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Sex Determination in Somatic and Embryonic Cells of the Pig by FISH and PCR

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FISH와 PCR에 의한 돼지 체세포 및 배아세포의 성 판정

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요 약

포유동물에 있어서 조기 성 판정기술은 축산에 있어서의 성별 육종프로그램이나 인간의 X-염색체 관련 열성유전병의 산전진단 등 여러 분야에 응용될 수 있다. 초기배에 대한 성 판정은 성염색체에 존재하는 특이적 염기서열을 증폭시키는 polymerase chain reaction (PCR)과 X와 Y 염색체에 대한 특이적 probe를 이용하는 fluorescent *in situ* hybridization (FISH)에 의하여 수행될 수 있다. 1992년과 93년, 2개년도에 걸쳐 본 연구실에서 돼지의 3.3 kb 웅성특이 DNA 절편(pEM39)을 cloning하였다. 본 연구는 pEM39가 성특이 DNA probe로 이용될 수 있는지를 조사하기 위해 PCR과 FISH를 이용하였다. 돼지 난자는 도축장에서 구입한 돼지 난소로부터 채취되었고, 체외배양후 체외수정되었다. 한편 처녀발생난자를 negative control로 이용하였다. 2 세포기의 수정란을 선발한 후 PCR을 통하여 DNA를 분석한 결과, 10개의 수정란 중 6개는 자성, 다른 4개는 웅성으로 판정되었으며. FISH를 수행한 결과, clone된 웅성특이 DNA 단편은 돼지 간조직과 초기배에서 웅성특이성을 보였다. 또한 FISH와 karyotyping을 수행한 결과 clone된 웅성특이 DNA 단편이 Y 염색체 q-arm의 heterochromatic region에 위치함을 알 수 있었다. 이러한 결과로 보아 clone된 웅성특이 DNA 단편이 초기배의 성을 조기판정하는데 있어 유용하리라 사료되며, PCR에 의한 초기배의 성 판정에 있어 신뢰할만한 지표가 될 수 있을 것이다.

I. INTRODUCTION

Control of the sex in domestic animals has significant effects on animal production. There have been many attempts to determine sex at the prenatal stage with limited success. Previous methods include immunological techniques using H-Y antigen (White et al., 1983) and biochemical analysis based on X-linked enzyme activity (Rieger, 1984; Williams, 1986). However, these methods fail to produce reliable results and require whole embryos for the analysis; thus, no offspring can be produced.

With the aids of recent development of molecular biological technologies, species- and Y chromosome-specific DNA repeats have been identified and some sequences analysed in many mammals including mouse (Nallaseth & Dewey, 1986; Nishoka & Lamothe, 1986), human (Coo-

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ke, 1976; Kunkel et al., 1976), pig (McGraw et al., 1988; Mileham et al., 1988; Jeon et al., 1994) and cattle (Ellis & Harpold, 1988; Reed et al., 1988). These repetitive sequences have been used as molecular probes in determining sex of preimplantation embryos either by amplification of specific sequences using polymerase chain reaction (PCR) or by fluorescent *in situ* hybridization (FISH). Such methods have been successfully applied to determining sex with a small number of blastomeres biopsied from early stage embryos (Handyside et al., 1990; Kunieda et al., 1992; Chong et al., 1993); thus, production of offsprings are possible with remaining embryos by subsequent *in vitro* development and transfer. Although PCR and FISH have been shown to be reliable and efficient, there still remain some limitations in application. Amplification failures are often found due to contaminations which plague polymerase chain reaction. In the second, it is impossible to determine the number of sex chromosomes; thus, the same results would be obtained in cases of numerical chromosomal anomalies and normal males. Although FISH allows direct visualization of DNA sequences of interest in metaphase or interphase nuclei, FISH failures are also possible, and they are more common in fetal cells than in adult cells (Grao et al., 1993).

