CLONING AND STEM CELLS
Volume 9, Number 3, 2007
© Mary Ann Liebert, Inc.
DOI: 10.1089/clo.2006.0090

Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.
Cloning and Stem Cells 2007.9:357-363.

An Epigenetic Modifier Results in Improved *In Vitro* Blastocyst Production after Somatic Cell Nuclear Transfer

YUNHAI ZHANG,[1,2,5] JUAN LI,[1,3] KLAUS VILLEMOES,[1] ANETTE M. PEDERSEN,[1] STIG PURUP,[4] and GÁBOR VAJTA[1]

ABSTRACT

The present study was designed to examine the effect of trichostatin A (TSA), an inhibitor of histone deacetylase, on development of porcine cloned embryos. Our results showed that treatment of cloned embryos derived from sow oocytes with 50 nM TSA for up to 24 h after the onset of activation could significantly improve blastocyst yield compared to the control (46.4 ± 4.6% vs 17.7 ± 4.9% for treated and untreated embryos, respectively; $p < 0.05$), whereas similar cleavage rate and total cell number per blastocyst were observed. In order to assess if the improvement is cell line specific, three cell lines were tested, and for all cell lines an enhancement in blastocyst development compared to their corresponding control was observed. Our data demonstrate that TSA treatment after somatic cell nuclear transfer in the pig can significantly improve the *in vitro* blastocyst production.

INTRODUCTION

PORCINE CLONING by somatic cell nuclear transfer (SCNT) provides a unique tool for basic research and biomedicine. The main reason for the latter is the special interests in using pigs as sources for human xenotransplantation or models for certain diseases as SCNT is so far the only practical way to produce targeted genetic modification in pigs (Lai et al., 2002; Vajta et al., 2007). Although dozens of nontransgenic and transgenic pigs have been cloned by SCNT, the efficiency is still unsatisfactory due to the poor *in vitro* embryo development and limited number of offspring obtained (Pratt et al., 2006). These problems may be attributed to incomplete reprogramming during cloning procedures resulting in errors at epigenetic level (Jaenisch et al., 2002; Wilmut 2002). Theoretically, an intervention to assist reprogramming of the transferred nucleus might be helpful to improve development of cloned embryos (Wilmut et al., 2002). Indeed, some previous studies in bovine cloning indicate that treatment of donor cells with certain epigenetic modifier such as the histone deacetylase inhibitors: sodium butyrate (Shi et

[1]Section of Population Genetics and Embryology, Department of Genetic and Biotechnology, Danish Institute of Agricultural Sciences, Tjele, Denmark.
[2]Department of Animal Reproduction, College of Animal Science and Technology, Anhui Agricultural University, Hefei People's Republic of China.
[3]Institute of Human Genetics, Aarhus University, Aarhus, Denmark.
[4]Section of Nutrition and Production Physiology, Department of Animal Health, Welfare and Nutrition, Danish Institute of Agricultural Sciences, Tjele, Denmark.

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.

al., 2003), and trichostatin A (TSA) (Enright et al., 2003), or the noncytotoxic transmethylation inhibitor: S-adenosyl homocysteine (Jeon et al., 2005) may enhance the *in vitro* blastocyst production; however, no *in vivo* results were reported so far. It should also be noted that not all studies found beneficial effects of treatment of somatic donor cells with DNA methylation inhibitor: 5-aza-2-deoxycytidine (Enright et al., 2003; Shi et al., 2003) or the histone deacetylase inhibitor TSA (Shi et al., 2003) to *in vitro* development of bovine embryos. Recently, considerable improvement in mouse cloning has been achieved by TSA treatment of cloned embryos after SCNT (Kishigami et al., 2006; Rybouchkin et al., 2006). In these reports, not only the blastocyst but also the full-term development rates were remarkably improved. Until now, however, it is unknown if TSA treatment of embryos could also benefit cloning techniques in species other than mouse, such as in pigs, and so far, no other reprogramming promoting agent was described as efficient in pigs. Consequently, the present study was designed to examine the effect of TSA treatment on *in vitro* developmental competence of porcine cloned embryos.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Preparation of somatic cells for SCNT

