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Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into *in vitro* matured oocytes

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Summary

The objective of this study was to evaluate *in vitro* and *in vivo* development of porcine *in vitro* matured (IVM) porcine oocytes fertilised by intracytoplasmic sperm injection (ICSI) and the possibility of producing transgenic embryos and offspring with this procedure. Activated ICSI oocytes had a higher pronuclear formation than non-activated ICSI oocytes (mean 64.8±17.3% vs 28.5±3.4%, $p<0.05$). When the zygotes with two pronuclei were cultured to day 2, there was no difference ($p<0.05$) in the cleavage rate (mean 60.0±7.0% vs 63.3±12.7%) between the two groups. The blastocyst rate in the activation group was significantly higher than that in the non-activation group (mean 30.0±11.6% vs 4.6±4.2%, $p<0.05$). After injection of the sperm transfected with DNA/liposome complex, destabilised enhanced green fluorescent protein (d2EGFP) expression was not observed on day 2 in either cleaved or uncleaved embryos. But from day 3, some of the embryos at the 2-cell to 4-cell stage started to express d2EGFP. On day 7, about 30% of cleaved embryos, which were in the range of 2-cell to blastocyst stage, expressed d2EGFP. However, for the IVF oocytes inseminated with sperm transfected with DNA/liposome complex, and for oocytes injected with sperm transfected with DNA/liposome complex, and for oocytes injected with DNA/liposome complex following insemination with sperm not treated with DNA/liposome complex, none of the embryos expressed d2EGFP. Sixteen day 4 ICSI embryos derived from sperm not treated with DNA/liposome complex were transferred into a day 3 recipient. One recipient delivered a female piglet with normal birthweight. After transfer of the ICSI embryos derived from sperm transfected with DNA/liposome complex, none of the four recipients maintained pregnancy.

Keywords: Green fluorescent protein, Intracytoplasmic sperm injection, *In vitro* matured oocytes, Transgene piglet

Introduction

Intracytoplasmic sperm injection (ICSI) has been used to produce offspring in humans (Palermo *et al.*, 1992, 1993; Van Steirteghem *et al.*, 1993*a*,*b*; Tesarik, 1996), mice (Ahmadi *et al.*, 1995; Kimura & Yanagimachi, 1995; Lacham-Kaplan & Trounston, 1995), rabbits (Hosoi *et al.*, 1998), cats (Pope *et al.*, 1997), cattle (Goto *et al.*, 1991) and sheep (Catt *et al.*, 1996). Development of the ICSI procedure has been of importance not only for study of the fertilisation process but also for treatment of male infertility.

In swine, there is a little information with regard to ICSI (Iritani, 1991; Catt & Rhodes, 1996; Lee *et al.*, 1998; Kolbe & Holtz, 1999; Kim *et al.*, 1998). Recently, Martin (2000) and Kolbe & Holtz (2000) reported that they obtained three piglets and one piglet derived from ICSI, respectively. But both of the experiments used *in vivo* matured oocytes and freshly collected sperm. There are limitations in using *in vivo* matured oocytes for commercial embryo production with regard to creation of transgenic pigs by using ICSI. It is important to use *in vitro* matured (IVM) oocytes as recipients for ICSI because an abundant supply of oocytes is available from the slaughterhouse, which could

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dramatically reduce the cost of production of transgenic animals. Recently, progress in *in vitro* production of pig embryos in our laboratory has resulted in a routine, chemically defined system for producing embryos that are developmentally competent (Abeydeera *et al.*, 2000). But compared with *in vivo* matured oocytes, IVM oocytes possess limited developmental potential when used for production of embryos and offspring. Therefore, it is uncertain whether IVM oocytes can be utilised in the production of ICSI piglets. So far, there has been no report on the birth of piglets when using IVM oocytes and long-term frozen sperm to perform ICSI.

