ニジマスより単離した新規サイトカインGSDFの発現および機能解析

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平成18年度

(2006)

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緒　論

始原生殖細胞は、胚発生初期の極めて早い時期に他の体細胞系列から分化し、独自の発生を遂げる。この細胞は、顆粒を多く含む直径 20 μ m 程度の円形細胞であり、大型の核を有するといった形態的特徴を有する（Houston and King, 2000）。また、始原生殖細胞は将来生殖巣が形成される場所とは離れた場所で初めて認められ、発生が進むに伴い生殖巣原基に移動し、そこで増殖・分化した後、卵や精子を形成することが知られている（Raz and Hopkins, 2002）。雄の始原生殖細胞は性分化の後、精巣内で精原細胞へと分化する。この中でも特に自己複製能と分化能を併せ持つ細胞集団は、精原幹細胞と呼ばれ、雄性個体が生涯にわたって極めて大量の精子を生産し続けるための源となっている（Aponte et al., 2005）。近年、ニジマス精巣内にも精原幹細胞が存在することが報告され、さらに、精原幹細胞は雄性の生殖細胞であるにも関わらず、雌配偶子への分化能も有していることが明らかになった（Okutsu et al., 2006）。

始原生殖細胞および精原幹細胞移植による魚類の代理親魚養殖技術は、近年世界的に増加している絶滅危惧種の保全や、クロマグロのように親魚の管理に多大な労力を必要とする魚種の種苗生産を簡略化する技術として注目されている（吉崎, 2006）。本技術は、まずドナー個体から始原生殖細胞または精原幹細胞を取り出し、近縁種の宿主個体腹腔への移植を行なう。腹腔に移植されたドナー生殖細胞は、宿主生殖腺へ自発的に移動した後、生着・増殖し、ドナー配

偶子に分化する（Takeuchi et al., 2003; Okutsu et al., 2006）。つまり、本法ではドナー種に由来する卵や精子を生産する宿主を作出できるため、得られた宿主を交配することでドナー種に由来する次世代を生産することが可能となる。例えば、クロマグロをドナーとした代理親魚養殖を行う場合、クロマグロの始原生殖細胞・精原幹細胞を近縁種であるマサバに移植することで、クロマグロ配偶子を生産するマサバの作出が期待できる。また、絶滅危惧種の始原生殖細胞・精原幹細胞を取り出し、液体窒素中にて凍結保存しておけば、もし当該種が絶滅してしまった場合でも、解凍後の細胞を近縁種に移植することで、絶滅種に由来する卵や精子を宿主が生産することが期待される。これにより、得られた宿主を交配することで絶滅種を復活させることが可能となる。さらに、遺伝的多様性の保全が重要視されている現在、本技術はそれを解決する策としても非常に有効である。地域個体群の個体数減少による遺伝子レベルでの多様性の減少は、環境の変化等に対応する適応能力を低下させることになり、集団の絶滅をも導くことが危惧される。また、当該種を水産資源として永続的に利用していくためには、遺伝的多様性に富む健全な集団を維持していかなければならない。そこで、絶滅に瀕している魚種や地域個体群の遺伝子解析を行い、各種ハプロタイプごとの始原生殖細胞・精原幹細胞を収集し、液体窒素中で凍結保存しておけば、遺伝的多様性を半永久的に維持することも可能となろう。

本技法の実用化を考えた場合、ドナー細胞に用いる始原生殖細胞や精原幹細

胞の供給が大きなネックになると予想される。すなわち、マサバにクロマグロを生ませる場合も、移植用の細胞を供給するためのクロマグロ個体が常に必要となる。そのため、上述した一連の技術のなかで、体外に取り出したドナー細胞を宿主個体に移植、もしくは凍結する前に、*in vitro* で増殖させるステップを介在させれば、ドナー種のごくわずかな細胞から大量の細胞を維持・保存することが可能となり、本技術がより有用な技術となると期待される。また、収集された細胞は小さなプラスチックチューブで保存できるため、遺伝的に多様な多くの個体から細胞を収集したとしても、非常にわずかなスペースで遺伝的多様性の維持を行うことが可能となる。

始原生殖細胞・精原幹細胞を *in vitro* 培養する際には、いかに培養中の細胞が生殖系列の細胞としての特徴を維持したままの状態で増殖できるかが重要である。本研究室ではこれまでにニジマス始原生殖細胞・精原幹細胞の培養系確立を目指して研究を行ってきたが、始原生殖細胞を *in vitro* で培養すると、培養９日目には増殖活性が低下し、培養を継続できないことが明らかになっている（伊原, 2003)。一方精原幹細胞を含む精原細胞集団は、始原生殖細胞に比べると比較的培養が容易な細胞であることが確認されたが、長期間安定した増殖速度を保つことはなく、培養日数の経過とともに増殖活性が減少してしまうという問題が存在している（識名, 2005)。体外に取り出した細胞が増殖活性を失う現象は、マウス始原生殖細胞の *in vitro* 培養系においても報告されている（Donovan et

al., 1986; Okubo et al., 1996; Chuma and Nakatsuji, 2001)。マウス生体内では、始原生殖細胞は 13.5 日胚頃まで活発に増殖した後、増殖を停止する。その後、雄では増殖休止期が保たれ、雌では減数分裂へと移行する（Wylie, 1999)。マウス始原生殖細胞の *in vitro* 培養を行なうと、12.5 日胚に相当する時期まで増加した後、減少しはじめることから、始原生殖細胞は *in vitro* では *in vivo* での増殖にほぼ対応した期間しか増殖できないと考えられている (Donovan et al., 1986; Okubo et al., 1996; Chuma and Nakatsuji, 2001)。しかし、膜結合型 stem cell factor (SCF) や leukemia inhibitory factor (LIF)、basic fibroblast growth factor (bFGF) との共存下で培養した場合、10 日間ほどで多能性幹細胞としての特徴を持つ EG 細胞へと脱分化し、増殖を続けるようになることが報告されている（Koshimizu et al., 1996; Donovan and De Miguel, 2003)。また、新生児マウスの精原幹細胞より樹立された germline stem cell (GS 細胞) は、培養の際に glial cell-derived neurotrophic factor (GDNF)を添加したことで、その株化に成功している（Kanatsu-Shinohara et al., 2003)。このように、何らかの液性因子との共存下でないと、細胞は増殖しないうえに、細胞を *in vitro* に取り出すと、細胞の特性が変わることも少なくない。SCF、LIF、bFGF および GDNF は分泌性タンパク質であるサイトカイン分子であるが、生体内にある多くの細胞はこのようなサイトカインによる増殖制御を受けていると考えられている。このことから、魚類始原生殖細胞・精原幹細胞も何らかのサイトカインによって、*in vivo* でその増殖等を制御されている可能

性が高い。よって、ニジマス生殖細胞を *in vitro* で増殖させ続けながら、生殖細胞系列としての特徴を維持したまま培養するには、その増殖を促進する因子や特徴を維持させる因子の存在が必須である。

魚類始原生殖細胞の分子レベルでの研究は、小型魚類であるゼブラフィッシュを中心として盛んに行なわれている。その結果、始原生殖細胞の移動に関与する分子が多数単離され、移動に関する詳細な分子機構が明らかになりつつある（Raz, 2004; Blaser et al., 2005）。しかし、増殖に関与している分子の報告はなく、魚類始原生殖細胞を *in vitro* 培養する際に有効な分子の情報は皆無である。そこで、生殖腺において特異的に発現する遺伝子を網羅的に探索することにより、包括的に分子レベルでの生殖細胞形成を捉えることが必要であろうと考えた。

本研究室では水産上重要魚種であるニジマスを材料として研究を行っており、*vasa* 遺伝子の転写制御領域と緑色蛍光タンパク質（Green Fluorescent Protein: GFP）遺伝子を融合させた発現コンストラクトをニジマスに導入することで、始原生殖細胞を可視化した *vasa*-GFP トランスジェニックニジマスを系統化している（Yoshizaki et al., 2000; Takeuchi et al., 2002）。ニジマスは胚が大きいため、緑色蛍光を指標に初期胚に存在する未熟な生殖腺（生殖隆起）を外科的に単離することが可能である。さらに、フローサイトメーターを用いた始原生殖細胞の大量収集、凍結保存、異種への移植、といった様々なアプローチも可能となっ

ている（Takeuchi et al., 2002; Takeuchi et al., 2004; Kobayashi et al., 2004 ; Kobayashi et al., 2006）。そこで本研究は、始原生殖細胞・精原幹細胞の in vitro 培養のための基礎情報を提供するため、ニジマス生殖腺において特異的に発現する遺伝子を網羅的に探索し、その機能を明らかにすることを目的とした。第一に、始原生殖細胞の周囲に存在する生殖隆起体細胞より分泌されるサイトカインがその増殖を制御していると予想し、生殖隆起体細胞において特異的に発現しているサイトカインの単離を行なった。まず、単離したニジマス初期胚の生殖隆起および、生殖隆起を除去した胚体を用いて、生殖隆起において特異的に発現する遺伝子を濃縮した cDNA サブトラクションライブラリーを作製した。続いて、cDNA サブトラクションライブラリーに含まれる偽陽性を除去するために、2次スクリーニングを行い、生殖腺に特異的な発現を示すクローン Gonadal Soma-Derived Growth Factor (GSDF)を単離した。第２に、*in situ* ハイブリダイゼーション・免疫染色によって GSDF の初期胚および成魚における発現組織、発現細胞の同定を行った。第３に、アンチセンスオリゴヌクレオチドを用いた翻訳阻害実験により、GSDF の初期胚における機能を解析した。第４に、組換え体を用いた培養実験により GSDF の精巣における機能を明らかにした。

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Abstract

Our understanding of the molecular mechanisms of primordial germ cell (PGC) proliferation in fish is rudimentary, but it is thought to be controlled by the surrounding somatic cells. We assumed that growth factors that are specifically involved in PGC proliferation are expressed predominantly in the surrounding genital ridge somatic cells. In order to isolate these growth factors, we compiled a complementary DNA (cDNA) subtractive library using cDNA from the genital ridges of 40-dpf rainbow trout embryos as the tester, and cDNA from embryos without genital ridges as the driver. This approach identified a novel cytokine, designated gonadal soma-derived growth factor (GSDF), which is a member of the transforming growth factor (TGF)-β superfamily. GSDF was expressed in the genital ridge somatic cells surrounding the PGCs during embryogenesis, and in both the granulosa and Sertoli cells at later stages. Inhibition of GSDF translation by antisense oligonucleotides suppressed PGC proliferation. Moreover, isolated testicular cells that were cultured with recombinant GSDF demonstrated dose-dependent proliferation of type-A spermatogonia; this effect was completely blocked by antiserum against GSDF. These results denote that GSDF, a novel member of the TGF-β superfamily, plays an important role for proliferation of PGC and spermatogonia.

