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In Vitro Culture Following Purfication of Mouse Spermatogenic Cells

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생쥐 정자세포의 분리와 체외 배양에 관한 연구

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요 약

본 연구는 생쥐의 곡세정관내에 존재하는 분화단계의 정자세포를 발생단계별로 분리하여, 체외에서 단기간 배양체계를 확립하기 위하여 실시하였다. 8 주령 이상된 생쥐의 정소로 부터 정소막을 제거시킨 후, collagenase (1mg/ml), trypsin (2.5mg/ml)를 처리하여 곡세정관을 간질세포와 분리하여 배양액에 부유시켰다. 부유세포는 Celcep장치를 이용하여 세포크기와 밀도 차이에 의해 분화 단계별로 분리하였다. 회수된 세포의 균질성은 haematoxylin/eosin 또는 orcein으로 염색한 후, 광학현미경하에서 확인한 결과 약 85% pachytene spermatocyte와 round spermatid을 성공적으로 분리해냈다. 따라서 sedimentation velocity에 의해서 생쥐의 spermatogenic cell의 발달단계별 분리가 가능함을 알 수 있었다. 이러한 방법으로 분리된 pachytene spermatogenic cell들은 DMEM 배양액에서 6일 이상 배양한 결과 약 36%의 생존율을 보였다. 따라서, 분화단계별 정자 세포의 분리 및 배양체계의 확립은 웅성생식세포의 발생과정에 따른 생리 또는 분자생물학적 현상을 규명함은 물론 세포융합기술의 이용에 의한 형질전환동물 생산에의 응용을 통해 가축에 있어서의 형질전환 생산효율의 개선에 기여할 수 있으리라 사료된다.

I. INTRODUCTION

Spermatogenesis represents a complex system of cellular differentiation involving mitotic stem cells proliferation, meiosis, and subsequent remolding of haploid spermatids to produce mature spermatozoa (Bellvé, 1979; Ewing et al., 1980). One of major ways of investigating is to isolate populations of testicular cells in the adult at various stages of development. The isolation of purified spermatids from rodent testis is most frequently achieved by sedimentation at unit gravity using the Celsep™ apparatus (Wolgemuth et al., 1985), or by sedimentation using an elutriator (Meistrich, 1977; Blanchard et al., 1991). These technique have been developed for the separation of differentiating cells such as spermatogenic cells into fractions enriched in specific cell types, for the study of characteristics of germ cell. However, such separation procedure have, in general, failed to provide ad-

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equate morphological evidence for the purity of isolated cell and result in contamination such as interstitial cells and fibroblast cells. Furthermore, the *in vitro* conditions necessary for the maintenance of germ cell development have not been realized. Therefore, the establishment of *in vitro* culture system, as well as purification of male germ cells, would greatly enhance our ability to study to stage-specific cellular integrations and paracrine regulation of spermatogenesis.

In vitro system for the study of mammalian spermatogenesis have been severly limited in the past since even organ cultures of testis fail to allow differentiation of male germ cells beyond the pachytene stage of the first meiotic prophase (Steinberger, 1975). Recent studies indicate that *in vitro* culture systems may facilitate more detailed analysis of early spermatogenic cell development (Haneji et al., 1983; Parvinen, 1982; Tres and Kierszenbaum, 1983), but the differentiation of haploid spermatids in these experiment has not yet documented. Although it is possible to obtain highly purified populations of mammalian cells at particular stages of spermatogenesis (Bellvé et al., 1977; Romrell et al., 1976), isolated male germ cells do not undergo extended differentiation *in vitro* using any culture techniques reported to date. However, these cells may be maintained *in vitro* for short-term studies to investigate a variety of metabolic events (Boitani et al., 1983; Grootegoed et al., 1982; Jutte et al., 1981; Stern et al., 1983).

