Zygote **12** (November), pp. 305–313. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002904 Printed in the United Kingdom

Detection of rare Leydig cell hypoplasia in somatic cell cloned male piglets

Mi-Rung Park[1], *Seong-Keun Cho*[1], *Jong-Yi Park*[1], *So-Young Lee*[1], *Yun-Jung Choi*[1], *Deug-Nam Kwon*[1], *Woo-Jin Son*[2], *Han-Geuk Seo*[3] *and Jin-Hoi Kim*[1]
GyeongSang National University, Jinju, GyeongNam, Korea

Date submitted: 12.5.04. Date accepted: 27.7.04

Summary

In this investigation, 22 cloned male piglets were obtained by male fetal fibroblast-cell-derived nuclear transfer. Eighteen of the cloned animals died. The two cell lines did not differ significantly with regard to efficiency of live piglet production. The gross anatomy of the testes of male piglets that died was normal. However, one piglet displayed Leydig cell hypoplasia (LCH). No anatomical defects were detected in the testes of other cloned male piglets. TUNEL analysis of the testis with LCH revealed significant apoptosis in the Leydig cells, while apoptosis was rarely detected in Sertoli cells and spermatogonia. In contrast, testes from the remaining 17 piglets that died appeared normal in size, and their Sertoli and Leydig cell numbers were comparable to those in control piglet testes. Although cloned piglets were derived from fibroblasts obtained from the same fetus, phenotypic instability between cells used for the production of somatic cell cloned piglets suggests that abnormalities in male cloned piglets are caused not by technical problems and/or reprogramming effects, but rather by epigenetically and/or genetically damaged cell-specific effects.

Keywords: Apoptosis, Leydig cell hypoplasia, Nuclear transfer, Pig, Somatic cell cloning

Introduction

Somatic cell cloning has been successfully achieved using both adult and fetal cells (Wilmut *et al*., 1997; Wells *et al*., 1997; Wakayama *et al*., 1998; Cibelli *et al*., 1998; Renard *et al*., 1999). Cloning is clearly of great benefit to commerce and conservation, given its potential applications in agriculture, animal and human medicine, and the propagation of rare animal species (Wall *et al*., 1997; Wilmut *et al*., 1998; Stice *et al*., 1998). Approximately 60–70% of mouse embryos generated by somatic cell nuclear transfer (scNT) survive to the blastocyst stage, in contrast to only 10–20% of embryos produced by embryonic stem cell NT (Wakayama *et al*., 1999). This is possibly mainly due to the fact that most somatic cells are in the G0 phase of the cell cycle, whereas most embryonic stem cells are in the S phase. Interestingly, embryonic stem cell NT embryos that reach the blastocyst stage have a 10- to 20-fold higher probability of developing to birth, compared with those derived from scNT (Renard *et al*., 1999, Wakayama *et al*., 1999; Hochedlinger & Jaenisch, 2002). However, a major limitation in current cloning procedures is inefficiency, since healthy offspring are generated at an extremely low frequency (Cibelli *et al*., 2002; Wilmut, 2002; Dinnyes *et al*., 2002).

Cloning creates phenotypic variations that are independent of genetic background. In other words, the expression of specific traits in some, but not all clones is affected (Ogura *et al*., 2002; Humpherys *et al*., 2002; Wilmut *et al*., 2002; Archer *et al*., 2003; Shi *et al*., 2003; Dean *et al*., 1998). Moreover, neonatal death occurs frequently in somatic cell cloning due to a variety of afflictions, including fetal overgrowth,

All correspondence to: Jin-Hoi Kim, Major of Dairy Science, Division of Applied Life Science, GyeongSang National University, Jinju, GyeongNam 660-701, Korea. e-mail: jinhoi@nongae.gsnu.ac.kr
[1] Major of Dairy Science, Division of Applied Life Science, GyeongSang National University, Jinju, GyeongNam 660-701, Korea.
[2] College of Veterinary Medicine, GyeongSang National University, Jinju, GyeongNam 660-701, Korea.
[3] Department of Pharmacology, College of Medicine, Gyeongsang National University, Jinju, GyeongNam 660-701, Korea.

placental malformations and a deficient immune system (Wakayama *et al*., 1998; Renard *et al*., 1999; McCreath *et al*., 2000).

Leydig cell hypoplasia (LCH) is a rare form of male pseudohermaphroditism with an autosomal recessive pattern of inheritance (Toledo *et al*., 1985; Schwartz *et al*., 1981; Martinez-Mora *et al*., 1991; Perez-Palacios *et al*., 1981; Lee *et al*., 1982). This gene defect in LCH was initially identified by Kremer *et al*. (1995). To date, a number of different LHR gene mutations have been highlighted in patients with LCH (Latronico *et al*., 1996; Toledo, 1992; Laue *et al*., 1996; Misrahi *et al*., 1997). These include missense and nonsense substitutions, and small and large deletions and insertions, all leading to a completely or partially inactive receptor molecule.

Developmental failure of scNT is due to an inability to reprogramme the epigenetic profile of the genetic donor nucleus to that of a normal zygotic nucleus (Dean *et al*., 2003; Carlson *et al*., 1992). Previous studies report that high methylation in sperm-derived sequences is rapidly demethylated in zygotes only hours after fertilization, whereas oocyte-derived maternal alleles are unaffected by this reprogramming (Dean *et al*., 2003). These alleles remain in the methylated status after fertilization, or become further methylated *de novo*. Based on this report, it is hypothesized that male somatic-cell-derived sequences are exposed to putative active demethylases in the oocytic cytoplasm, while female somatic-cell-derived sequences are protected from this reaction. Although the biological significance of genome-wide demethylation during preimplantation embryo development remains unclear, different types of epigenetic reprogramming may trigger distinct chemical reactions for embryo growth via subtle cascades of gene and protein activity that are blunted during cloning (Dean *et al*., 2003; Reik & Walter, 2001). Recently, we reported the successful cloning of female piglets using scNT (Yin *et al*., 2003). In the present study, we examine the developmental abilities of reconstructed male embryos. We report the generation of 22 live piglets using NT with two male fibroblast cell lines. Of the 18 piglets that died, one displayed LCH.