Previously, we have cloned a male-specific porcine DNA fragment of 3.3 kb (pEM39). Its male specificity was demonstrated by Southern and *in situ* hybridizations (Jeon et al., 1993, 1994). In this report, we employed PCR to determination of sex in preimplantation porcine embryos to evaluate applicability of pEM39 as a male-specific-probe. FISH was also applied to verify the sex determination procedure and to localize the male-specific probe.

Ⅱ. MATERIALS AND METHODS

1. Embryo preparation

Porcine ovaries were obtained from a local slaughter house and transported to the laboratory in 0.85% (w/v) saline. The cumulus-oocyte complexes were collected from follicles with 3~5 mm in diameter and matured *in vitro* (IVM) in NCSU23 (Petters and Reed, 1991) supplemented with 10% (v/v) porcine follicular fluid (pFF), 10 IU PMSG/ml, 10 IU hCG/ml, and 0.57 mM cysteine (Sigma Chemical Co., St Louis, MO, USA) for 44 h at 39℃ in an atmosphere of 5% CO_2 in air. Matured oocytes were fertilized *in vitro* (IVF) with porcine spermatozoa by the method of Kim et al. (1996). At 48 h after IVF, 2-cell embryos showing a healthy aspect were selected for further analysis.

2. Parthenogenetic activation of oocytes matured *in vitro*

Following IVM, the oocytes were suspended in 3% (w/v) sodium citrate solution to disperse cumulus cells and were freed from the cells by vortexing. Denuded oocytes were washed 3 times with TCM 199 containing 15% (v/v) FCS, incubated for 10 min at 39℃ in TCM 199 containing 15% (v/v) FCS and 10% (v/v) ethanol to induce parthenogenetic activation (Kim et al., 1994).

3. PCR amplification

Oligonucleotide primers used in this experiment were described previously (Jeon et al., 1995) and these amplify a 288 bp region on pEM39. This primer set is composed of PMS5 (5′-TCAGGCTCCCAAGCTCAATT-3′:sense primer) and PMS6 (5′-GGGCATTTTCAGCTCCACAC-3′ : antisense primer).

To each tube cotaining the 5 ㎕ of sample, 45 ㎕ of reaction mixture were added and overlaid

with 50 μl of mineral oil. The reaction mixture consisted of 1x PCR buffer (10 mM Tris-HCl pH 8.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.01% BSA), 100 μM of each dNTP (Promega), 50 pmol of each oligonucleotide primer, 2.5 units of *Taq* DNA polymerase (Promega). The PCR amplification was performed in a programmable DNA thermal cycler by 40 cycles of denaturation (94℃, 1 min), annealing (55℃, 1 min) and extension (72℃, 1 min) for 10 pg of genomic DNA sample (Kudo et al., 1993; Jeon, et al., 1995). Finally, samples were held at 72℃ for 5 min and cooled to 4℃.

The thermal condition for the embryonic sample was modified (Handyside et al., 1990; Guido et al., 1992; Kunieda et al., 1992; Jeon, et al., 1995). After 20 cycles of denaturation (94℃, 1 min), annealing (55℃, 1 min) and primer extension (72℃, 1 min), then 1 μl of the first amplified products was added into a freshly prepared PCR mixture. The second 40 cycles of PCR was performed under the same condition as that of the first amplification.

Two-cell embryo activated parthenogenetically and liver DNA from male and female pigs were used as controls. Amplification products (10 μl) were run on a 2% agarose gel stained with ethidium bromide and visualized on a UV transiluminator.

