

Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts

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The developmental potential of bovine fetal fibroblasts was evaluated using nuclear transfer. Fibroblasts from a 37-day-old fetus were fused to enucleated oocytes before activation. Nuclei of starved (cultured for 8 days in medium containing 0.5% serum) fibroblasts supported the development of reconstructed embryos to the blastocyst stage significantly better than those of non-starved fibroblasts (39% versus 20%; $P < 0.05$). When nuclear transfer morulae derived from starved or non-starved fibroblasts were used for re-cloning, the proportion of blastocysts (52 and 55%, respectively) obtained with these embryonic nuclei was significantly higher than it was with fibroblast nuclei used in the first round of nuclear transfer ($P < 0.05$ and $P < 0.001$, respectively). After transfer of blastocysts derived from non-starved and starved fibroblasts, respectively, 33% (1/3) and 78% (7/9) of recipients were pregnant on day 30 as assessed by ultrasonography. On day 90, the corresponding pregnancy rates were 33% (1/3) and 63% (5/8). Two live male twin calves, derived from non-starved fibroblasts, were delivered by Caesarean section at day 281 of gestation. This study demonstrates a positive effect of serum starvation on the efficiency of nuclear transfer using bovine fetal fibroblasts. The efficiency of nuclear transfer could be further increased by re-cloning.

Introduction

Nuclear transfer using embryonic cells as donors of genetic material is an important method both for producing cloned animals and for research purposes. However, this approach has little value for the introduction of new genes or genetic modifications. The ability to produce cloned offspring by nuclear transfer from a cell population that can be maintained in culture or under other appropriate conditions offers numerous advantages in the fields of research, agriculture and biotechnology. An established cell line provides the opportunity to modify the genomes and select the required cell populations before embryo reconstruction (for review, see Wolf *et al.*, 1998).

Although embryonic stem (ES) cell technology is established in mice and used for a plethora of research applications, so far no cell lines are available from livestock species that meet all characteristics of mouse ES cells, that is, germ-line transmission after generation of chimaeric animals (Stice and Strelchenko, 1996). However, bovine inner cell mass cells in primary culture – a cell lineage that has the potential to be maintained as ES cells – supported the birth of live offspring when used as donors for nuclear transfer (Sims

and First, 1993; Itoh *et al.*, 1998). In sheep, Campbell *et al.* (1996a) and Wells *et al.* (1997) were able to produce live cloned offspring from embryonic disc cells that had been maintained in culture for up to 13 and up to 9 passages, respectively.

A number of attempts have been made to use cells from later developmental stages for nuclear transfer. Live sheep have been produced by nuclear transfer using fetal fibroblasts (Schnieke *et al.*, 1997; Wilmut *et al.*, 1997) or adult mammary gland cells (Wilmut *et al.*, 1997). In these studies, it has been proposed that successful reprogramming of the donor chromatin from differentiated cells may relate to the serum starvation of donor cells and the concomitant arrest in G0 of the cell cycle. Similarly, pregnancies have been reported in cattle after transfer to recipients of cloned embryos derived from serum-starved fetal fibroblasts (Wells *et al.*, 1998) or muscle and skin fibroblast-like cells (Vignon *et al.*, 1998). From the latter study, the birth of a live calf has been announced (see Butler, 1998). However, live calves have also been born after nuclear transfer using non-starved fetal fibroblasts (Cibelli *et al.*, 1998).

Since the studies mentioned above used only cells either with or without serum starvation, the aim of the present study was to evaluate the effect of serum starvation on the efficiency of nuclear transfer using bovine fetal fibroblasts. In addition, some of these nuclear transfer embryos were used

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for re-cloning to study the consequences of a second round of nuclear reprogramming on embryonic development.

