The mammary gland: bioreactor for the production of recombinant proteins

REVIEW

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ABSTRACT

Biological systems for the expression of recombinant proteins have evolved from microorganisms, such as bacteria and yeast, to animal and plant cells. This evolution has been driven mainly by the specific post-translational modifications required for many recombinant proteins to display full biological activity. In this review we discuss the main advantages and drawbacks of the currently available expression systems, stressing the potential of the mammary gland as a biofactory. Several methods for the genetic modification of this organ have been developed; the choice of the method depends on the specific characteristics of the molecule to be expressed, and the time taken to secrete the final product. However, the mammary gland glycosylation machinery is limited to the synthesis of biantennary monosialylated complex oligosaccharides.

Keywords: mammary gland, recombinant proteins, glycosylation

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RESUMEN

La glándula mamaria: biofábrica para la producción de proteínas recombinantes. Los sistemas de expresión de proteínas recombinantes han evolucionado desde microorganismos como bacterias y levaduras hasta células de plantas y animales. Esto se debe a la necesidad de contar con hospederos que permitan la expresión de biofármacos proteicos con procesamientos postraduccionales específicos, que garanticen una actividad biológica similar a la de la proteína nativa. En esta revisión se presentan las principales ventajas y desventajas de los sistemas de expresión más utilizados en la actualidad, con especial énfasis en las capacidades de la glándula mamaria como biofábrica. Se han desarrollado varios métodos de modificación genética de este órgano; la utilización de uno u otro está condicionada fundamentalmente por las características específicas de la proteína de interés y la inmediatez en la obtención del producto final. Sin embargo, la glándula mamaria posee una «maquinaria» de glicosilación limitada, donde se sintetizan, sobre todo, estructuras oligosacarídicas de tipo complejo, biantenario y monosialilado.

Palabras claves: glándula mamaria, proteínas recombinantes, glicosilación

*I*ntroduction

The production of biopharmaceutical proteins by genetically transformed microorganisms, such as bacteria and yeast, has been established as a relatively inexpensive and safe process [1]. However, the biological activity of certain proteins undergo inadequate post-translational processes in those expression systems [2]. The biosynthetic machinery of eukaryotic cells is often required to produce drugs with high specific activity [3]. Mammalian cell expression systems have become viable strategies for the production of biologically active proteins [4], even though mammalian cell culture, and the methods used to achieve the stable genetic transformation of cell lines are costly and technically challenging processes [5].

Genetically modified animals expressing recombinant proteins in their tissues and secreting them into body fluids have been developed [6]. The mammary gland has been identified as an attractive alternative for the production of recombinant proteins requiring eukaryotic post-translational modifications [7]. Heterologous proteins synthesized in mammary epithelial cells are immediately secreted into the milk, from where they can be purified using relatively simple chromatographic procedures [8].

Genetic constructions, where the gene encoding the protein of interest is coupled to regulatory sequences, are required to transform mammals into biofactories. Regulatory elements limit protein expression in the mammary epithelial tissue during milk production [9]. DNA microinjection into unicellular embryos has been the technique of choice to generate transgenic animals that secrete recombinant proteins into their milk [10,11]. However, this methodology is technically challenging, expensive, and its application to livestock is inefficient. It has been estimated that the generation of one transgenic ruminant costs between 200 000 and 500 000 dollars [12, 13]. Besides, the time elapsed between DNA insertion in the embryo genome and the collection of the recombinant protein is too long [14]. The ectopic expression of the transgene is another limitation because it can drastically affect the viability of the transformed animal [15].

A great number of donor and receptor females are required for the generation of transgenic livestock. These are needed to successfully transform the embryos and implant them to obtain transgenic offspring. Additionally, the consumables and the equipment needed to transfer the DNA to the embryos and to culture them *in vitro* are expensive [16].

