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Differential Gene Expression in the Bovine Transgenic Nuclear Trasnsfer Embryos

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Abstract : The detrimental effects of gene transfection on embryo development and the molecular mechanism behind the differential expression of genes related to early embryo development were assessed in the production of transgenic cow embryos through somatic cell nuclear transfer (NT). Parthenogenetic, IVF, and transgenic NT embryos derived from α_1-antitrypsin transfected ear fibroblast cells was produced. To investigate the molecular mechanism behind lower developmental competence of transgenic NT embryos, the differential mRNA expression of three genes (IFN-τ, Oct4, Fgf4) in the 3 types of embryo (Parthenogenetic, IVF, transgenic NT) was examined. RNA was extracted from ten blastocysts derived from 3 types of embryos and reverse-transcripted for synthesis of the first cDNA. The quantification of 3 gene transcripts (IFN-τ, Oct4, and Fgf4) was carried out in three replicate by quantitative real-time reverse transcriptase PCR. Expression level of IFN-τ mRNA was significantly higher in transgenic NT embryos than parthenogenetic and IVF embryos ($P<0.05$). However, expression level of Oct4 and Fgf4 of transgenic NT embryos was significantly lower than IVF embryos ($P<0.05$). Altered levels of these three mRNA transcripts may explain some of the embryonic/fetal/neonatal abnormalities observed in offspring from transgenic NT embryos.

Key words **:** bovine, gene expression, nuclear transfer, transgenic.

Introduction

Cloning of bovine embryos via nuclear transfer (NT) from fetal or adult somatic donor cells has made great progress during the past few years (11,2,8,18,20,21,7,1). However, a high rate of embryonic, fetal, and neonatal abnormalities has been consistently observed, all of which are described by the term large offspring syndrome (2,8,1,18,21). The production of viable transgenic offspring using NT depends upon the successful combination of many factors, including oocyte quality and age, activation, reconstruction process, culture conditions, type of donor cell, donor cell transfection methods, cell cycle stage of both donor and recipient cells, and the viability of the transfected donor cell.

Normal expression of genes related to embryo development is required for reprogramming after NT. The molecular mechanisms underlying reprogramming are still largely unknown (10,24). Likely candidates include genetic imprinting (17) and epigenetic DNA modifications such as DNA methylation (13) and changes in chromatin configuration (9). Endogenous genes such as the transcription factor Oct-4, fibroblast growth factor (Fgf), and gp130 are expressed in a similar manner throughout the development of bovine NT embryos, while transcript levels are altered for several other growth factor and cytokine genes (3). The abnormal expression of developmentally important genes is suspected to have detrimental effects on embryonic development. The expression pattern of a set of marker genes in bovine NT-derived embryos has not yet been correlated with targeted modifications of the NT procedure.

In this study, the expression of genes related to embryo development, transcription of interferon-τ (IFN-τ), Octamer-binding transcription factor (Oct4), and fibroblast growth factor 4 (Fgf4) were compared in parthenogenetic, *in vitro* fertilization (IVF), and transgenic NT embryos for investigation of abnormal development competence of transgenic NT embryos. IFN-τ is associated with early differentiation and trophoblastic function (16), Oct4 is associated with pluripotent cell lineages in mammals and is expressed in the early embryonic stages of development and primordial germ cells (12), and Fgf4 transcription is associated with early development and natural inducer of limb mesenchyme proliferation (5). The identification of genes with predictable abnormal expression in transgenic NT embryos may provide genetic markers that can be used to assess transgenic embryo viability prior to transfer to recipient animals.

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Materials and Methods

Generation of transfected cell lines

In order to isolate ovine ear fibrobalst cells, high-performance Holstein cow was selected for donor cow. The external surface of ear was shaved and cleaned aseptically. A piece of skin tissue with about 100 mm^2 wide and 1 mm thick was biopsied and immediately immersed in sterile PBS (Life Technologies, Rockville, MD). The biopsied specimen was transferred into 3 rinsing baths of sterile PBS, and pieced tissue specimen was dispersed by exposure to 0.1% trypsin-EDTA for 1-2 hrs. The cell suspension was transferred into culture dish in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) in a humidified atmosphere of 5% CO_2 and 95% air. An expression plasmid for human α_1-antitrypsin (pGFP-αAT) was constructed by inserting a bovine beta-casein promoter (accession number : M55158.1), a green fluorescent protein (GFP) marker gene, and human αAT target gene (accession number : X01683) into a pcDNA3 plasmid (Life Technologies). For transfection, ear fibroblast cells were cultured to 50% confluency in a 35-mm culture dish and transfected with the plasmid using FuGene6® (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The transfected cells were cultured for another 4 days to induce chromosomal integration of the transgene. Before injection of the donor cells, transfected cells were collected by trypsinization. After centrifugation, the pellets were resuspended in PBS supplemented with 0.5% fetal bovine serum (FBS). GFP-expressing cells were selected under ultraviolet (UV) light using a standard fluorescein isothiocyanate filter set (FITC; excitation wavelength : 450-490 nm; B-mode filter, Nikon, Japan) and used for microinjection.

