Production of horse foals via direct injection of roscovitine-treated donor cells and activation by injection of sperm extract

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Abstract

We evaluated the effects of different donor cell treatments and activation methods on production of blastocysts after equine nuclear transfer. Nuclear transfer was performed by direct injection of donor cells, using a piezo drill, and standard activation was by injection of sperm factor followed by culture with 6-dimethylaminopurine. There was no difference in blastocyst development between embryos produced with roscovitine-treated or confluent donor cells (3.6% for either treatment). Addition of injection of roscovitine or culture with cycloheximide at the time of activation did not affect blastocyst development. Overall, transfer of eight blastocysts produced using roscovitine-treated donor cells and our standard activation protocol yielded three pregnancies, of which two (25% of transferred embryos) resulted in delivery of viable foals. Flow cytometric evaluation showed that roscovitine treatment significantly increased the proportion of cells classified as small, in comparison to growth to confluence or serum deprivation, but did not significantly affect the proportion of cells in G0/G1 (2N DNA content). Transfer of one blastocyst produced using roscovitine-treated donor cells, with addition of roscovitine injection at activation, yielded one pregnancy which was lost before 114 days' gestation. Transfer to recipients of two blastocysts produced using confluent donor cells with addition of cycloheximide at activation gave no resulting pregnancies. We conclude that roscovitine treatment of donor cells yields equivalent blastocyst production after nuclear transfer to that for confluent donor cells, and that direct injection of roscovitine-treated donor cells, followed by activation using sperm extract, is compatible with efficient production of viable cloned foals.

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Introduction

Somatic cell nuclear transfer (NT) has been used to produce live offspring in many large-animal species, including sheep, cattle, goats, and pigs. There are two reports of horse foals produced by NT from adult somatic cells (Galli *et al.* 2003, Lagutina *et al.* 2005); in addition, three mule foals have been produced by NT using fibroblasts obtained from a mule fetus (Woods *et al.* 2003, Vanderwall *et al.* 2004b).

Progress in NT in the horse is hampered by the low numbers of equine oocytes available for study and the lack of information on *in vitro* embryo production in this species. In addition, early studies on equine NT reported low rates of fusion of equine cytoplasts with donor cells (Li *et al.* 2002, Choi *et al.* 2002b). To make maximum use of the available oocytes, alternative methods yielding higher recombination rates have been developed in the

horse. These have included addition of sendai virus during fusion (Li *et al.* 2002), fusion after zona removal (Galli *et al.* 2003, Lagutina *et al.* 2005) and direct injection of the donor cell (Choi *et al.* 2002a). Of the recombination methods used, only fusion after zona removal has been shown to produce live young after nuclear transfer using adult somatic cells.

The reported rates of blastocyst production after equine NT have been low (0–16%; Galli *et al.* 2003, Li *et al.* 2004, Lagutina *et al.* 2005). Because rates of blastocyst production after intracytoplasmic sperm injection of *in vitro*-matured oocytes are acceptable (up to 38%; Hinrichs *et al.* 2005), the low blastocyst development after NT in the horse may be related to inefficiency in activation of equine oocytes. Effective methods for equine oocyte activation were not developed before work commenced on equine NT; methods commonly used in other species, including

exposure to calcium ionophore A23187, cycloheximide, ionomycin, ethanol, thimerosal, or 6-dimethylamino purine (6-DMAP), or injection of inositol 1,4,5-triphosphate, were reported to result in low activation or cleavage rates when used on horse oocytes (Hinrichs *et al.* 1995, Li *et al.* 2000, Carneiro *et al.* 2001, Choi *et al.* 2001). Activation procedures used in equine NT have included exposure to calcium ionophore A23187 or ionomycin, followed by treatment either with cycloheximide (Choi *et al.* 2002b, Galli *et al.* 2003, Li *et al.* 2003, Woods *et al.* 2003) or with cycloheximide plus 6-DMAP (Lagutina *et al.* 2005).

Because methods for oocyte activation commonly used in other species have not supported high blastocyst development in horse NT embryos, evaluation of other activation procedures is needed. Stallion sperm extract has been shown to induce calcium oscillations in horse oocytes similar to those found after intracytoplasmic sperm injection (Bedford *et al.* 2003). We obtained cleavage rates of 51% when equine NT oocytes were activated by injection of stallion sperm extract (Choi *et al.* 2002a), and cleavage rates up to 79% were obtained when recombined oocytes were cultured in the presence of 6-DMAP after sperm extract injection (Choi *et al.* 2004). Blastocyst production after this activation treatment has not been reported. Possible methods to increase activation after sperm extract injection include treatment with cycloheximide or roscovitine. Cycloheximide prevents recrudescence of maturation promoting factor (MPF; Cdk1/cyclin B) activity after an activation stimulus (Presicce & Yang 1994, Yang *et al.* 1994, Moos *et al.* 1996). Roscovitine, an inhibitor of ATP binding to MPF, has been used via this activity to induce activation of oocytes, either parthenogenetically or after NT (Mitalipov *et al.* 2001). In *Xenopus*, microinjection of roscovitine was found to be more effective for oocyte activation than was incubation in roscovitine-containing medium (Flament *et al.* 2000). To our knowledge, the effect of injection of roscovitine on oocyte activation in mammalian species has not been evaluated.

