Zinc is an essential trace element for spermatogenesis

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Zinc (Zn) plays important roles in various biological activities but there is little available information regarding its functions in spermatogenesis. In our current study, we further examined the role of Zn during spermatogenesis in the Japanese eel (*Anguilla japonica*). Human CG (hCG) was injected into the animals to induce spermatogenesis, after which the concentration of Zn in the testis increased in tandem with the progression of spermatogenesis. Staining of testicular cells with a Zn-specific fluorescent probe revealed that Zn accumulates in germ cells, particularly in the mitochondria of spermatogonia and spermatozoa. Using an in vitro testicular organ culture system for the Japanese eel, production of a Zn deficiency by chelation with *N,N,N′,N′*-tetrakis (2-pyridylemethyl)ethylenediamine (TPEN) caused apoptosis of the germ cells. However, this cell death was rescued by the addition of Zn to the cultures. Furthermore, an induced deficiency of Zn by TPEN chelation was found to inhibit the germ cell proliferation induced by 11-ketotestosterone (KT), a fish specific androgen, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), the initiator of meiosis in fish, and estradiol-17β (E2), an inducer of spermatogonial stem-cell renewal. We also investigated the effects of Zn deficiency on sperm motility and observed that TPEN treatment of eel sperm suppressed the rate and duration of their motility but that co-treatment with Zn blocked the effects of TPEN. Our present results thus suggest that Zn is an essential trace element for the maintenance of germ cells, the progression spermatogenesis, and the regulation of sperm motility.

apoptosis | germ cells | in vitro culture | Japanese eel | sperm motility

Zinc (Zn) is well known as an essential trace element for a variety of biological activities. In biological systems, Zn is present in protein-bound and ionic forms, and plays important roles in mediating the function and structure of proteins, and in maintaining physiological balance. In vertebrates, Zn accumulates in the testis at high levels which are comparable to those in liver and kidney (1). In epidemiological studies in human, the inhibition of spermatogenesis and sperm abnormalities have been observed in patients with Crohn's disease and nutritional disorders, both of which induce a Zn deficiency (1–3). In vivo experiments in rodents have also demonstrated that a Zn deficiency can cause severe damage to the testes such as atrophy of the testicular tubules and the inhibition of spermatid differentiation (4, 5). Moreover, there are some reports that exposure to Zn can alleviate testis damage by stresses such as heavy metals, fluoride, and heat (6). These findings suggest that the testes may harbor a Zn-incorporation system, and that Zn itself may exert protective effect against testicular injury and play an essential role in the maintenance of testicular functions. However, there has been no evidence reported to date that shows any direct effects of Zn upon spermatogenesis in vertebrates.

In contrast to spermatogenesis, the effects of Zn on sperm motility have been examined in a number of vertebrate and invertebrate species. In humans, sperm motility declines in association with increased Zn concentrations in the seminal plasma (7). Morisawa and Yoshida have also reported that Zn in the seminal plasma of human suppresses sperm motility, and that the removal of Zn by binding to a protein named semenogelin enhances motility (6). On the other hand, in sea urchin, treatment with the bivalent metal ion chelator, ethylenediamine tetra acetic acid (EDTA), inhibits sperm motility that is reversed by the addition of Zn (9). These results suggest that extracellular Zn indeed affects sperm motility but whether this is inhibitory or stimulatory appears to be species-specific. Additionally, it has been reported that Zn is present in sperm mitochondria and flagella (10, 11) but there had been no reports to date concerning the role of intracellular Zn upon sperm function.

To further study the role of Zn upon spermatogenesis in our current study, we chose Japanese eel (*Anguilla japonica*) as our animal model. In the Japanese eel in vivo, a complete pathway of spermatogenesis, from the spermatogonia stage to sperm maturation, can be induced by the injection of human CG (hCG; 12). Furthermore, we have developed a testicular organ culture system for the Japanese eel in our laboratory, which is the only currently available system of its kind in which the induction of complete spermatogenesis can be performed in vitro by the addition of 11-ketotestosterone or hCG (13, 14). By in vivo and in vitro analyses of spermatogenesis in the Japanese eel, we have previously further clarified the regulatory mechanisms underlying fish spermatogenesis (15, 16). Additionally, we have revealed the inhibitory effects of 4 trace elements (lead, molybdenum, rubidium, and arsenic) on fish spermatogenesis using our in vitro testicular organ culture system (17). In our present study, we again used the Japanese eel model to investigate the concentration and distribution of Zn in testis during spermatogenesis. Moreover we examined the effects of Zn addition and deficiency on spermatogenesis and sperm motility in vitro.