Preparation of recipient oocytes

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory in 0.9% (v/v) NaCl solution at 30 to 35°C. Follicular fluid and COCs from follicles 2 to 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10-ml disposable syringe. COCs with evenly granulated cytoplasm and enclosed by compact cumulus cells of more than three layers were selected, washed three times in HEPES-buffered tissue culture medium-199 (TCM-199; Life Technologies), and cultured for in vitro maturation (IVM) in bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS, 0.005 IU/ml FSH (Antrin, Teikoku, Tokyo, Japan), and 1 g/ml estradiol (Sigma Co., St. Louis, MO) in a humidified atmosphere of 5% CO_2 in air.

Production of embryos

Parthenogenetic embryos: After 24 h of maturation, oocytes were denuded by mouth pipetting. Next, mature oocytes were selected by the presence of the first polar body under a stereomicroscope. After 4 h of culture, mature oocytes were activated by incubation in handling medium containing 5 μm ionomycin (Sigma) for 4 min. Embryos were then extensively washed in TCM199-washing medium for 5 min before culture for 4 h in 2-mM 6-DMAP (Sigma) in mSOF for post-activation. Culture of parthenogenetic embryos was performed in 25 μl drops of mSOF overlaid with mineral oil at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

IVF embryos : The medium used for oocyte washing, sperm penetration, and IVF was TALP containing 3 or 6 mg/ml BSA, as described by Fukui (6). After maturation, mature oocytes were subsequently rinsed three times in 2-3 ml washing-TALP medium and rinsed once in IVF-TALP (Fukui). Five to seven oocytes were transferred into a 43 μl droplet of IVF-TALP under mineral oil. Frozen-thawed sperm were subjected to a swim-up procedure for 1 h in capacitation-TALP to recover the motile spermatozoa. The supernatant was centrifuged at 500 × g for 6 min and a 5 μl aliquot of the sperm pellet was added to the IVF-TALP (approximately 1×10^6/ml) droplets containing the oocytes. Fertilization was allowed to take place at 39°C in a humidified atmosphere of 5% CO_2 in air for 18-20 h. After culture, fertilized oocytes were denuded by gentle pipetting in a TCM-washing medium. Embryos were cultured in 25 μl drops of mSOF overlaid with mineral oil at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Transgenic NT embryos: Production of transgenic NT embryos was performed as described by Cho et al. (1). Briefly, after 18 h of maturation, oocytes were denuded and mature oocytes with a first polar body were selected for enucleation. Oocytes were enucleated by a squeezing method. Nuclear transfer was performed using transfected ear fibrobalst cells that expressed the GFP under UV light as donor cells, and then fused by an electric method. Reconstructed embryos were cultured for reprogramming followed by activation by a chemical method. At 4 h postactivation, embryos were cultured in 25 μl drops of mSOF overlaid with mineral oil at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and N_2 in air atmosphere.

mRNA Extraction, cDNA Synthesis and Real-time PCR

These procedures were performed as described in the previous study (19). Briefly, ten blastocysts of the 3 groups were pooled in 1 μl of PVA-PBS and immediately stored in liquid nitrogen until RNA isolation. Poly(A) mRNAs were extracted following the manufacturer's instructions using the Dynabeads mRNA Direct Kit (Dynal). After thawing, the samples were lysed in 50 μl of lysis/binding buffer (Dynal) at room temperature for 10 min. Then, 10 μl of Dynabeads oligo$(dT)_{25}$ were added to each sample. The beads were hybridized for 5 min and separated from the binding buffer using the Dynal magnetic separator. Poly(A) mRNAs and beads were washed in buffers A and B (Dynal), and were separated by adding 11 μl of diethyl pyrocarbonate-treated water. Poly(A) mRNAs were reverse transcribed in a total volume of 20 μl containing using 2.5 μl random hexamer primer, 1 × reverse transcription buffer, 20 IU of RNase inhibitor, 50 IU Moloney murine leukemia virus reverse transcriptase enzyme (Promega, Madrid, Spain), 5 mM $MgCl_2$, and 1 mM of each dNTP.

