

Successful production of offspring after superovulation and *in vitro* culture of embryos from domestic ferrets (*Mustela putorius furo*)

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In an effort to expand the use of ferrets as models for genetic disease, several experimental parameters that are required for successful genetic manipulation in this species were investigated. Optimum superovulation (19.3 ± 0.6 oocytes and embryos per female) was achieved after injections of 100 iu equine chorionic gonadotrophin (eCG) and 150 iu human chorionic gonadotrophin (hCG). The ovulation rate achieved by the treatment was more than double that induced by mating. Mating with a male immediately after hCG treatment did not significantly alter the number of oocytes ovulated or the number of embryos present, indicating that mating is not required for superovulation in ferrets. Of embryos harvested at the one-cell stage, 64.5% and 47.1% developed into blasto-

cysts when cultured *in vitro* in CZB or TCM-199 plus 10% fetal bovine serum (FBS) media, respectively. In contrast, only 17.1% of embryos cultured *in vitro* in NCSU-23 developed to the blastocyst stage. Both freshly retrieved and *in vitro* cultured embryos from cinnamon-coloured parents produced live young when transferred at the eight-cell stage into albino, pseudopregnant recipients. The percentage of kits delivered relative to embryos transferred was 61% for freshly retrieved embryos and 32% for embryos cultured *in vitro*. These results demonstrate successful embryo transfer in ferrets and provide a basis for further study of genetic modelling approaches in this species after embryo manipulation.

Introduction

The domestic ferret (*Mustela putorius furo*) has become an excellent model for biomedical studies in areas such as infectious diseases, neurological disorders and reproductive biology. Other studies have revealed that ferrets have marked similarities to humans in airway structure and cell biology (Plopper *et al.*, 1980; Leigh *et al.*, 1986; Curtis *et al.*, 1987; Sehgal *et al.*, 1996; Duan *et al.*, 1998). Furthermore, ferrets are a useful model for the study of lung infections caused by viruses such as influenza (Husseini *et al.*, 1983; Durchfeld *et al.*, 1991; Jakeman *et al.*, 1991; Leigh *et al.*, 1995; Fenton *et al.*, 1999). These studies indicate that ferrets may serve as an excellent animal model for the study of airway diseases in humans. Ongoing research in our laboratory has the eventual goal of generating a ferret model for the study of cystic fibrosis, an inherited disorder affecting about 1 in every 3000 newborns among the Caucasian population. The present study describes embryo manipulation in ferrets, as a prelude to initiating genetic modelling approaches in this species.

Natural ovulation in ferrets is induced by mating and occurs 24–36 h thereafter (Robinson, 1918; Hammond and Marshall, 1934). In the absence of any hormonal treatment, a virgin female in oestrus generally ovulates six to ten oocytes after mating with a male (Chang, 1965; Mead *et al.*, 1988). To date, only a few studies have investigated the effects of hormonal treatment on ovulation in ferrets, and none of these has achieved very high ovulation rates (Chang, 1965; Mead *et al.*, 1988; Amstislavskii *et al.*, 1997). Therefore, a more detailed investigation using different combinations of gonadotrophins is required to maximize the number of oocytes and embryos that can be retrieved from individual female ferrets.

The first successful embryo transfer was performed in rabbits (Heape, 1890), and has since been applied to a variety of domestic and wild animals (Day, 1979; Kremer *et al.*, 1979; Dickman, 1982; Moor, 1982; Newcomb, 1982; Nicholas, 1993), including ferrets (Chang, 1968; Kidder *et al.*, 1999b). However, there are no reports of embryo transfer in ferrets using embryos developed *in vitro*. The ability to culture ferret embryos *in vitro* and to produce live offspring by embryo transfer is critical for genetic manipulation of this species and the subsequent development of new animal models for human diseases.

