

New insights into the evolution, hormonal regulation, and spatiotemporal expression profiles of genes involved in the *Gfra1/Gdnf* and *Kit/Kitlg* regulatory pathways in rainbow trout testis

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Abstract The present study aimed to investigate whether the *Gfra1/Gdnf* and/or *Kit/Kitlg* regulatory pathways could be involved in the regulation of spermatogonial cell proliferation and/or differentiation in fish. Homologs of the mammalian *gfra1*, *gdnf*, *kitr*, and *kitlg* genes were identified in gnathostomes and reliable orthologous relationships were established using phylogenetic reconstructions and analyses of syntenic chromosomal fragments. Gene duplications and losses occurred specifically in teleost fish and members of the Salmoninae family including rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Some duplicated genes exhibited distinct spatiotemporal expression profiles and were differently regulated by hormones in rainbow trout. Undifferentiated type A spermatogonia expressed higher levels of *kitrb* and *kitra2* making them possible target cells for the gonadal *kitlgb* and somatic *kitlga* before the onset of spermatogenesis. Interestingly, *gdnfa* and *gdnfb* ohnologous genes were poorly expressed before the onset of spermatogenesis. The expression level of *gdnfb*

was correlated with that of the *vasa* gene suggesting that the late increased abundance of *gdnfb* during spermatogenesis onset was due to the increased number of spermatogonial cells. *gfra1a2* was expressed in undifferentiated type A spermatogonia whereas *gfra1a1* was mainly detected in somatic cells. These observations indicate that the germinal *gdnfb* ligand could exert autocrine and paracrine functions on spermatogonia and on testicular somatic cells, respectively. Fsh and androgens inhibited *gfra1a2* and *gdnfb* whereas *gfra1a1* was stimulated by Fsh, androgens, and 17α , 20β progesterone. Finally, our data provide evidences that the molecular identity of the male germ stem cells changes during ontogenesis prior to spermatogenesis onset.

Keywords *Oncorhynchus mykiss* · Spermatogenesis · Germ stem cell · *Gfra1* · *Gdnf* · *Kit* · *Kit ligand* · Stem cell factor

Introduction

Spermatogenesis is a highly organized process that allows undifferentiated spermatogonia to differentiate into spermatozoa. In mammals, undifferentiated type A spermatogonia are classified in three different main subpopulations. Single A spermatogonia (A_s) are isolated cells. Recent investigations suggest that only a subset of A_s spermatogonia expressing high levels of the Id4 protein is capable to colonize the seminiferous tubules, to self-renew, and to initiate new spermatogenetic waves after

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transplantation assays (Helsel et al. 2017). These cells should be considered as the spermatogonial stem cells (SSC). A paired spermatogonia (A_{pr}) derive from the symmetric division of the A_s spermatogonia but the daughter cells remain interconnected by a cytoplasmic bridge. Aligned A spermatogonia (A_{al}) result from symmetrical divisions of paired spermatogonia, and they successively form chains of 4, 8, and 16 interconnected spermatogonia. It has been shown that A aligned spermatogonia could revert to less-differentiated germ cells through spermatogonial cyst fragmentation. Using live imaging in vivo on the *ngn3/eGFP* mouse model, it has been observed that a chain containing eight A_{al} spermatogonia dividing synchronously were undergoing a fragmentation process leading to the apparition of one 12-cell cyst and two doublets of interconnected cells corresponding to A_{pr} . The latter gave rise to new A_{al} 4-cell cysts while the remainder 12-cell cyst underwent cell death (Nakagawa et al. 2010). These observations led to the assumption that the stock of A_s is also maintained in adult animal through clonal fragmentation with a continuous interconvert between equipotent A_s and syncytial A_{pr} and A_{al} cells (Hara et al. 2014). In consequence, A_{pr} and A_{al} should be considered as progenitor germ cells that maintain their stemness status while undergoing a reversible differentiation process.

Based on morphological studies, the population of undifferentiated spermatogonia is also heterogeneous in teleost fish species (Lacerda et al. 2010; Loir 1999). Although different nomenclatures were used, studies report the presence of multiple A_s spermatogonial subpopulations, syncytial doublets, and 4- to 64-cell cysts containing interconnected spermatogonia in adult fish testes. The presence of SSC in juvenile or adult fish has been evidenced through transplantation assays in salmonids (Bellaïche et al. 2014b; Okutsu et al. 2006), cichlids (Lacerda et al. 2010), and cyprinids (Nobrega et al. 2010). However, the subpopulation of fish SSC and the cellular dynamics leading to their homeostasis remain poorly understood.

SSC are not autonomous cells. It is well admitted that their survival and decision to self-renew or to differentiate into progenitors are under the control of a specific microenvironment provided by the “germinal niche” formed mainly by the Sertoli and peritubular cells in mammals. SSC and somatic cell interactions could be mediated directly through cell-cell contacts involving adherence molecules or indirectly through the secretion of soluble factors. In mammals, the glial cell line-

derived neurotrophic factor (Gdnf) is expressed by somatic cells located in the vicinity of the SSC and its action is mediated by the glial cell line neurotrophic factor receptor alpha 1 (Gfra1) and the multifunctional Ret co-receptor (Jing et al. 1996; Treanor et al. 1996). Invalidation of one *gdnf* allele in mouse results in spermatogenesis failure in aging animal due to the loss of SSC (Meng et al. 2000). Similarly, a reduction of *gfra1* gene expression in undifferentiated spermatogonia decreases SSC proliferation and promotes their differentiation. The transplantation of Gdnf-, Gfra1-, and Ret-deficient neonatal testes under the back skin of immunodeficient mice demonstrated that Gdnf/Gfra1 was a key physiological regulatory pathway regulating self-renewal and differentiation of SSC in adult mouse (Naughton et al. 2006).

Recent studies have addressed the potential involvement of the Gfra1 signaling pathway in the regulation of fish gametogenesis in cartilaginous and bony fishes. In dogfish (*Scyliorhinus canicula*), one type of *gfra1* transcript was detected in undifferentiated spermatogonia and the addition of mammalian Gdnf to cultured undifferentiated spermatogonia supports their long term survival and proliferation (Bosseboeuf et al. 2013; Gautier et al. 2014). However, no *gdnf* transcript was detected in the germinative zone of the dogfish testis. In bony fishes, *gfra1* and *gdnf* transcripts were identified in adult testes. In trout (*Oncorhynchus mykiss*), low expression levels of a *gfra1* transcript were detected mainly in undifferentiated spermatogonia (Bellaïche et al. 2014a; Nakajima et al. 2014). The expression of a *gdnf* gene copy named *gdnfb* was barely detected in the testes of immature animals. Its expression was evidenced not only in somatic cells (Bellaïche et al. 2014a) but also in germ cells (Bellaïche et al. 2014a; Nakajima et al. 2014). In addition, *gdnfb* transcript expression peaks rather lately in pre-spermiating male trout (Bellaïche et al. 2014a). The Gfra1 protein was detected on single undifferentiated spermatogonia of the Nile tilapia (*Oreochromis niloticus*) using a heterologous antibody (Lacerda et al. 2013) but not in carp (*Labeo rohita*) (Panda et al. 2011). In contrast to mammals, Fsh represses *gdnfb* expression in rainbow trout testes (Bellaïche et al. 2014a) whereas Fsh stimulates Gdnf in mammals (Tadokoro et al. 2002). In consequence, the physiological role of the Gfra1/Gdnf pathway remains unclear in adult fish gonads.

Interestingly, the testicular morphology of Gdnf-, Gfra1-, and Ret-deficient mice is normal before birth

(Meng et al. 2000). This suggests that other regulatory pathways are involved in the production of the initial stock of germ stem cells before birth and/or Gdnf-dependent SSC appear only after birth. In mouse, primordial germ cells (PGC) originate from a population of pluripotent epiblast cells that migrate towards the genital ridge at E10.5. The kit receptor and its cognate ligand named either kit ligand (Kitlg), steel factor, or stem cell factor (SCF) are essential for survival, proliferation, and migration of PGC (Runyan et al. 2006). Between E12.5 and E14.5, germ stem cells cease to proliferate due to the decreased release of soluble kit ligand. The germ stem cells differentiate into prospermatogonia and enter mitotic arrest (De Felici 2000). Similarly to mammals, a duplicated form of kit ligand, named kitlga, stimulates the proliferation of cultured PGC collected from 24-h zebrafish embryos (Fan et al. 2008). However, the *in vivo* spatial and temporal expression profiles of the genes involved in Kit/Kitlg pathway remain unexplored in fish.

The present study aimed to gain information on the possible involvement of the Kit/Kitlg and Gfra1/Gdnf regulatory pathways in the regulation of germ line stem cell homeostasis in rainbow trout during ontogenesis. We first identified homologous genes in rainbow trout and in other vertebrate species to validate the orthologous relationships and to understand the complex evolutionary history of the different receptors and cognate ligands. The cellular localization and temporal expression pattern of the transcripts encoding different receptors and cognate ligands were further examined in combination of known germ cell-specific transcripts. We provide evidence that the molecular identity of the germ stem cells evolves during ontogenesis and different combinations of receptor/ligand interactants may act sequentially prior and after spermatogenesis onset.

