Correlation of Oct4 and FGF4 Gene Expression on Peri-implantation Bovine Embryos Reconstructed with Somatic Cell

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ABSTRACT

This study was carried out to investigate the developmental rates of embryo reconstructed with different cell type and to estimate correlation of transcriptional level of octamer-binding transcription factor 4 (Oct4) and fibroblast growth factor 4 (FGF4) gene on peri-implantation stage embryos. Donor cells were transferred into perivitelline space of enucleated oocytes. The karyoplast-cytoplast couplets were accomplished by cell to cell fusion and activated with ionomycin and 6-dimethylaminopurine. Reconstructed embryos were co-cultured with bovine oviduct epithelial cells in CRlaa medium. There is no difference in blastocyst formation rate following nuclear transfer (NT) with fetal fibroblast cell (16/50; 32.0%), cumulus cell (16/49; 32.6%) and ear cell (17/52; 32.6%). The expression level of Oct4 and FGF4 in peri-implantation bovine embryo derived from in vitro fertilization (IVF) and NT were determined by reverse-transcription polymerase chain reaction (RT-PCR) technique. In peri-implantation of IVF result in a transient increased of FGF4 paralleled by an increased expression of Oct4. However, Oct4 gene was highly expressed in hatching blastocysts derived from NT compared to IVF. Also, FGF4 expression level in hatching blastocysts and outgrowth stage derived from NT was lower than that of IVF.

In conclusion, it is suggested that the different transcription patterns observed in nuclear transfer embryos may lead to a lower rate of embryo development, implantation and pregnancy.

(Key words : Nuclear transfer, Peri-implantation, Oct4, FGF4, RT-PCR)

I. INTRODUCTION

Despite the production of viable offspring from somatic cell nuclear transfer (NT) procedures in a number of species (Wilmut et al., 1997; Cibelli et al., 1998; Kato et al., Wakayama et al., 1998), the efficiency of animal production by cloning is still very low. Typically, less than half of all somatic cell clones develop to the blastocyst stage, and of those less than one third develop beyond implantation. Therefore, most cloned embryos are not able to develop to the late preimplantation or early postimplantation stage. It is considered that the NT embryos proceed inappropriate reconstruction with donor-recipient cell, which lead to a abnormal em-

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bryo development, and differential expression of mRNA transcript.

In order to increase the efficiency of animal cloning, researcher are now analysing gene expression of the nuclear transfer to find potential areas of improvement. Gene expression analysis using RT-PCR revealed a similar pattern of transcription between cloned and fertilized embryos in porcine (Koo et al., 2001) and bovine preimplantaton embryos (Van Stekelenburg-Hamers et al., 1994). In the cloned bovine blastocyst aberrant expressions have been detected in several genes, such as Mash 2, DNMT and HSP70. Expression of FGF4, and IL6 were detected in cloned embryo derived from granulosa (Daniels et al., 2000), but not fetal epithelial cells (Daniels et al., 2001).

A few of genes have been shown to be essential during preimplantation. Oct4 encodes a transcription factor required for mouse embryo development beyond the blastocyst stage (Ovitt and scholer, 1998). Expression of various genes including FGF4, Rex1, Sox2, OPN (Pesce and scholer, 2001) and putative downstream genes, Creatin kinase B, Histone H2A. Z and Ribosomal protein S7 (Du et al., 2001) is under the influences of Oct4 during early embryo development. FGF4 is target gene of Oct4 (Dailey et al., 1994; Yuan et al., 1995; Botquin et al., 1998) and is one of the few genes found to have aberrant levels in clones bovine blastocysts (Daniels et al., 2000, 2001). Transcription of Oct4 is associated with pluripotent cell lineages in mammals and this gene is expressed in the early embryonic stages of development and primodial germ cell. Whereas FGF4 has been shown to play important roles in either preimplantation, implantation or early post-implantation development in the mouse (Feldman et al., 1995). Also, this growth factor may be potentially important for the process of embryo implantation because it is stored within the extracelluar matrix and is thus easily available during embryo

invasion.

