Zygote **12** (May), pp. 133–141. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002667 Printed in the United Kingdom

Effects of the time interval between fusion and activation on *in vitro* rabbit nuclear transfer efficiency when nuclear donor cells are derived from older adults

R.P. Cervera and F. Garcia-Ximénez
Laboratory of Animal Reproduction and Biotechnology (LARB-UPV), Polytechnic University of Valencia, Spain

Date submitted: 12.12.03. Date accepted: 19.01.04

Summary

Cloning older adult rabbits can serve as a model in animal breeding, biodiversity preservation and in human therapeutic cloning. To establish the required exposure time of fibroblasts from these kind of animals to reprogramming factors, in the present study three different time intervals between fusion and activation were tested (30 min, 30-ADF group; 60 min, 60-ADF group; and 90 min, 90-ADF group). Vitrified epithelial fibroblasts derived from four older adult rabbit females (D1, D2, D3 and D4) and cultured from passages 0 to 4 were used as nuclear donors. Nuclear status of reconstructed embryos was not evaluated. No differences were observed in blastocyst rate (30-ADF 21% vs 60-ADF 19% vs 90-ADF 18%). Differences in hatching rates did not reach significance (30-ADF 11% vs 60-ADF 18% vs 90-ADF 18%). However, in the 60- and 90-ADF groups, embryos reached the blastocyst stage earlier than in the 30-ADF group (day 4: 40% and 50% vs 8%; $p > 0.05$). Moreover, the quality of blastocysts (good vs poor) was lower in the 30-ADF group (good: 30-ADF 38% vs 60-ADF 90% vs 90-ADF 90%; $p > 0.05$). Overall, these results suggest an unfavourable effect of the shortest exposure time tested (30 min). Differences between specimen origins were detected (blastocyst and hatching rates: D2 (26%; 25%) and D4 (25%; 27%) vs D1 (10%; 11%) and D3 (12%; 12%)), but significance were not reached. Effect of culture passage was not detected in any parameter studied.

Keywords: MPF, Older adults, Rabbit, Reprogramming, Somatic cloning

Introduction

Somatic cloning nuclear transfer (SCNT) has been proposed for establishing new productive rabbit and pig strains since it maximizes selection pressure but without increasing consanguinity (Silvestre *et al*., 2002). Recently, a cryobank of epithelial tissue samples from rabbit specimens with exceptional longevity and general disease resistance characters was founded in our laboratory to provide nuclear donors for SCNT. However, SCNT technologies in this species are still inefficient, especially with the most interesting cell type: fibroblasts from adults. Moreover no reference to the use of fibroblasts from older adult individuals as nuclear donors was found in the literature. In fact, live cloned rabbits have been obtained only in one experiment (Chesné *et al*., 2002), but using fresh cumulus cells as nuclear donors.

The donor's ageing can affect SCNT efficiency because somatic cells from older adult donors contain numerous gene mutations accumulated through many rounds of cell divisions, due to the long exposure time to mutagenic factors (Mukherjee & Thomas, 1997) and to changes in the patterns of genomic methylation (Holliday, 2000). In addition, *in vitro* cell life span decreases with the donor's ageing (Cristofalo *et al*., 1998). However, success in cloning aged/older adult animals in some species has demonstrated that cells from such animals can be sufficiently reprogrammed to support normal development to term (Kubota *et al*., 2000; Hill *et al*., 2000; Tian *et al*., 2000; Taneja *et al*., 2001).

All correspondence to: Rita P. Cervera Juanes, Laboratory of Animal Reproduction and Biotechnology (LARB-UPV), Polytechnic University of Valencia, Camino de Vera, 14, 46071, Valencia, Spain. Tel: +34 96 3879433. Fax: +34 96 3877439. e-mail: ritcer@dca.upv.es

In rabbits, as in other species, cells derived directly from tissue explant outgrowths (primary cultures) are less likely to support blastocyst development than cells from subcultures (Dinnyés *et al*., 2001), possibly because DNA in cultured cells is less methylated and may be more accessible to reprogramming factors (Kubota *et al*., 2000). However, genetic alterations may occur when *in vitro* culture is excessively prolonged (McCreath *et al*., 2000; Denning *et al*., 2001; Eggan *et al*., 2001; Ogonuki *et al*., 2002; Tamashiro *et al*., 2002). Therefore, short-term culture donor cells (< 10 passages) have commonly been used for nuclear transfer (Schnieke *et al*., 1997; Cibelli *et al*., 1998; Baguisi *et al*., 1999; Wells *et al*., 1999; Onishi *et al*., 2000; Polejaeva *et al*., 2000; Hill *et al*., 2000).

Both the individual origin and sex also affect development of SCNT embryos (Albeiro *et al*., 2001; Booth *et al*., 2003). Obviously, both factors should be taken into account when biodiversity preservation, strain foundation or, in the future, therapeutic somatic cloning are the objectives.

