Expression patterns of gdnf and gfrα1 in rainbow trout testis.

Expression patterns of *gdnf* and *gfrαl* in rainbow trout testis

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Abstract

In mice, glial cell line-derived neurotrophic factor (GDNF) is essential for normal spermatogenesis and *in vitro* culture of spermatogonial stem cells. In murine testes, GDNF acts as paracrine factor; Setoli cells secrete it to a subset of spermatogonial cells expressing its receptor, GDNF family receptor α1 (GFRα1). However, in fish, it is unclear what types of cells express *gdnf* and *gfrα1*. In this study, we isolated the rainbow trout orthologues of these genes and analyzed their expression patterns during spermatogenesis. In rainbow trout testes, *gdnf* and *gfrα1* were expressed in almost all type A spermatogonia (ASG). Noticeably, unlike in mice, the expression of *gdnf* was not observed in Sertoli cells in rainbow trout. During spermatogenesis, the expression levels of these genes changed synchronously; *gdnf* and *gfrα1* showed high expression in ASG and decreased dramatically in subsequent developmental stages. These results suggested that GDNF most likely acts as an autocrine factor in rainbow trout testes.

1. Introduction

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

Glial cell line-derived neurotrophic factor (GDNF) is a secretory factor produced in SSC niches in mice. GDNF is a distant member of the transforming growth factor-β (TGF-β) superfamily that was originally isolated from rat glioma cell-line supernatant as a trophic factor for midbrain neurons (Lin et al., 1993). It signals via a surface receptor complex composed of GDNF family receptor α1 (GFRα1) and Ret receptor tyrosine kinase (Ret) (Sariola and Saarma, 2003). In mouse testes, GDNF acts as paracrine factor secreted from Sertoli cells to undifferentiated spermatogonia expressing GFRα1 (Viglietto et al., 2000). Gene-targeted mice with one GDNF-null allele show a decreased total

45 number of germ cells and depletion of SSCs (Meng et al., 2000). To overcome the neonatal
46 lethality of *Gdnf* deficient mice, whole-testis transplantation has been performed
47 (Naughton et al., 2006). Transplanted *Gdnf*-deficient testes revealed that the disruption of
48 GDNF-mediated signaling results in a failure of spermatogenesis due to deficient SSC
49 self-renewal. Furthermore, a reduction of *Gfrα1* expression in type A spermatogonia
50 (ASG) induced a decrease of proliferation of SSCs and their phenotypic differentiation (He
51 et al., 2007). In contrast, testes that overexpress GDNF accumulate undifferentiated
52 spermatogonia (Meng et al., 2000; Grisanti et al., 2009). Taken together, these reports
53 indicate that GDNF-mediated signaling is essential for SSC proliferation and maintenance.
54 Thus, in mice, SSC niches have been well studied by focusing on the expression patterns
55 and functions of *gdnf* and *gfrα1*. However, information on the SSC niches in lower
56 vertebrates, including fish, is quite limited. Therefore, it is important to analyze the
57 expression patterns of *gdnf* and *gfrα1* in other vertebrates.
58 Rainbow trout (*Oncorhynchus mykiss*) is a suitable model fish for the following
59 reasons. First, there exist two transgenic rainbow trout strains: p*vasa-Gfp* and
60 p*inhibin-DsRed*. In p*vasa-Gfp* rainbow trout, spermatogonia are labeled by green

61 fluorescence protein (GFP) under the control of the *vasa*-gene regulatory region (Yoshizaki
62 et al., 2000b; Yano et al., 2008), which enables enrichment of ASG, including SSCs,
63 according to the intensity of green fluorescence (Okutsu et al., 2006a; Hayashi et al., 2012).
64 In p*inhibin-DsRed* rainbow trout, Sertoli cells are labeled by DsRed under the control of
65 the *inhibin*-gene regulatory region (Banba and Yoshizaki, unpublished data), which enables
66 enrichment of Sertoli cells according to the intensity of red fluorescence (Yagisawa and
67 Yoshizaki, unpublished data). Second, SSC activity can be evaluated by a spermatogonial
68 transplantation assay (Okutsu et al., 2006a). Third, the marker genes of each cell type,
69 Sertoli cells, *gsdf* (Sawatari et al., 2007); a Leydig cell, *3β-HSD* (Sakai et al., 1994); and
70 germ cells of each developing stage, *vasa*, *rtili* and *txndc6* (Yano et al., 2008; Rolland et al.,
71 2009). Therefore, as a first step to increase our knowledge of fish GDNF, we report the
72 cloning and expression analysis of rainbow trout GDNF and GFRα1 in this study.
73
74

2. Results

2.1. Cloning of rainbow trout gdnf *and* gfrα1 *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 *gfrα1* 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 GFRα1 orthologues such as an N-terminal signal peptide (Fig. 1A). Rainbow trout GFRα1 contained the 26 conserved cysteines corresponding to the regions of mouse and zebrafish

91 GFRα1s (Fig. 2A). A sequence comparison by BLAST analysis revealed that this isolated
92 gene was most similar to zebrafish *gfrα1a*. Phylogenetic analysis of GFRα members
93 clarified that rainbow trout GFRα1 belongs to the GFRα1 branch (Fig. 2B).
94
95 *2.2. Identification of cells expressing* gdnf *and* gfrα1 *by histology*
96 In fish, including rainbow trout, spermatogonia are classified morphologically as
97 type A or type B. The classification criteria are different from those of mouse
98 spermatogonia. ASG are singly isolated larger germ cells surrounded by Sertoli cells. Type
99 B spermatogonia (BSG) are smaller and organized into cysts where they synchronously
100 divide and develop into spermatocytes.
101 To identify what types of cells express *gdnf* and *gfrα1*, we performed *in situ*
102 hybridization on paraffin sections of immature testes containing only ASG from
103 9-month-old rainbow trout (body weight, 29.4 g; Gonadosomatic Index (GSI) (%) =
104 gonadal weight/body weight × 100, 3.73×10^{-2}). The results of *in situ* hybridization using a
105 *gdnf* probe showed that positive signals were detected in ASG (Fig. 3A and B), as
106 compared to sense probe control (Fig. 3C and D). Consistent with the expression of *gdnf*

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

111 It was difficult to completely eliminate the possibility that the above-mentioned
112 signals of *gdnf* detected in ASG were caused by diffused signals from Sertoli cells, since
113 Sertoli cells are located contiguously with spermatogonia and are very thin with extended
114 cytoplasms. To clarify this question, we performed *in situ* hybridization against dissociated
115 testicular cells smeared on glass slides. Cell smears were prepared with dissociated
116 testicular cells of 10-month-old p*vasa-Gfp* rainbow trout (body weight, 39.1±3.05 g; GSI,
117 $6.47\pm1.44\times10^{-2}$%). ASG were clearly distinguished by their green fluorescence (Fig. 3E).
118 *In situ* hybridization against smear preparations showed that 85.5±3.8% (N=4; 56, 103, 59,
119 and 57 ASG were randomly selected in each experiment) of ASG had clear signals of *gdnf*
120 mRNA (Fig. 3F).

121 Next, to identify the cells expressing *gfrα1*, we also performed *in situ* hybridization
122 and immunostaining on paraffin sections of immature rainbow trout testes. In immature

123 testes, *gfrα1* mRNA was localized in ASG (Fig. 5). In addition, GFRα1 protein (red in Fig.
124 6E and F) was also localized in ASG (green in Fig. 6D and F), which were singly isolated
125 by the GSDF-positive Sertoli cells (red in Fig. 6B and C) and whose cell cycle phases were
126 asynchronous (Fig. 6G-I).
127
128 *2.3. Expression analysis of* gdnf *and* gfrα1 *by RT-PCR*
129 From the above-mentioned results, it was difficult to completely role out the
130 possibility that some of the *gdnf* mRNA or protein also exist in both Sertoli cells and ASG.
131 Therefore, to further identify the cell types expressing *gdnf* and *gfrα1*, we performed
132 RT-PCR using GFP+, DsRed+, GFP- and DsRed-, and unsorted cells isolated from double
133 transgenic rainbow trout carrying p*vasa-Gfp* and p*inhibin-DsRed*. First, we identified cell
134 types enriched in each fraction by RT-PCR using the germ cell marker *vasa* (Yoshizaki et
135 al., 2000a), the Sertoli cell marker *gsdf* (Sawatari et al., 2007), and the Leydig cell marker
136 *3β-HSD* (Sakai et al., 1994). The results revealed that spermatogonia (*vasa*+), Sertoli cells
137 (*gsdf*+), and interstitial cells including Leydig cells (*3β-HSD* +) were enriched in GFP+,
138 DsRed+, and GFP- and DsRed- cell fractions, respectively (Fig. 7). An amplified signal of

139 *gdnf* was clearly detected in the GFP+ cells but not in the DsRed+ cells (Fig. 7). *Gfrα1*

140 transcripts were detected in GFP+ cells, and GFP- and DsRed- cells (Fig. 7).

