# Comparison of Developmental Capacity for Intra- and Interspecies Cloned Cat (*Felis catus*) Embryos

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**ABSTRACT** Interspecies nuclear transfer is an invaluable tool for studying nucleus–cytoplasm interactions; and at the same time, it provides a possible alternative to clone animals whose oocytes are difficult to obtain. In the present study, we investigated the possibility of cloning cat embryos using rabbit oocytes, and compared the developmental capacity; the timing of embryogenesis of the cat–rabbit cloned embryos with that of the cat–cat or the rabbit–rabbit cloned embryos. When cultured in M199, the rate of blastocyst formation of the cat–rabbit embryos was 6.9%, which was not significantly different than that of the cat–cat embryos (10.5%). However, the rate of blastocyst formation of rabbit–rabbit embryos (22.9%) was significantly greater than that of both the cat–rabbit and the cat–cat embryos ($P < 0.05$). The timing of the first three cleavages for the cat–rabbit embryos was similar to that of the rabbit–rabbit embryos, but significantly faster than that of the cat–cat embryos ($P < 0.05$), while the time to form blastocysts was similar to that of cat–cat embryos, but significantly slower than that of the rabbit–rabbit embryos ($P < 0.05$). Both M199 and SOF medium were evaluated for culturing cat–rabbit embryos; the rate of blastocyst formation in SOF (14.5%) was significantly greater than that in M199 (6.9%) ($P < 0.05$). These results demonstrate that: (1) the cat–rabbit embryos possess equal developmental capacity as cat–cat embryos; (2) the timing of the first three cleavages for the cat–rabbit embryos is recipient-specific, while the time to form blastocysts is donor nucleus-specific; and (3) SOF medium may be beneficial to overcome the morula-to-blastocyst block for cat–rabbit cloned embryos. *Mol. Reprod. Dev. 66: 38–45, 2003.*


**Key Words:** interspecies nuclear transfer; embryogenesis; cat

## INTRODUCTION

After the birth of the lamb, "Dolly" (Wilmut et al., 1997), successful cloning by somatic cell nuclear transfer (SCNT) has been achieved in mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999), bovine (Cibelli et al., 1998; Kato et al., 1998), pig (Polejaeva et al., 2000), cat (Shin et al., 2002), and rabbit (Chesne et al., 2002). The technique of SCNT provides not only a valuable tool to multiply animals of the same genetic traits, but a prospective alternative to save endangered animal species. However, the technique of SCNT is presently inefficient in this regard (Wilmut et al., 1997; Wakayama et al., 1998; Yanagimachi, 2002), because of the unavailability of species-specific competent recipient cytoplasm (Dominko et al., 1999); i.e., the unavailability of oocytes from endangered animals. Interspecies nuclear transfer, which is involved in transferring cell nuclei of one species into enucleated oocytes of another species, provides a possible approach to clone animal species whose oocytes are difficult to obtain. Previous studies have shown that oocyte cytoplasm from bovine (Dominko et al., 1999), sheep (White et al., 1999), and rabbit (Chen et al., 1999) are able to dedifferentiate somatic cell nuclei from sheep, pig, monkey, rat, and giant panda, and support early development of these interspecies cloned embryos to blastocysts. Lately, the successes of cloning gaur (Lanza et al., 2000) and mouflon (Loi et al., 2001) have demonstrated that it is practical to clone animal using the technique of interspecies nuclear transfer.

With respect to felines the domestic cat is the only group of this order that is not classified as an endangered species. The protocols of IVM–IVF–ET used for the domestic cat are effective for other feline species, and even full term development offspring have been obtained after transferring feline embryos into domestic cat uteri (Pope et al., 1993; Pope, 2000). It is generally accepted that the domestic cat is a useful model to study feline

