Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

Birth of normal kids after microinjection of pronuclear embryos in a transgenic goat (*Capra hircus*) production program in Brazil

V.J.F. Freitas[1], I.A. Serova[2], L.E. Andreeva[3], E.S. Lopes Júnior[1], D.I.A. Teixeira[1], M.F. Cordeiro[1], D. Rondina[1], N.R.O. Paula[1], I.J. Arruda[1], J.B. Lima Verde[1], G. Dvoriantchikov[2] and O. Serov[2]

[1]Laboratory of Physiology and Control of Reproduction, State University of Ceará, Av. Paranjana, 1700, 60740-000 Fortaleza, CE, Brazil
[2]Laboratory of Animal Biotechnology, Federal University of Rio de Janeiro, Ilha do Fundão, 21949-900 Rio de Janeiro, RJ, Brazil
[3]Institute of Molecular Genetics, Center of Gene Transfer into Eucaryotes, 123182 Moscow, Russia
Corresponding author: V.J.F. Freitas
E-mail: vjff@uece.br

Genet. Mol. Res. 2 (2): 200-205 (2003)
Received January 8, 2003
Accepted April 3, 2003
Published May 15, 2003

ABSTRACT. This pilot project was designed to determine if normal kids could be produced after microinjection in pronuclear embryos and subsequent transfer to recipients in a transgenic goat program in Brazil. Twelve donors of the Saanen breed and 17 recipients of an undefined breed were used. The estrus of both donors and recipients was synchronized by a standard progestagen treatment and superovulation obtained by six pFSH injections. Donors in estrus were mated with fertile Saanen bucks. Zygotes were recovered surgically by flushing oviducts. The recovered zygotes with visible pronuclei were microinjected with 500 to 1000 copies of the human G-CSF gene. Two or four embryos were surgically transferred into the oviducts of recipients. One recipient became pregnant and two kids were born. No transgenic goat was identified after PCR analysis. Even though transgenic goats were not obtained, this experiment establishes the basis of a synchronization and superovulation regimen for use in goats raised in Brazil, for the purpose

of collecting and manipulating the pronuclear embryos. This project also showed that microinjected one-cell goat embryos can survive to produce live young following surgical transfer.

Key words: Goat, Transgenesis, Pronuclear embryos, Human G-CSF

INTRODUCTION

Transgenesis can be defined as modification of the genetic information of an organism through recombinant DNA techniques. An attractive application of gene transfer is the production of transgenic dairy farm animals carrying genes that code for pharmaceutical, diagnostic or industrially important proteins that are expressed in the mammary gland (Maga and Murray, 1995).

Microinjection of DNA constructs into the pronuclei of zygotes has been the most common method for the generation of transgenic animals (Ebert and Schindler, 1993). The production of large transgenic ruminants (cattle) is, however, prohibitively expensive because of the long pregnancy period, small litter size and high maintenance costs of this livestock species. For these reasons, dairy goats are preferred as production animals (Denman et al., 1991; Ebert and Schindler, 1993; Gootwine et al., 1997). The feasibility of large-scale production of such goats for industrial applications has been confirmed (Edmunds et al., 1998).

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that stimulates the proliferation and the differentiation commitment of neutrophil precursor cells, and enhances some of the functional properties of mature neutrophils (Morstyn and Burgess, 1988). In human medicine, human G-CSF can be used to prevent neutropenia after intensive radio- or chemotherapy, to stimulate hematopoiesis after bone marrow transplant and to increase resistance to radiation. A small herd of transgenic goats could supply the human G-CSF needed in Brazil.

We tested the possibility of producing normal kids by microinjecting DNA that codes for human G-CSF into goat pronuclear embryos and transferring the embryos to recipient animals reared under tropical conditions in Brazil.

MATERIAL AND METHODS

Animals and hormonal treatments

We used 12 multiparous Saanen (donors) and 17 multiparous or nulliparous undefined-breed goats (recipients). The estrus was synchronized with vaginal sponges impregnated with 45 mg FGA (Synchro-part, Sanofi, France) maintained for 11 days. At the ninth day of progestagen treatment, the goats received intramuscular injections of 50 µg cloprostenol (Ciosin, Coopers, Brazil) and the first of six pFSH (Folltropin, Vetrepharm, Canada) injections with decreasing doses at 12-h intervals. The total dose for superovulation was 200 mg pFSH. Donors in estrus were mated with fertile Saanen bucks every 8 h (two or three times). Goats were considered to superovulate when they presented five or more corpora lutea.

The recipients received the same progestagen treatment for 11 days and intramuscular

injections of 50 µg cloprostenol and 200 IU eCG (Synchro-part, Sanofi) on the ninth day. A recipient was used if it presented one or more corpora lutea.

Embryo recovery and microinjection

Embryo recovery was performed 72 h after sponge removal. Donors were fasted 24 h prior to surgery. A low dose (0.1 mg/kg) of xylazine (Rompun, Bayer, Brazil) was injected intramuscularly as a preanesthetic drug. After a peridural injection of lidocaine (Anestésico L, Pearson, Brazil) a mid-ventral incision was made and the reproductive tract was exteriorized. The ovaries were observed for fresh ovulation sites to estimate the number of embryos. Zygotes were recovered by flushing both oviducts with sterile phosphate-buffered saline, as previously reported (Lee et al., 1997).

