Instituto de Biociências

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

"Identification of germ cell-intrinsic players (GDNF and long non-coding RNAs) involved in the spermatogonial stem cell fate of adult zebrafish"

LUCAS BENITES DORETTO

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Celular Estrutural e Funcional.

Prof. Dr. Rafael Henrique Nóbrega

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"Julio de Mesquita Filho"

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"Trabalhadores do Mundo" de Bertolt Brecht:

Trabalhadores do Mundo, uni-vos!

Lutai por um mundo novo e melhor!

A razão e a ciência iluminarão o caminho,

Se seguirdes a bandeira vermelha da revolução.

Trabalhadores do Mundo, uni-vos!

Lutai por um mundo novo e melhor!

Sem vós não há pão, não há vida,

Não há amor, nem liberdade.

Trabalhadores do Mundo, uni-vos!

Lutai por um mundo novo e melhor!

A morte há de vir um dia,

E nossas cinzas hão de unir-se às estrelas.

Mas o triunfo da nossa causa

Sobreviverá à própria morte.

Trabalhadores do Mundo, uni-vos!

Lutai por um mundo novo e melhor!

AGRADECIMENTOS

Como estudante de pós-graduação, sou plenamente consciente das inúmeras dificuldades que enfrentamos na vida acadêmica. Nossas jornadas são marcadas por longas horas de trabalho, bolsas de estudo escassas, baixa remuneração, falta de reconhecimento, desafios relacionados à saúde mental e a escassez de oportunidades no mercado de trabalho. No entanto, é crucial ressaltar que cada um de vocês desempenha um papel fundamental no avanço da ciência, na transformação da sociedade e no fortalecimento de nossa soberania nacional.

Neste momento, gostaria de expressar minha sincera gratidão a todos os meus colegas de trabalho, que têm sido uma fonte constante de apoio, colaboração e inspiração. Suas contribuições e dedicação são inestimáveis, e tenho a sorte de poder compartilhar essa jornada com pessoas tão talentosas e comprometidas.

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Além disso, expresso minha profunda gratidão ao meu orientador, cuja orientação sábia e experiente tem sido essencial para o meu crescimento acadêmico e profissional. Sua expertise, incentivo e disponibilidade para compartilhar conhecimento têm sido inestimáveis para minha formação como cientista. Sou grato pela confiança que você depositou em mim e pelo apoio constante ao longo dessa jornada.

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Atenciosamente,

Um colega cientista.

Summary

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Resumo

A espermatogênese é um processo controlado por células-tronco no qual uma única célula-tronco espermatogonial (SSC) se diferencia em muitos espermatozoides haploides. A divisão de uma SSC pode resultar em duas células tronco (divisão simétrica), ou em uma célula tronco e outra diferenciada (divisão assimétrica). Independentemente do tipo de divisão celular, o processo de auto-renovação e diferenciação é controlado por numerosos sinais provenientes do tecido somático circundante, assim como também de fatores intrínsecos da própria SSC. Nesse sentido, estudos recentes mostraram que RNAs não codificantes (microRNAs, cirRNAs, lncRNAs) podem desempenhar um papel fundamental na reprodução, em particular na regulação da determinação sexual, diferenciação sexual e gametogênese. Entre os RNAs não codificantes, os lncRNAs se destacam por desempenhar papel regulatório na transcrição de "genes mestres" envolvidos em vários processos biológicos, incluindo a diferenciação e manutenção da pluripotência de células-tronco. Considerando esse contexto, este estudo teve como objetivo entender a sinalização Gdnf-Gfrα1 em testículos de zebrafish combinando abordagens *in vivo, in silico* e *ex vivo*, além dos efeitos biológicos do Fsh em lncRNAs testiculares. Nossos dados revelaram que o Gdnf, fator derivado de célula germinativa, está envolvido na manutenção da pluripotência das células germinativas por meio da criação de nichos espermatogoniais, dando suporte ao desenvolvimento de cistos espermatogônicos e inibindo a diferenciação tardia de espermatogônias de maneira autócrina e parácrina. Além disso, neste estudo, identificamos 5161 novos lncRNAs dos quais 76 foram diferencialmente regulados pelo rzfFsh. Além disso, análises de enriquecimento das DEGs demonstrou que esses transcritos estão amplamente relacionados à sinalização e sistemas orgânicos, como o sistema endócrino. Quando focamos nos mRNAs, encontramos 270 DEGs, sendo 174 "up-regulated" 96 "down-regulated". Interessantemente, as vias mais enriquecidas estavam relacionadas à sinalização do hormônio da tireoide e esteroidogênese. Por fim, predições de interação entre lncRNAs-mRNA-proteínas mostraram que alguns lncRNAs podem interagir, e consequentemente, modular a expressão de genes de pluripotência de SSCs, como o *pou5f3, nanos3* e *nanog.* Em conjunto, nossos dados indicam uma provável atividade do Fsh em criar um ambiente mais favorável para a diferenciação de espermatogônias tronco.

Abstract

Spermatogenesis is a process controlled by stem cells in which a single spermatogonial stem cell (SSC) differentiates into many haploid spermatozoa. The division of SSC follows either the asymmetric model, where one stem cell produces a differentiated cell and a stem cell (self-renewal), or the symmetric model, where one stem cell produces two differentiated cells or two stem cells. Regardless of the model, the process of self-renewal and differentiation is regulated by numerous signals from the surrounding somatic tissue (the stem cell niche) and the germline stem cells themselves. Additionally, recent studies have shown that non-coding RNAs (microRNAs, circRNAs, lncRNAs) from the germline niche itself can play a crucial role in reproduction, particularly in the regulation of sexual determination, sexual differentiation, and gametogenesis. Among the non-coding RNAs, lncRNAs stand out for their regulatory role in the transcription of "master genes" involved in various biological processes, including the differentiation and maintenance of stem cell pluripotency. In this context, this study aimed to understand the Gdnf-Gfrα1 signaling in zebrafish testes by combining in vivo, in silico, and ex vivo approaches, as well as the biological effects of Fsh on testicular lncRNAs. Our data revealed that Gdnf, now a germ cell-derived factor, is involved in maintaining germline stem cell pluripotency through niche creation, supporting the development of spermatogonial cysts and inhibiting late spermatogonial differentiation in an autocrine and paracrine manner. Furthermore, in this study, we identified 5161 novel lncRNAs and 76 DEGs under the influence of rzfFsh, of which 46 were upregulated and 30 were downregulated. Additionally, enrichment analyses of the DEGs demonstrated that these transcripts are broadly related to signaling and organ systems, such as the endocrine system. When focusing on mRNAs, we found 270 DEGs, with 174 upregulated and 96 downregulated. Interestingly, the most enriched pathways were related to thyroid hormone signaling and steroidogenesis. Finally, predictions of interaction between lncRNAs-mRNAs-proteins showed that some lncRNAs can interact, and consequently, modulate the expression of pluripotency genes in SSCs, such as *pou5f3, nanos3,* and *nanog*. Collectively, our data indicate a likely activity of Fsh in creating a more favorable environment for spermatogonial stem cell differentiation.

1. General Introduction

1.1 *Spermatogenesis and Spermatogonial Stem cells*

Spermatogenesis is a stem cell-driven process by which a single spermatogonial stem cell (SSC) differentiates into many haploid spermatozoa (Nóbrega et al., 2009; Schulz et al., 2010). This process occurs in three phases: the mitotic or spermatogonial phase with successive rounds of mitotic duplication of the spermatogonia; the meiotic or spermatocytary phase, in which spermatocytes undergo the two meiotic divisions; and the third phase, spermiogenesis, where the haploid spermatids emerging from meiosis differentiate into flagellated spermatozoa (Schulz et al., 2010). Although many features are conserved among vertebrates, fish spermatogenesis (the focus of this project) takes place in the so-called cyst or spermatocyst (Figure 1B). The cyst is formed when a group of somatic Sertoli cells envelop a single SSC (Callard, 1996). As spermatogonia divide, the derived daughter cells remain interconnected by cytoplasmic bridges (Grier, 1993; Schulz et al., 2010; França et al., 2015). Another interesting feature of cystic spermatogenesis is that the Sertoli cell supports a single germ cell clone at once, whereas in amniotes (reptiles, birds, and mammals), depending on species, at least five germ cell clones at different stages of development are supported by a single Sertoli cell (Figure 1) (Schulz et al., 2010; França et al., 2015). Another difference is that fish Sertoli cells can continuously proliferate even after the onset of puberty (França et al., 2015).

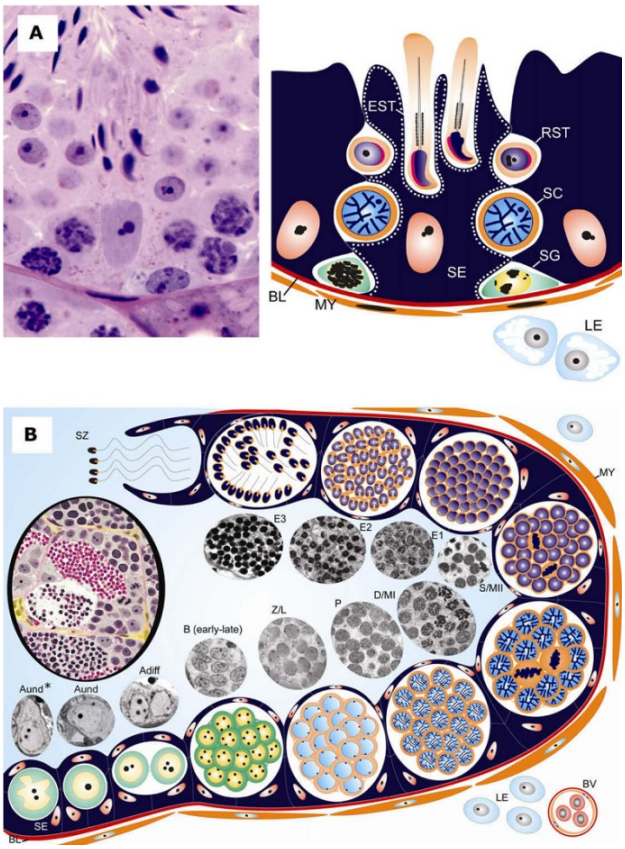


Figure 1. Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between cystic (B) and non-cystic (A) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. A: spermatogonia (SG); spermatocyte (SC); round spermatid (RST); and elongated spermatid (EST). B: type A undifferentiated spermatogonia (Aund); type A differentiated spermatogonia (Adiff); type B spermatogonia [B (early-late)]; leptotene/zygotenic primary spermatocytes (L/Z); pachytene primary spermatocytes (P); diplotene spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ). From: Schulz et al. (2010).