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Materials and Methods

Animals and housing conditions

Ferrets (6–24 months of age) with cinnamon- or albino-coloured coats were purchased from Marshall Farms (North Rose, NY). Female ferrets were in oestrus when delivered and non-vasectomized males were proven breeders. Four vasectomized male ferrets were also purchased from Marshall Farms and were used to induce pseudopregnancy in females. After vasectomy, each male was mated repeatedly before a sperm count was carried out to confirm the sterile state of the animal. Sterility, as inferred by aspermia and the lack of pregnancy after three sequential matings with an oestrous jill, was confirmed within 3 months of vasectomy in all four males. All ferrets were housed in separate cages under controlled temperature (20–22°C) and long day light cycles (16 h light:8 h dark).

Superovulation of ferrets

For superovulation, virgin female ferrets were injected i.p. with 50–150 iu eCG (Sigma G-4877; also referred to as PMSG), followed by an injection of 50–200 iu hCG (Sigma, C-1063) 72 h later. Virgin females were used because the litter size, and presumably the number of oocytes ovulated, decreases with subsequent pregnancies (McLain *et al.*, 1985). Each female was mated with one male or with two males in succession immediately after hCG treatment when embryos rather than haploid oocytes were needed. In experiments performed to determine the effect of mating on superovulation in ferrets, females were mated with vasectomized males. Female ferrets were subsequently killed by administration of an overdose of barbiturates (pentobarbital sodium administered i.p. at 50–100 mg kg⁻¹ body weight) so that oocytes or embryos could be harvested. The ovaries, oviducts and sections of the uterine horns were removed, and oocytes or embryos were flushed from the oviducts or uterine horns with Dulbecco's PBS (DPBS; 0.1 g CaCl₂ (anhyd) l⁻¹, 0.2 g KCl l⁻¹, 0.2 g KH₂PO₄ (anhyd) l⁻¹, 0.1 g MgCl₂·6H₂O l⁻¹, 8.0 g NaCl l⁻¹, 2.16 g Na₂HPO₄·7H₂O l⁻¹) + 2% (v/v) newborn calf serum (NCS; Sigma, N-4762).

Culture of ferret embryos

One- or two-cell embryos harvested from two or three superovulated, mated jills were divided evenly into three groups. The three groups of embryos were cultured simultaneously in one of the three types of medium: (i) TCM-199 + 10% FBS; (ii) NCSU-23 (Petters and Reed, 1991); and (iii) CZB (81.62 mmol NaCl l⁻¹; 4.83 mmol KCl l⁻¹; 1.18 mmol KH₂PO₄ l⁻¹; 1.18 mmol MgSO₄·7H₂O l⁻¹; 25.12 mmol NaHCO₃ l⁻¹; 1.70 mmol CaCl₂·2H₂O l⁻¹; 31.30 mmol sodium lactate l⁻¹; 0.27 mmol sodium pyruvate l⁻¹; 0.11 mmol EDTA (disodium salt) l⁻¹; 1.0 mmol glutamine l⁻¹; 5.0 mg BSA ml⁻¹, 100 iu penicillin G sodium salt ml⁻¹; 0.7 mg streptomycin ml⁻¹) (Chatot *et al.*, 1989). Repeated experiments were performed until a cumulative set of oocytes was derived from the total of nine individual

ferrets. Groups of five to ten embryos were placed in droplets of medium, covered with embryo culture grade mineral oil, and incubated at 38.5°C under 5% CO₂ and 95% air. Fresh medium was delivered to the embryos every 48 h and their development was examined under an inverted phase-contrast microscope. Embryos arrested at different stages of cleavage were recorded and quantitated for statistical analyses. Some of the *in vitro* cultured embryos at different stages of development were stained with 10 µg bisbenzimidazole ml⁻¹ (Hoechst 33342, Sigma B-2261) for 2–5 min before examination under UV.

