International Scholars Journals

African Journal of Pig Farming ISSN 2375-0731 Vol. 7 (1), pp. 001-004, January, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Review

Review of somatic cell nuclear transfer in pig

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Accepted 30 September, 2018

It is now more than 8 years, since the first cloned pig from nuclear transfer was reported. Success of somatic cell nuclear transfer (SCNT) in pig is still low compared to that in bovine. Embryonic and neonatal abnormalities of cloned piglets are probably a result of incorrect or incomplete reprogramming of the transferred donor cell nuclei. Recently, technical refinements have resulted in a considerable increase of SCNT efficiency. The current level of efficiency is already sufficient for special purposes with high scientific and commercial impact including xenotransplantation, generation of human disease models and for biomedical studies. In this paper, we review current pig cloning methodologies for the technical and potential applications.

Key words: Pig, somatic cell, nuclear transfer, oocyte and embryo.

INTRODUCTION

To date, cloned offspring by somatic cell nuclear transfer (Figure 1) have been successfully produced in a variety of mammals (Wilmut et al., 1997; Wakayama et al., 1998; Kato et al., 1998; Onishi et al., 2000). However, the success rate remains low and many important factors remain unclear especially in pig compared to other species (Wu et al., 2001). Usually, pig oocytes and embryos are very sensitive to stressors, physical change and chemical factors for nuclear transfer technology. The pig oocytes contain many lipids inside the cytoplasm which explain their sensitivity to environmental stressors. It seems that lipids in pig oocytes affect the development of embryos following somatic nuclear transfer technique (Nagashima et al., 1995). Several reports have indicated that the oocytes activation, embryo culture and embryo transfer and early embryo developmental stage seem to be the major problems of SCNT in pigs. However, there is a considerable improvement on the efficiency of SCNT in pigs and application such as cloned transgenic pigs to be used for xenotransplantation, are under intensive research (Dai et al., 2002; Lai et al., 2002b; Kolber-Simonds et al., 2004).

The objective of this review was to provide an overview of the somatic cell nuclear transfer in pig and summarize recent improvement on the efficiency of pig SCNT technology.

IN VITRO MATURATION (IVM) OF PIG OOCYTE

In SCNT technique, the oocytes are needed to use for recipient cytoplasm. Most of the recipient oocytes are matured *IN VITRO* or derived from *IN VIVO*. The first two reports using *IN VIVO* matured pig oocytes for SCNT reported successfully application of SCNT and they produced cloned piglet (Onishi et al., 2000; Polejaeva et

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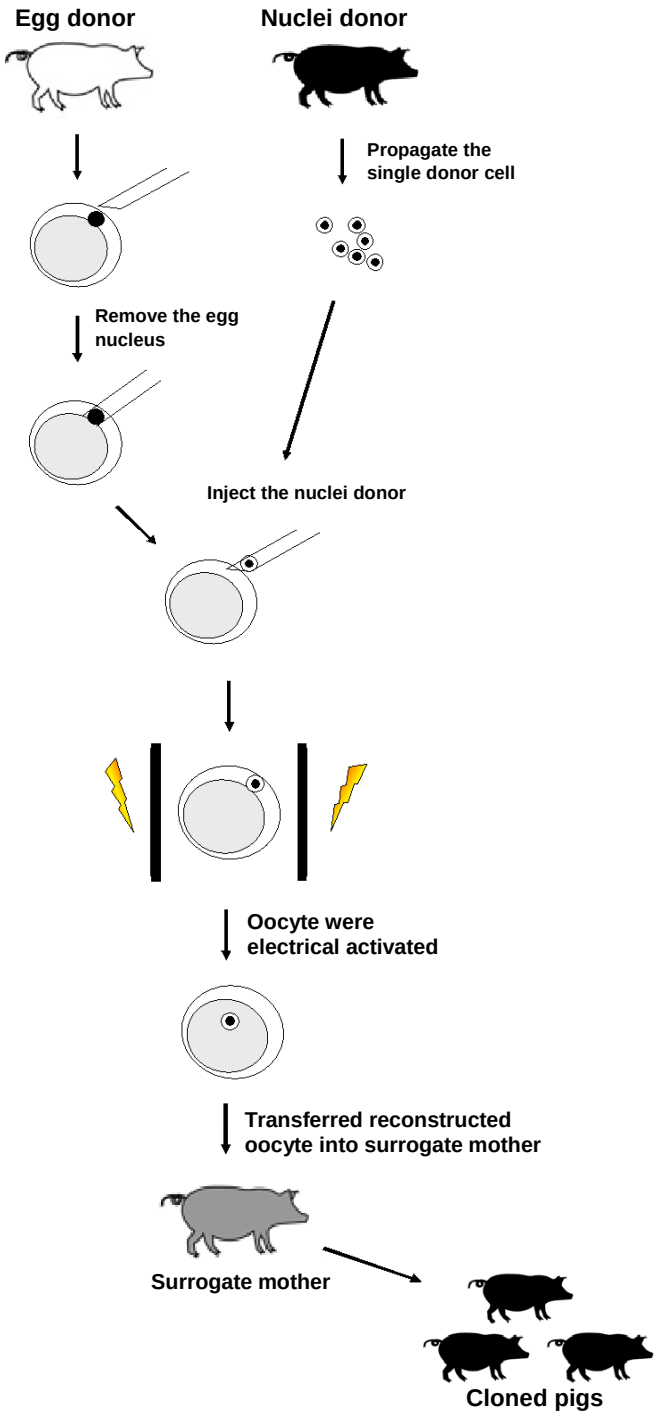

