Cloning and Transgenesis

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Goats (*Capra hircus*) as Bioreactors for Production of Recombinant Proteins Interesting to Pharmaceutical Industry

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Abstract

Goats are a particularly efficient mean of producing recombinant proteins since they produce considerable amounts of milk, and incur lower investment and maintenance costs than cows. Thus, the aim of this review is to present the state-of-the-art for obtaining transgenic goats producing recombinant proteins for further utilization in the pharmaceutical industry. Additionally, the approaches to directed site-specific integration of transgene as well as the economic interest for this activity will be discussed.

Keywords: Goat; Recombinant protein; Pharmaceuticals; Transgenesis

Abbreviations:

AT: Antithrombin; CHO: Chinese Hamster Ovary; CRISPR: Clustered, Regularity Interspaced, Short Palindromic repeats; Cas: CRISPR-Associated; EPO: Erythropoietin; GMA: Genetically Modified Animal; G-CSF: Granulocyte-Colony Stimulating Factor; GH: Growth Hormone; EGFR: Epidermal Growth Factor Receptor; ICSI: Intra Citoplasmatic Sperm Inject; SCNT: Somatic Cell Nuclear Transfer; SMGT: Sperm-Mediated Gene Transfer; TAL: Transcription Activator-Like; TALENs: Transcription Activator-Like Effector Nucleases; TNF: Tumor Necrosis Factor; tPA: Tissue Plasminogen Activator; ZFN: Zinc-Finger Nucleases

Introduction

Recombinant DNA technology has revolutionized the production of therapeutic proteins. Thus, genes of a great number of human proteins have already been identified and cloned, including clotting factors, growth hormone (GH), insulin, erythropoietin (EPO), among others. The first attempts to produce therapeutic proteins from cloned genes were made in yeast and bacteria. However, for many proteins this is not viable, because microorganisms are not capable to make the posttranslational modifications necessary for protein activity [1]. Although mammalian cell culture provides the posttranslational modifications, they are very expensive. Thus, the use of farm animals as bioreactors may be the better choice to produce recombinant therapeutic proteins in their mammary gland. For this activity, the term "pharming" (portmanteau of farming and pharmaceutical) was created referring to the use of genetic engineering to obtain a transgenic or genetically modified animal (GMA).

The use of GMA technology to domestic animals has been limited due to the high cost of this kind of research. Thus, the choice of species to be used as bioreactors depends on several factors (Table 1); however, the quantity of protein required and the timescale for production are key factors. Additionally, feasibility and the costs of keeping and breeding the animals should also be considered [2].

Species	Reference	Advantages	Disadvantages
Rabbit	[3]	Short generation interval Production of multiple offspring	Very low milk yield
Pig	[3]	Short pregnancy length Production of multiple offspring	Low milk yield Difficult DNA microinjection
Sheep	[3]	Short pregnancy length Production of multiple offspring	Low milk yield Difficult DNA microinjection
Goat	[4]	Short pregnancy length Production of multiple offspring Good milk yield	Difficult DNA microinjection
Cattle	[5]	Very good milk yield	Difficult DNA microinjection Long generation interval High maintenance cost

Table 1: Livestock mammal species obtained by transgenic technology, reference of pioneer report and the main advantages/disadvantages of its use as a bioreactor

Considering the advantages and disadvantages of each livestock species, goats appear as an excellent model for use as bioreactors. Additionally, it was from the milk of transgenic goats that was produced and approved the commercialization of the first human biological drug (antithrombin - AT). This approval occurred first in Europe, by The European Agency for the Evaluation of Medicinal

Products [6], and after in the US, by the Food and Drug Administration [7].

Generating Transgenic Goats

Production of transgenic livestock was demonstrated to be feasible almost three decades ago [3]. It became apparent almost immediately that the method used to produce the transgenic livestock had substantial limitations that would impede its use both for research and commercial applications. Nevertheless, transgenic goats have been obtained to date by two methods: pronuclear microinjection and somatic cell nuclear transfer (Figure 1).

