Recent Development in Embryo Technology in Pigs[a] - Review -

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ABSTRACT : Technologies on preimplantation porcine embryos have been developed quickly and significantly. Successful development of systems for culture of porcine zygotes to the blastocyst stage has made it possible to utilize follicular oocytes for *in vitro* production of embryos and thus stimulated research on various embryo technologies. Recent technological development of embryo cryopreservation, separation of X- and Y-bearing spermatozoa and non-surgical embryo transfer has also made it easy to utilize *in vivo*- and *in vitro*-produced embryos for artificial manipulation to produce clones and transgenic pigs. Further progress in overcoming various problems associated with each embryo technology will result in acceptable efficiency to utilize porcine embryos with a high or increased quality. Combining these technologies will accelerate further expansion of the swine industry not only for meat production but also for the production of therapeutic recombinant proteins and xonografts. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 6 : 966-975*)

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INTRODUCTION

The final goal of embryo technologies in animal production is efficient utilizations of embryos with excellent quality for various purposes. Due to a short period of generation, multiple piglets per litter and managemental characteristics, pigs are good species to manipulate in the field of biotechnological industries. Therefore, porcine embryos should be good materials to introduce a new genetic inheritance into individuals or herds and to deal in international trade. So far, however, effective techniques to recover and transfer porcine embryos have been limited to surgical procedures, which have been developed in the 1960's (Hancock and Hovell, 1962; Day, 1979), due to the anatomical characteristics of sow's cervix and uterus. Furthermore, progress in porcine embryo technologies had been restricted within narrow limits because of delayed development of techniques for producing normal embryos *in vitro*, culturing embryos from 1- and 2-cell stages and freezing embryos. However, recent development of such techniques has freed and accelerated the progress to utilize porcine embryos for practical use and for various research purposes. This review summarizes recent advancements of porcine embryo technologies and discusses the possible future researches.

PRODUCTION OF EMBRYOS *IN VITRO*

Growth of oocytes *in vitro*

In a porcine ovary, relatively larger number of primordial follicles exits as compared with other species such as humans, cattle and mice (Gosden and Telfer, 1987). It seems to be quite difficult to support growth of porcine oocytes *in vitro*. However, the research on this subject has recently become more active and physiological requirements of growing oocytes are being characterized (Christmann et al., 1994; Lazzari et al., 1994; Petr et al., 1994a, b; Price et al., 1995). Oocytes derived from preantral follicles can grow, mature and fertilize in vitro (Hirao et al., 1994) but the success rate is still low. The developmental ability of this type of oocytes following *in vitro* fertilization (IVF) has not been clarified. Further research on utilization of such oocytes in porcine embryo technologies will be important to reduce the cost and effort to produce large number of embryos.

Maturation of oocytes *in vitro*

Fully grown oocytes with uniform ooplasm and surrounded by a compact cumulus cell mass, which were collected from antral follicles of slaughtered prepubertal gilts, have been generally used for *in vitro* maturation (IVM). Although a high incidence of nuclear maturation of such oocytes is achieved *in vitro* in the presence of gonadotropins, an abnormally low incidence of male pronuclear formation after IVF has been routinely observed (see Niwa, 1993; Mattioli, 1994; Nagai, 1994; Funahashi and Day, 1996). However, this problem has been currently overcome by modifications of conditions for IVM (see Day and Funahashi, 1996; Funahashi and Day, 1997). The incidence of male pronuclear formation and glutathione content in porcine oocytes increase when thiols such as cysteine (Yoshida et al., 1993a), cysteamine (Grupen et al., 1995) and beta-mercaptoethanol (Funahashi and Day, 1997) are added to maturation

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media. The presence of cysteine in maturation medium is critical only between 42 and 48 h of culture both for glutathione synthesis and male pronuclear formation after sperm penetration in vitro (Sawai et al., 1997). Thus, the oxidative stress, especially during late stages of oocyte maturation may be involved in the failure of male pronuclear formation. On the other hand, the detrimental effects of an elevated NaCl concentration in maturation medium on histone H1 kinase activity (Funahashi et al., 1994c), microfilament organization (Funahashi et al., 1996) and glutathione content of oocytes have been demonstrated. Male pronuclear formation (Funahashi et al., 1994b) and subsequent embryonic development after IVF (Funahashi et al., 1994a) are also affected by the high NaCl concentration. The presence of organic osmolytes, such as taurine and sorbitol, in maturation media relieves the detrimental effects of a high concentration of NaCl (Funahashi et al., 1996).

Fertilization of oocyte *in vitro*

There are many reports describing successful IVF of porcine oocytes matured *in vivo* or *in vitro*. In most laboratories, freshly ejaculated spermatozoa are mainly used for porcine IVF. For repeated use of spermatozoa with the same characteristics for IVF, techniques for IVF by frozen-thawed (Nagai et al., 1988) and ejaculated (Wang et al., 1991; Abeydeera and Day, 1997) spermatozoa have developed.

Although, successful sperm penetration has been achieved, a high incidence of polyspermic penetration is one of the persistent problems in porcine IVF (see Niwa, 1993; Day and Funahashi, 1996; Nagai, 1996; Funahashi and Day, 1997). In most IVF systems, a relatively high number of spermatozoa is added to fertilization medium to make sure of inducing sperm capacitation. However, an increased number of spermatozoa per oocyte is known to be associated with a high incidence of polyspermic penetration (Rath, 1992; Coy et al., 1993). This may be due to gradual induction of sperm capacitation and acrosome reaction. Since the rate of sperm capacitation and acrosome reaction appears to be affected by fertilization media (Mattioli et al., 1996), the media in which capacitation could be induced in most spermatozoa at the same time would reduce the inidence of polyspermy. Limited success in reducing the incidence of polyspermic penetration has been reported by using porcine oviductal secretions (Nagai and Moor, 1990; Kano et al., 1994; Kim et al., 1996) or porcine follicular fluid (Funahashi and Day, 1993). On the other hand, sperm penetration is not blocked even after electrical activation of oocytes matured *in vitro* (Funahashi et al., 1993, 1995) by which cortical granules are known to be released (Sun et al., 1992). Therefore, the timing of cortical granule reaction (Kim et al., 1996; Wang et al., 1997) may be an important factor for preventing polyspermy. Another possible means to reduce the incidence of polyspermic penetration in porcine IVF may be to control the number of capacitated spermatozoa reaching to the oocytes.