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conservation (Swanson et al., 1996; Pope, 2000). However, even though SCNT has been successful in the cat, producing viable kittens (Shin et al., 2002), information of cat nuclear transfer remains limited. This may be partly attributed to the difficulty of enucleation of cat oocytes, since the cat oocyte is opaque and its plasma membrane is very fragile. Moreover, the availability of cat oocytes for nuclear transfer is a problem, since the technique of superovulating is inefficient (only 10–17 oocytes can be obtained per superovulated animal (Goodrowe et al., 1988; Swanson et al., 1995; Roth et al., 1997)). The rabbit is a widely used laboratory animal, and as many as 30–40 oocytes can be obtained from one superovulated female rabbit (our unpublished data). Previous studies in our laboratory have shown that panda–rabbit SCNT embryos can develop to blastocysts in vitro and implant in vivo (Chen et al., 1999, 2002). The results revealed that factors exist in rabbit oocyte cytoplasm for somatic nucleus reprogramming and dedifferentiation are not species-specific. Establishing a model of interspecies cloning cat embryos using rabbit oocyte cytoplasm would potentially provide greater insights into nucleus–cytoplasm interactions, as well as assist in the conservation of feline species.

Although interspecies cloning mammalian embryos have been reported in several species (Chen et al., 1999; Dominko et al., 1999; White et al., 1999), the characteristics of embryogenesis for these nucleo-cytoplasmic hybrids remain largely unknown. The timing of preimplantation embryogenesis, especially blastocyst formation varies among species in mammals (Bavister et al., 1983; Walker et al., 1992; Prather, 1993; Chesne et al., 2002), and the time to form blastocyst is critical for embryo survival since the period of uterine receptivity for implantation is transient (Feng and Gordon, 1997). Thus, understanding the characteristics of embryogenesis is essential for evaluating the developmental potential of interspecies cloned embryos.

In the present study, we investigate the possibility of cloning cat embryos using rabbit oocytes; and compared the developmental capacity and timing of embryogenesis for the intra-species and interspecies cloned cat embryos.

## MATERIALS AND METHODS

### Animal Care and Superovulation

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethical committee of the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Female Japanese big eared white rabbits (purchased from the Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were housed in stainless steel cages, and were fed rabbit fodder and water ad libitum. Rabbits were superovulated by administration of FSH and hCG (Institute of Zoology, Academia Sinica). Each rabbit was injected i.m. with 1 mg of FSH two times daily for 3 days and with 100 IU hCG (Institute of Zoology, Academia Sinica) i.v. 12 hr after the last FSH injection. Rabbits were killed 14 hr after the hCG injection. Cumulus masses were collected by flushing oviducts with M2 medium (Sigma Chemical Co., St. Louis, MO) and treated with 300 IU/ml hyaluronidase (Sigma Chemical Co.) in M2 medium to isolate the oocytes. Adherent cumulus cells were removed mechanically by gently aspirating the oocytes with a fine pipette. After washing in M2 medium for three times, the cumulus-free oocytes were transferred to M2 medium containing 7.5 μg/ml cytochalasin B (Sigma Chemical Co.), 7.5 μg/ml Hoechst 33342 (Sigma Chemical Co.), and incubated for at least 10 min. The oocytes were then used for micromanipulation.

Domestic cats (*Felis catus*) were housed in a room with a 12L:12D light schedule at 20–26°C. Commercial cat food was given to the animals two times daily and water ad libitum. Healthy female cats at 2–6 years of age were selected for superovulation, which was conducted as reported by Donoghue et al. (1993) and Kanda et al. (1995) with a slight modification. Briefly, each cat was injected i.m. with 200 IU of PMSG (Tianjin Biological Products Manufacture, Tianjin, China) and 96 hr later with 200 IU of hCG (Institute of Zoology, Academia Sinica). The cats were ovariectomized 84 hr after the hCG injection. Cumulus oocyte complexes were flushed from the oviduct with M2 medium; cumulus cells were removed mechanically after exposure to M2 medium containing 300 IU/ml hyaluronidase for 2 min. The cumulus-free oocytes were washed in M2 medium for at least three times and then used for nuclear transfer.

### Enucleation of the Cat and Rabbit Oocytes

The cumulus-free cat oocytes were transferred into M2 medium containing 7.5 μg/ml CB. A slit on the zona pellucida was made just over the first polar body using a sharp needle. The cat oocyte was rotated to put the first polar body at the 2 o'clock position and then pressed, forcing the first polar body and the adjacent cytoplasm containing the meiotic spindle out through the slit (Fig. 1a).

