# Expression of Placenta-Related Genes (Cdx2 and GATA6) in Cloned Porcine Development

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## ABSTRACT

Abnormal development and fetal loss during the post-implantation period are key concerns in the production of cloned animals by somatic cell nuclear transfer (SCNT). We hypothesized that the problems in cloned porcine offspring derived from SCNT are related to interactions between the conceptus and the endometrial environment. In the present study, we investigated expression patterns in the formation of placenta-related genes (Cdx2 and GATA6) in whole in vivo normal porcine embryos (from single cell to blastocyst) and each tissue of a normal fetus at Days 25, 35 and 55 by quantitative mRNA expression analysis using real-time PCR. The expression of Cdx2 and GATA6 mRNA increased to around the blastocyst stage. These genes were gradually decreased from the peri-implantation to post-implantation stage. Moreover, we examined the expression patterns of Cdx2 and GATA6 in Day 35 normal and SCNT cloned fetuses by the same methods. And, the level of Cdx2 and GATA6 gene expression in the extraembryonic tissue of SCNT was significantly higher than that of control tissues. From the present results, it can be postulated that the aberrant expression of Cdx2 and GATA6 genes in the endometrial and extraembryonic tissues at pre- and peri-implantation stages may be closely related to the lower efficiency of animal cloning.

(Key words : Cdx2, GATA6, Pre- and peri-implantation, Gene expression)

## INTRODUCTION

The cloning of mammals from an adult donor cell has sparked a flurry of research activities to improve cloning technology and to understand the fundamental mechanism of epigenetic reprogramming of a transferred somatic cell nucleus. As a result, many animals successfully cloned by somatic cell nuclear transfer (SCNT) have been conceived using ungulates, such as cattle (Kato *et al.*, 1998), pigs (Polejaeva *et al.*, 2000; Boquest *et al.*, 2002), and goats (Baguisi *et al.*, 1999).

The success rate of mammalian SCNT is low and usually only less than 2% of embryos transferred result in live births. But Kues and Niemann (2004) reported that cattle seem to be an exception to this viewpoint as levels of 15~20% can be reached. In any case, abnormalities in pre- and post-natal development such as embryonic, fetal and postnatal death and other developmental defects have been reported in SCNT animals (Chavatte-Palmer *et al.*, 2000; Yang *et al.*, 2002). These abnormalities include a wide range of symptoms, such as extended gestation length, oversized offspring, aberrant placental development, cardiovasculatory and respiratory problems, immunological deficiencies, problems with tendons, adult obesity, kidney and hepatic malfunctions, behavioral changes, and a higher susceptibility to neonatal diseases (Heiner Niemann *et al.*, 2008). When it comes to these various defects, Sawai *et al.* (2005) reported that the low efficiency of animal production by SCNT is considered to result from an incomplete reprogramming of the donor cell nucleus, which leads to abnormal expression of developmentally important genes.

Stroband *et al.* (1986) reported that development in the peri-attachment interval depends upon a conceptus-derived growth factor which was in porcine by histotrophic nutrition derived from the maternal uterine gland during early trophoblast elongation. Histotrophic factors appear to regulate the placental surface area available to each conceptus. Post-attachment conceptus growth and development subsequently come to depend

upon endometrial-placental interactions (Stroband *et al.*, 1986). By late pregnancy, a major increase in maternal blood supply to the uterus provides a boost to fetal nutrition. Indeed, porcine endometrial tissue is estimated to increase 15-fold during pregnancy and the uterus 100-fold when conceptus weights are included (Dyce *et al.*, 2002). For many years, various experiments have been conducted to improve litter sizes in pigs through identification of and selective breeding for genes responsible for uterine capacity and placental efficiency (i.e., the ratio of fetal weight to placental weight). However, there has been no marked improvement in that regard (Wilson ME *et al.*, 1999 Christenson and Leymaster, 2000) and nutritional studies have also failed to show consistent beneficial effects (Foxcroft, 1997). Thus the critical steps controlling successful interactions between porcine conceptuses and their endometrial environment have remained elusive.

