ISSN 2185-6230 (Online)

Journal of REPRODUCTION ENGINEERING

September 2012
Vol. 15, No. 1: 1–19

J. Reprod. Engineer.

Japan Society for
Reproduction Engineering

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J. Reprod. Engineer.
2012; Volume 15, Number 1

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J. Reprod. Engineer. 2012; 15: 1–5.
http://sre.ac.affrc.go.jp/Contents.htm

Journal of REPRODUCTION ENGINEERING JRE

= Research Note =

Co-cultured sperm remaining after *in vitro* fertilization is developmentally competent to term by intracytoplasmic injection in rats

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ABSTRACT

Although *in vitro* fertilization (IVF) protocol using cryopreserved sperm has been quite recently established in rats, the developmental competence of ICSI (intracytoplasmic sperm injection) oocytes is lower in rats than that in mice. In several mammalian species, ICSI is a way to routinely obtain offspring derived from zygotes fertilized by cryopreserved sperm. If spermatozoa remaining after IVF can be used for ICSI, large numbers of offspring derived from the same male rat can be obtained by treatment combining IVF and ICSI. In the present study, we examined whether spermatozoa remaining after IVF can be utilized for ICSI in rats. Spermatozoa after IVF were recovered and then cryopreserved in cryotubes. After thawing, the spermatozoa were used for ICSI (IVF-ICSI group). As a control, spermatozoa were frozen-thawed in straws and then used for ICSI. In the control, the rates of pronuclear formation, two-cell, and blastocyst stages were 88.6 ± 4.8%, 63.8 ± 8.3%, and 25.1 ± 5.5%, respectively. In the IVF-ICSI group, these rates were 90.4 ± 4.6%, 73.2 ± 7.7%, and 36.4 ± 5.1%, respectively. In the IVF-ICSI group, offspring were obtained by embryo transfer. The present results show that co-cultured sperm remaining after IVF can be used for ICSI and that this procedure can probably be applied to generate offspring from banked sperm in rats.

Key words: intracytoplasmic sperm injection, *in vitro* fertilization, rat

INTRODUCTION

In vitro fertilization (IVF) is now an indispensable technology in various fields, including human clinical medicine [8]. Moreover, IVF is only the procedure for the efficient production of genetically valuable experimental animals, such as transgenic and knockout animals from preserved germ cells, since many oocytes can be fertilized at the same time. In mice, an IVF protocol using cryopreserved sperm was established in the 1990s [21,22,24]. In rats, on the other hand, IVF using cryopreserved sperm has only quite recently succeeded [18]. Although the developmental ability of rat IVF oocytes is still lower than that of mice and further improvements are needed, it is expected to become a routine technology in the near future. In IVF, some spermatozoa are fertilized with oocytes whereas others are not. In our rat IVF protocols, 5×10^5 sperm/ml was used to inseminate 40–50 oocytes in a 200 µl drop [18], which means that less than 1% of the spermatozoa could fertilize those oocytes. In the general IVF protocol, these unsuccessful spermatozoa are discarded after IVF.

As an alternative method to IVF, intracytoplasmic sperm injection (ICSI) is an established technology, having succeeded in a number of mammalian species, including rats [1,2,4,7,12,15,20,23]. In rodents, ICSI has

Submitted: January 18, 2012
Accepted: January 25, 2012
Advance Publication in Website: January 28, 2012

been applied to new technologies, such as the generation of offspring using sperm derived from spermatogonial stem cells [19], embryonic stem cells, and induced pluripotent stem cells [5]. Also in rats, ICSI is used to obtain offspring routinely derived from genetic resources such as *N*-ethyl *N*-nitrosourea (ENU) mutagenesis [9]. For successful fertilization using a limited number of sperm, ICSI seems to be the most optimal technology. This leads us to the possibility that, if spermatozoa remaining after IVF can be used for ICSI, a much greater number of offspring derived from the same male rat will be obtained. Moreover, if IVF is unsuccessful for some unexpected reason, the remaining sperm could be easily preserved and then used for rescue by ICSI, which requires no cryoprotectants or complicated procedures [17]. For the production of genetically valuable rats, especially spontaneously mutated ones, the combination of IVF and ICSI will be a promising procedure. However, there have been very few studies in which these treatments were combined, at least in rats. Here we conducted a preliminarily whether or not spermatozoa remaining after IVF can be used for ICSI in rats.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. All procedures for the handling and treatment of the animals were conducted according to the guidelines established by the Animal Research Committee of Azabu University.

Animals

Wistar rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rats were housed in an environmentally controlled room with a 12-h dark/12-h light cycle at a temperature of 23 ± 2°C and humidity of 55 ± 5%, with free access to a laboratory diet and filtered water. Mature males (12–24 weeks old) and immature females (4–5 weeks old) were used as sperm and oocyte donors, respectively. Vasectomized male rats (15–30 weeks old) were used to induce pseudopregnancies. Although the vasectomized rats in our study were of the same strain as the other rats (Crlj: Wistar), we confirmed the sterility of the vasectomized male rats as part of this preliminary study.

Media

The culture medium for *in vitro* development of the ICSI oocytes was rat one-cell embryo culture medium (R1ECM) containing 76.7 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 10.0 mM sodium lactate, 0.1 mM glutamine, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (50x; Gibco BRL, Grand Island, NY, USA), 1% (v/v) MEM nonessential amino acid solution (100×; Gibco BRL) and 1 mg/ml PVA [10]. The base medium used for sperm collection and incubation was R1ECM-increased NaCl (110 mM) supplemented with 4 mg/ml of fatty-acid-free BSA instead of PVA (modified R1ECM, mR1ECM) [14]. For sperm cryopreservation, the freezing medium contained 23% (v/v) egg yolk, 8% (w/v) lactose monohydrate, antibiotics (1000 IU/ml penicillin G potassium, 1 mg/ml streptomycin sulphate), and 0.7% (v/v) Equex STM Paste (Nova Chemical Sales, Inc., Scituate, MA, USA) [13].

