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Expression patterns of *gdnf* and *gfr α 1* in rainbow trout testis

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15 **Abstract**

16 In mice, glial cell line-derived neurotrophic factor (GDNF) is essential for normal
17 spermatogenesis and *in vitro* culture of spermatogonial stem cells. In murine testes, GDNF
18 acts as paracrine factor; Sertoli cells secrete it to a subset of spermatogonial cells expressing
19 its receptor, GDNF family receptor $\alpha 1$ (GFR $\alpha 1$). However, in fish, it is unclear what types
20 of cells express *gdnf* and *gfr $\alpha 1$* . In this study, we isolated the rainbow trout orthologues of
21 these genes and analyzed their expression patterns during spermatogenesis. In rainbow
22 trout testes, *gdnf* and *gfr $\alpha 1$* were expressed in almost all type A spermatogonia (ASG).
23 Noticeably, unlike in mice, the expression of *gdnf* was not observed in Sertoli cells in
24 rainbow trout. During spermatogenesis, the expression levels of these genes changed
25 synchronously; *gdnf* and *gfr $\alpha 1$* showed high expression in ASG and decreased
26 dramatically in subsequent developmental stages. These results suggested that GDNF most
27 likely acts as an autocrine factor in rainbow trout testes.

28

29 **1. Introduction**

30 Germ line stem cells are the only cell lineage that undergo self-renewal and
31 distribute genetic material to subsequent generations. Spermatogonial stem cells (SSCs)
32 are a subset of undifferentiated spermatogonia and are critically important for
33 spermatogenesis because of their ability to self-renew and generate a large number of
34 sperm progenitors over a long reproductive period (Yoshida, 2010). Their self-renewal and
35 differentiation are believed to be controlled by secretory factors produced in SSC niches
36 (de Rooij, 2009; Oatley et al., 2011).

37 Glial cell line-derived neurotrophic factor (GDNF) is a secretory factor produced in
38 SSC niches in mice. GDNF is a distant member of the transforming growth factor- β
39 (TGF- β) superfamily that was originally isolated from rat glioma cell-line supernatant as a
40 trophic factor for midbrain neurons (Lin et al., 1993). It signals via a surface receptor
41 complex composed of GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) and Ret receptor tyrosine
42 kinase (Ret) (Sariola and Saarma, 2003). In mouse testes, GDNF acts as paracrine factor
43 secreted from Sertoli cells to undifferentiated spermatogonia expressing GFR $\alpha 1$ (Viglietto
44 et al., 2000). Gene-targeted mice with one GDNF-null allele show a decreased total

number of germ cells and depletion of SSCs (Meng et al., 2000). To overcome the neonatal lethality of *Gdnf* deficient mice, whole-testis transplantation has been performed (Naughton et al., 2006). Transplanted *Gdnf*-deficient testes revealed that the disruption of GDNF-mediated signaling results in a failure of spermatogenesis due to deficient SSC self-renewal. Furthermore, a reduction of *Gfral* expression in type A spermatogonia (ASG) induced a decrease of proliferation of SSCs and their phenotypic differentiation (He et al., 2007). In contrast, testes that overexpress GDNF accumulate undifferentiated spermatogonia (Meng et al., 2000; Grisanti et al., 2009). Taken together, these reports indicate that GDNF-mediated signaling is essential for SSC proliferation and maintenance. Thus, in mice, SSC niches have been well studied by focusing on the expression patterns and functions of *gdnf* and *gfral*. However, information on the SSC niches in lower vertebrates, including fish, is quite limited. Therefore, it is important to analyze the expression patterns of *gdnf* and *gfral* in other vertebrates.

Rainbow trout (*Oncorhynchus mykiss*) is a suitable model fish for the following reasons. First, there exist two transgenic rainbow trout strains: *pvasa-Gfp* and *pinhibin-DsRed*. In *pvasa-Gfp* rainbow trout, spermatogonia are labeled by green

fluorescence protein (GFP) under the control of the *vasa*-gene regulatory region (Yoshizaki et al., 2000b; Yano et al., 2008), which enables enrichment of ASG, including SSCs, according to the intensity of green fluorescence (Okutsu et al., 2006a; Hayashi et al., 2012). In *pinhibin-DsRed* rainbow trout, Sertoli cells are labeled by DsRed under the control of the *inhibin*-gene regulatory region (Banba and Yoshizaki, unpublished data), which enables enrichment of Sertoli cells according to the intensity of red fluorescence (Yagisawa and Yoshizaki, unpublished data). Second, SSC activity can be evaluated by a spermatogonial transplantation assay (Okutsu et al., 2006a). Third, the marker genes of each cell type, Sertoli cells, *gsdf* (Sawatari et al., 2007); a Leydig cell, *3 β -HSD* (Sakai et al., 1994); and germ cells of each developing stage, *vasa*, *rtili* and *txndc6* (Yano et al., 2008; Rolland et al., 2009). Therefore, as a first step to increase our knowledge of fish GDNF, we report the cloning and expression analysis of rainbow trout GDNF and GFR α 1 in this study.

2. Results

2.1. Cloning of rainbow trout *gdnf* and *gfra1* homologues

The cDNA sequence of rainbow trout *gdnf*, which contains the complete open reading frame (ORF), was obtained by RT-PCR using degenerate primers and subsequent 3'RACE PCR and 5'RACE PCR, and deposited in GenBank under accession number AB787266. The ORF was 711 bp and encoded 236 amino acids containing characteristic features of the TGF- β superfamily: an N-terminal signal peptide and seven conserved cysteines (Fig. 1A). BLAST analysis revealed that this sequence was most similar to the zebrafish *gdnf* orthologue. A phylogenetic analysis of the TGF- β superfamily clarified that rainbow trout GDNF belongs to the GDNF branch (Fig. 1B).