SSCs, a small population among type A undifferentiated spermatogonia, can either self-renew to produce more stem cells or differentiate into daughter cells dedicated to spermatogenesis (Schulz et al., 2010; Xie et al., 2020). The balance between SSC self-renewal and differentiation should be properly regulated to avoid stem cell tumors or spermatogenesis depletion (De Rooij, 2001). This regulation is also crucial for puberty and adult gonadal maturation in seasonal breeders. For example, SSC differentiation does not occur in juveniles but is activated during puberty or the beginning of a reproductive season. This switch in stem cell activity, from self-renewal to differentiation, is extremely relevant for aquaculture. In aquaculture, precocious puberty is considered one of the major economic problems limiting the further development of aquaculture species such as salmon, European sea bass, Atlantic halibut, and Chinese tongue sole among others (reviewed by Taranger et al., 2010; Carillo et al., 2015; Hagen et al., 2006; Ji et al., 2011). For example, in the European sea bass aquaculture, the high incidence (20-30%) of precocious sexual maturation in males during the first year of life (Begtashi et al., 2004) resulted in important economic losses (Taranger et al., 2010; Carillo et al., 2015). This is because precocious maturation results in non-marketable fish because growth and feed utilization are negatively affected by the energy invested in the production of gametes (Felip et al., 2006; 2008; Taranger et al., 2010; Carillo et al., 2015). Therefore, investigations to understand the physiological and molecular mechanisms controlling the switch in SSC activity are the basis for developing approaches to delay the start of pubertal testis maturation.

1.2 Endocrine and Paracrine Regulation of SSCs

SSCs are maintained in a specialized microenvironment in the testis known as the testicular niche (Nóbrega et al., 2010; Lacerda et al., 2012; De Siqueira-Silva et al., 2019). In a model laboratory species, zebrafish (*Danio rerio*), this microenvironment is composed of Sertoli cells, peritubular myoid cells, Leydig cells, and endothelial cells that are all near to SSCs and contribute to the niche (Nóbrega et al., 2010). The niche provides growth factors and cell-to-cell interactions that regulate SSC activity in the testis (Nóbrega et al., 2010). The production and release of these factors are modulated by reproductive hormones, such as Fsh (follicle-stimulating hormone) and androgens (Nóbrega et al., 2015; Safian et al., 2019). Information on the identity of these endocrine and paracrine factors and their mode of action is largely missing in vertebrates.

Gdnf (Glial cell line-derived neurotrophic factor) is a closely related member of the TGF-β superfamily which belongs to the Gdnf family of ligands (GFLs). This family of ligands consists of Gdnf, neurturin, artemin, and persephin (Airaksinen and Saarma, 2002). Gdnf exerts its biological roles by activating the Ret transmembrane receptor tyrosine kinase (RET) through the co-receptor Gfrα1 (Gdnf family receptor α1) (Airaksinen and Saarma, 2002). The importance of GDNF for SSC maintenance was unveiled by Meng and collaborators (2000) who showed that mice with impaired GDNF signaling exhibited a progressive loss of SSCs (Meng et al., 2000), while its pan-ectopic overexpression promoted germ cell hyperplasia, and ultimately tumors (Meng et al., 2001). Several studies have reported the presence of Gdnf and Gfrα1 homologs in different species of fish (Bosseboeuf et al., 2013; Gautier et al., 2014; Bellaiche et al., 2014; Nakajima et al., 2014; Wei et al., 2017; Zhao et al., 2018). Gdnf and Gfrα1 are expressed in type A undifferentiated and progressively decreased their expression during germ cell development as has been demonstrated in dogfish (*Scyliorhinus canicula*) (Gautier et al., 2014), rainbow trout (*Oncorhynchus mykiss*) (Nakajima et al., 2014; Bellaiche et al., 2014) and medaka (*Oryzias latipes*) (Zhao et al., 2018). Moreover, *in vitro* experiments have demonstrated that recombinant human GDNF promoted the proliferation and long-term maintenance of dogfish spermatogonia with stem characteristics (Gautier et al., 2014). Similar findings were found by Wei and collaborators (2017) who showed that two medaka Gdnf homologs, named Gdnfa and Gdnfb, can stimulate the proliferation of SG3, a spermatogonial cell line derived from adult medaka. On the other hand, expression analysis in rainbow trout revealed that *gdnfb* increased during the arrest of the spermatogenic cycle (end of germ cell proliferation and differentiation), suggesting that Gdnfb is likely involved in the repression of SSC differentiation rather than proliferation (Bellaiche et al., 2014).

1.3 *lncRNAs (long non-coding RNAs)*

RNA is a macromolecule responsible for transmitting and processing genetic information during transcription and translation (Zaha et al., 2014). While viral RNAs differ, all other RNAs are transcribed from genomic DNA. Over the years, a large number of RNAs that do not code for peptides or proteins have been identified, including non-mRNAs and non-coding RNAs (ncRNAs). Non-mRNAs are primarily found in bacteria, while ncRNAs are present in eukaryotic organisms (Wassarman et al., 1999). These ncRNAs are located in intergenic regions of the genome, which do not participate in protein coding. Initially, ncRNAs were considered junk RNAs due to their lack of a described function. However, identification of thousands of ncRNAs in humans and experimental models have demonstrated their roles in regulating various biological processes, including tumorigenesis and stem cell differentiation (Arrigo and Pulliero, 2015). There are two categories of ncRNAs based on their functions. Structural ncRNAs, including tRNAs, rRNAs, snRNAs, and snoR-RNAs, exert structural roles (Statello et al., 2021). Regulatory ncRNAs, such as siRNAs, miRNAs, piwi-RNAs, lncRNAs, and long intergenic ncRNAs, have regulatory roles on gene expression (Qu and Adelson, 2012) at various levels (Table 1).

RNA Type	Description	Function
mRNA	Messenger RNA	Carries the genetic information from DNA to the ribosomes for protein synthesis.
tRNA	Transfer RNA	Transfers specific amino acids to the ribosomes during protein synthesis.
snoRNA	Small nucleolar RNA	Guides chemical modifications of other RNAs, including rRNA and tRNA.
snRNA	Small nuclear RNA	Plays a role in the splicing and processing of pre-mRNA, forming the spliceosome.
sRNA	Small RNA	Involved in various regulatory functions, including gene silencing and post-transcriptional regulation.
CRISPR RNA	CRISPR RNA	Guides the Cas proteins in the CRISPR-Cas system for targeted gene editing.
miRNA	Micro RNA	Regulates gene expression by binding to complementary sequences in mRNA, leading to mRNA degradation or inhibition of translation.
siRNA	Small interfering RNA	Similar to miRNA, siRNA regulates gene expression through mRNA degradation or translational repression.
piRNA	PIWI-interacting RNA	Suppresses transposable elements and maintains genome integrity in germ cells.
lncRNA	Long non-coding RNA	Involved in diverse regulatory functions, including chromatin remodeling, gene expression regulation, and genomic imprinting.

Table 1. Functions of different classes of RNAs. Note the great diversity of ncRNA molecules in eukaryotes. Different types of RNAs indicated, mRNA (messenger RNA), tRNA (transfer RNA), snoRNA (RNA nucleolar), snRNA (nuclear), sRNA (small RNA), CRISPR (CRISPR RNA), miRNA (micro RNA), siRNA (interference RNA), piRNA (RNA that binds to the PIWI protein) and lncRNA (long non-coding RNA). Adapted fom Costa, 2017.

Analytical technologies such as CAGE, Chip-chip, Chip-seq, and RNA deep sequencing have allowed a better understanding of the complexity of the eukaryotic genome (Carninci, 2006; Gustincich et al., 2006). In mice, about one-third of transcripts are non-coding, and recently, studies have focused on long non-coding RNAs (lncRNAs). lncRNAs are longer than 200 nucleotides and have little or no ability to encode proteins (Cech and Steitz, 2014; Arrigo and Pulliero, 2015). While this limit may seem arbitrary, it distinguishes lncRNAs from other RNA categories. Recent studies have shown that lncRNAs regulate various cellular processes through their direct action on gene transcription (Hezroni et al., 2015). They can act in the promoter region or in other transcriptional regulatory regions, such as enhancers or locus control regions (Hezroni et al., 2015).

lncRNAs can be divided into three groups according to their action on gene expression (Figure 2). In the first group, known as "transcription only", only the transcription of the molecule is important, and the RNA produced does not present a characteristic function (Figure 2A). The only characteristic observed for this lncRNA group is the fact that they participate in the chromatin modification of the the locus that they are associated. Its sequence and structure itself are not important (Perry and Ulitsky, 2016) (Figure 2A). The second group, known as cis-acting RNAs, comprises lncRNAs whose function is related to the recruitment of transcripts that will act in genomic or spatially close regions (Figure 2B). Finally, the third group known as trans-acting RNAs that act independently of the site of their transcription, regulating the expression of other *loci* in the nucleus or acting on transcripts in the cell cytoplasm (Figure 2C). Many lncRNAs have functions strictly related to the nucleus, such as Xist, Neat1, and Malat1, however, most lncRNAs act in both nucleus and the cytoplasm, or only in the cytoplasm (Cabili et al., 2015; Derrien et al., 2012; Ulitsky e Bartel, 2013).

