Recent Advances in Cloning Technology in the Pig -Review-

K. Miyoshi* and E. Sato
Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University
1-1 Tsutsumidori-amamiyamachi, Aoba-ku, Sendai 981-8555, Japan

ABSTRACT : Production systems for cloned pigs are very important not only for an increase in production of superior animals but also for the production of knockout animals with organs that do not contain antigens for xenotransplantation or to analyze functions of isolated human genes. At present, however, effective systems have not been developed. We have tried to produce cloned pigs by transfering cultured cells into enucleated oocytes and obtained some cloned embryos. To develop a production system for cloned pigs, the basic technologies needed to support such an effort must be improved. *(Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 2 : 258-264)*

Key Words : Pig, Blastocyst, Cell Line, Nuclear Transfer, Totipotency

INTRODUCTION

To date, three different methods have been reported to produce cloned animals (figure 1). With the first method, 2- to 16-cell embryos were separated into two groups or morulae and blastocysts were cut in two to produce twins artificially. By using this method, identical twins have been obtained in the mouse, goat, sheep, bovine and horse (Willadsen, 1982). However, the number of cloned animals produced by this method is limited to two.

With the second method, embryonic stem (ES) cells were used. ES cells have an ability to proliferate *in vitro* with pluripotency and to form chimeras when introduced into early embryos (Evans and Kaufman, 1981). After mating germ-line chimeras, animals derived from ES cells can be obtained (Bradley et al., 1984). In the mouse, ES cells and gene targeting technologies have become routine procedures for introducing desired genetic change into the genome (Hooper, 1992). ES cells have also been reported in species other than the mouse, such as the hamster (Doetshman et al., 1988), American mink (Sukoyan et al., 1992), rabbit (Graves and Moreadith, 1993), rat (Iannaccone et al., 1994), pig (Wheeler, 1994), rhesus monkey (Thomson et al., 1995), bovine (Strelchenko, 1996) and human (Thomson et al., 1998). A limited number of reports have verified the pluripotency of ES cells by the production of chimeras in the rat (Iannaccone et al., 1994), pig (Wheeler, 1994) and rabbit (Schoonjans et al., 1996), but not germ-line chimeras. At present, therefore, ES cells can be used for the creation of cloned animals or targeted mutations only in the mouse.

Although a third strategy has been to consider using ES cells for nuclear transfer and to produce animals derived entirely from cultured cells, thus bypassing the chimeric step, no cloned animals have been obtained by this method. Recently, however, production of viable lambs from cells of an established embryonic cell line was achieved by inducing the cells to exit the normal cell division cycle and enter a quiescent or G0 state (Campbell et al., 1996). This result was all the more extraordinary because the cultured cells used were not ES cells but a more differentiated epithelial cell type. This raised the possibility that nuclei from other cell types can be similarly reprogrammed and yield offspring and in a subsequent study, lambs were produced from fetal fibroblasts and adult mammary-derived cells (Wilmut et al., 1997).

Development of the same cloning technique in the pig may be useful not only for an increase in the production of superior animals but also for the production of knockout animals with organs that do not contain antigens for xenotransplantation, or to analyze functions of isolated human genes. In this review, we will discuss recent advances in the production of cloned pigs by nuclear transfer of cultured cells.

STRATEGY FOR THE PRODUCTION OF CLONED PIGS

Our strategy for production of cloned pigs is shown in figure 2. To date, by using nuclear transfer, cloned animals have been produced from three different cultured cells: embryo-derived cells, fetal fibroblasts and adult cells (Wilmut et al., 1997). Fetal fibroblasts and adult cells can be obtained and cultured easily but they may not be suitable for homologous recombination which is essential to produce knockout animals (Arbone et al., 1994). In contrast, embryo-derived cells would be appropriate for

* Address reprint request to K. Miyoshi. E-mail: miyoshik@bios.tohoku.ac.jp.