DEVELOPMENT OF EMBRYOS *IN VITRO*

Culture systems

One- and two-cell porcine embryos had been known to fail in the development *in vitro* beyond the 4-cell stage (see Davis, 1985; Reed et al., 1992; Petters and Wells, 1993). This developmental block has been overcome by culturing embryos in sheep or mouse oviducts (Krisher et al., 1989; Prather, 1991), in the amniotic fluid of developing chick embryos (Ocampo et al., 1994), in medium supplemented with oviductal fluid (Archibong et al., 1989), in conditioned media with homologous porcine uterine cells (Poulin et al., 1997) and in co-culture systems with oviductal epithelial cells (White et al., 1989; Choi et al., 1995) or porcine cumulus or trophoblastic cells (Nagai and Takahashi, 1992). In a simple culture medium (modified Whitten's medium), when 1- and 2-cell embryos are cultured in the atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39℃, more than 80% of them have developed to the morula or blastocyst stage, and after transfer of embryos developed to the 4- to 8-cell stage to the recipients, normal piglets were obtained (Beckmann and Day, 1993). Since Whitten's medium (Whitten and Biggers, 1968) contains relatively low concentration of NaCl (68.49 mM), an increased NaCl concentration likely impairs the development of porcine embryos at the 4-cell stage. This has been confirmed by Galvin et al. (1993) who found that higher proportion of 1- and 2-cell embryos developed to the morula and blastocyst stages in Whitten's medium containing 68 mM (53.4%) than 100 mM (0%) NaCl in 5% CO_2 in air at 39°C. However, addition of 2 mM glutamine to the medium improved the early development of embryos in the presence of 100 mM, but not of 63 mM NaCl. The early development of embryos has also been supported in a simple medium supplemented with 5.55 mM glucose and 1 mM glutamine in the atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37℃ (Petters et al., 1990). Modification of this medium adding 7 mM taurine and 5 mM hypotaurine, named NCSU-23, produced very high proportion (93%) of morulae and blastocysts even in the atmosphere of 5% CO_2 in air at 37℃ (Petters and Reed, 1991). It is known that each of glutamine, taurine and hypotaurine works as an organic osmolyte. Beneficial effects of organic osmolytes likely rescue porcine embryos from the detrimental effect of a high NaCl concentration.

Furthermore, successful *in vitro* development of early porcine embryos has also been reported using modified Tyrode's solution supplemented with 10 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) in air at 39℃ (Hagen et al., 1991). Although it is not clarified why this medium supports the development, this finding may be important for commercial application of porcine embryo technologies because their culture system does not require supplementation of CO_2 during culture. However, higher developmental capacity of 1- and 2-cell embryos from Sinclair miniature pigs is observed in Whitten's medium in 5% CO_2 in air than in HEPES bufferred modified Tyrode's solution in air (Prather et al., 1995).

Development to hatching blastocysts

Even in various successful culture systems, the incidence of porcine embryos to develop to the peri-hatching blastocyst stage appears to be very low (Dobrinsky et al., 1996). An increased incidence of hatching has been demonstrated when embryos were co-cultured with porcine endometrial monolayer (Allen and Wright, 1984) or bovine uterine fibroblast (Kuzan and Wright, 1982) and when they were cultured in conditioned media with homologous porcine uterine cells (Poulin et al., 1997). Recently, the development of porcine zygotes to the blastocyst stage in a defined culture medium, Beltsville Embryo Culture Medium (BECM-3), has been reported by Dobrinsky et al. (1996). They demonstrated that both the mean cell number of blastocysts and the incidence of hatched blastocysts are increased when the zygotes were cultured in BECM-3-based media in the absence of BSA fraction V; Furthmore and that addition of fetal bovine serum to the medium by late Day 5 (at the late morula and blastocyst stages) increased the incidence (80%) of 2-cell embryos to develop to the hatched blastocyst stage. It has also been demonstrated that the incidence of hatching blastocysts is improved using BSA-free NCSU-23 medium which was supplemented with 20% fetal bovine serum when embryos were at the early morula stage, although supplementation of NCSU-23 with amino acids and insulin from 4 days after the start of culture enhanced only the development of embryos to the blastocyst stage (Koo et al., 1997a). Therefore, the presence of BSA in culture media beyond the morula stage seems to be detrimental for hatching of embryos and development of inner cell mass, but fetal bovine serum is stimulative.

Acquisition of developmental competence of IVM/IVF embryos

Although piglets from IVM/IVF oocytes have been produced after transfer them into the recipients (Mattioli et al., 1989; Yoshida et al., 1993b), the developmental competence of oocytes matured *in vitro* as determined by *in vitro* development to the blastocyst stage and the number of cells in a blastocyst after IVF is lower compared with those matured *in vivo* (Nagashima et al., 1996a). Culture conditions used for IVM of porcine oocytes affect their subsequent response to oocyte activation (Yamauchi et al., 1996). The ability of IVM/IVF porcine embryos to develop to the blastocyst stage *in vitro* has been improved by modification of maturation medium as reducing the concentration of NaCl (Funahashi et al., 1994a) or supplementing with cysteamine (Grupen et al., 1995) or organic osmolytes (Funahashi et al., 1996). Modification of various conditions between oocyte collection and germinal vesicle breakdown also improve embryonic development (Funahashi et al., 1997a, b). Reducing morphological variation in the germinal vesicles appears to enhance the developmental competence of porcine oocytes (Funahashi et al., 1997a). Exposure of oocyte-cumulus complexes to dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) for the first 20 h of culture for maturation does not affect the maturation of oocytes during a 44 h culture period and sperm penetration after IVM, but it does increase the homogeneity of oocyte nuclear maturation (Funahashi et al., 1997b). This treatment also improves embryonic development *in vitro* and, after surgical transfer of 2- and 4-cell embryos to the recipients, piglets have been produced with a high pregnancy rate and acceptable litter size (Funahashi et al., 1997b). The developmental competence of oocytes seems to be improved also by modification of IVM conditions after the germinal vesicle stage. The presence of a tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) from 20 to 44 h of culture for IVM enhances the competence of the oocytes to develop to the blastocyst stage without affecting factors associated with fertilization (Funahashi et al., 1997c). A culture system with treating oocytes with dbcAMP during the first 20 h of IVM and then with TIMP-1 from 20 to 44 h of IVM improves the ability of embryos to develop to the blastocyst stage to a more acceptable level (34%) (Funahashi et al., 1997c). A recent study has also demonstrated that co-culture of porcine oocyte-cumulus complexes with follicular shell pieces improved early embryonic development to the blastocyst stage after IVF (Abeydeera et al., 1996). In this IVM system, piglets were produced at a relatively high level of efficiency following surgical transfer of 2- to 4-cell embryos derived from IVM/IVF. The ability of IVM/IVF porcine embryos to develop to the blastocyst stage in simple media has also been improved.

