Zygote **17** (August), pp. 203–207. © 2009 Cambridge University Press
doi:10.1017/S0967199409005267 First Published Online 27 April 2009 Printed in the United Kingdom

Effect of recipient breed on delivery rate of cloned miniature pig

Ok Jae Koo[2], *Hee Jung Park*[2], *Dae Kee Kwon*[2], *Jung Taek Kang*[2], *Goo Jang*[2] *and Byeong Chun Lee*[1]
Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea.

Date submitted: 03.10.08. Date accepted: 24.12.08

Summary

The miniature pig is regarded as a better organ donor breed for xenotransplantation than other pig breeds because the size of their organs is similar to that of humans. To improve efficiency of cloned miniature pig production, we analysed the effect of breed difference between donor cells and embryo recipients on pregnancy rate and delivery rate. Cloned porcine embryos derived from domestic or miniature pig donor cells were transferred to domestic or miniature recipient pigs. Delivery rate was significantly higher when embryos reconstructed with miniature pig donor cells were transferred to miniature pig recipients as compared with that of embryos transferred to domestic pig recipients. However, pregnancy rates were similar between the two groups. The breed of donor cells, but not of embryo recipients, seems likely to affect litter size. From a 13 610 gene cDNA microarray, 1551 (11.7%) genes showed significantly different levels of expression between the fetuses of the two breeds. Vascular endothelial growth factor and c-kit ligand genes related to implantation and maintenance of pregnancy were significantly down-regulated in miniature pigs. In conclusion, the differential gene expression in fetuses interferes with proper fetal/maternal interactions, and results in late-stage pregnancy loss. Our results indicate that the miniature pig is the preferred embryo recipient breed than domestic pig for producing cloned miniature piglets.

Keywords: Breed, Delivery rate, Miniature pig, Recipient, Somatic cell nuclear transfer

Introduction

Pigs are regarded as a useful species for use in biomedical research due to their anatomic and physiologic similarities to humans (Svendsen, 2006). In particular, miniature pigs are used extensively because of their smaller body size (Wakai *et al*., 2008), and well defined genetic background (Yao *et al*., 2006) as compared with that of domestic pigs, such as Landrace, Yorkshire, Duroc, and their hybrids. Even so, domestic pigs still have the advantages of lower cost, higher prolificacy and wide availability (Estrada *et al*., 2008).

One of the most remarkable research fields using pigs is xenotransplantation. Recently, many kind of transgenic pigs have been produced for this purpose using the somatic cell nuclear transfer (SCNT) technique (Sprangers *et al*., 2008). Because their body weight is similar to that of humans (Lee *et al*., 2006), miniature pigs were selected as an appropriate cell donor for SCNT to produce transgenic pigs for xenotransplantation. However, the efficiency at producing cloned piglets using SCNT is still very low. Numerous factors have been shown to affect the efficiency of SCNT in the pig, including SCNT procedure (Miyoshi *et al*., 2000; Du *et al*., 2007), artificial activation conditions (Cheong *et al*., 2002; Zhu *et al*., 2002; Ziecik *et al*., 2005; Koo *et al*., 2008), stages of donor cells (Prather *et al*., 1999), and more.

A previous study that used *in vivo* produced embryos has documented that both the development of placenta and maintenance of pregnancy are influenced by fetal and maternal breed (Biensen *et al*., 1998). Thus, we hypothesized that breed difference between cell donor and recipient is an important factor that affects success

[1]All correspondence to: Byeong Chun Lee. Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151–742, Korea. Tel: +822 880 1269. Fax: +822 873 1269. e-mail: bclee@snu.ac.kr
[2]Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul, Korea.

of cloning of miniature pigs, as size of uterus and birth weight had been very different between miniature and domestic pigs in our past observation.

The breeding environment for miniature pigs and domestic pigs is different. Therefore, the selection of the correct breed for use as recipient of cloned embryos is a key decision to be made before establishing experimental or commercial animal facilities. Minimal information is available on the effect of breed difference between cell donors and embryo recipient pigs on producing cloned miniature pigs. For these reasons, the present study was performed to investigate effect of breed of recipient pig on production of cloned miniature pigs using SCNT.