After flushing, the number of structures was evaluated under a stereomicroscope (Nikon, Japan) and the zygotes were then transferred into TL-HEPES medium for evaluation under an inverted microscope with Nomarski optics (Olympus, Japan). The zygotes were classified as early, medium and late, according to the stage of formation of the pronuclei. No zygote centrifugation was used in this experiment. The zygotes clearly showing two pronuclei were microinjected with 500 to 1000 copies of the human G-CSF gene in a volume of 1 to 2 picoliters per zygote, and then immediately transferred into the oviducts of the recipients.

Embryo transfer and identification of transgenic goats

The same surgical procedure used for donors was also used for recipients. Two or four embryos were surgically transferred into one oviduct ipsilateral to the ovulated ovary, using a syringe connected to a sterile polyethylene tube, which was inserted into the oviduct lumen via the fimbria. Pregnancy diagnosis was performed by transabdominal ultrasonography at 40 days following embryo transfer.

The kids that were born were screened by PCR analysis, using genomic DNA extracted from the ears of 2-week-old kids according to the method indicated by Sambrook et al. (1989).

RESULTS

All (12/12) donors and 16/17 recipients showed estrus behavior after hormonal treatment. The mean interval (± SD) from the end of treatment to the onset of estrus was 17.7 ± 4.0 and 22.0 ± 4.4 h for donors and recipients, respectively.

Among the 12 donors, all responded to superovulation treatment. The ovulation rate (mean ± SD) was 17.4 ± 7.3. Altogether 144 oocytes/embryos were recovered, with a mean recovery rate of 69% (144/209). The fertilization rate was 65% (94/144) and most of the fertilized embryos (80%) were at the one-cell stage.

Early zygotes had small pronuclei, which were difficult to visualize. Middle zygotes had fairly visible pronuclei, positioned as a rule in the center of the zygote. This stage was the most suitable for microinjection (Figure 1). The late zygotes were also suitable for microinjection. They had very large pronuclei, positioned close to each other. A peculiar feature of goat zygotes is poor visibility of pronuclei (50-60%) due to poor translucence of the nucleoplasma.

Among 17 recipients, 2 to 13 ovulations were observed in 16 of them on the day of

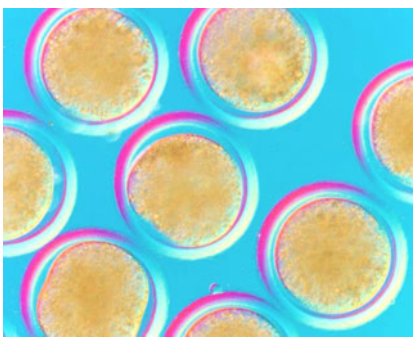

Figure 1. Pronuclear goat embryos before microinjection.

embryo transfer. The ovulation rate (mean ± SD) in the recipients was 5.3 ± 3.3. One recipient became pregnant and produced two kids (Figure 2).

No transgenic goat was identified after the PCR analysis.

Figure 2. Recipient goat and the two kids that were born (Irina and Iran).

DISCUSSION

Embryo viability of DNA-injected embryos was evaluated after embryo transfer and the overall efficiency for the production of transgenic goats (*Capra hircus*) was examined.

The superovulation treatment used in this experiment produced results comparable to those of other trials (Selgrath et al., 1990; Gootwine et al., 1997). In our experiment, hCG

together with FSH was used for the induction of an efficient and consistent ovulation rate (Lee et al., 2000). Folltropin has an extremely low LH activity, but its use alone was sufficient to induce superovulation in the goats that we used. Some researchers also use GnRH (Krisher et al., 1994) or LH (Baril et al., 1996) to help control the time of ovulation. Although these treatments increase the ovulation rate in these animals, they are not essential because FSH treatment alone was sufficient to induce ovulation.

In this study, a single injection of eCG (200 IU) was given to prepare the recipients. Although this dose was known to be nonsuperovulatory (Baril et al., 1993; Freitas et al., 1996), the ovulation rate was considered high when compared to other reports (Freitas et al., 1996, 1997). The number of one-cell fertilized embryos was similar to that found in a previous study (Selgrath et al., 1990). This indicates that the timing of collection of pronuclear embryos can be controlled by the removal of the vaginal sponge (which contains exogenous progestagen).

The pregnancy rate in this report (1/16) was lower than the results of previous reports (Gootwine et al., 1997; Lee et al., 2000). This could be due to the high number of nulliparous recipients used in this study, which are not the best recipients (Baril et al., 1995). It is also conceivable that the higher ovulation rate in induced recipients detrimentally affects the pregnancy rate (Lee et al., 2000).

The synchronism between donors and recipients, in the time of onset of estrus, is also essential to improve the rate of embryo survival due to the hormonal profile. Increased early embryonic loss and failure of implantation in recipients has been found in several mammal species, and this phenomenon could be linked to the hostile maternal endocrine environment (Moon et al., 1990).