Materials and Methods

Karyoplasts

Fetal fibroblasts were obtained from a 2.0 cm bovine fetus (37-day-old) of a 5-year-old German Simmental cow. The sex of the fetus was male as determined by PCR (Carvalho *et al.*, 1996). The isolated fetus was washed three times in Ca^{2+} - and Mg^{2+} -free PBS. The head and all internal organs were removed and the remnant tissues were cut into small pieces and dispersed by exposure to 0.1% (w/v) trypsin (Gibco, Grand Island, NY). The cell suspension was then transferred into 10 cm culture dishes containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal calf serum (Biochrom, Berlin), 2 mmol L-glutamine l^{-1} , 10^{-4} mol 2-mercaptoethanol l^{-1} , 2 mmol non-essential amino acids l^{-1} (Sigma, St Louis, MO), 100 iu penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (passage 0). The cells were cultured until sub-confluence (usually 2–3 days) at 37°C in 5% CO_2 in air and then frozen in 10% (v/v) dimethyl sulfoxide (Sigma) in culture medium and stored in liquid nitrogen. For experiments, the cells were thawed and cultured for up to four passages. Cells were used for nuclear transfer directly or after serum starvation (Campbell *et al.*, 1996a). The non-starved cells cultured until sub-confluence were used for nuclear transfer 2–3 days after passage. The culture medium was removed 1 day after passage to start serum starvation, the cells were washed three times with PBS and fresh medium containing 0.5% (v/v) FCS was added. The cells were then cultured for a further 8 days before use for nuclear transfer.

Immunofluorescence microscopy

Immunofluorescence detection of proliferating cell nuclear antigen (PCNA), which is an indicator of active DNA replication, was performed using non-starved or starved fibroblasts to evaluate whether the serum starvation altered the phase of the cell cycle of the donor cells. DNA replication (S phase) was also monitored using the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU).

Non-starved or starved cells were grown on gelatinized coverslips (18 mm \times 18 mm) for 2 or 8 days and then washed three times with PBS. Methanol fixation was carried out at -20°C for 6 min to recognize only the insoluble form of PCNA which is associated with the site of ongoing DNA synthesis during S-phase. Coverslips containing fixed cells were washed three times with PBS and covered with 50 μl fluorescein isothiocyanate (FITC)-conjugated mouse anti-PCNA antibody (1:50 in PBS containing 1% (w/v) BSA; (Dako, Hamburg)). After incubation for 1 h at 37°C in a humidified atmosphere, the coverslips were washed three times in PBS and once in distilled water. Alternatively, cells were incubated with 10 μmol 5-bromo-2'-deoxyuridine l^{-1} (Boehringer, Mannheim) at 37°C in a humid chamber for 45

min to detect BrdU incorporation. Cells were washed three times in PBS and fixed with 70% ethanol (in 50 mmol glycine buffer l^{-1} , pH 2.0) for 25 min at -20°C , and then the coverslips were washed three times in PBS and covered with 50 μl of a mouse monoclonal anti-BrdU antibody (1:10 in 66 mmol Tris-buffer l^{-1} containing 0.66 mmol MgCl_2 l^{-1} , 1 mmol 2-mercaptoethanol l^{-1} and nucleases for DNA denaturation; Boehringer). After incubation for 30 min at 37°C and washing in PBS, a FITC-conjugated sheep anti-mouse immunoglobulin (1:10 in PBS containing 1% (w/v) BSA; Boehringer) was added and cells were incubated for another 30 min at 37°C before final washing in PBS. The cells stained for PCNA, as well as for BrdU, were covered with 50 μl propidium iodide (1:250 in PBS; Sigma) and incubated for 5 min at room temperature and washed three times in PBS before mounting. Finally, the coverslips were mounted on glass slides in Vectashield (Vector Laboratories, Burlingame, CA) and sealed with nail varnish. Slides were viewed with a Zeiss Axiovert microscope fitted with a $\times 100$ oil immersion objective.