Oocyte collection and IVF

The females were superovulated by intraperitoneal injections of 30 IU equine chorionic gonadotropin (eCG; Nippon Zenyaku Kogyo Co., Tokyo, Japan) and 30 IU human chorionic gonadotropin (hCG; Asuka Pharmaceutical Co., Tokyo, Japan) at 48 h intervals, as reported by Fujiwara *et al*. [3]. The IVF protocol was carried out according to our previous report [18]. Fresh epididymal sperm (2 µl) was directly added in a paraffin-oil-covered 200 µl drop of mR1ECM (final sperm concentration: 5×10^5 sperm/ml) and cultured for 5 h. Twelve to 14 h after the hCG injection, the oviduct ampullae of the donor females were placed in oil, and cumulus-oocyte complexes (COCs) were collected from the oviductal ampullae. The COCs were transferred in the drops containing the sperm and co-cultured at 37°C under 5% CO_2 in air for 10 h. After the culture, COCs were transferred into a 100 µl drop of mR1ECM and cumulus cells were removed by being drawn up repeatedly into a fine pipette. The denuded oocytes were evaluated using an inverted phase-contrast microscope (IX70; Olympus, Tokyo, Japan). Oocytes having two pronuclei (2PN) and at least one sperm tail in the vitellus were considered fertilized. After IVF, 20 µl mR1ECM including spermatozoa (5×10^5 sperm/ml) were collected in a cryotube. The cryotube was just plunged into liquid nitrogen and stored until use.

ICSI

The spermatozoa in the straws were thawed in a 37°C water bath for 15 sec then diluted in 1 ml of

R1ECM containing 22 mM Hepes and 5 mM $NaHCO_3$ (Hepes-R1ECM). The spermatozoa were defined as the control. The sperm collection and freezing/thawing procedures were performed according to the methods described in our previous report [17] with some modifications. In brief, epididymal sperm were counted by a hemacytometer and then diluted to 5.0×10^7 sperm/ml with modified Niwa and Sasaki Freezing medium [13]. Sperm samples were loaded into 0.25-ml plastic straws (Fujihira Industry, Tokyo, Japan) and placed in a programmable freezer (Fujihira Industry) at 23°C. The straws were cooled to 5°C for 40 min (0.5°C/min). They were then exposed to liquid nitrogen vapor for 10–15 min, plunged into liquid nitrogen, and stored for at least 1 week. The preserved spermatozoa in the cryotube were thawed in a 37°C water bath for 15 sec then diluted in Hepes-R1ECM. This treatment was defined as the IVF-ICSI group. Spermatozoa in both groups were then separated into heads and tails by sonication for 10 sec at 25°C. The sonication treatment was performed using 100% power output (300 W, 40 kHz) of a UT-305 ultrasonic cleaner (Sharp Manufacturing Systems Corp., Osaka, Japan). More than 80% of the spermatozoa were separated into heads and tails by this sonication treatment, as described previously [17]. The spermatozoa were washed in Hepes-R1ECM by centrifugation (17,000 × *g* for 3 min) before ICSI.

The COCs were collected from the oviductal ampullae of the donor females using mR1ECM supplemented with 0.1% hyaluronidase. After the cumulus cells were removed, intracytoplasmic injection of sperm heads was carried out according to the method described in our previous study [17] with some modifications. In brief, about 10 oocytes were transferred into 20-µl drops of Hepes-R1ECM. A small volume (0.5 µl) of the sperm-head suspension was transferred to 2-µl drops of Hepes-R1ECM supplemented with 12% polyvinylpyrrolidone (MW 400,000), which was prepared close to the oil-covered drops for the oocytes. Microinjection of sperm heads into oocytes was performed using a Piezo-driven pipette (Prime Tech Ltd., Ibaraki, Japan) that was prepared from borosilicate glass capillary tubes (Sutter Instrument Co., Novato, CA, USA). The external diameter of the pipette tip was 2–4 µm. After the injection, the oocytes were cultured in mR1ECM up to 120 h. The rates of 2PN formation, cleavage, and blastocyst were evaluated at 6, 24, and 120 h, respectively. More than 70 oocytes were used in each group.

Embryo transfer

To evaluate the developmental competence *in vivo* of embryos derived from ICSI, one-cell embryos at 6–8 h after ICSI were transferred into the oviducts of recipients after the induction of pseudopregnancy as described previously [18]. Female recipient rats were mated with vasectomized males on day 0 to induce pseudopregnancy. On day 1, 9 or 10 of 2PN oocytes were transferred into each oviduct of the recipients. On day 22, the transferred females underwent Caesarean section to confirm pregnancy and the normality of the offspring.

Statistical analysis

Each experiment had at least three replicates. All percentage data were subjected to arcsine transformation before statistical analysis. Data were compared by one-way analysis of variance and Fisher's protected least significant difference test using StatView software (Abacus Concepts, Berkeley, CA, USA). A value of $P < 0.05$ was chosen to indicate statistical significance. Data are shown as means ± standard error of means (SEM).

RESULTS

In the control group, most (88.6 ± 4.8%) of the injected oocytes formed 2PN (Figure 1). More than half (63.8 ± 8.3%) of the embryos developed to the two-cell stage. Some (25.1 ± 5.5%) reached blastocyst stage. Also in the IVF-ICSI group, most (90.4 ± 4.6%) of the injected oocytes formed 2PN. The proportions of oocytes developed to the two-cell and blastocyst stages were 73.2 ± 7.7% and 36.4 ± 5.1%, respectively. There were no significant differences between the two groups at any developmental stage ($p > 0.05$).

Table 1 shows the *in vivo* development of ICSI oocytes. In the control group, 74 embryos were transferred to pseudopregnant recipients and six pups were delivered (12.7 ± 5.5%). On the other hand, in the IVF-ICSI group, 82 embryos were transferred to five recipients and six pups were obtained (9.4 ± 4.4%). There were no significant differences in developmental ability *in vivo* between the two groups ($p > 0.05$). These pups looked healthy and normal.

Table 1. *In vitro* development of ICSI oocytes after IVF

Treatment	No. of examined	No. of survived	No. of PN formed (%)	No. of 2 cell (%)	No. of blastocysts (%)
Control	93	80	73 (88.6 ± 4.8)	51 (63.8 ± 8.3)	19 (25.1 ± 5.5)
IVF-ICSI	78	45	41 (90.4 ± 4.6)	33 (73.2 ± 7.7)	16 (36.4 ± 5.1)

Table 2. *In vivo* development of ICSI oocytes after IVF

Treatment	No. of transferred embryos	No. of recipients	No. of offspring (%)
Control	71	5	6 (12.7 ± 5.5)
IVF-ICSI	80	5	8 (9.4 ± 4.4)

DISCUSSION

In the present study, we examined whether or not unfertilized spermatozoa after IVF can be used for ICSI. Our data showed high developmental ability in the oocytes injected with sperm remaining after IVF (IVF-ICSI group). Although there was no significant difference in developmental ability between the control and IVF-ICSI groups, the cleavage and blastocyst rates of the IVF-ICSI group were slightly higher than those of the control group. It has been demonstrated that the removal of the acrosomal membrane improves the developmental ability of ICSI oocytes in mice [11], rats [17], and pigs [6]. In this study, the spermatozoa in the IVF-ICSI group were totally cultured for 15 h (5 h for preincubation and 10 h for IVF) and then frozen-thawed before ICSI. Our previous study showed that incubation of frozen-thawed sperm for 5 h induces acrosome reaction in rats [16]. Therefore, incubation for 15 h seems to be sufficient to induce acrosome reaction, which may have contributed to the developmental ability of the oocytes in the IVF-ICSI group.

