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Production of Transgenic Goats, Carrying Human Coagulation Factor IX cDNA, by Nuclear Transfer of Transfected Fetal Fibroblasts

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***Abstract*— There are growing numbers of recombinant proteins that have been expressed in milk. Thus one can consider the placement of any gene of interest under the control of the regulatory elements of a milk protein gene in a dairy farm animal. Among the transgene introducing techniques, only nuclear transfer (NT) allows 100% efficiency and bypasses the mosaicism associated with counterpart techniques. Although live birth efficiency is very low in NT, dairy goats perform better than cattle and sheep in terms of fetal loss, prenatal morbidity and mortality. In this study, in an attempt to produce a transgenic goat carrying the human coagulation factor IX (hFIX) transgene, goat fetal fibroblasts were electroporated with a linearized marker-free construct in which the transgene was juxtaposed to β-casein promoter designed to secret the recombinant protein in goat milk. Two different lines of transfected cells were used as donors for NT to enucleated oocytes. Two transgenic goats were liveborn. DNA sequencing of the corresponding transgene locus confirmed authenticity of the cloning procedure. In conclusion, our study has provided the groundwork for a prosperous and promising approach for large-scale production and therapeutic application of hFIX expressed in transgenic goats.**

Keywords-Human Coagulation Factor IX, Goat, Transgenic, Somatic Cell Nuclear Transfer, Fibroblast

I. INTRODUCTION

For production of proteins with pharmaceutical importance, transgenic farm animals have become an attractive alternative to both microbial and cultured animal cell bioreactors. Transgenic rabbits, goats, sheep and cows have been developed as living bioreactors producing potentially high value biopharmaceuticals, commonly referred to as "pharming". The mammary gland is well suited for the production and expression of human recombinant proteins [1] such as; alpha-1-antitrypsin [2], fibrinogen [3], FIX [4], and antithrombin III [5], antibodies [6], human IGF1 [7], human NGF-b [8], human growth hormone [hGH] [9], human lactoferrin [10], human erythropoietin [11], human thrombopoietin [12] and human parathyroid hormone [3]. Interestingly, naturally complex proteins have been secreted in milk in a fully functional form [13].

Obvious benefits of using transgenic animals to provide human pharmaceuticals include: 1) high product yield, 2) low capital investment compared with cell culture techniques, 3) the ability to perform complex post-translational modifications (e.g., glycosylation and gammacarboxylation) and 4) elimination of reliance on products derived from human blood, which may contain pathogens (e.g., human immunodeficiency virus and hepatitis viruses) [14]. The generation of transgenic large ruminants (cattle) is, however, very expensive because of the long gestation period, small litter size and high maintenance costs of this livestock species. In contrast, goats are ideal for the transgenic production of therapeutic recombinant proteins because of their high yield of purified product, relatively short generation interval and production of multiple offspring [14]. For these reasons, the use of dairy goats as bioreactor animals is of particular interest.

Until recently, the only reliable method available for producing transgenic farm animals has been pronuclear microinjection. The success rate of this technique has been low, with 0.5–3% of microinjected embryos giving rise to transgenic offspring [15]. The emerging use of transfected cultured somatic cells as karyoplast donors for nuclear transfer (NT) has several advantages over microinjection, and has facilitated the generation of transgenic animals [5,16,17,18,19,20]. NT using transfected somatic cells allows the prescreening of cells for desirable genotypic characteristics which can reduce the number of animals (donors and recipients) used during the production of transgenic animals. One criterion is that the cell line used can be propagated and maintained in culture for sufficient time to allow for transfection, selection and characterization while remaining ”diploid”. A number of cell types have been used as donor cells for NT [21]. Fetal fibroblasts are generally the cell of choice for generation of transgenic cell lines; however, other cells types, including granulosa cells and skin fibroblast cells obtained from adult animals have been used [4,17,22,23, 24].

This study has used a transgene designed to express the human clotting factor IX [hFIX] protein in goats milk. Factor IX plays an essential role in blood coagulation and its deficiency results in hemophilia B [25]. This is an X-linked recessive condition which occurs at a frequency of about 1 in 30000 males. Patients are currently treated by injection of the hFIX concentrate prepared from pooled plasma from normal blood donors. This treatment is complicated by the risk of infection by blood-borne viruses such as those responsible for hepatitis and AIDS. Cloning of the gene for the hFIX [26,27] has provided alternative means for production of this protein and the prospect of gene therapy for hemophilia B based on recombinant DNA techniques [28].

