

Methods Paper

Nuclear Transfer in Goats Using *In Vitro* Matured Oocytes Recovered by Laparoscopic Ovum Pick-Up

HERNAN BALDASSARRE, CAROL KEEFER, BIN WANG,
ANTHOULA LAZARIS, and COSTAS N. KARATZAS

INTRODUCTION

THE PRODUCTION of the first transgenic mouse by pronuclear injection in 1981 (Gordon and Ruddle, 1981) stimulated the interest of animal scientists working with domestic livestock, since the potential of transgenic domestic livestock as bioreactors for the production of valuable biopharmaceuticals was immediately recognized. Relatively soon, transgenic sheep, pigs, and goats were produced (Wall et al., 1992); however, efficiencies of transgenic animal production in livestock were lower than that obtained in mice. Following pronuclear microinjection of *in vivo* derived goat zygotes, only two of 29 goats born were transgenic (Ebert et al., 1991). This rate of production has not changed despite improvements in techniques (Baldassarre et al., 2003). Cloning using nuclear transfer (NT) provides a much more efficient system than DNA microinjection for the production of transgenic goats as the donor cells can be screened for transgene incorporation into the genome, copy number, and integration sites prior to its use in NT (Keefer et al., 2001). This selection of the transgenic donor cell ensures that nearly all the progeny produced will be transgenic (Baldassarre et al., 2002). Cloning can also be used to propagate transgenic animals produced by DNA microinjection (Baguisi et al., 1999; Keefer et al., 2002a).

Successful production of transgenic goats not only requires care and skill in preparation of the

transgenic donor cell (Lazaris et al., in press), but also in the management of the oocyte donors and the embryo recipients. Reproductive management is important in maximizing the number and quality of the oocytes recovered from donors, as well as in the ability of the recipients to allow development to term of the NT embryos. In addition, excellent health status and animal husbandry practices are required to maximize the reproductive results and ensure compliance with regulatory guidelines and animal welfare recommendations.

MEDIA

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Oocyte

1. *Aspiration medium*. TCM199H (GIBCO) supplemented with 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 0.05 mg/mL kanamycin, 1% fetal calf serum, and 0.05 mg/mL heparin.

2. *Oocyte maturation medium*. TCM199H supplemented with bLH (0.02 U: Sioux Biochemicals, Sioux Center, IA), bFSH (0.02 U: Sioux Biochemicals), estradiol β -17 (1 μ g/mL), 0.2 mM sodium pyruvate, kanamycin (50 μ g/mL), 100 μ M cysteamine, and 10% heat inactivated goat serum (obtained from our own does).

Manipulation

1. *Manipulation medium.* EmCare® (ICP Chemicals, NZ) supplemented with 1% fetal calf serum (Gibco).

2. *Handling medium.* EmCare supplemented with 1 mg/mL BSA.

3. *Sorbitol fusion medium.* 0.25 M sorbitol, 100 μ M calcium acetate, 0.5 mM magnesium acetate, 0.1% BSA.

4. *Post fusion medium.* M119H supplemented with 10% FCS and 7.5 μ g/mL cytochalasin B.

5. *Activation medium 1.* G1.2 (VitroLife) supplemented with 5 μ M calcium ionomycin.

6. *Activation medium 2.* G1.2 supplemented with 7.5 μ g/mL cytochalasin B and 10 μ g/mL cycloheximide.

Embryo

1. *Culture medium.* G1.2 medium in microdrops (25 μ L) under lightweight mineral oil.

2. *Transport medium.* EmCare supplemented with 1% FCS.

METHODS

Donor animals

1. Does of various breeds (including BELE®, Saanen, Nubian, and crosses) are used as oocyte donors. Estrous are synchronized using intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix®, Upjohn, Canada) for 10 days combined with a luteolytic treatment of 125 μ g cloprostenol (Estrumate®, Schering, Canada) 48 h prior to sponge removal, which takes place at the time of LOPU.
2. Follicular development is stimulated by a gonadotrophin treatment consisting of 80 mg NIH-FSH-P1 (Folltropin-V®, Bioniche, Canada) and 300 IU eCG (Novormon®, Bioniche, Canada) administered intramuscularly 36 h prior to laparoscopic ovum pick-up.

