A novel culture system for mouse spermatid maturation which produces elongating spermatids capable of inducing calcium oscillation during fertilization and embryonic development

Hisataka Hasegawa · Yukihiro Terada ·
Tomohisa Ugajin · Nobuo Yaegashi · Kahei Sato

Received: 17 December 2009 / Accepted: 19 May 2010 / Published online: 5 June 2010
© Springer Science+Business Media, LLC 2010

Abstract

Purpose To establish an in vitro culture system for mouse round spermatids that models spermiogenesis and enables the assessment of oocyte activation ability.

Methods Round spermatids and Sertoli cells were isolated from testicular tissues of B6D2F1 male mice and co-cultured in the presence of testosterone and recombinant FSH. Cultured spermatids were examined for morphology and condensation of nuclei, fertilization and development rate, and Ca^{2+} oscillation pattern after ICSI.

Results The cultured spermatids elongated and resembled normal elongating spermatids in terms of both morphology and nuclear condensation. No significant differences in fertilization and development rates were observed between fresh and cultured elongating spermatids. Moreover, cultured spermatids showed similar Ca^{2+} oscillation patterns to fresh elongating spermatids during an initial stage in oocyte activation.

Conclusions These data suggest that a co-culture system of spermatids and Sertoli cells, supplemented with testosterone and recombinant FSH, supports normal differentiation

of round spermatids into elongating spermatids, as assessed by their morphology, nuclear condensation, and oocyte activation ability.

Keywords Spermatid · In vitro maturation · Co-culture · Calcium oscillation

Introduction

Since the first report of successful fertilization with round spermatid nuclei [1], numerous experiments using immature spermatogenic cells (spermatids and spermatocytes) have been conducted in laboratory animals [2–5]. The fertilization and development rates of oocytes injected with immature spermatids, however, are low [6–9]. One cause of this is the poor oocyte activation ability of immature spermatogenic cells.

Oocyte activation is characterized by a rise in cytoplasmic calcium concentration ($[\text{Ca}^{2+}]_i$) known as Ca^{2+} oscillation. In the mouse, Ca^{2+} oscillation persists for 10–15 min following fertilization and disappears when a pronucleus is formed [10]. This increase in $[\text{Ca}^{2+}]_i$ is a universal phenomenon observed in all animal species examined thus far [11–13]. Ca^{2+} oscillation is essential for activation at fertilization and for subsequent embryonic development [14].

Intracytoplasmic sperm injection (ICSI) has a high success rate provided that mature spermatozoa are used. If round spermatids are injected, the rates of oocyte activation and fertilization are much lower, likely because of their inability to activate the oocyte. This idea is supported by observations from in vitro models of spermatogenesis.

In vitro models that recapitulate the normal steps of spermatogenesis, in a manner that results in the acquisition

Capsule Co-culture of B6D2F1 round spermatids in vitro with Sertoli cells using culture media supplemented with testosterone and recombinant FSH produces spermatids with oocyte activation ability.

H. Hasegawa · K. Sato
Division of Cell Biological Science,
Graduate School of Bioresource Science, Nihon University,
Fujisawa, Kanagawa 252-8510, Japan

H. Hasegawa · Y. Terada (✉) · T. Ugajin · N. Yaegashi
Department of Obstetrics and Gynecology,
Tohoku University School of Medicine,
1-1, Seiryomachi, Aoba-ku,
Sendai, Miyagi 980-8574, Japan
e-mail: terada@mail.tains.tohoku.ac.jp

of oocyte activation ability, are key to fully understanding spermatogenesis. Moreover, a future role for such models in the application for human assisted reproductive technology (ART) is expected. One model utilizes mouse spermatogonia co-cultured with Sertoli cells, obtained from the testes of 13 to 18 day-old B6D2F1 mice [15]. In this culture system, the spermatogonia complete meiosis and differentiate into haploid round spermatids. Microinjection of these spermatids into an artificially activated oocyte results in fertilization and the development of a normal embryo. However, spermiogenesis is not addressed in this culture system. Our report is the first to demonstrate spermiogenesis in such a culture system.

