Research Article

Genetically modified animals for use in bio-pharmacology: from research to production

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Abstract

Introduction: In this review, the analysis of technologies for obtaining biologically active proteins from various sources is carried out, and the comparative analysis of technologies for creating producers of biologically active proteins is presented. Special attention is paid to genetically modified animals as bioreactors for the pharmaceutical industry of a new type. The necessity of improving the technology of development transgenic rabbit producers and creating a platform solution for the production of biological products is substantiated.

The advantages of using TrB for the production of recombinant proteins: The main advantages of using TrB are the low cost of obtaining valuable complex therapeutic human proteins in readily accessible fluids, their greater safety relative to proteins isolated directly from human blood, and the greater safety of the activity of the native protein.

The advantages of the mammary gland as a system for the expression of recombinant proteins: The mammary gland is the organ of choice for the expression of valuable recombinant proteins because milk is easy to collect in large volumes.

Methods for obtaining transgenic animals: The modern understanding of the regulation of gene expression and the discovery of new tools for gene editing can increase the efficiency of creating bioreactors for animals and help to obtain high concentrations of the target protein.

The advantages of using rabbits as bioreactors producing recombinant proteins in milk:

The rabbit is a relatively small animal with a short duration of gestation, puberty and optimal size, capable of producing up to 5 liters of milk per year per female, receiving up to 300 grams of the target protein.

Keywords

genetically modified animals, recombinant protein, bio-pharmacology.

Introduction

Research by Hammer et al. (1985) led to the creation of the first transgenic farm animals as producers of recombinant proteins (sheep, rabbits and pigs) (Hammer et al. 1985). Since then, a large number of transgenic animals have been obtained for the production of recombinant proteins for scientific purposes. Transgenic animals are bioreactors (TrB), which have been around for decades and used to produce therapeutic proteins. Currently, some of the proteins produced in these systems are in clinical trials now, and some of them have been already approved for sale. A good example is human factor IX (Atryn), which is used now to treat hemophilia B (Lubon and Palmer 2000) and C1-inhibitor (Ruconest) to prevent Quincke's edema.

This review considers the advantages of using transgenic animals for the production of recombinant proteins, explains the advantages of the mammary gland over other tissues (for the expression of recombinant proteins), describes the technologies for obtaining transgenic animals with an emphasis on animals producing recombinant proteins in milk, includes an experimental analysis of the already used promoters for the expression of recombinant proteins in milk and briefly outlines the current prospects of using transgenic rabbits as bioreactors for the production of recombinant proteins.

The advantages of using TrB for the production of recombinant proteins

Compared to some other systems for the production of recombinant proteins, TrB is the most attractive model because they are powerful tools to meet the growing demand for therapeutic recombinant proteins. The main advantage of using TrB is a low cost of obtaining valuable complex human therapeutic proteins in easily accessible fluids. Using the constructions of tissue-specific expression, large quantities of human recombinant proteins can be expressed and produced in the extracellular space, urine, seminal plasma, milk, and blood of large transgenic animals.

Proteins derived from TrB have a number of advantages compared to proteins from some other sources. First of all, the use of TrB for protein production can reduce the level of contamination of food with such contaminants as: HIV and viral hepatitis, compared to the level of contamination of proteins isolated directly from human blood. It would avoid such tragedies as the infection of hemophilia patients in Europe and Japan with HIV through the drugs based on donated blood.

The second important advantage of obtaining recombinant proteins using transgenic animals is the high safety of the activity of the native protein. The ability of transgenic animals to produce complex biologically active proteins in an efficient and economical way surpasses such capabilities in bacteria, mammalian cells, transgenic plants, and insects (Houdebine 2009). As we know, bacteria are limited in their ability to perform post-translational modifications that are required for many proteins (Balbas 2001; Swartz 2001). It is due to bacteria, because they cannot add carbohydrates to polypeptide chains and cannot generate the required proteins in their mature native structure. TrB mammary glands perform post-translational modifications of protein, such as: carboxylation, glycosylation, and amidation, which are useful for the full biological activity of many proteins (Houdebine 1995; Houdebine 2000). The use of eukaryotic cells (the cultured cells of mammals) in some cases helps overcome these problems. However, the cultivation of animal cells on an industrial scale is a very expensive technology. when compared with chemical synthesis, the production of therapeutically active peptides in the milk of transgenic animals also have a large number of advantages. The scales in which peptides can be chemically synthesized are limited by the considerations of a reactor size, the reagentizing and recycling of reagents, and costs of cleaning. Despite all the costs, TrB demonstrates the financial advantage over cell cultures and some other systems (Table 1, Table 2).

The advantages of the mammary gland as a system for the expression of recombinant proteins

The choice of an expression method of recombinant protein depends on its characteristics and the intended application of recombinant protein (Brondyk 2009). Milk is currently the most developed system for the creation of recombinant proteins from transgenic animal species. Other theoretically possible fluids and tissues, such as blood, egg white, seminal plasma, silk gland, and urine have fundamental disadvantages (Table3).

The mammary gland is the organ of the choice for the expression of valuable recombinant proteins because milk is easy to collect in large volumes. It is reported that foreign proteins are produced in transgenic milk in amounts of several grams per liter. Based on average daily milk expression levels and purification efficiencies, 5,400 cows would be required to produce 100,000 kg of human serum albumin needed worldwide annually; the production of 5,000 kg of α-antitrypsin (α-AT) would require 4,500 sheep; the production of 100 kg of monoclonal antibodies (mAbs) – 100 goats; the production of 75 kg of antithrombin III – 75 goats; and two pigs would be needed for the production of 2 kg of human clotting factor IX (Bösze and Hiripi 2012). In this way, milk is currently the best available bioreactor for the production of valuable recombinant proteins.

Methods for obtaining transgenic animals

A transgenic animal is an animal, whose genome contains an exogenous gene. Although modern methods of mole-

Table 1. Comparison of the different systems used to produce recombinant pharmaceutical proteins.