141

142 *2.4*. Gdnf *and* gfrα1 *localization during testis development*

143 To elucidate the expression patterns of *gdnf* and *gfrα1* during spermatogenesis, we

144 performed *in situ* hybridization against paraffin sections of 2-year-old rainbow trout testes

145 containing all stages of male germ cells (from ASG to spermatozoa). Developmental stages

146 of male germ cells were identified by morphological observation with hematoxylin-eosin

147 staining (Fig. 8A, D, G, J, and M) and the expression of marker genes for each

148 developmental stage (Fig. 8C, F, I, and L). As mentioned above, ASG are singly isolated

149 larger germ cells surrounded by Sertoli cells (Fig. 8A), and BSG are smaller and organized

150 into cysts (Fig. 8D). In addition, ASG, BSG, and spermatocytes express marker genes: *vasa*,

151 *rtili*, and *txndc6*, respectively (Fig. 8C, F, I, and L) (Yano et al., 2008; Rolland et al., 2009).

152 In maturing testes, ASG showed strong signals with the *gdnf* probe (Fig. 8B). As

153 germ cell development progressed, the expression of *gdnf* was dramatically decreased (Fig.

154 8E, H, and K). Finally, the expression of *gdnf* was not detectable in spermatids and

155 spermatozoa (Fig. 8N).

156 In maturing testis, *gfrα1* transcripts were detected in ASG (Fig. 9B). Similar to *gdnf*,

157 the expression of *gfrα1* 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 *gfrα1*. *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 *gfrα1* by *in situ* hybridization, immunohistochemistry, and RT-PCR revealed that *gfrα1* mRNA and protein were also expressed in almost all ASG. Furthermore, *in situ*

178 hybridization against paraffin sections of maturing rainbow trout testis containing all
179 developmental stages of male germ cells (from ASG to spermatozoa) showed that the
180 expression levels of *gdnf* and *gfrα1* changed synchronously; *gdnf* and *gfrα1* showed high
181 co-expression in ASG and decreased dramatically in subsequent developmental stages.
182 The expression pattern of *gdnf* is notably different from that of mice, whose *gdnf* is
183 the most well-studied to date. In mouse testes, *gdnf* is expressed in Sertoli cells and *gfrα1*
184 is expressed in a spermatogonial subset of cells (Meng et al., 2000; Viglietto et al., 2000;
185 Grisanti et al., 2009), and GDNF functions in the proliferation and maintenance of the
186 spermatogonial subset of cells in a paracrine manner. However, the present study clearly
187 showed that both rainbow trout *gdnf* and *gfrα1* were expressed in germ cells. Moreover,
188 *gdnf* expression is not observed in Sertoli cells in rainbow trout. Therefore, we concluded
189 that GDNF was not secreted from Sertoli cells as a SSC niche-factor in rainbow trout,
190 unlike in mammals. We currently do not know why the difference in cell types expressing
191 *gdnf* between mouse testes and rainbow trout testes arose during evolution. Interestingly,
192 rat *gdnf* is expressed in both Sertoli cells and germ cells (Fouchecourt et al., 2006). This
193 finding suggests that the last common ancestor of fish and mammals expressed *gdnf* in

194 both Sertoli cells and germ cells. Therefore, one hypothesis is that during fish radiation,
195 regulatory elements that allowed *gdnf* to be expressed in Sertoli cells mutated and became
196 inactivated in rainbow trout. Additionally, teleosts have a unique member of the TGF-β
197 superfamily of growth factors: gonadal soma-derived growth factor (GSDF), which is
198 expressed specifically in Sertoli cells. More importantly, GSDF potently promotes the
199 proliferation of ASG (Sawatari et al., 2007). Therefore, it might be that GSDF functionally
200 compensated for the roles of GDNF in Sertoli cells during fish radiation. To test these two
201 hypotheses, comparative analyses of the expression profiles and functional studies of both
202 GDNF and GSDF in other species are required.
203 GDNF promotes proliferation and suppresses differentiation of undifferentiated
204 spermatogonia in rodents (Meng et al., 2000; Naughton et al., 2006; He et al., 2007). In
205 mice, ASG are morphologically classified: A_{single} (A_s; isolated single cells), A_{paired} (A_{pr}; two
206 interconnected cells), and $A_{aligned}$ (A_{al}; 4, 8, 16, or 32 interconnected cells) (de Rooij, 2001).
207 The expression of *gfrα1* is restricted to A_s and A_{pr} spermatogonia corresponding to GDNF
208 functions. A_s spermatogonia in mice are morphologically similar to ASG in fish, since the
209 ASG are morphologically defined as a singly isolated larger germ cell surrounded by

210 Sertoli cells. Rainbow trout *gfrα1* is expressed in almost all ASG, and decreases
211 dramatically in subsequent developmental stages. This expression pattern resembles that of
212 mice. However, we recently discovered that *tubulin alpha chain* homolog is expressed in a
213 subpopulation of ASG, but not in the entire population, in rainbow trout (Hayashi et al.,
214 2012). Furthermore, in rainbow trout germ cell transplantation, only 4.6×10^{-2}% of
215 transplanted ASG are successfully incorporated into recipient gonads and act as SSCs
216 (Okutsu et al., 2006a). These findings indicate that rainbow trout ASG is a heterogeneous
217 population and that a part of ASG can possibly behave as SSCs. Therefore, the fact that
218 *gfrα1* is expressed in almost all ASG suggests that it cannot be used as a SSC marker,
219 unlike in mammals.
220 The addition of GDNF to SSC culture medium was the silver bullet in the
221 establishment of a mouse GS cell line (Kanatsu-Shinohara et al., 2003). As mentioned
222 above, GS cells can differentiate into functional sperm by transplanting them into
223 seminiferous tubules of recipient mice. Furthermore, the generation of transgenic mice
224 using GS cells has been reported (Kanatsu-Shinohara et al., 2006). Therefore, the
225 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 (*gfrα1* 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

237 **4. Experimental procedures**

238 *4.1. Cloning of rainbow trout* gdnf *and* gfrα1

239 Immature rainbow trout testes carrying only ASG were used for this study. Total

240 RNA isolation and cDNA synthesis were performed as previously described (Yano et al.,

241 2008). Polymerase chain reaction (PCR) was performed in 1× *La Taq* Buffer (Takara Bio

242 Inc., Shiga, Japan) with 0.5 units of Takara *La Taq* (Takara Bio Inc.), 1 μM of each primer,

243 1 μl of cDNA from the rainbow trout testes, 400 μM dNTPs, and 2.5 μM $MgCl_2$ in a total

244 volume of 10 μl.

245 A partial cDNA fragment of the rainbow trout *gdnf* homolog was amplified with the

246 degenerate PCR primers, gdnf F1 and gdnf R1, designed from the conserved regions of

247 zebrafish *gdnf* (AF329853 in GenBank: http://www.ncbi.nlm.nih.gov); the *Fugu rubripes*

248 genome sequence, which shows high sequence similarity to *gdnf* (chr4: 1926306-1926511

249 in the Tetraodon Genome Browser: http://www.genoscope.cns.fr/externe/); human *gdnf*

250 (L15306 in GenBank); and mouse *gdnf* (D88264 in GenBank). After determining the DNA

251 sequence of the partial cDNA fragment, 3'-rapid amplification of cDNA ends (3'-RACE)

252 PCR and 5'-RACE PCR were performed to isolate the complete open reading frame (ORF).

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

260 Rainbow trout *gfrα1* homolog was isolated by the same method. The degenerate
261 primers used for the RT-PCR were GFRa1 Fw and GFRa1 Rv. For 3'-RACE PCR primers,
262 GFRa1 3'RACE-1 and GFRa1 3'RACE-2 were used. For 5'RACE PCR, GFRa1
263 5'RACE-1 and GFRa1 5'RACE-2 were used as reverse primers (all primer sequences and
264 PCR conditions are listed in Supplementary Tables s1 and s2).

265 Phylogenetic analysis using the neighbor joining method (Saitou, N. and Nei, M.,
266 1987) for all known vertebrate orthologues. Dendrograms were produced with CLC
267 Sequence Viewer (Qiagen, Hilden, Germany).