To enucleate the MII rabbit oocytes, a small amount of cytoplasm from the area beneath the first polar body was aspirated using a 20–25 μm glass pipette, and

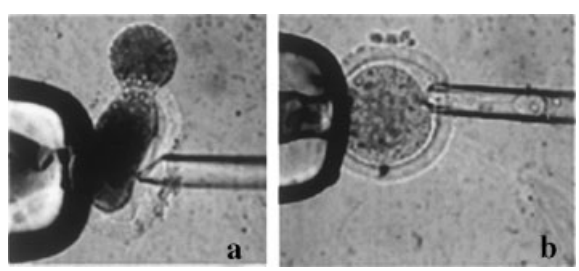


**Fig. 1.** Different approaches for enucleation of the cat or the rabbit oocyte. **a**: Enucleation of the cat oocyte by the "pressing method," (**b**) enucleation of the rabbit oocyte by the "aspirating method."

then the aspirated karyoplast was exposed to ultraviolet light to confirm the presence of nucleus (Fig. 1b). Only oocytes from which the chromosomes were removed were used for nuclear transfer.

### Culture of the Donor Cells

Cell culture and assessing procedures were described previously (Han et al., 2001). Briefly, the abdominal muscles were collected from a 3-year-old female domestic cat after anesthesia. Tissues were cut into pieces and digested with 0.25% trypsin (Gibco BRL, Grand Island, NY) for 30 min at 37°C. The digested cells and tissues were cultured in DMEM/F12 (Gibco BRL, NY) supplemented with 20% FBS (Gibco BRL) in a 5% $CO_2$ incubator at 37°C. Cells were passed at 70–80% confluence. The primary spindle-shaped cells confirmed as fibroblasts by immunochemical staining of vimentin proteins, were isolated for further culture. Cells at generation 3–8 were used as donors. The rabbit cumulus cells were collected after exposure to M2 medium containing 300 IU/ml hyaluronidase for 2 min, and washed at least three times in M2 medium before using as donor cells.

### Nuclear Transfer and Activation

A single cell was placed in the perivitelline space in close contact with the plasma membrane of an enucleated oocyte. The couplets were transferred to a fusion chamber containing 100 μl of fusion medium (0.25 M sorbitol, 0.5 mM $Mg(CH_3COO)_2$, 0.1 mM $Ca(CH_3COO)_2$, 0.5 mM HEPES, and 100 mg/100 ml BSA in deionized water) (Mitalipov et al., 1999). Fusion was induced by double DC pulses of 1.4 kv/cm for 80 μsec with an ECM2001 Electrocell Manipulator (BTX Inc., San Diego). Couplets were then washed in M199 (Gibco BRL) supplemented with 10% FBS (Gibco BRL) three times, and incubated in the same medium for 30 min at 38°C in a humidified air containing 5% $CO_2$. Couplets were checked for fusion with an inverted microscope, and the fused cells were activated by double DC pulses of 1.4 kv/cm for 40 μsec in the fusion medium. The activated embryos were washed in M199 + 10% FBS for at least three times before transferring them into the incubator for culturing.

### Parthenogenetic Activation of Rabbit Oocytes

Cumulus-free rabbit oocytes were transferred to the fusion medium and incubated in room temperature for 1 min, and then transferred to a fusion chamber containing 100 μl of fusion medium. Activation was achieved by applying double DC pulses of 1.4 kv/cm for 40 μsec with an ECM2001 Electrocell Manipulator (BTX, Inc., San Diego). The activated oocytes were washed in M199 + 10% FBS at least three times, and then transferred into the pre-equilibrated culture medium containing 10 μg/ml CB for suppression of polar body extrusion. The oocytes were cultured in this medium for 4 hr, and then transferred to medium without CB for further culturing.

### Embryo Culture and Assessment of Development

All cat–cat and rabbit–rabbit cloned embryos, parthenogenetic rabbit embryos, and part of the cat–rabbit cloned embryos were cultured in M199 + 10% FBS at 38°C in a humidified air containing 5% $CO_2$. Some cat–rabbit cloned embryos were cultured in synthetic oviduct fluid (SOF) medium supplemented with 1% minimal essential medium (MEM) nonessential amino acids and 2% essential amino acids (both Gibco BRL), and 10% FBS at the same culture conditions as cat–cat cloned embryos. The developmental stages of embryos were checked twice a day. To calculate the timing of embryogenesis, the hours and number of embryos at different cell stages were recorded twice a day after activation.

