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Developmental Ability and Transgene Expression of IVM/IVF Derived Porcine Embryos after DNA Microinjection

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DNA 미세주입 돼지 체외수정란의 발달능력과 유전자 발현

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요 약

본 연구는 체외에서 생산된 돼지수정란에 외래유전자를 미세주입후 체외 배 발달과 유전자 발현을 조사하기 위하여 실시하였다. 체외수정후 18~20 시간 사이에 LacZ 유전자와 산양 성장호르몬 유전자를 미세주입 하였으며, 체외 배 발달율과 유전자 발현은 미세주입후 9 일간 체외배양을 실시한 다음 조사하였다. 돼지수정란을 원심분리하여 전핵을 관찰한 결과 60.3%의 난자에서 전핵이 가시화 되었다. 또한 유전자가 미세주입된 수정란중 상실배와 배반포까지 발달한 비율은 각각 8.6, 9.1%로 대조구의 발달율 19.0, 20.8% 보다 유의하게 낮았다. 그러나, NCSU23 배양액에 4일간 배양후 EMEM 배양액으로 교체하여 배양한 결과, 배반포 및 부화배반포까지 높은 발달율(19.4%)을 나타내었다. X-gal 염색의 결과로서, LacZ의 발현을 나타낸 수정란의 비율은 상실배, 배반포 단계에서 40.0, 42.9%로 나타났으나, 이들 형질전환 수정란의 대부분은 mosaic 현상이 관찰되었다. 또한 PCR 분석에서, gGH 유전자가 도입된 수정란의 비율은 상실배, 배반포단계에서 45.0, 44.4%로 X-gal 염색의 결과와 유의한 차이가 없었다. 따라서 본 실험에서 얻어진 결과들은 체외에서 생산된 돼지수정란은 미세주입후에도 배반포 및 부화배반포까지 성공적으로 발달할 수 있다는 것을 입증하였다. 또한 체외성숙, 수정된 돼지수정란을 이용하여 형질전환 돼지 생산의 가능성을 시사하고 있다.

I. INTRODUCTION

Recent advances of *in vitro* maturation (IVM) and fertilization (IVF) system in livestock animals have considerably increased the availability of the embryos for the artificial manipulation of early embryo, such as production of clone and transgenic animal. However, in the pig, the progress toward efficient IVM/IVF has been limited due to a high incidence of polyspermy (Nagai, 1994; Funahashi et al., 1995; Kim et al., 1996e), and low incidences of male pronuclear formation (Yoshida et al., 1993) of *in vitro* developmemt of embryos at the early stages (Funahashi et al., 1994; Kim et al., 1996b). Kubisch et al. (1995b) demonstrated the developmental incidence of DNA microinjected IVM/IVF-derived porcine embryos reaching to the blastocyst was less than 3% with very high mosaicism.

Very recent studies have succeeded in the im-

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proving porcine IVM/IVF system. Maturing oocytes in NCSU23 medium and preincubation of oocytes with oviductal fluid have shown to decrease the polyspermic penetration and increase embryonic development following IVF (Funahashi et al., 1996; Kim et al., 1996a,c). In addition, Pollard et al. (1995) reported that dual cuture system for the porcine embryo significantly improved *in vitro* development of embryos to the blastocyst and hatched blastocyst stages. The objective of this study is to evaluate developmental ability *in vitro* and transgene expression of porcine embryos by improved IVM/IVF system.

Ⅱ. MATERIALS AND METHODS

1. *In vitro* maturation and fertilization

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 35~39℃ in Dulbecco's phosphate buffered saline supplemented with 5.54 mM D-glucose, 0.33 mM sodium pyruvate, 75 mg/ml potassium penicilin G and 50 mg/ml streptomycin sulphate (mDPBS). Oocyte-cumulus-complexes (OCC) were aspirated through an 18-gauge needle into a disposable 10 ml syringe from follicles 3 to 6 mm in diameter and washed three times with TL-HEPES medium. Groups of 50 OCC were matured in 500㎕ of NCSU 23 medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 10 IU/ml pregnant mare's serum gonadotrophin (Sigma, USA) and 10 IU/ml human chorionic gonadotrophin (Sigma) under paraffin oil at 39℃ for 44 h. After maturation, oocytes were incubated with sperm at a final concentration of 5×10^5 cells/ml for 6 h at 39℃ in an atmosphere of 5% CO_2 in air.