Indicator	Bacteria	Mammalian cells	Transgenic animals
Production level	++	+	++++
Investment cost	+++++	+	+++
Production cost	+++++	++	++++
Scaling-up ability	+++++	+	++++
Collection	+++++	+++++	++++
Purification	+++	++++	+++
Posttranslational modifications	+	++++	++++
Glycosylation	+	++++	++++
Stability of product	+++++	+++	++++
Contaminant pathogens	+++++	++++	++++
Products on the market	++++	+++++	+++

Note: Table adapted from (Houdebine 2009).

Table 2. Comparative estimated production cost between cell culture and transgenics.

Production scale (Kg/year)	System	Cost (dollars/gram product)
50	Cell culture	147
	Transgenics	20
100	Cell culture	48
	Transgenics	6

Note: Table adapted from (Margawati 2003).

Table 3. Comparison of the different transgenic animal species used to produce recombinant pharmaceutical proteins.

Points to consider	Production systems						
	Milk	Blood	Egg white	Seminal fluid	Urine	Silk cocoon	Others
Production level	+++++	+++++	+++++	+++	++	++	++
Investment cost	+++	+++	+++	+	+	+++	+++
Production cost	++++	++++	++++	++	+	+++++	++++
Scaling-up	++++	++++	++++	++	+	++++	+++
Collection	+++++	++++	+++++	+++	+++	+++++	+++++
Purification	+++	++	+++	++	++	+++	++
Effect on organism	+++	++	+++	+++	+++	++++	++++
Posttranslational modifications	++++	+++++	+++	+++	+++	++	++
Glycosylation	++++	++++	+++	+++	+++	++	++
Contaminant pathogens	+++	++	+++	+++	++	++++	++++
Products on the market	++++	+	++	+	+	++	+

Note: Table adapted from (Houdebine 2009).

cular biology make it possible to humanize animal proteins; currently, a gene transfer approach is used to create animal producers.

Method and procedure for obtaining foreign genes

The preparation of the transgene is the first step in the technology of transferring foreign genes. It is carried out by using the conventional DNA techniques, by cutting and ligating the DNA fragments, which results in the recombinant DNA (Huldiner 1996; Blanchard and Kelly 2005; Chrenek et al. 2010).

Typical transgenes contain the nucleotide sequences of the gene of interest with all the components that are necessary for efficient expression, including a promoter, artificial introns, and 3 'non-coding regions (Acquaah 2004). The transgene can be expressed in many tissues of a transgenic animal not only by using a promoter from a constitutively expressed gene, but also in certain tissue by

tissue-specific promoters, such as: a P2 adipocyte promoter (fat cells), a myosin light-chain promoter (muscle), an amylase promoter (acinar-pancreas), and an insulin promoter (islet beta cells) (Huldiner 1996).

The main method for obtaining a foreign gene of interest is a recombinant DNA technology, which includes three stages: 1) isolating the gene of interest; 2) cloning the target gene; 3) inserting the cloned gene into the host cell, most often, as a coding sequence (Bihon and Ayalew 2019).

Cloning is the process of introducing a foreign gene (called an insert) into vector (called plasmid). The legated ends of the vector and target DNA must be produced by the same enzyme – restriction endonuclease, in order to complement the insertions of the cut DNA into the vector and to be legated by the DNA ligase enzyme that covalently connects the sugar-phosphate backbone of the bases (Wilmut et al. 1997; Eghbalsaied et al. 2013). The vector containing the cloned gene is imbedded into the bacterial host cell for preparative plasmid production. The host cell containing the vector is called transformed cell (Blanchard and Kelly 2005). The transformation of bacteria with DNA plasmid of is carried out in several

ways: a) heat stroke (heating a solution containing cold calcium chloride with plasmids and normal bacteria at 42°C for 2-5 minutes, which increases the permeability of bacterial membranes for plasmids (Wilmut et al. 1997); b) electroporation (the use of a high voltage pulse temporarily destroys the host cell membrane, which allows the vector to enter the cell (Acquaah 2004). Later, the cloned transgenic cassette can be used as part of a plasmid (for insertion by homologous recombination or homologous repair) or in a linearized form (without a bacterial component for random insertion).

The insertion of a cloned gene into an animal cell can be carried out using different types of techniques:

- by using viruses (the ability of viruses to infect a susceptible cell and replicate made it possible to incorporate the desired DNA sequence into target cells (McKee et al. 1998);
- by using a gene gun (embedding foreign DNA segments into the host cell by firing gold particles coated with these DNA segments (Whitelaw and Sang 2005));
- by using microinjection (embedding a foreign DNA directly into the core of the host cell using a thin needle under a microscope (Houdebine 2002));
- by using liposomes (a small a membrane-bound vesicle (liposome) can contain vectors and transfer foreign DNA when fusing with a cell or nuclear membrane of the host cell (Whitelaw and Sang 2005)).

Tissue-specific promoters for the expression of recombinant proteins in milk

The key determinant providing the tissue specificity of the expression of a transgene is promoter (Shepelev et al. 2008). For the production of recombinant proteins in the mammary gland, a number of promoters of genes encoding milk proteins have been successfully used.

Promoters that make it possible to obtain the target protein in milk at a high level (up to dozen of grams per liter of milk) are the promoters of the following genes: goat β-casein, cow β-casein, cow α-s1-casein, rabbit whey acidic protein (WAP), human α-lactalbumin and sheep β-lactoglobulin.

The examples of the promoters used for obtaining recombinant proteins in milk are shown in Table 4.

Vectors used in the creation of transgenic animals

The expression of recombinant proteins in the milk of transgenic animals is controlled by the promoter regions of genes involved in the generation of specific milk proteins, such as: caseins (α, β, γ and κ), β-lactoglobulin, and α-lactalbumin.

The promoter region, located at the 5'-UTR (untranslated region) of the gene of interest, including tissue-speci-

fic enhancers for the mammary gland and the first non-coding exons and introns, has different sizes depending on the promoter used. Thus, the bovine α-lactalbumin and β-lactoglobulin promoters are used with a gene length of approximately 2.0 kbp and 2.8 kbp, respectively, and the casein promoters (αS1, αS2 and β) are typically used for genes of 3.1 kbp-14.2 kbp long (Bleck et al. 1998; Hyttinen et al. 1998).