Oocytes

Immature oocytes are collected by LOPU (Baldassarre et al., 1994). Compared to the surgical

recovery of ovulated (*in vivo* matured) oocytes from the oviduct, this procedure allows for a more homogenous quality and stage of development of the oocytes, as well as the possibility to repeat the procedure more times in the same donor. Compared to the use of abattoir-derived oocytes, this method may offer a higher oocyte quality as the oocytes are obtained from hormone-primed follicles (Baldassarre et al., 1994). Furthermore, there are safety advantages resulting from collecting the oocytes from goats of known health status (Baldassarre et al., 2002).

1. Donors are deprived of food and water for 24 h prior to laparoscopy. Anaesthesia is 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.
2. Follicular contents are aspirated while observing through the laparoscope. The laparoscopy equipment (Richard Wolf, Germany) is 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.
3. The follicle puncture set consists of a puncture pipette, tubing, a collection tube, and a vacuum pump. The puncture pipettes are prepared by fixing a 20-gauge short bevel hypodermic needle into the tip of an insemination pipette with instant glue. The collection tube is a 50-mL centrifuge tube with inlet and outlet ports in the stopper. These ports are connected to the aspiration pipette and to a vacuum pump (oil-less diaphragm-type vacuum pump, GAST model DOA-P104-AA, Fisher Scientific), respectively, with plastic tubing.
4. Vacuum pressure is regulated using a flow valve and measured as drops of collection medium per minute entering the collection tube. Flow rate is adjusted to 50–70 drops per minute.
5. The gas-sterilized puncture set is rinsed with collection medium prior to use.
6. A small volume of aspiration medium (0.5 mL) is aspirated into the collection tube prior to collecting the oocytes.
7. The anesthetized goat is restrained in a cradle in the standard position for laparoscopic insemination.

8. Trocars are inserted, and the abdominal cavity is filled with filtered air in order to facilitate visualization of the reproductive tract. The ovaries are exposed by pulling the fimbria in different directions using the grasping forceps.
9. The follicular contents are aspirated by puncturing follicles with the aspiration needle. At the end of the procedure, the surface of both ovaries are 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.

Oocyte maturation

1. The oocyte-cumulus complexes (OCC) are removed from the aspirant and washed through M199H supplemented with 10% heat-inactivated estrus goat serum. The numbers of denuded oocytes, OCC with one to two layers of cumulus cells, OCC with three or more layers of cumulus cells, and expanded OCC are determined and recorded. Those OCC that contained at least one layer of compact cumulus cells are placed into maturation medium.
2. OCC are cultured in groups of five to 15 to a drop (50 μ L volume) of maturation medium, which is covered with an overlay of mineral oil and incubated at 38.5–39°C in 5% CO₂.

Donor cells

There is considerable controversy as to whether it is better to synchronize donor cells in a growth arrested state (G0) or in a proliferating state (G1). In a few cases, cells at the metaphase stage have also been used. Serum starvation (low serum conditions) used to synchronize cells in G0 can have

detrimental consequences, such as induction of DNA fragmentation (Kues et al., 2000); however, cells can also be synchronized by maintenance in confluency (G0 arrest) or by exposure to various inhibitors (Bordignon et al., 2001; Gibbons et al., 2002). The critical issue is that the donor cell and the oocyte should be synchronized in their cell cycles.

1. Transfection of fetal fibroblasts is performed as previously described (Keefer et al., 2001; Lazaris et al., in press). Alternately, cells can be obtained from a transgenic animal (Baldassarre et al., 2002).
2. Cells ($0.5\text{--}1 \times 10^5$) are plated into 24- or 96-well plates and cultured in DMEM + 10% FBS until they reached 100% confluency.
3. The media is then replaced with low serum media (DMEM + 0.5% FBS + 20 μ g/mL gentamycin), and the cells are incubated at 38°C, 5% CO₂ for 4 days until day of nuclear transfer. Alternately, the cells can be maintained in confluency without serum starvation.
4. Just prior to cell transfer, the donor cells are collected by trypsinization using 0.05% trypsin-EDTA, washed twice, and resuspended in Em-Care containing 1% BSA.

Manipulation set-up

The manipulation system consists of an inverted microscope equipped with UV fluorescence (e.g., Zeiss Axiovert, Olympus, Leica DM IRB) and Narishige micromanipulators (M202) as illustrated in Figure 1.