Materials and methods

Phylogenetic analysis

Homologs of the candidate mammalian genes were searched in 25 vertebrate species by querying different types of nucleotide databases (genome, mRNA, and EST collections) hosted at the NCBI, EMBL and INRA institutes. The full amino acid sequences of the proteins deduced from the homologous genes were subsequently aligned using the CLUSTALW algorithm of the BioEdit

software and a phylogenetic reconstruction was carried out using the neighbor-joining method of the MEGA7 freeware.

Animals and tissue sampling

Male rainbow trout (*O. mykiss*) were obtained from the INRA experimental fish farm (PEIMA, Sizun, France) and kept in the laboratory facilities at 12 °C under natural photoperiod. In our rearing conditions, most of the male trout mature at 2 years old (60%) but precocious male puberty can occur at the end of the first year of life (40%). Fish were euthanized in 0.03% 2-phenoxyethanol. In order to validate the *gdnfa* primer sets, different organs (brain, pituitary, heart, muscle, gill, stomach, liver, intestine, skin, spleen, and kidney) were sampled from five pre-pubertal male rainbow trout (11 months of age).

The genetically male monosex population was generated by fertilizing eggs collected from two females with the sperm of one YY rainbow trout male. Offspring was maintained at 10 °C for 55 days and then reared at 12 °C. Gonads were collected from male alevins at 55, 90, 120, and 180 days of age. Five replicates comprising 250 gonads (at 55 and 90 days of age), 100 gonads (at 120 days of age), or 20 gonads (at 180 days of age) were generated. All samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C until RNA extraction.

Germ cell separation using centrifugal elutriation

Populations of undifferentiated spermatogonia were obtained from 40 testes of immature male trout (Stage I, 11 months of age) whereas B spermatogonia, spermatocytes, spermatids, and spermatozoa containing fractions were obtained from 10 testes of maturing males (stages III to IV, 18 months of age). Germ cell fractions were prepared as previously described (Loir and Sourdain 1994). Briefly, testes were minced and submitted to an enzymatic digestion in collagenase followed by mechanical dissociation. Cell suspensions were filtered through nylon membrane (50- and 32- μ m pore size) and centrifuged for 10 min at 100g. The cell pellets were suspended in L15 medium supplemented with 1% BSA and mixed with a 45% Percoll solution. Cells were centrifuged for 40 min at 500g and 20 min at 100g to remove erythrocytes. The upper cell layer was recovered and submitted to centrifugal elutriation (JE5

Beckman Instruments). Cell separation was performed at constant rotation speed (2000 rpm) and increasing flow rates in L15 medium supplemented with 0.5% BSA as described previously (Bellaïche et al. 2014b). Different germ cell fractions were collected and part of each fraction was fixed in Bouin's fixative, embedded first in agarose and then in Paraplast (Sigma). Then, 7- μ m sections were stained with Regaud's hematoxylin, orange G, and aniline blue to determine germ cell identity and enrichment as reported previously (Bellaïche et al. 2014b). The enrichment values were 90% for the type A spermatogonial fractions, 70% for the type B spermatogonial fractions, 70% for the spermatocytes fractions, and 95% for the spermatid/spermatozoa fractions. The remaining part of each fraction was rinsed in L15 medium containing 0.5% BSA, pelleted, and resuspended in TRIzol reagent. Samples were snap frozen and stored at -80°C until RNA extraction.

Real-time PCR analysis

Total RNA was isolated using the TRIzol reagent according to manufacturer's instructions (Invitrogen) and RNA concentrations were quantified using the NanoDrop ND-10 (Thermo Fisher Scientific, Courtaboeuf, France). RNA quality was determined using the Bioanalyser 2100 (Agilent Technologies, Massy, France). Five hundred nanograms total RNA was reverse transcribed using random hexamers and the GoScript™ Reverse Transcriptase (Promega). Quantitative polymerase chain reactions (qPCR) were conducted using the StepOne Plus thermocycler (Applied Biosystems). Primer sets were designed using online tools available at <https://eu.idtdna.com/calc/analyzer> and <https://eu.idtdna.com/scitools/Applications/RealTimePCR/>. Forward and reverse primers were chosen in different exons of the candidate genes to prevent amplification from genomic DNA. The primer sets are detailed in Table 1. PCR amplification was performed on 1/20 diluted first-strand cDNA templates, 1X Fast SYBR Green Master Mix (Applied Biosystems) fluorescent dye, and 300 nM of each forward and reverse primers. The cycling conditions were as follows: 95°C for 20s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. The efficiency of each primer set was about 100% as determined using twofold serial dilutions of pooled RT products. A melting curve analysis was carried out at the end of each run to control the amplification of a single PCR product. Gene expression levels

were measured on five independent biological samples in duplicate and normalized using *rs15* as described previously (Rolland et al. 2009). The relative abundance of the transcript was determined in arbitrary units relative to expression of the *rs15* gene using the comparative Ct method (Livak and Schmittgen 2001). A *t* test and a non-parametric Wilcoxon-Mann-Whitney were carried out to determine significant variations of the different transcript abundances.

Organotypic culture of testicular fragments

Testes were collected from immature male rainbow trout at stage III and cultured as previously reported (Sambroni et al. 2013). Note that the gonadal stage was later determined by histological examination of a testicular fragment fixed in Bouin's fixative. Testes were minced into 1 to 3 mm pieces with a tissue chopper and ten testicular fragments were randomly distributed (60–80 mg per well) onto Nunc polycarbonate membrane inserts into 24-well plates filled with 300 μ l per well of culture medium (synthetic L15 medium as modified by Loir and supplemented with 2% Ultrosor SF ($n = 6$ replicates per condition). Tissue fragments were incubated at 12°C for 96 h. A single dose (300 ng/ml) of three different androgens [11-ketotestosterone (11KT), testosterone (T), and 17α methyltestosterone (MT)] and one progestin [17α , 20β -Dihydroprogesterone (DHP)] was tested. Purified pituitary rainbow trout Fsh was also used at 500 ng/ml. The medium was changed every 2 days. At the end of the incubation, tissues were snap frozen and stored at -80°C in TRIzol reagent until RNA extraction.

Results

Phylogenetic reconstructions reveal species-specific gene duplication and losses in teleosts

The homologous genes corresponding to mammalian *gfal1*, *gdnf*, *kit*, and *kitlg* were searched in different vertebrate species and the orthologous relationships were first established using amino acid sequence alignments (Supplemental data 1, 2, 3 and 4) and phylogenetic reconstructions (Figs. 1 and 2), and then confirmed through analyses of syntenic chromosomal regions (Supplemental data 5 and 6).

Table 1 primers used in qPCR

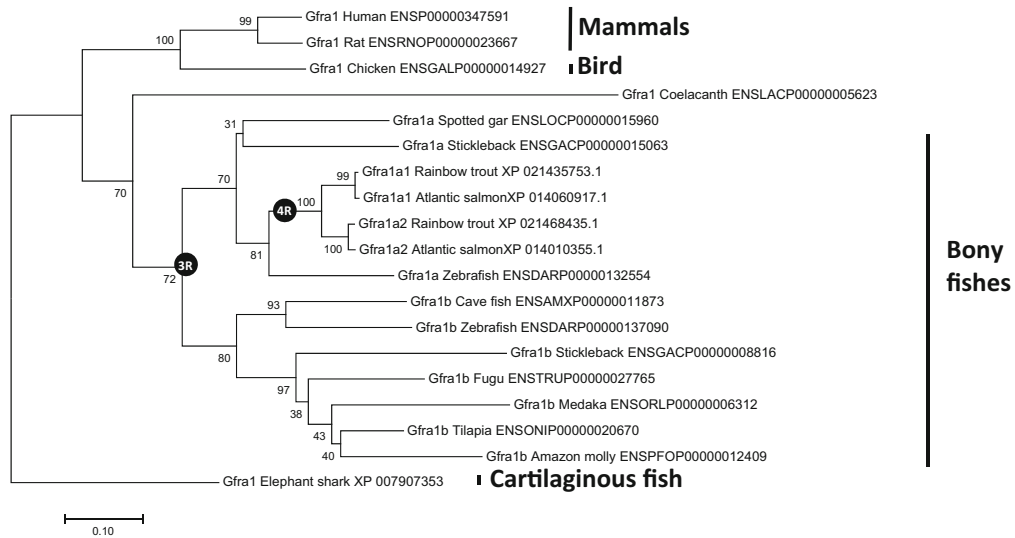
Genes	Sequences	Size bp	Amplicon bp	Tm °C	Concentration nM
<i>gdnfa1</i>	Fw: GTC-CGG-TGG-ATG-ATT-TGA-TTT-TC	21	100	60	300
	Rev: TTG-CTT-TAC-CGG-CAC-CGC-TTT-A	22			
<i>gdnfa2</i>	Fw: GAT-GTT-ACG-AAC-GTT-ATT-TAT-TAC	24	116	60	300
	Rev: GTT-CGC-CAA-AGC-CGA-GGC	18			
<i>gfra1a1</i>	Fw: CAT-GAG-GTC-CGA-TAT-TCG	21	159	60	300
	Rev: CTT-ATG-ATA-TTT-TAT-CGT-T	20			
<i>gfra1a2</i>	Fw: GCT-GGA-TGT-GTT-CTG-TAG-CCT-ATG	24	126	60	300
	Rev: GCG-GTT-GGG-ACC-AGA-AAG-GA	20			
<i>kitra1</i>	Fw: CTT-AAG-CCT-TCA-CTC-AAC-TGC-GG	23	200	60	300
	Rev: GGA-ATG-ACC-AGG-TGG-AGG-CCT	21			
<i>kitra2</i>	Fw: AGC-AAG-CCT-TCA-CTC-AGC-CATT	22	193	60	300
	Rev: GGA-ATG-ACC-AGG-TAG-GGG-TTG	21			
<i>kitrb</i>	Fw: ACA-GAC-CTG-TGA-CTT-ACA-A	19	171	60	300
	Rev: AGC-CGT-ACG-TCC-ACA-GTA-TAC-TGG	24			
<i>kitlga</i>	Fw: TTT-CCA-CTT-GGA-ACT-CAG-TTT-A	22	210	60	300
	Rev: CTC-CAA-GTT-CTT-CAT-GTG-AT	19			
<i>kitlgb</i>	Fw: TTC-TTG-GAT-ACT-ACA-CTG-GAT-ATT	24	191	60	300
	Rev: AAA-TGG-GGG-AAT-CAC-TTT-T	19			
<i>dnd</i>	Fw: AGA-AAC-GTC-AGC-TCT-GTC-TTG	21	150	60	300
	Rev: CAT-AGA-CCA-CCA-CTG-CAG-AC	20			
<i>nanos2</i>	Fw: CAA-AGT-CAT-CTG-CCC-ATT-CTG-A	22	142	60	300
	Rev: TCC-TGT-TTT-CTA-CTC-CAC-AGT-CC	23			
<i>vasa</i>	Fw: GGC-GTG-GCA-GCT-AGT-CAG-TTC-A	22	166	60	300
	Rev: CAC-ACA-AGT-CCC-ATG-GGC-AAA	21			
<i>gsdf1</i>	Fw: TCA-CAA-CCC-TCA-AGC-GCT-AGA	21	137	60	300
	Rev: ATG-AGA-GCT-CTG-CTG-TTA-TTG	21			
<i>rs15</i>	Fw: GTG-TGG-ACC-TGG-ACC-AGC-TT	20	91	60	300
	Rev: AGG-CCA-CGG-TTA-AGT-CTC-CT	20			
<i>rshl2</i>	Fw: CCA-ACAC-TGC-TCT-TGC-CAC-G	20	145	60	300
	Rev: TAC-ACC-ACG-CTG-AGG-GAA-CAC	21			