Results

Changes in the Levels and Distribution of Zinc (Zn) in the Testis of the Japanese Eel during Spermatogenesis. Before injection with hCG, the concentration of Zn in the testis of the Japanese eel was approximately 50 μg/g. After injection, the Zn concentration in the testis gradually increased, and the highest levels were observed on day 9. Thereafter, the concentration of Zn remained at high levels until day 18 (Fig. 1).

To detect the distribution of Zn in eel testes, an unfixed testicular fragment was stained with a fluorescence sensor for Zn(II), ZnAF-2DA. Strong fluorescent signals were obtained in the lobules but not in the interstitial tissue (Fig. 2*A* and *B*). We thus further investigated the distribution of Zn in testicular tissue using isolated cells. Germ cells were found to be strongly stained

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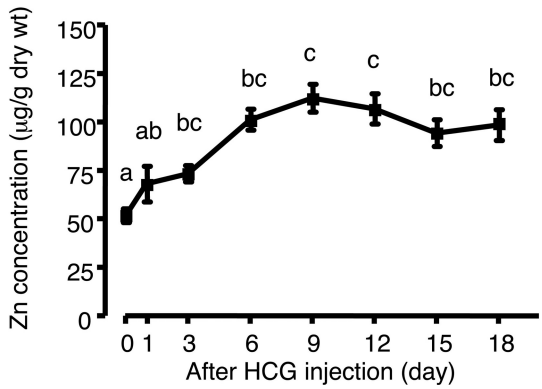

Fig. 1. Changes in the Zn concentrations in the testis of the Japanese eel after injection of human CG (hCG). The different letters indicate statistically significant differences ($P < 0.05$).

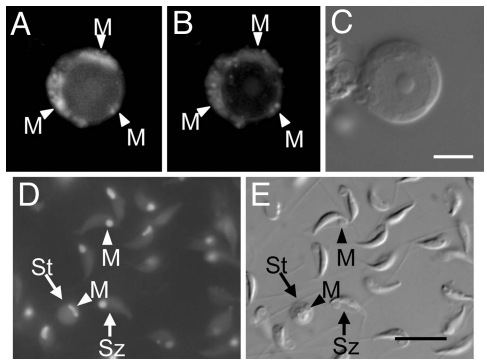

Fig. 3. Zinc distribution in germ cells of the Japanese eel. Zn was stained using ZnAF-2DA. Fluorescence images are shown for (*A*) Zn, and (*B*) mitochondria. (*C*) Bright field image of spermatogonia. (*D*) Zn fluorescence and (*E*) bright field image of spermatids and spermatozoa. M, mitochondria; St, spermatid; Sz, spermatozoa. (Scale bars: 10 μm.)

by ZnAF-2DA but Sertoli cells showed no signal (Fig. 2 *C* and *D*). When the germ cells were treated with 10 mM *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylemethyl) ethylenediamine (TPEN) for 1 h before staining with ZnAF-2DA, fluorescence was not detected (Fig. 2 *E* and *F*). We also stained the germ cells at various stages with ZnAF-2DA, that is, spermatogonia, spermatocytes, spermatids, and spermatozoa. ZnAF-2DA signals were detectable in spermatogonia, most notably in the mitochondria (Fig. 3 *A*–*C*). Additionally, the mitochondria of the spermatids and spermatozoa also displayed strong ZnAF-2DA signals (Fig. 3 *D* and *E*).

Effects of Zn and Zn Chelators on Japanese Eel Testes in Vitro. To investigate the putative key role of Zn during spermatogenesis, we analyzed the direct effects of Zn on the testis in the presence or absence of 11-ketotestosterone (KT). After culturing for 6 days, testicular fragments in the control group were found to be occupied by type A spermatogonia. Although the histological structure of the testicular fragments cultured with KT alone did not differ from the control group, the incorporation ratio of BrdU into the germ cells had significantly increased (Fig. 4), as also reported in our previous study (13). Treatment of the Japanese eel testicular fragments with any level of Zn with or without KT did not affect the histology of the testis or the BrdU index (Fig. 4*A*). Treatment with ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, calcium(II), disodium salt (Ca-EDTA), an extracellular Zn chelator, also did not affect the BrdU index or testicular morphology after 6 days in culture (Figs. 4*B* and 5*B*). In contrast, exposure to 0.01 and 0.1 mM TPEN, an intracellular chelator of Zn, inhibited BrdU-incorporation into germ cells (Fig. 4*B*), and induced germ cell death (Figs. 4*B* and 5*C*). Significantly, both the cell death and the inhibition of BrdU incorporation induced by TPEN was rescued by the addition of Zn (Figs. 4*B* and 5*D*). We further investigated the type of cell death that occurred using a TdT-mediated dUTP nick-end