Materials and methods

Chemicals and care of animals

All chemicals were obtained from Sigma–Aldrich Corp. unless otherwise stated. Gyeonggido Veterinary Service was responsible for breeding of the pigs in compliance with Gyeonggido Veterinary Service Institutional Animal Care and Use Committee, administered by National Veterinary Research & Quarantine Service.

Isolation and culture of donor cells

Ear tissues were obtained from three domestic and three miniature pigs, washed three times in DPBS (Invitrogen) and minced with a surgical blade. The minced tissues were dissociated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 0.25% (w/v) trypsin/1 mM EDTA (Invitrogen) for 1 h at 37 °C. Trypsinized cells were washed once in Ca^{2+}- and Mg^{2+}-free DPBS by centrifugation at 300 *g* for 2 min, and seeded onto 100 mm plastic culture dishes (Becton Dickinson). Subsequently, cells were cultured for 8 to 10 days in DMEM supplemented with 10% (v/v) FBS (Invitrogen) at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cultured cells at passages three to eight were used for SCNT.

In vitro maturation of porcine oocytes

Ovaries (mixed breed of Landrace, Yorkshire, and Duroc) were collected from a local slaughterhouse. Cumulus–oocyte complexes aspirated from 3 to 6 mm diameter of follicles were cultured in tissue culture medium 199 (TCM-199, Invitrogen), supplemented with 10 ng/ml EGF, 4 IU/ml serum gonadotropin (Foligon), 4 IU/ml chorionic gonadotropin (Choluron) and 10% porcine follicular fluid at 39 °C in a humidified atmosphere of 5% CO_2. After culturing for 22 h, COCs were washed then cultured in hormone-free medium for another 22 h. At 38 to 42 h of maturation culture, oocytes were freed from cumulus cells by repeated pipetting in 0.1% hyaluronidase.

Somatic cell nuclear transfer

For SCNT, a micromanipulation system (NT-88, Nikon-Narishige) attached to an inverted microscope (TE-2000, Nikon Instrument) was used. A cumulus-free oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm, presumably containing the metaphase-II chromosomes, were extruded by squeezing with the same needle. Enucleation was confirmed by staining with Hoechst 33342 during manipulation. Single fibroblast cells with a smooth surface were selected under a microscope and transferred into the perivitelline space of enucleated oocytes. These couplets were placed in a pulsing medium for 4 min and transferred to a chamber consisting of two electrodes overlaid with pulsing medium. The pulsing medium was 0.26 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Couplets were fused and activated simultaneously with a single 2 kV/cm DC pulse for 30 μs using a BTX Electro-Cell Manipulator 2001 (BTX, Inc.). Fused couplets were used for embryo transfer within 2 h without additional *in vitro* culture.

Embryo transfer and pregnancy diagnosis

In the laboratory, 120 to 150 of fused SCNT embryos were loaded into a sterilized 0.25 ml straw (Minitüb) and kept in a portable incubator (Minitüb) during transportation to the embryo transfer facility. An estrous-synchronized recipient was anesthetized using ketamine and xylazine for induction and 3% of isoflurane for maintenance. One oviduct was exposed by laparotomy. The straw containing the embryos was put directly into the oviduct of the recipient and embryos were expelled from the straw using one ml syringe (Becton Dickinson). Recipients were checked for pregnancy by transabdominal ultrasound examination on day 30 after embryo transfer. Landrace, and Yorkshire, and Duroc hybrids domestic pigs and miniature pigs were used for embryo recipient.

Microarray analysis and real-time RT PCR

Miniature pig and domestic pig fetuses derived from natural breeding were obtained by cesarean section at day 25 of gestation. For each breed, three fetuses from different litters were used for analysis. Each whole fetus was homogenized and used for RNA

https://doi.org/10.1017/S0967199409005267 Published online by Cambridge University Press

Table 1 Primers used for real-time RT-PCR.

