Gh is produced by the testis of Japanese eel and stimulates proliferation of spermatogonia

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Abstract

Gh plays important roles in development, somatic growth and gametogenesis in vertebrates. To determine the physiological role of Gh in reproduction in male teleosts, the expression of genes encoding Gh and the two Gh receptors (Ghrs) during spermatogenesis, and the action of Gh *in vitro* was examined using the Japanese eel (*Anguilla japonica*). *gh*, *ghr1* and *ghr2* mRNA transcripts were detected in all spermatogenic stages. *In situ* hybridization showed the presence of *ghr1* and *ghr2* mRNA in the germ cells. Immunohistochemistry using an antiserum against eel Gh indicated that Gh protein was localized to Sertoli cells surrounding the germ cells in early spermatogenesis. Recombinant eel Gh induced spermatogonial proliferation in a testis organ culture system, an effect that was independent from the production of steroid hormones or Igf1. This study identifies a role for eel Gh in the regulation of early spermatogenesis, particularly in the mitotic phase of spermatogenesis, that is not mediated by either steroid hormones or Igf1 production.

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Introduction

Gh is a pituitary hormone that has important physiological roles in the control of somatic growth, metabolism, and development in vertebrates (Björnsson 1997, Pérez-Sánchez 2000). The effects of Gh are primarily mediated by insulin-like growth factor 1 (Igf1), which is secreted from the liver and other tissues in response to Gh. In addition to these roles, Gh has been implicated, directly and indirectly, in the regulation of reproductive processes in several vertebrate taxa. In salmonid fishes, several studies have shown that growth rate at critical periods determines whether animals will undergo gonadal maturation and initiation of spermatogenesis or secondary ovarian follicle growth, an effect that may be mediated through plasma levels of Igf (Campbell *et al.* 2003, 2006). Gh and Igf1 have also been shown to act on the gonad to stimulate steroid production (Lubzens *et al.* 2010, Schulz *et al.* 2010) and Igf1 is involved in the regulation of final oocyte maturation (Lubzens *et al.* 2010). *igf1* mRNA is expressed in both teleost ovary and testis (Le Gac *et al.* 1996, Reinecke *et al.* 1997). However, *gh* gene is expressed in ovary and testis (Gomez *et al.* 1999, Li *et al.* 2005), not only Igf1 but also Gh promoted the incorporation of thymidine into sperm cells in cultured spermatogenetic testis (Loir &

Le Gac 1994, Loir 1999), raising the possibility that Gh may have paracrine actions on the regulation of gametogenesis in both sexes (Reinecke 2010). Gh receptor (Ghr) levels appear to be high at the onset of spermatogenesis and then decreased progressively suggesting that sensitivity of the testis to Gh changes during the reproductive cycle (Gomez *et al.* 1998).

Although Kishida *et al.* (1987) isolated Gh in the pituitary of Japanese eel (*Anguilla japonica*) and the cDNA of the Japanese eel Gh was cloned by Saito *et al.* (1988), the detailed actions of Gh in spermatogenesis are still unclear and the underlying physiological mechanisms are poorly understood. In this study, using the Japanese eel, we could assay the direct effects of Gh upon spermatogenesis. The male Japanese eel provides an excellent system for studying the mechanisms of spermatogenesis. Eel has a specific spermatogenetic pattern and under freshwater culture conditions the males of this species exhibit immature testes containing only non-proliferating type A and early type B spermatogonia. It has been reported, however, that injection with human chorionic gonadotropin (hCG) can induce all stages of spermatogenesis *in vivo* (Miura *et al.* 1991a). Furthermore, the Japanese eel is the only animal in which complete spermatogenesis has been induced by hormonal treatments using an *in vitro* organ culture

system (Miura *et al.* 1991b). In this study, using the eel system, *gh* and *ghr* gene expression in the testis and the action of Gh *in vitro* were examined to evaluate the biological function of Gh in spermatogenesis.

Results

Expression of *gh*, *ghr1* and *ghr2* mRNA during spermatogenesis

We used RT-PCR to examine the transcript levels of *gh*, *ghr1* and *ghr2* in developing testes. RT-PCR was performed by poly (A)⁺ RNA extracted from testes at 0, 1, 3, 6, 9, 12, 15, or 18 days after hCG treatment (Fig. 1). As a reference, samples were also analyzed for glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) mRNA. We found that *gh*, *ghr1* and *ghr2* mRNAs were present at all stages examined.

