

## Spermatogenesis-preventing substance in Japanese eel

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### SUMMARY

Under fresh-water cultivation conditions, spermatogenesis in the Japanese eel is arrested at an immature stage before initiation of spermatogonial proliferation. A single injection of human chorionic gonadotropin can, however, induce complete spermatogenesis, which suggests that spermatogenesis-preventing substances may be present in eel testis. To determine whether such substances exist, we have applied a subtractive hybridisation method to identify genes whose expression is suppressed after human chorionic gonadotropin treatment *in vivo*. We found one previously unidentified cDNA clone that was downregulated by human chorionic gonadotropin, and named it 'eel spermatogenesis related substances 21' (eSRS21). A homology search showed that eSRS21 shares amino acid sequence similarity with mammalian and chicken Müllerian-inhibiting substance. eSRS21 was expressed in Sertoli cells of immature testes, but disappeared after human chorionic gonadotropin injection.

Expression of *eSRS21* mRNA was also suppressed *in vitro* by 11-ketotestosterone, a spermatogenesis-inducing steroid in eel. To examine the function of eSRS21 in spermatogenesis, recombinant eSRS21 produced by a CHO cell expression system was added to a testicular organ culture system. Spermatogonial proliferation induced by 11-ketotestosterone *in vitro* was suppressed by recombinant eSRS21. Furthermore, addition of a specific anti-eSRS21 antibody induced spermatogonial proliferation in a germ cell/somatic cell co-culture system. We conclude that eSRS21 prevents the initiation of spermatogenesis and, therefore, suppression of eSRS21 expression is necessary to initiate spermatogenesis. In other words, eSRS21 is a spermatogenesis-preventing substance.

Key words: Spermatogenesis-preventing substance, Müllerian inhibiting substance, Gene expression screening, *In vitro* spermatogenesis, Eel

### INTRODUCTION

Spermatogenesis, the formation of sperm, is a complex developmental process that begins with the mitotic proliferation of spermatogonia and proceeds through extensive morphological changes that convert the haploid spermatid into a mature, functional spermatozoon. Although the process of spermatogenesis is the same in both mammalian and nonmammalian vertebrates, its control mechanisms are not well understood. In higher vertebrates, it is difficult to analyse the control mechanisms of spermatogenesis because the seminiferous tubules contain several successive generations of germ cells (Clermont, 1972), and few culture systems are available for induction of spermatogenesis *in vitro* (Abe, 1987).

Various reproductive styles and gametogenetic patterns exist among species of teleost. Japanese eel, for example, has a specific spermatogenetic pattern. Under fresh-water culture conditions, the male Japanese eel has immature testes containing only non-proliferated type A and early type B spermatogonia (Miura et al., 1991a). It has been reported, however, that human chorionic gonadotropin (hCG) injection can induce all stages of spermatogenesis *in vivo* (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 and a germ cell/somatic cell co-culture system (Miura et al., 1991b; Miura et al., 1991c; Miura et al., 1996). Thus, the eel testis provides an excellent system for studying the regulation of spermatogenesis.

Using these *in vivo* and *in vitro* systems in the eel, the following control mechanisms of spermatogenesis have been identified. The hormonal induction of spermatogenesis in eel testes is mediated via gonadotropin stimulation of Leydig cells, which produce 11-ketotestosterone (Miura et al., 1991a; Miura et al., 1991b). In turn, 11-ketotestosterone induces complete spermatogenesis from spermatogonia to spermatozoa through the action of Sertoli cells (Miura et al., 1991c). However, many details of this pathway remain unresolved. In particular, it seems that there are complex control mechanisms related to the initiation of spermatogenesis after 11-ketotestosterone stimulation.

It seems likely that key factors involved in the initiation of spermatogenesis are either expressed or suppressed by 11-ketotestosterone during hCG-induced spermatogenesis. Specifically, suppressive factors might have an inhibitory effect

on the progress of spermatogenesis in cultivated eel. In this paper, we describe the isolation of a cDNA that is downregulated by hCG stimulation and investigate its function in initiating spermatogenesis.

## MATERIALS AND METHODS

### Animals

Cultured male Japanese eels (1 year old; 180–200 g in body weight) were purchased from a commercial eel supplier.

Male eels were kept in 500 l circulating fresh-water tanks at 23°C. A single injection of human chorionic gonadotropin (hCG) dissolved in saline (150 mM NaCl) was administered to the eels intramuscularly at a dose of 5 IU per body weight. Fish were either sacrificed immediately or at 1, 3, 6, 9, 12, 15 and 18 days after hCG injection, and testes were collected for the extraction of poly(A)<sup>+</sup> RNA, in situ hybridisation, immunohistochemistry and western blotting. Poly(A)<sup>+</sup> RNA was extracted using a Fast Track kit (Invitrogen).

### Construction of a subtractive cDNA library

Subtractive cDNA libraries were constructed using poly(A)<sup>+</sup> RNA extracted from the testes of hCG-treated (3 days after hCG injection) (+) and untreated (–) eels. First strand of cDNA was synthesised with reverse transcriptase from 5 µg of each poly(A)<sup>+</sup> RNA using an oligo (dT) primer. The second strand of cDNA was synthesised with RNase H and *E. coli* DNA polymerase and repaired with bacteriophage T4 polynucleotide kinase and *E. coli* DNA ligase. After digestion with *Mbo*I, these cDNA fragments were subtracted from each other by a representational difference analysis (RDA) technique (Niwa et al., 1997). After three cycles of subtractive enrichment, enriched upregulated (+) and downregulated (–) cDNA fragments were packaged into *λ*zap II phage particles.

We predicted that the subtracted cDNA libraries would not include full-length cDNA clones, owing to digestion of the cDNA fragments by *Mbo*I. To obtain full-length cDNA clones, therefore, we constructed conventional cDNA libraries, using the same poly(A)<sup>+</sup> RNA as used for cDNA subtraction, that contained full-length cDNA clones. Non-digested cDNA was size-fractionated on a Sepharose CL4B column with a lower cutoff value of 500 bp and inserted into *λ*zap II arms after the addition of *Eco*RI adapters.

### Screening and cloning

Enriched (+) and (–) cDNAs were labelled using the Klenow fragment of *E. coli* DNA polymerase I and linker primer with [ $\alpha$ -<sup>32</sup>P]dCTP. About 10<sup>4</sup> plaques from each subtractive library were blotted onto two nylon membranes (High Bond N<sup>+</sup>; Amersham). To duplicate copies of the library, the radiolabelled (+) and (–) cDNA probes were hybridised separately for 18 hours at 65°C in hybridisation solution [5×Denhart's solution supplement with 6×standard sodium citrate (SSC), 0.1% sodium dodecylsulfate (SDS) and 100 µg/ml denatured, fragmented herring sperm DNA]. The clones that hybridised to (–) cDNA probe only were collected and purified, and plasmids were excision-rescued according to the manufacturer's instructions (Stratagene).

These partial cDNA fragments were labelled using the Random Primer Plus Extension Kit (NEN) with [ $\alpha$ -<sup>32</sup>P]dCTP. About 10<sup>5</sup> plaques from intact cDNA libraries were blotted onto nylon membranes and hybridised with 1×10<sup>7</sup> cpm of the probes for 18 hours at 65°C. These membranes were washed twice with 1×SSC/1% (w/v) SDS at 65°C for 1 hour.

The cDNA inserts from positively hybridised clones were collected and purified, and plasmids were excision-rescued as above. Sequence determination was performed on a ABI PRISM 310 DNA sequencer using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). Sequence analysis and comparison were

carried out using DNASIS software (Hitachi). The similarity search of the deduced amino acid sequence of the obtained cDNAs was done using the 'Fasta Sequence Similarity Search' protein query (Genome Net).

### Northern blot analysis

Poly(A)<sup>+</sup> RNA (1 µg) was electrophoresed on a 1% (w/v) agarose denatured gel and blotted onto nylon membranes. The membrane was washed briefly and then baked at 80°C for 1 hour. RNA integrity, as well as uniformity of loading and transfer, was monitored by Methylene Blue staining (Herrin and Schmidt, 1988) and hybridisation to an eel elongation factor I cDNA. The cDNA fragments used as probes were labelled by the Random Primer Plus Extension labelling kit (NEN) with [ $\alpha$ -<sup>32</sup>P]dCTP. The membrane containing poly(A)<sup>+</sup> RNA was hybridised with 1×10<sup>6</sup> cpm of the radiolabelled probe in hybridisation solution at 65°C for 18 hours, and was then washed with 1×SSC/0.01% SDS solution at 65°C for 1 hour. The membrane was exposed to an imaging plate for 24 hours and analysed using an image analyser (Fuji Photo Film, Japan).