Gene name	Primer sequence (5′–3′)	Annealing temperature (°C)	PCR fragment size (bp)	NCBI accession number
VEGF*	F: ACGACGAAGGTCGGAGTGT R: AAATGCTTTCTCCGCTCTGA	60	196	AF358502
c-kit ligand	F: TAAGCGAAATGGTGGAACAA R: GGGTTCTGGGCTCTTAGATG	60	188	L07786
Beta-actin	F: CATCACCATCGGCAACGA R: GTTGGCGTAGAGGTCCTTCCT	62	147	U07786

*Vascular endothelial growth factor.

extraction. Total RNA was extracted using the Easy-spin Total RNA Extraction Kit (iNtRON Biotechnology) by following supplier's instructions. An equal amount of RNA from three fetuses of each breed was mixed for further analysis to reduce individual effect. Microarray analysis was conducted using the Platinum pig 13K cDNA chip (Genocheck Inc.) according to a protocol described previously (Jung *et al*., 2004).

The same RNAs were used for real-time RT-PCR to confirm the microarray result. The 7300 real-time PCR system (Applied Biosystems) was employed and the amplifications were performed with SYBR Premix Ex *Taq* (Takara). All PCR products were analysed by the 7300 system SDS software version 1.3 (Applied Biosystems). The sequences of the primers used, the annealing temperatures, the size of expected PCR product and the sequence references are summarized in Table 1.

Statistical analysis

The chi-squared analysis was used to compare pregnancy and delivery rates among experimental groups. Litter size was analysed by Student's *t*-test using Prism software (GraphPad). A value of $p < 0.05$ was considered statistically significant. Microarray data were analysed by fold test using Genepix Pro 4.1 software (Axon Instruments Inc.). More than a two-fold of difference was considered statistically significant. Data on gene expression levels data from real-time RT-PCR were normalized to the internal control, beta-actin gene, and analysed by Student's *t*-test.

Results and Discussion

The present study demonstrated that the miniature pig is a more suitable recipient for producing cloned miniature pigs than the domestic pig. Cloned miniature piglets can be produced using both miniature pig and domestic pig recipients (Table 2). Pregnancy rates after transferring cloned miniature pig embryos into miniature and domestic recipient pigs were similar. However, delivery rates of miniature pig embryos transferred to miniature pig recipient were significantly higher than those transferred to domestic pig recipients. Our finding was similar to that of previous studies performed with Meishan and Yorkshire pigs using *in vivo* fertilized embryos (Biensen *et al*., 1998; Wilson *et al*., 1998). According to both previous reports, pregnancy rate was determined by the uterine environment up to day 90 of gestation, regardless of the fetal genotype. However, during late gestation, breed-specific mechanisms become more important due to fetal demand for nutrient uptake and increasing need for rapid waste removal. The significant difference in delivery rate in the present study, in spite of similar pregnancy rates, between two donor breeds may due to similar mechanisms suggested in the previous reports.

While previous reports used morphology and size of placenta as the criteria to compare placental differences between Meishan and Yorkshire pigs, we used a microarray assay to examine differences between miniature pig and domestic pig fetuses. In total, of 13 297 spots on the microarray chip, 1551 (11.7%) were significantly different between the two breeds (Fig 1). Among these, 252 (16.2%) genes were over-expressed and 1299 (83.8%) genes were down-regulated in miniature pig fetuses (please contact corresponding author for detailed data). Functionally, many genes that were related to transport, integral membrane proteins, protein binding and cell cycle regulation were differentially expressed between the two breeds. Successful implantation and maintenance of pregnancy requires complex processes and are tightly controlled by interplays between maternal and fetal factors (Huppertz, 2007). Thus, we assume that the low delivery rate after transfer of cloned miniature pig embryos to domestic pig recipients was a reflection of variation in gene expression between miniature pig and domestic pig fetuses.

