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Expression of early development-related genes in bovine nuclear transferred and fertilized embryos

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Summary

Cloning efficiency following somatic cell nuclear transfer is very low. In order to obtain insights into this problem, mRNA expression patterns of early development-related genes in nuclear transferred embryos were compared with those obtained from *in vivo* and *in vitro* fertilization. Semiquantitative reverse-transcription polymerase chain reaction assay was used to compare the gene expression of, the cell adhesion protein E-cadherin, interleukin -6, heat-shock protein 70.1 and bos taurus apoptosis regulator box-a (Bax). The relative abundances of glucose transporter-1, E-cadherin and interleukin-6 were significantly ($P < 0.05$) higher in *in vitro* fertilized morulae than *in vivo* derived morulae. Transcription of the gene encoding octamer-binding transcription factor 4 was higher in blastocysts obtained from *in vivo* fertilization than in those from *in vivo* blastocysts. The transcript for Bax was markedly upregulated in blastocysts derived from *in vitro* production and nuclear transfer procedures compared with *in vivo* fertilization. These results suggest that alterations in mRNA expression of early development genes are more associated with *in vitro* culture condition than the nuclear transfer procedure itself.

Keywords: Nuclear transfer, Gene expression, Bovine, Fertilization

Introduction

Offspring have been cloned from somatic cells in range of animals (Wilmut *et al*., 1997; Cibelli *et al*., 1998; Wakayama *et al*., 1998; Baguisi *et al*., 1999; Polejaeva *et al*., 2000). However, cloning efficiency is very low and abnormalities have been reported during embryonic, fetal, neonatal and postnatal development (Wilmut *et al*., 1997; Cibelli *et al*., 1998; Daniels *et al*., 2001). These problems are considered to be the result of incomplete reprogramming of the donor nucleus, which leads to abnormal expression of development-related gene expression. Previously, Daniels *et al*. (2000) observed that delayed and/or aberrant transcriptions of some of implantation related genes such as interleukin 6 (IL-6), fibroblast growth factor 4 (FGF4) and fibroblast growth factor receptor 2 (FGFr2) were found in some cloned embryos. More recently, Wrenzycki *et al*. (2001) observed that nuclear transfer (NT) affects mRNA expression of some development-related genes in NT embryos compared with *in vitro* fertilized (IVF) embryos.

Prolonged *in vitro* culture condition appeared to affect gene expression, which is associated with abnormal embryonic and fetal growth and development. The presence of exogenous proteins such as serum and bovine serum albumin (BSA) affects both mRNA expression and embryo viability (Wrenzycki *et al*., 1999; Rizos *et al*., 2002). Recently, Lazzari *et al*. (2002) showed a relationship between the incidence of early embryonic deviations and large offspring syndrome in *in vitro* produced bovine embryos.

Although both nuclear transfer procedure and *in vitro* culture condition seem to affect expression of development-related genes following NT, comparative experiments are largely lacking using *in vivo* derived embryos. The objective of the present study was to determine the relative abundance of gene transcripts in bovine nuclear transferred morulae and blastocysts, and to compare with their expression profiles with

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those of *in vivo* (VivoF) and IVF embryos with a semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) assay. In this paper, six genes were selected for their importance to preimplantation development in early embryonic development (octamer-binding transcription factor 4 (Oct4)), energy metabolism (glucose transporter-1 (Glut-1)), compaction (E-cadherin (E-cad)), trophoblastic function (interleukin-6 (IL-6)), stress (heat-shock protein 70 (Hsp)) and apoptosis (Bos taurus apoptosis regulator box-a (Bax)).

Materials and methods

Preparation of oocytes

Bovine cumulus oocyte complexes (COCs) with uniform ooplasm and compact cumulus cells were prepared in HEPES-buffered thyroids albumin lactate pyruvate (TALP) medium containing 0.1% polyvinylalcohol. Culture medium for *in vitro* maturation was Tissue Culture Medium 199 (TCM199; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (GIBCO, NY, USA), 0.2 mM sodium pyruvate, 1 μg ml^{-1} FSH and 1 μg ml^{-1} estradiol-17β. 30–50 COCs were cultured in 500 μl maturation medium at 39 °C and 5% CO_2.