Localization of *ghr1* and *ghr2* mRNA in testis

To determine the distribution of *ghr1* and *ghr2* mRNA expressions in testes, we performed *in situ* hybridization using digoxigenin (DIG)-labeled sense and antisense RNA probes. The signals obtained using of *ghr1* and *ghr2* antisense probes were detected mainly in germ cells of testes (Fig. 2A and D). In contrast, the sense probe did not hybridize to any testis cells (Fig. 2B and E).

Expression and localization of Gh protein in testis

To evaluate the presence of Gh protein during spermatogenesis, immunoprecipitation, and western blot analysis was performed by the anti-Gh antibody. Immunoreactive Gh protein was detected as a band of 19 kDa in testis extracts between 0 and 9 days after hCG injection, when the testis is occupied mostly by

spermatogonia, while Gh protein was not detectable between 12 and 18 days after hCG injection (Fig. 3A). In pituitary extracts, a band of the same molecular weight (19 kDa) was detected. To determine which cell types express Gh protein in the testis, immunohistochemistry was performed. The anti-Gh antibody stained the Sertoli cells (Fig. 3B) surrounding types A and B spermatogonia, which just started mitosis (Fig. 3D). In contrast, the preimmune serum used as a negative control showed no immunopositive cells (Fig. 3C).

Effects of recombinant Gh on spermatogenesis *in vitro*

To investigate the action of Gh on spermatogenesis, testicular fragments were cultured with various concentrations (0, 1, 10, and 100 ng/ml) of recombinant Gh (r-Gh) and/or 10 ng/ml of 11-ketotestosterone (11-KT) for 15 and 30 days. Before the experiment, the bioactivity of the r-Gh was assessed by testing the transcript levels of *ghr1*, *ghr2*, and *igf1* in a cell culture system for eel liver (Hayashi & Kumaga 2008, Moussavi *et al.* 2009) in response to r-Gh. Strong bands for *ghr1*, *ghr2*, and *igf1* were detected in testis after culture with r-Gh (Supplementary Figure 1, see section on supplementary data given at the end of this article). Following the culture, we then examined the proliferation of spermatogonia by adding 5-bromo-2-deoxyuridine (BrdU; 1 µM/well) to the cultures to label replicating DNA (Fig. 4A–C), counting cells with red/dark-stained nuclei as BrdU-positive cells. Before culture, all germ cells in the eel testis were type A spermatogonia undergoing stem cell renewal. After 15 days of culture, the percent of germ cells that were BrdU positive after treatment with 1 or 10 ng/ml r-Gh did not differ significantly from control levels. However, significantly more positive cells were found after culture with 100 ng/ml r-Gh ($P < 0.05$). Notably, significantly more

Figure 1 A schematic summary of stages of spermatogenesis in Japanese eel and the transcript levels of *gh*, *ghr1* and *ghr2* mRNA in developing testes determined by RT-PCR. RT-PCR was performed by poly (A)⁺ RNA extracted from testes at 0, 1, 3, 6, 9, 12, 15, or 18 days after hCG treatment. For reference, samples were also analyzed for *gapdh*. Numbers at the right indicate DNA size markers in bp.

Figure 2 Cellular localization of *ghr1* and *ghr2* mRNA in testis identified by *in situ* hybridization. Sections were hybridized with antisense probes for *ghr1* (A) and *ghr2* (D). Sections hybridized with sense probes as negative controls (B and E). Sections were stained with hematoxylin/eosin (C and F). Scale bar: 10 μ m. Arrowheads indicate germ cells.

BrdU-positive cells were found after culture of testis with 10 ng/ml 11-KT, compared with 100 ng/ml r-Gh (Fig. 4D).

After 30 days of culture, similar to control (Fig. 5A), testicular fragments treated with 100 ng/ml of r-Gh contain many type A spermatogonia (Fig. 5C), which are primary gonial cells undergoing mitosis. The mitotic chromosomes (Fig. 5C insert) indicate metaphase or anaphase stage of spermatogonia. These fragments, however, were not occupied by proliferated type B spermatogonia awaiting entry into meiosis (Fig. 5C). Mainly type B spermatogonia were observed after treatment with 11-KT (Fig. 5B). In 11-KT and Gh-treated testicular fragments (Fig. 5D), many more type B spermatogonia were present after treatment with a combination of 11-KT and Gh compared with 11-KT alone (Fig. 5B and D).

