Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type

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Abstract

The objective of the present work was to investigate and clarify the factors affecting the efficiency of somatic cell nuclear transfer (NT) in the horse, including embryo reconstruction, *in vitro* culture to the blastocyst stage, embryo transfer, pregnancy monitoring and production of offspring. Matured oocytes, with zona pellucida or after zona removal, were fused to cumulus cells, granulosa cells, and fetal and adult fibroblasts, and fused couplets were cultured *in vitro*. Blastocyst development to Day 8 varied significantly among donor cells (from 1.3% to 16%, $P < 0.05$). In total, 137 nuclear transfer-embryos were transferred nonsurgically to 58 recipient mares. Pregnancy rate after transfer of NT-embryos derived from adult fibroblasts from three donor animals was 24.3% (9/37 mares transferred corresponding to 9/101 blastocysts transferred), while only 1/18 (5.6%) of NT-blastocysts derived from one fetal cell line gave rise to a pregnancy (corresponding to 1/33 blastocysts transferred). Overall, seven pregnancies were confirmed at 35 days, and two went to term delivering two live foals. One foal died 40 h after birth of acute septicemia while the other foal was healthy and is currently 2 months old.

These results indicate that (a) the zona-free method allows high fusion rate and optimal use of equine oocytes, (b) different donor cell cultures have different abilities to support blastocyst development, (c) blastocyst formation rate does not correlate with pregnancy fate and (d) healthy offspring can be obtained by somatic cell nuclear transfer in the horse.

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Introduction

Somatic cell nuclear transfer has been the subject of much investigation in the last few years and, as a result, several mammalian species have been cloned (see Galli *et al.* 2003a for review). In Equids, the birth of three mule foals cloned from fetal cells (Woods *et al.* 2003) and one horse foal cloned from adult somatic cells (Galli *et al.* 2003b) has only recently been reported. Remarkably, all foals were born alive and survived to adulthood.

This relatively late and limited application of somatic cell nuclear transfer is a consequence of the limited information available in the literature on assisted reproductive techniques in Equids, in particular oocyte maturation and *in vitro* culture of early pre-implantation embryos. Indeed, the work that led to the cloning of mules (Wood *et al.* 2003) relied on *in vivo* matured oocytes that were transferred to the oviducts of recipient mares immediately after

nuclear transfer and activation. Only the work of Galli *et al.* (2003b) has been carried out completely *in vitro* up to the blastocyst stage.

One reason for the limited development of assisted reproduction techniques in Equids is the limited interest demonstrated by the horse industry, contrary to the cattle industry that still plays a very active role in the development and application of reproductive biotechnologies in ruminants. In general, few studies are available in the literature on equine *in vitro* embryo production (see reviews by Squires *et al.* 2003, Westhusin *et al.* 2003), and it is only recently that reports have been published on completely *in vitro* production of equine preimplantation embryos by means of *in vitro* oocyte maturation, fertilization by intracytoplasmic sperm injection (ICSI) and *in vitro* culture (Galli *et al.* 2002a, Lazzari *et al.* 2002a, Choi *et al.* 2004). These latter reports demonstrate the real possibility of applying a completely *in vitro* procedure for obtaining

horse ICSI blastocysts able to establish a pregnancy (Galli *et al.* 2001a, 2002b) and develop to live term offspring (present authors, unpublished data).