In vitro fertilization

Bovine oocytes matured for 22–24 h were fertilized in TALP medium supplemented with gentamycin (0.5 mg ml^{-1}, Sigma), heparin (30 μg ml^{-1}), hypotaurine (1.65 μg ml^{-1}), epinephrine (0.27 μg ml^{-1}; Sigma) and penicillamine (4.5 μg ml^{-1}; Sigma). Oocytes with cumulus cells were cultured with 2×10^6 motile sperm per ml for 6 h and transferred to drops of embryo culture medium, CR1aa medium (Rosenkrans *et al.*, 1993) supplemented with 3 mg ml^{-1} fatty-acid-free (FAF) BSA, 20 μl ml^{-1} MEM essential amino acids, 10 μl ml^{-1} MEM non-essential amino acids, 0.44 μg ml^{-1} Na pyruvate, 1.46 μg ml^{-1} glutamine and 25 μg ml^{-1} gentamycin.

Donor cell preparation

The cell line was derived from a surgical biopsy performed on a Korean bull's ear. Thin sections of the tissue were sliced into 1–3-mm pieces and explants were transferred into 100 mm dishes containing Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 15% FBS, 1% penicillin/streptomycin. Cell culture was performed at 39 °C in 5% CO_2 in air. When confluence was achieved at 14 days, cells were trypsinized for 3 min, the recovered cells centrifuged and the pellet resuspended at a concentration of 10^6 cell ml^{-1}. Culturing cycling cells with 10% and 0.5% FBS-supplemented medium for 5 days induced the synchronization of the cell cycle to G0/G1 stage.

Nuclear transfer

In vitro matured oocytes were enucleated with a 20 μm (internal diameter) glass pipette by aspirating the first polar body in CR1aa medium supplemented with 4.0 mg ml^{-1} pyruvate, 5.5 mg ml^{-1} lactate, 0.3% FAF-BSA (Sigma) and 7.5 μg ml^{-1} cytochalasin B (CCB; Sigma), and the second metaphase plate in a small volume of surrounding cytoplasm. Chromosome removal was confirmed by *bis*-benzimide (Hoechst 33342; Sigma) labeling under ultraviolet light. After enucleation, the donor cell was introduced through the same slit in the zona pellucida and the cell was wedged between the zona pellucida and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. After the injection, the reconstructed embryos remained in CR1aa medium until fusion.

Reconstructed embryos were electrically fused at 24 h after initiation of maturation (hpm) in buffer composed of 0.25 M mannitol (Sigma), 0.1 mM calcium, 0.1 mM magnesium and 0.01% polyvinylalcohol (Sigma). Fusion was performed at room temperature in a chamber filled with fusion buffer, with two stainless steel electrodes 3.2 mm apart. The reconstructed embryos were manually aligned with a fine glass needle so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with DC current of 1.6 kV cm^{-1} for 25 μs, two pulses each, delivered by an Electrocell Manipulator 2001 (BTX, San Diego, CA). After the electrical stimulus, the reconstructed embryos were washed in CR1aa medium. They were then checked for fusion by microscopic examination.

The fused embryos were cultured for 4 h in CR1aa medium before chemical activation. Activation was induced by incubation in CR1aa with 5 mM ionomycine (Sigma) for 4 min at 37 °C. Embryos were then extensively washed in CR1aa five times before being cultured in 1.9 mM 6-dimethyl-aminopurine (6-DMAP; Sigma).

In vitro culture of embryos

The reconstructed and *in vitro* fertilized embryos were cultured in a four-well dish of CR1aa medium containing 4.0 mg ml^{-1} pyruvate, 5.5 mg ml^{-1} lactate and 0.3% FAF-BSA, and a feeder layer of mouse fetal fibroblast cells (day 14). Embryos were cultured in a humidified incubator at 39 °C and 5% CO_2.