Two porcine fetal fibroblast cell lines (L1 and L2) were established from two approximately 40-day-old fetuses, while an ear skin fibroblast cell line (L3) was established from a newborn piglet. The procedure of primary cell culture, subculture, and cryopreservation of established cell lines were performed as described previously (Kragh et al., 2005). After thawing, cells at passage 3–8 were grown for 1 week to 100% confluence in wells of four-well dishes (Nunc, Roskilde, Denmark) in Dulbecco Modified Eagle Medium (DMEM, Gibco, Life Technologies, New York) supplemented with 10% fetal bovine serum (Gibco), at 39°C, under 5% CO_2 and 100% humidity. Just before SCNT, attached cells were washed twice with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) and incubated at 39°C for 5 min with 100 μL 0.25% Trypsin-EDTA (T4049). Then the dissociated cells were transferred to a centrifuge tube containing 500 μL HEPES-buffered Tissue Culture Medium 199 (TCM-199) supplemented with 2% cattle serum (CS; Danish Veterinary Institute, Frederiksberg, Denmark) and the fibroblast suspension was stored for approximately 0.5 h at room temperature (24–29°C) until use.

Oocyte collection and in vitro *maturation (IVM)*

Sow ovaries were collected from abattoirs and transported in physiological saline solution with antibiotics at 25–35°C to the laboratory within 4 h after slaughter. Cumulus-oocyte complexes (COCs) were aspirated from 3–6-mm follicles with an 18-gauge needle using vacuum suction. COCs with at least three layers of compact cumulus investment and even cytoplasm were selected and washed twice in HEPES-buffered TCM-199 plus 0.3% heparin (5000 IE/mL, LEO Pharma A/S, Denmark), 5% amphotericin, and 10% CS. Afterward, the selected COCs were matured in groups of 50–70 in 400-μL IVM medium in four-well dish at 38.5°C in 5% CO_2 in humidified air for 40–46 h. The IVM medium was bicarbonate-buffered TCM-199 supplemented with 15% (v/v) CS, 10% (v/v) porcine follicular fluid, 10 IU/mL eCG, 5 IU/mL hCG (both from Suigonan Vet; Skovlunde, Denmark), 0.8 mM L-glutamine, and 0.05 mg/mL gentamicin.

COCs were then transferred to 1 mg/mL hyaluronidase in TCM-199 and pipetted repeatedly for 2 min to denude cumulus cells. Oocytes with intact cell membrane and clear perivitelline space were selected for future manipulations.

Nuclear transfer

The solution used for micromanipulation was HEPES-buffered TCM-199 supplemented with 7.5 μg/mL cytochalasin B (CB) and 2% CS. Micromanipulation was performed with an inverted microscope equipped with manipulation systems and warm stage. The procedure of micromanipulation was the same as reported earlier (Zhang et al., 2006). A single 50-μL micromanipulation solution drop was made in the central area on a lid of 60-mm culture dish (Nunc, Denmark) and covered with mineral oil. Groups of 20–30 oocytes and nuclear donor cells were placed in this drop and incubated for 15–30 min in manipulation medium. Subsequently, one oocyte was fixed

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.

with a holding pipette (inner diameter: 25–35 μm and outer diameter: 80–100 μm), and the first polar body together with 20% of the adjacent cytoplasm presumptive containing metaphase plate were aspirated out with a beveled injection pipette (inner diameter: 20 μm). A somatic cell with diameter at 15–20 μm was injected subsequently into the perivitelline space through the same slot. Reconstructed couplets were then transferred into drops of HEPES-buffered TCM-199 and 2% CS, and covered with mineral oil for 0.5–1.5 h until fusion and activation.

