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Chapter 3

The Use of Reproductive Technologies to Produce Transgenic Goats

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Additional information is available at the end of the chapter

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Abstract

Recombinant DNA technology has revolutionized the production of therapeutic proteins. Thus, genes of a great number of human proteins have already been identified and cloned. The use of farm animals as bioreactors may be the better choice to produce recombinant therapeutic proteins. For this activity, the term "pharming" was created, referring to the use of genetic engineering to obtain a transgenic or genetically modified animal. Considering the advantages and disadvantages of livestock species, goats appear as a very good model. In addition, the first human commercially approved biological drug (antithrombin (AT)) was produced from the milk of transgenic goats. The aim of this chapter is to present various reproductive technologies used to obtain transgenic goats secreting recombinant proteins in milk. Initially, this chapter presents the methods for embryo production (in vivo and in vitro) to realize the DNA microinjection in pronuclear embryos. Thus, the techniques of superovulation of donors (in vivo embryo production) and ovarian stimulation for oocyte recovery (in vitro embryo production) are described. Also, the methods for DNA microinjection and embryo transfer are detailed in this chapter. Finally, this chapter describes the reproductive procedures used for obtaining transgenic goats by cloning.

Keywords: Transgenesis, DNA microinjection, Cloning, Assisted reproductive technologies, Embryo

1. Introduction

The transgenesis technique consists of introducing a DNA construction into the genome of a pluricellular organism, which then appears in most cells and, posteriorly, is transmitted to the offspring. Typically, the term "transgenesis" is used to plants and animals, whereas other

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organisms (yeast, bacteria, and cultured cells) harboring a foreign DNA fragment are named as "recombinant". The first gene transfer into mouse using isolated DNA showed that it was possible to generate animals stably harboring foreign DNA and to modify phenotypic properties [1, 2]. Subsequently, the use of gene microinjection was extended to pigs, sheep, and rabbits [3]. After 4 years, the gene replacement was achieved by homologous recombination [4].

Initially, in the genetic engineering area, the recombinant proteins were expressed in bacteria and yeast. Subsequently, it was proved that several human recombinant proteins might not be efficiently produced in those systems because human proteins do not undergo post-translational modifications in bacteria. Additionally, in yeast cells, the modifications those proteins suffer are different from those occurring in human cells. For these reasons, the great challenge was to develop expression systems ensuring correct post-translational modifications in recombinant proteins. According to the studies performed to date, transgenic animals offer attractive advantages to prepare recombinant proteins, such as low cost production and high protein quality (for review see [5]).

Application of genetic modified animals (GMA) technology to domestic species has been limited due to the high cost of this kind of research. Thus, the selection of species to be used as bioreactors depends on several factors (Table 1); however, the quantity of protein required and the timescale for production are key factors. Additionally, feasibility and the costs of keeping and breeding the animals should also be considered [6].

Species	Pros	Cons	Reference*
Rabbit	Short generation interval	Very low milk yield	[3]
Pig	Short pregnancy length Production of multiple offspring	Low milk yield Difficult DNA microinjection	[3]
Sheep	Short pregnancy length Production of multiple offspring	Low milk yield Difficult DNA microinjection	[3]
Goat	Short pregnancy length Production of multiple offspring Good milk yield	Difficult DNA microinjection	[9]
Cattle	Very good milk yield	Log generation interval High maintenance cost Difficult DNA microinjection	[10]

Table 1. The pros and cons for many animal species producing recombinant proteins in milk.

Considering the advantages and disadvantages of each livestock species, goats appear as an excellent model for their use as bioreactors. In addition, it was from the milk of transgenic goats that was produced and approved the commercialization of the first human biological

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drug (antithrombin (AT)). This approval first occurred in Europe, by The European Agency for the Evaluation of Medicinal Products [7], and after in the United States, by the Food and Drug Administration [8].

Transgenic goats are traditionally produced through the microinjection of a DNA construct into the pronuclei of early embryos (Figure 1). Since the first report on transgenic goats using this method to produce the human tissue plasminogen activator [9], several other human proteins have been similarly produced such as hepatitis B surface antigen [11], lysozyme [12], lactoferrin [13], and granulocyte colony stimulating factor (hG-CSF) [14]. Following the report of the birth of the first cloned sheep [15], new improvements in production of transgenic goats were obtained by the use of somatic cell nuclear transfer (SCNT), a method allowing to incorporate a DNA construct into target cells in culture using lipid-mediated transfection, followed by the selection of properly integrated donor cells for transfer. The efficiency obtained from the two methods is lower in nuclear transfer reconstructed embryos than in pronuclear microinjection, although all of the animals born are transgenic and most of them produce the recombinant protein in milk during induced lactation [16].