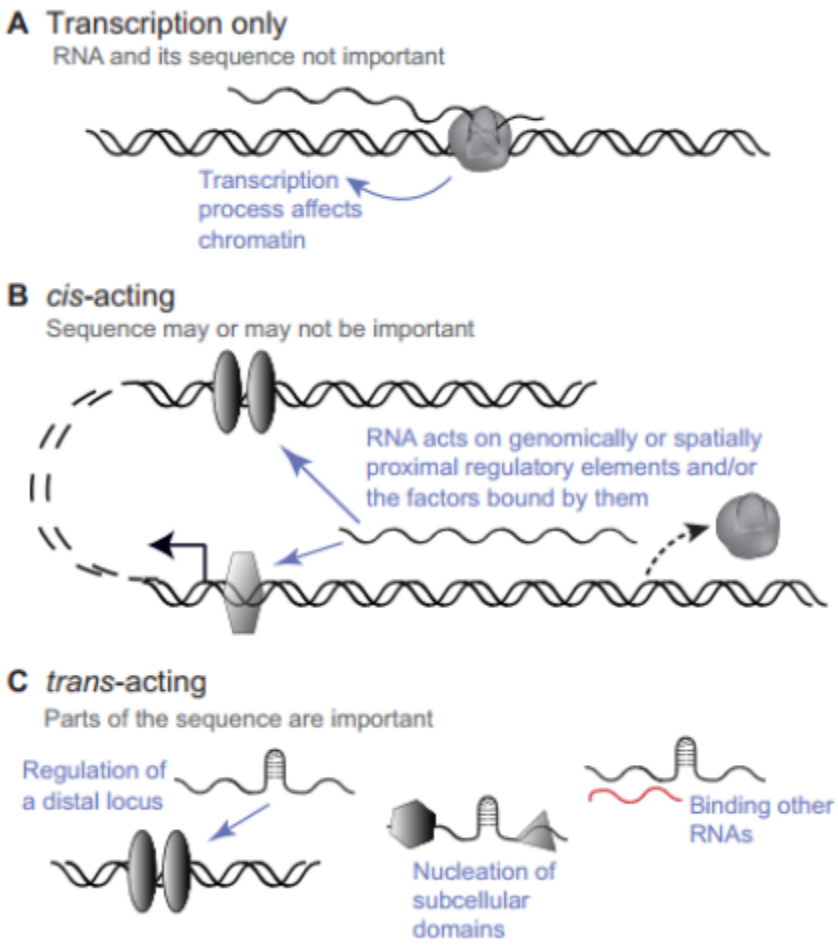


Figure 2. lncRNA modes of action. (A). For some lncRNA loci, the act of transcription itself plays a role in mediating lncRNA function, for example by affecting the underlying chromatin structure of the locus. In this context, the RNA product itself and its sequence are irrelevant. (B). In contrast, other lncRNAs act close to their transcription site, recruiting or repelling specific factors, which may or may not recognize the RNA in a sequence-specific manner. (W). Other lncRNAs leave their transcription site and act elsewhere, usually in sequence-specific or structure-dependent ways, via interactions with proteins and other RNA factors. Figure is taken from Perry and Ulitsky (2016).

Long non-coding RNAs (lncRNAs) have been increasingly associated with biological processes related to embryonic development and cell differentiation in adult mammals (Perry and Ulistsk, 2016). Although the molecular mechanisms that regulate these processes are still largely unknown, it is known that lncRNAs can control the transcription of "master" regulatory genes that play a crucial role in these processes (Perry and Ulitsky, 2016). Several mechanisms have been proposed for the regulation of lncRNAs in stem cell differentiation, as illustrated in Figure 3. LncRNAs can repress the early differentiation of stem cells by shaping the chromatin and creating a repressive environment (Figure 3A). Alternatively, lncRNAs can facilitate the binding of transcription factors, thereby promoting the process of differentiation (Figure 3B). In cases where the lncRNA target gene is also a transcription factor, lncRNA activity can be enhanced by the expression of the target gene, resulting in a positive feedback loop (Figure 3C). Furthermore, during the process of cell differentiation, lncRNAs can repress molecules necessary for the establishment of different cell lineages (Figure 3D).

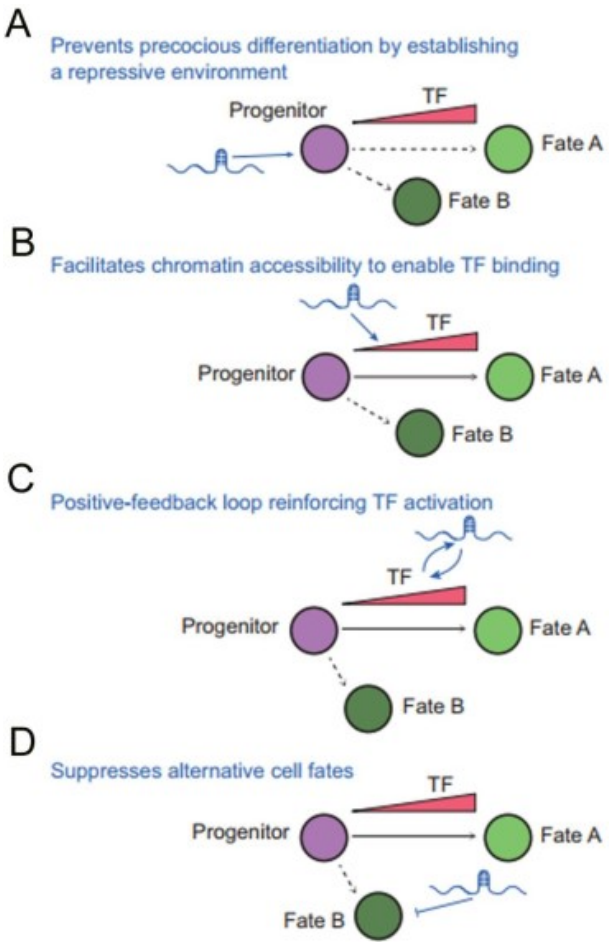
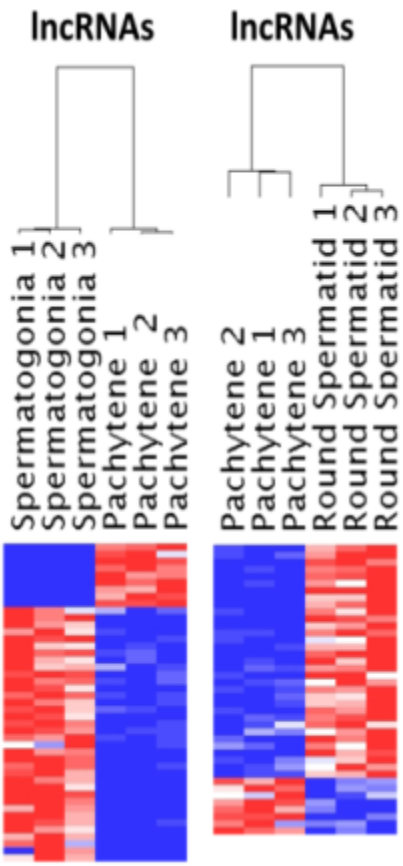


Figure 3. Main functions of lncRNAs in the regulation of stem cells. lncRNAs can play several roles during cell differentiation. Figure taken and adapted from Perry and Ulitsky (2016).

While lncRNAs are mainly involved in mechanisms of differentiation, several studies have suggested that they may also be associated with the maintenance of stem cells and the promotion of their undifferentiated state in various mammalian cell types, such as neuronal trunk cells (Ng et al., 2013; Aprea et al., 2013; Lin et al., 2014), epidermal cells (Kretz et al., 2012), cardiac cells (Klattenhoff et al., 2013), endodermal cells (Jiang et al., 2015; Kurian et al., 2015), endothelial cells (Boulberdaa et al., 2016), adipocytes (Sun et al., 2013), and hematopoietic cells (Deng et al., 2016).

Long non-coding RNAs (lncRNAs) have been identified as playing a key role in the spermatogenic process, and dysregulation of lncRNAs may be associated with male infertility (Wichman et al., 2017). Regulatory elements such as enhancers and regulatory ncRNAs specific for spermatogonia, spermatocytes and spermatozoa have been identified in mammals, indicating the significant impact of lncRNAs in spermatogenic regulation and maintenance of fertility (Choudhury et al., 2010; Wang et al., 2001; McCarrey et al., 1992; Penkner et al., 2005; Kuramochi et al., 2004; Hotta et al., 1995; Shima et al., 2004). Wichman and collaborators (2017) have identified lncRNAs expressed exclusively in different types of germ cell populations in mice, with differentially expressed lncRNAs found in spermatogonia and pachytenic spermatocytes. In this study, knockout of testis-specific lncRNA 1 resulted in reduced fertility in male mice. Another lncRNA, Tsx is important in the meiosis of germ cells of male mice (Anguera et al., 2011). lncRNAs have also been shown to participate in signaling pathways, such as Wnt, which is important for the maintenance of the spermatogenic process (Arun et al., 2012). In fish, a recent study demonstrated in *Nile tilapia* that the knockdown of the *igf3* gene results in the alteration of the expression of more than 124 different types of lncRNAs, indicating their fundamental role in the regulation of the spermatogenic process (Song et al., 2019).



Wichman et al. (2017) performed the knockout of the lncRNA "TSLrn1", or called "testis-specific lncRNA 1", found mainly in spermatogonia and in mice meiotic cells. The result is that all males had their fertility reduced. Anguera et al. (2011) also showed the importance of lncRNA Tsx in the meiosis of germ cells of male mice. Other studies described the participation of lncRNAs in signaling pathways, such as Wnt (Arun et al., 2012), important in the maintenance of the spermatogenic process (Takase et al., 2016; Sreenivasan et al., 2014).