This experiment establishes the basis of a synchronization and superovulation regimen for the purpose of collecting and manipulating the pronuclear embryo. This project also showed that microinjected one-cell goat embryos can survive to produce live young following manipulation, indicating that a project designed to produce transgenic goats is feasible.

To our knowledge, this is the first report in Brazil on the birth of kids after microinjection of goat pronuclear embryos, although we could not document the presence of the transgene in the kids. Further investigations are necessary to increase the number of offspring and, consequently, the possibility to obtain transgenic goats for human G-CSF production.

ACKNOWLEDGMENTS

The authors would like to thank M. Racine Távora for donating the recipient goats and the following research agencies: CNPq, FAPERJ and FUNCAP, for funding.

REFERENCES

Baril, G., Leboeuf, B. and **Saumande, J.** (1993). Synchronization of estrus in goats: the relationship between tiem of occurrence of estrus and fertility following artificial insemination. *Theriogenology 40*: 621-628.

Baril, G., Brebion, P. and **Chesné, P.** (1995). *Manuel de Formation Pratique Pour la Transplantation Embryonnaire Chez la Brebis et la Chevre.* FAO, Rome, Italy.

Baril, G., Pougnard, J.L., Freitas, V.J.F., Leboeuf, B. and **Saumande, J.** (1996). New method for controlling the precise time of occurrence of the preovulatory gonadotropin surge in superovulated goats. *Theriogenology 45*: 697-706.

Denman, J., Hayes, M., O'Day, C., Edmunds, T., Bartlett, C., Hirani, S., Ebert, K.M., Gordon, K. and **John, M.** (1991). Transgenic expression of human tissue-type plasminogen activator in goat milk: puri-

fication and characterization of the recombinant enzyme. *Biotechnology 9*: 839-843.

Ebert, K.M. and **Schindler, J.E.S.** (1993). Transgenic farm animals: progress report. *Theriogenology 39*: 121-135.

Edmunds, T., Van Patten, S.M., Pollock, J., Janson, E., Bernasconi, R., Higgins, E., Manavalan, P., Ziomack, C., Meade, H., McPherson, J.M. and **Cole, E.S.** (1998). Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. *Blood 12*: 4561-4571.

Freitas, V.J.F., Baril, G. and **Saumande, J.** (1996). Induction and synchronization of estrus in goats: the relative efficiency of one versus two fluorogestone acetate-impregnated vaginal sponges. *Theriogenology 45*: 1251-1256

Freitas, V.J.F., Baril, G., Martin, G.B. and **Saumande, J.** (1997). Physiological limits to further improvement in the efficiency of oestrous synchronization in goats. *Reprod. Fert. Dev. 9*: 551-556.

Gootwine, E., Barash, I., Bor, A., Dekel, I., Friedler, A., Heller, M., Zaharoni, U., Zenue, A. and **Shani, M.** (1997). Factors affecting success of embryo collection and transfer in a transgenic goat program. *Theriogenology 48*: 485-499.

Krisher, R.L., Gwazdauskas, F.C., Page, R.L., Russel, C.G., Canseco, R.S., Sparks, A.E.T., Velander, W.H., Johnson, J.L. and **Pearson, R.E.** (1994). Ovulation rate, zygote recovery and follicular populations in FSH-superovulated goats treated with PGF2α and/or GnRH. *Theriogenology 41*: 491-498.

Lee, W.K., Han, Y.M., Shin, S.T., Lee, D.H., Yoo, O.J. and **Lee, K.K.** (1997). *In vitro* development of DNA-injected embryos co-cultured with goat oviduct epithelial cells in Korean native goats (*Capra hircus aegagrus*). *Theriogenology 47*: 1115-1123.

Lee, C.S., Fang, N.Z., Koo, D.B., Lee, Y.S., Zheng, G.D., Oh, K.B., Youn, W.S., Han, Y.M., Kim, S.J., Lim, J.H., Shin, S.T., Jin, S.W., Lee, K.S., Ko, J.H., Koo, J.S., Park, C.S., Lee, K.S., Yoo, O.J. and **Lee, K.K.** (2000). Embryo recovery and transfer for the production of transgenic goats from Korean native strain, *Capra hircus aegagrus*. *Small Rum. Res. 37*: 57-63.

Maga, E.A. and **Murray, J.D.** (1995). Mammary gland expression of transgenes and the potential for altering the properties of milk. *Biotechnology 13*: 1452-1457.

Moon, Y.S., Yun, Y.W. and **King, W.A.** (1990). Detrimental effects of superovulation. *Semin. Reprod. Endocrinol. 8*: 232-241.

Morstyn, G. and **Burgess, A.W.** (1988). Hemopoietic growth factors: A review. *Cancer Res. 48*: 5624-5637.

Sambrook, J., Fritsch, E. and **Maniatis, T.** (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, NY, USA.

Selgrath, J.P., Memon, M.A., Smith, T.E. and **Ebert, K.M.** (1990). Collection and transfer of microinjectable embryos from dairy goats. *Theriogenology 34*: 1195-1205.