Therefore, this present study was conducted to evaluate the correlation of transcriptional level between Oct4 and FGF4 gene on peri-implantation stage embryos after IVF and NT.

II. MATERIALS AND METHODS

1. In Vitro Maturation (IVM) of Bovine Follicular Oocytes

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory in saline at $35 \sim 37℃$. Cumulus-oocyte complexes (COCs) were obtained from antral follicles of $2 \sim 8$ mm in diameter by a 10 ml syringe attaching 18 gauge needle. The COCs with evenly granulated cytoplasm and compact cumulus cells of more than 3 layers were selected and washed three times with TL-HEPES (Parrish et al., 1985) and the maturation medium drop subsequently. Ten COCs were transferred into a $50 \mu l$ of maturation medium under warm paraffin oil in a petri-dish equilibrated at $39℃$ with 5% CO_2 in air.

The maturation medium was TCM-199 (Gibco, Grand island, NY) supplemented with 25 mM sodium bicarbonate, 10% featal bovine serum (FBS; Gibco BRL), $0.22 \mu g/ml$ sodium pyruvate, $25 \mu g/ml$ gentamycin sulfate, $1 \mu g/ml$ estradiol-17β (Sigma Chemical Co., st. Louis, Mo) and $1 \mu g/ml$ FSH (Folltropin V; veterpharm, Canada). Culture was carried out at $39℃$, 5% CO_2 in air for $22 \sim 24$ hr.

2. In Vitro Fertilization (IVF) of Bovine Oocytes

Matured COCs were washed with Sperm-TALP (SP-TALP) medium and subsequently with Fertilization-TALP (Fert-TALP) medium (Rosenkians et al., 1993). After washing, COCs were transferred into a 44 μl droplet of Fert-TALP under paraffin oil. Motile spermatozoon were collected from frozen-thawed semen a discontinuous percoll gradient.

Final concentration of the sperm was adjusted to 2×10^6 Sperm/ml. Then $2 \mu l$ of heparin stock sloution and PHE stock solution (2 mM Phenicillamine, $20 \mu M$ hypotaurin and $1 \mu M$ epinephrine) were added into the droplet of Fert-TALP to induce sperm capacitation and to stimulate sperm motility, respectively. Sperm and oocytes were coincubated in a 50 μl drops of Fert-TALP under paraffin oil for 24 hrs at 39℃, 5% CO_2 in a humidified atmosphere.

3. Preparation of Donor Cells

Somatic donor cells were obtained from three different sources; adult cumulus, fetal fibroblasts and adult ear skin cell. Bovine fetal fibroblasts (BFFs) were isolated from a fetus of pregnant female bovine at 40 day in gestation. Twenty hours after the onset of maturation, cumulus cells were isolated by pipetting in TL-HEPES (Sigma) supplemented with 1 mg/ml hyaluronidase (Sigma) and transferred to DMEM for use as donor cells. Small pieces of biopsied ear tissue were obtained from an adult Korean native cattle (Hanwoo; Bos taurus coreanae). Fetal fibroblasts and adult ear skin cells were digested enzymatically in 0.05% trypsin-EDTA (Gibco) solution for 30 min at 39℃. After washing three times by a centrifugation at 1,000 rpm for 5 min each, the pellet was placed in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS and then placed in a plastic culture dish. After primary culture, the cells formed a confluent monolayer, the monolayer cells were subcultured for 2~8 passage in 5% CO_2 in air at 39℃. After three subpassages, cell stocks were frozen and stored in liquid nitrogen. Each passage required 5~6 days in culture. Individual cells were collected by trypsinization of the monolayer for 30 sec and were provided for NT.