TRANSFER OF EMBRYO

Successful embryo transfer techniques are expected

to reduce not only the high cost of shipping but also the risk of disease transmission, which are always involved in the case of transportation of animals or herds. Although embryo transfer in pigs has been accomplished with a high pregnancy rate (Polge, 1982), it is very restricted in surgical procedures developed more than three decades ago (Hancock and Hovell, 1962; Day, 1979). Practically, it is possible to produce piglets by embryo transfer following the international transportation of embryos (Niemann, 1989). However, the cost for surgical collection and transplantation of porcine embryos is still high for commercial application. Non-surgical embryo transfer has been continuatively conducted since a limited success at three decades ago (Polge and Day, 1968). Recently, successful production of piglets following non-surgical embryo transfer has been reported (Reichenbach et al., 1993; Galvin et al., 1994). To introduce embryos into the reproductive tract through cervix, Reichenbach et al. (1993) have used a disposable plastic spiral catheter, which is routinely used for artificial insemination, and a bovine embryo transfer cannula. Galvin et al. (1994) have used a disposable insemination spirette with an attached 3-way stopcock and embryos in 10-12 ml of culture medium have been expelled into the uterus: however, the pregnancy rates was low and litter size was also small compared with those obtained in surgical transfer. More recently, a set of instruments for non-surgical transfer of porcine embryo has been developed to improve both the farrowing rate and the average litter size (Li et al., 1996). The development of these non-surgical techniques for porcine embryo transfer will result in the effective utilization of another embryo technologies for practical use and scientific research.

As a substitute for IVF and *in vitro* culture of embryos, successful techniques for gamete intrafallopian transfer have been reported in pigs by Rath et al. (1994) who transferred *in vivo* matured denuded oocytes into the ampulla of the oviduct together with preincubated spermatozoa and observed relatively high rate (64%) of embryonic development to the morula/blastocyst stages and obtained piglets.

CRYOPRESERVATION OF EMBRYOS

Storage techniques of porcine embryos are essential not only for transporting them around the world but also for holding them until embryo transfer to a suitable recipient gilt. However, porcine embryos are known to be very sensitive to the temperatures below 15℃ (Polge, 1982). Subsequently, however, it was reported that the embryos at the expanded and hatched stages are more resistant to chilling (Nagashima et al., 1988a). Then, the research on cryopreservation of porcine embryos has rapidly progressed (see Nagashima et al., 1994a). Generally, porcine peri-hatching blastocysts are successfully frozen in a phosphate buffered saline (PBS) supplemented with 1.5 M glycerol and 15-50% fetal bovine serum (see Nagashima et al., 1994a). The birth of piglets from frozen-thawed peri-hatching blastocysts has been reported by several researchers (Hayashi et al., 1989; Kashiwazaki et al., 1991; Fujino et al., 1993). The freezability of porcine embryos seems to attain the summit immediately after hatching, because hatched blastocysts with a diameter of more than 300 μm can not survive after freezing (Nagashima et al., 1992a). Recently, it has been clearly demonstrated that piglets can be produced from 2- to 4-cell embryos frozen-thawed after removing the cytoplasmic lipid, showing that the sensitivity of porcine embryos to low temperatures is related to their cytoplasmic lipid content (Nagashima et al., 1994b, 1995).

Vitrification of porcine embryos has also been attempted. Expanded to hatched blastocysts can survive after cryopreservation in vitrification solution at -196℃ (Yoshino et al., 1993; Dobrinsky and Johnson, 1994). The embryos at the 2- to 4-cell stages are also successfully cryopreserved by vitrification after removal of cytoplasmic lipid (Nagashima et al., 1996b). In these techniques, however, zona pellucida which is the barrier to pathogens is removed after thawing in expanded to hatched blastocysts or partially broken before freezing in 2- to 4-cell stage embryos for removing cytoplasmic lipid. This may introduce various pathogens into the animals. Further improvement in the techniques for freezing of porcine embryos with intact zona pellucida should be needed for commercial use.

SEXING

Separation of X- and Y-bearing spermatozoa

Using flow cytometry, a method for sorting X- and Y-bearing mammalian sperm cells has been developed (Johnson, 1995; Johnson et al., 1996). It has been reported that the sorted boar spermatozoa can penetrate oocytes and viable embryos can be obtained after the gamete intrafallopian transfer (Rath et al., 1994). Recently, production of piglets preselected for sex following IVF with X-bearing spermatozoa sorted by flow cytometry has been reported by Rath et al. (1997). In their study, purity of sorting was 92% and 83% for X- and Y-bearing spermatozoa, respectively. After transfer of 92 IVF embryos produced from X-sorted spermatozoa into 2 recipients, 6 and 4 female piglets were delivered from each of the recipients. Additionally, when 2 gilts were intratubally inseminated with either X- or Y-sorted spermatozoa (2×10^5 cells per oviduct), 13 (85%) of 15 piglets produced were of the predicted gender. Further application of

the sorting method for separating X- and Y-bearing boar spermatozoa is being expected for the production of sexed embryos.

Sexing of embryos

Sex determination of porcine embryos has been done by chromosomal analysis of bisected trophectoderm cells from expanding blastocysts (Kato et al., 1987). The sex of porcine embryos has also been determined by using polymerase chain reaction (PCR; Pomp et al., 1995). Biopsied porcine 4-cell embryos are known to develop to the term after transfer of the embryos at the compacted morula to hatched blastocyst stages (Nakayama et al., 1997). Biopsied blastomeres of porcine embryos could be a good materials for chromosomal analysis or PCR analysis for sex determination. Although sexing of embryos would be of great advantage to various scientific researches, its commercial application to produce piglets of a preselected sex will require lowered cost as well as improved embryo transfer technology.

CLONING

It is possible to obtain piglets after transfer of bisected porcine embryos (Willadsen, 1982; Rorie et al., 1985). However, possibly due to less robust of porcine embryos, there are limited successful reports about production of identical twin piglets after transfer of bisected embryos (Polge, 1985; Nagashima et al., 1988b; Ash et al., 1989; Niemann and Reichelt, 1993). Recently, *in vitro* development of a blastomere derived from porcine embryos at the 4-cell and 8-cell stages to the blastocyst stage has been demonstrated (Niemann and Reichelt, 1993; Eckert et al., 1997).