Fusion and activation

A simultaneous fusion and activation (FAS) protocol was employed by using 0.3 M mannitol supplemented with 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.01% polyvinyl alcohol. Reconstructed couplets were equilibrated in the fusion solution for 4 min, and then groups of five couplets were placed in the fusion chamber (BTX microslide 1 mm fusion chamber; BTX, San Diego, CA) filled with fusion solution. Couplets were aligned manually using a fine needle to make the contact plane parallel to electrodes, then two, 100 μs, direct current pulse of 1.65 kV/cm with a 1-sec interval was applied using a CFS-150/B fusion machine (BLS, Budapest, Hungary). Subsequently, couplets were washed three times and cultured in PZM-3 plus 10 μg/mL CB, 10 μg/mL cycloheximide, and with or without TSA according to experimental design. Four hours later, fusion results were examined under a stereomicroscope. Fused couplets, that is, reconstructed embryos were transferred to Porcine Zygote Medium 3 (PZM-3; Yoshioka et al., 2002) with or without TSA (T-8522; see description of individual experiments).

Embryo culture and embryo quality evaluation

Embryo culture was conducted at 39°C under 5% CO_2, 5% O_2, 90% N_2, and 100% humidity. Cleavage and blastocyst rates were determined under a stereomicroscope after 48 h and 144–168 h *in vitro* culture, respectively. Some blastocysts were stained by 20 μg/mL Hoechst 33342 for 10 min, and then visualized under ultraviolet light by an inverted microscope and photographed. To assess inner cell mass (ICM) and trophectoderm (TE) cells, a simplified differential staining method (Thouas et al., 2001) was slightly modified. Briefly, the blastocysts were put into solution I (PZM3 plus 1% Triton X-100 and 100 μg/mL propidium iodide) for 15–25 sec or until TE visibly changed color to red and shrank slightly, and afterward these embryos were transferred to solution II (100% absolute ethanol plus 25 μg/mL Hoechst 33342) and put into refrigerator at 4°C overnight. Stained blastocysts then were mounted into a small drop of glycerol on a microscope slide under a cover slip and examined under an inverted microscope (Leica, Germany) equipped with epifluorescence. Cell numbers were counted using Java-based software designed by Villemoes (unpublished).

Experiments

In Experiment 1, the effect of TSA treatment on preimplantation development of sow oocytes-derived embryos was evaluated. Reconstructed embryos were randomly cultured in five groups: 50 nM for 10 h; 50 nM for 24 h; 50 nM for 36 h; 50nM for 48 h; and untreated control embryos (TSA treatment was started immediately after FAS). After being cultured in PZM-3 containing TSA for certain period, embryos were transferred to TSA-free PZM-3 for further culture. Rates of cleavage and blastocyst formation, as well as the total cell number per blastocyst were compared.

As in Experiment 1 treatment with 50 nM TSA for 24 h was found the most effective, in Experiment 2 we used this treatment for embryos derived from sow oocytes by using three different fibroblast cell lines (L1, L2, and L3) to test if the beneficial effect is cell line specific. The rates of cleavage and blastocyst formation, as well as the ICM and TE cell numbers of blastocysts derived from cell line L1 after TSA treatment and nontreatment were compared.

Statistical analysis

All experiments were replicated at least six times. In Experiment 1, the data were subjected to one-way ANOVA with SPSS (version 11.0 for Windows). Then least significant difference (LSD) was applied for post hoc multiple comparison among groups. In Experiment 2, a Student's *t*-test was employed to analyze the data between TSA-treated group and the according control group; while ANOVA was used to analyze the data among the TSA-treated groups, and nontreated groups. A value of $p < 0.05$ was considered to be significant.

TABLE 1. EFFECT OF TRICHOSTATIN A (TSA) EXPOSURE DURATION ON *IN VITRO* DEVELOPMENTAL COMPETENCE OF PIG CLONED EMBRYOS DERIVED FROM SOW OOCYTES

Group	*No. of embryos cultured*	*No. of cleaved embryos (%, mean ± SEM)*[a]	*No. of blastocysts (%, mean ± SEM)*[b]	*Total cell numbers of blastocysts (mean ± SEM)*
Control	100	77 (77.0 ± 3.1)	18 (17.7 ± 4.9)[c]	47 ± 4
50 nM, 10 h	106	81 (77.5 ± 5.5)	30 (28.6 ± 4.9)[cd]	51 ± 5
50 nM, 24 h	102	81 (79.2 ± 2.9)	38 (46.4 ± 4.6)[d]	54 ± 3
50 nM, 36 h	98	75 (75.4 ± 6.4)	39 (40.3 ± 6.9)[d]	49 ± 3
50 nM, 48 h	103	79 (76.0 ± 4.5)	38 (38.5 ± 5.7)[d]	46 ± 7

Values with different superscripts in the same column are significantly different ($p < 0.05$). Control means no TSA supplementation in culture media.