The direct transfer of foreign genetic material to mammary gland epithelial cells in adults is the strategy of choice to avoid the problems associated with

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traditional transgenesis. This alternative procedure will reduce both, production costs and the time frame required for the production of the biopharmaceutical [17]. The choice of an appropriate vector or vehicle to efficiently transfer the genes into mammary gland epithelial cells is a key factor for success.

Several research groups have tried to develope mammary gland expression systems based on the *in situ* transfer of genes to the secreting epithelia. A variety of delivery systems have been evaluated such as polyion complexes [18], receptor mediated endocytosis [19], gene gun [20] and viral vectors [21, 22]. Although the expression of the transgene in milk has been achieved, the low concentration of the product and its transient expression makes this system unsuitable as a production process.

Adenoviral vectors may facilitate the direct transfer of the genetic material to the target cells at the mammary epithelia. Those vectors have been successfully used in many gene therapy assays due to their high capacity to infect either quiescent or dividing cells [23]. On the other hand, cell culture multiplication systems have been optimized to yield high viral titers [24]. Besides, these viruses do not integrate into the host cell genome, and therefore the gene expression can not be negatively affected by the integration site [12]. Using the new method of adenovirus infection in the mammary gland, expression levels of milligrams per milliliters have been achieved in the milk of mice and goats. Those results could represent a starting point for systems development towards the production in of recombinant proteins the milk of genetically modified mammals. However, the limited time of transgene expression still persist as a considerable disadvantage of this method.

Expression of recombinant proteins

The development of recombinant DNA technology during the mid 1970's marked the starting point of a new biotechnology era. The generation of therapeutic proteins is the principal application of the biopharmaceutical industry [25]. In that sense, the production of recombinant proteins, and the development of animal models are priorities for many research groups.

Numerous organisms can be used as bioreactors for the production of heterologous proteins. Bacteria and yeast are the simplest and less expensive hosts, while artificial tissue culture and genetically modified organisms are the more complex and expensive ones [5]. The choice of the host microorganism for the expression of biopharmaceutical proteins depends on the type of post-translational modifications required to preserve the biological activity of the molecule.

Expression of recombinant proteins in prokaryotes

Escherichia coli, the subject of extensive genetic and physiologic studies, has been by far the most widely used bacteria for the production of recombinant proteins [26, 27]. Among the advantages of this microorganism as a bioreactor are: 1) a fast biomass generation due to a high growth rate, 2) its easy genetic manipulation, 3) the relatively low cost of the culture media and equipment, and, 4) its high efficiency for foreign DNA incorporation [28].

However, prokaryotes are not able to accomplish many of the post-translational modifications required for the production of biologically active recombinant proteins. Due to their reducing intracellular environment these hosts do not form disulfide bridges between cysteines. Additionally, no oligosaccharides are covalently linked to proteins and no tyrosine sulfatation occurs in this system [1].

The secretion of recombinant proteins in the extracellular milieu is not likely to occurs in bacteria. Proteins are frequently accumulated as insoluble aggregates in the cytoplasm, known as inclusion bodies. Chaotropic agents are required to extract the protein of interest out of those inclusion bodies, and this procedure leads to the denaturation of the polypeptide by disrupting its tertiary structure and reducing its biological activity [29].

Protein expression in lower eukaryotic microorganisms

As hosts for the production of heterologous proteins from eukaryotic origin, yeast exhibit important advantages compared to bacteria [30]. They combine the simplicity of bacterial expression systems and the low costs of the culture media, with an intracellular environment that is more suitable for post-translational processing and secretion [31].

Additionally, yeasts can glycosylate proteins, and this modification exerts a positive influence in the structural integrity, solubility and biological activity of the polypeptide chains [32,33]. However, the expression systems based on *Saccharomyces cerevisiae* yeast have been problematic in the industrial scaling up. Those problems are mostly related to the lack of stability in the copy number of the transforming plasmids in a high cell density environment [34].

