Transmeiotic differentiation of zebrafish germ cells into functional sperm in culture

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SUMMARY

Because cell culture systems are easily accessible for experimental genetic manipulation, male germ cell culture is of great usefulness in creating sperm vectors. This report describes that cultured male germ cells of zebrafish (Danio rerio) underwent mitosis and transmeiotic differentiation, including the entire process of meiosis, to develop into functional sperm. Enzymatically dissociated testicular cells containing germ cells were co-cultured on feeder cells derived from tumor-like testis, which exhibited features characteristic of Sertoli cells such as phagocytic activity and transcription of the Wilms’ tumor suppressor wt1 and sox9a genes. Germ cells formed a clump, divided by mitosis, and differentiated into flagellated sperm on the feeders. Expression of the germ cell marker gene vas was prolonged in co-culture with the feeders, compared with culture of dissociated testicular cells alone, indicating that the feeder cells stimulate proliferation of spermatogonia. When cultured germ cells/sperm with the feeders were used for in vitro fertilization, normal embryos were obtained. Addition of the thymidine analogue 5-bromo-2’-deoxyuridine (BrdU) into culture medium resulted in BrdU-positive sperm and four-cell stage embryos after in vitro fertilization. This culture system should prove useful not only in producing transfected functional sperm, but also in analyzing the regulatory function of testicular somatic cells on the mitosis and meiosis of male germ cells in vertebrates.

Key words: Male germ cell, Spermatogenesis, Zebrafish, Cell culture, Sertoli cell, Spermatogonia, Meiosis

INTRODUCTION

Sperm differentiate from spermatogonial stem cells through numerous complex processes. In mammals, spermatogonial stem cells are among the single undifferentiated A-type spermatogonia, termed Asingle (As) spermatogonia. As spermatogonia self-renew their stem cell identity and give rise to other undifferentiated A-type spermatogonia, which then proliferate to produce differentiating A-type spermatogonia. The differentiating A-type spermatogonia divide into Intermediate and, finally, B-type spermatogonia, then give rise to the more specialized meiotic spermatocytes. After the last DNA duplication, one spermatocyte produces four haploid spermatids by two successive nuclear divisions, namely meiotic division. Functional sperm are ultimately differentiated from postmeiotic haploid spermatids (reviewed by Kiger and Fuller, 2001).

Various in vitro systems have been used to represent several of these processes. When mouse male germ cells were co-cultured on a Sertoli-like cell line, premeiotic germ cells underwent the meiotic and postmeiotic differentiation into haploid spermatids expressing the protamine gene (Rassoulzadegan et al., 1993). Although immortalized mouse germ cells undergo meiosis without any supporting cells (Hofmann et al., 1994), the immortalization process probably altered the normal course of events. When isolated eel (Anguilla japonica) germ cells and somatic cells (mainly Sertoli cells) were co-cultured in pellets by centrifugation, the entire differentiation process from spermatogonia to spermatozoa (Miura et al., 1996), as well as the organ culture (Miura et al., 1991), was completed. These results imply that the initiation or completion of meiosis in normal male germ cells typically requires Sertoli cells or other somatic cells in vertebrates. In contrast with mouse, primary spermatocytes (in late prophase or metaphase of the first meiotic division) in lower vertebrates such as amphibians (reviewed by Abe, 1987) and fish (Saiki et al., 1997) differentiated into flagellated spermatids or functional sperm without any supporting cells. In lower vertebrates, differentiation from primary spermatocytes, which have probably undergone the G2-M phase transition of meiosis, into functional sperm does not seem to require interaction between the germ cells and somatic cells.

This study was designed to address the establishment of a male germ cell culture system for constructing a sperm vector, because cell culture systems are easily accessible for experimental manipulation, e.g. the introduction of DNA, chemical selection, retrovirus treatment, etc. For this purpose, the system should include the processes of both DNA duplication and differentiation into functional sperm.
Based on consideration of the nature of lower vertebrates and their popularity for genetic and developmental studies, zebrafish male germ cells were cultured on tumor-like testis-derived cells as feeder cells. This culture system allows germ cells to proliferate and to differentiate into functional sperm.

**MATERIALS AND METHODS**

**Fish stock**
Zebrafish *Danio rerio* wild-type and albino (*alb*-1/*alb*-1) (Lin et al., 1992) were obtained from Dr. N. Hopkins (Center for Cancer Research, Massachusetts Institute of Technology) and maintained as described (Westerfield, 1995).