To guarantee a high level and position-independent expression of the transgene, other regulatory elements (such as insulators) are inserted into the vector construct above the 5'-UTR. One of the examples of this is commercial vector pBC1, which includes insulators of the chicken β-globulin gene cluster (Invitrogen - Thermo Fisher Scientific).

The first noncoding exons and introns in the 3'-region of the 5'-UTR are different as they position mRNA in ribosomes for the start of translation and/or contain regulatory elements that can enhance the transcription of the gene (Rijinkels et al. 1998; Naruse et al. 2006). As a rule, these noncoding exons and introns are derived not only from milk protein genes, but can also be synthesized from the structural gene used in the transgene construct.

Most frequently, the gene of interest is inserted into the vector below the 5'-UTR as a cDNA sequence, either as a complete gene sequence (containing exons and introns), or as a mini-gene containing only a part of the instrons or even artificial introns. For recombinant proteins that are secreted with milk, it is necessary to include a signal peptide sequence, which, as a rule, is derived from the used transgene (in case the secreted protein is produced).

The pBC1 vector is universal and accumulated for various proteins in high concentrations in milk (see Table 5). It is necessary to take into consideration that there is no signal peptide in its sequence and it must be cloned within the transgene. This vector can be used for production of recombinant proteins in milk, which not secreted naturally secreted.

The 3'-UTR is inserted downstream of the transgene and can be synthesized not only from the milk gene used in the 5'-UTR, but also from the structural gene used in the transgene, or from another gene. This region provides the effective termination of transcription and the formation of stable mRNA encoding the target protein. In certain cases, the length of this region can reach 7.1 kbp, as in the vector pBC1 (Invitrogen), which includes introns, exons and the polyadenylation signal from goat β-casein. 3'-UTR may contain regulatory elements that improve transcription. The polyadenylation signal from bovine growth hormone, which is about 1 kbp, is 3'-UTR, which is usually inserted into expression vectors (Naruse et al. 2006).

To obtain transgenic farm animals, three classes of vectors are mainly used: bacterial artificial chromosomes (BAC), plasmids, and lentiviruses. The choice of a vector depends on the length of transgene. BAC vectors, which can contain DNA sequences up to 300 kbp in length are the main vectors. They are used when it is necessary to insert long sequences into the host genome, such as: com-

Table 4. Recombinant proteins produced from transgenic animals.

Protein	Promoter	Species	Other elements	Creation method	Level of production	Reference
Growth hormone	CMV promoter	mice	AdEasy adenoviral vector system	adenovirus infusion	up to 301 mkg/ml	Sánchez et al. 2004
Human α-glucosidase	bovine αs1-casein	rabbits			8 g/L	Bijvoet et al. 1999
	N-acetyl-β-glucosaminyl					Park et al. 2006
Alpha-fetoprotein	beta-casein	goats		nuclear transfer	1.09 mg/ml	Piedrahita et al. 1998
Antithrombin	goat beta-casein	goats	The cDNA coding for hAT-III (striped box) replaces the coding region of goat beta-casein, a milk-specific gene. This human cDNA is flanked by the promoter (6.2 kb) and by untranslated caprine beta-casein 3' sequences and downstream elements (7.2 kb). Black boxes indicate the noncoding exons of goat beta-casein gene.	p/m	3.5 mg/ml	Li et al. 2013
Antitransferrin receptor antibody-RNase fusion protein	beta-casein promoter	mice		p/m	0.8 g/L	Niavarani et al.2005
Bovine Follicle-Stimulating Hormone	bovine alpha-s1 casein	rabbits	cDN	p/m	5,354 mg/ml	Coulibaly et al. 2002
Coagulation factor IX	beta-casein	goats	cDNA, pBC1 (Invitrogen)	nuclear transfer using transfected fetal fibroblast cells	+	Amiri et al. 2013
Endogenous whey acidic protein (WAP) gene	rabbit WAP promoter	rabbits	rabbit WAP promoter and 39 flanking sequences	p/m	too low	Aguirre et al. 1998
Erythropoietin	rabbit WAP	mice	genomic	p/m	1,068 mIU/ml	Monzani et al. 2011
Erythropoietin	mouse WAP promoter	pigs	Human EPO genomic DNA was cloned using the mouseWAP promoter as a regulatory controller, and the SV40 T antigen poly-A as a poly-adenylation signal	p/m	877.9±92.8 IU/1 ml	Park 2007
Erythropoietin	rabbit WAP promoter	rabbits	rabbit WAP promoter and 3' flanking sequences	p/m	too low	Whitelaw and Sang 2005
Erythropoietin	rabbit WAP promoter	mice	cDNA under the 5' and 3' regulatory sequences of the rabbit whey acidicic protein gene	p/m	0.01 mg/L	Aguirre et al. 1998
Erythropoietin	rabbit WAP promoter	rabbits	cDNA under the 5' and 3' regulatory sequences of the rabbit whey acidicic protein gene	p/m	0.0003 mg/L	Rutovitz and Mayer 2002
hGH	CMV promoter	goats	genomic DNA	adenovirus infusion	0.311 mg/ml	Heyman et al. 1998
Human alpha antitrypsin	ovine beta-lactoglobulin promoter	sheep	The AATB construct comprises -4.0 kb of the 5' end of the ovine BLG clone SS1 (11, 12) fused to a minigene encoding human a1AT. Thick line, 5' BLG sequences; open box, BLG exon 1 sequences; hatched boxes, a1AT exons; thin lines, a1AT introns and 3' flanking regions. The position of the BLG TATA box and also the a1AT initiation codon, stop codon, and polyadenylylation site are shown.	p/m	63 grams/L	Carver et al. 1992
Human alpha antitrypsin	ovine beta-lactoglobulin promoter	mice	The AATB construct	p/m	7 g/L	Archibald et al. 1990
Human butyryl-cholinesterase	goat beta-casein promoter	goat	globin gene insulator; a 6.7-kb goat beta-casein gene promoter fragment, including the signal sequence in exon 2; a 1.7-kb human BChE cDNA fragment amplified from a huBChE cDNA clone (American Type Culture Collection (ATCC), Manassas, VA; catalog no. 65726); and a 6.1-kb fragment consisting of the -casein coding and 3 noncoding regions	p/m	5 g/L	Baldassarre et al. 2008; Baldassarre et al. 2008; Huang et al. 2008; Huldiner 1996
Human butyryl-cholinesterase	goat beta-casein promoter	mice	-globin gene insulator; a 6.7-kb goat-casein gene promoter fragment, including the signal sequence in exon 2; a 1.7-kb human BChE cDNA fragment amplified from a huBChE cDNA clone (American Type Culture Collection (ATCC), Manassas, VA; catalog no. 65726); and a 6.1-kb fragment consisting of the -casein coding and 3 noncoding regions	p/m	1.4 g/L	Huang et al. 2008
Human calcitonin	ovine beta-lactoglobulin promoter	mice	cDNA	p/m	+	Niemann et al. 2012

