Production of the First Cloned Camel by Somatic Cell Nuclear Transfer 1

Authors: Wani, Nisar A., Wernery, U., Hassan, F.A.H., Wernery, R., and Skidmore, J.A.

Source: Biology of Reproduction, 82(2) : 373-379

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.109.081083

BIOLOGY OF REPRODUCTION **82,** 373–379 (2010)
Published online before print 7 October 2009.
DOI 10.1095/biolreprod.109.081083

Production of the First Cloned Camel by Somatic Cell Nuclear Transfer[1]

Nisar A. Wani,[2,3] U. Wernery,[4] F.A.H. Hassan,[4] R. Wernery,[4] and J.A. Skidmore[3]

Camel Reproduction Centre,[3] Dubai, United Arab Emirates
Central Veterinary Research Laboratory,[4] Dubai, United Arab Emirates

ABSTRACT

In this study, we demonstrate the use of somatic cell nuclear transfer to produce the first cloned camelid, a dromedary camel (*Camelus dromedarius*) belonging to the family Camelidae. Donor karyoplasts were obtained from adult skin fibroblasts, cumulus cells, or fetal fibroblasts, and in vivo-matured oocytes, obtained from preovulatory follicles of superstimulated female camels by transvaginal ultrasound guided ovum pick-up, were used as cytoplasts. Reconstructed embryos were cultured in vitro for 7 days up to the hatching/hatched blastocyst stage before they were transferred to synchronized recipients on Day 6 after ovulation. Pregnancies were achieved from the embryos reconstructed from all cell types, and a healthy calf, named Injaz, was born from the pregnancy by an embryo reconstructed with cumulus cells. Genotype analyses, using 25 dromedary camel microsatellite markers, confirmed that the cloned calf was derived from the donor cell line and the ovarian tissue. In conclusion, the present study reports, for the first time, establishment of pregnancies and birth of the first cloned camelid, a dromedary camel (*C. dromedarius*), by use of somatic cell nuclear transfer. This has opened doors for the amelioration and preservation of genetically valuable animals like high milk producers, racing champions, and males of high genetic merit in camelids. We also demonstrated, for the first time, that adult and fetal fibroblasts can be cultured, expanded, and frozen without losing their ability to support the development of nuclear transfer embryos, a technology that may potentially be used to modify fibroblast genome by homologous recombination so as to generate genetically altered cloned animals.

assisted reproductive technology, camel, cloning, ovum pick-up/transport, somatic cell nuclear transfer

INTRODUCTION

Since the first report of a live mammal produced by nuclear transfer (NT) of a cultured cell line in 1996 [1], cloned mammals have been produced successfully in sheep [2], cattle [3], mouse [4], goat [5], pig [6], rabbit [7], cat [8], rat [9], horse [10], mule [11], dog [12], ferret [13], and buffalo [14] with different somatic cell types as nuclear donors. The growing list of species cloned, however, cannot obscure the fact that cloning remains inefficient compared with other assisted reproductive technologies, such as conventional embryo transfer, in vitro fertilization, or artificial insemination. Typically, only 1% to 5% of all cloned embryos transferred into surrogate mothers develop into viable offspring [15]. A number of approaches have been shown to improve the in vitro development of NT embryos, including better sources of recipient oocytes [16–18]; altering epigenetic marks in donor cells [19–21]; using chromatin transfer [22], serial NT [23], or sperm-mediated activation [24]; or aggregating somatic NT embryos [25]. However, significantly improved in vivo development has not been conclusively demonstrated for any of these treatments. Multiple factors, from recipient cytoplast preparation to transfer of cloned embryos to recipient females, influence success of each step in the nuclear-transfer process. It has been shown that oocyte source [26, 27], enucleation methods [28, 29], activation protocols and fusion methods [30], fusion timing [31, 32], and in vitro culture conditions have an overall effect on the efficiency of production of live cloned offspring.

The nuclear donor cell is undoubtedly a key component of the cloning process. Little is presently understood of the fundamental molecular and cellular events that could be involved in reprogramming the nucleus of an adult somatic cell. However, tissue of origin [33], stage of differentiation [34–36], age of donor [37], cell culture conditions and length [38–42], genotype [43–45], and transgenic modifications [46–47] have been shown to influence the development of reconstructed embryos. Live cloned calves have resulted from NT with cumulus cells [33]; granulose cells [31]; oviductal, uterine, and ovarian epithelial cells [33]; mammary gland cells [48]; muscle cells [49]; skin fibroblasts [33, 37, 38]; and blood cells [30, 36]. However, comparison of cloning efficiency from each donor cell type is difficult because of variations in nuclear-transfer procedures, laboratory and technician proficiencies, recipient oocyte source and quality, age and genotype of the donor animal, embryo culture systems, and surrogate female effects such as age, breed, nutrition, and season.