We considered the possibility that these causes for problems in cloned offspring derived from SCNT may also be related to interactions between porcine conceptuses and their endometrial environment. Accordingly, the present study sought to examine the expression pattern of placental formation-related genes (Cdx2 and GATA6) in whole in vivo normal porcine embryos (from 1 cell to hatched blastocyst) and each tissue of a normal fetus at Days 25, 35 and 55 by quantitative mRNA expression analysis using real-time PCR. In other words, Expression of interferon-tau (IFNT) is limited to the peri-attachment period, during which the blastocyst starts to elongate, and to attach to the uterine epithelium (Xie *et al.*, 1996; Klish *et al.*, 2006). So other studies reported that the numerous transcription factors thus far found as the potential regulators of IFNT gene are Cdx2 (Imakawa *et al.*, 2006) and GATA family (Yamaguchi *et al.*, 1999). Furthermore, we examined the expression patterns of Cdx2 and GATA6 in Day 35 normal and SCNT cloned fetuses using the same methods.

## MATERIALS AND METHODS

### Collection of Embryos and Conceptuses

Yorkshire gilts were monitored for estrus once a day by exposure to a healthy mature Yorkshire boar and mated 12 and 24 h after estrus detection (one boar was used for all natural mating). Pregnant gilts (3 to 5 animals/status per day) were hysterectomized through midventral laparotomy as previously described (Gries *et al.*, 1989). The day of the first mating was designated as Day 0. As shown in Fig. 1, each stage of the embryos from single ell to blastocyst at Day 6 and each day's conceptus (Days 12, 15 and 18 of gestation)

were flushed from the uterine horn with 30 ml of PBS supplemented with 1% FBS. Each conceptus collected in vivo was washed three times in PBS. For RNA preparation, only the conceptuses of identical morphologies were pooled and stored in 5 ul of diethyl pyrocarbonate-treated phosphate-buffered saline at −80℃ until use.

### Recovery of *In Vivo* (Control) and SCNT Fetuses

The cloned fetuses (on Day 35 only) were generated from fetal fibroblasts isolated from the F1 fetus produced by the crossing of inbred Duroc male and Landrace female pigs. And pig fetuses produced by natural mating (Control) or cloning (SCNT) were recovered from the uterus on Days 25, 35 and 55 (Day 0 indicates the day of natural mating or cloned embryo transfer). Samples of endometrium from each dam and liver from each fetus and extraembryonic tissue were isolated, frozen in liquid nitrogen, and stored at -80℃ until use. Samples of endometrium from each dam and liver from each fetus and extraembryonic tissue were isolated, frozen in liquid nitrogen, and stored at −80℃ until use.
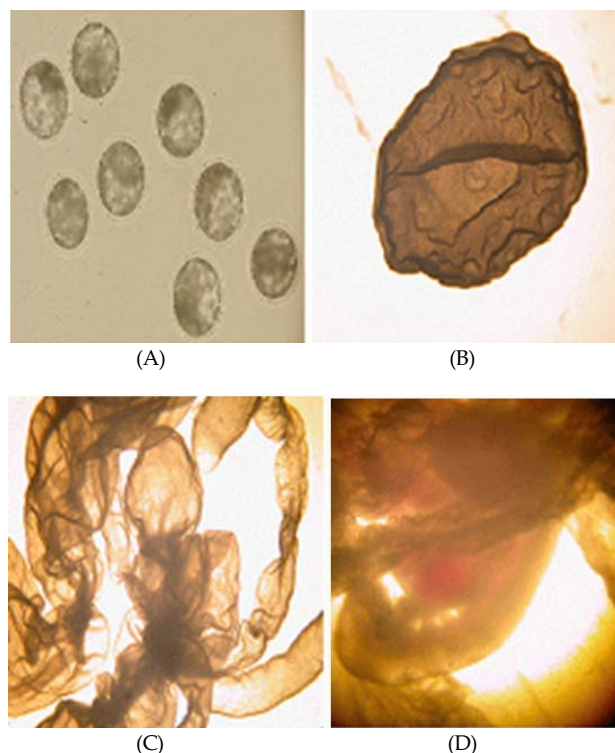


**Fig. 1. Morphology of conceptuses during peri-implantation development.** Conceptuses were obtained from bred gilts on Day 6, 8, 15, and 18 of gestation. (A) Blastocyst stage conceptus at Day 6 after insemination. (B) Blastodermic vesicles (blastocysts) of the pig at the beginning of elongation (8 days old). (C), (D) Conceptus attached by interloking mirovilli to form the epitheliochorial placenta (15 and 18 days old).