In addition, an ICSI-based method has been previously described for generation of *Xenopus* embryos in which sperm nuclei were first partially decondensed before mixing with DNA (Kroll & Amaya, 1996). Recently, Perry *et al.* (1999) introduced the ICSI procedure to produce transgenic mice. This study showed that membrane-disrupted mouse spermatozoa support the genomic integration and subsequent expression of exogenous DNA in offspring after ICSI. Also, Chan *et al.* (2000) reported that the injection of spermatozoa bound with exogenous DNA transmitted foreign DNA into rhesus monkey embryos. Since such a procedure does not require zygotes with a visible pronucleus, it may be a good alternative to pronucleus injection for the production of transgenic animals. This may be particularly useful in producing transgenic pigs because pronucleus injection in this species is often difficult due to the opaque ooplasm. Thus, in our experiments, we also employed an ICSI procedure to test the efficiency of transgenesis in pigs. Since liposomes could efficiently transfer DNA into sperm (Bachiller *et al.*, 1991), instead of disrupting the sperm membrane with Triton X-100 or NaOH, as used in previous experiments in mice (Perry *et al.*, 1999) and monkey (Chan *et al.*, 2000), we incubated the sperm with DNA pretreated with liposomes. The possibility of producing transgenic embryos and offspring by using the ICSI procedure with IVM oocytes and frozen sperm was evaluated.

Materials and methods

Preparation of recipient oocytes

Porcine ovaries were obtained from an abattoir and transported to the laboratory in a thermos filled with saline maintained at 30–35 °C. Follicular fluid from 3–6 mm antral follicles was aspirated by using an 18 gauge needle attached to a 10 ml disposable syringe. Cumulus–oocytes complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected and rinsed three times in TL-Hepes plus polyvinyl alcohol (PVA) (Abeydeera *et al.*, 2000). Approximately 50–70 COCs were put into each well of a 4-well multidish containing TCM-199 medium supplemented with PVA (0.1%), D-glucose (3.05 mM), sodium pyruvate (0.91 mM), penicillin (75 μg/ml), streptomycin (50 μg/ml), cysteine (0.57 mM), luteinising hormone (LH; 0.5 μg/ml), follicle stimulating hormone (FSH; 0.5 μg/ml) and epidermal growth factor (EGF; 10 μg/ml), and covered with mineral oil. The oocytes were cultured for 42–44 h at 39 °C in 5% CO_2 in air. After maturation, expanded cumulus cells were removed by vigorous pipetting in the presence of 0.3 mg/ml hyaluronidase. All chemicals were obtained from Sigma Chemical Company (St Louis, MO).

Preparation of sperm

A semen pellet was thawed and washed three times by centrifugation at 1900 *g* for 4 min in Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) supplemented with 0.1% bovine serum albumin (BSA); 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. At the end of the washing procedure, the sperm pellet was resuspended in *in vitro* fertilisation (IVF) medium (Abeydeera & Day, 1997).

Plasmic pd2 EGFP-N1 (Clontech, CA), which encodes a destabilised, red-shifted variant of wild-type GFP which has been optimised for brighter fluorescence and high expression in mammalian cells with a 2 h half-life, was linearised by endonuclease digestion with Tth111 I before being incubated with sperm. As described by Bachiller *et al.* (1991), 1 μg of lipofectin (Boehringer Mannheim, Germany) and 1 μg DNA were diluted in separated tubes containing 150 μl of IVF medium each. The dilutions were then mixed, and the resulting 300 μl drop was transferred into a Petri dish and covered with paraffin oil. After dispersal, a volume necessary (10–20 μl) to yield a final concentration of 1×10^6 sperm cells/ml was taken from the original suspension and added to the DNA–lipofectin mixture. The new suspension was incubated at 39 °C in 5% CO_2 in air, for 2–6 h for sperm injection, or 2–3 h for IVF.