In vivo production of bovine embryos

To produce *in vivo* compact morulae and blastocysts, Korean native cows were superovulated by intramuscular administration of 400 mg folltropin given in a

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Table 1 Information on the primers used for PCR

Gene		Primer sequences	Location	Fragment size (bp)	GeneBank accession no.
Globin*	Upper	5′-GCAGCCACGGTGGCGAGTAT-3′	241–260	257	X04751
	Lower	5′-GTGGGACAGGAGCTTGAAAT-3′	555–567		
Oct-4	Upper	5′-GGTTCTCTTTGGAAAGGTGTTC-3′	435–734	314	AF022987
	Lower	5′-ACACTCGGACCACGTCTTTC-3′	1336–1460		
E-cad*	Upper	5′-CTCAAGCTCGCGGATAACCAGAACAAAGAC-3′	1486–1515	332	X06339
	Lower	5′-AGGCCCCTGTGCAGCTGGCTCAAATCAAAG-3′	1785–1814		
Glut-1*	Upper	5′-AGCGTCATCTTCATCCCAGC-3′	741–760	556	M60448
	Lower	5′-CCACAATGCTCAGGTAGAC-3′	1262–1281		
IL-6	Upper	5′-CAGACTACTTCTGACCACTC-3′	171–190	475	X57317
	Lower	5′-GCTGAACTGCAGGAAATTCTC-3′	625–645		
Hsp	Upper	5′-TGATGCTGTTGTCCAGTCTG-3′	271–290	544	X53827
	Lower	5′-TAGCACGCTCACAAGCAGTA-3′	826–845		
Bax	Upper	5′-ACCAGCCTCGAGCAGATCATG-3′	40–60	372	U92569
	Lower	5′-CATGATGGTCCTGATCAACTC-3′	391–411		

*The oligonucleotide sequences of Globin, E-cad and Glut-1 have been previously published (Wrenzycki *et al.*, 2001).

series of decreasing doses over a 3–4 day period. Estrus was induced by intramuscular administration of 25 mg prostaglandin F_2 on the morning and evening of the third or fourth day of FSH treatment. Estrus detection was performed twice daily, beginning 24 h after the first prostaglandin F_2 injection. Donor cows were artificially inseminated 12 h and 24 h after first standing estrus, with semen from a proven Korean native bull. Embryos used in this study were recovered by nonsurgical uterine flushing of donor cattle on day 6 or day 8 of the cycle (day 0 being first standing estrus).

Poly(A) RNA extraction and reverse transcription

The mRNA was extracted according to Wrenzycki *et al*. (1999) and using a Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) following the manufacturer's instructions. Briefly, single morulae or blastocysts were washed in PBS, snap frozen in liquid nitrogen or at –80 °C for later use. When required, samples were lysed by 150 μl lysis/binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM DTT). Then, 1 pg rabbit globin mRNA (Sigma) per embryo was added to each tube as an internal standard. After vortexing for 5 min at room temperature, 20 μl prewashed Dynabeads oligo$(dT)_{25}$ was mixed with the lysate and annealed by rotating for 5 min at room temperature. The beads were separated employing a Dynal MPC-S magnetic particle concentrator, washed twice using 200 μl washing buffer A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS) and once with 100 μl washing buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). Poly$(A)^+$ RNAs were then eluted from the beads by incubation in 10 μl Tris-HCl (10 mM Tris-HCl, pH 7.5) at 65 °C for 2 min, and aliquots were used immediately for reverse transcription (RT). RT was performed for 50 min at 42 °C in a final volume of 20 μl consisting of 0.5 μg μl^{-1} oligo$(dT)_{12–18}$ (Gibco), 10 × RT buffer (200 mM Tris-HCl; pH 8.4, 500 mM KCl), 25 mM $MgCl_2$, 0.1 M DTT, 10 mM dNTP mix and SuperScript II RT (50 units; Gibco). Reactions were terminated by incubation at 70 °C for 15 min.