Only one piglet has been produced after surgical transfer of reconstituted embryos which were produced by fusing a blastomere obtained from 4-cell embryo with an enucleated, activated meiotic metaphase II oocyte (Prather et al., 1989). Although a number of papers has discussed on the basic factors influencing the success of nuclear transplantation in pigs (Prather et al., 1992; Niemann and Reichelt, 1993; Parry and Prather, 1995), the developmental ability of reconstituted embryos to the blastocyst stage has been very low (Nagashima et al., 1992b). The reason for the low developmental ability of the reconstituted porcine embryos is still unknown. However, it has been reported that porcine oocytes which were matured *in vitro* and parthenogenetical activated have poor ability of development even in the culture conditions which can support early embryonic development (Prather et al., 1991; Funahashi et al., 1994a). Improved parthenogenic development of IVM porcine oocytes to the blastocyst stage has recently been reported *in vivo* and *in vitro* by the modifications of IVM systems and methods for artificial oocyte activation (Jolliff and Prather, 1997; Machaty et al., 1997). Recent progress in culture techniques for IVM and early embryonic development and in techniques for oocyte activation will contribute to the improvement of the developmental ability of porcine embryos reconstituted by nuclear transplantation.

TRANSGENIC PIGS

The first transgenic pigs which express human growth hormone transgenes have been reported more than a decade ago (Hammer et al., 1985). Recent progress in gene transfer in pigs has been reviewed elsewhere (Pursel et al., 1990, 1996; Niemann and Reichelt, 1993; Martin and Pinkert, 1994). To produce transgenic pigs, several hundred copies of DNA have been directly microinjected into the pronuclei of zygotes after visualization of the pronuclei by centrifugation. Nowadays, it is possible to utilize porcine zygotes produced *in vitro* for the study on the transfer of foreign DNA (Kubisch et al., 1995; Koo et al., 1997b). Further efforts to combine the recombiant DNA technologies with nuclear transplantation will be required to achieve a rapid progress to produce transgenic pigs. Furthermore, measures for public acceptance about the practical application of recombinant DNA technology and its safety are also required.

Improvement of production traits

Transgenic pigs bearing genes aimed at improving production traits have been produced for growth hormone, cSKI and insulin like growth factor-I (Pursel et al., 1996). A number of those recombinant DNA has contained the regulatory region of the metallothionein gene as a promoter and enhancer (Hammer et al., 1985; Vize et al., 1988; Pursel et al., 1989) since an advantage of utilizing the metallothionein promoter is that the level of gene expression can be increased by supplementation of food or water with zinc (Palmiter et al., 1982). Marketing of transgenic pigs with elevated growth hormone would offer considerable potential value to both the producer and the consumer because those transgenic pigs gain weight more than 10% faster with higher feed efficiency and have much reduced carcass fat as compared to sibling controls (Pursel et al., 1996).

Utilization to xenotransplantation

Because of anatomical, physiological and immunological similarities of pigs to humans, recent progress in understanding the immunologic barriers to xenotransplantation (Lin and Platt, 1996) and a chronic lack of human tissues for transplantation, pigs are

being considered all the more as an inportant source for human organ replacement. A major barrier of human body to the transplantation of pig organ is the hyperacute rejection caused by pre-existing antibodies and complement. Development of transgenic pigs that do not express the alphaGal epitope and/or express a human complement inhibiting protein, such as decay-accelerating factor is expected to inhibit the hyperacute rejection by complement activation and consequently delay xonograft rejection (Cooper, 1996). The production of transgenic pigs expressing human decay-accelerating factor (Rosengard et al., 1995) and human complement-regulatory proteins, such as human CD59 (Byrne et al., 1997) and, as potential donors for clinical organ xenotransplantation has been reported. Recently, alpha1,2-fucosyltransferase transgenic pigs, which should have a reduced expression of Gal alpha(1,3)Gal, have been suggested as a good source of xenotransplantation for human (Cohney et al., 1997).

Establishment of animal factory to produce therapeutic proteins

Transgenic pigs expressing recombinant human protein C in milk have also been produced (Morcol et al., 1994; Subramanian et al., 1996; VanCott et al., 1997). Establishment of a herd of transgenic pigs producing such therapeutic recombinant proteins would offer distinct advantages for treating genetic or other disease. At present, however, the efficiency of gene-injected embryos that develop to transgenic pigs seems to be very low (0.1 to 4.0%) due to a definitive problem, mosaicism (Pursel et al., 1990, 1996).

Establishment of embryonic stem cell lines and gene-targetting

Another method for manipulating genes of porcine embryos is to make chimera by microinjection of embryonic stem (ES) cells manipulated genetically. Using this technology, it would be possible to produce 'knock-out' pigs. Rapid progress is being made in attempts toward maintaining porcine EC cell pluripotency (see Wheeler, 1994). Although micro-injection of inner cell mass (ICM) cells of porcine embryos into blastocoels has resulted in chimerism in female germ line which was assessed by a progeny test (Onishi et al., 1994), definitive ES cell lines so far generated seem to be very difficult to contribute to the germ line of chimeric pigs. Recently, the achievement of chimerism and transgenesis in domestic pigs at a fetal stage by using an ES cell line has been reported (Notarianni et al., 1997). The recent experiments in which ICM cells were introduced into tetraploid 4-cell pig embryos suggest that it may be possible to create a fetus derived from ICM cells or potentially stem cells (Prather et al., 1996). More efforts are required to improve the efficiency to produce transgenic chimeric pigs.

REFERENCES

Abeydeera, L. R. and B. N. Day. 1997. Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified Tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol. Reprod. 57: 729-734.

Abeydeera, L. R., H. Funahashi, T. C. Cantley, A. Rieke and B. N. Day. 1996. Co-culture with follicular shell pieces (FSP) increases the developmental competence of pig oocytes matured *in vitro*. Biol. Reprod. 54(Suppl. 1): 81 (abstr.).

Allen, R. L and R. W. J. Wright. 1984. *In vitro* development of porcine embryos in coculture with endometrial cell monolayer of culture supernatants. J. Anim. Sci. 59:1657-1661.

Archibong, A. E., R. M. Petters and B. H. Johnson. 1989. Development of porcine embryos from one- and two-cell stage to blastocysts in culture medium supplemented with porcine oviductal fluid. Biol. Reprod. 41:1076-1083.

Ash, K, G. B. Anderson, R. H. BonDurant, R. L. Pashen, K. M. Parker and T. Berger. 1989. Competition between split and non-manipulated embryos in the production of identical piglets. Theriogenology. 31:903-910.

