Birth of African Wildcat Cloned Kittens Born from Domestic Cats

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ABSTRACT

In the present study, we used the African Wildcat (Felis silvestris lybica) as a somatic cell donor to evaluate the in vivo developmental competence, after transfer into domestic cat recipients, of cloned embryos produced by the fusion of African Wildcat (AWC) fibroblast cell nuclei with domestic cat cytoplasts. Cloned embryos were produced by fusion of a single AWC somatic cell to in vivo or in vitro enucleated domestic cat cytoplasts. When the two sources of oocytes were compared, fusion rate was higher using in vivo–matured oocytes as recipient cytoplasts, but cleavage rate was higher after reconstruction of in vitro–matured oocytes. To determine the number of reconstructed embryos required per domestic cat recipient to consistently establish pregnancies, AWC cloned embryos were transferred within two groups: recipients (n = 24) receiving ≤25 embryos and recipients (n = 26) receiving ≥30 embryos. Twelve recipients (46.2%) receiving ≥30 embryos were diagnosed to be pregnant, while no pregnancies were established in recipients receiving ≤25 NT embryos. Also, to determine the influence of length of in vitro culture on pregnancy rate, we compared oviductal transfer on day 1 and uterine transfer on day 5, 6, or 7. Pregnancy rates were similar after transfer of embryos on day 1 (6/12; 50.0%), day 5 (4/9; 44.4%), or day 6 (2/5; 40.0%) to synchronous recipients, but the number of fetuses developing after transfer of embryos on day 1 (n = 17), versus day 5 (n = 4) or day 6 (n = 3) was significantly different. Of the 12 pregnant recipients, nine (75%) developed to term and fetal resorption or abortion occurred in the other three (25%) from day 30 to 48 of gestation. Of a total of 17 cloned kittens born, seven were stillborn, eight died within hours of delivery or up to 6 weeks of age, and two are alive and healthy. Perinatal mortality was due to lung immaturity at premature delivery, placental separation and bacterial septicemia. Subsequent DNA analysis of 12 cat-specific microsatellite loci confirmed that all 17 kittens were clones of the AWC donor male. These AWC kittens represent the first wild carnivores to be produced by nuclear transfer.

INTRODUCTION

The survival of most species in the Felidae family is considered at risk of extinction. The population decline can be attributed to poaching and human destruction of natural ecosystems. As populations decrease,
insularization eventually results in subpopulation extinctions caused by detrimental genetic and demographic effects (Nowell and Jackson, 1996a).

Domestic cats are a useful research model to develop assisted reproductive technologies for the conservation of endangered felids. During the last two decades, the feasibility of oocyte recovery (in vivo maturation), in vitro maturation (IVM), in vitro fertilization (IVF), in vitro embryo culture (IVC) and embryo transfer (ET) to recipients has been demonstrated (Pope, 2000; Luvoni, 2000). The domestic cat can also serve as a successful recipient of embryos from closely related small non-domestic cats as shown by the birth of Indian desert cat (Pope et al., 1993) and African Wildcat kittens (Pope et al., 2000) after transfer of IVC-derived embryos.

Recently, the feasibility of producing viable domestic cat offspring by nuclear transfer was demonstrated (Shin et al., 2002). Regarding endangered felids, nuclear transfer is a potentially valuable technique for assuring the continuation of species with few remaining numbers of animals. In fact, cloning could maintain genetic variability in endangered felids when there are few animals in a founder population by conserving the maximum number of alleles for future breeding projects (Seidel, 2001).

Inter-species nuclear transfer involves the transfer of a donor cell nucleus of one species into an enucleated oocyte of another species. Since a limited number of endangered feline oocytes are available, the ooplasm of the domestic cat oocyte can be used as the recipient cytoplast of a somatic cell nucleus of an endangered cat. Recently, we have shown that domestic cat ooplasm is a compatible host for somatic cell nuclei from an AWC and the reconstructed embryos do undergo development in vitro (Gómez et al., 2003). Although the feasibility of inter-species nuclear transfer has been demonstrated only three live endangered mammals have been produced: gaur (Lanza et al., 2000), moufflon (Loi et al., 2001), and banteng (Jansen et al., 2004) after transfer of NT embryos into domestic ruminant recipients.