The addition of oligosaccharide chains with more than 50 mannose residues (Man) generates a different glycosylation profile as compared to native proteins. Besides, terminal mannoses, α1,3 linked to the oligosaccharidic non-reducing terminal, increase the immunogenicity of the protein in mammals. Hypermannosylation can also modify the phar-macokinetics of glycoproteins, thus limiting their therapeutic efficiency [31].

Another host widely and successfully used for recombinant protein expression is the methylotrophic yeast *Pichia pastoris*. This microorganism is characterized by an efficient use of methanol as carbon and energy source, although itcan growth in a wide range of substrates [35]. Unlike *S. cerevisiae*, oligosaccharide chains with a lower degree of polymerization, and lacking α1,3 terminal mannoses are synthesized in *P. pastoris*. Since only high mannose oligosaccharides are produced, the ability of this host to express proteins having a biological activity that depends on a specific glycosyilation pattern, is quite limited [36].

The enzymatic glycosylation machinery has been recently modified in *P. pastoris*, generating strains that can assamble complex N-glycans. This is a novel

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alternative for the production of recombinant proteins with a specific and highly homogeneous glycosylation pattern in lower eukaryotes [37].

Plants as bioreactors

Due to its low cost, potential for scale up processes, and the simplicity of crops and their storage, genetically transformed plants are an economic option for current biopharmaceuticals production systems. A special interest has raised the production of vaccines and antibodies in plants [38, 39]. Since they lack oncogenic DNA sequences, plants are considered to be one of the safest production systems [40].

Despite these advantages, they are unable to generate complex glycosylation profiles which is a limitation of this expression system. Plants lack either the sialic acid synthesis pathway or the enzymes responsible for the elongation of oligosaccharide chains: β1,4-galactosyltransferase and sialyltransferase [41]. Furthermore, the addition of β1,2 xylose and α1,3 fucose residues to the oligosaccharide core renders the molecule allergenic immunogenic and this could limite their therapeutic use in humans [42].

Protein expression in mammalian cell cultures

Most of the therapeutic proteins, including blood proteins such as cytokines and immunoglobulins, structural proteins, hormones and lysosomal proteins, are directed to the lumen of the endoplasmic reticulum during translation and subsequently transported through the Golgi bodies to lysosomal compartments, the extracellular matrix or the blood [3]. Most modifications takes place in those compartments, including signal peptide removal, disulfide bridge formation, tyrosine sulfatation, carboxylation, methylation, hydroxylation, phosphorylation, N-glycosylation and O-glycosylation [43].

The bioactivity, pharmacokinetics, stability and solubility of several human pharmaceutical proteins, such as erythropoietin (hEPO), α1-antitrypsin, tissue plasminogen activator (tPA), coagulation factors VIII and IX, protein C and fibrinogen, are intimately related to the above mentioned post-translational modifications. Due to their structural peculiarities, these drugs should be produced in host cells harboring biosynthetic machineries similar to those of the original cells [4].

More than 60% of the recombinant proteins with pharmaceutical interest are currently produced in cell cultures [44]. Since the development of the first eukaryotic cell line in the 1960's [45] many cell types from different tissues and species have been routinely cultivated *in vitro*. However, due to the biosafety concerns associated with drug production, only a few cell lines have been genetically modified and used as bioreactors [46]. Chinese hamster ovary cells (CHO), murine myeloma derived cells (NSO), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retina derived cells (PERC6) have been the most extensively used so far [47].

Together with an adequate post-translational processing, secretion is one of the main advantages of biopharmaceutical production in mammalian cell cultures. Most of the molecules generated in this system, have been modified by genetic engineering techniques to promote their transfer to the extracellular milieu. This largely enables the purification of the molecule because there are fewer contaminant proteins in the metabolized culture medium [48]. The search for more efficient and specific procedures for cell culture has been greatly stimulated by the accelerated growth of the pharmaceutical industry [49, 50]. Although it is considered as the ideal expression system for complex proteins, the biological requirements of mammalian cell culture, as well as the complex technology involved, considerably increases production cost [5]. The use of genetically modified animals as biofactories or bioreactors for the production of recombinant products could be a solution to this conflict.