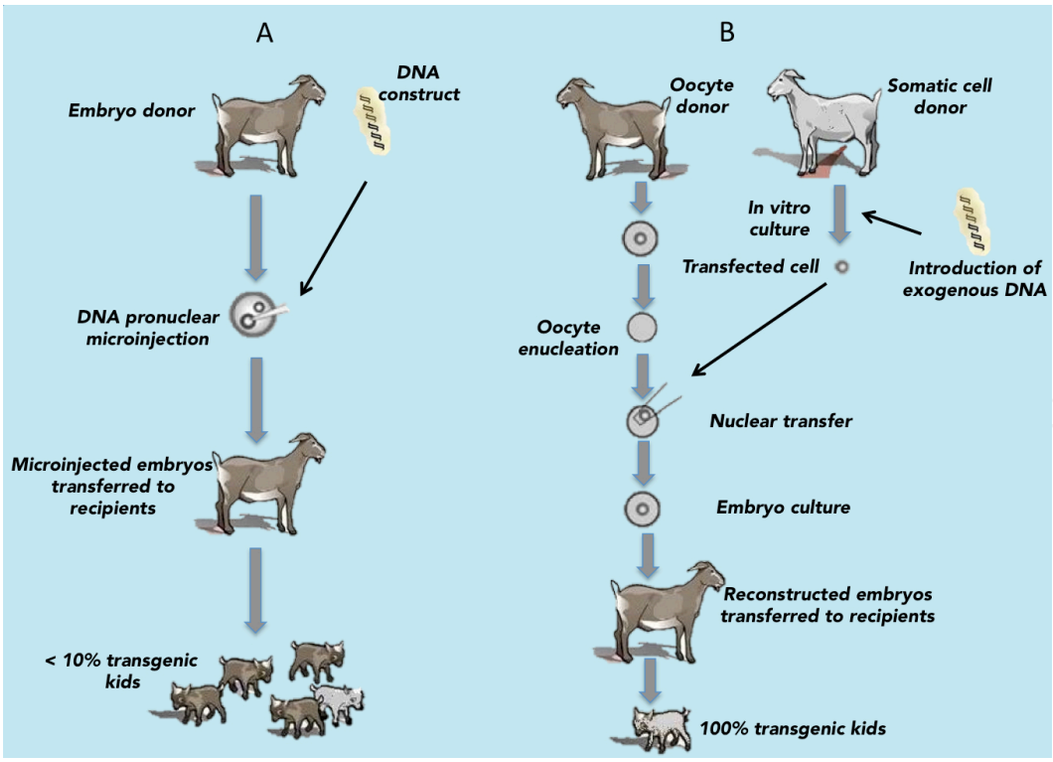


Figure 1. Traditional methods used to obtain transgenic goats. A: DNA pronuclear microinjection. B: Somatic cell nuclear transfer (SCNT).

2. Pronuclear microinjection

The intention of the production of the first genetically manipulated goat embryos was to obtain transgenic animals able to secrete pharmaceuticals in their milk, in particular the human tissue plasminogen activator [9]. Upon its success, several other human proteins have been produced in goats using pronuclear microinjection. However, the technique presents a poor overall efficiency, especially when compared to that obtained in mice. In goats, up to 1% of the injected zygotes give birth to a transgenic kid [17]. The method to produce transgenic goats using this method can be summarized in the following steps: obtaining pronuclear embryos (in vivo or in vitro), embryo microinjection with a DNA construct, transfer of microinjected embryos into recipient goats, and early pregnancy diagnosis by ultrasound. The methods presented here on microinjection technology to generate transgenic goats are based on our extensive experience in goat reproductive technology and are complemented by information from the literature.

2.1. In vivo embryo production

A closely timed protocol to recover pronuclear embryos was developed in our laboratory (Table 2); groups of four donors are used for reasons of efficiency. This protocol involves estrus synchronization with the use of vaginal sponges, superovulation with porcine follicle stimulating hormone (pFSH; Folltropin-V, Vetrepharm, Belleville, Canada), and the administration of gonadotrophin-releasing hormone (GnRH; Fertagyl; Intervet, Boxmeer, The Netherlands) to ensure precisely controlled ovulation [18]. In our experience, it is necessary to mate superovulated donors twice (at the beginning of estrus and 12 hours after) with the respective bucks to achieve acceptable fertilization rates. Using Saanen or Canindé goats, an average of 24 and 12 ovulations can be obtained, respectively. However, it is important to emphasize that FSH doses to obtain superovulation differ between breeds: we use 120 and 200 mg of pFSH for Canindé and Saanen females, respectively.

Embryos are recovered surgically 24 hours after the second mating. Animals are deprived of food and water for 24 hours prior to laparoscopy. Anesthesia is induced by administration of thiopental (20 mg/kg of body weight) intravenously and maintained by continuous infusion of 3% isoflurane, using an inhalational system with medical oxygen. A medial ventral incision is made, and the oviduct is flushed to harvest the embryos, with 15–20 mL of Dulbecco modified phosphate-buffered saline (DMPBS) using a catheter inserted in the uterotubal junction and connected to a syringe containing the medium. A plastic cannula (tom cat) is inserted in the infundibulum for flushing recovery into a Petri dish. The recovered medium is examined under a stereomicroscope (70–80×) for identification of ova and embryos. The average recovery rate (embryos per ovulation) is about 80% (>10 ova/embryo per donor) and, for Canindé breed, the fertilization rate is not lower than 75% (7–8 embryos per donor) [19, 20] (Figure 2).

Thereafter, the ova found are observed with an inverted microscope (300×) for a qualitative evaluation. Presumable zygotes are centrifuged (12000× *g* for 5 min) to allow a better visualization of the pronuclei, while ova are placed in drops of M16 medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS). Then, embryos presenting visible pronuclei

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are immediately microinjected, whereas the others are placed for in vitro culture at 38.5°C in a humidified atmosphere with 5% CO_2 for a short period. Goat zygotes show nontransparent cytoplasm and approximately 125–130 μm in diameter (Figure 3A and B). Nontransparent cytoplasm is due to the presence of a large amount of lipid granules that hinder the visualization of pronuclei. In addition, pronuclei seem to be visible sometimes, but this impression can, however, appear false after attentive examination with an inverted microscope equipped with interferential contrast optics (Nomarsky). The presence of the second polar body is a rather marked indication that the egg has been fertilized. The presence of the second polar body is typical for all zygote stages. It can be located in contact with the first polar body or not far away from it. The first polar body most often stays at the degradation stage; sometimes zygotes with three polar bodies occur if by that moment the division of the first polar body has already occurred [19].