1. Glass micromanipulation pipettes (holding, enucleation, and cell injection) can be pur-

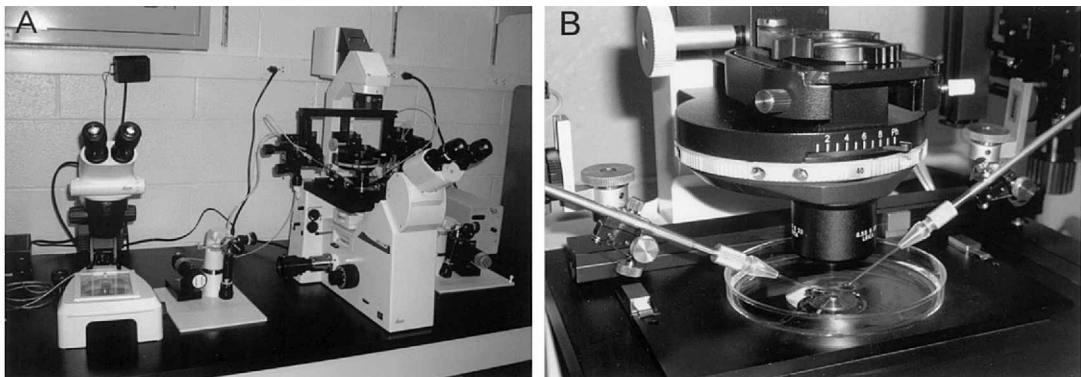


FIG. 1. (A) Micromanipulation set-up consisting of Leica DM IRB inverted microscope outfitted with Narishige manipulators and Eppendorf Cell Tram injectors. (B) Close-up of manipulation dish and microtools.

chased from a number of different distributors or produced using a needle puller (e.g., Sutter P97, Sutter Instruments), a microforge, and a beveler (e.g., Narisige EG4). The enucleation and cell injection pipettes are beveled at a 45-degree and have a small sharp spike on the tip to aid in penetrating the zona pellucida.

2. Suction and pressure are controlled using a micro syringe system (e.g., Cell Tram Vario, Eppendorf) connected by tubing to the micropipettes.
3. High-quality plastic Petri dishes (Optilux, Falcon) can be used to hold the manipulation drops. Approximately 150 μL of manipulation medium is placed in the center of the manipulation plate and spread to flatten out the drop. An overlay of lightweight mineral oil is placed over the drop to prevent evaporation and to provide a barrier to possible contamination.

Enucleation

1. After 23–24 h of maturation, the cumulus cells are removed by vortexing the OCCs for 1–2 min in a 1.5-mL cryovial containing 0.25 mL of EmCare[®] supplemented with 1 mg per mL hyaluronidase.
2. The denuded oocytes are washed in handling medium (EmCare supplemented with 1% FCS) and then returned to maturation medium.
3. Prior to enucleation, the oocytes are incubated in Hoechst 33342 (Sigma; 5 μg per mL handling medium) under an oil overlay for 15–30 min at 30–36°C in air atmosphere.
4. Five to 15 oocytes are placed into manipulation drops (EmCare supplemented with 1% FCS) covered with an overlay of mineral oil.
5. The cytoplasm adjacent to the polar body is briefly exposed (few seconds) to UV light (Zeiss Filter Set 01) to determine the location of the metaphase chromosomes.
6. The chromosomes and surrounding cytoplasm are removed by aspiration.
7. The pipette containing the aspirant is moved into UV light path to check for the presence of the chromosomes.

Cell transfer

1. Ten to 15 enucleated oocytes are placed into the manipulation drop.
2. Dispersed donor cells are placed either at the edge of the manipulation drop or in a small

adjacent drop. Small (<20 μm) donor cells are picked up with a manipulation pipette and slipped into perivitelline space of the enucleated oocyte.

3. Upon completion of the cell transfers, the couplets are placed into a dish of handling medium and placed onto a warming plate until fusion.

Fusion

1. Couplets are washed through a small Petri dish containing EmCare supplemented with 1 mg/mL BSA and then placed into a dish containing a 1:1 mixture of EmCare: sorbitol fusion medium. The couplets are allowed to settle to the bottom of the dish.
2. Couplets are moved to small dish containing the sorbitol fusion medium and allowed to settle to the bottom of the dish.
3. Two to four couplets are placed between the electrodes of the fusion chamber (500 μm gap fusion chamber, BTX). The couplets are manually aligned using a finely pulled, closed tip pipette. Each couplet is aligned such that the axis that runs through cell and oocyte is perpendicular to the electrodes.
4. The appropriate fusion pulse is applied. In this example, a brief fusion pulse (15 μsec) at 2.39 KV/cm was administered using a BTX Electrocell Manipulator 200.
5. The couplets are washed through a dish containing a 1:1 mixture of EmCare and sorbitol fusion medium and then through a dish of EmCare supplemented with 1 mg/mL BSA.
6. The couplets are placed into 25- μL drops of post fusion medium overlaid with mineral oil.
7. Couplets are observed after 1-h couplets for fusion. Couplets that have not fused are administered a second fusion pulse as described above.

