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Office
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TEL: +81-42-769-2339 FAX: +81-42-769-1762

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Editorial Office

National Institute of Agrobiological Sciences

Division of Animal Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan

TEL: +81-298-838-7447 FAX: +81-29-838-7408

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= Research Note =

Leukocyte count in follicular aspirate samples could be a prognostic biomarker of the developmental competence of human metaphase II oocytes

Takayuki ISHIKAWA[1,2,†], Masakazu DOSHIDA[1], Toshihiko KYOYA[1], Taeka TAKISAWA[1], Masahiro SAKURAI[2], Yumi HOSHINO[2], Kentaro TANEMURA[2], Koichi KYONO[1] and Eimei SATO[2]

[1]Kyono ART Clinic, Sendai 980-0014, Japan
[2]Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan.
[†] Correspondence: tishikawa1227@nifty.com

ABSTRACT

Multiple gestations markedly impact the health of children conceived via assisted reproductive technology (ART). The present study investigated the potential of leukocyte count in follicular aspirate (FA) samples as a follicular prognostic biomarker of the developmental competence of human metaphase II (MII) oocytes. FA samples were obtained from 27 ART patients during 27 natural menstrual cycles, Leukocytes were separated using density-gradient centrifugation and prepared the Diff-Quik stained smears. The 27 cycles were classified into 3 groups based on the developmental stage of the embryo (Group I, 7 oocytes (–); Group II, 6 unfertilized oocytes and 5 early cleavage embryos; Group III, 9 blastocysts) and evaluated estradiol, luteinizing hormone, and progesterone levels on the day of gonadotropin-releasing hormone agonist administration using enzyme immunoassay indicated that these levels did not correlate with developmental competence. The 27 cycles were divided into Categories A (19) and B (8) depending on the infiltrating leukocyte count in the follicular fluid (FF; less than 2.5×10^4 cells/follicle, and the value or more, respectively). Further classification and analysis of the 19 cycles classified as Category A into 3 groups (Group I, 6 oocyte (–); Group II, 5 unfertilized oocytes and 3 early cleavage embryos; Group III, 5 blastocysts) showed no significant differences among the groups regarding infiltrating mononuclear leukocyte (IML) count but a significantly increased infiltrating polymorphonuclear leukocyte (IPL) count in Group III compared to Groups I and II. These findings suggest that IPL count in FF is associated with the blastocyst formation of human MII oocytes, and thus it can be a predictor of the developmental competence of MII oocytes.

Key words: follicular fluid, human metaphase II oocytes, *in vitro* fertilization, infiltrating polymorphonuclear leukocyte

INTRODUCTION

Multiple gestations markedly impact the health and welfare of children conceived via assisted reproductive technology (ART). As twin pregnancies are associated with a higher risk of complications for both the mother and the fetus [1, 20, 21], reducing the incidence of multiple gestations is often desirable. One reasonable approach to reduce the disadvantage is single-embryo transfer (SET). With the development of SET, non-invasive and objective methods of oocyte/embryo selection have been required.

As oocyte maturation is controlled in the follicular

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microenvironment, age-related alteration of the microenvironment makes the human preovulatory oocyte susceptible to chromosomal disorders and cytoplasmic structural defects [8,9,25]. Because the follicular microenvironment is created by regulatory/metabolic factors present in the follicular fluid (FF) which is originated from follicular cells, detection of the existence of certain prognostic biomarkers in the FF might allow for prediction of oocyte viability. Higher levels of both follicular oxygen content [3,23,24] and vascular endothelial growth factor (VEGF) levels [14,16] in FF have been correlated with the developmental competence of human oocyte/embryo during *in vitro* fertilization (IVF) cycles. In addition, follicular concentrations of leptin [3,7], nitric oxide (NO) [3], high-density lipoprotein (HDL) cholesterol, HDL component proteins, and HDL micronutrients [5,6] have been reported to play important roles for in human oocyte/embryo development.

Although several studies have examined the presence of leukocytes in follicular aspirate (FA) samples obtained at oocyte retrieval in IVF cycles to determine their leukocyte populations [4,12,13,15,22], these studies used different methods of preparing the FA samples, estimating the leukocyte subset, and stimulating ovaries of patients, making the comparison of their results, which often differed, a difficult task. On the other hand, in their investigation of the leukocyte/erythrocyte ratio in FA samples and the leukocyte profile of both FA samples and peripheral blood in patients on the day of ovum pick up (OPU), Smith *et al.* (2005) concluded that leukocytes infiltrate the pre-ovulatory follicles [22]. Despite this finding, the origin of leukocytes remains unclear as a consequence of blood vessel damage during OPU.

Due to the mixed nature of the findings and the challenges faced in IVF research, no definitive predictor of oocyte viability has become widely used in clinical settings. This study evaluated infiltrating leukocyte count in FA samples as a prognostic biomarker of the developmental competence of human metaphase II (MII) oocytes.

The purpose of this experimental study is to evaluate the infiltrating leukocyte count in FF by examining FA samples obtained in natural cycles and to assess the potential of this variable as a predictor for developmental competence of human MII oocytes.

MATERIALS AND METHODS

Patients

FA samples were obtained during the 27 natural menstrual cycles of 27 patients with an unknown infertility factor undergoing an IVF cycle at the study site between December 2008 and October 2009 with the patients' informed consent.

Semen preparation

Semen samples were prepared using Sil-Select Plus (FertiPro N.V., Beernem, Belgium) in accordance with the manufacturer's protocol. After gradient centrifugation, washing and swim-up preparation were performed. Routine semen analysis was then performed according to World Health Organization guidelines [26].

Oocyte retrieval

Patients underwent transvaginal ultrasound and serum hormone analysis for estradiol (E_2), luteinizing hormone (LH), and progesterone (P_4) levels using enzyme immunoassay (Eclusys LH, E2 II and Progesterone II; Roche Diagnostics K.K, Tokyo, Japan). Hormone levels were measured from Day 13 to 16 of the menstrual cycle (Day 1: the first day of the menstrual cycle) on the day of gonadotropin-releasing hormone (GnRH) agonist (Buserecure; Fuji Pharma, Tokyo, Japan) administration. The schedule of oocyte retrieval was determined according to serum E_2, P_4, and LH levels. Final oocyte maturation was intranasally triggered with a GnRH agonist when the mean diameter of the dominant follicle was >18 mm under ultrasonography.

OPU was performed transvaginally at 36 h after GnRH agonist administration. For each cycle, the only dominant follicle was gently aspirated using a sterile syringe by a physician; no mechanical aspirators were used.