Animals as bioreactors

Several species have been genetically modified to express recombinant proteins in their body fluids [6]. An organism with a segment of foreign DNA inserted in the genome, and particularly in its germline, is defined as transgenic. The insertion of the genetic material occurs during the early stages of embryonic development, favoring its presence in the germline of the individual. Transgenic animals have been very useful in generating new animal models for experimentation and in producing recombinant proteins [51].

Recombinant proteins obtained form transgenic animals are usually expressed in the cells of a specific tissue and secreted into a specific body fluid. The efficiency of the system is given by the secretory capacity of the chosen tissue and by the ease in collecting the fluid containing the heterologous protein [52]. Biopharmaceutical proteins have been expressed in several body fluids: Human recombinant antibodies have been expressed in the blood of transgenic mice, rabbits, pigs and sheep [53, 54], and biologically active human α1-antitrypsin was also produced in the blood of transgenic rabbits [15].

When the recombinant protein is expressed in the blood its separation from the host counterpart is a common complication because the physico-chemical characteristics of these molecules are well conserved among vertebrates. Moreover, most proteins secreted into the blood are either unstable or drastically affect host survival; therefore, this system is considered inappropriate for biopharmaceuticals [12].

The expression of biopharmaceutical proteins in urine [55] and the seminal plasma [56] have recently been reported. However, due to its high secretory capacity and the ease of milk extraction, the mammary gland is the best suited organ for the production of recombinant proteins [57].

The mammary gland

Expression of recombinant proteins in the mammary gland

The mammary gland contains a tree of ducts embedded in a fat matrix and supported by a parenchyma. Those ducts rise from the nipple channel and diverge into thinner channels to end in a circular structure called alveoli (Figure 1A). The alveoli are covered by a layer of epithelial cells, which is in turn surrounded by a

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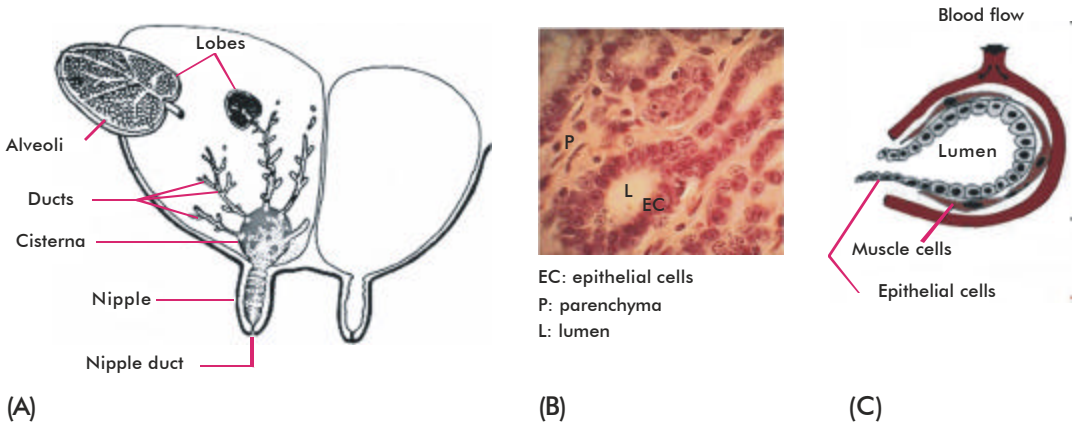

Figure 1. Morphological characteristics of the mammary gland. **A**: Schematic representation of the ruminant mammary gland. B: Mammary gland tissue of a lactating female: epithelial cells forming the alveoli. **C**: Representation of the mammary alveoli. (Taken from Internet: www.agrobit.com/images/udder2.gif).

layer of muscular cells sensitive to oxytocin stimulation (Figures 1B and 1C). Muscular cells contract in the presence of this hormone and the milk flows out of the alveoli through the ducts [58]. The terminal ducts lead to reservoirs called galactophores, located immediately after the nipple, where the milk is collected. In ruminants, galactophores are connected and expand to form cisterns where very high volumes of milk are stored. A group of ligaments and the connective tissue fix the gland to the body and the organ is covered by skin, forming the udder (Figure 1A) [59].