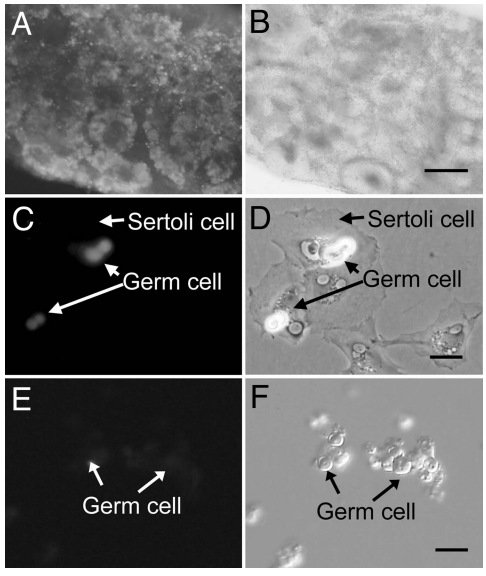

Fig. 2. The zinc distribution in the testis of the Japanese eel determined by staining with a Zn-specific fluorescent probe, ZnAF-2DA (*A*, *C*, and *E*). Bright field images are also shown (*B*, *D*, and *F*). (*A* and *B*) testicular fragments of the Japanese eel at 15 days after injection of hCG; (*C* and *D*) germ cells and Sertoli cells; (*E* and *F*) TPEN-treated germ cells. (Scale bars: *A* and *B*, 100 μm; *C*–*F*, 20 μm.)

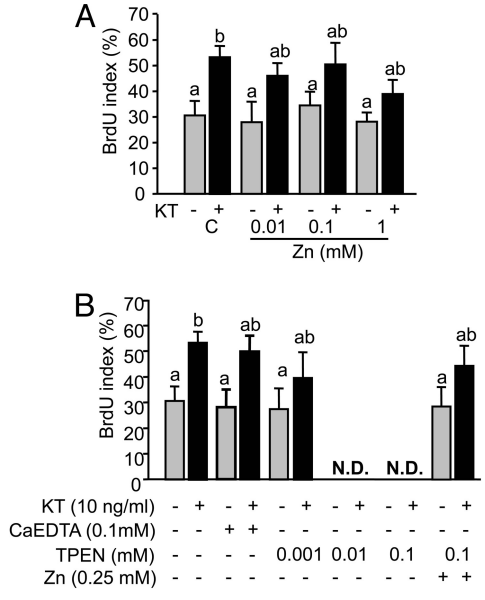

Fig. 4. Effects of Zn and Zn chelators on the early stages of spermatogenesis in vitro. The BrdU-labeling index was determined for germ cells in testicular fragments cultured with Zn (*A*) or Zn chelators (*B*) with or without KT. The number of BrdU-positive germ cells is expressed as a percentage of the total number of germ cells. C, control; Zn, $ZnCl_2$; KT, 11- ketotestosterone; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine; CaEDTA, ethylenediamine -*N*,*N*,*N*′,*N*′- tetraacetic acid, calcium(II), disodium salt, dihydrate. Results are given as the mean ± SEM. The different letters on the columns indicate statistically significant differences ($P < 0.05$).