Incomplete nuclear reprogramming is a major constraint to the in vitro developmental potential of cloned embryos (Kikyo and Wolffe, 2000). Most cloned blastocysts, regardless of species, are not able to maintain fetal development to term (Colman, 2000). Therefore, to maximize pregnancy rates in species such as the pig, up to 62 reconstructed embryos are transferred into each recipient (Polejaeva et al., 2000). In the domestic cat, we reported that more pregnancies occurred after uterine transfer of ≥52 IVC/IVC-derived embryos (Pope et al., 1993). In vivo developmental potential of cloned embryos may be even more susceptible to the stressful environment of in vitro culture. Although in vitro development of cat embryos, as measured by blastocyst development and number of cells per embryo, has improved (Pope et al., 1999; Gómez et al., 2001, 2003), the presence of perturbations affecting embryo viability such as nutritional imbalances, oxidative stress, and defective genomic imprinting must be acknowledged (Leese, 2002).

In the present study, we used the African Wildcat (Felis silvestris lybica) as a somatic cell donor to evaluate the in vivo developmental competence, after transfer into domestic cat recipients, of cloned embryos produced by the fusion of AWC fibroblast cell nuclei with domestic cat cytoplasts. Specifically, our primary goals were to (1) determine the minimal number of reconstructed embryos required per recipient to establish pregnancy; (2) evaluate the influence of in vitro culture interval on establishment and maintenance of fetal development in recipients after transfer of reconstructed embryos; and (3) evaluate factors affecting pre- and post-natal fetal survival and describe characteristics of the kittens.

MATERIALS AND METHODS

Animals

Domestic cats used as oocyte donors and embryo recipients were group-housed in environmentally controlled rooms with a 14-h/10-h light/dark cycle at 20–26°C. The antibody defined cats were purchased from Liberty Research, Inc. (Waverly, NY). The AWC male was housed in a separate room under the same conditions. The rooms were cleaned, and cats were fed once daily (Science Diet, Hill Pet Nutrition). Fresh water was available at all times. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Audubon Center for Research of Endangered Species as required by the Health Research Extension Act of 1985 (Public Law 99-158).
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Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Establishment of adult AWC cell line

The cell line was generated from skin tissue collected by biopsy from a 3-year-old male AWC. Collected tissue was finely cut into 1-mm² pieces and plated in a 75-cm² tissue-culture flask (Nunc, Denmark) containing 7 mL of Dulbecco Modified Eagles Medium (DMEM) supplemented with 50 μg/mL of gentamicin and 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) and cultured at 38°C in 5% CO₂/air. After 7–10 days of incubation, monolayer outgrowths with fibroblastic-like morphology were disaggregated with 2.5 mg/mL of pronase, re-suspended in DMEM with 10% FBS and 10% (v/v) dimethyl sulfoxide, and cooled at 1.0°C/min to −80°C (Mr. Frosty; Nathgene, Rochester, NY) before storage in liquid nitrogen (LN₂). To prepare fibroblasts as donor cells for NT, cells were thawed and cultured in DMEM containing 10% FBS for 1–3 passages. Then, when cells reached 80% confluency, FBS was reduced to 0.5% and cells were cultured for an additional 5 days (serum starvation) before cryopreservation and storage in LN₂.

Oocyte maturation

For in vitro maturation, cumulus-oocyte complexes (COCs) were recovered from ovaries of domestic cats within 2–5 h after ovariohysterectomy. Selected COCs were cultured in modified TCM-199 containing 1 IU/mL hCG, 0.5 IU/mL eCG, 10 μg/mL epidermal growth factor, and 3 mg/mL BSA (Fraction V, fatty acid free; Serum Laboratories, Kankakee, IL) for 24 h in 5% CO₂/air. After 7–10 days of incubation, monolayer outgrowths with fibroblastic-like morphology were disaggregated with 2.5 mg/mL of pronase, re-suspended in DMEM with 10% FBS and 10% (v/v) dimethyl sulfoxide, and cooled at 1.0°C/min to −80°C (Mr. Frosty; Nathgene, Rochester, NY) before storage in liquid nitrogen (LN₂). To prepare fibroblasts as donor cells for NT, cells were thawed and cultured in DMEM containing 10% FBS for 1–3 passages. Then, when cells reached 80% confluency, FBS was reduced to 0.5% and cells were cultured for an additional 5 days (serum starvation) before cryopreservation and storage in LN₂.