[a]Cleavage rate: No. of embryos cleaved/No. embryos cultured.

[b]Blastocyst rate: No. of blastocysts/No. embryos cultured.

SEM: Standard error of the mean.

RESULTS

As shown in Table 1, none of the four TSA treatments of sow oocyte-derived embryos significantly influenced cleavage rates. However, treatment with 50 nM TSA for 24 h (Fig. 1b), 36 h, or 48 h increased the blastocyst rate in comparison with the control (Fig. 1a; $p < 0.05$ for all). On the other hand, there was no difference regarding total cell numbers per blastocyst among groups.

As shown in Figure 2, for each cell line, the developmental rates of TSA-treated groups were always higher than those of the corresponding control groups in terms of blastocyst formation. However, there was no significant difference among 24 h, 50 nM TSA-treated blastocysts produced from three different cell lines, or among three corresponding controls. Regarding to the cloned embryos from L1 after the treatment with 50 nM TSA for 24 h, their ICM and TE cell numbers in blastocysts were 11.00 ± 3.16 and 47.00 ± 9.88 (mean ± SEM; $n = 6$, number of blastocyst examined), which was not significantly different from the control (7.56 ± 1.27 and 33.78 ± 5.78 for ICM and TE cells, $n = 6$).

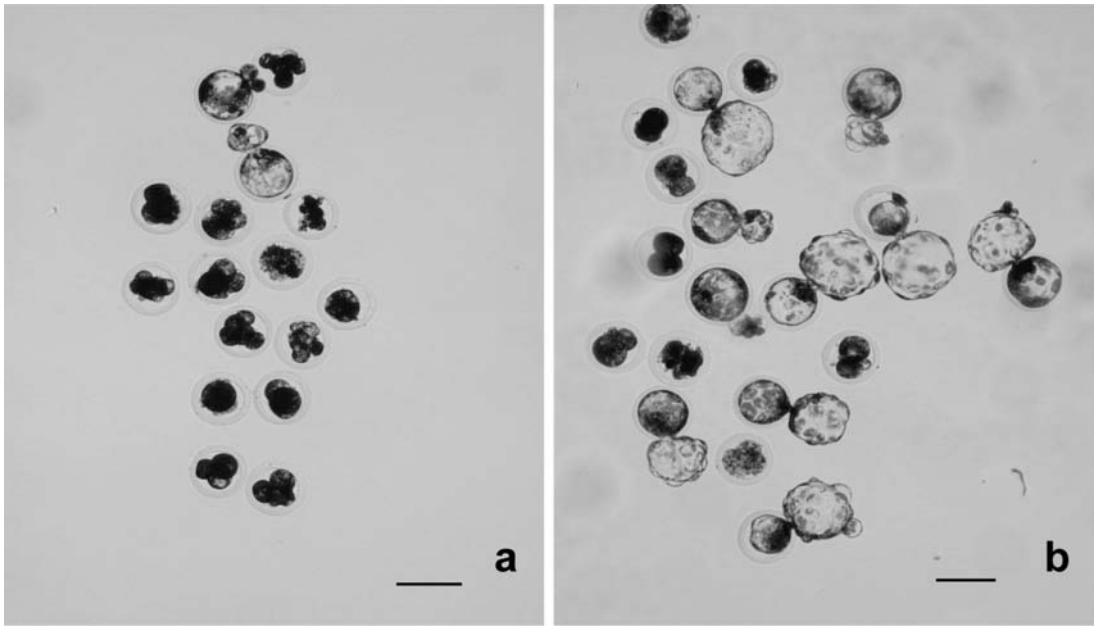

FIG. 1. Day 6 cloned blastocysts produced by somatic cell nuclear transfer combined without tichostatin A (TSA) treatment (**a**) or 50 nM for 20 h TSA treatment (**b**). Scale bar represents 150 microns.