We also isolated rainbow trout *gfra1* cDNA. The complete ORF was obtained by RT-PCR using degenerate primers and subsequent 3'RACE and 5'RACE PCR. The sequence was deposited in GenBank under accession number AB787265. It was 1131 bp and encoded 376 amino acids containing characteristic features of other GFR α 1 orthologues such as an N-terminal signal peptide (Fig. 1A). Rainbow trout GFR α 1 contained the 26 conserved cysteines corresponding to the regions of mouse and zebrafish

GFR α 1s (Fig. 2A). A sequence comparison by BLAST analysis revealed that this isolated gene was most similar to zebrafish *gfra1a*. Phylogenetic analysis of GFR α members clarified that rainbow trout GFR α 1 belongs to the GFR α 1 branch (Fig. 2B).

2.2. Identification of cells expressing *gdnf* and *gfra1* by histology

In fish, including rainbow trout, spermatogonia are classified morphologically as type A or type B. The classification criteria are different from those of mouse spermatogonia. ASG are singly isolated larger germ cells surrounded by Sertoli cells. Type B spermatogonia (BSG) are smaller and organized into cysts where they synchronously divide and develop into spermatocytes.

To identify what types of cells express *gdnf* and *gfra1*, we performed *in situ* hybridization on paraffin sections of immature testes containing only ASG from 9-month-old rainbow trout (body weight, 29.4 g; Gonadosomatic Index (GSI) (%) = gonadal weight/body weight $\times$ 100, 3.73×10^{-2}). The results of *in situ* hybridization using a *gdnf* probe showed that positive signals were detected in ASG (Fig. 3A and B), as compared to sense probe control (Fig. 3C and D). Consistent with the expression of *gdnf*

mRNA, immunostaining using anti-GDNF antibody revealed that GDNF (red in Fig. 4E and F) was also localized in ASG (green in Fig. 4D and F), which were singly isolated by the GSDF positive Sertoli cells (red in Fig. 4B and C) and whose cell cycle phases were asynchronous (Fig. 4D-G).

It was difficult to completely eliminate the possibility that the above-mentioned signals of *gdnf* detected in ASG were caused by diffused signals from Sertoli cells, since Sertoli cells are located contiguously with spermatogonia and are very thin with extended cytoplasm. To clarify this question, we performed *in situ* hybridization against dissociated testicular cells smeared on glass slides. Cell smears were prepared with dissociated testicular cells of 10-month-old *pvasa-Gfp* rainbow trout (body weight, 39.1 ± 3.05 g; GSI, $6.47 \pm 1.44 \times 10^{-2}\%$). ASG were clearly distinguished by their green fluorescence (Fig. 3E). *In situ* hybridization against smear preparations showed that $85.5 \pm 3.8\%$ (N=4; 56, 103, 59, and 57 ASG were randomly selected in each experiment) of ASG had clear signals of *gdnf* mRNA (Fig. 3F).

Next, to identify the cells expressing *gfra1*, we also performed *in situ* hybridization and immunostaining on paraffin sections of immature rainbow trout testes. In immature

testes, *gfra1* mRNA was localized in ASG (Fig. 5). In addition, GFR α 1 protein (red in Fig. 6E and F) was also localized in ASG (green in Fig. 6D and F), which were singly isolated by the GSDF-positive Sertoli cells (red in Fig. 6B and C) and whose cell cycle phases were asynchronous (Fig. 6G-I).

2.3. Expression analysis of *gdnf* and *gfra1* by RT-PCR

From the above-mentioned results, it was difficult to completely rule out the possibility that some of the *gdnf* mRNA or protein also exist in both Sertoli cells and ASG. Therefore, to further identify the cell types expressing *gdnf* and *gfra1*, we performed RT-PCR using GFP+, DsRed+, GFP- and DsRed-, and unsorted cells isolated from double transgenic rainbow trout carrying *pvasa-Gfp* and *pinhibin-DsRed*. First, we identified cell types enriched in each fraction by RT-PCR using the germ cell marker *vasa* (Yoshizaki et al., 2000a), the Sertoli cell marker *gsdf* (Sawatari et al., 2007), and the Leydig cell marker *3 β -HSD* (Sakai et al., 1994). The results revealed that spermatogonia (*vasa*+), Sertoli cells (*gsdf*+), and interstitial cells including Leydig cells (*3 β -HSD* +) were enriched in GFP+, DsRed+, and GFP- and DsRed- cell fractions, respectively (Fig. 7). An amplified signal of

gdnf was clearly detected in the GFP+ cells but not in the DsRed+ cells (Fig. 7). *Gfra1* transcripts were detected in GFP+ cells, and GFP- and DsRed- cells (Fig. 7).

2.4. *Gdnf* and *gfra1* localization during testis development

To elucidate the expression patterns of *gdnf* and *gfra1* during spermatogenesis, we performed *in situ* hybridization against paraffin sections of 2-year-old rainbow trout testes containing all stages of male germ cells (from ASG to spermatozoa). Developmental stages of male germ cells were identified by morphological observation with hematoxylin-eosin staining (Fig. 8A, D, G, J, and M) and the expression of marker genes for each developmental stage (Fig. 8C, F, I, and L). As mentioned above, ASG are singly isolated larger germ cells surrounded by Sertoli cells (Fig. 8A), and BSG are smaller and organized into cysts (Fig. 8D). In addition, ASG, BSG, and spermatocytes express marker genes: *vasa*, *rtili*, and *txndc6*, respectively (Fig. 8C, F, I, and L) (Yano et al., 2008; Rolland et al., 2009).

In maturing testes, ASG showed strong signals with the *gdnf* probe (Fig. 8B). As germ cell development progressed, the expression of *gdnf* was dramatically decreased (Fig. 8E, H, and K). Finally, the expression of *gdnf* was not detectable in spermatids and

155 spermatozoa (Fig. 8N).

156 In maturing testis, *gfra1* transcripts were detected in ASG (Fig. 9B). Similar to *gdnf*,
157 the expression of *gfra1* dramatically decreased in type B spermatogonia (Fig. 9E) and
158 became undetectable in spermatids (Fig. 9N). The developmental stages of germ cell
159 lineages were confirmed by the expression of marker genes (Fig. 9C, F, I, and L) and
160 morphological observation with hematoxylin-eosin staining (Fig. 9A, D, G, J, and M).