Table 1. Primers used for polymerase chain reaction

Genes	Primer sequences	Annealing T. (°C)	Fragment size (bp)	Genebank
Histone H2a	5' = GGTAAGGCTGGGAAGGACTC	60	181	NM174809
	3' = CGGTGAGGTACTCCAGGATG			
IFN-τ	5' = GAGCCCTCTCTTCATCAACCA	60	109	AF238612
	3' = TACGAAGGTGATGTGGCATCT			
Oct4	5' = CGAGTATCGAGAACCGAGTG	60	441	NM174580
	3' = CAGGGTTCTCTCCCTAGCTC			
Fgf4	5' = CGAGTGCAGGTTCAGAGAGA	60	151	NM001040605
	3' = GAGGAAGTGGGTGACCTTCA			

After denaturation of the secondary RNA structure at 70°C for 5 min, the cDNA was complete by the following conditions at room temperature for 10 min, at 42°C for 60 min to allow the reverse transcription of RNA, and at 93°C for 1 min to denature the enzyme. The quantification of 3 gene transcripts (IFN-τ, Oct4, and Fgf4) was carried out in three replicate by quantitative real-time reverse transcriptase PCR on the 7500 real-time PCR SYSTEM (Applied Biosystems, USA) using SYBR Green, a double-stranded DNA-specific fluorescent dye. The primer sequences for each gene was as Table 1. The reaction mixture of the total 20 μl volume consisted of 10 μl of 2 × buffer (containing modified DyNAmo hot start DNA polymerase, SYBR Green I, optimerase PCR buffer, 5 mM MgCl2 and dNTP mixture including dUTP; Molecular Probes), 2 μl of each primer (0.2 uM), 4 μl of double distilled water, and 2 μl of cDNA template. The PCR was conducted in an initial step of 94°C for 15 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The fluorescence values were determined after each elongation step. The dissociation curve was analyzed following the final cycle to determine the specificity of amplification. The melting protocol consisted of holding at 40°C for 60 s and then heating from 65 to 95°C, holding at each temperature for 0.1 s while monitoring fluorescence. To identify specific amplification of a single PCR product, the product was confirmed by 2% agarose gel electrophoresis. The comparative C_T method was used for quantification of expression levels.

Statistical analysis

All data wre analyzed by analysis of variance using the SAS package. Significant differences of relative expression for IFN-τ, Oct4 and Fgf4 genes were evaluated by using the Duncan test.

Results

Expression levels of IFN-τ, Oct4 and Fgf4 genes at blastocyst stage were comparatively evaluated between parthenogenetic, IVF, and transgenic NT embryos using quantitative real-time PCR. In comparison with expression level of IFN-τ gene, expression level of transgenic NT embryos was highy enhanced compared with parthenogenetic and IVF embryos (Fig 1).

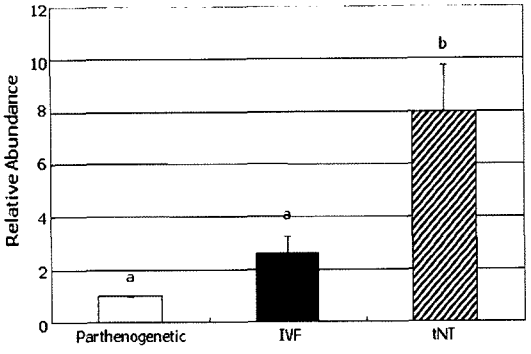

Fig 1. Comparative exrepssion levels of the IFN-τ gene between parthenogenetic, IVF and transgenic NT blstocysts. Quantification of gene expression levels is displayed as the mean ± S. E. a, b superscripts denote significant differences between parthenogenetic, IVF and transgenic NT embryos, repectively ($P < 0.05$)

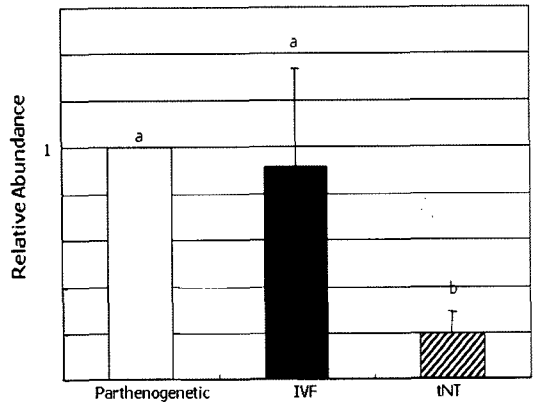

Fig 2. Comparative exrepssion levels of the Oct4 gene between parthenogenetic, IVF and transgenic NT blstocysts. Quantification of gene expression levels is displayed as the mean ± S.E. a, b superscripts denote significant differences between parthenogenetic, IVF and transgenic NT embryos, repectively ($P < 0.05$)