The technique of somatic cell NT (SCNT) is well advanced in cattle, when compared with most of the domestic animal species, because of the successful and repeatable procedures for in vitro oocyte maturation, oocyte activation, and in vitro embryo culture in this species. Each of these procedures represents a key step in the cloning process. Cloning by NT has a special significance in the genetic improvement of camelids. This technology can be used to produce animals with the highest potential for milk production or racing champions. Camel racing, which is a highly lucrative and well-organized sport, is an important traditional and economic activity in the Arabian Gulf states. There have been a few attempts at SCNT in camelids [50–52], but these were unsuccessful, mainly because of the limited basic information available about in vitro embryo production in these species. Optimization of the techniques for oocyte maturation [53], chemical activation of oocytes [54], and in vitro embryo culture [54, 55] in our

[1]Supported by His Highness General Sheikh Mohammed Bin Rashid Al-Makhtoum, Ruler of Dubai and Prime Minster of UAE.
[2]Correspondence: Nisar A. Wani, Reproductive Biology Laboratory, Camel Reproduction Centre, Post Box 79914, Dubai, United Arab Emirates 79914. FAX: 971 4 2238486;
e-mail: nwani@cvrl.ae or nawani@eim.ae

Received: 25 August 2009.
First decision: 2 September 2009.
Accepted: 28 September 2009.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

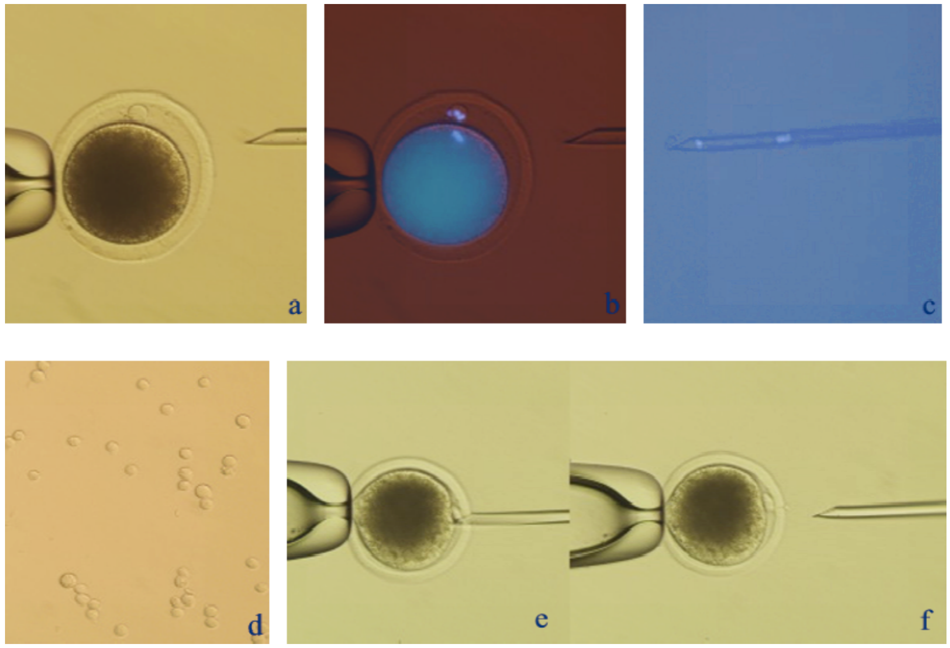

FIG. 1. Steps in the SCNT of dromedary camel. **a**) A mature oocyte with a visible polar body held with a pipette. **b**) Determining the location of metaphase chromosomes by a very short (1–2 sec) exposure to UV light. **c**) Exposing the pipette to UV light to confirm the presence of both metaphase and polar body chromatin. **d**) Donor cells after trypsinization and washing. **e**, **f**) Injection of the donor cell into the perivitelline space of the enucleated oocyte. Original magnification ×200.

laboratory during the past few years made the basis for studies on in vitro and in vivo development of SCNT embryos in camelids. In this report we describe the application of SCNT to produce the first camelid, a dromedary camel (*Camelus dromedarius*) calf named Injaz, cloned by SCNT. We evaluated three commonly used somatic donor cell types—cumulus, ear skin, and fetal fibroblasts—for their embryonic and fetal development in this species.

MATERIALS AND METHODS

All the chemicals and media were from Sigma unless otherwise indicated. Fetal calf serum (FCS) was from Gibco. Mature female dromedary camels aged 5–14 yr, maintained at the Camel Reproduction Centre, Dubai, were used as oocyte donors and recipients for NT embryos. They were in good physical condition, weighed approximately 450 kg, and were supplied with water and hay ad libitum. They were also fed a diet of mixed concentrates once daily. All procedures were performed in accordance with the government of United Arab Emirates' animal care and use guidelines.

Ovarian Stimulation, In Vivo Oocyte Maturation, and Ovum Pick-Up

The donor animals were induced to ovulate by administration of 20 μg of the GnRH analogue buserelin (Receptal; Hoechst Animal Health) when a dominant follicle (1.3–1.9 cm) was observed, after serial ultrasonography, on an ovary. Four days after ovulation, they were treated with a combination of 2500 IU equine chorionic gonadotropin (Folligon; Intervet Laboratories), given as a single intramuscular injection on Day 1 of the treatment protocol, and 400 mg porcine follicle-stimulating hormone (FSH) (Folltropin; Vetrepharm) injected twice daily in declining doses of 2 × 80 mg, 2 × 60 mg, 2 × 40 mg, and 2 × 20 mg over 4 days, also beginning on Day 1. The ovaries of all the donor camels were scanned on Day 4 after the start of treatment, and thereafter at intervals of 1 or 2 days until the majority of follicles had grown to between 1.3 and 1.8 cm in diameter. They were then given a single injection of 20 μg of buserelin 26 h before the ovum pick-up was scheduled.