Nuclear transfer and embryo culture

The nuclear transfer procedure was that described by Zakhartchenko *et al.* (1995), with substantial modifications concerning the timing of fusion and activation of recipient oocytes. At 18–20 h after maturation, oocytes were denuded of cumulus cells and enucleated within 2 h. A single cell suspension of starved or non-starved fibroblasts was prepared by trypsinization 10–15 min before nuclear transfer.

At 20–22 h after maturation, individual fibroblasts were transferred into the perivitelline space of enucleated oocytes, equilibrated for 5 min at 39°C in Zimmermann cell fusion medium (Robl *et al.*, 1987), and transferred to a fusion chamber consisting of two electrodes 500 μm apart, overlaid with fusion medium. The karyoplast–cytoplasm complexes (KCCs) were exposed to a double electric pulse of 2.1 kV cm^{-1} for 10 μs to initiate their fusion (Zakhartchenko *et al.*, 1995). KCCs were placed in Ham's F-12 medium (Sigma) supplemented with 10% (v/v) fetal calf serum and maintained at 39°C in a humidified atmosphere of 5% CO_2 in air. Fusion rates were determined 30–60 min after the fusion pulse.

At 24 h after maturation, the fused KCCs were activated by a 5 min incubation in 7% (v/v) ethanol followed by 5 h culture in 10 μg cycloheximide ml^{-1} and 5 μg cytochalasin B ml^{-1} (Presicce and Yang, 1994). KCCs were transferred into 100 μl drops of CR-1 medium (Rosenkrans and First, 1991) supplemented with 10% (v/v) oestrous cow serum, which were covered by paraffin oil, and cultured for 7–8 days at 39°C in a humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 . The proportions of blastocysts and hatched blastocysts, respectively, were recorded on days 7 and 8 of *in vitro* culture. Nuclear transfer experiments with starved and non-starved fibroblasts were replicated six times.

Some day 5 fibroblast-derived nuclear transfer morulae were disaggregated and used for a second round of nuclear transfer (re-cloning). In these experiments, enucleated

oocytes were activated 24 h after maturation as described above and then fused with embryonic cells at 31–32 h after maturation. Re-cloning experiments were replicated three times with donor embryos derived from both starved and non-starved fibroblasts.

Some day 8 blastocysts of excellent or good quality produced in the first round of nuclear transfer from starved fibroblasts were used for differential staining to count the numbers of cells in nuclear transfer embryos. Similar quality day 8 nuclear transfer blastocysts produced by the fusion of day 6 morula blastomeres with activated oocytes at 31–32 h after maturation served as controls. The inner cell mass (ICM) cells and trophectoderm (TE) cells were differentially stained with fluorochromes using the procedure described by Stojkovic *et al.* (1998).

Embryo transfer

Excellent or good quality embryos (classified in accordance with Lindner and Wright, 1983) that had been cultured *in vitro* for 7 days were transferred to synchronous recipients 7 days after standing oestrus which were treated by the injection of 2.0 ml Estrumate (Mallinckrodt Vet. GmbH, Burgwedel) to evaluate the viability of nuclear transfer blastocysts derived from fibroblast nuclei. The recipients were examined on day 30 after embryo transfer by ultrasonography for the presence of a conceptus, and by palpation per rectum at 42, 60 and 90 days of gestation.

DNA analysis

DNA samples were compared using microsatellite analysis to determine whether the retrieved calves have the same genotype as the donor fibroblasts. The analysis of microsatellite loci was performed using the StockMarks™ Kit for Cattle Bovine Paternity PCR Typing (PE Applied Biosystems, Weiterstadt) which is based on nine microsatellite loci and uses four-dye fluorescent labelling. Fluorescence data collected by GeneScan software were exported directly to the Genotyper software for automated genotyping (PE Applied Biosystems).