**Table 1. Primer sequences and cycling conditions used in Real-time RT-PCR**

| Gene | Primer sequence (5'-3') | Length (bp) | PCR condition |
|---|---|---|---|
| P-$\beta$-actin | F-ACGTGGACATCAGGAAGGAC<br>R-ACATCTGCTGGAAGGTGGAC | 210 | 60℃, 12s<br>45 cycles |
| p-Cdx2 | F-GCTTCTCTGGGCTGAATGTA<br>R-CCACTTCCCTTCACCATATC | 211 | 56℃, 4s<br>45 cycles |
| p-GATA6 | F-GGCCTCTACAGCAAGATGAA<br>R-TCATAGCAAGCGGTCGAG | 208 | 56℃, 15s<br>45 cycles |

### Total RNA Extraction from Tissues

Porcine liver, endometrial and extraembryonic tissues, etc., were homogenized with Trizol (Invitrogen, Carlsbad, CA, USA) and the phase was separated by adding chloroform (Sigma, St. Louis, MO, USA). RNA was then precipitated with isopropyl alcohol (Sigma), washed with 75% ethanol, and dissolved in DEPC water. The RNA was treated with RNas-free DNase I to remove possible contaminating DNA and stored at $-80$℃. Finally, a concentration of total RNA was quantified with a Nano Drop Spectrometer (ND-1000, MET, USA).

### First Strand cDNA Synthesis by Reverse Transcriptase

Extracted total RNA was employed for the synthesis of first-strand cDNA by reverse transcriptase using a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Briefly, RNA/primer mixtures were prepared in sterile 200 ul tubes (RNA/primer mixtures 10 ul RNA 2 ul, 50 uM oligo (dT) 1 ul, 10mM dNTP 1 ul, and DEPC-treated water 6 ul mixed and briefly centrifuged each component before use. Each sample was incubated at 65℃ for 5 min, and then placed on ice for at least 1 min. The reaction mixture was prepared by adding each component in the sample (9 ul mixture component; 10×RT buffer 2 ul, 25 mM MgCl$_2$ 4 ul, 0.1 M DTT 2 ul, and RNaseOUT$^{TM}$ Recombinant RNase Inhibitor 1 ul). The reaction mixture of 9 ul was added to each 10 ul RNA/primer mixture, mixed gently, and collected by brief centrifugation. Afterwards, the sample was incubated at 42℃ for 2 min, supplemented by 1 ul of SuperScript$^{TM}$II reverse transcriptase (50 U/ul) added to each tube except the no RT control, mixed and incubated at 42℃ for 50 min (total reaction mixture 20 ul). The reactions were terminated at 70℃ for 50 min and chilled on ice. They were collected by brief centrifugation. All cDNA samples were stored at $-20$℃ until amplification.

### Quantitative Real-Time PCR

Real-time PCR reactions were performed using Light-Cycler (Roche, Mannheim, Germany) and the PCR reactions were employed according to the real-time PCR machine manufacturer's instructions (Roche, Germany). The cDNA of the target genes and $\beta$-actin were then detected by real-time PCR using the specific primer pairs shown in Table 1. $\beta$-actin mRNA was used as an internal standard. The FastStart DNA SYBR Green I (Roche, Germany) contains a Taq DNA polymerase enzyme, reaction mix 10X conc. (with reaction buffer, SYBR Green, optimized PCR buffer, 5mM MgCl2, and a dNTP mix that includes dUTP. The PCR involved a preliminary denaturation program (95℃ for 10 min), followed by 45 cycles of amplification and quantification (95℃ for 10 sec, 56~64℃ for 30 sec, 72℃ for 30 sec with a single fluorescence measurement), after which a melting curve program was employed (65~95℃, with a heating rate of 0.2℃ per second and continuous fluorescence measurement). The fluorescence data were acquired after the extension step during PCR reactions containing SYBR Green 1. Thereafter, PCR products were analyzed by generating a melting curve.

Since the melting curve of a product is sequence-specific, it can be used to distinguish between nonspecific and specific PCR products. For the mathematical model, it is necessary to determine the crossing points (CP) for each transcript here, CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed by the 2-ddCt method (Livak and Schmittgen, 2001). The sizes of the PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light.

### Statistical Analysis

Data were analyzed by Duncan's multiple range test for mRNA expression assayed by quantitative real-time PCR. P values of less than 0.05 were considered to be statistically significant.