Figure 4. Heatmap of differentially expressed lncRNAs among spermatogonia, meiotic (pachytene), and post-meiotic (spermatid) cells of mice. Adapted from Wichman et al. (2017).

Information about the performance of lncRNAs in fish testes are still unknown. More recent, studies with common carp, *Cyprinus carpio*, demonstrated that the knockdown of igf3 (insulin-like growth factor 3), a gene responsible for controlling the axes of reproduction and growth in fish (Reinecke., 2010) and stimulates spermatogonial differentiation in zebrafish (Nóbrega et al., 2015), resulted in alteration of the expression of more than 124 different types of lncRNAs (Song et al., 2019). Similar to mammals, these results suggest that lncRNAs may also play a role in the regulation of the spermatogenic process in fish.

2. General Objective

The objective of this thesis is to better understand the regulatory mechanisms zebrafish spermatogonial niche, focusing on germ cell-derived factors (Chapter 1) and lncRNAs (Chapter 2).

Chapter 1

Gdnf Acts as a Germ Cell-Derived Growth Factor and Regulates the Zebrafish Germ Stem Cell Niche in Autocrine- and Paracrine-Dependent Manners

Lucas B Doretto, Arno J Butzge, Rafael T Nakajima, Emanuel R M Martinez, Beatriz Marques de Souza, Maira da Silva Rodrigues, Ivana F Rosa, Juliana M B Ricci, Aldo Tovo-Neto, Daniel F Costa, Guilherme Malafaia , Changwei Shao, Rafael H Nóbrega

Abstract

Glial cell line-derived neurotrophic factor (GDNF) and its receptor (GDNF Family Receptor α1-GFRα1) are well known to mediate spermatogonial stem cell (SSC) proliferation and survival in mammalian testes. In nonmammalian species, Gdnf and Gfrα1 orthologs have been found but their functions remain poorly investigated in the testes. Considering this background, this study aimed to understand the roles of the Gdnf-Gfrα1 signaling pathway in zebrafish testes by combining in vivo, in silico and ex vivo approaches. Our analysis showed that zebrafish exhibit two paralogs for Gndf (*gdnfa* and *gdnfb*) and its receptor, Gfrα1 (*gfrα1a* and *gfrα1b*), in accordance with a teleost-specific third round of whole genome duplication. Expression analysis further revealed that both ligands and receptors were expressed in zebrafish adult testes. Subsequently, we demonstrated that *gdnfa* is expressed in the germ cells, while Gfrα1a/Gfrα1b was detected in early spermatogonia (mainly in types A_{und} and A_{diff}) and Sertoli cells. Functional ex vivo analysis showed that Gdnf promoted the creation of new available niches by stimulating the proliferation of both type A_{und} spermatogonia and their surrounding Sertoli cells but without changing *pou5f3* mRNA levels. Strikingly, Gdnf also inhibited late spermatogonial differentiation, as shown by the decrease in type B spermatogonia and down-regulation of *dazl* in a co-treatment with Fsh. Altogether, our data revealed that a germ cell-derived factor is involved in maintaining germ cell stemness through the creation of new available niches, supporting the development of spermatogonial cysts and inhibiting late spermatogonial differentiation in autocrine- and paracrine-dependent manners.

1. Introduction

GDNF (Glial cell line-derived neurotrophic factor) is a closely related member of the TGF-β superfamily which belongs to the GDNF family of ligands (GFLs). This family of ligands consists of Gdnf, neurturin, artemin and persephin [1]. The importance of GDNF for spermatogonial stem cell (SSC) maintenance was unveiled by Meng et al. [2], who demonstrated that mice with impaired GDNF signaling exhibited a progressive loss of SSCs, whereas GDNF overexpression promoted germ cell hyperplasia and ultimately tumors [2]. Further studies showed that GDNF promoted in vitro expansion of mouse germline stem cells [3,4], this being considered an indispensable factor for long-term culture of SSCs for several species of rodents [3,5,6]. More recently, experiments using mice that ectopically expressed stage-specific GDNF in Sertoli cells revealed that GDNF increased SSC self-renewal by blocking differentiation rather than actively stimulating their proliferation [4]. Altogether, these studies in mammals demonstrated that GDNF is an important factor for SSC self-renewal, proliferation of the stem cell direct progenitors and maintenance of the SSC undifferentiated state (see the review in Parekh et al. [7]; see also Mäkelä and Hobbs [8]).

GDNF signaling occurs through binding the non-signaling co-receptor of the GDNF Family Receptor α1 (GFRα1), which is attached to the cell membrane by glycosylphosphatidylinositol-anchors [1]. The complex GDNF-GFRα1 associates with a single transmembrane RET receptor tyrosine kinase, leading to the activation of RET's intracellular kinase domain and the subsequent stimulation of different downstream cellular pathways [1]. In mammalian testes, GDNF is produced by testicular somatic cells, including Sertoli cells [2,9,10], peritubular myoid cells under the influence of androgens [11,12] and testicular endothelial cells, which seem to be the major GDNF-producing sources in mouse testes [13]. In rodents, GFRα1 is present in a subpopulation of single type A spermatogonia (A_s), which also expresses the inhibitor of DNA binding 4 (ID4) [14,15]. This subpopulation is considered the purest functional SSC population [14,15]. However, several other studies have demonstrated that GFRα1 is not exclusively detected in SSCs but is also expressed in types A paired (A_{pr}) and aligned (A_{al}) spermatogonia [16,17,18,19,20]. Similar expression patterns have been reported in other mammalian species, such as hamsters [21], pigs [22], collared peccaries [23,24], buffaloes [25], different equine species [26] and primates, including humans [27,28].

In nonmammalian species, particularly in fish, Gdnf/Gfrα1 homologs have been found in a limited number of species, such as dogfish (Scyliorhinus canicula) [29], rainbow trout (Oncorhynchus mykiss) [30,31,32] and medaka (Oryzias latipes) [33]. In these species, Gdnf and

Gfrα1 are co-expressed in type A undifferentiated spermatogonia, suggesting an autocrine mechanism for Gdnf-mediated functions in fish testes [30]. The physiological relevance of Gdnf for type A undifferentiated spermatogonia has been further demonstrated by in vitro studies showing that recombinant human GDNF (rh GDNF) promoted the proliferation and long-term maintenance of dogfish spermatogonia with stem characteristics [29]. Similar findings were reported by Wei et al. [34], who showed that two Gdnf homologs in medaka, named Gdnfa and Gdnfb, stimulated proliferation of SG3, a medaka spermatogonial stem cell line. On the other hand, studies in rainbow trout revealed that gdnfb mRNA levels increased during the arrest of the spermatogenic cycle (end of germ cell proliferation and differentiation), suggesting that Gdnfb is likely involved in the repression of SSC differentiation rather than proliferation [31].

Considering this background and the lack of knowledge about Gdnf-Gfrα1 signaling in fish, this study aimed to unravel the autocrine/paracrine roles of Gdnf on the zebrafish germ stem cell niche and to expand our knowledge about the critical factors involved in SSC activity as well as improve our abilities to predict the consequences of changes involved in the physiological mechanisms related to Gdnf. To these ends, we initially performed phylogenetic and synteny analyses for Gfrα1 and then investigated the testicular expression profiling of gdnf (gdnfa and gdnfb) and gfrα1 (gfrα1a and gfrα1b) transcripts in zebrafish testes. Subsequently, we identified the cellular types expressing Gdnf and Gfrα1 and assessed the biological effects of Gdnf through an ex vivo testis culture system. According to Oatley and Brinster [35], the impairment of SSC function disrupts spermatogenesis and causes subfertility or infertility in males; therefore, knowing the mechanisms that regulate SSC homeostasis is imperative for the conservation of species or for their use as experimental models in studies focusing on the treatment of pathological conditions affecting the reproductive organs in humans.

2. Material and Methods

2.1. Zebrafish Stocks

Sexually mature zebrafish (*Danio rerio*, outbred) (4–5 months old) were kept in 6 L water tanks in a recirculating system with controlled photothermal conditions (27 °C and 14 h of light and 10 h dark). Parameters such as salinity, pH, dissolved oxygen and ammonia were monitored daily in all tanks. Fish were fed twice a day using commercial food (Zeigler®, Gardners, PA, USA). Handling and experimentation were in accordance with Brazilian legislation regulated by the National Council for the Control of Animal Experimental (CONCEA) and the Ethical Principles in Animal Research of São Paulo State University (protocol no. 666-CEUA). Zebrafish is a tropical

freshwater fish species natural to rivers in Southern Asia [36,37,38] and has been considered a versatile model for reproductive biology [39], besides being used as a model for translational research in human health and disease [40]. Therefore, these aspects justify the choice of this species in our study.