In horse nuclear transfer, the availability of horse oocytes is a limiting factor due to the anatomy and physiology of the mare's ovary which makes this species a poor oocyte donor as compared with other large domestic species. Oocytes can be harvested from the ovaries of slaughtered mares (Zhang *et al.* 1989) or from ovum pick up (OPU) of live donors. On average it is possible to recover 3–4 immature oocytes per ovary from the ovaries of slaughtered animals (Galli & Lazzari 2001, Lazzari *et al.* 2002a); oocyte retrieval from live donors by OPU is slightly more variable and can be from 3 to 6 oocytes per session of OPU (Galli *et al.* 2002a, Lorenzo *et al.* 2002). The maturation rate of horse oocytes is also quite variable, averaging between 25 and 70% in published studies (Hinrichs & Williams 1997, Carneiro *et al.* 2001, Dell'Aquila *et al.* 2001, 2003, Bogh *et al.* 2002, Choi *et al.* 2002, Lorenzo *et al.* 2002). Interestingly, in the horse the recovered cumulus–oocyte complexes are a mixed population with either a compact or an expanded cumulus. While expanded cumulus–oocyte complexes are often discarded for *in vitro* embryo production in cattle and pigs, they represent about 40% (2060/5127, our observations) of the total population of recovered oocytes in the horse and seem to have some developmental competence for maturation (Dell'Aquila *et al.* 2003), cleavage (Choi *et al.* 2002) and blastocyst formation (Zhang *et al.* 1989). Another peculiar aspect specifically relevant to horse nuclear transfer is the reported (Choi *et al.* 2002, Li *et al.* 2002) low ability of oocytes to fuse with donor cells and the limited developmental competence of nuclear transfer (NT)-embryos *in vitro*. Even high DC voltage pulses of 2.2–2.5 kV/cm were able to fuse less than 60% of couplets, the cleavage rate was 35–50% and only about 2.5% of NT-embryos reached the blastocyst stage (Li *et al.* 2002).

The refinement of the *in vitro* culture conditions suitable for equine oocyte maturation and embryo development (Galli *et al.* 2002a, Lazzari *et al.* 2002a), the development of an adequate horse oocyte activation protocol (Lazzari *et al.* 2002b) and the application of zona-free manipulation for embryo reconstruction (Booth *et al.* 2001, Obach *et al.* 2003, Vajta *et al.* 2003), are all fundamental steps for the development of a successful *in vitro* procedure for somatic cell nuclear transfer in the horse. In this paper we describe, in detail, all the technical aspects involved in the *in vitro* production of cloned blastocysts in the horse. The objectives of our research concentrated on the optimization of the nuclear transfer procedure to make efficient use of the limited number of oocytes available in this species. Both expanded and compact cumulus–oocyte complexes were used as well as a modified zona-free method for embryo reconstruction that is known to increase the fusion rate in other species (Obach *et al.* 2003). Different sources of donor somatic cells were

compared in various experiments for their ability to develop to the blastocyst stage and to term.

Materials and Methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (Milan, Italy), and plastic-ware was from Nunc (Roskilde, Denmark).

Oocyte collection and *in vitro* maturation

Ovaries of slaughtered mares of mixed breeds were collected during the breeding season at 45° latitude (Italy, northern hemisphere). Cumulus–oocyte complexes (COCs) were recovered by a combination of scraping and washing of the inner wall of follicles that were between 0.5 and 3.0 cm in diameter with a Jacobson curette and a syringe filled with PBS. The fluid was recovered into Petri dishes containing Hepes-buffered TCM 199 supplemented with 1 mg/ml BSA and 10 µg/ml heparin. COCs were classified as either compact or expanded depending on cumulus and granulosa cell morphology (Fig. 1a,b), and transferred separately to maturation medium (Galli *et al.* 2001a, 2002a, 2002b). Briefly, the maturation medium was TCM 199 supplemented with 10% fetal calf serum (FCS), 1 µl/ml insulin, transferin, sodium selenite (ITS), 1 mM sodium pyruvate, 50 ng/ml long-epidermal growth factor, 100 ng/ml long-insulin-like growth factor-I and 0.1 IU/ml each of follicle-stimulating hormone and luteinizing hormone (Pergovet, Serono, Italy). COCs were matured for 22–24 h in 4-well plates at 38.5 °C in an atmosphere of 5% CO₂ in air.

Preparation of nuclear donor cells

Cumulus cells from COCs of mixed breeds after 22–24 h of maturation were collected after removal of the cumulus from oocytes in synthetic oviductal fluid-Hepes (SOF-Hepes) with hyaluronidase (1 µg/ml), trypsinized (2.5 mg/ml), pelleted by centrifugation at 200 g for 5 min, resuspended in SOF-Hepes and used for nuclear transfer within 2–3 h. Granulosa cells were collected during ovum pick up from a Haflinger mare (Galli *et al.* 2001b, 2002b), purified from blood cells on a Percoll gradient (45% in TCM 199), plated in 60 mm tissue culture dishes and cultured in DMEM with 10% FCS in 5% CO₂ and 5% O₂ in humidified air at 38.5 °C. After expansion of cells in culture they were either subcultured every 4 days or frozen in 10% DMSO and stored in liquid nitrogen.