## RESULTS

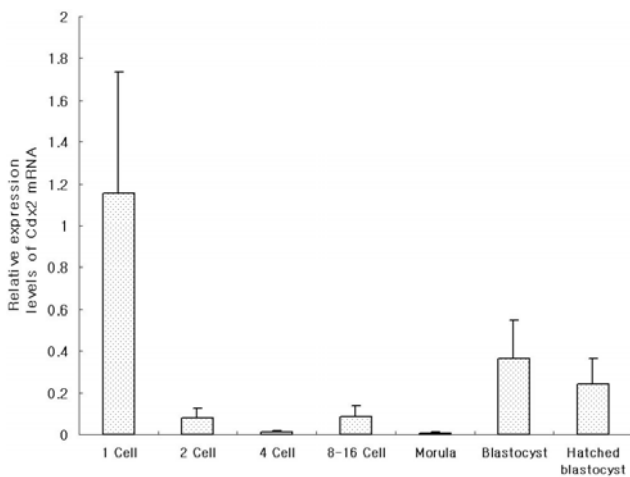### The Placenta-Related Gene (Cdx2 and GATA6) Expression in Porcine Pre-Implantation Development

**Fig. 2. Relative expression level of Cdx2 in porcine pre-implantation development.** Standard errors of means are indicated by bar. Conceptuses were obtained from bred gilts on Days 6, 8, 15, and 18 of gestation. Embryos from single cell to hatched blastocyst were collected from the uterine horn.
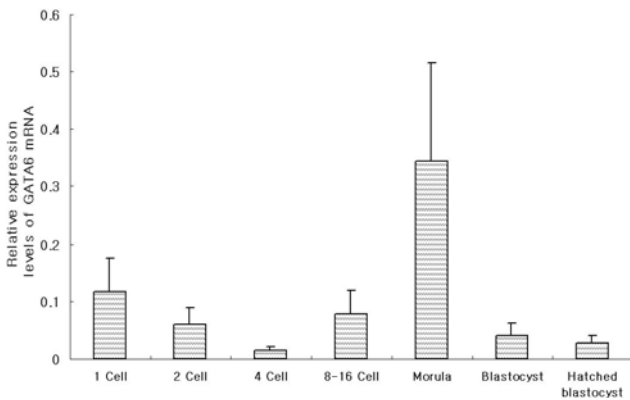


**Fig. 3. Relative expression level of GATA6 in porcine pre-implantation development.** Standard errors of means are indicated by bar. Embryos from single cell to hatched blastocyst were collected from the uterine horn.

The expression level of Cdx2 mRNA in the *in vivo* 1-cell embryos was significantly higher than the level in the embryos of 2- and 4-cell stages. The expression of the gene for 2-cell to morula stage embryos was lower. However, Cdx2 expression at the blastocyst and hatched blastocyst stage was slightly increased (Fig. 2).

The expression of GATA6 mRNA decreased gradually from 1 cell to 4 cells, and increased gradually from the 4-cell to the morula stage. Interestingly, at the morula stage, the expression level of GATA6 was remarkably higher than that of many other stages. However, GATA6 mRNA expression was significantly decreased at the blastocyst and hatched blastocyst stages (Fig. 3).
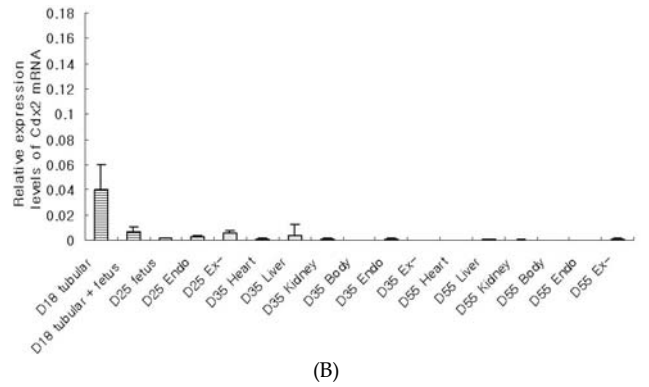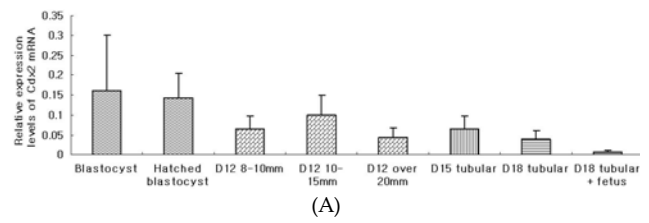


(A)



(B)

**Fig. 4. Relative expression level of Cdx2 in (A) peri- and (B) post-implantation developments.** Standard errors of means are indicated by bars. Endo, Endometrium; EX-, Extra-embryonic tissue. Bar colors are to divide the part of each day.