When an interphase donor nucleus is introduced into a high maturation promoting factor (MPF) milieu it undergoes nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC). Therefore, reprogramming factors present in the ooplasm could have free access to the DNA from the donor cell (Campbell *et al*., 1996; Bordignon *et al*., 1999, 2001; Kim *et al*., 2002). Emerging evidence from several species indicates that somatic cell nuclei, unlike embryonic blastomeres, need NEBD and PCC in order to be adequately reprogrammed (Campbell *et al*., 1996; Wilmut *et al*., 1997; Campbell, 2002), although nuclear reprogramming would occur later, when the nuclear envelope is re-formed and chromatin is decondensed (Stice & Robl, 1988; Kim *et al*., 2002). It is unclear, however, what the minimum time is that a somatic nucleus should be exposed to a high level of MPF for complete reprogramming (Wilmut *et al*., 1997; Wakayama *et al*., 1998, 1999). In fact, studies in which different time intervals between fusion and activation were compared are scarce. In pigs, Koo *et al*. (2000), using fetal fibroblasts as nuclear donors, assayed several time intervals between fusion and activation, obtaining a higher blastocyst rate with the shortest time interval assayed (2 h). Martínez-Díaz *et al*. (2002) obtained a higher blastocyst rate when fusion and activation occurred simultaneously, but using primary cultures of granulosa cells as nuclear donors. Wakayama *et al*. (1998), working in mice and using fresh granulosa cells as nuclear donors, obtained a higher rate of both blastocysts and cloned offspring when activation was delayed after fusion. In cattle, only Liu *et al*. (2001), using regenerated cloned fetal fibroblasts, assayed two time intervals between fusion and activation, obtaining a higher blastocyst rate with the longer time interval assayed. Thus, it may be proposed that the minimum period of exposure to ooplasmic factors required to reprogramme donor nucleus could be associated with species, cell type (*cattle*: fetal fibroblasts: Cibelli *et al*., 1998; mural granulosa cells: Wells *et al*., 1999; *pigs*: fetal cells: Koo *et al*., 2000; Betthauser *et al*., 2000; granulosa cells: Polejaeva *et al*., 2000; Martínez-Díaz *et al*., 2002; *sheep*: mammalian epithelium: Wilmut *et al*., 1997), and also with donor ageing, as in the case of older adult cells, in which a long period of exposure to reprogramming factors would be needed.

In the case of the rabbit, high blastocyst rates were obtained when fusion and activation occurred simultaneously: 30%, adult fibroblasts (Mitalipov *et al*., 1999); 23%, cumulus cells (Yin *et al*., 2000); 10–28%, adult fibroblasts (Dinnyés *et al*., 2001); 40%, fetal fibroblasts (Li *et al*., 2002). However, in this species only Chesné *et al*. (2002), using fresh cumulus cells as nuclear donors and delayed activation after fusion (1 h), obtained both a high blastocyst rate (47%) and the development to term of the first cloned rabbit. Perhaps this cell type requires lower reprogramming than others (Wakayama *et al*., 1998; Yin *et al*., 2000).

The aim of the present work was to study the effect of different time intervals between fusion and activation on *in vitro* embryo development when fibroblasts from older adult rabbit does were used as nuclear donors. The effect of individual origin and culture passage was also studied.

Materials and methods

Oocyte collection

Cumulus–oocyte complexes (COCs) were recovered in Hepes-buffered Ham's F-10 supplemented with 0.15% (w/v) bovine serum albumin (hereafter: H-Ham's) from oviducts of adult mixed-breed females at 13 h after ovulation induction treatment (20 μg i.m. GnRF Veterín-Receptal, Hoechst Russel Vet, Barcelona, Spain).

Cumulus cells were fully disaggregated by 5 min of incubation in hyaluronidase solution (2 mg/ml; H4272, Type IV-S, Sigma, Madrid, Spain) at 32 °C followed by gentle pipetting in bicarbonate-buffered Ham's F-10 supplemented with 20% (v/v) fetal bovine serum (FBS) (hereafter: B-Ham's) under equilibrated mineral oil. Denuded oocytes were held in this same medium in 5% CO_2 in air at 32 °C until use.

Nuclear donor cells

Fibroblasts used as nuclear donors in the present experiment were from four older adult rabbit does

https://doi.org/10.1017/S0967199404002667 Published online by Cambridge University Press

(at least 7 years old). These females were the best phenotypically evaluated from 25 rabbit does detected and cryobanked to date (within a base population of approximately 50 000 rabbit does), with regard to a parity value of at least 30 births and a mean value of at least 9 live-born pups in all births. Identity of the four donor does used was maintained throughout all steps of the cloning process.

Nuclear donor fibroblasts were obtained from cryobanked skin samples which had been vitrified according to the procedure described by Silvestre *et al*. (2002). Fibroblasts were seeded and cultured in 35 mm plates containing DMEM (Dulbecco's Modified Eagle's Medium, Cat. No. 31885, Invitrogen, Barcelona, Spain) supplemented with 20% FBS (hereafter: S-DMEM) and incubated at 37 °C in 5% CO_2 in air. Passages 0 to 4 were used as nuclear donors.

Just before nuclear transfer, donor cells were collected from culture plates by standard trypsinization, pelleted at 200 *g* for 5 min, re-suspended in bicarbonate-buffered Ham's F-10 without serum and maintained in this medium at 32 °C in 5% CO_2 until use.