### Estimating Karotype of the Cat–Rabbit Cloned Embryos

Cat–rabbit cloned embryos were assessed for karyotype at the blastocyst stage using an air-drying technique. Briefly, embryos at blastocyst stage were preincubated in 5 μg/ml colchicine (Sigma Co.) for 5–6 hr to maximize the number of cells in metaphase. Embryos were then exposed to a hypotonic solution consisting of 1% sodium citrate in deionised water for 30–50 min. Hypotonic treated embryos were loaded on the precooled glass slide, and fixed with a mixture of glacial acetic acid and methanol (1:3). The preparations were stained with Giemsa after being dried in air overnight. Chromosomes were counted using a light microscope.

### Synchronization of Recipients and Embryo Transfer

Healthy female domestic cats at 2–6 years of age were selected for synchronization. Each cat was injected (i.m.) with 0.3 mg FSH on the 1st day, 0.15 mg FSH for the next 3 successive days, and 100 IU hCG on the fifth day. Embryo transfer was conducted on the seventh day after the first FSH injection. Each recipient was anesthetized with 0.3–0.4 ml/kg (body weight) of "846" before laparotomy. Cat–rabbit cloned embryos at 2–4 cell stages were transferred into the oviducts of recipients. Each recipient received an injection of 50 IU LH (Institute of Zoology, The Chinese Academy of Sciences) after embryo transfer.

### Statistical Analysis

Percentages of embryos at different stages were compared between groups using Chi square analysis; the mean (±SEM) of hours for timing of embryogenesis was analyzed by *t*-test. Significant difference was determined at $P < 0.05$.

## RESULTS

### Karyotype Analysis and Developmental Capacity of the Cat–Rabbit Cloned Embryos

The cat–rabbit cloned embryos developed to blastocyst and were structurally similar to that of the rabbit–

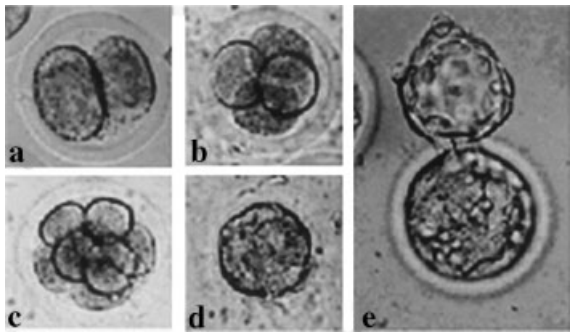


**Fig. 2.** Different developmental stages of the cat–rabbit cloned embryos. **a**: 2-cell; (**b**) 4-cell; (**c**) 8-cell; (**d**) morula; (**e**) hatching blastocyst.

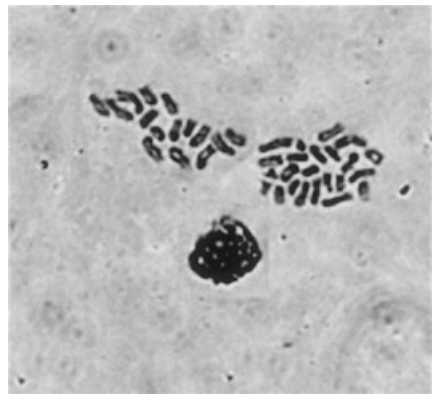

**Fig. 3.** The karyotype of cat–rabbit cloned blastocyst (2n = 38).

rabbit clone embryos (Fig. 2). The number of chromosomes in blastocyst cells was 38 (Fig. 3), which was the same as the cat karyotype (2n = 38), but different than that of the rabbit (2n = 44). The results of developmental rates for intra- or interspecies cloned cat embryos are given in Table 1. The fusion rate for cat oocytes as nucleus recipients was significantly lower than that of rabbit oocytes ($P < 0.05$). There was no significant difference in the rate of development for 2-cell, 8-cell, morula, and blastocyst between the cat–cat and the cat–rabbit cloned embryos ($P > 0.05$). For cat–rabbit and rabbit–rabbit cloned embryos, the developmental rates before morula stage were not significantly different; however, the blastocyst rate of rabbit–rabbit cloned embryos was significantly greater than that of both cat–rabbit and cat–cat cloned embryos ($P < 0.05$).

### Timing of Embryogenesis for Intra- and Interspecies Cloned Embryos and Rabbit Parthenotes

The time to attain different developmental stages was compared among cat–cat, cat–rabbit, rabbit–rabbit cloned embryos, and parthenogenetic rabbit embryos (Table 2).