Transfer of NT-derived embryos to recipients is associated with losses throughout gestation and in early postnatal life (Hill *et al.* 2000, Chavatte-Palmer *et al.* 2004). The incidence of fetal and neonatal loss associated with NT, and the abnormalities associated with such losses, have been best characterized in cattle, in which approximately 75% of initial pregnancies fail to be carried to term (Wells *et al.* 1999, Forsberg *et al.* 2002). Similarly, in the reports on pregnancies produced by equine NT, 75–100% of established pregnancies were lost, most in early gestation (Galli *et al.* 2003, Woods *et al.* 2003, Vanderwall *et al.* 2004a, Lagutina *et al.* 2005). In our laboratory in 2002, transfer of 4 NT blastocysts produced using fibroblasts grown to confluence resulted in one pregnancy, which was lost at 9 months gestation (Hinrichs and Choi, unpublished data). The high rates of loss associated with cloned fetuses are largely attributed to the

failure of the oocyte to effectively reprogram the donor cell nucleus; i.e. to transform the epigenetic markers of the transferred chromatin from those of a somatic cell to those of a zygote. The exact changes necessary for this, and the mechanisms by which the oocyte induces these changes, are currently unclear.

Gibbons and coworkers (2002) reported that higher proportions of viable NT calves were obtained when adult somatic donor cells were treated with roscovitine rather than by serum deprivation (7 calves from 62 blastocysts, 11%, vs 1 calf from 60 blastocysts, 2% respectively). This is a notable finding; treatments that increase production of viable NT offspring are important not only because they may shed light on the process of reprogramming, but also because they decrease the unwanted fetal losses associated with cloning. However, to our knowledge, no further studies on the use of roscovitine-treated donor cells for production of NT offspring have been reported. This may be due in part to the finding of Gibbons and coworkers (2002) that roscovitine treatment of bovine donor cells was associated with a significant decrease in the proportion of recombined oocytes that developed to the blastocyst stage (12% vs 21% for serum-deprived cells). In the cat, roscovitine treatment of donor cells did not affect the rate of blastocyst development (Gomez *et al.* 2003), but the ability of these blastocysts to produce pregnancies or offspring was not evaluated.

The present study was conducted to determine the rate of blastocyst development in equine NT embryos produced by direct injection of donor cells and activation by injection of sperm extract, using either confluent or roscovitine-treated donor cells. Flow cytometric analysis was used to evaluate the cell-cycle status of donor cells prepared by different donor cell treatments, and its relationship to cell size. Different methods for activation of recombined oocytes, including addition of cycloheximide treatment and roscovitine injection as described by Flament *et al.* (2000) were also evaluated. The viability of embryos produced by NT with roscovitine-treated donor cells was assessed after transfer of these embryos to recipient mares.

Materials and Methods

Experiment 1: Blastocyst production using roscovitine-treated or confluent donor cells

Oocyte collection and maturation

Ovaries were transported from two horse abattoirs to the laboratory at 20–32 °C (3–4 h transport time). All visible follicles were opened with a scalpel blade and the granulosa layer of each follicle was scraped using a 0.5 cm bone curette. All media components were obtained from Gibco Life Technologies, Inc. (Grand Island, NY) unless otherwise noted. The contents of the curette were washed into individual Petri dishes with Hepes-buffered TCM199 with Hanks' salts plus ticarcillin

(0.1 mg/ml; SmithKline Beecham Pharmaceuticals, Philadelphia, PA). The contents of the Petri dishes were examined using a dissection microscope at $\times 10$ – 20 . Oocyte–cumulus complexes were classified as compact, expanded or degenerating depending on the expansion of both mural granulosa and cumulus as described previously (Hinrichs & Williams 1997, Hinrichs & Schmidt 2000). Oocytes with any sign of expansion of either the cumulus or the mural granulosa (from having individual cells visible protruding from the surface to having full expansion with copious matrix visible between cells) were classified as expanded oocytes. Oocytes having both compact cumulus and compact mural granulosa were classified as compact oocytes. Because of their relative abundance, higher maturation rate, and equivalent developmental competence (Hinrichs *et al.* 2005), only expanded oocytes were used in these studies.

