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# MOLECULAR AND CYTOGENETIC CHARACTERIZATION OF HUMAN ALBUMIN TRANSGENIC GOAT FIBROBLASTS AS A SOURCE OF NUCLEI IN SOMATIC CLONING*

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**Abstract**

**Production of biopharmaceuticals in the mammary gland of domestic animals constitutes an important field of research in contemporary biotechnology. The objective of our investigations was to obtain goat transgenic fibroblast cells transfected with the assistance of the human albumin gene (*pWAPhAlb*) by the lipofection method for the needs of somatic cloning and to determine their molecular and cytogenetic characteristics. Transfection was carried out on 24 lines of goat fibroblasts and 19 lines in which integration of the introduced DNA was confirmed on the molecular level (PCR) were obtained. The method effectiveness reached 80%. Karyotype analysis (trypsin digestion, Giemsa stain) failed to show any changes in the chromosome number and structure (2n = 60, XX).**

**Key words: albumin, fibroblasts, transfection, nuclear transfer**

Obtaining transgenic domestic farm animals brings a number of advantages into such areas of life as medicine or agriculture. Among biomedical applications comprising, among others, xenotransplantation and a model animal, utilization of transgenic animals as bioreactors manufacturing therapeutic proteins is particularly important. Recombined proteins can be obtained in such body fluids as blood, urine or semen as well as in the white of birds' eggs and cocoons of silkworms. Due to high

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yields and ease of purification of the manufactured product as well as practicability of performing appropriate post-translation modifications and possibility of applying a number of regulatory sequences, the best system for manufacturing recombined proteins at the moment is the mammary gland of mammalian females, which is the source of milk (Massoud et al., 1991; Lipinski et al., 2003; Zbikowska et al., 2002; Love et al., 1994; Dyck et al., 1997; Tomita et al., 2003; Zhang et al., 2010; Yuan et al 2009). Scientists have already succeeded in obtaining recombined protein in almost all species of farm animals (Wells, 2010). When selecting the appropriate animal species, it is advisable to bear in mind milk yields of females, low milk allergy-inducing properties, short inter-generation interval, low rearing costs and the structure of the mammary gland. The species that meets all the above-mentioned requirements is the domestic goat (*Capra hircus* L.). So far scientists succeeded in obtaining the tissue plasminogen activator, antithrombin III and human monoclonal antibodies in the amount of 1–5 g/l (Ebert et al., 1994; Edmunds et al., 1998; DiTullio et al., 1995; Skrzyszowska and Samiec, 2006; Lenz et al., 2010). Antithrombin III manufactured by ATryn GTC Biotherapeutics, Framingham, MA, USA, was registered as a drug by the European Medicine Evaluation Agency (EMEA) in August 2006 (Niemann and Kues, 2007). This registration is a spectacular success showing real perspectives of practical utilization of recombinant biopharmaceuticals.

The most classical method of obtaining transgenic animals is microinjection of foreign DNA into the pronucleus of a fertilized oocyte. Other methods include techniques using stem cells and transferred sperm cells (Jura et al., 2008). Somatic cloning with the transplantation of transgenic cell nuclei is the most practical method of transgenesis (Lipinski et al., 2003). The first step is to introduce the transgene to donor cells by biolistics, electroporation, lipofection or using viral systems. Then, after integration analysis or even better – transgene expression, the transgenic nucleus is transferred into the enucleated oocyte, followed by short-term culturing and introduced into the synchronized recipient.

Albumin is a hydrophilic protein of 20–60 kDa, which occurs in the blood plasma making up 60% of all its proteins. It is coded by the *ALB* gene which, in humans, is found in the 4q11-q13 locus. It regulates oncotic pressure which participates in maintaining adequate proportions of water in blood and tissue fluids. It is also responsible for the transport of fatty acids, steroids and thyroid hormones.

The aim of investigations presented in this article was to obtain goat transgenic fibroblast cells transfected by the human albumin gene with the assistance of the lipofection method for purposes of somatic cloning and to perform their molecular and cytogenetic characteristic.