Unlike that of mouse, a fundamental problem to produce transgenic livestock is low efficiency and a limited supply of early embryos with the proper stages of development (Eyestone, 1994). Furthermore, it is very expensive and laborious to obtain zygote by superovulation and surgical collection. To overcome this problem, even though alternative methods such as retroviral vector, sperm vector, and stem cells were developed, application of these technique to livestock is still under examination. Very recently, it has been reported that developing male germ cells were successfully transfected and cultured *in vitro* to round spermatids having haploid chromosomes (Hoffman et al., 1992 and 1994). Ogura et al (1993 and 1994) reported that the mouse mature oocyte can be fertilized by fusion with a round spermatid and that the zygote is capable of development to term. Therefore, development of *in vitro* culture conditions for male germ cells could be contributed to enhance the production efficiency of transgenic livestock.

In this study, we report the purification of male germ cells for the culture *in vitro* from 8 to 12 weeks old mouse testis. The availability of this culture conditions represents a major breakthrough in our ability to produce transgenic livestock: the rationale is based on the assumption that the oocytes fused with the selected round spermatid harboring foreign DNA were expressed efficiently the foreign DNA, indicating that this scheme could be used to apply to transgenic livestocks.

Ⅱ. MATERIALS AND METHODS

1. Animals

Male adult (≥ 8 weeks) mice were used in this experiments. The mice were maintained at 22±1℃ under a 12h light-dark cycles with 70% humidity and given the commercial pellet diet and water *ad libitum*. All mice were acclimated for 1 week prior to the onset of experiment, then male mice with approximately 45g of body weight were used.

2. Reagents

Dulbecco's Modified Eagle's Medium (DMEM, low glucose), Eagle's minimal essential me-

dium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), nonessential amino acid, essential amino acid were obtained from GIBCO (Grand Island, New York). Bovine serum albumin (BSA), β-mercaptoethanol, trypsin, collagenase (type I), DNase-I, insulin, transferrin and percoll were purchased from Sigma Chemical Company (St. Louis, Missouri).

3. Preparation of cell suspensions

Testicular cells were isolated according to the methods developed by Romrell et al. or Bellvé and colleagues. Briefly, testes were recovered, decapsultaed and immediately placed into Erythrocyte-lysis buffer (155 mM NH_4Cl, 10mM $KHCO_3$, 2 mM EDTA, pH 7.2). Then, the tissue was dispersed and cut into small pieces in HBSS (Hank's Balanced Salt Solution: GIBCO). The suspensions were incubated with HBSS containing 1mg/ml of collagenase (Sigma), 2.5 mg/ml of trypsin (Gibco), and 20 μg/ml DNase (Deoxyribonuclease I, DN-EP: Sigma) at 37℃ for 15 min (Romrell et al., 1976; Bellvé et al., 1977). After twice washing in EKRB with 0.5% BSA (Bovine Serum Albumin, fraction V; Sigma), the cells were re-suspended in EKRB containing 1.0% BSA and DNase(1 μg/ml) and filtrated through 41 mm nylon mesh to remove cell aggregates. The cell concentration was determined using a hemocytometer and then adjusted to a final concentration of less 3×10^6 cells/ml.

4. Separation of spermatogenic cells

The spermatogenic cells were separated using sedimentation velocity at unit gravity provided by Eppendorf Celsep™ Cell Separation System. This system were gently provided by Dr. Karan Artz (University of Texas of Austin). Briefly, the technique of sedimentation velocity were slightly modified a procedure of the standard STA-PUT unit gravity sedimentation system proposed by Miller and Phillips (1969). To reduce the sedimentation rate, gradient materials used BSA in replaced with percoll in EKRB. After the gradient were formed, preparized single cell suspension at a concentration of 2 to 3×10^6 cells/ml in 1.0% BSA in EKRB are added into the separation cell loading chamber. After approximately 2 hours, fractions, numbered 1 to 68 starting from the bottom of the gradient and each containing 15 ml, were collected at a rate of 20~30 ml/min. Then, cells were washed with HBSS two times at 1,200 rpm for 8 min. To minimize microbial contamination, all glasses were autoclaved and separation apparatus were pre-treated with EO (ethlyene oxide) gas before use.

