The establishment of two transgenic goat lines for mammary gland hG-CSF expression


a Laboratório de Fisiologia e Controle da Reprodução, Faculdade de Veterinária, Universidade Estadual do Ceará, Fortaleza, Brazil
b Institute of Cytology and Genetics, Russian Academy of Sciences, Novosibirsk, Russia
c Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia
d Universidade Federal do Vale do São Francisco, Petrolina, Brazil
e Centro de Criação de Animais de Laboratório, Fiocruz, Rio de Janeiro, Brazil
f Laboratório de Imunologia e Bioquímica Animal, Faculdade de Veterinária, Universidade Estadual do Ceará, Fortaleza, Brazil
 g Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Fl. 33136, USA

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The production of recombinant human granulocyte-colony stimulating factor (hG-CSF) for therapeutic purposes relies on its expression in selected clones of transfected mammalian cells. Alternatively, this protein can be produced by targeted secretion in the milk of transgenic goats. Thus, the aim of this study was to produce founder transgenic goats expressing hG-CSF and to propagate a transgenic production herd. After DNA microinjection of in vitro-produced pronuclear embryos was performed, two founders were obtained (one male and one female, named 10 M and 12 F, respectively). The mean level of hG-CSF secreted in the milk from the 12 F goat, which was measured after a period of induced lactation, was 620.92 ± 179.93 μg/ml. In addition, the recombinant protein presented in vitro biological activity on differentiation of human umbilical stem cells to neutrophil granulocyte series. A total of nine kids (six from 10 M and three from 12 F) that carried the hG-CSF transgene were generated by outbreeding of the founders. In summary, we produced two transgenic goats with a stably integrated hG-CSF gene that were capable of secreting recombinant hG-CSF from the lactating mammary gland without causing any harm to the animals' health. Additionally, the founders proved to be fertile and capable of transmitting the hG-CSF gene to first generation progeny of each line. Additional investigations of the phenotypic and genotypic characteristics of the 10 M and 12 F lines are warranted.

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1. Introduction

The production of recombinant human proteins with pharmaceutical uses in the milk of transgenic animals is sometimes referred to as “gene pharming”. This technology can overcome the limitations facing traditional recombinant pharmaceutical protein production systems. The mammary gland is the preferred protein production site, due to the quantities of protein that can be produced and the ease of protein production (Niemann and Kues, 2007).

The generation of transgenic cattle is prohibitively expensive, due to the long gestation period, small litter size and high maintenance costs associated with cattle. For these reasons, the use of dairy goats as bioreactors provides
significant advantages. Several studies have demonstrated the production of transgenic goats for the secretion of valuable proteins in their milk (Ebert et al., 1991; Ko et al., 2000; Maga et al., 2006). In addition, the Food and Drug Administration’s market authorization of ATryn®, GTC Biotherapeutic’s recombinant human antithrombin, validated goat transgenic technology as a viable alternative method for the production of recombinant pharmaceutical proteins (Lavine, 2009).

Once a founder goat that has a stably integrated transgene and is correctly expressing the gene in the mammary glands has been produced, propagation of a transgenic herd may be initiated. The necessary size of the transgenic herd depends on the demand for that specific recombinant protein. When the demand only requires a few animals, the production of founder animals using a proven cell line could generate the needed supply. However, when large herds are needed to meet market demands, more traditional breeding or assisted reproductive technologies may be used. Embryos can be obtained from a transgenic female founder using either standard multiple ovulation and embryo recovery techniques or by in vitro embryo production. Moreover, male founders can be readily propagated using standard artificial insemination (AI) techniques (Keefer, 2004).