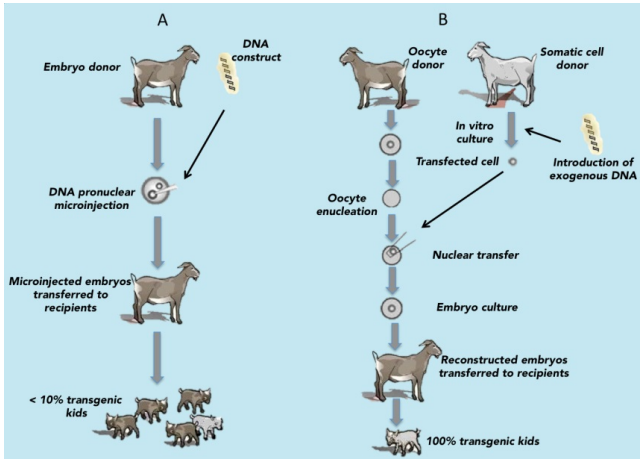

Figure 1: Traditional methods used to obtain transgenic goats: (A) pronuclear microinjection of DNA and (B) somatic cell nuclear transfer (SCNT)

Pronuclear microinjection

The aim of the first report on genetically manipulated goat embryos was to obtain transgenic animals that secreted pharmaceuticals, and in particular the human tissue plasminogen activator (tPA), in their milk [4]. After this first success, several other human proteins have been produced in goats using pronuclear microinjection. However, the overall efficiency of this technique is poor, especially when compared to that obtained in mice. In goats, around 1% or less of the injected zygotes gives birth to a transgenic kid [7].

Pronuclear microinjection has a simple concept: to inject a small volume containing the gene of interest into a pronucleus of a zygote, and then transfer the zygotes to the oviduct of a recipient. However, the microinjection is a procedure that requires a certain amount of dexterity and a significant amount of patience. In mouse, pronuclei are clearly visible during the latter phase of the zygotic stage of development. In other species (cattle, pigs and goats), it is necessary to centrifuge embryos.

During the experiments performed in our laboratory, goat zygotes showed non-transparent cytoplasm and approximately 125-130 μm in diameter (Figure 2A and B). Non-transparent cytoplasm is due to the presence of a large amount of lipid granules that hinder the visualization of pronuclei. In addition, pronuclei seem to be visible sometimes, but this impression can, however, appear false after attentive examination with an inverted microscope equipped with interferential contrast optics and using variable lighting. The presence of the second polar body is a rather marked indication that the egg has been fertilized. The presence of the second polar body is typical for all zygote stages. It can be located in contact with the first polar body or not far away from it. The first polar body most often stays at the degradation stage; sometimes zygotes with three polar bodies occur if by that moment the division of the first polar body has already occurred.

In goat embryos, the pronuclei can be visualized without centrifugation in approximately 30% of times [4] (Figure 2C). To facilitate visualization of pronuclei, all the fertilized eggs were subjected to centrifugation, which contributes to precipitation of the lipid granules. The pronuclei of the late zygotes were located closely to one another and mainly in the middle zone of the cytoplasm though closer to the dark pole with dark granules, one of them (male) was somewhat larger than the other (female) (Figure 2D and F). Their pronucleolars, unlike those in mouse, rabbit or swine pronuclei, were not visualized and morphologically rather resembled sheep and cow pronuclei. Both pronuclei could not be always simultaneously observed, and one of them could be located in the lipid granules. However, even after very careful examination of the centrifuged zygotes in a microscope with Nomarsky optics, the pronuclei could not always be clearly observed. Zygotes of different goat breeds differ in a degree of visualization of pronuclei. For instance, in Canindé goats the visualization was in almost 100% of examined zygotes, whereas in Saanen this rate was only slightly higher than 70% [8].

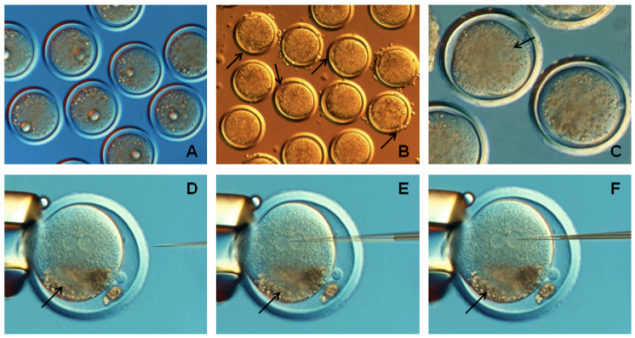

Figure 2: Morphology of goat oocytes and zygotes and details of pronuclear microinjection. A: unfertilized oocytes; secondary polar body is absent (×200). B: Saanen goat zygotes; arrows indicate secondary polar bodies and pronuclei are not visible (×200). C: Canindé goat zygotes; arrow indicates pronuclei visible without centrifugation (×300). D, E, F: DNA microinjection into centrifuged pronuclei of Canindé goat zygotes; arrow indicates pole with lipid granules. Microinjection was performed with an inverted microscope (×300) equipped with DIC (TE2000; Nikon, Japan) and a pair of micromanipulators (Narishige, Japan)