4. Oocyte Enucleation and Donor Cell Micro-

injection

Cumulus cells of matured oocytes were stripped of in TL-HEPES supplemented with 0.1% hyaluronidase and washed three times in TL-HEPES containing 0.1% BSA. The enucleation of recipient oocytes and the injection of donor cells were performed as previously described (Collas and barnes, 1994) with some modification. The oocytes were enucleated by aspirating the first polar body and M Ⅱ plate in a small volume of surrounding cytoplasm using a beveled pipette (25 μ m in diameter in CRIaa containing 0.3% bovine serum albumin (BSA) and 7.5 μM cytochalsin B (CB; Sigma)). Successful enucleation was confirmed by staining and the enucleated oocytes were incubated in modified CRIaa containing 0.3% BSA until injection of donor cell. One hour after enucleation, a $10 \sim 15 \mu$ m pipette (inner diameter) containing the cell was introduced through the slit on the zona pellucida formed during enucleation and expelled the cells into perivitelline space. Close contact of the donor cell membrane with the vitelline membrane of the cytoplast was visually confirmed prior to fusion.

5. Fusion and Activation

Reconstructed eggs were washed three times in fusion solution composed of 0.3 mM mannital, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Karyoplast-cytoplast couplets were manually aligned between two stainless steel electrodes (1 mm gap) in a fusion chamber filled with fusion solutin and fused by a double DC pulse (2.63 KV/cm for 30 μsec) delivered by a BTX Electrocell Manipulator 200 (BTX, Gentronics, San Diego, CA). Fusion of the couplets were evaluated after a 30 min incubation period in mCRIaa and then they were activated. Fused couplets were activated by a 5 min exposure to a drop of CRIaa supplemented with 5 μM ionomycin and then incubated 3 hr in 2 mM 6- dimethylaminopurine (6-DMAP; Sigma) in CRIaa containing 0.3%

BSA.

6. In vitro Culture of Reconstructed Oocytes

Cloned and fertilized embryos were cultured in 50 μl of CRlaa containing 0.3% BSA for 40~44 hrs. Oocytes were co-cultured with bovine oviductal epithelial cells in 50 μl of CRlaa containing 10% FBS for 6 days. All oocytes were cultured at 39℃, 5% CO_2 in air.

7. Embryonic Outgrowths

Cloned and fertilized embryos at the blastocyst and hatching blastocyst stage were placed in 60 mm dishes (Falcon 1007, Becton & Dickinson). Outgrowths were induced in DMEM supplemented 1% FBS, all blastocysts attached within 72 hrs. Subsequent outgrowth formation was defined by the observation of trophoblast cells spreading from attached blastocyst.

8. Analysis of Gene Expression using RT-PCR

The zonae pellucida of cloned and fertilized embryos were collected at blastocyst and hatching blastocyst stage were removed by brief exposure to TL-HEPES containing 0.5% protease. Single embryos were snap frozen in liquid nitrogen and stored at −70℃ prior to use. When required, samples were heated to 39℃ for 1 min, transferred directly to ice, and oligo dT was added. The mixed samples were incubated at 70℃ for 5 min and then chilled on ice. The incubated mixtures were transferred to RT premix tubes (Bionner) and diethyl-pyrocarbonate (DEPC)-treat water was added to fill up the reaction volume. RT reaction was performed at 42℃ for 60 min and 94℃ for 5 min. After RT, the reaction samples were stored at −20℃. PCR amplification was carried out on 10 μl of the RT product from embryo and PCR premix [1U Tag DNA polymerase, 250 μM dNTPs, 10 mM Tris -HCL, 40 mM KCl, 1.5 mM $MgCl_2$ (Bionner)].