### In situ hybridisation

For in situ hybridisation, a non-radioactive method using a digoxigenin (DIG)-labelled RNA probe was employed. A 296 bp cDNA of *esrs21-0392* (nucleotides 147–443 in *esrs21c19*) was subcloned into pBluescript II KS<sup>–</sup>. Sense and antisense RNA probes were transcribed in vitro using digoxigenin-labelled UTP (Boehringer Mannheim) and T3 or T7 RNA polymerase (Gibco BRL). The testicular fragments from hCG-untreated eel were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2) at 4°C for 18 hours, embedded in paraffin wax and cut into 5 µm serial sections. Deparaffinized sections were treated with proteinase K and acetylated. The sections were incubated with a hybridisation mixture of 200 µg/ml tRNA, 10 mM Tris-HCl (pH 7.4), 1×Denhardt's solution, 600 mM NaCl, 50% formamide, 0.25% SDS and 500 ng/ml probe. After hybridisation at 50°C for 18 hours, the sections were processed as follows: (1) 2×SSC/50% formamide at 50°C for 30 minutes; (2) 2×SSC at 50°C for 30 minutes; (3) RNase A treatment (5 µg/ml) at 45°C for 30 minutes; (4) 2×SSC at 50°C for 20 minutes twice; and (5) 0.2×SSC at 50°C for 20 minutes twice. Hybridised DIG-labelled probes were immunologically detected with a Nucleic Acid Detection kit (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 colour reaction, the slides were rinsed with 10 mM Tris-HCl (pH 8.0) containing 1 mM ethylenediamine tetraacetic acid (EDTA) and then stained nuclei by 0.2% Methyl Green, metachromatically. After counterstaining, the slides were mounted with cover glass.

### Production of polyclonal antibody

A PCR product encoding amino acids 1–243 of eSRS21 was inserted in the *Eco*RI site of the pGEX-2TK vector (Pharmacia), in frame with the *glutathione S-transferase* gene. Host bacteria carrying the recombinant constructs were grown at 37°C until log phase and induced with 1 mM IPTG to express the fusion proteins. After a 4 hour induction period, bacteria were harvested by BugBuster protein extraction reagent (Novagen). Recombinant protein purification was performed using glutathione sepharose 4B according to the manufacturer's instructions (Pharmacia). Female rabbits were immunised with 1 ml of PBS, 0.1% SDS (pH 7.5) containing 1 mg of the purified recombinant protein mixed with an equal volume of Freund's complete adjuvant. The rabbits received four immunisations at 2 week intervals by subcutaneous injection. Sera were collected after the fourth injection. The diluted antiserum (1/1,000) strongly reacted with purified antigen on Western blot analysis.

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 sulphate (SAS) in 0.01 M phosphate buffered saline (PBS) (pH 7.0) (40% SAS-serum). The precipitate was then collected and dissolved in 2 ml of PBS. Ion-exchange chromatography was carried out on DEAE-cellulose (DE52, Whatman) column (1×15 cm; bed volume approximately 10 ml) equilibrated with 0.0175 M PB (pH 6.8). The 40% SAS-serum was dialysed against the starting buffer and loaded onto the column. The IgG was eluted by 0.0175 M PB, the elutants were collected and the absorbance of the fractions was measured at 280 nm.

### SDS PAGE and western blot analysis

Fresh testicular samples were homogenised with physiological saline solution for eel (Miura et al., 1991a) at 4°C. The testicular homogenate was mixed with an equal volume of nonreducing (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 0.05% (w/v) Bromophenol Blue) or reducing sample buffer [nonreducing sample buffer with 10% (v/v) 2-mercaptoethanol]. The mixture was heated in a boiling water bath for 5 minutes, centrifuged at 9000 *g* for 5 minutes, and then the supernatant of the tissue extracts was collected. Samples were separated using SDS-PAGE on 10% gels. The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). After blotting, the membranes were incubated and shaken for 30 minutes in 5% skimmed milk in 20 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl (TBS) to block nonspecific binding sites. The blocked membranes were immersed overnight in 5% skimmed milk containing the primary antibody against eSRS21 diluted to 1:1000. After washing twice with TBS containing 0.025% Tween 20 (TTBS) and then with TBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (BioRad) diluted to 1:1000 in TBS for 2 hours at room temperature. After washing, HRP activity was visualised using a freshly prepared solution of 0.06% 4-chloro-1-naphthol in TBS containing 0.06% H<sub>2</sub>O<sub>2</sub>. To demonstrate the specificity of eSRS 21 antibody, the same procedures of Western blot analysis were carried out without primary antibody as a negative control.

### Immunoprecipitation

Testes (100 mg) from intact eels were homogenised with an equal volume of 0.25 M sucrose containing 10 µg/ml leupeptin and 100 mM phenylmethanesulphonyl fluoride. After centrifugation at 9000 *g* for 10 minutes, 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 50 µl of anti-eSRS21 antibody. After overnight incubation at 4°C, the immunocomplex was absorbed with 50 µl of protein A-Sepharose (Pharmacia) for 1 hour at 37°C. The beads were washed with TTBS by centrifugation at 1000 *g* for 10 minutes and treated with fivefold more concentrated SDS sample buffer. As a negative control, the same procedure was carried out without testicular samples. Proteins were separated by 10% acrylamide gels and blotted onto PVDF membranes, which were immunostained with an anti-eSRS21 antibody.

### Production of recombinant eSRS21

Recombinant eSRS21 using CHO *DHFR*<sup>-</sup> cells was produced by using the methods of Murata et al. (Murata et al., 1988). The eSRS21 cDNA was inserted into *pSD(X)* containing *pSV2dhfr* (Murata et al., 1988) in the correct orientation between the *SV40* early promoter and the poly(A)<sup>+</sup> signal. We designated this expression vector as *pSD(X)/esrs21c19*. *pSD(X)/esrs21c19* (10 µg) was transfected into CHO *DHFR*<sup>-</sup> cells by electroporation, according to the electroporation manual (BioRad). Transformants were selected after 2 weeks of growth at 37°C in selection medium without ribonucleotide and deoxyribonucleotides (MEM ALPHA medium, Gibco). Initially, transformants were grown in the selection medium with 100 nM methotrexate (MTX). The medium was then changed every 3 days until MTX-resistant transformants appeared, and the concentration of

MTX in the medium was gradually increased to 10 µM. Four days before collecting the conditioned medium, the culture medium was replaced without MTX and FCS. The same procedure was carried out using *pSD(X)* vector alone. The CHO cells were transfected with *pSD(X)/eSRS21*, and the conditioned medium was assayed by western blot analysis to check production and secretion of the recombinant protein.

Conditioned medium (80 ml) containing eSRS21 recombinant protein was concentrated by ammonium sulphate precipitation at 4°C. The precipitate was redissolved in 3 ml of distilled water and dialysed with distilled water. The dialysed protein concentrate was fractionated by ion exchange chromatography on a CM Sepharose<sup>TM</sup> Fast Flow column (Pharmacia) using a stepwise gradient of 0 to 1 M sodium chloride. The fraction containing eSRS21 was dialysed against PBS and then applied to a Sephadex<sup>TM</sup> G-25 gel-filtration column (Pharmacia). The eluted fractions contained recombinant eSRS21 as the major protein, which reacted with anti-eSRS21 antibody by western blotting, and a few minor protein contaminants. These partially purified samples were dialysed against physiological saline solution for eel and diluted with Leibovitz L-15 medium supplemented with 0.5% bovine serum albumin fraction V (Sigma) and 10 mM Hepes and adjusted to pH 7.4.

### Testicular organ culture techniques

Testicular organ culture techniques were performed as described (Miura et al., 1991b), with minor modifications. Freshly removed eel testes were cut into 1×1×0.5 mm pieces and placed on floats of 1.5% agarose covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. The agarose floats were substituted with basal medium for 18 hours before the start of culture. Testicular fragments were then cultured in 1 ml of the basal medium with 0, 0.005, 0.05, 0.5 and 5 ng/ml of recombinant eSRS21 and/or 10 ng/ml of 11-ketotestosterone, the concentration that is most effective for inducing spermatogenesis (Miura et al., 1991b), for 15 days at 20°C in humidified air. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin fraction V (Sigma), 1 mg/ml bovine insulin and 10 mM Hepes adjusted to pH 7.4. The medium was changed on day 7.