Nuclear transfer and artificial activation

Rabbit nuclear transfer was performed as described previously (Cervera & García-Ximénez, 2003) with the exception of the fusion and activation treatments applied. Fusion was performed at 16–19 h post-GnRF by the application of one DC pulse of 2.4 kV/cm for 60 μs in mannitol solution at 32 °C. Electrical pulses were delivered by a BTX Electrocell Manipulator 2001 (BTX, San Diego, CA). For activation, fused ooplast–donor cells (ODCs) were submitted, in the same mannitol solution (32 °C), to two DC pulses of 2.4 kV/cm for 60 μs, 8 min apart, either 30 min, 60 min or 90 min after the application of the fusion pulse and incubated for 1 h in B-Ham's containing 5 μg/ml CHX (Sigma) and 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) (Chesné *et al*., 2002) in 7% CO_2 in air at 39 °C under equilibrated mineral oil. After that, the embryos were cultured in B-Ham's in 7% CO_2 in air at 39 °C under equilibrated mineral oil.

At the end of the session, 1–5 h after the activation treatment, the presence of nuclear structures was recorded in reconstructed embryos (in all batches at the same time) as an indicator of an ongoing interphase.

In vitro development was assessed every 24 h throughout the culture period of 6 days. Nuclear status of reconstructed embryos was not evaluated.

Experimental design

Three experimental groups were established according to the activation delay from fusion treatment (ADF): 30 min (30-ADF), 60 min (60-ADF) and 90 min (90-ADF). Whatever the experimental group, it may be assumed that the electrical stimulus applied for fusion would be sufficient to start the reduction of the levels of MPF, which reached their basal values approximately 60–90 min later (Collas *et al*., 1995). In addition, the variability in the instant at which fusion occurs also makes the real period of exposure (and level of activity) to reprogramming factors variable.

The effect of these different time intervals between fusion and activation was evaluated on *in vitro* blastocyst development rate. Reconstructed embryo transfers were not carried out since in the rabbit the presence of a mucin coat is absolutely necessary to accomplish *in vivo* development to term, and therefore embryo transfer should be carried out at very early embryo development stages. Moreover, *in vivo* comparison of three experimental groups and the very low SCNT efficiency would require an unfeasible number of embryo transfers to detect any significant difference between experimental groups. Therefore, the comparison of two or more experimental groups was mainly carried out *in vitro* (Koo *et al*., 2000; Liu *et al*., 2001; Martínez-Díaz *et al*., 2002; Shin *et al*., 2002).

As indicated previously, four older adult rabbit does were used as nuclear donors, referred to as D1, D2, D3 and D4. Identified donor cells from culture passages 0 to 4 from these females were randomly used in all experimental groups.

Each experimental group was manipulated in a different order at each session to randomize the negative effects of *in vitro* postovulatory oocyte ageing on embryo development (Adenot *et al*., 1997).

Blastocyst and hatching rates were recorded on three successive days after nuclear transfer (days 4, 5 and 6).

At the end of the culture period (day 6) blastocysts were also classified, in a subjective manner, into two quality categories: good and poor blastocysts. Good blastocysts were defined as expanded or hatching blastocysts with a large blastocoelic cavity and an apparently large number of cells. Poor blastocysts showed a small blastocoelic cavity and an apparently low number of cells.

Statistical analysis

At least seven replicates were performed in all experimental groups. The results were analysed by the chi-square test. When a single degree of freedom was involved, Yates' correction for continuity was applied.

Results

The lower fusion rate obtained in the 90-ADF group could be explained by a reversion in cell fusion (Gaertig & Iftode, 1989), detected because fusion evaluation,

https://doi.org/10.1017/S0967199404002667 Published online by Cambridge University Press

Table 1 Effect of the time interval between fusion and activation on fusion, cleavage and blastocyst rates in rabbit reconstructed embryos, when fibroblasts from four different older adult does were used as nuclear donors

Experimental group	Number of ODCs	Fused/ODCs (%)	Cleaved/ cultured (%)	Blastocysts/ cultured (%)	Blastocysts/ cleaved (%)	Hatching blastocysts/ cleaved (%)
30-ADF	77	67/77 (78)[a]	55/63 (87)	13/63 (21)	13/55 (24)	6/55 (11)
60-ADF	71	56/71 (79)[a,b]	51/54 (94)	10/54 (19)	10/51 (20)	9/51 (18)
90-ADF	93	64/93 (69)[b]	51/56 (91)	10/56 (18)	10/51 (20)	9/51 (18)
Total	241	187/241 (78)	157/173 (91)	33/173 (19)	33/157 (21)	24/157 (15)

ODCs, ooplast–donor cells.
Within columns, data with different superscripts are significantly different ($p < 0.05$).

Table 2 Effect of the time interval between fusion and activation on timing of development of reconstructed embryos, evaluated as blastocyst rate at days 4, 5 and 6 after reconstruction

Experimental group	Development until blastocyst stage		
	at day 4 (%)	at day 5 (%)	at day 6 (%)
30-ADF	1/13 (8)	10/13 (77)	2/13 (15)
60-ADF	4/10 (40)	5/10 (50)	1/10 (10)
90-ADF	5/10 (50)	3/10 (30)	2/10 (20)
Total	10/33 (30)	18/33 (55)	5/33 (15)

carried out just before activation treatment, was done at a later time in this group.

The rate of the presence of nuclear structures did not differ between experimental groups (30-ADF 73% (47/64) vs 60-ADF 78% (42/54) vs 90-ADF 63% (35/56); data not shown in tables). The high rates of both the presence of nuclear structures and first cleavage (Table 1) showed that the combined electrical and chemical treatment applied is an efficient procedure for both activation and zygotic progression of rabbit nuclear transfer embryos.