PCR cycle for Oct4, were performed 94℃ for 5 min followed by 30 cycle embryos of 94℃ for 30 sec 59℃ for 30 sec, and 72℃ for 30 sec. Also PCR condition for FGF4 were 94℃ for 5 min followed by 30 cycle embryos of 94℃ for 20 sec, 60℃ for 20 sec, and 72℃ for 20 sec. 5 μl of the PCR products were visualized under ultraviolet light on 1.5% agarose gels. Primer sequences for Oct4 were (5'-3') GGTTCTCTTTGGAAAGGTG-TTC and ACACTCGGACCACCTTCTTTC. Also, Primer sequences for FGF4 were (5'-3') GTACG-GCTCGCCTTTCTTC and GAAGTGGGTGACC-TTCATGG.

9. Statistical Analysis

The data from at least three replication were pooled. Differences in the percentages of oocytes developed to particular stages were estimated by Student's t-test.

Ⅲ. RESULTS

1. Developmental Potential of Nuclear Transfer Embryos In Vitro

Development competence of bovine oocytes reconstructed with somatic cells was compared to IVF -derived embryos (Table 1). Although the cleavage rate of NT embryos (74.4% 140/198) was slightly lower than that of IVF embryos (84.3% 226/268) and activated embryos (82.2% 181/220), there was not significant difference. Developmental rate (37.1 % 52/140) to blastocyst stage of NT embryos was similar to that (41.5% 94/226) of IVF embryos and (36.4% 66/181) of activated embryos.

In vitro development of reconstructed bovine embryos produced by various somatic cell was showed in Table 2. Out of 97 (Fibroblast cell), 121 (Cumulus cell), 106 (Ear cell) bovine eggs reconstructed by NT, 50, 49, and 52 embryos were cleaved, respectively. Among them 16 (Fibroblast

Table 1. *In vitro* development of nuclear transferred bovine embryos by using somatic cell

No. of oocytes	No. (%) of		
	Fused	Cleaved	Blastocysts
Activation	220	–	181 (82.2)[a]	66 (36.4)[a]
IVF	268	–	226 (84.3)[a]	94 (41.5)[a]
NT	260	198 (72.3)	140 (74.4)[a]	52 (37.1)[a]

[a] Values with same superscripts within a column means no significant difference (P>0.05).

Table 2. *In vitro* development of nuclear transferred bovine embryos by using various somatic cells

Treatment	No. of oocytes	No. (%) of		
		Fused	Cleaved	Blastocysts
Fibroblast cell	97	72 (74.2)[a]	50 (69.4)[a]	16 (32.0)[a]
Cumulus cell	121	83 (68.5)[a]	49 (59.0)[a]	16 (32.6)[a]
Ear cell	106	73 (68.8)[a]	52 (71.2)[a]	17 (32.7)[a]

[a] Values with same superscripts within a column means no significant difference (P>0.05).

cell: 32.0%), 16 (Cumulus cell: 32.6%), and 17 (Ear cell: 32.7%) embryos developed to the blastocyst stage. There is no significant difference in the blastocyst rates between these NT embryos.

2. Developmental Potential of Nuclear Transfer Embryos *In Vivo*

Twenty two blastocyst derived from the NT embryo were transferred to 11 recipient cows, 7~9 days after the onset of oestrus (Table 3). Five of eleven surrogate cows became pregnant after embryo transfer. The frequency of pregnancy in the surrogate mothers was 45.4% by day 30, 36.3% by day 60, and 27.2% by day 90 of gestation.

3. Analysis of Gene Expression in Bovine Pre-implantation Embryos Derived from IVF and NT

With the profile of transcription for each gene determined in preimplantation embyos derived from IVF, the same RT-PCR techniques were applied to embryos reconstructed with somatic cell. Fig. 1 shows typical results obtained from preimplantation single embryos derived from IVF and NT. Expression of Oct4 and FGF4 were also detected in NT embryos with a similar pattern to IVF embyos.

4. Outgrowth of Bovine Peri-implantation Embryos Derived from IVF and NT

The present study was used outgrowth formation as an *in vitro* model for implantation and immediate postimplantation development of the embryo (Fig. 2). Cloned embryos were limited in their ability to form outgrowths, compared to IVF.