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.

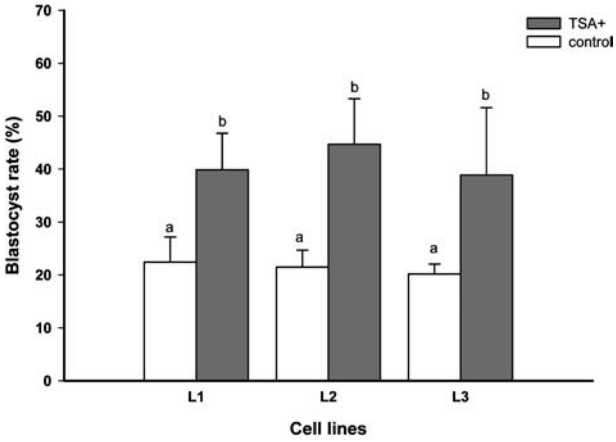

FIG. 2. Effect of trichostatin A (TSA)-supplementation on the rate of blastocyst formation in porcine cloned embryos derived from sow oocytes and different somatic cell lines. Different letters above the bars denote significant difference ($p < 0.05$). L1 and L2 fibroblast cell lines were from fetus, and L3 fibroblast cell line was from newborn pig ear skin. Different letters above the columns denote significant difference.

DISCUSSION

The present study demonstrates that TSA treatment of porcine SCNT embryos for certain duration and at appropriate concentration can considerably improve blastocyst yield without compromising the total cell number, as well as ICM and TE cell numbers per blastocyst.

SCNTs capable to produce 10%–30% blastocyst rates either from *in vivo* (Polejaeva et al., 2000) or *in vitro* matured sow (Betthauser et al., 2000) or prepubertal gilt (Park et al., 2004) oocytes, are generally regarded as appropriate to result in cloned offspring. However, to avoid the supposedly harmful effect of extended *in vitro* culture, embryos are usually transferred at the one- to two-cell stage. In our current study without TSA treatment, 20% of sow oocytes-derived reconstructed embryos have developed to the blastocyst stage, which was similar to other reports (Hyun et al., 2003; Im et al., 2004). However, when embryos were treated with TSA for a total of 24 h after FAS, the blastocyst rate has been increased to 46.4 ± 4.6%, considerably higher than that in any previously published report for porcine SCNT (37% and 31%; Lee et al., 2003; Onishi et al., 2000; respectively). In mouse cloning, Kishigami et al. (2006) obtained two- to five-fold increase in blastocyst rate based on cleaved embryos. Also in the mouse, Rybouchkin et al. (2006) obtained 81% blastocyst rate in the TSA treatment group, while only 40% could develop to blastocyst without TSA treatment. So according to our results, the supportive effect of TSA treatment has been also proved in porcine cloning. The increase in blastocyst rate may be the consequences of support of reprogramming by TSA when applied in appropriate concentration and duration during and after activation. TSA is a histone-deacetylase inhibitor which could enhance the pool of acetylated histones and DNA demethylation (Kishigami et al., 2006). Observations in mice cloning by TSA treatment showed that it is rather histone hyperacetylation than deacetylation, which actually improves reprogramming (Rybouchkin et al., 2006). Kang et al. (2001), and found that reprogramming of DNA methylation in porcine cloned embryos is controlled by recipient oocytes. However, demethylation and remethylation in satellite region or *PRE-1* sequences occurs a bit later than in *in vivo* controls. Accordingly, it is supposed that TSA treatment applied in a period without DNA replication has probably no direct impact on DNA demethylation (Rybouchkin et al., 2006). Therefore, further research is required to investigate if dynamics of molecular reprogramming events are improved in a similar way as *in vivo*. Anyway, similar beneficial effect of TSA-treatment on *in vitro* blastocyst development after SCNT implies that a more or less conservative reprogramming regulation mechanism may exist between murine and porcine cloned embryos.