Protein	Promoter	Species	Other elements	Creation method	Level of production	Reference
Human factor IX	b-casein promoter	mice	cDNA, pBC1 (Invitrogen, Carlsbad, CA, USA)	p/m	3% total soluble protein	Loftus and Rogers 1997
Human factor VIII	murine whey acidic protein promoter (mWAP)	rabbits	cDNA	p/m	0.599 IU/ml	Chrenek et al. 2007; Hofmann et al. 2004
Human growth hormone	CMV promoter	goats	AdEasy adenoviral vector system	adenovirus infusion	Up to 0.3 mg/ml	Sánchez et al. 2004
Human lactoferrin	CMV promoter	rabbits	cDNA	adenovirus infusion	2.3 mg/ml	Schmidhauser et al. 1990
Human lactoferrin	bovine alphaS1 casein	mice	hLF cDNA	p/m	1.7 mg/ml	Jongen et al. 2007; Ramos et al. 2011
Human lactoferrin	bovine alphaS1 casein	mice	genomic hLF	p/m	3.8 mg/ml	Jongen et al. 2007; Ramos et al. 2011
Human lactoferrin	including 90-kb and 30-kb 59 and 39 flanking regions	cattle	hLF genomic	fibroblast cells microinjection	2.56+-0.2 g/L) and 3.46+-0.4 g/L	Yu et al. 2012
Human lactoferrin	beta-casein promoter	mice	cDNA	p/m	0,2 mg/ml	Koles et al.2004
Human lactoferrin	goat beta-casein promoter	goats	cDNA, pBC1 (Invitrogen, Carlsbad, CA, USA)	p/m	0.765 mg/ml	Zhang et al.2009
Human protein C	mouse WAP promoter	rabbits	genomic	p/m	0.56 µg/ml	Chrenek et al. 2002
Human serum albumin	goat beta-casein promoter	mice	cDNA, SV40 polyadenylation signals	p/m	0.4 mg/ml	Yang et al. 2012
Human α-lactalbumin		cows	genomic	nuclear transfer	1.55 g/L	Wang et al. 2013
Spider silk	WAP	goats	5',3' WAP	p/m	-	Baldassarre et al. 2003
Growth hormone	rat WAP promoter	rabbits		p/m	10 µg/ml	Lipinski et al. 2012; Lisauskas et al. 2008
Nerve growth factor beta	CMV	goats	Recombinant replication-defective adenovirus	adenovirus infusion	196.8 mg/L	Ikawa et al. 1995
Growth hormone	WAP promoter	mice	7.2 kb genomic mWAP gene	p/m	4.77mkg/ml	Toledo et al. 2006; Whitelaw and Sang 2005
Factor IX	bovine ,B-lactoglobulin	mice	cDNA	p/m	120 mkg/ml	Zhang et al. 2008
Factor IX	bovine ,B-lactoglobulin	sheep	cDNA	p/m	1 mkg/ml	Zhang et al. 2008
Factor VIII made with von Willebrand factor	WAP (whey acidic protein) promoter	mice	ahead of the 1.7 kbp of mouse WAP 3' UTR containing the coding of the polyadenylation signal	p/m	200 ng/ml	Platenburg et al. 1994
Anti-HAV antibody	goat beta-casein promoter	mice	pBC1, H chain (HC) and L chain (LC) genes of a human IgG1 mAb against HAV were amplified by PCR from plasmids pHAVH3 and pHAVL3	p/m	32.2 mg/ml	Kim et al. 1997
Erythropoietin	CMV	goats	cDNA	adenovirus infusion	2 g/L	Turchiano et al. 2014
Interferon alpha 2b	cow beta-casein	mice	IFNa-2b, Jersey Cow beta-casein 5' regulation fragment and 3' regulation fragments were designed and synthesized with sequences from Genbank (Accession No.: AY255 838.1 and JN559864)	p/m	29.71 mkg/L	Limonta et al. 1995
Lactoferrin	bovine as1-casein	cows		microinjection	1.5–2.0 g/L	Berkel et al. 2002
	bovine as1-casein	goats		SCNT	Data not available	An et al. 2012
	goat b-casein (pbc1)	goats		SCNT	30 g/L rhLF	Yull et al. 1995
	goat b-casein (pbc1)	goats		microinjection	10 g/L hLf	Gordon 1996.
Lysozyme	bovine b-casein	cows		SCNT	0.0259 g/L	Yang et al. 2008
	bovine as1-casein	goats		microinjection	0.270 g/L	Margawat 2003
Human C1 inhibitor		rabbits				Kumar et al. 2001
Human extracellular SOD	Murine WAP	mice			3 g/L	Lipiński et al. 2003
Human IL-2	rabbit β-casein	rabbits			0.0005 g/L	Brem et al. 1994
Human insulin-like growth factor	bovine αs1-casein	rabbits			1 g/L	Wright et al. 1991
	bovine αs1-casein	rabbits			0.3 g/L	Kim et al. 1997
	bovine αs1-casein	rabbits			0.678 g/L	Coulibaly et al. 1999
Human tPA	bovine αs1-casein	rabbits			0.00005 g/L	Brem et al. 1995
Bovine chymosin	bovine αs1-casein	rabbits			1.5 g/L	Coulibaly et al. 2002
Equine chorionic gonadotropin	rabbit WAP	rabbits			0.022 g/L	McKee et al. 1998
Human interferon beta		rabbits			$2.2–7.2\times10^7$ IU/L	Yang et al. 2011

Notes: FSH: follicle stimulating hormone; IL-2: interleukin-2; NA: not available; SOD: superoxide dismutase; TNAP: tissue-non-specific alkaline phosphatase; tPA: tissue plasminogen activator; WAP: whey acidic protein.

plete genes and/or long 5 'and 3' UTRs. Using this class of vectors, transgenic cattle have been obtained (Yang et al. 2008).

