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Production of Transgenic Murine Embryos using Haploid Spermatids Transfected with EGFP Gene

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ABSTRACT

In this study, the production of transgenic embryo was attempted by microinjection of round spermatid cultured with foreign DNA. At first, the expression of haploid spermatids specific gene, mTP1 in mouse and hPrm2 in hamster spermatids were investigated by RT-PCR method in testes of young mice and hamster testis. The specific gene expression first appeared at 18 days post partum (dpp) in mice spermatid and 20 dpp in hamster spermatid. Therefore, the round spermatids isolated from 17 dpp mice and 19 dpp hamster were used for the introduction of foreign EGFP gene into haploid round spermatids. For the introduction of EGFP gene haploid round spermatids suspended in medium including EGFP gene were treated with a different electric field strength at 0.11, 0.18 and 0.44 kV/cm. After electrical stimulation, viability of testicular sperm cells and 67.6%, 66.4% and 49.9%, in mice and 62.6%, 57.9% and 27% in hamster, respectively. These values were significantly lower than those of non-treated control groups 80.5% in mouse and 69.1% in hamster. After 72 hrs culture, the highest expression rate of EGFP gene, 28.5% in mice and 32.1% in hamster were obtained from the spermatogenic cells electroporated by the field strength of 0.18 kV/cm. Then, the ability of fertilization and embryonic development of haploid spermatids transfected with foreign EGFP gene were estimated by the microinjection of spermatids into hamster oocytes. The male pronuclear formation rate (77.5%) was lower than non-treated control (80%), and the cleavage rate of the treated group (58.8%) was lower than control (65%). To prove the foreign EGFP integration in hamster embryos, 2-cell stage hamster embryos were subjected to the observation under the fluorescence microscope, and the PCR analysis. As a result, about 44% of 2-cell embryos were showed the integration of EFGP gene into their genome. Therefore, These results suggest the possibility to produce transgenic hamsters by microinjection of haploid spermatid transfected with foreign DNA.

(Key words : Haploid spermatids, EGFP gene, Mouse, Hamster, Electroporation, Microinjection)

Ⅰ. INTRODUCTION

Recently, of the various techniques, the use of sperm cells as vector vehicles for genetic transformation is remarked in the respect of transgenic efficiency and easy handing method. When DNA is added to the cell culture system, the DNA is integrated into the genome of the cells, expressed and transmitted to the progeny, producing new stable

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lines (Pellicer et al, 1980).

It was first reported that rabbit spermatozoa were enable to take up naked DNA present in the incubation medium (Brackett et al., 1971). Years later, Lavitrano et al. (1989) reported that mouse and porcine spermatozoa incubated with naked DNA could serve as vectors for introducing foreign DNA into ova and producing transgenic mice and pigs. The methods of production of transgenic animals were promoted by the development of IVF and ICSI techniques. In addition, Perry et al. (1999) reported that the skill using damaged sperm membrane interacting foreign DNA could be used to produce transgenic mice. However, if it is not able to use of particular aided devices to transport of DNA (such as electroporation and coprecipitation of DNA with calcium phosphate), transfection efficiency is quite low (Yanagimachi et al, 1995; 1998, Kimura et al., 1995). Thus, new technique for effective transport of foreign DNA is demanded to produce the transgenic animal efficiently.

In the spermatogenesis, diploid spermatogonia divides mitotically to provide a population of spermatocytes that proceed through meiosis to haploid spermatid. Specifically, when pachytene spermatocytes were divided into haploid round spermatids, binding histone proteins as nucleo proteins were substituted into protamines by mediated transition protein and compaction of spermatogenic cell (Green et al., 1994; Patricia et al., 1987). At this point, we have a confidence that introduction of foreign DNA may results in effective integration of foreign DNA into germ line.

The establishment of *in vitro* culture system is needed to facilitate the identification and characterization of the factors and genes that induce germ cell proliferation, meiosis and spermiogenesis. Many investigators conformed that the isolated male germ cells could be cultured *in vitro* for short -term period using simple combined medium (Joshi et al., 1990). Actually, Hue et al.,(1997; 1998) cultured rat germinal and sertoli cells for 3 weeks in coculture system. When *in vitro* culture of spermatogonia and spermatocytes with foreign DNA were successfully performed, spermatids and spermatozoa transfected with foreign DNA are able to be used as vector for DNA delivery into oocytes (Ogura et al., 1995).