In vitro steroid hormone production in eel testis in the presence of r-Gh

To investigate the effect of Gh on 11-KT, estradiol-17 β (E_2) and 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) production, testicular fragments were cultured with or without Gh for 24 h, and the concentration of each steroid hormone in testicular fragments was measured.

Although 11-KT production was induced by 1 U/ml of hCG, the positive control, Gh had no significant effect on 11-KT production (Fig. 6A). E_2 and DHP production was not affected by either r-Gh or hCG (Fig. 6B and C).

Effects of trilostane on the stimulation of spermatogonial proliferation by r-Gh, 11-KT, or hCG

To further investigate whether the effect of Gh on spermatogonial proliferation is mediated via steroid production, eel testes were cultured with the steroidogenesis inhibitor trilostane (0, 0.1, 1, and 10 μ g/ml), and/or hCG (0.05 IU/ml), 11-KT (10 ng/ml), or r-Gh (100 ng/ml) for 6 days (Fig. 7). Treatment with r-Gh, hCG, or 11-KT alone increased the percentage of BrdU-positive cells. However, the percentage of BrdU-positive cells after treatment with hCG was significantly decreased in the presence of 1 and 10 μ g/ml trilostane. By contrast, trilostane in the presence of 11-KT or Gh did not significantly alter the number of BrdU-positive cells compared with 11-KT or Gh alone.

Effect of Gh or 11-KT on levels of *igf1* in testis

RT-PCR analysis was used to examine the effects of culture of testicular fragments with 100 ng/ml of r-Gh

Figure 3 Expression and localization of Gh protein in testis using an anti-Gh antibody. (A) Immunoprecipitation, non-reducing SDS-PAGE, and western blot analysis of Gh protein expression during hCG-induced spermatogenesis. Samples were obtained from the testis at 0, 1, 3, 6, 9, 12, 15, and 18 days after hCG treatment and pituitary (P). Numbers at the left represent molecular size marker (kDa). (B) Testis with hCG injected after 6 days immunostained with anti-Gh antibody. Arrowheads indicate Sertoli cells. (C) The immunohistological sections stained with preimmune serum. (D) Testis section stained with hematoxylin and eosin. Arrows indicate type A spermatogonia. Scale bar: 10 μ m.

Figure 4 Effects of r-Gh on spermatogenesis *in vitro*. (A–C) Micrographs showing sections from testicular fragments cultured after 15 days in basal medium alone (A), with 11-KT (B), and with r-Gh (C). The cells with red/brown-stained nuclei are BrdU-positive cells. GA, type A spermatogonia; GB, late type B spermatogonia. Scale bar: 10 µm. (D) BrdU labeling index. The number of positively immunoreactive germ cells is expressed as a percentage of the total number of germ cells. Results are given as mean $\pm$ S.E.M. Values with different lowercase letter(s) are significantly different ($P < 0.05$).

alone, 10 ng/ml 11-KT alone, or co-treatment for 6 days on *igf1* mRNA levels. A control sample taken before the culture was also analyzed. In control and treatment of r-Gh and/or 11-KT, we could detect the levels of *igf1* mRNA (Fig. 8).

Discussion

Our results demonstrate that mRNAs encoding *gh* and *ghr1* and *ghr2* are present during spermatogenesis of Japanese eel, with *ghr1* and *ghr2* mRNA detectable in the germ cells by *in situ* hybridization. Previous work on rainbow trout has shown that *ghr* mRNA was expressed in the testis during the entire reproductive cycle, probably in somatic cells and in particular in Sertoli cells (Gomez *et al.* 1998). That study also demonstrated that pituitary Gh content gradually increased during gametogenesis and more abruptly in males during spermiation. In the chicken (Harvey *et al.* 2004), the presence of *GH* and *GHR* mRNA in the male reproductive system has been reported, with *GH* mRNA present in spermatogonia and primary spermatocytes of the seminiferous tubules but absent from secondary spermatocytes, spermatids, or spermatozoa. *GH* RNA was also not detectable in any somatic cells (smooth muscle fibers of the tubules, Sertoli cells, and interstitial cells) (Harvey *et al.* 2004). In eel, our results showed the presence of *ghr1* and *ghr2* mRNA in germ cells. Since Gh may have important roles in spermatogenesis, we investigated the presence of Gh protein in the testis during spermatogenesis using an anti-Gh