Our results showed that co-cultured sperm remaining after IVF could be utilized in ICSI in rats. Our results suggest that treatment with IVF and ICSI combined seems to be effective for the production of genetically valuable rats, especially those spontaneously mutated and stocked in gene banks.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research from JSPS (KAKENHI; 21780253 to J.I. and 22658085 to N.K.). This research was also partially supported by a research project grant awarded by the Azabu University Research Services Division to J.I. and N.K.

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J. Reprod. Engineer. 2012; 15: 7–12.
http://sre.ac.affrc.go.jp/Contents.htm

Journal of REPRODUCTION ENGINEERING JRE

= ミニレビュー =

精漿機能に着目した合成融解液の開発とそれを用いたブタ凍結精液の人工授精
Development of chemical defined thawing solution focused on the role of seminal plasma, and its application to artificial insemination of cryopreserved boar semen

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要旨

ブタ凍結精液による人工授精技術は、貴重な雄個体の遺伝資源保存や発情適期に人工授精が可能になるなど現場ニーズがあるにもかかわらず、実用化されていないのが現状である。我々はこれまでに、融解直後の精子に生じるクライオキャパシテーションが人工授精後の低受胎の原因であること、精漿はそれを抑制し、精漿含有融解液を用いることで高い繁殖成績が得られることを明らかとしてきた。しかし、精漿には伝染性ウイルス疾病を招く危険性があることから、精漿の機能を補完した合成融解液の開発が求められている。融解後のクライオキャパシテーションは細胞外の Ca^{2+} を取り込むことで助長され、精漿はそれを抑制していたことから、これらの現象に Ca^{2+} が関与していると考えられる。細胞外 Ca^{2+} キレーターである EGTA の融解液への添加は精子細胞内 Ca^{2+} 上昇と、それに起因するクライオキャパシテーションを抑制した。また、融解後の精子の運動率、体外および体内受精率も改善したことから、EGTA の有効性が確認された。しかし、EGTA 融解液による人工授精では、卵管内受精率は高いにもかかわらず、胎子の着床率は 51%と非常に低く、さらに、着床胎子においても、その死亡率が高かった。この結果から, 精漿には免疫抑制因子が存在し、これらが、精子が抗原となり遊走された白血球による胚の貪食を防ぐと仮説を立てた。精漿中から強い免疫抑制作用を有する cortisol を同定し、融解液への cortisol 添加は、凍結精液を人工授精後 24 および 48 時間の子宮腔内白血球数を有意に抑制し、正常妊娠モデルとなる「液状精液による人工授精」の子宮内白血球数と類似した動態を示した。この EGTA および cortisol を添加した合成融解液で人工授精した場合の着床率は飛躍的に向上し, 繁殖成績も受胎率が 84%、一腹産子数 10.1 頭と実用化レベルに達した。本ミニレビューでは、ブタ精漿は精子機能性のみでなく、子宮内の免疫系を制御するという新しい知見について解説すると共に、それを基に開発した凍結精液における合成融解液について紹介する。

キーワード: ブタ, 精漿, 凍結精液, 人工授精, 免疫

序論

ブタにおける凍結精液を用いた人工授精は、優秀な遺伝形質を有する雄ブタの後代を獲得するため一部の研究機関で行われているのみで、生産現場ではほとんど利用されていない。ブタにおける凍結精液技術は、優秀な雄個体の遺伝資源保存や発情適期に人工授精ができるなど経済的な価値は高いことからその開発研究は進められている[4,7,20,28,31]。しかし、人工授精による受胎率は 50%、一腹平均産子数は 5 頭程度と液状精液（受胎率 80%、一腹平均産子数 10 頭程度）と比較す

投稿日: 2012年6月10日
掲載決定日: 2012年8月6日
ウェブサイト事前公開日: 2012年8月8日

るとその差は歴然である[12,13]。この低繁殖性の主因は、精子凍結融解後の低い生存性および運動性であり、これには凍結時のダメージのみでなく、凍結融解時の温度ストレスによって生じる自発的なキャパシテーション(クライオキャパシテーション)が密接に関わっていることが報告されている[3,35]。近年、我々は、キャパシテーション抑制作用を有する精漿を融解液へ添加することでクライオキャパシテーションの指標となる精子タンパク質のチロシン残基のリン酸化と、その後誘起される先体反応を抑制でき、人工授精後の繁殖成績が向上したことを報告している[21,22]。この精漿含有融解液による人工授精は、液状精液と同等の繁殖成績を示すことが可能だが、精漿には豚繁殖・呼吸障害症候群(porcine reproductive and respiratory syndrome virus, PRRSV)、サーコウイルス II 型 (porcine circovirus type 2, PCV2)、パルボウイルス(porcine parvovirus, PPV)およびオーエスキーウイルス(Aujeszky's disease, ADV) など現在経済損失を増大させているウイルスが排出される[10,14,16]。したがって、このようなウイルスが存在しない SPF (specific pathogen-free)農場や非感染農場では疾病を蔓延させると懸念される。また、精漿成分は季節毎、個体毎によって大きく変動すること[19]から精漿含有融解液の性質を安定化させることが困難である。そのため、全ての農場で安定的かつ安全に使用可能にするためには精漿の正の作用を探究し、融解液に精漿を添加せずとも, 同等の機能を有する合成融解液の開発が求められる。これまでに、凍結融解精子における精漿の役割はほとんど明らかとされていない。本ミニレビューでは、精漿の機能解析を経て得られた知見を基に合成融解液を開発した我々の研究を紹介すると共に、それを用いた人工授精試験についても報告する。