Beckmann, L. S. and B. N. Day. 1993. Effect of media NaCl concentration and osmolarity on culture of the early stage porcine embryo and viability of embryos cultured in a selected superior medium. Theriogenology. 39:611-622.

Byrne, G. W., K. R. Mccurry, M. J. Martin, S. M. Mcclellan, J. L. Platt and J. S. Logan. 1997. Transgenic pigs expressing human CD59 and decay-accelerating factor produce an intrinsic barrier to complement-mediated damage. Transplantation. 63:149-155.

Choi, Y. H., S. Saito and N. Oguri 1995. *In vitro* development of porcine oocytes fertilized *in vitro* with spermatozoa preincubated in two different media. Theriogenology. 44:287-294.

Christmann, L, T. Jung and R. M. Moor. 1994. MPF components and meiotic competence in growing pig oocytes. Mol. Reprod. Dev. 38:85-90.

Cohney, S., I. F. McKenzie, Patton K, Prenzoska J, Ostenreid K, W. L. Fodor and M. S. Sandrin. 1997. Down-regulation of Gal alpha(1,3)Gal expression by alpha1,2-fucosyltransferase: further characterization of alpha 1,2-fucosyltransferase transgenic mice. Transplantation. 64: 495-500.

Cooper, D. K. C. 1996. Xenotransplantation - State of the art. Frontiers Biosci. 1:D248-265.

Coy, P, E. Martinez, S. Ruiz, J. M. Vazquez, J. Roca and J. Gadea. 1993. Environment and medium volume influence *in vitro* fertilization of pig oocytes. Zygote. 1: 209-213.

Davis, D. 1985. Culture and strage of pig embryos. J. Reprod. Fertil., Suppl. 38:115-124.

Day, B. N. 1979. Embryo transfer in swine. Theriogenology. 11:27-31.

Day, B. N. and H. Funahashi. 1996. *In vitro* maturation and

fertilization of pig oocytes. In: Beltsville Symposia in Agricultural Research, XX. Biotechnology's Role in the Genetic Improvement of Farm Animals. Ed. Miller, R. H, Pursel, V. G and Norman, H. D. American Society of Animal Science, Savoy, IL, USA. pp. 125-144.

Dobrinsky, J. R. and L. A. Johnson. 1994. Cryopreservation of porcine embryos by vitrification: a study of *in vitro* development. Theriogenology. 42:25-35.

Dobrinsky, J. R., L. A. Johnson and D. Rath. 1996. Development of a culture medium (BECM-3) for porcine embryos: effects of bovine serum albumin and fetal bovine serum on embryo development. Biol. Reprod. 55: 1069-1074.

Eckert, J., T. Tao and H. Niemann. 1997. Ratio of inner cell mass and trophoblastic cells in blastocysts derived from porcine 4- and 8-cell embryos and isolated blastomeres cultured *in vitro* in the presence or absence of protein and human leukemia inhibitory factor. Biol. Reprod. 57:552-560.

Fujino, Y. Y. Ujisato, K. Endo, T. Tomizuka, T. Kojima and N. Oguri. 1993. Cryoprotective effect of egg york in cryopreservation of porcine embryos. Cryobiology. 30: 299-305.

Funahashi, H. and B. N. Day. 1993. Effects of follicular fluid at fertilization *in vitro* on sperm penetration in pig oocytes. J. Reprod. Fertil. 99:97-103.

Funahashi, H. and B. N. Day. 1996. Current status of *in vitro* production of porcine embryos. In: Advances in Swine in Biomedical Research, Ed. Tombleson, M and Schook, L. Plenum Press, New York. pp. 491-502.

Funahashi, H. and B. N. Day. 1997. Advances in *in vitro* production of porcine embryos. J. Reprod. Fertil., Suppl. 52:271-283.

Funahashi, H., T. C. Cantley and B. N. Day. 1997a. Preincubation of oocyte-cumulus complexes before exposure to gonadotropins improves the developmental ability of porcine embryos matured and fertilized *in vitro*. Theriogenology. 47:679-686.

Funahashi, H., T. C. Cantley and B. N. Day. 1997b. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic AMP improves developmental competence following *in vitro* fertilization. Biol. Reprod. 57:49-53.

Funahashi, H., T. C. Cantley, T. T. Stumpf, S. L. Terlouw and B. N. Day. 1994a. *In vitro* development of *in vitro* matured porcine oocytes following chemical activation or *in vitro* fertilization. Biol. Reprod. 50:1072-1077.

Funahashi, H., E. W. McIntush, M. F. Smith and B. N. Day. 1997c. Effect of tissue inhibitor of metalloproteinase (TIMP-1) on early development of swine oocytes matured and fertilized *in vitro*. Theriogenology. 47:277 (Abstr.).

Funahashi, H., T. T. Stumpf, N. H. Kim and B. N. Day. 1994c. Effects of sodium chloride concentration in maturation media on histone H1 kinase (H1K) activity and cytoplasmic maturation of porcine oocytes. J. Reprod. Fertil., Abstr. No. 13:38(Abstr.).

Funahashi, H., T. T. Stumpf, S. L. Terlouw and B. N. Day. 1993. Effects of electrical stimulation before or after *in vitro* fertilization on sperm penetration and pronuclear formation in pig oocytes. Mol. Reprod. Dev. 36:361-367.

Funahashi, H., T. C. Cantley, T. T. Stumpf, S. L. Terlouw and B. N. Day. 1994b. Use of low salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after *in vitro* fertilization. Biol. Reprod. 51:633-639.

Funahashi, H., N. H. Kim, T. T. Stumpf, T. C. Cantley and B. N. Day. 1996. Presence of organic osmolytes in maturation medium enhances cytoplasmic maturation of porcine oocytes. Biol. Reprod. 54:1412-1219.

Funahashi, H., T. T. Stumpf, T. C. Cantley, N. H. Kim and B. N. Day. 1995. Pronuclear formation and intracellular glutathione content of *in vitro*-matured porcine oocytes following *in vitro* fertilization and/or electrical activation. Zygote. 3:273-281.

Galvin, J. M., D. B. Killian and A. N. V. Stewart. 1994. A procedure for successful nonsurgical embryo transfer in swine. Theriogenology. 41:1279-1289.

Galvin, J. M., A. N. V. Stewart and S. Meredith. 1993. Higher sodium chloride concentration can induce a four-cell block in porcine embryos. Theriogenology. 39: 224(Abstr.).