5. *In vitro* culture

Purified pachytene spermatocytes were washed twice in sterile HBSS prior to cell culture. Cells were incubated in DMEM (low glucose), EMEM, Ham's F12 or DMEM+Ham's F12 supplemented with 10% fetal bovine serum, 0.1 mM β-mercaptoethanol, 0.05 mM nonessential amino acid, 0.05 mM essential amino acid, 100 μg/ml penicillin, 100 U/ml streptomycin, and 25 ug/ml gentamycin in 24 well dish at 32℃ with 5% CO_2 in humidified air.

6. Evaluation of morphology and viability

After separation, cells were counted with a hemocytometer. Cellular viability was evaluated under light microscope with trypan blue exclusion and/or Live/Dead Eukolight™ viabilbity/cytotoxicity kit, which shows green color for living cells and a red color for dead cells, according to manufacture methods (Molecular probes, Inc.). Cell morphology were examined by differential interference contrast light microscopy. Fractionated cells were identified on air-dried smears in slide glass, fixed in Bouin's solution for 2 hours and stained with Hematox-

ylin & Eosin or Orcein.

7. Statistical analysis

A percentage of germ cell viability is presented as the mean ± SEM. The data were analyzed by one-way analysis of variance and Duncan's multiple-range test using SAS Institute software package (SAS Institue Inc., 1985). The difference was considered significant at $P<0.05$.

Ⅲ. RESULTS AND DISCUSSION

1. Isolation of testicular cells

Cell suspensions were obtained from mouse testes by incubation with collagenase and trypsin. After a 15-min incubation with collagenase, the connective and interstitial cells dissociated, and the seminiferous tubules were separated as individual tubules. During the 15-min incubation with trypsin (0.25%), the cytoplasmic bridges of cells were brokened and spermatogenic cells were isolated. This process is also induced by the mechanical pippeting, which minimized mechanical pressure on the testes to decrease the possibility of forcing nuclei from adjacent cells through innercellular bridges, thereby creating multinucleated spermatid. At this points, various cell types in these suspensions were observed by light micorscopy (×320, Fig. 2A). Yield of total male germ cells was highly variable between individuals, with a mean value of 3.5 to 4.5 $\times 10^{7}$ cells/testis. Viability of the cell was over 97% after separation.

2. Morphological typing of spermatogenic cells

The association of data from morphological identification allowed determination of the different cell populations. These compositions are outline in Table 1 and 2. The observation by phase contrast microscopy of the separated populations revealed difference of their cellular composition. According to their size and morphology, cells were classified as primary sper-

Table 1. Purity of mouse spermatogenic cells separated by sedimentation velocity at unit gravity

Cell types	Fraction No.	Purity
Pachytene spermatocyte	A 10~15	86.86±2.09
	B 2~7	82.87±2.49
Round spermatid (1~8)	A 39~43	89.13±2.97
	B 30~35	87.93±4.84
Residual body	A 51~55	82.76±2.92
	B 42~49	76.22±5.44

A: BSA gradient material

B: Percoll gradient material

Table 2. Percentage of various cell types in separated mouse germ cell fractions

	Pachytene fr.	Spermatid fr.	Residual body fr.
PS	86.86 ± 2.09	–	–
RS	–	89.13 ± 2.97	2.11 ± 2.88
CS	–	3.15 ± 0.80	9.97 ± 2.82
RB	–	2.97 ± 0.46	82.76 ± 2.92
OT	6.79 ± 1.96	5.20 ± 1.31	4.11 ± 2.54
MN	6.35 ± 1.19	0.29 ± 0.65	1.05 ± 2.35

PS: pachytene spermatocyte, RS: round spermatid, CS: condensing spermatid,

RB: residual body, OT: other material (Sertoli cell etc.), MN: multinuclear cell.