Somatic Cell Nuclear Transfer (SCNT)

SCNT, combined with molecular biology and cell culture methods, shows a variety of applications. Among the different areas, transgenesis is possibly the one that has benefited the most with the advances in this biotechnique in the sense of increasing the efficiency and reducing costs. Since the birth of Dolly sheep [9], the basic SCNT technology remains the same. It consists on the transfer of the donor

cell nuclei to enucleated oocytes with later reconstruction of the embryo through the cell fusion. By the use of SCNT technique it is possible to produce transgenic animals through the transfection of nuclei with vectors of DNA expression or by cloning transgenic founder animals [10].

In the SCNT method utilizing nuclei transfection, exogenous DNA is randomly incorporated into the genome using selective pressure. Moreover, transgenic cells can be completely characterized with respect to the integration region, integrated number of copies and integrity of the transgene before the nuclear transfer step. Although the capacity for development of the reconstructed embryos is low, the majority of the offspring are transgenic, making this technique more efficient than pronuclear microinjection [11]. However, the use of SCNT still has some limitations, as for example: reprogramming may be incomplete, resulting in embryonic loss, abortion, or abnormal development [12].

Alternative methods

Figure 3 shows the current state of transgenic technology development in goats showing the pioneer studies at each stage of success. While some problems inherent in traditional techniques still persist, some groups are working on alternative methods. Among these methods, some have proven to be feasible in other species, as for example, the sperm-mediated gene transfer (SMGT). Recently, it was shown that although goat spermatozoa can uptake DNA, the presence of seminal fluid partially inhibits it [13]. Before this study, other group [14] verified the possibility of using SMGT to produce transgenic goat embryos. In this work, the authors enhanced the technique by the use of intracytoplasmic sperm injection (ICSI) procedure. This study showed that the technique (in vitro fertilization vs. ICSI), sperm status (motile vs. live-immotile vs. dead) and to some extent DNA concentration affect embryo development, transgene transmission and expression. The results obtained by these two groups suggested that SMGT is applicable to goats. However, the genetic characterization of the resulting transgenic kids, such as mosaicism and transgene copy number, is required.

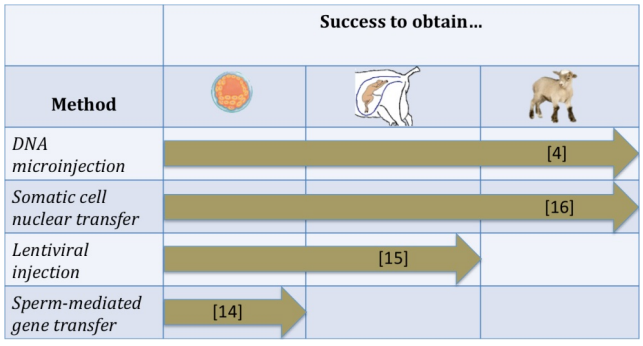

Figure 3: Success to obtain transgenic goat embryos, fetuses or offspring according to the different methods used

An alternative to gene inactivation by homologous recombination is gene knockdown by RNA interference. Thus, lentiviral vectors were used to deliver short-hairpin RNA expression cassettes targeting the prion protein mRNA in goat fibroblasts. These cells were posteriorly subsequently used for nuclear transplantation. The analysis of the transgenic fetuses (brain) confirmed the knockdown of the targeted mRNA and of the encoded PrP protein [15].

Transgene design and approaches to directed site-specific integration of transgene into animal genome

Typical design of transgenes used for generation of GMA includes three basic elements: 5'-flanking sequences of the milk gene, often together with non-translated exon 1 and intron 1; genomic region or cDNA coding protein of interest, and 3'-genomic flanking sequence of the milk gene including non-translated exon(s) and intron(s), and 3'-UTR or rare other genes, for example, growth hormone [16,17].