Statistical analysis

Differences between experimental groups were verified using chi-squared analysis (fusion rates and development

Table 1. Immunofluorescence staining of non-starved or starved bovine fibroblasts

Fibroblasts	Positive cells for PCNA (% ± SD)	Positive cells for BrdU (% ± SD)
Non-starved	214/845 (25.3 ± 1.3) ^a	204/888 (22.9 ± 2.7) ^a
Starved	47/848 (5.4 ± 1.1) ^b	34/846 (3.9 ± 0.8) ^b

BrdU, 5-bromo-2'-deoxyuridine; PCNA, proliferating cell nuclear antigen.

^aValues within columns with different superscripts differ ($P < 0.001$)

rates). Differences between the proportions of cells positively stained for PCNA and BrdU incorporation as well as ICM and total cell numbers were analysed by Mann–Whitney U test. $P < 0.05$ was considered significant.

Results

Effects of serum starvation on the cell cycle of fetal fibroblasts

Immunofluorescence detection of PCNA or BrdU showed that serum starvation significantly decreased the proportions of fibroblasts that were in S phase (5 and 4% of the starved fibroblasts versus 25 and 23% of non-starved fibroblasts, respectively, for PCNA and BrdU; $P < 0.001$; Table 1).

Effects of serum starvation on the development *in vitro* of reconstructed embryos

Fusion rates obtained with starved fibroblasts tended to be higher than those obtained with non-starved cells (Table 2). Nuclear transfer embryos derived from starved fibroblasts cleaved at a significantly ($P < 0.05$) higher rate than those derived from non-starved fibroblasts. The nuclei of starved fibroblasts supported the development to blastocysts and hatched blastocysts significantly ($P < 0.05$) better than those of non-starved fibroblasts.

Cell number analysis of day 8 nuclear transfer blastocysts

Morphologically, day 8 nuclear transfer blastocysts derived from fibroblasts ($n = 21$) were similar to those obtained from embryonic cells ($n = 17$). There were no significant differences ($P > 0.05$) in the numbers of inner cell mass cells (54.1 ± 11.3 and 50.9 ± 17.5 in fibroblast-derived

Table 2. Development *in vitro* of nuclear transfer embryos derived from non-starved or starved bovine fetal fibroblasts

Fibroblast type	Number of karyoplast–cytoplast complexes	Fused (%)	Cleaved* (%)	Blastocyst* (%)	Hatched blastocyst* (%)
Non-starved	242	174 (72) ^a	115 (66) ^a	35 (20) ^a	24 (14) ^a
Starved	254	205 (81) ^a	158 (77) ^b	80 (39) ^b	58 (28) ^b
Total	496	379 (76)	273 (72)	115 (30)	82 (22)

*Percentages were calculated on the basis of the number of fused embryos.

^aValues within columns with different superscripts differ ($P < 0.05$).

Table 3. Development *in vitro* of bovine embryos reconstructed with donor nuclei from fibroblast-derived nuclear transfer morulae

Source of donor morulae	Number of karyoplast-cytoplasmic complexes	Fused (%)	Cleaved* (%)	Blastocyst* (%)	Hatched blastocyst* (%)
Non-starved fibroblasts	65	58 (89)	50 (86)	32 (55)	23 (40)
Starved fibroblasts	103	91 (88)	73 (80)	47 (52)	36 (40)
Total	168	149 (89)	123 (83)	79 (53)	59 (40)

*Percentages were calculated on the basis of the number of fused embryos.

and embryonic cell-derived blastocysts, respectively) and total cells (132.3 ± 31.7 and 152.9 ± 37.5 in fibroblast-derived and embryonic cell-derived blastocysts, respectively).

Re-cloning of fibroblast-derived nuclear transfer embryos

When nuclear transfer morulae derived from starved or non-starved fibroblasts were used for re-cloning (Table 3), the rates of fusion, cleavage, and development to blastocysts and hatched blastocysts were not affected by the source of donor nuclei. The proportions of blastocysts obtained with embryonic nuclei from fibroblast-derived nuclear transfer morulae were significantly higher than those obtained with the fibroblast cell nuclei used in the first round of nuclear transfer (Fig. 1; starved: 52 versus 39%, $P < 0.05$; non-starved: 55 versus 20%, $P < 0.001$). In addition, re-cloning increased the proportion of hatched blastocysts (Table 3).