The epithelial cells covering the alveoli form the synthesis and secretion unit. Those cells receive hormone signals during lactation that activate the expression of genes encoding for specific proteins, which are then produced and secreted in the milk. Other molecules can also travel from the blood to the milk through the mammary epithelium [60].

Milk is an extremely abundant fluid, and in some mammals such as ruminants is very rich in proteins, producing from 200 g to 1 Kg of protein per day [14]. A variety of biologically active proteins such as tPA [61], the human growth hormone (hGH), [62] the neuronal growth factor [63], human protein C [64], human antithrombin (ATh) [65] and human lactoferrin [66] have been produced in the mammary gland (Table 1).

Transgenesis in the mammary gland

Back in 1987, Lothar Hennighausen and Heiner Westpal, from the National Institute of Health, USA, in association with Katy Gordon, from Integrated Genetics Institutes, set a milestone in the field of biotechnology when expressed human tissue plasminogen activator in the mammary gland of transgenic mice [61]. Afterwards, multiple studies to improve this methodology have been conducted.

The expression levels of heterologous proteins in the mammary gland depend on the expression casset-te and the method used to transfer genetic material. The strength of the promoter is a very important element. To be able to specifically drive the expression of the recombinant protein toward the mammary epithelial cells, the promoters naturally regulating protein expression in the milk have been preferred.

The relative strength of milk promoters varies among species. Kappa-casein and αS2-casein promoters are weak [12], while those of rat serum acid protein, (WAPr) [9], goat αS1-casein [67] and sheep β-lactoglobulin [68] are very strong (Table 1).

The amount of recombinant protein produced varies from one transgenic strain to the other even if the same expression cassette is used. This variability has been attributed to the so called «position effect», where transgene expression can be influenced by the chromatin, heterochromatin, or euchromatin region

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Table 1. Pharmaceutical proteins expressed in the milk of transgenic mammals [14]

Protein	Transgene source	Promoter	Expression levels
Cow			
Lactoferrin	genomic DNA	α–S1 bovine casein	Not available
β-lactoalbumin	Not determined	Not available	2.4 mg/mL
Goat			
Antithrombin III	Not determined	goat β casein	14 mg/mL
β-S1antitripsin	Not determined	goat β casein	20 mg/mL
Growth Hormone	Not determined	Retrovirus	1.2 x 10-4 mg/mL
Monoclonal antibody	genomic DNA	goat β casein	10 mg/mL
Tissue plasminogen Activator	cDNA	goat β casein	6 mg/mL
Pig			
Factor VIII	cDNA	murine WAP	3 mg/mL
Protein C	cDNA	murine WAP	1 mg/mL
Rabbit			
Calcitonin	Fusion Protein	bovine β-lactoglobulin	2.1 mg/mL
Superoxide dismutase	cDNA	murine WAP	2.9 mg/mL
Erythropoietin	cDNA	rabbit WAP	50 μg/mL
Erythropoietin	Fusion protein cDNA	bovineβ-lactoglobulin	50 μg/mL
Growth Hormone	genomic DNA	murine WAP	50 μg/mL
Insulinic Growth Factor	cDNA	bovine β-S1casein	1 mg/mL
Interleukine-2	genomic DNA	rabbit β-S1casein	0.5 μg/mL
Sheep			
a1 -antitrypsine	Minigene	ovine β-lactoglobulin	35mg/mL
Factor VIII	cDNA	ovine β-lactoglobulin	Not available
Factor IX	cDNA	ovine β-lactoglobulin	5 μg/mL
Fibrinogen	genomic DNA	ovine β-lactoglobulin	5 mg/mL

in which it is inserted [9, 69]. Expression cassettes including a combination of extensive regulatory sequences from promoters and regulators such as LCR (*locus control regions*) [70], *insulator* [71] and MAR (*matrix attachment regions*) have recently been developed [72]. Those regulatory elements can form active transcription domains, acting independently from the integration site.