## Material and methods

The experimental material comprised foetal fibroblasts as well as fibroblasts obtained from white (goats nos. 4 and 6) and brown (goat no. 5) breed goats. Sterile tissues were rinsed in PBS solution containing antibiotics (50 µg/ml gentamicin sul-

phate, 100 IU penicillin and 50 µg/ml streptomycin). Skin fragments were cut and digested in 5 ml trypsin-EDTA solution in PBS and then centrifuged. The cell sediment was suspended in enriched DMEM medium (20% FBS, 1% antibiotic/antimycotic (Gibco/Invitrogen)). Culturing was carried out at the temperature of 37°C at 5% $CO_2$ content. The total of 24 cell lines was obtained. Cultures of full confluence were passed (Lipiński et al., 2007).

The expression library established in the Lambda ZAP II vector was checked with the assistance of two oligonucleotides marked at the 5'end ($^{32}$P-γATP): NAL (5'-tgtcaaccccacacgcc-3') and CLAA2 (5'-CCATTTTTTATTAATCGATGCACAG-3') in order to select clones containing cDNA of human albumin. Human albumin cDNA was amplified on the template of the selected clone using, for this purpose, modified primers SPHAI (5'- CCTTTGGCAGCATGCAGTG-3') and CLAA2 (5'-CCATTTTTTATTAATCGATGCACAG-3') containing restriction sites *Aat*II and *Sph*I, respectively. The fragment obtained was cloned into pCR 2.1 vector (Introgen) and the clone obtained was designated as 5ALB. AATW1 (5'-GACCTCCGGGACGTCAAAGGAG-3') and SPHW2 (5'-CGAACAGCGCATGCTGTCGGC-3') primers containing, respectively, *Aat*II and *Sph*I restriction sites were amplified into a rat whey acidic protein (WAP) regulatory sequence. The reaction product was cloned into a pGEM vector (Promega) and the selected clone was designated pWAP. Both clones were sequenced using universal For/Rev primers. The selected 5ALB and pWAP clones were hydrolyzed by restriction enzymes (5ALB *Sph*I and *Cla*I as well as pWAP *Sph*I and *EcoR*I), fragments containing human albumin cDNA and WAP regulatory sequence were isolated, ligated in order to join both fragments with their sticky ends formed by the *Sph*I restriction enzyme and then cloned into pBluescript (Stratagene) hydrolyzed by *Cla*I and *EcoR*I restriction enzymes. After transformation, the clone containing appropriately connected DNA fragment was selected by colonial PCR employing CLAA3 (5'-ATCGATGCACAGAGAAAAG-3') and AATW2 (5'- GACGTCAAAGGAGTATGGGC-3') primers. The performed selection allowed selecting a clone which was designated as AW4Blusk.

In order to exchange the AAA codon for 525Lys into the AGA codon for ARG, the method of controlled mutagenesis was used employing a megaprimer in the PCR reaction (Lipiński et al., 2008). During the first PCR reaction the megaprimer was synthesized on the AW4Blusk gene construct using the KNMUTKR (5'- CAT ACG TTC CCA GAG AG -3') primer introducing the mutation and the KNMUTC (5'-GTG ATG TTA TAA GCC TAA GCA GC -3') primer. The reaction was carried out in the following conditions: 94ºC – 5 min., 94ºC – 45 sec., 52ºC – 30 sec., 72ºC – 50 sec., 72ºC – 5 min., 20 cycles. The 278 bp DNA fragment obtained served as a megaprimer for the second PCR reaction which allowed recovering the entire fragment of the albumin DNA (Figure 1). The second PCR reaction was conducted employing the KNMUTN (5'- GCA GAA TTC GUC TTG ACC TCC GGG ACG -3') primers and the megaprimer in the following conditions: 94ºC – 5 min., 94ºC – 45 sec., 65ºC – 60 sec., 72ºC – 60 sec., 72ºC – 5 min., 20 cycles (Figure 2). The PCR product 2815 bp long was cloned into the pBluescript vector and then re-cloned onto the AW4Blusk vector substituting the proper cDNA sequence by the modified one. The correctness of the sequence was confirmed by sequencing.