クライオキャパシテーションの抑制 −Ca^{2+}キレート剤 EGTA の効果−

射出精子におけるキャパシテーションは精子細胞内の Ca^{2+} の急激な増加によって誘起される[1,2]。この細胞内 Ca^{2+} の上昇は、細胞外からの Ca^{2+} 流入が初期のトリガーとなり、細胞内の Ca^{2+} ストアからイオンが放出されるためと考えられている[34,36]。一方、凍結融解後の精子においては、凍結融解刺激による原形質膜の物理的損傷により、Ca^{2+} チャンネルを介さずに直接細胞内へ流入すると考えられる。そこで、まず、ブタ凍結融解精子においても細胞内 Ca^{2+} が増加しているか否かを細胞内 Ca^{2+} 指示薬である Fluo-3/AM を用いて観察した。その結果、融解直後から精子頭部および中片部で強い Ca^{2+} シグナルが検出され、培養時間に依存してシグナル強度は増加した[23]。これらの精子ではタンパク質のチロシン残基のリン酸化が検出され、クライオキャパシテーションが誘起されており、さらに、培養液へ Ca^{2+} を添加することでそれらは増大した。一方、培養液に 10% (v/v)精漿を添加すると、Ca^{2+} 依存性のクライオキャパシテーションは完全に抑制されていた。つまり、融解時の精子に起こるクライオキャパシテーションは細胞外 Ca^{2+} により制御され, かつ精漿の作用により Ca^{2+} の流入を抑制していると示唆された。そこで、このような精漿の機能を模倣するため、細胞外 Ca^{2+} に高い親和性を示す 2 価イオンキレート剤であるグリコールエーテルジアミン四酢酸 (EGTA; *O,O'*-Bis (2-aminoethyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid))が細胞内 Ca^{2+} 上昇とクライオキャパシテーションにどのような影響を及ぼすか検討した。融解液への 6 mM EGTA 添加は、精漿添加時と同様にクライオキャパシテーションを抑制し、さらには、融解後に持続的に観察される細胞内 Ca^{2+} 上昇は認められず、その効果は長時間の培養でも保持されていた。これらの結果から、精漿の有するクライオキャパシテーション抑制作用は EGTA の融解液への添加で模倣できることが示された。

通常、融解液として用いられる Modena 液には 2 価イオンをキレートする EDTA が 6 mM 含まれている[8]。しかし, EDTA は Ca^{2+} に対する親和性が EGTA と比べて低く, 溶液中の Ca^{2+} を完全にキレートするためには, これ以上の高濃度を添加する必要がある。この高濃度(12 mM)の EDTA 添加は, 融解後の運動率を低下させた[23]。この結果は、EDTA は Ca^{2+} をキレートすると共に, 精子の運動能に関与していると報告されている Zn^{2+}, Mg^{2+} などの他の二価イオン[15,17,32]も同時にキレートしたためと推察される。一方で、EDTA には 2 価イオンをキレートすることで、抗酸化作用を示すという正の効果も存在する。実際、精子運動率および細胞膜正常率を指標にすると EGTA の効果は 6 mM EDTA の存在下で増強された。また、これら条件で培養した精子は、非常に高い運動率が長時間持続したことから、融解液へ EDTA と EGTA を併用することが重要であると結論づけた。このようにして開発した EDTA および EGTA を添加した融解液にて作出した凍結融解精子を体外受精および人工授精に供試したところ、通常使用される Modena 液で作出したものと比較して、いずれも有意に高い受精率を示したことから(図 1)、EGTA により抑制状態にあったキャパシテーションは受精時に可逆的に誘起され、これら精子は高い受精能を保持していることが明らかとなった。以上の結果から EDTA および EGTA 添加融解液は融解時に誘起されるクライオキャパシテーションを抑制し、高い運動率を維持させることで人工授精に有効なツールであることが示された。

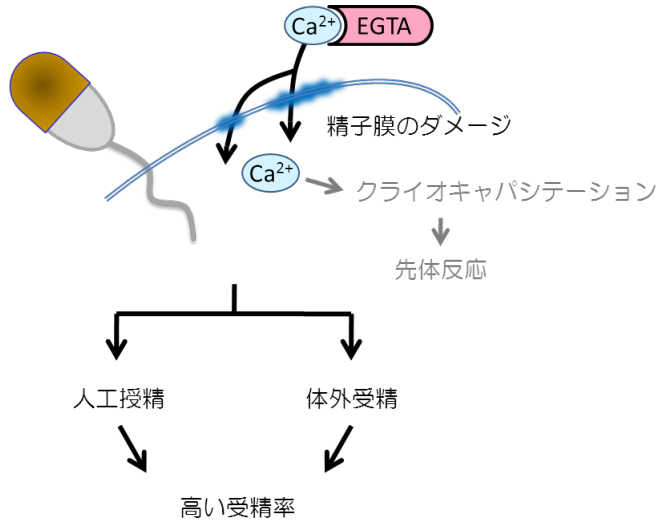
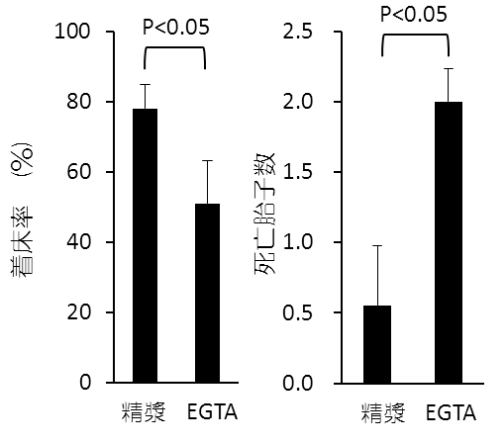


図 1. 融解液へ添加する EGTA の効果
融解液へ添加された EGTA は、細胞外 Ca^{2+} をキレートすることで、クライオキャパシテーションおよびその後誘起される先体反応を抑制する。これら EGTA の作用により、体外および人工授精後の受精率は改善される。



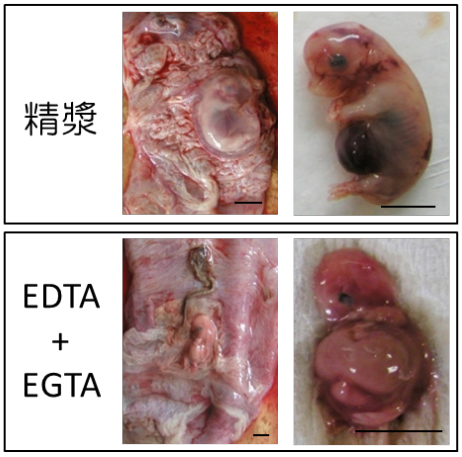


図 2. 精漿含有および EDTA と EGTA を含有した融解液により人工授精した着床率と胎子の様子
精漿を包含していない合成融解液では着床率が低く、さらには、着床胎子の死亡率が増加する。(スケールバー = 10 mm)

着床を促進する精漿中因子 −cortisol の免疫抑制作用−

上述した EDTA および EGTA 添加融解液を用いた人工授精で十分な数の着床胎子が得られるかを確認するため、PMSG-hCG により過排卵処理を施した雌ブタに 1 回(50×10^8 sperm/ml) 人工授精後、30 日後の子宮を回収し、着床率(胎子数/黄体数)を算出した。その結果、EDTA と EGTA 添加融解液を用いた場合、着床率は 51%と低く、対照とした精漿 10% (v/v) 含有した融解液を用いた場合では 78%と有意な差が認められた(図 2) [Okazaki and Shimada, unpublished data]。さらに、精漿を全く包含していない EDTA および EGTA 添加融解液では、着床胎子が免疫細胞による食作用を受け、死滅しているものが多く観察された。これらの結果から、精漿中に子宮内の免疫系を制御し、胚の着床を促進(あるいは正常化)させる因子が存在しているのではないかと推察した。