PCR amplification

PCR was carried out on 5 μl RT product from single embryos and 0.25 pg of rabbit globin mRNA. The reaction was performed in a final volume of 50 μl 10 × PCR buffer, 2.5 units Taq DNA polymerase (Takara Korea Biomedical, Seoul, Korea), 10 mM dNTP, 10 pM all sequence-specific primers using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, USA). The sequence of the primers, their approximate sizes and their GenBank accession numbers are listed in Table 1. PCR cycles were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 min, 55 °C for 1 min 30 s and 72 °C for 1 min 30 s. The reaction was ended by a final extension step of 10 min at 72 °C.

The RT-PCR products were visualized under ultraviolet light on 1% agarose (GIBCO) gels in 1 × TAE buffer containing 1 $\mu g\,ml^{-1}$ ethidium bromide (Sigma). The intensity of each band was assessed by densitometry using an image analysis program (LabWorks; UVP, Upland, CA). The relative abundance (RA) of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage.

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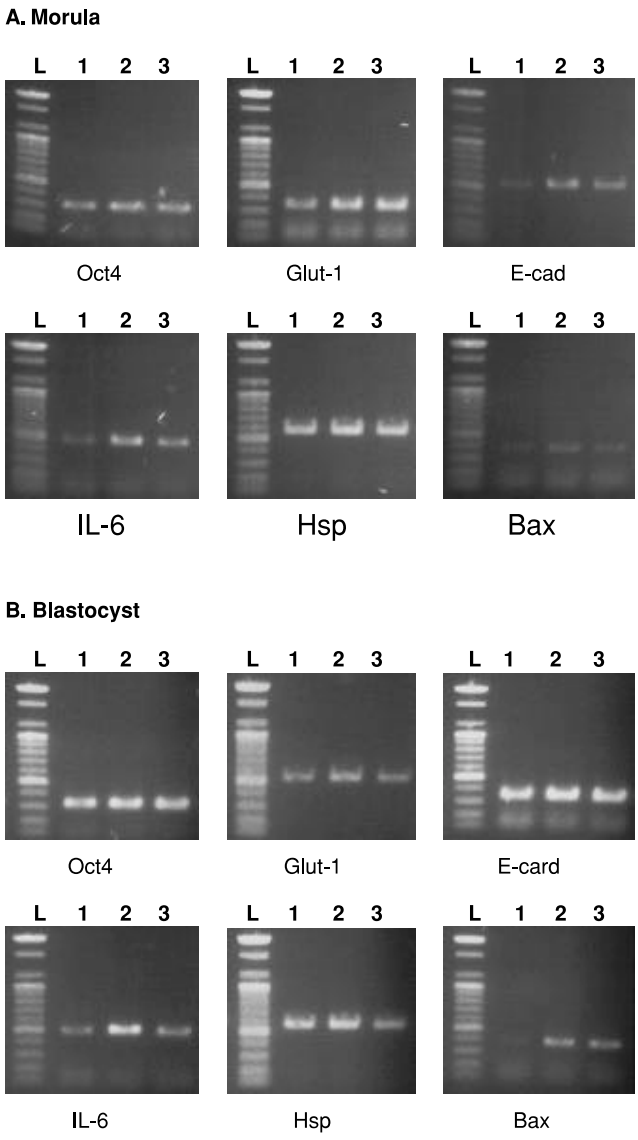

Figure 1 Representative gels produced from semiquantitative RT-PCR analysis of bovine nuclear transferred (lane 1), *in vitro* (lane 2) and *in vivo* fertilized embryos (lane 3).

Statistical analyses

The data were pooled from at least seven replicates. Differences in the relative abundance of transcripts developing to a particular stage were determined by one-way ANOVA procedures.