2.2. Sequence Analysis

The predicted amino acid sequences for Gfrα1a and Gfrα1b of D. rerio (Q98TT9 and Q98TT8, respectively), GFRA1 of Homo sapiens (P56159), Rattus norvegicus (Q62997) and Mus musculus (P97785) were obtained from the Universal Protein Resource (UniProt, accessed 09/12/2019) and aligned using the MEGA algorithm allocated in Geneious Pro 4.8.5 software [41]. For the phylogenetic analysis, we retrieved the protein sequences for GFRα1 (Gfrα1a and Gfrα1b) from the Universal Protein Resource (UniProt, accessed on 25 February 2020), the National Center for Biotechnology Information (NCBI, 25 February 2020) and Ensembl (accessed on 25 February 2020 [42]). For this analysis, we retrieved vertebrate sequences for GFRα1 and Growth arrest-specific protein 1 (GAS1) from humans (GAS1 as an outgroup). The predicted amino acid sequences were aligned using the Muscle algorithm [43] allocated in Geneious Pro 4.8.5 software [41]. The choice of the best fitting model of evolution was performed with SMS [44]. Phylogenetic reconstruction was determined by Bayesian methods implemented in Beast v1.7.0 software [45]. This step was carried out according to Geraldo et al. [46], with adaptations. Branch values were supported by posterior probabilities obtained by Bayesian analysis. For Bayesian methods, the burn-in was obtained through Tracer [45] using log likelihood scores, and data were compiled in TreeAnnotator [45] after trees that were out of the convergence area had been discarded. The visualization and the final tree edition were generated using FigTree v1.3.1 [45]. In the phylogenetic analyses, the proportion of invariable sites and γ-distributed rate variation across sites were estimated, and the substitution of rate categories set in four categories. The parameter settings used to reconstruct the phylogeny are shown in Table S2. To construct the synteny regions of GFRA1 (human), Gfrα1 (rat and mouse), gfrα1a and gfrα1b (zebrafish), we used the GenBank database, available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) (accessed on 25 February 2020) and Ensembl [42].

2.3. Expression Profiling of Gdnf (gdnfa and gdnfb) and Gfrα1 (gfrα1a and gfrα1b) Transcripts in Zebrafish Testes

To investigate the expression of *gdnfa* (glial cell-derived neurotrophic factor a), *gdnfb* (glial cell-derived neurotrophic factor b), *gfrα1a* (gdnf family receptor alpha 1a) and *gfrα1b* (gdnf family receptor alpha 1b) in zebrafish testes, total RNA from testes (n = 4 males) was extracted using an RNAqueous®-Micro kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA synthesis and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were performed as previously described [47]. The number of amplification cycles (Ct-cycle threshold) for *gdnfa*, *gdnfb*, *gfrα1a* and *gfrα1b* were determined through a StepOnePlus™ Real-Time PCR System (Thermo fisher, Waltham, MA, USA, EUA). Primers (Table 1) were designed based on zebrafish sequences available from the Genbank database.

Target Genes	Primer Sequences (5′–3′)	References
ef1α	GCCGTCCCACCGACAAG (Fw) CCACACGACCCACAGGTACAG (Rv)	Morais et al. [48]
b-actin	AGACATCAGGGAGTGATGGT (Fw) CAATACCGTGCTCAATGGGG (Rv)	Tovo-Neto et al. [49]
gdnfa	GAAGCTCCGGTCTGTATGGA (Fw) GGAGCTCAGGAGCAACAAAC (Rv)	This paper
gdnfb	AGGAGTAAATCAGTGGGCCAAA (Fw) AGTAGCTGAATATGAGCTCCTCC (Rv)	This paper
gfrα1a	TCGACTGGCTCCCATCTATTC (Fw) AGGTGTCATTCAGGTTGCAGG (Rv)	This paper
gfrα1b	CCTGTGCTTGATTTAGTGCA (Fw) GCATCCGTACTTTCCCAAAC (Rv)	This paper
igf3	TGTGCGGAGACAGAGGCTTT (Fw) CGCCGCACTTTCTTGGATT (Rv)	Morais et al. [48]
amh	CTCTGACCTTGATGAGCCTCATTT (Fw) GGATGTCCCTTAAGAACTTTTGCA (Rv)	García-Lopez et al. [50]
fshr	GAGGATTCCCAGTAATGCTTTCCT (Fw) TCTATCTCACGAATCCCGTTCTTC (Rv)	García-Lopez et al. [50]
pou5f3	GAGAGATGTAGTGCGTGTAT (Fw)	Tovo-Neto et al. [49]

Target Genes	Primer Sequences (5′–3′)	References
dazl	GCTCGTAATACTGTGCTTCA (Rv) AGTGCAGACTTTGCTAACCCTTATGTA (Fw) GTCCACTGCTCCAAGTTGCTCT (Rv)	Morais et al. [49]
sycp3l	AGAAGCTGACCCAAGATCATTCC (Fw) AGCTTCAGTTGCTGGCGAAA (Rv)	García-Lopez et al. [50]
gdnfa-ish	T7Rpps-CCGCAGTGAGAGCCCCG (Fw) T3Rpps-TCCCGTTAGGTCATATTGTTCCTC (Rv)	This paper

Fw, forward; Rv, reverse; T7Rpps–T7 RNA polymerase promoter sequence at its 5′-end (5′ CCGGGGGGTGTAATACGACTCACTATAGGG-3′), T3Rpps–T3 RNA polymerase promoter sequence at its 5′-end (T3′GGGCGGGTGTTATTAACCCTCACTAAAGGG-3′).

Table 1. Primers used for gene expression analysis (RT-qPCR) and to generate DNA templates for digoxigenin (DIG)-labeled cRNA probe synthesis for in situ hybridization (ISH) (Supplementary Materials).

2.4. Differential Plating Method

To obtain testicular cellular fractions (germ or somatic cell-enriched fractions), a differential plating method was carried out as previously described by Hinfray et al. [51]. To this end, testes (n = 20 males) were digested with 0.2% collagenase (Sigma Aldrich, San Luis, MI, USA) and 0.12% dispase (Sigma Aldrich, San Luis, MI, USA) [47]. Total cell suspension was submitted to a differential plating method, in which somatic cells adhere to the bottom of the plate, whereas germ cells either remain in suspension or only weakly associate with adhering somatic cells [51]. By using this approach, germ and somatic cell-enriched fractions can be obtained [51]. RNA from cell suspensions (total, germ and somatic cell-enriched fractions) was obtained using a PureLink® RNA Mini Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. cDNA synthesis was conducted using a SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers. The relative mRNA levels of pou5f3 (POU domain, class 5, transcription factor 3) (spermatogonia marker), vasa (spermatogonia marker), gdnfa, igf3 (insulin-like growth factor 3) (Sertoli cell marker), gfrα1a and gfrα1b were determined by qRT-PCR. β-actin and ef1 were used as housekeeping genes. The quantification cycle (Cq) values were determined in a StepOne system (Life Technologies, Carlsbad, CA, USA) using SYBR Green (Invitrogen, Carlsbad, CA, USA) and specific primers (Table 1), as described in section 2.3.

2.5. Immunofluorescence and Western Blot

Testes (n = 10 males) were fixed with 4% paraformaldehyde in PBS (Phosphate Buffered Saline) (1X, pH 7.4) for 1 h, embedded in paraplast (Sigma Aldrich, San Luis, MI, USA) and sectioned at 5 µm thickness. After deparaffinization and rehydration, sections were submitted to antigen retrieval by heating slides in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) until temperatures reached 95–100 °C in a microwave. To reduce background fluorescence, slides were incubated with NaBH4 (sodium borohydride—0.01g dissolved in 1 mL of distilled water) (Sigma Aldrich, San Luis, MI, USA) for 3 min. Subsequently, slides were rinsed with 1X PBS (pH 7.4) and incubated with the biotinylated primary antibody rabbit anti-zebrafish Gfrα1a (1:300, 1X PBS pH 7.4) at 4 °C overnight. Zebrafish polyclonal biotinylated antibody anti-Gfrα1a was synthesized by Rheabiotech (Campinas, SP, Brazil) using the specific antigen sequence 'RLDCVKANELCLKEPGCSSK' located at the N-terminus of zebrafish Gfrα1a (Figure 1). This antibody is also potentially able to recognize other Gfrα1 isoforms, such as GFRA1 in humans and rodents and Gfrα1b in zebrafish (Figure 1). After rising, the slides were incubated with Dylight 488 Streptavidin (BioLegend®, San Diego, CA, USA) (1:400) or Alexa Fluor 594 Streptavidin (BioLegend®, San Diego, CA, USA) (1:400) in 1X PBS (pH 7.4) for 60 min at room temperature. Subsequently, sections were counterstained with Hoechst (1:2000, 1X PBS pH 7.4) (Invitrogen, Carlsbad, CA, USA) or Propidium iodide (PI) (BioLegend®, San Diego, CA, USA) (1 mg/mL dissolved in distilled water) and mounted with ProLong Gold Antifade (Thermo Fisher Scientific, Waltham, MA, USA). Control sections were prepared by preadsorbing the zebrafish Gfrα1a antibody with the corresponding peptide (10 µg/1 µL of antibody, Rheabiotech, Campinas, SP, Brazil) or by omitting the primary antibody. Slides were photographed using a Leica SP5 laser scanning confocal microscope (Leica, Wetzlar, Hessen, Germany) from the Electron Microscopy Center, Institute of Biosciences, São Paulo State University (Botucatu, Brazil), and germ cells were classified according to Leal et al. [52].

Figure 1. GFRα1 predicted amino acid sequence alignment. Numbers at the top left of the sequences indicate amino acid positions, dashes indicate deletions and black boxes indicate shared sequences. The three cysteine-rich domains (D1–D3) (orange lines), 28 cysteine residues (*) (plus 2 in the terminal region) and two triplets (MLF and RRR) (green boxes) are highly conserved among humans, rodents and zebrafish. At the end of the alignment are the percentage identity values of zebrafish Gfrα1a and Gfrα1b in relation to the other corresponding sequences. The blue line indicates the amino acid sequence recognized by the zebrafish Gfrα1a antibody used in this study; the purple line indicates the putative motifs critical for binding to GDNF.