Figure 1. Somatic cell nuclear transfer (SCNT) in pig.

al., 2000). As a result, cloned piglet was also born using *IN VITRO* matured oocytes from the ovaries derived from slaughter house (Betthauser et al., 2000).

The North Carolina State University (NCSU) 23 and tissue culture medium (TCM) 199 have been compared for maturation of pig oocytes and no differences were found among them on supporting the development of cloned pig embryos (Hyun et al., 2003a). Several researchers are interested on improving oocyte and embryo developmental capacity by modify the

culture media and culture conditions to enhance the successful development of cloned embryos *IN VITRO*. Generally, the basic medium for pig oocytes is NCSU 23 or NCSU-37 (Petters and Wells, 1993).

For *IN VITRO* maturation, pig follicular fluid (pFF) was used to supplement in the IVM medium. The pFF is usually added to maturation media of pig oocytes to protecting oocytes from oxidative stress through a higher level of radical scavenging activity, resulting in the enhancement of cytoplasmic maturation for development competence after fertilization. The maturation medium that supplemented with pFF can enhanced development competence of pig maturation. The factors within the follicles may play an important role to support the oocytes during maturation (Schoevers et al., 2003; Algriany et al., 2004). However, pFF can be contaminated from numerous undefined factors with viral pathogens (Kim and Dubovi, 2003).

The addition of hormones, growth factors, vitamins, energy substrates, inorganic compound, cytokines and follicular fluid influences considerably maturation and subsequent development of pig oocytes (Abeydeera, 2002). There is a report that epidermal growth factor (EGF) plays an important role in nuclear and cytoplasmic maturation of pig oocytes (Grupen et al., 1997; Abeydeera et al., 2000). Moreover, exposure of cumulus-oocyte complexes (COCs) to dibutyryl cAMP (dbcAMP) for maturation increased developmental competent of pig oocytes (Funahashi et al., 1997). Recently, defined systems for *IN VITRO* production of pig embryos using a single basic medium were reported for blastocyst production from chemically defined pig gamete medium (PGM) and pig zygote medium (PZM). The media based on the composition of pig oviductal fluid and appropriately supplemented for specific stage of embryo development thus can be used for *IN VITRO* maturation, fertilization and culture in pig (Yoshioka et al., 2008).