The timing of embryogenesis for rabbit–rabbit cloned embryos and rabbit parthenotes was not significantly different ($P > 0.05$); however, the time to reach different developmental stages for cat–cat cloned embryos was significantly longer than that of both rabbit–rabbit cloned embryos and rabbit parthenotes ($P < 0.05$). For cat–rabbit cloned embryos, the time to reach the 2-cell, 4-cell, and 8-cell stages was similar to that of rabbit–rabbit cloned embryos ($P > 0.05$), but significantly shorter than that of cat–cat cloned embryos ($P < 0.05$). The time to reach the morula stage for cat–rabbit cloned embryos was shorter than that of cat–cat cloned embryos ($P < 0.05$) and longer than that of rabbit–rabbit cloned embryos ($P < 0.05$). Cat–rabbit cloned embryos usually remained at the morula stage for 2–3 days and then initiated blastocoele formation. The time to form blastocoele was similar to that of cat–cat cloned embryos ($P > 0.05$), but significantly later than that of rabbit–rabbit cloned embryos ($P < 0.05$).

### Developmental Capacity of the Cat–Rabbit Cloned Embryos in M199 and SOF Medium

The developmental capacity of cat–rabbit cloned embryos in M199 and SOF was evaluated. There was no significant difference among the rates of formation of 2-cell, 4-cell, 8-cell, and morula in SOF and in M199 medium ($P > 0.05$); however, the rate of formation of blastocysts was significantly greater in SOF medium than in M199 ($P < 0.05$) (Table 3).

### Results of the Embryo Transfer

A total of 82 cat–rabbit cloned embryos at 2-cell or 4-cell stage were transferred into oviducts of seven recipient cats. Due to the limited means of pregnancy detection, we only observed the behaviors of recipient cats and autopsied one recipient that died of disease after embryo transfer. Two recipients returned to estrous within 30 days. Four recipients were observed to return to estrous 36–47 days after embryo transfer, however, there were no palpable fetuses detected for these recipients. One recipient died of pneumonia

**TABLE 1. Developmental Capacity of Intra- and Interspecies Cloned Embryos**

| Embryo type | Number of NT | Fused (%) | Two-cell (%) | Four-cell (%) | Eight-cell (%) | Morula (%) | Blastocyst (%) |
|---|---|---|---|---|---|---|---|
| Cat–cat | 178 | 76 (42.7)$^{a}$ | 60 (78.9)$^{a}$ | 58 (76.3)$^{b}$ | 43 (56.6)$^{a}$ | 32 (42.1)$^{a}$ | 8 (10.5)$^{a}$ |
| Cat–rabbit | 301 | 189 (62.8)$^{b}$ | 127 (67.2)$^{a}$ | 110 (58.2)$^{a}$ | 84 (44.4)$^{a}$ | 68 (36.0)$^{a}$ | 13 (6.9)$^{a}$ |
| Rabbit–rabbit | 111 | 70 (63.1)$^{b}$ | 50 (71.4)$^{a}$ | 48 (68.6)$^{ab}$ | 37 (52.9)$^{a}$ | 27 (38.6)$^{a}$ | 16 (22.9)$^{b}$ |

Fused (%), fused oocytes/NT units; development rate of embryos at different stages, number of embryos/number of fused oocytes. Values with different superscripts within each column are significantly different (at least $P < 0.05$).

**TABLE 2. Hours (Mean ± SEM) to Reach Different Developmental Stages for Intra- and Interspecies Cloned Embryos and Rabbit Parthenotes**