Most of the plasmid vectors are from 3 kbp to 5 kbp in length. Plasmids allow inserting a sequence of DNA up to 20 kbp in length, and have been used to obtain transgenic animals (Kaushik et al. 2014). The commercial vector pBC1 with a DNA of 21.6 kbp long has been also used to obtain transgenic cattle (Yang et al. 2011).

The lentiviral vectors have a limited ability to insert DNA. The ideal size of the genetic material for being packaged in lentiviral elements is approximately 10 kbp in length. It is possible to produce lentiviruses with a proviral length of more than 18 kbp, but with an increase in the vector length, the virus titer significantly decreases. The decrease in titer appears at the level of viral encapsulation and/or because of the nuclear export restrictions of proviral RNA, but not at the level of packaging into lentiviral elements (Kumar et al. 2001). When creating lentiviral vectors, it is very important to limit the length of a vector. Virus titer can be increased by using cDNA or incomplete sequences of genes, including a shorter mammary specific promoter region and a polyadenylation signal, such as an α-lactalbumin promoter (Bleck et al. 1998) and a poly-A signal (Naruse et al. 2006) of bovine growth hormone. These modifications can increase the viral titer obtained from lentiviral vectors. The main viral elements that are the part of the lentiviral vector are the following: LTR, packaging signal, central DNA fragment and WPRE (Woodchuck Posttranscriptional Regulatory Element of woodchuck hepatitis virus). They have the size of approximately of 1.5 kbp; therefore, the total length of the transgene 5'- and 3'- UTR should not exceed 8.5 kbp (Hofmann et al. 2004).

Lentiviral vectors have been successfully used in transgenic mice for the expression of recombinant proteins in milk (Ramos et al. 2011), and for the expression of green fluorescent protein (GFP) in cows (Hofmann et al. 2004). In contrast to plasmid vectors, there are no commercial lentiviral vectors for the expression of recombinant proteins in milk. Although some of the authors have modified the commercial lentiviral vectors for this purpose (Ramos et al. 2011; Monzani et al. 2013; Monzani et al. 2015) (Fig. 1). Currently, there is success in the creation of transgenic cattle for the production of recombinant proteins in milk using lentiviral vectors (Monzani et al. 2013). It is expected that lentiviral vectors have the advantages over the other vectors because of a high percentage of transgene expression. Such a high expression of the transgene is conditioned by the integration of the lentivirus primarily into active transcriptional units in the host genome (Park 2007).

The use of plasmid vectors based on the transposon system has become an attractive alternative for the production of transgenic animals. Sleeping Beauty, piggyback and Tol2 transposons have been developed as gene transfer methods for vertebrates; the commercial vectors are available (Addgene). The gene of interest is cloned between the inverted terminal transposon repeats (ITRs), which carry the binding sites for the transposase enzyme. The insertion of the gene is carried out by the addition of the transposase enzyme from the second expression plasmid or a synthetic mRNA transposase. This system is capable to transpose transgenes up to 18 kbp in length, but the effectiveness of transposition is significantly reduced
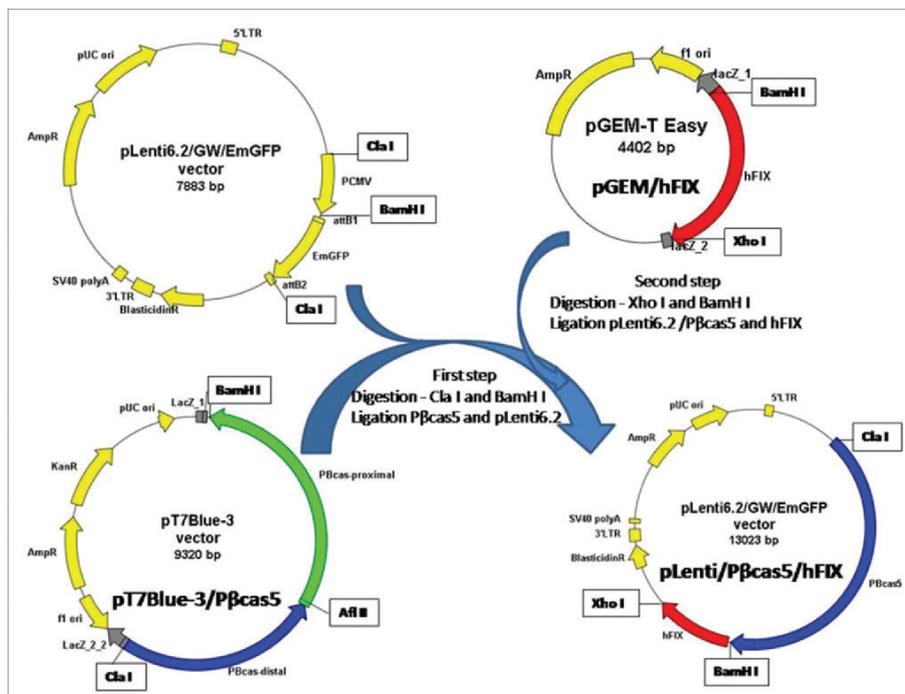

Figure 1. Lentiviral vector construct for the production of recombinant proteins with milk. Figure adapted from (Monzani et al. 2013).

with an increase in the size of the DNA strand, which probably depends on a type of cells used (Turchiano et al. 2014). Also the transposon plasmids for the production of transgenic pigs and rabbits have been developed (Ivics at al. 2014a; 2014b).