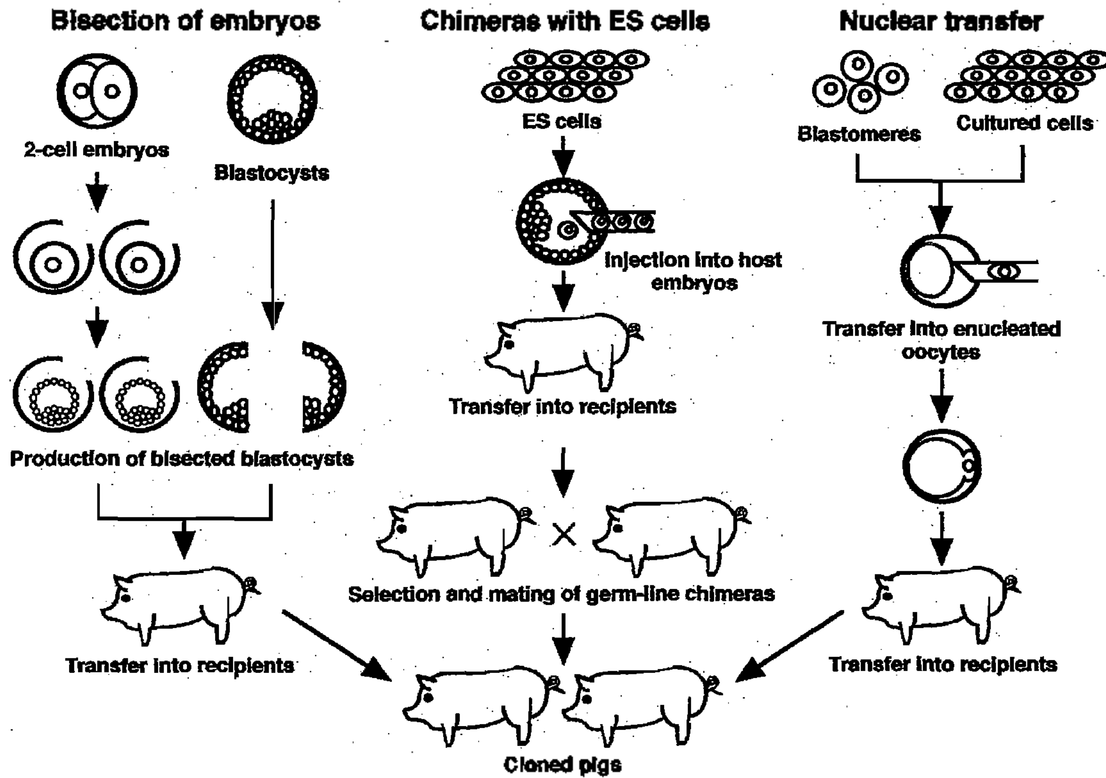

Figure 1. Methods for the production of cloned pigs

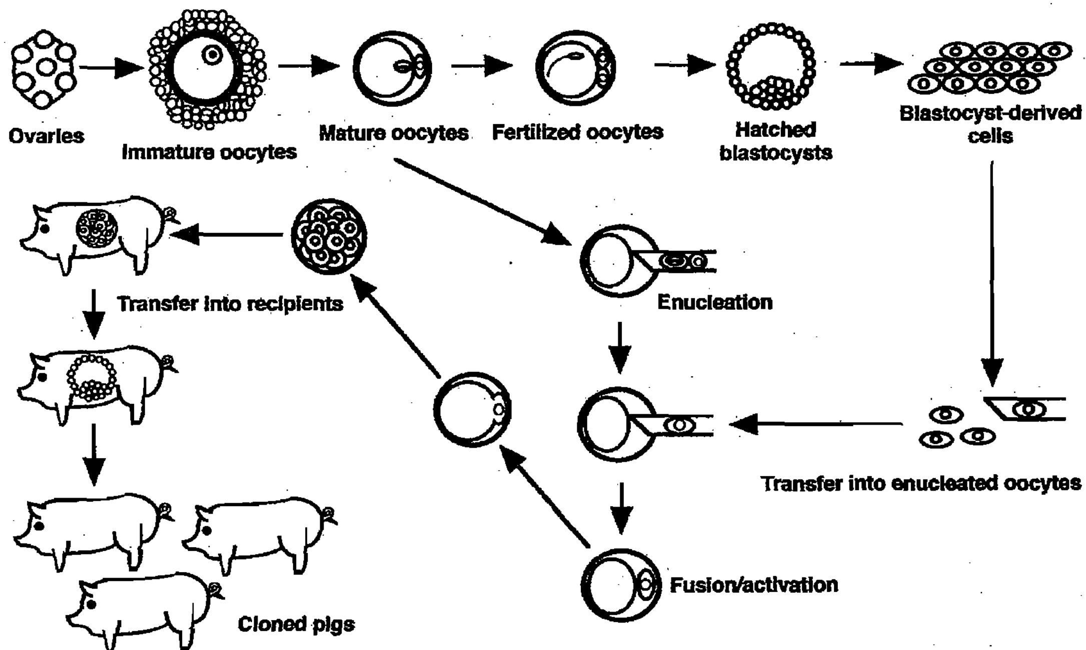

Figure 2. The strategy for the production of cloned pigs by nuclear transfer

homologous recombination because they are of the same origin as ES cells, so we tried to obtain a cell line from porcine embryos. To carry out experiments, a large number of mature oocytes and embryos are needed. Therefore, we used the *in vitro* maturation, fertilization and culture system to produce them from immature oocytes which can be obtained abundantly and easily from ovaries collected at a slaughterhouse. Hatched blastocysts are produced *in vitro* and a cell line is established from them. After proliferating *in*

vitro, the cells are transferred into enucleated *in vitro* matured oocytes. These reconstructed embryos are transferred into recipient females and develop into cloned pigs.