Adult fibroblasts were produced by organ culture of minced tissue from chest biopsies of adult animals of the Haflinger and Arabian breeds. Fetal fibroblasts were produced as described for adult fibroblasts from an aborted 6-month-old fetus cloned from an adult fibroblast cell line. Both adult and fetal fibroblasts were cultured and stored as described for granulosa cells. Cells were used at passage number 5 to 15.

Before using the cells in NT experiments, granulosa cells and fibroblasts were induced into quiescence by either serum starvation (0.5% FCS) for 1–3 days or by growth to confluence. A cell suspension was prepared by trypsinization of cell culture 30 min before nuclear transfer, washed and resuspended in SOF-Hepes.

Preparation of enucleated oocytes (cytoplasts) and NT-embryo construction

After 22–24 h of maturation the oocytes were denuded of cumulus cells by pipetting first in the presence of hyaluronidase, then placed in 0.25% trypsin for 1.5 min and afterwards in SOF-Hepes with 10% FCS where the cumulus cells were completely removed by pipetting. Finally, the oocytes were returned to maturation medium. For reconstruction of NT-embryos we used both the zona-enclosed and zona-free methods (Oback *et al.* 2003). For the zona-enclosed method, oocytes with an extruded polar body were stained with Hoechst 33342 in the presence of cytochalasin B (5 µg/ml). Enucleation was performed by the aspiration of the polar body and metaphase II plate in a minimal volume of ooplasm under UV light. All manipulations were in SOF-Hepes with 6 mg/ml BSA (Gardner *et al.* 1994), except fusion. Individual nuclear donor cells prepared as described above (cumulus cells 22–24 h of maturation or adult fibroblasts) were transferred into the perivitelline space of zona-enclosed cytoplasts with the enucleation pipette. The cytoplast–karyoplast couplets were transferred in 0.3 M mannitol (50 µM Ca and 100 µM Mg) solution and fused one or two times within a 15 to 30 min interval by a double DC pulse of 1.8–2.4 kV/cm applied for 30 µs at 26–27 h of maturation. For the zona-free method, the zona pellucida of oocytes with extruded polar bodies was digested with 0.5% pronase in PBS. Zona-free oocytes were enucleated under UV light with a blunt micropipette (with perpendicular break). All manipulations were performed in SOF-Hepes with 10% FCS. Subsequently, zona-free cytoplasts were individually washed for a few seconds in 300 µg/ml phytohemagglutinin P in TCM 199-Hepes and then quickly dropped over a single donor cell (Vajta *et al.* 2003) settled to the bottom of a drop of TCM 199 with 0.5% FCS. Formed cell couplets were washed in 0.3 M mannitol (50 µM Ca and 100 µM Mg) solution and fused one or two times at 15 to 30 min intervals by a single DC pulse of 1.2 kV/cm applied for 30 µs at 26–27 h of maturation.

Activation

Oocytes and NT-embryos were activated 1–2 h after fusion at 27–28 h of maturation. Oocytes and NT-embryos were treated with 8.7 µM ionomycin in SOF-Hepes for 4 min followed by 4 h culture in a combination of 1 mM 6-(dimethylamino) purine (6-DMAP) and 5 µg/ml cycloheximide in SOF supplemented with MEM essential

and non essential amino acids, 1 mM glutamine, and 4 mg/ml BSA (m-SOFaa) (Lazzari *et al.* 2002b).

Embryo culture

Zona-enclosed and zona-free embryos were cultured, respectively, in 20 µl culture drops and individually in 3 µl drops of m-SOFaa with 4 mg/ml BSA in 5% CO₂ and 5% O₂ in humidified air at 38.5 °C under mineral oil. Half of the medium was renewed on Day 3 with fresh m-SOFaa and on Day 5 with TCM 199 with 5% FCS and 5% Serum Replacement (Knockout SR, Gibco BRL) (Day 0 was the day of fusion and activation). Cleavage was assessed 48 h after activation, the rate of blastocyst formation was recorded at Day 7 and Day 8 when the embryos were either frozen or transferred to synchronized recipients (Fig. 1c,d).