Fig. 3 shows typical gene expression pattern

Table 3. Pregnancies following embryo transfer of NT blastocysts into synchronized recipient

|	No. of blastocysts transferred	No. (%) of recipients	No. (%) of pregnant recipient on day		
			30	60	90
NT	22	11	5 (45.4)	4 (36.3)	3 (27.2)
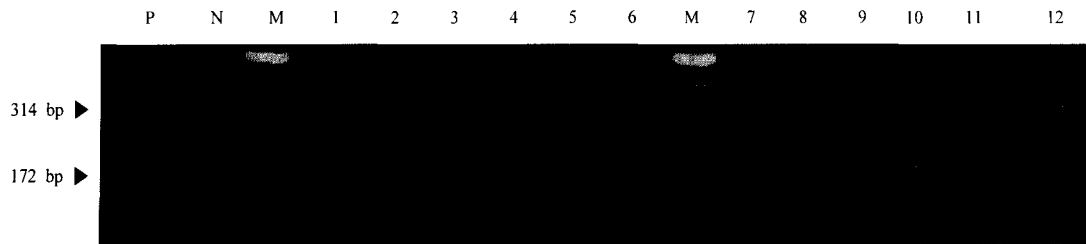
Fig. 1. The analysis of FGF4 (172 bp) and Oct4 (314 bp) transcripts in single bovine preimplantation stage embryos derived from IVF (1~6) and NT (7~12). M: size marker (100 bp ladder), P: positive control (liver tissue), N: negative control, Lane 1 and 7: 2 cell, Lane 2 and 8: 4 cell, Lane 3 and 9: 8 cell, Lane 4 and 10: 16 cell, Lane 5 and 11: morula, Lane 6 and 8: blastocysts.
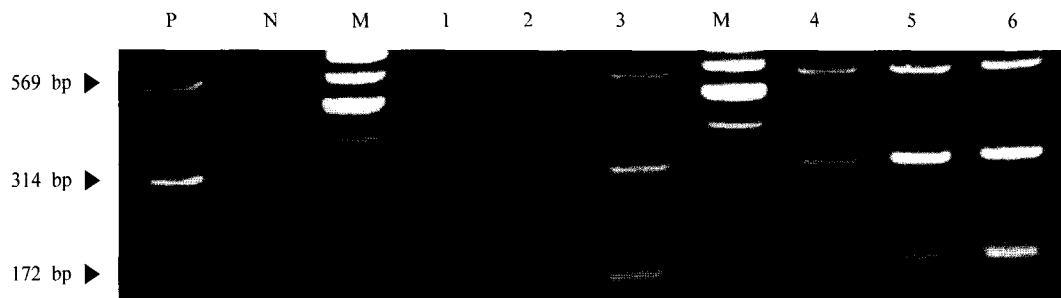
obtained from peri-implantation single embryos derived from IVF and NT. Oct4 and FGF4 mRNA were detected in early, hatching blastocysts and outgrowth at peri-implantation stage and amount of message relative to GAPDH mRNA unaffected by the treatment.