Cumulus cells of in vitro- and in vitro-matured oocytes were removed by vortexing in 1 mg/mL of hyaluronidase (type IV) for 5 min, followed by gentle mechanical pipetting. Denuded oocytes were placed in Tyrode's solution (Irvine Scientific, Santa Ana, CA) with 1% MEM nonessential amino acids, 3 mg/mL BSA, 15 mM NaHCO₃, 0.36 mM pyruvate, 2.2 mM calcium lactate, 1 mM glutamine, and 50 μg/mL gentamicin (IVC-1 medium) at 38°C in 5% CO₂ until further use.

Nuclear transfer

The protocol for nuclear transfer has been described previously (Gómez et al., 2003). Briefly, denuded metaphase II oocytes were incubated for 15 min at 38°C in Ca²⁺-free and Mg²⁺-free modified Tyrode's salt solution supplemented with 1% MEM nonessential amino acids, 3 mg/mL BSA, 30 mM NaHCO₃, 0.36 mM pyruvate, 1 mM glutamine, 50 μg/mL gentamicin (ECM medium), 20 μg/mL Hoechst 33342 and 20 μg/mL cytochalasin B (CCB). After incubation, oocytes were enculturated in ECM medium in which supplemental NaHCO₃ was reduced to 15 mM and to which 15 mM Hepes, 20 μg/mL CCB and 2 mg/mL sucrose were added. The first polar body and ~10% of the underlying cytoplasm were drawn into an enucleation pipette (20 μm, OD). Removal of the metaphase II plate was confirmed by brief exposure to epifluorescence microscopy.

A vial containing AWC fibroblast cells synchronized in G₀/G₁ phase by serum starvation (Gómez et al., 2003) was thawed prior to being used for NT, and a single AWC fibroblast cell was introduced into the perivitelline space of each enculturated oocyte. For fusion, each NT couplet, in a solution of 0.3 M mannitol and 0.1 mM MgCl₂, was placed between two stainless-steel electrodes (LF-101; Nepa Gene, Tokyo, Japan) that were controlled by micromanipulators. Membrane fusion was induced by applying a 3-sec AC pre-pulse of 20V, 1 MHz followed by two 30-μsec DC pulses of 240V/mm at intervals of 0.5 sec. Following the fusion pulses, couplets were washed and cultured in IVC-1 medium supplemented with 7.8 mM calcium lactate (IVC-1 + CaCl₂) for 30 min, at which time fusion was evaluated visually by confirming the presence or absence of the donor cell in the perivitelline space.
After 2–3 h of culture in IVC-1 + Ca\(^{2+}\), activation was induced by placing fused couplets between two electrodes in a fusion chamber containing 3 mL of a solution of 0.3 M mannitol, 0.1 mM Mg\(^{2+}\), and 0.05 mM Ca\(^{2+}\) and exposing them to two 60-μsec DC pulses of 120 V/mm. Then, couplets were incubated in 30-μL droplets of IVC-1 medium supplemented with 10 μg/mL cytochalasin and 5 μg/mL CCB at 38°C in 5% CO\(_2\) in air for 4 h.

Embryo culture

After activation, reconstructed embryos were cultured in 500 μL of IVC-1 medium in 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\) at 38°C. Some couplets were incubated in 30-μL droplets of IVC-1 medium supplemented with 10 μg/mL cytochalasin and 5 μg/mL CCB at 38°C in 5% CO\(_2\) in air for 4 h.

Embryo transfer

Cloned embryos were transferred into the oviducts or uteri of 50 gonadotropin-treated domestic short hair (DSH) recipients. Day 1 embryos were transferred into the oviducts of recipient females on day 1. The remaining couplets were cultured until day 2 or 3, at which time, after uncleaved couplets were recorded and removed, embryos were placed into 500 μL of fresh IVC-1 medium containing 1% MEM essential amino acids (IVC-1 + EAA). Then, on day 5, the number of morulae was recorded and all embryos were moved into Tyrode’s solution containing 1% NEAA, 2% EAA, 10% FBS, and the same supplements as in IVC-1 medium (IVC-2 medium) and cultured until transfer to a recipient.

Embryo culture

After activation, reconstructed embryos were cultured in 500 μL of IVC-1 medium in 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\) at 38°C. Some couplets were incubated in 30-μL droplets of IVC-1 medium supplemented with 10 μg/mL cytochalasin and 5 μg/mL CCB at 38°C in 5% CO\(_2\) in air for 4 h.