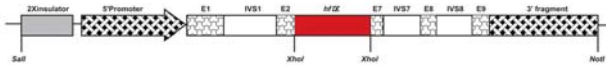
Thus, the aim of this study was to examine the efficiency of producing goats transgenic for human hFIX. This study

resulted in the production of twin transgenic goats that contained the hFIX gene; the first birth of a transgenic goat in Iran.

II. Materials and Methods

A. Production of gene construct

The cDNA encoding hFIX was constructed from liver cells by RT/PCR. The primers contained *XhoI* sites in the 5' end in order to synthesize hFIX cDNA and subcloning of pBC1 was designed. (Forward: 5'-CTCGAGCCACCATGCAGCGCGTGAACATGATC-3' Reverse: 5'-CTCGAGTCATTAAGTGAGCTTTGTTTTTTCCTTA -3'). PCR amplification consisted of 30 cycles with annealing at 58ºC for 30 sec and extension at 72ºC for 45 sec. The PCR product was cloned into the T vector (pTZ57R/T) followed by digestion and sequencing and subsequently cloned into pBC1 (Invitrogen) (Fig. 1).



B. Preparation of adult goat fibroblast cells

This step was carried out based on a method described by Yu et al. (2003). The ear of a female goat, 1.5 years of age was shaved and disinfected with 70% ethanol before a piece of skin was excised. The epidermis and hypodermis were removed with a scalpel blade and the dermis was cut into small pieces (2 × 2mm). Pieces of skin were put in culture dishes containing DMEM supplemented with 15% FBS and incubated at 38°C in a humid atmosphere containing 5% CO_2. The medium was refreshed every 2–3 days. When the cells reached confluency, after ten days of culture, tissue pieces were removed and cells were washed with PBS, and detached from dishes by adding trypsin/EDTA for 2 min at 38°C. Cells were trypsinized and frozen with 10% DMSO and stored in liquid nitrogen

C. Isolation and Transfection of the goat fetal fibroblast cells

The goat fetal fibroblast cells used as karyoplast donors were prepared as previously described [5] At 90% confluencey, about 10^7 cells were harvested and mixed with 10 to 40 µg of the linearize gene-targeted cassette, transferred into a 0.4 cm cuvette (Bio-Rad) and subjected to a pulse of 217-218 volts delivered by a Gene Pulser (Bio-Rad München, Germany). After transfection the cell/DNA-mix was incubated in DMEM medium containing 10% FBS for 15 min at room temperature, before being plated in 10 cm pettri- dishes in DMEM without selection. After 24 h, the culture medium was refreshed.

D. Identification of transfected by PCR and FISH

Three–five days after transfection, a part of the cells were considered for PCR analysis for identification of transfected cells. Genomic DNA was extracted by phenol/chloroform standard protocol and used as template for PCR.

Mini-prep extracted recombinant plasmid (pBC1-hFIX) was labeled base on a nick translation method using DNA Labeling kit (Vysis, Abbott Molecular) according to the manufacturer's instructions and the probe was hybridized on PCR-positive metaphase preparations according to standard FISH procedure. Positive cells were isolated and used for NT.

E. Nuclear transfer

Collected and processed oocytes were placed into manipulation drops (EmCare supplemented with 1% goat serum) and covered with an overlay of mineral oil. The zona pellucida was punctured and the polar body and metaphase plate was removed by aspirating a minimal amount of surrounding cytoplasm. After each individual oocyte was enucleated, the donor cell was transferred into the perivitelline space of the enucleated oocyte. Following reconstruction, karyoplast/oocyte couplets were transferred to TCM 199/goat serum and incubated at least 30 min prior to fusion and activation.

F. Fusion and activation

Fusion and activation were performed at room temperature. Couplets were manually aligned equidistant between two stainless steel electrodes of a 0.5 mm gap fusion chamber (Genetronics Biomedical, San Diego, CA, USA) overlaid with fusion buffer (sorbitol, 0.25 M, calcium acetate 100 µM, magnesium acetate 0.5 mM BSA, 0.1%) and fused by a single DC pulse (2.39 kV/cm for 25 µsec) delivered by a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). Couplets were evaluated for fusion after a 1 h incubation period in TCM and then activated.