Gosden, R. G. and E. Telfer. 1987. Number of follicles and oocytes in mammalian ovaries and their allometric relationships. J. Zool. 211:169-175.

Grupen, C. G., H. Nagashima and M. B. Nottle. 1995. Cysteamine enhances *in vitro* development of porcine oocytes matured and fertilized *in vitro*. Biol. Reprod. 53: 173-178.

Hagen, D. R., R. S. Prather, M. M. Sims and N. L. First 1991. Development of one-cell porcine embryos to the blastocyst stage in simple media. J. Anim. Sci. 69: 1147-1150.

Hammer, R. E., V. G. Pursel, C. E. Rexroad, R. J. Wall, D. J. Bolt, K. M. Ebert, R. D. Palmiter and R. L. Brinster. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature. 315:680-685.

Hancock, J. L. and G. J. R. Hovell. 1962. Egg transfer in the sow. J. Reprod. Fertil. 4:195-201.

Hayashi, S., K. Kobayashi, J. Mizuno, K. Saitoh and S. Hirano. 1989. Birth of piglets from frozen embryos. Vet. Rec. 125:43-44.

Hirao, Y., T. Nagai, M. Kubo, T. Miyano, M. Miyake and S. Kato. 1994. *In vitro* growth and maturation of pig oocytes. J. Reprod. Fertil. 100:333-339.

Johnson, L. A. 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. Reprod. Fertil. Dev. 7: 893-903.

Johnson, L. A., D. G. Cran, G. R. Welch and C. Polge. 1996. Gender preselection in mammals. In: Beltsville Symposia in Agricultural Research, XX. Biotechnology's Role in the Genetic Improvement of Farm Animals, Ed. Miller, R. H., Pursel, V. G. and Norman, H. D. pp. 151-164, American Society of Animal Science, Savoy, IL, USA.

Jolliff, W. J. and R. S. Prather. 1997. Parthenogenic development of *in vitro*-matured, in vivo-cultured porcine oocytes beyond blastocyst. Biol. Reprod. 56:544-548.

Kano, K., T. Miyano and S. Kato. 1994. Effect of oviductal epithelial cells on fertilization of pig oocytes *in vitro*. Theriogenology. 42:1061-1068.

Kashiwazaki, N., S. Ohtani, H. Nagashima, H. Yamakawa,

W. T. K. Cheng, A. T. Lin, RC. S. Ma and S. Ogawa. 1991. Production of normal piglets from hatched blastocysts frozen at -196 C. Theriogenology. 35:221 (abstr.).

Kato, Y., H. Nagashima, K. Shibata, M. Ishiwata and S. Ogawa. 1987. Microsurgical bisection of expanding blastocysts for the sexing of pig embryos. Jap. J. Anim. Reprod. 33:151-154.

Kim, N. H., H. Funahashi, L. R. Abeydeera, S. J. Moon, R. S. Prather and B. N. Day. 1996. Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during *in vitro* fertilization of porcine oocytes. J. Reprod. Fertil. 107:79-86.

Koo, D. B., N. H. Kim, H. T. Lee and K. S. Chung. 1997a. Effects of fetal calf serum, amino acids, vitamins and insulin on blastocoel formation and hatching of *in vivo* and IVM/IVF-derived porcine embryos developing *in vitro*. Theriogenology. 48:791-802.

Koo, D. B., N. H. Kim, J. G. Lim, S. M. Lee, H. T. Lee and K. S. Chung. 1997b. Comparison of *in vitro* development and gene expression of *in vivo*- and *in vitro*-derived porcine embryos after microinjection of foreign DNA. Theriogenology. 48:329-340.

Krisher, R. L., R. M. Petters, H. H. Johnson, B. D. Bavister and A. B. Archibong. 1989. Development of porcine embryos from the one-cell stage to blastocyst in mouse oviducts maintained in organ culture. J. Exp. Zool. 249: 235-239.

Kubisch, H. M., M. A. Larson, H. Funahashi, B. N. Day and R. M. Roberts. 1995. Pronuclear visibility, development and transgene expression in IVM/IVF-derived porcine embryos. Theriogenology. 44:391-401.

Kuzan, F. B. and R. W. J. Wright. 1982. Blastocyst expansion, hatching, and attachment of porcine embryos cocultured with bovine fibroblasts *in vitro*. Anim. Reprod. Sci. 5:57-63.

Lazzari, G., C. Galli and R. M. Moor. 1994. Functional changes in the somatic and germinal compartments during follicle growth in pigs. Anim. Reprod. Sci. 35: 119-130.

Li, J., A. Reike, B. N. Day and R. S. Prather. 1996. Technical note: Porcine non-surgical embryo transfer. J. Anim. Sci. 74:2263-2268.

Lin, S. S. and J. L. Platt. 1996. Immunologic advances towards clinical xenotransplantation. In: Advances in Swine in Biomedical Research, Ed. Tombleson, M and Schook, L, pp 147-162, Plenum Press, New York.

Machaty, Z., W. H. Wang, B. N. Day and R. S. Prather. 1997. Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. Biol. Reprod. 57:1123-1127.

Martin, M. J. and C. A. Pinkert. 1994. Production of transgenic swine. In: Transgenic Animal Technology: A Laboratory Handbook, Ed. Pinkert, CA, pp. 315-338, Academic Press, Boston.

Mattioli, M. 1994. Recent acquisitions in pig oocyte maturation and fertilization *in vitro*. Reprod. Dom. Anim. 29:346-348.

Mattioli, M., M. L. Bacci, G. Galeati and E. Seren. 1989. Developmental competence of pig oocytes matured and fertilized *in vitro*. Theriogenology. 31:1201-1207.

Mattioli, M., B. Barboni, P. Lucidi and E. Seren. 1996. Identification of capacitation in boar spermatozoa by chlortetracycline staining. Theriogenology. 45:373-381.

Morcol, T. et al. 1994. The porcine mammary gland as a bioreactor for complex proteins. Annals New York Acad. Sci. 721:218-233.

Nagai, T. 1994. Current status and perspectives in IVM-IVF of porcine oocytes. Theriogenology. 41:73-78.

Nagai, T. 1996. *In vitro* maturation and fertilization of pig oocytes. Anim. Reprod. Sci. 42:153-163.

Nagai, T. and R. M. Moor. 1990. Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized *in vitro*. Mol. Reprod. Dev. 26:377-382.

Nagai, T. and M. Takahashi. 1992. Culture of *in vitro* matured and fertilized pig oocytes. Proc. 12th Int. Congr. Anim. Reprod. 3:1324-1326.