Embryo transfer and foaling

Embryo transfer to recipients was performed over 3 breeding seasons (2002–2004). Haflinger mares were examined 2–3 times a week by ultrasound to determine the day of ovulation. Three to seven days after ovulation the mares received one to four Day 7 or Day 8 blastocysts (fresh or after thawing) by nonsurgical transfer. On Day 17 after ovulation the animals were examined (5 MHz linear probe, Sonovet 600, Medison) for pregnancy diagnosis and thereafter were examined weekly throughout the first trimester of pregnancy and later at monthly intervals until foaling.

All the experiments that involved the use of recipient animals were carried out under veterinary supervision in accordance with Decreto Legislativo 116/92 that regulates the use of animal experimentation in Italy.

Statistical analysis

Differences between the experimental groups were verified using the Chi-square test or Student's *t*-test. *P* < 0.05 was considered significant.

Results

Comparison of two methods of NT-embryo reconstruction

The development of NT-embryos reconstructed with either cumulus cells or adult fibroblasts by two different methods is summarized in Table 1. In this experiment only compact oocyte–cumulus complexes were used. The results indicate that more oocytes were fused and the cleavage rate was higher in the zona-free system (*P* < 0.05). The rate of blastocyst formation of the fused couplets was generally low (1.3–2.9% for cumulus cells and 4.2–6.4% for fibroblasts on Day 8) and did not differ significantly between groups. However, when comparing the efficiency of the two systems by calculating the percentage of enucleated oocytes subjected to fusion that developed

Figure 1 Equine cumulus–oocyte complexes of compact (a) and expanded (b) morphology before *in vitro* maturation. NT-horse blastocysts on Day 8 from zona-enclosed manipulation (c) and from zona free manipulation (d). Bars = 100 µm.

Table 1 Development of horse NT-embryos reconstructed by zona-enclosed versus zona-free methods. The number of replicates was ≥4.

Donor cells	Method of NT	No. of oocytes enucleated (%)	No. of couplets fused (%)	Cleaved (n (% of fused))	Blastocysts on Day 8	
					(n (% of fused))	(% of oocytes)
Cumulus	Zona-enclosed	113	78 (69) ^a	54 (69.2) ^c	1 (1.3)	0.9
	Zona-free	104	104 (100) ^b	88 (84.6) ^d	3 (2.9)	2.9
Adult fibroblasts	Zona-enclosed	181	120 (66.3) ^a	82 (68.3) ^c	5 (4.2)	2.7
	Zona-free	193	187 (97) ^b	159 (85.0) ^d	12 (6.4)	6.2

Values with different letters within columns differ significantly, *P* < 0.05 (Chi-square).

into Day 8 blastocysts (Table 1, blastocysts on Day 8, percent of oocytes), we found that the zona-free method is about 3.2 times more effective than the zona-enclosed method in the case of cumulus-derived NT-embryos and 2.3 times more effective in the case of fibroblast-derived NT-embryos.

Development of NT-embryos derived from oocytes with expanded and compacted cumulus before maturation

For this experiment we used adult fibroblasts from 3 different donor animals (female A, male B and male C) with a minimum of 3 replicates. Overall, 413 embryos from compacted and 197 from expanded cumulus–oocyte complexes were reconstructed and cultured in this experiment. The cleavage rate of NT-embryos derived from fibroblasts of male B was significantly higher in the compacted cumulus group (95.9% vs 84.6%), while no differences were observed for donors A and C. However, the development to the blastocyst stage was not affected by the type of oocyte used (compacted versus expanded: B, 6.0% vs 8.3%; A, 4.1% vs 4.6%; C, 1.8% vs 1.9%).