SOMATIC CELL NUCLEAR TRANSFER

It is a process known as nuclear transfer (NT) in which the nucleus of a donor cell is transferred into an oocyte. The first pig cloned by SCNT was reported by Prather et al. (1989). They used pronuclei exchange zygote and blastomere as a donor cell. Recipient cytoplasm derived from *IN VIVO* maturation was used and activated the reconstructed oocytes with electric before transfer to the oviduct for *IN VIVO* embryo culture. Blastocyst embryos were transferred to the recipient for develop into term. They received seven live pigs born from pronuclei exchange zygote and one live pig born from blastomere at 4-cell stage. Moreover, successful cloned pig by the use of somatic cell nuclear transfer technique has been reported by Polejaeva et al. (2000). They used granulosa cells as a donor cell with two-stage nuclear transfer technique that donor cells were fused to enucleated oocytes in the first stage, and the pronucleus-like structures formed were then subsequently transplantedinto *IN VIVO*-produced, enucleated zygotes. However, the successful production of cloned pig from a single-step nuclear transfer and the method is still wildly used in several laboratories to produce the SCNT pig (Betthauser et al., 2000; Onishi et al., 2000; Park et al., 2001; De Sousa et al., 2002; Yin et al., 2002).

Several researchers also reported the successful production of pig cloned with different donor cell types (Onishi et al., 2000; Betthauser et al., 2000; Bondioli et al., 2001; Park et al., 2002) and also they attempt to modify donor cells to improve the efficiency of nuclear transfer. The donor cell in SCNT can be prepared as synchronized (Onishi et al., 2000; Polejaeva et al., 2000) and non-synchronised donor cells (Betthauser et al., 2000; Bondioli et al., 2001). There success the nuclear transfer with both of *IN VIVO*-matured oocytes (Onishi et al., 2000; Polejaeva et al., 2000) and *IN VITRO*-matured oocytes (Betthauser et al., 2000). Many strategies to introduce the donor cell into cytoplasm of the recipient cytoplasm such as transferred by electrofusion (Polejaeva et al., 2000), by piezo-microinjection of isolated donor nuclei (Onishi et al., 2000), and also by whole cell injection (Lee et al., 2003b). Recently, the zona-free technique become of interest with zona removal before or after enucleation for fusion. (Booth et al., 2001; Kragh et al., 2004; Du et al., 2005).

ARTIFICIAL ACTIVATION

In an effort to establish the cloned embryos, activation is the part of important step to initial reconstructs oocytes and donor cells. Since sperm-mediated activation is absent in SCNT, an artificial activation is needed to initiate embryo development. Mostly in mammals, oocytes are arrested at metaphase II (MII) stage after ovulation and complete meiosis after fertilization. The MII arrest is characterized by MPF activity (Nurse, 1990). Since the sperm-induce the release from meiotic arrest by a signal transduction pathway, calcium, M-phase promoting factor (MPF) and its stabilizing molecules play a role during oocyte activation by inducing a transient increase in the intracellular free calcium concentration by artificial means.

For *IN VITRO* culture of pig embryos, oocytes are matured for around 42-44 h after the start of IVM, thus on this stage, a calcium transient is able to stimulate development (Machaty and Prather, 1998). There are many activation protocols to stimulate the oocyte such as electrical, chemical or mechanical activation. Simultaneous fusion/activation has been used for the generation of cloned pigs with targeted disruption of one or both alleles of the α-1,3-galactosyltransferase gene

(Dai et al., 2002; Lai et al., 2002a; Kolber-Simonds et al., 2004). The transgenic cloned pig expressing the green fluorescent protein were produced by triggering fusion and activation at the same time with an electrical stimulus (Park et al., 2001; Lai et al., 2002; Hyun et al., 2003b; Lee et al., 2005). Usually, high voltage direct current (DC) pulse is applied to generate an influx of extracellular calcium (Machaty and Prather, 1998).

In the absence of extracellular calcium ions, the DC pulse induces membrane fusion only. In this case, high MPF levels in the oocyte cytoplasm cause to nuclear envelope breakdown and premature chromosome condensation. In the case of chemical activation, the oocytes were exposed to the substrates such as cytochalasin B (Li et al., 2000; Park et al., 2002; Hoshino et al., 2005) to prevent the extrusion of the second polar body, cycloheximide (Lee et al., 2003a) or 6-dimethylaminopurine (DMAP; de la Fuente and King, 1998; Hölker et al., 2005) to inhibit the protein kinase after an induced calcium transient to promote activation or ionomycin (Betthauser et al., 2000; Boquest et al., 2002) that it forms a complex with calcium ions and transports through the plasma membrane and it can also stimulate calcium and induce a calcium influx similar electrical activation (Morgan and Jacob, 1994).