Our group initiated studies with the goal of producing the human granulocyte-colony stimulating factor (hG-CSF) in the milk of transgenic goats (Freitas et al., 2007). The hG-CSF protein is central to neutrophil-biased immune defenses, due to its regulatory roles in the growth, differentiation, survival and activation of neutrophils and their precursors (Barreda et al., 2004). Following its molecular cloning and subsequent production as a recombinant human protein (Nagata et al., 1986; Souza et al., 1986), hG-CSF has been the most widely used hematopoietic growth factor due to its proven efficacy against different forms of neutropenia and chemotherapy-induced leukopenia, as well as for mobilization of progenitor cells for autologous or allogeneic transplantation (Weltje et al., 1996).

In this study, we describe the production of transgenic founder goats for hG-CSF secretion, a partial description of the phenotypic characteristics of the founders, including milk hG-CSF production, and the propagation of a transgenic herd using AI and embryo transfer techniques.

2. Materials and methods

2.1. Animal ethics and biosafety

All animal protocols used in this work were approved by the Animal Ethics Committee of the State University of Ceará (CEUA/UECE 12/05) and Brazil’s Biosafety Technical National Committee (CTNBio 228/06). Additionally, all studies were conducted according to the guidelines for the ethical use of animals in research (ASAB, 2006).

2.2. Animals

To obtain the hG-CSF transgenic founders, Canidê goats were used for in vivo pronuclear embryo production. Eleven females were selected as embryo donors, which were fertilized by two males. Twenty goats of an undefined breed were used as the recipients for the microinjected embryos. To maintain the transgenic lines and generate progeny, the transgenic founders were bred to non-transgenic Canidê goats. For this step, seven non-transgenic females and two non-transgenic males were used to obtain the progeny of the male and female transgenic lines, respectively. Additionally, five females of an undefined breed were used as embryo recipients. All animals were housed indoors in groups of five animals per pen with controlled nutrition. To maintain animal welfare standards, goats also had access to Tifton (Cynodon dactylon) pasture for four hours a day. Goats were maintained with a high-quality concentrate (20% crude protein), water and mineral blocks.

2.3. Pronuclear embryo production

The embryo donors were subjected to a hormonal treatment for estrus synchronization and superovulation, as described by Moura et al. (2010). The recipient females received the same estrus synchronization treatment with an additional intramuscular injection of 100 µg of PMSG (Pitocen, Buenos Aires, Argentina) 48 h prior to the end of the treatment. Within 36 h after the hormonal treatment, bucks with proven fertility were mated to the donors. The donors were fasted 24 h prior to surgery, and the embryos were recovered 72 h after the hormonal treatment. To harvest the embryos, a medial ventral incision was made, and the oviduct was flushed with sterile phosphate-buffered saline (Freitas et al., 2007). The recovered medium was examined under a stereomicroscope (SMZ-800, Nikon, Kawasaki, Japan) for the identification of the ova/embryos.

2.4. DNA microinjection and embryo transfer

The pGoatcsGCSF expression vector with a long 5’ flanking sequence of goat os1-casein gene (CSN1S1) was previously designed and used to produce mice transgenic lines (Serova et al., in press). The pGoatcsGCSF construct was digested with the restriction enzymes Sall and Xhol and the DNA fragments were separated by electrophoresis in a 0.7% agarose gel using a Tris-acetate-EDTA (TAE) buffer. A 6391 bp DNA fragment (Fig. 1A) was isolated from the gel using QiaGen columns (QIAGEN Ltd., West Sussex, UK) according to the manufacturer’s recommendations. Purified DNA was dissolved in 0.01 M Tris–HCl 0.25 mM EDTA, pH 7.4 and injected in pronuclear goat embryos, as described earlier (Freitas et al., 2007). Thus, the harvested embryos were pooled together and placed in 100 µl droplets of M16 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Nutricell, Campinas, Brazil). After centrifugation (12,100 × g for 4–6 min), the zygotes with visible pronuclei were micromanipulated under an inverted microscope with DIC optics (Nikon TE2000, Kawasaki, Japan) using a pair of micromanipulators (Narisige, Tokyo, Japan). The embryos were cultured for 1–2 h at 37 °C with 5% CO2 to evaluate the post-injection survival. The surviving zygotes were maintained in culture media until they were transferred to recipients with at least one recent ovulation. Three to six embryos were transferred per recipient.