These studies were carried out to confirm the possibility that haploid spermatids could be used as vector carrying foreign DNA into oocytes. For this purpose, haploid spermatids were separated from testis a day before appearance of haploid spermatids. The spermatids electrically were stimulated in medium containing foreign DNA, cultured and injected into cytoplasm of oocytes. And then the integration of foreign DNA into embryos was investigated by PCR.

Ⅱ. MATERIALS AND METHODS

1. Extraction of RNA from Testicular Tissue

Tissue tearers were homogenized in Trizol (Gibco/BRL), the supernatant was placed at room temperature for 10 min and centrifuged at 12,000 rpm for 5 min at 4℃. The upper phase was transferred to a new 1.5 ml tube, added 0.5 ml of chloroform, and then vortexed. The upper phase separated by the centrifugation, was transferred to a fresh 1.5 ml tube, added 0.4 ml of isopropylalcohol, inverted for 2 min, and then subjected to centrifugation. The supernatant was decanted, and the precipitated total RNA was reprecipitated with 1 ml of 70% ethanol, and dissolved in water treated by diethylpyrocarbonate (DEPC). To analyze its purity and concentration, the ratio of A260/A280 was measured with a spectrophotometer (Beckmann, CA).

2. RT-PCR

First strand cDNA was synthesized from 2 μg total RNA and supernatant of testicular cells with

200 U Superscript Ⅱ Reverse Transcriptase (Gibco/BRL) and random hexamers in a reaction volume of 50 μl after treatment with DNase Ⅰ and then the volume was adjusted to 20 μl. PCR was carried out in a total volume of 50 μl using a Gene Amp PCR System 2400 Thermal Cycler (Perkin-Elmer, CT). Each reaction material contained 1 μl cDNA solution, 1 μl of each primer, 5 μl of 10×PCR buffer, 4 μl of 2.5 mM dNTPs, 1~2 units of EX Taq, and water to 50 μl. Primers sequence for each cDNA are as follow; mTP1, upstream primer 5'-CATG-AGGAGAGGCAAGAAC-3'; down stream primer 5'-GACTG GCATTTACCCACTC-3' (272bp). hPrm 2, upstream primer 5'-CATGGAC GTGAAGAGC-AAGG-3'; downstream primer 5'-CCTCCTGCGT-GATCT TCTG-3' (220 bp). The amplification profile for mTP1 gene consisted of 30 cycles of following three steps: at 94℃ for 30 sec (dissociation), at 61℃ for 30 sec (annealing) and at 72℃ for 1 min (extension), and for hPrm2 gene: at 94℃ for 30 sec (dissociation), at 55℃ for 30 sec (annealing) and at 72℃ for 1 min (extension). RT-PCR products were separated on a 2% agarose gel.

3. Preparation of EGFP Gene

pEGFP-N1 (Clontech, Palo Alto, CA) was isolated using Miniprep kit (Nucleogen) according to the manufacturer's protocol and linearized by EcoO109 Ⅰ restriction enzyme. Vector contained the gene encoding EGFP and neo R under the regulation of separate promoters.

4. Culture after Electoporation of Spermatogenic cells

Testes were removed from the 17 days old male ICR mice and 19 days old Syrian golden hamsters, and then cut into small fragments with a pair of fine scissors. These small fragments were treated with 0.5 mg/ml trypsin and 100 μg/ml DNase Ⅰ (Sigma. U.S.A.) in PBS for 10 min, centrifuged and then washed twice in PBS. These fragments were removed by filteration through a 30 μm nylon mesh. And sedimented cells were resuspended in DMEM/F12 medium. The linearied EGFP gene (20 μg/ml) was added to the cells suspension of 3×10^6 cells/ml and mixed. The cells suspension containing EGFP gene was transferred sterile disposable electroporation cuvettes and were subjected to electric stimulation one or more times at the desired voltage. The electroporated spermatogenic cells were cultured for 72 hrs in DMEM/F12 medium supplemented with 10% FBS, 10 μg/ml insulin, 5 ng/ml sodium selenite, 10 μg/ml transferrin, 50 ng/ml FSH, 10^{-7}M testosterone and 100 U/ml penicillin and 100 μg/ml streptomycin in 4 well dish at 32℃ in a humidity-saturated atmosphere of 95% air and 5% CO_2. The viability of spermatogenic cells were investigated by the 0.4% trypan blue exclusion test.

