Japanese Eel: A Model for Analysis of Spermatogenesis

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ABSTRACT—The Japanese eel has two characteristics advantageous for the study of the mechanisms controlling spermatogenesis. One is the possibility of artificial induction of the complete process of spermatogenesis from spermatogonial proliferation to spermiogenesis by exogenous gonadotropin injection, and the other is the possibility of inducing this process in an in vitro testicular organ culture or germ-Sertoli cell coculture system. Using the eel system, we analyzed the control mechanisms of spermatogenesis. In Japanese eel, the whole process of spermatogenesis is regulated by several sex steroid hormones. Spermatogonial stem cell renewal is promoted by estradiol-17β (the natural estrogen in vertebrates). Spermatogonial proliferation can be induced by 11-ketotestosterone, the main androgen in teleost. IGF-I is necessary for the action of 11-ketotestosterone in the initiation of spermatogenesis. The action of 11-ketotestosterone is mediated by other factors, such as activin B, produced by Sertoli cells. Although 11-ketotestosterone also induces meiosis and spermiogenesis, the control mechanisms of these processes are not clear. After spermiogenesis, immature spermatozoa undergo sperm maturation, thereby becoming capable of fertilization. Sperm maturation is regulated by 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP), which is progestogen in teleosts. The 17α,20β-DP acts directly on spermatozoa to activate the carbonic anhydrase existed in the spermatozoa. This enzymatic activation causes an increase in the seminal plasma pH, enabling spermatozoa to motile.

Key words: spermatogenesis, Japanese eel, androgen, estrogen, progestin

INTRODUCTION

Spermatogenesis, the formation of sperm that is highly adapted for delivering its genes to an egg, is a complex developmental process. It begins with the mitotic proliferation of spermatogonia, then proceeds through two meiotic divisions followed by spermiogenesis, during which the haploid spermatids develop into spermatozoa. Spermatozoa then undergo maturation, obtaining the ability to fertilize.

Although the process of spermatogenesis is the same in both mammalian and nonmammalian vertebrates, its control mechanisms are not well understood. Spermatogenesis is controlled by numerous hormones and unknown factors (Steinberger, 1971; Hansson et al., 1976; Callard et al., 1978; Billard et al., 1982; Cooke et al., 1998). In higher vertebrates such as mammals, it is difficult to analyze the control mechanisms of spermatogenesis because the seminiferous tubules contain several successive generations of germ cells (i.e., the testicular organization is complex; Clermont, 1972), and few culture systems are available for induction of spermatogenesis in vitro (Abe, 1987).

Among species of teleosts, various reproductive styles and gametogenetic patterns. Teleosts constitute the largest phylum (approximately 23,700 species) of living vertebrates (~48,200 species) (Nelson, 1994). Japanese eel, one of such species, has a special spermatogenetic pattern. Under culture conditions, male Japanese eel have immature testes (Fig. 1A) containing only non-proliferated type A and early type B spermatogonia (Miura et al., 1991a). This immature stage of the testis is attributed to insufficient gonadotropin in the eel pituitary (Yamamoto et al., 1972). However, a single injection of human chorionic gonadotropin (hCG) can induce the complete process of spermatogenesis from the proliferation of spermatogonia to spermiogenesis (Fig. 1B,C,D,E) (Miura et al., 1991a). Germ cell development is almost synchronous throughout the testis and the proliferation of spermatogonia,
meiosis, and spermiogenesis occur at definite times: 3, 12 and 18 days after hCG injection, respectively (Miura et al., 1991a). Furthermore, Japanese eel is the only animal in which complete spermatogenesis has been induced by hormonal treatment in vitro using an organ culture system (Fig. 2) and a germ-somatic cells coculture system (Fig. 3), respectively (Miura et al., 1991b,c, 1996). Therefore, the male Japanese eel provides an excellent system for studying the mechanisms of spermatogenesis. This review discusses our recent experimental observations, which indicate the possible molecular control mechanisms of spermatogenesis in Japanese eel.