2. Embryo manipulation and DNA microinjection

Embryo manipulations were performed in TL-HEPES medium. Zygotes were pipetting for several times to remove the cumulus cells and centrifuged at 15,000 rpm for 5 min to displace cytoplasmic lipids. The 5.5 kb SV40-Lac Z gene and 5.5 kb MT-Ⅰ/gGH gene was isolated from vector sequences by Pst Ⅰ digestion and EcoR Ⅰ/Hind Ⅲ digestion, and was used for DNA microinjection. Microinjection was performed under DIC optic on an inverted microscope (Zeiss) fitted with a micromanipulator (Leitz) at 18~20 h after IVF. Appoximately 1~2 pl containing 1~2 ㎍/ml of DNA solution was injected into the male pronucleus of the one cell embryos.

3. *In vitro* embryo development

One group of DNA injected embryos was transferred to 500 ㎕ of fresh NCSU23 with 0.4 % BSA (Fraction Ⅴ, Sigma) and cultured for 9 days. Another group of injected embryos was cultured in NCSU23 medium for 4 days, and then transferred into a Eagle's minimal essential medium (EMEM) at the morula stage for an additional 5 days at 39℃ in an atmosphere of 5% CO_2 in air. EMEM was prepared described by Pollard et al. (1995) and contained 4g/l glucose, 2× non-essential MEM-amino acids (Gibco), 2× MEM-vitamins (Gibco), 8.2mg/l insulin (Sigma) and 20% (v/v) FCS (Gibco).

4. X-gal staining

A histochemical staining procedure for *E. coli* β-galactocidase activity was used to detect expression of microinjected LacZ gene. Embryos were rinsed three times with PBS (pH 7.2) and then fixed for 10 min at 4℃ with 0.25% glutaraldehyde in PBS. After six times washes in PBS, the embryos were incubated in staining solution consisting of 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma), 1 mM Mg-

Cl_2, 10 mM potassium ferricyanide, and 10 mM potassium ferrocyanide in PBS.

5. PCR

Transgenic embryos were identified by using PCR analysis with following gGH specific primers: 5'-TCAGACTTGGAGCTGCTTCGC and anti 5'-GCGACACTTCATGACCCTCAG. The primers were selected based on GC content. The mixture was consisted of 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 μM of each four deoxynucleotides (dNTP), 1 μM of each primers and 1.5 unit of Taq polymerase (Boehringer Mannheim, Germany). PCR analysis was performed by using an automated temperature cycler (Ericomp, Inc., USA). Amplification was repeated for 30 cycles following the first cycles; denaturation at 95℃ for 5 min, annealing at 60℃ for 1 min and extension at 72℃ for 3 min. The repeated cycles were continued for another 30 cycles with 95℃ for 1 min, 60℃ for 1 min and at 72℃ for 2 min. The PCR reaction was terminated with a 10 min extension reaction at 72℃. The PCR products were separated electrophoretically in an 0.8% of agarose gel and photographed under ultraviolet light.