In this study, we demonstrate that co-culture of adult B6D2F1 round spermatids in vitro with mouse Sertoli cells produces spermatids with oocyte activation ability. The system employs a standard culture medium supplemented with testosterone and recombinant FSH. Under these conditions, round spermatids differentiate into elongating spermatids, exhibit Ca^{2+} oscillation following microinjection into the oocyte, and result in the production of normal blastocysts.

Materials and methods

Preparation of testicular cells

Testicular cells were aspirated from the testes of mature male B6D2F1 mice (8–10 weeks old) using a 27-gauge needle and syringe containing HEPES-buffered HTF medium (Hb-HTF), supplemented with 4 mg/mL bovine serum albumin (BSA). The aspirated testicular cells were then washed in Hb-HTF medium. Following centrifugation at 1500 g for 20 s, the pellet was resuspended in Hb-HTF medium. The cells were then incubated at 32°C before use.

Preparation of mature spermatozoa

Cauda epididymides, isolated from mature male B6D2F1 (8–13 weeks old) mice, were cut into small pieces in HTF medium supplemented with 4 mg/mL BSA to disperse the spermatozoa. Spermatozoa were then washed in HTF medium. Following centrifugation at 1500 g for 20 s, the pellet was resuspended in HTF medium. The spermatozoa were incubated at 37°C under 5% CO_2 in air for at least 30 min before use.

In vitro culture

Round spermatids and Sertoli cells were manually isolated from a testicular cell solution by using a micromanipulator and co-cultured at 32°C in an incubator with 5% CO_2 in air.

In selecting the round spermatids and Sertoli cells, we used size and form as the indices. For round spermatids, the size is 8–10 μm and the form is round. For Sertoli cells, the size is approximately 15 μm and the presence of fat droplets in the cytoplasm is a key feature. Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1×10^6 IU/L penicillin, 100 mg/L streptomycin, 8 mg/L kanamycin, 1 μM testosterone, 500 mIU/mL recombinant FSH (rFSH: Organon-Japan), and 5 $\mu\text{g/mL}$ epinephrine, was used as the culture medium. Each of about 20 round spermatids and Sertoli cells were put into a 5 μL culture medium drop.

As a control, round spermatids and Sertoli cells were cultured with DMEM supplemented with 10% FBS, 1×10^6 IU/L penicillin, 100 mg/L streptomycin and 8 mg/L kanamycin.

Nuclear staining

Propidium iodide (PI) DNA staining was used to examine the nuclei of the spermatids/spermatozoa. Round spermatids, elongating spermatids, epididymal spermatozoa, and elongating spermatids after in vitro maturation of round spermatids were fixed for 10 min in 3% formaldehyde. Fixed samples were washed in phosphate buffered saline (PBS) and stained for 30 min in PBS drops containing 1 $\mu\text{g/mL}$ PI. Stained samples were viewed using a confocal laser microscope (Olympus: X71, FV1000).

Preparation of oocytes

Mature female B6D2F1 mice (6–8 weeks old) were super-ovulated with intraperitoneal injections of 8 IU pregnant mare serum gonadotropin (PMSG; Teikokuzouki Co., Tokyo, Japan) followed by 8 IU human chorionic gonadotropin (HCG; Teikokuzouki Co., Tokyo, Japan) 48 h later. Mature oocytes were collected from the oviducts 17 h after HCG injection and were freed from their cumulus cells by treatment with 0.1% hyaluronidase in Hb-HTF. The cumulus-free oocytes were rinsed thoroughly and placed in drops of HTF covered with mineral oil.