The endocrine control of eel spermatogenesis

Eel spermatogenesis is also endocrinologically controlled, as is the case in other vertebrates. It is well established that in vertebrates, including fish, gonadotropins (GTHs) are the primary hormones regulating spermatogenesis (Nagahama, 1987). In most cases, however, it appears that GTHs do not act directly, but rather work through the gonadal biosynthesis of steroid hormones, which in turn mediate various stages of spermatogenesis (Nagahama, 1994).

1) The regulation of spermatogonial stem cell renewal

Spermatogonial mitosis can be categorized by spermatogonial stem cell renewal and spermatogonial proliferation toward meiosis (Clermont, 1972). Recently, it was indicated that estrogen is related to the regulation of the renewal of spermatogonial stem cells in eel (Miura et al., 1999b).

It is widely accepted that “estrogen” is a “female” hormone. However, it has been reported that estrogen exists in some male vertebrates (Schlinger and Arnold, 1992; Fasano and Pieratoni, 1993; Betka and Callard, 1998), and that its receptors are expressed in the male reproductive organs.
(Ciocca and Roig, 1995; Callard and Callard, 1987). Estradiol-17β (E2), a natural estrogen in vertebrates, was found in Japanese eel serum, and its receptor was expressed in the Sertoli cells (the only non-germinal elements within the seminiferous epithelium of the testes) during the whole process of spermatogenesis. These findings suggested that estrogen is related to the progress of spermatogenesis. We analyzed the action of E2 in spermatogenesis.

Mitosis of eel spermatogonial stem cells was promoted by the implantation of E2, but was suppressed by tamoxifen (an antagonist of estrogen). In vitro, 10 pg/ml of E2 was sufficient to induce spermatogonial stem cell division in cultured testicular tissue, confirming the in vivo observations. E2 treatments induced only spermatogonial stem cell renewal; they were not found to promote spermatogonial proliferation and meiosis. These findings clearly indicate that estrogen is an indispensable “male hormone”, and plays an important role in spermatogonial stem cell renewal.

Generally, E2 induces the target gene expression through its receptor, and the factor translated from this gene affects the biological process. Recently, using gene expression screening, we attempted to clone the cDNA that codes those factors induced or inhibited by E2 stimulation. As a result of this experiment, we obtained three cDNA clones (in preparation). The factor coded by some of these clones may act on spermatogonial renewal.

2) The regulation of spermatogonial proliferation toward meiosis and spermiogenesis

When GTH is secreted from the pituitary, spermatogonial mitosis switches from stem cell renewal to proliferation toward meiosis. As a matter of convenience, we call this point the initiation of spermatogenesis. It appears that in Japanese eel, GTH does not act directly on germ cells, but rather through the gonadal biosynthesis of 11-ketotestosterone (Miura 1991a,b). 11-ketotestosterone was first identified by Idler et al. (1961) as a major androgenic steroid in the male sockeye salmon (Oncorhynchus nerka). In various teleost fishes, this steroid has been shown to be synthesized in the testis following GTH stimulation, and high levels were detected in the serum during spermatogenesis (Billard et al., 1982). When 11-ketotestosterone was added to eel testicular organ culture, spermatogenesis from the proliferation of spermatogonia to spermiogenesis was induced (Miura et al., 1991b). The action of 11-ketotestosterone for spermatogenesis is not limited to the Japanese eel; it has been also recognized in goldfish (Kobayashi et al., 1991) and Japanese huchen (Amer et al., 2001). These findings indicate that 11-ketotestosterone is one of the factors involved in the initiation of spermatogonial proliferation toward meiosis.

However, it is believed that the action of 11-ketotestosterone is mediated by other factors produced by Sertoli cells, in which the androgen receptor exists (Ikeuchi et al., in press). It is possible that some of these factors are growth factors, such as insulin-like growth factor-I (IGF-I) and activin B.