2.5. Transgene detection

The hG-CSF transgene was detected in skin biopsies from the ears of two-week-old founders using PCR amplification according to the method reported by Freitas et al. (2007). The identification of transgenic animals carrying the DNA construct was conducted by polymerase chain reaction (PCR) with two pairs of primers: PrA (forward); 5’-AAAA GTA TAA TAA TGA GG-3’, PrB (reverse); 5’-GCT TGT ACT TTT GTA CGC ACC-3’; PrC (forward); 5’-TCT GCA AAA GCA GGA TAA AGG-3’, PrD (reverse); 5’-GCC AAG ACA TTC ACC ACC CAT CAG-3’. Primers PrA and PrD are complementary to the hG-CSF gene and to the 3’-polyA region of the bovine CSN1S1 gene, respectively, whereas PrB and PrC are complementary to the 5’-flanking sequence of the goat CSN1S1 gene and to the hG-CSF gene, respectively (Fig. 1A). Sizes of PCR products were 704 bp for PrA–PrB and 530 bp for PrC–PrD primer pairs. DNA samples were obtained by phenol–chloroform extraction according to the protocol described by Sambrook et al. (1989). The PCR amplifications were performed using 50 ng of genomic DNA, 2 µM of each primer and 0.25 U of GoTaq DNA polymerase (Promega, Madison, WI, USA). The thermal cycling was performed with the following steps: (a) 3 min at 95 °C, (b) 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and (c) 5 min at 72 °C. The amplicons were analyzed by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Amplification of the β-actin gene was performed as the reaction control. Additionally, genomic DNA samples from a non-transgenic goat and an hG-CSF transgenic goat (previously obtained by Freitas et al., 2007) were used as the negative and positive controls, respectively.
2.6 Hematological analysis, serum biochemistry, hG-CSF quantification and clinic evaluations

Transgenic founders (one male and one female) and non-transgenic goats (one male and one female) were subjected to hematological and serum biochemistry examinations of samples taken at regular intervals from 45 to 280 days of age. Blood samples were collected for both white blood cell (WBC) counts (total and differential) and serum biochemistry (urea, creatinine, glucose, aspartate transaminase-AST and alanine aminotransferase-ALT), which were performed using a Cell-Dyn 3700 hematology analyzer (Abbott Laboratories Diagnostic Division, IL, USA) and BT 3000 plus (Winer Lab, Rosário, Argentina), respectively. The data were presented as the mean ± SD qualitatively compared with the normal reference values for goats (Pugh, 2002). Additionally, six serum samples were used to determine the concentration of serum hG-CSF by ELISA assay (Human G-CSF ELISA kit, RayBiotech, Norcross, GA, USA) after a 1:10 (v:v) dilution in the standard diluent buffer provided by the manufacturer. Immediately before the blood collection, the following parameters were checked in the animals: rectal temperature, heart rate, respiratory rate, inspection of ocular mucosa and palpation of lymph nodes.

2.7 Induced lactation and quantification of hG-CSF in the milk

Lactation was hormonally induced in ten-month-old female goats (one transgenic and one non-transgenic) according to the protocol described by Cammuso et al. (2000). Briefly, estrogen (0.25 mg/kg, ECP, Pfizer, São Paulo, Brazil) and progesterone (0.75 mg/kg, Afisterone, Osasco, São Paulo, Brazil) were given on alternate days for a total of seven administrations. On day 13, prednisolone (0.40 mg/kg, Corti-dural 20, Avellaneda, Argentina) was injected daily for three days. Milk samples were collected for approximately 100 days. Six samples (selected at the beginning, middle and end of lactation) of whey fraction were obtained by centrifugation (17,900 × g for 15 min), and the concentration of hG-CSF was determined by ELISA assay (Hu G-CSF ELISA kit, Invitrogen, Camarillo, CA, USA) on a 1:100,000 (v:v) dilution of the whey fraction in the standard diluent buffer provided by the manufacturer.