Embryo transfer

Experienced jills only were used as recipients in embryo transfer studies to exclude the possibility of infertility among surrogate mothers. A pseudopregnant state was achieved in recipient albino females mated with vasectomized albino males 12–24 h after cinnamon-coloured jills (embryo donors) were mated. The time lag between the mating of donor and recipient jills ensured synchronization between the donors and recipients, as *in vitro* manipulation of embryos generally causes a developmental delay. Embryos developed *in vivo* were harvested from cinnamon-coloured jills at day 4 after mating and were at the 8–16-cell stage. These embryos were collected in warmed (38.5°C) mPBS (DPBS supplemented with 0.1% (w/v) D-glucose, 36 mg pyruvate l⁻¹ and 0.4% BSA) and were transferred immediately into the uterine horn of a surrogate albino female. Embryos collected from cinnamon-coloured females at the one-cell stage, 36–40 h after mating, were cultured in TCM-199 + 10% FBS to the 8–16-cell stage before they were transferred to a recipient female. On the day of transfer, the recipient albino female was anaesthetized by an injection of 25 mg ketamine kg⁻¹ and 4 mg xylazine kg⁻¹. A 3–4 cm incision was made along the midline of the abdomen to expose the uterus and the uterine horns. Between 6 and 19 embryos were delivered into the left uterine horn using a fine glass pipette with an inner diameter slightly larger than the embryos. After the surgical wound was sutured, the ferret was allowed to wake up in its own cage under close monitoring. Confirmation of pregnancy was performed by palpation 2–4 weeks after transplantation. The number of kits produced was recorded on the morning after parturition and again at weaning, 7 weeks later. The coat colour of the kits was used to confirm their genetic lineage.

Statistical analyses

Mean variances among different sample groups were determined by ANOVA. Comparison between two sample groups was made by the Student's *t* test. A significant difference was found when the *P* value was < 0.05.

Results

Domestic ferrets living in the wild normally give birth to

Table 1. The effectiveness of different doses of eCG and hCG in inducing ovulation in domestic ferrets during oestrus

Treatment (iu eCG/iu hCG)	Short-term study (6 weeks)			Long-term study (1 year)		
	Number of ferrets ^d	Total number of oocytes	Number of oocytes per jill (mean \pm SEM) ^e	Number of ferrets ^d	Total number of oocytes	Number of oocytes per jill (mean \pm SEM) ^e
0/0	3	18	6.0 \pm 1.2 ^a	7	62	8.9 \pm 2.5 ^a
50/50	3	19	6.3 \pm 0.7 ^a	3	19	6.3 \pm 0.7 ^a
100/100	3	60	20.0 \pm 1.7 ^c	3	60	20 \pm 1.7 ^c
100/150	3	56	18.7 \pm 2.3 ^c	20	397	19.9 \pm 1.1 ^c
150/150	3	45	15.0 \pm 3.1 ^b	9	135	15.0 \pm 1.0 ^b
150/200	3	46	15.3 \pm 1.5 ^c	6	100	16.7 \pm 0.9 ^c

^{abc}Within groups, values with the same letter are not significantly different in the superovulation rate ($P > 0.05$).

^dAll jills that were not treated with gonadotrophins were in oestrus and mated to stud males. All other jills treated with various doses of gonadotrophins were not mated and were in oestrus at the time of ovulation.

^eMean number of oocytes or embryos recovered from the Fallopian tubes and uterus 36–40 h after administration of hCG or mating.

between six and ten kits per litter. The present study investigated whether treatment with eCG to induce follicular maturation, followed by injection of hCG to induce ovulation, could increase the number of oocytes and embryos harvested from a single unmated oestrous female. In an initial set of experiments, cinnamon-coloured virgin female ferrets were allocated randomly into six treatment groups so that each group contained three animals with equivalent breeding background and reproductive status. Oocytes were harvested at 36 h after gonadotrophin treatment or mating (for untreated animals only) to ensure that most of the matured oocytes were ovulated. Results from these studies indicated that there was a significant increase in the ovulation rate when the concentration of gonadotrophins was increased from 50 iu eCG and 50 iu hCG (6.3 \pm 0.7) to 100 iu eCG and 100 iu hCG (20.0 \pm 1.7). Treatment with higher doses of eCG and hCG did not result in a further increase in the ovulation rate (Table 1). On the basis of the results from the initial set of experiments, a combination of 100 iu eCG and 150 iu hCG was chosen as the treatment for subsequent experiments. Data obtained from the initial phase of the experiments (in which three animals were included in each treatment group) were compared with a more comprehensive set of data from a long-term study over 1 year (Table 1). In the long-term study, oocytes were harvested at 36–40 h after gonadotrophin treatment or mating (for untreated animals only). In the long-term study, mating of untreated virgin ferrets in oestrus with stud males resulted in an ovulation rate of 8.9 \pm 2.5 oocytes per female. Treatment with gonadotrophins at the lowest concentrations, a combination of 50 iu eCG and 50 iu hCG, resulted in an ovulation rate of 6.3 \pm 0.7 oocytes from each female in the absence of mating, which did not differ significantly from untreated, mated females. Treatment with high doses of gonadotrophins, 150–200 iu of both eCG and hCG, resulted in more variable ovulation rates ranging from 15.0 \pm 1.0 to