Introduction

The regulatory mechanisms underlying germline development have been extensively investigated in many organisms, as this unique cell lineage plays an important role in transmitting genetic and developmental information to the next generation (McLaren, 2003). Primordial germ cells (PGCs) are the founder cells of both female and male gametes, from which all sexually reproducing organisms arise. Fish PGCs have been identified through isolation of genes related to *vasa*, the *Drosophila* gene that plays an essential role in germ cell determination, while mechanisms responsible for the specification and migration of PGCs have been illuminated thorough studies of the zebrafish, *Danio rerio* (Raz, 2003). During the early embryonic development of fish, as in other vertebrates, PGCs initially arise outside the gonadal region and migrate to the genital ridges, eventually coalescing with their somatic counterpart. Despite extensive knowledge of PGC specification and migratory behavior, however, the molecular basis of fish PGC proliferation has remained unclear. Moreover, the signaling molecules (such as growth factors) and pathways that are required for fish PGC proliferation have not been identified.

Several murine studies of PGC have shown that their proliferation, survival, and migration involve growth-factor signaling (McLaren, 2003). Tumor necrosis factor-α

(TNF-α) and basic fibroblast growth factor (bFGF) have been shown to stimulate PGC proliferation *in vitro* (Kawase et al., 1994; Resnick et al., 1992), while stem cell factor (SCF) is required for PGC survival both *in vivo* and *in vitro*, and, together with leukemia-inhibitory factor (LIF), stimulates PGC proliferation *in vitro* (De Felici, 2000). In mouse embryos, SCF is produced by the somatic cells surrounding the migratory and post-migratory PGCs, while the receptor, c-Kit, is expressed on the PGC surface (Godin et al, 1991). The receptor component of LIF and LIF-related cytokines (oncostatin M, interleukin [IL]-6, IL-11, and ciliary neurotrophic factor [CNTF]), known as gp130, is expressed in PGCs (Koshimizu et al., 1996). gp130-mediated signaling, together with c-Kit signaling, promotes PGC proliferation (Donovan and De Miguel, 2003). These data indicate that the signaling pathways that act through ligand-receptor binding have important effects on the proliferation of mouse PGCs.

The PGCs of mice and other mammals increase in number during migration, while those of fish tend to proliferate immediately before and after they reach the genital ridges (Yoshizaki et al., 2002). Based on this finding it is possible that, unlike in mice, the proliferation of fish PGCs requires close interaction with genital ridge somatic cells but not somatic cells located in their migration pathway, and that the two processes might also be mediated by different signaling pathways.

In the rainbow trout (*Oncorhynchus mykiss*), the PGCs reach the genital ridge at 20 days post-fertilization (dpf), while the proliferation phase starts from about 15 dpf and continues until sexual differentiation occurs at around 60 dpf (Okutsu and Yoshizaki, unpublished data). As the proliferation of PGCs takes place in or near the genital ridges, we hypothesized that molecules secreted by genital ridge somatic cells during the proliferation phase might play critical roles in PGC proliferation. This theory assumed that growth factors that are specifically involved in PGC proliferation are expressed predominantly in the surrounding genital ridge somatic cells. Therefore, in order to isolate these growth factors, we compiled a complementary DNA (cDNA) subtractive library using cDNA from the genital ridges of 40-dpf rainbow trout embryos as the tester, and cDNA from embryos without genital ridges as the driver.

In the current study, we isolated a novel growth factor, designated gonadal soma-derived growth factor (GSDF), from 40-dpf rainbow trout embryos, and showed that it is specifically expressed in gonadal somatic cells. In order to clarify the effect of GSDF on PGCs *in vivo*, we counted PGC numbers in GSDF-knockdown embryos. Furthermore, we examined the functional contribution of GSDF in the testis through *in vitro*-culture studies using isolated testicular cells.

Materials and Methods

Construction of subtractive cDNA library

Rainbow trout gametes were collected and used for artificial insemination as described previously (Takeuchi et al., 2001). The fertilized eggs were cultured in running water at 10°C for 40 days. In total, 500 paired genital ridges and 500 samples from embryos without genital ridges were collected from 40-dpf embryos, as described previously (Takeuchi et al., 2002). The tissues were immediately frozen at – 80°C. Total RNAs were extracted using an Isogen Kit (Nippon Gene, Tokyo, Japan), followed by the purification of poly A^+ RNAs using the Oligotex-dT 30 Super System (Takara Bio Inc., Shiga, Japan), according to the manufacturers' protocols. A subtractive cDNA library was constructed using a PCR-Select cDNA Subtraction Kit (Clontech, CA, USA), with 2 μg poly A^+ RNA extracted from the genial ridges as the tester, and poly A^+ RNA from the embryonic tissue without genital ridges as the driver, according to the manufacturer's protocol. Enriched genital ridge cDNA fragments were cloned into the pGEM-T Easy plasmid vector (Promega, WI, USA).

Identification of genes specifically expressed in gonads

Hybridization membranes were prepared by randomly selecting 600 plasmid cDNA

sequences from the subtractive library and purifying them by a Wizard SV96 Plasmid DNA Purification System (Promega) according to the manufacturer's protocol. The purified sequences were then blotted onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences, NJ, USA) according to the manufacturer's protocol. cDNA probes for differential screening were prepared from gonadal and various somatic tissues (gill, heart, liver, spleen, muscle, intestine, and kidney) from 12-month-old rainbow trout. The average values of the gonad–somatic index (GSI), which is the gonad weight/body weight (%), were 0.133 for females and 0.038 for males. Poly A^+ RNA was extracted from each tissue as described above, and 1.5 μg of each messenger RNA (mRNA) was labeled using a Hot Scribe First-Strand cDNA Labeling Kit (GE Healthcare Bio-Sciences) with [α-^{32}P] dCTP (MP Biomedicals, CA, USA). The nylon membranes were pre-hybridized with ULTRAhyb Hybridization Buffer (Ambion, TX, USA) for 30 min at 48°C, followed by hybridization with each radiolabeled cDNA probe for 20 h at 48°C. The membranes were then washed twice with 2×SSC/0.1% (w/v) sodium dodecyl sulfate (SDS) at 48°C for 20 min, 1×SSC/0.1% (w/v) SDS at 48°C for 20 min, 0.5×SSC/0.1% (w/v) SDS at 48°C for 20 min, and 0.2×SSC/0.1% (w/v) SDS at 48°C for 30 min. The membranes were exposed to imaging plates for 24 h and detection was performed using a BAS 1000 Imaging Analyzer (Fuji Photo Film, Tokyo, Japan). Clones

hybridized to gonadal cDNA probes were used for further screening.

Candidate clones were analyzed by Northern hybridization using total RNAs extracted from the ovary (average GSI = 0.129), testis (average GSI = 0.058), brain, gill, heart, liver, intestine, spleen, kidney and muscle tissues of one-year-old female and male rainbow trout. A 20 μg sample of each total RNA was blotted onto a nylon membrane, and inserts of candidate clones were labeled with [α-^{32}P] dCTP by the random-priming method using an Oligo Labeling Kit (GE Healthcare Bio-Sciences). The blotting and hybridization procedures followed the procedures described elsewhere (Yoshizaki et al., 1994). Detection was performed using the method detailed above. The cDNA sequences were determined using the methods reported by (Yoshizaki et al., 2000a). After sequencing the cDNA, 3′-rapid amplification of cDNA ends (RACE) was performed according to the method described previously (Yoshizaki et al., 2000a). 5′-RACE was carried out using a SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. The PCR product was purified, cloned into the pGEM T-Easy vector, and used for DNA sequencing.

Phylogenetic analysis

The predicted mature domains of GSDF, additional members of the transforming

growth factor (TGF)-β superfamily, and other mouse cysteine-knot cytokines (such as human chorionic gonadotropin [HCG] and brain-derived neurotrophic factor [BDNF]), were aligned using Molecular Evolutionary Genetics Analysis (MEGA) Ver. 3.1 software (http://www.megasoftware.net). The GenBank accession numbers of the aligned amino-acid sequences were as follows: mouse TGF-β1, AAH13738; mouse TGF-β2, AAH11055; mouse nodal, NP_038639; mouse bone-morphogenetic protein (BMP)-2, NP_031582; mouse BMP-3, NP_775580; mouse BMP-4, NP_031583; mouse BMP-5, NP_031581; mouse BMP-6, BAA03555; mouse BMP-7, AAP97721; mouse inhibin-β A-subunit, CAA49325; mouse inhibin-β B-subunit, CAA49326; mouse inhibin α-subunit, AAH79555; mouse anti-Muellerian hormone (MIS), CAA39424; mouse growth-differentiation factor (GDF)-1, BAA08660; mouse GDF-5, NP_032135; mouse GDF-6, AAH34862; mouse GDF-7, NP_038554; mouse GDF-9, AAH52667; mouse GDF-10, NP_665684; mouse glial cell line-derived NF (GDNF), BAA08660; *Xenopus* vg1, AAW30007; *Drosophila* 60A, AAA28307; HCG, AAD02325; and BDNF, AAH34862. Several teleost homologs of GSDF were isolated in this study and identified using expressed-sequence tag (EST) databases. Their accession numbers were as follows: Atlantic salmon (*Salmo salar*), CK897686; stickleback (*Gasterosteus aculeatus*), DW624794; dace (*Pimephales promelas*), DT308327; fugu (*Fugu*

rubripes), CA590677; and zebrafish, XP_706584.