For *in vitro* maturation, the oocytes were washed twice in maturation medium (TCM199 with Earle's salts, 5 mU/ml follicle stimulating hormone (FSH) (Sioux Biochemicals Inc., Sioux Center, IA), 10% fetal bovine serum (FBS) and 25 µg/ml gentamycin) containing 200 ng/ml insulin-like growth factor (IGF)-I (Sigma-Aldrich, Inc., St. Louis, MO). Oocytes were cultured at a ratio of 10 µl medium per oocyte in microdroplets of this medium under light white mineral oil (Sigma) at 38.2 °C in 5% CO₂ in air for 24 h. After culture, oocytes were denuded of cumulus by pipetting in 0.05% hyaluronidase in CZB-M (Choi *et al.* 2003). Those oocytes having a polar body were placed in CZB-H (Choi *et al.* 2003) and returned to the incubator until used for NT.

Nuclear transfer

Fibroblasts were obtained by culture of skin biopsies from a 23-year-old stallion. Cells in the 4th to 6th passage were utilized; through these passages, cultures had been frozen and thawed 1 to 2 times at the time of use. Cells were either grown to confluence, without serum deprivation, or were treated while actively growing with R-roscovitine (Sigma), 15 µg/ml for the 24 h preceding NT. Cells were trypsinized before use and washed and held in CZB-M without glutamine, non-essential amino acids or FBS, but supplemented with 2% polyvinylpyrrolidone (CZB-M/2% PVP) for a maximum of 30 min before being transferred to cytoplasts.

Oocytes having a first polar body after *in vitro* maturation were incubated for 10–15 min in CZB-H with 10% FBS containing 0.5 µg/ml Hoechst 33342 (Sigma) and 5 µg/ml cytochalasin B (Sigma). Oocytes were then held in CZB-M with a holding pipette (120–140 µm outer diameter) under an inverted microscope equipped with Eppendorf manipulators. The zona pellucida of the oocyte was drilled using an enucleation pipette (10–13 µm outer diameter) attached to a piezo drill (Prime Tech Ltd., Ibaraki, Japan), and the polar body and metaphase plate were aspirated into the enucleation

pipette. After enucleation, the resulting cytoplasts were held in CZB-H with 10% FBS. The injection of fibroblast cells into the enucleated equine oocytes was slightly modified from that of a previous report (Choi *et al.* 2002a). Briefly, the outside diameter of the injection pipette was 8–9 µm. Immediately before injection, a fibroblast cell of small to medium size (13–15 µm diameter) within the cell population (range of 11–23 µm diameter) was selected. The cell was held in CZB-M/2% PVP and was gently aspirated in and out of the injection pipette until the cell membrane was broken. Donor cell injection was carried out in a 100 µl drop of CZB-M with 10% FBS and reconstructed oocytes were held in CZB-H.

Recombined oocytes were then injected with 2–4 pL (volume estimated from the length of the liquid column and the diameter of the pipette) of sperm extract, prepared as previously described (Choi *et al.* 2002a). Oocytes were treated after sperm extract injection by culture in the presence of 2 mM 6-DMAP in CZB-H with 10% FBS for 4 h. They were then washed and were cultured in microdroplets of DMEM/F-12 (Sigma) with 10% FBS under oil in a humidified atmosphere of 5% O₂, 5% CO₂ and 90% N₂, at 38.2 °C. This medium provides up to 38% blastocyst development in ICSI-produced equine embryos (Hinrichs *et al.* 2005). Medium was changed every 2–3 days. Embryos were examined for cleavage on Day 2 or 3, and were evaluated on Day 7 and Day 8 of culture for development to the blastocyst stage.

Flow cytometric evaluation of donor cells

Donor cells in four treatments were evaluated by flow cytometry. Cells were prepared by growth to confluence or roscovitine treatment, as described above, or by serum deprivation (0.5% serum for 5 days), or were used from an actively growing culture. Treatments were synchronized so that cells in all four treatments were evaluated by flow cytometry on the same day. Four replicates were performed. To estimate the cell diameters corresponding to the divisions characterized on flow cytometry, the diameters of 100 cells from the growing fibroblast culture were measured using the 'Attributes-Length' feature of Axiovision 4 software (Carl Zeiss AG, Germany) on photomicrographs of random areas of the cell suspension.