The analysis of the phylogenetic tree of *gfra1* genes reveals that a single *gfra1* gene copy has been conserved in jawed animals including cartilaginous fishes, non-teleost bony fishes, birds, and mammals (Fig. 1). However, two orthologous gene copies, named *gfra1a* and *gfra1b*, were detected in most teleostean fish species suggesting this gene duplication event occurred during the teleost-specific third-round whole genome duplication (3R). Surprisingly, no *gfra1b* gene copy was discovered in species belonging to the Salmoninae family (rainbow trout and Atlantic salmon). However, the two salmonids gained an additional *gfra1a* gene copy. The two distinct *gfra1a* gene copies were named *gfra1a1* and *gfra1a2* in these two salmonids. Regarding the phylogenetic analysis of the Gdnf ligand (Fig. 1), a different evolutionary history is observed in gnathostomes although a single-copy gene

named *gdnf* is also conserved in cartilaginous fishes, non-teleost bony fish, birds, and mammals. In most teleost fish, two duplicated gene copies named *gdnfa* and *gdnfb* were identified. In contrast to *gfra1b* that was not identified in rainbow trout and Atlantic salmon genomes, *gdnfb* gene was maintained in Salmoninae as a single-copy gene.

A similar evolutionary history was observed with the genes involved in Kit/Kitlg pathway (Fig. 2). A single copy of the *kitr* and *kitlg* genes was identified in cartilaginous fishes elephant shark (*Callorhynchus milii*) and whale shark (*Rhincodon typus*), in non-teleost bony fishes, birds, and mammals. In teleost, the ancestral duplication 3R led to the emergence of two gene copies for *kitlg* (*kitlga* and *kitlgb*) and *kitr* (*kitra* and *kitrb*). The additional whole-genome duplication (4R) that occurred in Salmoninae resulted in the duplication of *kitlga*

Gfra1



Gdnf

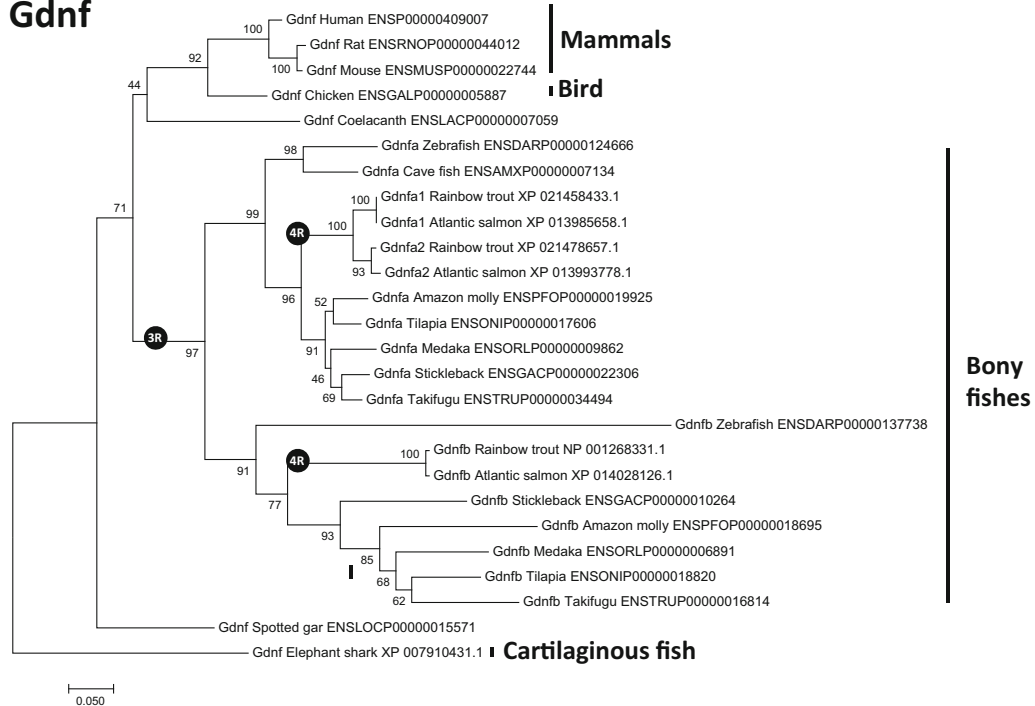


Fig. 1 Evolutionary history of Gfra1 receptors and cognate Gdnf ligands in gnathostomes. The evolutionary relationships of the genes encoding for distinct Gfra1 receptors and Gdnf ligands was examined using a sequence-based phylogenetic reconstruction. Amino acids sequences homologous to the mammalian Gfra1 receptor and Gdnf were searched using the tblastn algorithm in NCBI and ENSEMBL nucleotide databases including genomes, mRNA, and EST collections. Amino acid sequences deduced from the nucleotide sequences were aligned using the CLUSTALW multiple alignment software (Thompson et al. 1994). The phylogenetic tree

was built using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 (Kumar et al. 2016). The analysis of the amino acids sequences was carried using the Neighbor-Joining (NJ) method. The reliability of the inferred tree was estimated using the bootstrap method with 100 replications. The tree is represented as a rooted tree using the elephant shark sequences. The third (3R) and fourth (4R) whole genome duplications are indicated. The accession number of each protein is indicated after the common name of the species. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length

(*kitlga1* and *kitlga2*) and *kitra* (*kitra1* and *kitra2*) in rainbow trout and Atlantic salmon. The *kitrb* and *kitlgb* genes remained unique copy genes in these two salmonids.

In summary, genes encoding for the mediators of the Gfra1/Gdnf and Kit/Kitlg regulatory pathways are conserved during the evolutionary history of the gnathostomes suggesting they exert important physiological functions. However, two successive duplication events occurred, a first one in the ancestor of teleost fish and a second one in the ancestor of Salmoninae, resulting in the retention of additional gene copies or loss of paralogs (*gfra1b*). In order to further understand the biological significance of gene duplications and losses, we examined the spatial and temporal expression patterns of the duplicated gene copies in rainbow trout.

Cellular expression patterns of the candidate genes

Although not discriminating different ohnologous copies, our attempts to detect the cellular expression pattern of the transcripts on gonadal sections using the in situ RNA hybridization technique failed. In order to determine the cellular expression pattern of each gene copies using qPCR, we prepared total RNA from testes of immature animals and from enriched germ cell populations. The molecular identity of the different germ cell fractions was first validated by an expression profiling analysis of genes known to be expressed in germ cells or somatic cells (Supplemental data 7). As expected, the *vasa* transcript was detected in stage I testes and in all germ cell fractions. The *nanos2* and *dnd* genes were previously detected mainly in zebrafish undifferentiated spermatogonia using the in situ RNA hybridization technique (unpublished data). Both *nanos2* and *dnd* transcripts showed a two- to threefold increased accumulation in immature testis and undifferentiated type A spermatogonia compared to type B spermatogonia, meiotic, and post-meiotic germ cell fractions. The relative abundance of the *rshl2* gene was highest in spermatocytes and post-meiotic cell fractions. The expression levels of the Sertoli cell-specific *gsdf1* gene were highest in immature whole testes and were barely detected in germ cell fractions.