(A)



(B)

**Fig. 5. Relative expression level of GATA6 in (A) peri- and (B) post-implantation developments.** Standard errors of means are indicated by bars. Endo, Endometrium; EX-, Extra-embryonic tissue. Bar colors are to divide the part of each day.
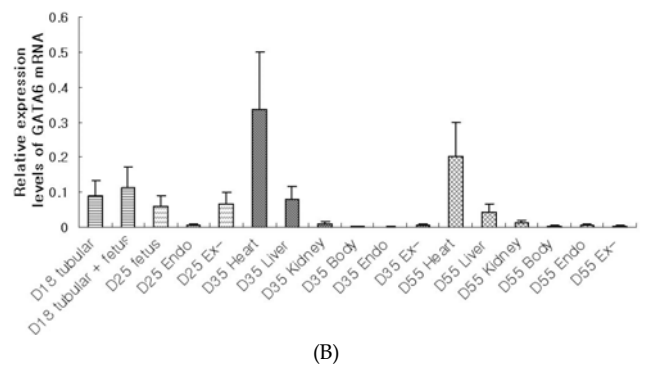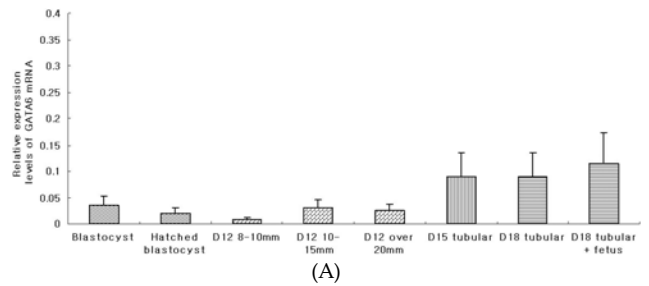
**The Formation of Placenta-Related Gene (Cdx2 and GATA6) Expression in Porcine Peri- and Post-Implantation Developments**
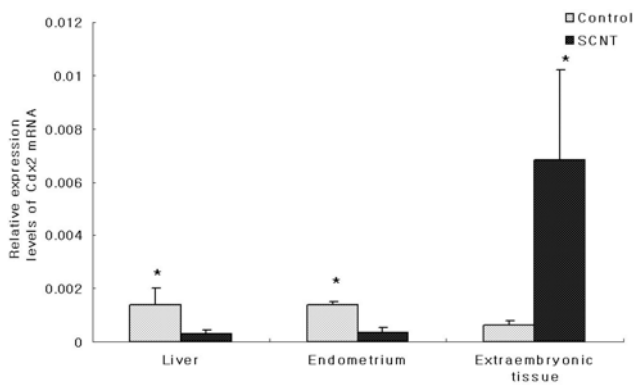
Fig. 6. Relative expression level of Cdx2 in liver, endometrium and extra-embryonic tissue of porcine fetus produced *in vivo* (Control) or by SCNT at Day 35. Standard errors of means are indicated by bar. Asterisks denotes significant difference from the control and SCNT ($p<0.05$).
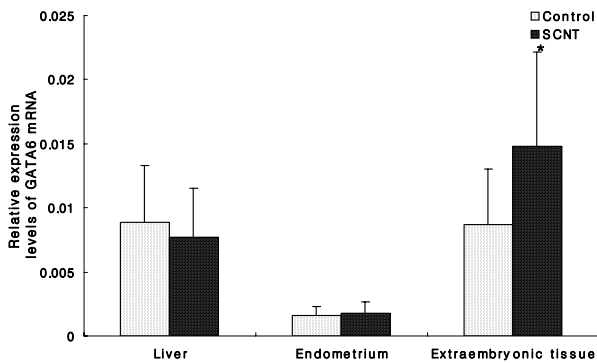


Fig. 7. Relative expression level of GATA6 in liver, endometrium and extra-embryonic tissue of porcine fetus produced *in vivo* (Control) or by SCNT at Day 35. Standard errors of means are indicated by bar. Asterisks denotes significant difference from the control and SCNT ($p<0.05$).