IGFs are known to be mediators of growth hormone action in vertebrates. In the rainbow trout testis, IGF-I is expressed in spermatogonia and/or Sertoli cells, and it binds to type 1 IGF receptors. Further, IGF-I stimulates DNA synthesis in spermatogonia (Loir, 1994; Loir and LeGac, 1994; LeGac et al., 1996). Although IGF-I is also necessary for the regulation of eel spermatogenesis, its role is to support the action of 11-ketotestosterone. More specifically, in Japanese eel...
It has been reported that FGF, BMP, PDGF and numerous other growth factors regulate the early stage of spermatogenesis in teleosts and mammals (Watanabe and Onitake, 1995; Zhao et al., 1996; Li et al., 1997; Kim and Fazleabas, 1998). Further investigation is needed for a better understanding of the relationship between growth factors and spermatogenesis in eel.

3) The entry of spermatogonia into meiosis

Following mitotic proliferation, type B spermatogonia differentiate into primary spermatocytes. Generally, the number of mitotic divisions of spermatogonia preceding meiosis are

eel 11-ketotestosterone is necessary for the induction of spermatogenesis, whereas IGF-I is necessary for the continuation of the process (Nader et al., 1999).

Activin B is a dimeric growth factor belonging to the transforming growth factor-like (TGFβ) superfamily, and is composed of two activin βB subunits. In the Japanese eel, activin B was found in the testis at the initiation of spermatogenesis after hCG stimulation, with its expression site restricted to Sertoli cells. Both transcription and translation of eel activin B were induced by 11-ketotestosterone stimulation in vitro. Further, activin B induced proliferation of spermatogonia, but its treatment could not induce meiosis and further spermatogenesis (Miura et al., 1995a, b; submitted for publication).

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Fig. 3. The eel germ-Sertoli cell coculture system.
species specific. In teleosts, a spermatogonial stem cell of medaka (Oryzias latipes) yields spermatocytes following 8 mitotic divisions; more specifically, 6 in Sakhalin taimen (Hucho perryi), 8 in masu salmon (Oncorhynchus masou), 6 in white spotted char (Salvelinus leucomaenis), 8 in goldfish (Carassius auratus) (Ando et al., 2000), 14 in guppy (Poecilia reticulata) (Billard, 1986), 5 or 6 in zebrafish (Danio rerio) (Ewing, 1972), and there are 10 mitotic divisions in Japanese eel (Miura et al., 1991a). Although the regulatory mechanisms of the initiation of meiosis are not yet clear, it has been shown that in Japanese eel there is a regulatory stage around the fifth mitotic division of spermatogonia prior to the cells entering meiosis (Miura et al., 1997). To cross this regulatory stage, some factors regulated by 11-ketotestosterone may be required. The key genes coding factors that showed unique expression during spermatogenesis have been considered. To identify these key genes, we isolated cDNA clones of stage-specific genes during eel spermatogenesis using cDNA subtraction and differential display methods (Miura et al., 1998, 1999a). As a result of these experiments, 28 independent cDNA clones showing unique expression patterns during spermatogenesis were obtained (Table 1). As a matter of convenience, we named these clones “eel spermatogenesis related substances (eSRSs)” cDNA. Among these eSRSs, 16 clones are up- or down-regulated by 11-ketotestosterone, the spermatogenesis inducing hormone. The initiation of meiosis may be regulated by some of these factors.

4) Determining whether a control mechanism exists for spermiogenesis

After two meiotic divisions, the germ cells develop into spermatids having small, round, and heterogeneous nuclei. The spermatids transform into spermatozoa through spermiogenesis. This process is characterized by remarkable morphological changes associated with the formation of a sperm head with condensed nucleus, a mid-piece, and a flagellum. Eel spermatozoon has an unusual figure (Fig. 4). It possesses a crescent-shaped nucleus with a flagellum consisting of a 9+0 axonemal structure (generally, the axonemal structure of the flagellum is 9+2), and a single large spherical mitochondrion with developed tubular cristae, which are attached to the caput end at one side of the sperm head (Todd, 1976; Miura et al., 1991a).