The specificity of promoters is also of importance. The physiology of the host can be seriously affected if the transgene is expressed in ectopic tissues during its development [15]. This is called a collateral effect of transgene ectopic expression. Some recombinant proteins, such as erythropoietin, cause severe damage to the host when expressed under the control of promoters lacking strict regulation [15, 17].

Genetic Transfer

The first methods used to transfer heterologous DNA to mammalian embryos were based on retroviral vectors. In 1974, Jaenisch [73] described the infection of mice blastocysts with the SV40 virus and the persistence of the viral genome integrated in adult animals, and in 1976 the mendelian transmission of the murine leukemia virus (MMLV) was documented in embryos microinjected with this retrovirus [74]. However, oncoretrovirus translation capacity is limited to dividing cells [75]. The infection of embryos in an early stage of development with retroviral vectors resulted in late integration and heterogeneous distribution, considerably affecting the efficiency of the method [76].

The limited capacity for the insertion of foreign DNA (less than 10 kb) is another drawback of retroviral vectors, restricting the cloning of genomic regions, large regulatory regions frequent in milk promoters, and distal regulatory elements [77]. Moreover, terminal repeats flanking the retroviral genome interferes with mammalian promoters, suppressing or reducing the expression of genes under their control [78]. This silencing phenomenon is mediated by methylation and occurs near the integration site [79].

Microinjection of DNA into unicellular embryos is other method used to transfer the foreign genetic material. Since its first description by Gordon in 1980 [11], this method has been the most universal technique for the horizontal transfer of genetic material. It consists of the injection of the foreign DNA into the pronucleus of unicellular embryos to facilitate the random insertion of the genetic material into the chromosome [80, 10]. This methodology has been applied to mice, hamsters, rabbits, pigs, sheep, goats and cows, without substantial variations during the last 25 years [12]. In the majority of these animals, the embryos are extracted from a donor female; the DNA is microinjected in one of the pronuclei of the zygote, and this is transferred to a receptor female to produce the transformed offspring. Cow oocytes should mature and be fertilized *in vitro* to increase the number of zygotes per donor female. The first offspring, or founders, are tested for the presence of the transgene in their genomes, and positive individuals are mated to produce homozygosis. The low integration efficiency of the transgene in ruminants and other species (less than 2% in pigs, sheep and goats; less than 1% in cows), as well as the long time period required needed to gather a small herd that produces the desired protein, are the main problems of microinjection to generate bioreactors (Table 2). Hence, many donors and receptor females are required to obtain an initial founder, increasing the cost of the process [6, 78].

Initially devised to creat an identical copy (clone) of an animal, the nuclear transfer of somatic cells (NTSC) [81] is considered an alternative procedure for transgenic animals. Applied to genetically transformed donor cells, NTSC enables the direct introduction of the transgene to enucleated mature oocytes. Donor cells are modified *in vitro* by transfecting foreign DNA before the genetic material is inserted and reprogrammed in the embryos. This enables the selection of transformed embryos using conventional molecular biology methods according to the integration site, the copy number, and the expression levels of the recombinant protein. The sex of the founders can also be selected with this methodology because it depends on the individual chosen as the tissue donor [82].

All founders will be transgenic and identical since they come from the same modified cell line. Those features would establish NTSC as the most efficient method in obtaining transgenic mammals as bioreactors, but there are, many limitations: the methodology is complex; it requires expensive equipment and consumables; and the efficiency is very poor in livestock (between 3 and 5% of the births of the cloned embryos implanted). NTSC generated transgenic calf fetuses are lost between days 35 and 60 of pregnancy. Fetal death is 60% higher than embryos generated from *in vitro* fertilization [83].