161

3. Discussion

We performed cloning and expression analysis of rainbow trout *gdnf* and *gfr α 1*. *In situ* hybridization and immunohistochemistry against paraffin sections and smear preparations of rainbow trout immature testes showed that *gdnf* mRNA and protein were expressed in ASG. For further confirmation, we performed RT-PCR for isolated ASG, Sertoli cells, and interstitial cells, and revealed the specific expression of *gdnf* only in ASG. Although almost all ASG had clear signals of *gdnf* mRNA in *in situ* hybridization against paraffin sections of immature testes, only about 85.5% of ASG had clear signals of *gdnf* mRNA against smear preparations. Using *in situ* hybridization against smear preparation analysis, it is sometime difficult to detect the staining signal in the all cells expressing target gene. Indeed, *vasa*, which is expressed in all ASG, was also detected in only about 93.5% of ASG using the same analytical method. In addition, we speculate that the expression level of *gdnf* is much lower than that of *vasa*. Therefore, we concluded that *gdnf* mRNA was expressed in almost all ASG in immature testes. Expression analyses of *gfr α 1* by *in situ* hybridization, immunohistochemistry, and RT-PCR revealed that *gfr α 1* mRNA and protein were also expressed in almost all ASG. Furthermore, *in situ*

hybridization against paraffin sections of maturing rainbow trout testis containing all developmental stages of male germ cells (from ASG to spermatozoa) showed that the expression levels of *gdnf* and *gfra1* changed synchronously; *gdnf* and *gfra1* showed high co-expression in ASG and decreased dramatically in subsequent developmental stages.

The expression pattern of *gdnf* is notably different from that of mice, whose *gdnf* is the most well-studied to date. In mouse testes, *gdnf* is expressed in Sertoli cells and *gfra1* is expressed in a spermatogonial subset of cells (Meng et al., 2000; Viglietto et al., 2000; Grisanti et al., 2009), and GDNF functions in the proliferation and maintenance of the spermatogonial subset of cells in a paracrine manner. However, the present study clearly showed that both rainbow trout *gdnf* and *gfra1* were expressed in germ cells. Moreover, *gdnf* expression is not observed in Sertoli cells in rainbow trout. Therefore, we concluded that GDNF was not secreted from Sertoli cells as a SSC niche-factor in rainbow trout, unlike in mammals. We currently do not know why the difference in cell types expressing *gdnf* between mouse testes and rainbow trout testes arose during evolution. Interestingly, rat *gdnf* is expressed in both Sertoli cells and germ cells (Fouchecourt et al., 2006). This finding suggests that the last common ancestor of fish and mammals expressed *gdnf* in

both Sertoli cells and germ cells. Therefore, one hypothesis is that during fish radiation, regulatory elements that allowed *gdnf* to be expressed in Sertoli cells mutated and became inactivated in rainbow trout. Additionally, teleosts have a unique member of the TGF- β superfamily of growth factors: gonadal soma-derived growth factor (GSDF), which is expressed specifically in Sertoli cells. More importantly, GSDF potently promotes the proliferation of ASG (Sawatari et al., 2007). Therefore, it might be that GSDF functionally compensated for the roles of GDNF in Sertoli cells during fish radiation. To test these two hypotheses, comparative analyses of the expression profiles and functional studies of both GDNF and GSDF in other species are required.

GDNF promotes proliferation and suppresses differentiation of undifferentiated spermatogonia in rodents (Meng et al., 2000; Naughton et al., 2006; He et al., 2007). In mice, ASG are morphologically classified: A_{single} (A_s ; isolated single cells), A_{paired} (A_{pr} ; two interconnected cells), and A_{aligned} (A_{al} ; 4, 8, 16, or 32 interconnected cells) (de Rooij, 2001). The expression of *gfra1* is restricted to A_s and A_{pr} spermatogonia corresponding to GDNF functions. A_s spermatogonia in mice are morphologically similar to ASG in fish, since the ASG are morphologically defined as a singly isolated larger germ cell surrounded by

Sertoli cells. Rainbow trout *gfra1* is expressed in almost all ASG, and decreases dramatically in subsequent developmental stages. This expression pattern resembles that of mice. However, we recently discovered that *tubulin alpha chain* homolog is expressed in a subpopulation of ASG, but not in the entire population, in rainbow trout (Hayashi et al., 2012). Furthermore, in rainbow trout germ cell transplantation, only $4.6 \times 10^{-2}\%$ of transplanted ASG are successfully incorporated into recipient gonads and act as SSCs (Okutsu et al., 2006a). These findings indicate that rainbow trout ASG is a heterogeneous population and that a part of ASG can possibly behave as SSCs. Therefore, the fact that *gfra1* is expressed in almost all ASG suggests that it cannot be used as a SSC marker, unlike in mammals.

The addition of GDNF to SSC culture medium was the silver bullet in the establishment of a mouse GS cell line (Kanatsu-Shinohara et al., 2003). As mentioned above, GS cells can differentiate into functional sperm by transplanting them into seminiferous tubules of recipient mice. Furthermore, the generation of transgenic mice using GS cells has been reported (Kanatsu-Shinohara et al., 2006). Therefore, the establishment of a fish GS cell line and the utilization of these cells can advance both basic

226 developmental biology and biotechnology (Okutsu et al., 2006b; Yoshizaki et al., 2011).

227 Although spermatogonia cultures have been attempted in fish (Shikina et al., 2008; Shikina

228 and Yoshizaki, 2010; Kawasaki et al., 2012), a fish GS cell line has not been established.

229 Considering the different expression patterns in testes (*gfra1* is expressed in ASG

230 including SSCs, and *gdnf* is also expressed in ASG), GDNF would not act as paracrine

231 factor in fish. Therefore, directly applying mammalian findings to fish is not adequate to

232 establish a fish GS cell line. However, it is still possible that GDNF acts as an autocrine

233 factor in fish testis, and functions in the proliferation and maintenance of undifferentiated

234 spermatogonia in fish. Further functional study is required to test the possibility that

235 GDNF is also a key factor in the establishment of fish GS cell lines.

236

4. Experimental procedures

4.1. Cloning of rainbow trout *gdnf* and *gfra1*

Immature rainbow trout testes carrying only ASG were used for this study. Total RNA isolation and cDNA synthesis were performed as previously described (Yano et al., 2008). Polymerase chain reaction (PCR) was performed in 1× *La Taq* Buffer (Takara Bio Inc., Shiga, Japan) with 0.5 units of Takara *La Taq* (Takara Bio Inc.), 1 μM of each primer, 1 μl of cDNA from the rainbow trout testes, 400 μM dNTPs, and 2.5 μM MgCl₂ in a total volume of 10 μl.