5. Preparation of Hamster Oocytes

The 5~8 weeks old female Syrian golden hamsters were superovulated by sequential injection of 30 IU PMSG and 30 IU hCG by 48 hours interval. Ooctyes were collected from oviducts at 15 hrs after hCG administration using hepes buffered Tyrode medium, freed from cumulus cells by treatment with 0.1% hyaluronidase, and then washed three times in Tyrode medium. The oocytes were transfected to each 50 μl drop of medium under paraffin oil at 37℃, 5% CO_2 in humidified atmosphere.

6. Microinjection of Haploid Spermatids into Oocytes

The cell suspensions were prepared in microinjection medium. A row consisting of 2 round droplets and 1 elongated droplet were placed along the centerline of the dish. An individual round spermatid was picked up from the droplet of spermatogenic cells by aspirating at the bottom of the

dish and then it was moved to the droplet containing the oocytes to be injected. An oocyte was held to the holding pipette and the injection pipette containing a round spermatid was inserted into the ooplasm of oocytes. At this time, moderate vacuum was established in order to rupture the oolemma and suck some of the ooplasm into the injection pipette. The aspirated ooplasm and round spermatid were expelled into the ooplasm with a minimum volume of medium.

7. PCR Analysis of Embryos

Embryos for PCR analysis were washed three times in sterile saline. Before PCR analysis, embryo samples were repeatedly frozen in liquid nitrogen and thawed in room temperature three times. Each reaction material contained 1~2 μl cDNA solution, 1 μl of each primer, 5 μl of 10 Ex Taq buffer, 4 μl of 25 mM dNTPs and 0.5 μl of Ex Taq. EGFP, upstream primer 5'-GACCACATGAAGCAGCACGA-3'; downstream primer 5'-TGCTCAGGTAGTGGTTGTCG-3' (345bp). The amplification cycle was 35 cycles of following three steps: 94℃ for 30 sec (dissociation), 60℃ for 30 sec (annealing), and 72℃ for 30 sec (extension). PCR products were separated on a 2% agarose gel.

8. Statistical Analysis

Each data was analyzed by T-test. The value of $P<0.05$ was considered to be statistically significant.

Ⅲ. RESULTS

1. Appearance Time of Haploid Spermatids in Mouse Testis

To identify the appearance period of *in vivo* haploid cells in mice, testicular tissues were obtained from 14 to 20 dpp mice. Each testis was subjected to mRNA analysis and RT-PCR with mTP1 gene known to be expressed at haploid stage. As a result, the specific band of 272 bp size indicating mTP1 was detected from amplified products of testicular tissues obtained from 18 to 20 dpp mice, but no band was observed from the testicular tissue of 14 to 17 dpp mice suggesting that spermatids in testis of mouse older than 18 days are haploid (Fig.

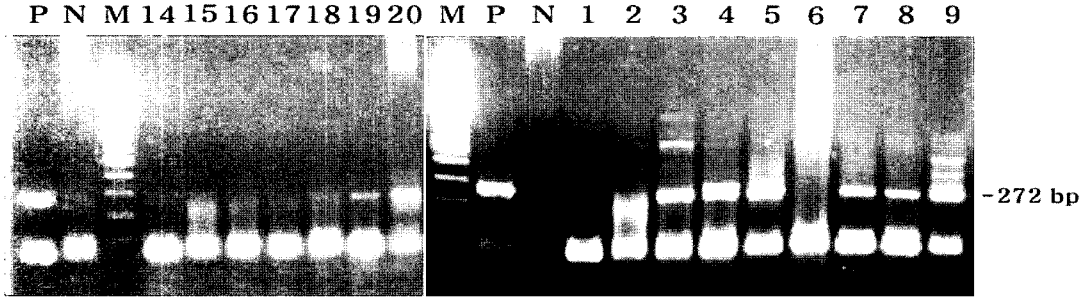

Fig. 1. RT-PCR results showing *in vivo* (A) and *in vitro* (B) appearance time of haploid spermatids cells in mice. (A) Lanes 14~20; testicular tissue from 14 to 20 dpp. (B) Lane 1~5; 16 dpp. Lane 1; 0 day cultured testicular cells, Lane 2; 1 day cultured testicular cells, Lane 3; 2 days cultured testicular cells. Lane 4; 3 days cultured testicular cells, Lane 5; 4 days cultured testicular cells. Lane 6~9; 17 dpp. Lane 6; 0 day cultured testicular cells, Lane 7; 1 day cultured testicular cells, Lane 8; 2 days cultured testicular cells, Lane 9; 3 days cultured testicular cells. M: 100 bp. P: positive control. N: negative control.

