

PRODUCTION OF TRANSGENIC GOATS BY PRONUCLEAR MICROINJECTION OF
IN VITRO PRODUCED ZYGOTES DERIVED FROM OOCYTES RECOVERED BY
LAPAROSCOPY

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ABSTRACT

Oocytes collected by laparoscopic ovum pick-up (LOPU) were successfully used to produce transgenic goats by pronuclear microinjection of in vitro zygotes. Estrus cycles of 109 donor goats were synchronized using intravaginal sponges impregnated with 60 mg of medroxyprogesterone acetate and treatment with 70 mg NIH-FSH-P1 and 300 IU eCG to stimulate follicular development. Follicles were aspirated under laparoscopic observation. In vitro maturation of oocytes was performed in M199 supplemented with hormones, kanamycin and 10% estrus goat serum. Following IVM, oocytes were cocultured with capacitated semen in TALP supplemented with 20% estrus goat serum for 15 to 20 h. The resulting zygotes were microinjected with a linear DNA fragment. In total, 3293 follicles were aspirated (15.7 ± 9 follicles aspirated per donor) and 2823 oocytes were recovered (13.4 ± 8 oocytes per donor). A total of 1366 zygotes were microinjected and transferred into 219 recipient goats by midventral laparotomy (average 6.2 embryos per recipient). A total of 150 kids were born, of which 9 (6M: 3F) were confirmed to be transgenic by PCR and Southern blotting analyses. These results demonstrate that acceptable transgenesis rates can be obtained in goats by DNA microinjection of in vitro produced zygotes.

Keywords: folliculocentesis, ovum pick up, transgenic, microinjection.

INTRODUCTION

The production of recombinant proteins in the milk of transgenic animals has attracted interest in the last few years due to the outstanding protein synthesis capacity of the mammary gland. As a result, transgenic animals are able to produce recombinant proteins in a more efficient manner than traditional systems based on microorganisms or animal cells (1-3). This economic opportunity has stimulated the development of a new industry, as well as methodologies for the improvement of efficiency in the production of transgenic animals.

Microinjection of DNA constructs into the pronuclei of zygotes has been the most common method for the generation of transgenic animals. While the procedure is somewhat reliable, it is rather inefficient (<10% transgenic offspring, unpredictable expression) making it costly and time consuming (3 - 6).

In cattle, for economic reasons, zygotes for DNA microinjection are produced following IVM/IVF of immature oocytes recovered from slaughterhouse ovaries. While this source of zygotes is inexpensive and readily available in almost unlimited quantities, it has the disadvantage of lower developmental potential and unknown health status of the donor animals (4, 7).

In goats, the method of choice for the generation of transgenic animals has been the microinjection of in vivo produced zygotes recovered from the oviduct 15 to 20 h after the estimated time of fertilization (8, 9). In this method, the donor goats are superovulated, inseminated or mated following heat detection, and the oviduct is flushed to collect the presumptive zygotes (8 - 10). Although this method results in zygotes of high developmental capacity, the procedure is characterized by a great deal of variability in the number of pronuclear stage zygotes recovered per donor. In addition, due to adhesion formation following laparotomy, the procedure results in limited repeat use of the donor animals. Furthermore, animal care organizations around the world are enforcing regulations that limit the number of surgical procedures that can be performed per animal to between one and four.

An alternative approach to overcome the limitations arising from the use of in vivo produced zygotes would be to collect oocytes from live animals of known health status using a less invasive technique. While extensive work has been published concerning laparoscopic ovum pick-up in sheep (11 - 15) and to some extent in goats (16 - 20), to our knowledge this technology has never been utilized for the production of transgenic goats. We describe here the production of transgenic goats by pronuclear microinjection of in vitro produced zygotes derived from oocytes retrieved by laparoscopic ovum pick up (LOPU).