18.0 \pm 1.2 oocytes from each unmated female. At intermediate doses of 100 iu eCG and 100 iu hCG, and 100 iu eCG and 150 iu hCG, ovulation rates were 20.0 \pm 1.7 and 19.9 \pm 1.1 oocytes, respectively. These results are in agreement with those obtained from the smaller number of animals in the initial phase of the study.

As mating induces ovulation in ferrets, the present study next investigated whether mating increases the number of oocytes obtained after hormonal treatment. According to previous studies (Table 1), it is apparent that mating is not the only factor responsible for ovulation. However, whether mating would increase superovulation rates further remains to be addressed. In an attempt to answer this question, two groups of oestrous jills were treated with eCG and hCG at two doses. One group of ferrets was mated with vasectomized males immediately after the hCG treatment, whereas the second group received hormone treatment only. In preliminary short-term studies, equal numbers of animals were evaluated in each of the four treatment groups (Table 2) until a total of three animals was obtained in each group. These studies were performed over a period of 4 weeks and the number of oocytes harvested from mated females at 36–40 h after mating was compared with the numbers of oocytes recovered from non-mated, hormone-treated females. The results obtained from this initial short-term study with matched numbers of jills, and data from our experiments performed over 1 year are shown (Table 2). A combination of either 100 iu eCG and 150 iu hCG or 150 iu eCG and 200 iu hCG did not result in significant differences between the mated and non-mated groups. These experiments demonstrated that mating does not significantly augment hormonal superovulation in ferrets. However, it was only possible to induce superovulation in oestrous ferrets, as nursing ferrets at 8 weeks after delivery produced only up to three oocytes despite treatment with eCG and hCG (data not shown).

The effects of different culture media on the *in vitro*

Table 2. Effect of mating on eCG- and hCG-induced ovulation in domestic ferrets during oestrus

Treatment (iu eCG/iu hCG)	Mating	Short-term study (4 weeks)			Long-term study (1 year)		
		Number of ferrets	Total number of oocytes or embryos	Number of oocytes or embryos per jill (mean \pm SEM)*	Number of ferrets	Total number of oocytes or embryos	Number of oocytes or embryos per jill (mean \pm SEM)*
100/150	No	3	56	18.7 \pm 2.3	20	397	19.9 \pm 1.1
	Yes	3	62	20.7 \pm 0.9	24	453	18.9 \pm 0.6
150/200	No	3	50	16.7 \pm 0.9	6	100	16.7 \pm 0.9
	Yes	3	54	18.0 \pm 2.5	4	80	20.0 \pm 2.7

*Mean number of oocytes or embryos recovered from the Fallopian tubes and uterus 36–40 h after the injection of hCG or mating.

development of ferret embryos recovered from super-ovulated, virgin females in oestrus that had been mated to stud males were examined. Most of the embryos used for studies *in vitro* were harvested at the one-cell stage. However, occasionally, embryos at the two-cell stage were also used. The sequential developmental stages of ferret embryos with reference to the time after hCG treatment are shown (Fig. 1). Of the embryos cultured in CZB medium, 64.5% developed to the morula stage 6–7 days after hCG treatment (Fig. 1; Table 3), and the same percentage of embryos developed to the blastocyst stage under these culture conditions. When TCM-199 medium supplemented with 10% FBS was used, 70.6% and 47.1% of cultured embryos developed into morulae and blastocysts, respectively. Although embryonic development in CZB and TCM-199 was largely similar, blastocyst formation occurred about 12 h earlier in TCM-199. Significantly fewer embryos developed to morula (28.6%) and blastocyst (17.1%) stages when cultured in the NCSU-23 medium. Several embryos were stained with Hoechst 33342 to determine whether embryos cultured *in vitro* had developed properly. Hoechst 33342 staining of embryos at the eight-cell, \geq 16-cell and blastocyst stages revealed numbers of nuclei corresponding to the developmental stages observed using light microscopy (Fig. 1). When the numbers of nuclei were counted in four *in vitro* cultured blastocysts, an average of 87.5 ± 19.2 nuclei per embryo was found (data not shown).