To confirm the microarray results and to further examine the mechanism(s) for the low delivery rate in domestic recipients of miniature pig cloned embryos, two important genes that are related to implantation

https://doi.org/10.1017/S0967199409005267 Published online by Cambridge University Press

Table 2 *In vivo* development of cloned porcine embryos derived from fetal fibroblasts of miniature or domestic pigs and transferred to miniature or domestic recipient pigs.

Donor breed	Recipient breed	No. of recipients	Pregnancy (%)	Delivery (%)	No. of piglets (mean litter size)
Domestic	Domestic	24	12 (50.0)[a]	6 (25.0)[a]	42 (7.0)[c]
Miniature	Domestic	34	9 (26.5)[a]	1 (2.9)[b]	1 (1.0)
Miniature	Miniature	35	16 (45.7)[a]	8 (22.9)[a]	20 (3.1)[d]

[a,b]Different superscripts in same column indicate significantly differences ($p < 0.05$).
[c,d]Significant different between domestic to domestic and miniature to miniature group. Miniature to domestic group was not used for statistically analysis due to restricted replication number.

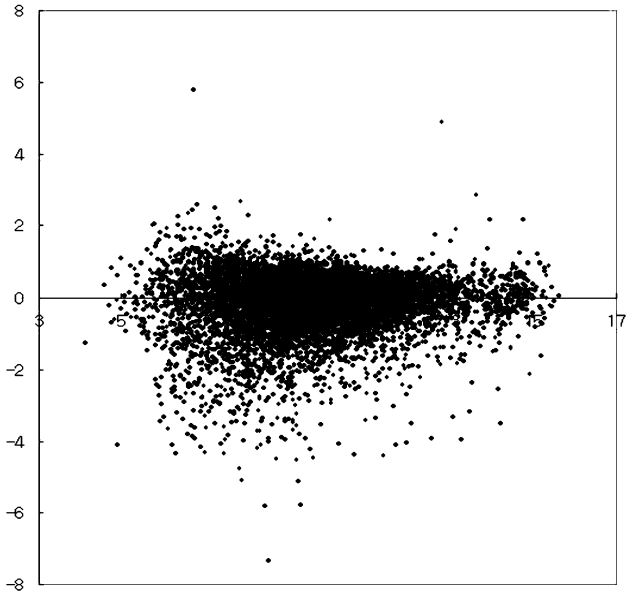

Figure 1 Differential gene expression levels in miniature pig and domestic pig fetuses. Microarray assay was used to examine differences between miniature pig and domestic pig fetuses.

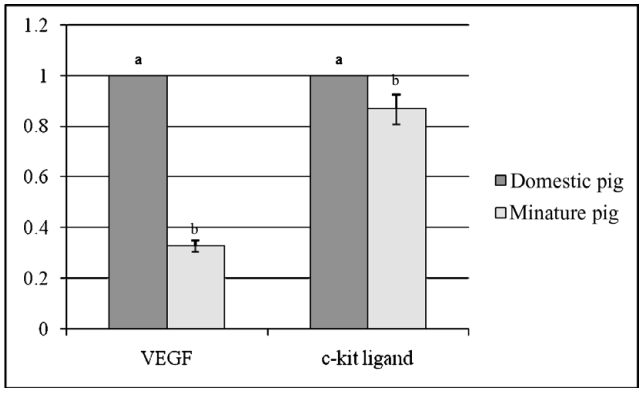

Figure 2 Expression of vascular endothelial growth factor (VEGF) and c-kit ligand gene in miniature and domestic pig fetuses. Gene expression levels were measured by RT-PCR. Different superscripts within the same gene group indicate statistically different values ($p < 0.05$).

and gestation were selected for further analysis. In the microarray analysis, vascular endothelial growth factor (VEGF) and c-kit both appeared to be down-regulated in miniature pig fetuses. VEGF is expressed from early to late gestation and is correlated with placental growth and vascular development (Vonnahme & Ford, 2004). c-kit protein is expressed in both the placenta and endometrium and plays important roles in the placental proliferation and differentiation (Horie *et al.*, 1992). As expected from microarray data, both genes were down-regulated in miniature pig fetuses when analysed by RT-PCR (Fig. 2). These differences in gene expression might be the cause of incompatibility between the two breeds and the result in low delivery rate of miniature pig fetuses transferred to domestic pig recipients.