Fig. 4 shows Oct4 versus GAPDH and FGF4 versus GAPDH signal strength ratios in early, hatching blastocysts, outgrowth embryo. Transcript of Oct4 gene was highly expressed in hatching blastocysts in NT group compared to IVF group. Expression level of Oct4 in outgrowth stage derived from IVF was higher than in outgrowth stage derived from NT. Transcripts of FGF4 were detected
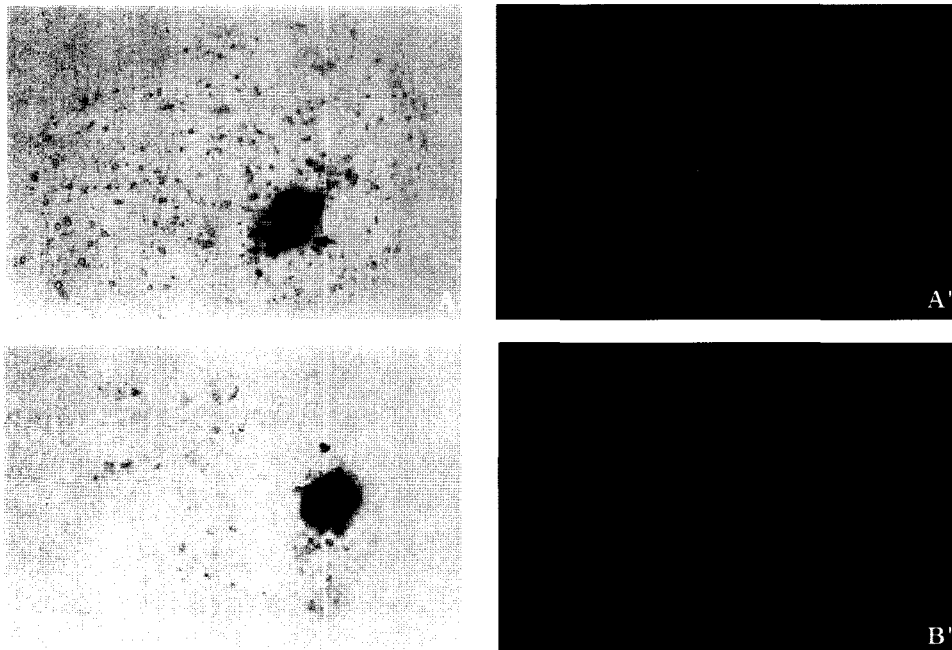
Fig. 2. Outgrowths from IVF (A, A') and NT (B, B'); A) the outgrowth at day 12 after IVF. B) the outgrowth at day 12 after NT. (A', B') the outgrowth were stained with Hoechst 33342.

Fig. 3. The analysis of GAPDH (569 bp), Oct4 (314 bp) and FGF4 (172 bp) transcripts in single bovine peri-implantation stage embryos derived from IVF (1~3) and NT (4~6). M: size marker (100 bp ladder), P: positive control (liver tissue), N: negative control, Lane 1 and 4: early blastocyst, Lane 2 and 5: hatching blastocyst, Lane 3 and 6: outgrowth stage.
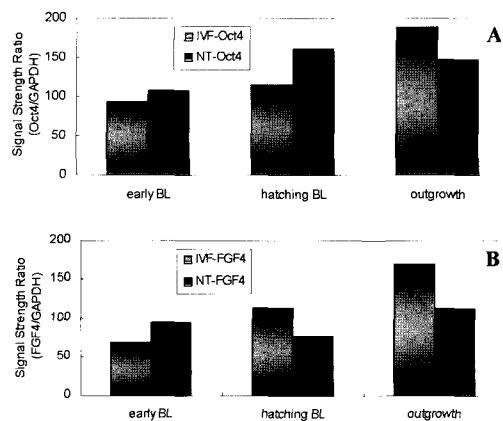
Fig. 4. Bar graph for signal strength ratios of Oct4 to GAPDH (A) and FGF4 to GA-PDH (B) for early blastocyst, hatching blastocyst and outgrowth stage group.

similar pattern to Oct4 derived from IVF. However, FGF4 expression level in hatching blastocysts and outgrowth stage derived from NT was lower than that of IVF. Correlation of Oct4 and FGF4 were different from IVF and NT on transcription pattern. In IVF, Oct4 play an essential role in transcriptional activation of the FGF4 gene. But the correlation of Oct4 and FGF4 in NT did not occurred.

IV. DISCUSSION

In order to determine the genetic causes of the

low efficiency of animal production using NT techniques, comparison was made on the transcription of Oct4 and FGF4 in bovine preimplantation and peri-implantation embryos derived from IVF and NT. The genes studied are known to have important functions during preimplantation and early postimplantation development in mammals. The intensity of gene expressed of embryo at different stages of development was estimated with PCR product. Hence, the profile of transcription for each gene was determined from early blastocyst to outgrowth stage of development in IVF and cloned embryos.