1A).

On the other hand, testicular tissues obtained from 16 to 17 dpp mice were cultured *in vitro* to confirm the appearance time of haploid spermatids *in vitro*. However, no differences in appearance period of mTP1 in testicular tissues was observed between *in vivo* and *in vitro* culture system (Fig. 1B). As shown in Fig. 1B, bands showing mTP1 appeared in the tissues obtained from 18 days dpp mouse even in *in vitro* culture system indicating that mice haploid spermatids appeared at 18 days dpp both *in vivo* and *vitro*.

2. Appearance Time of Haploid Spermatids in Hamster Testis

Identification of haploid cells *in vivo* in hamsters were conducted by the same method used in mice. Tissues of *in vivo* testis were obtained from 16 to 23 dpp hamsters, transferred to mRNA and subjected to RT-PCR with the hPrm2, which is known to appear at haploid stage. As a result, amplified products of testicular tissues obtained from hamster 20 dpp showed the specific band at 220 bp size but those prior to 20 dpp were not. Therefore, it could be said that haploid spermatids appear in the post 20 dpp hamster *in vivo* (Fig. 2A).

In next, *in vitro* cultured testicular tissues obtained from 16 to 21 dpp hamster were also used to identify haploid spermatids cells. mRNA of hPrm 2 were confirmed at 220 bp size in testicular tissues obtained from 17, 18, 19, 20 and 21 dpp hamster. Testicular tissues obtained from 17 and 18 dpp hamster were cultured *in vitro* for 2 and 1 day, respectively. These results suggest that haploid spermatids appear at 18 dpp hamster both in *in vivo* and *vitro* (Fig. 2B).

3. Viability of Spermatogenic Cells Transfected with EGFP Gene in Different Strength of Electrical Fields

The viability of mouse and hamster spermatogenic cells transfected with EGFP gene (20 μg/ml) in different strength of electric field is shown in Fig. 3. The viability was measured by 0.4% trypan blue exclusion test in complete medium at 4℃. As shown in Fig. 3, the viability of mouse testicular spermatogenic cells at 0 hr was 80.5% in non-electroporated group, whereas those of electroporated group were 68.7%, 66.4% and 49.9% of the 0.11, 0.18, and 0.44 kV/cm, respectively (Fig. 3A). On the other hand, the viability of hamster testicular spermatogenic cells at 0 hr were 69.1% in non-elec-

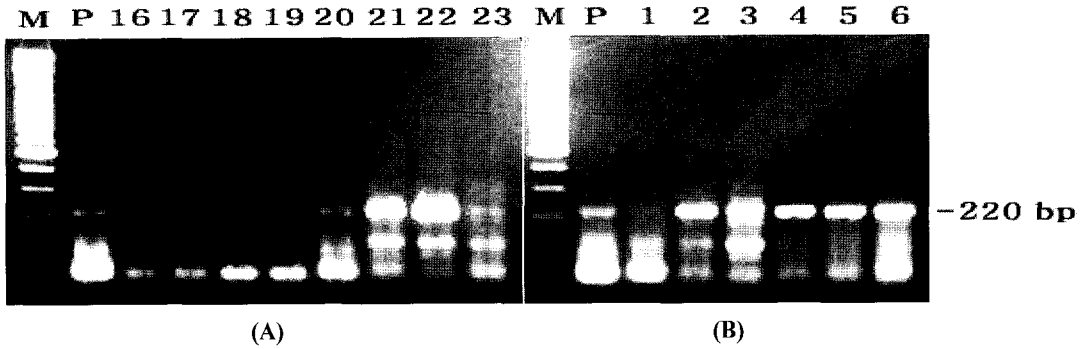

Fig. 2. RT-PCR results showing *in vivo* (A) and *in vitro* (B) appearance time of haploid spermatids in hamsters. (A) Lanes 16~23; testicular tissue from 16 to 23 dpp. (B) Lane 1~6; 2 days cultured testicular cells. Lane 1; 16 dpp, Lane 2; 17 dpp, Lane 3; 18 dpp, Lane 4; 19 dpp, Lane 5; 20 dpp, Lane 6; 21 dpp. M: 100 bp. P: positive control.