antibody. As in previous studies (Miura *et al.* 1991a), hCG-induced eel spermatogenesis, resulting in spermatogonial proliferation at 3 days after injection and meiosis by 12 days. Western blot analysis demonstrated that testicular Gh protein was present in the testis of spermatogonia stage (until 9 days post-injection of hCG), but was not detectable in the testis of spermatocytes stage and more advanced-stage germ cells. These results provided evidence that the testicular Gh is expressed in early spermatogenesis, and may have paracrine actions. The cellular site of expression of the Gh protein, analyzed through immunohistochemistry, was in the Sertoli cells surrounding the germ cells.

For determining potential actions of Gh on spermatogenesis, the eel is an ideal model because the mechanisms involved in eel spermatogenesis have been investigated in detail (Miura & Miura 2001). Moreover, the eel testicular culture system provides an opportunity to examine the direct action of Gh on spermatogenesis. We produced r-Gh, and determined its bioactivity before the experiment by showing that it induced *igf1*, *ghr1*, and *ghr2* mRNA levels in an eel liver cell culture system as reported previously (Hayashi & Kumaga 2008, Moussavi *et al.* 2009). Treatment of eel testicular fragments with r-Gh *in vitro* induced spermatogonial proliferation. The results strongly suggest that Gh participates in the regulation of early spermatogenesis in Japanese eel.

The presence of Igf1 specific binding sites in testicular germ cells has been reported in rainbow trout, and Igfs stimulated DNA synthesis in germ cells, apparently by interacting directly with these cells via a single Igf receptor (Loir & Le Gac 1994). Loir (1999) also demonstrated the potency of Gth1 (Fsh), Gh, Igf1I, and

Figure 5 Effects of r-Gh on spermatogenesis *in vitro*. (A–D) Micrographs showing sections from testicular fragments cultured for 30 days in basal medium alone (A), with 11-KT (B), r-Gh (C), and 11-KT and r-Gh (D). Insert (C) indicates spermatogonia with mitotic chromosomes (arrows). GA, type A spermatogonia; GB, late type B spermatogonia. Scale bar: 10 µm.

Figure 6 Effects of r-Gh on 11-KT (A), E₂ (B), and 17,20β-DHP (C) production by eel testicular fragments. Results are given as mean $\pm$ S.E.M.; $n=5$ per treatment; * $P<0.05$.

fibroblast growth factor-2 in stimulating the proliferation of spermatogonia present in primary cultures of total testicular cells prepared from trout testes at different stages of testis maturation.

In this study, long-term *in vitro* treatment with r-Gh alone induced mitosis of type A spermatogonia only. By contrast, treatment with 11-KT for 30 days induced the appearance of many type B spermatogonia, and the combination of 11-KT and Gh resulted in more type B spermatogonia compared with treatment with 11-KT alone. Thus, we conclude that a major action of Gh during early spermatogenesis in eel testis is the stimulation of proliferation of type A spermatogonia. The increase in the number of type B spermatogonia after co-treatment with r-Gh and 11-KT, compared with results with 11-KT alone, is most likely due to increased number of type A spermatogonia available for development into type B spermatogonia.

The potential mediation of the action of Gh on the testis via sex steroids was investigated using two approaches, examining if Gh stimulated 11-KT, E₂ or 17,20β-DHP production, and determining if inhibition of steroid production affected the action of Gh on spermatogonial proliferation. Although 11-KT production by testis fragments cultured for 24 h was induced by 1 U/ml of hCG, r-Gh did not affect 11-KT production, or production of E₂ or 17,20β-DHP. Using trilostane, a specific inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, we investigated whether Gh acts on spermatogenesis via the production and secretion of steroid hormone in testicular organ culture. Trilostane did not affect the action of r-Gh on stimulation of spermatogonial proliferation, determined by counting BrdU-labeled cells. Together, these results establish that the proliferative action of Gh on type A spermatogonia in the eel testis is independent of mediation via steroid hormones.