### Testicular germ cell/somatic cell co-culture system with anti-eSRS21 antibody

Testicular germ cell/somatic cell co-culture techniques were performed as described (Miura et al., 1996), with minor modifications. Germ cells and somatic cells containing mainly Sertoli cells were isolated from the testes of five eels by collagenase and dispase treatment. For each eel sample, aliquots of 0.5×10<sup>6</sup> of these isolated cells were suspended with 1 ml of basal culture medium containing 50 µg of purified anti-eSRS21 IgG. The cells were pelleted by centrifugation at 1000 *g* for 10 minutes at 20°C. After centrifugation, each pellet was incubated for 24 hours in a microcentrifuge tube (1.5 ml; Greiner) at 20°C. Five pellets were collected and analysed to give an initial control value, and another five pellets were transferred to 24-well culture dishes (Iwaki) and cultured with 1 ml of basal medium containing 50 µg of anti-eSRS21 IgG for 15 days at 20°C in humidified air. For control experiments, 10 pellets were treated with an equal amount of normal rabbit IgG instead of anti-eSRS21 IgG at all the relevant stages, and five of these 10 control samples were cultured with 11-ketotestosterone for 15 days as a positive control. The medium was not changed during the culture period.

### Detection of proliferating germ cells

To detect the proliferating cells, labelling with 5-bromo-2-deoxyuridine (BrdU) was carried out according to the manufacturer's instructions (Amersham Pharmacia Biotech), with minor modifications. Testicular fragments or pellets of testicular germ cell/somatic cells were incubated with BrdU (1 µl/well) for the last

12 hours of culture. After culture, samples were fixed in Bouin's solution, and 5 µm paraffin wax-embedded sections were cut and stained immunohistochemically. Sections were then counterstained with Delafield's Haematoxylin. The number of immunolabelled germ cells was counted and expressed as a percentage of the total number of germ cells.

**Statistics**

Data analysis was carried out by the Sceirer, Ray, and Hare extension of the Kruskal-Wallis test (a two-way ANOVA design for ranked data), followed by the post-hoc Benferroni adjustment. A value of  $P < 0.05$  per number of comparisons was considered to be significant.

**RESULTS**

**cDNA cloning**

To isolate downregulated cDNA from eel testes, two populations of double-stranded cDNA were prepared from testes of eel that had been either untreated (-) or treated (+) with a single injection of hCG for 3 days, and an enriched subtractive (-) cDNA library was constructed. Approximately  $10^4$  clones from this library were screened by differential hybridisation, using each subtractive cDNA as a probe, and roughly 1,000 clones that hybridised only to the (-) cDNA library were picked up. These clones were composed of six non-crosshybridising cDNA fragments. We thought that some of these cDNA fragments may not be full length, as they might have been digested by the *Mbo*I enzyme. We therefore probed for full-length cDNA clones using a complete cDNA library that was constructed from nondigested cDNA inserted

into the *λzapII* arm. Fragments that hybridise to the same full-length cDNA clones are usually derived from the same mRNA. This analysis identified three downregulated cDNA clones: *eSRS3* and *eSRS4*, which have been reported previously (Miura et al., 1998), and *eSRS21*, which is newly isolated and on which we focus in this study.

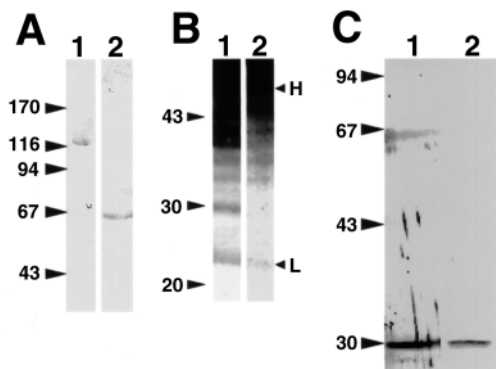
The screening of approximately  $2 \times 10^6$  plaques from an intact eel testicular cDNA library yielded positive clones for *eSRS21*, and the clone with the longest insert was subjected to sequence analysis.

**Characterisation of eSRS21**

*esrs21c19*, the longest cDNA clone of *eSRS21*, has a 2017 bp insert (DDBJ/EMBL/GenBank Accession Number, AB074569). The sequence contains a long open reading frame encoding 614 amino acids (Fig. 1). Three potential N-linked glycosylation sites are located at Asn (77) to Ala (80), Asn (144) to Gly (147) and Asn (398) to Ala (401). The N-terminal

**Fig. 1.** Comparison of the deduced amino acid sequence of eSRS21 with the sequence of Müllerian-inhibiting substance (MIS) from chicken (Eusèbe et al., 1996), human (Cate et al., 1986) and mouse (Münsterberg et al., 1991). This alignment was performed using Clustal W software (Thompson et al., 1994). Numbering of the amino acid residues relates to eSRS21. Red characters indicate residues that are conserved throughout all sequences. Grey characters indicate residues that are the same for 3/4 sequences. Asterisks indicate seven conserved cysteine residues. Dash represents a gap in the sequence introduced to maximise alignment; arrowhead indicates the signal sequence cleavage site. Underlined residues denote a possible N-glycosylation site.