For the Western blot analysis, testes (n = 10 males) were homogenized in an extraction TBST buffer (10 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20) containing a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany). Subsequently, the homogenate was incubated on ice for 15–20 min before sonication (3 × 1 min on ice) and centrifuged at 4000 rpm at 4 °C for 20 min in order to determine the total protein concentration by means of a NanoVue spectrophotometer (GE Healthcare, Chicago, IL, USA). A total of 40 µg protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein extracts were blotted onto a nitrocellulose membrane (Amersham, Little Chalfont, UK) blocked with 3% non-fat milk diluted in 1X Tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-HCl, pH 7.6.) for 1 h, and incubated with the primary antibody rabbit anti-zebrafish Gfrα1a (1:500, Rheabiotech, Campinas, SP, Brazil) at 4 °C overnight. The membrane was washed with TBS and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Santa Cruz Biotechnologies, Dallas, TX, EUA) for 2 h. After washing, blots were developed with a chemiluminescence substrate kit (Pierce ECL Western Blotting Substrate, GE Healthcare, Chicago, IL, USA) and the signal was captured by a CCD camera (ImageQuant LAS 4000 mini®, GE Healthcare, Chicago, IL, USA).

As controls, some membranes were alternatively incubated with primary antibodies that had been preadsorbed with the respective peptides.

2.6. Recombinant Human GDNF

To evaluate the effects of Gdnf on zebrafish spermatogenesis (see below), a rhGDNF purchased from PeproTech® (London, UK) (reference no. 450-10; https://www.peprotech.com/en/recombinant-human-gdnf#productreviews)(accessed on 20 February 2020) was used. We used a recombinant human hormone because the recombinant zebrafish Gdnf is not commercially available. In addition, rhGDNF has previously been used in fish [53]. The rhGDNF was dissolved in sterile Lebovitz medium (L-15) (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 100 µg/mL and subsequently aliquoted and stored at −20 °C until use. After identifying the binding sites between rhGDNF and human GFRA1A, a 3D structure model was built to predict the interaction sites between rhGDNF and zebrafish Gfrα1a (Q98TT9). The 3D protein structure used was obtained through SWISS-MODEL (swissmodel.expasy.org), with multiple target sequences representing different subunits of a hetero-oligomer (hetero-2-2-mer), and the quality of the modeling was analyzed by means of a Ramachandran plot generated with Rampage software [50]. The template (4ux8.1) and the final model were viewed in the software Pymol (the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

2.7. Testis Tissue Culture

The effects of rhGDNF on zebrafish spermatogenesis were investigated using a previously established ex vivo culture system [52]. In this system, one testis (left) was incubated in the presence of rhGDNF (100 ng/mL, based on Gautier et al. [53]) and its contra-lateral (right) in the basal culture medium (L-15). The culture medium was changed every 3 days, and after 7 days, testes were collected for histomorphometrical analysis via a BrdU (bromodeoxyuridine) (Sigma Aldrich, San Luis, MI, USA) incorporation assay and gene expression (RT-qPCR) (see below). Additional cultures were carried out to assess the interaction of Gdnf with Fsh-mediated effects on the zebrafish spermatogonial phase [54]. To this end, zebrafish testes (n = 10 males) were incubated with recombinant zebrafish Fsh (rzfFsh) (100 ng/mL [55]) (U-Protein Express B.V; Utrecht, the Netherlands) in the presence or absence of rhGDNF (100 ng/mL) for 7 days. After the culture period, testes were collected for RT-qPCR analysis. For histomorphometry, zebrafish testicular explants (n = 10) were fixed in 4% buffered glutaraldehyde at 4 °C overnight,

dehydrated, embedded in historesin Technovit 7100 (Wehrheim, Germany), sectioned at 4µm thickness and stained with 0.1% toluidine blue to estimate the frequency of the different germ cell cysts using a high-resolution light microscope (Leica DM6000 BD, Leica Microsystems, Wetzlar, Germany). In this analysis, five histological fields for each animal were randomly selected for counting the frequency of germ cell cysts (type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (SPG B), spermatocytes (SPCs) and spermatids (SPTs)), as previously described [47,52].

To investigate the effects of rhGDNF on germ cell proliferation, BrdU (100 µg/mL) was added during the final 6 h of incubation. After incubation, zebrafish testes (n = 10) were fixed at 4 °C overnight in freshly prepared methacarn (60% (v/v) absolute ethanol, 30% chloroform and 10% acetic acid) for 4 h. Subsequently, testes were dehydrated, embedded in Technovit 7100 (Wehrheim, Germany), sectioned at 4 µm thickness and used for BrdU immunodetection, as previously described [47,55]. The mitotic index or BrdU incorporation ratio of types A_{und}, A_{diff} and Sertoli cells was determined by counting the BrdU-positive and BrdU-negative cells in a total of 100 cells for the same cellular type, as described previously [47,48,55].

For RT-qPCR, total RNA from testicular explants (n = 20 males) was extracted using the same method described in section 2.3. The relative mRNA levels of *gdnfa, gfrα1a, gfrα1b, amh* (anti-Müllerian hormone), *igf3, fshr* (follicle stimulating hormone receptor), *pou5f3, dazl* (deleted in azoospermia-like) and *sycp3l* (synaptonemal complex protein 3) were evaluated. The mRNA levels of the targets (Cts) were normalized by β-actin levels, expressed as relative values of basal expression levels, according to the $2^{-(\Delta\Delta CT)}$ method. Primer sequences are indicated in table 1.

2.8. In Silico Analysis of Putative Regulatory Sequences Upstream Human GDNF, Mouse Gdnf and Zebrafish Gdnfa

To retrieve the putative regulatory sequences of upstream human GDNF (NM_000514.4), mouse Gdnf (NM_010275.3) and zebrafish gdnfa (NM_131732.2), the transcription start site (TSS) was found in the Eukaryotic Promoter Database (EPD), and the promoter regulatory regions (3' to 5') were prospected by means of the flanking regions (2000 bp) extracted from NCBI. The cAMP response elements (CRE, four different sequences), the androgen receptor binding site (AR, full and half sequences), several NF-kB-binding sites, N-Box, E-Box, TATA-Box and GC-Box (Table S3) were prospected using sequences described in the literature [7,56,57,58,59,60,61].

2.9. Statistical Analyses

Data were initially checked for deviations from variance normality and homogeneity through the Shapiro–Wilk and Bartlett's tests, respectively. Significant differences between two groups were identified using a paired Student's t-test, at 5% probability. Comparisons of more than two groups were performed with one-way ANOVA followed by Student–Newman–Keuls test, at 5% probability. Graphpad Prism 7.0 (Graphpad Software, Inc., San Diego, CA, USA) was used for the statistical analysis.

3. Results

3.1. Sequence Analyses, Phylogenetic Tree and Genomic Organization of Zebrafish Gfrα1a and Gfrα1b

Sequence analysis revealed that both predicted zebrafish Gfrα1a and Gfrα1b have sequence characteristics of Gfrα family members, such as the three cysteine-rich domains (D1-3), 28 cysteine residues (plus 2 in the terminal region), and two triplets (MLF and RRR) in the domain D2 (Figure 1). Sequence alignment of zebrafish Gfrα1a and 1b with different GFRA1s (human and rodent) revealed that the three cysteine-rich domains (D1, D2, D3) are highly conserved among the species, highlighting, in particular, the conserved residues and motifs in the domain D2 critical for binding to GDNF and eliciting downstream cellular pathways (Figure 1). Sequence analyses also demonstrated that zebrafish Gfrα1a and 1b have 67.1% identity to each other, and zebrafish Gfrα1a showed a higher identity with mammalian GFRA1 (61.7%, 61.1% and 60.9% similarity to human, rat and mouse GFRA1, respectively) than zebrafish Gfrα1b (57.4%, 57.2% and 57% identity to human, rat and mouse GFRA1, respectively) (Figure 1).

Phylogenetic analysis further confirmed that both zebrafish Gfrα1a and Gfrα1b are related to other fish Gfrα1a and Gfrα1b predicted sequences, respectively, and that these isoforms diverge and form two separate fish-specific subclades (estimated posterior probability = 1) (Figure 2A). On the other hand, the GFRA1 sequences from other vertebrates (mammals, birds, reptiles, amphibians and Chondrichthyes) are clustered and form a separate clade to the fish Gfrα1 (estimated posterior probability = 0.851) (Figure 2A).