| Embryo type | Two-cell | Four-cell | Eight-cell | Morula | Blastocyst |
|---|---|---|---|---|---|
| Cat–cat | $27.0 \pm 2.5^{a}$ (52) | $33.9 \pm 11.1^{a}$ (50) | $68.6 \pm 7.4^{a}$ (37) | $100.2 \pm 34.5^{a}$ (25) | $144.3 \pm 21.3^{a}$ (8) |
| Cat–rabbit | $19.4 \pm 1.9^{b}$ (60) | $24.5 \pm 3.9^{b}$ (60) | $43.2 \pm 9.1^{b}$ (60) | $70.3 \pm 13.3^{b}$ (60) | $135.0 \pm 15.1^{a}$ (16) |
| Rabbit–rabbit | $20.8 \pm 0.8^{b}$ (50) | $25.5 \pm 3.8^{b}$ (48) | $37.5 \pm 7.1^{b}$ (37) | $52.1 \pm 8.7^{c}$ (27) | $90.9 \pm 15.9^{b}$ (16) |
| Rabbit parthenotes | $19.0 \pm 2.3^{b}$ (40) | $21.4 \pm 8.0^{b}$ (40) | $38.2 \pm 10.5^{b}$ (40) | $58.4 \pm 13.1^{c}$ (32) | $94.8 \pm 24.1^{b}$ (14) |

Values with different superscripts within each column are significantly different (at least $P < 0.05$). Values in parentheses are number of embryos calculated.

20 days after embryo transfer. Upon autopsy, the uterus was swollen and several small corpra lutea of pregnancy were present on both ovaries. The uterine lumen contained a syrup-like fluid; no distinct fetus structure was found (Table 4).

## DISCUSSION

Because cat oocytes contain many lipid granules and are opaque; it is difficult to view their meiotic spindles without altering the properties of the cytoplasm. In addition, the cat plasma membrane is fragile and easy to break during enucleation. In these experiments, we enucleated the cat oocytes by pressing the first polar body and 1/4 adjacent cytoplasm through a slit made in the zona pellucida. This manipulation avoided piercing the ooplasma membrane directly by an aspirating pipette and ensured the minimal trauma to the oocytes. The rate of successful enucleation was greatly improved by this "pressing method" in comparison to the "aspirating method," one normally used for rabbit oocyte enucleation (data not shown) (Fig. 1). The fusion rate for cat–cat NT couplets was only 42.0%, significantly lower than that of cat–rabbit and rabbit–rabbit NT couplets. This may be in part due to the larger perivitelline space of enucleated cat oocytes. Usually, more cytoplasm was removed by "pressing method" than that of "aspirating method." However, the developmental rate of 2-cell, 8-cell, or morula for cat–cat cloned embryos was not significantly different than that of both cat–rabbit and rabbit–rabbit cloned embryos. This result suggests that removal of one-fourth of oocyte cytoplasm may not affect the developmental potential for the NT cat embryos.

Interspecies nuclear transfer is an invaluable tool for studying nucleus–cytoplasm interactions; at the same time, it provides a possible alternative to clone animals whose oocytes are difficult to obtain. The oocytes used for interspecies nuclear transfer should be easy to obtain, able to dedifferentiate other species somatic nuclei, and support development of the hybrid embryos. As far as we are aware, only oocytes from bovine (Dominko et al., 1999), sheep (White et al., 1999), and rabbit (Chen et al., 1999, 2002) have been used for interspecies nuclear transfer. Rabbit is a widely used laboratory animal, and as many as 40 oocytes can be obtained from just one female rabbit after superovulation. Rabbit oocytes are also semi-transparent; their spindles are just adjacent to the first polar body 13–16 hr after hCG injection, and their spindles can even be detected with DIC microscopy (our unpublished data). Previous studies have shown that the rabbit oocyte cytoplasm can dedifferentiate panda somatic cell nuclei and support the early development of panda–rabbit cloned embryos to blastocyst (Chen et al., 1999, 2002). In the present study, we successfully cloned cat embryos using enucleated rabbit oocytes, and show that the developmental capacity for these hybrid embryos is comparable to that of intra-species cloned cat embryos. These results demonstrate that rabbit oocytes can be used as nucleus recipients of somatic cells to clone cat embryos, and these cat–rabbit cloned embryos possess equal developmental capacity compared with those of cat–cat cloned embryos.

