen et al. 2002a, Lee et al. 2003, Van Cott et al. 2004, Gil et al. 2008) and erythropoietin (Cheng et al. 2002) have so far been excreted in the milk of transgenic cloned sheep, goats and pigs (Table 1). The transgenic approach can also provide a practical means of humanizing the milk of cloned ruminants via onset of expression in their mammary glands of foreign (xenogeneic) genes encoding the bionutriceuticals/bionutraceuticals, which include different recombinant human milk proteins. The modification of milk protein composition through random co-integration of additional copies of genetically-engineered bovine β- and κ-casein genes into genomic DNA caused the cow's milk casein content and its physicochemical properties, and thereby its dietary as well as nutritional

value, to be similar to those of human milk (Brophy et al. 2003).

Alfa-1-antitrypsin ($\alpha_1$-AT) is the protease inhibitor, which is a member of the serpin family. Its physiological substrate is the neutrophilic elastin. Inhibiting the enzymatic activity of the elastase and accelerating its half-life period, $\alpha_1$-AT thereby prevents acute or chronic tissue damage by this protease. The gene mutation-related lack of $\alpha_1$-AT is the cause of congenital (inherent) defect variant of emphysema in humans. Before the therapeutic $\alpha_1$-AT protein can be utilized in the biopharmaceutical industry, it has to be subjected first of all to precise quantitative and/or qualitative analyses for the frequency of posttranslational modifications (particularly glycosylation) in the secretory cells of lactogenic vesicles. Subsequently, the transgenically-produced $\alpha_1$-AT has to undergo preliminary pharmacokinetic tests and predictable characteristics of biocatalytic and pharmacological activities, and finally a series of preclinical trials (Jang et al. 2006). Not until then can such purified pharmaceuticals of recombinant human $\alpha_1$-AT synthesized by udder bioreactors of genetically-engineered cloned ruminants be used in clinical programs involving substitutional therapy and multipreventive prophylaxis not only for different variants of emphysema, but also for mucoviscidosis (cystic fibrosis), another hereditary mono-gene disease of the respiratory epithelium in pulmonary alveoli and bronchi (McCreath et al. 2000, Boaglio et al. 2006). In turn, recombinant human antithrombin III-$\alpha$, which has been extracted from the milk or beestings of transgenic SCNT-derived livestock species, could be applied as a blood anticoagulation agent (inhibitor of thrombin activity) in cardiologic surgery (Baguisi et al. 1999, Zhou et al. 2005).

Genetically-modified pharmaceuticals including blood clotting/coagulation factor VIII (i.e., antihemophilic globulin/factor A – AHG or plasma prothrombokinase) and blood clotting/coagulation factor IX (i.e., Christmas factor, antihemophilic globulin/factor B or plasma thromboplastin factor – PTF), which have been provided with the milk/colostrum suspension of cloned goats, sheep, cattle, pigs or rabbits, could be utilized in the substitutional therapy and prophylactic treatments of haemophilia types A and B, respectively (Paleyanda et al. 1997, Schnieke et al. 1997, Pipe 2005, Yan et al. 2006, Chrenek et al. 2007).

The function of erythropoietin, which is the polypeptide growth factor synthesized by renal endocrine cells, is the stimulation of proliferative/replicative activity, induction of maturation, and differentiation of haematopoietic stem cells. The direct regulatory action of this tissue hormone is to initiate and enhance the generation of red blood corpuscles in the erythropoiesis process of the haematopoietic pathway (Mikus et al. 2004). Therefore, recombinant human

erythropoietin (rhEPO), which has been isolated from the milk or beestings of transgenic cloned small or large ruminants, could provide a practical means of clinical intervention involving temporary or permanent and complete overcoming of anaemia triggered by congenital or acquired kidney failure. Furthermore, rhEPO together with vascular endothelial growth factor (VEGF) could be suitable for hormonal induction of assisted neovascularization/angiogenesis processes in both injured tissues and angioplasty (Aguirre et al. 1998, Cheng et al. 2002).