2.8 Western blotting analysis

The whey proteins from milk samples that had been diluted 1:5 (v:v) in water were separated on a 12.5% polyacrylamide gel using sodium dodecyl sulfate (SDS-PAGE) electrophoresis according to the method described by Laemmli (1970). Next, the proteins were electrophoretically transferred onto a Hybond P PVDF membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) using the protocol described by Sambrook et al. (1989). The membrane was blocked with a solution of 5% nonfat dried milk in PBS (w/v) with 0.1% (v/v) Tween 20 and incubated in 2 μg/ml anti-human G-CSF monoclonal antibody (Sigma–Aldrich, Saint Louis, MO, USA) at 25 ºC for 2.5 h. After incubation with the primary antibody, the membrane was then incubated in a 1:10,000 dilution of anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., Saint Louis, MO, USA) at 25 ºC for 1 h. The membrane was developed using a BCIP/NBT substrate solution (SigmaFast, Sigma Chemical Co., Saint Louis, MO, USA).
2.9. Estimation of hG-CSF biological activity in the milk

Samples of umbilical human cord blood were diluted 1:2 in TCM199 (Sigma, USA) for hematocrit reduction. Total nucleated cells were obtained after sedimentation of erythrocytes with Hespan (Hydroxyethyl starch, McGaw Inc., Irvine, CA, USA) added at a ratio of 1:6; they were then homogenized and kept at room temperature for 30 min. The top layer, rich in nucleated cells, was transferred to another tube and mixed. Plates were previously prepared with 1 ml of “Iscove’s” medium containing 3% agar (Sigma, USA) and 20% calf fetal serum (Sigma, USA), creating a semi-solid layer to prevent cell adhesion. The prepared plates were seeded with $5 \times 10^5$ total nucleated cells in 1 ml of Iscove’s medium containing 2% agar at 38 °C. The plates were fixed with formalin for at least 12 h. The colonies (groups of cells with more than 20 cells) were quantified using the inverted microscope (Nikon TE2000, Kawasaki, Japan). For morphological identification of cells, the layers of agar were transferred to slides, dehydrated, and stained by May-Grünwald–Giemsa. The G-CSF activity was expressed in colony-forming units (CFU). The significance of the differences between groups was assessed by ANOVA followed by Tukey test ($P < 0.01$).

2.10. Transgene transmission to progeny

Before F1 offspring from the founder had been obtained, the transgene was detected in the spermatozoa of the 10-month-old transgenic male founder by PCR using the method previously described for skin biopsies. The strategy used to obtain the founder offspring is demonstrated in Fig. 1B. Non-transgenic Canindé female goats were estrus-synchronized and made pregnant using AI or natural breeding with the founder male. After estrus synchronization and superovulation (as described in Section 2.3), the founder female was fertilized by a non-transgenic Canindé buck, and the embryos were recovered and evaluated under a stereomicroscope according to established guidelines (Stringfellow and Givens, 2009). After evaluation, the embryos were transferred to non-transgenic goats of an undefined breed. After the birth of the founders' progeny, both the sexual proportion and the transgene transmission efficiency were analyzed by Pearson’s chi-squared test.

3. Results

All of the goats that were used as embryo donors responded to the hormone treatment and produced 109 total embryos or ova, including 79 (72.5%) embryos in the pronuclear stage. After microinjection and embryo transfer, 12 recipients became pregnant and produced 18 kids, including one male (10 M) and one female (12 F) that carried the hG-CSF transgene (Fig. 2). Thus, the transgenic rate was 11.1% (2/18) based on offspring produced and 3.3% (2/61) based on the number of microinjected/transfered embryos. The two transgenic founders remained healthy throughout the experimental period and thereafter. At 280 days of age, both the male and female were sexually active. The male demonstrated a normal libido and his semen contained normal sperm cells that carried the hG-CSF transgene (Fig. 3).