IVF procedure

The denuded oocytes were washed three times in IVF medium and 35 oocytes were placed in each 50 μl drop of the same medium that had been covered with warm paraffin oil in a 35×10 mm^2 polystyrene culture dish. The dishes were kept in the incubator for about 30–45 min until spermatozoa were added for fertilisation. After appropriate dilution, 50 μl of this sperm suspension was added to 50 μl of the medium that contained oocytes to give a final sperm concentration of 1.5×10^5 cells/ml. Oocytes were co-incubated with spermatozoa for 5–6 h at 39 °C, in 5% CO_2 in air.

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ICSI procedure

Oocytes were centrifuged in TL-Hepes plus 0.3% BSA at 1500 *g* for 10 min to permit visualisation of sperm following injection (Rho *et al.*, 1998). ICSI was conducted in 300 µl drops of TL-Hepes plus 0.3% BSA under mineral oil (M 8410; Sigma, St Louis, MO). Movement of live sperm was slowed down by adding 2 µl of sperm suspension to a 4 µl drop of TL-Hepes plus 0.3% BSA and 10% polyvinylpyrrolidone (PVP; MW 360 000).

ICSI was conducted with an inverted microscope with a warm stage and Hoffman optics at ×200. Sperm were individually immobilised by scoring the tail. A single sperm was aspirated from the sperm droplet and moved to the drop containing oocytes. The oocyte was captured by a holding pipette and immobilised with its polar body at either the 6 or the 12 o'clock position as described by Martin (2000). The sperm was injected directly into the cytoplasm of an oocyte in TL-Hepes plus 0.3% BSA with a 6–8 µm inner diameter glass pipette as described by Nagy *et al.* (1995).

Parthenogenetic activation of oocytes

For electrical activation, oocytes were equilibrated for 5 min in 0.3 M mannitol solution supplemented with 0.5 mM Hepes, 0.01% BSA, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, and transferred to a chamber consisting of two electrodes 1 mm apart which was overlaid with the same activation solution. Oocytes were exposed to a single pulse of 1.2 kV/cm for 30 µs, on a BTX Electrocell manipulator 200 (BTX, San Diego, CA).

Embryo culture and transfer

After fertilisation or ICSI the embryos were cultured in NCSU-23 with 4 mg/ml BSA for 14–16 h. The zygotes were centrifuged at 150 *g* for 10 min and pronuclear number was checked by rotating the embryos captured by holding pipette with an injection pipette under an inverted microscope one by one. Some of the 1-cell embryos with two pronuclei were cultured in the same NCSU for 7 days. Cleavage and blastocyst rates were checked on day 2 and day 7, respectively. Blastocysts were stained with Hoechst 33342 to determine the nuclear number. Some of the 1-cell embryos were cultured *in vitro* for 4 days. The 8-cell to morula stage embryos were surgically transferred into the uterus of non-bred day 3 recipient pigs to allow the pigs to continue pregnancy.

Experimental design

Experiment 1
To establish whether activation is necessary for the normal development of ICSI oocytes matured *in vitro*, some ICSI oocytes were electrically activated, 0.5–1.5 h after microinjection, and some ICSI oocytes were not activated. *In vivo* matured oocytes derived from two gilts were also used as recipient oocytes for ICSI to compare the developmental ability between *in vivo* and *in vitro* matured oocytes. IVF of IVM oocytes was used as controls.

Experiment 2
To determine whether ICSI could be used to produce transgenic embryos, the sperm incubated with the d2EGFP DNA/liposome complex were injected into the cytoplasm of IVM oocytes. IVF oocytes inseminated with sperm treated with DNA/liposome complex were used as a control. In another control group, we injected the DNA/liposome complex (1–2 pl) directly into the cytoplasm of oocytes and then the oocytes were parthenogenetically activated. All the embryos were cultured in NCSU for 7 days. The embryos were checked with an ultraviolet microscope on days 2, 4 and 7 to determine whether EGFP had been expressed.