Fibroblasts from the four different culture treatments were harvested by trypsinization, washed once with DMEM/F-12 with 10% serum and once with PBS + 0.2% BSA. Stock solutions of 1 mg/ml of ribonuclease-A (Sigma), 2.5 mM propidium iodide (PI) and 1% Triton-X in phosphate-buffered saline were prepared. Cell suspensions were dispersed at approximately 5 million/ml, then 40 µl of the cell suspension was combined with 6.4 µl of ribonuclease-A, 6.4 µl of PI, and 20 µl of the Triton-X/PBS solutions. Final concentrations of ribonuclease-A, PI and Triton-X were 88 µg/ml, 0.22 mM, and 0.27%

respectively. This suspension was incubated at room temperature for 10 min prior to evaluation by flow cytometry.

Stained cell suspensions were evaluated using a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer, equipped with an air-cooled Argon ion 488 nm laser. Cells were gated using forward (FSC) and side scatter (SSC) characteristics and the PI-stained cells were excited and emission was detected on detector FL2 (log units). Acquisition was performed in Cell Quest (Becton Dickinson, Mountain View, CA) and analysis was performed using WinList software (Verity Software House Inc., Topsham, ME).

Two-dimensional cell analyses of relative size and of DNA content were performed using FSC and FL2 emission cell characteristics respectively. Cell populations were gated based on three (small, medium, and large) size populations using relative forward scatter measures and three populations based on DNA content (2N, intermediate, and 4N). FSC gives an estimation of relative cell size but cannot be used to calculate actual cell dimensions (Shapiro, 2003). Overall, nine cell populations (3×3) were created (Fig. 1). Population dimension (box size) was fixed between samples. Variation in cell location between samples was minimized by applying the Auto-Position feature of the WinList software to the medium 2N cell population, thereby adjusting the box location to the median value of that cell population.

The proportion of cells in each treatment that were categorized as small, medium and large, the proportion of cells having fluorescence associated with 2N, intermediate, and 4N DNA content, and the proportion of cells in each DNA content group within each

Figure 1 Flow cytometric distribution of fibroblast cell types based on size (forward light scatter, FSC) and DNA content (2N, intermediate, and 4N) as measured by propidium iodide (FL2) fluorescence.

size category were evaluated by one-way analysis of variance (ANOVA) after arcsin square root transformation, with the Kruskal–Wallis one-way ANOVA on ranks used if unequal variances were found after transformation. The Holm–Sidak procedure was used for pairwise comparisons. Analysis was performed using SigmaStat 2.0 statistical software (Jandel Scientific, San Rafael, CA).

Experiment 2: Transfer of NT embryos produced from roscovitine-treated or confluent donor cells; effect of cycloheximide at activation

Fibroblasts from a 20-year-old stallion were used as nuclear donor cells. The fibroblasts were grown in culture and used at passage 7 to 10; through these passages, cultures had been frozen and thawed 3–4 times at the time of use. Oocyte maturation, fibroblast treatments (confluence and roscovitine treatment), NT procedures, and embryo culture were performed as described for Experiment 1, except that IGF-I was not included in oocyte maturation medium. During 6-DMAP culture after injection of sperm extract, cycloheximide, 10 µg/ml, was added to the 6-DMAP-containing medium for half of the recombined oocytes in each donor cell treatment. Embryo culture and assessment was performed as for Experiment 1.

A herd of 12 Quarter-horse type mares, aged 5–15 years and weighing 400–500 kg, was maintained to provide embryo recipients. Mares were kept in paddocks with run-in sheds, and were fed hay and sweet feed with free-choice water. Work with these mares was performed according to the *United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and was approved by the Laboratory Animal Care Committee at Texas A&M University. Blastocysts (one per mare) were transferred transcervically to the uteri of recipient mares which had ovulated from 1 day before to 4 days after the date of NT, or that were in anestrus or seasonal transition and were treated with a biorelease formulation of estradiol 17β (BETPharm, Lexington, KY), 10 mg i.m., 2 days before the day of NT and with a biorelease form of progesterone (BETPharm), 1.5 g i.m., 3 days after the day of NT. The mares' reproductive tracts were monitored by ultrasonography per rectum from Day 11 to Day 16 after transfer to detect presence of an embryonic vesicle. When an embryonic vesicle was seen, the mares were administered biorelease progesterone, 1.5 g i.m., weekly until either accessory corpora lutea were present, or until after day 100 of gestation.

Experiment 3: Co-injection of roscovitine for activation

Oocyte recovery and maturation procedures, fibroblast preparation, and NT procedures were as described for

Experiment 2 except that either DMEM/F-12 or CZB-M was used as the micromanipulation medium. All fibroblasts used for NT were prepared by roscovitine treatment. Recombined oocytes were divided into three activation treatments. Oocytes in the control treatment were activated by injection of sperm extract and then culture with 6-DMAP, as performed in Experiment 1. In the 25 μ M roscovitine treatment, roscovitine was added to the sperm extract to a final concentration of 25 μ M and was injected into the oocyte at the time of sperm extract injection. In the 50 μ M roscovitine treatment, roscovitine was added to the sperm extract to a final concentration of 50 μ M and was similarly co-injected. The co-injected oocytes were then treated with 6-DMAP and cultured as for control oocytes.