In the present study, Cdx2 expression levels were higher at the early peri-implantation development, while the expression of Cdx2 was gradually decreased from the peri-implantation to post-implantation stage (Fig. 4A, B).

The pattern of expression of GATA6 mRNA increased gradually from the blastocyst stage up to the tubular stage on Day 18, and tended to decrease gradually from the tubular stage on Day 18 to the Day 25, 35 and 55 stages. However, the GATA6 mRNA expression levels of each heart tissue on Days 35 and 55 were remarkably higher than for the other tissues (Fig. 5A, B).

**Relative Expression Levels of Cdx2 and GATA6 in Liver, Endometrium and Extra-Embryonic Tissue of Porcine Fetus Produced In Vivo or by SCNT at Day 35**

The expression of the Cdx2 gene in normal liver and endometrial tissue was significantly higher than that of the cloned groups ($p<0.05$) in the fetus liver and endometrium.

The level of Cdx2 gene expression in the extra-embryonic tissue of SCNT was significantly higher than that of the control ($p<0.05$) (Fig. 6).

The level of GATA6 in the control was significantly higher than that of SCNT (P$<0.05$) in the fetus liver. The relative abundance of GATA6 mRNA in the endometrium did not differ between the control and the SCNT. In the extra-embryonic tissue, the mRNA expression of the GATA6 gene in SCNT was significantly higher than that of the control ($p<0.05$) (Fig. 7).

## DISCUSSION

As a result of somatic cell nuclear transfer (SCNT), placental abnormalities and failed implantation are characterized phenotypes that occur in many species. The primary cause of these developmental abnormalities is thought to be due to the incomplete reprogramming of the donor cell, as a cause of epigenetic and genetic modifications throughout embryonic development (Latham, 2004; Piedrahita *et al*., 2004). Furthermore, in mouse clones, abnormally large placentas resulting from placental hyperplasia of the spongiotrophoblast or basal layer are often observed at term (Ono *et al*., 2001; Humphreys *et al*., 2002; Ogura *et al*., 2002). Similarly, many cloned ovine and bovine placentas at Day 35 post transfer display vascularization and hypovascularization of the placenta (Hill *et al*., 2000; De Sousa *et al*., 2001).

Here, we have examined the mRNA expression of Cdx2 and GATA6 during early development from 1-cell to each tissue at Day 55.

First, our primary focus of this work was to examine the aspect of Cdx2 and GATA6 mRNA expression in whole implantation development. Ralston and Rossant (2008) reported that Cdx2 is initially ubiquitously expressed, and becomes progressively upregulated in outside, future trophectoderm cells prior to blastocyst formation in mice. Besides, Cdx2 is necessary for development of murine trophoblast and in Cdx2 knockout mice, the trophectoderm epithelium fails to be maintained (Strumpf *et al*., 2005). Also, the findings of another paper that GATA6 is required for the development of extraembryonic tissue are supported by the generation of chimeric embryo in mice (Beddington and Robertson, 1989). In addition, GATA6 is expressed in the inner cell mass (ICM) of mouse blastocysts and is mandatory for survival past the blastocyst stage (Koutsourakis *et al*., 1999). And other studiesin murine ES cells suggest that GATA6 is an essential factor formation of the extraembryonic endoderm (Li *et al*., 2004).

Anyway, our Cdx2 data show that mRNA expression at the single-cell stage is sharply higher than those of other stage embryos in pre-implantation. But Ralston and Rossant (2008) reported that early stage embryos should have lower expression levels. That report examined only mRNA expression of Cdx2 in the 2-cell stage. In any case, our data clearly contradicted the previous report (Ralston and Rossant, 2008). We have no clear explanation for this difference. But, it is worth noting that other cell stages showed normal mRNA expression levels as compared with the previous report (Ralston and Rossant, 2008). Our data showed that Cdx2 levels increased to around the blastocyst stage, suggesting that Cdx2 mRNA expression is related to blastocyst formation. In GATA6 mRNA expression levels, we also supposed that this result is very similar to our Cdx2 data. As might be expected of these results, they are similar to the trends for Cdx2 mRNA expression with the exception of the morula stage. However, in whole expression levels, GATA6 mRNA expression was lower than Cdx2 mRNA expression.