Expression level of IFN-τ gene in the parthenogenetic embryos was significantly lower that other 2 embryos ($P < 0.05$). In the expression level of Oct4 gene, that of transgenic NT embryos

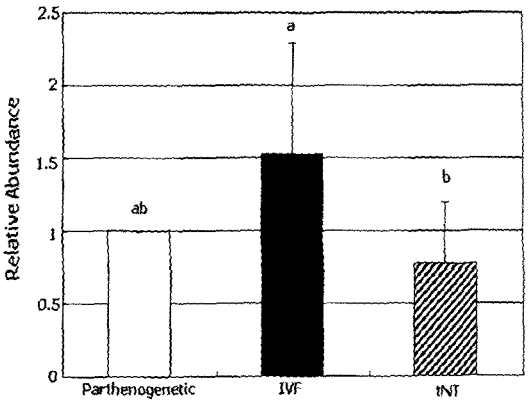

Fig 3. Comparative exrepssion levels of the Fgf4 gene between parthenogenetic, IVF and transgenic NT blstocysts. Quantification of gene expression levels is displayed as the mean± S.E. a, b superscripts denote significant differences between parthenogenetic, IVF and transgenic NT embryos, repectively ($P < 0.05$)

was significantly lower than other 2 embryos (Fig 2; $P < 0.05$). Expression level of Fgf4 gene was significantly higher in the IVF embryos than trasngesnic NT embryos (Fig 3; $P < 0.05$).

Discussion

In this study, in order to determine the possible genetic causes of the low efficiency of animal production with transgenic NT, transcription of three genes (IFN-τ, Oct4, and Fgf4) was evaluated in bovine preimplantation embryos derived from parthenogenetic activation, IVF, and transgenic NT procedures. These genes are known to have important functions during pre-implantation, early postimplantation development or both in mammals and, hence, have the potential to be used as genetic markers of embryo viability.

We found that the transcription of genes was different in blastocysts derived from parthenogenetic, IVF, and transgenic NT methods. IFN-τ is responsible for maternal recognition of pregnancy and plays a crucial role in placentation (14, 22). IFN-τ mRNA was found in blastocysts not in morulae and it is commonly believed that a higher amount of IFN-τ mRNA is an indicator of poor-quality blastocysts (23). Significantly higher expression level of IFN-τ in the transgenic NT embryos was associated with abnormal development of transgenic NT embryos.

Oct4 is a mammalian POU transcription factor expressed by early embryo cells and activity of Oct4 is essential for the identity of the pluripotent founder cell population in the mammalian embryo (12). Oct4 expression pattern suggests that Oct4 is associated with the status of totipotency, both in the preimplantation embryo and in in vitro culture (15). In this study, significantly lower expression of Oct4 gene in the transgenic NT embryos suggests that totipoency of transgenic NT embryos are not enough for normal development.

In the present study, expression of Fgf4 was detected with a high level in IVF embryos, similar with previous studies (3,4). Additionally, lower expression levels in both parthenogenetic and transgenic NT embryos also similar with previous studies (4). Higher expression of Fgf4 gene in the IVF embryos and somatic cells (4) and lower expression in the transgenic NT embryos showed that efficient reprogramming is needed for normal development of transgenic NT.

In conclusion, we observed relatively lower development rates and different mRNA expression patterns of developmentally important genes in transgenic NT embryos compared to non-transgenic NT embryos. Such early deviations in gene expression patterns in transgenic NT-derived embryos warrant further investigation, including following the embryos through postimplantation and neonatal development, to improve our understanding of the causative mechanism behind abnormalities and elevated mortality after transfer of transgenic NT embryos. mRNA phenotyping provides a useful tool to unravel the effects of NT at the molecular level. These findings should help reduce the incidence of large offspring syndrome and increase the proportion of viable calves.