Recombined oocytes were cultured and assessed, and transferred to recipient mares, as described for Experiment 2; all recipient mares were ovulatory. Some blastocysts were not transferred due to lack of available recipient mares.

Results

Experiment 1: Blastocyst production using roscovitine-treated or confluent donor cells

One-hundred and fifty-five ovaries were processed, and 741 follicles were scraped to recover oocytes. From these, 206 Ex, 139 Cp and 32 degenerating oocytes were recovered. For this experiment, 206 Ex oocytes were used. Four oocytes were broken during removal of cumulus cells after maturation; 138 oocytes (68%) were seen to have polar bodies and were subjected to NT. Twenty seven oocytes were lysed during enucleation, donor cell injection, or sperm factor injection and 111 recombined oocytes were cultured. The cleavage and blastocyst development rates for embryos produced using confluent or roscovitine-treated donor cells are presented in Table 1. There was no difference in cleavage rate or in blastocyst development rate between the two donor cell treatments (2/55, 3.6% vs 2/56, 3.6% respectively).

Representative scatterplots of forward scatter (size) vs fluorescence (DNA content) for fibroblasts obtained from growing, roscovitine-treated, confluent or serum-deprived cultures are presented in Fig. 2. By comparing the proportions of cells classified as small, medium and large in the growing culture treatment with the distribution of measured diameters of cells in that treatment, we estimated that these size categories were equivalent to cells of 11 to 14, 15 to 19 and 20 to 23 μ m

diameter respectively. Roscovitine-treated cultures had proportionately more small cells, and fewer medium cells, than did the confluent or serum-deprived treatments ($P < 0.05$, Table 2). There were no significant differences among treatments in the proportions of cells in the 2N (G1/G0), Intermediate (S) or 4N (G2) DNA categories, or in the proportions of these DNA categories within the different cell sizes (Tables 3 and 4).

Experiment 2: Transfer of NT embryos produced from roscovitine-treated or confluent donor cells; effect of cycloheximide at activation

From 410 ovaries processed, 2952 follicles were scraped to recover oocytes and 903 Ex, 564 Cp, and 190 degenerating oocytes were recovered. From 873 Ex oocytes placed in maturation culture, 17 were broken during removal of cumulus cells after maturation, and 526 oocytes (61%) were seen to have polar bodies. After manipulation, 442 oocytes were successfully recombined and injected with sperm factor; 423 of these were cultured for Experiment 2, and 19 were used for another study. The rates of cleavage and blastocyst formation for oocytes reconstructed with either confluent or roscovitine-treated donor cells and activated after SE injection by culture either in 6-DMAP alone or in 6-DMAP + cycloheximide are presented in Table 5. There was no significant difference in rates of cleavage or blastocyst development among treatment groups. The blastocyst development rate in the donor-roscovitine/6-DMAP treatment (4/105, 4%) tended to be higher than that for the confluence/6-DMAP treatment (0/106; $P = 0.059$) and the donor-roscovitine/6-DMAP + cycloheximide treatment (0/104; $P = 0.062$).

Two mares received embryos from the confluent/6-DMAP + cycloheximide treatment; neither established pregnancy. Of four mares that received embryos from the donor-roscovitine/6-DMAP treatment, one was found to have an embryonic vesicle on later examination by ultrasonography per rectum. The vesicle was first visible at Day 12 (Day 0 = day of NT), and developed normally on evaluation by palpation and ultrasonography per rectum. Ultrasonographic measurement of orbital and aortic diameters at 356 days (normal gestation 335–345 days) indicated that the fetus was small for a term foal. These measurements increased over the following weeks to those compatible with a term foal (Reef 1998). The mare's plasma progesterone rose normally (from 4 ng/ml to 9 ng/ml) over the 4 weeks prior to parturition. A male foal was delivered with minimal

Table 1 Cleavage and blastocyst development after nuclear transfer with confluent or roscovitine-treated fibroblasts.