Many actions of systemic Gh are mediated through its action in increasing Igf1 synthesis, with the liver being the main source of circulating Igf1 (Peter & Marchant 1995, Ohlsson *et al.* 2009). However, among the teleosts, there is evidence for gonadal expression of *igf1* genes (Perrot *et al.* 2000, Fukada *et al.* 2003), raising the possibility that the identified effect of r-Gh on eel testis could be due to the local production of Igf1. The lack of effect of r-Gh on levels of testis *igf1* mRNA suggests the possibility of Igf1 does not mediate the action of Gh on spermatogonial proliferation. Thus, Gh, acting through Ghr1 and Ghr2 may not require a secondary hormonal mediator.

Figure 7 Effects of the steroidogenesis inhibitor trilostane on hCG, 11-KT, and r-Gh induced spermatogenesis in Japanese eel *in vitro*. Eel testes were cultured with trilostane (0, 0.1, 1, and 10 μ g/ml), and/or hCG (0.05 IU/ml), 11-KT (10 ng/ml), or r-Gh (100 ng/ml) for 6 days. The percentages of late type B spermatogonia per total number of germ cells are compared among the different treatments. Results are given as mean $\pm$ S.E.M.; $n=5$ per treatment; * $P<0.05$.

Figure 8 Testis *igf1* gene expression after treatment with r-Gh. Testicular fragments were cultured with r-Gh and/or 11-KT for 6 days. Control samples were cultured without treatment. Numbers at the right indicate DNA size markers in bp. For reference, samples were also analyzed for *gapdh*.

In summary, the result of these studies lead to a new hypothesis of action of Gh on germ cells, wherein Gh is produced by the Sertoli cells and interacts with Ghr on germ cells to stimulate renewal proliferation of type A spermatogonia. This action of Gh is independent of steroid hormone production. Our results thus suggest that Gh, produced by Sertoli cells, plays an important paracrine role in spermatogenesis, promoting the mitotic phase of eel spermatogenesis.

Materials and Methods

Ethics statement

All experiments were conducted in strict accordance with the institutional animal ethics guidelines of Ehime University and were approved by the Animal Experimental Committee of Ehime University (Protocol Number 128, Permit Number H19-001).

Animals

Cultured male Japanese eels (180–200 g in body weight) were purchased from a commercial eel supplier and kept in a freshwater tank at 23 °C until use. A single injection of hCG dissolved in saline (150 mM NaCl) was administered intramuscularly to the eels at a dose of 5 IU/g body weight. Seven to ten eels per group were either killed immediately or 1, 3, 6, 9, 12, 15, and 18 days after hCG injection, and testes were collected for the extraction of poly (A)⁺ RNA, *in situ* hybridization, immunohistochemistry, and western blotting.

RT-PCR

Poly (A)⁺ RNA was extracted from 1 g of eel testes after hCG injection by the acid guanidium isothiocyanate–phenol–chloroform extraction method by Sepasol-RNA 1 super (Nacalai Tesque, Kyoto, Japan). After DNase I treatment, 0.5 µg of poly (A)⁺ RNA was transcribed by Superscript II (Invitrogen) using an oligo(dT) primer. The cDNA was amplified by PCR with *gh* primers (sense primer: 5'-TTACTCC-GACTCCATCCCTA-3'; antisense primer: 5'-CAGGG-TGCAGTTGCTTCTA-3'), eel *ghr1* primers (sense primer: 5'-ATCCTGGTCTACATCCAGCCCAT-3'; antisense primer: 5'-TCGTCCTTGAAGCCCAGGAA-3'), eel *ghr2* primers (sense primer: 5'-CAGTACCGCGAGAAGACTAG-3'; antisense primer: 5'-GTCCCTGAGGGAGGGGTCATAAGGAGT-3'), eel

igf1 (sense primer: 5'-AGCCTTGCCAGGTTTCACTG-3'; antisense primer: 5'-AAACCCACAGGCTATGGATC-3'); and eel *gapdh* primers (sense primer: 5'-GCCATCAACGACCCGTT-CATCG-3'; antisense primer: 5'-GTGCAGGACGGGTTGCT-GAC-3'). The *gh* and *igf1* PCR cycling parameters were as follows: 23 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min. The *ghr1* and *ghr2* PCR cycling parameters were as follows: 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. The GAPDH PCR cycling parameters were as follows: 25 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 2 min. The PCR products were separated on a 2% agarose gel, which was then stained with ethidium bromide.