The expression profiles of the genes involved in the Gfra1/Gdnf regulatory pathway are presented in Fig. 3. Transcripts corresponding to *gfra1a1* and *gfra1a2* gene copies were detected in immature whole testis and in undifferentiated type A spermatogonia. Note that the

gfra1a1 transcript was predominantly detected in whole immature testes compared to undifferentiated type A spermatogonia suggesting that this transcript is also expressed in somatic cells. The expression levels of the *gdnfb* and *gdnfa1* transcripts were higher in undifferentiated type A spermatogonia compared to whole immature testis. No expression of *gdnfa2* transcript was detected in the testis of immature male trout. Taken together, our data indicate that the main source of Gdnf ligands originated from the germinal compartment itself. The Gdnfa1 and Gdnfb ligands could exert an autocrine regulation on undifferentiated type A spermatogonia through their binding to the germinal Gfra1a2 receptor and a paracrine action on somatic cells through the activation of the Gfra1a1 receptor.

The cellular expression profiles of the genes involved in Kit/Kitlg pathway are detailed in Fig. 4. The *kitrb* transcript showed a sixfold increase of its abundance in undifferentiated type A spermatogonia compared to immature whole testes. The *kitrb* transcript was barely detected in the other germ cell fractions. The *kitra1* and *kitra2* were also predominantly expressed in undifferentiated type A spermatogonia. We designed a primer set capable to amplify both *kitlga1* and *kitlga2* gene copies. The *kitlga* transcripts were significantly detected in whole testes but not in germ cell fractions indicating that *kitlga1* and/or *kitlga2* are expressed in somatic cells. In contrast, *kitlgb* was mainly detected in undifferentiated type A spermatogonia and immature testes. In summary, there are two distinct sources of Kit ligand in testes: *kitlga* and *kitlgb* transcripts arise mainly from somatic testicular cells and undifferentiated type A spermatogonia, respectively. The high expression levels of *kitrb*, *kitra1*, and *kitra2* in undifferentiated type A spermatogonia indicate that this cell type could be the target cells.

Histological analysis of the male gonads and temporal gene expression patterns during ontogenesis

To determine the temporal expression patterns of the candidate genes in the testes using qPCR, an all-male rainbow trout offspring was produced and gonads were dissected out at 55, 90, 120, and 180 days post fertilization (dpf). Note that in our rearing conditions, 45% of the male trout sexually matured precociously at 1 year of age. Histological observations of testicular sections indicated that undifferentiated type A spermatogonia were single cells in the male gonads from 55 to 90 dpf

(Supplemental data 8). The organization of the seminiferous tubules and additional clusters of two to four undifferentiated type A spermatogonia were observed from 120 dpf onwards only in sexually maturing males. The appearance of type B spermatogonia was observed between 120 and 180 dpf.

A gene expression profiling of the sample panel was first carried out with control genes to examine the evolution of the Sertoli cells and spermatogonial populations before and after spermatogenesis onset (Supplemental data 9). The expression levels of the *vasa* gene remained unchanged between 55 and 90 dpf. This indicates that the proportion of undifferentiated type A spermatogonia remains constant in the male gonads during this time period. Interestingly, the expression levels of the *nanos2* and *dnd* genes were significantly increased between 55 and 90 dpf indicating that the initial spermatogonia had undertaken a differentiation process. This population of spermatogonia was referred as prospermatogonia. Concomitantly with the apparition of clustered type A spermatogonia (named progenitors), the abundance of the *dnd* and *nanos2* transcripts increased drastically between 90 and 120 dpf. The *dnd* and *nanos2* gene expression levels reached a plateau between 120 and 180 dpf whereas *vasa* gene expression levels continued to strongly increase. This reveals that the proportion of progenitors reaches a plateau between 120 and 180 dpf while other spermatogonial cell populations differentiate and proliferate (type B spermatogonia). In contrast to germ cell-specific genes, *gsdf1* gene expression levels showed moderate variations.

The expression patterns of the genes involved in the *Gfra1/Gdnf* regulatory pathway are presented in Fig. 5. The *gfra1a1* and *gfra1a2* transcripts showed distinct patterns of expression. *gfra1a2* expression was highest at 55 dpf and then decreased sharply between 55 and 90 dpf. In contrast, *gfra1a1* gene expression increased between 90 and 120 dpf. The temporal expression pattern of *gdnfb* was similar to *vasa* suggesting that this gene could be expressed concomitantly with active spermatogonial proliferation and/or differentiation. The *gdnfa1* gene expression appeared similar to *gfra1a2* but the variations were rather low. In summary, we did not observe obvious correlation between the expression patterns of the genes encoding for the *Gfra1* receptors and their cognate ligands. However, a clear shift of expression between *gfra1a1* and *gfra1a2* occurred between 55 and 120 dpf suggesting that the two receptors could be

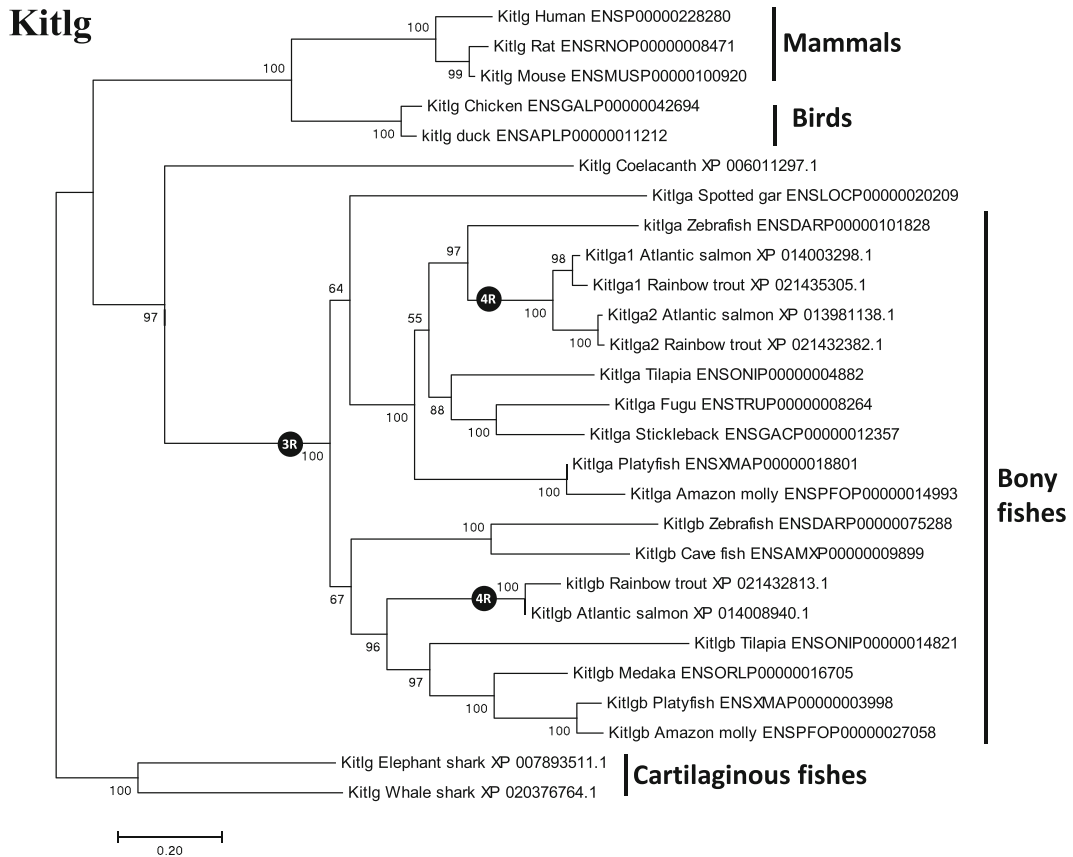
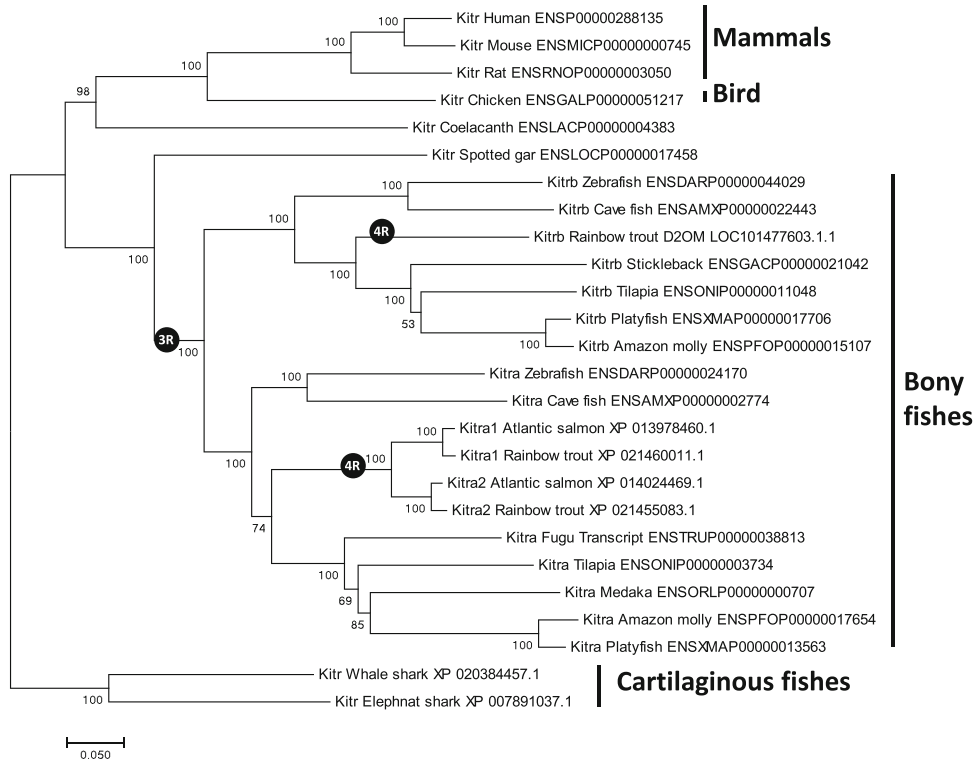
expressed in different germ cells or are differently regulated. Our data indicate that *gdnfa1* and *gdnfb* are poorly expressed before spermatogenesis onset. The progressive increase of *gdnfb* gene expression from 90 to 180 dpf favors a late role of the *Gfra1/Gdnfb* regulatory pathway during spermatogenesis onset.