Conventional *in vitro* fertilization and embryo culture

Conventional *in vitro* fertilization (cIVF) was performed with subsequent embryo culture [10]. In brief, all incubations were performed at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Oocyte-cumulus complexes (OCCs) were inseminated at a final concentration of 1×10^5 motile sperm/ml at approximately 40 h post-trigger and incubated in Medi-Cult IVF medium (MediCult a/s, Jyllinge, Denmark) for 20 h. Inseminated oocytes were cultured in Global medium (Life Global, Guilford, CT, USA) from day 2 to 6 post-fertilization. Blastocysts were assessed by Gardner's criteria [11] and vitrified on day 5

Table 1. Preovulatory serum E_2, LH and P_4 levels in patient on the day of GnRH-agonist administration

	Oocyte(-)	Unfertilized/ early cleavage stage	Blastocyst	*P* value
Group (n)	I (7)	I (11)	III (9)	
E_2 (pg/ml)	304.6 ± 89.8	306.0 ± 95.0	242.8 ± 53.8	NS
LH (mIU/ml)	7.8 ± 8.7	12.8 ± 6.7	8.2 ± 3.0	NS
P_4 (ng/ml)	0.42 ± 0.16	0.54 ± 0.25	0.63 ± 0.36	NS

E_2; estradiol, LH; luteinizing hormone, P_4; progesterone. Serum hormone levels are given as means ± SD. Non-repeated ANOVA method. NS: no statistical significance.

Table 2. Comparison of infiltrating leukocyte count in FF classified by developmental competence of MII oocytes

Developmental stage	Groups	Cycles (n)	IML count (10^2 cells/follicle)	IPL count (10^2 cells/follicle)
Oocyte(-)	I	6	28.5 ± 22.0[a]	5.7 ± 7.2[c]
Unfertilized	II	2	111.9 ± 22.1[b]	8.4 ± 9.2[c]
Early cleavage stage	II	6		
Blastocyst	III	5	159.9 ± 62.6[b]	106.6 ± 30.7[d]

IPL count; infiltrating polymorphonuclear leukocyte count, IML count; infiltrating mononuclear leukocyte count, oocyte (-); no oocyte was retrieved.
IML and IPL counts are expressed as means ± SD. Tukey-Kramer method; [a-b, c-d]$P<0.01$

or 6 using Cryotop (Kitazato Bio Pharma, Fuji, Japan).

Leukocyte preparation

Leukocytes were separated from the FA samples using Polymorphprep (Axis-Shield, Oslo, Norway) in accordance with the manufacturer's protocol and centrifuged 2 times at 500 × *g*. After the leukocytes had been suspended in 100 µl of PBS, 1 µl suspensions were applied to prepare smears. The smears were fixed in methanol and stained with Diff-Quik (DQ) (CYSMEX, Kobe, Japan) in accordance with the manufacturer's protocol, with 2 separate smears prepared for each cycle. The stained smears were examined microscopically at × 400 or × 1,000 magnification and the leukocytes were classified as lymphocytes, neutrophils and eosinophils.

Erythrocyte and leukocyte counts in FA samples

Based on the erythrocyte count in the FA samples and the blood cell populations in the peripheral blood of the patients on the day of OPU, the putative peripheral blood volume and peripheral leukocyte count in the FA samples were estimated. The infiltrating mononuclear leukocyte (IML) count in the FF was determined by subtracting the peripheral blood mononuclear leukocyte count from the mononuclear leukocyte count in the FA samples. The infiltrating polymorphonuclear leukocyte (IPL) count in the FF was determined using a same method. As the FA samples contained varying amounts of flashing medium in each cycle, the leukocyte counts were expressed in terms of mean ± standard deviation (SD)/follicle.

Grouping of cycles

The 27 cycles were classified into Category A (less than 2.5×10^4 cells/ follicle) and Category B (greater than 2.5×10^4 cells/follicle) depending on peripheral blood leucocyte count in the FA samples. The cycles that were classified as Category A were further classified into 3 groups based on the developmental stage of the embryo (Group I; no oocyte was retrieved, Group II; an unfertilized oocyte or an embryo at the early cleavage stage, and Group III; a blastocyst).

Statistical analysis

Significant differences in hormone levels were evaluated by non-repeated measures ANOVA. On the other hand, infiltrating leukocyte counts in FF among the experimental groups were evaluated by Tukey-Kramer method. *P* values < 0.01 were considered to indicate statistical significance.

RESULTS

Patient age and oocyte retrieval

The mean patient age was 33.6 ± 3.1 years (range: 27–38 years). OPU was performed for 27 cycles.

cIVF and embryo culturing

In 20 of the 27 cycles, 1 oocyte was retrieved and cIVF was performed. As the results of cIVF and subsequent *in vitro* culturing, 14 fertilized oocytes (14/20, 70.0%) developed to the early cleavage stage and 9 blastocysts (9/14, 64.3%) were obtained on day 5 or 6. All blastocysts were classified as 6 Grade 4-blastocysts and 3 Grade 3-blastocysts, and vitrified them in liquid nitrogen.

Serum hormone levels

The 27 cycles in which OPU was performed were classified into 3 groups based on the developmental stage of the embryos and statistically evaluated. As shown in Table 1, evaluation of E_2, LH, and P_4 levels indicated that these levels did not correlate with the developmental competence.

Peripheral blood leucocyte count in FA samples

Peripheral blood leucocyte count (peripheral blood volume) in the FA samples was less than 2.5×10^4 cells/follicle (< 4 μl) in 19 cycles (Category A) but greater than 2.5×10^4 cells/follicle (> 6.8 μl) in 8 cycles (Category B).

Infiltrating leukocyte count in FA samples

The 19 cycles that were classified as Category A were further classified into 3 groups based on the developmental stage of the embryos. As shown in Table 2, although there were no significant differences between Group II and III with regard to IML count, there was a significant increase in the IPL count of Group III compared to that in Groups I and II. While IPL count ranged from 0.78 to 1.59×10^4 cells/follicle in Group III cycles, it was less than 0.29×10^4 cells/follicle in all Group I and II cycles. Due to the large number of peripheral blood leucocytes in the FA samples in all category B cases and the difficulty in determining IPL count on the stained smears, IPL count could not be determined accurately for this category.

DISCUSSION

Oocyte maturation is known to be controlled a follicular microenvironment that is composed of follicular cells and regulatory/metabolic factors present in the FF. Based on this knowledge, several investigators have investigated levels of O_2, VEGF, NO, leptin, HDL cholesterol, HDL component proteins, and HDL component micronutrients in FF as predictors of the developmental competence of human oocytes/embryos in IVF cycles [3,5–7,14,16,23,24]. In contrast, other investigators have focused on the presence of leucocytes in FF to determine leukocyte populations and the number of leukocytes in FA samples obtained at OPU in IVF cycles. However, the investigators have obtained mixed results due to their use of different methods and examination of different cycle characteristics patient populations, and could not determine the origin of leukocytes as a result of the presence of erythrocytes in the FA samples. Despite these challenges, in their investigation of the leukocyte/erythrocyte ratio in FA samples and the leukocyte profile of both FA samples and peripheral blood in patients on the day of OPU, Smith *et al.* (2005) concluded that leukocytes infiltrate the pre-ovulatory follicles [22].

In recognition of these findings and challenges, all FA samples examined in this study were obtained from patients in natural cycles, and contamination of the FA samples by peripheral blood due to blood vessel damage during OPU was considered. The putative peripheral blood volume was estimated based on erythrocyte count in both the FA samples and the peripheral blood, and the leukocyte count in the FA samples determined by subtracting the putative peripheral blood leukocyte count from the leukocyte count in the FA samples. Analysis of FA samples containing varying amounts of peripheral blood in which the peripheral blood leucocyte count was less than 2.5×10^4 cells/follicle revealed a stronger correlation between blastocyst formation of MII oocytes and IPL count in FA samples obtained from follicles nurturing MII oocytes that developed to the blastocysts (Group III) compared with that of Groups I and II. On the other hand, the peripheral blood leucocyte count of category B exceeded 2.5×10^4 cells/ follicle, leading to difficulty in estimating the IPL count due to the large number of peripheral blood leucocytes on the DQ-stained smears.