In situ hybridization

A 750-base pair (bp) cDNA fragment of *Gsdf* (nucleotides 2,578-3,328 of *Gsdf*) was subcloned into the pGEM T-easy vector. Sense and antisense RNA probes were transcribed *in vitro* using digoxigenin-labeled uridine triphosphate (UTP; Roche, Mannheim, Germany), and SP6 or T7 RNA polymerase (Promega). Whole-mount *in situ* hybridization was performed as described previously (Yoshizaki et al., 2000a) using sense and antisense RNA probes. For the *in situ* hybridization of tissue sections, rainbow trout embryos and gonads at various developmental stages were fixed in Bouin's solution at 4°C for 12 h, embedded in paraffin wax, and then sliced into 5 μm serial sections. The paraffin sections were dewaxed and dehydrated by passing them through a xylene-ethanol series. The sections were incubated with a hybridization mixture of 50 μg/ml tRNA, 50% formamide, 5×SSC (pH 4.5), 50 μg/ml heparin, 1% SDS and 1 μg/ml probe. After hybridization at 65°C for 18 h, the sections were processed as follows: twice in 5×SSC/50% formamide at 65°C for 30 min, three times in 2×SSC/50% formamide at 65°C for 30 min, 1×SSC/25% formamide/1×TBST at 65°C for 10 min, three times in 1×TBST at room temperature for 5 min, lastly in blocking solution

(Roche) at room temperature for 1 h. The sections were then incubated with the Fab fragment of an anti-DIG-alkaline phosphatase-conjugated antibody (Roche), diluted to 1:2000 with blocking solution, for 16 h at 4°C. After the nitroblue tetrazolium (NBT; Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) color reaction had been performed, the slides were mounted using Entellan neu (Merck KGaA, Darmstadt, Germany). Some sections were counterstained by Nuclear Fast Red (NFR; Vector Laboratories, CA, U.S.A) for 3 hours after NBT/BCIP color reaction.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNAs were extracted from unfertilized eggs and whole embryos at 2.5, 7.0, 10, 15, 20 and 30 dpf and various tissues (brain, gill, heart, liver, intestine, spleen, kidney, muscle, ovary and testis) from 12-month-old rainbow trout as described above. First-strand cDNA was synthesized using Ready To Go You-Prime First-Strand Beads (GE Healthcare Bio-Sciences) with an oligo (dT) primer. The PCR reaction was carried out with *Gsdf*-specific primers. The sense primer was located between nucleotides 2,189 and 2,213 (5′-TCAGAAGCTTCGAGACATTAAATGA-3′), while the antisense primer was located between nucleotides 2,334 and 2,360 (5′-AATATCGTCCCAAAAATAGCATCAA-3′). The

PCR reaction was performed at 94°C for 2 min, followed by 40 cycles of 20 sec at 94°C, 20 sec at 56°C, and 20 sec at 72°C, followed by a final elongation step at 72°C for 3 min. The PCR products were electrophoresed on a 2% agarose gel.

Production of polyclonal antibody

A PCR product encoding amino acids 27-67 of GSDF was inserted in the *Bam* HI and *Sal* I sites of the pET32 vector (Novagen, Darmstadt, Germany). Host bacteria carrying the recombinant constructs were grown at 37°C until log phase, and then induced to express the fusion proteins with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After an 8 h induction period, the bacteria were harvested and the recombinant protein was extracted by the B-PER Bacterial Protein Extraction Reagent (Pierce, IL, USA). Recombinant protein purification was performed using an Ni-NTA gel (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 1.5 mg of purified protein was immunized four times into a rabbit (Keari Co. Ltd., Osaka, Japan). Serum was collected after the fourth injection.

Protein extraction and western blot analysis

Gonadal and somatic tissues (brain, gill, heart, liver, intestine, spleen, kidney and muscle) from adult rainbow trout were prepared for protein extraction. Each tissue was homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 6.8) and 10% glycerol. The lysate was centrifuged at 11,000 rpm for 15 min at 4°C. The supernatants were then collected, and the absorbance was measured at 280 nm. The supernatant containing 30 μg protein was used for the subsequent SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis, as described previously (Boonanuntanasarn et al., 2002). The primary antibody against GSDF was diluted to 1:5000, and the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology Inc., CA, USA) was diluted to 1:1000.

Immunohistochemistry

Tissue sections of rainbow trout embryos and gonads at different stages of development were fixed in Bouin's solution at 4°C for 12 h, embedded in paraffin wax, and then sliced into 5 μm serial sections. Immunohistochemistry was performed as described previously (Yoshizaki et al., 2000b). The primary antibody against GSDF was diluted to 1:500, and the

secondary HRP-conjugated goat anti-rabbit IgG antibody was diluted to 1:200.

Antisense experiments

Antisense gripNA oligonucleotides against nucleotides –15 to +3 (antisense pNA1; 5′-CATTTTTGGAAAGATTGT-3′) and +1 to +18 (antisense pNA2; 5′-AAAGTGCGCAAAATACAT-3′) of *Gsdf* mRNA (sequences complementary to the predicted start codon are underlined) were obtained from Active Motif Inc (CA, USA). Five-base mismatch pNAs, 5mis-pNA1 (5′-CATATTTCGTAACATAGT-3′) and 5mis-pNA2 (5′-AATCTGCGGATATTACAT-3′), were used as negative controls (modified sequences are underlined).

In order to diminish individual variation in PGC number during the antisense experiments, we used gametes obtained from clonal rainbow trout strains that had been maintained at the Nagano Prefectural Fisheries Experimental Station (Nagano, Japan). Gamete collection and insemination were performed as described previously (Takeuchi et al., 2002). Fertilized eggs were activated in 1 mM reduced glutathione solution (pH 8.0) to prevent hardening of the chorion (Yoshizaki et al., 1991). Green fluorescent protein (GFP)-rt/*vasa* RNA (Yoshizaki et al., 2005) together with pNA solution was injected into live

embryos in order to visualize the PGCs. A 2 nl sample of each mixture (containing pNA [20 μg/μl] and GFP-rt/*vasa* RNA [400 ng/μl] in distilled water) was microinjected into the blastodisc of one-cell-stage embryos 2-7 h after fertilization, using the method described by (Yoshizaki et al., 1991). Microinjected eggs were cultured at 10°C. To investigate whether the pNAs interfered with gene expression, the numbers of GFP-positive PGCs in the trout embryos were examined at 20 and 30 dpf under a BX-50 fluorescent microscope, with a BX-FLA attachment and U-MWIB2 filter sets (Olympus, Tokyo, Japan). We counted the number of total PGC of seven embryos from each group. The numbers of PGCs were counted and expressed as a percentage of the average number of PGCs compared with the number in the control embryos without pNA injection.

In order to detect the apoptotic PGCs, we performed DAPI staining. 5μm sliced tissue sections were prepared described above. The paraffin sections were dewaxed and dehydrated by passing them through a xcylen-ethnol series. The sections rinsed with PBS followed by staining NFR for 3 hours. After staining by NFR, the sections were incubated with DAPI staining solution for 15 min. The slides were mounted using Entellan neu. The slides were examined under a BX-50 fluorescent microscope, with a BX-FLA attachment and U-MWU filter sets (Olympus, Tokyo, Japan).

Preparation of conditioned medium (CM) containing recombinant GSDF

CM containing recombinant GSDF was produced from rainbow trout gonad (RTG-2) cells derived from fibroblasts (Wolf and Quimby, 1962). The GSDF cDNA was inserted between the medaka β-actin promoter (Takagi et al., 1994) and the SV 40 poly A^+ signal. This expression vector was transfected into RTG-2 cells by the TransFast Transfection Reagent (Promega), according to the manufacturer's protocol. The transformants were selected after approximately three months of culture at 20°C using the G418 sulfate agent (Promega) in Eagle's Minimum Essential Medium (MEM; Nissui Seiyaku, Tokyo, Japan) supplemented with 5% fetal bovine serum (FBS), 25 mM Hepes and 2 mM L-glutamine adjusted to pH 7.4. After selection with G418 sulfate, the transformed RTG-2 cells were cultured in basal medium modified for spermatogonia, consisting of Leibovitz L-15 medium (Invitrogen, CA, USA) supplemented with 10% FBS and 25 mM Hepes (pH 7.8). CM containing GSDF recombinant protein (GSDF-CM) was collected after six days of culture, and centrifuged at 13,000 rpm for 15 min at 4°C to remove cellular debris. As a control, CM was also prepared from non-transfected RTG-2 (RTG-2-CM). The production and secretion of recombinant GSDF were confirmed by Western blot analysis using CM that had been concentrated 150 times with an ULTRAFREE-0.5 Centrifugal Filter Device-Biomax-5K

(Millipore, MA, USA) according to the manufacturer's protocol.

In vitro culture of spermatogonia

A testicular suspension for use in the *in vitro* culture was prepared from the testes of eight-month-old *vasa*-GFP transgenic rainbow trout (Takeuchi et al., 2002; Yoshizaki et al., 2000b). The samples were dissociated using 2 mg/ml collagenase H (Roche) and 1,000 U/ml dispase (Sanko Junyaku, Tokyo, Japan) in a serum-free basal medium for approximately 7 h at 10°C, with gentle pipetting every 30 min. The cell suspension containing both spermatogonia and somatic cells was then washed twice with basal culture medium. The cells were seeded at a concentration of 2×10^4 per well in a 96-well gelatin-coated dish containing 200 μl samples of various media. To investigate the dose-dependent effect of recombinant GSDF on spermatogonia, 20, 40 and 100 μl aliquots of GSDF-CM were supplemented with basal medium, and the total volume was adjusted to 200 μl by adding plain medium. A 100 μl sample of RTG-2-CM was also added as a negative control. In order to suppress GSDF activity, antisera against GSDF containing 15 or 45 μg IgG was added to the culture medium containing 100 μl GSDF-CM. The samples were cultured for six, nine and 12 days at 10°C, and the medium was changed every three days.