The timing of preimplantation embryogenesis, especially blastocyst formation is species-specific and varies among species in mammals. The timing of embryogenesis in early embryo development is important to the survival of the embryo, and it is a critical parameter for predicting successful embryogenesis (McKiernan and Bavister, 1994). In these experiments, the timing of embryogenesis of rabbit–rabbit cloned embryos was not significantly different than that of rabbit parthenotes; however, cat–cat cloned embryos was significantly different than that of both rabbit–rabbit cloned embryos and rabbit parthenotes. Even though the development of embryos from the one-cell stage to blastocysts in vitro is generally slower than the time-course for development in vivo (McKiernan et al., 1994), our results

**TABLE 3. Developmental Rate of Cat–Rabbit Cloned Embryos in M199 and SOF Medium**

| Medium type | Number of NT | Fused (%) | Two-cell (%) | Four-cell (%) | Eight-cell (%) | Morula (%) | Blastocyst (%) |
|---|---|---|---|---|---|---|---|
| M199 | 301 | 189 (62.8)[a] | 127 (67.2)[a] | 110 (58.2)[a] | 84 (44.4)[a] | 68 (36.0)[a] | 13 (6.9)[a] |
| SOF | 279 | 166 (59.5)[a] | 110 (66.3)[a] | 100 (60.2)[a] | 77 (46.4)[a] | 68 (41.0)[a] | 24 (14.5)[b] |

Fused (%), fused oocytes/NT units; development rate of embryos at different stages, number of embryos/number of fused oocytes. Values with different superscripts within each column are significantly different (at least $P < 0.05$).

**TABLE 4. The Results of Transferring Cat–Rabbit Cloned Embryos Into Domestic Cat**

| Recipient | Number of cat–rabbit embryos | Days of return estrous | Results of autopsy |
|---|---|---|---|
| R8 | 14 | 27 | ND |
| R12 | 7 | 20 | Died with pregnant luteins and swollen uterus presented |
| R14 | 11 | 24 | ND |
| R19 | 14 | 47 | ND |
| R25 | 10 | 38 | ND |
| R26 | 14 | 43 | ND |
| R27 | 12 | 36 | ND |

ND, autopsy was not done and pregnancy cannot be determined.

showed that the time frame before blastocyst is a relatively settled issue in one species regardless of the SCNT embryos or parthenotes if they were cultured in the same condition in vitro. The different rates of embryogenesis between cat and rabbit species provide a useful model for studying the rates of development of early embryogenesis of interspecies cloned embryos.

The timing of embryogenesis for interspecies cloned embryos is not clear. Is it oocyte recipient-specific or donor nucleus-specific? Dominko et al. (1999) reported that the timing of the first two cleavage divisions was not different in NT embryos produced regardless of the donor nucleus species when bovine oocytes were used as recipients. They explained their results as the cause of similar times for the first embryonic cleavages in several mammalian species (Dominko et al., 1999). However, our results showed that the timing of the first three cleavages for cat–rabbit cloned embryos was significantly faster than that of cat–cat cloned embryos, whereas it was much similar to that of rabbit–rabbit cloned embryos. The results cannot be explained by a similar timing of the first cleavages for mammalian embryos. The timing of early embryogenesis for cat–rabbit cloned embryos was similar to that of the oocyte recipient, indicating that factors that control the timing of early cleavages may exist in the ooplasm, and that the donor nuclei may not play an active role in determining early cleavage times in interspecies cloned embryos. From these results, we may propose that the timing of early cleavages is recipient-specific for interspecies cloned embryos. Our results also show that the time to reach the morula stage for cat–rabbit clone embryos was shorter than that of cat–cat cloned embryos yet longer than that of the rabbit–rabbit cloned embryos. This indicates that the time to reach morula stage is neither recipient-specific, nor donor nucleus-specific, and that both the cytoplasm and nucleus have effects on the development of the interspecies cloned embryos during the morula stage. However, the time to form blastocysts for cat–rabbit cloned embryos was similar to that of cat–cat cloned embryos, but significantly different than that of rabbit–rabbit cloned embryos. These results indicate that the time to reach blastocyst stage for interspecies cloned embryos is donor nucleus-specific. This result is consistent with that of the sheep–cow, pig–cow, and monkey–cow cloned embryos (Dominko et al., 1999).