268

269 *4.2*. In situ *hybridization*

270 A 711-base pair (bp) (AB787265 in GenBank, nucleotides 38-749) and a 987 bp

271 (AB787266 in GenBank, nucleotides 1-987) cDNA fragment of rainbow trout *gdnf* and

272 *gfrα1* were each subcloned into the pGEM T-easy vector (Promega, Madison, WI). Sense

273 and antisense RNA probes were transcribed *in vitro* using digoxigenin-labeled uridine

274 triphosphate (UTP; Roche, Mannheim, Germany) and SP6 or T7 RNA polymerase

275 (Promega). Designs for the *vasa* probe, *rtili* probe, and *txndc6* probe were described

276 previously (Yano et al., 2008; Nagasawa et al., 2010; Kise et al., 2012). For *in situ*

277 hybridization of tissue sections, rainbow trout testes at various developmental stages were

278 fixed in Bouin's solution at 4°C for 16 h, embedded in paraffin wax, and then sliced into

279 4-μm serial sections. The paraffin sections were dewaxed and rehydrated by passing them

280 through a xylene-ethanol series. After rehydration, sections were incubated in 4%

281 paraformaldehyde (PFA)/PBS for 20 min. After washing twice for 5 min in PBST, sections

282 were treated with Proteinase K (Roche) in PBST for 12 min at 37°C. The concentrations of

283 Proteinase K were 3 μg/ml and 1 μg/ml for *gdnf* mRNA and *gfrα1* mRNA detection,

284 respectively. Next, sections were washed three times in PBST for 2 min and then

post-fixed in 4% PFA/PBS for 20 min. After washing twice in PBST for 5 min, sections were acetylated in 0.125% acetic anhydride in Tris-HCl (pH 8.00) for 20 min. Next, sections were washed in PBST for 2 min, followed by incubation in prehybridization buffer (5×SSC (pH 4.5)/50% formamide) for 2 h. Then, sections were incubated with a hybridization mixture of 50 μg/ml yeast tRNA, 50% formamide, 5× SSC, 50 μg/ml heparin, 1% SDS, and 1 μg/ml probe. The temperatures of hybridization were 65°C and 60°C for *gdnf* mRNA and *gfrα1* mRNA detection, respectively. After hybridization for 18 h, the subsequent process was as follows: incubation 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 (pH 7.5) at 65°C for 10 min, three times in 1× TBST at room temperature for 5 min, then 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), then diluted to 1:500 with blocking solution for 1 h at room temperature. After the nitroblue tetrazolium (NBT; Roche) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) color reaction was performed, the slides were mounted using Entellan Neu (Merck KGaA, Darmstadt, Germany). Some

301 sections were counterstained by Nuclear Fast Red (NFR; Vector Laboratories, Burlingame,
302 CA) for 30 min after the NBT/BCIP color reaction.
303
304 *4.3. Immunohistochemistry*
305 Immature testes from p*vasa-Gfp* rainbow trout were fixed in 10% picric acid-4%
306 PFA/PBS at 4°C for 16 h, embedded in paraffin wax, and then sliced into 4-μm sections.
307 The paraffin sections were dewaxed and rehydrated by passing them through a
308 xylene-ethanol series. After rehydration, sections were treated with HistoVT One solution
309 (Nacalai, San Diego, CA) for antigen retrieval at 90°C for 20 min. For reduction of
310 non-specific antibody binding, sections were then blocked by Block-Ace (DS Farmer
311 Biomedical, Osaka, Japan) for 30 min. Next, sections were incubated with primary
312 antibodies overnight at 4°C, followed by washing three times in PBST for 5 min and
313 incubation with secondary antibody for 1 h at room temperature. Sections were washed
314 three times in PBST for 5 min and sealed by Vectashield Hard Set Mounting Medium
315 (Vector Laboratories). Paraffin sections were immunostained using the following
316 antibodies: rabbit anti-GDNF antibody (sc-328; Santa Cruz Biotechnology, Santa Cruz,

317 CA; 1:100), rabbit anti-GFRα1 antibody (ab84106; Abcam Inc., Cambridge, UK; 1:500),
318 mouse anti-GFP antibody (11 814 460 001; Roche; 1:1000), rabbit anti-phospho-Histone
319 H3 (PH3) antibody (06-570; Merck Millipore, Billerica, MA; 1:1000), and rabbit
320 anti-GSDF antibody (Iwasaki and Yoshizaki, unpublished, 1:5000) as a primary antibody.
321 Anti-GFRα1 antibody was pre-absorbed with acetone powder to improve the specificity.
322 As a secondary antibody, goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 546 (Life
323 Technologies, Carlsbad, CA; 1:200) and goat anti-mouse IgG conjugated to Alexa Fluor
324 488 or 546 (Life Technologies; 1:200) were used. Anti-GSDF antibody was diluted with
325 Can Get Signal immunostain solution B (Toyobo Co., Osaka, Japan), and others were
326 diluted with solution A (Toyobo Co.).
327
328 *4.4. Smear preparation*
329 For smear preparation, immature testes from p*vasa-Gfp* rainbow trout were used.
330 Testes were dissected into small pieces using scissors, then incubated in 1 ml of 0.5%
331 trypsin (Worthington Biochemical Corp., Lakewood, NJ) in PBS (pH 8.2) containing 5%
332 FBS, 1 mM $CaCl_2$, 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, $6.47\pm1.44\times10^{-2}$%) whose spermatogonia and Sertoli cells were

349 labeled by the expression of GFP and DsRed under the control of the *vasa*-gene
350 regulatory region and the *inhibin*-gene regulatory region, respectively. These double
351 transgenic rainbow trout were obtained by crossing transgenic rainbow trout carrying
352 p*vasa-Gfp* (Yoshizaki et al., 2000b; Yano et al., 2008) and those carrying p*inhibin-DsRed*
353 (Banba and Yoshizaki, unpublished data). At this age, germ cells were mostly ASG. Cell
354 sorting was performed as previously described (Hayashi et al., 2012). For DsRed
355 detection, a 488 nm sapphire laser and 575 nm band-pass filter were used.
356
357 *4.6. RT-PCR*
358 After the cell sorting, GFP+ cells, DsRed+ cells, GFP- and DsRed- cells, and
359 unsorted cells were subjected to conventional RT-PCR. Extraction of total RNA was
360 performed using 6×10^4 cells of each sample as previously described (Hayashi et al., 2012).
361 First-strand cDNA was synthesized using Ready To Go You-Prime First-Strand Beads
362 (GE Healthcare Life Sciences, Picataway, NJ) with an oligo (dT) primer. RT-PCR was
363 performed for a spermatogonia marker, *vasa* (Yoshizaki et al., 2000a; Yano et al., 2008); a
364 Sertoli cell marker, *gsdf* (Sawatari et al., 2007); a Leydig cell marker, *3β-HSD* (Sakai et

365 al., 1994); *gdnf*; *gfrα1*; and an internal control, *β-actin* (all primer sequences and PCR

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

367

368 **References**

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464 **Figure Legends**

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

471

472 Fig. 2. (A) Deduced amino acid sequence of rainbow trout GFRα1. The signal peptide is
473 indicated in italics. The conserved 26 cysteines are indicated by underlining. (B)
474 Phylogenetic analysis using the neighbor joining method for all known vertebrate GFRαs.
475 The bar represents genetic distance. Values at branching points represent bootstrap values
476 (Replicates: 10,000).

477

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

480 probe (C and D). Blue: *in situ* hybridization positive signal, Pink: Nuclear Fast Red for
481 counterstaining. B and D are high magnifications of the insets in A and C, respectively. A
482 smear preparation made with testicular cells isolated from p*vasa-Gfp* immature rainbow
483 trout was also stained by *in situ* hybridization using *gdnf* anti-sense probe (E and F). E:
484 GFP-fluorescent image. F: Corresponding bright field image. The cells labeled by green
485 fluorescence are ASG (arrowheads). Scale bars, 50 μm (A and C), 10 μm (B, D, E, and F).
486
487 Fig. 4. Distribution of GDNF protein in immature testis. Serial sections of immature testis
488 from p*vasa-Gfp* rainbow trout were stained with antibodies against GFP (green in A, C, D,
489 and F); GSDF, a marker gene of Sertoli cells (red in B and C); GDNF (red in E and F); and
490 PH3, a mitotic cell marker (red in G). C and F are merged images of A and B, and D and E,
491 respectively. All GFP-positive germ cells were singly isolated by GSDF (red in B and
492 C)-positive Sertoli cells. In addition, the cell cycle phases of adjacent cells surrounded by
493 the broken yellow lines were not synchronous (D-F). Asterisks indicate the same cell
494 between serial sections (D-F and G). Scale bar, 10 μm.
495

Fig. 5. Expression pattern of *gfrα1* mRNA in immature testis. The sections of immature testis were stained by *in situ* hybridization using a *gfrα1* 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 μm (A and C), 10 μm (B and D).