The hematological analyses of WBCs in the peripheral blood of transgenic founders and non-transgenic controls from 45 to 280 days are shown in Table 1. Regardless of the relatively high average values for transgenic goats, neutrophil counts were not stable over time, decreasing from 45 to 160 day-of-old. Subsequent counts have reached values near the upper limit of the normal range for goats.

**Fig. 2.** The identification of hG-CSF transgenic goats using PCR analysis. The amplicons produced by PCR from genomic DNA with the PrA/PrB (AB), PrC/PrD (CD) or β-actin (Act) primers were run on an agarose gel and visualized by ethidium bromide staining. The lanes contain PCR products from the 10 M founder (lane 2), a non-transgenic goat (lane 3), the 12 F founder (lane 4) and hG-CSF transgenic goat (lane 6) previously obtained by Freitas et al. (2007). Amplification of the β-actin gene was served as the positive control, and omission of the template served as the negative control (lane 5). The molecular weight marker is a 100 bp ladder (lanes 1 and 7).

**Fig. 3.** The detection of the hG-CSF gene in the spermatozoa of the 10 M transgenic founder using PCR analysis. The amplicons produced by PCR from genomic DNA with the PrA/PrB (AB), PrC/PrD (CD), or β-actin (Act) primers were run on an agarose gel and visualized by ethidium bromide staining. The lanes contain PCR products from semen DNA (lanes 2 and 3) or skin DNA (lanes 4 and 5) from the founder (lanes 2 and 4) or a non-transgenic goat (lanes 3 and 5). Amplification of the β-actin gene served as the positive control, and omission of the template served as the negative control (lane 6). The molecular weight marker is a 100 bp ladder (lanes 1 and 7).
Both the transgenic and the non-transgenic females produced milk after the hormonal induction of lactation. The average concentration of hG-CSF produced by the transgenic female was 620.92 ± 179.93 μg/ml of milk. According to western blot analysis, two different forms of hG-CSF were detected in the milk of the transgenic goat, as shown in Fig. 4. After an image analysis using a protein ladder as a reference, the upper and lower bands of hG-CSF had apparent molecular weights of 19 and 16.5 kDa, respectively. The hG-CSF produced in bacteria was detected as a single band of 17 kDa. Additionally, the milk collected from a non-transgenic female did not exhibit any hG-CSF bands.

Table 1
Analysis of white blood cells (WBC) in hG-CSF transgenic and non-transgenic goats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>WBC</th>
<th>Granulocytes</th>
<th>Basophils</th>
<th>Eosinophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>Male</td>
<td>27.29</td>
<td>21.86</td>
<td>0.02</td>
<td>0.61</td>
<td>4.47</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>21.84</td>
<td>14.83</td>
<td>0.06</td>
<td>0.54</td>
<td>5.93</td>
<td>0.47</td>
</tr>
<tr>
<td>Non-transgenic</td>
<td>Male</td>
<td>10.99</td>
<td>4.79</td>
<td>0.07</td>
<td>0.52</td>
<td>5.32</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>5.91</td>
<td>2.49</td>
<td>0.06</td>
<td>0.09</td>
<td>3.02</td>
<td>0.24</td>
</tr>
<tr>
<td>Normal range</td>
<td></td>
<td>4.0–13.0</td>
<td>1.2–7.2</td>
<td>0–0.12</td>
<td>0.05–0.65</td>
<td>2.0–9.0</td>
<td>0–0.55</td>
</tr>
</tbody>
</table>

a The mean (±SD) of 18 observations per animal are presented for both transgenic (one male and one female) and non-transgenic (one male and one female) goats.
b Pugh (2002).