Day	Time	Event in	
		Donors	Recipients
−10	Morning	Insertion of vaginal sponge (60mg medroxyprogesterone acetate)	
−2	8:00 a.m. 8:00 p.m.	50 μg cloprostenol + 50 mg pFSH 50 mg pFSH	50 μg cloprostenol + 300 iu eCG
−1	8:00 a.m. 8:00 p.m.	30 mg pFSH 30 mg pFSH	
0	8:00 a.m. 8:00 p.m.	20 mg pFSH + sponge removal 20 mg pFSH	Sponge removal
1	8:00 p.m.	100 μg GnRH + first natural mating	Estrus detection
2	8:00 a.m.	Second natural mating	Estrus detection
3	8:00 – 10:00 a.m. 2:00 – 5:00 p.m.	Surgical embryo recovery	Transfer of microinjected embryos

GnRH, gonadotrophin-releasing hormone; pFSH, porcine follicle- stimulating hormone.

Table 2. Time schedule to produce pronuclear goat embryos for subsequent DNA microinjection.

The pronuclei of goat zygotes are visible without centrifugation in only 30% of times (Figure 3C). To facilitate visualization of pronuclei, all the fertilized eggs are subjected to centrifugation, which contributes to precipitation of the lipid granules. The pronuclei of late zygotes are located closely to one another, usually in the center of the cytoplasm though closer to the pole with dark granules; one of the pronuclei (male) is somewhat larger than the other (female) (Figure 3D and F). Pronuclei in goat, unlike those in mouse, rabbit, or swine, are not visualized; morphologically, they rather resembled sheep and cow pronuclei. The two pronuclei may not be always simultaneously observed, and one of them can be located in the lipid granules. However, even after very careful examination of the centrifuged zygotes in a microscope with

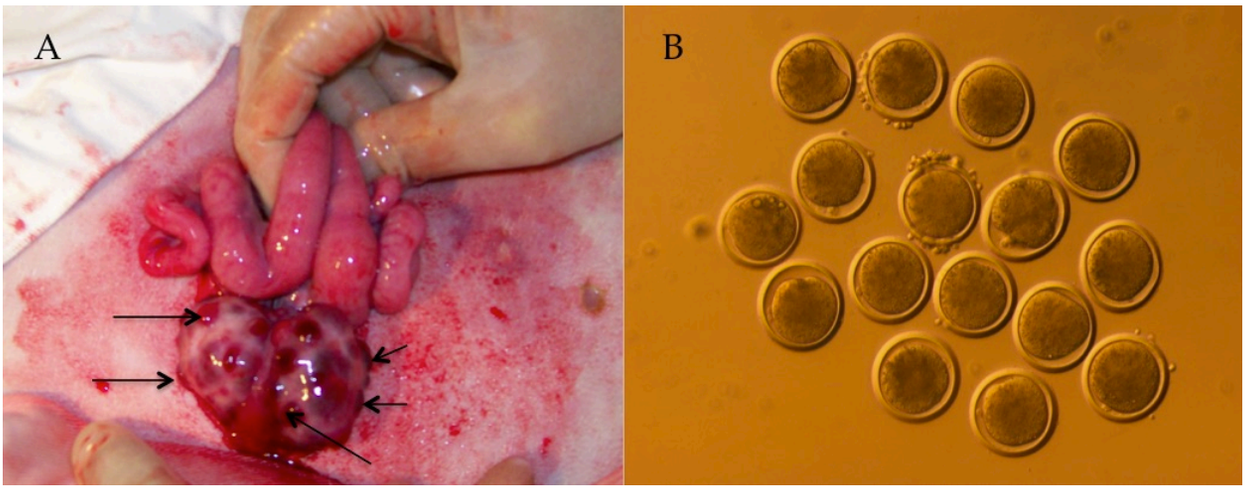


Figure 2. Embryo recovery in goats. A: Evaluation of ovarian response (arrows show some ovulation points) for further oviduct flushing. B: Presumable embryos observed under stereomicroscope.

Nomarsky optics, the pronuclei are not always clearly observed. Zygotes of different goat breeds differ in the degree of visualization of pronuclei. For instance, in Canindé goats, the pronuclei visualization is possible in almost 100% of examined zygotes, whereas in Saanen this rate was only slightly higher than 70% [20].