Fig. 6. Distribution of GFRα1 protein in immature testis. Serial sections of immature testis from p*vasa-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); GFRα1 (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 μm.

Fig. 7. RT-PCR analysis of *gdnf* and *gfrα1*. cDNA from GFP+, DsRed+, and GFP- and

512 DsRed- cells from testes of double transgenic rainbow trout carrying p*vasa-Gfp* and

513 p*inhibin-DsRed* genes were used. β-actin was used as an internal control for RT-PCR

514 amplification. Lane NC was a negative control containing no cDNA template.

515

516 Fig. 8. Expression patterns of *gdnf* during spermatogenesis. A-C, D-F, G-I, J-L, and M-N

517 are serial sections, respectively. Sections of testis from 2-year-old rainbow trout were

518 stained by *in situ* hybridization using a *gdnf* anti-sense probe (B, E, H, K, and N).

519 Developmental stages of germ cell lineages were confirmed by morphological observation

520 with hematoxylin-eosin staining (A, D, G, J, and M), and marker genes of each

521 developmental stage (C, *vasa*; F, *rtili*; I and L, *txndc6*). ASG, type A spermatogonia; BSG,

522 type B spermatogonia; P-SC, primary spermatocyte; S-SC, secondary spermatocyte; ST,

523 spermatids. Scale bars, 10 μm.

524

525 Fig. 9. Expression patterns of *gfrα1* during spermatogenesis. A-C, D-F, G-I, J-L, and M-N

526 are serial sections, respectively. Sections of testis from 2-year-old rainbow trout were

527 stained by *in situ* hybridization using a *gfrα1* anti-sense probe (B, E, H, K, and N).

528 Developmental stages of germ cell lineages were confirmed by morphological observation
529 with hematoxylin-eosin staining (A, D, G, J, and M), and marker genes of each
530 developmental stage (C, *vasa*; F, *rtili*; I and L, *txndc6*). ASG, type A spermatogonia; BSG,
531 type B spermatogonia; P-SC, primary spermatocyte; S-SC, secondary spermatocyte; ST,
532 spermatids. Scale bars, 10 μm.
533
534 Fig. 10. Expression patterns of GDNF and GFRa1 in mouse and fish. In mouse, GDNF is
535 expressed in Sertoli cells, and GFRa1 is expressed in spermatogonia, and GDNF acts as
536 paracrine factor. In fish, both GDNF and GFRa1 are expressed in spermatogonia, not in
537 Sertoli cells. GDNF most likely acts as autocrine factor in fish. Arrows indicate GDNF
538 secretion patterns.
539

Figure 1
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A

MKLWDTFTTC FVLLSSVHTS PLRNRPSTKR TRASESLHDF PPMQLSIFST
KSPETAYREE RSVETQYNMV ELQPEQFEDV VDFIKVTISR LKSSLHLGTG
SRIRMKRERR KGGKGATRGK DQRERSGSGR GRGRGRGGRG QGCLLKQIHL
NVTDLGLGYQ TSEEMIFRYC SGPCRNSETN YDKILNNLTQ NKRLLPETPP
HACCRPVAFD DDLSFLDDHL MYHTMKKHSA RRCGCV

B

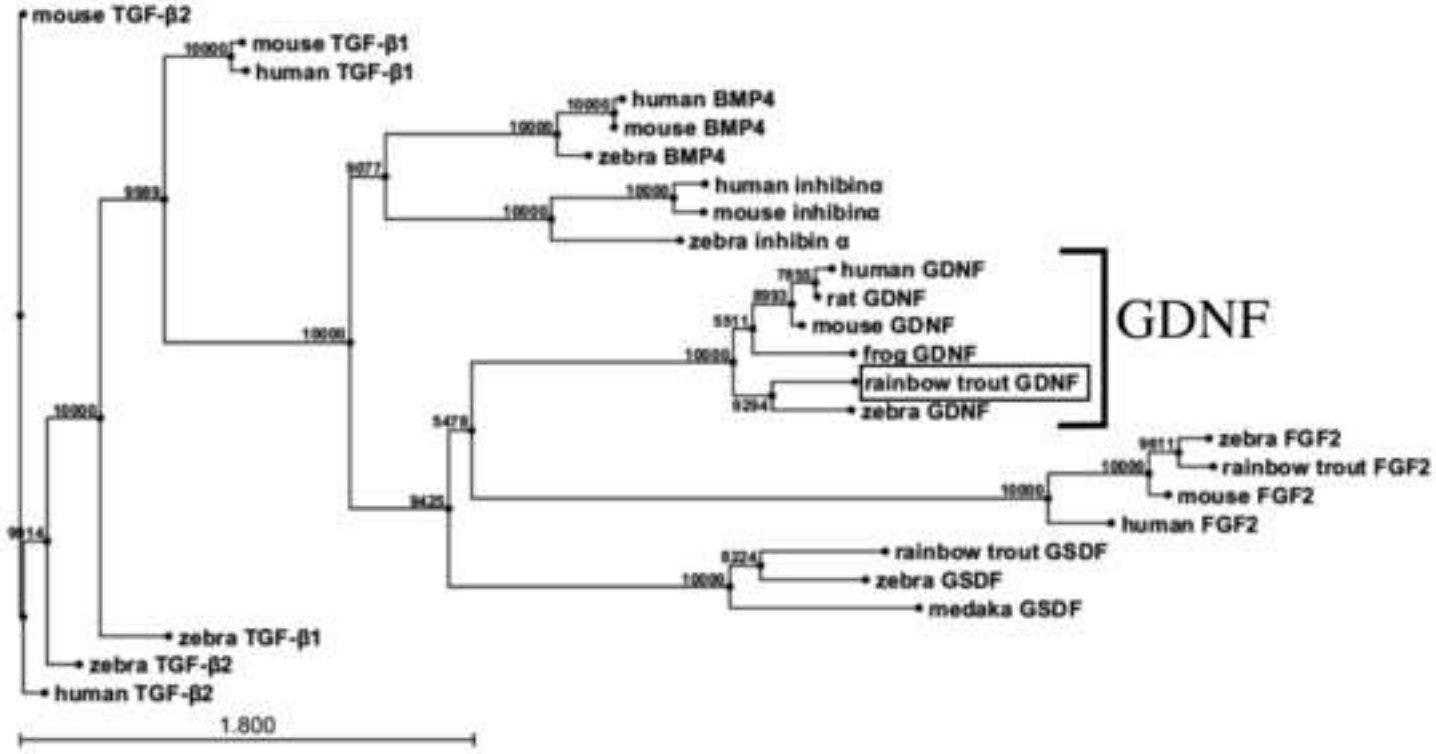

Figure 1

Figure 2
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A

MIFVTLYVVL PLLDVLYAQE NALSGPNRLD CVKASEQCMK EQGCSTKYRT
MRQCVAGGKE RNFSMVAGLE AQDECRSAID AVKQSPLYNC RCKRGMKKEK
NCLRIYWGIY QTLQGNDFLE DSPYETMNSR LSDMFRLAPI ISGEPAVTRE
NNCLNAAKAC NLNDTCKKYR SAYISPCTSR VSTAEVCNKR KCHKALRQFF
DKVPPKHSYG MLFCSCPAGD QSACSERRRQ TIVPVCSYED KEKPNCLSLQ
ASCKTNYICR SRLADFFANC QSEPRSLSGC LKENYADCLL SYSGLIGTVM
TPNYLRSPKI SVVPYCDCSS SGNGKEECDK FHRVLHRQHL PPQSHPRVWE
RDRRGSVAAH ASGADHHLHH HSFPEG