ESSENTIAL TECHNOLOGIES FOR THE PRODUCTION OF CLONED PIGS

Our strategy for the production of cloned pigs relies on several basic technologies, none of which has been developed to a satisfactory level. This prevents us from developing a production system for cloned pigs. The present status of the individual technologies is as follows.

IN VITRO MATURATION, FERTILIZATION AND CULTURE OF OOCYTES

Until recently, it was very difficult to produce porcine blastocysts from immature oocytes by *in vitro* maturation, fertilization and culture. One of the most prominent obstacles was that few normal fertilized oocytes are obtained by *in vitro* fertilization. Almost all immature oocytes liberated from ovarian follicles could resume meiosis and reach metaphase II in culture. Although the matured oocytes could be penetrated *in vitro* by spermatozoa, low rates of male pronuclear formation and a high incidence of polyspermy were observed.

It was concluded that the failure of male pronuclear formation in porcine penetrated oocytes can be attributed to incomplete cytoplasmic maturation of oocytes and so conditions of maturation have been improved. Media used for maturation of porcine oocytes were generally supplemented with sera such as fetal calf serum (FCS) (Mattioli et al., 1988a, b; Yoshida et al., 1990; Galeati et al., 1991; Wang et al., 1991), newborn calf serum (Nagai and Moor, 1990) and pig serum (Eng et al., 1986; Zheng and Sirard, 1992). However, it was reported that FCS inhibits maturation of porcine oocytes in a modified Krebs-Ringer bicarbonate solution supplemented with FSH and can not improve male pronuclear formation after sperm penetration *in vitro* (Naito et al., 1988). In contrast, when oocytes matured in pig follicular fluid (pFF) supplemented with FSH were inseminated *in vitro*, male pronuclei formed in 81% of penetrated oocytes (Naito et al., 1988). Funahashi and Day (1993a) also showed that supplementation of 10% FCS or newborn-piglet serum in modified tissue culture medium-199 (mTCM-199) appears to be detrimental to cytoplasmic maturation, as the male pronuclear formation was lower than in 10% pFF or 0.4% polyvinylalcohol. In mouse (Calvin et al., 1986) and hamster (Perreault et al., 1988; Perreault, 1990) oocytes, it had been suggested that synthesis of glutathione during oocyte maturation is a prerequisite for male pronuclear formation. A series of experiments (Yoshida et al., 1992, 1993a, b; Yoshida, 1993) clearly indicated that the addition of cysteine as a substrate of glutathione to the maturation medium is an important factor for male pronuclear formation of porcine oocytes after sperm penetration. Porcine oocytes were frequently cultured in maturation media supplemented with different combinations of gonadotrophins and oestradiol, because beneficial effects of these components on nuclear maturation of porcine oocytes had been reported (Meinecke and Meinecke-Tillmann, 1979; Racowsky and McGaughey, 1982). Funahashi and Day (1993b) found that the incidence of male pronuclear formation is greatly improved when oocyte-cumulus complexes are cultured in a maturation medium with PMSG, hCG and oestradiol for 20 h followed by 20 h culture without hormones.

It has been suggested that the rate of polyspermy can be reduced by controlling the conditions in the medium used for the treatment of spermatozoa and fertilization of oocytes. Nagai and Moor (1990) showed that when spermatozoa are preincubated for 2.5 h with oviduct cells, the rate of polyspermy is reduced without a marked reduction in the penetration rate. It was also indicated that addition of pFF to mTCM-199 with 0.4% bovine serum albumin (BSA) during sperm preincubation alone reduces the incidence of polyspermy (Funahashi and Day, 1993c). Recently, it was reported that the polyspermy was suppressed without the preincubation of spermatozoa by using modified Tris buffered medium as a fertilization medium (Abeydeera et al., 1997).