All experiments were performed for at least three times.

matocytes, round spermatids, elongated spermatocytes (Fig. 1). The pachytene spermatocytes were the largest germ cells having a round shape, single nucleus (Fig. 1A). They had a diameter of about 16 μm. While, round spermatids could be easily distinguished from elongating ones, the nuclei of which were protruded, and formed elongated spermatids, which had lost their flagellum during cell preparation. While the round spermatids were distinguished from pachytene spermatocytes by being smaller, nucleated cells (Fig. 1C, D). These cells had a diameter of 10 μm and a nucleus or condensed chromatin within the nucleus of each round spermatid. The small anucleated residual bodies contain large granules and vesicles and have an average diameter of 6.5 μm (Fig. 1F). The condensing spermatids are readily identified by

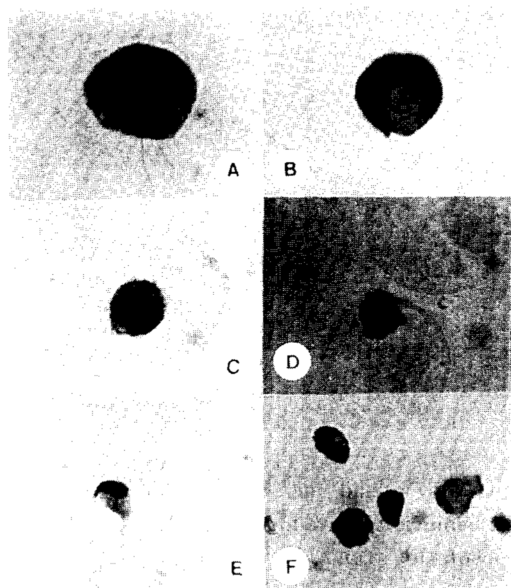

Fig. 1. Heamatoxylin & Eocin stained mouse testis cells.
A: pachtene spermatocyte, B: binucleated cell, C,D: round spermatid (1~8), E: elongating spermatid, F: residual body.

their nuclear morphology (Fig. 1E). The nuclei were at various stages in acquisition of the characteristic sickle shape of late spermatids.

A more accurate and objective determination of composition of the different population was performed by haematoxylin & eosin or orcein staining as shown in Fig. 3. It allowed classification of the cells as pachytene, round spermatids, and elongating /elongated spermatids under light microscopy (Table 1 and 2). Unlike the Holstein (1976) classification, round spermatids corresponded to spermatids from stages 1~8, elongating spermatids to those from stages 9~16, and elongated ones to those from stages 17~24. Stages 9 is an intermediate stages and behaves either as round or elongating spermatids.

3. Separation of spermatogenic cells

Separation of germ cell fractions from the crude cell suspension is achieved by unit gravity sedimentation using Celsep™ separation system. After sedimentation through the BSA or Percoll gradients, 15ml fractions were collected and centrifuged. When the resuspended cell pellets were examined by DIC microscopy, the fractions were divided into three separated populations: pachytene spermatocytes, round spermatids and residual bodies. The purity of three fractions were summarized in Table 1. The pachytene spermatocytes were obtained by pooling fractions 10 to 15. The fraction showed purity of 86% pachytene spermatocytes (Fig. 2B, 3A), but also contaminated multinucleated round, which were discernible by their multiple nuclei and the presence of acrosomes (Fig 1B). The round spermatid fraction were obtained by pooling fractions 39 to 43 had 89% purity (Fig. 2C, 3B). The condensing spermatid were recovered from 44 to 50 fractions. The fraction of 51 to 55 were anucleate cytoplasm, which showed approximately