B

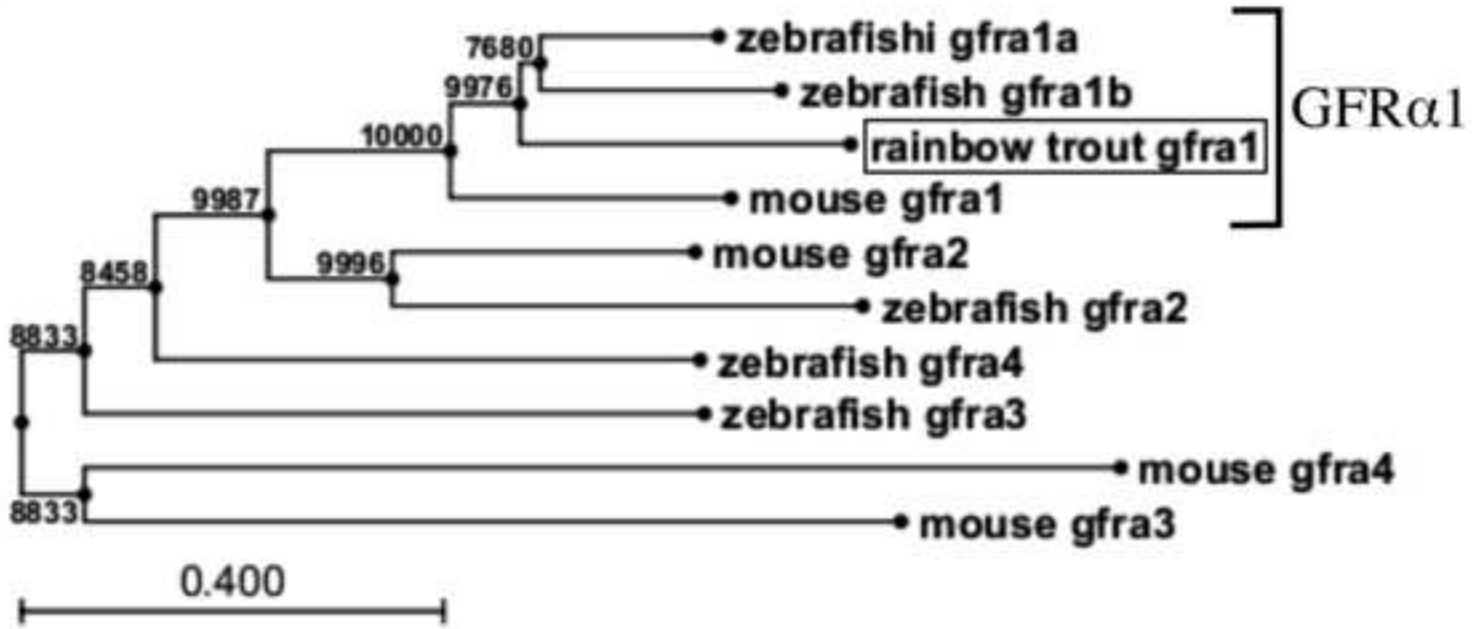

Figure 2

Figure 3

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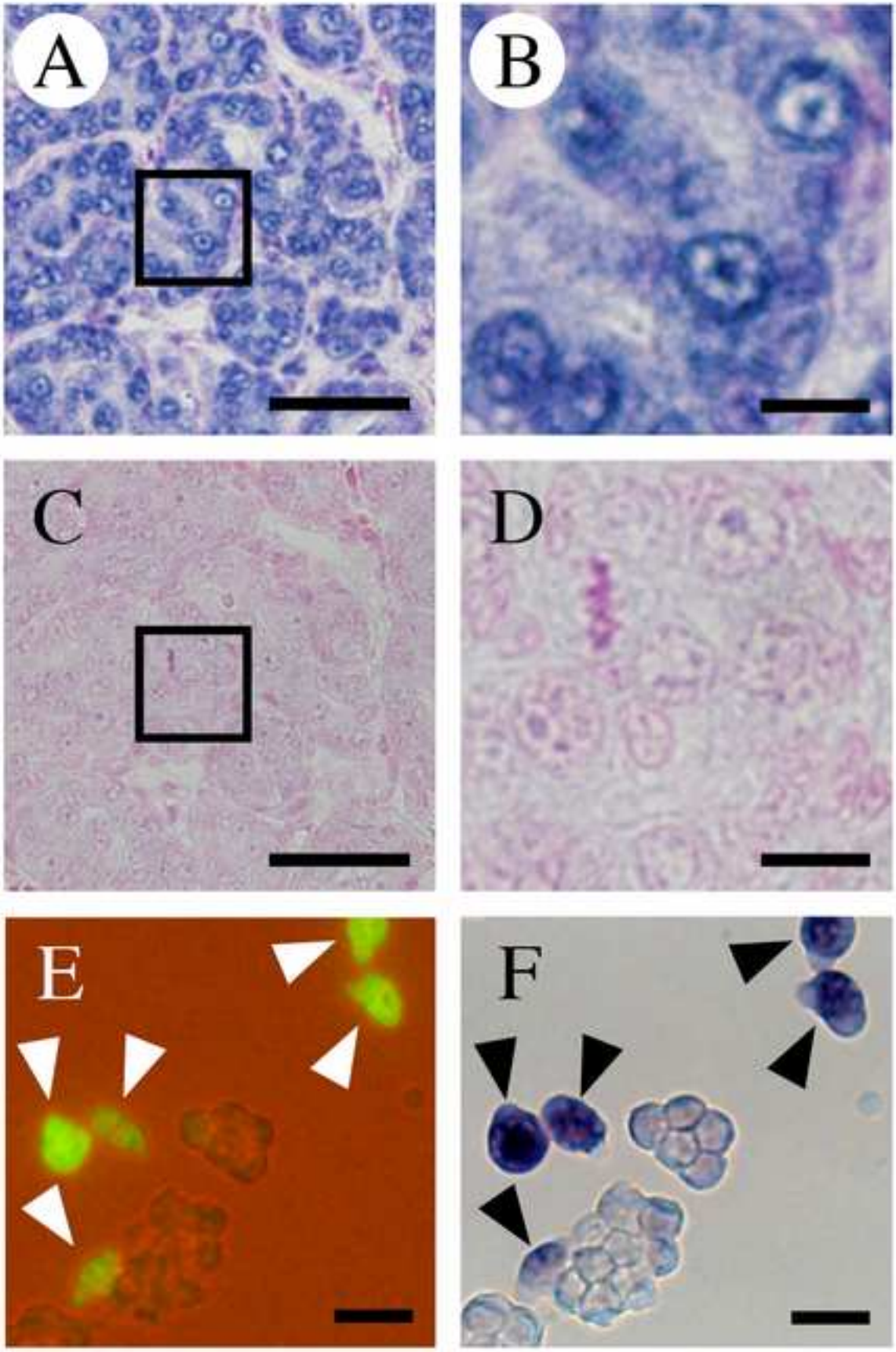

Figure 3

Figure4
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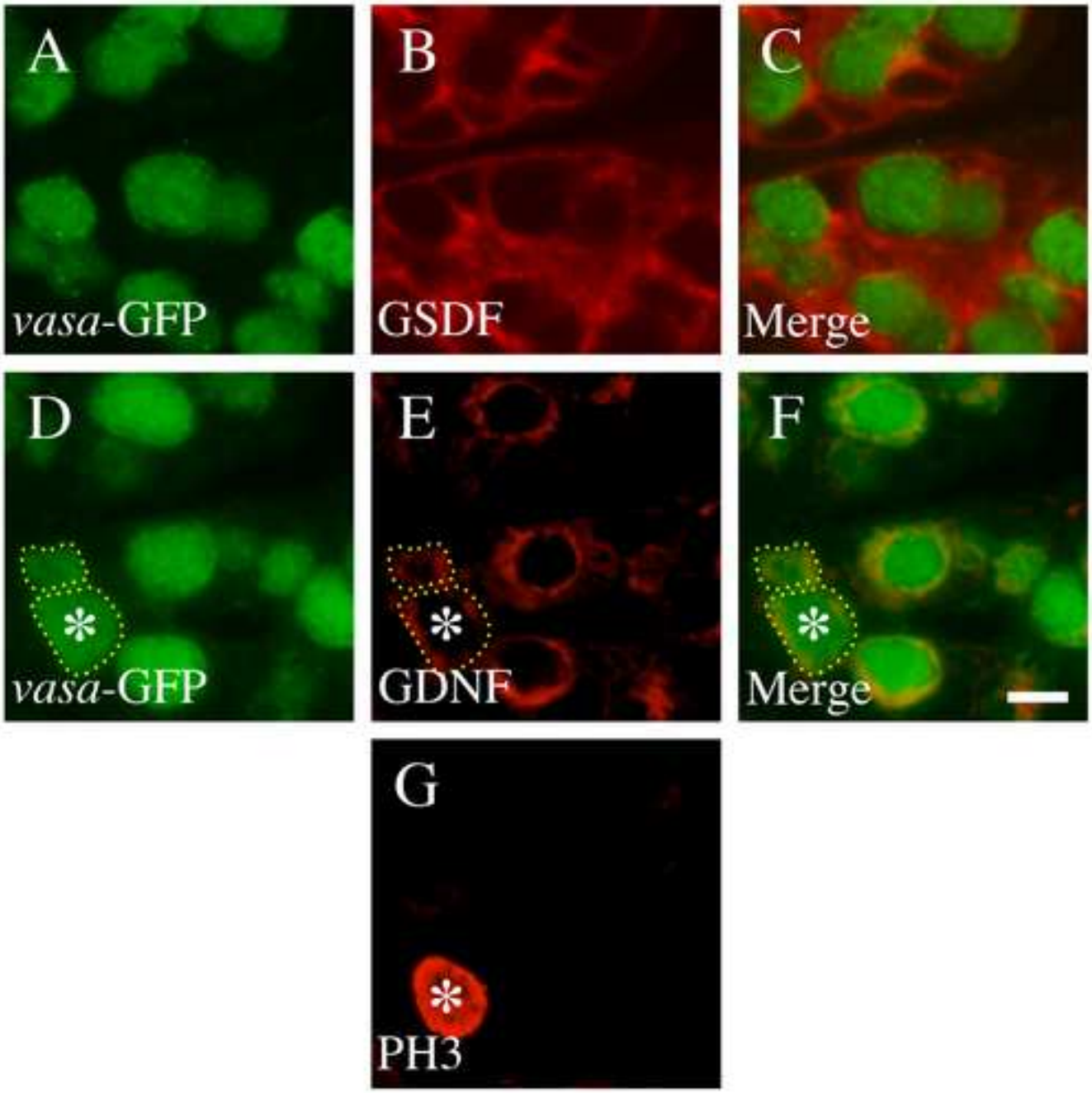