There were no significant differences among experimental groups as regards blastocyst rate, either in cultured or in cleaved nuclear transfer embryos (Table 1). Hatching rate was lower in the 30-ADF group, but this difference did not reach significance (Table 1).

The majority of nuclear transfer blastocysts were obtained at days 4 and 5 (30% and 55%, respectively). Most of the day 4 blastocysts belonged to the 60-ADF and 90-ADF groups (4/10 and 5/10 respectively vs 1/13 in the 30-ADF group; Table 2), although these differences did not reach significance, possibly due to the small final number of nuclear transfer embryos that reach blastocyst stage.

The rate of blastocysts categorized as good (foll owing the criteria described in Materials and Methods) was lower in the 30-ADF group (30-ADF 38% (5/13) vs 60-ADF 90% (9/10) and 90-ADF 90% (9/10); $p > 0.05$, data not shown in tables), although these differences did not reach significance, possibly due to the small number of nuclear transfer embryos that reach blastocyst stage.

The individual effect of each female on donor cell lysis, fusion and *in vitro* embryonic development rates was also studied, regardless of the time interval between fusion and activation and the cell culture passage. Donor cell lysis rate was higher in the case of female D4, but differences only reached significance when compared with female D3, possibly due to the small number of ODCs (Table 3). In the case of

Table 3 Effect of nuclear donor female on cell lysis, fusion and embryonic development rates

	Female			
	D1	D2	D3	D4
ODCs	97	62	65	17
Donor cell lysis/ODCs (%)	6 (6)[a,b]	3 (5)[a,b]	1 (2)[b]	3 (18)[a]
Fused/ODCs (%)	61/97 (63)[b]	55/62 (89)[a]	57/65 (88)[a]	14/17 (82)[a,b]
Cleaved/cultured (%)	56/59 (95)	40/50 (80)	50/52 (96)	11/12 (92)
Blastocysts/cultured (%)	6/59 (10)	13/50 (26)	6/52 (12)	3/12 (25)
Blastocysts/cleaved (%)	6/56 (11)	13/40 (33)	6/50 (12)	3/11 (27)
Hatching blastocysts/cleaved (%)	6/56 (11)	10/40 (25)	6/50 (12)	3/11 (27)

ODCs, ooplast–donor cells.
Between columns, data with different superscripts are statistically different ($p < 0.05$).

https://doi.org/10.1017/S0967199404002667 Published online by Cambridge University Press

Table 4 Effect of cell culture passage on cell lysis, fusion and embryonic development rates

	Cell culture passage				
	P0	P1	P2	P3	P4
ODCs	51	64	16	72	38
Donor cell lysis/ODCs (%)	6/51 (12)	3/64 (5)	1/16 (6)	0	3/38 (8)
Fused/ODCs (%)	42/51 (82)	53/64 (83)	14/16 (88)	49/72 (68)	29/38 (76)
Cleaved/cultured (%)	29/36 (81)	44/48 (92)	10/12 (83)	47/49 (96)	27/28 (96)
Blastocysts/cultured (%)	9/36 (25)	10/48 (21)	2/12 (17)	7/49 (14)	5/28 (18)
Blastocysts/cleaved (%)	9/29 (31)	10/44 (23)	2/10 (20)	7/47 (15)	5/27 (19)
Hatching blastocysts/cultured (%)	5/36 (14)	9/48 (19)	2/12 (17)	4/49 (8)	5/28 (18)
Hatching blastocysts/cleaved (%)	5/29 (17)	9/44 (20)	2/10 (20)	4/47 (9)	5/27 (19)

ODCs, ooplast–donor cells.

female D1 fusion rate was significantly lower than with females D2 and D3, while this difference did not reach significance when compared with female D4 (Table 3).

No differences were observed in cleavage rate among the four females used. Blastocyst and hatching rates both in cultured and in cleaved nuclear transfer embryos were higher for females D2 and D4 than for D1 and D3 (Table 3), although levels of significance were not reached.

No differences were detected in any of the recorded parameters according to the cell culture passage (Table 4), with the exception of a slightly (but not significantly) higher donor cell lysis rate in P0.

Discussion

In a previous work, our blind enucleation efficiency indirectly estimated by extrusion of the second polar body (PB2) before, throughout and after the electrical treatment, was approximately 40% (Cervera & García-Ximénez, 2003). In the present study, the same blind enucleation method was applied; however, only 4% (9/241; data not shown in tables) of reconstructed embryos extruded PB2, regardless of the experimental group, due to the short time interval between fusion and activation treatments (Collas & Robl, 1991; Escribá & García-Ximénez, 2000), and also due to 6-DMAP suppressing PB2 extrusion (Liu *et al*., 1998; Liu & Yang, 1999). Consequently, in the present study around 40% of reconstructed embryos would retain the oocyte nuclear material. But in these cases, cell-cycle asynchrony between somatic donor nucleus and resident nuclear material may interfere negatively with developmental ability to blastocyst stage. This situation could be different when the donor nucleus is an early embryo blastomere (Modlinski & Smorag, 1991), where there is great synchrony between nuclear cycles in the two nuclei; this could be the reason for the high blastocyst rate obtained in these cases. However, despite the inefficiency of blind enucleation we decided to apply this enucleation technique in our laboratory because it avoids the negative effects of ultraviolet light and Hoechst staining and also allows the manipulation time and time of exposure to CCB to be reduced (Cervera & García-Ximénez, 2003).