The only hormone preparation applied in human dwarfism treatment and recognized by the World Health Organization (WHO) is the growth hormone (somatotropin), isolated from bacteria. This hormone preparation has excellent biological characteristics, but is relatively expensive (Lee et al. 1996, Nagasawa et al. 1996). Although it is entirely a functional product, it is different from the natural one, since proteins in prokaryotic cells do not undergo glycosylation. Because the efficacy of a human protein is generally dependent on both its amino acid composition as well as various post-translational modifications, many recombinant human proteins can only be obtained in a biologically active conformation when produced in mammalian cells. Apparently, post-translational modifications precise enough to form functional protein as it normally occurs in the human organism can take place only in blood cells and also mammary gland-descended secretory epithelial cells of transgenic animals as well as in different types of *in vitro* cultured mammalian cells used as a source of multicellular bioreactors providing human therapeutic proteins (biopharmaceuticals). Hence, mammalian cell culture systems are often used for the expression of genetically-transformed proteins. However, in spite of its many advantages, this approach is generally known for limited production capacity and high costs. In contrast, the production of recombinant human proteins in the milk of transgenic farm animals, particularly cattle, presents a safe alternative without the constraint of limited protein output (Bösze et al. 2008). Moreover, compared to cell culture, production in milk is very cost-effective. Although transgenic farm animal technology was still in its infancy two decades ago, today it is on the verge of fulfilling its potential of providing therapeutic proteins which can not otherwise be produced in sufficient quantities or at affordable cost (Brink et al. 2000, Niemann and Kues 2007).

Growth hormone can be easily obtained by extraction from human pituitary glands. The costs of hormone isolation in this way are not high, but the availability of material is limited and there is a risk of contamination of purified protein samples/extracts by human infectious agents and the spread of prions or human immunodeficiency virus (HIV) and hepatitis C virus (HCV) particles. An alternative method is the

possibility of using transgenic animals as bioreactors producing the recombinant human growth hormone (rhGH) in milk (Devinoy et al. 1994, Limonta et al. 1995, Lee et al. 1996, Nagasawa et al. 1996). A construct containing regulatory elements of the rabbit *WAP* gene linked with the human growth hormone (*hGH*) gene was applied by Devinoy and co-workers (1994) to generate transgenic mice. The level of human therapeutic somatotropin in the milk of genetically-engineered mice reached a concentration ranging from 4 mg/ml to 22 mg/ml. An equally efficient secretion of human growth hormone to the milk of transgenic mice was achieved under the control of the rat β-casein gene promoter (Lee et al. 1996). In turn, Limonta et al. (1995) created transgenic rabbits, whose secretory epithelial cells in mammary gland-derived lactogenic vesicles were able to express the fusion transgene including the *WAP* gene promoter linked to the exon sequence of the *hGH* structure gene. The efficiency of transgenesis was 23.4% and 2% in relation to the litter number and to the number of microinjected zygotes, respectively. The rhGH concentration in the milk of genetically transformed rabbits reached a level of 50 mg/ml in the best case.

The production of recombinant human growth hormone in the milk of a cloned transgenic cow at levels of up to 5 g/l has been reported by Salamone et al. (2006). Additionally, the haematological and somatometric parameters of the cloned transgenic cow are within the normal range for the breed and it is fertile and capable of producing normal offspring. In turn, Skrzyszowska et al. (2006) developed the novel technique of chimaeric somatic cell cloning which led to the production of rabbit genetically-engineered chimaeric embryos as well as both chimaeric and non-chimaeric offspring since the donor nuclear transfer of ear cutaneous fibroblast cells originating from an adult *Tg(Wap-GH1)* transgenic heterozygous female rabbit took place within only one blastomere of 2-cell embryos while the second one remained intact. The live cloned female rabbit, which had been generated by the use of this original method, had integrated the *Tg(Wap-GH1)* fusion gene in the nuclear genome of different tissue samples originating from ear-derived skin and from various organs such as the liver, kidneys, heart, lungs and gonads, and also from skeletal muscles. These results confirmed that the phenomenon of transgenic chimaerism was not revealed by PCR analysis of genomic DNA samples isolated from all the above-mentioned tissue biopsies. The transcriptional and translational activity of the *Tg(Wap-GH1)* gene construct containing the full length of the genomic (exon) sequence for human growth hormone (*hGH*) structure gene under rat whey acidic protein promoter was targeted at secretory epithelial cells of mammary gland-descended lactogenic vesicles (Skrzyszowska et al. 2006) (Table 1).