Pregnancy complications due to the giant fetus syndrome are other frequent causes of progeny loss. Lung anomalies and metabolic deficiencies in either the fetuses or neonates increase the mortality rate. Those problems are associated to deficiencies in the reprogramming of the diploid genome, and are reflected

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Table 2. Chronology of recombinant protein production in the mammary gland of transgenic strains from different species [14]

	Rabbit	Pig	Sheep	Goat	Cow
Gestation period (months)	1	4	5	5	9
Sexual maturity (months)	5	6	8	8	15
Time from introduction of transgene to the beginning of lactation (months)					
Female Founder Lactation induced in puberty	-	-	-	-	-
Natural Lactation	7	16	18	18	33
Male Founder Lactation induced in puberty (daughters)	-	-	22	22	45
Natural Lactation (daughters)	15	28	31	31	57
Average progeny	8	10	1-2	1-2	1
Annual Yield of milk production (L/year)	4-5 [a]	300 [b]	500	800	8000
Production of the recombinant protein /female/year (kg)	0.02	1.5	2.5	4	40

[a] Average of 3 lactations per year

[b] Average of 2 lactations per year

as lethal changes during the development of cloned embryos [84].

Retroviral vector mediated gene transfer of early embryos (lentitransgenesis) was revived in the last five years with the use of lentiviral vectors [85]. Lentiviruses are a family of retroviruses infecting either quiescent or dividing cells [86], they are highly stable and no genetic silencing has been observed thus far [87]. A 60% efficiency in transgenic mice [88], 70% in swine [89], and 100% in cows [90] has been achieved using lentiviruses.

In situ genetic modification of the mammary gland epithelia

There are still many limitations in creating transgenic farm animals expressing heterologous proteins. As described above, microinjection is a low efficiency technique. Moreover, the process from embryo microinjection to producing the transgenic progeny may last years in the most productive species (Table 2), and costs are high [91].

In situ genetic modification of the mammary gland can be a solution to the limitations of transgenesis. The method consists of the direct transfer of DNA to mammary gland epithelial cells from an adult female. Once the genetic modification takes place the recombinant protein is secreted in the milk. The procedure is simplified by the morphology of the mammary gland: the direct communication along the thin ducts from the alveoli to the nipple gives direct access from the outside to the epithelial cells without the need for surgery. A slender catheter can be introduced through the nipple to the galactophore to infuse antibiotics, pharmaceuticals, or genetic material to the mammary gland [17].

In situ genetic modification can be applied to every mammalian species independently of their genetic background, enabling the insertion of multiple expression cassettes. Additionally, there is no need to use tissue specific promoters to restrict the expression to the mammary gland, and finally the time frame from the genetic modification to the final product is greatly reduced [17]. The low efficiency of the genetic modification, and the low productivity due to the transient nature of gene expression, are the main restrictions of this method.

Two main procedures for *in situ* genetic modification of the mammary gland have been developed in recent years: physico-chemical and viral vectors. Several physico-chemical methods for the direct transfection of mammalian epithelia have been used: *in situ* transfection with polyions complexes such as DEAE-dextran and poly-L-lysine [18], receptor mediated endocytosis [19], and gene gun mediated transfection [20, 92]. In some cases the insertion of the transgene and the expression of the recombinant protein have been documented, but the expression levels were so low that any of these methods were considered suitable for large scale production of proteins in the mammary gland.

Retroviral vectors have also been used for the direct introduction of genes into mammary epithelial cells. The ability of these vectors to insert themselves into the host genome was exploited to generate animal models for breast cancer and gene therapy, by directly infusing the virus through the nipple channel [93-95]. A replication incompetent retrovirus, a hybrid between MMLV and Gibbon Leukemia Virus, containing the hGH gene, was injected in the mammary gland of goats with induced lactation. Recombinant hGH was collected in the milk of the treated animals at levels of 60 ng/mL on the first day of lactation, decreasing during the course of the next days reaching a plateau of 12 ng/mL between days 9 and 15 [21]. This study demonstrate the capacity of retroviral vectors for *in situ* transduction of the mammary gland to express recombinant proteins, however the expression levels are still too low to be recommended as practical application [21].