**Cell culture**
Testicular cell culture medium (TCCM) was based on zebrafish growth medium (Westerfield, 1995). To obtain optimal proliferation of testicular cells, human chorionic gonadotropin (final concentration of 5 IU/ml, Sigma), pregnant mare’s serum gonadotropin (2 IU/ml, Sigma), and human chorionic gonadotropin (2 IU/ml, Sigma) were obtained from Dr. N. Hopkins (Center for Cancer Research, Massachusetts Institute of Technology) and maintained as described (Westerfield, 1995).

Zebrafish under a dissecting microscope and treated with 0.5% trypsin-EDTA (Gibco BRL). Prior to the addition of germ cells, tumor-derived cells were repeatedly replated by using 0.05% trypsin-EDTA with 500 U/ml of collagenase/dispace (Sigma) in L-15 for 2 hours. After centrifugation at 500 g for 4 minutes, the majority of the cell suspension was centrifuged at 35 g for 10 minutes. The supernatant was disciminated and the centrifugation pellet was resuspended in TCCM supplemented with 3% fetal bovine serum (FBS, Gibco BRL) or!7% cruican carp (Carassius carassius) serum. The cells were then plated on gelatin-coated dishes (Corsterol) or feeder cells, and incubated at 28°C in air. Typically, four testes were added to 1000-fold diluted suspension of polystyrene beads (Sigma LB-1; average diameter, 1.1 μm) was added, plates were further incubated at 28°C for 16-20 hours. Internalization of beads was determined by phase contrast microscopic observation after extensive washing of the cell layer.

**RT-PCR analysis**
Poly (A)+ RNA was isolated from cultured cells using the mRNA Isolation Kit (Roche) and treated with RNase-free DNase I (Roche). The reverse transcribes and the PCR products of poly (A)+ RNA (0.1 μg) were obtained using the TaKaRa RNA PCR Kit (Takara, Japan) with sox9a (Chiang et al., 2001), the Wilms’ tumor suppressor wt1 (S. I. Smith, M. Down, M. Power and A. W. Boyd, personal communication; GenBank Accession Number AF 144550), gata1 (Detrich et al., 1995) and vas (Yoon et al., 1997) primers for 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 90 seconds. The sox9a primers (CCCTAGCGTGGGGGATCCCGCCG and GGTGCTGGTATCAGGTCGCGGA), the wt1 primers (ACCTTGGGCGAAGCTTCCACCTTCCCG and ATGAGGATCGTCCGATCTTGTGC), the gata1 primers (CCTCTGGATGCGGCTGCAACAAAGATATT and CATGCAGATACAGGAGTACCGTCTTGTGTC), and the vas primers (AGCATGCGCGTGGTGACCTGCGCGAAGAAAC and TCACTCGCTCTCTAAATCGATCCTCCCG) amplified a 446 bp fragment, a 476 bp fragment, a 424 bp fragment and a 588 bp fragment, respectively.

**Competitive PCR for vas and α-tubulin** (Bormann et al., 1998) was carried out using a competitor according to the Competitive DNA Construction Kit (Takara, Japan). The vas primers amplified a 588 bp of the transcript and a 501 bp of the competitor. The α-tubulin primers (AACTCTATCCGGCAACCCACACC and AGGCCAGATCCCACCTGGGAACCAAC) generated a 552 bp of the transcript and a 470 bp of the competitor. The PCR products were analyzed on 0.7% agarose (Sigma) plus 1.5% agarose (Wako, Japan) containing 0.5 μg/ml of ethidium bromide. The density of the fluorescence was analyzed using a Densitograph system (Atto, Japan).

**Incorporation and detection of BrdU**
5-bromo-2-deoxyuridine (BrdU, Sigma; 15 μg/ml) was added to the TCCM-FBS medium for the first 2 days. After culture, cells were fixed with acid-alcohol (90% ethanol: 5% acetic acid: 5% water) at room temperature for 30 minutes. After centrifugation at 500 g for 4 minutes, the cells were suspended in 0.01% poly-L-lysine (Nacalai tesque, Japan) and spread on a glass slide.