Transcripts of Oct4 and FGF4 were not readily detectable in granulosa cell cDNA. Hence, the evaluating transcription of these genes in NT and IVF embryos is the critical factor when assessing the effects of nuclear reprogramming in cloned embryos (Daniels et al., 2000). Previous studies have shown that development beyond the blastocyst stage depends on Oct4 (Nichols et al., 1998) and the Oct4 level determines the fate of embryonic stem cells *in vitro* (Niwa et al., 2000). Expression of Oct4 at the RNA and protein levels was observed in bovine oocytes and embryos until day 10 of development, consistent with a role in early embryos development, as has been postulated in the mouse (Rosner et al., 1990). The present study was

used outgrowth formation as an in vitro model for implantation and immediate postimplantation development of the embryo. Expression of Oct4 was observed in peri-implantation until day 12. One of the most interesting candidate targets of Oct4 is FGF4. FGF4 gene has an octamer-containing enhancer in its 3' noncoding region and has been demonstrated to respond to Oct4 in a Sox2-dependent fashion (Curatola and Basilico, 1990; Yuan et al., 1996; Ambrosetti et al., 1997). Expression of FGF4 is co-expressed with Oct4 in the ICM and epiblast (Ma et al., 1992; Niswander and Martin, 1992). Co-expression of Oct4 and FGF4 suggests functional interaction which may be important for regulation of ICM versus trophectoderm formation (Ovitt and Scholer, 1998). Transcription of Oct4 and FGF4 was consistently detected in preimplantation embryos reconstructed with somatic cell in a similar pattern to that detected in IVF embryo. In the analysis of FGF4, no transcripts were detected from the oocytes to the 8-cell stage embryos, but consistently detected at the morula and blastocyst stage embryos derived from IVF (Daniels et al., 2000). Also, in the case of NT, transcription of FGF4 was not detected preimplantation stage (Daniels et al., 2000). In peri-implantation stage embryos derived from IVF revealed a transient increase of FGF4 paralleled by an increased expression of Oct4. Therefore, Oct4 derived from IVF plays an essential role in transcriptional activation of the FGF4 gene. However, expression pattern of Oct4 and FGF4 in the embryo in IVF group was different from in the embryo in NT group. In adequate maintenance of Oct4 derived from NT expression reflects improper regulation of FGF4. Work is currently being carried out using differential gene expression studies to identify further genes which are aberrantly expressed in nuclear transfer embryos in comparison to in vitro and in vivo produced embryos.

In the present study, transcript of FGF4 was detected with a high efficiency from hatching blastocyst to outgrowth stage embryos derived from IVF. Interestingly, FGF4 transcript was not detected in a significant number of parthenogenetic blastocysts (Daniels et al., 2001). Other mammals including cow, parthenogenetic embryos fail to produce viable pregnancies following transfer to recipient (Fukui et al., 1992). Mouse embryos that are homozygous for targeted disruptions of the FGF4 gene were shown to die shortly after implantation (Feldman et al., 1995). Hence, the lack of FGF4 transcription in the nuclear transfer is associated with a group of embryo which are not developmentally viable. From the results obtained in the present studies, abnomal pattern of Oct4 and FGF4 in somatic cell bovine clones suggests that developmental competence of clones is already compromised at the peri-implantation stage and reflected in subsequent development. The failure of cloned bovine embryo to develop implantation is related to an incorrect lineage determination in the peri-implantation stage as directed by Oct4 and FGF4. This method will provide a powerful research tool for examine other genes essential for peri-implantation and postimplantation development of embryos.

V. REFERENCES

1. Ambrosetti, D. C., Basilico, C. and Dailey, L. 1997. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol. Cell. Biol., 17:6321-6329.
2. Botquin, V., Hess, H., Fuhrmann, G., Anastassiadis, C., Gross, M. K., Vriend, G. and Scholer, H. R. 1998. New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct4 and Sox2.