4. FISH onto the paraffin-embedded sections

Paraffin-embedded sections of porcine liver tissue were obtained by the method of Brahic and Ozden (1992). The 3.3 kb *Eco*RI-fragment of pEM39 was labelled with DIG-dUTP. The sections were treated with 30 μl of 100 μg/ml proteinase K and fixed in 0.4% formaldehyde for 5 min at 4℃. The 10 μl of probe-cocktail (1x Denhart's solution, 5% dextran sulfate, 0.2 mg/ml of sonicated salmon sperm DNA, 4x SSC, 50% formamide and 1 μg DIG-labelled probe/ml) was layered onto each tussue section. Tissue slides were allowed hybridization for 12 h at 42℃. Washing and detection of hybrids was performed as follows : two washes for 5 min in 2x SSC at RT, followed by 10 min in 0.1x SSC at 42℃; a dip into buffer 1 (100 mM maleic acid pH 7.5, 150 mM NaCl); treatment with 20 μl of buffer 2 (1% blocking reagent in buffer 1) for 15 min at 37℃; a wash in buffer 1; treatment with 200 μl of 1:100 diluted with anti-DIG-fluorescein Fab fragment for 1 h at 37℃; three washes in buffer 1 for 1 h. Finally, slides were counterstained with 15 μl of antifade solution containing 1 μg/ml of propidium iodide. Slide were analysed by fluorescence microscopy.

5. Blastomere fixation and FISH

Detailed procedures of blastomere isolation have been carried out as described previously (Lee et al., 1995). Briefly, the zona pellucida was removed by an acid Tyrode solution treatment. Zona-free embryos were then incubated in Ca^{2+}- and Mg^{2+}-free PBS supplemented with 4 mg/ml bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA) to isolate blastomeres.

Blastomeres were spread and fixed, following the method of Tarkowski (1966) with modifications as described by Santaló et al. (1986). Blastomeres were fixed in a few drops of absolute methanol, and refixed in a methanol : glacial acetic acid (3:1). Prior to hybridization, slides were treated with 10 μl of 20 μg/ml RNase A for 1 h at 37℃, washed four times in 2x SSC at RT for 2 min each, dehydrated through a cold ethanol series (70, 85, 100%) for 2 min each, air-dried and processed immediately for FISH. Slides were denatured at 70℃ for 5 min in 70% formamide in 2x SSC, and rinsed in cold 70, 85 and 100% ethanol for 2 min each. Probe-cocktail was denatured at 70℃ for 5 min and placed im-

mediately on ice for longer than 2 min. Hybridization was caried out in 20 μl of the probe cocktail for 12 h at 42℃. Washing and detection of hybrids was performed as follows : three washes in 50% formamide in 2x SSC and in 2x SSC at 45℃ for 10 min each; treatment with 25 μl of 1:100 diluted with anti-DIG-fluorescein Fab fragment for 30 min at 37℃; two washes in 2x SSC at 45℃ and in 0.1% NP-40 in 2x SSC at RT for 5 min each. Finally, slides were counterstained with 15 μl of antifade solution containing 1 μg/ml of propidium iodilde. Slide were analysed by fluorescence microscopy.

6. Localization onto the metaphase plate

The metaphase plate were prepared from lymphocytes through *in vitro* culture method (Gosden et al., 1992). C-banding was performed using the method of Summer (1972). For the localization of pEM39 sequence, FISH was carried out to the metaphase plates (Tomsen et al, 1992). Briefly, slides were incubated in RNase (10mg/ml). Metaphase spaeads were denatured at 38℃ for 16 h with about 100 ng of probe in 50 μl of hybridization solution. Primed *in situ* hybridization (PRINS; Tomsen et al, 1992) was performed onto the metaphase plate for a rapid localization. PRINS labelling was carried out by incubating the metaphase slide for 5 min at 95℃ in a heating block (Lab-line, II, USA) with 50 μl of reaction mixture containing 500 pmols of PMS1 (5′-GGTCCCTTATGTGTAGGTGG-3′; Jeon et al., 1995) or pEM39 fragment digested with *Alu* I and *Hae*III, 10 nmol each of dATP, dCTP, dGTP, 2.5 nmol of DIG-11-dUTP, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% BSA and 1 unit *Taq* DNA polymerase. The slide was then incubated at 55℃ or 70℃ for 1 h in a humid chamber. The reaction was terminated by washing in 50 mM EDTA (pH 8.0) for 5 min at 60℃ and twice for 1 h with TN buffer (4x SSC, 0.1% Tween 20) at RT.