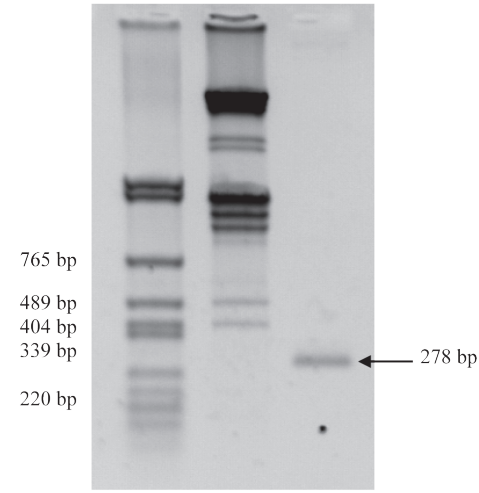


Figure 1. The first PCR product (megaprimer) obtained after amplification with primers KNMUTKR and KNMUTC. Homogeneous fragment indicated by an arrow

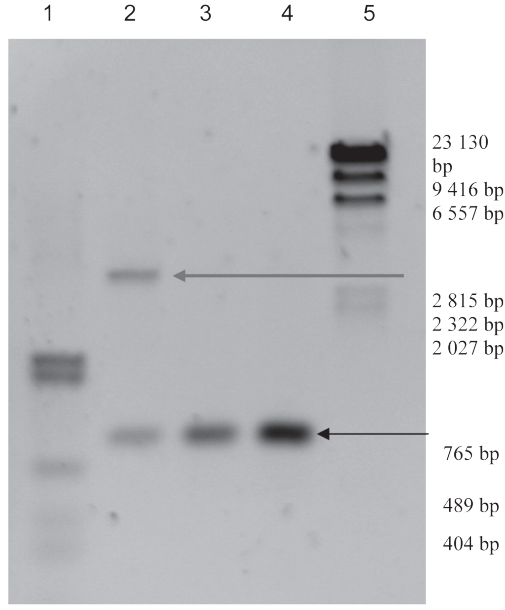


Figure 2. The second PCR product obtained after amplification with megaprimer and KNMUTN primer. Lanes 1 and 5 – size markers; lanes 2, 3, 4 – PCR products obtained after amplification with different amounts of megaprimer. Non-specific products are indicated by a black arrow, PCR product is indicated by a red arrow

The *pWAPhAlb* gene construct was introduced by the lipofection method in co-transfection with the pTracer-EF/Bsd A (Invitrogen) plasmid containing a resistance gene against antibiotic ampicillin. After attaining confluence at the level of 50%, the culturing medium was changed into pure DMEM and after 2 hours lipofection was performed using lipofectamine 2000 (Invitrogen). 5–10 µg of gene construct *pWAPhAlb* was added to a test tube with 500 µl of pure DMEM medium and, simultaneously, 16 µl of lipofectamine was added to another test tube containing 500 µl of pure DMEM medium. Everything was incubated for 5 min. at room temperature. The content of both test tubes was mixed and incubated for 20 min. at room temperature. Everything was added to cell cultures and cultured for 24 hours. The medium was changed into selective (5 µg/ml blasticidine S). After 8–10 days, the medium was changed into non-selective. Cultures of full confluence were utilized for DNA isolation employing the Qiagen DNA Kit. Cytogenetic preparations were prepared by adding colcemid (0.05 µg/ml), induction of osmotic shock (0.075 M KCl) and then fixing the material in a mixture of methanol and acetic acid.

The analysis of transgene integration was performed by the PCR method employing two pairs of primers of which F primers were complementary for the WAP promoter, while R primers were complementary for the sequence of the structure gene. Using the WAP-F15 (5'- TTC CCC AAA GTC TTC CTC CT- 3') and Alb-R11 (5'- CTG AGC AAA GGC AAT CAA CA -3') pair of primers we amplified the region comprising 250 base pairs, whereas the WAP-F16 (5'- TGC ATG TGT CCA AGA GGA AG -3') and Alb-R12 (5'- GCT GAC TCA TCA GCA ACA CA -3') pair of primers was employed to amplify the region which embraced 400 pairs of bases. The reaction was performed in the following conditions: 94ºC – 5 min., 94ºC – 45 sec., 56ºC – 45 sec., 72ºC – 60 sec., 72ºC – 10 min., 30 cycles. Products were fractionated in 1.5% agarose gel.