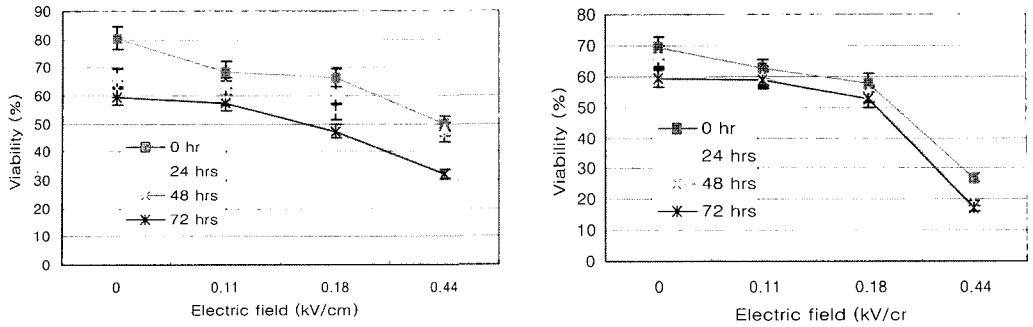

Fig. 3. Viability of spermatogenic cells during culture after different strength of electrical fields ($P<0.05$).

troporated group and 62.6%, 57.9% and 27% in electroporated group at 0.11, 0.18 and 0.44 kV/cm, respectively (Fig. 3B).

4. Expression of EGFP Gene in Rate of Spermatogenic Cells Treated in Different Strength of Electric Fields

In order to confirm the optimal electrical strength for the integration of EGFP in spermtogenic cells, testicular spermatogenic cells were cultured for 72 hrs after different strength of electrical field and then emitted under the green fluorescence microscope. The highest expression rates of EGFP gene in mouse and hamster spermatogenic cells, 20.5% and 32.1%, were obtained from the cells cultures for 72 hrs with 0.18 kV/cm electric field, respectively (Fig. 4A, 4B). Expression rate was increased with the elevating strength of electric field and reached maximum at 0.18 kV/cm and then declined thereafter. This decline may be due to the voltage -induced cell killing.

5. Culture of Spermatogenic Cells Isolated from Testis Tissues

Spermtogenic cells, sperm cells and somatic cells were cultured in medium for 3 days. As shown Fig. 5, spermatogenic cells were morphologically differentiated into 2 types of cells, haploid round spermatids and elongated somatic cells. The former was used as material for the transfection of EGFP gene and microinjection into oocytes.

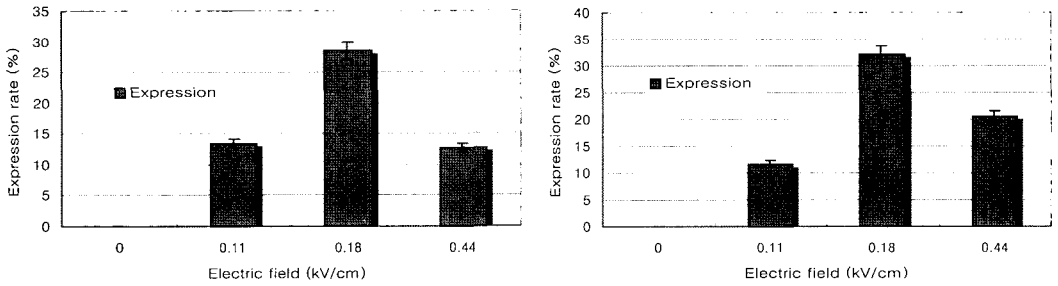

Fig. 4. Expression rate of EGFP gene in mouse (A) and hamster (B) spermatogenic cells cultured for 72 hrs after stimulation with different strength of electric field ($P<0.05$).