Materials and methods

Isolation and culture of porcine somatic cells

Two male cell lines were obtained from F1 fetuses derived from two different dams (Yorkshire) that were inseminated by the same sire (Landrace). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 in air at 37 °C. After they had reached confluence, cells were passaged. Donor cells were employed for NT between passages 8 and 15 within 3 days after reaching confluence.

In vitro maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory at 25–35 °C. Antral follicles (2–6 mm in diameter) were aspirated with an 18-gauge needle. Aspirated oocytes with an evenly granulated cytoplasm and surrounded by at least three uniform layers of compact cumulus cells were selected and washed three times in TL-Hepes with 0.1% polyvinyl alcohol (PVA). Oocytes were cultured in four-well plates containing NCSU-23 medium (500 µl/well) supplemented with 10% porcine follicular fluid, 0.6 mmol/l cysteine, 1 mmol/l dibutyryl cyclic adenosine monophosphate (dbcAMP, Sigma), and 0.1 IU/ml human menopausal gonadotropin (hMG, Teikokuzoki, Tokyo, Japan) for 20 h. Oocytes were further cultured without dbcAMP and hMG for another 18–24 h, as reported previously (Yin *et al*., 2003).

Nuclear transfer

NT was performed as reported previously (Yin *et al*., 2003). Briefly, matured eggs with a first polar body were cultured in medium supplemented with 0.05 mol/l sucrose for 1 h. Sucrose was used to enlarge the perivitelline space of eggs. Treated eggs were moved to medium supplemented with 5 mg/ml cytochalasin B, and metaphase II oocytes were enucleated 40–42 h after maturation using a bevelled 30 µm diameter glass pipette. A single donor cell (pFF1 or PFF2) was injected into the perivitelline space of each enucleated egg. Groups of oocytes were fused and activated with two direct current pulses of 150 V/mm for 50 µs in 0.28 mol/l mannitol supplemented with 0.1 mM $MgSO_4$, 100 µM $CaCl_2$ and 0.01% PVA (Sigma) using a BTX Electrocell Manipulator 200 (BTX, San Diego, CA). Eggs that were simultaneously activated and fused using pFF1 or pFF2 donor cells were cultured in medium for 6 days in an atmosphere of 5% CO_2 and 95% air at 39 °C, or for 2 days followed by transfer into the oviducts of recipient gilts. In the latter case, embryos were either harvested 4 days after transfer or allowed to develop to term.

Chromosome analysis

All chromosome analytic procedures have been described previously (Clouston *et al*., 2002). In brief, somatic-cell-derived NT blastocysts were synchronized by overnight incubation in medium supplemented with thymidine at a final concentration of 0.5 mg/ml. Embryos were transferred the following morning to

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

fresh medium for 2 h before the addition of colcemid at a final concentration of 0.1 μg/ml for the last 5–6 h of culture. Blastocysts were placed in a hypotonic solution for 10 min at room temperature, and embryos were gradually fixed by slowly adding an equal volume of 3:1 methanol:acetic acid fixative. After 5 min, embryos were transferred to a fresh dish of 3:1 fixative for at least 20 min. Next, embryos were individually transferred to a 3:3:1 methanol:acetic acid:water solution for 1–2 min before disaggregation of cells in a small volume of 70% acetic acid on a polylysine-coated microscope slide. Slides were left on a warm surface, and chromosomes were examined by microscopy.

Genomic DNA isolation, bisulfite treatment, PCR amplification and restriction analysis

All procedures, satellite region primer and PRE-1 primer sequences have been described previously (Dean *et al*., 2001). In brief, male and female somatic-cell-derived NT blastocysts were obtained by *in vitro* or *in vivo* culture, and subjected to PCR analysis. Band intensities were calculated using a Tina 20 image analyser.

Embryo transfer, pregnancy determination and microsatellite analysis

Gilts (Duroc × Yorkshire) at least 8 months old were used as recipients. Estrus synchronization of recipients was carried out as reported previously (Yin *et al*., 2003). Somatic cell NT embryos were surgically transferred into oviducts of synchronized recipients. The pregnancy status of recipients was determined by ultrasound between days 30 and 35. For parentage analysis, DNA was extracted from ear punches or tail clippings of both recipients and newborn piglets, respectively, as well as donor cells. Six porcine DNA microsatellite markers (*SWR1120*, *SWR308*, *SW66*, *SW1311*, *SW1327* and *SW936*) were used to confirm that the genetic identity of the cloned piglets was that of the donor cells used for NT.

TUNEL assay

All TUNEL (TdT-mediated dUTP-X Nicked End Labelling) procedures were performed according to the instructions of the manufacturer of the *in situ* Cell Death Detection Kit (TMR red; Roche, Mannheim, Germany). Pig testes were fixed in 4% (w/v) paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4). Organs were washed in PBS and dehydrated in ethanol (70 %, 90% and 100 %) before embedding in paraffin wax. Testicular sections (5 μm) were rehydrated (xylene 5 min; ethanol 100 %, 95 %, 70% 2 min each) and washed in distilled water, prior to TUNEL staining. Sections were incubated for 15 min with 20 μg/ml proteinase K at room temperature. After washing with PBS (×1), endogenous peroxidase activity was blocked with 2% H_2O_2 for 5 min. Sections were washed three times with PBS (×1) and incubated for 60 min at 37 °C in a moist chamber with the TUNEL mix (0.3 U/μl calf thymus terminal deoxynucleotidyl transferase, 0.007 nmol/μl biotin dUTP, 1 mM cobalt chloride in 1× reaction buffer in distilled water). After washing (four PBS baths of 5 min each at RT), sections were saturated for 10 min in 2% bovine serum albumin (BSA) at RT. This was followed by incubation for 30 min at 37 °C in a moist chamber with Extra Avidin peroxidase antibody (1:20 dilution). After three washes in PBS, detection was performed with DAB (1.24 mg DAB, 25 μl of 3% $NiCl_2$, 152 μl of 1 M Tris-HCl (pH 7.5) in 2 ml distilled water). Slides were mounted in Crystal Mount (Biomeda, Foster City, CA).