MATERIALS AND METHODS

Animals

A total of 210 LOPU sessions were performed on 109 goats that were assigned as oocyte donors. The donors were of various breeds including the Nigerian Dwarf, Nubian, Saanen and the Angora breeds. At the time of LOPU they were between 3 and 24 months of age. A total of 219 goats were used as recipients of microinjected embryos. Recipient goats were of standard breeds (mainly Nubian, Alpine and Boer crosses) and were between the ages of 1 to 4 years at the time of embryo transfer. All animals were housed indoors throughout the experiment and fed good quality hay.

Treatment of Donor Animals

Estrus cycles of donors were synchronized using an intravaginal sponge containing 60 mg medroxyprogesterone acetate (Veramix®, Upjohn, Canada) for 10 days combined with a luteolytic treatment of 125 µg cloprostenol (Estrumate®, Schering, Canada) 48 h prior to sponge removal, which took place at the time of LOPU. In addition, follicular development was stimulated by a gonadotrophin treatment consisting of 70 mg NIH-FSH-P1 (Folltropin-V®, Vetrepharm, Canada) and 300 IU eCG (Equinex®, Ayerst, Canada) administered intramuscularly 36 h prior to LOPU.

Treatment of Recipients

Recipients were heat synchronized using intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix®, Upjohn, Canada) for 10 days combined with a luteolytic treatment of 125 µg cloprostenol (Estrumate®, Schering, Canada) and 300 IU eCG (Equinex®, Ayerst Labs. Canada) administered intramuscularly 24 h prior to sponge removal, which took place approximately 15 h prior to LOPU in donors.

Anesthesia

Donors and recipients were deprived of food and water for 24 h prior to laparoscopy. Anaesthesia was induced with intravenous administration of 0.35 mg/kg body weight of diazepam (Diazepam®, Sabex, Canada) and 5 mg/kg body weight of ketamine (Ketalean®, Bimeda-MTC, Canada), and maintained with Isoflurane (Isoflo®, Abbot, Canada) via endotracheal intubation.

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, USA)

Laparoscopic Ovum Pick Up (LOPU)

Oocytes were recovered by aspiration of follicular contents under laparoscopic observation. The laparoscopy equipment used (Richard Wolf, Germany) was composed of a 7 mm telescope, light cable, light source, 7 mm trocar for the laparoscope, an atraumatic grasping forceps, and two 5 mm trocars. The follicle puncture set was composed of a puncture pipette, tubing, a collection tube, and a vacuum pump. The puncture pipettes were prepared by fixing a 20 G short bevel hypodermic needle into the tip of an insemination pipette with instant glue. The collection tube was a 50 mL centrifuge tube with inlet and outlet ports in the stopper. These ports were connected to the aspiration pipette and to a vacuum pump, respectively, with plastic tubing. The vacuum pressure was regulated with a flow valve and measured as drops of collection medium per minute entering the collection tube, and was adjusted to 50 to 70 drops per minute. The complete puncture set was gas sterilized and rinsed with collection medium prior to use. The collection medium was TCM 199 supplemented with 0.05 mg per mL Heparin (Hepalean®, Organon Teknica,

Canada) and 1% (v:v) fetal calf serum; the collection tube contained 0.5 mL of this medium to receive the oocytes.

The anesthetized goats were restrained in a cradle in the standard position for laparoscopic insemination. Trocars were inserted and the abdominal cavity was filled with filtered air in order to facilitate visualization of the reproductive tract. The ovaries were exposed by pulling the fimbria in different directions using the grasping forceps and the follicular contents were aspirated by puncturing follicles with the aspiration needle. At the end of the procedure, the surface of both ovaries was flushed with warm heparinized saline using an insemination gun introduced through one of the 5 mm trocars, in order to clean any blood from the follicle puncture site. The procedure was repeated 1 to 4 times in the same animals throughout the experiment.

Washing and Grading of Oocytes

Follicular contents were poured into a Petri dish and observed under a stereomicroscope. The cumulus-oocyte complexes (COCs) were located and washed in M199 supplemented with penicillin (100 IU/mL), streptomycin (0.1 mg/mL), kanamycin (0.05 mg/mL) and 0.5% bovine serum albumin. They were then transferred into the IVM drops and graded based on their cellular vestments and cytoplasm uniformity, as follows:

Grade 1: Multilayered compact cumulus and evenly granulated cytoplasm.