The evaluation of the constructed vectors

Before the generation of transgenic animals as bioreactors, it is important to evaluate the construction of the transgene product, its ability to respond to hormonal induction, and its ability to express the recombinant protein of interest. The production of the recombinant protein in the milk of transgenic cattle requires a long period of time from birth of an animal to the first cycle of lactation. Therefore, it is important to evaluate the efficiency of the vector in the production of the recombinant protein. The cost of producing transgenic cattle can amount from $300,000 to $500,000 per animal, using the pronucleus microinjection method, or tens of thousands of dollars when using lentiviral gene transfer into the perivitelline space of bovine oocytes (Hofmann et al. 2004). It is necessary to optimize the use of time and resources when creating transgenic cattle. The initial and important step in this process is the evaluation of the expression of the vector.

The production of transgenic mice has been successfully carried out using various methods. These animals are used as the main model of animal transgenesis because of their high reproductive potential and a short period of time required to reach sexual maturity, mating and lactation. The first transgenic mice were obtained in 1980 by microinjection of recombinant DNA into the pronuclei fertilized eggs (Gordon et al. 1980). To obtain the first expression of the recombinant protein in mouse milk, the β-lactoglobulin gene of sheep was microinjected into fertilized oocytes (Simons et al. 1987). In 1990, transgenic mice were chosen as a model to study the expression of a recombinant protein in milk (Aguirre et al. 1998). Though transgenic mice are the best model for evaluating recombinant protein in milk, their creation depends on the qualification of the technical attendants for microinjection and embryo transplantation, as well as on the level of biosafety of the vivarium.

The biology of the mammary gland can also be reproduced using primary mammary epithelial cells (MECs), separated from the extracellular matrix, which form the monolayer cell cultures. In addition to cell-cell and cell-substrate interaction, MEC cultures synthesize and eliminate a large amount of milk proteins. The stages of growth and differentiation are controlled by various proteins and steroid hormones (Schmidhauser et al. 1990). These characteristics make MEC cultures an attractive system for replicating mammary gland biology. In 1998, Ilan et al. (1998) suggested that MEC cultures could be an alternative to transgenic mice models to assess the potential gene constructs for expression in the mammary gland of transgenic farm animals. Some other studies have used MEC cultures to assess the expression of the established vectors (Monzani et al. 2011; Monzani et al. 2013; Kaushik et al. 2014; Monzani et al. 2015). Although, transgenic mice can be the best model for transgenic farm animals, MEC culture is a strategy that is easier to implement than the creation of transgenic mice. The MEC culture demonstrates certain advantages, such as lower cost and no necessity for a vivarium to create and maintain transgenic mice.

Strategies for creating transgenic animals. The methods of transgenesis

Effective methods of introducing foreign DNA must exclude chemical or physical mutagenesis, because of the foreign gene that must be in a relatively stable form. The methods of transgenesis can be gene transfer via gonads, microinjection, and the use of stem cells, sperm vectors, somatic cells, and retroviruses (Miao 2012).

Sperm-mediated gene transfer method, based on the inherent ability of sperm to bind and internalize exogenous DNA molecules and transfer them to the oocyte during fertilization, is used as an alternative to microinjection (Rutovitz and Mayer 2002; Houdebine 2002; Miao 2012). Some of the advantages over other methods are higher efficiency, lower cost and the simplicity of use, no need for either embryo manipulation or expensive equipment. However, this method leads to extremely variable results in different animal species (Chrenek et al. 2010).

Using the sperm-mediated gene transfer (SMGT), transgenic pigs were produced (firstly, sperm was incubated with plasmid DNA) (Lavitrano et al. 2002). However, this method showed the limited integration of exogenous DNA into the host genome. The improved modifications of the sperm transformation method were developed, including the use of lentiviruses (Zhang et al. 2012). It was initially reported that using bovine sperm to produce blastocysts in vitro SMGT is ineffective (Eghbalsaied et al. 2013). The modification of this method by using spermatogonial stem cells offers a new method for obtaining transgenic animals.

Transgenesis via gonads is carried out using transfection of spermatogonia in situ by introducing transgenes into the seminiferous tubules or by in vitro transfection of germ cell progenitors followed by transplantation into the testicles of the host. The cells produced from the testis, transplanted into the testis of infertile males, colonize the testicles of the host, generate sperm and start the production of offspring (Chrenek et al. 2010). The obtained transgenic spermatozoid can be used in in vitro fertilization methods (IVF) or in intracytoplasmic sperm injections (ICSI). This method was successfully used in mice (Kanatsu-Shinohara 2008), but it has not been developed

for the conditions of in vitro cultivation of large animals (Niemann et al. 2012).

Transgenesis through fertilized eggs or embryos can be carried out by the microinjection of DNA and the transfer gene using retroviruses (adenoviruses) or stem cells.

DNA microinjection is a microsurgical procedure performed on a single cell to introduce a foreign DNA into the cytoplasm or nucleus. In this procedure, the injection is carried out into the male pronucleus of the embryos because of their large size (Houdebine 2002; Chrenek et al. 2010). Until recently, it was the only successful method of obtaining genetically modified livestock. However, this method is not effective because only 3-5% of the injected embryos received the transgene (Markkula and Huhtaniemi 1996; Whitelaw and Sang 2005; Miao 2012). Moreover, it is necessary to transfer a large number of microinjected embryos for the production of several transgenic offspring. One embryo can be injected with 200 to 500 copies of the gene construct (Chrenek et al. 2010). In such a strategy, the DNA fragment that contains the transgene is randomly inserted into the genome of the recipient organism during the natural processes of formation by genomic DNA breaks and their reparation. Linear DNA fragments containing the transgene, both intact and those that have undergone nonspecific cleavage in the cell, can be inserted into different parts of the genome. Also, the number of transgene copies in the insertion site varies widely. Moreover, the integration process can occur at different stages of embryonic development, which leads to mosaicism of primary transgenic animals, that is to the presence of the transgene not in all cells of the body. The presence of the transgene in the genome of germ cells and the transfer of genomic DNA is necessary to obtain a line of animals carrying the transgene (Markkula and Huhtaniemi 1996; Rutovitz and Mayer 2002; Blanchard and Kelly 2005; Shepelev et al. 2008). The obtained embryos containing the transgene must be cultured in vitro within 24 hours and be implanted in a "pseudopregnant" surrogate mother (Chrenek et al. 2010). The use of transgenic technologies in livestock is limited. That is related to the long gestation period, high maintenance costs, mainly due to non-transgenic pregnancies and transgenic animals that expresses recombinant protein (Niemann et al. 2012). Despite all these difficulties, the pronuclear DNA microinjection has been successfully used to produce transgenic cattle (Berkel et al. 2002).