Results

Representative gel photos of mRNA expression patterns in NT, IVF and VivoF embryos are shown in Fig. 1. The relative abundances of six gene transcripts in cloned morulae and blastocysts compared with those in IVF and VivoF embryos are shown in Fig. 2. The relative abundance of *Glut-1* mRNA is higher in *in vitro* generated morulae than those in VivoF embryos. Similarly, *IL-6* and *E-cad* mRNA expression were increased in IVF morulae compared with those in VivoF morulae. However, the differences between *in vitro* and VivoF expression of *Glut-1*, *IL-6* and *E-cad* were not statistically significant at the blastocyst stage. The relative abundance of *Oct-4* mRNA is higher in IVF blastocyst than in VivoF embryos. The relative amounts of *Hsp* mRNA displayed similar patterns in embryos from all production systems. The relative abundance of Bax transcript was significantly ($P < 0.05$) higher in both morulae and blastocysts derived from NT than in VivoF embryos. Especially, it was markedly higher in IVF and NT groups than the VivoF control group at the blastocyst stage.

Discussion

The present study compared the transcription of six genes, *Oct4*, *Glut-1*, *E-cad*, *IL-6*, *Hsp* and *Bax* in preimplantation bovine embryos derived from IVF, NT and VivoF. The genes are known to have important functions during preimplantation, early postimplantation development or both in mammals and thus have the potential to be used as genetic markers of embryo viability. The successful reprogramming of a somatic cell following NT would result in an embryo with the same profile of gene transcription as that seen in embryos produced by IVF. In the present study, we observed similar amount of transcription of six development-related genes in NT embryos compared with those in IVF embryos. This suggested that reprogramming of the somatic cell NT, at least by the method used here, was sufficient in these embryos to produce the correct pattern of embryonic transcription of the six genes used here.

Oct-4 is a prime candidate for an early developmental control gene encodes a transcription factor required for embryo development (Scholer, 1998; Ovitt and Scholer, 1998; Kirchhof *et al*., 2000). In the mouse, a targeted deletion of *Oct-4* resulted in lack of inner cell mass of the blastocysts and death shortly after implantation (Nichols *et al*., 1998). *Oct-4* expression was shown in preimplantation embryos, but it is downregulated during formation of the blastocysts (Palmieri *et al*., 1994). In the present study, the relative levels of *Oct-4* mRNA in both IVF- and NT-derived blastocysts were greater than those of *in vivo* derived embryos. Higher expression of *Oct-4* in NT- and IVF-derived blastocysts than in *in vivo* embryos might be due to failure of appropriated downregulation during their *in vitro* development.

Higher expressions of *IL-6*, *E-cad* and *Glu-1* were observed in IVF morulae than in *in vivo* fertilized embryos.