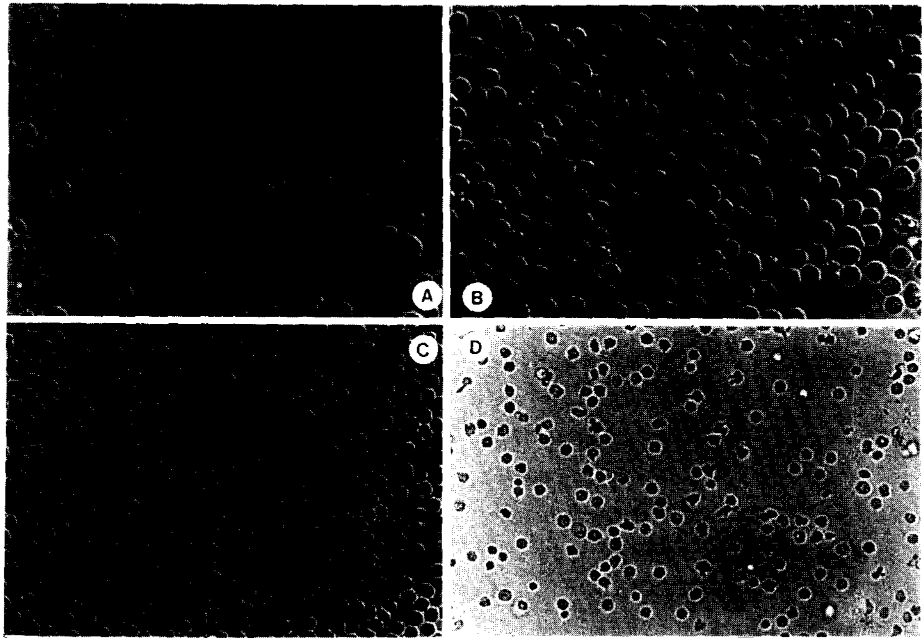

Fig. 2. Photomicrographs of isolated mouse spermatogenic cell populations. Seminiferous cell suspensions were prepared (A) and separated into populations of pachytene spermatocytes (B), round spermatids (C), and Residual bodies with condensing spermatids (D). Shown are representative fields of each cell populations as viewed by DIC microscopy.

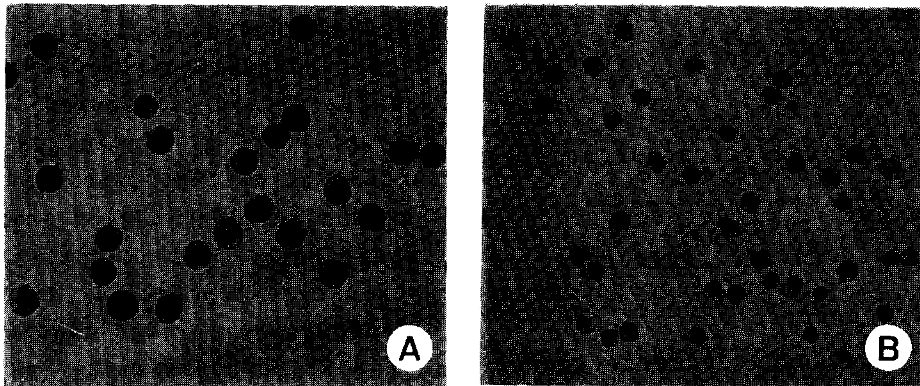

Fig. 3. Purified pachytene spermatocytes and round spermatids stained with Orcien. A: population of pachytene spermatocytes, B: round spermatids.

82% purity (Fig, 2D).

4. Culture of primary spermatocytes

By assaying trypan blue exclusion and Live/Dead kit (Fig. 4), the viability of cultured cells were examined at 48 h intervals after plating on 24 well dish in DMEM, EMEM Ham's F12 and DMEM-Ham's F12 respectively. Initial viabilities of the isolated male germ cells were over 95%. In DMEM medium, about 36.8% of pachytene cells was successfully cultured for 6 days (Fig. 5) and some of cells were developed

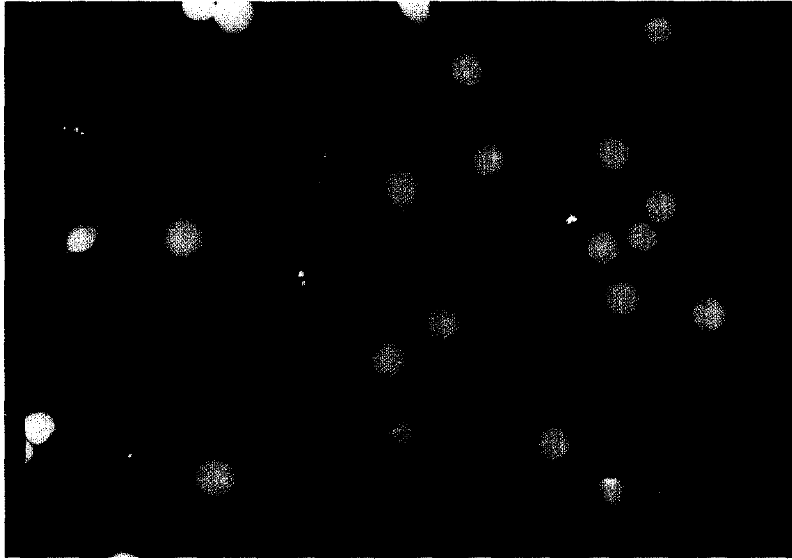

Fig. 4. The viability of cultured pachytene spermatocytes for 4 days by assaying LIVE/DEAD ket : Green color; live cell, Red color; dead cell.