In teleosts, it is not yet clear whether regulation mechanisms exist in spermriogenesiss. In medaka, it was possible to induce complete spermiogenesis without hormonal treatment in in vitro cultured germ cells (Saiki et al., 1997), indicating that there is no regulatory mechanism in medaka spermiogenesis. However, the figure of the Japanese eel spermatozoo produced by in vitro testicular organ culture and germ cell-Sertoli cell coculture with 11-ketotestosterone is not exactly similar to the fertilizable spermatozoo of eel produced by hCG injection in vivo. Based on this discrepancy, it seems conclusive that regulation mechanisms exist in the spermiogenesis of Japanese eel.

5) Induction of sperm maturation

In some species, once the spermatozoo in the testis have completed spermiogenesis, they are not yet capable of fertilizing eggs. In salmonids, the spermatozoo in the testis and in the sperm duct are immotile. If spermatozoo from the sperm duct are diluted with fresh water, they become motile; in contrast, the testicular spermatozoo remain immotile after dilution with fresh water. Thus, spermatozoo acquire the ability of motility during their passage through the sperm duct.

Sperm maturation, the phase during which non-functional gametes develop into mature spermatozoo (fully capable of vigorous motility and fertilization) involves only physiological, not morphological, changes. In salmonids, sperm maturation (the acquisition of sperm motility) has been induced by increasing the seminal plasma pH (approximately to pH 8.0) in the sperm duct, which results in elevation of intrasperm cAMP levels (Morisawa and Morisawa, 1988; Miura et al., 1992). Similar results have been reported for Japanese eel spermatozoo by Miura et al. (1995c) and Ohta et al. (1997).

Sperm maturation is also regulated by the endocrine sys-

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**Table 1.** List of cDNA clones obtained by gene expression screening and their expressional patterns.

<table>
<thead>
<tr>
<th>eSRS No.</th>
<th>homologous protein</th>
<th>size (kb)</th>
<th>up- or down-regulated during hCG induced spermatogenesis</th>
<th>up- or down-regulated by 11-KT stimulation in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>actin β</td>
<td>3.4</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>2</td>
<td>unknown</td>
<td>3.4</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>3</td>
<td>ZP2</td>
<td>1.4</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>4</td>
<td>ZP3</td>
<td>1.6</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>5</td>
<td>cathepsin S</td>
<td>0.85</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>6</td>
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<td>1.5</td>
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<td>–</td>
</tr>
<tr>
<td>7</td>
<td>unknown</td>
<td>1.3</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>8</td>
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<td>1.4</td>
<td>down</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>PCNA</td>
<td>1.6</td>
<td>up</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
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<td>0.5</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>11</td>
<td>histone H1</td>
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<td>up</td>
<td>up</td>
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<tr>
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<td>up</td>
<td>–</td>
</tr>
<tr>
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<td>unknown</td>
<td>–</td>
<td>up</td>
<td>–</td>
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<tr>
<td>14</td>
<td>unknown</td>
<td>–</td>
<td>up</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
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<td>up</td>
</tr>
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<td>16</td>
<td>HMG2</td>
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<td>up</td>
</tr>
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<td>histone H2A</td>
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<td>up</td>
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<td>tubulin α</td>
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</tr>
<tr>
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<td>up</td>
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<td>up</td>
<td>up</td>
</tr>
<tr>
<td>21</td>
<td>TGFβ family</td>
<td>2.4</td>
<td>down</td>
<td>down</td>
</tr>
<tr>
<td>22</td>
<td>carbonic anhydrase</td>
<td>1.5</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>23</td>
<td>thymidylate synthase</td>
<td>1.2</td>
<td>up</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>fatty acid binding protein</td>
<td>1.0</td>
<td>up</td>
<td>up</td>
</tr>
<tr>
<td>25</td>
<td>prothymosin α</td>
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<td>–</td>
</tr>
<tr>
<td>26</td>
<td>ictacalcin</td>
<td>0.8</td>
<td>up</td>
<td>–</td>
</tr>
<tr>
<td>27</td>
<td>cytochrome C</td>
<td>0.8</td>
<td>up</td>
<td>–</td>
</tr>
<tr>
<td>28</td>
<td>calnexin</td>
<td>2.3</td>
<td>up</td>
<td>–</td>
</tr>
</tbody>
</table>
tem. In some teleosts including Japanese eel, it is suggested that 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) is related to the regulation of sperm maturation (Miura et al., 1991d, e, 1992). 17α,20β-DP has also been identified as the maturation-inducing hormone of salmonid oocyte (Nagahama and Adachi, 1985). 17α,20β-DP does not act directly on the sperm; its action is mediated through an increase in the seminal plasma pH, which in turn increases the sperm content of cAMP, thereby allowing the acquisition of sperm motility (Miura et al., 1991d, 1992, 1995c). However, the mechanisms involved in the increase of the seminal plasma pH by 17α,20β-DP remain unclear. Recently, we attempted to elucidate these mechanisms. As mentioned above, although eSRS22 is one of the factors cloned by the testicular cDNA subtraction method, this factor is related to regulation of the increase in pH (in preparation). In the Japanese eel, eSRS22 is a homologue of carbonic anhydrase (CA). CA catalyzes the reversible hydration of carbon, and is involved in the regulation of ion and acid-base balance in various fluids and tissues (Carter, 1972; Maren, 1967). In Japanese eel, eSRS22/CA protein was expressed in the spermatids and spermatozoa membranes.