Experiment 3
To determine whether IVM oocytes can be utilised in the production of ICSI piglets, the 4-day cultured embryos derived from zygotes with two pronuclei were transferred into the uterus of the day 3 natural oestrus recipient pigs. To explore the possibility of generation of transgenic piglets by using the ICSI procedure, we also transferred the embryos derived from sperm transfected with DNA/liposome complex into recipient pigs.

Statistical analysis

All dependent variables were analysed for normality using the Wilks–Shapiro test (SAS, Cary, NC). Cleavage rate and blastocyst rate were arcsine-transformed to achieve a normal distribution. Treatment effects were analysed with the GLM procedure of SAS. All data were expressed as mean ± SEM. A probability of $p<0.05$ was considered to be statistically significant.

Results

In vitro development of ICSI embryos

After 14–16 h of culture, of 141 surviving ICSI oocytes activated before culture (ICSI ± activation), 88 formed two pronuclei (PN) (mean 64.8±17.3%) (Fig. 1*A*). The others had one or no PN and were discarded. When the zygotes with 2 PN were cultured to day 2, 53 embryos (mean 60.0±7.0%) cleaved. Twenty-six blastocysts (Fig.

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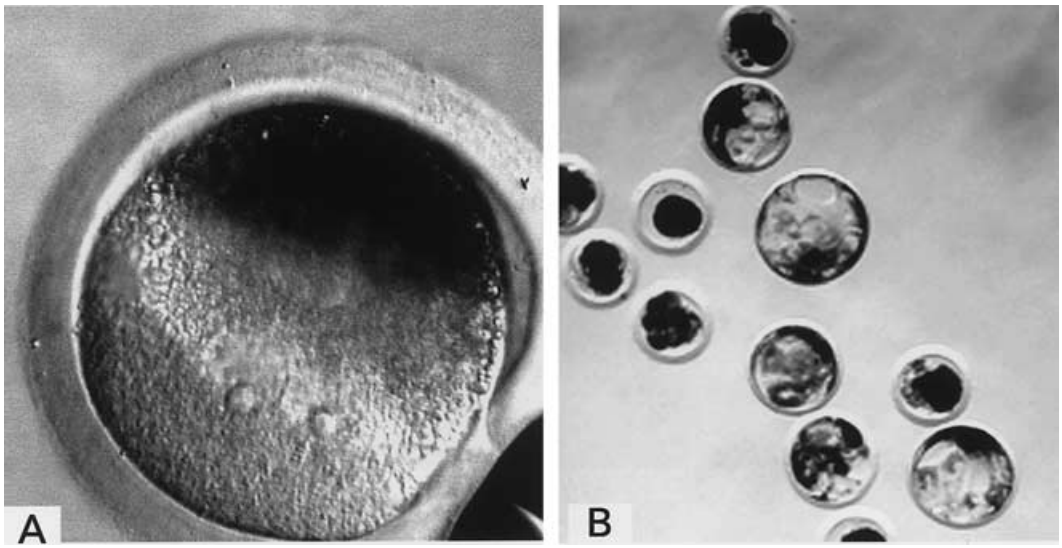

Figure 1 (*A*) Zygote with two apposed pronuclei derived from ICSI with untransfected sperm 14 h after activation (×400). (*B*) Blastocysts derived from ICSI with untransfected sperm 7 days after culture.

1*B*) were obtained on day 7 (mean 30.0±11.6%). In the group of ICSI oocytes without activation (ICSI + non-activation), of 150 oocytes surviving the ICSI, only 43 formed 2 PN (mean 28.5±3.4%), which was significantly lower than the proportion in the activated ICSI oocyte group ($p<0.05$). Although there was no difference in the cleavage rate (mean 63.3±12.7%) between the two groups, the blastocyst rate of the ICSI activation group was significantly higher than that in the ICSI + non-activation group (mean 4.6±4.2%) ($p<0.05$). In the ICSI + *in vivo* matured oocyte group, the 2 PN zygote rate (80.0%), cleavage rate (75.0%) and blastocyst rate (30.0%) were not different from those in the ICSI + activation group. For the IVF group, of 191 zygotes, 65 formed 2 PN (mean 33.8±11.5%), which is significantly lower than in the ICSI + activation group. Most of the others had three or more pronuclei. Only the zygotes with 2 PN were cultured further. The cleavage rate (mean 60.7±5.2%) and blastocyst rate (mean 35.5±9.8%) were not significantly different from those in the ICSI group. There were no statistical differences in nuclear number of the blastocysts among the four groups (Table 1).