Developmental competence of horse NT-embryos derived from different cell types

We tested the developmental ability of NT-embryos derived from cultured granulosa cells, cumulus cells, and adult and fetal fibroblasts from various donor animals and in parallel with the developmental ability of parthenogenetically activated matured (MetII) oocytes (Table 2). There was no difference in the rate of cleavage between parthenogenetic and NT-embryos derived from different donor cell types obtained by the zona-free method. The developmental capacity of NT-embryos to form blastocysts on Day 8 differed among donor cells (from 1.3% to 16%, *P* < 0.05). NT-embryos derived from adult fibroblasts of male B and from fetal fibroblasts (cloned fetus from male C) were better able to support development to blastocysts and this rate was similar to the rate of parthenogenetic development (Table 2).

Embryo transfer, pregnancies and offspring (Table 3)

In total, 137 blastocysts were transferred to 58 recipient mares. The average pregnancy rate per mare was 17.2%

Table 2 Development of horse NT-embryos derived from different types of nuclear donor cells. The number of replicates was ≥ 4 .

Donor of cells for NT	Cells	Method of NT	Number of embryos	Cleaved* (n (%))	Blastocysts on Day 8 (n (%))
Different females	Cumulus cells	ZE or ZF	182	142 (78.0)	4 (2.20) ^{a,c}
Female A	Granulosa cells	ZE or ZF	67	49 (73.1)	0 (0) ^a
Female A	Adult fibroblasts	ZE or ZF	139	111 (79.9)	7 (5.0) ^{a,b,c}
Male B	Adult fibroblasts	ZF	120	113 (94.2)	19 (15.8) ^d
Male C	Adult fibroblasts	ZF	616	560 (90.9)	8 (1.3) ^c
Cloned fetus of male C	Fetal fibroblasts	ZF	144	138 (95.5)	23 (16) ^d
Parthenogenetic control			91	80 (87.9)	12 (13.2) ^{b,d}

ZE, zona-enclosed; ZF, zona-free.

Values with different letters within columns differ significantly, $P < 0.05$ (Chi-square).

*Statistics were not applied because the first three groups were manipulated with both ZE and ZF methods and the last four groups only with ZF method and therefore they were not directly comparable. No difference was observed within the first three and within the last four groups.

(10/58) on Day 17 and per blastocyst was 7.3% (10/137). The pregnancy rate per mare/per blastocyst was 0%/0%, 5.6%/3.0% and 24.3/8.9% for NT-embryos derived from cumulus cells, fetal fibroblasts and adult fibroblasts respectively. Six pregnancies from NT-embryos obtained from adult cells were confirmed at Day 35. The highest pregnancy rates were obtained in recipients when Day 7 and Day 8 NT-blastocysts were transferred on Day 4 or 5 of the cycle (data not shown). A single pregnancy derived from NT-embryos reconstructed with fetal fibroblasts (derived from a previously aborted fetus from male C, Galli *et al.* 2003b) aborted at 180 days and one pregnancy from NT-embryos reconstructed from adult fibroblasts of male C was lost at 231 days of gestation. The two late abortions were both from the same genotype (male C), one from adult cells and one from fetal cells. Remarkably, both shared the same abnormality (see Fig. 2b and d) on the umbilical cord with a cystic structure filled with allantoic fluid; this cyst was likely to be a dilatation of the uracus and was the only abnormality observed in otherwise normal fetuses and placentae at necropsy. The placenta of the fetus originating from the fetal cells presented an area without villi (see Fig. 2a). The fetus was aborted at 180 days (Fig. 2c) and was apparently normal at necropsy. Two pregnancies developed to term and delivered two live foals from the same male genotype (B). The first foal was delivered after 344 days of pregnancy; it weighed 49 kg while the placenta was 4.6 kg. This foal was apparently normal, stood up on its own, and nursed within 2 h after birth. However, 24 h after birth he developed a mas-

sive diarrhea that rapidly caused the death of the foal 40 h after birth. Laboratory analysis and anatomo-pathological examination confirmed that the foal developed a hemorrhagic enteritis that evolved into septicemia, while no other gross morphological abnormalities were reported at necropsy. The second foal was delivered after 329 days of gestation; it weighed 42 kg and the placenta was 4.1 kg. Like the previous foal, this one stood up on its own, nursed within 2 h after birth and developed well without any sort of problems (Fig. 3). At present he is 2 months old and growing very well.