To determine karyotype of the cells, dried cytogenetic preparations were subjected to the treatment with trypsin solution for a time determined experimentally. Then they were rinsed in order to stop proteolysis and stained using Giemsa solution. Karyograms were elaborated with the assistance of the Ikaros software (Ikaros, Metsystems).

## Results

The molecular analysis of the transgene showed proper amplification of target fragments (Figures 1 and 2).

The total of 24 cell lines was subjected to transfection and the selection with blasticidine survived 19 lines, so the efficiency of the lipofection method was at the level of 80%. After the termination of the selection process, cell vitality was assessed and we also checked if the expression of the green fluorescent protein (GFP) reporter, took place. Both GFP as well as the blasticidine resistance gene present in the pTracer-EF/Bsd A (5987 bp) vector are under the control of the CMV promoter, which ensures their constitutive expression. The GFP expression provided an indirect proof of the integration of the pTracer-EF/Bsd A vector with the DNA genome

because transitional episomal expression in the cytoplasm can also take place. In addition, it also allows assuming that the *pWAPhAlb* gene construct became integrated too, because plasmids subjected to co-transfection usually integrate at the same sites. The presence of the reporter in gene constructs makes work easier thanks to rapid, live screening of transfected cells. A positive picture in all 19 lines was shown in UV light. The integration analysis of the introduced transgene was carried out employing polymerase chain reaction and revealed its integration in all 19 investigated cell lines (Figure 3). Additionally performed karyotype analysis failed to reveal any changes (2n = 60,XX) (Figure 4).

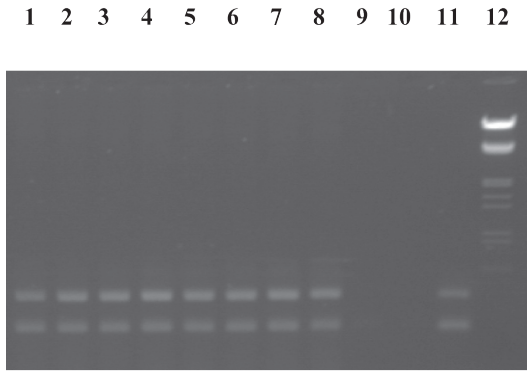


Figure 3. *pWAPhAlb* transgene integration analysis in goat fetal fibroblast lines. PCR method was employed to amplify products 250 or 400 bp long. Lane 1–8 – goat fetal fibroblast lines after transfection; lane 9 – negative control (without DNA); lane 10 – negative control (non-transgenic animals); lane 11 – positive control (*pWAPhAlb* plasmid); lane 12 – size marker (λ DNA/ HindIII, EcoRI)

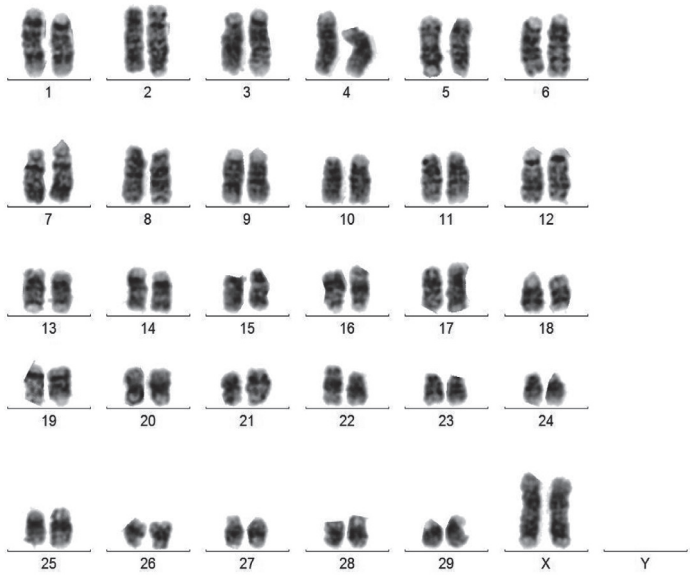


Figure 4. Normal karyotype of analysed transgenic cell line no 18 (60,XX)

## Discussion

The research results obtained corroborate the usefulness of the lipofection application as an effective and efficient method of obtaining transgenic lines of farm animals. The choice was based on the experience of a research team, indicating that this is an easy and effective procedure which allows transfection of different cell types without restrictions on the length of the introduced DNA (Oliviera et al., 2005). Gene constructs placed inside liposomes are characterized by stability and are protected against the activity of nucleolytic enzymes. Positively charged complexes, developed as a result of mutual interactions of lipids and DNA, penetrate into cells by way of endocytosis. The total of 24 goat fibroblast lines was subjected to lipofection to obtain 19 lines in which integration of the introduced DNA was confirmed. The method efficiency amounted to 80% and confirmed experimental results of the research team as well as our own investigations regarding cell lines of other animal species.