As it involves the participation of both leukocytes and inflammatory mediators, ovulation, the terminal stage of oocyte maturation, shares several similarities with a local inflammatory reaction. In fact, previous studies have found that several inflammatory genes are expressed in preovulatory granulosa cells (GCs) and

cumulus cells (CCs) [17]. Specifically, the concentration of IL-8 (known as typical chemokine CXCL8) has been found to be higher in dominant follicles of the late follicular/ovulatory phase compared with those of the mid-follicular phase, and the capability of GCs to synthesize IL-8 to be up-regulated [18,19]. It is possible that the infiltrating polymorphonuclear leucocyte (IPML) populations of the FA samples examined in this study may have reflected the integrity of the microenvironment of the ovulatory follicle, specifically that of an environment that promoted oocyte maturation and ensured developmental competence of the human MII oocyte.

In conclusion, this is the first study to reveal that the IPL count of FA samples might be associated with the ability of human MII oocytes to develop to blastocysts. Although further studies are required using larger samples before IPL count can definitively defined a predictor in clinical settings and as a subset of IPL count populations in FA samples, the findings of this study indicate that IPL count may be a predictor of the developmental competence of human MII oocytes.

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= ミニレビュー =

ピエゾマイクロマニピュレータを用いた発生工学技術について
The technology for developmental engineering with piezo micromanipulator

岩元 正樹
Masaki IWAMOTO

プライムテック株式会社 先進技術開発チーム，〒300-0841 茨城県土浦市
iwamoto@primetech-jp.com

要旨

発生工学とは、哺乳類に限らず様々な生物種の生殖細胞や胚に人為的な実験操作を加えた後、発生を進行させ成体を作出する技術開発と、作出した成体を用いて基礎および応用研究を行うことを目的としている。これにより、哺乳動物を中心に発生機構の解明が進むと同時に、生物学、医学、畜産学、実験動物学等への応用が進展してきている。発生工学研究は、発生学、細胞生物学および分子生物学の各種の技術や知識を基本として行われており、哺乳類胚において特に必要とされる基本的な技術として1)初期胚の作出・体外培養技術、2)生殖細胞や胚への顕微鏡下での操作（マイクロマニピュレーション）や遺伝子操作、3)操作した胚を、仮親へ移植し個体を作出するために関連する技術の3つの技術が挙げられる。その中でも、2)の顕微鏡下での操作や遺伝子操作については、マイクロマニピュレーション技術が基本技術となり、顕微授精、核移植、DNA注入およびES (embryonic stem)細胞注入に応用できる。

従来、このマニピュレーション技術は、その操作に職人技が必要となり、習得に時間がかかり、新たな発生工学技術の進展には、簡便な操作方法の開発が必要とされた。1990年代前半にプライムテック株式会社より開発・発売されたピエゾ駆動式マイクロマニピュレータ(piezo micro manipulator; PMM)は、セッティング方法を習得すれば、従来から必要とされたその職人技の習得が必要なく、容易に操作ができる。PMMは、ピエゾ(圧電素子； piezo electric elements)を伸縮させることによって得られる慣性力を動力としたステップ状の駆動原理に基づき、0.1 μm以下の超微動域の動作が可能となる。そのため、卵子や胚の弾性のある透明帯や伸展性の高い卵細胞膜でも微細ピペットをスムースに挿入可能となり、顕微操作による損傷を最小にすることで、操作者による差もほとんどない。また、従来からマニピュレーション操作に使用されていたガラスマイクロピペットは、卵子や胚への穿刺性を高めるために、先端を鋭利に研磨しスパイクと呼ばれる棘状に仕上げたものが用いられていたが、この先端の形状が卵子への穿刺性に大きく影響する。さらに、穿刺する際、卵子の変形や卵細胞質の流動等のストレスのため、崩壊および変性する割合が高くなる。一方、PMMを用いた場合、ガラスマイクロピペットの先端は、平らであり特殊な加工が必要ないため、先端形状による穿刺性への影響は、ほとんどない。また、卵子や胚への穿刺する際の変形や卵細胞質の流動もなく穿孔することが可能となり、穿刺後の崩壊や変性を軽減できる。

PMMの使用用途は、上記の基本的なマニピュレーション操作の顕微授精、核移植、ES細胞注入、バイオプシー、アシストハッチング等に利用され、哺乳動物卵子のみならず魚介類卵子等への応用もされ幅広く使用されている。本稿では、PMMを用いた発生工学技術について、これまでの報告例をまとめたので紹介する。

キーワード: ピエゾマイクロマニピュレータ, PMM, ICSI, 顕微注入, 核移植

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顕微授精(卵細胞質内精子注入法, intracytoplasmic sperm injection; ICSI)

顕微授精は、卵子に対して人為的に精子を授ける技術である。顕微授精には、機械的に卵子の透明帯に穴を開けて精子が侵入し易くする透明帯開孔法、卵子囲卵腔内に直接精子を注入する囲卵腔内精子注入法および卵細胞質内に精子を直接注入する卵細胞質内精子注入法(以下ICSI)等の技術があるが、現在ではICSIが、顕微授精と同じ意味として使われることが多くなっている。

様々な生物種でICSIを用いた研究が行われているが、哺乳動物で初めて報告したのはハムスター卵子にハムスター精子を注入し前核形成を観察したUehara & Yanagimachi (1976)[1]である。その後、ウサギ(1988)[2]、ウシ(1990)[3]およびヒト(1992)[4]では産子の作出も報告されている。マウスにおいては、卵子の特性から、ハムスターやヒトと同様の手法では、精子を卵細胞質内に顕微注入した後の生存率が著しく低下し、受精率および胚盤胞形成率も低下するため、長年難しい技術とされてきた。しかし、1995年にKimura & Yanagimachiが報告したマウスのICSIでは、ピエゾマイクロマニピュレータ(piezo micro manipulator; PMM, プライムテック社製)を用いることで、生存率が大幅に改善され多くのマウス産子が得られた[5]。その理由は、マウス卵子の卵細胞質膜の伸展性が高いため、従来法で用いるピペットの先端の鋭利な形状では卵子への穿刺の際、変形や損傷等の悪影響が生じるのに対し(図1A)、PMMを用いた場合は、取り付けたピペットの先端が平坦な形状のため、その様な悪影響が少ないうえに、安定した穿刺性が得られるためであった(図1B、図2)[5]。その後、マウスICSIは、PMMによるマウス精子の不動化を行うと同時に頭部と尾部を切断し、頭部のみを注入する一般的な手法となった[6]。