Embryo transfer was performed using ferrets with cinnamon-coloured coats as embryo donors and ferrets with albino-coloured coats mated with vasectomized albino males as recipients. In three of a total of six embryo transfer experiments, freshly retrieved 8–16-cell embryos were transferred immediately to the uterine horn of the recipient. All embryos harvested from donor jills were at the 8–16-cell stage and were of normal morphology. No deteriorated oocytes or dead embryos were recovered. All three female recipients became pregnant, and 61% (33 of 54) of the embryos transferred developed to term (Table 4). The average litter size was 11 ± 5 . Fifty per cent (27 of 54) of the transferred embryos produced live kits at birth, of which 26% (14 of 54) survived beyond week 8 (at which

time the experiment was terminated). Three additional embryo transfer experiments were performed using embryos cultured *in vitro*. In these experiments, embryos were harvested at the one- or two-cell stage and were cultured in TCM-199 + 10% FBS media for 3 days, by which time most of the embryos had developed to the eight-cell stage. Embryos of normal morphology were then transferred to albino female recipients. All three female recipients became pregnant and 32% (12 of 38) of the transferred embryos developed to term (Table 4). The average litter size was 4 ± 2 . Only 21% (8 of 38) of the transferred embryos resulted in live kits at birth, of which 16% (6 of 38) survived beyond week 8 of weaning. In all cases, the kits from both experimental groups (*in vivo* and *in vitro* matured embryos) were born with pink-coloured skin, and all kits surviving to or beyond 18 days after birth showed an obvious cinnamon-coloured coat (Fig. 2).

Discussion

Superovulation, embryo culture and embryo transfer were investigated in ferrets as a prerequisite to embryo manipulation and the development of ferret models for the study of genetic diseases such as cystic fibrosis. The results of this study demonstrate that treatment of domestic ferrets in oestrus with an optimum combination of eCG (100 iu) and hCG (150 iu) administered at an interval of 72 h induced an ovulation rate that was more than double that achieved by mating. The average number of oocytes (19.9) that could be harvested from the oviducts and uterine horns was significantly greater than the numbers reported in ferrets treated with 90 iu hCG alone (Chang, 1965; Mead *et al.*, 1988). The mean number of oocytes produced varied significantly (as determined by ANOVA) between the experimental group receiving 100 iu eCG and 150 iu hCG and the groups receiving higher doses of eCG and hCG. However, there was no significant decrease in the rate of ovulation at a dose of 200 iu hCG. This finding is in contrast to a report by Mead *et al.* (1988) in which higher doses (150 or 300 iu) of hCG resulted in a decrease in the average number of corpora lutea formed. Results from the present