Results

We established two fibroblast cell lines from two male fetuses derived from different dams of the same strain but sharing the same sire. The effects of the donor cells on the ability of scNT embryos to develop to the blastocyst stage after 6 days of *in vitro* culture were evaluated, and results were analysed statistically. Table 1 shows the numbers of blastocysts that developed when the two types of fibroblast-derived cells (pFF1 and pFF2) were used as donors for NT. Blastocyst formation rates during *in vitro* culture were similar to those of the *in vivo* cultured scNT group, whereas slightly fewer scNT blastocysts were recovered after *in vivo* culture, and had many more cells

Table 1 Development of porcine scNT embryos constructed using male fetal fibroblasts

Culture type		No. of oocytes fused (r)	No. cleaved (%)	No. of blastocysts (%)	Cell numbers of blastocysts (range)
In vivo		230*	–	27 (12)†[b]	99.2 ± 55.7 (47–160)[c]
In vitro	pFF1	132 (4)	100 (76)[a]	19 (14)[b]	33.2 ± 10.21 (19–52)[d]
	pFF2	150 (3)	112 (75)[a]	18 (16)[b]	33.8 ± 9.48 (17–54)[d]

r, replication ($p < 0.05$)
*No. of embryos transferred into recipients. For *in vivo* experiments, PFF1 were used as donor cells.
†No. of embryos recovered from the uterus of recipients at 4 days after embryo transfer.
[a–d]Rows with different superscripts within a column differ ($p < 0.05$).

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

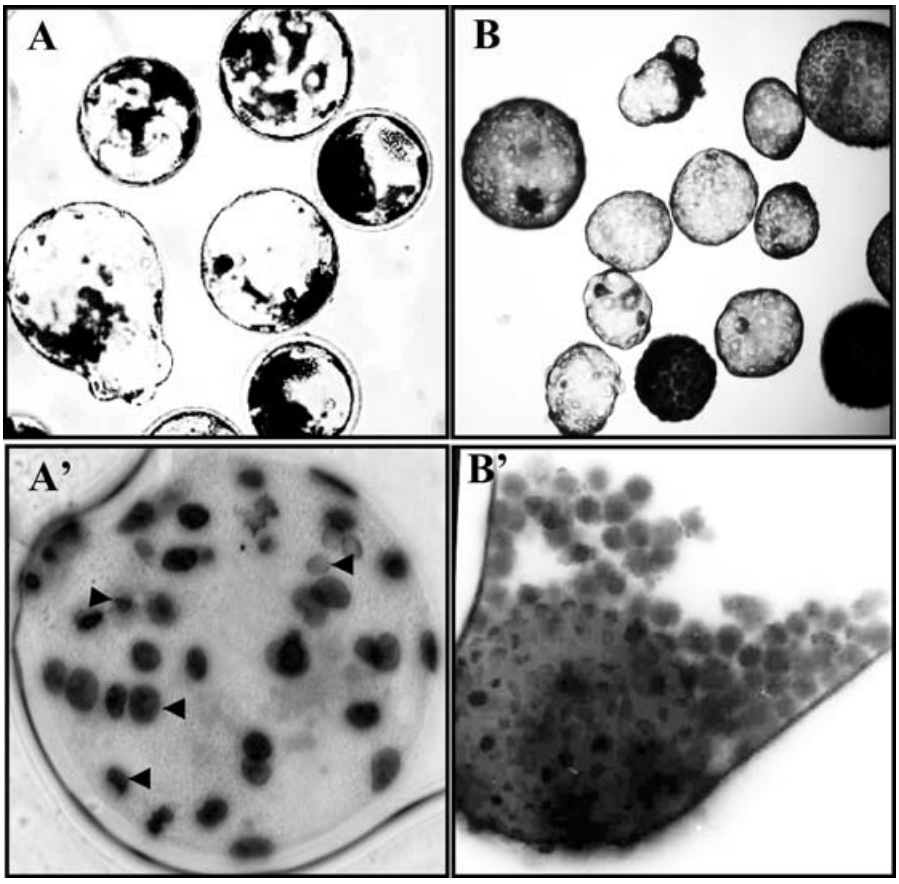

Figure 1 Representative scNT blastocysts derived from *in vivo* cultured (*B*) and *in vitro* cultured scNT embryos (*A*). *In vivo* cultured scNT blastocysts (*B'*) display a higher cell number and more homogeneous cell morphology than *in vitro* cultured scNT blastocysts (*A'*). The arrowheads indicate a heterogeneous cell population in scNT blastocysts.

on average, compared with the *in vitro* culture regimen (Table 1). This finding signifies the effectiveness of the culture system. Representatives of the scNT blastocysts cultured *in vivo* and *in vitro* are shown in Fig. 1. *In vivo*-derived scNT blastocysts divided equally well and bore a slightly larger number of cells, compared to *in vitro* cultured scNT blastocysts.

To evaluate the quality of scNT embryos that developed into blastocysts, we compared the chromosome number and methylation patterns of at least 20 NT blastocysts. As shown in Fig. 2, IVF-derived and *in vitro* cultured scNT blastocysts displayed higher methylation patterns (84.5 ± 1.69 vs 66.0 ± 3.11) compared with *in vivo* cultured scNT blastocysts (20.3 ± 12.44) (Fig. 2), implying that epigenetic reprogramming of cloned embryos is very sensitive to culture conditions. In contrast to the methylation patterns, chromosome numbers of scNT and IVF embryos were similar (Table 2).