Fused couplets were activated by a 5 min exposure to 5 µM ionomycin and then incubated for 5 h in 2 mM 6-dimethylaminopurine prepared in global medium (Vitrolife). Following activation, the reconstructed embryos were washed and cultured under oil in 50 µl droplets of global medium for 72 h (four- and eight-cell stages) prior to transfer to recipient females.

G. Embryo transfer

Four- and eight-cell stage embryos were surgically transferred to synchronized recipients on day two of their estrus cycle (day 0=estrus). Immediately prior to transfer, embryos were placed in global medium supplemented with 10% FBS. Utilizing a 3.5 Fr G Tom Cat Catheter attached to a 1 ml syringe, 4-6 embryos were transferred into the oviduct of each recipient.

H. Transgene analysis of offspring

PCR reactions were performed on the extracted genomic DNA of two goats with specific primers designed for amplification of the gene construct. Subsequently, the PCR products were sequenced.

I. Statistical analysis

Statistical analyses were confirmed by ANOVA. Differences between experimental groups were evaluated

with the T-test. Data were expressed as mean±SD and P<0.05 was considered significant.

III. RESULTS

A. Oocyte maturation, embryo reconstruction and embryo development

F Seventy one percent of the 1488 recovered oocytes reached the MII stage (Table I). A total of 568 couplets from adult fibroblast cells were produced, which resulted in an overall reconstruction rate of 54.9±13.6 (568 couplets/1074 mature oocytes) for this experiment. The overall fusion and cleavage rates were 77.5±14.9 and 68.5±15.0, respectively.

A total of 21 recipient does received an average of 5 embryos. Three of the recipients maintained gestation until two months. The pregnancy rate at 30 days of gestation was 14.28% (3/21).

Following a maturation interval of 18 to 22 h, 71.6±12.0 of 1231 recovered oocytes reached the MII stage. A total of 439 couplets from fetal fibroblast cells were produced, resulting in an overall reconstruction rate of 54.5±19.1 (439 couplets/863 mature oocytes) for this experiment. The overall fusion and cleavage rates were 74.2±17.5 and 61.3±16.6, respectively.

The development of 439 NT couplets reconstructed from donor cells were assessed (Table I). The rate of fusion of NT embryos reconstructed was 74.2±17.5. Of the 344 fused embryos, 208 (61.3±16.6) cleaved and were subsequently 64 embryos transferred to 12 recipients.

TABLE I. RATE OF OOCYTE MATURATION, EMBRYO RECONSTRUCTION AND EMBRYO DEVELOPMENT RATES IN GOAT TRANSGENIC ADULTS AND FETAL FIBROBLAST CELLS (MEAN±SD).

Donor cell	Fetal fibroblast cells	Adult fibroblast cells
No. ovaries	690	649
No. oocytes recovered	1231	1488
No. (%) oocytes matured	863 (71.6±12.0)	1074 (70.5±12.7)
No. (%) couplets produced	439 (54.5±19.1)	568 (54.9±13.6)
No. (%) couplets fused	344 (74.2±17.5)	435 (77.5±14.9)
No. (%) embryos	208 (61.3±16.6)	285 (68.5±15.0)
No. embryos transferred	64	150
No. recipients	12	21
No. (%) does pregnant, day 30	2 (16.6)	3 (14.28)
No. healthy offspring	2	0

B. Verification of transfected fibroblast cells

A total of 120 cultured plates of transfected fibroblast cells were analyzed by PCR and subsequently by single-cell PCR reactions, among them three plates appeared as positive.

The positive samples were subjected for, FISH, by using whole gene construct, as a probe .As the results indicates the transgene was integrated in a single position in two out of three cells and at two sites in the third one (Fig. 2). The first two lines of the fetal fibroblast cells were used for NT.

C. Embryo transfer and cloned transfer offspring

An average of five embryos was transferred into 21 and 12 recipients in experiments 1 and 2, respectively. Two pregnancies were detected in group2 and no pregnancies were detected in group 1 (Table I). One recipient maintained conceptuses throughout gestation. Twin transgenic kids were born at 147 days of gestation. Both offspring were healthy and vigorous at birth, and weighed 2 kg and 2.5 kg. The pregnancy rate, based on the number of pregnant recipients per total number of recipients, was 16.6%.