Development *in vivo* of fibroblast-derived nuclear transfer blastocysts

After transfer of blastocysts derived from non-starved and starved fibroblasts, respectively, 33% (1/3) and 78% (7/9) recipients were pregnant on day 30 as assessed by ultrasonography (Table 4). One recipient of a starved fibroblast-derived blastocyst was slaughtered on day 60 to evaluate the morphology of the fetus. This fetus did not show any obvious abnormalities. Another fetus derived from a starved fibroblast was aborted on day 78 of gestation. Pregnancy was confirmed on day 90 in 1/3 (33%) and in 5/8 (63%) recipients that received blastocysts derived from non-starved and starved fibroblasts, respectively. In the starved-fibroblasts group, abortion had to be induced between 5 and 6 months of gestation in two recipients owing to

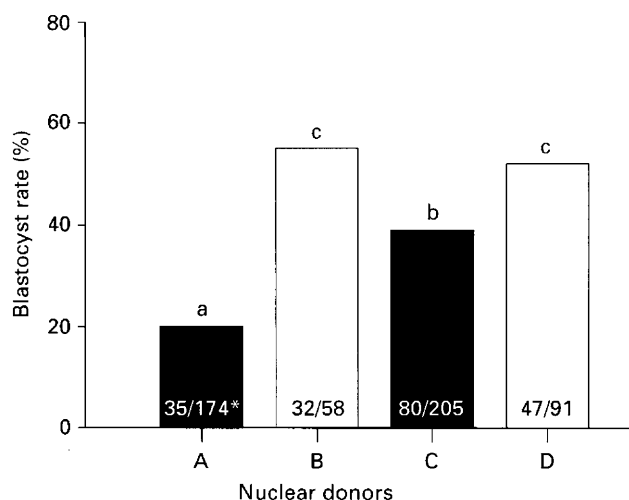


Fig. 1. Effect of first and second rounds of nuclear transfer on development *in vitro* of non-starved and starved fibroblast-derived embryos. A, non-starved fibroblasts; B, non-starved fibroblast-derived morulae; C, starved fibroblasts; D, starved fibroblast-derived morulae. ^{abc}Values with different superscripts differ (chi-squared, $P < 0.05$). *Blastocysts from fused karyoplast-cytoplasmic complexes.

hydroallantois. Each of these recipients carried one normal and one oversized fetus. DNA microsatellite analysis confirmed that all these clones originated from the fibroblast cell line (Table 5). One recipient aborted a starved fibroblast-derived fetus at the eighth month of pregnancy and the second one, carrying the same type of fetus, was induced to abortion at the last month of pregnancy owing to hydroallantois. Two live male twin calves, derived from non-starved fibroblasts, were delivered by Caesarean section on day 281 of gestation. The birth weights of these calves

Table 4. Development *in vivo* of nuclear transfer bovine embryos derived from non-starved or starved fetal fibroblasts

Fibroblast type	Number of transferred blastocysts	Number of recipients	Number pregnant on day (%)				Number of fetuses on day (%)*				Number of calves (%)*
			30	60	90	210	30	60	90	210	
Non-starved	7	3	1/3 (33)	1/3 (33)	1/3 (33)	1/3 (33)	2 (28)	2 (28)	2 (28)	2 (28)	2 (28)
Starved	16	9	7/9 (78)	6/8 (75)**	5/8 (63)	2/8 (25)	9 (56)	9 (56)	7 (44)	2 (13)	0

*On the basis of the number of transferred blastocysts.