eSRS21	MW---S---RRVMRA-MLLLL-SCATATL <sup>▼</sup> PQQ <sup>*</sup> EGGGGMDRILKPD <sup>*</sup> R-PRPDQTDGGEDGA	52
ChickenMIS	MKAALGVLLP-Y---LVLLLR <sup>*</sup> S-T--ALPRK <sup>*</sup> GTPG--AE-AV---L-EQ--SL-SEEMGL	
HumanMIS	MR---D--LP-LTS-LALVL-S-ALGAL-L-GT-----E-AL---RAE <sup>*</sup> EP-AV-GTS-GL	
MouseMIS	MQ---G---PHL-SPLVLLL-A-TMGAV-L--QP-----E-AV-----ENL-AT-NTR-GL	
eSRS21	WGSERENED---P---RSR---SDD-AAETAD-Q-----NSTA--RPDPEP-----	87
ChickenMIS	GAK-RENVSRAR <sup>*</sup> PGLAVR <sup>*</sup> PKMTAA <sup>*</sup> SRLP-EPAAS <sup>*</sup> CTQADGSS <sup>*</sup> LQWPLG <sup>*</sup> GL-EGFVCR <sup>*</sup> L	
HumanMIS	-IF-REDLD--WP-----P-----GI---PQEP <sup>*</sup> L--C-----LVA--LG--DS-----	
MouseMIS	-IF-LED-E-LWP-----P-----S--PPEP <sup>*</sup> L--C-----LVT--VRG--EG-----	
eSRS21	EPDPR--TP-SGTGPCFGLVVEHEAVGRD <sup>*</sup> LLSAVLE-GWGREREPEQEDL <sup>*</sup> TRFGLCS <sup>*</sup> DPD	142
ChickenMIS	KMD-RNEVPPSHL-EVVGVLTRYE---SAFIK-VLRAGWE-HRSPE---T-FGLCPGD	
HumanMIS	NGS-S--SP--L-RVVGALSAYE---QAF <sup>*</sup> LGA <sup>*</sup> VQ <sup>*</sup> RRARW <sup>*</sup> G-PRDLA---T-FGVCNTGD	
MouseMIS	NTS-R--AS---L-RVVGGLNSYE---YAFLEAVQESRWG-PQDLA---T-FGVSS <sup>*</sup> T-D	
eSRS21	GNSTG-PNAALSALAKGVEKDRG-C---LHVLHPTKEL-WGADGEHQ <sup>*</sup> RRELT <sup>*</sup> LRFLP--	195
ChickenMIS	AQTVLRP--LQQQEHLAG-PGMGLQRF <sup>*</sup> LILH-LEE <sup>*</sup> VKW---EAQ--PR-LW <sup>*</sup> RF <sup>*</sup> LFFH	
HumanMIS	RQAAL-P--SLRR <sup>*</sup> LGAWL-RDPG-C-QRLV <sup>*</sup> LH-LEE <sup>*</sup> VTW---EPT--PS-LRFQEP--	
MouseMIS	SQATL-P--ALQR <sup>*</sup> LGAWL-GETG-E-QQLV <sup>*</sup> LH-LAE <sup>*</sup> VIW---EP <sup>*</sup> E--LL-LK <sup>*</sup> FQEP--	
eSRS21	PPLHARL-G--GL--APVLV <sup>*</sup> FALGG-TASTG-G-M---RVSF-GSPS <sup>*</sup> LLPHR <sup>*</sup> QTVCLSA	242
ChickenMIS	AEV--GRLLG--ELHVA-LLLF-YPGSP--TGRWAEGRQELLVEGT-GL-TQE <sup>*</sup> SLCF <sup>*</sup> TR	
HumanMIS	PP---GGA-GPEL--A-LLVL-YPG-P---G---P---EVT <sup>*</sup> VTRA-GL-PGA <sup>*</sup> SLCP <sup>*</sup> SR	
MouseMIS	PP---GGA-SRWEQ--A-LLVL-YSG-P---G---P---QVT <sup>*</sup> VGT-GL-RGT <sup>*</sup> QLCP <sup>*</sup> TR	
eSRS21	GTRYAVL-TRRQTKD-HSHGQQG-QGGQGHV <sup>*</sup> LRI <sup>*</sup> TV <sup>*</sup> EANQ-AEEGEKRS <sup>*</sup> LQD <sup>*</sup> LLT <sup>*</sup> GEGV	298
ChickenMIS	DTQYLLRAS--V-AAVSSSSKLSFQLS-LTIR-H-HGDGAPLSLME-TQQ <sup>*</sup> LLFGSD	
HumanMIS	DTRYLVL-A---V-D-REPAGAWR-GSGLA-LTLQ-P-RGED-SRLSTAR-IQAL <sup>*</sup> LF <sup>*</sup> GDD	
MouseMIS	DTRYLVL-T---V-D-FPAGAWS-GFLI-LTLQ-PSR-EG-ATLSIDQ-LQAL <sup>*</sup> LF <sup>*</sup> GSD	
eSRS21	GRGTQG-P-LLLFQ--SR-ILDSA-D---SPSNG-L--APEVSPS---PGF-LA--P--	339
ChickenMIS	EKCFTRMTPVLLLLAGSFP <sup>*</sup> EG-DAAAPSSYLSASGVVDVAP-Y-PQLSPPH <sup>*</sup> PAEL <sup>*</sup> PFH	
HumanMIS	HRCFTRMTPALLLL--PR-S-EPA-P---LPAHQ <sup>*</sup> LD <sup>*</sup> TV <sup>*</sup> P-F-PP---PRP-SAE-LEE	
MouseMIS	SRCFTRMTPTLVVL---PP-A-EPS-P---QPAHQ <sup>*</sup> LD <sup>*</sup> TM <sup>*</sup> P-F-PQ---PGL-SLE-PE-	
eSRS21	--S-----RT-F-H--FLCVLQK <sup>*</sup> F-LSEAL---S-PEKAGSVPAPAAPVSLD--SIQS	378
ChickenMIS	TTASPANTSP <sup>*</sup> TPGDSGQFLGVL <sup>*</sup> T-LFIRVLS <sup>*</sup> PRSE <sup>*</sup> PTQPS-S--Q--HWLDF <sup>*</sup> MMET	
HumanMIS	--S-P-----PS-ADP--FLET <sup>*</sup> LTRL-VR-AL--RV-PPARAS-A-PR--LALD <sup>*</sup> PDALAG	
MouseMIS	--ALP-----HS-ADP--FLET <sup>*</sup> LTRL-VR-AL--RG-PLTQAS-N-TQ--LALD <sup>*</sup> PGALAG	
eSRS21	LP-P-LTLGASSSESLLGLLNS <sup>*</sup> APT <sup>*</sup> LSF <sup>*</sup> -PRWAGELLE---H-RVELD--LQP-P--	428
ChickenMIS	LPHQ-L-LNLS <sup>*</sup> ETAALER-LVQSEEP <sup>*</sup> SVLLF-FQEGGAGLE-Q-HFG---D--WQPEGT <sup>*</sup> V	
HumanMIS	FP-QGL-VNLS <sup>*</sup> SDPAALER-LLDGEE <sup>*</sup> PLLLLR <sup>*</sup> ETAATTGDPAPLH-D-PTSAPAA <sup>*</sup> A--G--	
MouseMIS	FP-QGL-VNLS <sup>*</sup> SDPAALGR-LLDWEE <sup>*</sup> PLLLLS <sup>*</sup> ETAAT <sup>*</sup> TEREPI <sup>*</sup> RLH-G-PASAPAA <sup>*</sup> G--	
eSRS21	LVAVLR <sup>*</sup> LRLE-EAARAAREATGGCGL-GD-RLQR <sup>*</sup> LREL <sup>*</sup> GSL--PGE <sup>*</sup> GAAPPADK-GSDR	480
ChickenMIS	L-Q-LLMG-KLQAVI <sup>*</sup> HELRD <sup>*</sup> IPEFQ-A-NTGLFQHL--LSFCY <sup>*</sup> HPP-G--PAA-G-G-Q-L	
HumanMIS	L-A-RRVAELQAAAE <sup>*</sup> RLS <sup>*</sup> PLGPATAP-LIARL--LALC--PG-G--P-G-GLG-D-D	
MouseMIS	L-Q-RRVAVELQAAAE <sup>*</sup> RLD <sup>*</sup> PLPPT-APPLIARL--LALC--PN-D--SRG-S-G-D-D	
eSRS21	E--A-QYR <sup>*</sup> ALLL <sup>*</sup> KALQ <sup>*</sup> TVLGAWE <sup>*</sup> EERSL <sup>*</sup> R-SARGGQ-E--GSGKG-CG <sup>*</sup> RLQ <sup>*</sup> PLSV <sup>*</sup> LK	532
ChickenMIS	PPGSGK <sup>*</sup> LHALLL <sup>*</sup> KALQ <sup>*</sup> TVRARWQE-R--RKVLR--QNR--SARHQ-AH <sup>*</sup> CR <sup>*</sup> LQ <sup>*</sup> ELT <sup>*</sup> IDL <sup>*</sup> R	
HumanMIS	P-----LRALLL <sup>*</sup> KALQ <sup>*</sup> GLRVEWRG-RDPRG <sup>*</sup> GRA-Q-RSAGATAAD <sup>*</sup> PCAL <sup>*</sup> RELS <sup>*</sup> VDL <sup>*</sup> R	
MouseMIS	P-----LRALLL <sup>*</sup> KALQ <sup>*</sup> GLRAE <sup>*</sup> WHG-REG <sup>*</sup> RGRTRA-Q-R--GDKGQ <sup>*</sup> AD <sup>*</sup> PCAL <sup>*</sup> RELS <sup>*</sup> VDL <sup>*</sup> R	
eSRS21	-SY--VLEPAT--AA <sup>*</sup> IN <sup>*</sup> NC <sup>*</sup> EG <sup>*</sup> PC <sup>*</sup> FLAEAN---NHAILLSHEQ <sup>*</sup> SQ <sup>*</sup> PLGRS <sup>*</sup> PC <sup>*</sup> VFL	583
ChickenMIS	-DRNFIVMP-TVVAA-NNCE <sup>*</sup> G <sup>*</sup> PC <sup>*</sup> RL <sup>*</sup> PLS <sup>*</sup> TRV <sup>*</sup> FGYHS <sup>*</sup> HTV <sup>*</sup> LLLMQ <sup>*</sup> ERGS <sup>*</sup> PLQ <sup>*</sup> RAP <sup>*</sup> CC <sup>*</sup> VFV	
HumanMIS	AERS-VLIPET-YQA-NNCQ <sup>*</sup> G <sup>*</sup> VG <sup>*</sup> WF <sup>*</sup> QSD <sup>*</sup> RN <sup>*</sup> PRYGN <sup>*</sup> HVV <sup>*</sup> LLLMQ <sup>*</sup> ARGAAL <sup>*</sup> ARP <sup>*</sup> CC <sup>*</sup> VFT	
MouseMIS	AERS-VLIPET-YQA-NNCQ <sup>*</sup> AC <sup>*</sup> RW <sup>*</sup> FQ <sup>*</sup> SD <sup>*</sup> RN <sup>*</sup> PRYGN <sup>*</sup> HVV <sup>*</sup> LLLMQ <sup>*</sup> ARGAAL <sup>*</sup> ARP <sup>*</sup> CC <sup>*</sup> VFT	
eSRS21	RYAD-LQVVELKAGTEITIK <sup>*</sup> PNMVATR <sup>*</sup> CG <sup>*</sup> CR	614
ChickenMIS	RYSDQL-IISVSAEGL <sup>*</sup> VRK <sup>*</sup> PNMVAAE <sup>*</sup> CG <sup>*</sup> CR	
HumanMIS	AYAGKL-LISLSE <sup>*</sup> ERISA <sup>*</sup> HV <sup>*</sup> PNMVATE <sup>*</sup> CG <sup>*</sup> CR	
MouseMIS	AYAGKL-LISLSE <sup>*</sup> ERISA <sup>*</sup> DHV <sup>*</sup> PNMVATE <sup>*</sup> CG <sup>*</sup> CR	