Donors were sedated with 0.7–1 ml of detomidine hydrochloride 10 mg/ml (Domosedan; Orion Pharma) and were made to sit in sternal recumbency. The perineum region was washed with surgical scrub and dried with a towel. For oocyte collection, an electronic convex transducer with an attached needle guide (UST-994P-5; Aloka) was used. Sterile lubricant (KY lubricating jelly; Johnson and Johnson) was applied on the transducer, which was guided through the vulva and into the cranial-most portion of the vagina. The free hand was placed into the rectum to manipulate the ovary and position it against the vaginal wall over the face of the transducer. A 17-gauge, 55-cm single-lumen needle (Cook) was placed in the needle guide of the ultrasound probe and advanced through the vaginal fornix and into the follicle. Follicular fluid was aspirated using a regulated aspiration pump (IVF Ultra Quiet, Model V-MAR-5100; Cook) set at a vacuum of 55 mm Hg. The contents of all follicles >10 mm in diameter were aspirated into 50- or 15-ml conical tubes containing embryo-flushing media (IMV) supplemented with heparin (10 000 IU/L). Aspirates were transferred to Petri dishes to search for and evaluate the cumulus-oocyte complexes using a stereomicroscope.

Preparation of Recipient Cytoplasts

The cumulus-oocyte complexes obtained were denuded from the surrounding cumulus cells by manual pipetting in the presence of hyaluronidase (1 mg/ml), and oocytes with an extruded first polar body (Fig. 1a) were selected for enucleation. The selected oocytes were placed into the manipulation medium (Hepes-TCM-199 + 10% FCS) supplemented with 7.5 μg/ml of cytochalasin B and 5 μg/ml of bisbenzamide for 20 min before micromanipulation. Location of the metaphase chromosomes was determined by a brief exposure (1–2 sec) to ultraviolet (UV) light (Fig. 1b) and the polar body, along with the metaphase II plate, was removed by aspiration with a 25-μm-inner-diameter beveled pipette under an inverted microscope equipped with an Eppendorf micromanipulator (TransferMan NK2). Exposing all the removed cytoplasm to UV light and checking for the presence of the removed metaphase plate confirmed successful enucleation (Fig. 1c).

Preparation of Donor Karyoplasts

Tissues from aborted fetuses (50- and 100-day-old) were enzymatically digested with 0.25% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) for 30 min, and the disaggregated cells were washed three times in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS by centrifugation at 500 × *g* for 5 min and then placed in culture in 60-mm tissue culture dishes under a humidified 5% CO_2 in air atmosphere at 38.5°C.

Cumulus cells from 5- to 10-mm-diameter follicles of a slaughtered animal were washed three times in DMEM supplemented with 10% FCS by centrifugation at 500 × *g* for 5 min and then placed in culture in a 60-mm tissue culture dish under a humidified 5% CO_2 in air atmosphere at 38.5°C.

The ear skin biopsies were taken aseptically from two adult camels (one male and one female) in sterile Dulbecco phosphate buffer saline. After proper washing, the tissue was cut into small pieces and cultured in dishes containing DMEM supplemented with 10% FBS. The explants were removed after proliferation and establishment of fibroblasts.

In all the above cell types, once a confluent fibroblast monolayer was obtained it was passaged with an enzymatic solution (0.25% trypsin and 0.05% EDTA) for 5 min. All the cell lines were frozen after the second passage. For use as nuclear donors, the cells were thawed, passaged, and used from third to ninth passage. The cells either were serum starved by culture in DMEM plus 0.5% FCS for 72 h or were cultured after confluency for 72 h before being used as donor nuclei.

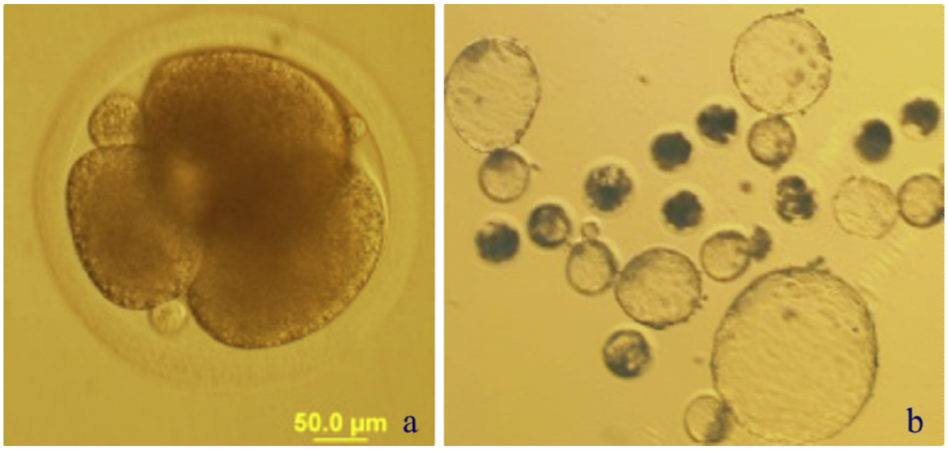

FIG. 2. Development of SCNT embryos in dromedary camel: four-cell embryo on Day 2 (**a**) and blastocysts observed hatching out on Day 7 (**b**) of culture. Bar = 50 µm.