A partial cDNA fragment of the rainbow trout *gdnf* homolog was amplified with the degenerate PCR primers, *gdnf* F1 and *gdnf* R1, designed from the conserved regions of zebrafish *gdnf* (AF329853 in GenBank: <http://www.ncbi.nlm.nih.gov>); the *Fugu rubripes* genome sequence, which shows high sequence similarity to *gdnf* (chr4: 1926306-1926511 in the Tetraodon Genome Browser: <http://www.genoscope.cns.fr/externe/>); human *gdnf* (L15306 in GenBank); and mouse *gdnf* (D88264 in GenBank). After determining the DNA sequence of the partial cDNA fragment, 3'-rapid amplification of cDNA ends (3'-RACE) PCR and 5'-RACE PCR were performed to isolate the complete open reading frame (ORF).

Two specific primers, GDNF 3'RACE-1 and GDNF 3'RACE-2, were synthesized as forward primers for 3'-RACE PCR. The adapter primers AP1 and AP2 were used as the reverse primers for 3'-RACE PCR. 5'-RACE PCR was performed using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA) (Zhu et al., 2001) according to the manufacturer's protocol, with universal primer A mix (UPM) and nested universal primer A (NUP) as forward primers, and GDNF 5'RACE-1 and GDNF 5'RACE-2 as reverse primers.

Rainbow trout *gfral* homolog was isolated by the same method. The degenerate primers used for the RT-PCR were GFRa1 Fw and GFRa1 Rv. For 3'-RACE PCR primers, GFRa1 3'RACE-1 and GFRa1 3'RACE-2 were used. For 5'RACE PCR, GFRa1 5'RACE-1 and GFRa1 5'RACE-2 were used as reverse primers (all primer sequences and PCR conditions are listed in Supplementary Tables s1 and s2).

Phylogenetic analysis using the neighbor joining method (Saitou, N. and Nei, M., 1987) for all known vertebrate orthologues. Dendrograms were produced with CLC Sequence Viewer (Qiagen, Hilden, Germany).

4.2. In situ hybridization

A 711-base pair (bp) (AB787265 in GenBank, nucleotides 38-749) and a 987 bp (AB787266 in GenBank, nucleotides 1-987) cDNA fragment of rainbow trout *gdnf* and *gfr α 1* were each subcloned into the pGEM T-easy vector (Promega, Madison, WI). 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). Designs for the *vasa* probe, *rtili* probe, and *txndc6* probe were described previously (Yano et al., 2008; Nagasawa et al., 2010; Kise et al., 2012). For *in situ* hybridization of tissue sections, rainbow trout testes at various developmental stages were fixed in Bouin's solution at 4°C for 16 h, embedded in paraffin wax, and then sliced into 4- μ m serial sections. The paraffin sections were dewaxed and rehydrated by passing them through a xylene-ethanol series. After rehydration, sections were incubated in 4% paraformaldehyde (PFA)/PBS for 20 min. After washing twice for 5 min in PBST, sections were treated with Proteinase K (Roche) in PBST for 12 min at 37°C. The concentrations of Proteinase K were 3 μ g/ml and 1 μ g/ml for *gdnf* mRNA and *gfr α 1* mRNA detection, respectively. Next, sections were washed three times in PBST for 2 min and then

285 post-fixed in 4% PFA/PBS for 20 min. After washing twice in PBST for 5 min, sections
286 were acetylated in 0.125% acetic anhydride in Tris-HCl (pH 8.00) for 20 min. Next,
287 sections were washed in PBST for 2 min, followed by incubation in prehybridization
288 buffer (5×SSC (pH 4.5)/50% formamide) for 2 h. Then, sections were incubated with a
289 hybridization mixture of 50 µg/ml yeast tRNA, 50% formamide, 5× SSC, 50 µg/ml
290 heparin, 1% SDS, and 1 µg/ml probe. The temperatures of hybridization were 65°C and
291 60°C for *gdnf* mRNA and *gfral* mRNA detection, respectively. After hybridization for 18
292 h, the subsequent process was as follows: incubation twice in 5× SSC/50% formamide at
293 65°C for 30 min, three times in 2× SSC/50% formamide at 65°C for 30 min, 1× SSC/25%
294 formamide/1× TBST (pH 7.5) at 65°C for 10 min, three times in 1× TBST at room
295 temperature for 5 min, then in blocking solution (Roche) at room temperature for 1 h. The
296 sections were then incubated with the Fab fragment of an anti-DIG-alkaline
297 phosphatase-conjugated antibody (Roche), then diluted to 1:500 with blocking solution for
298 1 h at room temperature. After the nitroblue tetrazolium (NBT; Roche) and
299 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) color reaction was performed, the
300 slides were mounted using Entellan Neu (Merck KGaA, Darmstadt, Germany). Some

sections were counterstained by Nuclear Fast Red (NFR; Vector Laboratories, Burlingame, CA) for 30 min after the NBT/BCIP color reaction.