Treatment	<i>n</i>	Cleaved (%)	Blastocysts day 7	Blastocysts day 8	Total blastocysts (%)
Confluent	55	40 (73)	1	1	2 (3.6)
Roscovitine	56	44 (79)	0	2	2 (3.6)

Figure 2 Representative scatterplots of forward scatter (representing size) and FL2 emission (representing DNA content) for fibroblasts in the four cell treatment groups.

assistance on March 13, 2005, at 389 days gestation. The colt was 27 kg at birth; anticipated foal weight for this size mare was 35–40 kg. The colt was bright and alert but somewhat slow to stand (3 h) and nurse effectively (8 h). The placenta and umbilical cord were normal on gross and histological examination. The colt was placed prophylactically on oxygen supplementation for one day, was given

colostrum by nasogastric tube, and was supplemented with milk after nursing by nasogastric tube for 2 days. He weighed 55 kg at 2 weeks of age, and has continued to develop normally. Typing for 13 equine microsatellites confirmed that the colt was of the same DNA type as the donor fibroblasts (Table 6). The colt is 11 months of age at the time of writing.

Table 2 Percentage of cells categorized as small, medium and large in the four donor cell treatment groups (mean $\pm$ s.d.)

	Donor cell treatment			
	Actively growing	Roscovitine treated	Confluent monolayer	Serum deprived
% Small cells	26.4 $\pm$ 4.4 ^{ab}	30.7 $\pm$ 2.9 ^a	24.7 $\pm$ 4.0 ^b	21.1 $\pm$ 2.2 ^b
% Medium cells	58.8 $\pm$ 4.3 ^{ab}	53.3 $\pm$ 4.1 ^a	61.5 $\pm$ 6.2 ^{bc}	67.6 $\pm$ 3.8 ^c
% Large cells	14.8 $\pm$ 0.4	15.9 $\pm$ 2.5	13.8 $\pm$ 3.0	11.3 $\pm$ 2.4

Within rows, values with different superscripts are significantly different.

Table 3 Percentage of cells having fluorescence associated with 2N, intermediate and 4N DNA content (mean $\pm$ s.d.) in four donor cell treatment groups

	Donor cell treatment			
	Actively growing	Roscovitine treated	Confluent monolayer	Serum deprived
2N	72.8 $\pm$ 4.4	73.9 $\pm$ 8.4	77.8 $\pm$ 8.4	84.5 $\pm$ 9.9
Intermediate	15.3 $\pm$ 6.6	14.2 $\pm$ 6.8	9.3 $\pm$ 1.4	8.3 $\pm$ 7.3
4N	11.9 $\pm$ 7.7	11.9 $\pm$ 3.6	12.9 $\pm$ 7.1	7.2 $\pm$ 6.5

Differences among groups were not statistically significant.

Table 4 Percentages of cells in the different DNA content groups within size categories (mean $\pm$ s.d.) for four donor cell treatment groups.

Size category	DNA category	Actively growing	Roscovotine treated	Confluent monolayer	Serum deprived
Small	2N	96.2 $\pm$ 1.4	95.6 $\pm$ 4.2	97.1 $\pm$ 1.6	98.0 $\pm$ 1.6
	Intermediate	2.8 $\pm$ 1.6	3.3 $\pm$ 3.8	1.5 $\pm$ 0.6	1.3 $\pm$ 1.2
	4N	1.0 $\pm$ 1.0	1.1 $\pm$ 0.8	1.3 $\pm$ 0.1	0.7 $\pm$ 1
Medium	2N	73.6 $\pm$ 6.6	75.4 $\pm$ 11	79.6 $\pm$ 11.8	88.8 $\pm$ 8.6
	Intermediate	16.4 $\pm$ 5.4	14.2 $\pm$ 7	9.4 $\pm$ 4.4	6.8 $\pm$ 7
	4N	10.0 $\pm$ 9.6	10.4 $\pm$ 6.4	11.0 $\pm$ 7.4	4.4 $\pm$ 5.4
Large	2N	27.8 $\pm$ 9.0	25.5 $\pm$ 4.8	32.3 $\pm$ 5.2	36.0 $\pm$ 22.8
	Intermediate	32.9 $\pm$ 21.4	35.3 $\pm$ 12.8	24.9 $\pm$ 13.2	29.2 $\pm$ 18.6
	4N	39.3 $\pm$ 14.2	39.2 $\pm$ 10.8	42.7 $\pm$ 12.2	34.8 $\pm$ 20.8

Differences among groups were not statistically significant.

Experiment 3: Co-injection of roscovotine for activation

From 195 ovaries processed, 1332 follicles were scraped to recover oocytes and 397 Ex, 299 Cp and 72 degenerating oocytes were recovered. From 397 Ex oocytes placed in maturation culture, 14 were broken during removal of cumulus cells after maturation, and 238 oocytes (62%) were seen to have a polar body. After manipulation, 199 oocytes were successfully recombined and injected with sperm factor; 144 of these were cultured for Experiment 3 and 55 were used for another study. The cleavage and blastocyst development rates for the control activation (sperm extract + 6-DMAP), 25 μ M roscovotine injection and 50 μ M roscovotine injection treatments are presented in Table 7. There were no significant differences in cleavage or blastocyst development rates among groups.