Another problem with the production of porcine blastocysts *in vitro* was that the development of fertilized oocytes is blocked at the 4-cell stage (Davis and Day, 1978). To overcome this developmental arrest, effects of various factors on *in vitro* development of *in vivo* fertilized porcine oocytes have been examined. Oxaloacetate, phosphoenolpyruvate, pyruvate or lactate can be used as an exclusive energy source by early cleavage stage mouse embryos (Brinster, 1965). Lactate and pyruvate are generally included in media formulations and lactate is thought to be required for optimal development (Biggers, 1987), In the pig, however, lactate inhibited embryo development (Davis and Day, 1978; Stone et al., 1984; Davis, 1985) and neither lactate nor pyruvate was required for embryo development (Petters et al., 1990; Petters and Reed, 1991). In the absence of lactate and pyruvate, glucose and glutamine supported embryonic development to the morula and blastocyst stages (Petters et al., 1990). Hypotaurine and/or taurine addition significantly improved embryonic development *in vitro* (Petters and Reed, 1991). Based on these results, a medium designated NCSU-23 was developed

for culture of porcine *in vivo* fertilized embryos to the blastocyst stage (Petters and Reed, 1991; Reed et al., 1992). NCSU-23 could be applied not only to the culture of *in vitro* produced embryos but also to the *in vitro* maturation of immature oocytes (Wang et al., 1997). Addition of hyaluronic acid stimulated blastocyst formation of *in vivo* fertilized embryos (Miyano et al., 1994) and electro-activated oocytes (Kurebayashi et al., 1995) or of *in vitro* fertilized embryos (Kano et al., 1998) that had been matured *in vitro*. Recently, it was reported that the kind of BSA is an important factor for porcine embryonic development: essentially fatty acid free BSA supported development, but fraction V BSA had a detrimental effect (Dobrinsky et al., 1996). Moreover, addition of FCS at the late morula/early blastocyst stage stimulated hatching of blastocysts although FCS was detrimental to the development until embryos reached the late morula/early blastocyst stage (Dobrinsky et al., 1996).

NUCLEAR TRANSFER

The *in vitro* developmental ability of porcine nuclear transfer embryos reconstituted with post-embryonic genome activation stage nuclei is very limited and up to now only one piglet has been generated as a result of nuclear transfer (Prather et al., 1989). This piglet was derived from the fusion of single 4-cell blastomeres with metaphase Ⅱ oocytes and was one of 88 reconstructed embryos that had been transferred. It was shown that the porcine nuclear transfer embryos reconstituted with 8- to 16-cell stage nuclei and enucleated mature oocytes cleaved *in vitro* at rates of 36-43% but developmental rates beyond the morula stage were only 4.5-7% (Saito et al., 1992; Terlouw et al., 1992). Recently, Nagashima et al. (1997) reported that 28% of the nuclear transfer embryos reconstituted with morula stage nuclei developed to morula and 15% formed blastocysts by using preactivated oocytes as recipient cytoplasts and inducing fusion of blastomeres and cytoplasts at 6 h after the electric activation.

FREEZING OF EMBRYOS

Reconstructed embryos must be transferred into recipient females to obtain cloned offspring, but suitable recipients are not always prepared because it is required that the developmental stage of embryos and the pregnant stage of recipients be synchronous. Therefore, a technique for the storage of embryos without affecting their viability until they are transferred is necessary. Perihatching stage embryos survived after cryopreservation and piglets were obtained from cryopreserved embryos (Kashiwazaki et al., 1991; Fujino et al., 1993). Until recently, however, porcine embryos with intact zona pellucida could not be frozen successfully. Earlier studies showed that porcine embryos degenerate when cooled to temperatures of 5-10℃ (Wilmut, 1972; Polge et al., 1974) and 15℃ had been identified as the critical temperature at which damage occurs (Polge, 1977). Damage to membrane lipids had been identified as the major reason for the lack of survival of porcine embryos after exposure to decreased temperatures or freezing (Polge and Willadsen, 1978; Wilmut, 1986). In addition, it was suggested that the high lipid content of porcine embryos may lead to uneven intracellular ice formation, which was considered the major cause of degeneration after freezing and thawing (Toner et al., 1986). Recently, it was shown that porcine early cleavage-stage embryos survive cryopreservation following removal of cytoplasmic lipid (Nagashima et al., 1995).

EMBRYO TRANSFER

At present, piglets have not been obtained by transfer of blastocysts produced from immature oocytes *in vitro*. When blastocysts developed *in vitro* from *in vivo* or *in vitro* fertilized oocytes were transferred into recipient females, embryos derived from *in vivo* fertilized oocytes developed offspring but the transfer of embryos derived from *in vitro* fertilized oocytes did not result in pregnancies (Rath et al., 1995). On the other hand, it was shown that embryos derived from *in vitro* matured and fertilized oocytes can develop to piglets when they are transferred into recipients at the 2-cell stage (Funahashi et al., 1997). These results suggest that if any of the systems of *in vitro* maturation, fertilization and culture are incomplete, the viability of oocytes/embryos is affected at all stages. When either *in vitro* maturation/fertilization or *in vitro* culture is used, embryos can develop to piglets after transfer because the amount of damage is relatively small. However, the damage to embryos produced by using *in vitro* maturation, fertilization and culture is extensive so they do not develop to offspring.