4.3. Immunohistochemistry

Immature testes from *pvasa-Gfp* rainbow trout were fixed in 10% picric acid-4% PFA/PBS at 4°C for 16 h, embedded in paraffin wax, and then sliced into 4-μm sections. The paraffin sections were dewaxed and rehydrated by passing them through a xylene-ethanol series. After rehydration, sections were treated with HistoVT One solution (Nacalai, San Diego, CA) for antigen retrieval at 90°C for 20 min. For reduction of non-specific antibody binding, sections were then blocked by Block-Ace (DS Farmer Biomedical, Osaka, Japan) for 30 min. Next, sections were incubated with primary antibodies overnight at 4°C, followed by washing three times in PBST for 5 min and incubation with secondary antibody for 1 h at room temperature. Sections were washed three times in PBST for 5 min and sealed by Vectashield Hard Set Mounting Medium (Vector Laboratories). Paraffin sections were immunostained using the following antibodies: rabbit anti-GDNF antibody (sc-328; Santa Cruz Biotechnology, Santa Cruz,

CA; 1:100), rabbit anti-GFR α 1 antibody (ab84106; Abcam Inc., Cambridge, UK; 1:500),
mouse anti-GFP antibody (11 814 460 001; Roche; 1:1000), rabbit anti-phospho-Histone
H3 (PH3) antibody (06-570; Merck Millipore, Billerica, MA; 1:1000), and rabbit
anti-GSDF antibody (Iwasaki and Yoshizaki, unpublished, 1:5000) as a primary antibody.
Anti-GFR α 1 antibody was pre-absorbed with acetone powder to improve the specificity.
As a secondary antibody, goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 546 (Life
Technologies, Carlsbad, CA; 1:200) and goat anti-mouse IgG conjugated to Alexa Fluor
488 or 546 (Life Technologies; 1:200) were used. Anti-GSDF antibody was diluted with
Can Get Signal immunostain solution B (Toyobo Co., Osaka, Japan), and others were
diluted with solution A (Toyobo Co.).

4.4. Smear preparation

For smear preparation, immature testes from *pvasa-Gfp* rainbow trout were used.
Testes were dissected into small pieces using scissors, then incubated in 1 ml of 0.5%
trypsin (Worthington Biochemical Corp., Lakewood, NJ) in PBS (pH 8.2) containing 5%
FBS, 1 mM CaCl₂, and 15 U/ml DNase (Sigma-Aldrich, St. Louis, MO) for 2 h at 10°C.

During the incubation, gentle pipetting was applied to enhance physical dissociation. The cell suspension was washed twice with Leibovitz's L-15 medium (Life Technologies) (pH 7.8) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 25 mM HEPES and antibiotics (50 mg/ml ampicillin, 50 U/ml penicillin, and 50 mg/ml streptomycin; Wako Pure Chemical Industries, Tokyo, Japan). Then the cell suspension was filtered through a 42 µm pore-size nylon screen to eliminate non-dissociated cell clumps. A cell suspension containing 2×10^4 dissociated cells was fixed with the same volume of Tissue-Tek Ufix (Sakura Finetech USA Inc., Torrance, CA) at room temperature for 5 min. Then, smear preparations were made on a glass slides (Mas-GP type A; Matsunami Glass, Tokyo, Japan) with fixed cell suspensions using Cytospin 4 (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions.

4.5. Cell sorting

Dissociated cell suspensions were prepared by the method used for smear preparation. Testes were collected from 10-month-old transgenic rainbow trout (body weight, 39.1 ± 3.05 g; GSI, $6.47 \pm 1.44 \times 10^{-2}\%$) whose spermatogonia and Sertoli cells were

labeled by the expression of GFP and DsRed under the control of the *vasa*-gene regulatory region and the *inhibin*-gene regulatory region, respectively. These double transgenic rainbow trout were obtained by crossing transgenic rainbow trout carrying *pvasa-Gfp* (Yoshizaki et al., 2000b; Yano et al., 2008) and those carrying *pinhibin-DsRed* (Banba and Yoshizaki, unpublished data). At this age, germ cells were mostly ASG. Cell sorting was performed as previously described (Hayashi et al., 2012). For DsRed detection, a 488 nm sapphire laser and 575 nm band-pass filter were used.

4.6. RT-PCR

After the cell sorting, GFP⁺ cells, DsRed⁺ cells, GFP⁻ and DsRed⁻ cells, and unsorted cells were subjected to conventional RT-PCR. Extraction of total RNA was performed using 6×10⁴ cells of each sample as previously described (Hayashi et al., 2012). First-strand cDNA was synthesized using Ready To Go You-Prime First-Strand Beads (GE Healthcare Life Sciences, Picataway, NJ) with an oligo (dT) primer. RT-PCR was performed for a spermatogonia marker, *vasa* (Yoshizaki et al., 2000a; Yano et al., 2008); a Sertoli cell marker, *gsdf* (Sawatari et al., 2007); a Leydig cell marker, *3β-HSD* (Sakai et

365 al., 1994); *gdnf*, *gfra1*; and an internal control, *β -actin* (all primer sequences and PCR

366 conditions are listed in Supplementary Tables s1 and s2).

367

References

- de Rooij, D.G., 2001. Proliferation and differentiation of spermatogonial stem cells. *Reproduction* 121, 347-354.
- de Rooij, D.G., 2009. The spermatogonial stem cell niche. *Microsc Res Tech* 72, 580-585.
- Fouchecourt, S., Godet, M., Sabido, O., Durand, P., 2006. Glial cell-line-derived neurotrophic factor and its receptors are expressed by germinal and somatic cells of the rat testis. *J Endocrinol* 190, 59-71.
- Grisanti, L., Falciatori, I., Grasso, M., Dove, L., Fera, S., Muciaccia, B., Fuso, A., Berno, V., Boitani, C., Stefanini, M., Vicini, E., 2009. Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem Cells* 27, 3043-3052.
- Hayashi, M., Sato, M., Iwasaki, Y., Terasawa, M., Tashiro, M., Yokoyama, S., Katayama, N., Sadaie, S., Miwa, M., Yoshizaki, G., 2012. Combining next-generation sequencing with microarray for transcriptome analysis in rainbow trout gonads. *Molecular reproduction and development* 79, 870-878.
- He, Z., Jiang, J., Hofmann, M.-C., Dym, M., 2007. Gfra1 silencing in mouse

384 spermatogonial stem cells results in their differentiation via the inactivation of RET
 385 tyrosine kinase. *Biology of reproduction* 77, 723-733.