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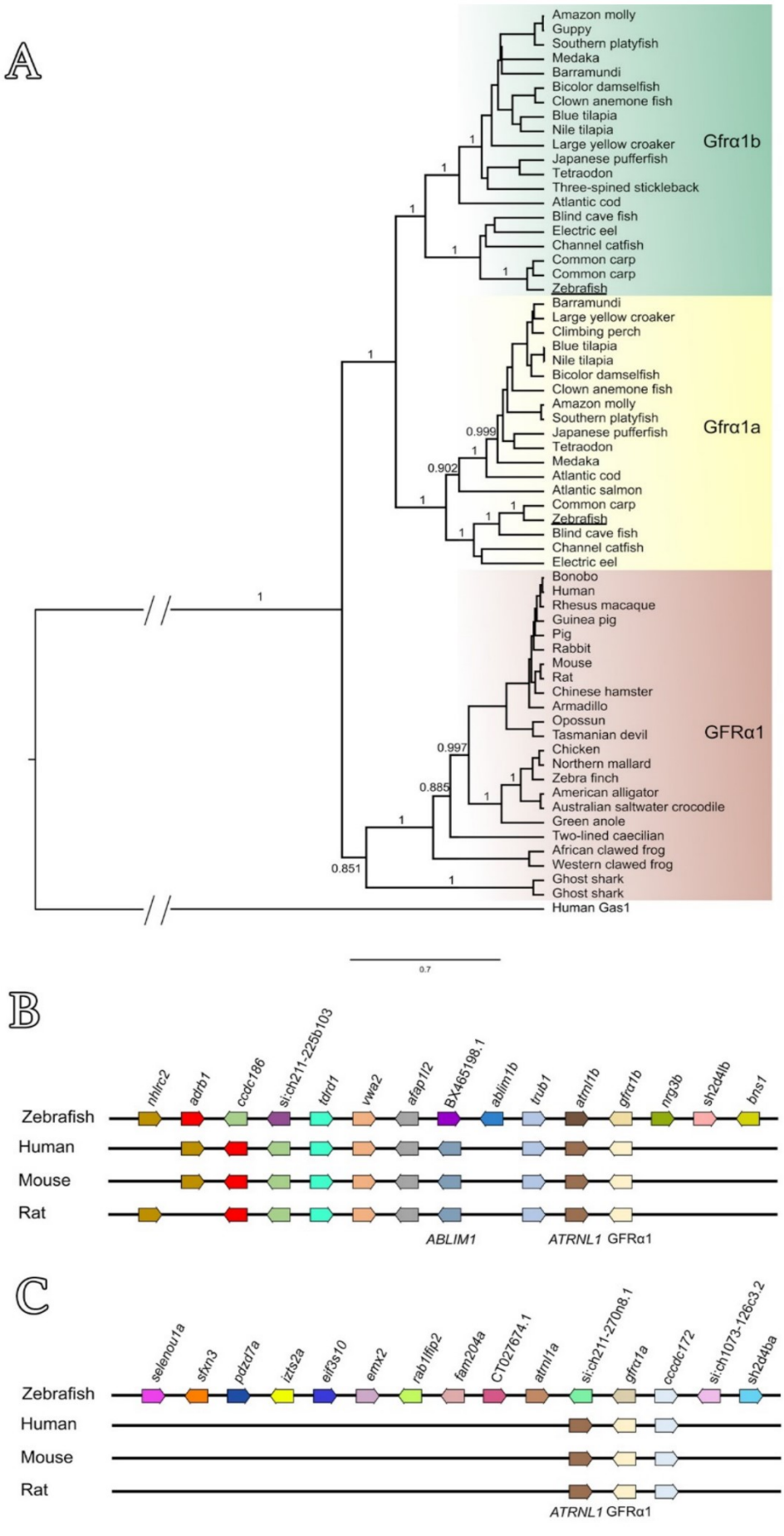
A
Amazon molly
Guppy
Southern platyfish
Medaka
Barramundi
Bicolor damselfish
Clown anemone fish
Blue tilapia
Nile tilapia
Large yellow croaker
Japanese pufferfish
Tetraodon
Three-spined stickleback
Atlantic cod
Blind cave fish
Electric eel
Channel catfish
Common carp
Common carp
Zebrafish
Gfrα1b
Barramundi
Large yellow croaker
Climbing perch
Blue tilapia
Nile tilapia
Bicolor damselfish
Clown anemone fish
Amazon molly
Southern platyfish
Japanese pufferfish
Tetraodon
Medaka
Atlantic cod
Atlantic salmon
Common carp
Zebrafish
Blind cave fish
Channel catfish
Electric eel
Gfrα1a
Bonobo
Human
Rhesus macaque
Guinea pig
Pig
Rabbit
Mouse
Rat
Chinese hamster
Armadillo
Opossun
Tasmanian devil
Chicken
Northern mallard
Zebra finch
American alligator
Australian saltwater crocodile
Green anole
Two-lined caecilian
African clawed frog
Western clawed frog
Ghost shark
Ghost shark
GFRα1
Human Gas1
1
0.999
0.902
0.997
0.885
0.851
0.7
B
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adrb1
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tdrd1
vwa2
afap1l2
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trub1
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bns1
Zebrafish
Human
Mouse
Rat
ABLIM1
ATRNL1 GFRα1
C
selenou1a
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gfra1a
cccdc172
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sh2d4ba
Zebrafish
Human
Mouse
Rat
ATRNL1 GFRα1

Figure 2. (**A**) Phylogenetic analysis of GFRα1 predicted amino acid sequences across vertebrates. Zebrafish Gfrα1a and 1b (both underlined) are clustered with other fish-specific Gfrα1a (yellow box) and Gfrα1b (green box) sequences, respectively, forming two separate subclades. Note that the GFRA1 sequences from other vertebrates (mammals, birds, reptiles, amphibians and Chondrichthyes) formed a separate clade (brown box). Branch values represent posterior probabilities obtained by Bayesian analysis (see Table S1). (**B**, **C**) Genomic organization and synteny comparisons among human GFRA1, rodents Gfrα1 and zebrafish gfrα1b (**B**) or zebrafish gfrα1a (**C**). The syntenic regions were analyzed according to the alignment of the target genes and genomic annotation available in the GenBank database (National Center for Biotechnology Information and Ensembl).

A cross-species comparison of chromosome neighboring genes revealed that both the zebrafish gfrα1a- and gfrα1b- containing regions are syntenic to human GFRA1- and rodent Gfrα1-containing regions (Figure 2B). This analysis also showed that the zebrafish gfrα1b gene (chromosome 12, NC_007123.7) showed a larger group of syntenic genes (8 out of 14 genes analyzed) when compared with zebrafish gfrα1a (chromosome 13, NC_007124.7) (2 out of 14 genes analyzed) (Figure 2C).

3.2. Expression Profiling in Zebrafish Testes and Identification of Gdnfa-, Gfrα1a- and Gfrα1b-Expressing Cells

RT-qPCR analyses revealed that both ligands (gdnfa and gdnfb) and receptors (gfrα1a and gfrα1b) were expressed in zebrafish testes, although with different numbers of amplification cycles (i.e., values of cycle threshold (Ct)) (Figure 3). As the Ct for gdnfb is greater than 30, this value indicates lower amounts for this target nucleic acid in zebrafish testes (Figure 3).

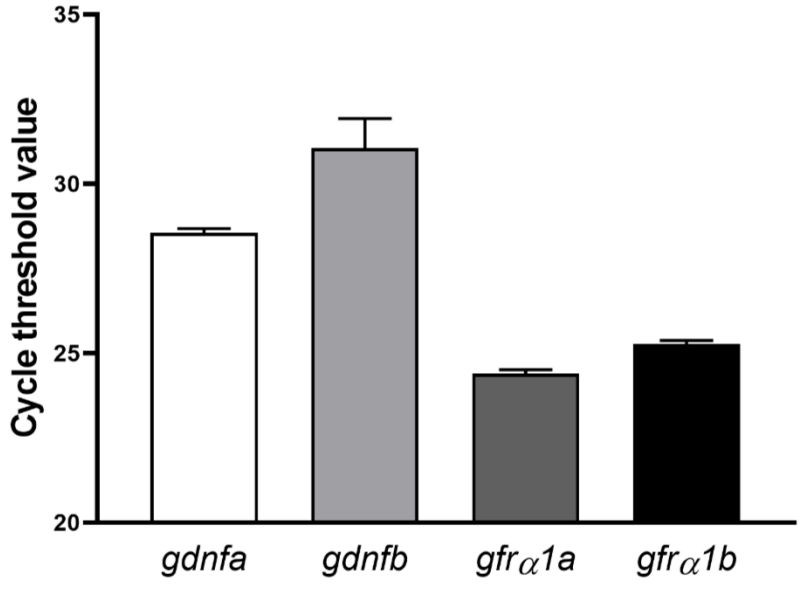

Figure 3. Number of amplification cycles (cycle threshold (Ct)) for both ligands (gdnfa and gdnfb) and receptors (grfα1a and grfα1b) in zebrafish testes. Bars represent the mean ± SEM (n = 4) for each transcript.

Considering the lower amounts of gdnfb transcripts in zebrafish testes, we focused our analysis on gdnfa. We tried to identify the cellular types expressing gdnfa mRNA in zebrafish testes by employing in situ hybridization with a specific antisense cRNA probe (Table 1, Figure S1) and RT-qPCR using RNA from isolated testicular cell populations (germ and somatic cell-enriched populations) (Figure 4). The first approach showed that gdnfa is expressed in germ cells (Figure S1). Nevertheless, due to limited resolution, it was not possible to unravel whether the signal was present or not in the Sertoli cells (Figure S1). This was attributed to the fact that cytoplasmic extensions of Sertoli cells protrude towards the lumen of a cyst in between the germ cells, making it difficult to accurately locate the signal. The precise identification of gdnfa expression sites was then accomplished through RT-qPCR using testicular cell populations obtained after the differential plating method (Figure 4A–E). In this approach, expression analysis showed higher transcript levels for gdnfa in the germ cell-enriched population when compared to the levels found in the total testicular cell suspension (Figure 4D,E). When analyzing the testicular somatic cell population, we found that gdnfa mRNA levels decreased significantly as compared to the levels observed in the germ cell fraction (Figure 4D,E). To confirm this result, we performed proper controls using specific markers for germ (vasa and pou5f3) and Sertoli cells (igf3). For the germ cells, we used vasa, which is a germ cell marker mostly expressed in early germ cells, including types A_{und}, A_{diff} and B spermatogonia [47]. We showed that vasa was expressed in the germ cell-enriched population, although with levels not significantly higher as compared to the total cell suspension (Figure 4D,E). On the other hand, vasa was not expressed in the testicular somatic cell fraction (Figure 4D,E). For pou5f3, a marker of types A_{und}, A_{diff} and B spermatogonia (Souza, Doretto and Nóbrega (unpublished data)), we showed higher mRNA levels in the germ cell-enriched fraction, but no expression in the somatic cell population (Figure 4D,E). For the Sertoli cells, we used igf3, which is a growth factor produced by Sertoli cells [54]. igf3 was not expressed in the germ cell population but it was detected in the somatic cell fraction with levels comparable to those found in the total cell suspension (Figure 4D,E).