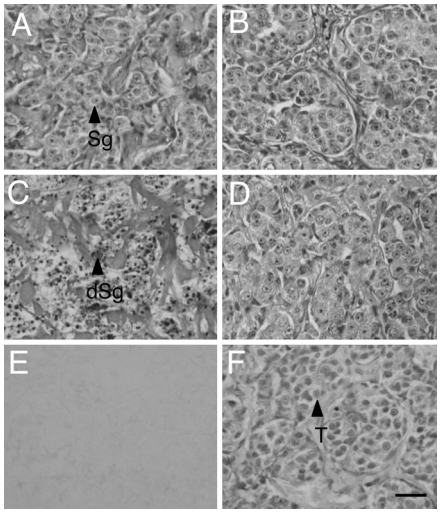

Fig. 5. Light micrographs of testicular fragments after culture with Zn chelators for 6 days. (*A–D*) Hematoxylin and eosin-stained testicular fragments cultured for 6 days. (*A*) control; (*B*) cultured with 0.1 mM CaEDTA; (*C*) cultured with 0.01 mM TPEN; (*D*) cultured with 0.1 mM TPEN and 0.25 mM $ZnCl_2$. (*E* and *F*) One-day cultures of testicular fragments subjected to a TUNEL assay. (*E*) control; (*F*) cultured with TPEN. Dark stained cells are TUNEL-positive (*E* and *F*). Sg, spermatogonia; dSg, dead spermatogonia; T, TUNEL-positive cells. (Scale bar: 20 μm.)

labeling (TUNEL) assay after a 1-day culture in the presence of TPEN. In the control and KT-treatment groups, no cell staining was observed (Fig. 5*E*). However, TUNEL-positive germ cells were detectable after treatment with 0.01–0.1 mM TPEN with or without KT (Fig. 5*F*).

We also investigated the effects of 0.001 mM TPEN, a dose that does not cause cell death, upon KT-induced spermatogenesis using our testicular organ culture system. Treatment with this dosage for 6 days had no effects on the histological structure of the testicular fragments. In contrast, after 15 days of this treatment, the testicular fragments were found to only have type A spermatogonia, although those cultured with KT alone contained the more progressed germ cells, type B spermatogonia (Fig. 6 *A–C*). Importantly, the addition of Zn led to a recovery of spermatogenesis, such that the TPEN/KT treated cultures resembled those exposed to KT alone (Fig. 6*D*).

Effects of Zn on the Germ Cell Proliferation Induced by Various Steroid Hormones. In the Japanese eel, KT, 17α,20β-dihydroxy-4-pregnen-3-one (DHP), and estradiol-17β (E2) induce DNA

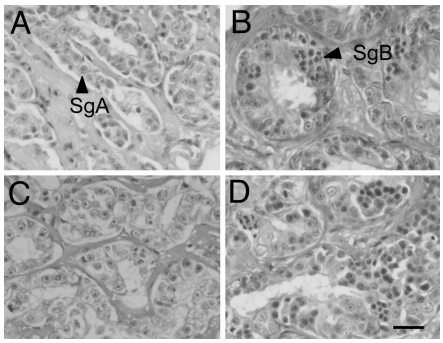

Fig. 6. Light micrographs of testicular fragments after culture with 10 ng/mL KT and 0.001 mM TPEN for 15 days. (*A*) Control; (*B*) cultured with 10 ng/mL KT; (*C*) cultured with KT and 0.001 mM TPEN; (*D*) cultured with KT, TPEN, and 0.0025 mM $ZnCl_2$. SgA, type A- spermatogonia; SgB, type B-spermatogonia. (Scale bar: 20 μm.)

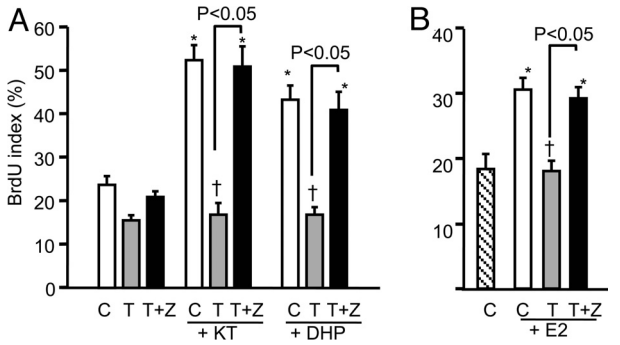

Fig. 7. Effects of a low dose TPEN upon germ cell proliferation in vitro. Testicular fragments were cultured with 0.001 mM TPEN and/or 10 ng/mL KT or DHP for 6 days (*A*) or cultured with TPEN and/or 1 ng/mL E2 (*B*). (*C*) Testicular fragments cultured without TPEN as a control for each steroid hormone; T, with TPEN; T+Z, with TPEN and Zn. KT, 10 ng/mL 11- ketotestosterone; DHP, 10 ng/mL 17α,20β-dihydroxy-4-pregnen-3-one; E2, 1 ng/mL estradiol-17β. Asterisks indicate significant differences from the negative control ($P < 0.05$). Daggers indicate significant differences from the control for each steroid hormone treatment ($P < 0.05$).