Ⅲ. RESULTS AND DISCUSSION

The two step PCR method has been used for the detection of Y-specific single copy genes such as *SRY and ZFY* (Giudo et al., 1992; Kunieda et al., 1992; Handyside et al., 1990). In this experiment with th embryonic sample, the two step PCR method was used to enhance amplification signals. Among 10 samples analysed, four male-specific amplification signals were identified whereas no signals were produced in both female samples and parthenogenetically activated embryos (Fig. 1). Amplification products were of expected size (288 bp). It has been suggested that misclassification be often associated with amplification failure. In this study, inclusion of parthenogentically activated embryos could eliminate possibility of amplification failure; thus, confirming successful PCR. Although autosomal (gender-common) sequences could be included in the parallel PCR to verify the presence of DNA even in the absence of Y-specific sequences (Takahashi et al., 1992; Miller et al.,

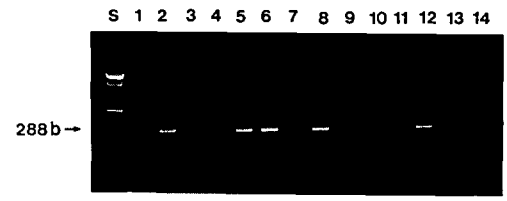

Fig. 1. PCR amplification of the embryonic samples. Lane s, size marker (100 bp DNA ladder; GIBCO BRL); 1 to 10, IVF 2-cell stage embryos; 11, 10 pg of purified female DNA; 12, 10 pg of purified male DNA; 13, 2-cell embryo activated parthenogenetically; 14, negative control (no DNA sample). 288 b, 288 bp product. Sample 2, 5, 6 and 8 were male embryos.

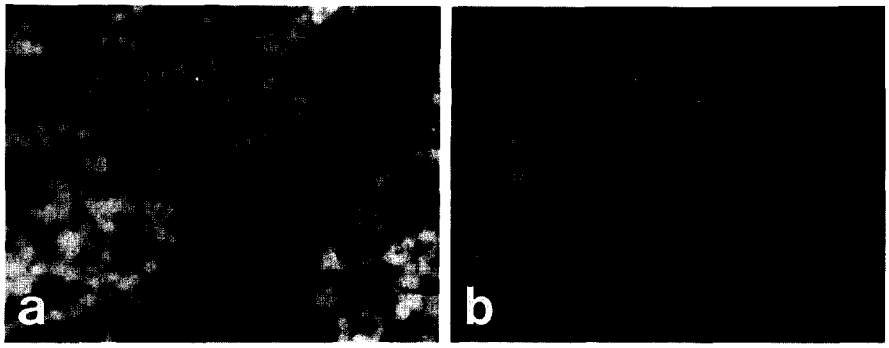

Fig. 2. FISH onto the liver cell plates of male and female. a, male; b, female. ×200.

1993), our results demonstrate at least high sensitivity and specificity of sex determination methods by PCR.

FISH has also been widely used to determine sex of preimplantation embryos (Munné et al., 1994) and offsprings (Agrawala et al., 1992). In this study, FISH was applied to refine the sex determination procedure and to localize the male-specific probe. Results confirmed specificity of the male-specific DNA (pEM39) in FISH as DIG-labelled pEM39 probe hybridized to the liver tissues of male and female revealed the specificity to the male cells (Fig. 2a). No positive signal was shown in female tissues (Fig. 2b).

FISH was also used in sexing embryos. A total of 18 blastomeres were obtained from 9 embryos, 2 blastomeres per embryo, and chromosome spreads prepared (Fig. 3). Positive hybridization signals were appeared in 3 embryos which were classified as male, while no hybridization was apparent in 5 embryos (Fig. 3). In one embryo, the results of FISH were contradictory; while one blastomere was identified as male, the other was classified as female. However, these results indicate that the pEM39 DNA sequence is male-specific although the results of FISH were contradictory in one embryo.