Microinjection of spermatids/spermatozoa

Microinjection with round spermatids, elongating spermatids, epididymal spermatozoa, and elongating spermatids after in vitro maturation of round spermatids was performed using a piezo-micromanipulator (Suruga Seiki Co., Shizuoka, Japan). The oocytes were loaded into a pipette and the zona pellucida was penetrated using piezo pulses. After expulsion of a segment of zona pellucida from the pipette, the oocytes were moved to the tip of the injection pipette, which was inserted into the ooplasm as the

oolemma was punctured by application of a single piezo pulse. The sperm at the tip of the pipette was injected into the ooplasm with a minimal amount of medium. After injection, the oocytes were incubated in HTF medium at 37°C under 5% CO₂ in air. The oocytes were checked 6 h after ICSI for fertilization. Oocytes with two pronuclei and a second polar body were considered to be fertilized. The fertilized oocytes were separated and allowed to develop to the blastocyst stage.

Measuring $[Ca^{2+}]_i$ of oocytes

Ca^{2+} responses of oocytes injected with spermatids/spermatozoa were examined by fluorometric $[Ca^{2+}]_i$ measurements. Before injection, oocytes were loaded with the fluo-3 acetoxymethyl ester (Fluo-3/AM; CS21, Calcium Kit-Fluo3, Dojin) in dimethylsulfoxide (final concentration 44 μ mol/L in HTF) with 0.02% Pluronic F-127 for 30 min at 37°C. Loaded and injected oocytes were examined using a confocal laser microscope (Olympus: X71, FV1000), and $[Ca^{2+}]_i$ was recorded.

Statistics

Each experiment was performed at least three times, and statistical significance was assessed using the Chi-Square test. P values less than 0.05 were considered statistically significant.

Results

Differentiation of round spermatids

Cultured round spermatids changed their morphology, as shown in Fig. 1. Round spermatids differentiated into elongating spermatids after two days of culture. The differentiation rate of the round spermatids into elongating spermatids was 16.0% (15/94). In the controls, the differentiation rate of round spermatids to elongating spermatids was 6.3% (6/95).

Condensation of spermatid nuclei

Stained nuclei of spermatids/spermatozoa are shown in Fig. 2. In round spermatids, the DNA was diffusely distributed throughout the cytoplasm (Fig. 2a). In elongating spermatids, however, it was condensed in the center or at the edge of the cell (Fig. 2b). In epididymal spermatozoa, staining was localized at the head region with a completely condensed nucleus (Fig. 2c). In elongating spermatids after in vitro maturation of round spermatids, staining was observed at the center or edge of the cell (Fig. 2d), as was

Fig. 1 Round spermatids (a) differentiated into elongating spermatids (b) with oval morphology following co-culture with Sertoli cells (c) for two days. Bar=10 μ m

the case for elongating spermatids, indicating that the nuclear condensation accompanied the other morphological changes in these in vitro cultured cells. Condensation of nuclei was obtained in all elongating spermatids after in vitro maturation of round spermatids.

Development of oocytes injected with spermatids/spermatozoa

The fertilization and development rates of oocytes injected with spermatids/spermatozoa are summarized in Table 1. When epididymal spermatozoa were injected, 41 of 55 (74.5%) oocytes were fertilized, and 13 of 55 (23.6%) oocytes developed to the blastocyst stage. When round spermatids were injected, no oocytes were fertilized. When elongating spermatids were injected, 12 of 64 (18.5%) oocytes were fertilized, and 2 of 64 (3.1%) oocytes developed to the blastocyst stage. When elongating spermatids after in vitro maturation of round spermatids were injected, 9 of 59 (15.3%) oocytes were fertilized, and 1 of 59 (1.7%) oocytes developed to the blastocyst stage. There were no significant differences in the fertilization and development rates between elongating spermatids which had matured in vivo and elongating spermatids after in vitro maturation of round spermatids (Chi-Square test).