386 Kanatsu-Shinohara, M., Ikawa, M., Takehashi, M., Ogonuki, N., Miki, H., Inoue, K.,
 387 Kazuki, Y., Lee, J., Toyokuni, S., Oshimura, M., 2006. Production of knockout mice
 388 by random or targeted mutagenesis in spermatogonial stem cells. *Proceedings of the*
 389 *National Academy of Sciences* 103, 8018-8023.

390 Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S.,
 391 Shinohara, T., 2003. Long-term proliferation in culture and germline transmission of
 392 mouse male germline stem cells. *Biology of reproduction* 69, 612-616.

393 Kawasaki, T., Saito, K., Sakai, C., Shinya, M., Sakai, N., 2012. Production of zebrafish
 394 offspring from cultured spermatogonial stem cells. *Genes Cells* 17, 316-325.

395 Kise, K., Yoshikawa, H., Sato, M., Tashiro, M., Yazawa, R., Nagasaka, Y., Takeuchi, Y.,
 396 Yoshizaki, G., 2012. Flow-cytometric isolation and enrichment of teleost type A
 397 spermatogonia based on light-scattering properties. *Biol Reprod* 86, 107.

398 Lin, L.-F., Doherty, D.H., Lile, J.D., Bektesh, S., Collins, F., 1993. GDNF: a glial cell
 399 line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260,

400 1130-1132.

401 Meng, X., Lindahl, M., Hyvönen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W.,
402 Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., 2000. Regulation of cell
403 fate decision of undifferentiated spermatogonia by GDNF. *Science* 287, 1489-1493.

404 Nagasawa, K., Shikina, S., Takeuchi, Y., Yoshizaki, G., 2010. Lymphocyte antigen 75
405 (Ly75/CD205) is a surface marker on mitotic germ cells in rainbow trout. *Biol*
406 *Reprod* 83, 597-606.

407 Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A., Milbrandt, J., 2006. Glial cell-line
408 derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem
409 cell fate. *Biology of reproduction* 74, 314-321.

410 Oatley, M.J., Racicot, K.E., Oatley, J.M., 2011. Sertoli cells dictate spermatogonial stem
411 cell niches in the mouse testis. *Biol Reprod* 84, 639-645.

412 Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., Yoshizaki, G., 2006a. Testicular germ
413 cells can colonize sexually undifferentiated embryonic gonad and produce functional
414 eggs in fish. *Proc Natl Acad Sci U S A* 103, 2725-2729.

415 Okutsu, T., Yano, A., Nagasawa, K., Shikina, S., Kobayashi, T., Takeuchi, Y., Yoshizaki, G.,

416 2006b. Manipulation of fish germ cell: visualization, cryopreservation and
 417 transplantation. *J Reprod Dev* 52, 685-693.

418 Rolland, A.D., Lareyre, J.J., Goupil, A.S., Montfort, J., Ricordel, M.J., Esquerre, D., Hugot,
 419 K., Houlgatte, R., Chalmel, F., Le Gac, F., 2009. Expression profiling of rainbow
 420 trout testis development identifies evolutionary conserved genes involved in
 421 spermatogenesis. *BMC genomics* 10, 546.

422 Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing
 423 phylogenetic trees. *Molecular biology and evolution* 4, 406-425.

424 Sakai, N., Tanaka, M., Takahashi, M., Fukada, S., Mason, J.I., Nagahama, Y., 1994.
 425 Ovarian 3 beta-hydroxysteroid dehydrogenase/delta 5-4-isomerase of rainbow trout:
 426 its cDNA cloning and properties of the enzyme expressed in a mammalian cell.
 427 *FEBS Lett* 350, 309-313.

428 Sariola, H., Saarma, M., 2003. Novel functions and signalling pathways for GDNF. *J Cell*
 429 *Sci* 116, 3855-3862.

430 Sawatari, E., Shikina, S., Takeuchi, T., Yoshizaki, G., 2007. A novel transforming growth
 431 factor-beta superfamily member expressed in gonadal somatic cells enhances

432 primordial germ cell and spermatogonial proliferation in rainbow trout
 433 (*Oncorhynchus mykiss*). Dev Biol 301, 266-275.

434 Shikina, S., Ihara, S., Yoshizaki, G., 2008. Culture conditions for maintaining the survival
 435 and mitotic activity of rainbow trout transplantable type A spermatogonia. Molecular
 436 reproduction and development 75, 529-537.

437 Shikina, S., Yoshizaki, G., 2010. Improved in vitro culture conditions to enhance the
 438 survival, mitotic activity, and transplantability of rainbow trout type a spermatogonia.
 439 Biol Reprod 83, 268-276.

440 Viglietto, G., Dolci, S., Bruni, P., Baldassarre, G., Chiariotti, L., Melillo, R.M., Salvatore,
 441 G., Chiappetta, G., Sferratore, F., Fusco, A., Santoro, M., 2000. Glial cell line-derived
 442 neutrotrophic factor and neurturin can act as paracrine growth factors stimulating
 443 DNA synthesis of Ret-expressing spermatogonia. Int J Oncol 16, 689-694.

444 Yano, A., Suzuki, K., Yoshizaki, G., 2008. Flow-cytometric isolation of testicular germ
 445 cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent
 446 protein gene driven by trout vasa regulatory regions. Biol Reprod 78, 151-158.

447 Yoshida, S., 2010. Stem cells in mammalian spermatogenesis. Dev Growth Differ 52,

448 311-317.

449 Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y.,

450 2011. Spermatogonial transplantation in fish: A novel method for the preservation of

451 genetic resources. *Comparative biochemistry and physiology. Part D, Genomics &*

452 *proteomics* 6, 55-61.

453 Yoshizaki, G., Sakatani, S., Tominaga, H., Takeuchi, T., 2000a. Cloning and

454 characterization of a vasa-like gene in rainbow trout and its expression in the germ

455 cell lineage. *Molecular reproduction and development* 55, 364-371.

456 Yoshizaki, G., Takeuchi, Y., Sakatani, S., Takeuchi, T., 2000b. Germ cell-specific

457 expression of green fluorescent protein in transgenic rainbow trout under control of

458 the rainbow trout vasa-like gene promoter. *Int J Dev Biol* 44, 323-326.