Activation

At 2–3 h after application of the first fusion pulse, the fused couplets are activated using the calcium ionomycin and cycloheximide (Liu et al., 1998).

1. Briefly, couplets are incubated for 5 min in 2 mL of EmCare containing 5 μM calcium ionomycin (Sigma).
2. They are then incubated for 5 min in 2 mL of EmCare containing 30 mg BSA per ml.

TABLE 1. RESULTS FROM NUCLEAR TRANSFER IN GOATS USING *IN VITRO* TRANSFECTED FETAL FIBROBLASTS AND CUMULUS-GRANULOSA CELLS FROM TRANSGENIC DONOR ANIMALS

| <i>Type of donor cell</i> | <i>FF (transfected)</i> | <i>Cumulus-granulosa</i> |
|---------------------------------------|-------------------------|--------------------------|
| Oocytes processed | 1700 | 595 |
| Embryos reconstructed and transferred | 830 | 256 |
| Recipients transferred | 74 | 23 |
| Initial pregnancy | 21 (28%) | 8 (35%) |
| Pregnancy losses | 5 | 1 |
| Total kids born | 26 | 9 |
| Total live kids | 17 | 8 |
| Kids alive/embryos transferred | 2% | 3% |

Data from Baldassarre et al., 2002.

3. The stimulated embryos are washed through EmCare and then are cultured for 5 h in embryo culture medium containing cycloheximide and cytochalasin B.
4. The Embryos are then washed in handling medium and placed into culture drops.

Embryo culture

1. Reconstructed zygotes are cultured in G1.2 under an oil overlay at 38.5–39°C in 6% CO₂, 6% O₂, 88% N₂.
2. Both one-cell and cleaved (2-cell) embryos are transferred on day 1 (day 0 = day of fusion) into synchronized recipients (Keefer et al., 2002b).
3. Small cryovials (1.5 mL) are used to transport the embryos from the laboratory to the farm for transfer into recipients. The vials are filled with 1 mL of pre-warmed EmCare supplemented with either BSA or serum.
4. All the embryos for transfer into one recipient (~10 day 1 embryos) are placed together in one vial.
5. The vial is labeled with the number and code for the NT embryos, and is placed into the pre-warmed portable incubator.
6. At the farm laboratory, the vials are maintained in the portable incubator until embryo transfer, which is performed surgically. Surgery should be scheduled to begin immediately upon arrival of the embryos at the facility.

Embryo transfer

1. Adult goats of various breeds (including Boer, Saanen, Nubian, and Kinder crosses) are used as recipients. They are heat synchronized using intravaginal sponges containing 60 mg medroxyprogesterone acetate for 10 days com-

2. Estrus is detected and recorded in recipients at 12-h intervals, between 24 and 60 h from sponge removal. Ideally, recipients will have ovulated 0–24 h prior to embryo transfer.
3. Recipient goats are deprived from food and water prior to surgery and anesthetized following procedures previously described for donors.
4. Prior to the transfer of the reconstructed embryos, recipient goats are examined laparoscopically to confirm the presence of at least one recent ovulation.
5. A midventral laparotomy is then established and the reproductive tract is exteriorized to allow introduction of the catheter containing the



FIG. 2. Cloned kids derived from transgenic cumulus-granulosa cells.

embryos into the oviduct ipsilateral to the ovulations, through the fimbria. The number of NT-embryos transferred per recipient varies between 8 and 12.

6. Goats remain under close monitoring for the first 72 h after laparotomy, after which they were transferred back to their pens.

Pregnancy detection

Pregnancy is detected by ultrasound using a SonoVet® 600 scanner (Medison Corp., Korea) with a transrectal 7.5-MHz linear array probe at 28 and 56 days following embryo transfer.