One blastocyst resulting from the 50 μ M roscovotine injection treatment was transferred, and this resulted in a vesicle first visualized at Day 14. The conceptus appeared small and irregular in shape for stage, but an embryo with heartbeat was seen on Day 31 (heartbeat expected to be visualized on Day 25). This fetus appeared to develop normally to Day 94. The heartbeat could not be identified on examination on Day 108, and the pregnancy was lost at pasture between Days 108 and 115. The fetus and placenta were not found.

Two pregnancies resulted from transfers of four embryos in the control activation group. One embryo resulted in a vesicle first visualized on ultrasonography per rectum on Day 13. This conceptus appeared to develop normally, but was aborted at 239 days gestation.

Necropsy revealed a normal male fetus and placenta, with the exception of an edematous umbilical cord and focal nodules of necrosis in the liver. Typing for 13 equine microsatellites confirmed that the fetus was of the same DNA type as the donor fibroblasts (Table 6). The second vesicle was first visualized on ultrasonography per rectum on Day 12. This embryo continued to develop normally thereafter, and resulted in birth of a male foal at day 340 of gestation (May 29, 2005). The colt was delivered without assistance, and was 40.5 kg at birth. The colt was active and had normal times to stand (30 min) and nurse (2.5 h). The umbilical cord appeared slightly enlarged (2.7 cm diameter) and was edematous; however, the placenta was normal on both gross and histological examination. The umbilical stump remained large, and was excised surgically when the colt was 12 days old. Typing for 13 equine microsatellites confirmed that this colt was of the same DNA type as the donor fibroblasts (Table 6). The colt has continued to develop normally and is 8.5 months old at the time of writing.

Discussion

These results demonstrate that NT using direct injection of donor cells and activation by injection of sperm extract is compatible with production of both blastocysts and foals. The foals produced in this study represent the third and fourth viable foals reported from adult somatic cell NT in the horse. To our knowledge, these are the first cloned offspring produced using direct injection of the donor cell and activation with sperm extract. This

Table 5 Cleavage, blastocyst development, and pregnancy rates after nuclear transfer with different donor cell treatments and activation protocols.

Fibroblast treatment	Activation treatment	n	Cleaved (%)	Blastocysts (%)	Blastocysts transferred	Pregnancies	Viable foals
Roscovotine	SE/6-DMAP ^a	105	92 (88)	4 (4)	4	1	1
Roscovotine	SE/6-DMAP + cycloheximide	104	86 (83)	0	0	0	0
Confluence	SE/6-DMAP	106	83 (78)	0	0	0	0
Confluence	SE/6-DMAP + cycloheximide	108	84 (78)	2 (2)	2	0	0

^aSE/6-DMAP: injection of sperm extract followed by culture in the presence of 6-dimethylaminopurine.

Table 6 Results of typing of equine microsatellites for fibroblasts from the donor animal, liver tissue from the aborted fetus, and hair follicle cells from the two foals.

Satellite	Donor	Fetus	Foal 1	Foal 2
ASB17	FR	FR	FR	FR
VHL20	IR	IR	IR	IR
HTG10	M	M	M	M
HTG4	M	M	M	M
AHT5	IM	IM	IM	IM
AHT4	HJ	HJ	HJ	HJ
HMS3	MP	MP	MP	MP
HMS6	LM	LM	LM	LM
HMS7	MO	MO	MO	MO
LEX3	N	N	N	N
LEX33	MQ	MQ	MQ	MQ
ASB2	NO	NO	NO	NO
ASB23	JK	JK	JK	JK

provides an alternative technique for consideration in species that have been difficult to clone successfully.

We evaluated the use of roscovitine for donor cell synchronization because this treatment was previously shown to significantly increase the production of viable NT calves (Gibbons *et al.* 2002). In contrast to the situation in cattle (Gibbons *et al.* 2002), blastocyst production in our study was equivalent between roscovitine-treated and confluent donor cells (Experiment 1). Direct injection of roscovitine-treated donor cells in combination with our standard activation protocol resulted in an overall blastocyst formation rate (Experiments 1, 2 and 3 combined) of 11/210 (5.2%). While we achieved an insufficient number of pregnancies with confluent donor cells to directly compare the viability of blastocysts produced from the two donor cell sources, the rate of production of viable foals (two foals from eight blastocysts; 25%) compares favorably with that of the two previous reports on production of NT foals from adult fibroblasts (one foal from 17 blastocysts, 6%, and one viable foal from 101 blastocysts, 1%; Galli *et al.* 2003, Lagutina *et al.* 2005), and to the overall proportion of viable calves resulting from transfer of NT-derived embryos in cattle (6%; Wells 2005). That two viable offspring were achieved from three pregnancies in this treatment is noteworthy, given the 75–100% losses previously reported in horse NT (Galli *et al.* 2003, Woods *et al.* 2003, Vanderwall *et al.* 2004b, Lagutina

et al. 2005) and the low production of viable cloned offspring in other species.