Embryo transfer

Cloned embryos were transferred into the oviducts or uteri of 50 gonadotropin-treated domestic short hair (DSH) recipients. Day 1 embryos were transferred into the oviducts of recipients, some of which had served as oocyte donors on day 0 (n = 2) and others of which served as recipients only after induction of ovulation (n = 14) using a lower dose of FSH (1–3 IU) and a higher dose of LH (5 IU) than the oocyte donors received. For oviductal embryo transfer, the abdominal cavity was visualized via a 5-mm endoscope inserted just above the umbilicus, and a 16 gauge × 6.35 cm stainless steel trocar/cannula was inserted transabdominally near the midline ~5 cm below the umbilicus. Then, a 14.5-cm tom cat catheter (3.5 French, Sherwood Medical, St. Louis, MO) was passed through the 16-gauge catheter and directed through the infundibulum overlaying the ovary and into the upper ampullary portion of the oviduct. A 50-cm length of sterile polyethylene tubing (PE 10, no. 427400, Becton Dickinson, Sparks, MD) containing the embryos near the lower tip was threaded through the tom cat catheter, and the embryos were expelled into the oviduct using positive pressure from a 1-mL syringe.

The recipients of day 5, 6, and 7 embryos were gonadotropin-treated females that had undergone laparoscopic oocyte aspiration 5–7 days previously. Briefly, one uterine horn was exteriorized through a 1.5-cm mid-ventral incision and punctured with a sterile 16-gauge round-tipped, short bevel trocar ~1 cm from the anterior tip. Embryos were aspirated in ~50 μL of IVC-2 medium into a 14.5-cm tom cat catheter using a 1-mL plastic syringe. The catheter was then threaded 4–5 cm into the uterine lumen before depositing the embryos (Pope et al., 1994).

Pregnancy detection and parturition

Each recipient was checked for pregnancy status using abdominal ultrasonography on day 21–23 after ovulation or oocyte aspiration. Pregnant recipients were monitored by ultrasonography on a weekly basis until day 60, at which time daily ultrasonographic examinations were made to check for fetal movement and heart beat. Pregnant recipients were allowed to complete gestation, at which time kittens were delivered by Caesarean section, either (1) as an intervention after onset of vaginal bleeding and/or occurrence of labor symptoms or (2) on a pre-scheduled day before symptoms of parturition were seen. In an effort to reduce the onset of premature parturition, some pregnant cats (n = 3) were orally administered altrenogest (0.08 mg/kg, Regu-Mate, Hoechst; Kustritz, 2001) daily from days 55 to 67 of gestation. Also, in an attempt to reduce perinatal kitten mortality (n = 7) cats were treated with four injections (im), 12 h apart, of betamethasone (0.1 mg/kg, CelestoneSoluspan, Schering) starting at varying times after day 60 of gestation.

Microsatellite analysis

To determine the clonal status of all kittens, DNA was extracted using standard phenol/chloroform techniques from the fibroblast cells and blood of the AWC donor, blood of the recipient dam, umbilical cord blood of live kittens, or spleen of dead kittens. Standard sodium hydroxide isolation was used to isolate DNA from cheek swabs of live kittens. Feline derived microsatel-
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RESULTS

Embryo production after NT

A total of 2,565 in vivo (n = 1751) and in vitro (n = 814) matured domestic cat oocytes were mechanically enucleated and electrically fused to frozen-thawed AWC fibroblasts. Fusion efficiency was higher when in vivo-matured oocytes (1696/1751; 96.8%) were used as recipient cytoplasts as compared with in vitro-matured oocytes (736/814, 90.4%, p < 0.05). In contrast, cleavage rate of embryos reconstructed with in vitro-matured oocytes (435/511, 85.1%) was higher than that of embryos reconstructed with in vivo-matured oocytes (944/1194, 79.1%, p < 0.05). Some reconstructed couplets (n = 727) were not included in the cleavage rate evaluation because they were transferred to domestic cat recipients on day 1 before cleavage occurred.

Embryo transfer and pregnancies

To determine the number of reconstructed embryos that should be transferred per recipient to consistently establish pregnancies, AWC cloned embryos were transferred into domestic cat recipients within two groups: (1) recipients (n = 24) that received fewer than 25 embryos (mean = 19.4; range = 8–25), and (2) recipients (n = 26) that received more than 30 embryos (mean = 41.7; range = 31–59). Also, to determine the influence of length of in vitro culture on pregnancy rate we compared the transfer of day 1 embryos into the oviduct (n = 588) with transfer of day 5 (n = 467), day 6 (n = 335), and day 7 (n = 142) embryos into the uterus. Embryos were at the 1–2-cell stage on day 1, morulae on day 5 and day 6, and morulae and blastocysts on day 7 of culture.