Next, we compared the efficacy of cloning piglets by using the two male-derived fibroblasts as donor cells (Table 3). scNT eggs at the 1- to 4-cell stages were surgically transferred into oviducts of 25 recipient gilts. The pregnancies of the recipients were terminated approximately 118 days later. Using the pFF1 and pFF2 donor cell lines, cloned male piglets were produced with an average success rate of one clone out of every 215 and 220 attempts, respectively. The average birth weight of cloned piglets was 0.84 ± 0.25 kg (0.45–1.25 kg) for the pFF1 donor cell line, and 0.83 ± 0.35 kg (0.58–1.08 kg) for the pFF2 donor cell line. Parentage analysis using five porcine DNA microsatellite (MS) markers (*SWR1120*, *SWR308*, *SW66*, *SW1311* and *SW1327*) was performed on DNA obtained from tail clippings or ear punches of scNT piglets and surrogate recipients to confirm that the genetic identity of the piglets was that of the donor cell line used (Fig. 3)

Out of the 22 somatic cloned piglets born, 18 died within 1 week of birth. One piglet displayed LCH. A TUNEL assay of this sample revealed increased apoptosis in Leydig cells, but little apoptosis in Sertoli cells and spermatogonia (Fig. 4). In contrast, testes from the remaining 17 dead cloned piglets appeared normal in terms of size and shape. Moreover, testicular somatic cell numbers in the 17 piglets were not significantly affected. While *in vitro* cultured scNT and IVF blastocysts displayed slightly variable fluctuating methylation patterns, those of scNT piglets produced

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

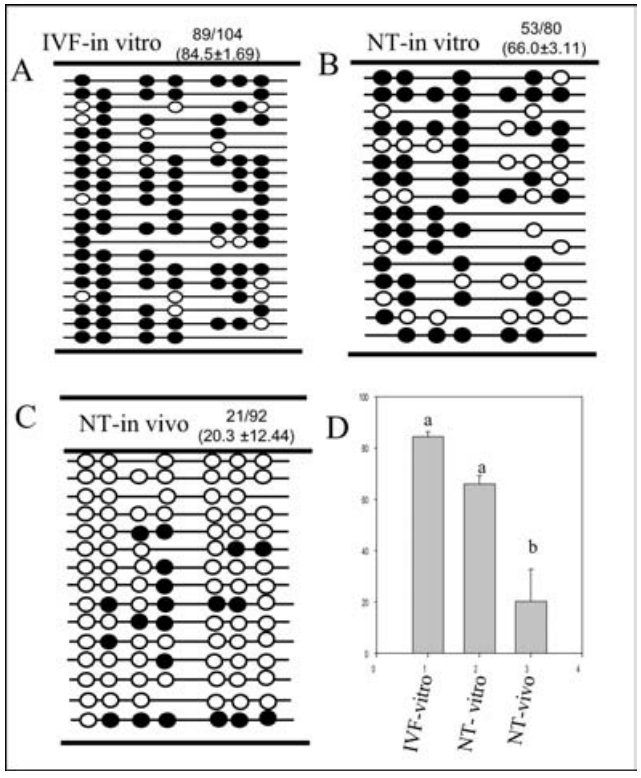

Figure 2 Comparison of the methylation status of PRE-1 sequences in scNT and IVF blastocysts. Methylation profiles of CpG dinucleotides of PRE-SINE sequences in IVF-derived blastocysts (*A*), *in vitro* cultured scNT blastocyst (*B*) and *in vivo* cultured scNT blastocyst (*C*). Open and filled circles indicate unmethylated and methylated CpGs, respectively. Specific CpG sites are absent from the PRE-SINE sequences in some clones due to mutations in particular copies. Numbers in parentheses indicate the proportion of methylated CpG sites relative to all the CpG sites counted. (*D*) Data from (*A*), (*B*) and (*C*) are presented as mean ± SEM. [a,b]Rows with different superscripts within a column differ ($p < 0.0033$).

Table 2 Chromosomal analysis of porcine embryos produced by nuclear transfer

	No. of blastocysts investigated	No. of embryos (%)		
		Haploid	Diploid	Others*
IVF	17	0	9 (53)	8 (47)
scNT	17	0	11 (65)	6 (35)

*Others includes embryos with unidentified and mixed multiple chromosomes.

by two different cells, pFF1 and pFF2, were not significantly different (Fig. 3).

Discussion

This is the first report to identify LCH in somatic cell cloned animals. Here we describe the birth of 22 live piglets cloned by NT using cultured male fetal fibroblasts. Of these, 18 died within 1 week. The remaining four cloned piglets, which are currently 8 months old, appear quite healthy. All piglets had low birth weights. Although the gross anatomy of the testes of cloned male piglets was normal, one exhibited LCH. No other anatomical defects were detected in the testes of the other cloned male piglets.

Table 3 Efficacy of male clone production after transfer of cloned embryos

Cell types	No. of embryos transferred	No. of recipients	No. of pregnancies	No. of piglets	Efficiency per embryo
pFF1	4302	23	5 (22)	20*	1/215
pFF2	440	2	2 (100)	2†	1/220

*Seventeen of 20 piglets died either due to one specific factor (crushing to death (1), meningitis (1) and diarrhoea (1)) or multiple risk factors (meningitis and congestion (1); meningitis and leg abnormalities (3); meningitis, congestion and leg abnormality (1); stillbirth, congestion and leg abnormality (1); stillbirth, short face and Leydig cell hypoplasia (1)) and unknown factors (7).
†One of the two piglets died due to meningitis and leg abnormalities.