Variability in expression of transgenes directed by the promoter of various milk genes was reported by many investigators [16,18,19]. Most researchers believe that incorrect transgene expression, in particularity ectopic and high variability occurs mainly due to its random insertion into the recipient genome [16-18,20]. It is presumed that transgene expression depends on the chromatin environment in which it is located, a phenomenon known as the position-effect. Meanwhile, for successful creation of transgenic goats effective as bioreactors it is required the expression of transgene exclusively in mammary gland.

The criteria for correct transgene expression under control of a milk gene promoter include: i) the expression must be restricted to the lactating mammary gland, without ectopic expression; ii) the expression must take place in all of the epithelial cells of the mammary gland, without cell mosaicism; and iii) there must be low variability in transgenic expression between animals originated from different founders.

Since almost 30 years passed from the first generation of farm animals [3], many efforts were applied to reach the criteria. For instance, in the first researches on transgenic animals including goat promoters of the milk genes fused with cDNA coding human protein were used. However, genomic sequences of the gene of interest provide higher levels than cDNA sequences presumably due to regulatory elements located within introns [21]. Also, during the search for optimal transgene designs diverse variants of the milk genes promoters were tested. As a rule, an increase of the promoter size prompt increase transgene expression [16]. The most difficult problem is to overcome the ectopic transgene expression. Progress in identification of the regulatory sites within the promoters of milk genes allowed partially resolving the problem [22].

Here is pertinent to note that parameters of expression of the transgene tested in transgenic mice may be different when the same transgene is introduced in genome of another species. For instance, we did not observe expression of the human Granulocyte-Colony Stimulating Factor (G-CSF) after birth of transgenic mice in other tissue except the mammary gland [23] whereas goats carrying the same transgene showed leukocytosis (due to elevated number of neutrophils) at birth and persisted throughout their life [24]. It is obvious that the neutrophilia in the transgenic goats is a result of expression of the human G-CSF in fetuses before birth. Despite conservatism in organization of the milk genes in mammals one cannot exclude that they may have distinct functions. This is supported by the finding that hG-CSF secretion into milk of transgenic mice was at a lower concentration when compared to transgenic goats [23,25].

A long genomic DNA fragment expressed in bacterial artificial chromosomes or yeast artificial chromosomes often provides correct expression of the transgene [26]. However, long DNA fragments can be fragmented during the microinjection procedure.

Although not yet used in goats, three new approaches were developed allowing creating site-specific endogenous gene modifications in cell cultures and animals. The first is zinc-finger nucleases (ZFN) technology basing on joint of the transcriptional zinc-finger factor and the nuclease domain of Fok I. The hybrid protein links a DNA binding domain of the zinc-finger type to the Fok I nuclease and, hence, induces double-strand breaks at preselected genomic sites [27]. Combining the ZFN-technology with nuclear transfer, Whyte et al. [28] have generated piglets with the targeted GFP transgene. It was the first communication on farm animals carrying the site-specific knock out mutations.

Similarly to ZFNs, the transcription activator-like (TAL) effector nucleases (TALENs) are able to create double-strand breaks site-specific manner and then are either sealed by homologous recombination with mutant, synthetic oligodeoxynucleotides or by non-homologous end-joining repair giving rise often deletions and insertions [29].

The third approach to genome editing is based on the use of clustered, regularity interspaced, short palindromic repeats (CRISPR) together with CRISPR-associated (Cas) which provide an adaptive microbial immune response against viruses and plasmids [30,31]. The first communications on generation of knockout mice by CRISPR/Cas-mediated gene editing have practically appeared simultaneously [32,33].

In general, the ZFN, TALEN and C CRISPR/Cas technologies open perspectives for site-specific genome editing and potentially can be applied to many other species including goats.

Market for Recombinant Proteins from Goats

The global protein therapeutics market reached US$ 138.3 billion in 2012 and this market is expected to increase to nearly US$ 179.1 billion in 2018 [34]. This can be an interesting point for companies who want to use the "goat model" as bioreactor. However, even though it was always considered as a highly-perspective approach, the number of new commercial recombinant proteins that have successfully reached the market still counts only two products [35]. The first reason to this is the level of technology of the production of transgenic animals. Even though it has reached significant developments and lots of improvements were made, it still takes a long time to find out and test which design of the mammary expression system should be used in order to produce a particular protein. Some of the attempts towards this direction are still being unsuccessful, especially in the case of highly-active human proteins or those having very complex posttranslational modifications [36].