Table 2
Parameters of serum biochemistry in hG-CSF transgenic and non-transgenic goats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Glucose (mg/dl)</th>
<th>AST (U/l)</th>
<th>ALT (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic</td>
<td>Male</td>
<td>37.00</td>
<td>0.85</td>
<td>53.50</td>
<td>89.17</td>
<td>18.67</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>42.89</td>
<td>0.74</td>
<td>59.57</td>
<td>46.00</td>
<td>16.28</td>
</tr>
<tr>
<td>Non-transgenic</td>
<td>Male</td>
<td>40.89</td>
<td>0.82</td>
<td>55.11</td>
<td>97.00</td>
<td>28.72</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>34.44</td>
<td>0.76</td>
<td>56.28</td>
<td>99.78</td>
<td>18.17</td>
</tr>
<tr>
<td>Normal range</td>
<td></td>
<td>21.4–42.8</td>
<td>0.7–1.8</td>
<td>50–75</td>
<td>43–132</td>
<td>15–52</td>
</tr>
</tbody>
</table>

a The mean (± SD) of 18 observations per animal are presented for both transgenic (one male and one female) and non-transgenic (one male and one female) goats.
b Boyd (1984), Kaneko et al. (1997).

Fig. 4. The expression of hG-CSF in the milk of the 12 F female transgenic goat. Milk from the transgenic goat (lanes 2 and 5) and a non-transgenic goat (lanes 4 and 7), as well as an E. coli-derived, non-glycosylated hG-CSF (lanes 3 and 6) was subjected to SDS-PAGE (A) and western blotting (B) analyses. The molecular weight standards are shown in lane 1. The arrows in panel (B) show the positions of the non-glycosylated (lower band) and glycosylated (upper band) forms of hG-CSF in the milk of the transgenic goat. The E. coli-derived hG-CSF contained only the non-glycosylated band.
To determine the biological activity of hG-CSF in milk, we examined its effect on precursor cells of human umbilical cord blood in the colony-generating test (Fig. 5). Addition of the milk of the transgenic goat containing 50 ng/ml of hG-CSF produced a significant increase in the number of the colonies when compared to negative control with milk of non-transgenic goat (12.2 ± 4.2 versus 1.2 ± 0.9 CFU, P < 0.01). The same effect was produced by recombinant bacterial G-CSF given in the same concentration (14.3 ± 2.9 versus 1.2 ± 0.9 CFU, P < 0.01). Morphological examination of the colonies showed that they were composed of characteristic granulocyte cells at different stages of differentiation (Fig. 6).

The efficiency of transgene transmission from the founding goats (10 M and 12 F) to their progeny is presented in Table 3. To date, a total of nine transgenic (four females and five males) and ten non-transgenic (five females and five males) kids have been produced by the founders. In both 10 M and 12 F lines, the transgene segregated equally among the male and female offspring and no significant differences (P > 0.05) were found between the observed values and the expected 1:1 ratio.

4. Discussion

In this study, we report the generation and partial characterization of two transgenic goat lines for hG-CSF expression in milk. Two transgenic founders were generated by pronuclear microinjection with a DNA construct that contained the hG-CSF gene fused to goat αs1-casein promoter. The founders were healthy, fertile and able to transmit their transgenes to the F1 progeny. Additionally, the female founder was able to express the recombinant hG-CSF protein in her milk.

The transgenic rate after pronuclear microinjection, obtained in this study, was higher than the previously reported range of 7–10% described for goats (Baldassarre et al., 2004; Lee et al., 2000). The transgenic rate observed in this study was also higher than that in our previous reports, in which one transgenic Saanen goat represented 8.3% of the kids born and 0.7% of the transferred embryos (Freitas et al., 2007). Formerly, we reported that Canindé goats had a more efficient pronuclear embryo yield and a higher rate of successful microinjection than did the Saanen breed (Moura et al., 2010). Thus, in the present work, the Canindé goats may have produced higher-quality pronuclear embryos. This approach could explain the improved transgenic rate, but other factors, such as the DNA construct used, cannot be ruled out.

Fig. 5. The colony-forming assay performed on human umbilical cord blood. Negative control (milk of non-transgenic goat); milk of transgenic goat (containing 50, 100 and 200 ng/ml of hG-CSF) and positive control (recombinant hG-CSF; Filgrastim, 50 ng/ml).