The successful genetic modification of the mammary gland in cloned cattle has recently resulted in the introduction of another potentially therapeutic protein that is defined as a bile salt-stimulated lipase (BSSL) into cow's milk composition (Chen et al. 2002b) (Table 1). The bile salt-stimulated lipase is an orally active enzymatic protein which is normally produced by the human pancreas and is present in human breast milk and which helps break down fats to make them more available for use by the digestive system. This enzymatic protein can play a significant role in replacement therapy for patients with pancreatic insufficiency (including cystic fibrosis patients) and for premature infants who do not receive human breast milk. Transgenic cattle expressing the recombinant human BSSL (rhBSSL) in the secretory epithelial cells of udder lactogenic vesicles have the potential to provide large quantities of rhBSSL in their milk for this application. Chen et al. (2002b) reported the transfection of bovine fetal fibroblast cells with gene constructs containing the human *BSSL* genomic sequence under the promoter of the ovine β-lactoglobulin (*BLG*) gene and subsequent use of these genetically-transformed nuclear donor cells in the somatic cell cloning technique for highly efficient production of a total of seven *rhBSSL* transgenic calves (Table 1).

The domestic goat, as a species with a relatively great biodiversity of dairy breeds, which possesses high genetic merit and milk yield, can be a valuable tool for embryo gene engineering (Clark 1998). Herds of cloned dairy goats can be ideal for the transgenic production of recombinant xenogeneic (human) proteins not only because of their high milk yield and consequently high concentration of certain biopharmaceutical products, which have been purified from the milk suspension, but also because they are characterized by a relatively short generation interval and low incidence of the *scrapie* prion disease (Wrathall 2000).

Baguisi et al. (1999) as well as Reggio et al. (2001) first reported the application of SCNT technology to the propagation of genetically-transformed goats. A total of eight transgenic does, which carried a transgene targeting the expression of recombinant human antithrombin III-α (rhAT) on the lactogenic vesicles of the mammary gland under the promoter of caprine β-casein, were generated (Table 1). At concentrations of genetically-engineered therapeutic protein of 1-5 g/l which have been reproducibly achieved with various animal models of transgenic ruminants, herds of cloned goats of manageable size might easily yield up to 300 kg of purified recombinant product per year. The availability of several transgenic cloned females with completely identical genetic backgrounds expressing, in the mammary gland, the gene that encodes rhAT will help improve the biochemical charac-