The use of embryonic stem cells (ESCs) and embryonic germ cells (EGCs) is a promising method for the generation of transgenic animals. The meaning of this method is in injecting the desired gene into pluripotent stem cells, followed by their embedding into the blastocyst cavity (Robertson 1991; Miao 2012), as a result of which chimeric animals are born (Gordon 1996; Markkula and Huhtaniemi 1996; Miao 2012). This method requires an adult transgenic animal to check for the presence of the desired transgene, thus, making the testing at the cell stage impossible (Evans and Kaufman 1981; Margawati 2003).

As an alternative, modified ESCs were used in sandwich aggregation with unviable diplokaryotic morulae to obtain transgenic mice completely derived from ESCs (Hadjantonakis et al. 2002). These techniques have been used for cattle and chimeric animals have been created (Iwasaki et al. 2000; Furusawa et al. 2013).

However, the effort of creation of pluripotent ESCs from bovine embryos has failed. Obtaining the transgenic bovine chimeric offspring is only possible by using the nuclear transfer method, when transgenic embryonic stem cells were obtained from modified fetal bovine fibroblasts (Cibelli et al. 1998). Transgenic porcine chimeras were generated using colonies obtained from primordial germ cells (PGCs) (Piedrahita et al. 1998). PGCs can become ESCs by the differentiation in vitro or in vivo (Piedrahita 1998). Induced pluripotent stem cells (iPSCs) are stem cells that can be obtained by reprogramming cells using gene transduction or the treatment of somatic cells with recombinant proteins. Studies have shown that PGCs obtained by in vitro differentiation of iPSCs produce functional gametes as well as healthy offspring (Imamura et al. 2014). This is a promising approach, because modified somatic cells can be used to obtain iPSCs using a methodology that does not integrate the OCT4, SOX2, c-MYC and KLF4 genes into the genome of previously modified somatic cells. The iPSCs carrying the transgene of interest can be used to generate PGCs that can be differentiated into gametes by in vivo or in vitro methods and then injected into the blastocoel of the blastocyst to generate chimeras.

Another method of transgenesis is **the transfer of the nucleus of a somatic cell**. This method includes the transfer of the nucleus of a somatic cell into the cytoplasm of an enucleated ovum to reprogram its cytoplasmic factors with the formation of a zygote (Wilmut and Whitelaw 1994; Campbell et al. 1996; McKee et al. 1998, Ball and Peters 2004). Subsequently, the zygote must be artificially placed in the uterus of the surrogate mother (Heyman et al. 1998; Denning et al. 2001). This method is successfully used for the transgenesis of various animal species, except humans (Heyman et al. 1998; McCreath et al. 2000; Kuroiwa et al. 2002).

Virus-mediated gene transfer is another promising methodology for obtaining transgenic animals. Retroviruses (adenoviruses) are the RNA viruses with a reverse transcriptase enzyme that produces DNA from RNA (Blanchard and Kelly 2005). Viral transduction is carried out by injection of viral elements into the perivitelline space of oocytes or zygotes, or by removing the zona pellucida and culturing in a medium containing the virus. Viruses are capable to integrate into DNAs of the host and copy their own proteins (Rutovitz and Mayer 2002; Whitelaw and Sang 2005; Chrenek 2010). It leads to the formation of chimeras because not all cells can receive the transgene (Blanchard and Kelly 2005; Miao 2012). Final homozygous transgenic animals can be obtained only after 5 generations as a result of inbreeding (Chrenek et al. 2010). Lentiviral gene transfer into oocytes using the

commercial FUGW vector has been successfully used to obtain transgenic farm animals (Hofmann et al. 2004). This methodology was established more than twenty years ago. Although it seems to be perspective for obtaining transgenic farm animals, until now it has not been used for the efficient production of biopharmaceuticals in milk. The main disadvantages of this method are the limitation on the size of the injected DNA, the inability to replicate in early embryonic cells, the lower efficiency when compared to other methods that is combined with the risk of the formation of new pathogens (Rutovitz and Mayer 2002).

Transgenic markers and transgenesis screening

To check the incorporation of the transgene into cells, it is possible to include markers of visual, positive or negative selection. It increases the efficiency of transgenesis by identifying true transgenes (Blanchard and Kelly 2005).

β-galactosidase, firefly luciferase, secreted placental alkaline phosphatase and green fluorescent protein (GFP) are the currently available transgenic markers. Even before embryo implantation, GFP is an ideal marker for the selection of transgenic embryos after gene transfer (Ikawa et al. 1995).

Enzymes that inactivate aminoglycoside antibiotics, such as neomycin or kanamycin, are widely common positive selection markers used to select transgenic cells, which is especially important in molecular biology (when integrating the construction into the culture of genomic cells according to the mechanism of homologous recombination), when the efficiency of transfer of gene constructs is low and a pool of many cells is required for transfection (Howard et al. 2001).

A PCR analysis is not always effective in relation to the primary transgenic animals, especially in the case of a high degree of mosaicism. In this case, it is necessary to analyze the offspring or the methods needed to enhance the signal without increasing the risk of nonspecific reactions (Bihon and Ayalew 2019).

Southern blotting is the most widely used method of testing for a transgene in host animals. It includes segregation of DNAs by means of restriction enzymes and an analysis by agarose gel electrophoresis. The DNA is then denatured with a strong base or acid and applied to a membrane, followed by hybridization with a DNA probe to the gene of interest. If the gene of interest (or its fragment) is present, the blotter membrane captures the probe and illuminates the gene (Blanchard and Kelly 2005).

Western blotting is used to find a transgenic protein produced by animals. SDS-polyacrylamide gel is used for electrophoresis. If the protein is small, it moves to the positive pole and is applied to the nitrocellulose membrane. It is then incubated with the primary antibody, which adheres to the transgenic protein to form a protein-antibody complex. Visualization is carried out by hybridization with a secondary antibody that produces a color. The presence of transgenic protein forms a dark band on the film (Khalsa et al. 2000; Bihon and Ayalew 2019).