The second reason is caused by the existence of patents on all "blockbusters" of the recombinant protein market, which are still being produced in other systems, such as bacteria or Chinese Hamster Ovary (CHO) cells. Presumably because of this fact, pioneers of the industry had to concentrate on orphan drugs. Efforts taken to bring an orphan drug produced by a transgenic animal to the market were higher than those for a blockbuster produced in conventional expression systems, but its commercial benefit was usually less, which makes such business quite risky. A number of companies went bankrupt since the beginning of the era of transgenic animal bioreactors, which serves as a good proof of it. Even the most notable of them had to struggle. Just recently, a key-player of the industry, GTC-Biotherapeutics (USA), which goat-derived recombinant human AT (ATryn®) was the first one to reach the market, had to cut about 50 of their employees and make two loans from its French partner company "LFB Biotechnologies". On January 2013, GTC-Biotherapeutics announced that it was acquired by LFB and changed its company name to "rEvo".

So far the only goat-derived recombinant human protein on the market is still the above-described human ATIII. However, the available data indicate that there are few dozens of various recombinant proteins produced in goats to date. Despite the fate of some of these projects is unknown, several of these proteins were reported to reach the stage of clinical trials.

Human blood serum proteins are of significant medical importance and the need of their use is constantly growing. This made them a desirable aim for bioreactor industry [18]. Thus, since the 90's, blood clotting factors were produced by transgenic goats. Most of the mentioned recombinant blood proteins were developed in USA by GTC-Biotherapeutics. This is quite unsurprising, since GTC-Biotherapeutics is the biggest company on the market and it has a U.S. patent, issued in 2006 and expiring in 2021, that covers the production of all therapeutic proteins in the milk of transgenic mammals.

However, activities in this field are also carried out in other countries, such as Iran, where goats expressing human factor IX were produced [37]. Another important and rapidly growing segment is the production of therapeutic human monoclonal antibodies. Many of them are successfully expressed in other recombinant production systems, but levels of their production can be higher with the use of transgenic goats. Despite the fact that in published articles there are almost no data on transgenic goats producing monoclonal antibodies, some sources, such as GTC-Biotherapeutics 2010 annual report [38], evidences that goats expressing human monoclonal antibodies against CD20 receptor, CD137 receptor, tumor necrosis factor (TNF) and epidermal growth factor receptor (EGFR) already exists. Moreover, methods for the purification of recombinant antibodies from goat milk have been described [39].

Recombinant hormones, growth factors and cytokines is a more complex task, since they have high biological activity and may have adverse effect on the health of transgenic animal [23]. Nevertheless, transgenic goats expressing G-CSF [24,40] and EPO [36] were already produced.

Developments of other commercially important proteins expressed in transgenic goats are also the subject of interest. Thus, several groups have reported the creation of transgenic goats expressing high levels of human lactoferrin [41,42]. Supported by the US Ministry of Defense, an interesting project was conducted by PharmAthene Inc., who has created a herd of goats expressing human butyrylcholinesterase [43]. It is assumed, that this enzyme will be successfully used as an antidote against organophosphorus poisons.

It seems that after all these years the industry is close to accumulate its critical mass of developments, which as we hope will finally turn into a number of new goat-derived proteins on the market.

Conclusions

Even though new different strategies are being developed for laboratory animals, in goats, the pronuclear microinjection and SCNT remain the most used tools for obtaining transgenic animals. It can be concluded that currently there is no perfect transgene design that could be reliable to provide correct tissue-specific expression of transgene at high level.

Concerning the use of transgenic goats in pharmaceutical industry, the level of technology is growing and guidelines regulating the use of these animals and quality assessment for milk-derived recombinant proteins are getting more established, which makes the whole way easier than it was before. Furthermore, since patents on many of the pharma "blockbusters" will soon expire, one can expect the appearance of their biosimilars. Since then the cost of recombinant protein production will become the main criteria, making transgenic goats a very competitive production system.

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