teristics of this therapeutic glycoprotein, whose carbohydrate structure is modified post-translationally in the epithelial cells of lactogenic vesicles. This can facilitate its extraction and purification from milk. It may also be suitable for increasing the total volume and concentration of purified rhAT in the milk suspension probes per lactation period. This latter possibility seems to result from the elevation of the milk yield of transgenic prepubertal does subjected to hormonal induction of lactation. Thereby, hormonally-enhanced propensity for the milk-mediated rhAT excretion appears to be affected by the reduction of the time necessary to obtain adequate quantities of this recombinant biopharmaceutical either for preclinical or clinical tests in humans (Cammuso et al. 2000). The high expression profile of rhAT measured with its concentration or enzymatic activity (i.e., 5.8 g/l or 20.5 U/ml at Day 5, and 3.7 g/l or 14.6 U/ml at Day 9 of 33-day period of milking) detected in the milk of one nuclear-transferred goat, in which lactation had been prematurely induced at the age of two months for two weeks, indicates that onset of transcriptional activity for the integrated *rhAT* transgene copies may be accelerated significantly. Consequently, the secretion of genetically-engineered proteins by epithelial cells in the mammary gland may be initiated earlier (Table 1). This involves the shortening to 8-9 months of the time interval between nuclear donor cell line transfection and rhAT expression in the milk of prepubertal cloned does. Milk production performance, which reaches 160 ml during the whole period of hormonally-induced (i.e., prematurely-triggered and prolonged) lactation, is not only sufficient to predict the genetically transformed protein concentration per volume unit, but, above all, is also adequate for the repeatability of both quantitative measurements and the diagnostic tests for purification level and pharmacokinetic activity of the biologically-active recombinant protein, when its secretion levels in milligrams per milliliter of milk suspension are achieved (Baguisi et al. 1999).

To recapitulate, the obvious benefits of using genetically-engineered SCNT animals to synthesize human therapeutic proteins in the mammary gland (udder) include, among others, high production yields, low capital investment compared with cell culture techniques of animal cellular or microbial bioreactors (which involve high capital costs, expensive culture media and low yields) and lack of disadvantageous post-translational modifications versus improper folding and high purification costs, respectively (Bösze et al. 2008). Another benefit involves elimination of reliance on products derived from human blood, which may contain pathogens such as HIV and hepatitis viruses (HCV, HBV). On the other hand, among the disadvantages of this technology are the ability of genetically-engineered proteins to perform complex post-translational modifications, e.g., glycosylation, phosphorylation and γ-carboxylation in the secretory epithelial cells of the udder, as well as chelate formation in milk micella lipoprotein clusters, which can decrease considerably the efficiency of their extraction and purification from the milk suspension (Clark 1998, Salamone et al. 2006).

## Commercialisation of the first pharmaceutical protein produced in the milk of large transgenic cloned farm livestock species as an important milestone of gene engineering and reproductive biotechnology

In January 2004, the Genzyme Transgenic Corporation (GTC) Biotherapeutics firm put forward to the European Medicines Agency a proposal requesting permission for introduction on the market of a biopharmaceutical preparation commercially named Atryn®. In August of 2006, following a positive opinion by the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency, also defined as the European Agency for the Evaluation of Medicinal Products (EMEA), the European Commission approved Atryn® for prophylactic treatment of patients with congenital antithrombin deficiency, undergoing high-risk surgical or childbirth procedures. The European Commission approval followed the positive opinion expressed by the EMEA on June 2, 2006. The positive opinion had been based on an extensive review of the application submitted by GTC Biotherapeutics (GTCB) in January 2004 (Echelard et al. 2005, Melican et al. 2005). In the United States, the Food and Drug Administration (FDA or USFDA), which is a government agency of the Department of Health and Human Services, approved Atryn® in the first quarter of 2009 for the prevention of peri-operative and peri-partum thromboembolic events in hereditary antithrombin deficient (HAD) patients.

Atryn® is therefore both the first ever transgenically produced therapeutic protein and the first recombinant antithrombin (AT) product that has been approved through the centralized procedure for the pharmaceutical market not only in the European Union, but also in the world. Furthermore, it is now the first ever biotechnological product derived from genetically-engineered animals and the first recombinant AT preparation that has already been certified by the FDA as fit for use in the biopharmaceutical industry and passed as suitable/safe for pharmacological treatment (pharmacotherapy) in humans. Therefore, Atryn® has been authorized for sale on the USA pharmacy market. Along with the approval of Atryn®, the FDA's Center for Veterinary Medicine also ap-

proved GTCB's New Animal Drug Application, the first of its kind to regulate genetically-engineered animals. This is now required for a recombinant technology used to develop transgenic animals, such as the cloned goats that produce recombinant antithrombin. GTCB has granted Lundbeck Inc. (formerly OVATION Pharmaceuticals, Inc.) the right to market Atryn® in the U.S. and pursue further clinical development. The companies expected Atryn® to be available in the United States during the second quarter of 2009 (Echelard et al. 2006, Niemann and Kues 2007).