6. Statistical analyses

Statistical analyses of data from at least three replicate trials were carried out by analysis of variance (ANOVA) and Fisher's protected least significant difference test. The percentage data were subjected to arcsine transformation before statistical analysis. Probability of $P<0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. *In vitro* embryo development after DNA microinjection

Pronuclei were visible in 60.3% zygotes when examined between 18 and 21 h after IVF. In 80% of them, 1 and 2 pronuclei could be seen. The remaining 20% of embryos were found to contain more than 2 pronuclei. Among microinjected embryos, 412 embryos were cleaved. Percentages of DNA microinjected embryos and non-injected embryos that developed to the blastocyst or hatched blastocyst stage in dual culture systems (NCSU23 and EMEM) were significantly higher than those in NCSU23 medium alone (Table 1, $P<0.05$). The procedure of mic-

Table 1. Effect of DNA microinjection on development of IVM/IVF embryos the during 9 days of culture in NCSU-23 alone or NCSU23-EMEM

Treatment	Culture medium	No. of embryos examined	No. (%) of embryos developed to						
			1cell	2cell	4cell	8cell	Morula	Blastocyst (BL)	Hatching & hatched BL
Injected	NCSU23	232	32 (13.8)	42 (18.1)	48 (20.7)	69 (29.7)	20 (8.6)	21 (9.1)[a]	0 (0)
	NCSU23 -EMEM	180	20 (11.1)	21 (11.7)	38 (21.1)	41 (22.8)	25 (13.9)	31 (17.2)[b]	4 (2.2)
	Subtotal	412	52 (12.6)	63 (15.3)	86 (20.9)	110 (26.7)	45 (10.9)	51 (12.4)	4 (0.9)
Non-injected	NCSU23	226	10 (4.4)	20 (8.8)	40 (17.7)	66 (29.2)	43 (19.0)	47 (20.8)[c]	0 (0)
	NCSU23 -EMEM	208	9 (4.3)	20 (9.6)	32 (15.4)	48 (23.1)	25 (12.0)	64 (30.8)[d]	10 (4.8)
	Subtotal	434	19 (4.4)	40 (9.2)	72 (16.6)	114 (26.3)	68 (15.7)	111 (25.6)	10 (2.3)

[a,b] $P<0.05$, [c,d] $P<0.05$

roinjection decreased *in vitro* development of embryos to the blastocyst stage in both culture systems ($P<0.05$).

2. Expression of microinjected gene

The expression rates of the exogenous LacZ gene microinjected embryos were summarized in Table 2. The proportions of embryos expressed LacZ were 40.0, 42.9% at the morula and blastocyst stages, respectively. But, non-injected embryos showed no staining throughout the preimplantation development. Most of embryos expressing transgene were mosaics (Table 2). After X-gal staining, the embryos showed blue with various degrees of intensity. For example, blue spots were observed in the cytoplasm of some blastomeres (Fig. 1).

PCR analysis of whole embryos following microinjection demonstrated that microinjected MT-gGH gene were integrated into the chromosome of embryos. The specific band for gGH gene were generated from all embryos at the 2~8 cell stages (Fig. 2A) but were not generated from half of embryos at the morula and blastocyst stages (Fig. 2B). Integration rates were 100% at the 2~8cell stages and 45, 44.5% at the morula and blastocyst stages, respectively (Table 3).

Table 2. The expression of LacZ gene after 9 days of culture following microinjection

Stage of embryos examined	No. of embryos examined	No. (%) of embryos expressed LacZ gene	No. (%) of mosaicism
Morula	40	16 (40.0)	14 (87.5)
Blastocyst	42	18 (42.9)	18 (100)