Blastocyst rate and/or blastocyst cell number are the most frequent parameters used to evaluate the *in vitro* efficiency of SCNT (Mitalipov *et al*., 1999; Tao *et al*., 1999; Tani *et al*., 2000; Koo *et al*., 2000; Dinnyés *et al*., 2001; Kühholzer *et al*., 2001; Kasinathan *et al*., 2001; Matsuda *et al*., 2002; Shin *et al*., 2002; Martínez-Díaz *et al*., 2002). Reaching blastocyst stage reflects the embryo's ability to survive to such stage, which is especially interesting since it goes through the maternal–zygotic transition and continues embryo development. However, reaching the blastocyst stage does not guarantee normality in ploidy, nor nuclear integrity and complete reprogramming of these 'self-selected' blastocysts (King, 1990; Munne *et al*., 1995). Some additional indicators of both normal ploidy and a sufficient level of nuclear reprogramming are the timing of blastocyst formation (King *et al*., 1987; Totey *et al*., 1996; Booth *et al*., 2003; Khorram *et al*., 2000) and hatchability (Khorram *et al*., 2000). In our case, timing of development, hatchability and blastocyst morphology, although not significant, are consistently markedly lower in the 30-ADF group of blastocysts. This fact may be due not to a different rate of ploidy defects derived from a different rate of enucleation failure between groups, but to alterations in both imprinted (Humphreys *et al*., 2001) and non-imprinted (Daniels *et al*., 2001; Wrenzycki *et al*., 2001) gene expression that could derive from such a short time interval between fusion and activation in this group. This can be especially determinant when the donor cell is from an older adult specimen, as in the present work.

https://doi.org/10.1017/S0967199404002667 Published online by Cambridge University Press

In the present study, differences observed between experimental groups can only be due to the different period of exposure of the somatic donor nucleus to reprogramming factors, even in these non-enucleated embryos, since the time of exposure to MPF does not affect the oocyte chromatin.

The negative effect of a short period of exposure to reprogramming factors detected by other authors (Wakayama *et al*., 1998; Wells *et al*., 1998; Koo *et al*., 2000; Shin *et al*., 2002) and by ourselves in the present work, could unacceptably limit the required time for integration of reprogramming factors to the donor chromosomes, and in consequence the level of reprogramming reached when the maternal–zygotic transition (MZT) concludes. At this time, the donor DNA should epigenetically be in an embryonic-like status able to express information required to carry out successful further post-transcriptional development. In fact, in our case, differences detected between nuclear donor cell origins emerged only at the blastocyst stage but not at earlier stages of development (cleavage stage). This same phenomenon was also detected between the three time intervals tested, since differences between them only emerged at the hatching blastocyst stage but not at the earliest stages. This reinforces the need for sufficient donor DNA reprogramming when the MZT concludes.

From a practical point of view, and although no differences between 60-ADF and 90-ADF groups were detected in any of the parameters evaluated, the slight oocyte ageing prior to activation in the 90-ADF group that could negatively affect further embryo development makes the use of a 60 min time interval between fusion and activation advisable in future work.

CHX and 6-DMAP accelerate the exit from metaphase II stage in artificial activation, but with potentially detrimental side effects on the cell cycle (Soloy *et al*., 1997; Meyer & Kim, 1997). For this reason, and given that rabbit zygotes enter S phase very early after activation, the period of exposure to these chemicals was reduced to 1 h (Chesné *et al*., 2002). After the reconstructed embryos were removed from this treatment they showed a dark cytoplasm and membrane distortion that disappeared some time later (personal observations). It is noteworthy that, contrary to the observations by Chesné *et al*. (2002), nuclear structures were not detected immediately after such treatment.

Individual identity of cell donors should be considered in SCNT applications such as animal breeding, biodiversity preservation and therapeutic cloning. The similar rates of cell lysis observed in the four nuclear donors showed that cells were affected in the same manner by the technical treatments (electrical stimulation, trypsinization, *in vitro* culture). However, the lower fusion rate obtained with D1 cells suggests differences in biological characteristics, such as membrane structure and/or properties (lipid and integral protein composition, surface charge density or mechanical viscoelasticity) (Sowers, 1992).

As each donor female was randomly used in each experimental group throughout the study, the lower blastocyst and hatching rates obtained when fibroblasts from females D1 and D3 were used as donors suggest possible differences in the methylation pattern of the donor cells (Renard *et al*., 2002), or, perhaps, differential nucleocytoplasmic interactions between donor cells and recipient cytoplasts (Booth *et al*., 2003).

In the present work, the higher donor cell lysis rate (12%) obtained with culture passage P0 suggests that these cells are more susceptible to the electrical pulse than cells from later passages, but no references have been found that relate to this. On the other hand, in our case the reduced number of culture passages used (until P4) avoided the detection of a possible effect of *in vitro* culture on the epigenetic status of somatic cells, as was proposed by Kubota *et al*. (2000) and Dinnyés *et al*. (2001).