To detect proliferating cells, 5-bromo-2'-deoxyuridine (BrdU) labeling (Sigma, MO, USA) was carried out according to the manufacturer's instructions, with minor modifications. The primary antibody against BrdU (mouse anti-BrdU monoclonal antibody; Chemicon, CA, USA) was diluted to 1:500, and the secondary goat anti-mouse IgG-Fluor Alexa546 antibody (Invitrogen) was diluted to 1:100. Testicular cells were incubated with 18 μM BrdU during the last 24 h of the six-day, nine-day and 12-day culture periods. The samples were then fixed in 4% paraformaldehyde for 25 min at 4°C, and rinsed with phosphate-buffered saline (PBS). After washing, the samples were stained immunohistochemically. The number of GFP-positive spermatogonia showing BrdU immunoreactivity was counted and expressed as a percentage of the total number of GFP-positive spermatogonia. All experiments were replicated three times.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by the Duncan multiple-range test.

Results

Isolation of genital ridge-specific genes from rainbow trout embryos

Of the 600 subtractive cDNA clones analyzed by differential screening, 42 were found to be expressed predominantly in the gonadal tissues. These candidate clones were screened by Northern blot analysis using various adult tissues, and 9 of the 42 showed strong signals in the gonads (data not shown). DNA sequencing revealed that only one of these clones had a signal peptide. We therefore designated this clone GSDF and used it for all further analyses.

The full-length sequence of GSDF contained a 3,328-bp insert cDNA, with an open reading frame (ORF) of 648 bp. The molecular weight was consistent with that estimated from the results of the Northern blot analysis. The ORF encoded a protein containing 215 amino acids, with a calculated molecular mass of 23,486 Da (Fig. 1A). The amino-terminal regions of the clone were rich in hydrophobic amino-acid residues, and were followed by a potential cleavage site comprising Gly and Lys (amino-acid residues 18 and 19; Fig. 1A). A phylogenetic analysis of the mature domain of the cysteine-knot cytokines (TGF-β superfamily, HCG and BDNF) revealed that the GSDF sequence belonged to the TGF-β superfamily branch (Fig. 1B). Although GSDF showed the highest identity (24%) with

zebrafish BMP-7, it was not a member of the BMP family (Fig. 1B). Furthermore, GSDF did not form a subcluster with other known TGF-β superfamily members, suggesting that the GSDF isolated in this study was a novel member of the TGF-β superfamily. Orthologous sequences of trout GSDF were also found in Atlantic salmon, stickleback, dace, fugu, and zebrafish (Fig. 1B)

Gsdf mRNA expression during germ cell development

In situ hybridization of 30-dpf embryos showed that the *Gsdf* mRNA was specifically expressed in genital ridge somatic cells that had direct contact with PGCs (Figs. 2A-F). In 40-dpf, 50-dpf, and 60-dpf embryos, the genital ridge somatic cells were multi-layered (Figs. 2G-L). In these embryos, *Gsdf*-positive signals were detected only in genital ridge somatic cells other than epithelial cells (Figs. 2J-L). In previtellogenic and vitellogenic ovaries, only granulosa cells expressed *Gsdf* mRNA (Figs. 3A-D). In one-year-old testes, which contained mainly type-A spermatogonia, *Gsdf*-positive signals were specifically detected in the Sertoli cells (Figs. 3E and F). In two-year-old testes, which contained all types of spermatogenic cells, *Gsdf*-positive signals were predominantly detected in the Sertoli cells located around the spermatogonia (Figs. 3G-J). By contrast, no

hybridization signal was observed in any cells when the sense probes were applied (data not shown). We were unable to detect *Gsdf* mRNA in embryos before 30 dpf, suggesting that the expression level was relatively low during the early stages of development. By RT-PCR, *Gsdf* mRNA was first detected at 2.5 dpf, which coincided with the onset of zygotic transcription in the rainbow trout embryos. The signal increased in strength until 7 dpf, and remained stable thereafter (Fig. 4).

GSDF protein expression during germ cell development

To determine the distribution of GSDF, we performed Western blot analysis and immunohistochemistry. As shown in Fig. 6A, two major bands of approximately 23 kDa were detected in the ovary and testis. These two bands might be caused by post-translational modification such as glycosilation or processing. RT-PCR analysis performed using adult tissues proved that mRNA is localized specifically in gonadal tissues (Fig. 5). Since we could not detect any amplicons by RT-PCR of heart mRNA, the faint band observed in heart sample of Western blot can be an artifact. Anti-GSDF staining of paraffin sections of 45-dpf embryos revealed that the GSDF protein was exclusively localized in genital ridge somatic cells other than epithelial cells (Fig. 6B), and was not detected earlier than 45 dpf. In the ovary, the

GSDF protein was specifically detected in the granulosa cells (Figs. 6C and D). In the testes of 120-dpf and two-year-old fish, the GSDF protein was localized in the Sertoli cells (Figs. 6E-G), particularly those surrounding type-A spermatogonia (Fig. 6E).

Inhibition of GSDF translation by antisense gripNA

To analyze the effect of GSDF on PGC development during early embryogenesis, we injected several gripNA oligonucleotides (pNAs), together with GFP-rt/*vasa* RNA, into the fertilized eggs of the clonal rainbow trout strains. No obvious abnormalities were found in either the pNA-injected or control-injected embryos, according to external observations (Fig. 7A). The inhibition of GSDF translation by antisense pNAs resulted in a decrease in the number of PGCs (Figs. 7B and C). At 20 dpf, the average numbers of PGCs in the antisense pNA1-injected and pNA2-injected embryos were 49% and 27% lower, respectively, than that of the control embryos without pNAs (average number of PGC, 56) (Fig. 7E). At 30 dpf, the average numbers of PGCs in the antisense pNA1-injected and pNA2-injected embryos were 59% and 64% lower, respectively, than that of the control (average number of PGC, 64) (Fig. 7E). By contrast, both types of 5mis-pNA-injected embryo were unaffected in PGC number (Figs. 7D and E). Furthermore, ectopic PGCs were not found in antisense pNA-injected

embryos. In order to confirm that the PGCs of the antisense pNA-injected embryos did not undergo apoptosis, we performed DAPI staining on paraffin sections of 20 dpf-embryos. We observed five embryos of each group. All PGCs showed normal nuclear morphology (Figs. 7F and G) and did not show apoptotic morphology such as nuclear condensation or fragmentation. These results provide clear evidence that GSDF controls the PGC number in rainbow trout embryos.

Effect of GSDF on spermatogonial growth

To reveal the function of GSDF in the testis, we performed an *in vitro* culture of isolated type-A spermatogonia. CM containing recombinant GSDF was produced from RTG-2 cells. As shown in Fig. 8 A and B, abundant recombinant GSDF was produced and secreted into the CM, and non-transfected RTG-2 cell also produced a small amount of GSDF. Various concentrations of GSDF-CM were added to the testicular cell culture. CM containing the recombinant GSDF promoted the proliferation of type-A spermatogonia in a tendency of dose-dependent manner (Fig. 8C). Although RTG-2-CM alone had mitogenic effects on the type-A spermatogonia (Fig. 8C), GSDF-CM induced a dramatic increase in this activity (Fig. 8C). Furthermore, we confirmed the specificity of this proliferation-enhancing effect on

type-A spermatogonia by the addition of antiserum against GSDF (Fig. 8D), which suppressed the activity. By contrast, adding the control rabbit preimmune serum did not reduce the proliferation-enhancing effect on the type-A spermatogonia (Fig. 8D).

Discussion

Sequence comparisons and phylogenetic analysis of the mature domain of GSDF suggested that this protein was a member of the TGF-β superfamily. However, it could not be easily assigned to any of the known subfamilies of this group: the TGF-β, BMP and activin subfamilies. A homology search using the EST databases of several teleosts identified GSDF-like sequences from the Atlantic salmon, stickleback, dace, fugu, and zebrafish (with amino-acid identities of 90, 44, 39, 37 and 32%, respectively). Furthermore, the teleost GSDFs formed an independent cluster that was distinct from any other known subfamilies. Notably, the non-piscine databases (human, mouse, chicken, and *Xenopus*) did not include any homologous sequences showing high similarity to GSDF. These facts suggest that GSDF is a unique gene that exists only in teleosts.

A whole-genome duplication event is thought to have occurred at the base of the teleost radiation (Meyer and Schartl, 1999). An extra gene created by such a genome duplication could be preserved by obtaining a novel function via a process known as neo-functionalization (Furutani-Seiki and Wittbrodt, 2004). Therefore, it is conceivable that GSDF, which has been found specifically in teleosts, might have originated via a whole-genome duplication event at the base of the teleost radiation, and might have acquired

a novel expression pattern and function during subsequent teleost evolution, namely gonadal somatic cell-specific expression and the enhancement of germ cell proliferation.

A characteristic feature of the members of the TGF-β superfamily is the presence of seven cysteines, which form a three-dimensional structure and dimer in the carboxy-terminal region of the mature protein; the exception to this rule is GDF-9, which contains only six cysteine residues (Kingsley, 1994). Notably, GSDF lacks the seventh cysteine residue, which forms a cysteine knot in the monomer with the third cysteine residue (Lawrence, 1996). In addition, although GSDF has a fourth cysteine residue for dimerization, we were unable to detect the dimerized molecule using Western blot analysis with tissue extracts or recombinant GSDF produced by RTG-2 cells. This strongly indicated that GSDF does not form a dimer, and adopts a different conformation to the other known members of the TGF-β superfamily.

In the ovary and testis, *Gsdf* showed specific and high expression levels in granulosa and Sertoli cells, respectively. According to the results of the *in situ* hybridization, the restricted expression of *Gsdf* mRNA was first detectable at 30 dpf, which was slightly before the time of hatching. At this stage, only the somatic cells surrounding the PGCs expressed *Gsdf*. This confirmed that the genital ridge somatic cells of pre-hatched embryos contained at least two different cell lineages: germ cell-supporting cells, which were expected

to be *Gsdf*-positive; and the progenitors of Sertoli or granulosa cells, and stromal cells, which were *Gsdf*-negative. Moreover, mRNA and protein showed similar expression patterns, suggesting that GSDF expression was regulated mainly at the transcriptional level.