人工授精後に子宮内に遊走される白血球は、子宮腔内に残留した死滅精子を貪食し、子宮環境を整える役割がある[18]。一方、Rozeboom *et al.* [29,30]は、これらの白血球が精漿存在下では人工授精後 24 時間以内に消失することを報告している。また、マウスおよびヒト精漿には、種々のサイトカイン・ケモカインや抗炎症性ステロイドホルモンなどが含まれていることが示されている[9,24,33]。したがって、精漿中の免疫抑制因子が、人工授精後の精子が抗原となって起こる細胞性免疫能を抑制し、胚が子宮へ到達した時にはその免疫力は低下し、白血球による胚への侵襲を抑制していると考えた。しかし，ブタ精漿中の免疫抑制因子と人工授精後の子宮内環境、さらには、それらが繁殖成績にどのように関与しているかについては全く明らかとされていない。そこで，ステロイドホルモンを中心に精漿中免疫抑制因子の同定を試みた。その結果、精漿中には免疫抑制作用を有する cortisol が 0.92 ng/ml 濃度で存在することが EIA により明らかとなった。cortisol は prostagrandin (PG)、leukotriene などの炎症性物質を阻害する[6]他、細胞質に局在する glucocorticoid receptor (GR)に結合し，核内へ移行後、NF-κB responsive element を負に制御することで炎症性サイトカイン(*Il-1*、*Il-2*、*Il-5*、*Il-6* など)遺伝子の転写を抑制する。さらには，抗炎症性サイトカイン遺伝子(*Lipocortin, Il-1R antagonist, Iκ–β*) を転写させることで，初期自然免疫の際に遊走される好中球などの白血球遊走を阻害する[5]。この cortisol の子宮内免疫抑制作用を、人工授精後の子宮腔内白血球数を指標に検討した。人工授精後 6 時間で子宮内に多核白血球 (polymorphonuclear leukocyte, PMN) が遊走され、その

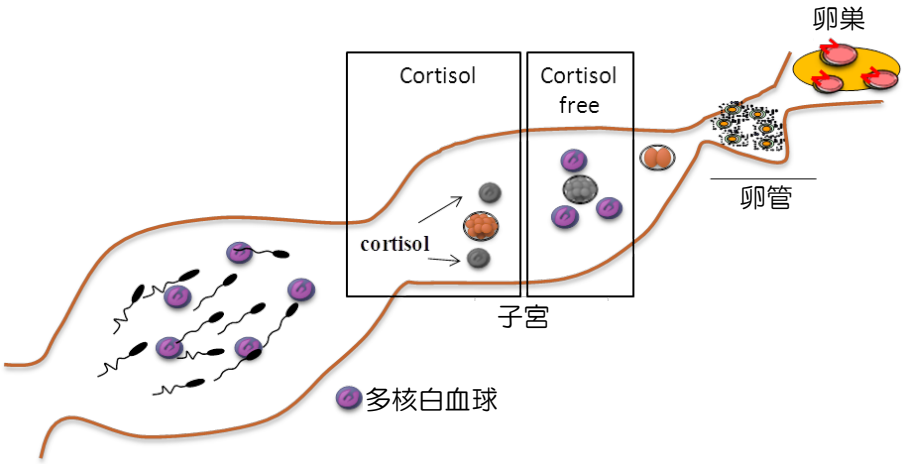


図 3. 注入する cortisol と子宮内免疫系および胚の生存について
人工授精後、精子を異物認識し、子宮内は多核白血球で充満される。人工授精時に子宮内へ注入される cortisol は、細胞性免疫を抑制し、胚の生存を担保する。

数は、液状精液、cortisol 無添加および添加凍結融解精液いずれを注入した処理区間でも有意な差は認められなかったが、人工授精後 24 および 48 時間後において、cortisol 無添加凍結融解精液を注入したそれは有意に高い値を示し、子宮内へ cortisol を注入することでこれらは精漿を含有する液状精液と同水準にまで低下した。これらの結果から、精漿中に含まれる cortisol は子宮内で精子など異物に対して遊走される好中球を人工授精後 24 時間以内で減少させる免疫抑制作用をもつことが明らかとなった（図 3）。

精漿中の生理活性物質が子宮内膜細胞に作用し、着床サイトでのリンパ球誘引および分化を促すことで着床を成立させることもマウスやヒトで報告されている。着床期の子宮粘膜中のリンパ球は、父性抗原をもつ半同種移植片(semi-allograft) である胎子を侵襲しないため、着床サイトのリンパ球が細胞障害作用の低下した子宮内特異的な uterine NK-cell (uNK)と $CD4^+$ helper T-cell (Th2) へと分化している。近年、Robertson *et al.* [25,26,27]および Gutsche *et al.* [11]は、精漿中の TGF-β と IL-8 が子宮粘膜細胞に作用することが着床サイトでのリンパ球集積と分化に重要であると報告している。我々はブタにおいて、精漿中に炎症性サイトカインであるマクロファージ遊走阻止因子 (MIF) や IL (interleukin)-17 を、抗炎症性サイトカインとして IL-13 を検出していること、着床期の子宮粘膜細胞に T-cell マーカーである $CD3^+$ cell が散在していることを見いだしている[Okazaki, unpublished data]ことから、ブタにおいても着床に重要な子宮粘膜中の T-cell が存在し、精漿がそれらに何らかの役割を果たしているかもしれない。今後、このような視点で精漿が胚の着床に果たす役割を研究していく必要がある。

2 価イオンキレート剤(EDTA+EGTA)および cortisol 含有融解液による人工授精テスト

私これまでの研究で、cortisol が子宮腔内の白血球数を減少させ，細胞性免疫能を抑制させることが明らかとなったことから、自然発情中の雌ブタへ EDTA、EGTA および cortisol 含有合成融解液を用いて凍結融解精液の人工授精を行い、その繁殖成績を算出した。その結果、人工授精後 30 日齢における子宮内の胎子着床率は 83%へと向上し、その値は、精漿を 10% (v/v)包含した融解液を用いた場合(78%)と遜色ないものであった。さらに、現場実証試験として、81 頭の自然発情中の雌ブタに、3 回の人工授精（5×10^9 sperm/回）を実施した。受胎率は 80%で、一腹平均産子数は 10.1 頭という好成績を示した。対照区として、これまでに開発してきた精漿含有融解液を用いて人工授精した成績、受胎率 81%、一腹平均産子数 10.3 頭と比較しても有意な差は認められなかったことからも、本研究で開発された合成融解液は産業上においても十分利用可能なものであると示された。