Fig. 6. Analysis in light microscopy of the colonies formed after addition of transgenic goat milk. The morphology of the colonies indicates that their cells belong to the neutrophil granulocyte series. (A) 50 ng/ml; (B) 100 ng/ml; and (C) 200 ng/ml of hG-CSF.

Table 3

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of pregnancies</th>
<th>No. of offspring</th>
<th>Offspring by sex (%)</th>
<th>Transgenic ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>10 M</td>
<td>7</td>
<td>11</td>
<td>6(54.5)</td>
<td>5(45.5)</td>
</tr>
<tr>
<td>12 F</td>
<td>5</td>
<td>8</td>
<td>4(50.0)</td>
<td>4(50.0)</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>19</td>
<td>10(52.6)</td>
<td>9(47.4)</td>
</tr>
</tbody>
</table>

The transgenic ratios for each line were compared with the expected value of 50% at a significance level of 5%.

a Percentage of the total offspring

b Percentage of the transgenic animals.
The capability to hormonally induce the mammary gland to produce milk prior to the onset of natural lactation can provide early milk for the detection of the preliminary expression levels of recombinant proteins from a transgenic line (Maga and Murray, 1995). The ability to decrease the length of time to milk production could significantly impact the decision-making process regarding an animal’s genetic value within a transgenic founder development program. In our study, the 12 F founder produced more than 600 µg/ml of hG-CSF in its milk during an induced lactation. The actual quantity of hG-CSF in the milk was approximately 12 times greater than the yields obtained with the transgenic goat produced by Ko et al. (2000). Moreover, although we did not induce lactation in the F1 females, these animals may also express hG-CSF in their milk similar to the female founder, since the expression of a transgene is essentially at a constant level in the different individuals of a line over number of generations (Colman, 1996).

Before the expression cassette was introduced into goat embryos, the expression of hG-CSF directed by goat αs1-casein was investigated in mice (Serova et al., in press). Thus, the pGoatcasGCSC vector expression was able to direct tissue-specific secretion of hG-CSF into the milk of transgenic mice in the range of 19–40 µg/ml. The variability in milk hG-CSF concentration between murine lines was low, in spite of the different integration sites of the transgene in the founders, and was significantly lower than levels in the previously generated transgenic mice with the pGCm1 and pGCm2 expression vectors, in which hG-CSF concentration levels varied from 0.008 to 1000 µg/ml (Dvorianchikov et al., 2005).

Lee et al. (2000) also generated transgenic goats (one male and one female) for the production of hG-CSF. The authors used the goat β-casein promoter to regulate the expression of the cassette injected into the embryos. The transgenic female was able to produce hG-CSF in milk at levels of up to 50 µg/ml during natural lactation (Ko et al., 2000). The authors reported that the transgene was not detected in the sperm DNA of the male transgenic goat.

In the present work, we confirmed that transgenic goats were true founders, as they were able to produce transgenic descendants. To predict the birth of transgenic offspring from the 10 M line, we tested the presence of the foreign gene in the spermatooza before mating 10 M with non-transgenic females. Thus, as expected, transgenic kids were obtained at a rate of 54.4% (6/11). Because most founding animals obtained by pronuclear microinjection are hemizygous for the transgene, we would expect the transgene to be inherited by 50% of the offspring. Our results were not significantly different from the expected transgenic ratio for the first generation. Therefore, this line apparently transmits the hG-CSF transgene in a Mendelian fashion. Despite the apparent lower transgenic ratio in the 12 F first generation (37.5%), this ratio also did not differ significantly from the expected 1:1 ratio. Additionally, although the sex ratio in transgenic offspring did not differ significantly from the expected value, the number of observations remained low and will require further monitoring.