With regard to Cdx2 and GATA6 mRNA expression levels in peri- and post-implantation, our evidence suggests a difference in expression pattern between Cdx2 and GATA6. Interestingly, mRNA expression levels of both genes were significantly opposed at Day 18. Accordingly, it would seem that these genes make up for each other. In addition, before and after this period (at Day 18), both genes may be relate with placental formation and then play an important role in placental formation or development between Day 15 and Day 18. Our data also indicate that overexpression of GATA6 occurred in heart tissues of the fetus at Days 35 and 55. Because GATA6 has been reported to be related to heart development in mice, Moreover, transcription levels were highly expressed and implantation was almost complete at Day 21 (Gove *et al.*, 1997).

Second, our work aimed to examine the aspect of Cdx2 and GATA6 mRNA expression in the liver and extraembryonic tissue of porcine fetuses and in endometria produced *in vivo* or by SCNT at Day 35. In this study, our data suggest that Cdx2 and GATA6 mRNA expression is significantly different between *in vivo* (control) and by SCNT. Especially, both the liver of the fetus and the endometrium show a similar trend in Cdx2 and GATA6 expression levels, but extraembryonic tissue has a different level. This data indicated that mRNA level is expressed abundantly in SCNT extraembryonic tissue. In light of this result, overexpression of Cdx2 in SCNT might regulate some crucial processes associated with placentation. (Humpherys *et al.*, 2002; Inoue *et al.*, 2002; Ogawa *et al.*, 2003; Yamazaki *et al.*, 2003). More than 50 imprinted genes have been identified in mice and/or humans (Dean *et al.*, 2003), and many of them are involved in regulation of fetal growth. However, we could not completely rule out the function of the other genes except for imprinting genes.

Results from this study will improve the understanding of placental abnormalities, which is one of many problems to clone production by SCNT. In other words, it can be postulated that expression of Cdx2 and GATA6 genes in the endometrium and extraembryonic tissue of pre- and peri-implantation may be closely related with the formation of placentas. But, it is difficult to explain only mRNA results. Furthermore, functional studies such as siRNA-mediated knockdown are needed to further elucidate the roles of factor such as Cdx2 and GATA6 in porcine.

## REFERENCES

1. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y (1999): Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17:456-461.
2. Bedding RSP, Robertson EJ (1989): An assessment of the developmental potential of the embryonic stem cells in the midgestation mouse embryo. Development 105:733-737.
3. Boquest AC, Grupen CG, Harrison SJ, Mcllfatrick SM, Ashman RJ, d'Apice AJ, Nottle MB (2002): Production of cloned pigs from cultured fetal fibroblast cells. Biol Reprod 66:1283-1287.
4. Chavatte-Palmer P, Heyman Y, Renard JP (2000): Cloning and associated physiopathology of gestation. Gynecol Obstet Fertil 28:633-642.
5. Christenson RK, Leymaster LA (2000): Effects of selection for ovulation rate or uterine capacity on gravid uterine, farrowing and weaning traits in swine. J Anim Sci 78 (Suppl. 1):202(Abstr).
6. Dean W, Santos F, Reik W (2003): Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. Sem Cell Dev Biol 14:93-100.
7. De Sousa PA, King T, Harkness L, Young LE, Walker SK, Wilmut I (2001): Evaluation of gestational deficiencies in cloned sheep fetuses and placenta. Biol Reprod 65:23-30.
8. Dyce KM, Sack WO, Wensing CJG (2002): Textbook of Veterinary Anatomy. NL, Elsevier Science.
9. Foxcroft, GR (1997): Mechanisms mediating nutritional effects on embryonic survival in pigs. J Reprod Fertil Suppl 52:47-61.
10. Gove C, Walmsley M, Nijjar S, Bertwistle, D, Guille M, Partington G, Bomford A, Patient R (1997): Over-expression of GATA-6 in xenopus embryos blocks differentiation of the heart precursors. EMBO J 16: 355-368.

11. Gries LKR, Giesert D, Zavy MT, Garrett JE, Morgan GL (1989): Uterine secretory alterations coincident with embryonic mortality in the gilt exogenous estrogen administration. J Anim Sci 67:276-284.

12. Heiner Niemann, X Cindy Tian, W Allan King and Rita S F Lee (2008): Epigenetic reprogramming in embryonic and foetal development upon somatic cell nuclear transfer cloning. Reproduction 135:151-163.

13. Hill JR, Burghard RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME (2000): Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 63:1787-17794.