In mammalian species, the maternal–zygotic transition (MZT) occurs at earlier embryonic stages and is often associated with embryo compaction and developmental blocks (Frei et al., 1989; Telford et al., 1990). This MZT, which is species-dependent and occurs at a particular stage of development, is characterized by a large increase in detectable transcription (Telford et al., 1990; Campbell, 1999). The completion of MZT leads the nucleus to take full control of the embryo development. For somatic nuclear transfer embryos, there is a similar transition associated with the nucleus reprogramming (Campbell, 1999); however, we do not know whether the time of this transition is recipient-specific or nucleus-specific in interspecies cloned embryos. The time of MZT for the cat is not clear, and it may occur at the 5- to 8-cell stage in vivo (Swanson et al., 1994, 1996), while the MZT for the rabbit occurs at the 8- to 16-cell stage (Manes, 1973; Henrion et al., 1997). In the present experiment, most of the cat–rabbit cloned embryos compacted at the 16-cell stage. They reached the morula stage the third day following activation and more than half of them arrested at morula stage for 2–3 days and subsequently degenerated. Only a small portion of these morulae could develop to blastocysts. We contend that in this instance there is a morula-to-blastocyst block for cat–rabbit cloned embryos. Our results imply that the MZT for cat–rabbit cloned embryos might occur at 16-cell stage and that cat nuclei take full control of the hybrid embryos after the morula stage. The time of MZT for the interspecies cloned cat embryos is likely oocyte recipient-specific; however, this requires further verification by more specific studies.

When cultured in M199, the rate of blastocyst formation of cat–rabbit cloned embryos was not significantly different than that of cat–cat cloned embryos; however, the rate of blastocyst formation of rabbit–rabbit embryos was significantly greater than that of both cat–rabbit and cat–cat cloned embryos ($P < 0.05$). These results indicate that M199 medium is not an ideal medium for culturing cat embryos. In the cat, IVF-derived embryos can develop readily to morula stage under a variety of culture conditions, but the morula-to-blastocyst block in vitro remains largely resistant to these culture condition variations (Kanda et al., 1995; Swanson et al., 1996). In addition, previous studies reveal that the morula-to-blastocyst block in vitro coincides with the timing of in vivo embryo compaction (at ~32-cell stage), blastocoele formation, and the transition from the oviductal to the uterine environment (Swanson et al., 1996). Results from our experiment showed that the developmental capacity of the cat–rabbit cloned embryos was not significantly different before morula stage in M199 and SOF medium. This demonstrates that interspecies cloned cat embryos possess similar developmental characteristics to that of IVF-derived cat embryos, and that these interspecies cloned embryos also can develop to morula stage

in different culture media. SOF is a chemical defined medium, it has been widely used to culture embryos from various mammalian species (Walker et al., 1996; Krisher et al., 1999; Freistedt et al., 2001; Donnay et al., 2002). Freistedt et al. achieved about 60% blastocyst rate (No. of blastocsyts/No. of cleavages) using the modified SOF medium to culture IVF-derived cat embryos (Freistedt et al., 2001). Our results also showed that the rate of blastocyst formation in SOF was significantly improved when compared with rates obtained with M199, suggesting that the SOF medium may be beneficial to overcome the morula-to-blastocyst block for cat–rabbit cloned embryos.

The true indication of successful reprogramming of somatic cells in nuclear transfer embryos is the production of live offspring (Campbell, 1999). In order to further evaluate the developmental capacity of cat–rabbit cloned embryos, we transferred these hybrid embryos into the oviducts of synchronized cats. The results showed that two of the seven recipients returned to estrous. Four recipients returned to estrous 36–47 days of embryo transfer, and, we could not determine whether or not these animals were pregnant. In another experiment, we co-transferred cat–rabbit cloned embryos with panda–rabbit cloned embryos into the oviducts of synchronized cats. One recipient died after 21 days of embryo transfer, and autopsy found that the recipient carried six fetuses, two of them were from panda–rabbit cloned embryos, which was confirmed by microsatellite DNA analysis (Chen et al., 2002). We speculate that the other four fetuses were from the cat–rabbit cloned embryos. Although, no viable fetus was obtained in this experiment, the results suggest that interspecies cloned cat embryos transferred into recipient cats might arrest at a very early stage after implantation. Whether or not these interspecies cloned embryos are able to develop to term in cat uteri is a question that requires further study.

In conclusion, cat fibroblast nuclei can be dedifferentiated in enucleated MII rabbit oocytes, and these interspecies cloned embryos possess equal developmental capacity as intraspecies cloned cat embryos during preimplantation development. The timing of the first three cleavages for cat–rabbit cloned embryos was recipient-specific and the time to reach the blastocyst stage was donor nucleus-specific. SOF medium may be beneficial to overcome the morula-to-blastocyst block for cat–rabbit cloned embryos.

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