NT, Fusion, and Activation

Trypsinized and washed donor cells (Fig. 1d) were transferred into the perivitelline spaces of enucleated oocytes with a 25-µm micropipette (Fig. 1, e and f). Cell couplets were washed in fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.05% fatty acid-free BSA) and fused by two DC pulses of 100 V/cm for 15 µs each using an Eppendorf electroporator at room temperature. Couplets were removed from the fusion chamber and put back into Hepes-TCM-199 to score fusion success and detect detached or lysed donor cells. Reconstructs were activated 1 h postfusion with 5 µM ionomycine followed by exposure to 6-dimethylaminopurine (6-DMAP) for 4 h, as described previously for the camel oocytes [54]. The activated oocytes were then transferred to 500 µl of embryo culture medium I (modified potassium simplex optimization medium with essential and non-essential amino acids [KSOMaa] supplemented with 1% BSA) and cultured at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 in air. On Day 2 (Day 0 = day of activation) the cleaved embryos (Fig. 2a) were transferred into 500 µl of embryo culture medium II (modified KSOMaa supplemented with 10% FCS) and cultured under the same conditions until Day 7. The proportion of oocytes that cleaved was recorded on Day 2, and those that reached morula and blastocyst stages were recorded on Day 7 of culture.

Embryo Transfer

Day 7 hatching/hatched blastocysts (Fig. 2b) were transferred nonsurgically into the left uterine horn of recipient camels at Day 6 of their luteal phase. An initial pregnancy examination was performed using transrectal ultrasonography between Days 14 and 16 (Day 0 = day of ovulation), followed by examinations at approximately weekly intervals until about Day 60 of gestation, and then at monthly intervals. The following endpoints were noted at each pregnancy examination: 1) presence of the embryonic vesicle, 2) evidence of an embryo proper within the vesicle, and 3) presence or absence of an embryonic heartbeat once the embryo proper was evident.

Microsatellite Analysis

To identify the calf derived from donor cells, a microsatellite analysis of genomic DNA from the various samples was performed with 25 microsatellite markers. These assays were performed with DNA extracted from the frozen ovarian tissue, from frozen donor cells, and from blood of the surrogate mother, the calf, and an unrelated dam. These markers are used routinely for parentage verification and individual identification. Tests were independently performed at the Molecular Biology and Genetics Laboratory of CVRL, Dubai.

TABLE 1. Proportion of successfully fused cytoplast-donor couplets with cumulus, skin, and fetal fibroblast cells, 1 h after their fusion.

Cells used[a]	Total couplets	Fused reconstructs (% mean ± SEM)
CC-111205	75	79.8 ± 5.4
SKF-311208	98	88.6 ± 4.6
SKF-B10	70	92.1 ± 2.9
FF-040407	101	88.0 ± 3.8
FF-270207	75	92.8 ± 2.7

[a] CC, cumulus cells; SKF, skin fibroblasts; FF, fetal fibroblasts.

Statistical Analysis

The data are presented as percentage mean ± SEM. The proportions of couplets fused, cleaved embryos, and blastocysts produced from different cell lines were analyzed by ANOVA with Fisher protected least significant difference test (MINITAB statistical software, Minitab Ltd.). All the percentage data were arcsine transformed before analysis. Experiments using different cell lines were replicated 7–9 times.

RESULTS

Five cell lines—a cumulus, two adult skin fibroblasts, and two fetal fibroblasts—were generated and used as karyoplasts in the present experiment. No difference ($P > 0.05$) was observed in the proportion of successfully fused cytoplast-donor couplets between the groups utilizing cumulus, skin fibroblasts, or fetal fibroblasts as donor cells (Table 1). About 68%–80% of the embryos reconstructed with different cell types cleaved (Table 2) with no significant difference between the groups ($P > 0.05$). The proportions of embryos developing to the blastocyst stage tended to be lower from both the fetal fibroblast cell lines (29% from cultured reconstructs and 39%–42% from the cleaved embryos), but were not significantly different ($P > 0.05$) from the proportions of blastocysts obtained from cumulus (44% from cultured reconstructs and 64% from cleaved embryos) or from skin fibroblasts (43%–52% from cultured reconstructs and 34%–40% from cleaved embryos). In total, 402 nuclear-transfer embryos were reconstructed from the five cell lines and placed into culture, producing a total of 139 Day 7 blastocysts (35%).

Embryo Transfer and Pregnancy Detection

All viable Day 7 blastocysts were transferred to synchronized recipients on Day 6 after ovulation, either singly or in pairs depending on the quality of the embryos. From the five cell lines used we observed a higher vesicle formation in recipients with embryos reconstructed from cumulus cells (46%), followed by skin fibroblast (18% and 29%). Only three pregnancies were achieved from the 39 blastocysts produced from the two fetal fibroblast cell lines; these were lost around Day 60 of gestation. One out of ten pregnancies achieved from embryos reconstructed with skin fibroblast is continuing at an advanced stage of gestation (Table 3). Out of the six pregnancies achieved with cumulus cells, two were lost between Days 14 and 20 and three between Days 75 and 120 of gestation. The sixth pregnancy resulted in the birth of a live

TABLE 2. Development of the SCNT embryos after their reconstruction with different cell lines in dromedary camel.

Cells used[a]	Total reconstructs cultured	Cleaved (% mean ± SEM)	Blastocysts from (% mean ± SEM)	
			Cleaved embryos	Total reconstructs
CC-111205	58	72.3 ± 8.06	63.88 ± 8.66	44.38 ± 5.43
SKF-311208	87	80.23 ± 4.9	42.73 ± 5.22	34.29 ± 4.48
SKF-B10	64	74.1 ± 11.3	51.68 ± 5.54	39.88 ± 9.2
FF-040407	85	71.34 ± 6.08	41.87 ± 5.56	28.69 ± 3.9
FF-270207	69	68.07 ± 8.17	39.5 ± 11.7	29.04 ± 8.0

[a] CC, cumulus cells; SKF, skin fibroblasts; FF, fetal fibroblasts.

female calf named Injaz (Fig. 3) on April 8, 2009. The fetus developed to full term (378 days), and parturition proceeded naturally without any assistance. The calf weighed 32 kg at birth and was (and continues to be) developmentally normal.