synthesis in germ cells thereby initiating spermatogenesis, meiosis, and spermatogonial stem-cell renewal, respectively (13, 16, 18). To elucidate at the stages of spermatogenesis at which Zn functions, that is, spermatogonial stem-cell renewal, spermatogonial proliferation or meiosis, we examined the effects of a 0.001 mM concentration of an intracellular Zn chelator on the germ cell proliferation induced by 10 ng/mL KT, 1 ng/mL E2, and 10 ng/mL DHP. These doses of KT, DHP and E2 were previously shown to be optimal for the induction of DNA synthesis in germ cells in vitro involving the initiation of spermatogenesis, meiosis, and spermatogonial stem-cell renewal, respectively (13, 16, 18). The rates of BrdU incorporation increased after treatment with KT and DHP for 6 days. However, treatment with 0.001 mM TPEN decreased the levels of BrdU incorporation induced by DHP or KT (Fig. 7*A*). To then investigate the effects of Zn on E2-induced germ cell proliferation, eel testes were cultured with E2 with or without 0.001 mM TPEN for 15 days according to the method of Miura et al. (18). Treatment with E2 alone significantly increased the number of BrdU-positive germ cells, whereas E2 in combination with 0.001 mM TPEN suppressed germ cell proliferation. This inhibition by TPEN was rescued by Zn treatment (Fig. 7*B*).

Effects of Zn Deficiency on Sperm Motility. In the mitochondria of Japanese eel sperm, strong ZnAF-2DA signals were observed. Hence, we analyzed the effects of Zn chelators on the rate and duration of eel sperm motility. Treatment of the sperm with Ca-EDTA did not alter their motility rate or duration at any concentration (Fig. 8*A* and *B*). In contrast, the addition of TPEN decreased both the motile rate and duration in a dose-dependent manner: 0.1–1 mM TPEN was found to be an effective concentration range for both indices. Furthermore, treatment with 1 mM Zn treatment rescued the inhibition of sperm motility by 1 mM TPEN (Fig. 8 *A* and *B*).

Discussion

Some previous studies have reported that a high concentration of Zn is detectable in testis, and that a Zn deficiency inhibits spermatogenesis and causes sperm abnormalities (5, 19). However, there are currently few reports that address the function of Zn during spermatogenesis in any detail. We thus investigated in our current study the distribution of Zn in testis and the direct effects of Zn upon spermatogenesis using an in vitro testicular organ culture model derived from the Japanese eel.

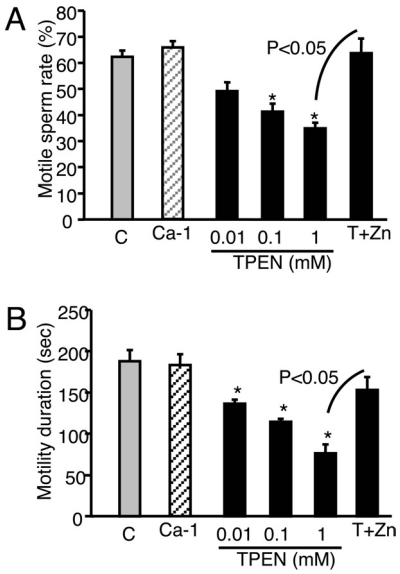

Fig. 8. Effects of Zn chelators on the motility of Japanese eel sperm. (*A*) Ratio of motile sperm; (*B*) duration of sperm motility. C, control; Ca-1, incubated with 1 mM Ca-EDTA; T+Z, incubated with 1 mM TPEN and 1 mM $ZnCl_2$. Asterisks indicate statistically significant differences from the control.