Genes Dev., 12:2073-2090.

3. Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de Leon F. A. and Robl, J. M. 1998. Cloned transgenic calves produced form nonquiescent fetal fibroblasts. Science, 280:1256-1258.

4. Curatola, A. M. and Basilico, D. 1990. Expression of the k-FGF proto-oncogene is controlled by 3' regulatory elements which are specific for embryonal carcinoma cells. Mol. Cell. Biol., 10: 2475-2489.

5. Dailey, L., Yuan, H. and Basilico, C. 1994. Interaction between a novel F9-specific factor and octamer-binding proteins is required for cell type restricted activity of the fibroblast growth factor 4 enhancer. Mol. Cell. Biol., 14:7758-7769.

6. Daniels, R., Hall, V. J. and Trounson, A. O. 2000. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol. Reprod., 63:1034-1040.

7. Daniels, R., Hall, V. J., French, A. J., Korfiatis, N. A. and Trounson, A. O. 2001. Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. Mol. Reprod. Dev., 60:281-288.

8. Du, Z., Cong, H. and Yao, Z. 2001. Identification of putative downstream genes of Oct -4 by suppression-subtractive hybridization. Biochem. Biophys. Res. Commun., 282:701-706.

9. Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiara, T. M. and Goldfarb, M. 1995. Requirement of FGF4 for postimplantation mouse development. Science, 267:246-249.

10. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J. Y., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. Science, 282: 2095-2098.

11. Koo, D. B., Kang, Y. K., Choi, Y. H., Park, J. S., Kim, H. N., Kim, T., Lee, K. K. and Han, Y. M. 2001. Developmental potential and transgenic expression of porcine nuclear transfer embryos using somatic cells. Mol. Reprod. Dev., 58:15-21.

12. Ma, Y. G., Rosfjord, E., Huebert, C., Wilder, P., Tiesman, J., Kelly, D. and Rizzino, A. 1992. Transcriptional regulation of the murine k-FGF gene in embryonic cell lines. Dev. Biol., 154:45-54.

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

14. Niswander, L. and Martin, G. R. 1992. FGF4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development, 114:775-769.

15. Niwa, H., Miyazaki, J. and Smith, A. G. 2000. Quantitative expression of Oct3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet., 24:372-376.

16. Ovitt, C. E. and Scholer, H. R. 1998. The molecular biology of Oct4 in the early mouse embryo. Mol. Hum. Reprod., 4:1021-1031.

17. Parrish, J. J., Susko-Parrish, J. L. and First, N. L. 1985. Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. Theriogenology, 24:537-549.

18. Pesce, M. and Scholer, H. R. 2001. Oct4: Gatekeeper in the beginnings of mammalian development. Stem Cells, 19:271-278.

19. Rosenkrans, C. F., Zeng, G. Q., McNamara, G. T., Scfoff, O. K. and First, N. L. 1993. Development of bovine embryos in vitro as affected by energy substrates. Biol. Repord., 49:459-

462.

20. Rosner, M. H., Vigano, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. J. and Staudt, L. M. 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature, 345:686-692.

21. Van Stekelenburg-Hamers, A. E., Revel, H. G., van Inzen, W. G., de Loos, F. A., Drost, M., Mummery, C. L., Weima, S. M. and Trounson, A. O. 1994. Stage-specific appearance of the mouse antigen TEC-3 in normal and nuclear transfer bovine embryos: Re-expression after nuclear transfer. Mol. Reprod. Dev., 37:27-33.

22. Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R. and Yanagimachi, R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature, 394:369-374.

23. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. S. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature, 385:810-813.

24. Yuan, H., Corbi, N., Basilico, C. and Dailiy, L. 1996. Developmental-specific activity of the FGF 4 enhancer requires the synergistic action of Sox2 and Oct3. Genes Dev., 9:2635-2645.