ステロイドホルモンである cortisol の注入は，胎子と母体へ副作用を及ぼす懸念があるが、子宮へ注入する cortisol 量は，ブタの炎症の治療時に投与する量に比較して 1/10,000 程度であり、精漿に含有する量と注入量が同程度であること、かつ、cortisol は代謝分解が早いこと、産子の一週齢平均体重、その後の発達も正常であることから、本融解液を用いた人工授精は安全面においても影響はないと考えられる。

謝辞

今回の試験遂行にあたり、豚の飼養管理および人工授精に御協力頂いた工藤 一男、伊東 昭司、藤原 弘

樹 氏に感謝申し上げます。なお，本成果は，生物系特定産業技術研究支援センターにおけるイノベーション創出基礎的研究推進事業により行われたものである。

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J. Reprod. Engineer. 2012; 15: 13–19.
http://sre.ac.affrc.go.jp/Contents.htm

Journal of REPRODUCTION ENGINEERING JRE

= Mini Review =

Recent advance of germ cell production *in vitro* from pluripotent stem cells

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INTRODUCTION

Primordial germ cells (PGCs), precursors of the oocytes and the spermatozoa, accomplish a unique developmental program to finally acquire totipotency. During the program, there are a number of biologically significant processes such as expression of pluripotency-specific genes, epigenetic reprogramming and meiosis. A flaw in any of these processes can cause developmental disorders and infertility. Understanding the whole process of PGC development is thereby of particular importance for not only basic biology but also clinical issues. How PGCs execute the unique program has, however, remained unclear, mainly due to limitation of experimental materials; for example the number of nascent PGCs in an embryo is less than 50. To overcome the limitation, we recently developed a culture system that produces a robust number, nearly a million, of PGC-like cells, of which potential is almost equivalent to nascent PGCs in embryos. This review briefly introduces the significance of developmental processes of PGCs, summarizes the culture system, and discusses possible applications towards understanding a whole process of PGC development.

Key words: primordial germ cells, pluripotent stem cells, epiblast, BMP4, spermatogenesis

PGC development *in vivo*, its specification, reprogramming and meiosis

In the mouse, the major model organism of mammals, PGCs are specified in the post-implantation embryo by signaling molecules, in contrast to other experimental organisms, in which a germ cell determinant complex preexists in the ooplasm [7,14]. PGC fate is induced in the pluripotent epiblast by bone morphogenetic protein 4 (BMP4), a secreted molecule, from the adjacent extraembryonic ectoderm [17]. Around embryonic day (E) 6.25 about six cells of the posterior proximal epiblast start to express *Prdm1/Blimp1*, a zinc finger transcriptional repressor, and these *Prdm1/Blimp1*-positive cells are lineage-restricted to become PGCs [26]. Prdm1/Blimp1 is a master regulator for PGC specification, as PGCs in *Prdm1/Blimp1*-deficient embryos impair repression of somatic cell genes, thereby resulting in severe disruption of PGC development at the early stage. This study suggests that Prdm1/Blimp1 endows epiblast cells to PGCs by repressing somatic gene expression program; however, direct target(s) of the transcription repressor remain unclear. Following *Prdm1/Blimp1* expression, PGC-specific genes, such as *Prdm14*, *Nanos3*, *Dnd1*, *Tdrd5*, and *Stella* etc. start to be expressed in PGCs [16,41]. At the specification, PGCs are located at the posterior end of the primitive streak, and then start to migrate along the developing hindgut

Submitted: August 16, 2012
Accepted: September 18, 2012
Advance Publication in Website: September 19, 2012

towards the genital ridges, the future testes or ovaries.

When epiblast cells give rise to PGCs, their cellular status transforms from a pluripotent epiblast state to a unipotent PGC state: it appears that PGCs soon after the specification commit to unipotent germ cell lineage that is designated to either the oocytes or the spermatozoa. It has been an enigma why PGCs keep unipotency despite of gene expression involved in pluripotency, such as *Oct4*, *Nanog* and *Sox2*. On the other hand, it is known that PGCs cultured under specific conditions reacquire pluripotency by transforming into pluripotent embryonic germ (EG) cells [22,28]. To address how PGCs switch their cell state, it seems important to reveal in detail the function of key pluripotent transcription factors, such as Oct4, Sox2 and Nanog, as well as germ cell specific transcription factors not expressed in the pluripotent cells.

While migrating towards the genital ridges, epigenetic marks, such as histone modification and DNA methylation, are extensively reorganized in the PGC genome (epigenetic reprogramming). Global changes in histone modifications, such as a decrease in histone 3 lysine 9 dimethylation (H3K9me2) and an increase in histone 3 lysine 27 trimethylation (H3K27me3), occur specifically in the PGC genome [9,32,33]. Although the biological significance of the global changes of histone modification is largely unclear, it is feasible that the changes are necessary for acquisition of totipotency at the terminal products. A clue is served from *Prdm14*-deficient embryos in which H3K9me2 levels remain high and H3K27me3 levels stay low [42]. Of interest, *Prdm14*-deficient PGCs are incapable of giving rise to pluripotent EG cells, suggesting that the global changes of histone modification (and maybe also Prdm14 itself) provide a permissive epigenetic environment enabling cells to maintain their potential pluripotency [42].

An accessible and reliable hallmark of epigenetic reprogramming is X-chromosome reactivation in female PGCs. In general, one of two X-chromosomes in the female embryo is inactivated, so that amount of gene transcript from X-chromosome is equivalent to the male embryo. The exception is PGCs, in which the inactive X-chromosome starts to reactivate [4,5,35]. The non-coding RNA *Xist*, an functional marker of X-chromosome inactivation, is downregulated in some PGC precursors as early as E7.0, but only at E10.5 have the majority of PGCs lost *Xist* [35]. Disappearance of *Xist* is followed by a gradual loss of H3K27me3 enrichment on the inactive X-chromosome [5], and instead an increase in a global level of H3K27me3. Molecular mechanisms underlying X chromosome reactivation are largely unclear. Recent reports raise the possibility that X-chromosome reactivation is possibly a consequence of the expression of pluripotency genes. For example, *Xist* is repressed by Oct4, Sox2 and Nanog in embryonic stem (ES) cells [23], indicating that *Xist* is downregulated in PGCs by the transcription factors expressed.