In contrast to the *Gfra1/Gdnf* regulatory pathway, we observed similar expression patterns between the *kitra2*, *kitrb*, and *kitlgb* genes (Fig. 6). These three genes were expressed at higher levels at 55 dpf and a sharp decrease of gene expression was observed from 90 to 180 dpf. The *kitra1* gene expression was hardly detectable and showed no significant variation during this time period. The expression profile of *kitlga* was similar to that of the *gsdf1* gene. Our data indicate that the *Kit/Kitlg* regulatory pathway could be mainly active before spermatogenesis onset.

Hormonal regulation of the candidate genes

The hormonal regulation of the genes involved in the two candidate regulatory pathways was examined (Fig. 7). The addition of Fsh to cultured testicular explants slightly decreased *gdnfb*, *gdnfa1*, and *gfra1a2* but increased *gfra1a1* gene expression. *gfra1a1* gene expression was mainly increased by 17α , 20β dihydroprogesterone and by testosterone (T) and 17α methyl testosterone although to a lesser extent. This indicates that Fsh action on *gfra1a1* is mediated by sexual steroids. Similarly, Fsh action on *gdnfb* and *gdnfa1* is mediated by sexual steroids. The *gdnfb* transcript expression was slightly decreased by the different sexual steroids used whereas *gdnfa1* gene expression was decreased only by T and 11KT. Only *gfra1a2* was not regulated by any sexual steroids. No effect of the different hormones was observed on the expression levels of the *kitra1*, *kitra2*, *kitlga* and *kitlgb* genes (data not shown).

Fig. 2 Evolutionary history of the Kit receptors and cognate Kit ligands in gnathostomes. The evolutionary relationships of the genes encoding for Kit receptors and cognate Kitlg ligands was examined using a sequence based phylogenetic reconstruction as described in Figure legend 1. The tree is represented as a rooted tree using the whale shark and elephant shark sequences. The third (3R) and fourth (4R) whole-genome duplications are indicated. The accession number of each protein is indicated after the common name of the species. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length



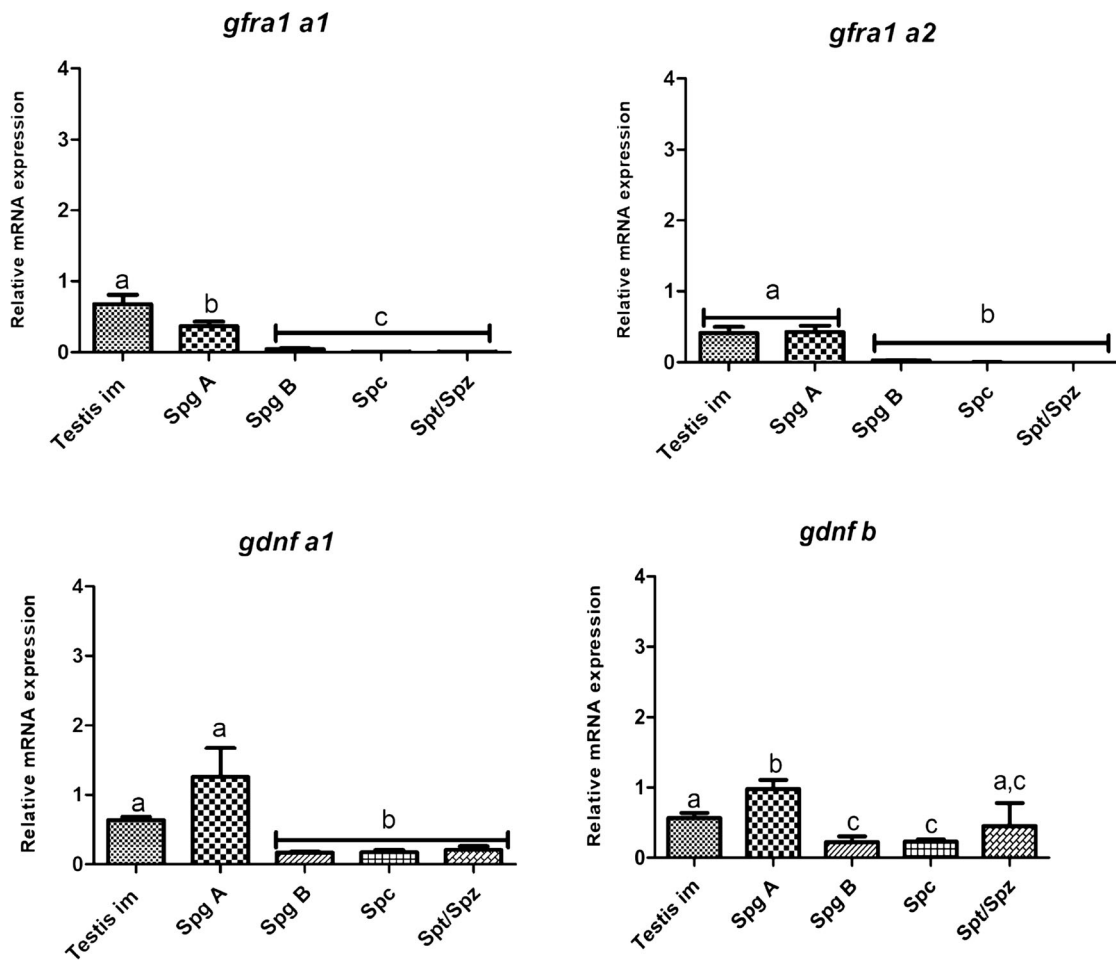


Fig. 3 Cellular expression patterns of the genes involved in the Gfra1/Gdnf regulatory pathway. Gene expression was determined using qPCR from a panel of samples including whole testes (Testes Im) and enriched A spermatogonia (Spg A) collected from immature male rainbow trout at stage I. Additional samples include B spermatogonia (Spg B), spermatocytes (Scytes), and small post-meiotic germ cells (Spt/Spz: secondary spermatocytes, spermatids, and spermatozoa) collected from maturing males. The *gfra1a2*,