Ca^{2+} oscillation-inducing abilities of spermatids/spermatozoa

The representative response patterns of $[Ca^{2+}]_i$ following spermatid/spermatozoon injection are shown in Fig. 3. No significant change in $[Ca^{2+}]_i$ was observed in the sham-injected oocytes (Fig. 3b) or oocytes injected with round spermatids (Fig. 3c). In contrast, a significant increase in $[Ca^{2+}]_i$ was seen in oocytes injected with spermatozoa, and a steady Ca^{2+} oscillation pattern was observed (Fig. 3a).

Fig. 2 Nuclear staining (red) of spermatids/spermatozoa. **a** Round spermatids: DNA is diffusely distributed throughout the entire cytoplasm. **b** Elongating spermatids: DNA is condensed at the center or edge of the cytoplasm. **c** Epididymal spermatozoa: DNA is localized at the head region with a completely condensed nucleus. **d** Elongating spermatids after in vitro maturation of round spermatid: DNA is condensed at the center or edge of the cytoplasm

With the injection of elongating spermatids, an increase in $[Ca^{2+}]_i$ was detected, and a weak, unstable Ca^{2+} oscillation pattern was observed (Fig. 3d). A similar Ca^{2+} oscillation pattern was observed in oocytes injected with elongating spermatids after in vitro maturation of round spermatids (Fig. 3e).

Discussion

A number of models for spermatogenesis have been developed and a few include in vivo components. Toyooka et al. induced embryonic stem cells to differentiate into germ cells in vitro. Subsequent transplantation of these cells into seminiferous tubules resulted in the production of mature spermatozoa [16]. Spermatozoa capable of producing live offspring following ICSI have also been generated by transplanting primordial germ cells into seminiferous tubules [17].

In a study utilizing in vitro culture alone, spermatogenic cells isolated from postnatal day 13–18 mice were co-cultured with Sertoli cells, and differentiated into round spermatids. These cells lacked nuclear condensation [15]. In both that model and the transplantation model, while both meiosis and germ cell differentiation were completed, differentiation past the round spermatid stage in vitro was not observed. The ability of the spermatid to cause Ca^{2+} oscillation is attained once it differentiates past the round stage [18]. In this study, we focused on the spermiogenesis of the B6D2F1 mouse. We isolated round spermatids and co-cultured the Sertoli cells in standard culture conditions. As we were able to produce sperm differentiation past the round spermatid stage, we then assessed Ca^{2+} oscillation during an initial stage in oocyte activation after microinjection as a marker of functional maturation.

In our model, the differentiation of round spermatids was produced by co-culture with Sertoli cells from adult

Table 1 Development of embryos injected with spermatids/spermatozoa

Cell type	No. of embryos	No. of Fertilized*	No. of embryos developed into			
			2-cell	4-cell	8-cell/morula	blastocyst
Spermatozoa	55	41(74.5%)	39(70.9%)	30(54.5%)	19(34.5%)	13(23.6%)
Round spermatid	40	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Elongating spermatid	64	12(18.5%) ^a	11(17%) ^c	5(7.8%) ^e	2(3.1%) ^g	2(3.1%) ⁱ
Elongating spermatid after in vitro maturation of round spermatid	59	9(15.3%) ^b	7(11.9%) ^d	4(6.8%) ^f	3(5.1%) ^h	1(1.7%) ^j

*No. of embryos observed 2pronuclei & 2nd polar body

a vs. b, c vs. d, e vs. f, g vs. h, i vs. j; $P < 0.05$ (Chi-Square test)

Fig. 3 $[Ca^{2+}]_i$ in oocytes during an initial stage in oocyte activation following spermatid/spermatozoon injection. **a** Oocytes injected with epididymal spermatozoa: A marked increase in $[Ca^{2+}]_i$ and a steady Ca^{2+} oscillation pattern were observed. **b** Sham-injected oocytes: No significant change in $[Ca^{2+}]_i$ was observed. **c** Oocytes injected with round spermatids: No significant change in $[Ca^{2+}]_i$ was observed. **d**