In vitro development of ICSI embryos derived with the sperm transfected with GFP DNA

After injection of the sperm transfected with DNA/liposome complex, followed by electrical activation, the cleavage rate of the zygotes was 58.4±18.1 (102/180) on day 2, and the blastocyst rate was 17.8±13.1% (34/102) of the total surviving ICSI oocytes on day 7. On day 2, d2EGFP expression was not discerned in either cleaved or uncleaved embryos. But from day 3, some of cleaved embryos at the 2-cell to 4-cell stage started to express d2EGFP (Fig. 2*A*,*B*). The quality of some green embryos looked poor, and unevenly cleaved green embryos were frequently observed (Fig. 2*C*). On day 7, of 102 cleaved embryos, 30 were green. The green embryos were in the range of 2-cell to blastocyst stage (Fig. 2*E*,*F*). Seven green blastocysts were obtained of the 34. In some of the embryos, only partial blastomeres were green. However, for the IVF oocytes inseminated with sperm transfected with DNA/liposome complex, and for oocytes injected with DNA/liposome complex following insemination with sperm without treatment of DNA/liposome complex, none of the cleaved embryos

Table 1 *In vitro* development of ICSI embryos

Treatment	No. examined	Embryos with 2 embryos (%)	Rate of cleavage (%)	Rate of blastocysts (%)	Nuclear no. of blastocyst
ISCI + activation	141	88 (64.8±17.3)^a	53 (60.0±7.0)^a	26 (30.0±11.6)^a	23.5±1.3^a
ICSI + non-activation	150	43 (28.5±3.4)^b	28 (63.3±12.7)^a	2 (4.6±4.2)^b	18.0±1.4^a
IVF + IVM	191	65 (33.8±11.5)^b	39 (60.7±5.2)^a	24 (35.5±9.8)^{a}a	26.0±5.0^a
ICSI + *in vitro*	25	20 (80.0±0)^a	15 (75.0±0)^a	6 (30.0±0)^a	23.0±7.0^a

Values with different superscripts in the same column are significantly different ($p<0.05$).

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expressed d2eGFP at any culture time or embryo stage, although the cleavage rate and blastocyst rate were not significantly different from those in the ICSI group (p>0.05) (Table 2).

ICSI embryo transfer

Sixteen 8-cell to morula stage embryos obtained from culture of 4-day zygotes with 2 PN derived from ICSI with sperm not treated with DNA/liposome complex, were transferred into a day 3 recipient. The recipient was pregnant, and delivered a female piglet and a mummy on day 120 (day 0 = date of oestrus of recipient pig). The birthweight was 1765 g. The placenta was 375 g. Immediately after birth, the piglet died (Fig. 3). An autopsy indicated that all organs were anatomically normal. After transfer of the ICSI embryos derived from sperm transfected with DNA/liposome complex, of the four recipients, two had a normal cycle, one had 35 day cycle and one had 75 day cycle. But none of them maintained pregnancy to give birth (Table 3).