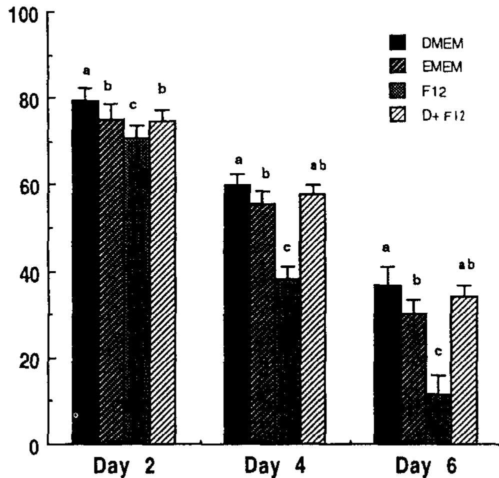

Fig. 5. Viability of mouse spermatogenic cells cultued by different media. Germ cells were seeded at a density of 10^5 cells/well in 24-well dish and cultured for 2, 4, 6 days in EMEM, DMEM, Ham's F-12, DMEM-Ham's F12 medium, respectively. Initial viabilities prior to culture were always in excess of 97% ($^{a, b \& c.}$ $p<0.05$).

to secondary spermatids and round spermatids (in preparation). The optimal cell number for culture is approximately 1×10^5 cells/dish, but low cell densities than 1×10^5 cell/dish showed a decreased cell viability (data not shown). However, in EMEM conditions used Gerton, all of pachytene cells were died within 48 hrs after *in vitro* culture (data not shown). Very recently, it has been reported that developing male germ cells were successfully transfected and cultured *in vitro* to round spermatids having haploid chromosomes (Hoffman et al., 1992 and 1994). Ogura et al (1993 and 1994) reported that the mouse mature oocyte can be fertilized by fusion with a round spermatid and that the zygote is capable of development to term. Taken together, these results and our data suggested that the oocytes fused with the selected round spermatid harboring foreign DNA will express efficiently the foreign DNA, indicating that this scheme will be used as a feasible tools for application of transgenic livestocks.

In conclusion, we began to study the possibility of spermatogenic cell for sperm-mediated gene transfer, which it allows the foreign DNA into spermatogenic cells at various stages of differentiation using the technique such as direct injection and intracytoplasmic sperm injection. In DMEM medium, about 36.8% of pachytene cells was successfully cultured for 6 days (Fig. 5) and some of cells were developed to secondary spermatids and round spermatids (in preparation). The optimal cell number for culture is approximately 1×10^5 cells/dish, but low cell densities than 1×10^5 cell/dish showed a decreased cell viability (data not shown).

Ⅳ. SUMMARY

This study was carried out to establish the *in vitro* short-term culture system of developing male germ cells by purifing germ cells of various stages. The decapulated testicular cells were incubated with collagenase (1mg/ml) and trypsin (2.5mg/ml) in HBSS. After separating male germ cell, the separated germ cells were stained with heamatoxylin/eosin and determined developing stages under light microscopy. The purity of pachtene spermatocytes and round spermatid were 85%, respectively. Yield of total male germ cells was highly variable between individuals, with a mean value of 3.5 to 4.5 $\times10^7$ cells/testis. Viability of the cell was over 97% after separation. In DMEM medium, the optimal cell number for culture is approximately 1×10^5 cells/dish, but low cell densities than 1×10^5 cell/dish showed a decreased cell viability. Furthermore, about 36.8% of pachytene cells was successfully cultured for 6 days and some of cells were developed to secondary spermatids and round spermatids. Therefore, our data suggested that this culture conditions will be utilize as a feasible tools to produce transgenic livestock using techniques such as intracytoplasmic injection and cell fusion.

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