In rabbits, rates of blastocyst development reached by both embryonic (3–44%: Collas & Robl, 1990, 1991; 9–17%: Modlinski & Smorag, 1991; 44%: Yang *et al*., 1992; 8–23%: Adenot *et al*., 1997; 9–16%: Piotrowska *et al*., 2000) and somatic nuclear transfer (*adult fibroblasts*: 30%: Mitalipov *et al*., 1999; 10–28%: Dinnyés *et al*., 2001; *cumulus cells*: 23%: Yin *et al*., 2000; 3%: Cervera *et al*., 2002; 47%: Chesné *et al*., 2002; 7%: Cervera & García-Ximénez, 2003; *fetal fibroblasts*: 8–40%: Li *et al*., 2002; 17–24%: Matsuda *et al*., 2002; 1%: Cervera & García-Ximénez, 2003; *older adult fibroblasts*: 10–26%: present work) are highly variable in both cases and not very different between them. This suggests that although reprogramming is absolutely required in the case of somatic cells (but not in blastomeres from early embryos), it is not the only factor determining nuclear transfer efficiency. Slight variations in most of the technical steps and working conditions could surely also be responsible for this low final efficiency. Thus, technical steps and working conditions (osmolarity, pH, light, Hepes buffer, temperature) should be refined in future work.

In conclusion, our results have shown that fibroblasts from older adult rabbits support blastocyst development, and that better hatching rate, blastocyst timing and morphology were obtained when donor nuclei were exposed to reprogramming factors for the longer periods of time tested (60 and 90 min). However, due the greater oocyte ageing and the possible reversion of fusion in the 90-ADF group, an interval between fusion and activation of 60 min will be applied. Cloning older adult rabbits may be significant as a model in animal breeding,

biodiversity preservation and human therapeutic cloning.

Acknowledgements

The authors thank Mr Neil Macowan for revising the English version of the manuscript. This work was supported by CICYT (AGL 2000-0595-C03-01).

References

Adenot, P.G., Szollosi, M.S., Chesné, P., Chastant, S. & Renard, J.P. (1997). *In vivo* aging of oocytes influences the behaviour of nuclei transferred to enucleated rabbit oocytes. *Mol. Reprod. Dev*. **46**, 325–36.

Albeiro, R., Brero, A., Motlik, J., Cremer, T., Wolf, E. & Zakhartchenko, V. (2001). Remodeling of donor nuclei, DNA-synthesis, and ploidy of bovine cumulus cell nuclear transfer embryos: effect of activation protocol. *Mol. Reprod. Dev*. **59**, 371–9.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. & Echelard, Y. (1999). Production of goats by somatic cell nuclear transfer. *Nat. Biotech*. **17**, 456–61.

Betthauser, J., Forsberg, E., Augenstein, M., Childs, L., Eilertsen, K., Enos, J., Forsythe, T., Golueke, P., Jurgella, G., Koppang, R., Lesmeister, T., Mallon, K., Mell, G., Misica, P., Pace, M., Pfister-Genskow, M., Strelchenko, L., Voelker, G., Watt, S., Thompson, S. & Bishop, M. (2000). Production of cloned pigs from *in vitro* systems. *Nat. Biotech*. **18**, 1055–9.

Booth, P.J., Viuff, D., Tan, S., Holm, P., Greve, T. & Callesen, H. (2003). Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. *Biol. Reprod*. **68**, 922–8.

Bordignon, V., Clarke, H.J. & Smith, L.C. (1999). Developmentally regulated loss and reappearance of immunoreactive somatic histone H1 on chromatin of bovine morula-stage nuclei following transplantation into oocytes. *Biol. Reprod*. **61**, 22–30.

Bordignon, V., Clarke, H.J. & Smith, L.C. (2001). Factors controlling the loss of immunoreactive somatic histone H1 from blastomeres nuclei in oocyte cytoplasm: a potential marker of nuclear reprogramming. *Dev. Biol*. **233**, 192–203.

Campbell, K.H.S. (2002). *Principles of Cloning*, pp. 391–9. San Diego: Academic Press.

Campbell, K.H.S., McWhir, J., Ritchie, W.A. & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380**, 64–6.

Cervera, R.P. & García-Ximénez, F. (2003). Oocyte age and nuclear donor cell type affect the technical efficiency of somatic cloning in rabbits. *Zygote* **11**, 151–8.

Cervera, R.P., Saeed, A.M., Silvestre, M.A., Escribá, M.J. & García-Ximénez, F. (2002). Comparison of different electrofusion treatments to be used in rabbit somatic cloning. *Invest. Agr.: Prod. Sanid. Anim*. **17**, 49–58.

Chesné, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. & Renard, J.P. (2002). Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotech*. **20**, 366–9.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de León, F.A. & Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–8.

Collas, P. & Robl, J.M. (1990). Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. *Biol. Reprod*. **43**, 877–84.

Collas, P. & Robl, J.M. (1991). Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol. Reprod*. **45**, 455–65.

Collas, P., Chang, T., Long, C. & Robl, J. (1995). Inactivation of histone H1 kinase by Ca^{2+} in rabbit oocytes. *Mol. Reprod. Dev*. **40**, 253–8.

Cristofalo, V.J., Allen, R.G., Pignolo, R.J., Martin, B.G. & Beck, J.C. (1998). Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. *Proc. Natl. Acad. Sci. USA* **95**, 10614–9.

Daniels, R., Hall, V.J., French, A.J., Korfiatis, N.A. & Trounson, A.O. (2001). Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. *Mol. Reprod. Dev*. **60**, 281–8.

Denning, C., Dickinson, P., Burl, S., Wylie, D., Fletcher, J. & Clark, A.J. (2001). Gene targeting in primary fetal fibroblasts from sheep and pig. *Cloning and Stem Cells* **2**, 251–3.