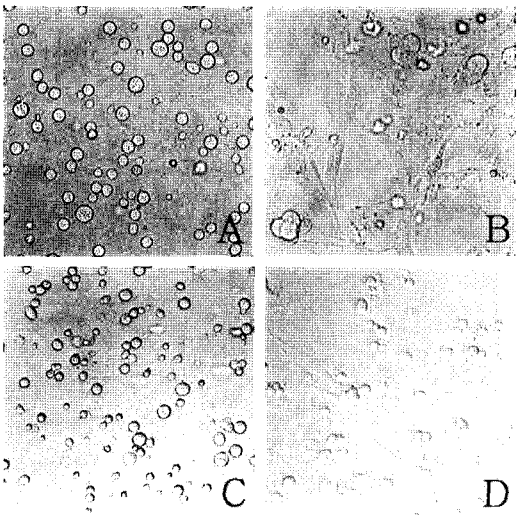

Fig. 5. Testicular cells and cultured haploid spermatids of mouse and hamster (×200). (A) spermatogenic cells separated from 17 dpp mouse testis. (B) 3 days cultured haploid spermatids from 17 dpp mouse testis. (C) spermatogenic cells separated from 19 dpp hamster testis. (D) 3 days cultured haploid spermatids from 19 dpp hamster testis.

6. Introduction EGFP Gene into Embryos by Haploid Spermatid Transfected with EGFP Gene

Injection of haploid spermatids transfected with foreign DNA into oocytes were attempted in mouse oocytes. Since, no cleaved mouse embryo was obtained by microinjection of haploid spermatids, in next hamster oocytes were subjected to microinjection of haploid spermatids transfected with EFGP gene. Haploid spermatids were injected into hamster oocytes using micromanipulator. The cleavage rate of control group was 65%, and that of the electroporated group was 58.8% (Table 1). The embryos developed up to 2-cell stage were subjected to observation under fluorescence microscope. However, any evidence showing expression of EGFP gene was observed. Therefore, in next embryos developed to 2-cell stage were subjected to PCR to confirm

Table 1. *In vitro* development of hamsters oocytes after microinjection of haploid spermatids into cytoplasm

Treatment	No. of oocytes	Embryo development rate (%)	
		2PN	2 cell
Control	60	48(80)	39(65)
Electroporation	97	75(77.5)	57(58.8)

* PN : pronucleus

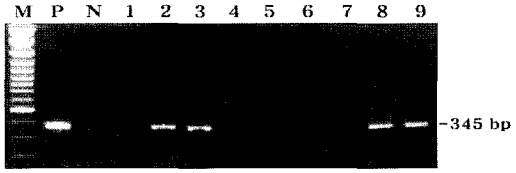

Fig. 6. PCR analysis of *in vitro* produced hamsters 2-cell embryos after microinjection of haploid spermatids transfected with EGFP gene. Lanes 1~10: hamsters 2-cell embryos. M: 100 bp. P: positive control. N: negative control.

the integration of foreign gene into embryo by microinjection of haploid spermatids into ooctyes. Fig. 6 shows the results of PCR analysis. As shown in Fig. 6, integration rate of EGFP gene into 2-cell embryos by microinjection of haploid spermatids transfected with foreign DNA was confirmed to be about 44% of embryos.

Ⅳ. DISCUSSION

EGFP gene has been used as a genetic marker for the genetic transformation of the mouse and hamster embryos. The study of EGFP expression continually has proceeded by many investigators (Brackett et al., 1971, Lavitrano et al., 1989, Perry et al. 1999). However, poor EGFP gene expression of the microinjected mouse and hamster oocytes has been encountered. This problem may be due to the

difficulty of transfection of foreign gene into spermatozoa cells. *In vitro* development and expression rate of foreign DNA in microinjeted oocytes with spermatozoa was very low in the mouse and hamster (Yanagimachi et al, 1995; 1998, Kimura et al., 1995). The reports have pointed out that sperm cells transfected with foreign gene may cause the embryonic development and expression rate to be low. As well known, DNA of mature spermatozoon is too tightly compacted by protamine to be penetrated by foreign DNA. To avoid, this protamine barrier, immature spermatozoa could be used. However, diploid spermatozoa can not bring normal fertilization. This is the reason why the haploid spermatids are needed to introduce foreign DNA into embryos. So we tried to identify the appearance time of haploid spermatids *in vivo* or *in vitro*. The genes, mTP1 and hPrm2 are known as spermatid specific gene in mouse and hamster (Bunick et al., 1990, Murry et al., 1991, Corzett et al., 1999, Green et al., 1994, Hecht et al., 1987; 1990). By investigating the appearance time of mTP1 and hPrm2 gene, the appearance time of haploid spermatids was confirmed to be 18 dpp in mouse and 20 dpp in hamster. In the hamster, there was a gap of one day between *in vivo* and *in vitro* spermatogenic cells (Fig. 1, 2). According to this result, the spermatogenic cells collected from 17 dpp mouse and 19 dpp hamster testes were used for next experiment.