In a recent report, the domestic pig was recommended as a embryo recipient for production of cloned miniature pigs due to their ability to accommodate more fetuses (Kurome *et al.*, 2008). However, according to our observations, litter size tends to be influenced more by the cell donor breed than by the embryo recipient breed. Domestic pigs used in the present study were Landrace, and Yorkshire and Duroc hybrids, and their normal litter size varies from eight to 15. Litter size of miniature pigs normally is from four to six. In the present study, mean litter size was significantly lower in miniature pig embryos transferred to miniature pig recipients as compared with that of domestic pig embryos transferred to domestic pigs (3.1 ± 0.2 vs. 7.0 ± 1.0, respectively; mean ± S.E.M.). However, we did not see a specific pattern when litter size was categorized according to recipient breeds groups. In the present study, litter size was even reduced in miniature donor to domestic recipient group, as compared with miniature to miniature group. Therefore, we conclude that litter size was not improved by using domestic pigs as recipients of cloned miniature pig embryos.

A limitation of the present study is that we didn't obtain data from transfer of domestic pig cloned embryos to miniature pig recipients; hence, the effect of breed difference on pig cloning is not explained fully.

https://doi.org/10.1017/S0967199409005267 Published online by Cambridge University Press

However, the present study clearly showed that the use of miniature pigs, as recipients, improved delivery rate of cloned miniature piglets.

For successful xenotransplantation, animal facilities built to human-use standards are required. Miniature pigs are easier to maintain in a germ-free or specific pathogen-free state. Their genetic background is more defined and they are more likely to meet the standards necessary to obtain approval for human use. In addition, an estrus synchronization procedure for the miniature pig has already been established (Kurome *et al*., 2008), making it feasible to use as a recipient species for cloned embryos. Accordingly, we recommend the miniature pig is a better embryo recipient than domestic pigs to use for producing cloned miniature piglets.

Acknowledgements

This study was financially supported by Korean MEST through KOSEF (grant #M10625030005–07N250300510) and BK21 programme. We thank Sung Sik Lee, Nam Yong Paek, Young Hee Nam, Moon Young Ko, and many others from the Gyeonggido Veterinary Service for valuable contributions in animal husbandry and discussion. We also thank Genocheck Inc. for technical and statistical support for microarray and real-time RT-PCR.

References

Biensen, N.J., Wilson, M.E. and Ford, S.P. (1998). The impact of either a Meishan or Yorkshire uterus on Meishan or Yorkshire fetal and placental development to days 70, 90, and 110 of gestation. *J. Anim. Sci.* **76**, 2169–76.

Cheong, H.T., Park, K.W., Im, G.S., Lai, L., Sun, Q.Y., Day, B.N. and Prather, R.S. (2002). Effect of elevated Ca^{2+} concentration in fusion/activation medium on the fusion and development of porcine fetal fibroblast nuclear transfer embryos. *Mol. Reprod. Dev.* **61**, 488–92.

Du, Y., Kragh, P.M., Zhang, Y., Li, J., Schmidt, M., Bogh, I.B., Zhang, X., Purup, S., Jorgensen, A.L., Pedersen, A.M., Villemoes, K., Yang, H., Bolund, L. and Vajta, G. (2007). Piglets born from handmade cloning, an innovative cloning method without micromanipulation. *Theriogenology* **68**, 1104–10.

Estrada, J.L., Collins, B., York, A., Bischoff, S., Sommer, J., Tsai, S., Petters, R.M. and Piedrahita, J.A. (2008). Successful cloning of the Yucatan minipig using commercial/occidental breeds as oocyte donors and embryo recipients. *Cloning Stem Cells* **10**, 287–96.

Horie, K., Fujita, J., Takakura, K., Kanzaki, H., Kaneko, Y., Iwai, M., Nakayama, H. and Mori, T. (1992). Expression of c-kit protein during placental development. *Biol. Reprod.* **47**, 614–20.