RESULTS

Results from our transgenic founder generation and propagation program by NT are shown in Table 1. Nine transgenic cell lines were used in that data set; eight of these lines were generated by transfecting fetal fibroblasts derived from two fetuses, and one was generated from cumulus-granulosa cells obtained from a transgenic doe. The largest source of variation in pregnancy establishment and maintenance, as well as neonatal viability, appeared to be the cell line. This was demonstrated in a larger data set compiled using over 40 different cell lines in which rates were 0–89% for initial pregnancy, 0–67% for pregnancy after 60 days, 0–67% for maintenance of pregnancy to term, and 0–67% perinatal kid mortality (data not shown). Furthermore, a decrease in initial pregnancy rate and an increase in pregnancy losses in recipients transferred out-of-season (spring–summer) were observed as compared to those transferred during the reproductive season (fall–winter, data not shown). While most term pregnancies resulted in healthy kids (Fig. 2), diverse types of abnormalities affecting the heart, liver, and/or kidneys were observed following necropsy of those clones that were born dead or died shortly after birth. Live kids from some lines have manifested behavioral abnormalities, including “aggressive feeding,” in which the clones were very demanding and aggressive at feeding, and “selective feeding,” in which the clones were delayed in their willingness to switch to solid feed. Clones from some other lines have shown susceptibility to contracting respiratory infections. In most cases, these conditions improved dramatically and/or disappeared after 1 year of age.

ACKNOWLEDGMENTS

The information presented in this manuscript is the result of the efforts of several dedicated teams at Nexia Biotechnologies, including molecular and cell biology (Anthoula Lazaris, Rebecca Keyston, Martin Leduc, Annie Bilodeau), embryology (Carol Keefer, Bin Wang, Isabelle Begin, Bhim Bhatia, Khalid Rao), assisted reproduction (Hernan Baldassarre, Janice Pierson, Nathalie Neveu, Fabienne Cote, Renate Reijmers), and animal health and husbandry (Nacereddine Kafidi, Duncan Hockley, Jaime Lapointe, Laura Sneek, Julie Begin, Carol-Anne Carriere).

REFERENCES

- Baguisi, A., Behboodi, E., Melican, D.T., et al. (1999). Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* **17**, 456–461.
- Baldassarre, H., de Matos, D.G., Furnus, C.G., et al. (1994). Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. *Anim. Reprod. Sci.* **35**, 145–150.
- Baldassarre, H., Wang, B., Kafidi, N., et al. (2002). Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. *Theriogenology* **57**, 275–284.
- Baldassarre, H., Wang, B., Kafidi, N., et al. (2003). Production of transgenic goats by pronuclear microinjection of *in vitro* produced zygotes derived from oocytes recovered by laparoscopy. *Theriogenology* **59**, 831–839.
- Bordignon, V., Clarke, H.J., and Smith, L.C. (2001). Factors controlling the loss of immunoreactive somatic histone H1 from blastomere nuclei in oocyte cytoplasm: a potential marker of nuclear reprogramming. *Dev. Biol.* **233**, 192–203.
- Ebert, K.M., Selgrath, J.P., DiTullio, P., et al. (1991). Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Biotechnology NY* **9**, 835–838.
- Gibbons, J., Arat, S., Rzczidlo, J., et al. (2002). Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. *Biol. Reprod.* **66**, 895–900.
- Gordon, J.W., and Ruddle, F.H. (1981). Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science* **214**, 1244–1246.
- Keefer, C., Lazaris, A., Keyston, R., et al. (2002a). Cloning using somatic cells for the production and propagation of transgenic Nigerian Dwarf and Dwarf-crossbred goats. *Theriogenology* **57**, 422.
- Keefer, C.L., Baldassarre, H., Keyston, R., et al. (2001). Generation of dwarf goat (*Capra hircus*) clones following nuclear transfer with transfected and nontrans-

- fectured fetal fibroblasts and *in vitro*-matured oocytes. *Biol. Reprod.* **64**, 849–856.
- Keefe, C.L., Keyston, R., Lazaris, A., et al. (2002b). Production of cloned goats after nuclear transfer using adult somatic cells. *Biol. Reprod.* **66**, 199–203.
- Kues, W.A., Anger, M., Carnwath, J.W., et al. (2000). Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. *Biol. Reprod.* **62**, 412–419.
- Lazaris, L., Keyston, R., Karatzas, C.N., et al., in press. Transgenesis using nuclear transfer in goats. In *Cell Reprogramming and Transgenesis by Nuclear Transfer*. P.J. Verma and A.O. Trounson, eds. (Humana Press, Totowa, NJ).
- Liu, L., Ju, J.C., and Yang, X. (1998). Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev.* **49**, 298–307.
- Wall, R.J., Hawk, H.W., and Nel, N. (1992). Making transgenic livestock: genetic engineering on a large scale. *J. Cell. Biochem.* **49**, 113–120.

Address reprint requests to:

Dr. Carol Keefe

Department of Animal and Avian Science

University of Maryland

College Park, MD 20742-2311

E-mail: ckeefe@umd.edu