Oocytes injected with elongating spermatids: A weak increase in $[Ca^{2+}]_i$ and an unstable Ca^{2+} oscillation pattern were observed. **e** Oocytes injected with elongating spermatids after in vitro maturation of round spermatids show a Ca^{2+} oscillation pattern similar to that observed in oocytes injected with elongating spermatids which had matured in vivo

B6D2F1 mice, in a standard media consisting of DMEM supplemented with testosterone and recombinant FSH. The nuclear staining of the elongating spermatids after in vitro maturation of round spermatids resembled that of elongating spermatids, indicating that nuclear condensation had occurred in vitro. It is plausible that the improved efficacy of this system was the result of the meticulous isolation of the spermatids and Sertoli cells.

The round spermatid of the B6D2F1 mouse lacks the capacity for fertilization in the absence of artificial oocyte activation [3]. Unlike the round spermatids of ICR mice, which induce Ca^{2+} oscillation and result in successful fertilization when injected, round spermatids from B6D2F1

mice fail to trigger Ca^{2+} oscillation [19]. To date, all conventional spermatogenic studies employing haploid germ cells from B6D2F1 mice have employed artificial oocyte activation. In this study, we found that while oocytes injected with round spermatids had the same $[Ca^{2+}]_i$ as non-injected oocytes, injection of elongating spermatids after in vitro maturation of round spermatids caused an increase in $[Ca^{2+}]_i$ during an initial stage in oocyte activation similar to that observed following injection with elongating spermatids which had matured in vivo. It will be necessary to determine the changes in further sequential Ca^{2+} oscillation after ICSI.

Using our novel in vitro culture system, we successfully induced the differentiation of round spermatids of B6D2F1

mice into elongating spermatids which had oocyte activation ability. No significant differences in fertilization and developmental rates were observed between the oocytes injected with elongating spermatids after in vitro maturation of round spermatid and those injected with elongating spermatids which had matured in vivo. In this study, we have shown that the B6D2F1 round spermatid, which is normally incapable of fertilizing an oocyte, can be matured in vitro such that it fertilizes and activates an oocyte in vitro. The spermatids produced in our system closely resemble elongating spermatids with regard to their nuclear condensation and oocyte activation/ Ca^{2+} oscillation-inducing ability.

Our culture model has advantages over previously reported systems. First, unlike a previously described coculture system [20], both the spermatids and the Sertoli cells used in our model are from the same species, which more closely approximates physiologic conditions. Second, our method is simple and utilizes standard, easily obtainable reagents. We anticipate that these features will result in the adoption of our culture conditions to a variety of experiments designed to elucidate the molecular pathways and processes of spermatogenesis.

For future implication of our model in ART, there are some important considerations. First, there is a difference in centrosomal inheritance between the mouse and human. Mouse sperm does not have a centrosome while functional centrosomes exist in the mouse oocyte, and these centrosomes function in mouse fertilization. In contrast, although the centrosome does not exist in a human oocyte, the sperm centrosome functions during human fertilization. Investigation of centrosomal function in the human immature spermatid is needed. Second, there is the matter of the sperm factor for oocyte activation. The most powerful candidate is sperm-specific Phospholipase C (PLC) zeta as the factor of the oocyte activation [21]. It is reported that PLC zeta was undetected in sperm from a patient who had failed ICSI [22]. It is necessary to examine the appearance of PLC zeta of immature spermatids which are cultured in vitro. Finally, with in vitro spermatogenesis, it is a problem whether the genomic imprinting of spermatids is normal or abnormal. Normal genomic imprinting is critical in producing normal offspring; thus whether the genomic imprinting of spermatids cultured in vitro is normal or abnormal should be examined.