Our present analyses show that the Zn concentration in the testes of the Japanese eel gradually increases following an injection with hCG, and peaks on day 9 after this induction. Additionally, using a fluorescent Zn probe, strong signals were observed in germ cells, particularly spermatogonia, but not in the interstitial tissue or Sertoli cells. Similar to our present findings, Sørensen et al. have previously demonstrated by autometallography (AMG) that Zn is present in spermatogonia and primary spermatocytes in mouse (20). We previously demonstrated in our laboratory that a single injection of hCG first induced spermatogonial proliferation, then initiated meiosis on about day 12, and induced spermiogenesis on day 18 postinjection (12). Taken together therefore, our current data and previous findings suggest that Zn accumulates in the testis during early spermatogenesis, and may play a key role in the regulation of the spermatogonial proliferation and in the meiosis of germ cells. In the germ cells of other vertebrates, some Zn transporters have been observed. In rat, metallothionein (MT) was detected in spermatocytes (21) and other reports have shown that a testis-specific metallothionein-like protein (tesmin) is also present in these cells (22, 23). Additionally, Chi et al. have demonstrated that Zn accumulates in sperm in the mouse and that the Zn-exporter, ZnT-7, is present in the mouse testis, suggesting that Zn may be supplied to the germ cells via ZnT-7 (24). We speculate therefore that Zn may be accumulated in the germ cells of the Japanese eel via such transporter molecules.

In our present experiments in eel, Zn was found to accumulate prominently in the mitochondria in spermatogonia, spermatids and spermatozoa. In mouse, as detected via the AMG technique, sperm mitochondria were also previously shown to accumulate Zn, similar to our current results (10). Costello et al. have reported that Zn is imported into the mitochondria of prostate and liver cells in the form of a Zn-ligand complex such as Zn-citrate and Zn-MT (25, 26). Additionally, the membrane type Zn transporter protein ZnT-1 is expressed in the mitochondria of mouse spermatozoa (21). Taken together, there is now ample evidence to suggest that mitochondria may harbor a transporting system for Zn, and that Zn itself may have an important role to play in mitochondrial function in germ cells. In addition to mitochondria, Zn has also been detected in other areas of the cytoplasm in eel spermatogonia. In rat, Zn accumulates in the cytoplasm of both spermatogonia and spermatocytes (20). Furthermore, murine ZnT-7 is present in Golgi apparatus of spermacytes and spermatids (24). Thus, other organelles and cytosolic compartments may also accumulate Zn as part of its germ cell functions.

To further clarify the role of Zn in germ cells, we also investigated the effects of Zn and intra/extracellular Zn chelators on spermatogenesis using our in vitro testicular organ culture system develop from the Japanese eel. The results of these experiments demonstrated that treatment with the intracellular chelator TPEN caused germ cell death, which was blocked by the addition of Zn. This suggests that Zn is an essential trace element for the maintenance of germ cells. We performed a TUNEL assay using cultured testes and found that TPEN specifically caused apoptotic death in germ cells. There are some reports that a Zn deficiency causes apoptosis in various cell and tissue types. In human lymphocytes and rat hepatocytes for example, treatment with TPEN causes DNA fragmentation (27, 28). Interestingly, an in vitro and in vivo Zn deficiency was shown to induce caspase-3 activity in human mast cells and rat embryos, respectively (29, 30). Furthermore, treatment with Zn induces the antiapoptotic protein Bcl-2 and inhibits apoptosis in U947 cells (31). Caspase-3 and Bcl-2 in mitochondria have important roles in mitochondrial apoptosis; caspase-3 is released after cell damage and induces apoptosis, whilst Bcl-2 suppresses the apoptotic response (32). In our present study using a fluorescent Zn probe, we found that Zn accumulates in the mitochondria of germ cells and this may underpin its protection of these cells from apoptosis. However, the molecular mechanisms of how Zn regulates caspase-3 and Bcl-2 in mitochondria remain unclear at present. Some studies have addressed the correlation between Zn and apoptosis and suggest that Zn may function as an antioxidant in cells (32). Further studies will be necessary to clarify the role of Zn in the maintenance of germ cells.

We additionally investigated the influence of mild Zn deficiency on spermatogonial stem-cell renewal, spermatogonial proliferation, and meiosis in vitro. In a previous study, we reported that KT, E2, and DHP induce spermatogenesis, spermagonial stem-cell renewal, and meiosis in eel germ cells, respectively (13, 15, 16). In our present report, TPEN was found to inhibit all steroid hormone-induced DNA synthesis in the testes of the Japanese eel. These results suggest that Zn has an important role in DNA synthesis involving mitotic cell proliferation and meiosis. A previous study using 3T3 cells has reported that treatment with the Zn chelator, diethylenetriaminepentaacetic acid, decreases the mRNA expression and activity of thymidine kinase, after which DNA synthesis was inhibited in 3T3 cells (33). Furthermore, steroid hormone receptors such as progestin, androgen, and estrogen receptors all harbor Zn finger motifs within their structures (34). Other transcription factor genes containing Zn-finger motifs are also expressed during spermatogenesis (35). These findings suggest therefore that during steroid hormone-induced DNA synthesis, germ cells may incorporate Zn to activate a number of specific enzyme and Zn finger proteins, which are functionally disrupted by TPEN. Further analyses will be necessary to clarify the role of Zn on the functions of steroid hormone receptors and transcription factors during spermatogenesis.