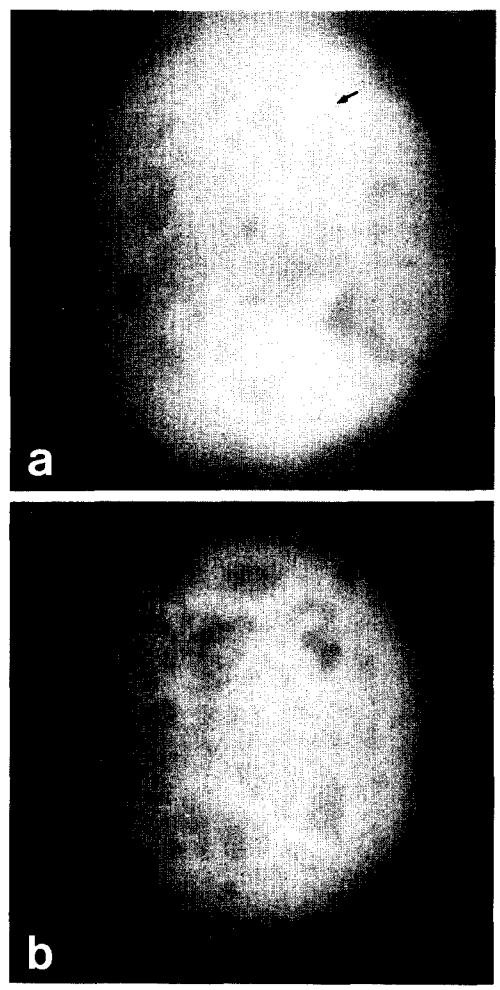

Fig. 3. FISH onto the embryonic cells. a, male; b, female. ×200.

It was possible to detect signals from a single male cell by FISH. This may be due to that pEM39 suquence exists only in male DNA or has more highly repeats on the male genome than female. It is thought that pEM39 sequence might be only located on the Y-chromosome considering differences between male and female genome in the constitution of sex chromosome.

To obtain metaphase chromosome for the karyotyping and localization of the cloned sequence, lymphocytes were cultured *in vitro*. The lymphocyte has large nucleus comparing to other cell types and is easily activated by mitogens such as phytohematoglutinin during culture period. The Y chromosome is easily identifiable due to its relatively small size. Thus, it could be easily discriminated after FISH. After C-banding of early metaphase chromosome, strong bands were shown in an entire long arm of Y chromosome (Yq region; data not shwon). As shown in Fig. 4, a positive signal was shown in male metaphase plates whereas no signal was identified in female ones. The signal was derived from distal arm of Yq region which was proved as heterochromatic region in the previous emperiments. Thus, pEM39 sequence must be located to the heterochromatic region of Yq.

PRIN is based on sequence specific annealing of an unlabelled oligonucleotide *in situ*. In this study, the PRINS was employed to verify the specificity of the pEM39 sequence. While pEM39 sequence digested with *AIu* I and *Hae*III hybridized only to the Y chromosome (Fig. 5a), the PMS1 (Jeon et al., 1995) oligonucleotide recognized sequences located on the Y chromosome as well as autosomal centromeric regions although intensity of the signals were weak (Fig. 5b). It is likely that the appearance of autosomal signals came from non-specific amplification of the primer under the low stringent condition at 55℃. At higher stringency (at 70℃) and using longer probes, a signal was obtained from the Y chromosome only. The results suggest that pEM39 is located only on the Y chromosome. This was consistent with the previous FISH experiments using random labelled probe. This result is in agreement with that of Tomsen et al. (1992) using PDYZIS sequence from McGraw's sequence (McGraw et al., 1988) as a PRINS porbe.