In situ hybridization

For *in situ* hybridization, a non-radioactive method by a DIG-labeled RNA probe was employed. A 405 bp cDNA fragment of eel *ghr1* (GenBank accession number AB180476, nucleotides 896–1301) and a 426 bp cDNA fragment of eel *ghr2* (GenBank accession number AB180477, nucleotides 802–1227) were subcloned into pBluescript II KS-. Sense and antisense RNA probes were transcribed *in vitro* using DIG-RNA labeling Mix (Roche) and T7 and Sp6 RNA polymerase (Promega). Testicular fragments of each five eels were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 4 °C for 18 h, embedded in paraffin wax and cut into 8 µm serial sections. *In situ* hybridization was performed with a Microprobe Staining System (Fisher Scientific, Tokyo, Japan) according to the manufacturer's protocol. The sections were incubated in hybridization mixture of 0.2 µg/µl tRNA, 10 mM Tris–HCl (pH 7.4), 1×Denhardt's solution, 10% Dextran sulfate, 600 mM NaCl, 50% formamide, 0.25% SDS, and 2 µg/ml probe. After hybridization at 50 °C for 12 h, the sections were washed with 5×SSC and 2×SSC twice. Hybridized DIG-labeled probes were immunologically detected with anti-DIG-alkaline phosphatase antibody, diluted titer of 1:2000 (Boehringer Mannheim) according to the manufacturer's instructions. The sections were incubated with the Fab fragment of an anti-DIG-alkaline phosphatase-conjugated antibody (Boehringer Mannheim). After the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate color reaction, the slides were rinsed with AP chromogen buffer (*In Situ* Hybridization DAB/Fast Red kit; Falma Co., Ltd, Tokyo, Japan).

Production of r-Gh

R-Gh was prepared by a method reported previously (Ozaki *et al.* 2006) with minor modifications. Gh was subcloned into pQE vector (Qiagen). The recombinant pQE-Gh was expressed in *Escherichia coli* following induction by isopropyl β-D-thiogalactopyranoside and purified from the bacterial lysate by affinity chromatography using the metal chelate adsorbent nickel-nitrilotriacetic acid agarose (Qiagen). The fusion protein was eluted with elution buffer (8 M urea, 0.1 M sodium phosphate, 10 mM Tris, pH 4.0) and refolded according to the previously reported method (Sugimoto *et al.* 1991, Paduel *et al.* 1999, Sandowski *et al.* 2000). R-Gh was assayed by western blot and a specific band of 24 kDa was detected as reported previously by Ozaki *et al.* (2006).

Production of a polyclonal antibody against recombinant eel Gh

Female rabbits were immunized with 1 ml PBS containing 1 mg of the purified recombinant protein mixed with an equal volume of Freund's complete adjuvant. The rabbits received four immunizations at 2-week intervals by s.c. injection. Sera were collected after the fourth injection. In western blot analysis the diluted antiserum (1/1000) strongly reacted with purified antigen. The IgG of normal rabbit serum and of immunized rabbit serum was purified as follows. Pooled serum (15 ml) was mixed with the same volume of 80% saturated ammonium sulfate (SAS) in 0.01 M PBS (pH 7.0) (40% SAS-serum). The precipitate was then collected and dissolved in 2 ml PBS. Ion exchange chromatography was carried out on DEAE-cellulose (DE52, Whatman, Kent, UK) column (11.5 cm; bed volume ~10 ml) equilibrated with 0.0175 M PB (pH 6.8). The 40% SAS-serum was dialyzed against the starting buffer and loaded onto the column. The IgG was eluted with 0.0175 M PB and the elutants were collected. The absorbance of the fractions was measured at 280 nm.