Another example for determining the presence of transgenic proteins is enzyme immunoassay by determining the amount of proteins in serum, blood and urine. If the sample contains a transgenic protein, it reacts with the antigen and gets stained (Blanchard and Kelly 2005). DNA hybridization and PCR are also capable to detect transgenes.

Application of CRISPR-Cas systems in animal transgenesis

The revolutionary changes have occurred in the field of modification over the last decade, due to the possibility of highly efficient directed genome editing and a significant simplification of this technology after the discovery of the CRISPR/Cas9 system (Shepelev 2008). Protein Cas9 is RNA-dependent DNA endonuclease, a unique enzyme that introduces double-stranded breaks in DNA, which is in a complex with a protein and programmed by the molecule of RNA (Barrangou and van der Oost 2013).

Using the CRISPR/Cas9 technology, it is possible to create transgenic animals with the integration of a transgene into a given place of the genome, which, with the use of homologous repair, determines the controlled number of copies of the transgene. In particular, one of the most promising approaches to the creation of animals producing recombinant proteins in milk is the targeted integration of a transgene using the CRISPR/Cas9 system into the region of genes coding milk proteins in such a way that the expression of the transgene is controlled by the endogenous regulatory sequences of the recipient animal. The use of such technologies will simplify and standardize technologies for obtaining transgenic animal producers of recombinant proteins. It will make the transgenesis process more efficient and reduce the cost of obtaining economically valuable transgenic animals (Shepelev 2008).

The advantages of using rabbits as bioreactors for producing recombinant proteins in milk

The mammary glands of transgenic animals are the best available bioreactors because they can express many interesting recombinant proteins with high efficiency and full biological activity. Currently, it has led to the popularity of this technology and its successful use in various animals.

Transgenic mice can only be used as a predictive model for utility assessment of expression constructs and studying the properties of expressed proteins. However, they currently cannot accommodate commercial needs,

because they are unfit as bioreactors for the production of large quantities of recombinant proteins.

The criteria for selecting the best suitable producer animal species are based on the quantity of proteins required per year, in addition to the other factors, such as the prelactation period of animals, their maintenance and the amount of milk produced. The features of milk secretion in farm animals are given in Table 5.

When compared to some other kinds of large farm animals, the rabbit is a relatively small animal with a short gestation period, puberty period and an optimal size. Rabbits produce the desired protein only 8 months after starting the injection of transgene (Table 5).

Rabbit breeding can be carried out under the certain barrier conditions that are free from pathogens. There are no identified prion diseases of rabbits, unlike cattle (Loftus and Rogers 1997). Therefore, the transgenic rabbit system is safe for the production of therapeutic proteins.

Another selection criteria is the quality composition of rabbit milk. The protein concentration in rabbit milk is 14% compared to 5% in cow milk. A lactating female rabbit can produce 170-220 g of milk per day and give up to 10 kg of milk per year in semi-automatic hygienic milking conditions (Bosze et al. 2003). The expression levels of transgenic protein can run to 20 grams per liter. The rabbit system is ideal for the production of up to 50 kg of protein per year for small and medium-sized facilities. Thus, the transgenic rabbit system is a cheaper alternative to livestock, because rabbits are smaller and cheaper to keep.

In rabbits, caseins are the main proteins that make up milk. The concentration of caseins in rabbit milk is more than 60 mg/ml, while the concentration of whey acidic proteins (WAP) in milk is 15 mg/ml. Therefore, the aS1- and b-casein promoters and the WAP promoter, along with the b-lactoglobulin promoter, are widely used to drive tissue-specific expression of recombinant proteins in transgenic rabbits. Recombinant human proteins produced by transgenic rabbits include the human a1-antitrypsin, interleukin-2, tPA, erythropoietin, insulin-like growth factor-1, extracellular superoxide dismutase, growth hormone, aglucosidase, miacalcin, chorionic gonadotropin, protein C, and chymosin (Table 5).

It should be noted that transgenic rabbits or the recombinant proteins that they produce are not always functional or practical because of their low expression levels. However, these findings have laid the foundations for possible technological developments that will allow large quantities of human therapeutic proteins to be produced and used in future.

The production of transgenic rabbits is an advantageous technique for the production of recombinant proteins. In this connection, there have been developed the models in which rabbits are used as fast bioreactors for the production of therapeutic proteins used in biomedical studies (Fan and Watanabe 2003).

Thus, considering both economical and hygienical aspects, rabbits are advantageous for the expression of recombinant proteins in the mammary gland. Currently, there is a positive trend of using transgenic rabbits as producers of recombinant proteins by researchers and pharmaceutical companies.

Conclusion

After 30 years of research and development, the first medicines based on biologically active proteins from the milk of transgenic animals have appeared on the pharmaceutical market. The modern understanding of the regulation of gene expression and the discovery of new tools for gene editing can significantly increase the efficiency of creating animal bioreactors and obtain high concentrations of the target protein.

Special attention should be paid to the creation of full cycle solutions in order to minimize the time from the idea or order for the production of the target protein to obtaining an end-product.

The most promising is the embedding of transgenic cassettes in the region of the alphaS1-casein gene, while the cassette may contain its own highly effective milk promoter.

Rabbits are a unique tool that combines the ability to produce up to 5 liters of milk per year per female, which allows you to get up to 300 grams of the target protein. Thus, the milk of one bioreactor rabbit can replace up to 150,000 liters of donated blood.

Conflict of interests

The authors declare no conflict of interests.

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Table 5. Comparison of transgenic milk expression systems in different species.

Species	Gestation (months)	Maturation (months)	Milk yield per lactation (L)	Elapsed months from microinjection to milk
Mouse	0.75	1	0.0015	3–6
Rabbit	1	5-6	1–10	7-8
Pig	4	7-8	200–400	15-16
Sheep	5	6–8	200–400	16–18
Goat	5	6–8	600–800	16–18
Cow	9	15	6000–8000	30–33

Note: Table adapted from (Wang et al. 2013).

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