Grade 2: 1 to 3 layers of cumulus cells and evenly granulated cytoplasm.

Grade 3: No cellular vestments or heterogeneous cytoplasm.

COCs of grades 1 and 2 were selected for subsequent use.

Maturation of Oocytes

In vitro maturation was performed in 50 μ L drops of maturation medium under mineral oil. Maturation medium consisted of M199 supplemented with bLH (0.02 U/mL; Sioux Biochemicals, USA), bFSH (0.02U/mL; Sioux Biochemicals, USA), estradiol β -17 (1 μ g/mL), sodium pyruvate (0.2 mM), kanamycin (50 μ g/mL) and 10% heat-inactivated estrus goat serum (21). IVM

was performed at 39°C in humidified atmosphere with 5% CO₂ in air for 24 to 27 h.

In Vitro Fertilization

The expanded cumulus cells were partially removed from matured COCs by repeated pipetting or dissection using a needle. Oocytes were washed in fertilization medium and transferred to 40 µL drops of fertilization medium under mineral oil. Fertilization medium consisted of TALP medium supplemented with 20% heat-inactivated estrus goat serum.

Fresh semen was collected from one male of the Nigerian Dwarf breed into a Falcon tube using an artificial vagina on the day of IVF and was kept in the dark at 20 °C for 2 to 3 hr. Two mL of warm (37 to 38°C) Defined Medium supplemented with 6 mg/mL fatty acid free BSA (mDM) were added to the tube and mixed with the semen. A 100 µL aliquot of the semen mix was overlaid on top of a 45:90% Percoll gradient which was then centrifuged at room temperature for 30 minutes at 500 x g. The pellet was resuspended in 4 mL of mDM and centrifuged for 10 minutes at 70 x g. The washed pellet was resuspended in capacitation medium containing 0.5mM 8-bromo-cAMP, 100 nM ionomycin and 10 µg/mL heparin in 1 mL of mDM (B. Bhatia and C.L. Keefer, unpublished data). The sperm-capacitation mixture was incubated at 39° C for 15 min. The sperm concentration was adjusted to 10 X 10⁶ sperm per mL and five µL were added to the fertilization drops containing the oocytes (final sperm concentration; 1 x 10⁶ sperm per mL). The IVF drops were then incubated at 39°C in a humidified atmosphere incubator with 5% CO₂ in air.

Microinjection of Zygotes

Following coculture of the gametes for 15 to 20 h the presumptive zygotes were observed for pronuclear (PN) formation using a microscope equipped with DIC optics. In order to improve PN visualization, oocytes were microcentrifuged at 15600 xg for 3 to 4 minutes. Zygotes with visible PN were microinjected with the transgene which was a linear DNA fragment free of plasmid backbone sequences and consisted of WAP 5'- and 3'-end controlling elements fused to a spider silk encoding sequence (1 to 2 ng/mL). Apparent

swelling of the microinjected nucleus was the criterion for successful microinjection.

Embryo Transfer

Successfully microinjected zygotes were transferred into the oviducts of recipient goats that were first examined laparoscopically to confirm at least one recent ovulation. A midventral laparotomy was established and the reproductive tract was exteriorized for the transfer of embryos contained in a Tomcat® catheter (Sovereign, Canada). The catheter was introduced into the oviduct through the fimbria. The number of microinjected embryos transferred per recipient varied between 5 and 10.

Pregnancy Detection

Pregnancy was detected by ultrasound using a SonoVet® 600 scanner (Medison Corp., Korea) with a transrectal 5MHz linear array probe at 28 and 56 d following embryo transfer.

Identification of Transgenic Animals

Genomic DNA was extracted from the blood or skin of 2-week-old putative transgenic offspring using standard molecular biology techniques (22). The DNA was screened for the presence of the transgene by PCR using two different sets of primers specific to the transgene.