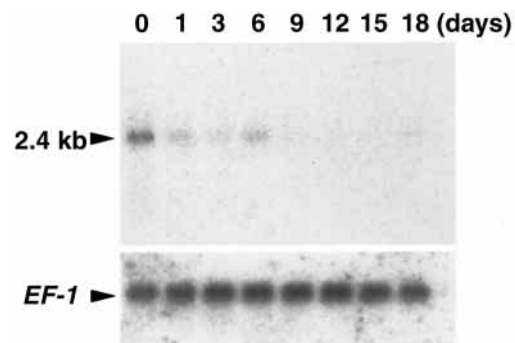


**Fig. 2.** Characterisation of eSRS21 protein using an anti-eSRS21 antibody. (A) Western blot analysis of the immature cultivated eel testis under non-reducing (lane 1) and reducing (lane 2) conditions. (B) Testicular immunoprecipitation using anti-eSRS21 (lane 1). Lane 2 shows the negative control without testicular samples. H and L indicate heavy and light chains of IgG, respectively. (C) Western blot analysis of CHO cells transfected with *pSD(X)/esrs21c19* (lane 1) and its conditioned medium (lane 2) under reducing conditions. Numbers on the left represent molecular size markers (kDa).

regions of this clone are rich in hydrophobic amino acid residues, which is characteristic of signal peptides. According to the (-3, -1) rule (von Heijne, 1984), in combination with the signal peptidase cleavage pattern (von Heijne, 1986), there is likely to be a cleavage site for signal peptidase located between Pro (22) and Gln (23).

Database searches showed that the C-terminal 129 amino acids of eSRS21 share amino acid sequence similarity with Müllerian inhibiting substance (MIS) (Fig. 1). Overall, eSRS21 shares 43.3, 41.3 and 40.7% similarity, respectively, with the amino acid sequence of MIS from chicken (Eusèbe et al., 1996), human (Cate et al., 1986) and mouse (Münsterberg and Lovell-Badge, 1991). In the C-terminal region of eSRS21, there are seven conserved cysteine residues, which are features of the TGF $\beta$  superfamily (Massagué, 1990).

To confirm the existence of the eSRS21 protein, we carried out western blot analysis and immunoprecipitation on immature eel testes using an anti-eSRS21 antibody (Fig. 2). eSRS21 was detected as a band of 60 kDa in western blotting under reducing conditions. Under non-reducing conditions, however, the eSRS21 band shifted to 120 kDa (Fig. 2A). Immunoprecipitation also revealed a 30 kDa band, which was not detected in the negative control (Fig. 2B). However, this band did not appear in western blot analysis (Fig. 2A). We also assayed CHO cells transfected with *pSD(X)/esrs21c19* and their conditioned medium by western blot analysis under reducing conditions (Fig. 2C). Although the 30 kDa and 60 kDa bands that react with anti-eSRS21 were detected in the transfected cells, only the 30 kDa band was found in the conditioned medium. This 30 kDa band did not shift to a higher mass under non-reducing conditions (data not shown). By contrast, none of these bands was detected in the mock transformants or their conditioned medium. Although all western blot analyses were carried out without primary antibody against eSRS21 for the negative control, no band could be detected except for the bands of IgG in immunoprecipitation (data not shown).



**Fig. 3.** eSRS21 mRNA expression in developing testes. Northern blot analysis was performed using poly(A)<sup>+</sup> RNA extracted from the testes 0, 1, 3, 6, 9, 12, 15 and 18 days after hCG treatment. Northern blot of *elongation factor 1 (EF1)*, which serves as reference, is given underneath the figure.

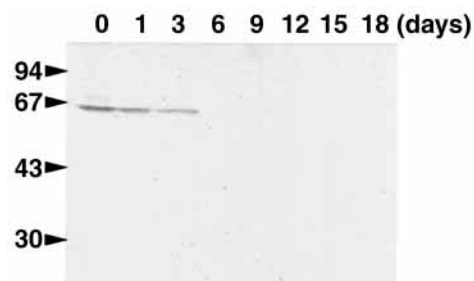
### Sequential changes of eSRS21 transcription and translation in spermatogenesis

Northern blot analysis was used to examine how the transcript levels of testicular *eSRS21* change during hCG-induced spermatogenesis (Fig. 3). Before hCG injection, 2.4-kb mRNA transcripts were prominent in testis. This signal was immediately suppressed by hCG injection, and was not detected in testis for 9-18 days after hCG injection. As an analysis control, the same northern blots were re-probed with a labelled *elongation factor 1 (EF1)* cDNA and the same amount of *EF1* transcripts was detected in all samples.

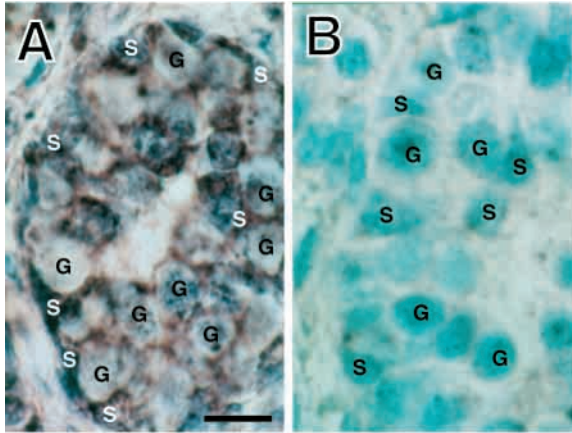
To evaluate how levels of eSRS21 protein change during spermatogenesis, western blot analysis was performed using an anti-eSRS21 antibody (Fig. 4). eSRS21 protein was detected before hCG injection, but protein levels decreased drastically on six days after hCG injection and no eSRS21 protein was detected on 12 days. By contrast, none of these bands was detected in negative control without primary antibody (data not shown). Thus, eSRS21 is present in untreated eel testes containing spermatogonial stem cells, and its transcription and translation is downregulated by hCG injection.

### Localisation of eSRS21 mRNA in testis

To determine the distribution of *eSRS21* mRNA expression in testis, we performed in situ hybridisation using DIG-labelled



**Fig. 4.** Western blot analysis of eSRS21 protein expression during hCG-induced spermatogenesis using an anti-eSRS21 antibody. Samples were obtained from the testis at 0, 1, 3, 6, 9, 12, 15 and 18 days after hCG treatment. Numbers on the left represent molecular size markers (kDa).



**Fig. 5.** Cellular localisation of eSRS21 mRNA in the immature cultivated eel testis by in situ hybridisation. (A) Section hybridised with antisense probes. (B) Section hybridised with sense probes as a negative control. Sections were stained nuclei by 0.2% Methyl Green, metachromatically. G and S indicate germ cell and Sertoli cell, respectively. Scale bar: 10 µm.

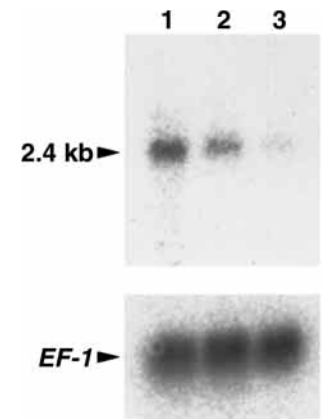
sense and antisense RNA probes (Fig. 5). The signal obtained with the antisense probe was detected mainly in Sertoli cells in testes. By contrast, the sense probe did not hybridise to any cells in the testes.