Similarly, delayed activation was effective in supporting term development after demecolcine assisted enucleating (Yin et al., 2002; Kawakami et al., 2003) or mechanical activation when the donor nuclei were microinjected directly into the recipient cytoplasm (Onishi et al., 2000; Watanabe et al., 2005; Takeda et al., 2006). It has been suggested that nuclear reprogramming is more successful if chromosomes of the nuclear donor cell are exposed to the recipient oocyte cytoplasm for an extended period of time (Miyoshi et al. 2000; Tani et al. 2001; Shin et al. 2002). Furthermore, development after nuclear transfer may be superior under delayed activation conditions compared to simultaneous fusion/activation (Yin et al., 2003). All the activation protocols mentioned above stimulate embryonic development by triggering a single calcium transient. However, the availability of current methods to induce repetitive calcium transient in pig oocyte is limited.

EMBRYO CULTURE AND TRANSFER

In order to establish pregnancies, the pig embryos were transfer to the recipient in early embryo development stage between one-cell to eight-cell stage with a many number of embryo. Most of embryo numbers were transferred more than 100 embryos to one recipient to avoid the negative effects of the *IN VITRO* system that seems to be effect to pig embryos more than other species. However, the pregnancy rate remains low and the fetus is almost lost during gestation (Chae et al., 2006).

Numerous studies aimed to improve the culture medium for preimplantation of embryo that can produce the cloned pig after transfer to the recipient. The result is considered to better understanding of pig embryonic development. The culture medium for pig embryo culture is NCSU-23 (Onishi et al., 2000; Polejaeva et al., 2000), NCSU-37 (Kikuchi et al., 2002) and Pig Zygote Medium (PZM)-3 (Yoshioka et al., 2002; Im et al., 2004). Recently, the result found that PZM-3 was superior in order to support the development of pig embryos, especially the embryos derived from SCNT. It seems that the PZM-3 could better improve the efficiency of pig embryo to develop to preimplantation stage compared to NCSU-23 (Im et al., 2004). Moreover, there is a report to study effect of bovine serum albumin (BSA) and polyvinyl alcohol (PVA) supplementation in NCSU-23. They found that both of them could enhance the production of pig embryo (Roh and Hwang, 2002).

Regarding to culture condition under temperature and gases control incubator, the pig embryos cultured in an atmosphere with 5% CO_2 in air showed the developmental rates and total cell numbers of blastocysts higher than embryos cultured under 5% CO_2, 5% O_2 and 90% N_2 (Machaty et al., 1998). Recently, there are reports showing that low oxygen concentration (five-seven %) can enhance the pig embryo development from *IN VITRO* culture (Im et al., 2004; Sage et al., 2005). However, there were no differences developing in embryo development, total cell number and apoptosis incident under low and high oxygen concentration from *IN VITRO* fertilization and parthenogenetic pig embryos (Ock et al., 2005).

In addition, the modify culture conditions for cloned pig embryos by supplementation of various growth factors such as platelet activating factor (Kidson et al., 2004), EGF (Lee et al., 2004) showed significant increased of blastocyst rate and total cell number in pig SCNT embryos. Moreover, drop and Well of the Well (WOW) (Vajta et al., 2000) including the drop culture technique are modified culture systems that seem to be beneficial to establish the pig SCNT embryos (Taka et al., 2005). Regarding the attempts to modify the culture system for *IN VITRO* system, the ideal way used for cloned embryos in future experiment and in the physical environment may also have profound effect to *IN VITRO* development.