Glycosylation is undoubtedly one of the most important post-translational modifications for therapeutic proteins (Fussenegger et al., 1999) and plays an important role in many biological processes including protein stability, serum half-life, immune response, intracellular signaling and cell–cell/extracellular matrix interaction (Varki et al., 1999). In the present study, the milk of the transgenic founder contained two bands that corresponded to recombinant hG-CSF by western blotting analysis. Although specific biochemical analysis was not performed, we assumed the upper band to be a glycosylated (or more glycosylated) form of hG-CSF and the lower band to be a non-glycosylated (or less glycosylated) form of the protein, when compared with the pattern of E. coli-derived (non-glycosylated) hG-CSF band. The presence of both hG-CSF forms in the milk of transgenic animals has already been described in mice (Dvorianchikov et al., 2005) and goats (Ko et al., 2000). Ko et al. (2000) reported that the intensity of the non-glycosylated hG-CSF band was stronger than that of the glycosylated band, as evaluated by densitometry. However, the glycosylation of recombinant proteins in the mammary gland is a particularly complex issue (Houdébine, 2000, 2009), and the mechanism responsible for the presence of two bands with different intensities in the milk of our transgenic goat remains to be investigated.

The human G-CSF protein specifically regulates the in vivo proliferation and differentiation of neutrophil granulocyte precursor cells from the bone marrow (Nagata and Fukunaga, 1991). In the present work, the two forms of hG-CSF mixed in the milk of our transgenic founder (12 F) presented an in vitro biological activity comparable to E. coli-derived recombinant hG-CSF and statistically greater than negative control (non-transgenic milk). The morphological examination of colonies showed characteristic granulocyte cells at different stages of differentiation. These results indicate the functional validity of hG-CSF in the transgenic goat milk as was observed by Serova et al. (in press) using the same expression vector in mice.

In light of this recombinant protein being produced in the mammary gland, the potential for systemic in vivo biological activity in the transgenic animal is a possibility and has previously been reported (Massoud et al., 1996; Ko et al., 2000; Kim et al., 2006). Therefore, the serum levels of hG-CSF were evaluated in the transgenic goats to investigate the possibility of ectopic hG-CSF expression. Leukocyte parameters were evaluated because this cytokine can act on blood cells in the granulocytic lineage. Interestingly, despite the measured dynamic changes in WBC and neutrophil counts prior to 280 days of life, the transgenic goats in the present study had no detectable of serum hG-CSF. Despite the use of a highly sensitive ELISA assay, ectopic expression of serum hG-CSF below 1 pg/ml cannot be ruled out. However, this level probably would not explain the blood cell changes observed in transgenic goats because only hG-CSF serum levels higher than 1 ng/ml have been reported to produce granulocytosis and lymphocytosis in transgenic mice (Yamada et al., 1996). Moreover, our transgenic founders have no changes in other granulocytic and non-granulocytic cell counts, which, together with the non-transgenic animals, were
within the normal ranges for goats (Pugh, 2002). Another possibility is that transgene expression in tissues other than the mammary gland occurs and is able to alter the blood cellularity through a non-endocrine action. However, Serova et al. (in press) used RT-PCR and immunofluorescent analysis to confirm the precise tissue-specific expression of hG-CSF in the mammary glands of four mice lines produced with the same DNA construct used in the present work.

For the 10 M line, we do not have any information regarding hG-CSF expression in the milk, as induced lactation was not performed in the male founder to avoid deleterious effects on reproductive parameters. Additionally, lactation was not hormonally induced in the F1 progeny of this line because a study investigating the puberty and reproductive characteristics of these animals is currently underway.

In summary, we produced two transgenic goats with a stably integrated hG-CSF gene that were capable of secreting recombinant hG-CSF from the lactating mammary gland without causing any harm to the animals’ health. Additionally, both the male and female transgenic goats are true founders as they were proven to be fertile and capable of transmitting the hG-CSF gene to first generation progeny of each line. Additional investigations regarding the phenotypic and genotypic characteristics of the 10 M and 12 F lines are warranted. We believe that the experiments presented here provide an efficient strategy that may be useful for the industrial production of recombinant hG-CSF protein using transgenic goats.

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