#### Effects of 11-KT on eSRS21 mRNA expression in eel testes in vitro

Northern blot analysis was used to examine the effects of 11-KT on *eSRS21* mRNA expression in the eel testicular organ culture system (Fig. 6). A concentration of 10 ng/ml 11-KT was used, because this concentration has been found to be most effective in inducing spermatogenesis in vitro (Miura et al., 1991a; Miura et al., 1991b). *eSRS21* transcripts were prominent in testis before cultivation. In cultured testis, expression of *eSRS21* did not change for 6 days in control systems without hormones; however, the signal strength of *eSRS21* mRNA decreased markedly in 11-KT-treated cells. As an analysis control, the same Northern blot was re-probed with a labelled *EF-1* cDNA, and the same amounts of *EF-1* transcripts were detected in all samples.

#### Effects of recombinant eSRS21 on spermatogenesis in vitro

To investigate the action of eSRS21 in spermatogenesis, testicular fragments were cultured with various concentration of recombinant eSRS21 (r-eSRS21; 0.005, 0.05, 0.5 and 5 ng/ml) and/or 10 ng/ml of 11-KT for 15 days. We then measured the proliferation of spermatogonia by exposing the testicular tissue to BrdU to check for replicating DNA (Fig. 7). Before cultivation, all germ cells in the eel testis were type A and early type B spermatogonia (which are spermatogonial stem cells), and the BrdU index was  $10.3 \pm 0.6\%$  on stem cell renewal. In the positive controls, treatment with 11-KT alone stimulated DNA replication (BrdU index =  $43.4 \pm 1.3\%$ ) and proliferation of spermatogonia. When testicular fragments were cultured simultaneously with r-eSRS21 and 11-KT, the increase in BrdU index caused by 11-KT stimulation was suppressed by r-eSRS21 in a dose-dependent manner, with



**Fig. 6.** Northern blot analysis of mRNA from cultured testicular fragments for eSRS21. Testicular fragments were cultured without (lane 2) or with (lane 3) 10 ng/ml 11-ketotestosterone for 6 days. Lane 1 shows the initial control before culture. Northern blot of *elongation factor 1* (*EF1*), which serves as reference, is given underneath the figure.

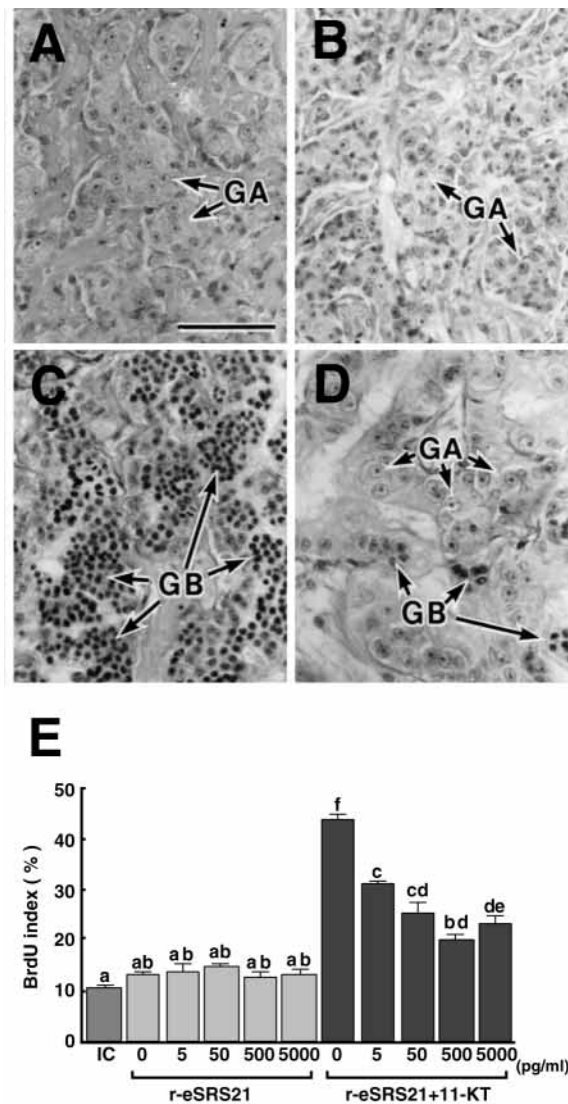
peak suppression ( $19.8 \pm 1.3\%$ ) occurring at 0.5 ng/ml recombinant protein. Treatment with r-eSRS21 alone, however, did not affect either the BrdU index or any spermatogenic process during the experimental period.

#### Effects of anti-eSRS21 antibody on spermatogenesis in vitro

To investigate further the action of eSRS21 in initiating spermatogenesis, germ cell/somatic cell pellets were cultured with or without purified anti-eSRS21 antibody for 15 days (Fig. 8). Before cultivation, all germ cells in the pellets were type A and early type B spermatogonia. In the positive controls, 11-KT treatment alone stimulated the proliferation of spermatogonia, and as a result late type B spermatogonia were detected in the pellets, indicating that this experimental system functions normally. Treatment with anti-eSRS21 antibody also stimulated spermatogonial proliferation, and as a result several cysts constructed by late type B spermatogonia (35%) were observed in the pellets. By contrast, control germ cell/somatic cell pellets cultured with normal rabbit IgG had only type A and early type B spermatogonia.

## DISCUSSION

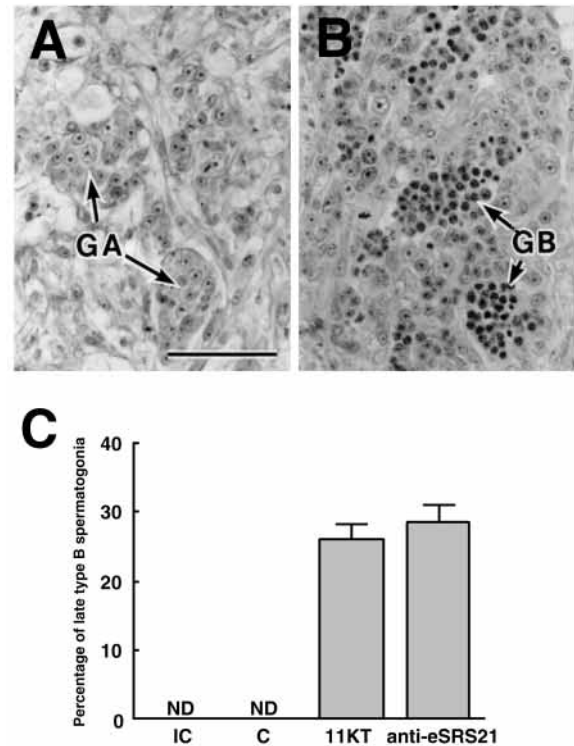
Under fresh-water cultivation conditions, male Japanese eel have immature testes containing only non-proliferated type A and early type B spermatogonia (Miura et al., 1991a). This immature stage of the testis is attributed to insufficient gonadotropin (GTH) in the eel pituitary (Yamamoto et al., 1972). Although a single injection of hCG can induce the complete process of spermatogenesis from the proliferation of spermatogonia to spermiogenesis, GTHs do not cause this induction directly, but rather work through the gonadal biosynthesis of 11-KT, which in turn mediates the start of spermatogenesis (Miura et al., 1991a; Miura et al., 1991b; Nagahama, 1994). It is believed that the action of 11-KT is mediated by other factors produced by Sertoli cells, which also contain androgen receptor (Ikeuchi et al., 2001). Alternatively, however, it is possible that factors that suppress the progress of spermatogenesis are expressed in the testis when the fish are in fresh water. In other words, eel spermatogenesis may be initiated by the downregulation of the genes encoding suppressive factors. On the basis of this hypothesis, we used gene expression cloning to isolate cDNA clones that show



**Fig. 7.** The effects of recombinant-eSRS21 (r-eSRS21) on 11-ketotestosterone-induced spermatogenesis in Japanese eel in vitro. (A-D) Microphotographs show the testicular sections from fragments cultured in basal medium alone (A), and with r-eSRS21 (B), 11-ketotestosterone (C) and r-eSRS21 and 11-ketotestosterone together (D). GA and GB indicate type A spermatogonia and late type B spermatogonia, respectively. Scale bar: 50  $\mu$ m. (E) BrdU index. The number of positively immunoreacted germ cells is expressed as a percentage of the total number of germ cells. IC, initial controls; 11-KT, 11-ketotestosterone. Results are given as the mean  $\pm$  s.e.m. Values with the same lowercase letter(s) are not significantly different ( $P < 0.05$ ).

suppressed expression after hCG treatment. As a result of this cDNA cloning, we succeeded in identifying *eSRS21*.