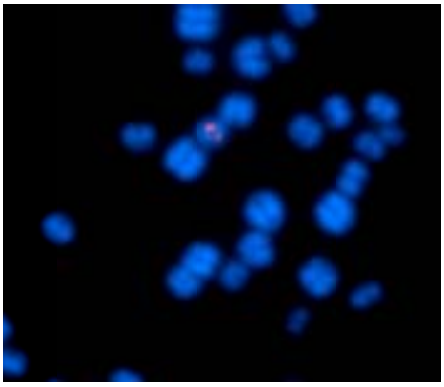


Figure 2. Single integration site of one of the transfected cell lines mapped with FISH analysis

D. Live born transgenicity confirmation

A DNA fragment of the expected sizes corresponding to specific subfragments of the hFIX coding region was amplified when PCR was carried out on the genomic DNA of the live born goats (Fig. 3). Comparison of the PCR products DNA sequences against the GeneBank database was performed, using the BLAST program [29], indicating the presence of the hFIX transgene in the two live born goats.

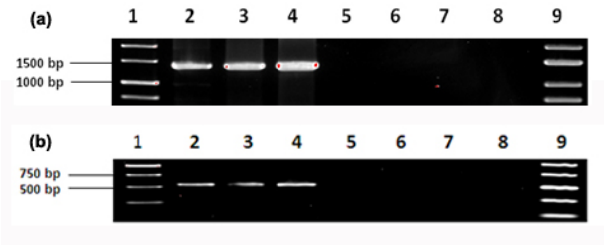


Figure 3. (a) 1386 bp (b) 537 bp fragments amplified from the genomic DNA of the live born goats with different pairs of primers. Lanes 1: 1 Kb DNA ladder, Lanes 2: Goat no.1 (Shangoole), Lanes 3: Goat no.2 (Mangoole), Lanes 4: Positive control (gene construct), Lanes 5, 6, 7: Negative control (normal goats), Lanes 8: Negative control (without template), Lanes 9: Express DNA ladder

had been a provision since the onset of recombinant DNA technology a number of years ago. Improvements in transgenesis methodologies have made it possible to virtually produce any desirable known protein. Not long after the generation of the first transgenic mice, attention was focused on farm animals as biofactories and soon, theories were developed and implemented [13,30,31]. Over the past two decades, production of transgenic livestock has been proven to be feasible and the outcome has shed light on new possibilities in different fields, particularly the pharmaceutical industry [13].

The present study is meant to report live birth of a twin pregnancy, a major success obtained thus far in an attempt to generate transgenic goats with the ability to secrete hFIX in milk. The following paragraphs address the logic behind each choice in the experimental steps throughout the project; including hFIX as the recombinant protein, the animal system as the appropriate bioreactor system, mammary gland as the secretory organ, the use of goats as the appropriate livestock, NT as the transgene introducing technique, choice of marker-free transgenesis strategy and a final discussion of the results.

The advent of recombinant DNA technology has paved the way to a prosperous future for the cure of patients with hemophilia B or Christmas disease. These patients are currently treated by replacement therapy with infusion of factor IX concentrate purified from pooled plasma from healthy blood donors. This infusion is risky due to the potential infection by blood-borne viruses such as HBV and HIV. The cloning of the corresponding gene for hFIX has provided a tool for alternative approaches such as synthetic production of hFIX in bioreactor systems and gene therapy.

Bioreactors for the synthesis of recombinant proteins are gradually shifting from cells to animals. Currently more than 60% of recombinant proteins are produced in cell culture systems [32]. Although many cell types from different sources have been successfully cultured *in vitro* since the first establishment of the eukaryotic cell line, only a few genetically modified cell lines have been used as bioreactors owing to the biosafety concerns related to drug production [33]. Biological requirements of cell cultures in addition to the sophisticated technology required have significant production costs at the industrial level. Therefore, the search for a more efficient and ideal expression system for proteins has led to the use of genetically modified animals as bioreactors for the production of recombinant products.