Discussion

Whether the spermatozoon enters the oocyte by injection or by insemination, oocyte activation is a prerequisite for decondensation of the sperm head and formation of a male pronucleus. Susceptibility to artificial activation of oocytes may vary with different species. Bovine oocytes, unlike those of other species (mouse, hamster, rabbit and human), are rarely activated by the mechanical stimulation of microinjection alone; an additional activation stimulus is therefore required during ICSI (Keefer *et al.*, 1990; Chen & Seidel, 1979). The susceptibility of pig oocytes to artificial activation may also vary with the sources of the oocyte. Extra artificial activation might not be necessary if using *in vivo* matured ova for ICSI. Martin (2000) showed that the fertilisation rate of ICSI oocytes, judged by cleavage, was about 70%, and that 38% of the oocytes cleaved formed a blastocyst. A recipient received 22 ova and delivered three live piglets. In another similar experiment attempting to produce ICSI piglets by using *in vivo* matured oocytes, only about 26% of *in vivo* matured oocytes injected with ejaculated, *in vitro* capacitated spermatozoa were fertilised. Of four recipients, only one that had received ^{32}Ca-ionophore-treated embryos remained pregnant and delivered a single piglet (Kolbe & Holtz, 2000). These two experiments demonstrated that artificial activation might be optional for the development of ICSI embryos derived from *in vivo* matured oocytes. Berardino *et al.* (2000) showed that, by using *in vivo* matured oocytes, non-activated ICSI oocytes developed to the blastocyst stage to a limited extent (0.9%), and none of the oocytes activated with ethanol developed to blastocyst stage after 7 days of culture. In contrast the oocytes activated with ionophore developed to the blastocyst stage at a higher rate (11.4%) but no ICSI piglets were reported in their experiment. In our experiment electrical activation was employed. As shown in Table 1, in the ICSI + activation group, the fertilisation rate, judged by the formation of two pronuclei, the proportion of cleavage and the proportion of embryos that developed to blastocysts, was comparable with that derived of *in vivo* oocytes that were not artificially activated, in this and other experiments (Kim *et al.*, 1998; Martin, 2000). This is also similar to the results observed following culture of *in vitro* matured/*in vitro* fertilised porcine oocytes (Abeydeera, 1997; Wang *et al.*, 1997). But without activation, the fertilisation rate of ICSI *in vitro* matured oocytes was reduced dramatically (64.8±17.3% vs 28.5±3.4%, p<0.05), and only a limited number of zygotes with two pronuclei developed to the blastocyst stage (30.0±11.6% vs 4.6±4.2%, p<0.05). Our result indicates that *in vitro* matured porcine oocytes need exogenous activation for embryo development, and that electrical activation is a suitable method for producing ICSI embryos.

To evaluate the long-term *in vivo* developmental ability of ICSI embryos derived from IVM oocytes and frozen sperm, after culture for 4 days (vs 24 h in Martin's (2000) experiments and 40 h in Kolbe and Holtz's (2000) experiments), we transferred 16 ICSI embryos at the 8-cell to morula stage derived from zygotes with two pronuclei into a day 3 recipient. Pregnancy was successfully maintained and a female piglet and a mummy with skeleton were delivered. It is thought that in pigs at least four embryos in the uterus of a surrogate mother sow are needed to induce and maintain pregnancy (Polge *et al.*, 1996). We did not induce the

Table 2 *In vitro* development of ICSI and IVF embryos derived from sperm transfected with GFP

Treatment	No. examined	Rate of cleavage (%)	Rate of blastocysts (%)	Rate of green embryos (%)
ICSI	180	102 (58.4±18.1)[a]	34 (17.8±13.1)[a]	30 (29.4)
IVF	176	76 (43.3±7.2)[a]	41 (22.7±6.4)[a]	0
DNA injection + IVF	38	23 (60.6±0.8)[a]	8 (21.1±1.5)[a]	0

Values with different superscripts in the same column are significantly different (p<0.05).