In some teleosts including eel, it is suggested that a progesterone receptor exists in the spermatozoon (Gosh and Thomas, 1995; Thomas et al., 1997). If eSRS22/CA is related to sperm maturation, its function seems to be correlated with 17α,20β-DP and eSRS22/CA activity. Therefore, the relationship between 17α,20β-DP and eSRS22/CA was examined using intratesticular sperm incubation in vitro. The pH value of the artificial seminal plasma (ASP) was increased by 17α,20β-DP treatment. Moreover, acetazoleamide, a specific inhibitor of CA or anti-eSRS22 specific antibody, suppressed the increase in pH value induced by 17α,20β-DP stimulation.

Fig. 4. Electron micrographs of eel spermatozoon. A, The whole image by scanning micrograph (Bar, 1 μm); B, sagittal section of spermatozoon (Bar, 1 μm); C, cross section of flagella (Bar, 0.1 μm); and D, cross sections of sperm heads. Arrowheads indicate the 9 sets of microtubules (Bar, 1 μm).
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These findings suggested the following possible mechanisms involved in sperm maturation. $17\alpha,20\beta$-DP acts directly on spermatozoa and induces the activation of eSRS22/CA; this enzymatic activation causes an increase in the seminal plasma pH, and spermatozoa subsequently acquire the motile ability. In masu salmon (*Oncorhynchus masou*), $17\alpha,20\beta$-DP stimulates the CA activity in spermatozoa, and causes an increase in the pH value of the ASP *in vitro*.

**Conclusion**

Fig. 5 illustrates the spermatogenetic cycle and its predicted regulatory mechanisms in the Japanese eel. By establishment of testicular organ culture and use of molecular biology techniques, analysis of the control mechanisms of eel spermatogenesis has advanced remarkably, and the eel system has proven to be an advantageous system for the study of spermatogenesis. Investigation of the Japanese eel has led to the discovery of several interesting aspects of spermatogenesis. It is highly possible that further investigations of eel spermatogenesis will lead to a better understanding of the general aspects of spermatogenesis.

Recently, environmental pollution by chemicals (collectively known as endocrine disrupters) has been shown to stimulate or block various biological processes (Colborn *et al*., 1993), and to interfere with the sensitive hormonal pathways that regulate the reproductive functions. Especially in male animals, exposure to estrogenic compounds (environmental estrogens or exestrogens) can lead to reduced gonad size, feminization of genetic males, and low sperm count and/or quality (Sharpe, 1993; Sumpter, 1995). Japanese eel will also provide an excellent system for analysis of the negative effects of environmental disrupters on spermatogenesis.

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