Gene Expression Patterns 14 (2014) 111–120

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: www.elsevier.com/locate/gep

Expression patterns of *gdnf* and *gfrα1* in rainbow trout testis

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ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form 18 January 2014
Accepted 23 January 2014
Available online 8 February 2014

Keywords:
GDNF
GFRα1
Rainbow trout
Spermatogonial stem cell
Spermatogenesis

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; Sertoli 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.

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 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 *Gfrα1* 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 *gfrα1*. 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 *gfrα1* in other vertebrates.

Rainbow trout (*Oncorhynchus mykiss*) is a suitable model fish for the following reasons. First, there exist two transgenic rainbow trout strains: p*vasa-Gfp* and p*inhibin-DsRed*. In p*vasa-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 p*inhibin-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.,

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http://dx.doi.org/10.1016/j.gep.2014.01.006
1567-133X/

2007); a Leydig cell, *3β-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 *gfrα1* in this study.

1. Results

1.1. 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 GFRα1s (Fig. 2A). A sequence comparison by BLAST analysis revealed that this isolated gene was most similar to zebrafish *gfrα1a*. Phylogenetic analysis of GFRα members clarified that rainbow trout GFRα1 belongs to the GFRα1 branch (Fig. 2B).

1.2. Identification of cells expressing gdnf and gfrα1 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 *gfrα1*, 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 cytoplasms. 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 p*vasa-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 ± 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 *gfrα1*, we also performed *in situ* hybridization and immunostaining on paraffin sections of immature rainbow trout testes. In immature testes, *gfrα1* mRNA was localized in ASG (Fig. 5). In addition, GFRα1 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).

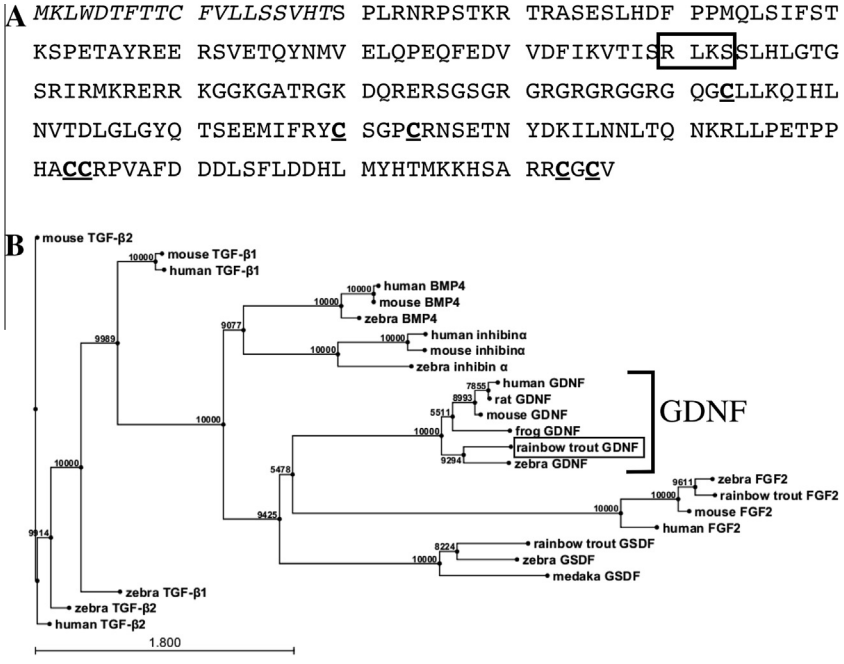

Fig. 1. (A) Deduced amino acid sequence of rainbow trout GDNF. The signal peptide is indicated in italics. The six conserved cysteine residues are indicated by underlining. The box indicates the consensus sequence for proteolytic processing in the constitutive secretion pathway. (B) Phylogenetic analysis 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).