Huppertz, B. (2007). The feto-maternal interface: setting the stage for potential immune interactions. *Semin. Immunopathol.* **29**, 83–94.

Jung, J.W., Park, J.S., Hwang, J.W., Kang, K.S., Lee, Y.S., Song, B.S., Lee, G.J., Yeo, C.D., Kang, J.S., Lee, W.S., Jeon, K.S., Um, C.H., Kim, Y.S., Oh, M.J., Youn, J.P., Li, P., Park, J.E. and Hwang, S.Y. (2004). Gene expression analysis of peroxisome proliferators- and phenytoin-induced hepatotoxicity using cDNA microarray. *J. Vet. Med. Sci.* **66**, 1329–33.

Koo, O.J., Jang, G., Kwon, D.K., Kang, J.T., Kwon, O.S., Park, H.J., Kang, S.K. and Lee, B.C. (2008). Electrical activation induces reactive oxygen species in porcine embryos. *Theriogenology* **70**, 1111–8.

Kurome, M., Ishikawa, T., Tomii, R., Ueno, S., Shimada, A., Yazawa, H. and Nagashima, H. (2008). Production of transgenic and non-transgenic clones in miniature pigs by somatic cell nuclear transfer. *J. Reprod. Dev.* **54**, 156–63.

Lee, E., Lee, S.H., Kim, S., Jeong, Y.W., Kim, J.H., Koo, O.J., Park, S.M., Hashem, M.A., Hossein, M.S., Son, H.Y., Lee, C.K., Hwang, W.S., Kang, S.K. and Lee, B.C. (2006). Analysis of nuclear reprogramming in cloned miniature pig embryos by expression of Oct-4 and Oct-4 related genes. *Biochem. Biophys. Res. Commun.* **348**, 1419–28.

Miyoshi, K., Saeki, K. and Sato, E. (2000). Improvement in development of porcine embryos reconstituted with cells from blastocyst-derived cell lines and enucleated oocytes by optimization of reconstruction methods. *Cloning* **2**, 175–84.

Prather, R.S., Boquest, A.C. and Day, B.N. (1999). Cell cycle analysis of cultured porcine mammary cells. *Cloning* **1**, 17–24.

Sprangers, B., Waer, M. and Billiau, A.D. (2008). Xenotransplantation: where are we in 2008? *Kidney Int.* **74**, 14–21.

Svendsen, O. (2006). The minipig in toxicology. *Exp. Toxicol. Pathol.* **57**, 335–9.

Vonnahme, K.A. and Ford, S.P. (2004). Differential expression of the vascular endothelial growth factor-receptor system in the gravid uterus of Yorkshire and Meishan pigs. *Biol. Reprod.* **71**, 163–9.

Wakai, T., Sugimura, S., Yamanaka, K., Kawahara, M., Sasada, H., Tanaka, H., Ando, A., Kobayashi, E. and Sato, E. (2008). Production of viable cloned miniature pig embryos using oocytes derived from domestic pig ovaries. *Cloning Stem Cells* **10**, 249–62.

Wilson, M.E., Biensen, N.J., Youngs, C.R. and Ford, S.P. (1998). Development of Meishan and Yorkshire littermate conceptuses in either a Meishan or Yorkshire uterine environment to day 90 of gestation and to term. *Biol. Reprod.* **58**, 905–10.

Yao, S.K., Zhang, Q., Sun, F.Z. and Liu, P.Q. (2006). Genetic diversity of seven miniature pig breeds (strains) analyzed by using microsatellite markers. *Yi Chuan* **28**, 407–12.

Zhu, J., Telfer, E.E., Fletcher, J., Springbett, A., Dobrinsky, J.R., De Sousa, P.A. and Wilmut, I. (2002). Improvement of an electrical activation protocol for porcine oocytes. *Biol. Reprod.* **66**, 635–41.

Ziecik, A.J., Biallowicz, M., Kaczmarek, M., Demianowicz, W., Rioperez, J., Wasielak, M. and Bogacki, M. (2005). Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* **51**, 379–84.