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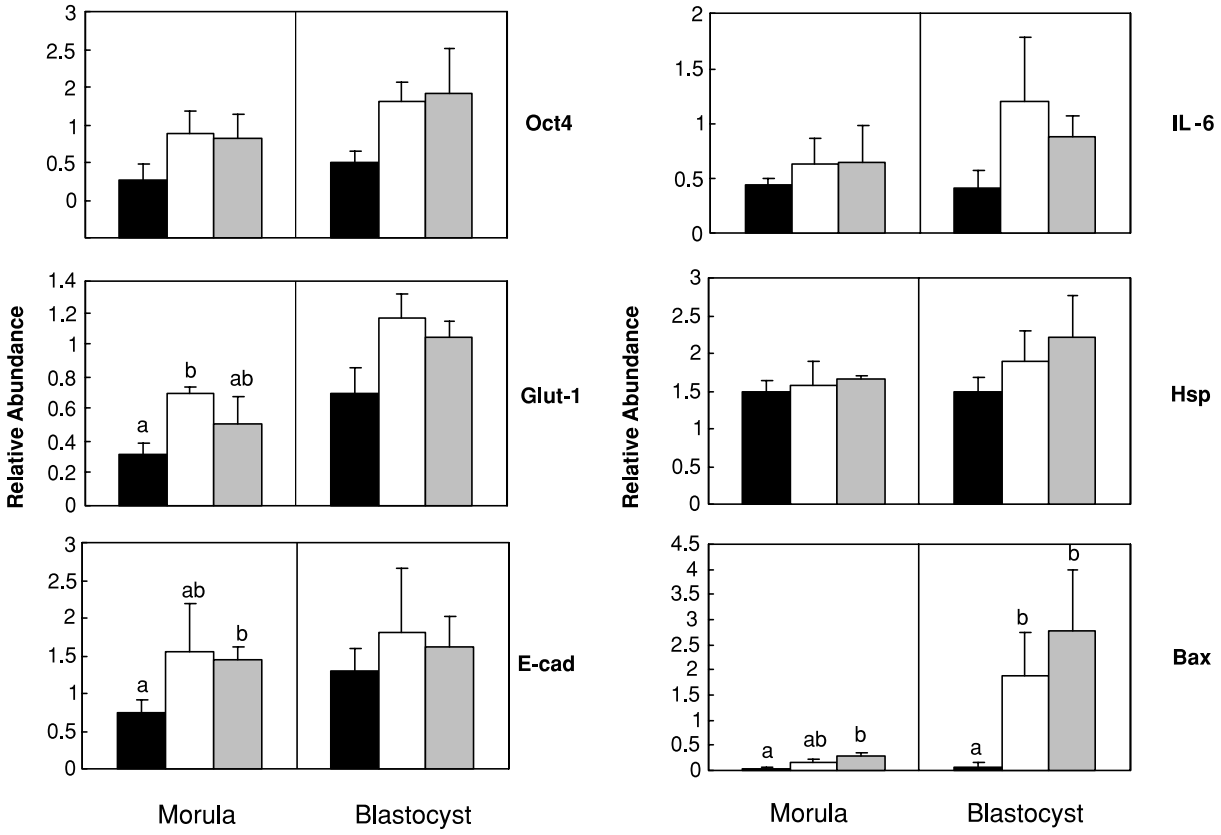

Figure 2 Relative abundance of various developmental important gene transcript (values shown as means SEM) in bovine morulae and blastocysts. Black bar: VivoF, open bar: IVF, gray bar: NT. Bars with different superscripts within transcripts differ significantly ($P < 0.05$).

These three genes are important for early preimplantation development of embryos. E-cad, a principal molecular component of the adherent junction, associates with the compaction of morulae and blastocyst formation (Nagafuchi *et al*., 1987). E-cad plays key roles in differentiation of the trophectoderm and supports further embryonic development (Watson *et al*., 1999).

Like E-cad, IL-6 appeared to support embryonic compaction, blastocyst formation, hatching and embryo–mother signaling (Meisser *et al*, 1999; Desail *et al*., 1999). Glut-1 is known to regulate glucose transport in morulae and blastocysts (Rieger *et al*., 1992). There were no clear high concentrations of the transcripts of these genes in embryos following *in vitro* culture. Higher levels of gene transcription might be an endogenous mechanism for providing increased substrate to cope with increased demand arising from various stresses under *in vitro* culture condition.

In the present study, we observed that *Bax* transcripts were significantly increased in NT-derived embryos at both morulae and blastocyst stages compared with the VivoF counterpart. Previous studies have shown that overexpressed *Bax*, a member of the *Bcl-2* gene family, plays key roles in regulating apoptosis, accelerated to apoptotic death in cells (Cory and Adams, 1998; Oltvai *et al*., 1993). Furthermore, expression of this apoptotic protein was higher in low-quality oocytes and degenerated embryos than in good-quality embryos (Yang *et al*., 1997). These reports showed that *Bax* mRNA production has been considered a suitable candidate for evaluating the quality of embryos. Higher expression of Bax in both NT- and IVF-derived embryos suggests that inappropriate environments during their *in vitro* development cause lower quality of embryos for further development.