Besides the global change of the histone modification, genome wide DNA demethylation also occurs specifically in PGCs. Along the genome-wide DNA demethylation, parental state of genomic imprinting is erased. It is apparent that DNA demethylation, specifically erasure of genomic imprinting, is required for acquisition of totipotency, as incomplete erasure of genomic imprinting causes developmental disorders [1]. Much attention has been paid to understand how such genome wide DNA demethylation is achieved. In general, there are two pathways of DNA demethylation, passive and active pathways. The former is a replication-dependent manner, in which 5-methylcytosine (5mC) is diluted in the absence or under the prevention of maintenance DNA methyltransferase (Dnmt1) or/and its associated molecules, such as Uhrf1 [34,39]. The latter involves enzymes that directly modify and/or remove 5mC and of which the mechanism has not been fully elucidated [29]. Recently it has been proposed that DNA demethylation is an active process occurring through base-excision repair, which may in turn trigger the extensive chromatin changes of PGCs in the genital ridge [9,10]. This study also suggests the possibility that Tet-family proteins, which catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, play a role in genome-wide DNA demethylation in PGCs. This study, however, does not exclude a possibility that passive DNA demethylation is involved in the genome wide demethylation. Further analysis will provide insights into the molecular mechanism.

At E12.5, PGCs differentiate in a sex-dependent manner. PGCs in the female embryo undergo meiosis, whereas those in the male embryo start to arrest cell cycle at G1. The sex-dependent differentiation of PGCs is triggered by somatic cells of mesonephros and gonad. In female embryos, somatic cells in mesonephros

produce retinoic acid that induces meiosis in PGCs through a specific gene expression such as *Stra8* [2,15]. PGCs entering meiosis, then pass through leptotene, zygotene and pachytene, and finally arrest at diplotene at the birth. Some primordial follicles start to grow at puberty. In male embryos, PGCs arrest at G1 phase, called gonocytes, from about E13.5 to birth. After birth, gonocytes attach to the basement membrane of seminiferous tubules and then resume cell cycle to proliferate [27]. Some gonocytes differentiate into spermatogonial stem cells that produce spermatozoa throughout life.

Reconstitution of PGC specification *in vitro* development *in vivo*

As described above, there are a number of issues in PGC development that remain to be addressed. The main reason for this is the limited number of nascent PGCs, where the specification and the early process of epigenetic reprogramming occur. To address the biological significance of the PGC-specific gene expression signature and epigenetic reprogramming, a culture system to generate a large number of PGCs *in vitro* would be required for molecular and biochemical analysis. So far, several attempts have been made to generate germ cells from mouse ES cells [8,13,24,36]. These methods are mostly based on undirected differentiation of ES cells as monolayer culture or embryoid bodies and selection of rare germ cell-like cells. However, the ideal *in vitro* system would induce germ cell fate rather than select for randomly differentiated cells and it would recapitulate the earliest stages of PGC specification in an ordered manner. Most importantly, PGCs produced *in vitro* must be functional, which would otherwise essentially remain unclear whether PGC specification and epigenetic reprogramming *in vitro* recapture properly those *in vivo*. The gold standard to evaluate the function of germ cells is whether they give rise to healthy offspring. The studies mentioned above did not succeed in generating such fertile gamete [8,13,24,36].

Recently, we developed a robust culture system using ES cells to produce a large number of PGC-like cells (PGCLCs), of which the potential is almost equivalent to nascent PGCs *in vivo* [12]. Notably, the culture system recaptures the developmental process *in vivo*, as ES cells differentiate, by culturing with Activin A and basic fibroblast growth factor (bFGF), into epiblast-like cells (EpiLCs) and then EpiLCs differentiate into PGCLCs in response to BMP4. Based on the criterion of gene expression, EpiLCs mirror epiblast cells *in vivo*. Considering that ES cells mimic to some extent the inner cell mass (ICM) of preimplantaion blastocysts, it can be interpreted that the culture system recapitulates *in vitro* the differentiation process from ICM to postimplantation epiblast. Under a culture condition with a set of cytokines, which can produce functional PGCs from epiblast *ex vivo*, PGCLCs were induced at an efficient rate (more than 40%). This PGCLC induction strictly depends on BMP4, as withdrawal of BMP4 from the culture totally abolished the induction, faithfully recapitulating PGC specification *in vivo*. Characteristics of PGCLCs are indistinguishable from those of PGCs *in vivo*, based on criteria of gene expression, epigenetic status and potential to differentiate into fertile sperm. Microarray analyses showed that the transcriptome in PGCLCs mirrors that of early PGCs *in vivo*. Interestingly, PGCLCs express properly genes characteristic of early PGC development, but not marker genes expressed at late PGC development, such as *Mvh* and *Dazl*, as well as at meiotic-specific genes, such as *Stra8* and *Sycp3*. This indicates that under the culture condition PGCLCs arrest their development at a stage corresponding to E8–9.5 PGCs *in vivo*. At E8–9.5, PGCs *in vivo* arrest cell cycle at G2 phase and hardly proliferate while migrating in hind gut endoderm. Consistently, PGCLCs arrest their cell cycle at G2 phase and the number of PGCLCs is only slightly increased. These results indicate that additional (and still uncharacterized) growth factor(s) are required for exit of the G2 arrest and for further proliferation. Epigenetic reprogramming in PGCLCs occurs at a similar manner of that in PGCs *in vivo*, as a decrease in histone 3 lysine 9 dimethylation (H3K9me2) and an increase in histone 3 lysine 27 trimethylation (H3K27me3) were observed in the PGCLC genome. Most importantly, when PGCLCs were transferred into testis of germ cell-less W/Wv males, PGCLCs gave rise to fertile spermatozoa. Fertilized eggs with the spermatozoa developed fully to healthy offspring with the normal size of placentas. The offspring, both male and female, grew normally and had the ability to bear the next generation. These results clearly demonstrate that PGCLCs are properly derived from EpiLCs in the culture system. Taken together, the culture system reconstitutes

developmental processes from ICM to PGCs, which will contribute to address many issues that remain unclear due to the limitation of the material.