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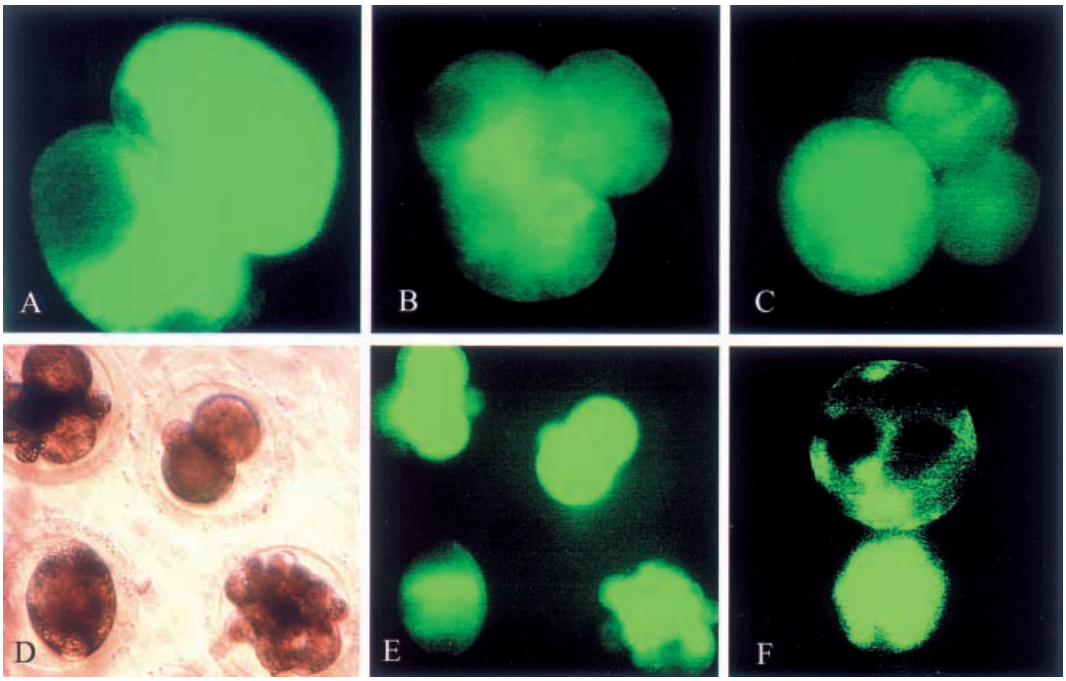

Figure 2 (*A*) A 2-cell embryo expressing d2EGFP derived from ICSI with transfected sperm 3 days after culture (×400). (*B*) A 4-cell embryo expressing d2EGFP derived from ICSI with transfected sperm 3 days after culture (×400). (*C*) An unevenly cleaved embryo expressing d2EGFP derived from ICSI with transfected sperm 3 days after culture (×400). (*D*) Poorly developing embryo derived from ICSI with transfected sperm on day 7 (bright field, ×200). (*E*) The same embryo as in (*D*) (epifluorescent field, ×200). (*F*) A green blastocyst derived from ICSI with transfected sperm on day 7. The lower green clump is the hatched part of the blastocyst (×400).

pregnancy of the pig by injection of oestradiol as Kolbe & Holtz (2000) described. This suggested that, of 16 embryos transferred, at least 4 fetuses had passed 12–15 days, which is the crucial time for maternal recognition of pregnancy. Besides the mummy, at least two other embryos may have been lost during gestation. Although the piglet died when delivered, the birthweight and placenta weight were in the normal range for a newborn piglet, and no anatomical abnormality was found. The fact that delivery was postponed for 5 days, which could be caused by insufficient signal from a single fetus to stimulate uterine contraction, might have contributed to the death.

To our knowledge, this is the first report of a piglet derived from ICSI of *in vitro* matured oocytes with frozen sperm. The successful birth of the ICSI piglet in this experiment opens the way for generation of transgenic pigs by the sperm-mediated transfer of DNA via ICSI using IVM oocytes and frozen sperm. Compared with *in vivo* matured oocytes and freshly collected sperm, the adoption of IVM oocytes and frozen sperm may dramatically reduce the financial and labor costs of producing a transgenic pig by the ICSI procedure.