In conclusion, our study suggests that alterations in mRNA expression of early developmental genes are associated more with *in vitro* culture condition than with nuclear transfer itself. Further experiments are needed to find out how the *in vitro* environment affects the preimplantation development of NT-generated embryos.

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References

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., Meadé, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. & Echelard, Y. (1999). Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* **17**, 456–461.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A. & Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–1258.

Cory, S. & Adams, J.M. (1998). Matters of life and death: programmed cell death at cold spring harbor. *Biochim. Biophys. Acta* **1377**, 25–44.

Daniels, R., Hall, V. & Trotmson, A.O. (2000). Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biol. Reprod.* **63**, 1034–1040.

Desail, N., Scarrow, M., Lawson, J., Kinzer, D. & Goldfarb, J. (1999). Evaluation of the effect of interleukin-6 and human extracellular matrix on embryonic development. *Hum. Reprod.* **14**, 1588–1592.

Kirchhof, N., Carnwath, J.W., Lemme, E., Anastassiadis, K., Schöler, H. & Niemann, H. (2000). Expression pattern of Oct-4 in preimplantation embryos of different species. *Biol. Reprod.* **63**, 1698–1705

Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H. & Galli, C. (2002). Cellular and molecular deviations in bovine *in vitro*-produced embryos are related to the large offspring syndrome. *Biol. Reprod.* **67**, 767–775.

Meisser, A., Cameo, P., Islami, D., Campana, A. & Bischofl, P. (1999). Effects of interleukin-6 (IL-6) on cytotrophoblastic cells. *Mol. Hum. Reprod.* **5**, 1055–1058.

Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K. & Takeichi, M. (1987). Transformation of cell adhesion properties by exogenously introduced *E-cadherin* cDNA. *Nature* **329**, 341–343.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H. & Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391.

Oltvai, Z.N., Milliman, C.L. & Kormeyer, S.J. (1993). Bcl-2 heterodimerizes *in vitro* with a conserved homology, Bax, that accelerated programmed cell death. *Cell* **74**, 609–619.

Ovitt, C.E. & Scholer, H.R. (1998). The molecular biology of Oct-4 in the early mouse embryo. *Mol. Hum. Reprod*. **4**, 1021–1031.

Palmieri, S.L., Peter, W., Hess, H. & Scholer, H.R. (1994). Oct4 transcription factor is differentially expressed in the mouse embryo during establishment of the first two extraembryonic cell lineages involved in implantation. *Dev. Biol.* **166**, 259–67.

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.

Rieger, D., Loskutoff, N.M. & Betteridge, K.J. (1992). Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured *in vitro*. *J. Reprod. Fertil.* **95**, 585–595.

Rizos, D., Lonergan, P., Boland, M.P., Arroyo-Garcia, R., Pintado, B., de la Fuente, J. & Gutierrez-Adan, A. (2002). Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol. Reprod.* **66**, 589–595.

Rosenkrans, C.F., Zeng, G.Q., McNamara, G.T., Schoff, P.K. & First, N.L. (1993). Development of bovine embryos *in vitro* as affected by energy substrates and inorganic ions. *Biol. Reprod.* **49**, 459–462.

Scholer, H.R. (1998). New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. *Genes Dev.* **12**, 2073–2090.

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**, 369–374.

Watson, A.J., Westhusin, M.E., DeSousa, P.A., Betts, D.H. & Barcroft, L.C. (1999). Gene expression regulating blastocyst formation. *Theriogenology* **51**, 117–133

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. & Campbell, K.H.S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.

Wrenzycki, C., Herrmann, D., Carnwath, J.W. & Niemann, H. (1999). Alteration in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Mol. Reprod. Dev.* **53**, 8–18.

Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R. & Niemann, H. (2001). Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol. Reprod.* **65**, 309–317.

Yang, J., Liu, X. & Bhalla, K. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. *Science* **275**, 1129–1136.