Questions to be addressed by the *in vitro* culture system

The culture system allows us to address several key questions to understand nature of PGC development. One would be molecular mechanism(s) of how BMP4 induces PGC fate in the epiblast. BMP signaling is transduced by Smad proteins. Once BMP binds to its receptors, Smad1, 5 and 8, which belong to receptor-regulated Smads (R-Smads), are activated through their serine-phosphorylation, bind to Smad4, the common mediator Smad (co-Smad), localize into nuclei and then act as a transcriptional factor. Consistently, it has been shown that disruption of *Smad1* and *5*, but not *Smad8*, genes result in attenuation of PGC specification [3,11,37]. Therefore, it would be very useful to identify the target genes of the R-Smad proteins to understand the molecular mechanism of BMP4 signaling on PGC specification. EpiLCs are suitable for ChIP analysis for isolating the target genes, since millions of EpiLCs can be easily prepared. Likewise, isolating target gene(s) of *Prdm1/Blimp1* is also of particular interest. Prdm1/Blimp1 is known as a master transcription repressor that directs terminally differentiated B-lymphocytes to antibody-secreting plasma cells [38]. It is feasible that this transcription repressor orchestrates PGC differentiation from pluripotent epiblast. Therefore, to reveal target genes of Prdm1/Blimp1 will be important to illustrate the gene expression network controlling PGC specification. Apart from Smad1 and Prdm1/Blimp1, other key transcription factors such as Oct4, Nanog and Sox2 are also worth investigating further to understand why PGCs are unipotent despite of pluripotent gene expression. Furthermore, to draw an accurate epigenome landscape of PGCs will be important to broaden our understanding of the contribution of a chracteristic pattern of histone modifications to PGC development. Specifically, comparison of the landscape between PGCs and pluripotent ES or/and EG cells will help to understand epigenetic nature of unipotency with potential pluripotency, which is observed in developing PGCs.

As described above, PGCLCs arrest their cell cycle at G2 phase, indicating that the growth factors in the culture medium are not sufficient to support further proliferation and differentiation. Considering PGC development *in vivo*, PGCs exit the G2 arrest after E9 and enter into genital ridge while proliferating. It is possible that somatic cells surrounding PGCs produce factor(s) supporting PGC proliferation and differentiation. Alternatively, biological activity of the recombinant proteins used in the culture is not as potent as those *in vivo*. Stem cell factor (SCF), a ligand of c-kit, is known to support proliferation of PGCs. However, the biological activity of SCF depends on its form; membrane-bound SCF has higher activity in supporting PGC proliferation than a secreted form of SCF [19,21]. Since soluble SCF is used in the culture system, its activity might not be enough to support proliferation of PGCLCs. As described above, PGCLCs do not express later PGC marker genes, such as *Mvh* and *Dazl*, and meiotic marker, *Sycp3*. It is reported that these genes start to be expressed according to DNA demethylation in PGCs [18]. These observations indicate that PGCLCs do not complete DNA demethylation, a hallmark of epigenetic reprogramming. To find factor(s) or condition(s) supporting proliferation and differentiation it is important to develop a culture system that supports the exit of G2 arrest, later marker gene expression, epigenetic reprogramming and meiosis entry.

Gametogenesis in vitro from PGCLCs

A goal of developing the culture system is to reconstitute *in vitro* the entire process of germ cell development. Although PGCLCs are fully potent, successful differentiation into spermatozoa still depends on environmental cues from the testis *in vivo*. Although it seems impossible to reconstitute all the environmental cues *in vitro*, a recent study presented an *ex vivo* culture system, in which the entire process of spermatogenesis was reproduced in a piece of neonatal testis cultured on an agarose block [30]. Mature spermatozoa obtained by this *ex vivo* culture method were functional, and could fertilize eggs that developed normally into healthy offspring. Furthermore, it was shown that germline stem cells, spermatogonial stem cells that proliferate indefinitely *in vitro* while keeping spermatogenic potential, gave rise to haploid cells by transplantation into W/Wv or busulfan-treated testis, followed by the *ex vivo* culture [31]. The *ex vivo* culture is therefore a bypass through which PGCLCs give rise to mature

spermatozoa in culture. This is a first step towards reconstituting the entire process of germ cell development.

Besides spermatogenesis, reconstitution of oogenesis *in vitro* from PGCLCs is also of particular importance. To date there is no defined stem cell population in oogenesis. However, it has been reported that reconstituted ovaries, which are reaggregates of dissociated fetal gonads containing PGCs, are able to produce fertile oocytes, when transplanted into the kidney capsule [20]. This suggests in theory that PGCLCs could possibly give rise to fertile oocytes in reconstituted ovaries as well. Although apart from nuclear transfer transplantation is the only method so far to obtain mature oocytes from PGCs, it is known that neonatal oocytes in primordial follicles can differentiate into mature oocytes under certain culture conditions *in vitro* [6,25]. In this culture system, however, efficiency of successful maturation to fully potent oocytes is relatively low, suggesting that the culture conditions are suboptimal even for isolated primordial follicles. Refinement of culture conditions using primordial follicles may precede establishment of a culture system using PGCLCs.

Application of the *in vitro* culture system to human ES cells

Successful production of functional PGCs from pluripotent stem cells may lead to an idea of applications to human reproductive medicine. Without mentioning the ethical issues, there are, however, technical obstacles to the direct application of our developed culture system to human PGC production from human ES cells. First of all, human and mouse ES cells are essentially different in responsiveness to growth factors. For example, bFGF promotes self-renewal of human ES cells, whereas it promotes differentiation of mouse ES cells to EpiLCs. Likewise, ActivinA/Nodal also promotes self-renewal of human ESCs through upregulation of *Nanog* expression [40], whereas that signal promotes differentiation of ESCs into EpiLCs with downregulation of *Nanog*. Furthermore, mouse ES cells can self-renew stably under a serum- and feeder-free condition, whereas this condition has not been firmly established in human ES cell culture. It is known that mouse ES cells cultured with pharmacological inhibitors of MEK and GSK3β remain in the ground state [43], which allows the ES cells to differentiate homogenously in response to a set of growth factors. In contrast, a culture condition placing human ES cells in the ground state has not yet been identified. Finding such a culture condition may be necessary for producing a large number of PGCLCs from human ES cells. Most importantly, there is no tool to evaluate whether human PGCLCs from human ES cells are functional. This obstacle may be overcome in part by using primate ES cells.

Concluding remarks

Here, we discussed several aspects of PGC development *in vivo* and *in vitro*, as well as possible future applications. PGCs are the sole lineage that acquires totipotency, by definition self-contained entities that can give rise to the whole organism. Totipotency is essentially different from pluripotency in the sense that pluripotency is a capability of differentiating into all cell lineages that compose the body, but do not support the formation whole organism formation (including the placenta). How germ cells acquire totipotency is the everlasting question for researchers concerning reproductive biology. We think that the culture system that we have successfully developed will contribute to provide new theoretical and empirical *insights* into the nature of totipotency.

Acknowledgements

This study was supported, in part, by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; by PRESTO; by JST-CREST/ERATO; by the Takeda Science Foundation.

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Journal of REPRODUCTION ENGINEERING　生殖工学会誌
2012年9月発行
発行者: 日本生殖工学会 (SRE)
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発行所: 日本生殖工学会 (SRE)
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