Nagai, T., T. Takahashi, H. Masuda, Y. Shoya, M. Kuwayama, M. Fukushima, S. Iwasaki and A. Hanada. 1988. *In-vitro* fertilization of pig oocytes by frozen boar spermatozoa. J. Reprod. Fertil. 84:585-591.

Nagashima, H., H. Yamakawa and H. Niemenn. 1992a. Freezability of porcine blastocysts at different peri-hatching stages. Theriogenology. 37:839-850.

Nagashima, H., H. Yamakawa and S. Saito. 1992b. Transplantation of porcine blastomere nuclei into oocytes collected from prepubertal gilts. J. Reprod. Dev. 38:73-78.

Nagashima, H., C. G. Grupen, R. J. Ashmen and M. B. Nottle. 1996a. Developmental competence of *in vivo* and *in vitro* matured porcine oocytes after subzonal sperm injection. Mol. Reprod. Dev. 45:359-363.

Nagashima, H., Y. Kato, H. Yamakawa and S. Ogawa. 1988a. Survival of pig hatched blastocysts exposed below 15℃. Jap. J. Anim. Reprod. 34:123-131.

Nagashima, H., J. Katoh, K. Shibata and S. Ogawa. 1988b. Production of normal piglets from microsurgically split morulae and blastocysts. Theriogenology. 29:485-495.

Nagashima, H., N. Kashiwazaki, R. J. Ashman, C. G. Grupen and M. B. Nottle. 1995. Cryopreservation of porcine embryos. Nature. 374:416.

Nagashima, H., M. Kuwayama, C. G. Grupen, R. J. Ashmen and M. B. Nottle. 1996b. Vitrification of porcine early cleavage stage embryos and oocytes after removal of cytoplasmic lipid droplets. Theriogenology. 45:180 (abstr.).

Nagashima, H., N. Kashiwazaki, R. Ashman, C. Grupen, Seamark, R. F. and M. Nottle. 1994a. Recent advances in cryopreservation of porcine embryos. Theriogenology. 41:113-118.

Nagashima, H., N. Kashiwazaki, R. J. Ashman, C. G. Grupen, R. F. Seamark and M. B. Nottle. 1994b. Removal of cytoplasmic lipid enhance the tolerance of porcine embryos to chilling. Biol. Reprod. 51:618-622.

Nakayama, E., A. Onishi, T. Kojima and K. Totsukawa 1997. Farrowing of normal piglets by transfer of biopsied porcine 4-cell embryos following *in vitro* culture. Anim. Sci. Technol. 68:105-106.

Niemann, H. 1989. To South America in a thermos flask: first experiences of international transportation of pig embryos. Tierzuchter. 41:336-337.

Niemann, H. and B. Reichelt. 1993. Manipulating early pig embryos. J. Reprod. Fertil., Suppl. 48:75-94.

Niwa, K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in the pig. J. Reprod. Fertil.,