Twelve of 26 (46.2%) recipient cats receiving more than 30 NT embryos were diagnosed to be pregnant when examined by ultrasonography at 21–23 days after induction of ovulation or aspiration of follicular oocytes. In contrast, none of the recipients receiving less than 25 embryos (n = 24) were diagnosed pregnant (Table 1). Although, no differences were observed in pregnancy rates after transfer of ≥30 embryos on day 1 (6/12, 50.0%), day 5 (4/9, 44.4%), or day 6 (2/5, 40.0%) to synchronous recipients, a significant difference was found in the number of fetuses implanted after transfer embryos on day 1 (n = 17) versus day 5 (n = 4) or day 6 (n = 3; p < 0.05; Table 1). Six of 7 (85.7%) pregnant cats with multiple fetuses were recipients of day 1 embryos.

Birth of AWC cloned kittens and histological analysis

Of the 12 pregnant recipients, nine (75%) pregnancies developed to term (>60 days). In the

Table 1. Effects of transfer day and number of embryos on occurrence of pregnancy in domestic cat recipients receiving African wildcat (AWC) cloned embryos

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<th>AWC NT embryos</th>
<th>Average per recipient</th>
<th>Recipients</th>
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<tr>
<td>Embryos per recipient</td>
<td>ET, day</td>
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<td>≤25</td>
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<td>Total</td>
<td>1552</td>
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three (25%) other pregnancies, a total of five fetuses were present at day 21–23, four of which were reabsorbed between days 30 and 40, and one which was spontaneously aborted on day 48 of gestation (Table 2).

Six of the nine term pregnancies, consisting of a total of eight fetuses, were not hormonally treated during the last 7–10 days of pregnancy (Table 2). Two of these recipients each had a single kitten that died in utero within ≤24 h of delivery by Caesarean section on days 64 and 67 of gestation. Histological analysis of the two kittens showed no indications of infectious disease. No inflammatory or other degenerative lesions were recognizable in the tissue sections and all organs appeared to be developing normally. One of the two kittens had moderate haemorrhage within the choroids plexus of the brain, possibly due to hypoxia that occurred after placental separation.

The other four recipients delivered six kittens by Caesarean section on days 62–67 of pregnancy after vaginal bleeding occurred. Of the six kittens, four experienced respiratory failure shortly after delivery and resuscitation efforts were not successful, one kitten lived for 36 h and one kitten had died in utero at ~45 days of gestation.

Analysis of the kitten that died 36 h after delivery showed multifocal moderate to severe acute ulcerative enterocolitis associated with rod-shaped bacteria, and squames were present within the alveolar spaces of the lungs. These results suggest that the kitten had complications caused by bacterial septicemia of clostridial origin.

The kitten that died in utero on ~45 days of gestation showed gross physical abnormalities, including incomplete closure of the ventral body wall musculature with abdominal organ exteriorization, severe cerebella and cerebellum aplasia and no recognizable central nervous system. All the other kittens that died shortly after delivery had a normal physical appearance except for one that showed incomplete closure of the ventral body wall musculature with abdominal

<table>
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<tr>
<th>Pregnant recipients</th>
<th>Embryos transferred</th>
<th>Total fetuses</th>
<th>Fetuses reabsorbed</th>
<th>AWC kittens (days 48–70)</th>
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<td>ET transferred n</td>
<td>days 21–23</td>
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<td>Total</td>
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*Pregnant cats showed vaginal bleeding.
*Pregnant cats treated with progesterone and betamethasone.
*Died in utero just before delivery.
*Died in utero on day 45–50 of gestation.
*Kittens with body wall defect.
*Kittens died within 36–72 h after birth.
*Gómez ET AL.
organ exteriorization. Histological evaluation of the lungs of the kittens revealed variable collapse of alveolar spaces and presence of epithelial squames within the lumen of alveolar spaces, indicating that alveoli were not inflated and the lungs were immature. Thus, prenatal and early neonatal mortality appears to have been primarily due to lung immaturity resulting from the spontaneous initiation of premature parturition.