PREGNANCY AND FARROWING

Although, the cloned pigs were established from *IN VITRO* production, the overall efficiency is still very low in pig compared to cattle, sheep and mouse. Failures of SCNT pregnancy are associated with placental abnormalities, such as placentomegaly, reduced vascularisation, hypoplasia of trophoblastic epithelium, and altered

basement membrane (Hill et al., 2000). Pregnancy has already been achieved with non-surgical transfer of *IN VITRO*-produced pig embryos (Suzuki et al., 2004). These may be due to intrinsic differences between pigs and these other species, or to differences in protocols for embryo production used. Furthermore, the larger litter size in pigs may also limit fetal growth to a greater extent than in cattle and sheep (Young et al., 1998). Especially that the number of pig embryos transferred to the recipient is very high than in another species (Koo et al., 2004). Mostly pig embryos were transferred at the one-cell; two-four-cell or four-eight-cell stages and 50-150 embryos were transferred to one recipient. In pigs, there is a need for a signal of three or more embryos to maintain pregnancy (King et al., 2002). In case, of SCNT, this signal is weak, there are reports on the use of enhance and maintain the signal of pregnancy (Polejaeva et al., 2000; Lai et al., 2002b) or in vitro embryos by mating of recipient (Onishi et al., 2000; Lai et al., 2002b; Pan et al., 2006) or by hormone injection after embryo transfer (Walker et al., 2002; Harrison et al., 2004; Pan et al., 2006) that were successfully used in producing cloned pigs.

APPLICATION OF PIG SOMATIC CELL NUCLEAR TRANSFER (SCNT)

The mainly purpose of pig SCNT is related on the biomedical applications to human and also for xenotransplantation to replace organs or tissues by using the pig as an animal models for human diseases to therapy study because pig has a relatively similar organ size, anatomy, and physiology to that of human. Furthermore, their genome is much closer to human compared with mouse. The gene expression pattern of pigs is more closely similar to those of a human (Forsberg, 2005). However, there is a need to improve and increase the efficiency of pig SCNT and develop cloned pig with genetic modification that the organs will not cause an immunological response and destroy transplanted tissue when transferred to human. In addition, initial development and validation of the lab procedures, the availability of reliable and efficient methods for producing viable pig cloned following nuclear transfer technique still needs to be accomplished.

ACKNOWLEDGEMENTS

The authors thank the Hungarian-South African Bilateral Agreement Project (NRF-DST, TET OMFB-00302/2008), ARC-NRF-RT21000 and EU FP6 (MEDRAT-LSHG-CT-2005-518240 and MRTN-CT-2006-035468) EU FP7 (AniStem, PIAP-GA-2011-286264) projects for supporting the work resulting in this review article.

REFERENCES

Abeydeera LR (2002). In vitro production of embryos in swine. Therio. 57: 257-273.

Abeydeera LR, Wang WH, Cantley TC, Rieke A, Murphy CN, Prather RS, Day BN (2000). Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. Therio. 54: 787-797.

Algriany O, Bevers M, Schoevers E, Colenbrander B, Dieleman S (2004). Follicle size-dependent effects of sow follicular fluid on *IN VITRO* cumulus expansion, nuclear maturation and blastocyst formation of sowcumulus oocytes complexes. Therio. 62: 1483-1497.

Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enow J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M (2000). Production of cloned pigs from *IN VITRO* systems. Nat. Biotechnol. 18, 1055-1059.

Bondioli KR, Ramsoondar JJ, Williams BL, Costa C, Fodor, WL (2001). Cloned pigs generated from cultured skin fibroblasts derived from a H-transferase transgenic boar. Mol. Reprod. Dev. 60: 189–195.

Booth PJ, Tan SJ, Holm P, Callesen H (2001). Application of the zona-free manipulation technique to porcine somatic nuclear transfer. Cloning Stem Cells 3: 191-197.

Boquest AC, Grupen CG, Harrison SJ, McIlfatrick SM, Ashman RJ, d'Apice AJ, Nottle, MB (2002). Production of cloned pigs from cultured fetal fibroblast cells. Biol. Reprod. 66: 1283-1287.