Immunoprecipitation and western blot analysis

Fresh testicular samples from five fish per group of 1, 3, 6, 9, 12, 15, and 18 days after hCG injection and pituitary samples were homogenized with an equal volume of 0.25 M sucrose containing 10 mg/ml leupeptin and 100 mM phenylmethanesulfonyl fluoride. After centrifugation at 9000 *g* for 10 min, the supernatant was incubated with 10 volumes of immunoprecipitation buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.5) containing the anti-Gh antibody. After overnight incubation at 4 °C, the immunocomplex was absorbed with protein A-Sepharose (Pharmacia) for 1 h at 37 °C. The beads were washed with TTBS by centrifugation at 1000 *g* for 10 min and treated with fivefold more concentrated non-reducing SDS sample buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, and 0.05% (w/v) bromophenol blue). The mixture was heated in a boiling water bath for 5 min, centrifuged at 9000 *g* for 5 min, and then the supernatant of the tissue extracts was collected. Proteins were separated by 12.5% acrylamide gels and blotted onto PVDF membranes, which were immunostained with the anti-Gh antibody.

Immunohistochemistry

Eel testicular fragments were fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.2) at 4 °C for 18 h, embedded in paraffin wax, and cut into 5 µm serial sections. The sections were deparaffinized in xylene and hydrated in a graded ethanol series. Immunohistochemical analysis was performed by an ABC-AP-alkaline phosphatase substrate kit I (Red) (Vector Laboratories, Burlingame, CA, USA), and sections were counterstained with Delafield's hematoxylin.

In vitro testicular organ cultures

Testicular organ culture techniques were carried out following Miura *et al.* (1991a, 1991b) with minor modifications. Testes from five eels were used in *in vitro* experiments representing

five replicates per treatment. Freshly removed testes were cut into 1 × 1 × 0.5 mm pieces and placed on 1.5% (w/v) agarose (Agarose S; Wako, Inc., Osaka, Japan) cylinders covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. Testicular fragments (*n*=2) from each fish were randomly assigned to each control and treatment group and then cultured for 15 or 30 days with or without 0.01–1 mM r-Gh, in combination with or without 10 ng/ml 11-KT.

Detection of proliferating germ cells

To detect proliferation, germ cells were labeled with BrdU according to the manufacturer's instructions (Amersham Bioscience). In brief, 1 µM BrdU was added to the culture well 18 h before the termination of culture. After culture, samples were fixed in Bouin's solution and embedded in paraffin wax and sections (5 µm) were immunohistochemically stained with anti-BrdU antibody, diluted titer of 1:1000, and then counterstained with Delafield's hematoxylin. The number of immunolabeled germ cells was expressed as a percentage of total number of germ cells (BrdU index).

Production of 17,20β-DHP, 11-KT, and E₂ by testis incubated with r-Gh

For each incubation, 20 mg of eel testicular fragments were transferred into 500 µl of eel Ringer's solution contained within each tissue culture plate well. Testes from five eels were used in *in vitro* experiments representing five replicates per treatment. The fragments were incubated with r-Gh (10, 100, and 1000 ng/ml) or with hCG (0.01, 0.1, and 1 IU/ml) as positive control. After incubation for 24 h at 20 °C, concentrations of 11-KT and 17,20β-DHP in the Ringer's solution were measured using time-resolved fluoroimmunoassay according to Yamada *et al.* (1997). E₂ production was determined using ELISA (Asahina *et al.* 1995, Rahman *et al.* 2000).

Effects of trilostane on the action of r-Gh and hCG on eel spermatogenesis

To investigate whether Gh acts directly on spermatogenic processes or via the stimulation of steroid hormone production, five replicates testicular fragments from five eels were cultured with or without various concentrations (0.1, 1, and 10 mg/ml) of either trilostane, a steroid hormone synthesis inhibitor that inhibits 3β-HSD, r-Gh (100 ng/ml), hCG (0.05 IU/ml), or 11-KT (10 ng/ml) for 15 days. The medium was changed on day 7. After culture, media were retained for steroid assay, and testicular fragments were fixed in Bouin's solution for histological examination.

Statistical analysis

All values presented are expressed as mean ± S.E.M. Data analysis was carried out by the Scheirer, Ray, and Hare extension of the Kruskal–Wallis test (a two-way ANOVA design for ranked data), followed by *post hoc* Bonferroni adjustment. A probability of *P* < 0.05 was considered statistically significant.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0203>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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