In the present study we used 50 nM TSA as a major concentration according to suggestions of Kishigami et al. (2006). Initially, when the preliminary study was conducted by us, we applied 50 nM TSA for 10 h, a similar protocol to what is effective in mice cloning, to treat porcine cloned embryos. Although there was a slight increase in blastocyst development, no statistical significance was obtained (Table 1). Therefore, we increased the duration of treatment from 10 h that was efficient in mouse (Kishigami et al., 2006) to 24–48 h for the treatment of sow oocyte-derived cloned embryos considering that a longer time may required for porcine oocytes to accumulate enough acetylation level, which might be critical for subsequent fine-tuned reprogramming regulation. After the most effective treatment was defined in porcine cloning, we also have investigated if this beneficial effect is cell line-specific, since Kishigami et al. (2006) reported that no beneficial effect was obtained when mouse cloned embryos

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.

derived from ES cells were treated, whereas a two- to five-fold increase in blastocyst development was obtained when cumulus cells, tail-tip cells, spleen cells, and neural stem cells were used. In the present study, 50 nM TSA was used for 24-h treatment for reconstructed embryos derived from three different cell lines: L1 and L2 from two independent fetuses, and L3 from newborn piglet ear skin. Embryos originated from all cell lines had a considerable increase in blastocyst formation compared to the corresponding untreated controls, and no differences were observed among the three TSA-treated groups.

It is argued that measuring the potential of a cloned blastocyst to generate a viable clone or ES cells provides more defined readouts for cloning efficiency than just development through cleavage stages (Hochedlinger and Jaenisch 2006). In porcine cloning, it is proposed that 10 high-quality blastocysts are enough to produce one cloned piglet (Nagashima et al., 2003), leaving only slightly more embryos to develop to term than the minimum four required to induce and maintain pregnancy (Polge et al., 1966). Very recently, nonsurgical embryo transfer using both *in vivo* and *in vitro* porcine embryos has been successfully performed (Cuello et al., 2005; Martinez et al., 2004; Suzuki et al., 2004), although a more reliable and efficient method should be developed (Pratt et al., 2006). Meanwhile, cloned piglets also have been produced successfully using SCNT combined with cryopreservation of blastocysts (Li et al., 2006). Accordingly, it seems to be reasonable to suppose that a reliable and effective *in vitro* blastocyst production system using SCNT and TSA treatment is of great value as the demands increase, especially in national or international embryo movement and pig breeding or human medicine and basic researches.

In conclusion, our data suggest that TSA treatment after SCNT in pigs can considerably improve the blastocyst production *in vitro*. However, further research is needed to evaluate the long-term effects of TSA treatment on *in vivo* developmental competence, and afterward, the growth and behavior of cloned piglets in the future.

ACKNOWLEDGMENTS

We thank Dr. Peter Kragh and Professor Ingrid Bogh for their valuable suggestions in preparation of this manuscript, and thank Ruth Kristensen and Annette Nielsen for their excellent technical assistance. We are grateful to Dr. T. Wakayama's encouragement. This work was supported by grants from Danish Research Council for Technology and Production (Production of a transgenic porcine model of Alzheimer disease) and Danish Research Council for Health and Disease (Production of genetically modified pigs as human disease models).

REFERENCES

Betthauser, J., Forsberg, E., Augenstein, M., et al. (2000). Production of cloned pigs from in vitro systems. Nat. Biotechnol. 18, 1055–1059.

Cuello, C., Berthelot, F., Martinat-Botte, F., et al. (2005). Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. Anim. Reprod. Sci. 85, 275–286.

Enright, B.P., Kubota, C., Yang, X., et al. (2003). Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2′-deoxycytidine. Biol. Reprod. 69, 896–901.

Hochedlinger, K., and Jaenisch, R. (2006). Nuclear reprogramming and pluripotency. Nature 441, 1061–1067.

Hyun, S.H., Lee, G.S., Kim, D.Y., et al. (2003). Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos. Theriogenology 59, 1641–1649.

Im, G.S., Lai, L., Liu, Z., et al. (2004). In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61, 1125–1135.

Jaenisch, R., Eggan, K., Humpherys, D., et al. (2002). Nuclear cloning, stem cells, and genomic reprogramming. Cloning Stem Cells 4, 389–396.