Chae JI, Cho SK, Seo JW, Yoon TS, Han YM, Yu K (2006). Proteomic analysis of the extraembryonic tissue from cloned porcine embryos. Mol. Cell Proteo. 5: 1559-1566.

Dai Y, Vaught TD, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL (2002). Targeted disruption of the α-1,3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol. 20: 251-255.

De la Fuente R, King WA (1998). Developmental consequences of karyokinesis without cytokinesis during the first mitotic cell cycle in bovine parthenotes. Biol. Reprod. 58: 952-962.

De Sousa PA, Dobrinsky JR, Zhu J, Archilbald AL, Ainslie A, Bosma W, Bowering J, Bracken J, Ferrier PM, Fletcher J, Gasperini B, Harkness L, Johnston P, Ritchie M, Ritchie WA, Travers A, Albertini D, Dinnyess A, King TJ, Wilmut, I (2002). Somati cell nuclear transfer in the pig: control of pronuclear formation and integration with improved method for activation and maintenance of pregnancy. Biol. Reprod. 66: 642-650.

Du Y, Kragh PM, Zhang X, Purup S, Yang, H, Bolund L, Vajta G (2005). *IN VITRO* combining partial zona digestion and oocyte trisection with sequential culture. Cloning Stem Cells 7: 199-205.

Forsberg EJ (2005). Genetically modified swine. In 'Proceedings of the Swine in Biomedical Research Conference', 27–29 January 2005, Chicago, IL. p. 40.

Funahashi H, Cantley TC, Day BN (1997). Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *IN VITRO* fertilization. Biol. Reprod. 57: 49-53.

Grupen CG, Nagashima H, Nottle, MB (1997). Role of epidermal growth factor and insulin-like growth factor-I on porcine oocyte maturation and embryonic development *IN VITRO*. Reprod. Fertil. Dev. 9: 571-575.

Hölker M, Petersen B, Hassel P, Kues WA, Lemme E, Lucas-Hahn A, *IN VITRO* Niemann H (2005). Duration of maturation of recipient affects blastocyst development of cloned porcine embryos. Cloning Stem Cells 7: 35-44.

Hoshino Y, Uchida M, Shimatsu Y, Miyake M, Nagao Y, Minami N, Yamada M, Imai H (2005). Developmental competence of somatic cell nuclear transfer embryos reconstructed from oocytes matured *IN VITRO* with follicle shells in miniature pig. Cloning Stem Cells 7: 17-26.

Hyun S, Lee G, Kim D, Kim H, Lee S, Nam D, Jeong Y, Kim S, Yeom S, Kang S, Han J, Lee B, Hwang, WS (2003b). Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. Biol. Reprod. 69: 1060-1068.

Hyun SH, Lee GS, Kim DY, Kim HS, Lee SH, Kim S, Lee ES, Lim JM, Kang SK, Lee BC, Hwang WS (2003a). Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos. Therio. 59: 1641-1649.

Im GS, Lai L, Liu Z, Hao Y, Wax, D, Bonk A, Prather RS (2004). *IN VITRO* development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Therio. 61: 1125-1135.

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: 2095-8.

Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, AkitaT, Nagai T (2002). Successful piglet production after transfer of blastocysts produced by a modified *IN VITRO* system. Biol. Reprod. 66: 1033-1041.

Kim SG, Dubovi EJ (2003). A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood and follicular fluid samples. Biologicals. 31: 103-106

King TJ, Dobrinsky JR, Zhu J, Finlayson HA, Bosma W, Harkness L, Ritchie WA, Travers A, McCorquodale C, Day BN, Dinnyes A, De Sousa A, Wilmut I (2002). Embryo development and establishment of pregnancy after embryo transfer in pigs: coping with limitations in the available of viable embryos. Reproduction 123: 507-515.

Kolber-Simonds D, Lai L, Watt SR, Denaro M, Arn S, Augenstein ML, Betthauser J, Carter DB, Greenstein JL, Hao Y, Im GS, Liu Z, Mell GD, Murphy CN, Park KW, Rieke A, Ryan DJJ, Sachs DH, Forsberg EK, Prather RS, Hawley R (2004). Production of α-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proc. Natl Acad. Sci. USA 101: 7335-7340.