Dinnyés, A., Dai, Y., Barber, M., Liu, L., Xu, J., Zhou, P. & Yang, X. (2001). Development of cloned embryos from adult rabbit fibroblasts: effect of activation treatment and donor cell preparation. *Biol. Reprod*. **64**, 257–63.

Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W.M. 3rd, Yanagimachi, R. & Jaenisch, R. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc. Natl. Acad. Sci. USA* **98**, 6209–14.

Escribá, M.J. & García-Ximénez, F. (2000). Use of a variable electrical pulsing sequence in rabbit oocyte activation. *Reprod. Nutr. Dev*. **40**, 261–9.

Gaertig, J. & Iftode, F. (1989). Rearrangement of the cytoskeleton and nuclear transfer in *Tetrahymena thermophila* cells fused by electric field. *J. Cell. Sci*. **93**, 691–703.

Hill, J.R., Winger, Q.A., Long, C.R., Looney, C.R., Thompson, J.A. & Westhusin, M.E. (2000). Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. *Biol. Reprod*. **62**, 1135–40.

Holliday, R. (2000). Somatic mutations and aging. *Mutat. Res*. **463**, 173–8.

Humphreys, D., Eggan, K., Akutsu, H., Hochedlinder, K., Rideout, W.M. 3rd, Biniszkiewicz, D., Yanagimachi, R. & Jaenisch, R. (2001). Epigenetic instability in ES cells and cloned mice. *Science* **293**, 95–7.

Kasinathan, P., Knott, J.G., Moreira, P.N., Burnside, A.S., Jerry, D.J. & Robl, J.M. (2001). Effect of fibroblast donor cell age and cell cycle on development of bovine nuclear transfer embryos *in vitro*. *Biol. Reprod*. **64**, 1487–93.

Khorram, O., Shapiro, S.S. & Jones, J.M. (2000). Transfer of nonassisted hatched and hatching human blastocysts after *in vitro* fertilization. *Fertil. Steril*. **74**, 63–5.

Kim, J.M., Ogura, A., Nagata, M. & Aoki, F. (2002). Analysis of the mechanism for chromatin remodeling in embryos

reconstructed by somatic nuclear transfer. *Biol. Reprod*. **67**, 60–6.

King, W.A. (1990). Chromosome abnormalities and pregnancy failure in domestic animals. *Adv. Vet. Sci. Comp. Med*. **34**, 229–50.

King, W.A., Guay, P. & Picard, L. (1987). A cytogenetical study of 7-day-old bovine embryos of poor morphological quality. *Genome* **29**, 160–4.

Koo, D.B., Kang, Y.K., Choi, H.Y., Park, J.S., Han, S.K., Park, I.Y., Kim, S.U., Lee, K.K., Son, D.S., Chang, W.K. & Han, Y.M. (2000). *In vitro* development of reconstructed porcine oocytes after somatic cell nuclear transfer. *Biol. Reprod*. **63**, 986–92.

Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M. & Yang, X. (2000). Six cloned calves produced from adult fibroblasts cells after long-term culture. *Proc. Natl. Acad. Sci. USA* **97**, 990–5.

Kühholzer, B., Hawley, R.J., Lai, L., Kolber-Simonds, D. & Prather, R.S. (2001). Clonal lines of transgenic fibroblast cells derived from the same fetus result in different development when used for nuclear transfer in pigs. *Biol. Reprod*. **64**, 1695–8.

Li, G.P., Chen, D.Y., Lian, L., Han, Z.M., Zhu, Z.Y. & Seidel, G.E. Jr. (2002). Rabbit cloning: improved fusion rates using cytochalasin B in the fusion buffer. *Mol. Reprod. Dev*. **61**, 187–91.

Liu, L. & Yang, X. (1999). Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activated bovine oocytes. *Biol. Reprod*. **61**, 1–7.

Liu, L., Ju, J.C. & Yang, X. (1998). Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. *Mol. Reprod. Dev*. **49**, 298–307.

Liu, L., Shin, T., Pryor, J.H., Kraemer, D. & Westhusin, M. (2001). Regenerated bovine fetal fibroblasts support high blastocyst development following nuclear transfer. *Cloning* **3**, 1–8.

Martinez-Díaz, M.A., Ikeda, K. & Takahashi, Y. (2002). Effects of cycloheximide treatment and interval between fusion and activation on *in vitro* development of pig nuclear transfer embryo. *Reprod. Fertil. Dev*. **14**, 191–7.

Matsuda, J., Takahashi, S., Ohkoshi, K., Kaminaka, K., Kaminaka, S., Nozaki, C., Maeda, H. & Tokunaga, T. (2002). Production of transgenic chimera rabbit fetuses using somatic cell nuclear transfer. *Cloning and Stem Cells* **4**, 9–19.

McCreath, K.J., Howcroft, J., Campbell, K.H., Colman, A., Schnieke, A.E. & Kind, A.J. (2000). Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405**, 1066–9.

Meyer, L. & Kim, S.H. (1997). Chemical inhibitors of cyclin-dependent kinases. *Methods Enzymol*. **283**, 113–28.

Mitalipov, S.M., White, K.L., Farrar, V.R., Morrey, J. & Reed, W.A. (1999). Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-triphosphate. *Biol. Reprod*. **60**, 821–7.