Genotyping of Cloned Offspring

Genomic DNA was isolated from the blood of the cloned animal and compared with genomic DNA samples isolated from the donor cell line, the ovarian tissue, the surrogate mother, and an unrelated female. All of the 25 microsatellite markers observed were similar between the calf, the donor cell line, and the donor ovary (Table 4), showing that the calf was indeed a clone from the donor cells used.

DISCUSSION

The present study reports, for the first time, establishment of pregnancies and birth of the first cloned camelid, a dromedary camel (*C. dromedarius*), by use of SCNT.

The efficiency of SCNT, as measured by the proportion of successfully fused cytoplast-donor couplets and their development to blastocyst stage, did not differ between the groups utilizing cumulus, skin fibroblasts, or fetal fibroblasts as donor cells in the present study. The parameters used to fuse the cytoplast-donor couplets were selected after trying many DC (75–200 V/cm) and time (10–60 μs) combinations in our preliminary studies (unpublished observations), which could be one of the reasons for a higher fusion rate when compared with earlier studies in llamas [50] and dromedary camels [51, 52]. The proportion of NT embryos that cleaved in the present study was also higher than that of earlier studies in llamas [50] and dromedary camels [51, 52]. We observed a 29%–46% blastocyst production rate, whereas in earlier studies in llamas [50] none of the embryos grew past the morula stage, and only 14%–15% of the dromedary reconstructs developed to blastocyst stage [51]. In addition, in our earlier study using the zona-free method of NT, only 8%–9% of the cleaved embryos developed to the blastocyst stage [52]. Our results in the present study are, however, similar to reports of successful nuclear-transfer experiments in cattle with cumulus cells by Kato et al. [33], granulosa cells by Wells et al. [31], and ear skin fibroblasts by Kubota et al. [38], who reported blastocyst development rates that ranged from 30% [38] to 49% [33].

Many factors, including the source of recipient cytoplast [26, 27], enucleation methods [28, 29], and activation protocols [30], have been shown to have an overall effect on the efficiency of the cloning process. In the present study, in vivo-maturated oocytes were used, compared with other studies [50, 51] in which oocytes were matured in vitro. In vivo-matured oocytes have been reported to have a higher developmental potential when compared with their in vitro-matured counterparts in cattle [56]. We collected oocytes from preovulatory follicles of animals after several days of treatment with FSH in the present study. The role of FSH in the acquisition of developmental competence of oocytes is primarily associated with its effect on follicular growth, as several days of treatment are required to obtain oocytes of higher competence [57]. In vitro-matured oocytes used in other studies [51, 52] were collected from 2- to 10-mm follicles of slaughterhouse ovaries, which usually come from a heterogeneous group of animals that are either old or unproductive. The oocytes from these ovaries do not undergo normal preovulatory development such as selection and growth, which are accompanied by a change in pulsatile release of luteinizing hormone and FSH, leading to prematuration [58]. The reconstructs, in the present study, were activated by a protocol using ionomycine/6-DMAP, which has been optimized and standardized for this species [54], whereas in the llama study [50] ionomycin/cycloheximide and in the dromedary study [51] calcium ionophore/6-DMAP protocols adopted from other species were used.

Enucleation has been accomplished successfully in a range of species by labeling the oocyte DNA with Hoechst 33342 [59], by enucleation under the Spindle View System (Pol-Scope image) [60], or by the aid of chemicals like demecolcine [61]. In the present study, oocytes were stained with Hoechst 33342 in order to aid to locate the chromatin and its visualization in the pipette under epifluorescence during the enucleation process, whereas in another study on dromedary camel NT [51], oocytes were enucleated without the aid of any

TABLE 3. Pregnancies established after the transfer of cloned blastocysts obtained by SCNT using different cell lines in dromedary camel.

Cells used for reconstruction[a]	Total embryos transferred	No. of recipients	Pregnant by			Follow-up
			Day 15 (%)	Day 30 (%)	Day 60 (%)	
CC-111205	26	13	6 (46)	4 (31)	4 (31)	Three lost between Days 75–120, whiles one gave birth to a live calf.
SKF-311208	29	17	3 (18)	2 (12)	2 (12)	One lost after Day 60 whiles other one is carrying (+8 months).
SKF-B10	45	24	7 (29)	7 (29)	4 (17)	All lost between Days 60–100.
FF-040407	24	15	2 (13)	2 (13)	2 (13)	Both lost after Day 60.
FF-270207	15	10	1 (10)	0	0	

[a] CC, cumulus cells; SKF, skin fibroblasts; FF, fetal fibroblasts.

FIG. 3. The first cloned camelid, a female dromedary camel calf named Injaz: on the day of birth (**a**); and 2 mo old (**b**), growing well (photograph taken on 8 June 2009).

of the above-mentioned methods, assuming that the metaphase spindle was visible during the nucleation process. In our observations, dromedary camel oocytes are dark because of their high lipid content, like porcine and buffalo oocytes, making it impossible to see the metaphase spindle under an inverted microscope without an aid. The removal of oocyte chromatin prior to NT is of crucial importance in order to 1) avoid aneuploidy, with its detrimental effects on later development; 2) eliminate any genetic contribution of the recipient cytoplasm; and 3) exclude the possibility of parthenogenetic activation and embryo development without the participation of the newly introduced nucleus.