Kimura & Yanagimachiの報告により、PMMを用いた顕微授精は、国内外の多くの研究者によって、その応用は一気に広がり、ヒト(1998)[7]、ラット(2002)[8]、ウシ(2002)[9]、ハムスター(2002)[10]、ブタ(2003)[11]、メダカ(2009)[12]等で報告されている(表1)。Hirabayashi *et al.* [8]は、ラットでPMMを用いたICSI成功例を報告している中で、ピペットの先端外径を従来使用されていた7−10 μmから 2−4 μmに最小にすることで生存率および受胎率を改善していた。ピペット外径を最小にすることで、精子注入時の培養液注入量を最小限にしたことが生存率および受胎率改善の要因であると報告している。上述のようにPMMの穿刺性は、ピペットの先端形状の影響を受け難いため、先端外径を小さくしても卵子への安定した穿刺性が得られたことも成功要因の一つとして考えられる。Yamauchi *et al.* [10]は、ハムスターにおいてPMMを用いたICSIでの産子作出に成功している。ハムスター卵子は、実験室の電灯等の短波長の光により胚発生等に悪影響が及ぼされることが報告されており、体外およびインキュベーターの外での操作を迅速に行うことが必要となる。そこでYamauchi *et al.*は、マニピュレーションを行う実験室の電灯や顕微鏡の光源からの短波長の光を卵子や胚に長時間照射しないことで生存率と受精率を改善した。Nakai *et al.* [11]は、ブタにおいて初めて体外成熟卵子を用いたICSIの産子作出の成功例を報告している。ブタの体外受精において多精子受精が問題とされており、Nakai *et al.*のPMMを用いたICSIは、その問題を解決する1つの手段となった。

ICSIはヒトでの不妊治療でも、Palermoの報告[4]以来、一気に臨床応用され、同時に技術革新も進んできている。Yanagida *et al.* [7]は、PMMを用いたヒトのICSIを従来法と比較し、PMMを用いた手法が卵子生存率および受精率が有意に改善されることを報告している。現在では技術革新も進み、従来法でのヒトICSIでも高い生存率と受精率が得られるようになっているが、PMMとピペット外径を最小にした極薄肉管ピペットの組み合わせにより、卵子への注入時の負担を軽減させることが可能となり、さらに生存率を高めることができたとの報告もされている[13]。

Kimura & Yanagimachiが報告したこの技術は、上述のような他の動物種への応用以外にも、フリーズドライ精子によるICSI [14]、精子ベクターによる遺伝子組換え動物の作製[15]およびクローンマウスの作出[16]での基盤となった。

ES細胞注入

発生工学において、胚への遺伝子操作は重要な技術の1つである。1980年にマウスにおいては、直接受精卵の前核にDNAを注入する方法で遺伝子改変個体を作出する技術が開発されたが、特定の遺伝子配列に対する操作が不可能であった。一方、マウスにおいて胚性幹(embryonic stem; ES)細胞が樹立され(1987)[17]、このES細胞をマウス胚に移植することでキメラ個体を介してES細胞の形質をその子孫に伝達する技術が1980年代に確立された[18]。さらにES細胞に対してジーンターゲッティングと呼ばれる染色体の特定の遺伝子配列を自由に変換する遺伝子組み換え操作が行えるようになり、遺伝子組換えマウスの作出が可能となった[19,20]。このキメラマウスを用いたジーンターゲッティング(ノックアウト)法により、胎児や成体における形態形成、免疫、器官形成、脳神経機能等の分子メカニズムの解析が進み、基礎的な研究が進展している。

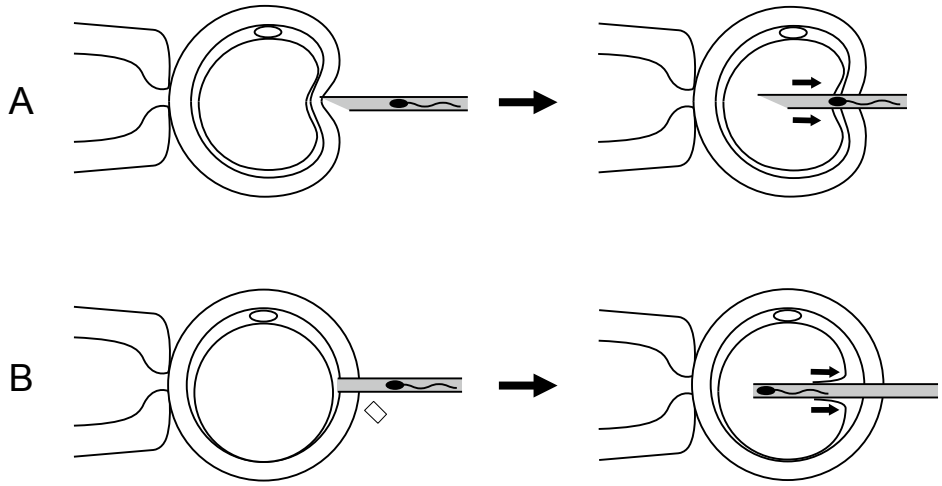

図1. 穿刺方法
A: 従来法の鋭利なピペットによる卵細胞質への穿刺状況
B: PMMを用いた先端が平らなピペットによる卵細胞質への穿刺状況

Kawase *et al.*は、マウス胚盤胞に組換えES細胞を注入する際に、PMMを用いる手法を報告している(2001)[21](図3)。Kawase *et al.*の報告では、通常法とPMMを用いた場合において、キメラ産子率では差はないものの、ES細胞注入後の生存率においてPMMを用いた方が有意に高くなることが示されていた。また、1時間当たりの操作数もPMMを用いた場合は、通常法の約3倍の数の胚に操作が可能となり、効率良くキメラマウスを作出できるようになっていた。Kawase *et al.*は、通常法では胞胚腔が極力大きい拡張胚盤胞を選別して穿刺するのに対して、PMM法は胞胚腔の大きさに関係なく安定して穿刺できることも利点として挙げている。

体細胞核移植

1997年に世界で初めての体細胞クローン動物の作出が、Wilmut *et al.*によってヒツジで報告されて以来[22]、現在までに、ウシ[23]、マウス[16]、ヤギ[24]、ネコ[25]、ウサギ[26]、ラバ[27]、ウマ[28]、ラット[29]、シカ、イヌ[30]、オオカミ[31]、フェレット[32]、ラクダ[33]においてクローンの作出成功例が報告されている。また、筆者らのグループは、2000年にブタの体細胞クローンの作出成功例を世界で初めて論文報告している[34]。体細胞クローン技術の利用としては、畜産分野において優秀な形質を持つ家畜の大量生産や家畜育種改良の効率化が上げられる。また、通常の畜産上の応用以外にも希少(野生)動物の保護、遺伝子組み換え技術を利用した有用物質生産のため動物工場化、移植用臓器の生産等の幅広い応用が期待されている。