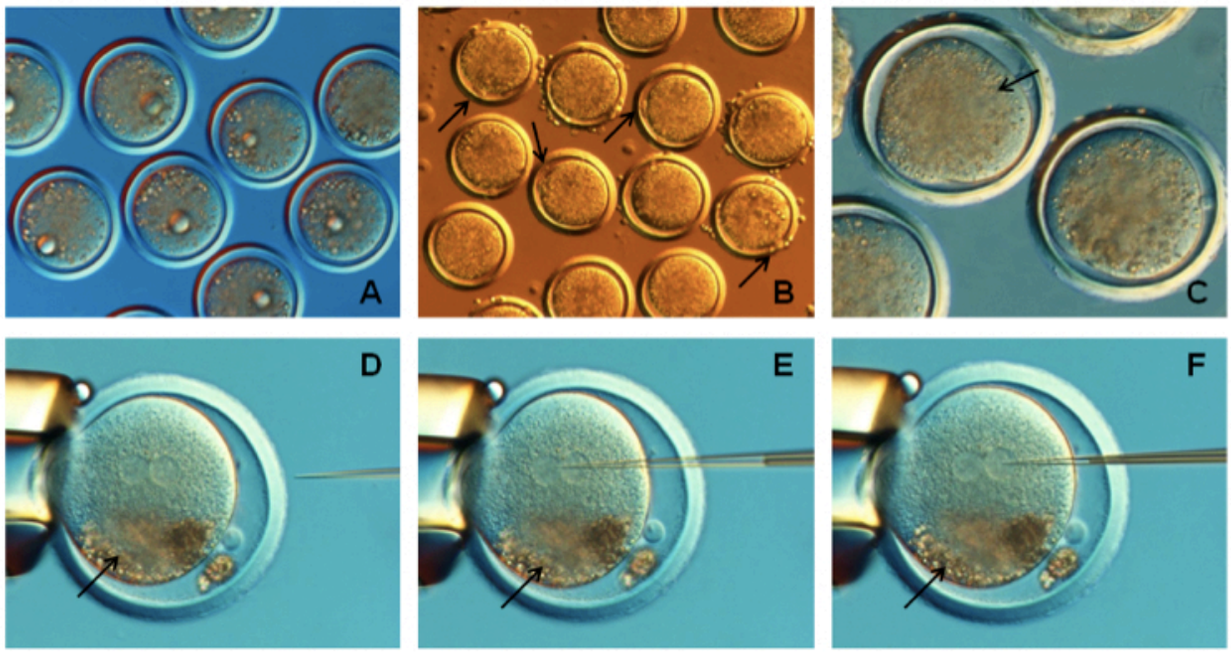


Figure 3. Morphology of goat ova and embryos with microinjection details. A: Unfertilized oocytes; the secondary polar body is absent (×200). B: Zygotes; arrows indicate the secondary polar bodies but pronuclei are not visible (×200). C: Zygotes; arrow indicates pronuclei visible without centrifugation (×300). D, E, and F: Microinjection into centrifuged pronuclei of zygotes; arrow indicates the pole with lipid granules.

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2.2. In vitro embryo production

Although the in vivo production method results in zygotes of high developmental capacity, the procedure is characterized by a great deal of variability in the number of pronuclear embryos. In addition, this technique causes adhesion formation following laparotomy and, consequently, limits the repeat use of the donors. Laparoscopic ovum pick-up (LOPU) followed by in vitro embryo production appears as an efficient method for the exponential dissemination of high genetic value sheep and goats [21]. Similarly described for bovines, the use of in vitro-matured oocytes is also advantageous in goats [22]. A year-round provision of immature oocytes can be obtained from hormone-primed goats by LOPU, contrasting to the seasonal fluctuations observed in the quantity of oocytes obtained from slaughterhouse-derived ovaries. Thus, the laparoscopic approach provides a minimally invasive, efficient means of obtaining immature oocytes for subsequent embryo production and microinjection.

The estrous cycle of goats should be manipulated with gonadotropins. Different hormonal protocols have been tested to improve the oocyte production in quantity and quality obtained from goats submitted to LOPU [22, 23]. These protocols enable the synchronous recruitment of a large population of follicles. Progestagen impregnated vaginal sponges (45 mg MAP, Progespon; Syntex, Buenos Aires, Argentina) are employed for 10 days to synchronize the female cycle; they should be left in the vagina until the moment for oocyte aspiration to avoid ovulations. Indeed, a dose of prostaglandin $F_{2\alpha}$ analog (50 μg cloprostenol, Ciosin; Coopers, São Paulo, Brazil) is used at the seventh day of progestagen treatment to promote luteolysis. In a breed-dependent total dose (120–200 mg), pFSH is administered in five injections (decreasing doses), 12 hours apart, beginning at prostaglandin injection. A 24-hour interval between the last pFSH administration and LOPU is used. Goats can be treated seven or more times using this protocol without a decrease in the ovarian response (Table 3).

Session	Punctured follicles	Recovered COCs	Recovery rate (%)
1	21.3 ± 7.1	12.9 ± 5.1	60.8 ± 21.6
2	21.8 ± 8.3	15.6 ± 5.9	71.6 ± 12.7
3	20.0 ± 5.7	15.2 ± 5.1	75.8 ± 11.7
4	16.5 ± 7.9	12.9 ± 6.8	78.8 ± 14.4
5	16.3 v 4.8	11.9 ± 3.8	73.3 ± 9.6
6	19.0 ± 5.9	13.9 ± 3.4	73.2 ± 16.0
7	14.9 ± 5.8	10.6 ± 3.4	70.9 ± 16.4

Table 3. Punctured follicles, recovered cumulus–oocyte complexes (COCs), and recovery rate in goats hormonally treated for seven times per session [25]. The values are mean (±SD) and percentages.