LCH in humans is induced by a mutation in the luteinizing hormone (LH) receptor gene that blocks the ability of fetal Leydig cells to respond to placental hCG (Latronico *et al.*, 1996). This results in inappropriate fetal androgen production. Consequently, Leydig cells do not mature into adult type cells at puberty, and fail to generate sufficient androgens for normal secondary sex differentiation. This imbalance in steroid hormone production affects spermatogenesis and spermiogenesis, ultimately resulting in male infertility. To identify LH gene mutations, genomic DNA was extracted from LCH piglet tissues and exons 1 to 11, and the adjacent exon–intron boundaries of the LH receptor gene, were amplified by PCR. A comparison of the sequence with that of a control pig LH receptor gene disclosed no point mutations or deletions (data not shown). This finding suggests that LCH in the cloned piglet arises from a molecular mechanism distinct from that inducing LCH in humans.

The differences between relatively high blastocyst development *in vitro* or *in vivo* and the low pregnancy rate achieved after embryo transfer suggest that some scNT blastocysts fail to develop further within the uterus. In this investigation, a success rate of one male clone out of every 214 attempts was achieved (Table 3). Inappropriate genome-wide methylation in early events of preimplantation scNT embryos may influence embryonic survival during gestation (Dean *et al.*, 2001, 2003; Carlson *et al.*, 1992; Reik &

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

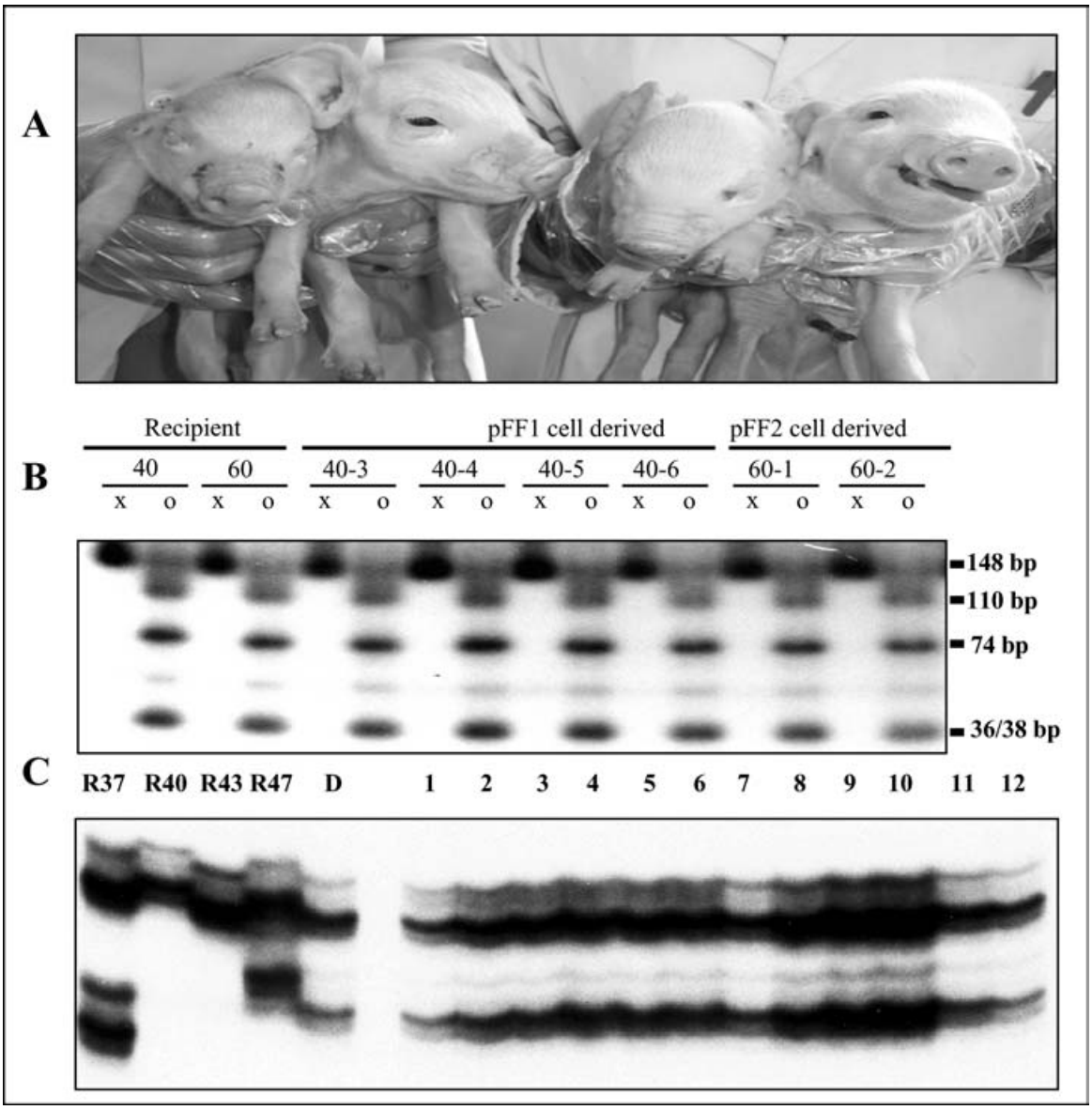

Figure 3 Microsatellite analysis of cloned male piglets. (*A*) scNT piglets. (*B*) Methylation patterns were measured by *Taq*I digestion of radiolabelled products amplified from bisulfate-treated genomic DNA. Each lane shows either intact (crosses) or *Taq*I-digested DNA bands (circles). Although the bands are stronger in pFF1-cell-derived male scNT piglets compared with pFF2-cell-derived male scNT piglets, no significant differences in the methylation profiles of CpG dinucleotides of PRE-SINE sequences were detected. (C) Data obtained using the microsatellite marker, SW1120. Microsatellite alleles of NT-derived piglets (lines 1–12) derived from donor cells (D) were compared with those of recipient sows (R37, R40, R43 and R47). R indicates recipients.