Our current findings demonstrate that treatment with TPEN decreases sperm motility in the Japanese eel. Consistently in this regard, studies of human sperm have also demonstrated that diethyldithiocarbamate, which is an intracellular Zn chelator, inhibits sperm motility and decreases sperm velocity (36). These results suggest that intracellular Zn is important for sperm motility. As mentioned above, the mitochondria in the sperm of

the Japanese eel accumulate Zn. The ATP synthesized by the mitochondria is required for sperm flagella motility (37). Hence, Zn may have a function in mitochondrial ATP synthesis. Additionally, carbonic anhydrase (CA) is necessary for eel sperm motility, and this enzyme is expressed in the sperm membrane. CA catalyzes the reversible hydration of carbon and regulates the pH in various fluids. After spermiation, CA in the eel spermatozoa is activated after which it increases the pH and then induces sperm motility (15). CA is also known to be a Zn-binding protein, and its activity is dependent on the Zn concentration (38). Additionally, the removal of Zn from Zn-protein complexes extracted from human U87 human glioblastoma-astrocytoma cells by TPEN inhibited the function of the transcription factor, Sp1 (39). Although there is currently no information on effects of TPEN on CA activity, we speculate that TPEN may inhibit sperm motility by sequestering Zn away from this enzyme in sperm.

In conclusion, the results of our present study demonstrate that the Zn concentration in testis increases during spermatogenesis, and that Zn accumulates mainly in germ cells but not in either interstitial tissue or Sertoli cells. Our in vitro testicular organ culture experiments also demonstrated that a Zn deficiency causes the inhibition of DNA synthesis in germ cells, and induces an apoptotic response. Additionally, a Zn deficiency was found to suppress sperm motility in the Japanese eel animal model. These results suggest that Zn is an essential trace element for the maintenance and regulation of both spermatogenesis and sperm motility. However, the detailed mechanisms of Zn action during spermatogenesis remain to be clarified in further studies.

Materials and Methods

Animals. Cultivated male Japanese eels (180–200 g) were purchased from a commercial supplier and kept in a freshwater tank at 23 °C until use.

Measurement of Zn in Testis During Spermatogenesis. A previous report has indicated that hCG injection of a cultivated Japanese eel induces a complete cycle of spermatogenesis (11). Hence, these animals were injected with 1,000 IU/eel of hCG following anesthetization by ethylbenzoate. After injection, the fish were kept in a freshwater tank at 23 °C for 1, 3, 6, 9, 12, 15, and 18 days. Thereafter, hCG-injected eels ($n = 5$ for each day) were anesthetized and dissected, and the testes were collected and stored at −30 °C until measurement of Zn concentration. Before the experiments, testicular fragments were sampled from 5 uninjected eels as an initial control group. The testicular samples were dried for 12 h at 80 °C. For the analysis of Zn, dried testes were digested with HNO_3 in a microwave oven (ETHOS D, Milestone S.r.l.). The concentration of Zn was then measured using an inductively coupled plasma-mass spectrometer (ICP-MS; HP-4500, Hewlett-Packard).

Distribution of Zn in the Testis. We stained both the testicular fragments of the Japanese eel and the cells derived from these tissues with a Zn-specific probe. For this purpose, testis samples collected from the eels were cut into 100-μm sections in ice-cold eel Ringer's solution using a Vibratome 3000 (Vibratome). Testicular cells were also prepared according to Miura et al. (40, 13, 16) for Zn staining. Briefly, testes were harvested and testicular cells were isolated by collagenase and dispase treatments. After treatment with DNase I, testicular cells were cultured in plastic culture dishes at 20 °C overnight and both fibroblasts and interstitial cells were allowed to adhere to the bottom of the dish, thus separating these cells from germ cells and Sertoli cells. The germ cells and Sertoli cells were then collected from the culture dishes and plated in collagen-coated dishes at 20 °C overnight. After this overnight culture, only the Sertoli cells adhere to the bottom of the dish. Thereafter, germ cells were collected in a test tube, and both the germ cells and Sertoli cell preparations were used to analyze the Zn distribution. Sertoli cells and germ cells could be identified using a variety of distinguishing characteristics and specific marker expression. Sertoli cells attached and spread to the bottom of the dish, whereas germ cells did not attach and appeared spherical in shape. Furthermore, only germ cells express the progenstin receptor. We separated germ cells and Sertoli cells using this method previously (16).