LOPU starts in the morning of the 10th day of progestagen treatment, after sponge removal. Briefly, goats are deprived of food and water for 36 hours and 24 hours prior to LOPU, respectively. Using a similar protocol for anesthesia to that previously described for embryo

recovery, the female is placed on a cradle at a 45° angle to avoid accidents at trocars punction (Figure 4A). One trocar is used to pass the endoscope into the abdominal cavity, cranial to the udder, and to the left of the midline. Once the abdominal cavity is expanded with air, a second trocar inserted in the right side is used to introduce atraumatic grasping forceps. Using gentle manipulation, the uterine horns are pulled to allow visualization of each stimulated ovary. A third trocar, inserted in the midline, is used to pass the oocyte aspiration needle (Figure 4B). The system comprises a 5-mm laparoscope associated to a 22-G needle and a vacuum pump regulated to 35 mmHg. Follicles of 2–7 mm are punctured from the side, the needle paralleling the base of the follicle (Figure 4C). The collection medium is composed of HEPES buffered TCM 199 supplemented with 20 IU/mL heparin and 40 mg/mL gentamicin sulfate. To avoid adherences, after LOPU, each ovary is flushed with 100 mL of heparinized saline.



Figure 4. Structure to perform the laparoscopic ovum pick-up (LOPU) in goats. A: Room for oocyte recovery showing the inhalation anesthesia equipment ①, goat on a cradle ②, video-laparoscopy equipment ③, and vacuum pump ④. B: Points for insertion of the endoscope, follicular puncture needle, and the atraumatic grasping forceps. C: Moment of the follicular puncture with needle in a vacuum system.

As shown in Figure 5, collected COCs are visualized under a stereo zoom microscope and graded according to the classification used in our laboratory [24]. Only oocytes graded as 1 and 2 are considered acceptable and used for in vitro maturation (IVM). COCs are then washed four times and transferred into maturation medium consisting of TCM 199 supplemented with 10 ng/mL epidermal growth factor (EGF) and 100 μM cysteamine in four-well Petri dishes, each well containing 45–50 oocytes in 500 μL of maturation medium. COCs are incubated for 24 hours at 38.5°C and 5% CO_2.

Oocyte maturation includes meiotic resumption and progression to the fertilizable stage of metaphase II after emission of the first polar body and related events in oocyte cytoplasm and surrounding cumulus cells. Therefore, the result of IVM depends on the intrinsic quality of immature oocytes, but the maturation conditions can widely modulate the final competence

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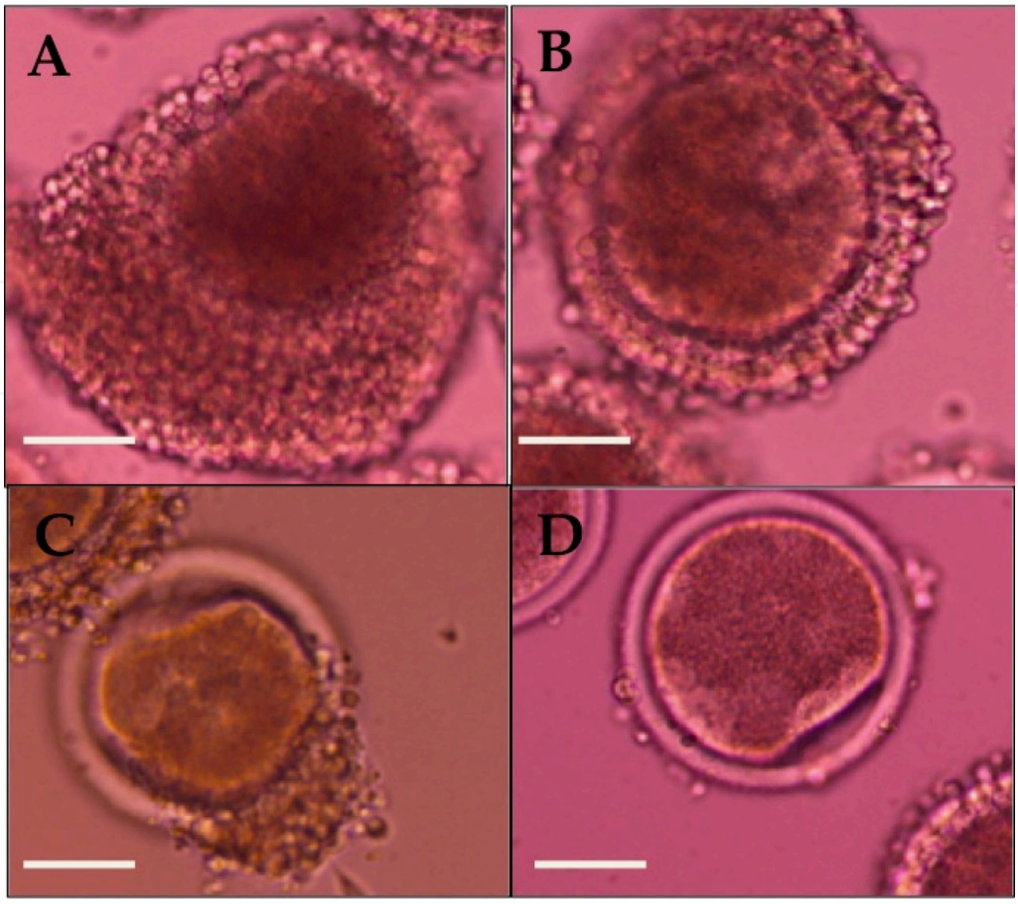


Figure 5. Criteria for grading *cumulus*–oocyte complexes (COCs) recovered from goats. A: Grade 1 COCs show multilayered compact *cumulus* and finely granulated oocyte cytoplasm. B: Grade 2 COCs show one to three layers of *cumulus* cells and finely granulated oocyte cytoplasm. C: Grade 3 COCs show incomplete or no cellular investment or heterogeneous oocyte cytoplasm. D: Grade 4 oocyte evidences an abnormal shape and heterogeneous oocyte cytoplasm, whereas apoptotic oocytes are surrounded by jelly-like *cumulus*-corona cells investment. Scale bar represents 50 μm.

of IVM oocytes. The IVM procedure is commonly performed using TCM 199 enriched with amino acids and glucose, supplemented with hormones and heat-inactivated serum. To make IVM simpler, more safe, and repeatable, our laboratory proposed a maturation medium using just defined compounds—TCM 199 supplemented with 10 ng/mL EGF and 100 mM cysteamine—and obtained good results in embryo development of adult goat oocytes [26].