Suppl. 48:49-59.
Notarianni, E., S. Laurie and K. Sathasivam. 1997. Incorporation of cultured embryonic stem cells into transgenic and chimeric, porcine fetuses. Int. J. Dev. Biol. 41:537-540.
Ocampo, M. B., L. C. Ocampo, T. Mori, J. Ueda, H. Shimizu and H. Kanagawa. 1994. Blastocyst formation of pig embryos derived from *in vitro* fertilization of *in vitro* matured pig oocytes in the amniotic fluid of a developing chick embryo. Anim. Reprod. Sci. 37:65-73.
Onishi, A., K. Takeda, M. Komatsu, T. Akita and T. Kojima. 1994. Production of chimeric pigs and the analysis of chimerism using mitochondrial deoxyribonucleic acid as a cell marker. Biol. Reprod. 51:1069-1075.
Palmiter, R. D., R. J. Brinster, R. E. Hammer, M. E. Trumbauer, M. G. Rosenfeld, N. C. Birnberg and R. M. Evans. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature. 300:611-615.
Parry, T. W. and R. S. Prather. 1995. Carry-over of mRNA during nuclear transfer in pigs. Reprod. Nutr. Dev. 35: 313-318.
Petr, J., J. Rozinek, J. J. Fulka and F. Jilek. 1994a. Influence of cytoplasmic microinjection on meiotic competence in growing pig oocytes. Reprod. Nutr. Dev. 34:81-87.
Petr, J., O. Tepla, R. Grocholova and F. Jilek. 1994b. Inhibition of meiotic maturation in growing pig oocytes by factor(s) from cumulus cells. Reprod. Nutr. Dev. 34: 149-156.
Petters, R. M. and M. L. Reed. 1991. Addition of taurine or hypotaurine to culture medium improves development of one- and two-cell pig embryos. Theriogenology. 35:253 (abstr.).
Petters, R. M. and K. D. Wells. 1993. Culture of pig embryos. J. Reprod. Fertil., Suppl. 48:61-73.
Petters, R. M., B. H. Johnson, M. L. Reed and A. E. Archibong. 1990. Glucose, glutamine and inorganic phosphate in early development of the pig embryo *in vitro*. J. Reprod. Fertil. 89:269-275.
Polge, C. 1982. Embryo transplantation and preservation. In: Control of Pig Reproduction, Ed. Cole, DJA and Foxcroft, G. R. pp. 277-291, Butterworth Scientific, London.
Polge, C. 1985. How does embryo manipulation fit into present and future pig reproduction? J. Reprod. Fertil. Suppl. 33:93-100.
Polge, C. and B. N. Day. 1968. Pregnancy following non-surgical egg transfer in pigs. Vet. Rec. 82:712.
Pomp, D., B. A. Good, R. D. Geisert, C. J. Corbin and A. J. Conley. 1995. Sex identification in mammals with polymerase chain reaction and its use to examine sex effects on diameter of day-10 or -11 pig embryos. J. Anim. Sci. 73:1408-1415.
Poulin, S., J. P. Laforest, M. A. Forlier, E. Perras and M. A. Sirard. 1997. Effects of conditioned media on porcine embryos at different stages of development. Theriogenology. 47:1337-1345.
Prather, R. S. 1991. Culture of porcine embryos from the one- and two-cell stages to the blastocyst stage in sheep oviducts. Theriogenology. 35:1147-1151.
Prather, R. S., M. M. Sims and N. L. First. 1989. Nuclear transplantation in early pig embryos. Biol. Reprod. 41: 414-418.
Prather, R. S., T. T. Stumpf and L. F. Rickords. 1992. Nuclear transplantation as a method of producing genetically identical livestock. Anim. Biotech. 3:67-79.
Prather, R. S., P. A. Eichen, D. K. Nicks and M. S. Peters. 1991. Artificial activation of porcine oocytes matured *in vitro*. Mol. Reprod. Dev. 28:405-409.
Prather, R. S., K. E. Hoffman, R. A. Schoenbeck and T. T. Stumpf. 1996. Characterization of DNA synthesis during the 2-cell stage and the production of tetraploid chimeric pig embryos. Mol. Reprod. Dev. 45:38-42.
Prather, R. S., M. L. Boice, J. Gibson, K. E. Hoffman and T. W. Parry. 1995. *In vitro* development of embryos from sinclair miniature pigs: a preliminary report. Theriogenology. 43:1001-1007.
Price, C. A., P. D. Carriere, B. Bhatia and N. P. Groome. 1995. Comparison of hormonal and histological changes during follicular growth, as measured by ultrasonography, in cattle. J. Reprod. Fertil. 103:63-68.
Pursel, V. G., M. B. Solomon and R. J. Wall. 1996. Genetic engineering of swine. In: Advances in Swine in Biomedical Research, Ed. Tombleson, M and Schook, L, pp. 189-206, Plenum Press, New York.
Pursel, V. G., D. J. Bolt, K. F. Miller, C. A. Pinkert, R. E. Hammer, R. D. Palmiter and R. L. Brinster. 1990. Expression and performance in transgenic pigs. J. Reprod. Fertil. Suppl. 40:235-245.
Pursel, V. G., C. A. Pinkert, K. F. Miller, D. J. Bolt, R. G. Campbell, R. D. Palmiter, R. J. Brinster and R. E. Hammer. 1989. Genetic engineering of livestock. Science. 244:1281-1288.
Rath, D. 1992. Experiments to improve *in vitro* fertilization techniques for *in vivo*-matured porcine oocytes. Theriogenology. 37:885-896.
Rath, D., L. A. Johnson, G. R. Welch and H. Niemann. 1994. Successful gamete intrafallopian transfer (GIFT) in the porcine. Theriogenology. 41:1173-1179.
Rath, D., L. A. Johnson, J. R. Dobrinsky, G. R. Welch and H. Niemann. 1997. Production of piglets preselected for sex following *in vitro* fertilization with X and Y chromosome-bearing spermatozoa sorted by flow cytometry. Theriogenology. 47:795-800.
Reed, M. L., M. J. Illera and R. M. Petters. 1992. *In vitro* culture of pig embryos. Theriogenology. 37:95-109.
Reichenbach, H. D., J. Modl and G. Brem. 1993. Piglets born after transcervical transfer of embryos into recipient gilts. Vet. Rec. 133:36-39.
Rorie, R. W., S. A. Voelkel, C. W. McFarland, L. L. Southern and R. A. Godke. 1985. Micromanipulation of day-6 porcine embryos to produce split-embryo piglets. Theriogenology. 23:225(Abstr.).
Rosengard, A. M., N. R. Cary, G. A. Langford, A. W. Tucker, J. Wallwork and D. J. White. 1995. Tissue expression of human complement inhibitor, decay-accelerating factor, in transgenic pigs. A potential approach. Transplantation. 59:1325-1333.
Sawai, K., H. Funahashi and K. Niwa. 1997. Stage-specific requirement of cysteine during *in-vitro* maturation of porcine oocytes for glutathione synthesis associated with male pronuclear formation. Biol. Reprod. 57:1-6.
Subramanian, A., R. K. Paleyanda, H. Lubon, B. L. Williams,

F. C. Gwazdauskas, J. W. Knight, W. N. Drohan and W. H. Velander. 1996. Rate limitations in post-translational processing by the mammary gland of transgenic animals. Annals New York Acad. Sci. 782:87-96.

Sun, F. Z., J. Hoyland, X. Huang, W. Mason and R. M. Moor. 1992. A comparison of intercellular changes in porcine eggs after fertilization and electroactivation. Development. 115:947-956.

VanCott, K. E., H. Lubon, C. G. Russell, S. P. Butler, F. C. Gwazdauskas, J. Knight, W. N. Drohan and W. H. Velander. 1997. Phenotypic and genotypic stability of multiple lines of transgenic pigs expressing recombinant human protein C. Transgenic Res. 6:203-212.

Vize, P. D., A. E. Michalska, R. Ashman, B. Lloyd, B. A. Stone, P. Quinn, J. R. E. Wells and R. F. Sermark. 1988. Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth. J. Cell Sci. 90:295-300.

Wang, W. H., M. Hosoe and Y. Shioya. 1997. Induction of cortical granule exocytosis of pig oocytes by spermatozoa during meiotic maturation. J. Reprod. Fertil. 109:247-255.

Wang, W. H., K. Niwa and K. Okuda. 1991. *In-vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. J. Reprod. Fertil., 93:491-496.

Wheeler, M. B. 1994. Development and validation of swine embryonic stem cells: a review. Reprod. Fertil. Dev. 6: 563-568.

White, K. L., K. Henke, L. F. Rickords, L. L. Southern, D. L. J. Thompson and T. C. Wood. 1989. Early embryonic development *in vitro* by coculture with oviductal epithelial cells in pigs. Biol. Reprod. 41:425-430.

Whitten, W. K. and J. D. Biggers. 1968. Complete development *in vitro* of the pre-implantation stages of the mouse in a simple chemically defined medium. J. Reprod. Fertil. 17:399-401.

Willadsen, S. M. 1982. Micromanipulation of the large domestic species. In: Mammalian Egg Transfer, Ed. Adams, CE, pp 185-210, CRC Press, Boca Raton.

Yamauchi, N., H. Sasada, S. Sugawara and T. Nagai. 1996. Effect of culture conditions on artificial activation of porcine oocytes matured *in vitro*. Reprod. Fertil. Dev. 8: 1153-1156.

Yoshino, J., T. Kojima, M. Shimizu and T. Tomizuka. 1993. Cryopreservation of porcine blastocysts by vitrification. Cryobiology. 30:413-422.

Yoshida, M., K. Ishigaki, T. Nagai, M. Chikyu and V. G. Pursel. 1993a. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol. Reprod. 49:89-94.

Yoshida, M., Y. Mizoguchi, K. Ishigaki, T. Kojima and T. Nagai. 1993b. Birth of piglets derived from *in vitro* fertilization of pig oocytes matured in vitro. Theriogenology. 39:1303-1311.