**Embryos** obtained by in vitro fertilization with the sperm were dechorionated by pronase (Westerfield, 1995) and fixed according to the above methods. Embryos were embedded and sectioned using standard paraffin histological procedures below a temperature of 58°C. The detection of BrdU was performed with a Cell Proliferation Kit (Amersham). After blocking (2 hours at room temperature, 2% Roche blocking reagent in PBS with 0.1% Tween 20) (Rodaway et al., 1999), whole-mount embryos were treated with the Cell Proliferation Kit (Amersham) with the following modification: 3 × dilution of the antibody PBST, an overnight incubation with antibody and an overnight washing.

**In vitro fertilization with cultured sperm**
After culture, cells were collected by normal trypsin treatment. The supernatant medium, the PBS wash and the trypsin medium were combined. BSA (0.5%) and Hepes (10 mM from 1 M stock, pH 7.9) were then added to prevent unintentional activation of unfertilized eggs (Sakai et al., 1997). To concentrate the sperm, the cell suspension was centrifuged at 500 g for 4 minutes. The majority of the supernatant was discarded. The pellet was then resuspended in the remaining medium (<100 μl) and stored on ice. Unfertilized eggs were collected according to the method of Westerfield (Westerfield, 1995). The suspension was added and the dish was shaken gently for 2 minutes to mix well. PBS (100 μl) was added gradually with shaking. After an additional 2 minutes, 5 ml of fish water (Westerfield, 1995) was gradually added.
RESULTS

When enzymatically dissociated testicular cells containing germ cells were plated in testicular cell culture medium supplemented with 3% fetal bovine serum (TCCM-FBS) or 7% crucian carp (Carassius carassius) serum (TCCM-crucian serum), germ cells were found to only barely attach to the gelatin-coated dish. Most of the germ cells appeared to attach to somatic cells after somatic cells had attached themselves to the dishes, suggesting that testicular somatic cells were required for the culture of male germ cells. However, despite efforts to culture testicular somatic cells, cell growth slowed after several passages, resulting in a failure to derive proliferating cells from normal testis.

A spontaneous tumor-like hypertrophied testis happened to be found among hundreds of albino zebrafish. After enzymatic dissociation and plating with TCCM-FBS, cells of the tumor-like testis attached to the gelatin-coated dish and continued proliferating. The cells, designated ZtA6, contained several morphological types, including epithelial and fibroblast cells (Fig. 1A). To characterize the cells, phagocytic activity was analyzed by the uptake of latex beads, based on the phagocytic nature of mammalian Sertoli cell lines (Tokuda et al., 1992; Rassoulzadegan et al., 1993). Many of the cells showed phagocytic activity (Fig. 1B). The ZtA6 cells expressed transcripts of Sertoli cell marker genes, sox9a (Chiang et al., 2001) and the Wilms’ tumor suppressor wt1 (Pelletier et al., 1991), while scarcely any gata1, which is found in Sertoli cells in the mouse (Yomogida et al., 1994), was detected (Fig. 2). Expression of the germ cell marker gene vas (Yoon et al., 1997) was slightly found in ZtA6 cells. These results indicate that ZtA6 cells contain a considerable number of Sertoli cells. After several replatings, cells were used as feeder cells in a co-culture with enzymatically dissociated cells of normal testis.

For the culture of male germ cells, two different kinds of culture medium, supplemented with FBS or crucian carp serum, were used. When the TCCM was supplemented with crucian serum, comparatively fewer germ cells attached to the feeder cells, although in vitro fertilization studies indicate that male germ cells cultured in TCCM-crucian serum resulted in more fertilized eggs than did TCCM-FBS-cultured sperm (data not shown). Thus, enzymatically dissociated cells of normal testis containing germ cells were suspended in TCCM-FBS and plated on mitomycin C-treated ZtA6 cells as feeder cells. After 2 days, because there was no further increase in germ cell attachment to the ZtA6 cells, the medium was then changed to TCCM-crucian serum supplemented with 50 ng/ml of 11-ketotestosterone. 11-Ketotestosterone, the major teleost androgen (Idler et al., 1961; Fostier et al., 1983), has been shown to induce spermatogenesis in organ culture of eel testis (Miura et al., 1991). Under these conditions, germ cells formed a clump and divided by mitosis (Fig. 3). The clump became larger (Fig. 3A-F) until the appearance of flagellated sperm (day 9, Fig. 3G), then it decreased in size (Fig. 3H-L). Flagellated sperm with nuclei similar in size to those of mature sperm could be found attached to the clump after 9 days of culture (Fig. 3G,J,K) and in suspension by 15 days (Fig. 3M). Given that the clumps had disappeared by about 20 days, spermatogonial stem cells do not seem to proliferate under these conditions.