Motile sperm from frozen/thawed semen are separated by centrifugation (700× *g* for 15 min) on 2 mL of Percoll discontinuous density gradient (45%/90%). Then, dilution of viable sperm in fertilization medium to achieve a final concentration of 2.0×10^6 sperm/mL is performed. The matured COCs are transferred into plates containing washing medium. The washing and fertilization medium are synthetic oviduct fluid (SOF) medium containing 10% of heat inactivated estrus goat serum, 5 mg/mL heparin, and 4 mg/mL gentamicin. Groups of 45–50 oocytes are transferred into four-well Petri dishes with 450 mL of fertilization medium and 50 mL of sperm suspension is added to each well. Sperm and oocytes are co-incubated for 18 hours at 38.5°C in a humidified atmosphere of 5% CO_2 in air [27, 28]. Oocytes are then stripped

off cumulus cells and centrifuged to facilitate pronuclear visualization. All procedures for DNA microinjection are similar to those made during the in vivo embryo production.

3. Somatic cell nuclear transfer

The current knowledge on SCNT results from the hard work and curiosity of scientists who began more than 100 years ago. Hans Spemann was the first to demonstrate that salamander nuclei were pluripotent up to 16-cell stage [28]. The fascinating study demonstrated the production of twin larvae by cutting the embryo in half using his son's hair strand. Further research from other groups confirmed that nuclei from early amphibian embryos transferred to appropriate cytoplasm were totipotent. The further experiments tried to transfer nuclei from more advanced species; disappointingly, the results were not encouraging. Following the lead, Illmensee and Hoppe [29] reported successful nuclear transfer of embryonic nuclei in mice by directly injecting inner cell mass into enucleated zygotes. Willadsen [30, 31] reported for the first time the nuclear transfer using enucleated, metaphase-II sheep oocytes. After 10 years, Campbell et al. [32] reported the production of first offspring using transfer of cultured embryonic cell line derived from day 9 in vivo–produced sheep embryo. This significant study demonstrated that differentiated cells have the ability to originate a new individual and cells could be induced to enter a quiescent state. Finally, in 1997, the first mammal cloned from an adult, differentiated cell was named Dolly; it was produced by transferring a differentiated somatic cell into an enucleated mature oocyte [15]. Since Dolly, several other mammalian species have been cloned.

Different approaches have been conducted to modify donor cells and for nuclear transfer to improve the efficiency of the technique. These manipulations were focused on donor cells, including (a) the synchrony of the cell cycle stage among donor cells and the synchrony between donor cells and recipient oocytes; (b) the use of somatic cells from donors of various ages, tissue origins, passages, and culture conditions; (c) the transfer of stem cells with epigenetic marks; and (d) the drug-induced modification of the epigenetic marks in donor cells. The efficiency of nuclear transfer has been dramatically improved from the initial success rate, although none of these efforts eliminated the common problems associated with nuclear transfer, suggesting the need for further studies on nuclear reprogramming to better understand the underlying mechanisms of reprogramming and consequently to enhance the ability to reprogram differentiated somatic nuclei.

Metaphase II enucleated oocytes are most frequently used as recipient cytoplasm in SCNT studies. Two sources are available for these stage oocytes: (a) in vivo mature oocytes collected directly from animals and (b) aspirated oocytes collected from the ovaries of live or slaughtered animals and cultured in vitro for maturation (as described for microinjection). The selection of the source of recipient oocyte depends on the experimental species. In some species, the developmental competence is similar for both the oocyte sources. Conversely, in other species, the developmental competence is rather variable for in vivo or in vitro recipient oocytes.

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The use of recombinant somatic cell lines for nuclear transfer shows several advantages: it allows the introduction of transgenes by traditional transfection methods, increases the efficiency of transgenic animal production to 100%, and overcomes the problem of founder mosaicism. Additionally, SCNT protocols may also be used to clone transgenic founders obtained by pronuclear microinjection. Several steps of SCNT technique to produce transgenic goats are described in Table 4.