We previously found that the number of rainbow trout PGCs increases approximately 1.9 times between 15 (average number, 29.5; n=10) and 20 dpf (average number, 56; n=10) (Okutsu and Yoshizaki, unpublished data). In the gene-knockdown experiment, we found no sign of nucleus condensation or fragmentation, which is a typical symptom of apoptotic cells, in the PGCs. This suggested that the PGCs of the antisense pNA-injected embryos had not undergone apoptosis. Indeed, the approximate 50% loss of PGCs caused by antisense-pNA injection coincided with the increase in PGC numbers between 15 and 20 dpf, proving that the inhibition of *Gsdf* translation suppressed PGC proliferation. However, due to the technical limitations of *in situ* hybridization, we were unable to detect *Gsdf*-expressing cells in embryos before 30 dpf. Nonetheless, our gene-knockdown results allowed us to predict that *Gsdf* was expressed in the somatic cells adjacent to PGCs at 20 dpf or even earlier. Indeed, PGCs already existed adjacent to the future genital ridge at 17 dpf. Moreover, although PGCs were not incorporated into the genital ridges until 20 dpf, the progenitors of the genital ridge-somatic cells were located proximal to the

PGCs, and GSDF might have regulated PGC proliferation even before they reached the genital ridges. It was clear that GSDF was not essential for PGC migration as no ectopically located PGCs were found in the gene-knockdown experiment. Taken together, our findings suggest that GSDF plays an important role in the proliferation of PGCs.

Our *in vitro*-culture experiments clearly indicated that GSDF-CM had a specific proliferation-enhancing effect on the spermatogonia. This was shown by the dose-dependent effect of GSDF-CM, and the fact that the addition of specific antiserum against GSDF completely suppressed activity. Indeed, the BrdU index of the antiserum-treated group showed an even weaker effect than that of the control. This result suggested that the antiserum blocked the function of GSDF that was endogenously produced and secreted by RTG-2 cells.

The spermatogonial culture experiments used immature testes that contained only single type-A spermatogonia. Recently, Okutsu et al. (2006) reported the existence of a stem cell population in trout spermatogonia. Based on the present results, we cannot determine whether the germ cell proliferation caused by GSDF stimulation affected spermatogonial stem cells (SSCs) or differentiated spermatogonia that had already lost their stem cell activity, because it is difficult to distinguish between these cells based on morphological observations alone. In mammalian testes, SSCs are morphologically distinct, as the only spermatogonia

without an intracellular bridge are single type-A spermatogonia (De Rooij and Russell, 2000). Therefore, we believe that GSDF most likely enhanced the proliferation of SSCs in the rainbow trout testes. Our data showing that GSDF is predominantly expressed in the Sertoli cells surrounding single type-A spermatogonia support the results of the *in vitro*-culture experiments.

The predominant expression of GSDF in granulosa cells led us to consider it as a potential regulator of reproductive function in the ovary. As the oocytes did not proliferate, we believe that the GSDF function in the ovary differs from that in the testis. In fish, multiple functions of activin, another member of the TGF-β superfamily, have been reported, including the induction of oocyte maturation (Pang and Ge, 2002) and spermatogonial proliferation (Miura and Miura, 2001). It is possible that GSDF also has multiple functions.

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Figure legends

Fig. 1. GSDF is a distinct and novel member of the TGF-β superfamily. (A) Deduced amino-acid sequence of GSDF. The signal peptide is indicated in italics. The six conserved cysteine residues are indicated by underlining. (B) The unweighted pair-group method with arithmetic mean (UPGMA) tree for the TGF-β superfamily and GSDF-like proteins of teleosts. The tree was rooted using mouse HCG.

Fig. 2. *Gsdf* mRNA is specifically expressed in gonadal somatic cells of embryos. *Gsdf* expression revealed by whole-mount *in situ* hybridization: A, lateral view of a 30-dpf embryo; B, ventral view of a 30-dpf embryo. *Gsdf* expression revealed by section *in situ* hybridization (D, J, K and L). C and D show sagittal sections of a 30-dpf embryo. E and F are high magnification of C and D, respectively. G-L show transverse sections of sexually undifferentiated gonads. *Gsdf* expression was restricted to the genital ridge somatic cells surrounding the PGCs (arrowheads) at 30 dpf (C and D), 40 dpf (G and J), 50 dpf (H and K), and 60 dpf (I and L). The boxes in the schematic drawings in the insets show the location of each picture (A-C). The dotted lines in D indicate the outline of the genital ridge. The asterisks indicates GSDF-negative cell. The scale bars represent 300 μm (A and B), 15 μm

(C-L), respectively.

Fig. 3. *Gsdf* mRNA is specifically expressed in gonadal somatic cells of ovary and testis. *Gsdf* expression revealed by section *in situ* hybridization (B, F and H). C, D, I and J are high magnification images of A, B, G and H, respectively. *Gsdf* was expressed in the granulosa cells (gc) of the one-year-old ovary (A and B), and the Sertoli cells (arrows) surrounding the spermatogonia (arrowheads) of the one-year-old testis (E and F) and two-year-old testis (G and H). A and G show serial sections of B and H, respectively. A and E were stained by hematoxylin and eosin. G-J were stained by Nuclear Fast Red. oc, oocyte; sg, spermatogogonia; sc, spermatocyte; st, spermtid; sp, sperm. The scale bars represent 200 μm (A and B), 25 μm (C and D), 20 μm (E and F), 100 μm (G and H) and 50 μm (I and J) respectively.

Fig. 4. *Gsdf* expression begins before the formation of the genital ridge. PCR amplification was performed using *Gsdf*-specific primers. cDNAs from unfertilized eggs and whole embryos (2.5, 7, 10, 15, 20, and 30 dpf) were used for PCR. *Gsdf* expression started at 2.5 dpf. β-actin was used as an internal control for RT-PCR amplification. Lane PC was a positive

control using ovary cDNA. Lane NC was a negative control containing no cDNA template.

Fig. 5. *Gsdf* expression restricts only in gonadal tissues. PCR amplification was performed using *Gsdf*-specific primers. cDNAs from various tissues (brain, gill, heart, kidney, liver, intestine, spleen, muscle, ovary and testis) were used for PCR. *Gsdf* expression restricted only in gonadal tissues. β-actin was used as an internal control for RT-PCR amplification. Lane NC was a negative control containing no cDNA template.

Fig. 6. GSDF protein is specifically expressed in gonadal somatic cells. (A) Western blot analysis of the GSDF protein. Protein samples (30 μg) extracted from the indicated tissues were added to each lane and immunostained with a specific antibody against GSDF. The immunohistochemical identification of GSDF-positive cells was carried out on the tissue sections using a specific antibody against GSDF (B-G). GSDF expression was restricted to the genital ridge somatic cells surrounding the PGCs (arrowheads) in the transverse section of the 45-dpf gonad (B). GSDF was expressed in the granulosa cells (gc) of the one-year-old ovary (C and D) and the Sertoli cells (arrows) surrounding the spermatogonia (arrowheads) of the 120-dpf testis (E) and the two-year-old testis (F and G). D and G are high magnification

views of C and F, respectively. The scale bars represent 20 μm (B), 100 μm (C), 50 μm (D), 15 μm (E), and 40 μm (F and G), respectively. oc, oocyte; sg, spermatogonia; sc, spermatocyte; st, spermtid.

Fig. 7. Knockdown of GSDF by antisense pNA leads to a loss of PGCs. (A) External observation of 20 dpf embryos. No obvious abnormalities were seen in the following: control, control embryo without pNA injection; antisense, antisense pNA1-injected embryo; and 5mis, 5mis-pNA1-injected embryo. The scale bars represent 2 mm. Fluorescence images of the trunk region from a control embryo injected without pNA (B), an embryo injected with antisense pNA1 (C), and an embryo injected with 5mis-pNA1 (D). I indicates the area of the intestine showing auto-fluorescence. Arrowheads indicate the GFP-positive PGCs. The scale bars represent 50 μm. (E) Effects of antisense-pNA injection on the number of PGCs in 20-dpf and 30-dpf embryos. The percentage of GFP-positive PGCs in the pNA-injected embryos compared with the control embryos without pNA injection is shown by the y-axis. The results are given as the mean ± SEM. Values with the different lowercase letters are significantly different from one another ($P<0.05$). (F) (G) Nuclear staining of PGCs (arrows) of antisense-pNA1 injected embryo (F) and 5mis-pNA1 injected embryo (G). left, DAPI;

right, Nuclear Fast Red (NFR). The scale bars represent 10 μm.

Fig. 8. Recombinant GSDF enhances the proliferation of spermatogonia *in vitro*. Western blot analysis of RTG-2 lysate proteins (A) and secreted proteins from RTG-2 (B) using a specific antibody against GSDF. M indicates the molecular-weight marker. RTG-2 indicates the lysate protein extracted from non-transfected RTG-2 cells. GSDF-RTG-2 indicates the lysate protein extracted from the GSDF-transfected RTG-2 cells. RTG-2-CM indicates the CM from non-transfected RTG-2 cells. GSDF-CM indicates the CM from GSDF-transfected RTG-2 cells. P indicates 30 μg protein extracted from the one-year-old testis. (C and D) The proliferation of spermatogonia was quantified by a BrdU-incorporation assay using recombinant GSDF in a type-A spermatogonial culture system. GSDF had a dose-dependent effect on the proliferation on type-A spermatogonia (C). Specific antiserum against GSDF inhibited the proliferative effect of GSDF on the type-A spermatogonia (D). The results are given as the mean ± SEM. Values with the different lowercase letters are significantly different from one another ($P<0.05$). Control, basal medium; RTG2-50%, basal medium containing 50% RTG-2-CM; GSDF-10%, basal medium containing 10% GSDF-CM; GSDF-20%, basal medium containing 20% GSDF-CM; GSDF-50%, basal medium

containing 50% GSDF-CM; anti-15, GSDF-50% containing 15 μg IgG from antiserum; anti-45, GSDF-50% containing 45 μg IgG from antiserum; pre-45, GSDF-50% containing 45 μg IgG from preimmune serum.