References

- Ogura A, Yanagimachi R. Round spermatid nuclei injection into hamster oocytes form pronuclei and participate in syngamy. *Biol Reprod*. 1993;48:219–25.
- Ogura A, Matsuda J, Yanagimachi R. Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc Natl Acad Sci USA*. 1994;91:7460–2.
- Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development*. 1995;121:2397–405.
- Sasagawa I, Yanagimachi R. Comparison of methods for activating mouse oocytes for spermatid nucleus transfer. *Zygote*. 1996;4:269–74.
- Sofikitis NV, Toda T, Miyagawa I, Zavos PM, Pasyanos P, Mastelou E. Beneficial effects of electrical stimulation before round spermatid nuclei injections into rabbit oocytes on fertilization and subsequent embryonic development. *Fertil Steril*. 1996;65:176–85.
- Tesarik J, Rolet F, Bami C, Sedbon E, Thorel J, Tibi C, et al. Spermatid injection into human oocytes? Clinical application in the treatment of infertility due to non-obstructive azoospermia. *Hum Reprod*. 1996;11:780–3.
- Fishel S, Green S, Hunter A, Lisi F, Rinaldi L, Lisi R, et al. Human fertilization with round and elongated spermatids. *Hum Reprod*. 1997;12:336–40.
- Vanderzwalmen P, Zech H, Birkenfeld A, Yemini M, Bertin G, Lejeune B, et al. Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation. *Hum Reprod*. 1997;12:1203–13.
- Sousa M, Barros A, Takahashi K, Oliveira C, Silva J, Tesarik J. Clinical efficacy of spermatid conception: analysis using a new spermatid classification scheme. *Hum Reprod*. 1999;14:1279–86.
- Jones KT, Carroll J, Merriman JA, Whittingham DG, Kono T. Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependent. *Development*. 1995;121:3259–66.
- Miyazaki S, Shirakawa H, Nakada K, Honda Y. Essential role of the inositol 1, 4, 5-trisphosphate receptor/ Ca^{2+} wave and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev Biol*. 1993;158:62–78.
- Stricker SA. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol*. 1999;211:157–76.
- Runft LL, Jaffe LA, Mehlmann LM. Egg activation at fertilization: where it all begins. *Dev Biol*. 2002;245:237–54.
- Ajduk A, Malagocki A, Maleszewski M. Cytoplasmic maturation of mammalian oocytes: development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. *Reprod Biol*. 2008;8:3–22.
- Marh J, Tres LL, Yamazaki Y, Yanagimachi R, Kierszenbaum AL. Mouse round spermatids developed in vitro from preexisting spermatocytes can produce normal offspring by nuclear injection into in vivo-developed mature oocytes. *Biol Reprod*. 2003;69:169–76.
- Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. *Proc Natl Acad Sci USA*. 2003;100:11457–62.
- Chuma S, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, et al. Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. *Development*. 2000;132:117–22.
- Yanagida K, Yazawa H, Katayose H, Kimura Y, Hayashi S, Sato A. Oocyte activation induced by spermatids and the spermatozoa. *Int J Androl*. 2000;23:63–5.
- Yazawa H, Yanagida K, Sato A. Oocyte activation and Ca^{2+} oscillation-inducing abilities of mouse round/elongated spermatids and the developmental capacities of embryos from spermatid injection. *Hum Reprod*. 2001;16:1221–8.
- Movahedin M, Ajeen A, Ghorbanzadeh N, Tiraihi T, Valojerdi MR, Kazemnejad A. In vitro maturation of fresh and frozen-thawed mouse round spermatids. *Andrologia*. 2004;36:269–76.
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, et al. PLC zeta: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development*. 2002;129:3533–44.
- Yoon SY, Jellertette T, Salicioni AM, Lee HC, Yoo MS, Coward K, et al. Human sperm devoid of PLC, zeta 1 fail to induce Ca^{2+} release and are unable to initiate the first step of embryo development. *J Clin Invest*. 2008;118:3671–81.