Koo DB, Kang YK, Park JS, Park JK, Chang WK, Lee KK, Han YM (2004). A paucity of structural integrity in cloned porcine blastocysts produced *IN VITRO*. Therio. 62: 779-789.

Kragh PM, Vajta G, Corydon TJ, Purup S, Bolund L, Callesen H (2004). Production of transgenic porcine blastocysts by hand-made cloning. Reprod. Fertil. Dev. 16: 315–318.

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS (2002b). Production of α-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295: 1089-1092.

Lai L, Park KW, Cheong HT, Kühholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS (2002a). Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. Mol. Reprod. Dev. 62: 300-306.

Lee GS, Hyun SH, Kim HS, Kim DY, Lee SH, Lim JM, Lee E, Kang SK, Lee BC, Hwang, WS (2003a). Improvement of a porcine somatic cell nuclear transfer technique by optimizing donor cell and recipient oocyte preparations. Therio. 59: 1949-1957.

Lee GS, Kim HS, Hyun SH, Lee SH, Jeon HY, Nam DH, Jeong YW, Kim S, Kim JH, Han JY, Ahn C, Kang SK, Lee BC, Hwang WS (2005a). Production of transgenic cloned piglets from genetically transformed fetal fibroblasts selected by green fluorescent protein. Therio. 63: 973-991.

Lee JW, Wu SC, Tian XC, Barber M, Hoagland T, Riesen J, Lee KH, Tu CF, Cheng WT, Yang X (2003b). Production of cloned pigs by whole-cell intracytoplasmic microinjection. Biol. Reprod. 69: 995-1001.

Lee MS, Kang SK, Lee BC, Hwang WS (2005b). The beneficial effects of insulin and metformin on *IN VITRO* developmental potential of porcine oocytes and embryos. Biol. Reprod. 73, 1264-1268.

Lee SH, Kim DY, Nam DH, Hyun SH, Lee GS, Kim HS, Lee CK, Kang SK, Lee BC, Hwang WS (2004). Role of messengerRNAexpression of platelet activating factor and its receptor in porcine *IN VITRO*-fertilized and cloned embryo development. Biol. Reprod. 71: 919-925.

Li GP, Tan JH, Sun QY, Meng QG, Yue KZ, Sun XS, Li ZY, Wang HB, Xu LB (2000). Cloned piglets born after nuclear transplantation of embryonic blastomeres into porcine oocytes matured *IN VIVO*. Cloning 2: 45-52.

Macháty Z, Day BN, Prather RS (1998). Development of early porcine embryos *IN VITRO* and *IN VIVO*. Biol. Reprod. 59: 451-455.

Macháty Z, Prather RS (1998). Strategies for activating nuclear transfer oocytes. Reprod. Fertil. Dev. 10: 599–613.

Miyoshi K, Saeki K, 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: 175–184.

Morgan A, Jacob R (1994). Ionomycin enhances Ca2+ influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane. Biochem. J. 300: 665-672.

Nagashima H, Kashiwazaki N, Ashman RJ, Grupen C, Nottle, MB (1995). Cryopreservation of porcine embryos. Nature 374, 416.

Nurse P (1990). Universal control mechanism regulating onset of M-phase. Nature 344: 503-508.

Ock SA, Kim JG, Shi LY, Jin HF, Mohana Kumar B, Choe SY, Rho GJ (2005). Comparison of development and quality of porcine embryos cultured in different oxygen concentrations. Reprod.Fertil. Dev. 17: 223.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC (2000). Pig cloning by microinjection of fetal fibroblast nuclei. Science 289: 1188-1190.

Pan D, Zhang Y, Sun X, Zhang J, Li X, Li Y, Gu Z, Dai Y, Wu C, Li N (2006). Cloned pigs derived from somatic cell nuclear transfer *IN VITRO* 839-844.

Park KW, Cheong HT, Lai LX, Im GS, Kühholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS (2001). Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim. Biotechnol. 12: 173–181.