Fig. 1

A

*MYFAHFVMMLVLFGCSLG*KSFVLQSSEKEPAAATGSAVLHTDRCHG
ELLNDIRKTLIGALNLQQEPQVAADRLTAIREQWKTAFSAIPHKTQ
DKAVALTQAEGPAADNSSGLICCPLASQIFLKDLGWENWVIYPESF
TYVQCSPCKSRLDLSPSRCPSHAPPAQDTPSQMPCCQTTSTEHVPF
LYMDEFSTLTIPSVQLTRACGPGNPQLPAED

B

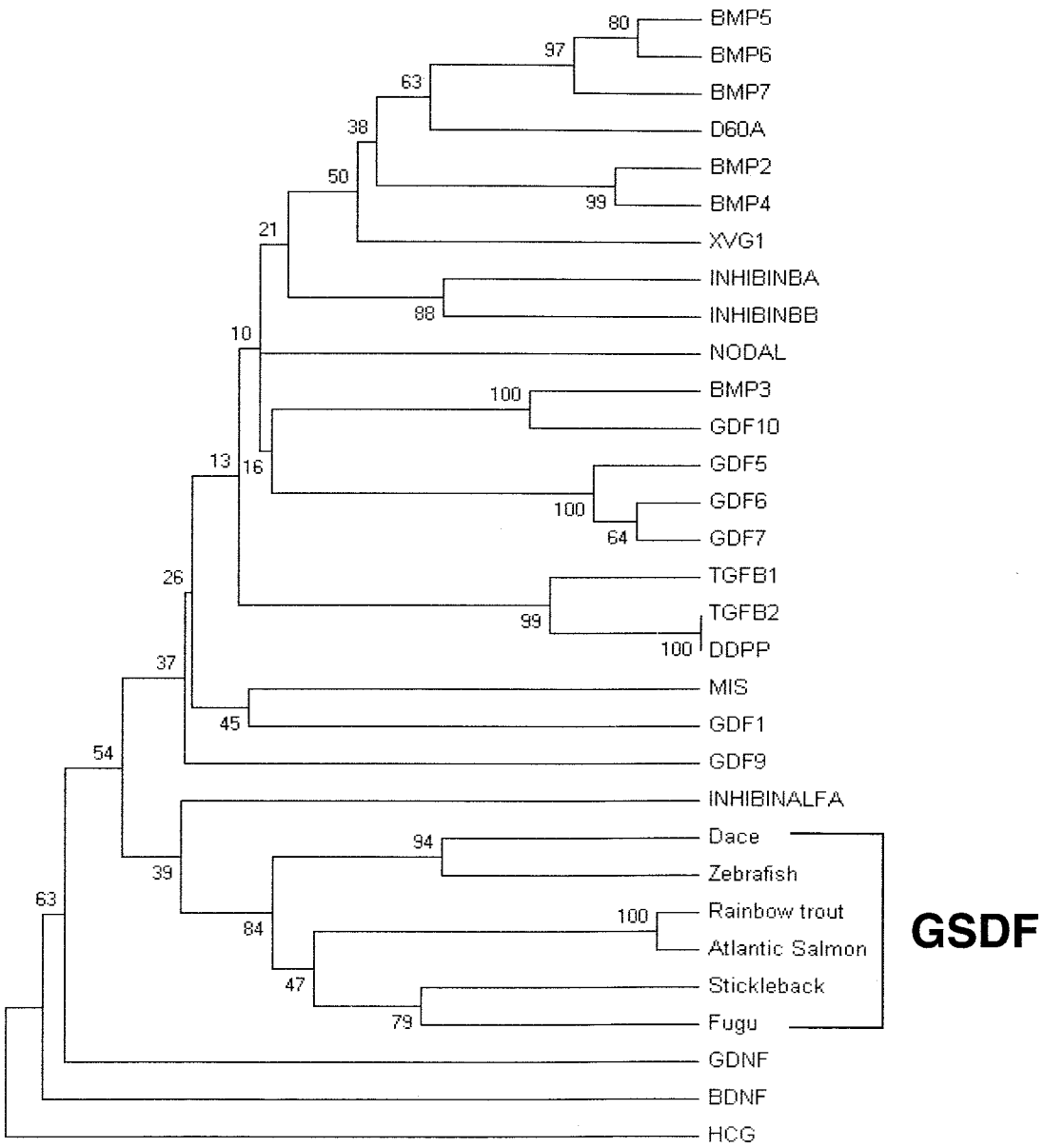


Fig. 2

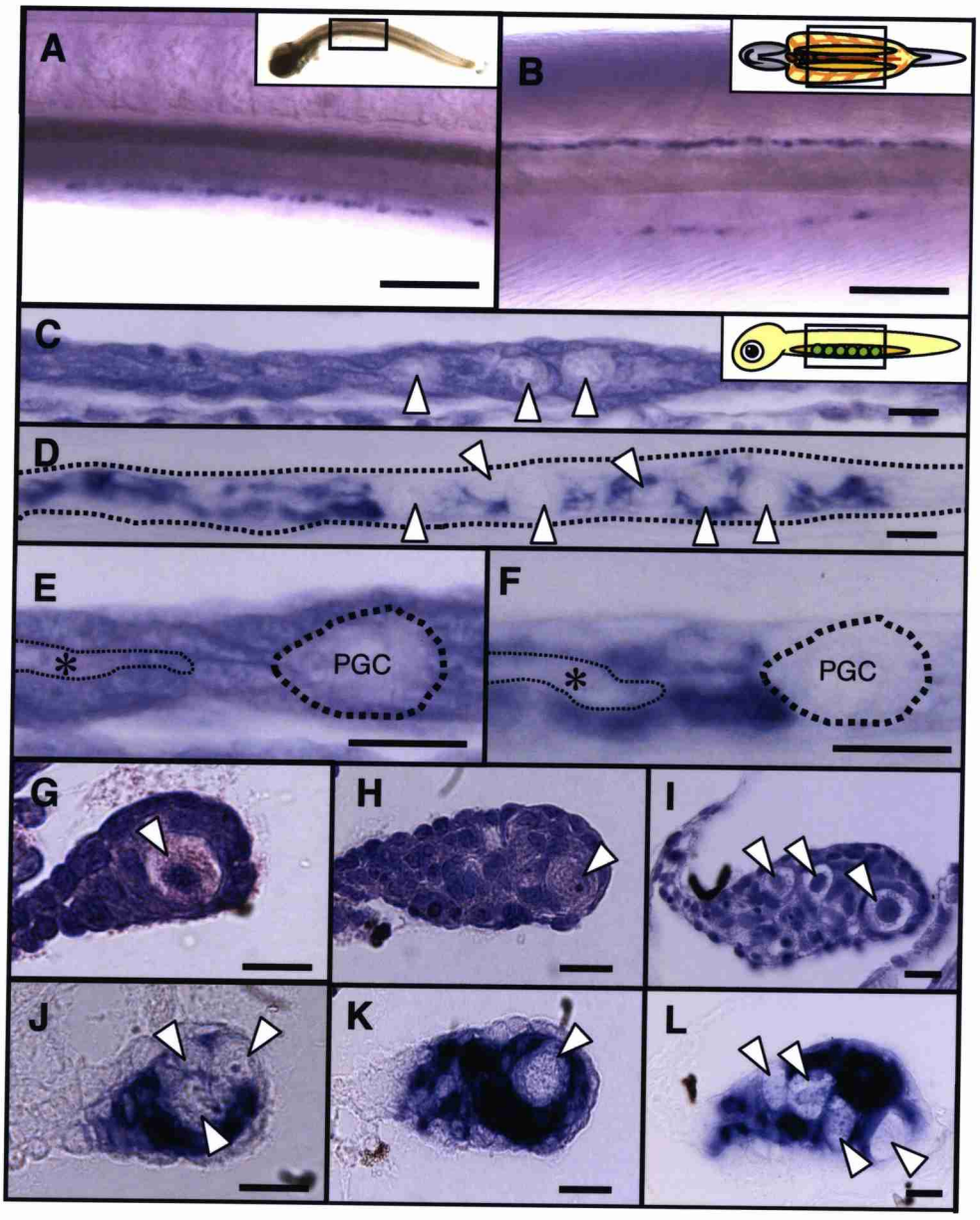
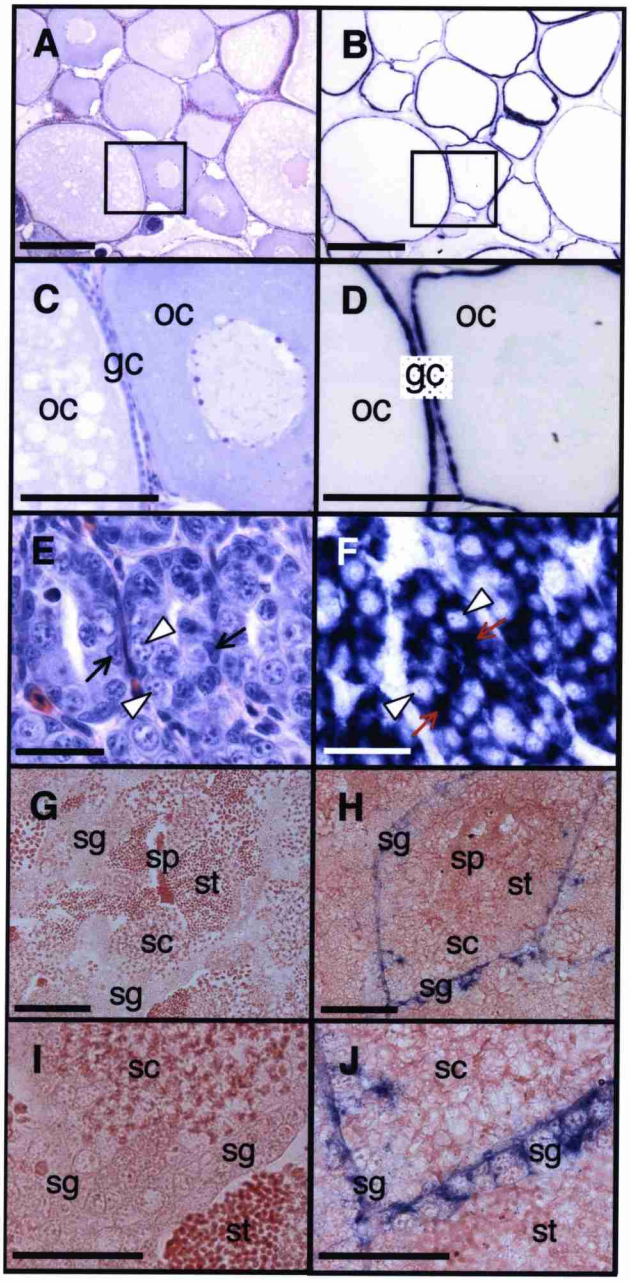