Recombinant human antithrombin (rhAT), which is the biologically-active ingredient of Atryn®, is a 432 amino acid glycoprotein with a molecular weight of approximately 57.215 kDa. Its molecular formula is: $C_{2191}H_{3457}N_{583}O_{656}S_{18}$. The amino acid sequence of rhAT is identical to that of human plasma-derived antithrombin III-α. Recombinant human antithrombin and plasma-descended antithrombin III-α both contain six cysteine residues forming three disulphide bridges and 3-4 N-linked carbohydrate moieties. The glycosylation profile of rhAT is different from plasma-derived antithrombin III-α, which results in an increased heparin affinity. When assayed in the presence of excess of heparin the potency of the recombinant product is not different from that of plasma-retrieved product. As compared to plasma-descended antithrombin III-α, Atryn® has a shorter half-life and more rapid clearance (approximately nine and seven times, respectively). Atryn® does not contain any preservatives nor is it formulated with human plasma proteins. Furthermore, rhAT is affinity purified using a heparin immobilized resin and contains no detectable heparin (<0.0002 IU heparin per IU antithrombin) in the final product (Echelard et al. 2005, Patnaik and Moll 2008).

Recombinant human AT is isolated and purified from the milk of genetically-engineered does descended from several generations of the first somatic cell cloned goats in the world created by Baguisi et al. (1999). The goats, in the udder of which the rhAT is synthesized, are United States Department of Agriculture (USDA) certified *scrapie*-free, and controlled for specific pathogens. Using GTCB technology, recombinant antithrombin is purified using both conventional and proprietary methods. The rhAT production process also incorporates one viral treatment step and one viral removal step (nanofiltration) (Echelard et al. 2005).

Atryn® is, then, a recombinant AT indicated for the prevention of peri-operative and peri-partum thromboembolic events, including pulmonary embolism (PE) and deep vein thrombosis (DVT), in hereditary antithrombin deficient (HAD) patients. Patients with a hereditary antithrombin deficiency (HAD) are prone to developing blood clots/coagulations. The prevalence of HAD in the general population is approximately one in $2 \times 10^3$ to one in $5 \times 10^3$. Half of HAD patients may experience a thrombosis before 25 years of age and up to 85% may suffer a thromboembolic event by age 50. The hereditary deficient population had previously been dependent on plasma-derived antithrombin products for use during high-risk procedures such as surgery or childbirth. GTCB's recombinant form of antithrombin III-α offered an alternative to treatment with plasma-derived product and a consistent availability of product throughout the European Union, once reimbursement rates were obtained (Echelard et al. 2006, Patnaik and Moll 2008).

Negotiation of the reimbursement rates with each country's health system and establishment of sales and marketing efforts in Europe were carried out by GTCB's partner, LEO Pharma A/S. GTCB continued to produce the Atryn® preparation, receiving a transfer price from LEO as well as a royalty on commercial sales. Market launch was targeted for the second quarter of 2007 as reimbursement rates had been finalized. LEO also made a non-refundable $ 2 million milestone payment to GTCB for receipt of the European market authorization (Echelard et al. 2005, 2006).

In addition to the prophylactic aversion of the risk of development of acute peri-operative or peri-partum venous thromboembolisms (VTEs) in HAD patients, rhAT has been used off-label to treat heparin-resistance in cardiac surgery and sepsis. It is a promising adjuvant for immunosuppression in organ transplantation, and may have a role as an anti-angiogenic, anti-tumor and anti-viral agent. Recombinant human AT has clear safety advantages over human plasma-derived AT III-α, such as the avoidance of infection transmission (Konkle et al. 2003, Patnaik and Moll 2008).