In the present study, we did not observe any difference between the proportions of embryos developing to blastocysts from skin fibroblasts or cumulus cells; however, there are some controversial reports regarding the development of embryos from such cells in cattle. Kato et al. [33] reported that the blastocyst development of ear cell-derived embryos is higher compared to that of embryos derived from cumulus cells. However, results from the studies by Xue et al. [62] and Batchelder et al. [16] indicate that cumulus cells and granulosa cells lead to more blastocysts compared to ear skin fibroblast cells. In the present study, proportions of reconstructs developing to blastocysts tended to be lower from the fetal fibroblast cells, possibly because of some undetected genetic abnormality in these cell lines. However, the development of NT embryos has been reported to differ among donor cell lines, even if they are derived from the same tissue or organ, because of some unknown reasons [33]. The reprogramming of a donor nucleus in the cytoplasts seems to be dependent on their genetic characteristics, and thus their selection may be important to enhance NT efficiency.

Early embryonic losses varied from 33% to 100% with the embryos from different cell lines in the present study. However, in dromedaries maintained under natural conditions, the incidence of early embryonic loss, mostly occurring during the first 2 mo of pregnancy, is also about 30%–40%, which needs to be taken into consideration [63]. In cattle, SCNT pregnancy loss from Day 30 to term also varies from 67% [48] to 93% [49], and the proportion of viable cloned offspring produced from embryos transferred varies from 0.1% to 6% for most laboratories, with occasional reports of greater success (7%–40%) [33]. We obtained one viable offspring from 26 embryos transferred to 13 recipients (8%) using cumulus cells

TABLE 4. Microsatellite analysis of the cloned camel (Injaz), donor cells, ovarian tissue, surrogate mother and a random female.

DNA microsatellites	Clone (Injaz)	Donor cell line	Ovarian tissue	Surrogate mother	Random female
CMS009	248/260	248/260	248/260	248/252	247/260
CMS036	159/161	159/161	159/161	161/161	161/161
CMS121	185/187	185/187	185/187	171/183	170/170
CMS013	267/269	267/269	267/269	265/269	267/269
CMS016	201/203	201/203	201/203	201/201	201/225
CMS018	176/182	176/182	176/182	176/182	182/182
CVRL01	234/240	234/240	234/240	208/240	218/218
CVRL02	209/209	209/209	209/209	209/211	203/209
CVRL04	147/147	147/147	147/147	135/135	135/147
CVRL05	161/167	161/167	161/167	171/173	161/173
CVRL06	218/218	218/218	218/218	218/218	218/218
CVRL07	294/294	294/294	294/294	290/290	282/282
CVRL08	225/225	225/225	225/225	225/225	225/225
GLM7	213/213	213/213	213/213	213/213	213/213
LCA18	229/231	229/231	229/231	229/231	235/235
LCA63	233/237	233/237	233/237	231/237	231/237
LCA66	239/243	239/243	239/243	243/243	243/243
LCA68	217/217	217/217	217/217	217/217	217/217
LCA08	248/250	248/250	248/250	248/250	248/250
LCA82	111/113	111/113	111/113	113/113	113/113
LGU76	241/247	241/247	241/247	241/249	247/251
VOLP03	175/177	175/177	175/177	151/151	149/173
VOLP67	156/180	156/180	156/180	150/178	152/178
YWLL08	135/173	135/173	135/173	135/167	135/135
YWLL38	187/193	187/193	187/193	187/187	187/193

as donor karyoplasts. The gestation length for the cloned pregnancy, in the present study, was in the normal range (315–440 days) for this species [63] in contrast to the longer gestation period reported for cloned pregnancies in cattle [38] and buffalo [14]. The birth weight of the calf was also in normal range (26–45 kg) for the species [63], no abnormality was detected in the placenta or calf at birth or afterward, and the calf is growing normally.

In conclusion, the present study reports, for the first time, establishment of pregnancies and birth of the first cloned camelid, a dromedary camel (*C. dromedarius*), by use of SCNT. This has opened doors for the amelioration and preservation of genetically valuable animals like high milk producers, racing champions, and males of high genetic merit in camelids. We also demonstrated, for the first time, that adult and fetal fibroblasts can be cultured, expanded, and frozen without losing their ability to support the development of NT embryos, a technology that may potentially be used to modify fibroblast genome by homologous recombination so as to generate genetically altered cloned animals. At present, an overall low efficiency of live births produced remains a major obstacle to beneficial applications of NT technology. Strategies to aid selection of relatively undifferentiated cells in a given culture system and to identify and optimize the different steps of NT procedures warrant future development and research in this species.

ACKNOWLEDGMENTS

The authors would like to thank H.H. General Sheikh Mohammed bin Rashid Al Maktoum, Ruler of Dubai, for his moral and financial support. His advice during the course of this study has been invaluable. We also thank Dr. Ali Ridha, administrative director, for his unconditional support and cooperation. His encouragement throughout the course of this study has made it possible to achieve the target. We are also thankful to Mr. A.N. Siddiqui, Mr. M. Billah, and Mr. Aejaz Hussain for their help in handling and treatment of animals.