THE STATUS OF THE PRODUCTION OF CLONED PIGS

Based on results of previous studies, we developed a production system for porcine hatched blastocysts by *in vitro* maturation, fertilization and culture (figure 3, Miyoshi et al., 1999) and established porcine hatched blastocyst-derived cells which had an epithelial-like morphology (unpublished data). This morphology was very similar to that of ovine blastocyst-derived cells that are totipotent by nuclear transfer and can produce viable offspring (Campbell et al., 1996; Wilmut et al.,

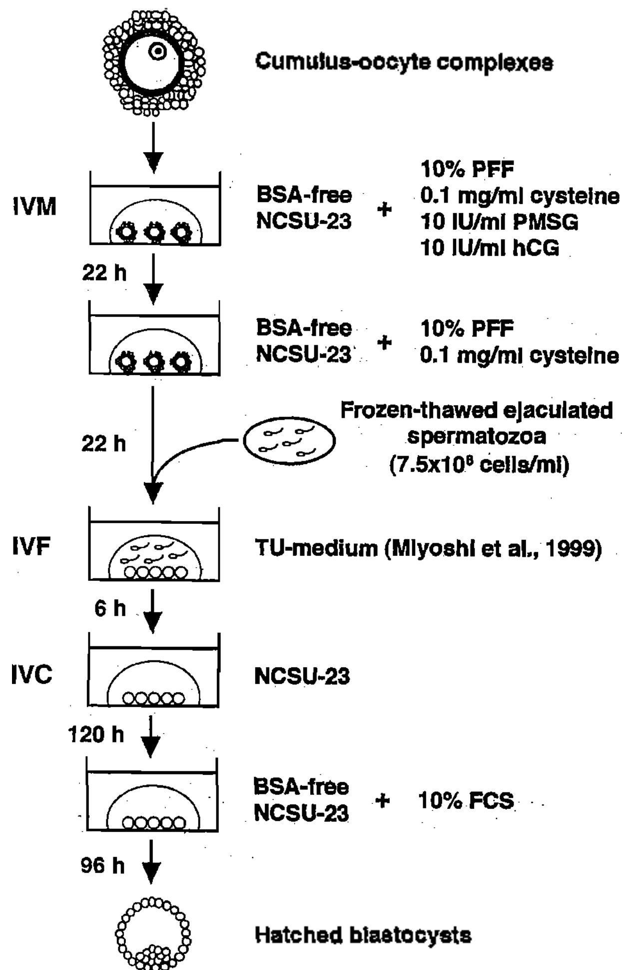

Figure 3. The system for producing porcine hatched blastocysts from immature oocytes *in vitro*

1997; Wells et al., 1997), and was stable up to at least passage 30.

The hatched blastocyst-derived cells were fused with enucleated *in vitro* matured oocytes (Miyoshi and Sato, 1999). In general, a chamber consisting of parallel wires has been used for fusion (Nagashima et al., 1997) but this chamber was not suitable for fusion of hatched blastocyst-derived cells and enucleated oocytes. In contrast, when cell-oocyte complexes were sandwiched between wires arranged in a straight line, the percentage of fused embryos was high (unpublished data). When reconstructed embryos were cultured *in vitro*, some of them cleaved and developed to the blastocyst stage. Further experiments in which reconstructed embryos are transferred into recipient females to examine their ability to develop into offspring are in progress.

CONCLUSION

Production systems for cloned pigs are very important not only for an increase in production of superior animals but also for the production of knockout animals with organs that do not contain antigens for xenotransplantation, or to analyze functions of isolated human genes, but effective systems have yet to be developed. One prominent obstacle is a lack of information on the basic technologies which are needed to support this study. These basic technologies need to be developed to a satisfactory level before a production system for cloned pigs can be developed.

ACKNOWLEDGMENTS

This work was supported by grants for Encouragement of Young Scientists from the Japan Society for the Promotion of Science (JSPS), JSPS-RFTF 97L00904 for the "Research for the Future" Program from JSPS and for the Program for Promotion of Basic Research Activities for Innovative Bioscience. K.M. is a recipient of the JSPS Fellowship (No. 9801517).

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