Fig. 3


A
B
C
oc
gc
oc
D
oc
gc
oc
E
F
G
sg
sp
st
sc
sg
H
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sp
st
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sg
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J
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sg
st

Fig. 4

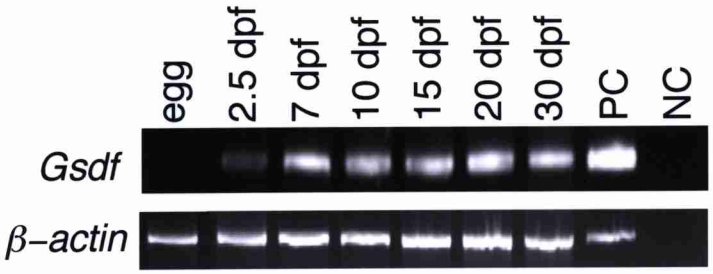


Fig. 5

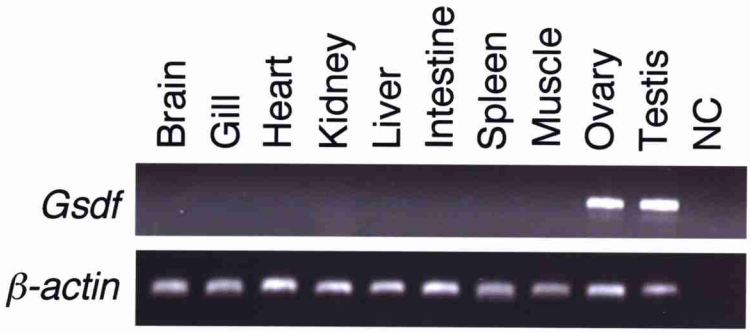

Brain
Gill
Heart
Kidney
Liver
Intestine
Spleen
Muscle
Ovary
Testis
NC
Gsdf
β-actin

Fig. 6

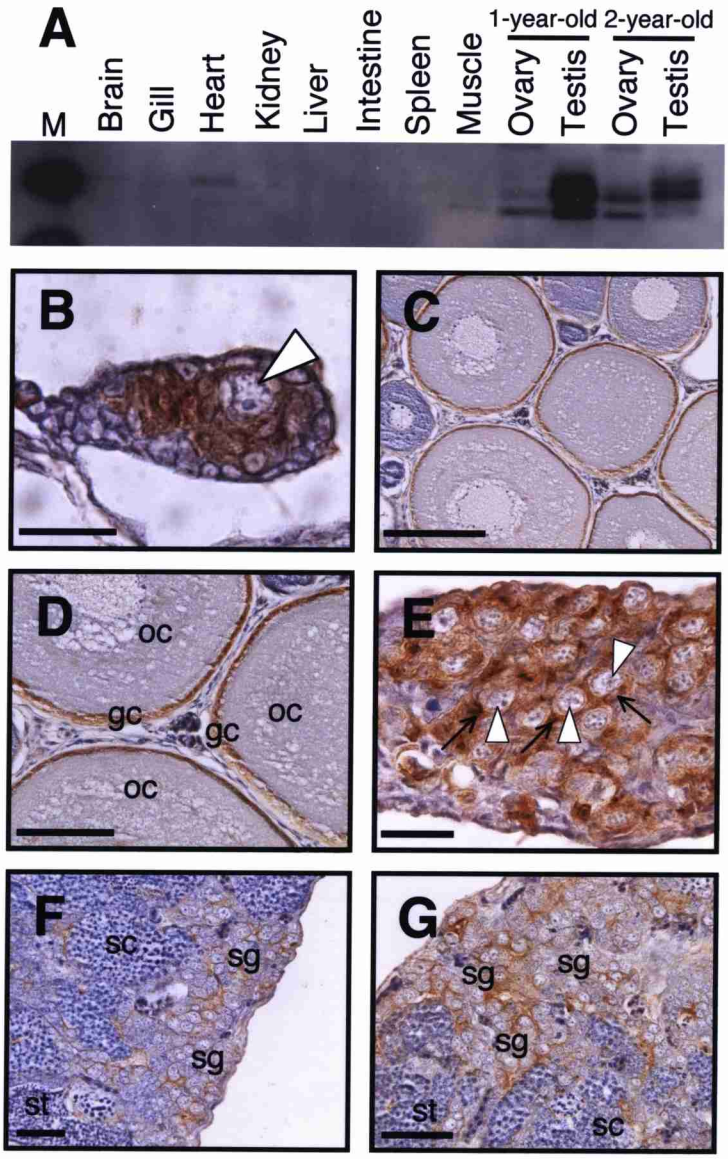

A
M
Brain
Gill
Heart
Kidney
Liver
Intestine
Spleen
Muscle
1-year-old
Ovary
Testis
2-year-old
Ovary
Testis
B
C
D
oc
gc
gc
oc
oc
E
F
sc
sg
sg
st
G
sg
sg
sg
st
sc

Fig. 7

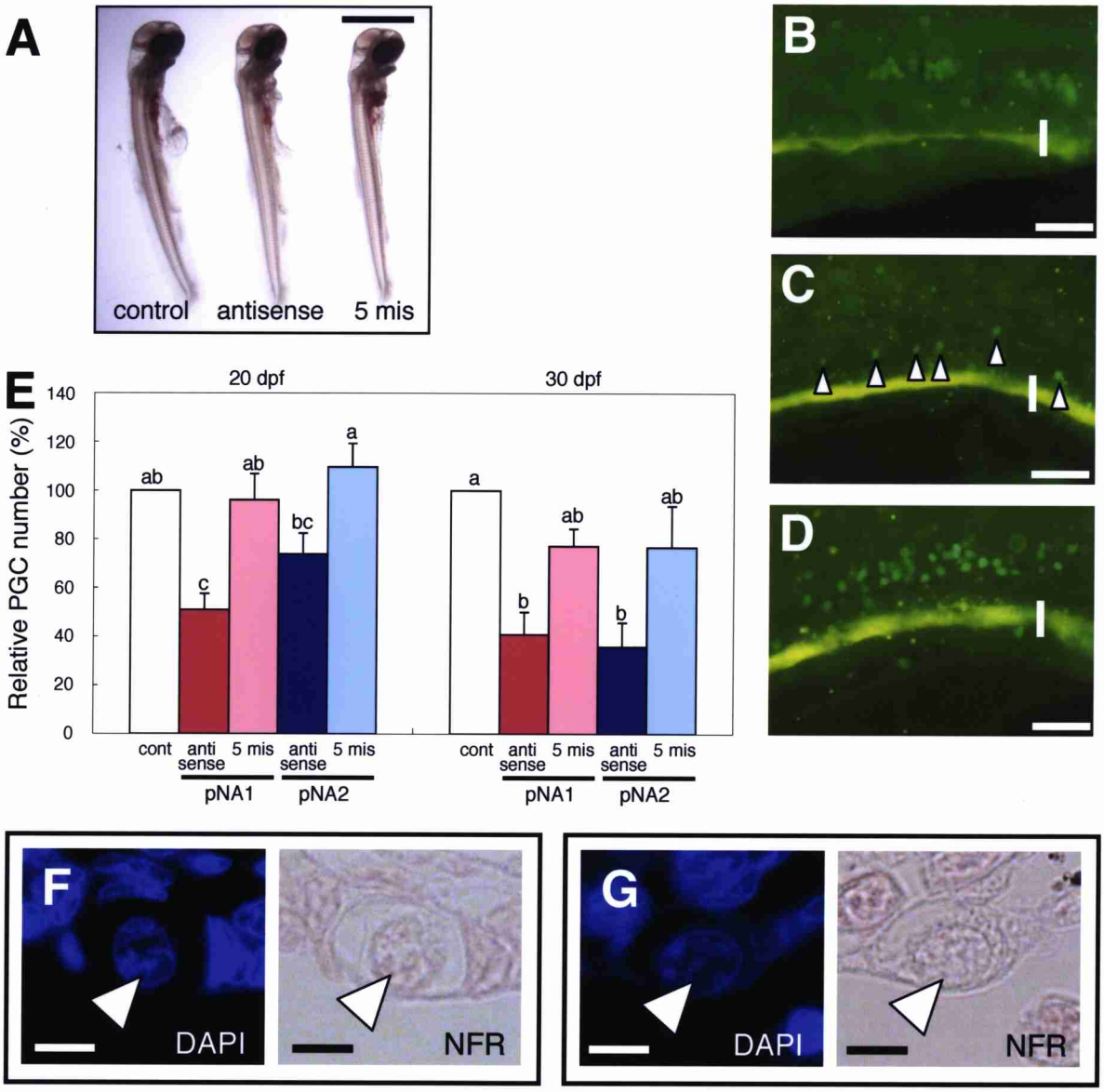
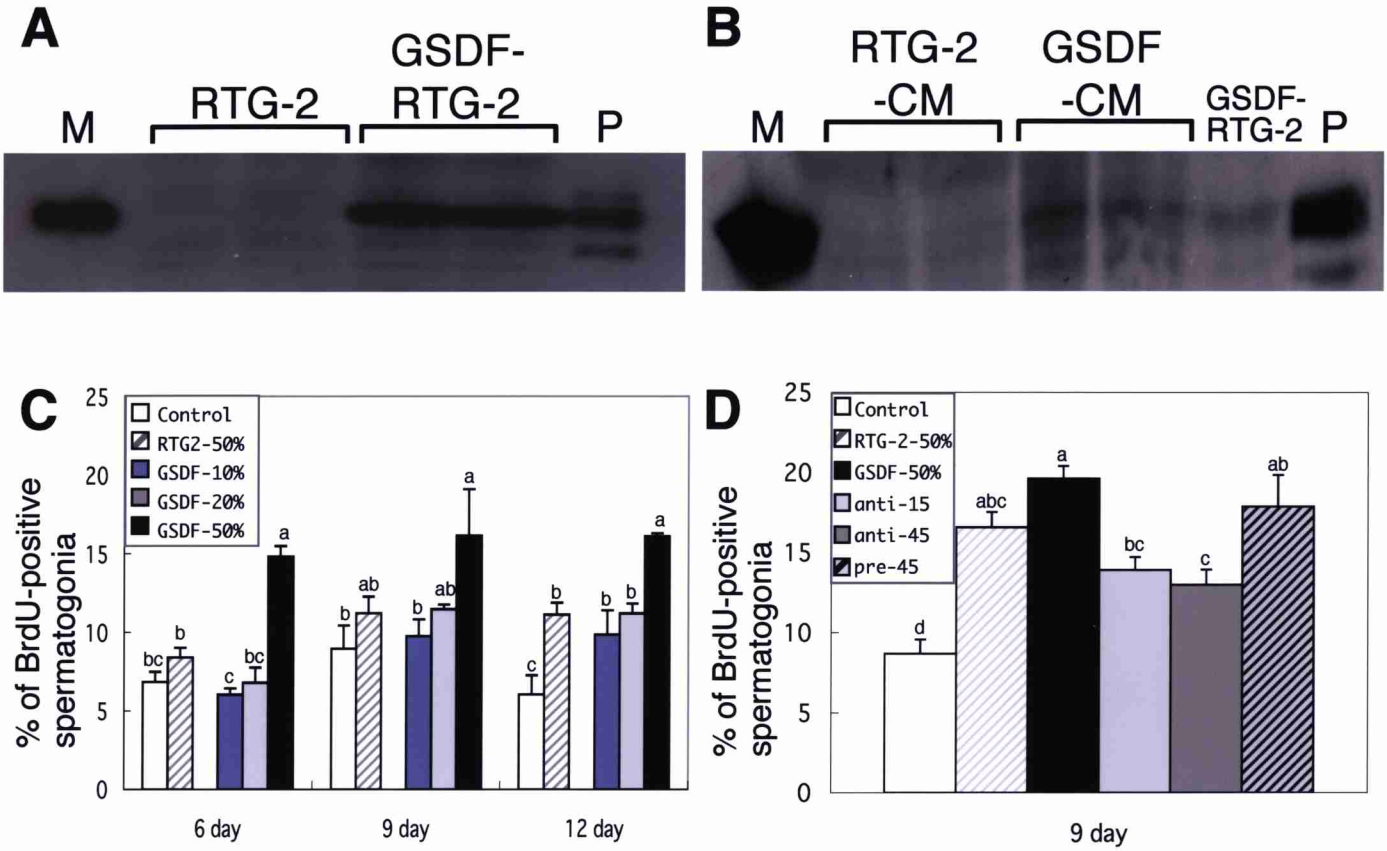