REFERENCES

1. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380:64–67.
2. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385: 810–813.
3. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of single adult. Science 1998; 282:2095–2098.
4. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369–374.
5. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999; 17:456–461.
6. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Suyapa B, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000; 407: 86–90.
7. Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L, Renard JP. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 2002; 20:366–369.
8. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M. A cat cloned by nuclear transplantation. Nature 2002; 415:859.
9. Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J. Generation of fertile cloned rats by regulating oocyte activation. Science 2003; 302:1179.
10. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G. A cloned horse born to its dam twin. Nature 2003; 424:635.
11. Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, Meerdo LN, Pate BJ. A mule cloned from fetal cells by nuclear transfer. Science 2003; 301:1063.
12. Lee BC, Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Shamim MH, Kim JJ, Kang SK, Schatten G, Hwang WS. Dogs cloned from adult somatic cells. Nature 2005; 436:604.
13. Li Z, Sun X, Chen J, Liu X, Wisely SM, Zhou Q, Renard JP, Leno GH, Engelhardt JF. Cloned ferrets produced by somatic cell nuclear transfer. Dev Biol 2006; 293:439–448.
14. Shi D, Lu F, Wei Y, Cui K, Yang S, Wei J, and Liu Q. Buffalos (*Bubalus bubalis*) cloned by nuclear transfer of somatic cells. Biol Reprod 2007; 77: 285–291.
15. Wilmut I, Beaujean N, De Sousa PA, Dinnyes A, King TJ, Paterson LA, Wells DN, Young LE. Somatic cell nuclear transfer. Nature 2002; 419: 583–587.
16. Batchelder CA, Hoffert KA, Bertolini M, Moyer AL, Mason JB, Petkov SG. Effect of nuclear-donor cell lineage, type and cell donor on development of somatic cell nuclear transfer in cattle. Cloning Stem Cells 2005; 4:238–254.
17. Gao S, Czirr E, Chung YG, Han Z, Latham KE. Genetic variation in oocyte phenotype revealed through parthenogenesis and cloning: correlation with differences in pronuclear epigenetic modification. Biol Reprod 2004; 70:1162–1170.
18. Hiiragi T, Solter D. Reprogramming is essential in nuclear transfer. Mol Reprod Dev 2005; 70:417–421.
19. Enright BP, Kubota C, Yang X, Tian XC. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-20-deoxycytidine. Biol Reprod 2003; 69:896–901.
20. Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT, Wakayama T. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun 2006; 340:183–189.
21. Shi W, Hoeflich A, Flaswinkel H, Stojkovic M, Wolf E, Zakhartchenko V. Induction of a senescent-like phenotype does not confer the ability of bovine immortal cells to support the development of nuclear transfer embryos. Biol Reprod 2003; 69:301–309.
22. Sullivan EJ, Kasinathan S, Kasinathan P, Robl JM, Collas P. Cloned calves from chromatin remodeled in vitro. Biol Reprod 2004; 70:146–153.
23. Ono Y, Shimozawa N, Ito M, Kono T. Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol Reprod 2001; 64:44–50.
24. Schurmann A, Wells DN, Oback B. Early zygotes are suitable recipients for bovine somatic nuclear transfer and result in cloned offspring. Reproduction 2006; 132:839–848.
25. Boiani M, Eckardt S, Leu NA, Scholer HR, McLaughlin KJ. Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? EMBO J 2003; 22:5304–5312.
26. Bruggerhoff K, Zakhartchenko V, Wenigerkind H, Reichenbach H, Prelle K, Schernthaner W, Alberio R, Küchenhoff H, Stojkovic M, Brem G, Hiendleder S, Wolf E. Bovine somatic cell nuclear transfer using recipient oocytes recovered by ovum pick-up: effect of maternal lineage of oocyte donors. Biol Reprod 2002; 66:367–373.
27. Piedrahita JA, Wells DN, Miller AL, Oliver JE, Berg MC, Peterson AJ, Tervit HR. Effects of follicular size of cytoplast donor on the efficiency of cloning in cattle. Mol Reprod Dev 2002; 61:317–326.
28. Vajta G, Lewis IM, Hyttel P, Thouas GA, Trounson AO. Somatic cell cloning without micromanipulators. Cloning 2001; 3:89–95.
29. Oback B, Wierseman AT, Gaynor P, Laible G, Tucker FC, Oliver JE, Miller AL, Troskie HE, Wilson KL, Forsyth JT, Berg MC, Cockrem K, et al. Cloned cattle derived from a novel zona-free embryo reconstruction system. Cloning Stem Cells 2003; 5:1–2.
30. Galli C, Lagutina I, Vassiliev I, Dutchi R, Lazzari G. Comparison of microinjection (piezo-electric) and cell fusion for nuclear transfer success with different cell types in cattle. Cloning Stem Cells 2002; 4:189–196.
31. Wells DN, Misica PM, Tervit HR, Vivanco WH. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. Reprod Fertil Dev 1998; 10:369–378.
32. Akagi S, Adachi N, Matsukawa K, Kubo M, Takahashi S. Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol Reprod Dev 2003; 66:264–272.
33. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J Reprod Fertil 2000; 120:231–237.
34. Heyman Y, Chavatte-Palmer P, LeBourhis D, Camous S, Vignon X, Rinanrd JP. Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol Reprod 2002; 66:6–13.
35. Hochedlinger K, Rideout WM, Kyba M, Daley GQ, Blelloch R, Jaenisch