Walter, 2001). Moreover, this observation may be true across species. Accordingly, we analysed whether *in vivo* cultured scNT embryos experience different degrees of methylation compared with *in vitro* cultured scNT embryos. Interestingly, methylation patterns of *in vivo* cultured scNT embryos ($n = 27$) were considerably different from those of *in vitro* cultured scNT ($n = 30$) and IVF blastocysts ($n = 29$) (Fig. 2). This finding suggests that different culture conditions lead to incomplete nuclear reprogramming and result in developmental failure of cloned embryos. The development of cloned embryos can be improved due to partial repair of incomplete reprogramming by treatment with exogenous compounds such as dimethylsulfoxide (DMSO; Chung *et al*., 2003). However, this does not imply that DMSO treatment increases cell numbers of cloned blastocysts. Our data are inconsistent with a previous report showing that pig scNT preimplantation embryos display lower methylation levels (Kang *et al*., 2001). This discrepancy may be due to the individual culture conditions or activation methods used.

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

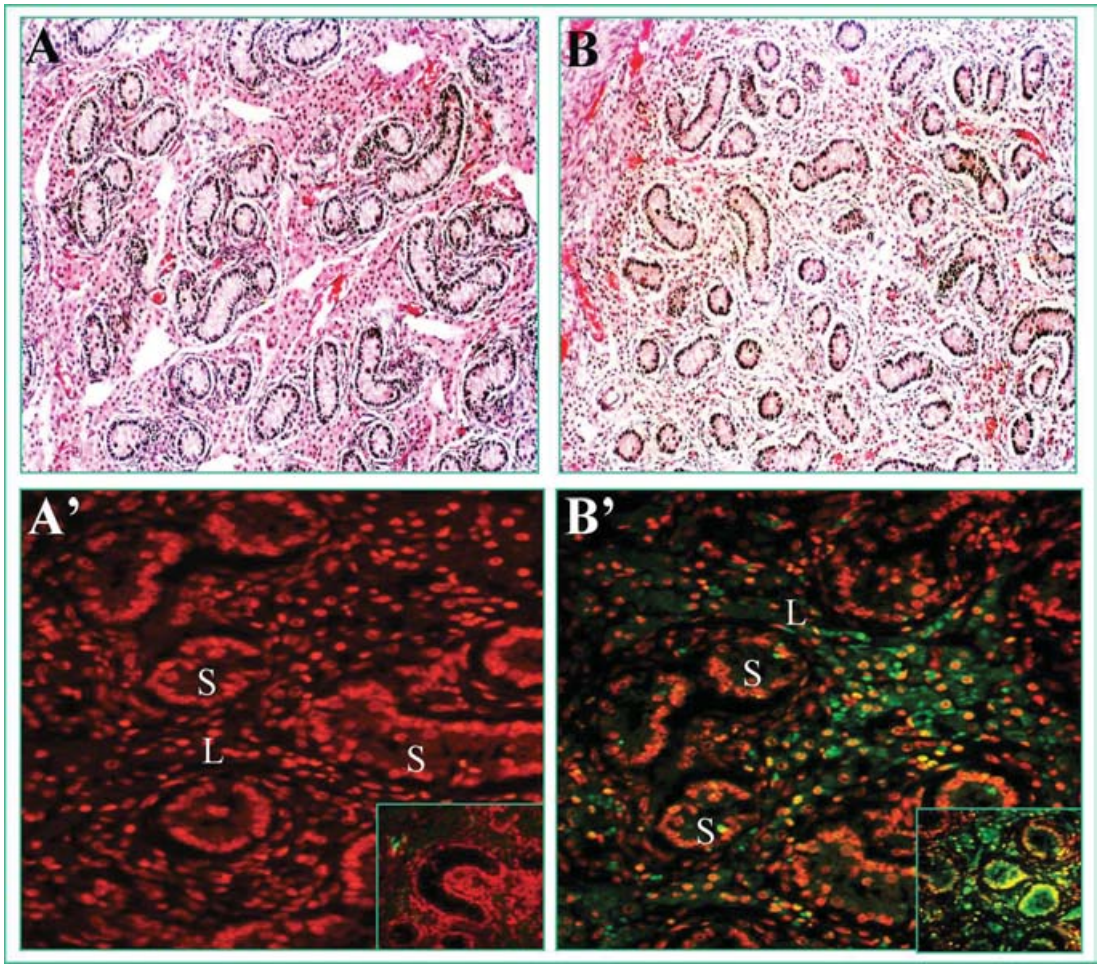

Figure 4 Hypoplasia of Leydig cells in a male cloned piglet. Haematoxylin and eosin staining disclosed that a cloned male piglet (*B*) has few Leydig cells in comparison with other age-matched somatic cell cloned piglets (*A*). TUNEL assay using the testis of the male cloned piglet with LCH (*B'*) revealed intensive apoptosis, especially of Leydig cells, although Sertoli cells displayed minor apoptosis (insert in *B'*). However, signals were not detected in the testes of control and other cloned male piglets (*A'*). The green and yellow colours signify apoptosis of most somatic cells. S and L indicate Sertoli and Leydig cells, respectively.

In vivo cultured scNT blastocysts divided equally well and contained more cells than their *in vitro* counterparts (Fig. 1). Normally, embryo division is activated by precise changes in calcium levels choreographed by a chemical, oscillin, brought by the sperm into the egg (Sasagawa *et al*., 1997). *In vitro*, the timings of these changes may vary. As a result, *in vitro* cultured scNT embryos develop into blastocysts with heterogeneous cell morphology. Next, we examined the *in vivo* and *in vitro* development of *in vitro* matured porcine oocytes reconstructed by fusion with somatic cells. While the cell numbers of *in vitro* cultured scNT blastocysts in this study were relatively high, cell numbers of *in vitro* cultured scNT blastocysts were a third of those observed following *in vivo* transfer (Table 1). This observation suggests that *in vitro* and *in vivo* culture conditions result in different signals for embryo growth and division that are blunted during *in vitro* culture.

The mechanism by which the culture system induces reprogramming of early embryos following scNT remains unclear. Our data show that despite extensive global methylation levels observed in cloned male embryos, some develop to term. This finding suggests that extensive genome-wide methylation in somatic cell cloned pig embryos does not fully impair pig embryogenesis, but reprogramming of scNT embryos may be a critical factor. Therefore, we propose that the reason for the poor quality of *in vitro* cultured scNT porcine embryos lies with the culture system rather than developmental defects caused by the technique itself.