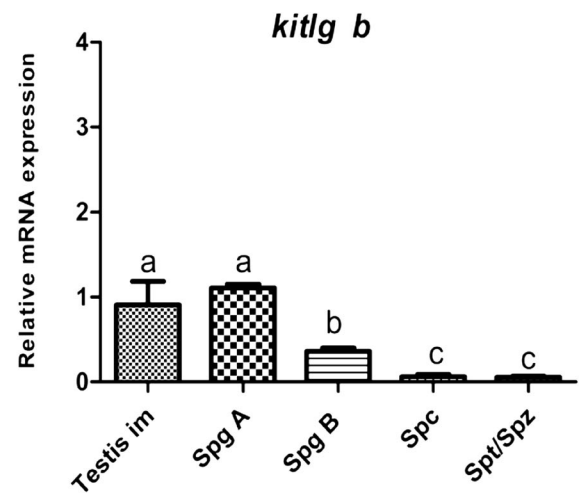
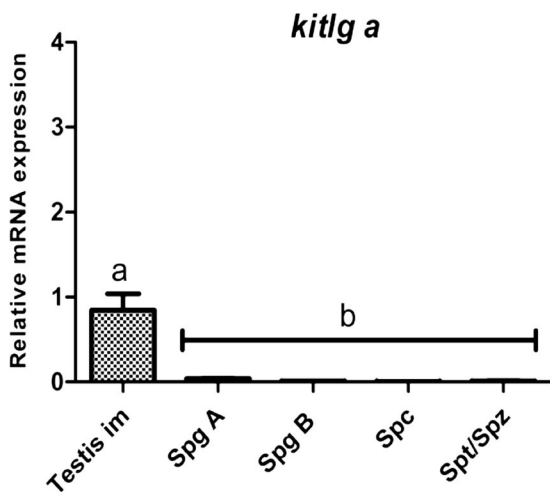
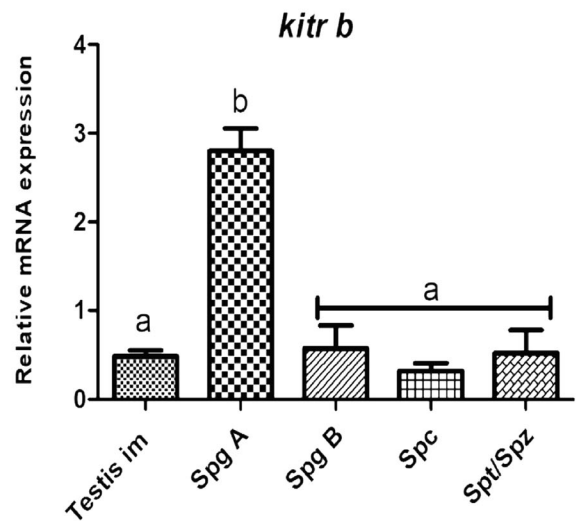
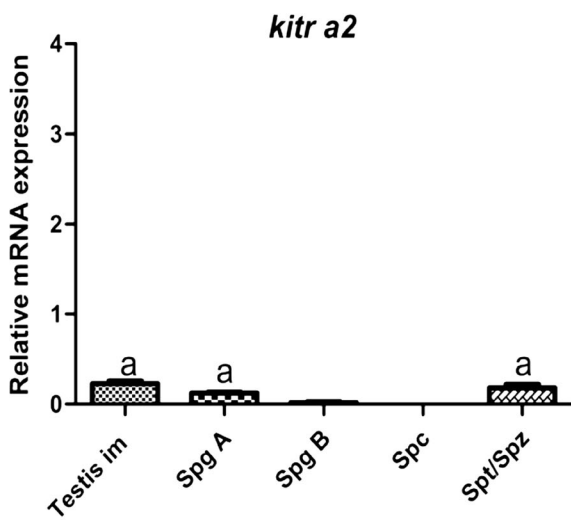
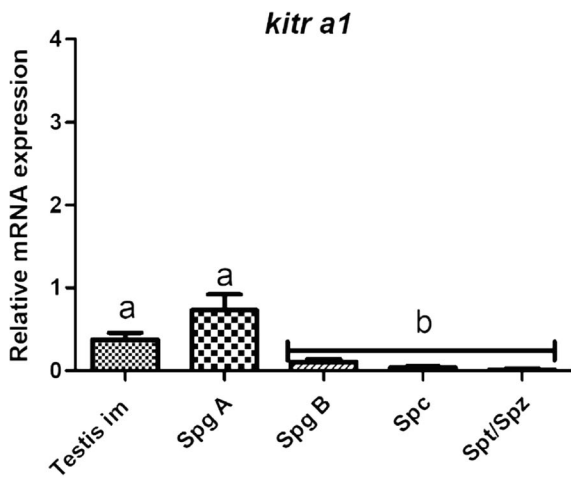
gdnfa1, and *gdnfb* genes are predominantly expressed in undifferentiated type A spermatogonia. The *gfra1a1* transcript is accumulated in undifferentiated type A spermatogonia but higher expression levels are observed in whole testes. This suggests that *gfra1a1* is also expressed in testicular somatic cells of immature male trout. Statistical analyses were performed using confidence intervals. The different statistical groups are represented by letters a, b, and c ($p < 0.05$). Values represent means \pm SEM of five replicates ($n = 5$)

Discussion

Genes involved in the Gfra1/Gdnf and Kit/Kitlg regulatory pathways show a complex evolutionary history in teleost fish and in salmonids

Using phylogenetic reconstructions and analyses of syntenic chromosomal fragments, we demonstrate that genes involved in Gfra1/Gdnf and Kit/Kitlg regulatory pathways are conserved in gnathostomes as single-copy genes with the exception of teleost fish in which gene duplications and losses have occurred. It is well admitted

Fig. 4 Cellular expression patterns of the genes involved in the Kit/Kitlg regulatory pathway. Gene expression was determined using qPCR from a panel of samples including whole testes (Testes Im) and enriched A spermatogonia (Spg A) collected from immature male rainbow trout at stage I. Additional samples include B spermatogonia (Spg B), spermatocytes (Scytes), and small post-meiotic germ cells (Spt/Spz: secondary spermatocytes, spermatids, and spermatozoa) collected from maturing males. The *kitrb*, *kitra1*, and *kitlg* genes are predominantly expressed in undifferentiated type A spermatogonia. In contrast, much higher expression levels of the *kitlg* transcript are observed in whole testes. This suggests that *kitlg* is expressed mainly in testicular somatic cells. The different statistical groups are represented by letters a, b, and c ($p < 0.05$). Values represent means \pm SEM of five replicates ($n = 5$)