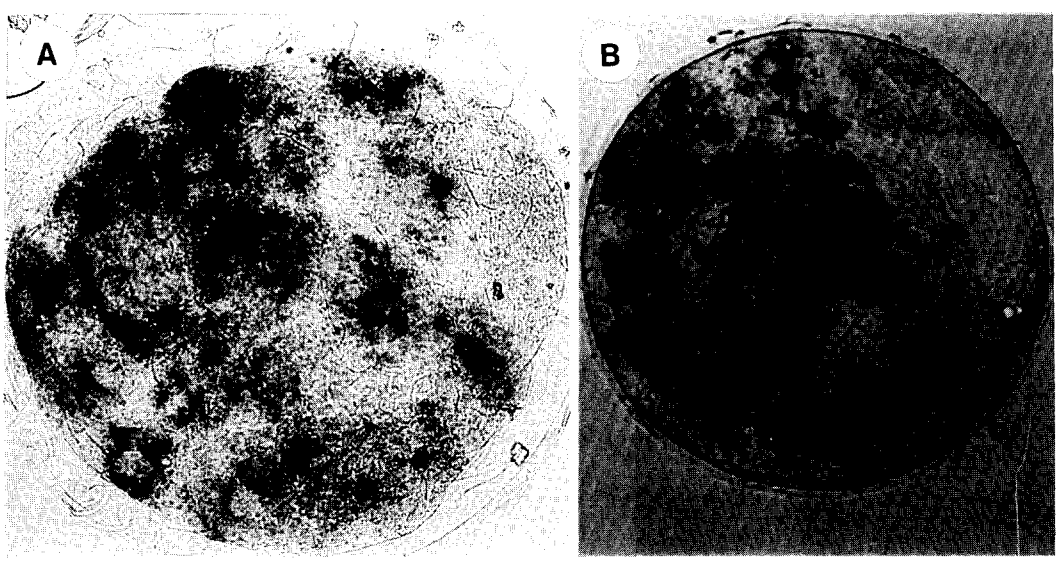

Fig. 1. The β-galactosidase detection by X-gal staining at the morulae and blastocyst stages ($\times$ 400 and $\times$200).
A. morulae, B. blastocyst

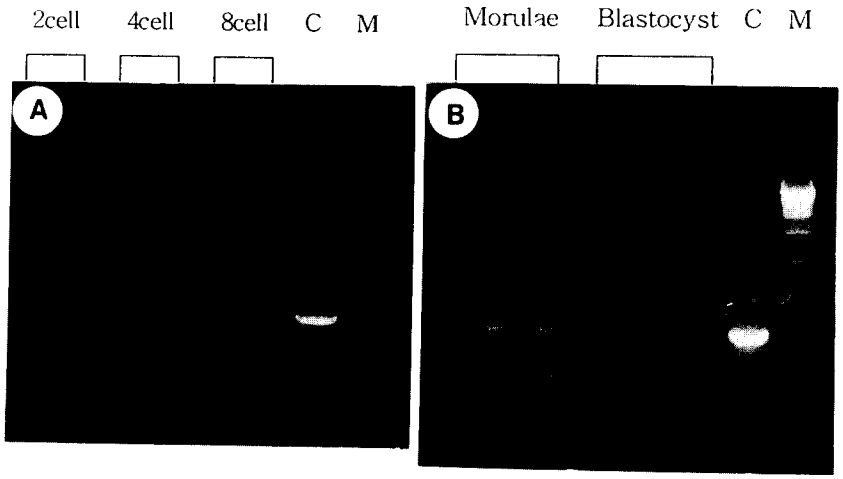

Fig. 2. Detection of microinjected gGH genes in individual embryos.
PCR products were applied individually on the wells of 0.8% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Table 3. PCR analysis of porcine embryos injected with gGH gene (%)

Stage of embryos examined	Embryos containing gGH gene
2-cell	25/25 (100)
4-cell	25/25 (100)
8-cell	25/25 (100)
Morula	9/20 (45)
Blastocyst	8/18 (44.4)

Ⅳ. DISCUSSION

In this study, we found a relatively higher rate of *in vitro* development of IVM-IVF porcine embryos microinjected with foreign gene upto the blastocyst stage. The rate was significantly higher than the previous data reported by Kubish et al. (1995b). The reason why we got the higher rate might be caused by using different culture medium, NCSU23, for oocyte maturation and *in vitro* culture of IVM-IVF derived zygotes in this experiment. The NCSU medium has been developed for the *in vitro* culture of porcine embryos by Petters and Wells (1993). A major difference of NCSU23 from other media is the presence of organic osmolytes, such as taurine, hypotaurine and sorbitol. Recently it was known that the addition of organic osmolytes to the maturation medium enhanced cytoplasmic maturation which was reflected by glutathione content and cytoskeleton assembly of oocytes at the end of maturation and by developmental rate to the blastocysts. (Funahashi et al., 1996; Kim et al., 1996d,f). Therefore, our data suggest that adoptation of a modified medium for oocyte maturation as well as *in vitro* culture of embryo may improve developmental competence and following microinjection of foreign DNA.