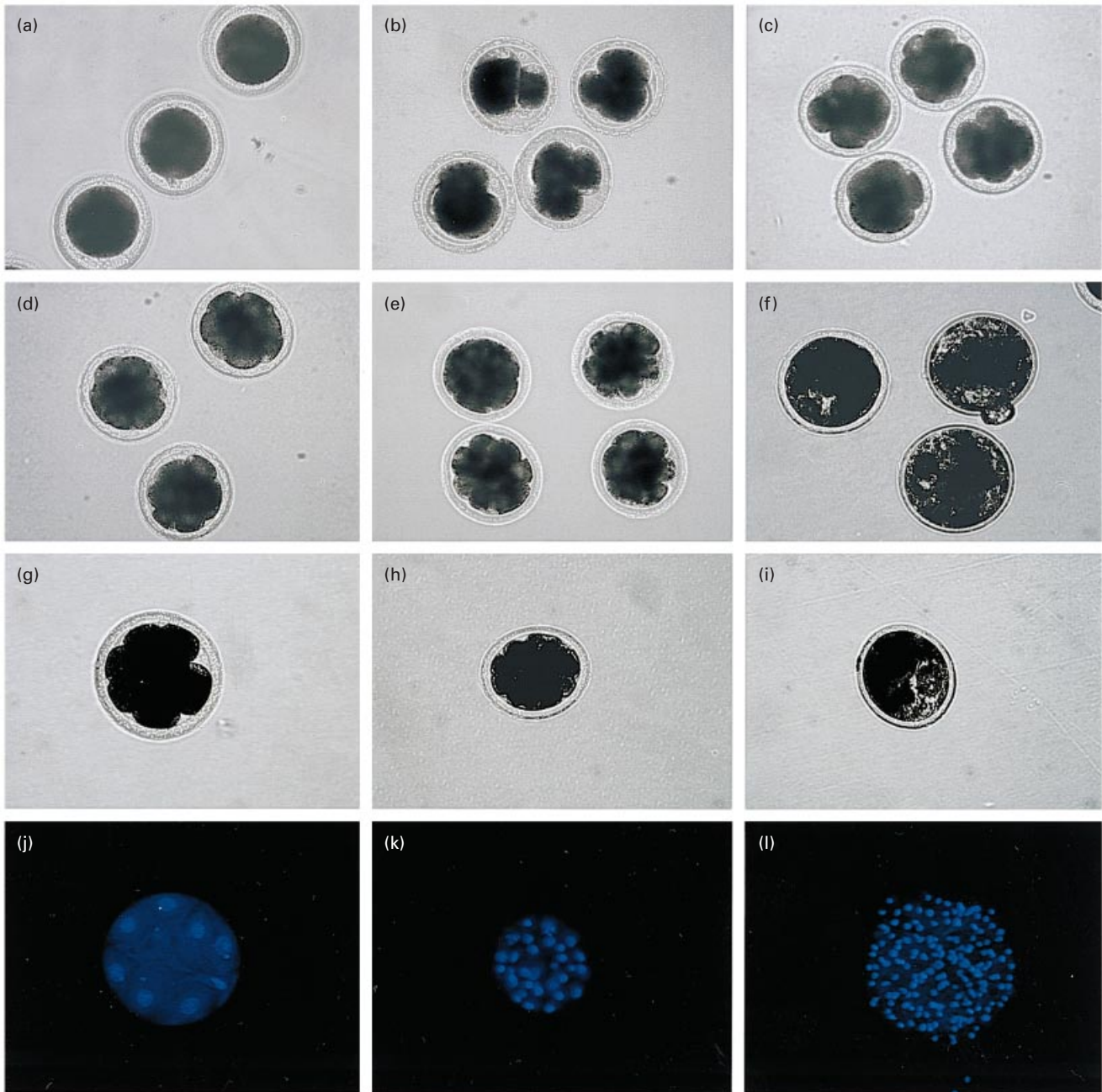


Fig. 1. Ferret oocytes and embryos at different stages of development. Fertilized oocytes at the pronuclear stage were harvested 40 h after hCG injection from mated cinnamon-coloured females that had been treated with eCG and hCG to induce superovulation. Embryos were cultured *in vitro* in TCM199/FBS medium. One-cell embryos at harvest (a). *In vitro* cultured embryos at the (b) two- to four-cell stage (day 3 after mating), (c) five- to eight-cell stage (day 4 after mating), (d) 9–16-cell stage (day 5 after mating), (e) 17–32-cell (morula) stage (day 6 after mating) and (f) blastocyst stage (> day 7 after mating). (g–l) Hoechst 33342 stained nuclei of individual blastomeres from ferret embryos cultured *in vitro*. Embryos at the eight-cell stage (g,j), 30-cell stage (h,k) and blastocyst stage (126 cells) (i,l). Nomarski images shown in (g), (h) and (i) correspond to Hoechst-stained embryos in (j), (k) and (l), respectively. Note that a coverslip was placed on the Hoechst-stained embryos to flatten the tissues so that all blastomere nuclei could be visualized in one plane of focus; hence, position of nuclei in Nomarski images without coverslips does not correlate with fluorescent images.