Because the germ cell marker gene vas (Yoon et al., 1997) is strongly expressed in zebrafish spermatogonia and spermatocytes prior to the first meiotic division (S. Maegawa et al., personal communication), as in the mouse (Fujiwara et al., 1994 and Xenopus (Komiya et al., 1994), the expression of vas was determined in dissociated testicular cells cultured with or without feeder cells. Competitive PCR analysis showed that vas expression in the co-culture with ZtA6 cells was stronger than that without ZtA6 cells on day 2 (Fig. 4), suggesting that ZtA6 cells aid the attachment of spermatogonia and primary spermatocytes. On days 5 and 8, the co-culture showed that vas was still strongly expressed, in contrast to the culture without ZtA6 cells (Fig. 4). By comparison, in diluted samples of the co-culture to the culture of dissociated testicular cells alone on day 8, five- to eightfold more vas transcript was found in the co-culture when compared with the culture alone. These results indicate that ZtA6 cells not only aid the attachment, but also maintain spermatogonia and primary spermatocytes.

To determine whether DNA duplication occurs in this culture system, thymidine analogue 5-bromo-2’-deoxyuridine (BrdU) incorporation experiments were performed. BrdU was added to the culture medium for the first 2 days of culture.

![Fig. 1. Derivation of ZtA6 feeder cells from tumor-like hypertrophied testis. (A) ZtA6 cells included heterogeneous testicular cell types, including epithelial and fibroblast cells. (B) Many cells showed phagocytic activity in terms of the uptake of latex beads, which is characteristic of mammalian Sertoli cell lines (Tokuda et al., 1992; Rassoulzadegan et al., 1993). Scale bars: 50 μm.](image)

![Fig. 2. Expression of Sertoli cell and germ cell marker genes in ZtA6 cells by RT-PCR. ZtA6 cells expressed the marker genes of Sertoli cells, zebrasoma sox9a and the Wilms’ tumor suppressor wt1, but scarcely any gata1. Germ cell marker vas was detected at low levels.](image)
At five days, positive cells with large nuclei were found by immunohistochemistry for BrdU, but there were no positive flagellated sperm (Fig. 5A). After 8-9 days of culture, BrdU-positive nuclei similar in size to those of mature sperm were present (Fig. 5C). BrdU-positive flagellated sperm were also found (Fig. 5E). Nuclear shape and size of the flagellated sperm were similar to those of control mature sperm (Fig. 5F).

When wild-type germ cells/sperm cultured for a total of 12 days were used to in vitro fertilize albino eggs, embryos with normal melanocytes were obtained (Table 1). The embryos developed normally, and generated their progeny with half wild-type when mated with albinos. BrdU-treated sperm cultured for a total of 11 days (BrdU for the first 2 days) resulted in a four-cell stage BrdU-positive embryo, as determined in both whole-mount (Fig. 6A) and sections (Fig. 6B). The four-cell stage was chosen for BrdU detection because unfertilized eggs never reach the four-cell stage. The late stage, when the chromosomes are condensed, was also chosen because the positive signal becomes weaker after the cleavage of blastomeres. Two positive signals in each blastomere indicates that the chromosomes have already divided. Whole-mount detection revealed that more than half the fertilized embryos exhibited obvious positive signals (Table 1).

Fig. 3. Co-culture of dissociated testicular cells on ZtA6 feeder cells. Male germ cells were attached on the feeder cells as a clump. (A-L) The same single clump was observed every day from 3 days to 14 days of culture. (M) On day 15, the clump had disappeared; however, an aggregation of flagellated sperm was found in suspension. (N) Control normal mature sperm. As the germ cells divided, the clump became larger (A-F) until the appearance of flagellated sperm on day 9 (G). It then decreased in size (H-L). The nuclear sizes of flagellated sperm (arrowhead) on days 9 (G), 12 (J) and 13 (K) are similar to those of normal mature sperm (N). Scale bar: 10 μm.