体細胞クローンの作出における一般的な核移植方法は、第二減数分裂中期の未受精卵子の染色体を除去し(除核)、その除核卵子細胞質に細胞周期をG1/G0期に同期化した体細胞核を導入する。その後、発生を開始させるための受精の疑似刺激としての薬剤や電気刺激による活性化処理を行う。体細胞核移植において核の導入法は、電気融合法と顕微注入法の2つが挙げられる。電気融合法は未受精卵子の除核後、体細胞核を電気刺激により卵細胞質内に融合・導入する方法である。最初の体細胞クローン作出成功例のヒツジやウシにおいて、この電気融合法を用いて核の導入を行い、体細胞クローンの作出に成功している。その後、マウス[35]やブタ[36]においても電気融合法による体細胞クローン作出成功例が報告されている。電気融合を行った後、再度電気刺激もしくは薬剤処理により活性化を促すが、ウシにおいてはその処理のみでは活性化が困難であるため、タンパク質合成阻害剤を併用している。一方、マウスにおいては、卵子の活性化が容易に誘発されるため、電気融合法では融合液のCa^{2+}濃度等の条件を厳密に設定しない限り、核の導入と同時に活性化を受け易くなる。Wakayama *et al.* [16]により開発された顕微注入法は、Kimura & Yanagimachiが報告したPMMを用いたICSIを応用した体細胞核を直接、除核卵子の細胞質内に核を導入する方法である(図4)。この手法は、体細胞核導入時に卵子の活性化を誘発しない。そのため、体細胞核が未受精卵子の細胞質内に存在する卵成熟促進因子(maturation promoting factor; MPF)の影響を受けることになり、染色体凝縮(premature chromosome condensation; PCC)を起こす。このPCCが体細胞クローンマウスの作出効率に関連することも報告されている。ラットに関しては、プロテアーゼ阻害剤であるMG132で卵子を処理することでMPF活性の低下を抑制し、PMMを用いた核移植により体細胞クローンの作出に成功している[29]。

表1. ピエゾマイクロマニピュレータを用いたICSIの報告例

動物種	発表者	内容	発表年	文献番号
マウス	Kimura & Yanagimachi	世界初のPMMを用いたマウスICSI成功例	1995	5
ヒト	Yanagida et al.	ヒトのICSI	1998	7
マウス	Wakayama et al.	マウスフリーズドライ精子によるICSI成功例	1998	14
マウス	Perry et al.	マウス精子ベクターによるICSIでの組換え個体作出成功例	1999	15
ラット	Hirabayashi et al.	ラットのICSI	2002	8
ウシ	Horiuchi et al.	ウシのICSI	2002	9
ハムスター	Yamauchi et al.	ハムスターのICSI	2002	10
ブタ	Nakai et al.	ブタのICSI	2003	11
メダカ	Otani et al.	メダカのICSI	2009	12

表2. ピエゾマイクロマニピュレータを用いた体細胞核移植の報告例

動物種	発表者	内容	発表年	文献番号
マウス	Wakayama et al.	体細胞クローン作出	1998	16
ブタ	Onishi & Iwamoto et al.	体細胞クローン作出	2000	34
マウス	Wakayama et al.	体細胞核移植胚からES細胞樹立	2001	41
ウシ	Ushijima et al.	核移植胚作出	2002	37
ウシ	Galli et al	体細胞クローン作出	2002	38
ラット	Zhou et al.	体細胞クローン作出	2003	29
マウス	Kishigami et al.	体細胞クローン作出、HDACi使用	2006	47
マウス	Rybouchkin et al.	体細胞クローン作出、HDACi使用	2006	48
マウス	Inoue et al.	体細胞クローン作出、*Xist*-KO細胞	2010	49
マウス	Matoba et al.	体細胞クローン作出、*Xist*-siRNA使用	2011	50

HDACi: Histone deacetylase inhibitor, *Xist*-KO: *Xist*遺伝子ノックアウト, *Xist*-siRNA: RNA干渉により*Xist*遺伝子の発現抑制

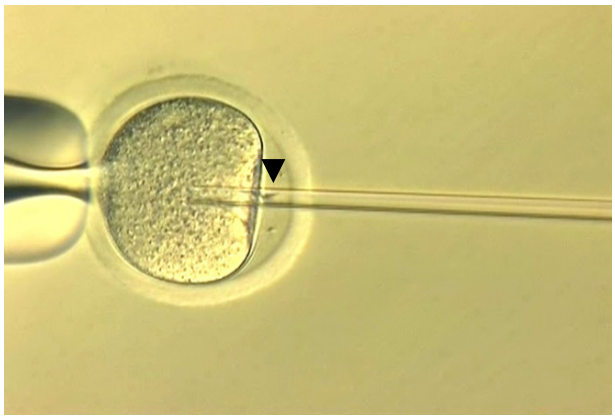

図1. PMMを用いたマウスICSI
矢頭: マウス精子頭部

筆者らは、ブタの体細胞核移植において以下の2つの理由からPMMを用いた顕微注入法を選択した。1つ目の理由としてブタ卵子も、マウス同様に電気刺激により容易に活性化を受けるため、核の導入と活性化を分け、体細胞核が卵子細胞質内のMPFによりPCCを生じることが重要と考えた。2つ目の理由としてブタは多胎のため、妊娠率を高めるために多数の胚移植用クローン胚が必要であると考えられた。多数のクローン胚を迅速かつ簡便に作出することが必要と考えられ、PMMを用いた顕微注入法は最適であった。筆者らは、これらの理由から顕微注入法による体細胞クローンブタの作出を試み、国内初のクローンブタ作出に成功した。この結果から、顕微注入法によりブタでも体細胞クローンの作出が可能であることが確認できた。

ウシの核移植では、電気融合法が多く用いられているが、ドナー細胞の直径が小さい場合において融合率が低下する場合がある。そこでUshijima *et al.* [37]は、ドナー細胞の直径が小さい場合に、融合率への影響を受けないPMMを用いた顕微注入法がウシ体細胞核移植でも使用できるか検討を行った。その結果、ウシの核移植において顕微注入法を用いた場合でも、胚盤胞を作出できることを報告している。さらに、体細胞核を注入する際に、細胞内カルシウム導入剤であるinositol 1,4,5 triphosphate (IP3)を同時に注入することで胚盤胞発生率が向上する結果を示している。また、Galli *et al.* [38]は、PMMによる顕微注入法と電気融合法によるウシ核移植胚の産子への発生率を比較し、差がないことを報告している。

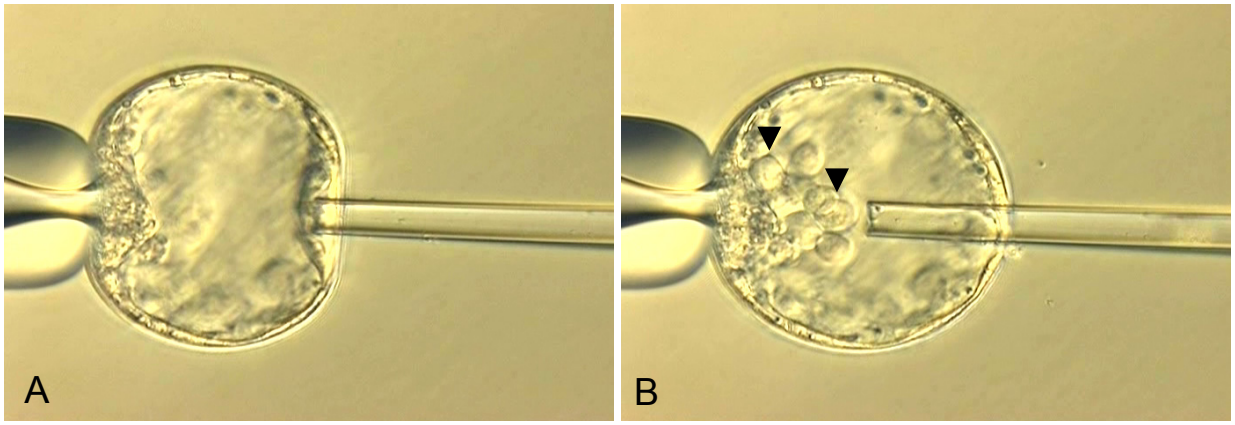

図3. ピエゾマイクロマニピュレータを用いたマウス胚盤胞へのES細胞注入操作
AからBの手順で操作を行う。A:マウス胚盤胞の透明帯を穿刺した直後の写真。B: マウス胚盤胞の栄養膜外胚葉を穿刺した後、ES細胞を注入した直後の写真。矢頭: 注入されたマウスES細胞。