### The perspectives of somatic cell cloning and transgenesis of farm livestock species for human nutrition/dietetics technologies

Milk as a nearly perfect food source because of its balanced protein, fat, carbohydrate, and mineral content represents a fundamental dietary ingredient in many societies, and is consumed not only in its natural form, but also in a wide variety of processed products. Intensive cattle crossbreeding strategies, nutritional management and quantitative genetics have resulted in a steady improvement in milk yield, but have not generated major changes in milk protein composition. It has been found that the biochemical composition of human milk differs considerably from the composition of both the milk of cattle and of small ruminants. Human milk contains much less total protein (10 g/l) than cow's milk (33 g/l), 70% of which consists of the

fraction of whey proteins including, above all, lactoferrin and lysozyme (i.e., mucopeptide glycohydrolase) (Hyvönen et al. 2006, Yu et al. 2006). In contrast to bovine milk, the concentration of caseins in human milk is considerably lower. The only casein fractions, whose level is higher in the human milk suspension, are β- and κ-caseins. For this reason, genetic modification of the cattle milk composition through at least partial substitution of its own endogenous protein fractions with human proteins or induction of overexpression of β- and κ-casein variants can lead to humanization of cow's milk.

A glass of bovine milk (about 250 ml) contains 8.0 grams of protein, with caseins comprising 78 to 80% of this amount. Caseins are one of the most valuable components of milk because of its nutritional value and processing properties. Therefore, casein is a prime target for the improvement of milk composition. The casein fraction of cow's milk comprises four proteins: $\alpha_{S1}$- and β-casein (10 g/l each), $\alpha_{S2}$-casein (3.7 g/l), and κ-casein (3.5 g/l), which exist naturally in a number of protein variants (isoforms). They are aggregated into large spherical particles known as casein colloidal micelles, whose structure and stability govern many of the complex physicochemical properties of milk. Relatively small changes in casein ratios can affect the micelle structure and thus can have substantial effects on the functional properties of milk. For this reason, a higher casein concentration would not only increase a valuable milk component but could simultaneously improve milk characteristics (Hitchin et al. 1996, Choi et al. 2001). Together with fat, casein proteins are responsible for the specific white chalky children's milk "mustache". One of the important functions of caseins is to bind and sequester calcium phosphate and magnesium cations within the colloidal micelles. The outer surface of these is enriched with post-translationally modified (i.e., glycosylated) κ-casein, which is cleaved by chymosin used in cheese making to destabilize the micelles and form the curd. Increased content of κ-casein proteins has been related to a reduction of the micelle size and to enhanced heat stability and cheese making/ripening properties. The interior of the micelle is composed of the highly phosphorylated $\alpha_{S1}$-, $\alpha_{S2}$-, and β-casein proteins which bind the otherwise insoluble calcium phosphate. As one of the predominant milk proteins, β-casein is thereby implicated in determining the levels of milk $Ca^{2+}$ ions. Additionally, elevated β-casein concentration has been correlated positively with the processing properties, involving reduced rennet clotting time and raised whey expulsion (Rijnkels et al. 1998, Baranyi et al. 2007, Pampel et al. 2008).