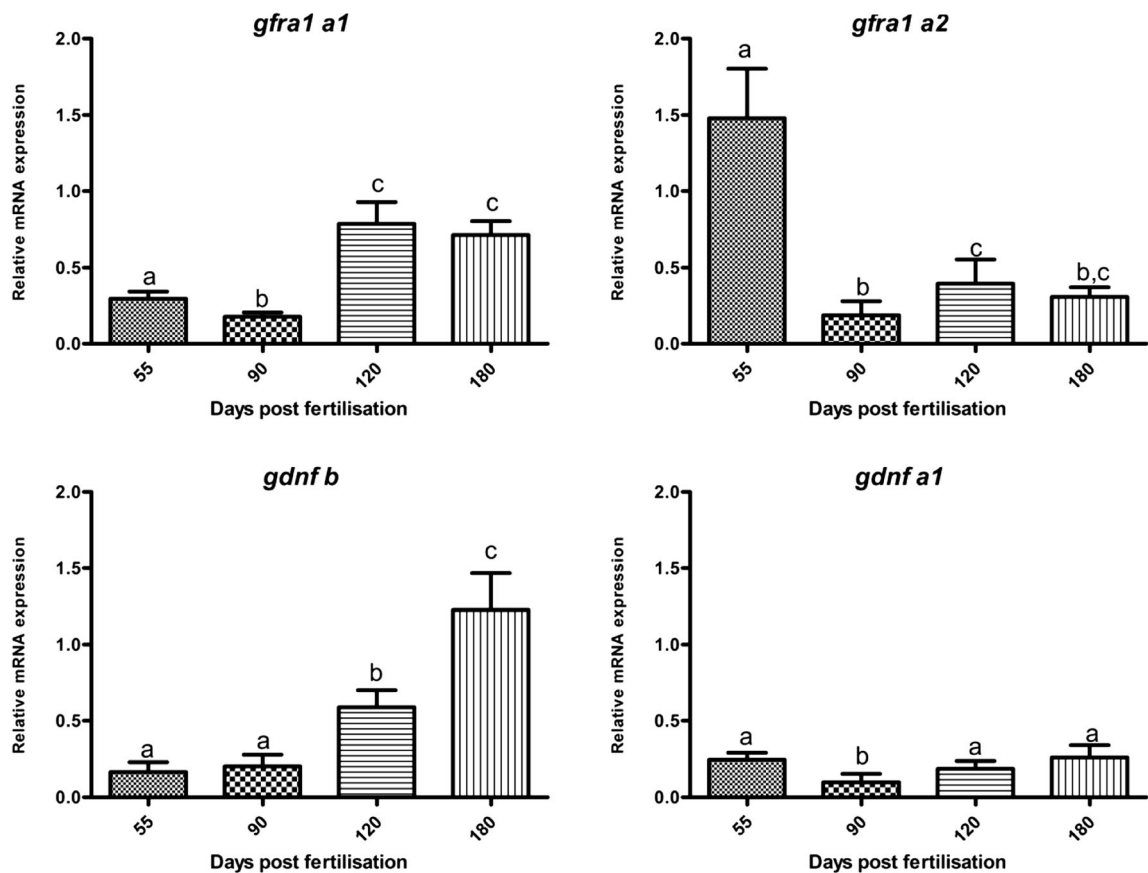


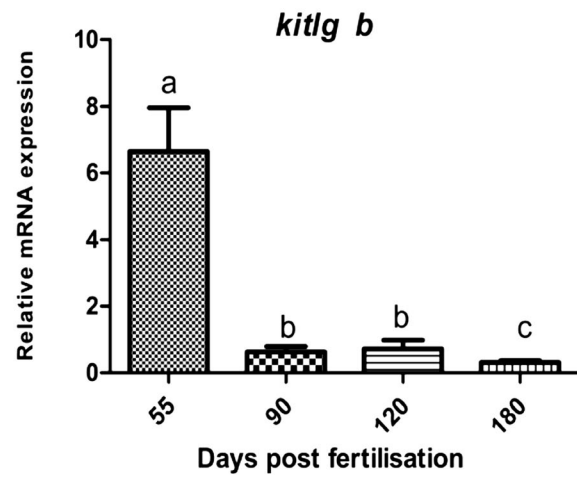
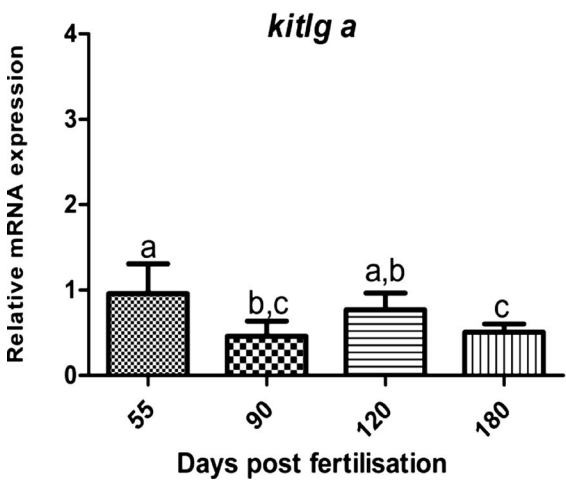
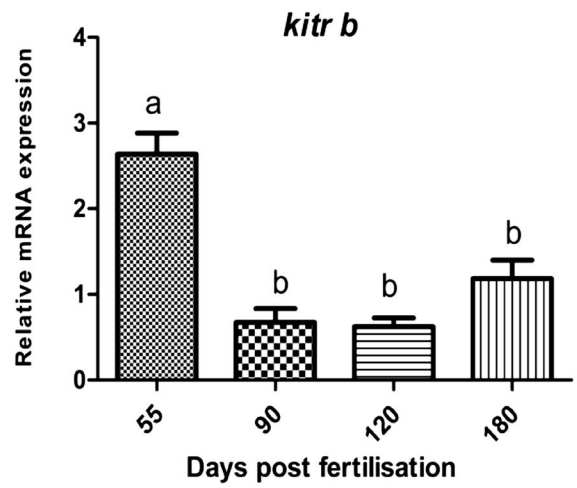
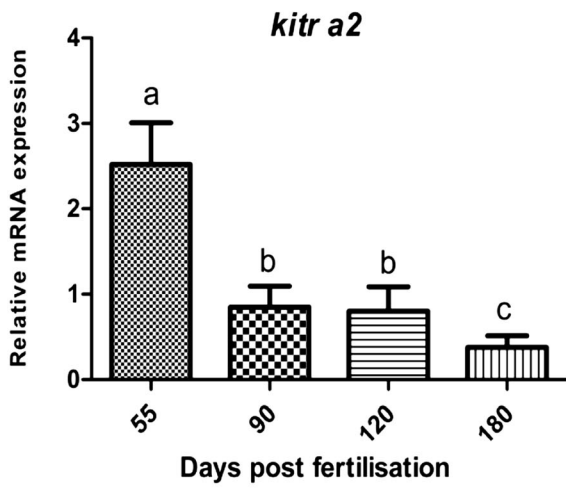
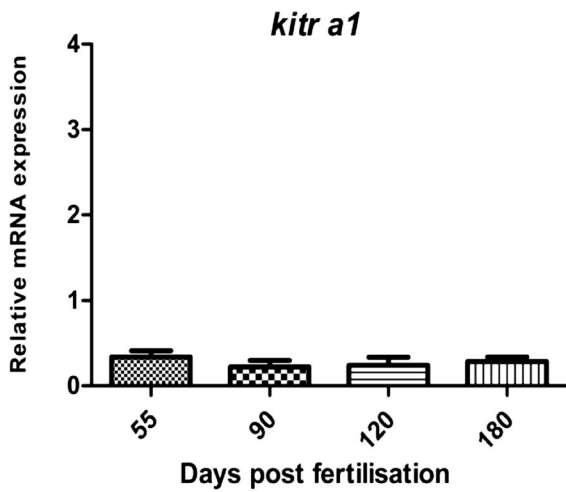
Fig. 5 Temporal expression profiles of the genes involved in the Gfra1/Gdnf regulatory pathway. Gene expression was monitored in the male gonads during rainbow trout ontogenesis using qPCR. Gonadal samples were collected at 55, 90, 120, and 180 days post fertilisation (dpf) from an all-male trout population. Note that *gfra1a2* expressions levels drop significantly after 55 dpf and before spermatogenesis onset. The expression levels of *gfra1a1*

and *gdnfb* increase significantly at 120 dpf concomitantly with the increased number of spermatogonia. The *gdnfa1* gene expression pattern shows almost no variation during the time period examined. The different statistical groups are represented by letters a, b, and c ($p < 0.05$). Values represent means \pm SEM of five replicates ($n = 5$)

that the ancestor of the teleost fish has been submitted to a third round of whole genome duplication (Amores et al. 1998; Meyer and Van de Peer 2005). This event is likely responsible for the duplication of the ancestral *gfra1*, *gdnf*, *kitr*, and *kitlg* genes. In agreement with an additional fourth round of whole-genome duplication in rainbow trout and in Atlantic salmon (Berthelot et al. 2014; Lien et al. 2016), we observed an additional duplication of *gfra1a*, *gdnfa*, *kitra*, and *kitlga* in these two salmonids. Whole-genome duplications are followed by gene retentions but also by gene losses to restore a diploid status (Brunet et al. 2006). This implies that duplication of *gfra1b*, *gdnfb*, *kitrb*, and *kitlgb* occurred in the common ancestor of the two salmonid species but one copy of the *gdnfb*, *kitrb*, and *kitlgb* genes was lost. More, our data

indicate that the two *gfra1b* gene copies were lost. Note that *ngr3b* and *trub1* that were surrounding *gfra1b* in the ancient fish species localized to different chromosomes (Supplemental data 5) in the two salmonid genomes

Fig. 6 Temporal expression profiles of the genes involved in the Kit/Kitlg regulatory pathway. Gene expression was monitored in the male gonads during rainbow trout ontogenesis using qPCR. Gonadal samples were collected at 55, 90, 120, and 180 days post fertilisation (dpf) from an all-male trout population. Note that *kitrb*, *kitra2*, and *kitlgb* show similar expression profiles with a sharp decrease of their relative abundances at 90 dpf before spermatogenesis onset. The *kitra1* and *kitlga* transcripts show almost no variation of their relative abundances during the time period examined. The different statistical groups are represented by letters a, b, and c ($p < 0.05$). Values represent means \pm SEM of five replicates ($n = 5$)



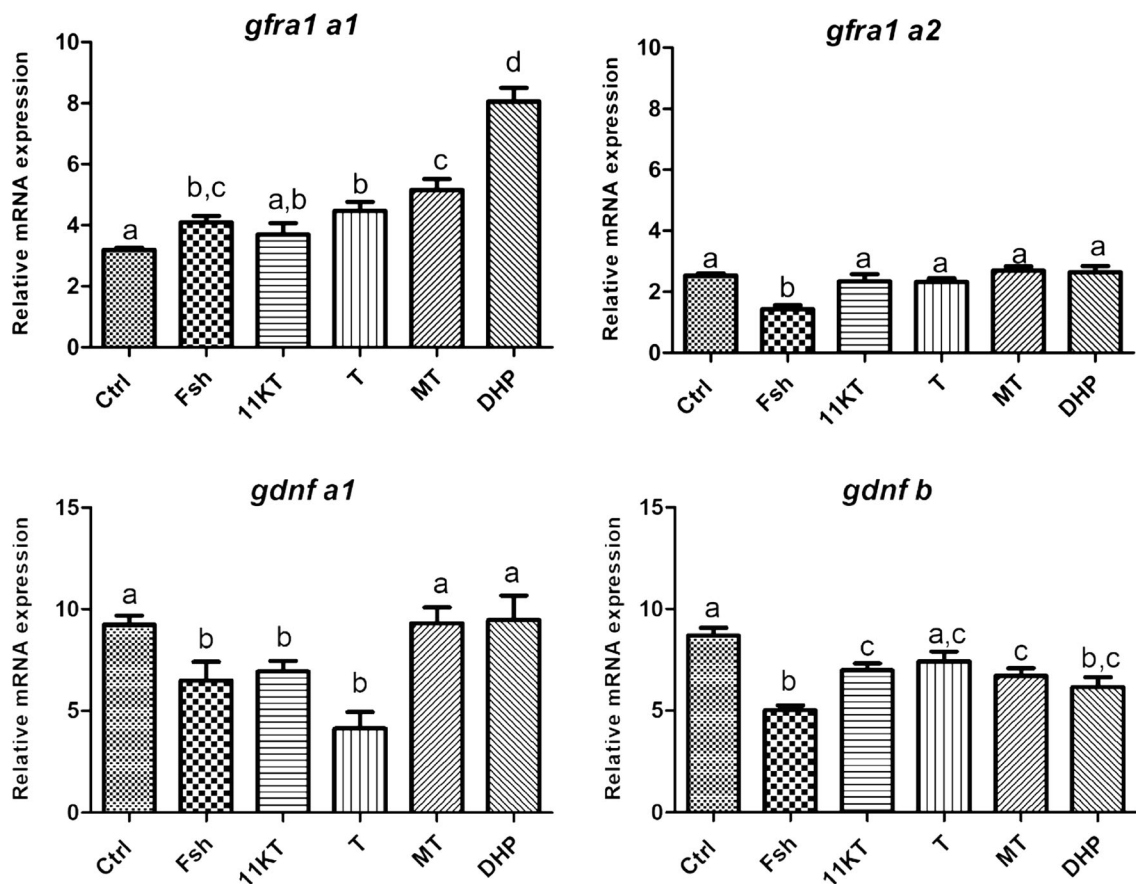


Fig. 7 Hormonal regulation of the genes involved in the *Gfra1*/*Gdnf* regulatory pathway. Testicular explants collected from maturing rainbow trout males (stage III) were incubated for 4 days in the presence or absence of purified Fsh (300 ng/ml), 11-ketotestosterone (11KT 300 ng/ml), testosterone (T 300 ng/ml), 17 α methyl testosterone (MT 300 ng/ml) and 17 α -20 β dihydroprogesterone (DHP 300 ng/ml). Gene expression levels were measured using qPCR. Note that *gfra1a* gene expression is

stimulated by Fsh and sexual steroids including androgens and progestogens. In contrast, *gdnfb* gene expression is negatively regulated by Fsh and sexual steroids. Note that *gdnfa1* is also negatively regulated by Fsh and by physiological androgens only. Different letters indicate significantly different levels of relative abundance at $p < 0.05$. Values represent means \pm SEM of six replicates ($n = 6$)

suggesting that *gfra1b* could have been lost because of its location close from the breaking spot of the chromosomal fragment. The present study highlights that gene content is different in salmonids compared to other bony fish but the biological significance remains to be further investigated.