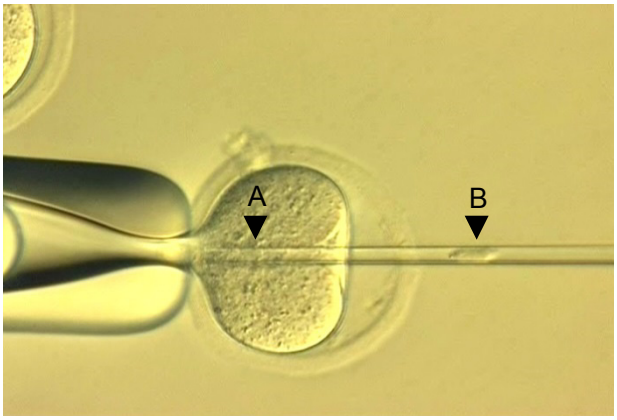

図4. ピエゾマイクロマニピュレータを用いたマウス核移植操作(体細胞核注入)
矢頭A: 保持した除核卵子に注入するマウス体細胞核。矢頭B: 次の卵子に注入するためにピペット内に吸引されているマウス体細胞核。

Wakayama *et al.*が開発したPMMを用いた核移植技術は、マウスを中心に一般的に確立された手法[39, 40]となっており、胚発生の基礎研究や遺伝子組換え動物の作出等に幅広く利用されている(表2)。さらに、Wakayama *et al.* [41]は、核移植胚からES細胞を樹立することに成功し、ntES (embryonic stem cell via nuclear transfer)細胞分離技術も確立した。この技術はヒトにおいても応用されており、再生医療への利用が期待されている[42]。また、筆者らは、顕微注入法による体細胞クローンブタの作出技術を遺伝子組換えブタの作出に利用した。遺伝子組換えブタは、医療用モデルブタとして、臓器移植モデル[43,44]、再生医療用モデル[45]およびヒト疾患モデル[46]が作出されている。

体細胞クローン技術は、発生工学技術が結集された複合技術である。胚操作、培養および胚移植技術等が必要とされ、どれか一つでも不完全であると個体の作出は困難となる。PMMは、その中の胚操作(核移植)において安定した穿刺性と操作性を与え、技術としては安定したレベルまで達しているものと思われる。しかし、これらの発生工学技術がある程度のレベルまで確立されている動物種においても体細胞クローンの作出成功率は、現在においてもきわめて低いという問題点を抱えている。この問題点は、どの動物種においても多少の差はあるものの、同様である。体細胞クローンの作出効率が低い主たる原因としては、体細胞核のリプログラミングがその後の胚発生に大きく影響する可能性が挙げられ、その制御が重要とされている。近年、脱アセチル化酵素阻害剤(histone deacetylase inhibitor; HDACi)であるトリコスタチンA等を核移植胚の発生初期に添加することで胚発生の改善やクローンの作出効率が向上するとの報告が数多くなされている[47,48]。また、核移植初期胚ではX染色体の不活化で重要な働きのある*Xist*遺伝子が過剰に発現する問題が報告され、その過剰な発現を*Xist*遺伝子ノックアウト[49]もしくはRNA干渉により*Xist*遺伝子の発現を抑制し、体細胞クローンの作出効率を改善したとの報告もある[50]。しかし、現状では大幅な改善までには至っていないことから、リプログラミングの制御には、さらなる手法の改善が必要と考えられる。近年、

再生医療の分野では、Yamanaka *et al.*により開発されたiPS (induced pluripotent stem)細胞が注目されている[51]。iPS細胞は、分化多能性に関連する4遺伝子を線維芽細胞に導入し、ES細胞様に誘導することで作出される。4遺伝子が線維芽細胞をリプログラムすることで多能性幹細胞に誘導しているものと考えられている。このiPS細胞研究におけるリプログラミングは、体細胞クローン技術に共通する課題として参考にする必要がある。

今後の展望と課題

これまで解説してきたようにPMMは、近年の発生工学・生殖工学の著しい伸展に大きく貢献している。特にマウスにおけるICSIおよび核移植による基礎的な発生機構の解明やヒトの不妊治療でのICSIで多く利用されている。PMMを用いた発生工学技術は、安定した穿刺性と操作性から、マウスやブタ等の多胎動物では多数の卵子に対して短時間で簡便な操作が可能となり、ヒトの不妊治療では、1個の卵子に対して優しい操作を行うことが可能となる。一方、従来から行われているマニピュレーション技術は、先に述べたように操作者の癖や経験に基づく感覚が重要な職人技が必要とされる。そのため、基礎研究分野では、安定したデータの取得までの時間が必要となる上に、データの精度にも影響が生じる可能性がある。また、不妊治療施設では、近年の晩婚化に伴う高齢出産の影響で治療件数が増大し、マニピュレーション操作等についての新人教育を行う余裕のない状況が出てくることが予想される。そのような中で、PMMの安定した操作技術法をさらに発生工学・生殖工学分野で共有することにより、発生生物学の基礎研究分野でのデータの信頼性を向上させ、不妊治療分野では新人のマニピュレーション教育に最適な技術となるものと考えられる。筆者らは、PMMによる発生工学技術のさらなる進展を目指した研究開発を継続すると同時に本誌日本生殖工学会を含む各関係学会と連携を取り、PMMを用いた発生工学技術についての研修事業などを手掛けて、若手研究者や不妊治療施設の培養士等の育成や技術支援を行い、研究および不妊治療分野のさらなる技術発展に貢献したい。

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= Mini Review =

Prospects of female germ cell production *in vitro*

Katsuhiko HAYASHI† and Mitinori SAITOU

Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
†Correspondence: khayashi@anat2.med.kyoto-u.ac.jp

ABSTRACT

Eggs are the only cell type harboring totipotency, and thus play a fundamental role in the creation of new individuals. There are multiple and complex regulations in the production of eggs, named oogenesis, and errors in this process can cause a number of clinical issues, such as infertility, ovarian cancer and developmental disorders. Thus the understanding and *in vitro* reconstitution of oogenesis are of biological and clinical importance. Recently, we produced fully potent eggs from pluripotent stem cells, i.e., embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs and iPSCs differentiate *in vitro* into primordial germ cells (PGCs), the precursor of egg cells, and are in turn matured to eggs by transplantation into adult female mice with fetal gonadal somatic cells. This review introduces the method and discusses applications of this technology to reproductive biology.