Three pregnant cats that were hormonally treated during the last 10–12 days of gestation delivered eight kittens by Caesarean section. The four kittens that survived were born on 6 August 2003, 15 November 2003, and 12 January 2004 at 69, 65, and 67 days of gestation, respectively (Table 2).

Of the four kittens that did not survive, two died in utero within hours of delivery on days 64 and 66. One kitten that died within a few minutes after delivery had incomplete closure of the ventral body wall musculature with abdominal organ exteriorization. Another kitten died in utero on ~50 days of gestation and was being resorbed at delivery.

Pathology analysis of one kitten that died in utero on day 64 revealed diffuse adrenal congestion and mild nephrosis. Necrosis of the renal tubular epithelium could be induced by endotoxic shock. The hypoxia may have been caused by cardiovascular collapse and release of nephrotoxins before death. Therefore, prenatal death was possibly due to hypoxia that occurred after placenta separation and is probably analogous to the prenatal death occurring in pregnant recipients that were not hormonally treated during the final stage of gestation.

Two kittens, currently 7 and 3 months of age (Fig. 1) are healthy and developing normally. Of the other two kittens, one died at 56 h after birth and one died at 6 weeks of age. Histological analysis of the kitten that died at 56 h after birth indicated a diffuse moderate septic and suppurative pneumonia with abnormally high bacterial counts in several organs. The bacteria may have gained entry through the urachus and extended into the urinary bladder.

The kitten that died at 6 weeks of age had a congenital megaeosophagus and acute suppurative pneumonia. The primary cause of death was pneumonia, likely a result of aspiration of formula during bottle-feeding.

As shown in Table 2, the birth average weight of live kittens (92.3 g; range = 71.9–105.0 g) was similar to the average weight of kittens that died during the peri- and post-parturient period (87.4 g; range = 50.0–111.0 g). Phenotypically, all cloned kittens (n = 17) were African Wildcats. Subsequent DNA analysis of 12 cat-specific microsatellite loci performed with “blind” testing at the School of Veterinary Medicine, University of California, Davis, confirmed that they were identical to DNA of the AWC donor male (“Jazz”; Table 3).

**Placental analysis**

A total of 11 placentae were analyzed. Macroscopic analyses showed that five placentae had
patches of serous-lacy tissue replacing normal placental tissue. Microscopic analysis of these five placentae showed that areas of the placental epithelium were reduced in thickness and had shorter and thicker papillary projections of merely a thin band of edematous fibrous connective tissue. These observations indicate the occurrence of multifocal placental atrophy with fibrous tissue replacement. Curiously, these abnormalities were not present in all the placental sections analyzed; some sections were histologically normal.

In the present study, we demonstrated that AWC kittens can be produced after transfer of embryos derived by fusion of adult somatic cells from one species with enucleated oocytes of a closely related species (domestic cat). We have shown that, under our current laboratory conditions, transfer of at least 30 NT embryos per recipient was required for establishing a pregnancy. Also, the length of in vitro culture affected the number of embryos that successfully underwent fetal development. The primary causes of peri-natal mortality were placental separation, lung immaturity at premature delivery and bacterial septicemia.

We obtained pregnancies only in recipients that received ≥30 NT embryos. A possible explanation for the high number of embryos required for producing pregnancies is that most of the reconstructed embryos had inadequate nuclear remodeling or incomplete reprogramming (Gómez et al., 2003). We previously demonstrated in AWC reconstructed embryos that 18% were fragmented and unable to undergo chromatin remodeling, 38% arrested at the 8–10-cell stages and 44% and 24% reached the morula and blastocysts stages, respectively (Gómez et al., 2003). In this study, NT embryos were reconstructed from the same AWC somatic cell line and oocytes were similar in type and source to those used in previous NT experiments. Then, from the mean number of embryos transferred on day 1 (40.5), day 5 (46.3), and day 6 (36.6), we can estimate that at least 10 embryos should develop to the blastocyst stage. Based on these values, we suggest that, using our current methods, to consistently establish pregnancies in domestic cats receiving AWC NT-derived embryos, at least 30 embryos should be transferred per recipient. If it is assumed that NT-derived cat blastocysts may be less developmentally competent than IVF-derived cat blastocysts, these numbers directly correlate with the minimal number of IVF-derived embryos required to reliably establish pregnancies in domestic cat recipients.