A previous study reported that phenotypic instability in swine results from scNT (Archer *et al*.,

https://doi.org/10.1017/S0967199404002904 Published online by Cambridge University Press

2003). However, other groups have observed a few problems (Bondioli *et al*., 2001; De Sousa *et al*., 2002; Walker *et al*., 2002; Yin *et al*., 2002). Additionally, in cattle, the donor cell type is responsible for some of the abnormal phenotypes observed, as cells derived from adults result in more abnormalities (Heyman *et al*., 2002). In this study, 9 of the 22 somatic cell cloned male piglets exhibited phenotypic instability (unpublished data). Our data imply that male clones have more variation-susceptible traits than expected for genetically identical animals, and that trait variability associated with phenotypic abnormality may be induced by damage to fetal fibroblasts used as donor cells. However, the issue of whether donor cell types cause multi-organ failure and sudden early death in cloned males remains to be investigated.

Cloning creates variations independent of genetic background. These procedures can affect specific traits in some, but not all clones (Ogura *et al*., 2002; Humpherys *et al*., 2002; Wilmut *et al*., 2002; Archer *et al*., 2003; Shi *et al*., 2003; Dean *et al*., 1998). The present data reflect sex and cell type differences, even when donor cells are derived from the same fetus. Many scientific researchers oppose the cloning of human beings, primarily due to the high failure rate and/or because the technology for analysing genetic abnormalities used in routine prenatal diagnosis cannot detect problems in epigenetic reprogramming (Kang *et al*., 2001). Although various types of somatic cells have been successfully employed to generate somatic-cell-derived clones, our results confirm that the safety and long-term adverse biological effects of cloning require further investigation.

Acknowledgements

We apologize for not being able to cite all the primary literature. We thank Dr Patrick Hughes (Bioedit) for assistance with manuscript preparation. This work was supported by BioGreen from RDA, Republic of Korea. M.-R.P., Y.-J.C. and D.-N.K. are recipients of a scholarship from the BK21 program granted by the Ministry of Education, Korea.

References

Archer, G.S., Dindot, S., Friend, T.H., Walker, S., Zaunbrecher, G., Lawhorn, B. & Piedrahita, J.A. (2003). Hierarchical phenotypic and epigenetic variation in cloned swine. *Biol. Reprod*. **69**, 430–36.

Bondioli, K., Ramsoondar, J., Williams, B., Costa, C. & Fodor, W. (2001). Cloned pigs generated from cultured skin fibroblasts derived from a H-transferase transgenic boar. *Mol. Reprod. Dev*. **60**, 189–95.

Carlson, L.L., Page, A.W. & Bestor, T.H. (1992). Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. *Genes Dev*. **6**, 2536–41.

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

Cibelli. J.B., Campbell, K.H., Seidel, G.E., West, M.D. & Lanza, R.P. (2002). The health profile of cloned animals. *Nat. Biotechnol*. **20**, 13–14.

Chung, Y.G., Ratnam, S., Chaillet, J.R. & Latham, K.E. (2003). Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol. Reprod*. **69**, 146–53.

Clouston, H.J., Herbert, M., Fenwick, M., Murdoch, A.P. & Wolstenholme, J. (2002). Cytogenetic analysis of human blastocysts. *Prenatal Diagn*. **22**, 1143–52.

Dean, W., Bowden, L., Aitchison, A., Klose, J., Moore, T., Meneses, J.J., Reik, W. & Feil, R. (1998). Altered imprinted gene methylation and expression in completely ES-cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125**, 2273–82.

Dean, W., Santos, F., Stojkovic, M., Zakhartchenko, V., Walter, J., Wolf, E. & Reik, W. (2001). Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc. Natl. Acad. Sci*. USA **98**, 13734–8.

Dean, W., Santos, F. & Reik, W. (2003). Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. *Semin. Cell Dev. Biol*. **14**, 93–100.

De Sousa, P.A.J., Dobrinsky, R., Zhu, J., Archibald, A.L., Ainslie, A., Bosma, W., Bowering, J., Bracken, J., Ferrier, P.M., Fletcher, J., Gasparrini, B., Harkness, L., Johnston, P., Ritchie, M., Ritchie, W.A., Travers, A., Albertini, D., Dinnyes, A., King, T.J. & Wilmut, I. (2002). Somatic cell nuclear transfer in the pig: control of pronuclear formation and integration with improved methods for activation and maintenance of pregnancy. *Biol. Reprod*. **66**, 642–50.

Dinnyes, A., De Sousa, P., King, T. & Wilmut, I. (2002). Somatic cell nuclear transfer: recent progress and challenges. *Cloning Stem Cells* **4**, 81–90.

Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous, S., Vignon, X. & Renard, J.P. (2002). Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol. Reprod*. **66**, 6–13.

Hochedlinger, K. & Jaenisch, R. (2002). Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* **415**, 1035–8.

Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E.S., Golub, T.R. & Jaenisch, R. (2002). Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc. Natl. Acad. Sci*. USA **99**, 12889–94.

Kang, Y.K., Koo, D.B., Park, J.S., Choi, Y.H., Kim, H.N., Lee, K.K. & Han, Y.M. (2001). Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. *J. Biol. Chem*. **276**, 39980–4.

Kremer, H., Kraaij, R., Toledo, S.P.A., Post, M., Fridman, J.B., Hayashida, C.Y., Van Ree, M., Milgrom, E., Ropers, H.H., Mariman, E., Themmen, A.P.N. & Brunner, H.G. (1995). Male pseudohermaphroditism due to a homozygous

missense mutation of the luteinizing hormone receptor gene. *Nat. Genet*. **9**, 160–4.