Key words: primordial germ cells, pluripotent stem cells, oogenesis

Introduction

Eggs, the terminally differentiated cells of the female germ cell lineage, are established through a series of processes. All eggs (and sperm) originate from primordial germ cells (PGCs), the founder cell population of the germ cell lineage that is specified in mice from the pluripotent epiblast around embryonic day (E) 6.5 (Figure 1) [5,26,27]. Soon after specification, PGCs start to migrate, while proliferating, toward fetal gonads that are the future ovaries (or testes). Early differentiation of PGCs is accomplished in a sex-independent manner, but once the PGCs reach the fetal gonads they begin to differentiate in a sex-dependent manner. In females, PGCs in the gonads, thereafter called oogonia, proliferate to expand the pool of future oocytes by around E13.5 and then cease mitosis and enter meiosis. It is essential for PGCs to settle in the gonads to properly differentiate into oocytes, since ectopic PGCs, which are mainly caused by aberrant migration, would trigger apoptosis and eventually be eliminated from the embryo proper [24]. The gonadal environment is important not only in terms of survivability, as described above, but also for further differentiation of the oogonium; gonadal somatic cells contain precursors of granulosa and theca cells, both of which are essential compartments for ovarian follicles [3]. Based on morphological observation, the oogonia interact with surrounding somatic cells and then form primordial follicles, which are the most immature type of ovarian follicle. This process is thought to be controlled by a gonadotrophin-independent mechanism, mainly because null-mutation of gonadotrophin receptors does not abrogate the primordial follicle formation [2,16]; however, aside from this detail it remains elusive how the differentiation of oogonia is regulated.

Apart from understanding the mechanism of oogenesis, an ultimate goal of reproductive biologists is to reconstitute oogenesis *in vitro*, so that an unlimited number of functional oocytes can be generated. Such *in vitro* reconstitution of oogenesis would open possibilities in basic biology as well as clinical application. Despite of making efforts to achieve oogenesis *in vitro*, yet there is no robust culture system that produces an unlimited number of functional oocytes. As a prerequisite to the

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establishment of oogenesis *in vitro*, we must first achieve a comprehensive understanding of the mechanisms underlying oogenesis *in vivo*.

Our recent studies have shown that the PGC specification can be reconstituted *in vitro* using mouse embryonic stem cells (ESCs) as well as induced pluripotent stem cells (iPSCs) [12]. ESC/iPSC-derived PGCs, called PGC-like cells (PGCLCs), are capable of differentiating into spermatozoa and oocytes that give rise to healthy individuals through *in vitro* fertilization [11,12]. In this review, I describe the partial reconstitution of female germ cell development using ESC/iPSC-derived PGCLCs and discuss further issues to be resolved before the method can be applied to other species and ultimately used to reconstitute the entire process of oogenesis *in vitro*.

PGC specification *in vitro*

ESCs and iPSCs are a valuable source for production of gametes *in vitro*, as they are capable of proliferating indefinitely while maintaining their ability to differentiate into all cell lineages of the embryo proper, including germ cells. A number of attempts have been made to generate germ cells *in vitro* from ESCs and iPSCs [8,14,19,29]. Despite the intensive efforts, a culture system that produces a robust number of functional PGCs, the earliest step of germ cell development, has not been established. The reason for the unsuccessful attempts may be that it was not adequately determined whether the PGC specification in the culture system properly reconstituted that *in vivo*. Since PGC specification is a highly orchestrated process, it seems essential to properly reproduce it *in vitro*.

In mice, the manner of PGC specification is epigenesis; PGCs are specified from the pluripotent cell population in response to inductive signals triggered by intrinsic and extrinsic signals [6,15]. Therefore, PGCs originate from cells that are poised to differentiate into various somatic cell lineages. The germ cell fate is induced in the proximal epiblast by direct BMP4 signals from the extraembryonic ectoderm [17,22]. At E6.25, approximately 6 cells of the posterior proximal epiblast start to express *Prdm1/Blimp1*, a zinc finger transcriptional repressor, and these *Prdm1*-positive cells are lineage-restricted to become PGCs [23]. One day later, about 40 PGCs are located at the posterior end of the primitive streak, and begin to migrate into the allantois and along the developing hindgut toward the genital ridges [23, 25].

Recently we have developed a culture system in which PGC specification processes are reconstituted in vitro by using ESCs/iPSCs [11−13]. In this system, the ESCs/iPSCs first differentiate into a novel type of cells harboring the post-implantation epiblast status, called epiblast-like cells (EpiLCs), by inducing the transient differentiation under a defined condition with bFGF and activin (Figure 1). The EpiLCs differentiate robustly into PGCLCs in response to BMP4 (Figure 1). The manner of differentiation from ESCs/iPSCs to PGCLCs is highly similar, if not identical, to that from the inner cell mass (ICM) of the blastocyst to PGCs *in vivo*, based on the criteria of gene expression and epigenetic status. Importantly, PGCLCs are functional, as they differentiate into fertile sperm upon transplantation into testis. Eggs fertilized with PGCLC-derived sperm give rise to healthy pups that eventually grow up to fertile adults, further demonstrating that PGCLCs are fully potent.

Oocyte production from pluripotent stem cells

Based on the results of gene expression analyses, it is likely that PGCLCs at day 6 of induction culture correspond to PGCs at E9.5 *in vivo*, at which time sexually dimorphic PGCs migrate in the hindgut toward the genital ridges. PGCLCs under the induction culture condition no longer differentiate or proliferate, but instead, eventually decrease in number for an unknown reason, suggesting another signal(s) is required for further differentiation of PGCLCs. Interestingly, PGCLCs, when aggregated with E12.5 female gonadal somatic cells, resume proliferation and differentiation [11]. Among several tissues, only gonadal somatic cells showed a robust effect on the proliferation and differentiation of PGCLCs. This suggests that gonadal somatic cells produce factor(s) essential for PGC proliferation and differentiation. It is noteworthy that the PGCLC induction condition includes stem cell factor (SCF), which is a factor known to promote PGC proliferation, indicating that gonadal somatic cells produce a crucial factor(s) other than SCF. PGCLCs with gonadal somatic cells showed several features observed in oogonia: expression of later germ cell-maker genes such as *Mvh* and *Dazl*, erasure of parental epigenetic