Step	Description
1. Isolation of fibroblast cell line	The primary goat fibroblast cells used as karyoplast donors are obtained by biopsy. Tissues are minced, washed, and transferred into culture flasks. Cells are cultured in TCM 199 supplemented with 10% fetal bovine serum (FBS), nucleosides, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol as an antioxidant, and 50 μg/mL gentamicin. After three subpassages, cells are frozen and stored in liquid nitrogen.
2. Oocyte recovery	Oocytes can be obtained from follicle-stimulating hormone (FSH)-stimulated animals (as described by laparoscopic oocyte collection) or slaughterhouse ovaries
3. Oocyte enucleation	Cumulus–oocyte complexes (COCs) are vortexed at 18 to 22 hours post in vitro maturation (IVM) in TL-HEPES containing 0.6 mg/mL hyaluronidase for removal of the cumulus cells. Oocytes are washed in modified TL-HEPES (TL-HEPES supplemented with 10% FBS), selected based on the presence of a polar body, and labeled with 3 μg/mL Hoechst 33342 for 1 min. Mature oocytes are enucleated in inverted microscope with micromanipulators and epifluorescent illumination.
4. Donor cell preparation	Fibroblast cells are trypsinized, washed, and held (<1.5 hours) in modified TL-HEPES immediately before transfer into the recently enucleated oocytes.
5. Reconstruction and fusion	A single fibroblast cell is injected into the perivitelline space of each enucleated oocyte. Karyoplast–cytoplast couplets are manually aligned between two stainless steel electrodes in a microslide fusion chamber filled with fusion buffer (0.3 M mannitol, 0.1mM $MgSO_4 7H_2O$, 0.05mM $CaCl_2$, 0.5mM HEPES and 4mg/mL BSA) and fused by a single DC pulse (1.30 kV/cm for 25 μsec) delivered by electrofusion equipment. Fused couplets are activated by a 5-min exposure to 5 μM ionomycin, washed extensively in modified TL-HEPES, and then incubated for 3 hours in 2 mM 6-dimethylaminopurine. Following activation, reconstructed embryos are cultured for 36 hours prior to transfer to recipient females.

Table 4. Brief methodology for the use of somatic cell nuclear transfer (SCNT) technique to produce transgenic goats.

There are no major changes in the technical characteristics of SCNT methods for farm animals since the pioneer description of the first successful embryonic cloning [31]. Researchers working in embryology consider the *zona pellucida* as important for the correct embryonic development. However, this technique requires the use of micromanipulators, highly skilled labor, and very expensive equipment. Some years ago, other forms of the technique (zona-free procedures) were described [36]. For clear distinction, the term hand-made cloning (HMC)

was used for the micromanipulation-free SCNT method. Several publications with zona-free NT techniques agree that these methods are easier to learn and do not require skilled workforce, originating embryos with a final quality comparable with that of traditional cloning [37, 38]. Our group obtained a transgenic female goat founder by pronuclear microinjection, which expressed acceptable levels of the hG-CSF in the milk [14]. Currently, the objective of our group is to increase the number of transgenic animals by cloning techniques. Therefore, using skin fibroblast primary culture cells collected from the transgenic founder goat as nucleus donor cells, the HMC cloning procedure was used (Figure 6) to test the developmental potential of cloned transgenic goat embryos [39].

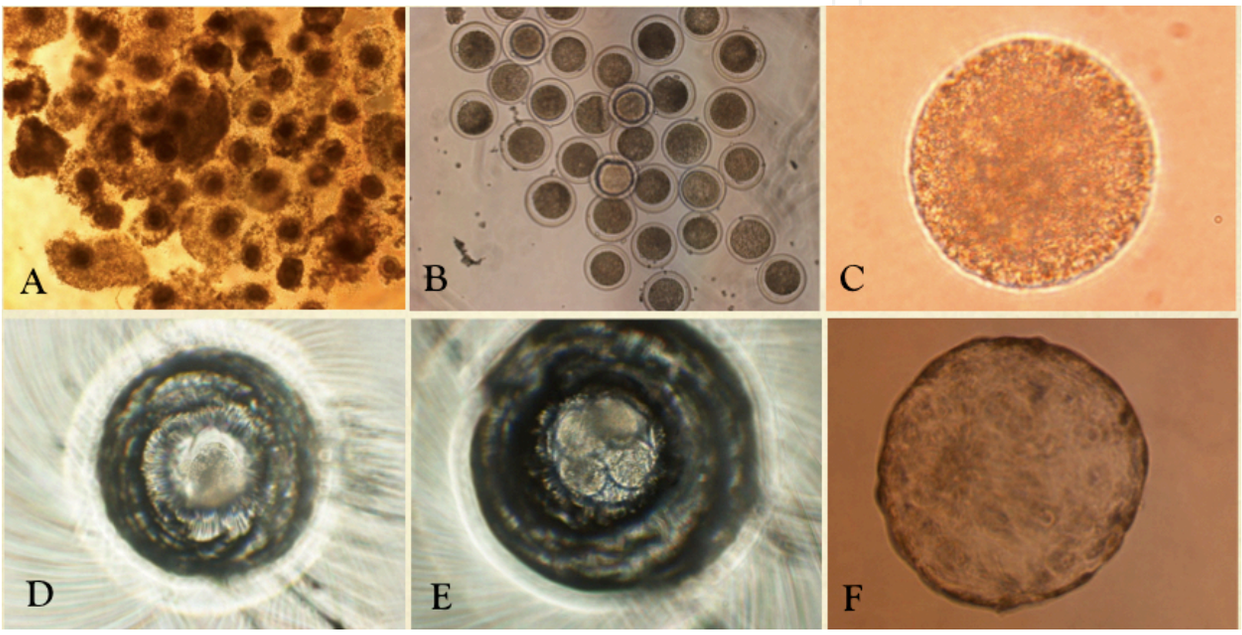


Figure 6. In vitro production steps of goat hand-made cloned embryos using donor cells from a transgenic founder goat. A: *Cumulus*–oocyte complexes after 20 hours of in vitro maturation (200×). B: In vitro–matured oocytes based on evaluation of polar body (200×). C: Zona-free oocyte after removal of the *zona pellucida* (600×). D: Transgenic one-cell stage cloned embryo on day 1 of culture into a microwell (400×). E: Transgenic four-cell stage cloned embryo on day 2 of culture into a microwell (400×). F: Transgenic cloned blastocyst on day 7 of development (600×).