Latronico, A.C., Anasti, J., Arnhold, I.J., Rapaport, R., Mendonca, B.B., Bloise, W., Castro, M., Tsigos, C. & Chrousos, G.P. (1996). Brief report: testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene. *N. Engl. J. Med*. **334**, 507–12.

Laue, L.L., Wu, S.-M., Kudo, M., Bourdony, C.J., Cutler, G.B. Jr, Hsuehm, A.J.W. & Chan, W.-Y. (1996). Compound heterozygous mutations of the luteinizing hormone receptor gene in Leydig cell hypoplasia. *Mol. Endocrinol*. **10**, 987–97.

Lee, P.A., Rock, J.A., Brown, T.R., Fichman, K.M., Migeon, C.J. & Jones, H. Jr (1982). Leydig cell hypofunction resulting in male pseudohermaphroditism. *Fertil. Steril*. **37**, 675–9.

Martinez-Mora, J., Saez, J.M., Toran, N., Isnard, R., Perez-Iribarne, M.M., Egozcue, J. & Audi, L. (1991). Male pseudohermaphroditism due to Leydig cell agenesis and absence of testicular LH receptors. *Clin. Endocrinol. (Oxf.)* **34**, 485–91.

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.

Misrahi, M., Meduri, G., Pissard, S., Bouvattier, C., Beau, I., Loosfelt, H., Jolivet, A., Rappaport, R., Milgrom, E. & Bougneres, P. (1997). Comparison of immunocytochemical and molecular features with the phenotype in a case of incomplete male pseudohermaphroditism associated with a mutation of the luteinizing hormone receptor. *J. Clin. Endocrinol. Metab*. **82**, 2159–65.

Ogura, A., Inoue, K., Ogonuki, N., Lee, J., Kohda, T. & Ishino, F. (2002). Phenotypic effects of somatic cell cloning in the mouse. *Cloning Stem Cell* **4**, 397–405.

Perez-Palacios, G., Scaglia, H.E., Kofman-Alfaro, S., Saavedra, D., Ochoa, S., Larraza, O. & Perez, A.E. (1981). Inherited male pseudohermaphroditism due to gonadotrophin unresponsiveness. *Acta. Endocrinol. (Copenh.)* **98**, 148–55.

Reik, W. & Walter, J. (2001). Genomic imprinting: parental influence on the genome. *Nat. Rev. Genet*. **2**, 21–32.

Renard, J.P., Chastant, S., Cesne, P., Richard, C., Marchal, J., Cordonnier, N., Chavatte, P. & Vignon, X. (1999). Lymphoid hypoplasia and somatic cloning. *Lancet* **353**, 1489–91.

Sasagawa, I., Tomaru, M., Adachi, Y., Kubota, Y. & Nakada, T. (1997). Simultaneous injection of round spermatid nuclei from mice and hamster oscillogen can initiate the normal development of mouse embryos. *J. Urol*. **158**, 2006–8.

Schwartz, M., Imperato-McGinley, J., Peterson, R.E., Cooper, G., Morris, P.L., MacGillivray, M. & Hensle, T. (1981). Male pseudohermaphroditism secondary to an abnormality in Leydig cell differentiation. *J. Clin. Endocrinol. Metab*. **53**, 123–7.

Shi, W., Zakharchenko, V. & Wolf, E. (2003). Epigenetic reprogramming in mammalian nuclear transfer. *Differentiation* **71**, 91–113.

Stice, S.L., Robl, J.M., Ponce de Leon, F.A., Jerry, J., Golueke, P.G., Cibelli, J.B. & Kane, J.J. (1998). Cloning: new breakthroughs leading to commercial opportunities. *Theriogenology* **49**, 129–38.

Toledo, S.P. (1992). Leydig cell hypoplasia leading to two different phenotypes: male pseudohermaphroditism and primary hypogonadism not associated with this. *Clin. Endocrinol. (Oxf.)* **36**, 521–2.

Toledo, S.P., Arnhold, I.J., Luthold, W., Russo, E.M. & Saldanha, P.H. (1985). Leydig cell hypoplasia determining familial hypergonadotropic hypogonadism. *Prog. Clin. Biol. Res*. **200**, 311–14.

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

Walker, S.C., Shin, T., Zaunbrecher, G.M., Romano, J.E., Johnson, G.A., Bazer, F.W. & Piedrahita, J.A. (2002). A highly efficient method for porcine cloning by nuclear transfer using *in vitro*-matured oocytes. *Cloning Stem Cells* **4**, 105–12.

Wall, R.J., Heyman, P., Kerr, D., Pintado, B. & Wells, K. (1997). Transgenic animal technology. *J. Androl*. **18**, 236–9.

Wells, D.N., Misica, P.M., Day, T.A. & Tervit, H.R. (1997). Production of cloned lambs from an established embryonic cell line: a comparison between *in vivo*- and *in vitro*-matured cytoplasts. *Biol. Reprod*. **57**, 385–93.

Wilmut, I. (2002). Are there any normal cloned mammals? *Nat. Med*. **8**, 215–16.

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

Wilmut, I., Young, L. & Campbell, K.H. (1998). Embryonic and somatic cell cloning. *Reprod. Fertil. Dev*. **10**, 639–43.

Wilmut, I., Beaujean, N., de Sousa, P.A., Dinnyes, A., King, T.J., Paterson, L.A., Wells, D.N. & Young, L.E. (2002). Somatic cell nuclear transfer. *Nature* **419**, 583–6.

Yin, X.J., Tani, T., Yonemura, I., Kawakami, M., Miyamoto, K., Hasegawa, R., Kato, Y. & Tsunoda, Y. (2002). Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. *Biol. Reprod*. **67**, 442–6.

Yin, X.J., Cho, S.K., Park, M.R., Im, Y.J., Park, J.J., Bhak, J.S., Kwon, D.N., Jun, S.H., Kim, N.H. & Kim, J.H. (2003). Nuclear remodeling and the developmental potential of nuclear transferred porcine oocytes under delayed-activated conditions. *Zygote* **11**, 167–74.