It has been demonstrated that the simple culture media containing BSA can support the development of pig zygote to the blastocyst stage (Menino and Write, 1982; Beckmann and Day, 1993), but limited to the continued development

and hatching of cultured blastocysts. Recently, Pollard et al. (1995) found the marked increase in developmental incidence of zygotes to the expanded or hatched blastocysts in which zygotes were cultured in CZB to the morula stage and in pig-MEM for an additional 72 h. In the present study, although we used the different culture medium, we also observed the increase in the incidence of development to the hatched blastocysts.

It has been known that microinjection of forign DNA into the porcine embryo decreased the developmental rate to the blastocyst as compared to the control (Hammer et al., 1986; Hajdu, 1994). Further more, the microinjection of DNA itself was more detrimental to embryos compared with buffer injected embryos. These results suggest that chemical and /or physical contamination of the DNA preparation were being intergrated into vital regions of the genome in such way that embryonic development was inhibited (Williams et al., 1992). In this study we demonstrated that the exogenously introduced SV 40-lacZ gene was expressed in morula and blastocyst with extremely high incidence of mosaicism. This finding was consistant with a previous report by Kubish et al. (1995b) who observed that the genes could be transferred into *in vitro* generated porcine embryos with predominantly mosaic. The exact mechanism by which DNA microinjection causes mosaicism is not known. It has been suggested that such mosaicism results from the regulatory events controlling transcription or translation that can silence expression selectively in some blastomeres and not in others (Steven et al., 1989; Kothary, 1989; Takeda and Toyoda, 1991), however, Burdon and Wall (1992) observed a mosaic distribution of an injected gene construct after PCR analysis of individual mouse blastomeres. Powell et al (1992) showed that bovine zygote have a high ability to generate large ligation products from cytoplasmically injected DNA. Such concatamerization might ultimately lead to circularization which could make the exogeneous DNA more resistance to exonuxleolytic degradation and likely to be asymmetrically distributed at cell division (Bevilacqua et al., 1992; Kubish et al., 1995a). Therefore, nonintergrated DNA may be transiently transcribed in the embryos and subsequently degraded as development progresses (Kubish et al., 1995b).

In conclusion, our results suggest that IVM /IVF porcine embryos may be able to develop in culture and express a microinjected transgene. Such as other species, transgenic embryos appear to be predominantly mosaic which is likely to be a significant obstacle to increasing the efficiency of generating transgenic pigs.

V. SUMMARY

In the present study, we investigated developmental ability and transgene expression of IVM /IVF derived porcine embryos following microinjection with SV40-LacZ. A total of 412 IVM /IVF derived embryos were used to examine developmental ability and transgene expression following DNA microinjection. After centrifugation, pronuclei were visible in 60.3% when examined between 18~21h after IVF. Development and transgene expression were assessed after 9 days in culture. The percentages of injected embryos reaching to the morula and blastocyst were significantly lower ($P<0.05$) than those of non-injected control embryos. However, the percentages of DNA microinjected embryos and non-injected embryos that developed to the blastocyst or hatched blastocyst stage in dual culture systems (NCSU23 and EMEM) were significantly higher ($P<0.05$) than those in NCSU23 medium alone. As the re-

sult of X-gal staining, the proportion of positive embryos was 40~43% in morula and blastocyst stage embryos, however, mosaicism has been observed in the most putative transgenic morulae and blastocysts. In the PCR analysis, the percentages of embryos integrated gGH gene were 45.0 and 44.4% in morula and blastocyst stage, respectively. These results suggest that improved IVM/IVF system and culture condition increased the embryo viability and expression of a microinjected transgene.

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