459 Zhu, Y., Machleder, E., Chenchik, A., Li, R., Siebert, P., 2001. Reverse transcriptase

460 template switching: A SMART™ approach for full-length cDNA library

461 construction. *Biotechniques* 30, 892-897.

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Figure Legends

Fig. 1. (A) Deduced amino acid sequence of rainbow trout GDNF. The signal peptide is indicated in italics. The six conserved cysteine residues are indicated by underlining. The box indicates the consensus sequence for proteolytic processing in the constitutive secretion pathway. (B) Phylogenetic analysis using the neighbor joining method for all known vertebrate GDNF orthologues. The bar represents genetic distance. Values at branching points represent bootstrap values (Replicates: 10,000).

Fig. 2. (A) Deduced amino acid sequence of rainbow trout GFR α 1. The signal peptide is indicated in italics. The conserved 26 cysteines are indicated by underlining. (B) Phylogenetic analysis using the neighbor joining method for all known vertebrate GFR α s. The bar represents genetic distance. Values at branching points represent bootstrap values (Replicates: 10,000).

Fig. 3. Expression pattern of *gdnf* mRNA in immature testis. Sections of immature testis were stained by *in situ* hybridization using a *gdnf* anti-sense probe (A and B) and sense

probe (C and D). Blue: *in situ* hybridization positive signal, Pink: Nuclear Fast Red for counterstaining. B and D are high magnifications of the insets in A and C, respectively. A smear preparation made with testicular cells isolated from *pvasa-Gfp* immature rainbow trout was also stained by *in situ* hybridization using *gdnf* anti-sense probe (E and F). E: GFP-fluorescent image. F: Corresponding bright field image. The cells labeled by green fluorescence are ASG (arrowheads). Scale bars, 50 μ m (A and C), 10 μ m (B, D, E, and F).

Fig. 4. Distribution of GDNF protein in immature testis. Serial sections of immature testis from *pvasa-Gfp* rainbow trout were stained with antibodies against GFP (green in A, C, D, and F); GSDF, a marker gene of Sertoli cells (red in B and C); GDNF (red in E and F); and PH3, a mitotic cell marker (red in G). C and F are merged images of A and B, and D and E, respectively. All GFP-positive germ cells were singly isolated by GSDF (red in B and C)-positive Sertoli cells. In addition, the cell cycle phases of adjacent cells surrounded by the broken yellow lines were not synchronous (D-F). Asterisks indicate the same cell between serial sections (D-F and G). Scale bar, 10 μ m.

Fig. 5. Expression pattern of *gfr α 1* mRNA in immature testis. The sections of immature testis were stained by *in situ* hybridization using a *gfr α 1* anti-sense probe (A and B) and sense probe (C and D). Blue: *in situ* hybridization positive signal, Pink: Nuclear Fast Red for counterstaining. B and D are high magnifications of the insets in A and C, respectively. Scale bars, 50 μ m (A and C), 10 μ m (B and D).

Fig. 6. Distribution of GFR α 1 protein in immature testis. Serial sections of immature testis from *pvasa-Gfp* rainbow trout were stained with antibodies against GFP (green in A, C, D, F, G, and I); GSDF, a marker gene of Sertoli cells (red in B and C); GFR α 1 (red in E and F); and PH3, a mitotic cell marker (red in H and I). C, F, and I are merged images of A and B, D and E, and G and H, respectively. All GFP-positive germ cells were singly isolated by GSDF (red in B and C)-positive Sertoli cells. In addition, the cell cycle phases of adjacent cells surrounded by the broken yellow lines were not synchronous (D-I). Asterisks indicate the same cell between serial sections (D-F and G-I). Scale bar, 10 μ m.

Fig. 7. RT-PCR analysis of *gdnf* and *gfr α 1*. cDNA from GFP+, DsRed+, and GFP- and

DsRed- cells from testes of double transgenic rainbow trout carrying *pvasa-Gfp* and *pinhibin-DsRed* genes were used. β -actin was used as an internal control for RT-PCR amplification. Lane NC was a negative control containing no cDNA template.

Fig. 8. Expression patterns of *gdnf* during spermatogenesis. A-C, D-F, G-I, J-L, and M-N are serial sections, respectively. Sections of testis from 2-year-old rainbow trout were stained by *in situ* hybridization using a *gdnf* anti-sense probe (B, E, H, K, and N).

Developmental stages of germ cell lineages were confirmed by morphological observation with hematoxylin-eosin staining (A, D, G, J, and M), and marker genes of each developmental stage (C, *vasa*; F, *rtili*; I and L, *txndc6*). ASG, type A spermatogonia; BSG, type B spermatogonia; P-SC, primary spermatocyte; S-SC, secondary spermatocyte; ST, spermatids. Scale bars, 10 μ m.

Fig. 9. Expression patterns of *gfra1* during spermatogenesis. A-C, D-F, G-I, J-L, and M-N are serial sections, respectively. Sections of testis from 2-year-old rainbow trout were stained by *in situ* hybridization using a *gfra1* anti-sense probe (B, E, H, K, and N).

Developmental stages of germ cell lineages were confirmed by morphological observation with hematoxylin-eosin staining (A, D, G, J, and M), and marker genes of each developmental stage (C, *vasa*; F, *rtili*; I and L, *txndc6*). ASG, type A spermatogonia; BSG, type B spermatogonia; P-SC, primary spermatocyte; S-SC, secondary spermatocyte; ST, spermatids. Scale bars, 10 μ m.

Fig. 10. Expression patterns of GDNF and GFRa1 in mouse and fish. In mouse, GDNF is expressed in Sertoli cells, and GFRa1 is expressed in spermatogonia, and GDNF acts as paracrine factor. In fish, both GDNF and GFRa1 are expressed in spermatogonia, not in Sertoli cells. GDNF most likely acts as autocrine factor in fish. Arrows indicate GDNF secretion patterns.