**One recipient was slaughtered on day 60.

Table 5. Microsatellite analysis of nine loci using the StockMarks™ kit

Locus/allele	BEF	CC1-98	RM1-98	CCI324	CCII324	RM324	CCI325	RM325
TGLA57 1	93.36*	93.37	86.67	93.44	93.42	93.27	93.28	93.37
TGLA57 2	97.85	97.88	93.41	97.87	97.83	93.27	97.82	95.52
TGLA73 1	112.89	112.81	116.69	112.90	112.77	116.60	112.75	116.53
TGLA73 2	118.80	118.70	118.68	118.68	118.73	116.60	118.64	118.61
MGTG4B 1	131.95	131.99	132.02	131.87	131.77	131.88	131.85	131.72
MGTG4B 2	131.95	131.99	134.11	131.87	131.77	131.88	131.85	146.90
AGLA293 1	228.11	228.30	220.02	227.99	227.90	224.01	227.92	227.69
AGLA293 2	228.11	228.30	224.06	227.99	227.90	227.90	227.92	238.48
TGLA48 1	74.01	74.14	72.06	74.10	74.10	71.93	74.00	71.81
TGLA48 2	74.01	74.14	72.06	74.10	74.10	71.93	74.00	73.90
TGLA122 1	138.31	138.21	146.79	138.30	138.19	138.31	138.19	146.77
TGLA122 2	146.84	146.78	146.79	146.81	146.81	150.89	146.81	146.77
TGLA263 1	116.21	116.37	118.68	116.18	116.12	105.81	116.12	105.78
TGLA263 2	118.37	118.49	118.68	118.27	118.30	118.35	118.20	118.17
TGLA53 1	151.01	151.12	157.58	150.89	151.02	159.50	150.89	159.62
TGLA53 2	167.86	167.96	165.95	167.75	167.82	159.50	167.73	165.73
TGLA126 1	112.25	112.08	112.21	112.15	112.13	110.31	112.10	110.25
TGLA126 2	112.25	112.08	119.85	112.15	112.13	110.31	112.10	110.25

BEF: fibroblast cell line; CC1-98, CCI324, CCII324, CCI325: retrieved cloned calves; RM1-98, RM324, RM325: recipient mothers.

Both alleles (1 and 2) from each locus are shown. Microsatellite loci TGLA227 and MGTG7 amplified poorly in all samples and were not considered for paternity testing. Numbers are fragment lengths in base pairs as calculated by computer-aided analysis using an internal lane molecular size standard provided with the StockMarks™ Cattle Bovine Paternity PCR Typing Kit (PE Applied Biosystems).

were 31 and 50 kg. The larger calf died 3 days after birth owing to insufficient pulmonary function, while the other is apparently normal and healthy.

Discussion

The effects of serum starvation and re-cloning were studied to optimize the efficiency of nuclear transfer using cultured bovine fetal fibroblasts. The proportion of nuclear transfer blastocysts derived from fibroblast cell nuclei ranged from 20% (non-starved) to 39% (starved) and was in the same range as values obtained after fusion of blastomeres from day 5 (19%) or day 6 morulae (34%) with pre-activated aged (42–45 h after maturation) oocytes (Zakhartchenko *et al.*, 1997). The advantages of using pre-activated versus non-activated cytoplasts depend on the cell cycle stage of donor nuclei. If donor cells are in mitosis (Kwon and Kono, 1996; Liu *et al.*, 1997) or in the G1 phase (Collas *et al.*, 1992a; Cheong *et al.*, 1993) of the cell cycle, nuclear transfer is most efficient with non-activated cytoplasts. However, transfer of nuclei in S or G2 phase to non-activated cytoplasts with high concentrations of maturation promoting factor leads to nuclear envelope breakdown (NEBD) and premature chromatin condensation (PCC), resulting in damaged chromatin or tetraploidy, respectively (Campbell *et al.*, 1996b). In contrast to embryonic cells which are mainly in S phase (Barnes *et al.*, 1993), fetal fibroblasts were most efficient in combination with non-activated cytoplasts (Wells *et al.*, 1998). However, with pre-activated cytoplasts, nuclear transfer using fibroblasts or fibroblast-like muscle and skin cells was relatively inefficient (7–10% development to blastocysts; Vignon *et al.*, 1998).