As mentioned above, it is thought that transcription of the gene encoding a key factor regulating spermatogenesis is controlled by 11-KT, a spermatogenesis-inducing steroid, and that this factor is expressed in Sertoli cells in which the receptor for 11-KT is located. In this study, we found that *eSRS21* mRNA was expressed in the Sertoli cells of immature eel testis in which the spermatogenesis process is resting. Moreover, *eSRS21* gene expression was downregulated not



**Fig. 8.** The effect of anti-eSRS21 antibody treatment on spermatogonial proliferation using a germ-somatic cell co-culture system. (A,B) Microphotographs show the pellets of germ cells/somatic cells cultured with anti-eSRS21 IgG and IgG from normal rabbit serum, respectively. GA and GB indicate type A spermatogonia and late type B spermatogonia, respectively. Scale bar: 50  $\mu$ m. (C) The rate of appearance of proliferated germ cells (late type B spermatogonia). IC, initial controls; C, controls; 11KT, 11-ketotestosterone; anti-eSRS21, anti-eSRS21 IgG. Results are given as the mean  $\pm$  s.e.m. Values with the same lowercase letter(s) are not significantly different ( $P < 0.05$ ).

only by hCG injection in vivo, but also by 11-KT treatment in vitro. These findings suggest that *eSRS21* fulfils the criteria defined for a key factor that regulates spermatogenesis.

The *esrs21c19* clone was isolated from a normal cDNA library constructed from nondigested cDNA fragments inserted into a  $\lambda$ zapII vector. A homology search of the predicted amino acid sequence showed that *eSRS21* shares comparatively high similarity with Müllerian-inhibiting substance (MIS). Furthermore, *eSRS21* also has seven conserved cysteine residues in its C-terminal region, a feature of the TGF $\beta$  superfamily (Massagué, 1990), and forms dimers in situ as observed by the 120 kDa band in western blots run under non-reducing conditions. These findings indicate that *eSRS21* may be the eel homologue of MIS and a member of the TGF $\beta$  superfamily.

Under reducing conditions, testicular *eSRS21* exhibits two forms with different molecular masses: one of 60 kDa and another of 30 kDa. CHO cells transfected with *esrs21c19* cDNA also produce these two forms of recombinant protein. However, conditioned medium from the transfected CHO cells contains only the 30 kDa form. Although further investigation is needed, it seems likely that *eSRS21* is produced as a 60 kDa protein in Sertoli cells but is secreted as 30 kDa protein after

proteolytic processing, similar to the processing that occurs with members of the TGF $\beta$  superfamily (Massagué, 1990). Although the testicular 30 kDa protein was detected by immunoprecipitation, it was difficult to detect by using western blot analysis. This suggests that the quantity of 30 kDa protein is less than 60 kDa protein in situ condition. There may be some regulational mechanisms in this proteolytic processing from 60 kDa to 30 kDa form.

In general, growth factors belonging to the TGF $\beta$  superfamily are disulphide-linked homo- or heterodimers (Massagué, 1990). Although the testicular 60 kDa eSRS21 forms a dimer, the 30 kDa secreted recombinant eSRS21 from CHO cells is monomeric. From our results, however, it is not clear whether the testicular 30 kDa eSRS21 is monomeric or dimeric. Although mammalian CHO cells may not faithfully construct teleostean eSRS21, it seems likely that eSRS21 does not need to form a dimer, because its monomeric recombinant retains biological action in eel spermatogenesis.

MIS, also called anti-Müllerian hormone (AMH), is a glycoprotein that is expressed early in gonadal differentiation of the mammalian male. MIS was identified and later purified on the basis of its ability to induce regression of primordium of female genitalia primordium, the Müllerian duct, in mammalian embryos (Balanchard and Josso, 1974). MIS is also produced by Sertoli cells of the fetal and adult testis and by ovarian granulosa cells after birth (Balanchard and Josso, 1974; Vigier et al., 1984). In ovary, MIS blocks meiosis (Takahashi et al., 1986), leading to a loss of germ cells (Vigier et al., 1987; Behringer et al., 1990), and inhibits the transcription of aromatase (Vigier et al., 1989) and LH receptor (di Clemente et al., 1994). MIS also affects the function of the adult testis by blocking the differentiation of mesenchymal into Leydig cells (Behringer et al., 1990; Behringer et al., 1994; Mishina et al., 1996) and by decreasing the expression of steroidogenic enzymes (Racine et al., 1998; Rouiller-Fabre et al., 1998; Teixeira et al., 1999); however, MIS does not affect germ cell development in mammalian males. Thus, MIS is a growth factor with multiple functions in mammalian reproduction. Because teleosts do not have a Müllerian duct (Hoar, 1957), we propose that eSRS21 may share similar functions with MIS except for regression of the Müllerian duct in male eels.

Using recombinant eSRS21 protein produced from a CHO expression system, we investigated the effect of eSRS21 on 11-KT-induced in vitro spermatogenesis. Adding recombinant eSRS21 to culture media suppressed the spermatogonial proliferation induced by 11-KT, with peak suppression occurring at 500 pg/ml. Thus, eSRS21 is able to suppress 11-KT-induced spermatogenesis. Furthermore, removing eSRS21 secreted from Sertoli cells using an anti-eSRS21 antibody induced spermatogonial proliferation in a testicular germ cell/somatic cell co-culture system. This indicates that under normal conditions, eSRS21 inhibits the start of spermatogenesis in Japanese eel. In mammal, MIS inhibits the growth of ovarian epithelial cells, ovarian cancer cells and breast cancer cells (Ha et al., 2000; Segev et al., 2000). Taken together, these observations suggest that MIS and its homologous protein, eSRS21, have a role in suppressing cell proliferation.

It has been reported that MIS affects breast cancer cells through an NF $\kappa$ B-mediated pathway, which leads to the

inhibition of cell growth (Segev et al., 2000). When MIS suppresses androgen synthesis in Leydig cell in mammalian fetal testis, however, its signal transduction directly mediates downregulation of the mRNA expression of cytochrome P450c17 $\alpha$  hydroxylase/C<sub>17-20</sub> lyase. (Teixerira et al., 1999). In future, it will be necessary to establish whether eSRS21 inhibits spermatogonial proliferation by directly affecting germ cells, or by mediating its actions through either the production of steroid hormones other than 11-KT or via the production of growth factors such as BMP8B and activin B, which are known to affect spermatogonial proliferation (Zhao et al., 1996; Miura et al., 1995).

We propose that eSRS21 should be called not 'MIS' but 'spermatogenesis-preventing substance (SPS)' for the following six reasons: (1) eSRS21 is expressed in the Sertoli cells of immature eel before the initiation of spermatogenesis; (2) the initiation of spermatogonial proliferation corresponds with the disappearance of eSRS21 expression; (3) eSRS21 expression is downregulated by 11-KT, which is a spermatogenesis-inducing steroid; (4) recombinant eSRS21 prevents spermatogenesis induced by 11-KT treatment in in vitro organ culture; (5) removing naturally occurring eSRS21 protein from testis using a specific antibody induces the initiation of spermatogenesis in an in vitro germ cell/somatic cell co-culture; and (6) teleosts do not have a Müllerian duct.

Although the molecules involved have not been identified, evidence for the existence of hormones or factors that suppress spermatogenesis, especially meiosis, has been shown for polychaeta and mammalia (Bertout, 1984; Gondos et al., 1996; Hayashi et al., 2000). We suggest that eSRS21/SPS may be one of these factors in Japanese eel.

As mentioned above, in fresh-water conditions eel spermatogenesis arrests at an immature stage prior to initiation of spermatogonial proliferation. This immature stage seems to be maintained by the expression of SPS in eel Sertoli cells in fresh-water conditions. When eels downmigrate to the ocean, spermatogenesis resumes and progresses to maturation (Larsen and Dufour, 1993). We propose that during eel migration, spermatogenesis is resumed through a suppression of SPS expression caused by an increase in 11-ketotestosterone, which is induced by gonadotropin stimulation.