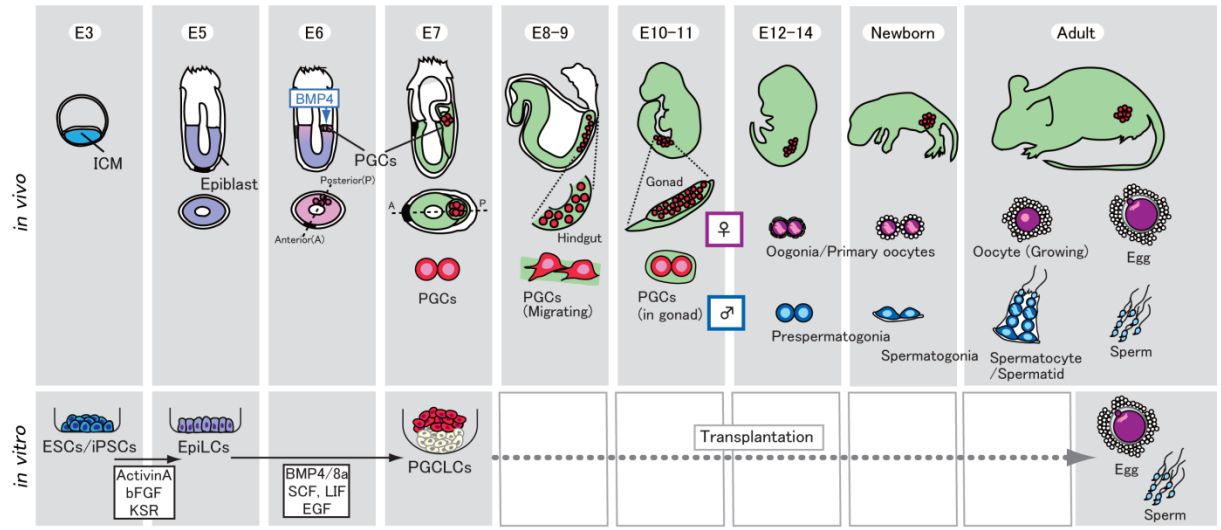

Figure 1. Germ cell development in mice and reconstitution *in vitro*
Primordial germ cells (PGCs) are specified from pluripotent epiblast in response to BMP4, and then start to migrate along hindgut toward genital ridges. After reaching genital ridges, PGCs differentiate either oogonia or prespermatogonia depending on the sex of the embryo. Using pluripotent stem cells, we recently developed a culture system that reconstitutes PGC specification in vitro. Although PGCs *in vitro* (PGC-like cells: PGCLC) are capable of differentiating into functional sperm and oocyte, differentiation process after reaching genital ridges are accomplished in vivo where PGCLCs are transplanted. Therefore, reconstitution in vitro of the process would be the next step for establishing a culture system that reconstitutes an entire process of germ cell development *in vitro*.

status in imprinting gene loci, and entrance into the meiotic prophase. To validate oogenesis in PGC-gonadal somatic cell aggregation, hereafter called reconstituted ovary, we transferred them into the ovarian bursa of immunocompromised mice according to previous studies [10,18]. At 4 weeks after transplantation, growing follicles containing PGCLC-derived oocytes were observed in the reconstituted ovary. Since follicles in reconstituted ovaries show almost no ovulation, the PGCLC-derived oocytes are collected by mechanically puncturing the follicles and then are subjected to an *in vitro* maturation culture. Most of the collected PGCLC-derived oocytes entered the fully-grown germinal vesicle (GV) stage and reached a diameter of approximately 70 µm. These GV oocytes were capable of resuming meiosis and of reaching the metaphase II (MII) stage, when oocytes are ready for fertilization. *In vitro* fertilization using sperm from wild-type males and PGCLC-derived oocytes yielded fertilized eggs. The resultant fertilized eggs gave rise to apparently healthy pups through transplantation into surrogate mothers, demonstrating the PGCLC-derived oocytes are potent, though not as fully potent as oocytes *in vivo* (see below for details). These pups grow up and show reproductive ability with normally sized litters.

Problems to be solved

Although the production of functional oocytes appeared to be successful, several problems remain to be solved. In principle, it is clear that, compared to production from oocytes *in vivo*, successful production of pups from PGCLC-derived oocytes is less efficient [11]. Among the several possible reasons for the low productivity, we found that PGCLC-derived oocytes frequently exhibited aberrant fertilization; half of the fertilized eggs possessed three pronuclei composed of two maternal pronuclei and a paternal pronucleus. This suggests that PGCLC-derived oocytes have a limited ability to extrude the second polar body upon fertilization. The detailed reasons for the deficient second polar body extrusion remain to be clarified. In addition to this deficiency, we found embryos absorbed at a later stage of pregnancy in the uteri of surrogate mothers, suggesting that the totipotency of PGCLC-derived oocytes is limited. We consider that the reason for these deaths was an error in epigenetic reprogramming during oogenesis.

Since reconstituted ovaries were transferred to produce the oocytes, the environmental cues that organize oogenesis in the reconstituted ovary remain essentially unclear (Figure 1). To reconstitute the entire process of oogenesis using pluripotent stem cells, the

next step would be to develop a culture system in which oocytes mature within the reconstituted ovary. This is challenging work, as no report has so far succeeded in the production of functional oocytes from PGCs, except by using nuclear transfer; Obata *et al*. succeeded in generating pups by transferring the nuclei of oocytes that had matured in an *in vitro* culture from PGCs, into enucleated fully grown oocytes from adult ovaries, followed by *in vitro* fertilization [21]. Another study showed that, despite the low efficiency, the neonatal primordial follicles matured *in vitro* to functional oocytes [4,20]. Collectively, these results underscore the necessity of developing a culture system in which PGCLCs in the reconstituted ovary differentiate into primary oocytes of a stage consistent with neonatal primary oocytes *in vivo*.

Nevertheless, the successful production of oocytes from ESCs/iPSCs via transplantation arouses interest for its potential application to other animals, especially humans. Needless to say, there are both ethical and technical steps that have to be overcome. Focusing on the technical steps, first it should be taken into consideration that the features of ESCs/iPSCs are different between humans and mice [9]. Recent studies have focused on identifying culture conditions under which human ESCs might become similar to mouse ESCs [7]. The development of such culture conditions might ultimately allow us to apply PGCLC production using mice to that using human ESCs. However, the functional validation of mouse PGCLCs by transplantation into ovaries is not readily applicable to human PGCLCs. An alternative may be to use non-human primate ESCs for both the production and functional validation of PGCLCs.

Concluding remarks

Here I introduced a culture system that produces functional oocytes from ESC/iPSC-derived PGCLCs. As described above, the culture system reconstitutes *in vitro* early PGC specification and subsequent early differentiation of oogonia. The latter process requires female gonadal somatic cells. From a biological point of view, it is of particular interest to know interaction between the PGCLCs and somatic cells at a molecular level. Since several factors, such as Wnt4 and R-Spondin, have been identified as determinants for ovary differentiation [1,28,30], it is worth testing whether these factors can compensate for the function of gonadal somatic cells in the differentiation of PGCLCs. If not, this would suggest that the differentiation of oogonia require other factors, which must be produced by gonadal somatic cells. Identification of the factors and reconstitution of oogonia differentiation under defined conditions are of practical importance, particularly with respect to human application, since it is impossible to obtain a sufficient number of gonadal somatic cells from human embryos. Nevertheless, we have reported for the first time that ESC/iPSC-derived PGCLCs are capable of differentiating into functional oocytes that can give rise to fertile individuals. This is a first step towards the establishment of a culture system that entirely reconstitutes oogenesis *in vitro*.

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〒252-5201 相模原市中央区淵野辺1-17-71
麻布大学 獣医学部 動物繁殖学研究室内
TEL: 042-769-2339 FAX: 042-769-1762


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