4. Embryo transfer and pregnancy diagnosis

Crossbred recipient goats can be selected from those exhibiting estrus 48–72 hours prior to the scheduled embryo transfer. Nuclear transfer embryos are transferred into oviduct's recipients. For the embryo transfer procedure, we used the same general anesthesia protocol as for oocyte collection. Likewise, goats are placed in dorsal recumbence for midventral laparotomy procedure. The ovaries are then examined for evidence of ovulation; thereafter, the uterus is exteriorized, and embryos are transferred into the oviduct (2-day embryos) or the uterus (7-day embryos) ipsilateral to the ovary with the most ovulation points. Embryos (5–10/recipient)

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are transferred via a small plastic catheter through the tubal ostium, the catheter gently advanced into the oviduct.

The need for early diagnosis of pregnancy in goats, especially for valuable embryos, as in the case of transgenesis and cloning, lead to the common application of transrectal or abdominal real-time B-mode ultrasonography (US) for pregnancy examination. The distension of the uterus, the evidence of embryonic vesicles or the embryo, the fetal heartbeat, and placentomes are positive signals for pregnancy. Ideal time for abdominal scanning is between 40 and 60 days. Prior to 40–45 days, the transducer may have to be placed higher in the inguinal region, but if transrectal US is used the diagnosis is anticipated up to 25–30 days. In our studies, transrectal US examinations of recipients begin on day 25 of gestation, whereas additional US assessments are performed at intervals of 7 and 10 days until unequivocal pregnancy diagnosis. The fetal heartbeat is normally used to diagnose pregnancy and fetus viability. Goats with pregnancy confirmed are monitored at 2-week intervals until day 90, when they are separated from the others and placed in groups (two or three) per pen. Monitoring of pregnant recipients past day 45 is performed through abdominal US (Figure 7).

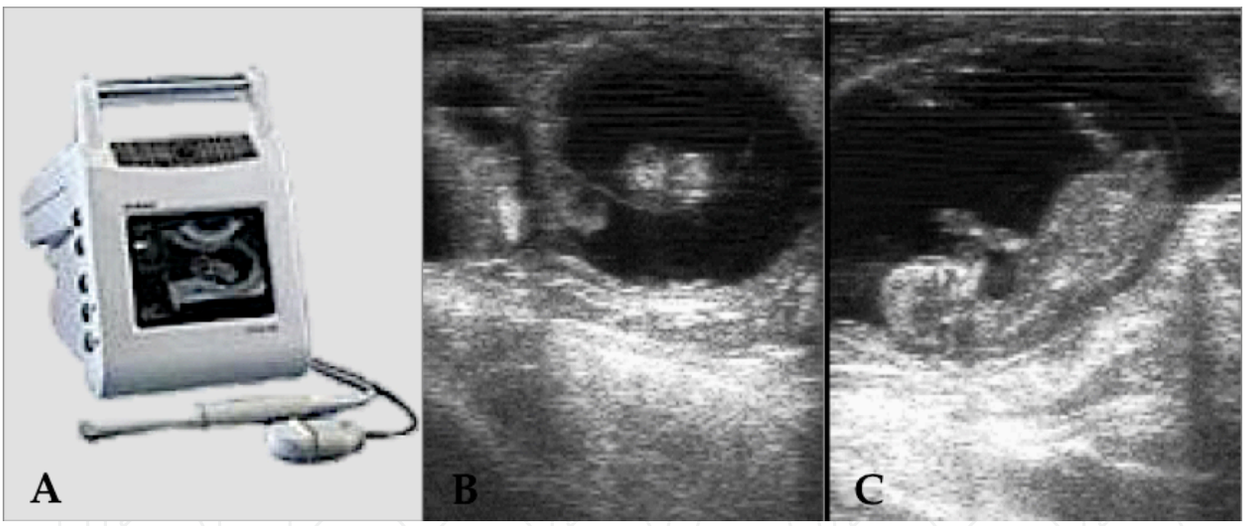


Figure 7. Pregnancy diagnosis in recipient goats. A: Real-time ultrasonography (US) equipment and its probes for transrectal and abdominal examination. Pregnant recipients at 25 (B) and 45 days (C) after embryo transfer.

Real-time US is also currently used for other kind of assessments, such as to verify and monitor the ovarian follicular dynamics, the time of ovulation, and to accurately estimate the number of corpora lutea at days 6–7 after induced superovulation in goats. Nevertheless, studies focusing the superovulation response in goats, particularly, to verify early ovulatory response (*corpora haemorraghica* (CH)), are very limited, and the accuracy of the method with this objective has not been assessed. Thus, our group proposed to assess the efficiency of real-time US as a noninvasive method to estimate the ovulation ratio after superovulation in goats by counting the number of CH's in the ovaries. The sensitivity, specificity, and total efficiency of

the ultrasonographic estimation of ovulations in a goat were 100.0% [40]. Thus, transrectal real-time US is a valuable method for assessing the ovulatory response and can be used to select donor goats in a transgenesis program or to estimate embryo yield after superovulation.

5. Conclusions

Research in producing transgenic animals currently uses goats for its lower cost and precise genomic alterations. This statement is reinforced by the approval and the commercialization of the first human biological drug from the milk of transgenic goats.

Nuclear transfer protocols have evolved, which make it possible to obtain transgenic goats efficiently. Also, it seems that goats generated by SCNT do not suffer from the health problems reported in sheep and cattle. Moreover, the HMC technique does not require the use of micromanipulators and accessories. Also, the HMC procedure does not request micromanipulators or related equipment, making the method accessible to laboratories with limited funding.

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