R. Nuclear transplantation, embryonic stem cells and the potential for cell therapy. Hematol J 2004; 5:S114–S117.
36. Panelli S, Damiani G, Galli C, Sgaramella V. Rearranged genomes of bovine blood cells can allow the development of clones till late fetal stages, but rare unrearranged genomes have greater potential and lead to adulthood. Gene 2004; 334:99–103.
37. Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol Reprod 2000; 62:1135–1140.
38. Kubota C, Yamakuchi H, Todoroki J, Mizoshita K, Tabara N, Barber M, Yang X. Six cloned calves produced from adult fibroblast cells after long-term culture. Proc Natl Acad Sci U S A 2000; 97:990–995.
39. Ogura A, Inoue K, Ogonuki N, Noguchi A, Takano K, Nagano R, Suzuki O, Lee J, Ishino F, Matsuda J. Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. Biol Reprod 2000; 62: 1579–1584.
40. Cho JK, Lee BC, Park JI, Lim JM, Shin SJ, Kim KY, Lee BD, Hwang WS. Development of bovine oocytes reconstructed with different donor somatic cells with or without serum starvation. Theriogenology 2002; 57: 1819–1828.
41. Gao S, McGarry M, Ferrier T, Pallante B, Gasparrini B, Fletcher J, Harkness L, De Sousa P, McWhir J, Wilmut I. Effect of cell confluence on production of cloned mice using an inbred embryonic stem cell line. Biol Reprod 2003; 68:595–603.
42. Powell AM, Talbot NC, Wells KD, Kerr DE, Pursel VG, Wall RJ. Cell donor influences success of producing cattle by somatic cell nuclear transfer. Biol Reprod 2004; 71:210–216.
43. Rideout WM III, Wakayama T, Wutz A, Eggan K, Jackson-Grusby L, Dausman J, Yanagimachi R, Jaenisch R. Generation of mice from wild-type and targeted ES cells by nuclear cloning. Nat Genet 2000; 24:109–110.
44. Eggan K, Akutsu H, Loring J, Jackson-Grusby L, Klemm M, Rideout WM III, Yanagimachi R, Jaenisch R. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc Natl Acad Sci U S A 2001; 98:6209–6214.
45. Inoue K, Ogonuki N, Mochida K, Yamamoto Y, Takano K, Kohda T, Ishino F, Ogura A. Effects of donor cell type and genotype on the efficiency of mouse somatic cell cloning. Biol Reprod 2003; 69:1394–1400.
46. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de León FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998; 280:1256–1258.
47. Arat S, Gibbons J, Rzucidlo SJ, Respess DS, Tumlin M, Stice SL. In vitro development of bovine nuclear transfer embryos from transgenic clonal lines of adult and fetal fibroblast cells of the same genotype. Biol Reprod 2002; 66:1768–1774.
48. Zakhartchenko V, Alberio R, Stojkovic M, Prelle K, Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R, Düchler M, Steinborn R, Mueller M, Brem G, et al. Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. Mol Reprod Dev 1999; 54: 264–272.
49. Shiga K, Fujita T, Hirose K, Sasae Y, Nagai T. Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese black bulls. Theriogenology 1999; 52:527–535.
50. Sansinena MJ, Taylor SA, Taylor PJ, Denniston RS, Godke RA. Production of nuclear transfer llama (*Lama glama*) embryos from in vitro matured llama oocytes. Cloning Stem Cells 2003; 3:191–198.
51. Khatir H, Anouassi A. Preliminary assessment of somatic cell nuclear transfer in the dromedary (*Camelus dromedarius*). Theriogenology 2008; 70:1471–1477.
52. Wani NA, Skidmore JA, Wernery U. Preliminary studies on the development of reconstructed embryos after nuclear transfer in dromedary camel (*Camelus dromedarius*). Reprod Fertil Dev 2009; 21:128–128.
53. Wani NA, Nowshari MA. Kinetics of nuclear maturation and effect of holding ovaries at room temperature on in vitro maturation of camel (*Camelus dromedarius*) oocytes. Theriogenology 2005; 64:75–85.
54. Wani NA. Chemical activation of in vitro matured dromedary camel (*Camelus dromedarius*) oocytes: optimization of protocols. Theriogenology 2008; 69:591–602.
55. Wani NA. In vitro embryo production in camel (*Camelus dromedarius*) from in vitro matured oocytes fertilized with epididymal spermatozoa stored at 4°C. Animal Reprod Sci 2008; 111:69–79.
56. Van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, Van de Weijden GC, Dieleman SJ. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled pre-ovulatory LH surge. Theriogenology 1999; 52:335–349.
57. Sirard MA. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology 2001; 55:1241–1254.
58. Hyttel P, Fair T, Callesen H, Greve T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 1997; 47:23–32.
59. Smith LC. Membrane and intracellular effects of ultraviolet irradiation with Hoechst 33342 on bovine secondary oocytes matured in vitro. J Reprod Fertil 1993; 99:39–44.
60. Liu L, Oldenbourg R, Trimarchi JR, Keefe DL. A reliable, noninvasive technique for spindle imaging and enucleation of mammalian oocytes. Nat Biotechnol 2000; 18:223–225.
61. Gasparrini B, Gao S, Ainslie A, Fletcher J, McGarry M, Ritchie WA, Springbett AJ, Overström EW, Wilmut I, De Sousa PA. Cloned mice derived from embryonic stem cell karyoplasts and activated cytoplasts prepared by induced enucleation. Biol Reprod 2003; 68:1259–1266.
62. Xue F, Tain XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H. Aberrant patterns of X chromosome inactivation in bovine clones. Nat Genet 2002; 31:216–220.
63. Tibary A, Anouassi A. Theriogenology in Camelidae, anatomy, physiology, pathology and artificial breeding. Mina, UAE: Abu Dhabi Printing Press; 1997.