Fig. 4. Competitive PCR analysis of \( \text{vas} \) and \( \alpha \)-tubulin expression in a co-culture of dissociated testicular cells with ZtA6 feeder cells and in a culture of dissociated testicular cells alone. Co-culture was used with cDNA from a co-culture of dissociated testicular cells with mitomycin C-treated ZtA6 cells. The control used cDNA from dissociated testicular cells cultured alone plus the same ZtA6 cells as that used in the co-culture cultured separately, making it equivalent to the co-culture. Two hundred times more \( \text{vas} \) cDNA was used than \( \alpha \)-tubulin cDNA. As duplicate experiments resulted in the same pattern, this figure shows the results from one experiment. The number below the photos indicates the ratio of sample density to competitor density.
DISCUSSION

The results of present study show that functional sperm are differentiated through DNA duplication on feeder cells derived from tumor-like testis in culture. In addition to observations regarding which germ cells divide by mitosis, the results suggest that functional sperm are differentiated from spermatogonia. This male germ cell culture system should provide an excellent opportunity to analyze the regulatory mechanisms of male germ cell development and to create sperm-based vectors. The zebrafish is a major vertebrate model genetic system, and a large number of mutants, including those that are spermatogenesis deficient (Bauer and Goetz, 2001), are available. Because cell culture is amenable to genetic analysis, combining mutants together with in vitro culture could lead to new insights regarding vertebrate spermatogenesis.

The culture described herein represents parts of mitotic processes, except those of spermatogonial stem cell proliferation, and whole meiotic processes of the male germ cell, i.e. initiation of meiosis, G2-M phase transition of a meiotic spermatocyte, etc. Owing to the similarity of each stage of spermatogonium under microscopic observation and the lack of specific markers, it was difficult to identify the stage of the starting germ cells. Because the vas gene is highly

<table>
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<th>Experiment</th>
<th>Total number of eggs exposed to culture</th>
<th>Number of fertilized eggs</th>
<th>Number of BrdU-positive eggs</th>
<th>Number of normally developed eggs</th>
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<td>1*</td>
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<td>4</td>
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<td>5†</td>
<td>177</td>
<td>13</td>
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*Cultured for 12 days without BrdU treatment. BrdU detection was not performed.
†Cultured for 11 days with BrdU treatment. All of the fertilized eggs were fixed at the four-cell stage.
expressed in spermatogonia and spermatocytes prior to the first meiotic division (Fujiiwara et al., 1994; Komiya et al., 1994) (S. Maegawa et al., personal communication), prolongation of vas expression in co-culture with feeder cells indicates that feeder cells stimulate proliferation of spermatogonia, which is confirmed by the observations that germ cells divide by mitosis. The organ-culture experiment of eel testis reveals that A-type or early B-type spermatogonia progress into further stages in response to stimulation of gonadotropin or 11-ketotestosterone with the involvement of Sertoli cell’s factor activin B (Miura and Miura, 2001). The present culture covers at least from division of B-type spermatogonia to further stages, or may cover the same developmental stages of spermatogonia as the eel-culture system. Methods or mutants to obtain a certain stage of spermatogonia would allow to analyze the role of feeder cells with the involvement of specific factors stimulating spermatogonial division and differentiation.

Sertoli cells are known to interact with germ cells by the production of many molecules or by mechanisms that mediate cell junctions and adhesion (reviewed by Jegou, 1993; Sharpe, 1993). The best characterized of these molecules is the Kit tyrosine kinase receptor-ligand system, which is essential for proliferation of primordial germ cells in the mouse (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). The ligand expressed in Sertoli cells is required for differentiation of spermatogonial stem cells (Ogawa et al., 2000; Ohta et al., 2000), and for either entering or completing meiosis (Vincent et al., 1998). In contrast to the mouse, zebrafish kit is not essential for primordial germ cell development, and the mutant sparse exhibits no qualitative defects in fertility (Parichy et al., 1999). As in hox clusters (Amores et al., 1998) and other many genes (Postlethwait et al., 1998), there may be another kit gene generated by a whole genome duplication event hypothesized to have occurred at the base of teleost evolution. Many issues remain to be explored in terms of a new kit-ligand system, if it exists, or the substitutive receptor-ligand system in zebrafish, and in terms of general factors that essential to the development of male germ cells in vertebrates. Because heterogeneous cells derived from a tumor-like testis were used as feeder cells in present study, it was difficult to ascertain which cell types had the special function for the development of male germ cells. Subsequently, clonal cell lines from this heterogeneous mix will be established to analyze the function of specific feeder cell types. The cells will be amenable to isolation of secreted factors and specific expressed genes, facilitating the analysis of the regulatory functions of testicular somatic cells on the mitosis and meiosis of male germ cells in vertebrates.