Discussion

In our study we found that the use of the zona-free method for nuclear transfer embryo reconstruction significantly increased the fusion rate - up to nearly 100% after two rounds of cell fusion. Interestingly, this result was favorable compared with the combination of cell fusion and treatment with inactivated Sendai virus of zona-enclosed couplets (87%; Li *et al.* 2002). This effect might be the result of both the cleaner cytoplasmic membrane that favored a closer contact between the oocyte and the somatic cell membrane as well as the reduced DC field required to achieve successful fusion (1.8–2.4 kV/cm vs 1.2 kV/cm). A high DC field also caused some couplets to lyse (our observations). This low-strength field is also a possible explanation why we did not find differences in the cleavage rate between parthenogenetic and nuclear transfer zona-free embryos. This was not the case for

Table 3 Embryo transfer and pregnancy

Donor cells	Horse	No. of embryos	No. of recipients	Pregnancy (n (%))	35 days (n (%))	3 months (n (%))	6 months (n (%))	Offspring (n (%))
Cumulus 22 hIVM	*	3	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fetal fibroblasts§	C	33	18	1 (5.6)	1 (5.6)	1 (5.6)	1 (5.6)	0 (0)
Adult fibroblasts	A	4	2	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)
Adult fibroblasts	B	71	23	6 (26.1)	4 (17.4)	3 (13)	2 (8.7)	2 (8.7)
Adult fibroblasts	C	26	12	2 (16.7)	2 (16.7)	1 (8.3)	1 (8.3)	0 (0)
Adult fibroblasts	Total	101	37	9 (24.3)	6 (16.2)	4 (10.8)	3 (8.1)	2 (5.4)

*cumulus cells of oocytes from slaughtered mares; § fetal fibroblasts derived from an aborted cloned fetus of male C.

Chi square, $P < 0.05$ statistical differences.

Figure 2 (a) Placenta of the fetus derived from an NT-horse blastocyst from fetal cells of male C; the arrow indicates the area without villi. (b) Umbilical cord of the same placenta with an allantoic fluid filled cystic structure (bar = 10 cm). (c) Fetus of the placenta in (a) aborted at 180 days of gestation (bar = 20 cm). (d) Umbilical cord of the fetus derived from an NT-horse blastocyst from an adult fibroblast of male C aborted at 231 days of gestation showing the same type of abnormality (bar = 10 cm).

Figure 3 The foal derived from an NT-horse blastocyst from an adult cell of male B next to his Haflinger surrogate mother.

zona-enclosed embryos as reported by Choi *et al.* (2003). For *in vivo* matured oocytes manipulated within the zona, Woods *et al.* (2003) reported that 90% fusion could be achieved after successive 2.25 kV/cm DC pulses until fusion occurred or the oocyte lysed. In our experience, bovine couplets subjected to more than two successive DC pulses to achieve fusion have low developmental competence. In our study, we also observed an associated increase in the cleavage rate that was reflected in an increased development to the blastocyst stage.

In our experience in the horse, contrary to other species, expanded cumulus–oocyte complexes maintain some developmental competence after *in vitro* fertilization by ICSI, although oocytes with a compacted cumulus give rise to more embryos (Galli *et al.* 2000). However, the classification between compacted and expanded cumulus is not well defined in the literature and therefore the selection can be influenced by subjective criteria applied by different people. This may explain why, in another study, no difference in development was observed between compact and expanded oocyte–cumulus complexes (Tremoleda *et al.* 2003). In nuclear transfer embryos, as shown in this study, no differences were observed between the two types of cumulus–oocyte complexes and therefore the only reason to grade oocytes before maturation is the fact that expanded oocytes mature 2–4 h earlier than compact oocytes so they can be manipulated earlier.

Amongst the several types of cells tested in the zona-free method (cumulus cells, adult and fetal fibroblasts) there were no observed differences in the cleavage rate nor were there observed differences with parthenogenetic activation in contrast to what has been reported by others (Choi *et al.* 2003, Li *et al.* 2003). Subsequent embryo development to the blastocyst stage was, however, much lower ranging from 0 to 16% on average.