To enhance milk composition and milk processing efficiency by elevation of the casein concentration in milk, Brophy et al. (2003) have introduced additional copies of the genes encoding bovine β- and κ-casein (*CSN2* and *CSN3*, respectively) into *in vitro* cultured female bovine fetal fibroblasts. Using nuclear transfer with four independent transgenic nuclear donor cell lines or one parental, unmodified somatic cell line, a total of 11 transgenic and 7 non-transgenic cloned calves were generated, respectively (Table 1). To evaluate the expression and secretion of the transgene-derived β- and κ-casein fractions into milk suspension, genetically modified and unmodified (control) nuclear-transferred heifers were hormonally induced into lactation. Analysis of skim/defatted milk samples, which had been collected following hormonal stimulation of heifers, revealed substantial expression and secretion into milk of caseins that were the translation products of mRNA molecules synthesized by genomic DNA exon sequences of transgene-transmitted *CSN2* and *CSN3* alleles. Compared to the non-transgenic control individuals, nine cows, representing two high-expressing transgenic founder lines, produced milk with slightly (8-20%) higher β-casein levels (15.8-20.9 mg/ml vs 14.3-14.9 mg/ml), twofold higher κ-casein concentrations (8.4-14.1 mg/ml vs 5.0-5.8 mg/ml), and a markedly altered κ-casein to total casein ratio (0.26-0.42 vs 0.15-0.21) (Table 1). These results indicate that it is feasible to considerably alter a major component of milk in high producing dairy cows by a random co-insertion and co-integration of *CSN2* and *CSN3* transgene copies into the genomic DNA of a single chromosomal locus and thereby to improve the functional properties of milk. There was no correlation between the expression levels of β- and κ-caseins and the *CSN2* and *CSN3* transgene copy numbers. This suggests locus-dependent transcriptional activity of the transgenes, since the copies of two gene constructs have been built randomly as large concatemeric structures into a single chromosome. Brophy et al. (2003) generated transgenic cloned cows that overexpress casein variants, resulting in a 30% increase in the total milk casein or a 13% increase in total milk protein.

Genetically-transformed dairy breeds of cattle, goats and sheep secreting milk with either heterologous pharmaceutical proteins or a significantly altered composition of endogenous casein proteins or whey proteins such as β-lactoglobulin or α-lactalbumin are becoming a reality (Zuelke 1998, Pampel et al. 2007). Designer milk, speciality milk or humanized milk produced by transgenic ruminant bioreactors may be competing in the next decade to capture part of the global dairy product market. The concept of modifying milk biochemical composition by augmenting the protein content of milk through increased casein gene dosage in the cow nuclear genome has been postulated for many years (Jenkins and McGuire 2006, Sabikhi 2007). A functional consequence of co-transfection of gene constructs encoding additional fractions of casein proteins can be an increase in the

ratio of κ-casein to β-casein or a concomitant increase of all caseins by transferring casein locus. This results in both elevation of protein and calcium content in the genetically-engineered milk and reduction of colloidal micelle size as well as enhancement of milk heat stability. A further functional outcome of modification of casein genes by adding the genomic sequences that encode phosphorylation sites is an increase in calcium level, micelle size and stability of the milk. This can lead to enhanced amphophilicity of β-casein, which thus enlarges/amplifies its emulsifying and foaming properties. Introduction of additional genomic sequences encoding protease (chymosin) cleavage sites into the casein gene *locus* can cause the improvement of cheese-ripening process. On the other hand, deletion from the β-casein gene of exon regions responsible for the formation of the protease (plasmin) site in its translation product can induce an increase in β-casein emulsifying properties as well as elimination of the bitter flavour in produced cheese (Hitchin et al. 1996, Laible et al. 2007, Pampel et al. 2008). Introduction of supplementary functional copies of genes encoding regulatory immunoproteins of the milk such as lysozyme, lactoferrin or lysostaphin can enhance the antimicrobial immunosupressive activity of such genetically-modified milk (Hyvönen et al. 2006, Yu et al. 2006).

## Conclusions

The production of therapeutic human proteins is one of the major successes of biotechnology. Animal cells are required to synthesize proteins with the appropriate post-translational modifications. Different transgenic mammalian species are being used for this purpose. Milk from transgenic animals is the best candidate as a source of recombinant human proteins on an industrial scale (Niemann and Kues 2007, Kind and Schnieke 2008).