14. Humpherys D, Eggan K, Akutsu H, Friedman A, Hochedlinger K, Yangimachi R, Lander ES, Golub TR, Jaenisch R (2002): Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc Natl Aced Sci USA 99: 12889-12894.

15. Imakawa K, Kim M-S, Matsuda-Minehata F, Ishida S, Iizuka M, Suzuki M, Chang K-T, Echternkamp SE, Christenson RK (2006): Regulation of the ovine interferon-tau gene by a trophoblastspecific transcription factor, Cdx2. Mol Reprod Dev 73:559-567.

16. Inoue K, Kohda T, Lee J, Ogonuki N, Mochida K, Noguchi Y, Tanemura K, Kaneko-Ishino T, Ishino F, Ogura A (2002): Faithful expression of imprinted genes in cloned mice. Science 295:297.

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

18. Klisch K, Boos A, Friedrich M, Herzog K, Feldmann M, Sousa N, Beckers J, Leiser R, Schuler G (2006): The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition. Reproduction 132:791-798.

19. Koutsourakis M, Langeveld A, Patient R, Beddington R & Grosveld F (1999): The transcription factor GATA6 is essential for early extraembryonic development. Development 126:723-732.

20. Kues WA, Niemann H (2004): The contribution of farm animals to human health. Trends in Biotechnology 22:286-294.

21. Latham KE (2004): Cloning: questions answered and unsolved. Differentiation 72:11-22.

22. Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402-408.

23. Ono Y, Shimozawa N, Ito M, Kono T (2001): Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. Biol Reprod 64:

44-50.

24. Ogawa H, Ono Y, Shimozawa N, Sotomaru Y, Katsuzawa Y, Hiura H, Ito M, Kono T (2003): Disruption of imprinting in cloned mouse fetuses from embryonic stem cells. Reproduction 126:549-557.

25. Ogura A, Inoue K, Ogonuki N, Lee J, Kohda T, Ishino F (2002): Phenotypic effects of somatic cell cloning in the mouse. Cloning Stem Cells 4:397-405.

26. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407:86-90.

27. Piedrahita JA, Mir B, Dindot S, Walker S (2004): Somatic cell cloning: The ultimate form of nuclear reprogramming? J Am Soc Nephrol 15:1140-1144.

28. Ralston A, Rossant J (2008): Cdx2 downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryos. Developmental Biology 313:614-629.

29. Sawai K, Kageyama S, Moriyasu S, Hirayama H, Minamihashi A, Onoe S(2005): Analysis of mRNA transcripts for insulin-like growth factor receptors and binding proteins in bovine embryos derived from somatic cell nuclear transfer. Cloning Stem Cells 7:189-198.

30. Stroband HW, Taverne N, Langenfeld K, Barends PM (1986): The ultrastructure of the uterine epithelium of the pig during the estrous cycle and early pregnancy. Cell Tissue Res 246:81-9.

31. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J (2005): Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132:2093-2102.

32. Wilson ME, Biensen MJ, Ford SP (1999): Novel insight into the control of litter size in pigs, using placental efficiency as a selection tool. J Anim Sci 77:1654-1658.

33. Xie S, Nagel RJ, Green J, Beckers JF, Roberts RM. (1996): Trophoblast-specific processing and phosphorylation of pregnancy-associated glycoprotein-1 in day 15 to 25 sheep placenta. Biol Reprod 54:122-129.

34. Yang L, Xue F, Tian XC, Du F, Kubota C, Taneja M, Dinnyes A, Dai Y, Levine H, Lii S, Kuo B, Buckley A, Buckley D, Chen C, Xu X, Coss D, Walker AM (2002): Maternal prolactin composition can permanently affect epidermal T cell function in the offspring. Dev Comp Immunol. 26:849-860.

35. Yamaguchi H, Ikeda Y, Moreno JI, Katsumura M, Miyazawa T, Takahashi E, Imakawa K, Sakai S, Christenson RK (1999): Identification of a functional transcription factor AP-1 site in the sheep interferon t gene that mediates a response to PMA in JEG3 cells. Biochem J 340:767-773.

36. Yamazaki Y, Mann MR, Lee SS, Marh J, McCarrey

JR, Yanagimachi R, Bartolomei MS (2003): Reprogra-
mming of primordial germ cells begins before mig-
ration into the genital ridge, making these cells ina-
dequate donors for reproductive cloning. Proc Natl
Acad Sci USA 100:12207-12212.