Figure 4

Figure 5
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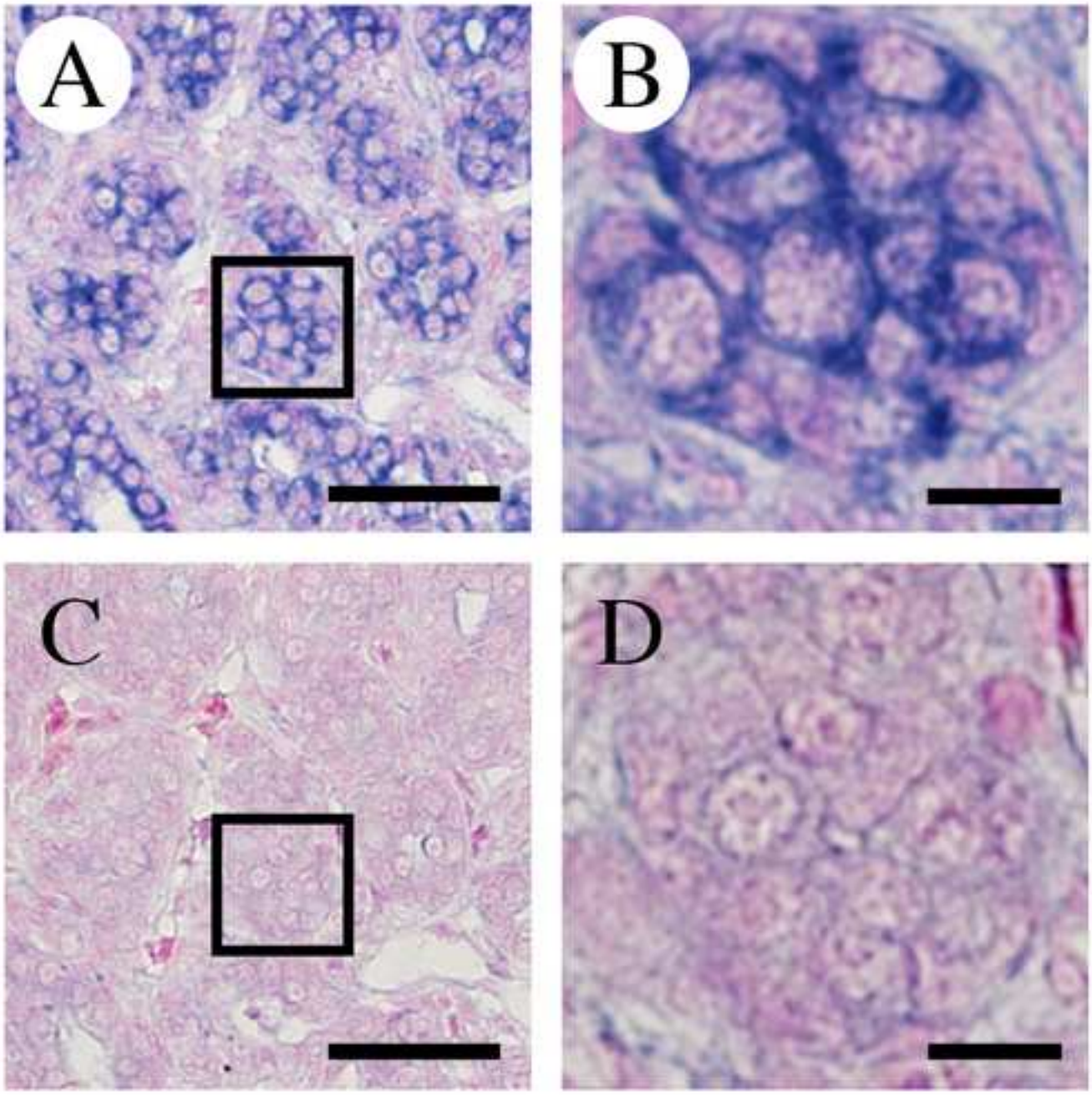

Figure 5

Figure6
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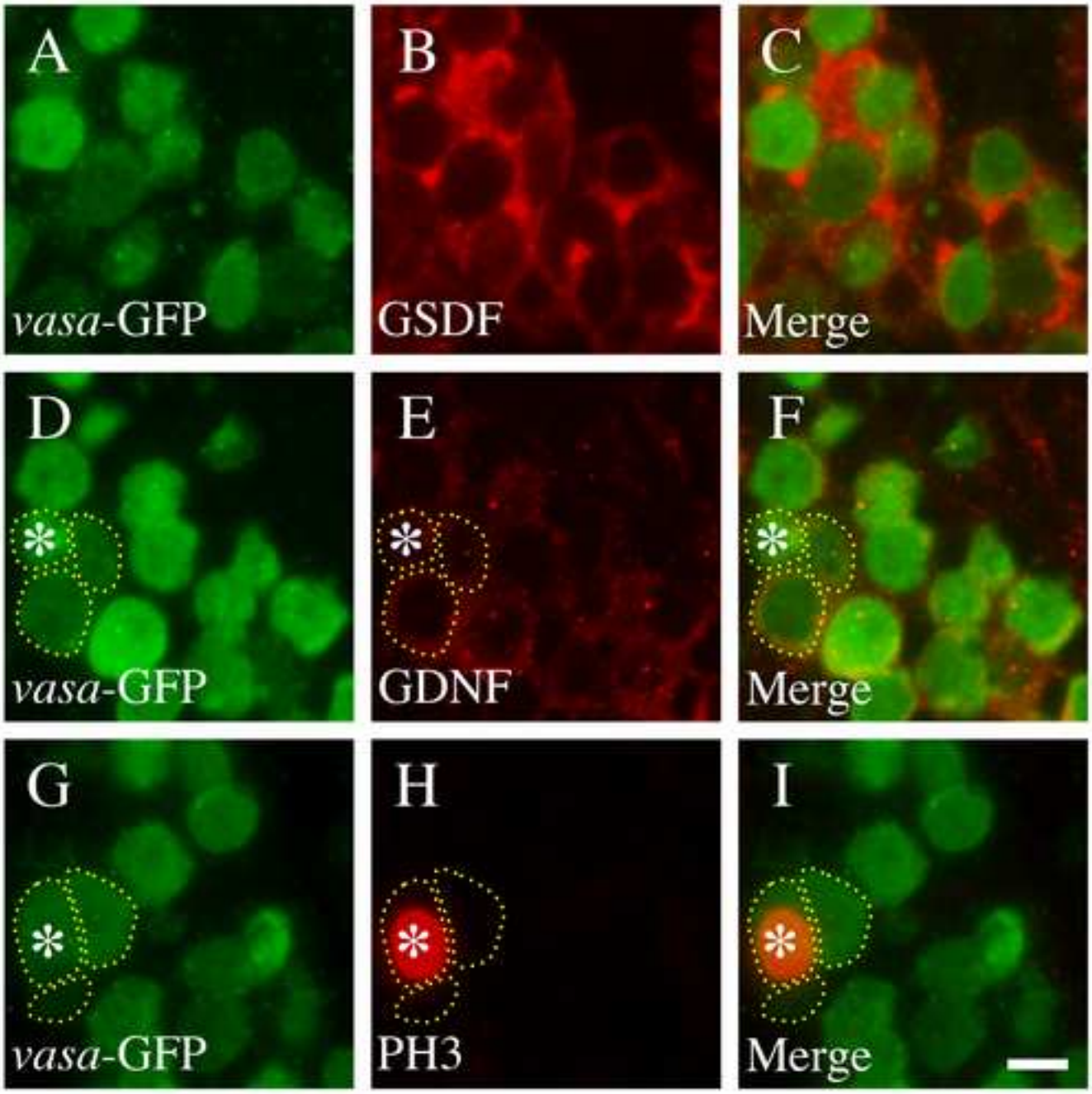