Before staining of the germ cells, they were attached to a polyL-lysine coated glass slide. Testicular fragments, attached germ cells and Sertoli cells were then washed 3 times in eel Ringer's solution, and incubated with 1 μM of a permeable Zn-specific probe, Zn-AF 2DA (41) in eel Ringer's solution for 45 min at 20 °C. After this incubation, the cells were washed again in the Ringer's solution for 1 h at 20 °C and analyzed by fluorescence microscopy. The mitochondria of the spermatogonia were stained using MitoTracker Red (Invitrogen Co. Ltd.) according to the manufacturer's instructions with minor modifications before staining with Zn-AF 2DA.

In Vitro Testicular Organ Cultures. Organ cultures were prepared in accordance with the method of Miura et al. (13, 42). Male Japanese eels were dissected after anesthetization with ethylbenzoate. The testes were then collected, placed in ice-cold eel Ringer's solution and dissected into small pieces. Testicular fragments were placed on nitrocellulose membranes on top of cylindrical 1.5% agarose gels and set into a 24-well culture plate. Thereafter, 1 mL of Leibovitz' L-15 culture medium (Invitrogen Co. Ltd.) for eels (13) was added into each well with or without 0.01–1 mM $ZnCl_2$ (Zn), 0.001–0.1 mM TPEN, or 0.001–0.1 mM Ca-EDTA, which are intracellular and extracellular chelators of Zn, respectively, in combination with or without 10 ng/mL KT. The concentrations of Zn and chelators used in the in vitro experiments were based on the results obtained from the Zn measurement in the testis. Testicular fragments were incubated for 6 or 15 days and then fixed Bouin's solution for histological analysis.

Analysis of the Effects of a Mild Zn Deficiency upon Germ Cells. Testicular fragments were cultured with 0.001 mM TPEN in combination with 10 ng/mL KT, 1 ng/mL E2, and 10 ng/mL DHP for 6 or 15 days. Thereafter, testicular fragments were fixed and their histology was analyzed as described above.

Detection of Germ Cell Proliferation. The proliferation of Japanese eel germ cells was analyzed by immunohistochemical detection of 5-bromo-2-deoxyuridine (BrdU, Amersham Pharmacia Biotech) incorporation into replicating DNA. After culture for 6 or 15 days, testicular fragments were labeled with a 0.5 μl/mL BrdU solution for 18 h at 20 °C, and fixed in Bouin's solution. The fixed testicular fragments were then embedded in paraffin, cut into 4-μm sections, and subjected to immunohistochemistry with a mouse monoclonal anti-BrdU antibody.

TdT-Mediated dUTP Nick-End Labeling (TUNEL) Assay. For the detection of apoptosis, the TUNEL assay was performed. One-day cultured testicular fragments were fixed in Bouin's solution, cut into 5 μm-thick paraffin sections and then analyzed using an In Situ Cell Death Detection Kit (Roche Diagnostics, Ltd.) according to the manufacturer's instructions.

Effects of Zn on Sperm Motility. Eel sperm was collected after injection of the animals with hCG as described by Ohta et al. (43) and diluted 1:10,000 with artificial seminal plasma (149.3 mM NaCl, 15.2 mM KCl, 1.3 mM $CaCl_2$, 1.6 mM $MgCl_2$, and 10 mM $NaHCO_3$, adjusted to pH 8.2, see 43). The diluted sperm were then treated with 0.01–1 mM TPEN or 0.01–1 mM Ca-EDTA with or without 1 mM $ZnCl_2$ for 12 h at 4 °C. Thereafter, the sperm motility rate in seawater was measured as described previously (43). The duration of sperm motility was measured from 15 s after dilution in seawater until all movement had ceased completely.

Statistical Analysis. The results presented in this study are expressed as the mean ± SEM. In instances where the data did not distribute normally, these values were converted to a logarithmic scale. Differences between the means were analyzed by 1-way analysis of variance followed by a Bonferroni multicomparison test. Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc.). In all cases, significance was set at $P < 0.05$.

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