Milk is currently the best available bioreactor [34,35,36,37,38]. Secretion to the extracellular environment as the main strategy for the production of recombinant proteins with pharmaceutical interest has long been the subject of biotechnology. Transgenes in animals are expressed in the specialized cells of a tissue and the product is secreted into a body fluid. The efficiency of the bioreactor is a function of the secretory capacity of the tissue and the ease in collecting the fluid containing the transgene product [39]. Transgenic products have been expressed in several body fluids; however, due to its high secretory capacity and the ease of milk extraction, the mammary gland is the best appropriate organ for the production of recombinant proteins [40].

Detailed studies have demonstrated that milk is a milieu within which various recombinant proteins, even complex molecules, can be secreted in fully functional forms [3,41,42,43,44]. There is also a report on fully functional hFIX produced from a transgenic ewe showing that the mammary gland is capable of performing all the post-translational modifications necessary to convert the nascent polypeptide chain into its active protein form [45] and therefore a transgenic goat can be a suitable means of hFIX production.

The choice of the bioreactor system not only depends on the secretory performance and the type of post-translational modifications, but on several other issues, including economical advantages, health related concerns, the ease in handling the research, and future industrial maintenance, to name a few. Several animals can be exploited as bioreactors for the production of recombinant proteins, considering that large scale production is attainable with dairy animals. Goats with short breeding generation intervals and multiple offspring per pregnancy are preferred when compared to other farm animals. More importantly, goat-to-human transmittable diseases are less severe than other farm animals, such as cows. Pregnancy complications, giant fetus syndrome, lung anomalies, metabolic deficiencies and in general terms, problems with prenatal and neonatal morbidity and mortality associated with NT are less frequent in goats than sheep and cattle. All the above mentioned issues were the criteria of this investigation to choose goats as the proper bioreactor [13].

The NT technique has been proven to be efficient in goats [5]. This approach was also successfully used to obtain a transgenic ewe with the hFIX gene [4]. Apparently the method outweighs its counterpart techniques in a few aspects: for cloning in comparison with pronuclear microinjection, fewer animals are needed to provide the same number of transgenic ewes [4]. Unlike microinjection, the transfection takes place in culture which allows for the selection of cellular clones according to desired transgene copy number and integration site. Female sex selection is another advantage of NT which offers a 100% certainty instead of 50% and thus at least one generation closer to manipulated milk production. NT also provides the opportunity to choose between donors of high genetic value with proven performance. More importantly, among the transgene introducing techniques, only NT allows homogeneity of the produced animal and circumvents the mosaicism associated with counterpart techniques.

The fusion of the karyoplast into an enucleated oocyte and subsequent activation are important steps for production of live offspring by NT. While electronic fusion of the donor karyoplast into the enucleated oocyte is the most common method used, many different methods for activation and the time of the activation step for the initiation of embryo development in domestic species have been demonstrated [46]. This study showed that the recommended method for the activation of reconstructed oocytes was to expose them to ionomycin for 5 min and 6DMAP for 5 h. Those oocytes which were activated with this protocol showed satisfactory cleavage and development.

The synchronization of the recipient with the embryo age will have a direct effect on the probability of pregnancy. In our study four- to eight-cell embryos were transferred to the recipients who had undergone the estrus phase in the previous 48-60 h. We estimated that at the time of embryo transfer, the recipients had ovulated about 60 h prior to transfer. Synchronization between the recipients and the development stage of reconstructed embryos was an effort to compensate the slow development of reconstructed embryos. Additionally, 16.6 and 14.2% of the recipients became

pregnant from fetal and adult fibroblast cells, respectively and consequently, 8% of fetal fibroblast cells resulted in birth. This pregnancy rate was based on the total number of pregnant recipients to the total number of recipients was more than the pregnancy rate achieved in the study of Baguisi et al., (1999).

Surprisingly, our results showed that coat color in goats is an X-linked trait or epigenetically controlled regarding imprinted genes, because the two kids were genetically proven to be clones of the same donor and yet differentially colored.

In essence, numerous reports have been published on the work on the expression of hFIX in transgenic animals; however, to date no report has been published on transgenic goats incorporating the gene for hFIX. Here we report the first transgenic goats harboring the expression cassette for hFIX. Although a significant progress has been made in producing the modified goats of interest, complementary studies are required to ensure efficient transgene expression. The transgenic strain-to-strain variability in expression which is attributed to a phenomenon called position effect is the main reason. In addition, biochemical and biophysical characteristics of the resultant hFIX including post-translational modifications, protein functionality and activity remain to be identified.

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