Modlinski, J.A. & Smorag, Z. (1991). Preimplantation development of rabbit embryos after transfer of embryonic nuclei into different cytoplasmic environment. *Mol. Reprod. Dev*. **28**, 361–72.

Mukherjee, A.B. & Thomas, S. (1997). A longitudinal study of human age-related chromosomal analysis in skin fibroblasts. *Exp. Cell Res*. **235**, 161–9.

Munne, S., Alikani, M., Tomkin, G., Grifo, J. & Cohen, J. (1995). Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil. Steril*. **64**, 382–91.

Ogonuki, N., Inoue, K., Yamamoto, Y., Noguchi, Y., Tanemura, K., Suzuki, O., Nakayama, H., Doi, K., Ohtomo, Y., Satoh, M., Nishida, A. & Ogura, A. (2002). Early death of mice cloned from somatic cells. *Nat. Genet*. **30**, 253–4.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. & Perry, A.C.F. (2000). Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**, 1188–90.

Piotrowska, K., Moslinski, J.A., Korwin-Kossakowski, M. & Karasiewicz, J. (2000). Effects of preactivation of ooplasts or synchronization of blastomere nuclei in G1 on preimplantation development of rabbit serial nuclear transfer embryos. *Biol. Reprod*. **63**, 677–82.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. & Campbell, K.H. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407**, 86–90.

Renard, J.P., Zhou, Q., Le Bourhis, D., Chavatte-Palmer, P., Hue, I., Heyman, Y. & Vignon, X. (2002). Nuclear transfer technologies: between successes and doubts. *Theriogenology* **57**, 203–22.

Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A. & Campbell, K.H. (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278**, 2130–3.

Shin, M.R., Park, S.W., Shim, H. & Kim, N.H. (2002). Nuclear and microtubule reorganization in nuclear-transferred bovine embryos. *Mol. Reprod. Dev*. **62**, 74–82.

Silvestre, M.A., Saeed, A.M., Escribá, M.J. & García-Ximénez, F. (2002). Vitrification and rapid freezing of rabbit fetal tissues and skin samples from rabbits and pigs. *Theriogenology* **58**, 69–76.

Soloy, E., Kanka, J., Viuff, D., Smith, S.D., Callesen, H. & Greve, T. (1997). Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. *Biol. Reprod*. **57**, 27–35.

Sowers, A.E. (1992). Mechanisms of electroporation and electrofusion. In *Guide for Electroporation and Electrofusion* (ed. D.C. Chang, B.C. Chassy, J.A. Saunders & A.E. Sowers), pp. 119–38. California: Academic Press.

Stice, S.L. & Robl, J.M. (1988). Nuclear reprogramming in nuclear transplant rabbit embryos. *Biol. Reprod*. **39**, 657–64.

Tamashiro, K.L., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J.L., Wortman, M.D., Seeley, R.J., D'Alessio, D.A., Woods, S.C., Yanagimachi, R. & Sakai, R.R. (2002). Cloned mice have an obese phenotype not transmitted to their offspring. *Nat. Med*. **8**, 262–7.

Taneja, M., French, R. & Levine, H. (2001). Clinical and pathological status of cloned calves born pre-term (abstract). *Theriogenology* **55**, 293.

Tani, T., Kato, Y. & Tsunoda, Y. (2000). Developmental potential of cumulus cell-derived cultured cells frozen in

a quiescent state after nucleus transfer. *Theriogenology* **53**, 1623–9.

Tao, T., Boquest, A.C., Machaty, Z., Petersen, A.L., Day, B.N. & Prather, R.S. (1999). Development of pig embryos by nuclear transfer of cultured fibroblast cells. *Cloning* **1**, 55–62.

Tian, X., Xu, J. & Yang, X. (2000). Normal telomere lengths found in cloned cattle. *Nat. Genet*. **26**, 272–3.

Totey, S.M., Daliri, M., Appa Rao, K.B.C., Pawshe, C.H., Taneja, M. & Chillar, R.S. (1996). Differential cleavage and developmental rates and its correlation with the cell number and sex ratio of buffalo embryos generated *in vitro*. *Theriogenology* **45**, 521–33.

Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. & Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–74.

Wakayama, T., Rodriguez, I., Perry, A.C., Yanagimachi, R. & Mombaerts, P. (1999). Mice cloned from embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **96**, 14984–9.

Wells, D.N., Misica, P.M., Tervit, H.R. & Vivanco, W.H. (1998). Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod. Fertil. Dev*. **10**, 369–78.

Wells, D.N., Misica, P.M. & Tervit, H.R. (1999). Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod*. **60**, 996–1005.

Wilmut, I., Schnieke, A.E., McWhir, A., Kind, A.J. & Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–13.

Wrenzycki, C., Herrmann, D., Keskintepe, L., Martins, A., Sirisathien, S., Brackett, B. & Niemann, H. (2001). Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Hum. Reprod*. **16**, 893–901.

Yang, X., Jiang, S.E., Kovacs, A. & Foote, R.H. (1992). Nuclear totipotency of cultured rabbit morulae to support full-term development following nuclear transfer. *Biol. Reprod*. **47**, 636–43.

Yin, X.J., Tani, T., Kato, Y. & Tsunoda, Y. (2000). Development of rabbit parthenogenetic oocytes and nuclear-transferred oocytes receiving cultured cumulus cells. *Theriogenology* **54**, 1469–76.

https://doi.org/10.1017/S0967199404002667 Published online by Cambridge University Press