As in mice (Wakayama & Yanagimachi 2001), pigs (Kühholzer *et al.* 2001, Yin *et al.* 2002, Lee *et al.* 2003) and cattle (Kato *et al.* 2000, Servely *et al.* 2003) we have found a significant difference in the developmental potential of NT-embryos derived from different cell cultures. Blastocyst formation rates with some donors were low and comparable to those found by Li *et al.* (2002). However, with one adult and one fetal cell line the development to the blastocyst stage was comparable to the parthenogenetic controls (15.8 and 16% vs 13% respectively) and comparable with *in vivo* data on development of cloned mule blastocysts on Day 14 (12%; Woods *et al.* 2003). These data indicate that the source of the donor nuclei for nuclear transfer is a major source of variability in Equids as in other species.

Interestingly, the only fetus derived from fetal fibroblasts was aborted at the same time as the fetus derived from the genetically identical adult fibroblasts and had the same type of abnormality on the umbilical cord (see Fig. 2). The number of transfers with cumulus cell-derived embryos was small in this study and did not result in any pregnan-

cies. However, we believe that this cell type can successfully serve as a nuclear donor in horse cloning as in cattle (Wells *et al.* 1998, Kato *et al.* 2000), mice (Wakayama & Yanagimachi 2001), rabbits (Chesne *et al.* 2002), goats (Keefer *et al.* 2002) and in mules where NT-embryos derived from cumulus cells were able to implant in recipient mares and to survive to Day 80 of pregnancy (Vanderwall *et al.* 2004).

Pregnancy losses reported here are typical of cloned pregnancies in other species (Heyman *et al.* 2002, Chavatte Palmer *et al.* 2004) as well as those reported by Woods *et al.* (2003) with cloned mule embryos. Fifty-seven percent (4/7) of NT-pregnancies from adult fibroblasts were lost between Days 17 and 35, in comparison with less than 15% of early embryonic death in normal breeding (Villahoz *et al.* 1985, Ball 1993). Two pregnancies resulted in the natural birth of two male foals. The average development to term was 5.4% and depended on donor cell culture as in other cloned animals (see Wilmut *et al.* 2002 for a review).

The first ever cloned foal (Galli *et al.* 2003b) that was born from a Haflinger female adult fibroblast NT-embryo is now about 20 months old. Incidentally, she was born to the recipient that served as the nuclear donor for the nuclear transfer experiment and therefore she is the genetic copy of the surrogate mother. Interestingly, the outcome of this experiment is contrary to the idea that the maintenance of gestation depends on adequate immunological recognition of fetal antigens by the mother (Szekeres-Bartho 2002).

The number of observations made on advanced pregnancies that aborted is limited; however, the pattern is similar to that of other species whereby abnormalities have a higher incidence in the extraembryonic tissues. In our case, the umbilical cord was abnormal, which was the same observation reported by us for a previous abortion (Galli *et al.* 2003b). One placenta had an area without villi, which is compatible with normal pregnancies; appearance and thickness were normal. Also the placentae of the pregnancies that went to term were normal and the weight was in the normal range (LeBlanc 1997). The foal that died perinatally developed septicemia. This pathological situation is described in horse neonatology and can be a cause of death for the foal. It is difficult to correlate this event to the fact that it was a cloned offspring; however, it is known that perinatal mortality of cloned offspring in other species is higher than normal (Chavatte-Palmer *et al.* 2002, Cibelli *et al.* 2002).

In conclusion, we have investigated the developmental capacity of NT-embryos derived from cumulus cells and from adult and fetal fibroblasts to develop *in vitro* and of embryo and fetal development *in vivo* following embryo transfer into recipients. We have shown that different cell cultures possess different capacities for supporting the pre-implantation development of horse NT-embryos and that blastocyst formation rate does not correlate with or predict future pregnancy fate. The zona-free method for embryo

reconstruction proved very efficient in increasing the fusion rate and the efficient use of oocytes. In this study, oocytes with expanded and compact cumulus had equal ability to support the preimplantation development of NT-embryos. Finally, horse embryos cloned from adult fibroblasts were able to implant and develop to term.

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