study also demonstrated that mating does not significantly augment hormonal superovulation in ferrets. A treatment regimen of 100 iu eCG and 150 iu hCG was chosen in all of

our downstream studies primarily due to the fact that high doses of gonadotrophins can adversely affect the quality of superovulated oocytes or embryos, and the proper

Table 3. Effect of different culture media on development of ferret embryos *in vitro*

Medium	Number of embryos ^a	Morulae (6 days after hCG)		Blastocysts (> 7 days after hCG)	
		Number	%	Number	%
CZB	31	20	64.5	20	64.5
NCSU-23	35	10	28.6	6	17.1
TCM199 + 10% FBS	34	24	70.6	16	47.1

^aOnly one- or two-cell embryos of normal morphology were used for analysis. Embryos were derived from nine independent jills.

Table 4. Embryo transfer in domestic ferrets

	Experiment number					
	1	2	3	4	5	6
Embryos cultured <i>in vitro</i>	No	No	No	Yes	Yes	Yes
Number of embryos transferred	19	17	18	6	16	16
Number of live kits	13	3	11	6	0	2
Number of dead kits	3	3	0	0	2	2
Number of live kits at week 8 post partum	5	3	6	6	0	0

All embryos were harvested from cinnamon-coloured female ferrets mated with males of the same colour. All recipients of embryo transfer were albino. Embryos developed *in vivo* were harvested at the eight-cell stage and were transferred immediately. Embryos developed *in vitro* were harvested at the one- to two-cell stage and were cultured until they reached the eight-cell stage before being transferred to the recipients. All six embryo transfer experiments resulted in pregnancy of recipient albino females.

development of embryos (Whyman and Moore, 1980; Moor *et al.*, 1985; Ertzeid and Storeng, 1992). This treatment regimen resulted in the recovery of a smaller percentage of abnormal oocytes and embryos. However, the overall rate of blastocyst formation after culture *in vitro* and the birth rate after embryo transfer were not optimal. Further refinement of the superovulation strategies, such as the use of single or multiple injections of FSH, treatment with GnRH antagonists, and the use of recombinant gonadotrophins, may ensure more balanced FSH:LH ratios, and thus improve the quality of oocytes and embryos produced. In general, determination of a satisfactory superovulation method in ferrets as reported here, which simplifies oocyte collection procedures, should benefit the future application of *in vitro* fertilization or somatic cell cloning in ferrets.

The present study is the first systematic analysis of the *in vitro* culture conditions required for the development of ferret embryos. CZB and TCM199/FBS, two of the most commonly used media for embryo culture in other species, provided optimum culture conditions, in which a high percentage of embryos developed to the morula or blastocyst stages in an atmosphere of 5% CO₂ and 95% air at 38.5°C. However, embryos cultured in TCM199/FBS developed into blastocysts 12 h more rapidly than did embryos cultured in CZB. It is possible that the CZB medium contains factors that cause developmental delay in

ferret embryos cultured *in vitro*. An incubation temperature of 38.5°C was chosen in accordance with the normal body temperature of ferrets (37.8–39.4°C). The NCSU-23 medium, which is ideal for the development of pig embryos *in vitro* (Petters and Reed, 1991; Petters and Wells, 1993), produced significantly poorer results, despite the fact that ferret and pig embryos are similar in size, morphology and pigmentation. Chang (1968) reported that only morulae were found in the uterus of ferrets at day 6 after mating, and that blastocysts did not appear until day 7 after mating. This conclusion was supported by Marston and Kelly (1969), although Hamilton (1934) reported blastocyst formation at day 6 after mating. In the present study, embryos that had developed to the morula stage after culture *in vivo* could be identified at day 6 after mating, and blastocysts first appeared at day 7 after mating, thus supporting the findings of Chang (1968) and Marston and Kelly (1969). In addition, the timing of embryo development to the eight-cell stage (day 4 after mating) was identical for embryos developed *in vivo* and *in vitro*, indicating that the *in vitro* culture conditions used in the present study provided an environment similar to that found *in vivo*.