In conclusion, the entire sequence of pEM39 is Y-specific as shown by PCR and PRINS. Results also indicate that methods used in this stud-

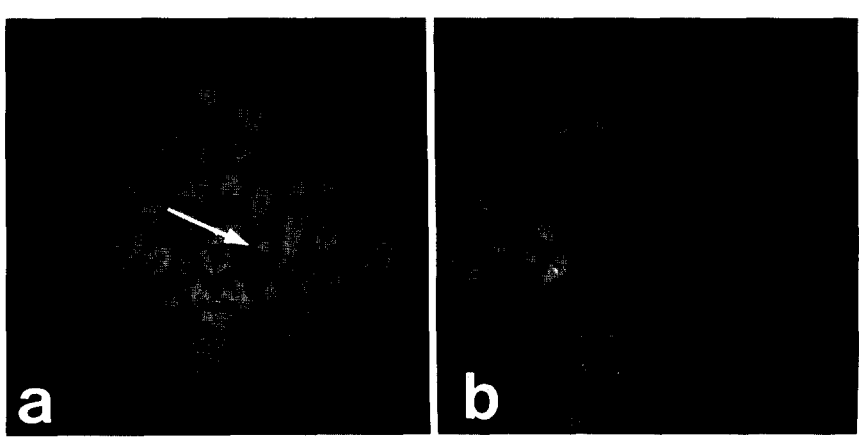

Fig. 4. FISH onto a metaphase plate. a, male; b, female. A white arrow indicates a positive signal on the Y chromosome. ×1,000.

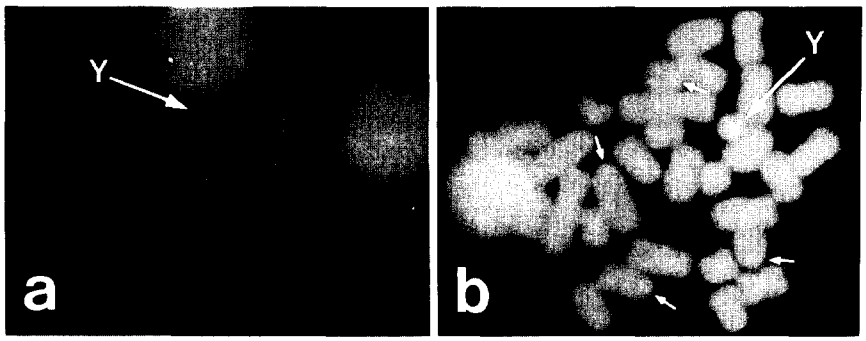

Fig. 5. PRINS onto the male metaphase plates. a, PRINS using long probe (pEM39 cut with *Alu* I and *Hae* III); b, PRINS using short probe (PMS1). White arrows indicate positive signals. ×1,000.

y can be applicable to the sexing of the porcine embryos with high efficiency. Currently, we have been using gender-common sequences in PCR to minimize incidence of misclassification. If sex determination of embryos are carried out at the preimplantation stages, assisted reproduction procedures can be more efficient.

Ⅳ. SUMMARY

Predetermination of sex in mammalian species has many aspects of application including the prenatal diagnoses of genetic disorders in humans and sex-selected breeding programs in the animal industry. Embryos sexing can be carried out using the polymerase chain reaction (PCR) to amplify specific sequences present in the sex chromosomes, or by fluorescent *in situ* hybridization (FISH) of specific probes to the X and Y chromosomes. A 3.3 kb porcine male-specific DNA fragment (pEM39) was cloned previously in our laboratory. In this study, FISH and PCR methods were employed to examine if the pEM39 can be used a sex-specific DNA probes. Porcine ovaries were obtained from a local slaughter house and oocytes collected. All oocytes were subjected to *in vitro* maturation followed by *in vitro* fertilization. Parthenogenetically activated embryos were served as a negative control. Embryonic samples were collected at the 2-cell stages and PCR was performed to analyze DNA. Among 10 embryos examined, four embryos were identified as males and six were females. The cloned male-specific DNA fragment showed male-specificity for the cells in the liver tissue and the porcine early embryos by FISH. It was also demonstrated that the cloned male-specific DNA is localized on the heterochromatic region of the long arm in the Y chromosome (Yq) as shown by the FISH and karyotyping. The results suggest that the cloned male-specific DNA fragment may be useful for predetermination of sex with a few embryonic cells. The porcine male-specific sequence can be a reliable index for embryo sexing by PCR.

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