Microinjection of foreign DNA into the pronuclei of fertilized oocytes (zygotes) has predominantly been used for the generation of transgenic livestock. This technique works reliably, but is inefficient and results in random integration and variable expression patterns in the transgenic offspring. Nevertheless, remarkable achievements have been made with this technique. By targeting expression to the mammary gland, numerous xenogeneic (heterologous) recombinant human proteins have been produced in large amounts which could be purified from the milk of transgenic goats, sheep, cattle and rabbits. Recent developments in the technology of somatic cell nuclear transfer and its merger with the growing genomic data of different farm livestock species allow a targeted and regulatable transgenic production. The method of zygote intrapronuclear microinjection of gene constructs is now being replaced by more efficient protocols based on SCNT which also permit targeted genetic modifications. Although the generation of transgenic farm animals has recently become easier mainly with the technique of somatic cell cloning using transfected nuclear donor cells, this point remains a limitation as far as cost is concerned. Moreover, a certain number of technical problems remain to be solved before the various systems are optimized.

Numerous experiments carried out for the last fifteen years have shown that the expression of the transgene in milk is predictable only to a limited extent. This is clearly due to the fact that the expression vectors are not constructed in an appropriate manner. This undoubtedly is due to the fact that all the signals contained in genes have not yet been identified. Different species of mammals determine various levels of transgene expression in milk. Mice are routinely used to evaluate the gene constructs to be transferred into larger animals. Mice can also be used to prepare amounts as high as a few hundred mg of recombinant proteins from their milk. Rabbits appear adequate for amounts not higher than 1 kg per year. For larger quantities, goats, sheep, pigs and cows are required (Niemann and Kues 2007). Gene constructions sometimes result in poorly functional expression vectors. Therefore, the vectors carrying the genes coding for the therapeutic proteins of interest are of unpredictable efficiency. One possibility relies on the identification of the major important elements required to obtain a satisfactory transgene expression. Improvement of these vectors includes the choice of efficient promoters, introns and transcription terminators, the addition of matrix attached regions (MAR) and specialized chromatin sequences (SCS) to enhance the expression of the transgenes (e.g., chromatin openers) and to insulate them from the chromatin environment (i.e., gene insulators). Promoter sequences from a number of different milk protein genes have been used to target expression to the mammary gland, although significant problems remain with regard to achieving transgene expression levels consistent with commercial exploitation. The other possibility consists in using long genomic DNA fragments contained in yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) vectors. Lentiviral vectors and small interfering ribonucleic acid (siRNA) technology are also becoming important tools for transgenesis. A certain number of recombinant human proteins with complex structures (e.g., formed by several subunits undergoing association, being correctly folded, assembled, cleaved, glycosylated, γ-carboxylated, and so on) have been obtained at levels sufficient for industrial exploitation. In other cases, the mammary cellular machinery seems insufficient to promote all the post-translational modifications. In

a certain number of cases, the xenogeneic therapeutic proteins produced in milk have deleterious effects on the mammary gland function or in the animals themselves. This comes independently from ectopic expression of the transgenes and from the transfer of the recombinant proteins from milk to blood. One possibility for eliminating or reducing these side-effects may be to use systems inducible by an exogenous molecule such as tetracycline, allowing the transgene to be expressed only during lactation and strictly in the mammary gland. The purification of recombinant proteins from milk is generally not particularly difficult. This may not be the case, however, when the endogenous proteins such as serum albumin or antibodies are abundantly present in milk. This problem may be still more crucial if proteins are produced in blood. Among the biological contaminants potentially present in the recombinant proteins prepared from transgenic animals, prions are certainly those raising the main concern. The selection of animals chosen to generate transgenics on one hand and the elimination of potentially contaminated animals on the other, thanks to recently defined quite sensitive tests, may reduce the risk to an extremely low level. The available techniques for producing pharmaceutical proteins in milk can also be used to optimize the milk composition of farm animals, to add nutriceuticals to milk and potentially to reduce or even eliminate some mammary gland-related infectious diseases (Melo et al. 2007, Kind and Schnieke 2008).

## Acknowledgments

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