A
control
antisense
5 mis
B
C
D
E
20 dpf
30 dpf
Relative PGC number (%)
0
20
40
60
80
100
120
140
ab
c
ab
bc
a
a
b
ab
b
ab
cont
anti sense
5 mis
anti sense
5 mis
pNA1
pNA2
F
DAPI
NFR
G
DAPI
NFR

Fig. 8


A
M
RTG-2
GSDF-RTG-2
P
B
M
RTG-2 -CM
GSDF -CM
GSDF-RTG-2
P
C
% of BrdU-positive spermatogonia
Control
RTG2-50%
GSDF-10%
GSDF-20%
GSDF-50%
6 day
9 day
12 day
D
% of BrdU-positive spermatogonia
Control
RTG-2-50%
GSDF-50%
anti-15
anti-45
pre-45
9 day

総　括

本研究では、硬骨魚類にのみ存在する分子である新規サイトカイン GSDF の単離に成功した。また、GSDF が始原生殖細胞および A 型精原細胞の増殖促進能を有することを明らかにした。本研究で単離した GSDF は、魚類始原生殖細胞の増殖を促進する因子としては世界で初めて単離されたサイトカインであり、今後、魚類始原生殖細胞研究への貢献が期待される。さらに、本研究で得られた知見は、今後の魚類始原生殖細胞および精原幹細胞の培養技術の発展、ひいては代理親魚養殖技術の大幅な効率化に大きく貢献できるものと予想される。

魚類始原生殖細胞および精原幹細胞の *in vitro* 培養は、代理親魚養殖技術への貢献のみならず、遺伝子ターゲティング技法の確立にもつながる。遺伝子ターゲティング技法は、魚類育種や遺伝子ノックアウト技法への利用が期待されている（吉崎, 2002)。従来のマイクロインジェクション法やエレクトロポーレーション法による遺伝子改変は、導入した外来遺伝子がランダムに宿主染色体に挿入されるため、外来遺伝子の発現量や発現部位の正確な調節が困難であるうえ、内在遺伝子の機能に予測不可能な影響を与える可能性も否定できなかった（Devlin et al., 2001)。一方、遺伝子ターゲティング技法は「相同遺伝子組換え」を利用することで、染色体上の任意の場所に正確に外来遺伝子を挿入入可能であり、その発現制御も正確に行われると考えられる。また、相同遺伝子組換えが行われた細胞を *in vitro* で選別することができるため、外来遺伝子に由来する

有用形質を保持する個体を、短期間かつ小さなスペースで得ることが可能になる。このような技術を実用化するため、魚類における胚性幹細胞（embryonic stem cell; ES 細胞）株樹立の試みが、多くの研究者によってなされてきた。しかし、形態学的、生化学的特徴はマウス ES 細胞と類似した細胞株が、メダカやゼブラフィッシュの胞胚細胞から樹立されてはいるものの（Helmrich and Barnes, 1999; Hong et al., 2000; Fan et al., 2004）、長期間培養したこれらの細胞が宿主内で生殖細胞系列に分化したという報告はない。遺伝子ターゲティング技法を完成させるためには、用いる培養細胞が ES 細胞のような全能性を有することは必須条件ではなく、生殖細胞系列のみに分化できる能力を備えていれば良い。この考えに基づくと、本技術で用いるべき細胞は生殖細胞系列への分化が決定づけられた細胞、すなわち、始原生殖細胞および精原幹細胞であると言える。実際にマウスにおいては、始原生殖細胞由来の株化細胞（embryonic germ cell; EG 細胞）が樹立されており、生殖細胞系列への分化能のみならず、ES 細胞同様に全能性を有することが明らかになっている（Matsui et al., 1992; Durcova-Hills et al., 2001）。さらに近年樹立された、新生児マウスの精原幹細胞由来の GS 細胞は、全能性を有していないものの、生殖系列への分化が決定付けられており、本細胞を用いた遺伝子ノックアウトマウスも作出されている（Kanatsu-Shinohara et al., 2005a）。また、ES 細胞は長期培養によって生殖細胞系列への分化能を失うことがあるが、GS 細胞は 2 年以上もの長期培養後も正常な状態を保つことができ、その安定性

はES細胞に比べて非常に高いことも報告されている（Kanatsu-Shinohara et al., 2005b)。このように、生殖細胞系列への分化が決定付けられた始原生殖細胞・精原幹細胞は、遺伝子ターゲティングを行うにあたって非常に強力なツールとなる。魚類においてもこれらの細胞の培養細胞株が樹立されることで、従来、全く利用不可能であった遺伝子ターゲティング技術が魚類でも確立することが期待される。

現在、ニジマスA型精原細胞の全てが精原幹細胞であるのか、またはその一部のみが精原幹細胞であるのかは不明である。しかし、本研究室で行われたニジマスA型精原細胞の移植実験より、移植されたA型精原細胞の少なくとも一部が幹細胞能を保持していたことが示されている（鈴木 2005)。以上のことから、GSDFの刺激により増殖したA型精原細胞が精原幹細胞である可能性が示唆されるが、今後は移植実験により、増殖したA型精原細胞が幹細胞能を保持しているか否かを確認することが急務である。また、GSDFはA型精原細胞に対する高い増殖促進効果を示したものの、その株化には至らなかった。本研究の培養実験に用いたconditioned mediumに含まれる組換えGSDFは非常にわずかな量であったと考えられるため、今後、昆虫細胞等により生産された高濃度の組換え体を培養系に用いることで、A型精原細胞のより高い増殖が期待される。

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謝　辞

本研究の遂行および論文の作製にあたり、終始丁寧な御指導、御鞭撻、および御校閲を賜りました東京海洋大学海洋科学部海洋生物資源学科水族養殖学研究室教授竹内俊郎教授、および同水族生理学研究室助教授吉崎悟朗博士に厚くお礼申し上げます。

また、材料の入手にあたり、御便宜を図って下さった東京海洋大学水圏科学フィールド教育研究センター大泉ステーション三井拓也技官、熊沢　勝技官、佐久間賢志技官、ならびに、長野県水産試験場環境部細江　昭氏、小原昌和氏、降幡　充氏に深く感謝いたします。

さらに、実験を行うにあたって有益な御助言を頂いた東京海洋大学先端科学技術研究センター助手竹内　裕博士に深く感謝いたします。最後に、研究遂行に際して適切な御助言と御指導を頂いた水族養殖学研究室・水族生理学研究室の学生、卒業生の皆様に対し、心からの感謝の意を表します。