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## REFERENCES

- Abe, S.-I. (1987). Differentiation of spermatogenic cells from vertebrates in vitro. *Int. Rev. Cytol.* **109**, 159-209.
- Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D. and Brinster, R. L. (1990). Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. *Nature* **345**, 167-170.
- Behringer, R. R., Finegold, M. J. and Cate, R. L. (1994). Müllerian-inhibiting substance function during mammalian sexual development. *Cell* **79**, 415-425.
- Bertout, M. (1984). Spermatogenesis in *Nereis* as a model for the study of endocrine control. *Fortschr. Zool.* **29**, 115-122.
- Blanchard, M. and Josso, N. (1974). Source of anti-Müllerian hormone synthesized by the fetal testis: Müllerian inhibiting activity of fetal bovine Sertoli cells in culture. *Pediatr. Res.* **8**, 968-971.



- Cate, R. L., Mattaliano, R. J., Hession, C., Tizard, R., Farber, N. M., Cheung, A., Ninfa, E. G., Frey, A. Z., Gash, D. J. and Chow, E. P. (1986). Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell* **45**, 685-698.
- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals. Seminiferous epithelium cycle and spermatogonial renewal. *Int. Rev. Cytol.* **70**, 27-100.
- di Clemente, N., Goxe, B., Remy, J. J., Cate, R. L., Josso, N., Vigier, B. and Salesse, R. (1994). Inhibitory effect of AMH upon the expression of aromatase and LH receptors by cultured granulosa cells of rat and porcine immature ovaries. *Endocrine* **2**, 553-558.
- Eusèbe, D., di Clemente, N., Rey, R., Pieau, C., Vigier, B., Josso, N. and Picard, J. (1996). Cloning and expression of the chick anti-Müllerian hormone gene. *J. Biol. Chem.* **271**, 4798-4804.
- Gondos, B., Byskov, A. G. and Hansen, J. L. (1996). Regulation of the onset of meiosis in the developing testis. *Ann. Clin. Lab. Sci.* **26**, 421-425.
- Ha, T. U., Segev, D. L., Barbie, D., Masiakos, P. T., Tran, T. T., Dombkowski, D., Glander, M., Clarke, T. R., Lorenzo, H. K., Donahoe, P. K. et al. (2000). Müllerian inhibiting substance inhibits ovarian cell growth through an Rb-independent mechanism. *J. Biol. Chem.* **275**, 37101-37109.
- Hayashi, T., Kageyama, Y., Ishizaka, K., Kihara, K. and Oshima, H. (2000). Sexual dimorphism in the regulation of meiotic process in the rabbit. *Biol. Reprod.* **62**, 1722-1727.
- Herrin, D. L. and Schmidt, G. W. (1988). Rapid, reversible staining of northern blots prior to hybridization. *BioTechniques* **6**, 196-197.
- Hoar, W. S. (1957). The gonads and reproduction. In *The Physiology of Fishes*, Vol. I (ed. M. E. Brown), pp. 287-321. New York: Academic Press.
- Ikeuchi, T., Todo, T., Kobayashi, T. and Nagahama, Y. (2001). Two subtypes of androgen and progesterone receptors in fish testes. *Comp. Biochem. Physiol.* **129B**, 449-455.
- Larsen, L. O. and Dufour, S. (1993). Growth, reproduction and death in lampreys and eels. In *Fish Ecophysiology* (ed. J. C. Rankin and F. B. Jensen), pp. 72-104. London: Chapman & Hall.
- Massagué, J. (1990). The transforming growth factor-beta family. *Annu. Rev. Cell Biol.* **6**, 597-641.
- Mishina, Y., Rey, R., Finegold, M. J., Matzuk, M. M., Josso, N., Cate, R. L. and Behringer, R. R. (1996). Genetic analysis of the Müllerian-inhibiting substance signal transduction pathway. *Genes Dev.* **10**, 2577-2587.
- Miura, C., Miura, T., Yamashita, M., Yamauchi, K. and Nagahama, Y. (1996). Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. *Dev. Growth Differ.* **38**, 257-262.
- Miura, T., Kudo, N., Miura, C., Yamauchi, K. and Nagahama, Y. (1998). Two testicular cDNA clones suppressed by gonadotropin stimulation exhibit ZP- and ZP3- like structures in Japanese eel. *Mol. Reprod. Dev.* **51**, 235-242.
- Miura, T., Miura, C., Yamauchi, K. and Nagahama, Y. (1995). Human recombinant activin induces proliferation of spermatogonia in vitro in the Japanese eel *Anguilla japonica*. *Fisheries Sci.* **63**, 434-437.
- Miura, T., Yamauchi, K., Nagahama, Y. and Takahashi, H. (1991a). Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. *Zool. Sci.* **8**, 63-73.
- Miura, T., Yamauchi, K., Takahashi, H. and Nagahama, Y. (1991b). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proc. Natl. Acad. Sci. USA* **88**, 5774-5778.
- Miura, T., Yamauchi, K., Takahashi, H. and Nagahama, Y. (1991c). Human chorionic gonadotropin induces all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Dev. Biol.* **146**, 258-262.
- Münsterberg, A. and Lovell-Badge, R. (1991). Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. *Development* **113**, 613-624.
- Murata, M., Eto, Y., Shibai, H., Sakai, M. and Muramatsu, M. (1988). Erythroid differentiation factor is encoded by the same mRNA as that of inhibin  $\beta_A$  chain. *Proc. Natl. Acad. Sci. USA* **85**, 2434-2438.
- Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.* **38**, 217-229.
- Niwa, H., Harrison, L. C., DeAizpura, T. J. and Cram, D. S. (1997). Identification of pancreatic  $\beta$  cell-related genes by representational difference analysis. *Endocrinology* **138**, 1419-1426.
- Racine, C., Rey, R., Forest, M. G., Louis, F. and Ferre, A. (1998). Receptors for anti-Müllerian hormone on Leydig cells are responsible for its effects on steroidogenesis and cell differentiation. *Proc. Natl. Acad. Sci. USA* **95**, 594-599.
- Rouiller-Fabre, V., Carmona, S., Abou-Merhi, R., Cate, R. L., Hebert, R. and Vigier, B. (1998). Effect of anti-Müllerian hormone on Sertoli and Leydig cell functions in fetal and immature rats. *Endocrinology* **139**, 1213-1220.
- Segev, D. L., Ha, T. U., Tran, T. T., Kenneally, M., Harkin, P., Jung, M., MacLaughlin, D. T., Donahoe, P. K. and Maheswaran, S. (2000). Müllerian inhibiting substance inhibits breast cancer cell growth through an NF $\kappa$ B-mediated pathway. *J. Biol. Chem.* **275**, 28371-28379.
- Takahashi, M., Koide, S. S. and Donahoe, P. K. (1986). Müllerian inhibiting substance as oocyte meiosis inhibitor. *Mol. Cell. Endocrinol.* **47**, 225-234.
- Teixeira, J., Fynn-Thompson, E., Payne, A. H. and Donahoe, P. K. (1999). Müllerian-inhibiting substance regulates androgen synthesis at transcriptional level. *Endocrinology* **140**, 4732-4738.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic acids Res.* **22**, 4673-4680.
- Vigier, B., Picard, J. Y., Campargue, J., Forest, M. G., Heyman, Y. and Josso, N. (1984). Production of anti-Müllerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* **114**, 1315-1320.
- Vigier, B., Wang, Z., Magre, S., Tran, D. and Josso, N. (1987). Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it in vitro. *Development* **100**, 43-55.
- Vigier, B., Forest, M. G., Eychenne, B., Bezdard, J., Garrigou, O., Robel, P. and Josso, N. (1989). Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proc. Natl. Acad. Sci. USA* **86**, 3684-3688.
- von Heijne, G. (1984). How signal sequences maintain cleavage specificity. *J. Mol. Biol.* **173**, 243-251.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage site. *Nucleic Acids Res.* **14**, 4683-4690.
- Yamamoto, K., Hiroi, O., Hirano, T. and Morioka, T. (1972). Artificial maturation of cultivated male Japanese eel by Synahorin injection. *Bull. Jpn. Soc. Fish.* **38**, 1083-1090.
- Zhao, G. Q., Deng, K., Labosky, P. A., Liaw, L. and Hogan, B. L. M. (1996). The gene encoding bone morphogenetic protein 8B (BMP8B) is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev.* **10**, 1657-1669.