Domestic cat recipients receiving day-7 blastocysts (mean = 7.4) and morulae (mean = 8.3) on day 7 post-oocyte retrieval did not establish pregnancies. During this portion of the study we were transferring an average of 10 blastocysts per cat. We do not think that the longer in vitro culture interval was the primary reason for the developmental failure because we have produced domestic cat kittens after transfer of either 10 or 11 IVF-derived day-7 blastocysts per recipient (Pope et al., 2003). Further experiments should determine if pregnancies can be established after transferring AWC cloned blastocysts.

In sheep, more lambs were produced after early transfer (day 2 of IVF embryos as compared to later transfer at day 6 (Walmsley et al., 2004). Similarly, in the present study, a higher percentage of NT embryos developed into fetuses when the transfer was done after a shorter period of culture (day 1, n = 17/486, 3.5%) as compared with embryos that were cultured for longer periods.
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(day 5, n = 4/417, 1.0%; day 6, n = 3/183, 1.6%). From these results, we suggest that the developmental potential of some NT embryos is “rescued” by transferring them to recipient cats as unclefted couplets and two-cell embryos; thereby minimizing the detrimental effects of in vitro culture (Leese, 2002). Although more embryos implanted after transfer on day 1, fetal mortality by day 48 of gestation also increased (6/17, 35.3%) as compared to embryos transferred on day 5 (1/4; 25.0%) or day 6 (0/3; 0%). Also, three kittens that were born after embryo transfer on day 1 had incomplete closure of the ventral body wall musculature with abdominal organ exteriorization. Because few fetuses were evaluated, we cannot conclude that the increase in fetal mortality or the physical abnormalities were directly related to the shorter period of culture. However, we can speculate that losses occurring after transfer on day 1 are not found after the transfer of embryos on day 5 or 6 because the developmental potential of such embryos is sufficiently reduced by the extended in vitro culture interval that they are incapable of further development in utero.

Although embryo viability was demonstrated, embryo survival after transfer was quite modest (1.0–3.5%) and was similar to the overall efficiency of cloning reported in other species (1.0–4.0%; Wilmut and Paterson, 2003). In addition to incomplete reprogramming of the differentiated nucleus, another factor that may contribute to low cloning efficiency is variation in oocyte quality. Both in vitro-matured and in vitro-matured oocytes have been used as recipient cytoplasts for production of cloned animals. For example, in sheep, higher blastocyst development rates, pregnancy rates and embryo survival were reported when in vitro-matured or in vitro-matured oocytes were used as recipient cytoplasts of AWC clones (Gómez et al., 2003). A primary purpose of the present study was to find out if AWC kittens could be produced by transfer of NT embryos to domestic cat recipients. Because we were unable to produce NT pregnancies after transferring ≤17 embryos per recipient in a previous study (Gómez et al., 2003), in the present study in order to transfer ≥25 embryos per recipient, for most transfers it was necessary to combine reconstructed embryos derived from both in vitro-matured and in vivo-matured domestic cat cytoplasts. The one domestic cat recipient receiving NT embryos derived from in vivo-matured cytoplasts did establish pregnancy and produce a live kitten. We do not know if the other NT kittens originated from embryos derived from in vitro-matured or in vitro-matured cytoplasts. Certainly, the comparative developmental competence of in vitro-matured versus in vivo-matured cat cytoplasts requires investigation.

Several developmental abnormalities occur in pregnancies from somatic cell nuclear transfer–derived embryos, including a high rate of abortion during early gestation and a number of perinatal complications. One complication we encountered in some cats was the occurrence of vaginal bleeding by day 62–67 of pregnancy without overt signs of labor. Although the kittens died shortly after delivery from respiratory failure, a condition usually associated with premature delivery. In domestic cats, serum progesterone levels begin a slow decline by day 50 of pregnancy, progressing to a precipitous decrease during the last week prior to parturition (Verhage et al., 1976). Although we did not measure serum progesterone levels in pregnant recipients, it seems possible that, since the recipient domestic cats were gestating AWC cloned fetuses, fetoplacental endocrine signaling may have been inadequate. As a result, progesterone levels may have shown an abnormally early decrease, starting a cascade of pre-parturient events that resulted in vaginal bleeding. Consequently, with the last three preg-
nant recipients, in an effort to reduce perinatal kitten mortality, a synthetic progestagen (altrenogest) was orally administered. Altrenogest has been shown to maintain gestation in ovariec-
tomized mares and dogs (Hirtrichs et al., 1986; Eilts et al., 1994). Since pregnancy was success-
fully maintained (until elective Caesarean sec-
tion) in cats treated with the synthetic progesta-
gen, luteal insufficiency may be implicated in the occurrence of premature vaginal bleeding. Ma-
ternal administration of betamethasone is widely used to improve fetal lung maturation (Senat,
2002; Celik et al., 2002). Recipients administered both synthetic progestagen and betamethasone
during the latter part of gestation produced a to-
tal of five live kittens. Thus, while the improve-
ment in fetal survival rate cannot be conclusively attributed to the treatment combination of pro-
gestagen and betamethasone, the present results do provide at least circumstantial evidence of their effectiveness.