Figure 1
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Figure 1

Figure 2
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A

MIFVTLYVVL	PLLDVLYAQE	NALSGPNRLD	<u>C</u> VKASEQCMK	EQG <u>C</u> STKYRT
MRQ <u>C</u> VAGGKE	RNF ^S SMVAGLE	AQDE <u>C</u> RSAID	AVKQSPLYNC	R <u>C</u> KRGMKKEK
N <u>C</u> LRIYWGIY	QTLQGNDFLE	DSPYETMNSR	LSDMFRLAPI	ISGEPAVTRE
NN <u>C</u> LNAAKAC	NLNDT <u>C</u> KKYR	SAYISP <u>C</u> TSR	VSTAEV <u>C</u> NKR	K <u>C</u> HKALRQFF
DKVPPKHSYG	MLF <u>C</u> S <u>C</u> PAGD	QSAC <u>C</u> SERRRQ	TIVPV <u>C</u> SYED	KEKPN <u>C</u> LSLQ
AS <u>C</u> KTNYI <u>C</u> R	SRLADFFAN <u>C</u>	QSEPRSLSG <u>C</u>	LKENYAD <u>C</u> LL	SYSGLIGTVM
TPNYLRSPKI	SVVPY <u>C</u> D <u>C</u> SS	SGNGKEE <u>C</u> DK	FHRVLHRQHL	PPQSHPRVWE
RDRRGSVAAH	ASGADHHLHH	HSFPEG		

B

Figure 2

Figure 3
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Figure 3

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Figure 9

Figure 10

Supplementary Table s1. The primer sequences for degenerate PCR, RACE-PCR and RT-PCR.

Primer name	Primer sequence (5'-3')
gdnf Fw1	TAARGAGGARCTGATYTTYMGVTAYTG
gdnf Rv1	TGAATGCTTYTYAGVTRTRTGRTA
GDNF 3'RACE-1	TGACCTTGCAGGAACTCGGAAACGA
AP1	CCATCCTAATACGACTCACTATAGGGC
GDNF 3'RACE-2	TGAAACGCCACCTCACGCTTG
AP2	CTATAGGGCACGCGTGGT
UPM	CTAATACGACTCACTATAGGGC
GDNF 5'RACE-1	TCAAGCGTGAGGTGGCGTTTC
NUP	AAGCAGTGGTATCAACGCAGAGT
GDNF 5'RACE-2	TTCGTTTCCGAGTTCCTGCAAGGTC
gdnf-check Fw	GAAACAGCCTACAGGGAAGA
gdnf-check Rv	GAAGGACAGGTCATCGTCAA
GFR α 1 Fw	TATGAAGAARGARAAGAACRGCCTGCG
GFR α 1 Rv	TCWGCWARRCGAGATCTGCAGATGTA
GFR α 1 3'RACE-1	TGCCTGCGCATCTATTGTGCGGAATCT
GFR α 1 3'RACE-2	AACGATTTCTGGAGGACTCCCCTTA
GFR α 1 5'RACE-1	TGAAATAATGGGGGCCAGTCT
GFR α 1 5'RACE-2	TGGAGTCCTCCAGGAAATCGTT
gfra1-check Fw	ACAGCTATGGCATGCTGTTCTGTTCCCT
gfra1-check Rv	GCAGTCTGCGTAGTTCTCCTTAAGACA
vasa-check Fw	TCTTCAGAGAGATGGGGCAAGTCATC
vasa-check Rv	TCCCATATCCAGGACCACACGCACATT
gsdf-check Fw	TGACTGCCATCAGAGAGCAATGGAAGA
gsdf-check Rv	TGCTCTGTAGAAGTGGTCTGGCAGCA
3 β -HSC-check Fw	TTGGACTGGGCCATGTCTCT
3 β -HSC-check Rv	ATGCTGCTGGTGTAGATGAAGGA
β actin-check Fw	ACTACCTGATGAAGATCCTG
β actin-check Rv	TTGCTGATCCACATCTGTTG

Supplementary Table s2. The reaction conditions for degenerate PCR, RACE-PCR and RT-PCR.

Gene	Objective	Forward primer	Reverse primer	Number of cycle	Denaturing		Annealing		Extention	
					Temperature(°C)	Duration(sec)	Temperature(°C)	Duration(sec)	Temperature(°C)	Duration(sec)
gdnf	degenerate-PCR	gdnf Fw1	gdnf rv1	35	94	30	45	30	72	30
	3'RACE-PCR	GDNF 3'RACE-1	AP1	35	94	30	55	30	72	120
	3'RACE-PCR	GDNF 3'RACE-2	AP2	35	94	30	65	30	72	120
	5'RACE-PCR	UPM	GDNF 5'RACE-1	first 5cycle	94	30	72	60	two step PCR	
				second 5cycle	94	30	70	60	two step PCR	
				last 25cycle	94	30	63	30	72	120
	5'RACE-PCR	NUP	GDNF 5'RACE-2	35	94	30	56	30	72	120
	RT-PCR	gdnf-check Fw	gdnf-check Rv	38	94	30	64	30	72	30
gfra1	degenerate-PCR	GFRα1 Fw	GFRα1 Rv	35	94	30	58	30	72	60
	3'RACE-PCR	GFRα1 3RACE-1	AP1	35	94	30	58	30	72	90
	3'RACE-PCR	GFRα1 3RACE-2	AP2	35	94	30	58	30	72	90
	5'RACE-PCR	UPM	GFRα1 5'RACE-1	first 5cycle	94	30	65	30	72	60
				second 5cycle	94	30	63	30	72	60
				last 25cycle	94	30	56	30	72	60
	5'RACE-PCR	NUP	GFRα1 5'RACE-2	35	94	30	58	30	72	60
	RT-PCR	gfra1-check Fw	gfra1-check Rv	40	94	30	66	30	72	30
vasa	RT-PCR	vasa-check Fw	vasa-check Rv	30	94	30	62	30	72	30
gsdf	RT-PCR	gsdf-check Fw	gsdf-check Rv	25	94	30	62	30	72	30
3β-HSD	RT-PCR	3β-HSD-check Fw	3β-HSC-check Rv	28	94	30	64	60	72	30
β-actin	RT-PCR	βactin-check Fw	βactin-check Rv	30	94	30	58	30	72	30