Hoechst 33342 staining of embryos cultured *in vitro* to different stages of development in TCM199/FBS revealed a positive correlation between the number of nuclei and the developmental stage observed under light microscopy, and



Fig. 2. Cinnamon-coloured ferret pups born to an albino surrogate female. Embryos from cinnamon-coloured ferrets were collected at the eight-cell stage at 100 h after hCG treatment and were transferred immediately into a pseudopregnant albino ferret that had mated with a vasectomized male. The photos of the cinnamon-coloured kits with their albino surrogate mother were taken at (a) day 4 and (b) day 18 after birth. The pigmented coats and nails of these pups (which darkened further as the kits matured) show prominent differences from those of their surrogate mother.

also indicated proper development. The average number of nuclei in embryos at the blastocyst stage, as determined in this study at the beginning of day 7 after hCG treatment, were in agreement with the findings of Kidder *et al.* (1999a), further demonstrating the ability of TCM199/FBS to support the development of ferret embryos *in vitro*. However, whether media tested in the present study will support the development of ferret embryos beyond the blastocyst stage remains to be elucidated. The percentage of live offspring produced after *in vitro* culture and embryo transfer was low, indicating that not all well-developed blastocysts under *in vitro* culture conditions have the potential to develop to term once transferred to the uterus. Further modification of *in vitro* culture conditions should provide better environmental conditions for development of ferret embryos.

The six embryo transfer experiments all resulted in pregnancy of the recipients and only one female failed to deliver live young. For the recipient that did not produce live kits, two dead kits of apparently normal development were found in the collection pan of the cage. It is possible that these kits had been pushed out of the nesting pan by the mother. The high rate of pregnancy in ferrets after embryo transfer is in agreement with the rate (90%) reported by Kidder *et al.* (1999b). When eight-cell embryos were transferred immediately after harvest, the percentage of embryos that developed to term (61%) was twice as high as that found after transfer of embryos cultured *in vitro* (32%). One of the recipients of the embryos cultured *in vitro* gave birth to only one live and one dead kit, even though more fetuses were detected by palpation. Therefore, a Caesarean section was performed within 24 h after the start of the labour. Two more kits were delivered manually and, one was still alive. When the uterine horns were exposed, eight placental discs were found, indicating that resorption

of at least four implanted embryos had occurred. In the only ferret that produced live young from all of the transferred embryos cultured *in vitro*, all embryos were delivered to the left uterine horn, whereas the other two ferrets received embryos in both uterine horns. Hence, it is possible that the increased surgical manipulation of the uterus results in increased trauma and aborted fetuses. Furthermore, under *in vitro* culture conditions considered optimal in this study, only 64% of the one- or two-cell embryos developed to the blastocyst stage. Although all eight-cell embryos cultured *in vitro* looked morphologically normal at the time of transfer, at least one-third lacked the ability to develop to term. It is also possible that the number of embryos transferred affects the overall efficiency of embryo transfer in ferrets. Ferrets in the wild generally ovulate six to ten oocytes and produce similar numbers of live kits. When six *in vitro* cultured embryos were transferred to females, all six embryos developed to term and survived beyond week 8 after birth. Therefore, transferring too many embryos at one time may adversely affect the chance of survival of individual embryos. However, when fresh embryos were transplanted, large numbers (13 and 11) of live kits were born, indicating that ferrets are capable of carrying more than ten embryos. Given the limited number of experiments to date, it is difficult to draw firm conclusions about the optimum number of embryos in a given transfer experiment.

In the present study, the overall survival rate of ferret kits after birth was low. A closer observation of the postnatal development of ferret kits revealed that a subgroup grew significantly more slowly than their siblings, mainly as a result of their inability to feed. These kits normally died within 2 weeks of birth. The relatively large litter size apparently contributed to the high mortality rate among newborns, as all kits in the two smallest litters (three and six)

survived beyond week 8. Other factors that may affect the success of embryo survival include seasonal variations in reproductive physiology, and the age and number of previous pregnancies.

In summary, experiments in the present study have begun to optimize parameters required for genetic manipulation of ferret embryos. This increased understanding of embryo development and transfer may allow for successful development of new animal models for genetic diseases using techniques such as somatic cell nuclear transfer and transgenic technology.

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