Placental abnormalities are a major cause of mortality in cloned animals and likely contribute to a number of perinatal complications in farm animals (Cibelli et al., 2002). Abnormal placenta-
ues have been reported in cloned mice, cattle and sheep (Shimozawa et al., 2003; Hill et al., 2000;
Hartwich et al., 2003) and may be a result of aber-
rant gene expression (Suemizu et al., 2003). In our study, histological analysis of placental tissue re-
vealed a reduction in thickness and multifocal at-
rophy. In fact, some kittens died from hypoxia af-
fter placental separation, with respiratory failure as a secondary consequence. Accordingly, it is plausible that placental abnormalities/dysfunction were either directly or indirectly responsible for the perinatal deaths of the cloned kittens. To im-
prove success rates and reduce neonatal deaths, basic studies are needed to identify factors caus-
ing abnormal placentation.

Developmental anomalies occur with a greater frequency in cloned animals than in the average population. Diagnostic pathology of cloned lambs that died after birth revealed several cloning-related abnormalities, some of which were analogous to congenital human diseases (Rhind et al., 2003). For example, four of eight cloned lambs exhibited fetal overgrowth and a body wall defect. These abnormalities are linked to Beckwith-Wiedemann syndrome (B-WS), a hu-
man genetic syndrome characterized by abdom-
inal wall defects, visceromegaly, macroglossia, pre-
and postnatal overgrowth and neonatal hypoglycemia. This disorder involves the dereg-
ulation of several genes, including insulin-like growth factor, type 2 (IGF2) and cyclin-depen-
dent kinase inhibitor 1C (CDKN1C; Murrel et al.,
2001). Recently, in humans, an association was found between babies produced by in vitro tech-
nology and the incidence of B-WS (DeBaun et al.,
2003). In our study, a major abnormality found in three cloned kittens was incomplete closure of
ventral body wall musculature with exterioriza-
tion of abdominal organs; however, pathology as-
sociated with overgrowth was not observed.
Likewise, knockout mice for CDKN1C have been found to manifest an anterior wall defect but do
not exhibit fetal overgrowth or other features of
B-WS (Fitzpatrick et al., 2002). Whether body wall pathology is caused by gene deregulation or is
merely a consequence of another wall anomaly is
not known.

The influence of possible mitochondrial het-
eroplasmy and/or a deleterious mtDNA effect on neonatal death of the cloned AWC kittens cannot be discounted. Indeed, in future studies the pre-
cise mitochondrial inheritance pattern in AWC cloned kittens will be evaluated to determine its influence on survival rate.

The effectiveness of our cloning procedure was
shown by the birth of healthy AWC kittens. These kittens represent the first wild carnivores to be produced by NT and further expand the grow-
ing list of species in which cloned offspring have been produced. Our success may be attributable to improvements in the embryo culture system
(Gómez et al., 2003), refinements made to the nu-
clear transfer procedure (Gómez et al., 2003), in-
creasing the number of embryos transferred, re-
ducing the culture interval before transfer and
modification of current protocols successfully used in other species to manage pregnancy and
improve viability of offspring (Ptak et al., 2002).

While there are major limitations to current cloning technologies, knowledge about nuclear transfer as one of the assisted reproduction tech-
niques potentially useful for the conservation of endangered species, which in some cases, may of-
fer the prospect of species continuation rather
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than extinction. Future research should focus on defining and understanding the mechanisms responsible for the abnormalities, so that efficiency is improved and neonatal death rate reduced. We expect advances in understanding the molecular events for reprogramming of the donor genome will contribute to clarifying the derivation of developmental defects in cloned embryos.

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