Above mentioned spermatogenic cells were cultured to determine in a chemically defined medium supplemented with hormones, FSH and testosterone which is known to be necessary for normal spermatogenesis (Hue et al., 1998, Tesarik et al., 2000, Orth et al., 1998, Hecht et al., 1997). During culture, spermatogenic cells were maintained their normal shapes for 3 days in close association with sertoli cells. Thus, the attempt to induce transfection of foreign DNA was accomplished during the division of the spermatogenic cells. When the isolated spermtogenic cells were transfected with EGFP gene in the electric field of different strength, the highest expression rate was obtained in 0.18 kV/cm (Fig. 4). This result confirmed the results reported by Yamazaki et al., (1998) and Masaaki et al., (1995). According to this result the transfection of EGFP gene into spermatogenic cells was performed in the electric field strength using spermatogenic cells obtained from 17 dpp mouse and 19 dpp hamster testes.

After transfection of EGFP gene, the spermatogenic cells and gene were cultured for 72 hrs. During this culture, spermatogenic cells were morphologically differentiated into 2-types of cells, haploid spermatids and somatic cells (Fig. 5). The spermatids were selected under microscope and then injected into oocytes by micromanipulator. However, only hamster spermatids cells were selected in normal fertilization and 2-cell embryos. In case of mouse, the chromosomes were lysed following microinjetion because of extensively damaged oolemma of oocytes. This result also reconfirm the reports of Yanagimachi et al. (1995; 1997). Even though hamster oocytes were fertilized by microinjection of haploid spermatid transfected with EGFP gene, *in vitro* culture of the embryos is no easy. In order to overcome 2-cell block, hamster oocytes were cultured in defined medium containing amino acid, phosphate and glucose (Bavister et al., 1988; 1990; 1991; 1992). Whereas, no embryos developed more than 2-cell stage were obtained (Table 1). Unfortunately, foreign EGFP gene introduced into oocytes is never expressed in 2-cell stage embryos. Therefore, the embryos were subjected to PCR analysis to confirm the integration of EGFP gene in oocytes. As a result, about 44% of embryos microinjected with transfected spermatids were confirmed to have foreign EGFP gene (Fig. 6). This result suggests that haploid spermatids could be used as a

vector of foreign DNA, EGFP gene.

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요 약

EGFP 유전자가 도입된 반수체 정자세포에 의한 형질전환 설치류 난자의 생산

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본 연구의 목적은 외래 EGFP 유전자를 분화이전의 웅성생식세포에 도입한 후 이를 난모세포내에 미세주입하여 형질전환동물을 생산하는 기술을 개발하는 데에 있다. 이를 위하여 반수체 정자세포에서 특이적으로 발현하는 생쥐의 mTP1과 햄스터의 hPrm2 유전자 발현 시기를 RT-PCR로 조사한 결과 그 시기는 생쥐와 햄스터에서 각각 18일령과 20일령으로 확인되었다. 이에 따라 외래 유전자의 침입이 용이한 감수분열 직전단계인 17일령의 생쥐와 19일령의 햄스터 정자세포를 EGFP 유전자가 포함된 배양액에 부유시킨 다음, 전기자극을 부여한 결과 0.18 kV/cm의 전기자극을 가한 후 72시간 배양한 정자세포의 28.5%와 32.1%에서 EGFP 유전자가 발현되는 것으로 확인되었다. EGFP 유전자가 도입된 반수체 정자의 수정 및 발달 능력을 검증하기 위하여, 이들 정자세포를 햄스터 난자 내에 미세주입하였으나, 형광현미경하에서는 EGFP 유전자의 발현은 관찰할 수 없었다. 이에 이들 난자를 공시하여 PCR 분석을 실시한 결과, 약 44%의 수정란에서 EGFP 유전자의 존재가 확인되었다.

이러한 결과로 보아 반수체 정자세포는 외래 유전자를 난자 내에 도입하기 위한 운반체로 이용될 수 있을 것으로 생각된다.

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