Jeon, B.-G., Perrault, S.D., Rho, G.-J., et al. (2005). Developmental potential and reprogramming efficiency of bovine embryos cloned with adult fibroblasts treated by a demethylating agent, s-adenosyl-homocysteine. Reprod. Fertil. Dev. 18, 130.

Kang, Y.K., Koo, D.B., Park, J.S., et al. (2001). Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. J. Biol. Chem. 276, 39980–39984.

Kishigami, S., Mizutani, E., Ohta, H., et al. (2006). Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem. Biophys. Res. Commun. 340, 183–189.

Kragh, P.M., Du, Y., Corydon, T.J., et al. (2005). Efficient in vitro production of porcine blastocysts by handmade cloning with a combined electrical and chemical activation. Theriogenology 64, 1536–1545.

Lai, L., Kolber-Simonds, D., Park, K.W., et al. (2002). Pro-

duction of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295, 1089–1092.

Lee, J.W., Wu, S.C., Tian, X.C., et al. (2003). Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol. Reprod. 69, 995–1001.

Li, R., Lai, L., Wax, D., et al. (2006). Cloned transgenic Swine via in vitro production and cryopreservation. Biol. Reprod. 75, 226–230.

Martinez, E.A., Caamano, J.N., Gil, M.A., et al. (2004). Successful nonsurgical deep uterine embryo transfer in pigs. Theriogenology 61, 137–146.

Nagashima, H., Fujimura, T., Takahagi, Y., et al. (2003). Development of efficient strategies for the production of genetically modified pigs. Theriogenology 59, 95–106.

Onishi, A., Iwamoto, M., Akita, T., et al. (2000). Pig cloning by microinjection of fetal fibroblast nuclei. Science 2891, 1188–1190.

Park, M.R., Cho, S.K., Park, J.Y., et al. (2004). Detection of rare Leydig cell hypoplasia in somatic cell cloned male piglets. Zygote 12, 305–313.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., et al. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407, 86–90.

Polge, C., Rowson, L.E., and Chang, M.C. (1996). The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. J. Reprod. Fertil. 12, 395–397.

Pratt, S.L., Sherrer, E.S., Reeves, D.E., et al. (2006). Factors influencing the commercialisation of cloning in the pork industry. Reprod. Suppl. 62, 303–315.

Rybouchkin, A., Kato, Y., and Tsunoda, Y. (2006). Role of histone acetylation in reprogramming of somatic nuclei following nuclear transfer. Biol. Reprod. 74, 1083–1089.

Shi, W., Hoeflich, A., Flaswinkel, H., et al. (2003). Induction of a senescent-like phenotype does not confer the ability of bovine immortal cells to support the development of nuclear transfer embryos. Biol. Reprod. 69, 301–309.

Suzuki, C., Iwamura, S., and Yoshioka, K. (2004). Birth of piglets through the non-surgical transfer of blastocysts produced in vitro. J. Reprod. Dev. 50, 487–491.

Thouas, G.A., Korfiatis, N.A., French, A.J., et al. (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod. Biomed. Online 3, 25–29.

Vajta, G., Zhang, Y., and Machaty, Z.N. (2007). Somatic cell nuclear transfer in pigs: recent achievements and future possibilities. Reprod. Fertil. Dev. 19, 403–423.

Wilmut, I. (2002). Are there any normal cloned mammals? Nat. Med. 8, 215–216.

Wilmut, I., Beaujean, N., de Sousa, P.A., et al. (2002). Somatic cell nuclear transfer. Nature 419, 583–586.

Yoshioka, K., Suzuki, C., Tanaka, A., et al. (2002). Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66, 112–119.

Zhang, Y.H., Pan, D.K., Sun, X.Z., et al. (2006). In vitro developmental competence of pig nuclear transferred embryos: effects of GFP transfection, refrigeration, cell cycle synchronization and shapes of donor cells. Zygote 14, 239–247.

Address reprint requests to:
Dr. Yunhai Zhang
Blichers Allé 12B
Tjele, DK-8830, Denmark

E-mail: yunhaizhang2005@gmail.com

Cloning and Stem Cells 2007.9:357-363.
Downloaded from online.liebertpub.com by Uc Davis Libraries University of California Davis on 01/17/15. For personal use only.