Adenoviral vectors are currently being explored in gene therapy clinical trials [23, 96], and they represent a powerful tool for *in situ* transduction of the mammary gland. After infecting their target cells, adenoviruses do not integrate into the cellular genome, but remain as independent or episomal entities in the nuclei. Therefore, the expression cassettes bearing the genetic information are not exposed to the integration site depending genetic silencing. Multiple copies or independent genes can thereby be introduced into mammary epithelial cells by infusing this viral vector through the nipple channel.

Average expression levels of 1.31 mg/mL for recombinant hGH [93] and 2.2 mg/mL for recombinant hEPO [94] were achieved after adenovirus mediated *in situ* transduction of mouse mammary gland during the final stages of pregnancy. The same procedure yielded 1.35 g/L of hEPO in goat milk [95]. Adenovirus mediated *in situ* transformation of mammary glands renders high concentrations of recombinant proteins, such as hEPO, which is lethal to the host when expressed in transgenic animals [15, 98].

The short period of active expression (10 days in mice and 8 days in goats), and the impossibility of reinfecting the same host with the adenoviral vector, are the main drawbacks of this methodology. The later explained by the strong immunity generated against the viral proteins expressed in the transformed cells [22, 100]. However, the high yield of recombinant protein per animal; the short time frame between the injection of the gene and the production of the protein; and the possibility producing proteins that are toxic for transgenic hosts, reflect the potential of this procedure as a productive system [98, 99].

Glycosylation of recombinant proteins expressed in the mammary gland

The expression of recombinant proteins in the mammary gland tends to modify the glycosylation pattern of native proteins toward specific structures of this tissue [98, 101]. The native plasmatic hAT contains complex diantennary oligosaccharides with lactosamine ramifications (Gal-GlcNAc), disialylated with N-acetyl neuraminic acid (Neu5Ac). However, a change in the N-glycosylation pattern is observed when this protein is expressed in the mammary gland of transgenic goats, is characterized by the presence of uncommon diantennary structures, with lactose-diamine ramifications (GalNAc-GlcNAc), and monosialylated by the binding of N-glycolyl neuraminic acid (Neu5Gc) [101].

Similar modifications to the glycosylation pattern were observed in hEPO expressed in the milk of

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mice and goats [98] (Montesino *et al.*, manuscript in preparation).

This glycosylation pattern, characteristic of the mammary gland, can affect the biological activity of proteins expressed in the milk of genetically modified mammals. Moreover, these modifications in the carbohydrate moiety can trigger the immune response in patients treated with milk derived pharmaceuticals. A clear example is the monosaccharide Neu5Gc or Hanganutziu-Deicher antigen, which is not present in human glycoproteins [103].

The biological function of the modified glycosylation pattern of glycoproteins expressed in the mammary gland is yet to be defined, although it could be related to a mechanism of cellular economy. During lactation, this gland works like a factory, secreting huge amounts of proteins to the lumen of the cistern. The "simplification" of the enzymatic machinery of the endomembrane system responsible for the glycosylation of secreted glycoproteins could facilitate the transport and secretion of those proteins.

Yet, the mammary gland has certain limitations as an expression system: first, a method to achieve an efficient genetic transfer to farm animals has to be developed; and second, the glycosylation pattern of this organ limits the production of certain recombinant proteins having biological activity that depend on multiantennary and polysialylated structures. However, the development of novel methods for genetic transfer such as lentitransgenesis, or *in situ* adenoviral infection of the mammary gland, could become valid alternatives for the wide use of this expression system.

A more thorough knowledge of the molecular events modulating the profile of glycoprotein isoforms in the mammary gland would enable the genetic manipulation of exoglycosidases and glycosyltransferases participating in oligosaccharide synthesis. These modifications would lead to phenotypes that can produce recombinant glycoproteins with the required biological activity.

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