Liposomes have long been used to introduce a variety of molecules into living cells. To investigate the potential use of sperm cells as vectors to transfer exogenous DNA via the fertilisation of oocytes into the germ line of mice. Bachiller *et al.* (1991) used liposomes to transfect DNA into the sperm head. Although the

Table 3 Results of ICSI embryo transfer

Sperm	Zygotes with 2 pronuclei (%)	Cleavage rate (%)	Embryos transferred	Recipient no.	Pregnancy results
DNA–	24/48 (50)	17/24 (70.8)	16	266–3	A piglet born on day 120
DNA+	36/47 (76.6)	25/36 (69.4)	24	262–2	Cycle, 21 days
DNA+	56/80 (70.0)	40/56 (71.4)	40	45	Extended cycle, 75 days
DNA+	32/42 (76.2)	16/32 (50.0)	16	93	Cycle, 20 day
DNA+	39/70 (55.7)	20/39 (51.3)	18	35	Extended cycle, 36 days

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Figure 3 The piglet derived from ICSI with IVM oocytes and untransfected frozen sperm.

DNA transfer into sperm mediated by liposomes was very efficient, they were unable to generate transgenic mice by this method.

We monitored the expression of d2EGFP by using an epifluorescence microscope in embryos that had been cultured for 2 and 7 days after sperm injection. The cleavage rate and the blastocyst rate of embryos were similar to those derived from ICSI with untransfected sperm. On the day 7, blastomeres in 29.4% of embryos expressed EGFP. But when transfected sperm were used to inseminate the oocytes, although the cleavage and blastocyst rates were high, no embryos expressed EGFP. In contrast to co-injection with sperm, injection of a similar or greater quantity of DNA/liposome complex alone could lead to high cleavage and blastocyst rates, but none of the resulting embryos exhibited EGFP expression.

The failure to produce transgenic embryos by using transfected sperm to inseminate oocytes directly might be due to the following reasons. First, not all liposome-treated sperm displayed exogenous DNA-specific staining (80%; Bachiller *et al*., 1991), and sperm without internalized DNA may be more efficient at fertilising oocytes. Second, the binding of DNA to spermatozoa appears to be a reversible process, which is stimulated by polycations and inhibited by polyanions, and unidentified factor(s) present in the seminal liquid (Lavitrano *et al.*, 1992). DNA might be lost during penetration of the zona pellucida and oocyte membrane. Third, part of the DNA bound to spermatozoa is internalised in the sperm heads and is mainly localised in the acrosome of sperm (Horan *et al.*, 1991; Camaioni *et al.*, 1992; Lavitrono *et al.*, 1992). During the acrosome reaction DNA may be lost with cytoplasm during rupture of the acrosome. By using the ICSI procedure, no matter where and how the DNA exists in or on the sperm, it can be transferred into the cytoplasm of the oocytes. Therefore, the above limitation could be overcome by the ICSI procedure.

To determine whether genomic integration could be demonstrated in live offspring, we also transferred the embryos (4-cell to morula stage, with or without GFP expression) cultured for 4 days to day 3 open recipient pigs. Unfortunately, although two of the gilts had extended cycles, none of the four recipients maintained pregnancy to delivery. The deleterious effect of GFP expression on the subsequent development of the embryos might have contributed to the failure of pregnancy in our experiment. Of 30 cleaved green embryos obtained on day 7, most of them stopped developing before the morula stage, and only 7 green blastocysts were formed. This kind of phenomenon was observed by Perry *et al.* (1999) in mice and Murakami *et al.* (1999) in cattle embryos derived from nuclear transfer. It is suggested that, to obtain GFP transgenic piglets, more embryos need to be transferred into a recipient. Other reporter genes, which have no deleterious effect on the development of embryos, might be better alternatives to further explore the feasibility of producing transgenic pig by ICSI or IVM oocytes.

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