Figure 6

Figure7
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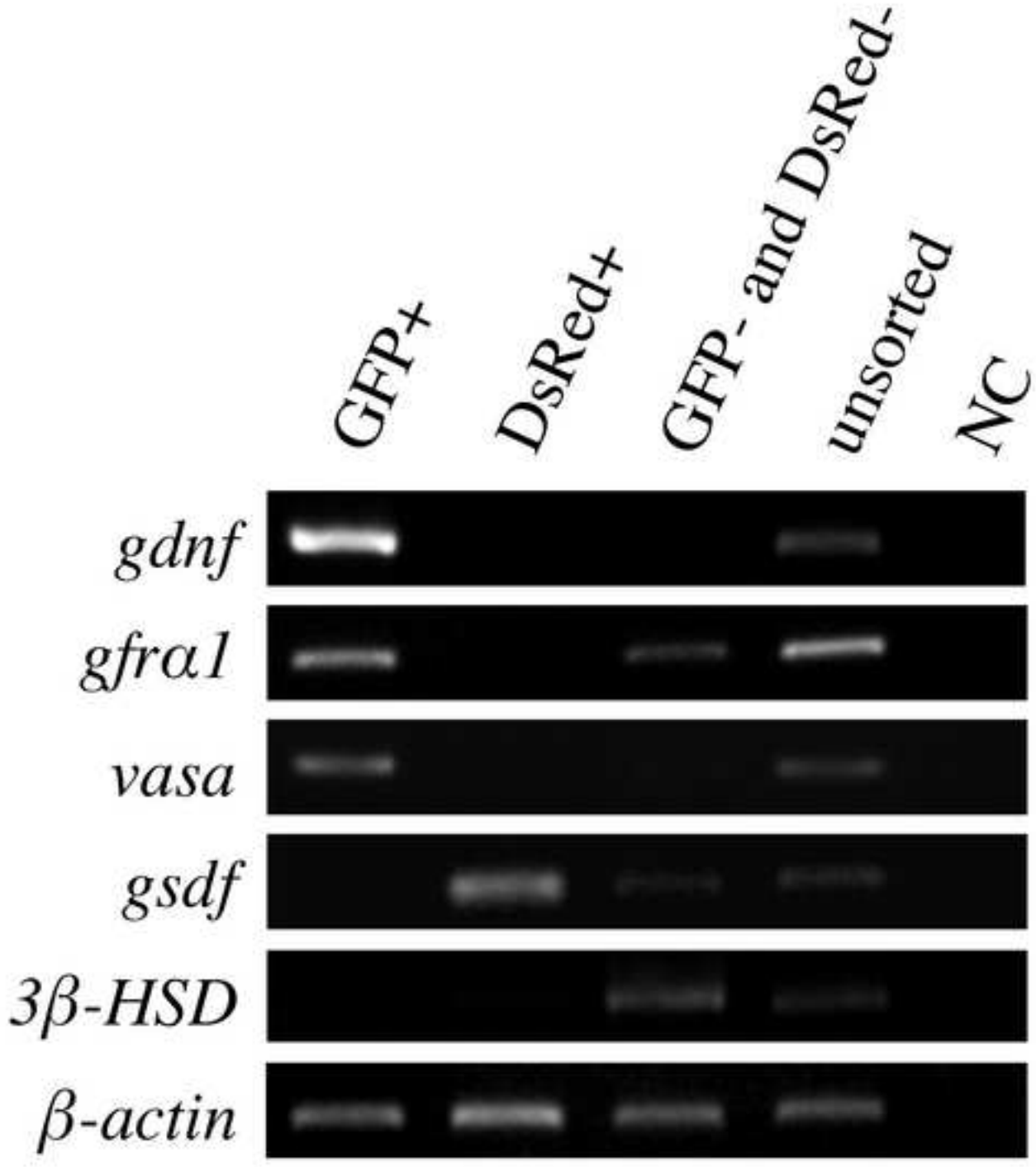

Figure 7

Figure 8

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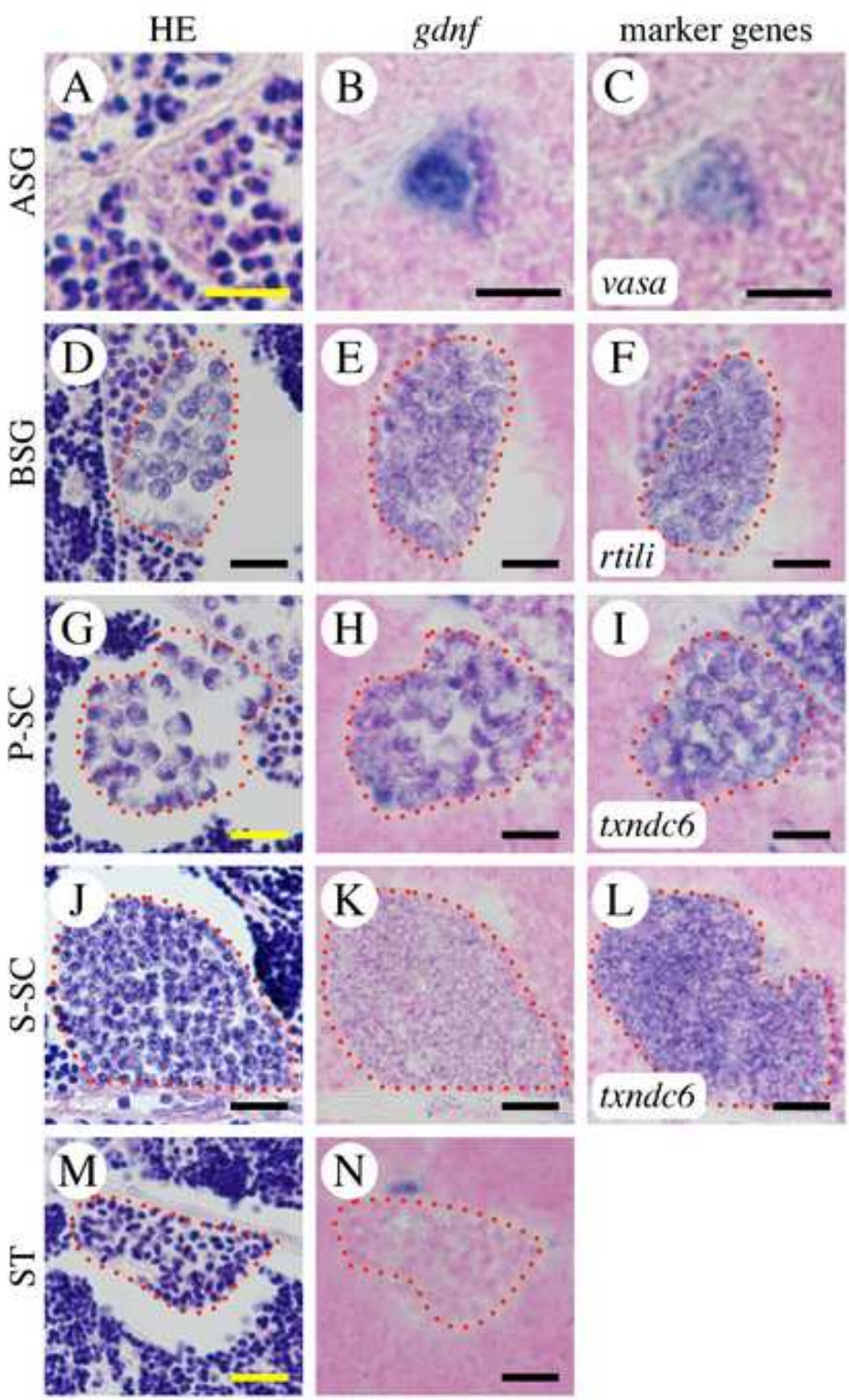