Confirmation of all PCR positive animals was performed using Southern blotting analysis and the Roche Molecular Biochemicals DIG system for detection.

Statistical Analysis

The first LOPU (LOPU 1) was not preceded by any other surgery or hormonal treatments. In order to determine if repetition of LOPU had a detrimental effect on follicular response or oocyte recovery, results from LOPU 1 were

used as "Control" and were compared to the results obtained in LOPU 2, 3 and 4 using Student's t test analysis.

RESULTS

A total of 3293 follicles were aspirated (15.7 ± 9 follicles aspirated per donor) and 2823 oocytes were recovered (13.4 ± 8 oocytes per donor) resulting in an average recovery rate of 85.7%. A large variation in follicle number was observed among the donors subjected to the LOPU procedure, which may be partially attributed to the age and breed of the animals used. The animals of the Nigerian Dwarf and Angora breeds were adults while the goats of the Nubian and the Saanen breeds were very young (3 to 5 months of age). As a result, the effect of breed and age on follicles aspirated could not be ruled out and, hence, statistical analysis was not attempted. Table 1 summarizes the results of follicles aspirated and oocytes recovered by LOPU.

With respect to the quality of the COCs recovered, 1386 (49%) grade 1 and 1237 (44%) grade 2 were used subsequently in IVM.

Of 2823 oocytes recovered, 2611 were evaluated, 2505 matured in vitro (95%) and 1612 were at the pronuclear stage \sim 15h after IVF (62% of oocytes). An additional 187 zygotes were polyspermic (7%) and were discarded.

A total of 1482 zygotes were microinjected, of which 1366 were transferred into 219 recipients (average 6.2 embryos per recipient). The initial pregnancy rate was 50% with 109 recipients identified pregnant at day 28. At term, 91 does gave birth to 150 kids (1.7 kids per recipient). Pregnancy loss (17% of the recipients pregnant) occurred mainly between days 30 and 60 of gestation.

Of the kids born, 9 (6M: 3F) were found to be transgenic by PCR and Southern blotting analyses. The transgenesis rate was 6% of the kids born, 0.6% of the microinjected zygotes and 0.3% of the total oocytes recovered.

The effect of repetition of the LOPU procedure on follicle numbers aspirated and oocyte recovery was analyzed in 14 Nigerian Dwarf donor goats that were subjected to LOPU four consecutive times. The average interval between

aspirations in these animals was approximately 60 days. No surgical sequels to the animal's fertility that could be detrimental for further LOPU were observed. The number of follicles aspirated and COCs recovered from these animals are shown in Table 2.

Notably, the second LOPU session resulted in a significantly higher number of follicles available for aspiration and oocytes recovered when compared to LOPU 1 using the Student's t test ($P < 0.05$).

DISCUSSION

This paper reports for the first time the production of transgenic goats by pronuclear microinjection of in vitro produced zygotes. The application of LOPU technology to animals of known health status provides an attractive method for the generation of transgenic goats.

Pronuclear microinjection of zygotes following IVM/IVF of LOPU retrieved oocytes has resulted in comparable transgenesis rates to those obtained when using in vivo-produced zygotes (8,9,10). An average of 6.2 microinjected zygotes were transferred per recipient compared to 3.6 microinjected in vivo zygotes transferred per recipient in previous studies (8). This increase in the number of microinjected zygotes transferred per recipient has resulted in similar pregnancy rates (~50%) and kids born per recipient (~1.7) to those obtained with in vivo produced zygotes. Hence, the lower developmental capacity of in vitro produced zygotes was partially compensated for in this study by means of an increased number of microinjected zygotes transferred per recipient.

An added advantage of the LOPU methodology over the utilization of in vivo produced zygotes is the improved control over the stage of development of the zygotes at the time of microinjection. Oviduct flush of superovulated donors often results in the recovery of embryos that are early (no visible pronuclei) or late (2 cell-stage) in development, as well as unfertilized oocytes (8,10).