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References

1. Cho, J.K., Lee, B.C., Park, J.I., Lim, J.M., Shin, S.J., Kim, K.Y., Lee, B.D. and Hwang, W.S. 2002. Development of bovine oocytes reconstructed with different donor somatic cells with or without serum starvation. Theriogenology 57: 1819-1828.
2. 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 from non-quiescent fetal fibroblasts. Science 280: 1256-1258.
3. Daniels, R., Hall, V. and Trounson, A.O. 2000. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol. Reprod. 63: 1034-1040.
4. Daniesl R. Hall VJ, French AJ, Korfiatis NA, Trounson AO. Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. Mol. Reprod. Dev. 2001 60: 281-288.
5. Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM, Goldfarb M. Requirement of FGF-4 for postimplantation mouse development. Science. 1995 13; 267: 246-249.
6. Fukui, Y. 1990. Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine embryos matured in vitro. Mol. Reprod. Dev. 26: 40-46.
7. Hwang, W.S., Shin, T.Y., Park, J.I., Roh, S.H., Lim, J.M., Shin, S.J., Cho, J.K. and Lee, B.C. 1999. Production of Holstein and Korean Native Cattle Cloned by Somatic Cell Nuclear Transfer (SNT) in Korea. Transgenic Res. 8: 482(abstr).
8. 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.
9. Koide, T., Ainscough, J., Wijgerde, M. and Surani, M.A. 1994. Comparative analysis of Igf-2/H19 imprinted domain: identification of a highly conserved intergenic DNase I hypersensitive region. Genomics 24: 1-8.
10. Kono, T. 1997. Nuclear transfer and reprogramming. Rev. Reprod. 2: 74-80.
11. Kruip, T.A.M. and den Daas, J.H.G. 1997. In vitro produced and cloned embryos: effects on preganancy, parturition and offspring. Theriogenology 47: 43-52.
12. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998 30; 95: 379-391.
13. Razin, A. and Shemer, R. 1995. DNA methylation in early development. Hum. Mol. Genet. 4: 1751-1755.
14. Roberts RM, Cross JC & Leaman DW 1992 Interferons as hormones of pregnancy. Endocrine Reviews 13 432–452.
15. Scholer HR, Balling R, Hatzopoulus AK, Suzuki N, Gruss P. Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. EMBO J 1989; 8: 2551-2557.
16. Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev. 2007; 19: 65-78.
17. Surani, A., Kothary, R., Allen, N.D., Singh, R.B., Fundele, R., Ferguson-Smith, A.C. and Barton, S.C. 1990. Genome imprinting and development in the mouse. Dev. Suppl. 89-98.
18. Vignon, X., LeBourhis, D., Chesne, P., Marchal, J., Heyman, Y. and Renard, J.P. 1999. Development of bovine nuclear transfer embryos reconstituted with quiescent and proliferative skin fibroblasts. Theriogenology. 51: 216.
19. Wee G, Koo DB, Song BS, Kim JS, Kang MJ, Moon SJ, Kang YK, Lee KK, Han YM. Inheritable histone H4 acetylation of somatic chromatins in cloned embryos. J Biol Chem. 2006 3; 281: 6048-6057.
20. Wells, D.N., Misica, P.M., Tervit, H.R. and 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: 369-378.
21. Wells, D.N., Misica, P.M. and Tervit, H.R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod. 60: 996-1005.
22. Wrenzycki, C., Herrmann, D., Carnwath, J. W. and Niemann, H. 1999. Alterations 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
23. Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R. and Niemann, H. 2001. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol. Reprod. 65: 309-317.
24. Young, L.E., Sinclair, K.D. and Wilmut, I. 1998. Large offspring syndrome in cattle and sheep. Rev Reprod. 3: 155-163.

소 형질전환 복제란의 유전자 이상발현 규명

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서울대학교 치과대학 치학연구소 BK21 치의학생명과학사업단**

요 약 : 체세포 핵이식을 통한 형질전환 소를 생산 시 초기 수정란 발육 시 발생하는 주요 유전자의 이상 발현을 규명을 목적으로 본 연구를 수행하였다. 형질전환 복제수정란의 낮은 발육능의 원인을 규명하기 위해 하기 위하여 단위발생란, 체외수정란 및 형질전환 복제수정란에서 초기 배 발육에 중요한 유전자인 IFN-t, Oct4 및 Fgf4 유전자의 발현량을 비교분석하였다. RNA는 각각 10개 수정란에서 추출한 후 reverse-transcription하여 first cDNA를 합성하고 이를 가지고 실시간 중합효수 연쇄반응을 실시하였다. IFN-t 유전자의 발현은 형질전환 복제란에서 유의적으로 높게 나왔다 ($P<0.05$). 그러나 Oct4 및 Fgf4 유전자의 경우 형질전환 복제란에서 체외수정란에 비해 유의적으로 낮게 나옴을 확인하였다. 이상의 결과로, 형질전환 복제수정란의 낮은 발육능이 원인이 발육에 중요한 유전자의 이상 발현에 기인된다고 사료된다.

주요어 : 소, 유전자 발현, 핵이식, 형질전환