Figure 8

Figure 9
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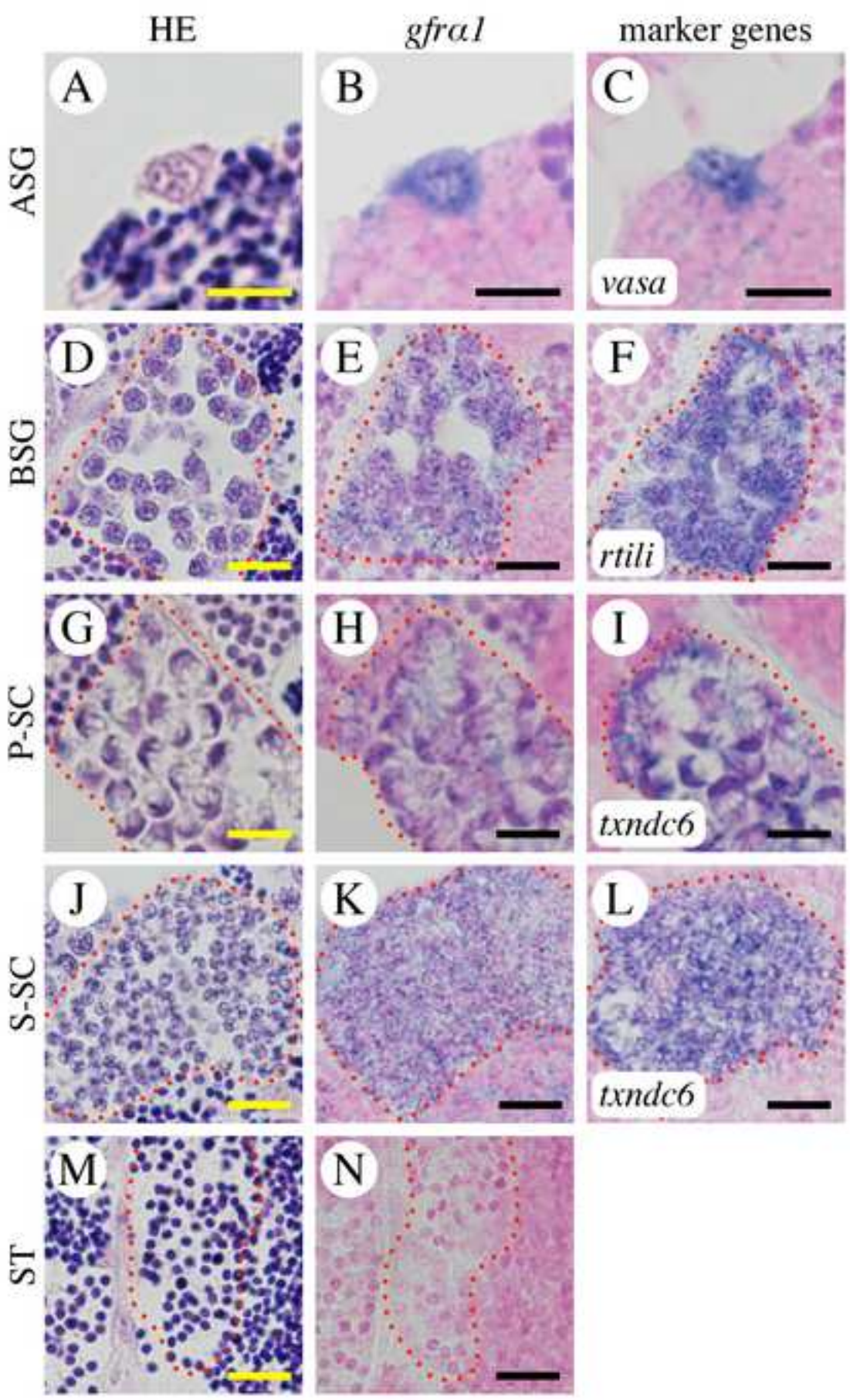

Figure 9

Figure10
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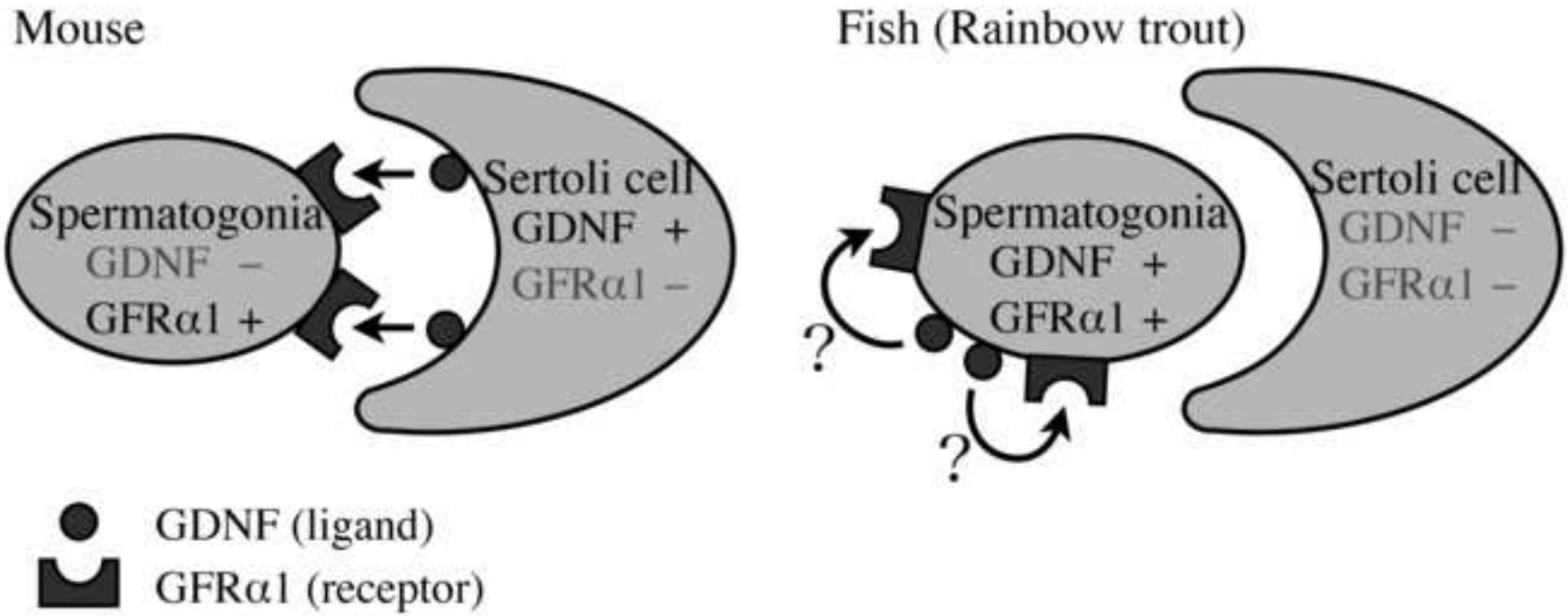

Figure 10

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

Primer name	Primier sequence (5'-3')
gdnf Fw1	TAARGAGGARCTGATYTTYMGVTAYTG
gdnf Rv1	TGAATGCTTYYTYAGVRTRTGRTA
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
GFRα1 Fw	TATGAAGAARGARAAGAACRGCCTGCG
GFRα1 Rv	TCWGCWARRCGAGATCTGCAGATGTA
GFRα1 3'RACE-1	TGCCTGCGCATCTATTGTGCGGAATCT
GFRα1 3'RACE-2	AACGATTTCCTGGAGGACTCCCCTTA
GFRα1 5'RACE-1	TGAAATAATGGGGGCCAGTCT
GFRα1 5'RACE-2	TGGAGTCCTCCAGGAAATCGTT
gfrα1-check Fw	ACAGCTATGGCATGCTGTTCTGTTCCT
gfrα1-check Rv	GCAGTCTGCGTAGTTCTCCTTAAGACA
vasa-check Fw	TCTTCAGAGAGATGGGGCAAGTCATC
vasa-check Rv	TCCCATATCCAGGACCACACGCACATT
gsdf-check Fw	TGACTGCCATCAGAGAGCAATGGAAGA
gsdf-check Rv	TGCTCTGTAGAAGTGGTCTGGCAGCA
3β-HSC-check Fw	TTGGACTGGGCCATGTCTCT
3β-HSC-check Rv	ATGCTGCTGGTGTAGATGAAGGA
βactin-check Fw	ACTACCTGATGAAGATCCTG
βactin-check Rv	TTGCTGATCCACATCTGTTG

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

					Denaturing		Annealing		Extention	
Gene	Objective	Forward primer	Reverse primer	Number of cycle	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
gfrα1	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	gfrα1-check Fw	gfrα1-check Rv	40	94	30	66	30	72	30
vasa	RT-PCR	vasa-chek 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