Park KW, Lai L, Cheong HT, Cabot R, Sun QY, Wu G, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather, RS (2002). Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. Biol. Reprod. 66: 1001-1005.

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 prduced by nuclear transfer from adult somatic cells. Nature. 407: 86-90.

Prather RS, Sims MM, First NL (1989). Nuclear transplantation in early pig embryos. Biol. Reprod. 41: 414-418.

Roh S, Hwang WS (2002). In vitro development of porcine parthenogenetic and cloned embryos: comparison of oocyte-activating techniques, various culture systems and nuclear transfer methods. Reprod. Fertil. Dev. 14: 93–99.

Sage D, Hassel P, Petersen B, Mysegades W, Westermann P, Lucas-Hahn A, Niemann H (2005). Improved in vitro development of porcine embryos produced by nuclear transfer, IVF and parthenogenesis. Reprod. Fertil. Dev. 17: 181.

Schoevers EJ, Kidson A, Verheijden JH, Bevers MM (2003). Effect of follicle-stimulating hormone on nuclear and cytoplasmic maturation of sow oocytes in vitro. Therio. 59: 2017-2028.

Shin MR, Park SW, Shim H, Kim NH (2002). Nuclear and microtubule reorganization in nuclear-transferred bovine embryos. Mol. Reprod. Dev. 62: 74-82.

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

Taka M, Iwayama H, Fukui Y (2005). Effect of the well of the well (WOW) system on in vitro culture for porcine embryos after intracytoplasmic sperm injection. J. Reprod. Dev. 51: 533-537.

Takeda K, Tasai M, Iwamoto M, Akita T, Tagami T, Nirasawa K, Hanada H, Onishi A (2006). Transmission of mitochondrial DNA in pigs and progeny derived from nuclear transfer of Meishan pig fibroblast cells. Mol. Reprod. Dev. 73: 306-312.

Tani T, Kato Y, Tsunoda, Y (2001). Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. Biol. Reprod. 64: 324-330.

Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trounson AO, Callesen H (2000). New method for culture of zona-included or zonafree embryos: the Well of the Well (WOW) system. Mol. Reprod. Dev. 55:

256-264.

Walker SC, Shin T, Zaunbrecher GM, Romano JE, Johnson GA, Bazer FW, Piedrahita, JA (2002). A highly efficient method for porcine cloning by nuclear transfer using in vitro-matured oocytes. Cloning Stem Cells, 4: 105-112.

Watanabe S, Iwamoto M, Suzuki S, Fuchimoto D, Honma D, Nagai T, Hashimoto M, Yazaki S, Sato M, Onishi A (2005). A novel method for the production of transgenic cloned pigs: electroporation-mediated gene transfer to non-cultured cells and subsequent selection with puromycin. Biol. Reprod. 72: 309-315.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell, KH (1997). Viable offspring derived from fetal and adult mammalian cells. Nature. 385: 810-3.

Wu D, Luo S, Wang Y, Zhuang L, Chen Y, Peng C (2001). Smads in human trophoblast cells: expression, regulation and role in TGFbeta-induced transcriptional activity. Mol. Cell Endocrinol. 175: 111-121.

Yin XJ, Cho SK, Park MR, Im YJ, Park JJ, Jong SB, Kwon DN, Jun SH, Kim NH, Kim JH (2003). Nuclear remodelling and the developmental potential of nuclear transferred porcine oocytes under delayed-activated conditions. Zygote 11: 167-174.

Yin XJ, Tani T, Yonemura I, Kawakami M, Miyamoto K, Hasegawa R, Kato Y, Tsunoda Y (2002). Production of cloned pigs from adult *SOMATIC* cells by chemically assisted removal of maternal chromosomes. Biol. Reprod. 67: 442-446.

Yoshioka K, Suzuki C, Onishi A (2008). Defined system for in vitro production of porcine embryos using a single basic medium. J. Reprod. Dev. 54: 3: 208-213.

Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002). Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol. Reprod. 66: 112-119.

Young LE, Sinclair KD, Wilmut I (1998). Large offspring syndrome in cattle and sheep. Rev. Reprod. 3: 155-163.