In order to enhance efficiency and improve the somatic cloning method with a transfer of the cell nucleus and to increase the level of foetal survivability, it is necessary to take care of the appropriate preparation of the donor cell. The basis of investigations is the analysis of the transgene incorporation at molecular level and cytogenetic characterization, as confirmed by this study. It is essential to conduct a general karyotype evaluation of transfected cells to eliminate from further investigations lines of abnormal chromosome number and structure. It should be remembered that the frequency of chromosomal aberrations increases with the frequency of the number of passes and, therefore, cells used in this kind of studies as well as those sampled for cloning should be at the lowest number of passes. Cytogenetic studies of transgenic cell lines are very difficult. Following transfection, cells are characterized by a very low degree of proliferation and, consequently, it is very difficult to obtain chromosomal material from them (Watanabe et al., 2005).

Cytogenetic analysis of transgenic animals is based, among others, on a direct physical mapping of the transgene, karyotype evaluation, homozygosity in consecutive generations as well as on the assessment of the number of copies of the built-in transgene (FISH on chromatin fibres). Stable integration of transgenes with the animal genome occurs most frequently in the telomere region as well as in the region of repeat sequences. Structurally, the genome of eukaryotic organisms is organized into two chromatin classes: euchromatin and heterochromatin, the latter of which comprises telomere regions and centromeres. Both telomeres and sub-telomere regions are rich in repeated sequences and poor in transcriptionally active regions. The function of these regions, stabilizing genome structure, may exert a negative influence on the expression of transgenes integrated not far away (Perrod and Gasses, 2006). Decreased expression levels may be caused by changes in the spatial structure of the integration site, histone deacetylation or DNA methylation (Pedram et al., 2006; Rincón-Arano et al., 2007). However, our own investigations regarding transgene expression analysis did not confirm the appearance of the so-called telomerase position effect (TPE), neither in the transgenic CMVFUT boar in which the transgene was mapped to the 14q28 locus nor in the transgenic WAPhGH rabbit in which the

transgene was mapped to the 7q26-27 locus (Jura et al., 2004; Lipiński et al., 2003). Despite the fact that both loci are situated in chromosomal regions rich in repeat sequences, a high level of transgene expression is observed in animals because a transcriptionally active structural-functional domain may develop at the integration site (Rincón-Arano et al., 2007).

Another important area of investigations on the improvement of the method of somatic cloning is the control of early symptoms of apoptosis of the transfected cells and embryos. Such analyses allow rejection of apoptotic cells and, consequently, lead to the increase in survivability and percentage of positive embryo implantations (Samiec and Skrzyszowska, 2008).

**Competing interests**

The authors declare that they have no competing interests.

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**Molekularna i cytogenetyczna charakterystyka transgenicznych fibroblastów kozy transfekowanych ludzkim genem albuminy jako źródła jąder komórkowych w klonowaniu somatycznym**

STRESZCZENIE

Produkcja biofarmaceutyków w gruczole mlekowym zwierząt domowych stanowi istotny rozdział we współczesnej biotechnologii. Celem badań było uzyskanie transgenicznych komórek fibroblastów

kozy transfekowanych ludzkim genem albuminy metodą lipofekcji na potrzeby klonowania somatycznego oraz dokonanie ich molekularnej i cytogenetycznej charakterystyki. Transfekcje przeprowadzono na 24 liniach komórkowych. Analiza integracji (PCR) potwierdziła obecność transgenu w komórkach 19 linii. Efektywność transfekcji wyniosła 80%. Analiza kariotypu (trawienie trypsyną, barwienie barwnikiem Giemsa) komórek linii transgenicznych nie wykazała zmian w liczbie i strukturze chromosomów (2n = 60, XX).