Gibbons *et al.* (2002) attributed the significantly higher production of viable offspring that they observed after roscovitine treatment of donor cells to improved synchronization of the cells into G0/G1. Roscovitine is a purine that blocks the ATP-binding site of specific cyclin-dependent kinases (Cdks). Roscovitine inhibits not only Cdk1, the activity associated with use of this compound for suppression of maturation and activation, but also Cdk2, Cdk5, Cdk7, and other Cdks (Meijer *et al.* 1997, McClue *et al.* 2002). Through these activities, roscovitine at low concentrations (<20 µM) suppresses the G1/S and G2/M transitions in cultured fibroblasts, thus arresting cells in G1 or G2 (Alessi *et al.* 1998). However, we found that roscovitine-treated cells were no more likely to have 2N DNA than were serum-deprived or confluent cells. Boquest *et al.* (1999) reported that in serum-deprived or confluent cultures of porcine fibroblasts, 93–95% of small cells were 2N (G0/G1), and we found a similar situation in roscovitine-treated fibroblasts. When selection for size, as is done when selecting donor cells for NT, was considered (Table 4), we found that 95–98% of small cells were in G0/G1 (2N), regardless of donor cell treatment. These data suggest that the beneficial effect of roscovitine noted by Gibbons *et al.* (2002) was due to changes in some quality of the cells, perhaps related to the ability of the cell to be reprogrammed, rather than to increased synchrony of cells in G0/G1. A qualitative effect of roscovitine treatment is supported by our finding that a significantly greater proportion of cells in this treatment were classified as small. This finding is interesting; one would expect that cessation of growth due to deprivation of serum for 5 days or to crowding would affect cell size more profoundly than would arrest of the cell cycle via roscovitine for 24 h, during which time most other cellular machinery should be functional. Further work is warranted on the nuclear and cytoplasmic characteristics of cells prepared under different donor cell synchronization treatments, and their relationship to nuclear reprogramming and embryo viability after nuclear transfer.

The 5% rate of blastocyst formation achieved in this study with direct injection of donor cells and activation

Table 7 Cleavage, blastocyst development and pregnancy rates after nuclear transfer using roscovitine-treated fibroblasts and activation with three different treatments.

Activation treatment	n	Cleavage (%)	Blastocysts (%)	Blastocysts transferred	Pregnancies	Viable foals
Control ^a	49	41 (84)	5 (10)	4	2	1
25 ^b	55	42 (76)	2 (4)	0	0	0
50 ^c	40	29 (73)	1 (3)	1	1	0

^aControl treatment: sperm factor injection followed by incubation with 6-dimethylaminopurine.

^b25: Control activation treatment plus injection of 25 µM roscovitine.

^c50: Control activation treatment plus injection of 50 µM roscovitine.

with sperm extract and 6-DMAP is similar to that of previous reports on equine NT (Galli *et al.* 2003, Li *et al.* 2004, Lagutina *et al.* 2005). This low rate indicates that further work to improve blastocyst development in this species is essential. We evaluated the effect of addition of cycloheximide treatment or of roscovitine injection at the time of activation on blastocyst development. Cycloheximide has been shown to significantly increase the activation rate in equine oocytes treated with calcium ionophore (Hinrichs *et al.* 1995). The combination of cycloheximide and 6-DMAP is commonly used in other species (Liu *et al.* 2004), and this combination, after ionomycin treatment, was used in production of one of the previous NT foals (Lagutina *et al.* 2005). However, addition of cycloheximide to the activation protocol in our study did not increase blastocyst development. Roscovitine, as an inhibitor of MPF, has been shown to be an effective activation agent in rhesus oocytes (Mitalipov *et al.* 2001). We chose injection of roscovitine rather than culture with roscovitine as an activation treatment in this study because injection was reported to be the more effective treatment in *Xenopus* (Flament *et al.* 2000), and injection was simply performed in our system by addition of roscovitine to the sperm extract solution. However, this treatment did not increase blastocyst development rates, and resulted in a conceptus that developed abnormally in early gestation and was lost before 114 days. Further work on the timing of the roscovitine injection, or on roscovitine culture rather than injection, is needed to fully evaluate the effect of roscovitine on activation of horse oocytes after NT.

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