Duplicated gene copies resulting from the recent 4R duplication of the salmonid genome exhibit similar or different spatiotemporal expression profiles and are differently regulated by reproductive hormones

Numerous studies have reported that the ancient third round of whole genome duplication in teleost led either to similar or different cell-specific expression patterns of the duplicated gene copies (Rolland et al. 2009).

Therefore, it is not surprising to observe that *kitlga* and *kitlgb* were expressed in somatic and germinal cells, respectively. However, information on changes of gene expression profiles resulting from the recent fourth round whole genome duplication in salmonids remains scarce. The present study shows that *gfra1a1* was mainly expressed in somatic testicular cells as evidenced by higher expression levels observed in whole immature testes compared to that of undifferentiated type A spermatogonia. In contrast, *gfra1a2* was mainly expressed in undifferentiated type A spermatogonia. More, the *gfra1a1* and *gfra1a2* ohnologs are expressed differently during ontogenesis and are differently regulated by Fsh and sexual steroids. These observations indicate that the fourth round of whole genome duplication in salmonids was

followed by a different cell-specific distribution and regulation of the duplicated *gfra1a* gene copies. Such changes together with discrete amino acids differences in gene product open the way for subfunctionalization or neofunctionalization (Ohno 1970). One could hypothesize that the duplicated genes in fish fulfill all the functions and regulation of the ancestral gene (subfunctionalization) or acquire new functions (neofunctionalization). In salmonids, the gain of an additional *gfra1a* gene copy with a different regulation may have been sufficient to compensate the loss of *gfra1b*.

In contrast to *gdnfa1*, no expression of *gdnfa2* was detected in any rainbow trout tissues examined including brain and ovary, two major tissues expressing Gdnf in mammals (data not shown). Further investigations will be required to determine whether the fourth whole-genome duplication resulted in the pseudogenization of the *gdnfa2* gene copy in salmonids.

The Kit/Kitlg regulatory pathway could act on germ stem cells prior to spermatogenesis onset

Gene expression profiling during ontogenesis revealed that the *kitrb*, *kitra*, and *kitlgb* transcripts were co-expressed with the highest relative abundance measured at 55 dpf. These genes are likely expressed in germ stem cells at this age since the corresponding transcripts were predominantly detected in undifferentiated type A spermatogonia collected from immature male trout. In contrast to mammalian species in which Kitlg is expressed by somatic cells, our data indicate that *kitlgb* exerts an autocrine regulation on the male germ stem cells before spermatogenesis onset that occurs between 90 and 120 dpf in precocious males. In mouse, *kitr* and *kitlg* genes are also expressed before spermatogenesis onset in the embryonic gonads where they have pleiotropic functions. The *kitr* and *kitlg* genes support PGC proliferation and cell survival through a decreased germ cell apoptosis (Dolci et al. 1991; Runyan et al. 2006). In zebrafish, it has been demonstrated that a feeder layer expressing Kitlga stimulated the proliferation of PGC collected from 1 dpf embryos but a Kitlgb conditioned medium had no effect. Interestingly, the sharp decreased expression of *kitlgb* in trout was not followed by a decreased number of spermatogonia as revealed by monitoring the expression levels of the *vasa* gene between 55 and 90 dpf. In parallel, note that the expression levels of the somatic *kitlga* were rather unchanged during the time period examined. Altogether, the observations gained in

zebrafish and rainbow trout suggest that Kitlgb may have another function than to stimulate germ cell proliferation. In mouse, both *kitr* and *kitlg* genes encode for cell-surface proteins and they play a major role in the adhesion of the PGC to the somatic cells (Pesce et al. 1997). Further investigations will be required to determine whether *kitrb*, *kitra1*, and *kitlgb* have a similar role in fish gonads before spermatogenesis onset.

The Gfra1/Gdnf regulatory pathway could act on germ cells and somatic cells

The present study reveals that both *gfra1a1* and *gfra1a2* are expressed in rainbow trout undifferentiated type A spermatogonia collected from 11-month immature males. Therefore, undifferentiated type A spermatogonia are likely target cells of the Gdnf ligands. In mammals, Gdnf is required for the maintenance of SSC in vivo and in vitro in long-term cultures of spermatogonia. Gdnf that originates from Sertoli and/or peritubular cells promotes SSC proliferation and inhibits their differentiation through a complex cascade of molecular events (Braydich-Stolle et al. 2005; He et al. 2008). In contrast to mammals, our data provide evidences that Gdnf ligands could exert an autocrine action because *gdnfa1* and *gdnfb* are also expressed in these germ cells. Interestingly, *gdnfb* expression levels appeared similar to that of the *vasa* gene between 55 and 180 dpf in rainbow trout male gonads. This similarity suggests that *gdnfb* is co-regulated with *vasa* in the different spermatogonia or both proteins are involved in the same biological process.

We observed that *gfra1a1* was expressed at higher levels in whole immature testes compared to that of undifferentiated type A spermatogonia, suggesting that this gene is also expressed in somatic testicular cells. In mammals, the Gfra1/Gdnf regulatory pathway is known to influence germ stem cell homing (Kanatsu-Shinohara et al. 2012). Gdnf stimulates the proliferation of immature Sertoli cells through its binding to Gfra1 and interaction with the neural cell adhesion molecule (NCAM) co-receptor that mediates a complex intracellular signaling (Yang and Han 2010). NCAM mediates adhesion between Sertoli cells and gonocytes in cocultures from testes of neonatal rats (Orth and Jester Jr. 1995). In contrast to mammals, Sertoli cells continue to proliferate in the testes of adult fish during spermatogenesis (Schulz et al. 2005). In consequence, one could speculate that the increased *gdnfb* expression observed after spermatogenesis onset could be a germ cell derived

signal that promotes the proliferation of surrounding Sertoli cells to provide an appropriate homing with nutritional and structural support. Note that *gfra1a1* was stimulated by Fsh and other sexual steroids. Fsh and sexual steroids are major mitotic factors for Sertoli cells in mammals (Pitetti et al. 2013). However, further investigations will be required to identify the testicular cells that express *gfra1a1* in rainbow trout and the effect of Gdnf ligands on these target cells.

The molecular identity of the male germ cells changes during rainbow trout ontogenesis and spermatogenesis onset

In the present study, gene expression profiling shows that the molecular identity of germ stem cells changes during ontogenesis. The *kitrb* transcript was expressed at much higher levels at 55 dpf and was restricted to undifferentiated type A spermatogonia collected at 11 months of age. These observations suggest that *kitrb* is highly expressed in the early germ stem cell population. Other genes that include *kitra2*, *kitlgb*, and *gfra1a2* showed a similar expression pattern. In contrast, *nanos2* and *dnd* gene expression levels were low in this germ cell population. It is unlikely that the decreased expression of *kitrb*, *kitra2*, *kitlgb*, and *gfra1a2* genes at 90 dpf could result from a decreased number of germ cells because *vasa* gene expression remained stable and *nanos2* and *dnd* gene expression was increased between 55 and 90 dpf. Altogether, our data suggest that the initial male germ cell population observed at 55 dpf likely differentiates to form a new population of germ stem cells at 90 dpf. Interestingly, in mouse, PGC that reach the gonads between E10.5 and E12.5, stop to proliferate by E14.5 because they do not perceive the Kitlg signal. These cells become prospermatogonia and are arrested in G0/G1 of the cell cycle from E14.5 until 1–2 days post-partum (Busada et al. 2014; Western et al. 2008). Our observations in rainbow trout show that germ cells decrease *kitr* transcripts expression, but further investigations will be needed to demonstrate whether the initial germ stem cells observed at 55 dpf in the male gonads could differentiate to become “prospmatogonia” between 55 and 90 dpf. We previously observed that *nanos2* and *dnd* were mainly expressed in zebrafish paired A spermatogonia using the in situ hybridization technique (unpublished data). In consequence, we hypothesize that the sharp increased expression levels of *nanos2*, *dnd*, and *vasa* genes between 90 and 120 dpf reflects the differentiation and

proliferation of this population of progenitors. This population of progenitors likely reaches a plateau between 120 and 180 dpf because *nanos2* and *dnd* gene expression levels remain constant during this time window. Between 120 and 180 dpf, *vasa* expression levels continue to increase indicating that other types of spermatogonia proliferate within the gonads.

In conclusion, spatiotemporal gene expression profiling carried out in the present study provides evidences that both Kit/Kitlg and Gfra1/Gdnf regulatory pathways could be active in the male gonads of rainbow trout during ontogenesis. An autocrine Kit/Kitlg regulatory pathway is likely active on germ stem cells before 55 dpf and involved the *kitrb*, *kitra2*, and *kitlgb* genes. A constant paracrine action of Kitlga should not be excluded. An autocrine Gfra1/Gdnf regulatory pathway is also likely activated at 55 dpf though the involvement of *gfra1a2*, *gdnfa1*, and *gdnfb* genes. Again, an additional paracrine action of the regulatory pathway involving *gfra1a1* is likely active in somatic testicular cells and is regulated by Fsh and sexual steroids. Further investigations will be required to precise the functional effects of these regulatory pathways on germ cell survival, proliferation and or differentiation.

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