It has been suggested that NEBD and PCC are essential for

the reprogramming of gene expression (Collas *et al.*, 1992b) and are important processes for the development of nuclear transfer embryos to term (Cheong *et al.*, 1994), and it has been argued that prolongation of the period of exposure of the donor nucleus after loss of its nuclear envelope to cytoplasmic factors enhances the developmental potential of the reconstructed embryo (Szöllosi *et al.*, 1988). In cattle, Wells *et al.* (1998) obtained a significantly higher proportion of blastocysts by fusion of fibroblasts with recipient cytoplasts 4–8 h before activation than when reconstructed embryos were activated and fused simultaneously (52 versus 25%). These authors suggest that the increase in embryo development obtained is due to the facilitation of nuclear remodelling and reprogramming.

In the present study, the development of nuclear transfer embryos to the blastocyst stage was significantly improved when donor fibroblasts were serum starved. Immunofluorescence staining showed that the proportion of fibroblasts in S phase of the cell cycle decreased significantly after serum starvation. Serum starvation has been shown to synchronize donor cells in G0 of the cell cycle (Wilmot *et al.*, 1997). The chromatin of quiescent nuclei has been reported to undergo a number of modifications, including a reduction in transcription and chromatin condensation (Whitfield *et al.*, 1985). Such modifications may change the chromatin structure in such a way as to make it more readily reprogrammed after nuclear transfer.

However, an acceptable rate of development to the blastocyst stage was also obtained without serum starvation of donor fibroblasts, indicating that this treatment is not necessary for nuclear reprogramming. Cibelli *et al.* (1998) have produced cloned transgenic calves from non-starved transfected fetal fibroblasts. On the basis of the fact that rapid cell divisions occur in early embryonic development and the

assumption that this may be an indication of relatively undifferentiated cells, these authors selected mitotically active cells for nuclear transfer. However, the development to blastocysts (12%) was relatively low in this study. Furthermore, the proportion of cells in S phase has been overestimated due to inappropriate fixation procedures (reviewed in Campbell, 1998).

In addition to effects of serum starvation the efficiency of re-cloning of fetal fibroblast-derived nuclear transfer morulae was evaluated. When embryonic cells were used as initial nuclear donors, live calves were obtained from third generation nuclear transfer embryos (Stice and Keefer, 1993; Takano *et al.*, 1997). A study by Ectors *et al.* (1995) did not reveal differences in developmental potential between first and second generation clones, while Lewis *et al.* (1998) reported increased developmental rates after the third round of nuclear transfer. In amphibians, development has been enhanced by the transfer of donor nuclei into oocytes followed by re-cloning (Orr *et al.*, 1986). Differentiated nuclei developed to advanced tadpoles when the nuclei were transferred back into enucleated eggs after the initial transfer into the oocytes; nuclei that were not transferred back to the egg had lower developmental potential. These findings and the results of the present study suggest that an additional round of nuclear transfer exposes the donor nucleus more extensively to conditioning factors in oocyte cytoplasm, increasing the developmental potential of second generation nuclear transfer embryos.

Studies on bovine nuclear transfer using blastomeres as donor nuclei have shown a high incidence of pregnancy loss and placental abnormalities. The greatest number of established pregnancies was lost between day 30 and day 90 of gestation (Willadsen *et al.*, 1991). In the present study, from eight established pregnancies, only one was lost by abortion before day 90. However, developmental abnormalities including fetal oversize and hydroallantois occurred around or after mid-gestation. Further studies are necessary to unravel the potential causes of these abnormalities which may be due to experimental manipulations of the early embryonic stages.

In conclusion, the present study demonstrates a positive effect of serum starvation on the efficiency of nuclear transfer using bovine fetal fibroblasts. The efficiency of nuclear transfer may be further increased by re-cloning.

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