In cattle, slaughterhouse ovaries are the most common source of in vitro produced zygotes for DNA microinjection since first reported by Krimpenfort

et al (1991). More recently, Eyestone et al (1999) reported that 36,500 zygotes needed to be microinjected in order to produce 18 transgenic calves. In short, transgenic calves were 8% of the total born but 0.05% of the microinjected zygotes. In contrast, efficiencies of transgenesis in the present study expressed as transgenic animals born per microinjected zygote are higher. This difference may be attributed to the fact that the embryos were transferred to recipients immediately after microinjection without prolonged in vitro culture.

Overall, follicles aspirated and oocytes recovered by LOPU were similar to those reported previously in goats (16, 17, 18, 20). Results in calves (24) and sheep (13), as well as in goats (Baldassarre et al, unpublished), have demonstrated that a combination of FSH and eCG in a single treatment 36 h prior to LOPU rather a multiple treatment regime is preferable because of simplicity and efficient follicular response.

Preservation of cumulus vestments in the recovered oocytes was good, suggesting that the needle size and the vacuum pressure were properly selected. These results are in agreement with those previously reported in sheep (12).

Of special importance was the confirmation that the LOPU procedure can be repeated in the same donor within a short period of time without significant surgical sequels or decrease in response. This is in agreement with the results reported by Stangl et al (25) in sheep, who performed up to 20 LOPU sessions in a group of ewes and found no detrimental effects on the fertility of these sheep at the end of the experiment. Graff et al (17) have proposed transvaginal ultrasound-guided aspiration (TUGA) as a preferred system for the recovery of oocytes from valuable animals. However, the rate of recovery of oocytes was significantly lower than those obtained by LOPU (4.3 oocytes/donor). These authors recommended TUGA over LOPU, because they suggested that LOPU had a greater risk for post-surgical adhesion formation. Our results indicate that the utilization of appropriate aspiration techniques and grasping instruments may minimize trauma to the reproductive tract resulting in few if any surgical sequels. As a result, we believe LOPU may be preferable over TUGA for the propagation of valuable animals and endangered species. The ability to repeat the LOPU procedure in the same donor animals at least four times without any decrease in the numbers of oocytes recovered

is a main advantage over the recovery of in vivo zygotes by laparotomy. As a result the LOPU procedure allows for better utilization of donor animal resources and higher number of transgenic animals born per donor goat.

In conclusion, our results indicate that efficient transgenesis rates can be obtained in goats by DNA microinjection of in vitro produced zygotes. In addition, we have demonstrated that LOPU is a reliable and effective technique for the recovery of goat oocytes for production of zygotes.

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Table 1. Follicles were aspirated and cumulus-oocyte complexes were recovered by laparoscopic ovum pick up (LOPU) from goats of four breeds. Values reported are total recovered from each breed and means and SD per goat.

Breed of goat (n)	Laparoscopic		Aspirated follicles	Cumulus-oocyte
	ovum pick ups practiced		Total for breed (mean + SD/goat)	Total for breed (mean + SD/goat)
Nigerian Dwarf (61) (11.3±5)		149	1918 (12.8±5)	1699
Saanen	(16)	19	469 (24.7±20)	365 (19.2±13)
Nubian	(23)	33	813 (24.6±10)	671 (20.3±10)
Angora (9.8±4)	(9)	9	93 (10.3±4)	88
Total (13.4±7)	(109)	210	3293 (15.5±9)	2823

Table 2 . Follicles aspirated and oocytes recovered from a group of 10 goats subjected to 4 consecutive laparoscopic ovum pick up sessions at approximately 60 day-intervals.

	Total follicles aspirated (mean + SD/goat)	Total oocytes recovered (mean + SD/goat)
LOPU session 1	177 (12.6±5)	160 (11.4±5)
LOPU session 2	251 (17.9±6)	228 (16.3±6)
LOPU session 3	199 (14.2±3)	181 (12.9±3)
LOPU session 4	156 (11.1±3)	123 (8.8±3)

Values in the same line with different superscripts are different (t test, P<0.05).