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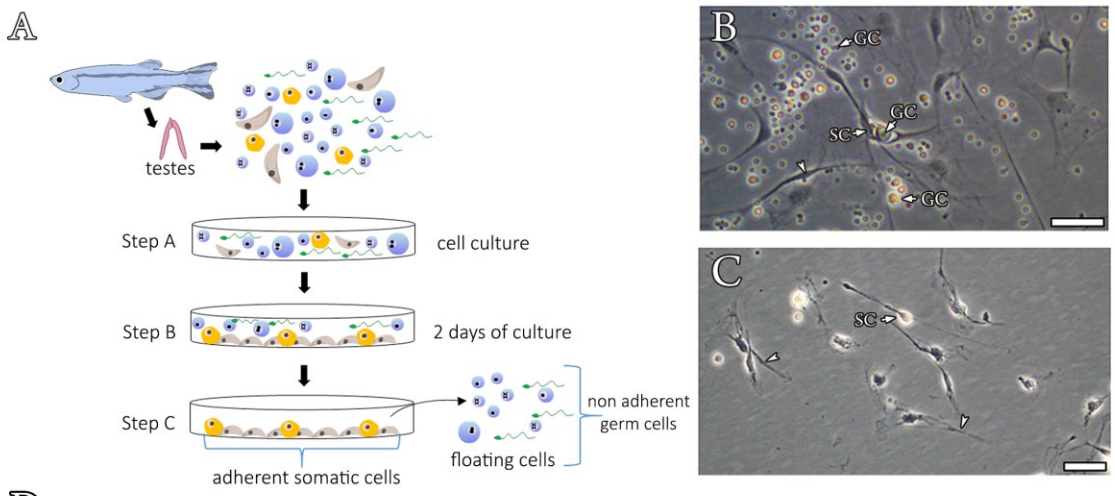
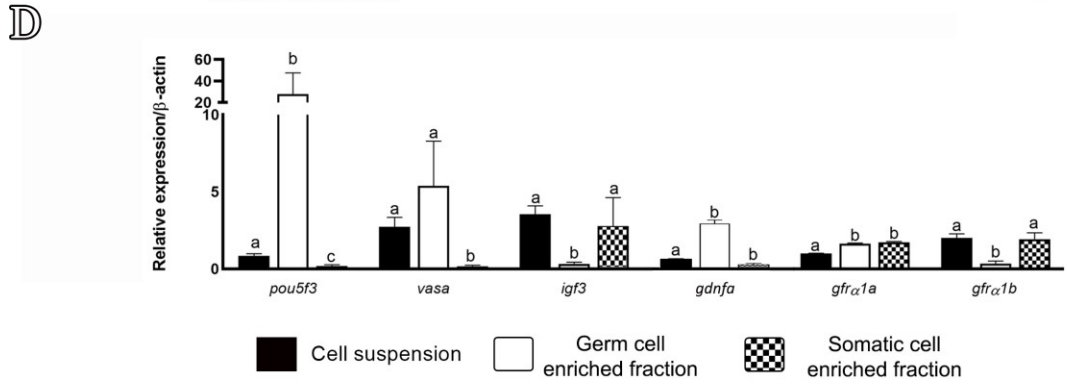
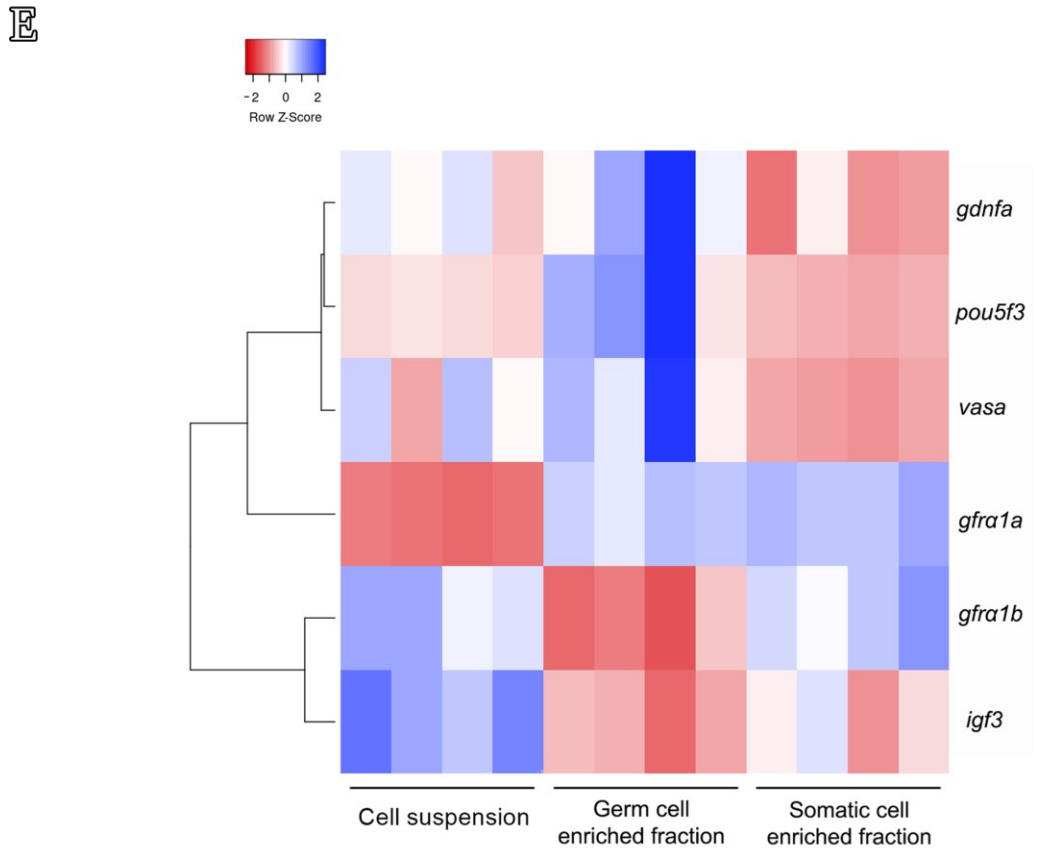

A
testes
Step A
cell culture
Step B
2 days of culture
Step C
adherent somatic cells
floating cells
non adherent germ cells
B
GC
SC
C
SC


D
Relative expression/β-actin
pou5f3
vasa
igf3
gdnfa
gfrα1a
gfrα1b
Cell suspension
Germ cell enriched fraction
Somatic cell enriched fraction


E
Row Z-Score
gdnfa
pou5f3
vasa
gfrα1a
gfrα1b
igf3
Cell suspension
Germ cell enriched fraction
Somatic cell enriched fraction

712

Figure 4. Differential plating method and expression analysis of the cellular enriched fractions. (**A**) Scheme showing the steps of the differential plating method, according to Hinfray et al. [51]. Briefly, a total testicular cell suspension was harvested (step A) in L-15 culture medium, and after 2 days of culture, only somatic cells (Sertoli cells, brown triangular shapes; Leydig cells, yellow oval shapes) adhere to the bottom of the plate (step B), while germ cells (blue shapes) remain floating or loosely attached to the bottom of the plate (step C). After washing steps, germ cells (floating and weakly attached to the somatic cells) can be removed, leaving the adherent somatic cells at the bottom of the plate. The firmly attached somatic cells can be obtained after extensive washing with trypsin. (**B**) Total testicular cell suspension after 2 days of culture. Note the somatic adherent cells (SCs) with cytoplasm extensions towards different germ cells (GC). (**C**) After washing, note that only somatic adherent cells (SCs) remain attached to the bottom of the plate. Scale bars: 20 µm. (**D**,**E**) Gene expression analysis of isolated zebrafish testicular cell populations: total cell suspension (black bar), germ cell-enriched population (white bar) and testicular somatic cells (hatched bar). Cells were obtained from three independent experiments. Bars represent relative mRNA levels of target genes expressed as mean ± SEM; different letters indicate significant differences between the cell populations (one-way ANOVA followed by the Student–Newman–Keuls test). (**E**) Heat map illustrating the relative mRNA levels of pou5f3, vasa, igf3 gdnfa, gfrα1a and gfrα1b according to different cell populations. Data shown are log2 values (relative quantification) relative to the average expression. Each colored cell in the heat map represents the standardized relative gene expression value for each sample. Genes (rows) are hierarchically clustered using Pearson correlation and the distance metric. The higher expression values are displayed in blue, moderate expression values in shades of white (light blue and light red) and lower expression values in red.

We also expressed our data in a heat map and genes were hierarchically clustered using Pearson correlation and the distance metric (Figure 4E). We showed through this analysis that genes such as vasa, *pou5f3* and gdnfa were hierarchically clustered in the germ cell fraction and separated from *igf3* and *gfrα1b*, which were clustered in the somatic cell fraction (Figure 4E). gfrα1a was expressed in both germ and somatic cell fractions (Figure 4D,E).

3.3. Localization of Gfrα1a Protein in Zebrafish Testis

Gfrα1a was detected in all generations of zebrafish spermatogonia, although the staining pattern varied among them according to the developmental stage (Figure 5A,C–E). The Gfrα1a signal was finely dispersed in the cell surface and cytoplasm of type A_{und} spermatogonia (Figure 5C) and later became more aggregated, forming intensely stained spots in type A_{diff} spermatogonia (Figure 5D). In type B spermatogonia, the Gfrα1a signal became finely dispersed again (Figure 5E) and gradually decreased as the number of spermatogonia B increased within the cyst until it became undetectable in the meiotic and post-meiotic cysts (Figure 5A). Furthermore, Gfrα1a was also found in Sertoli cells contacting germ cells at different stages of development (Figure 5A,B (inset) and Figure S1F,G). This result was also confirmed by the expression of both gfrα1a and gfrα1b in

the somatic cell-enriched population (Figure 4D). Altogether, these two bodies of evidence support the presence of Gfrα1a and 1b in zebrafish Sertoli cells. The specificity of the antibody (anti-zebrafish Gfrα1a) was confirmed by immunoblots (Figure 5F) and control sections either by using a preadsorbed antibody with the corresponding peptide or omitting the primary antibody (Figure S2). It is important to mention that the immunofluorescence signal should not be limited to Gfrα1a, since the antibody could potentially recognize part of zebrafish Gfrα1b (see the blue line in Figure 1).

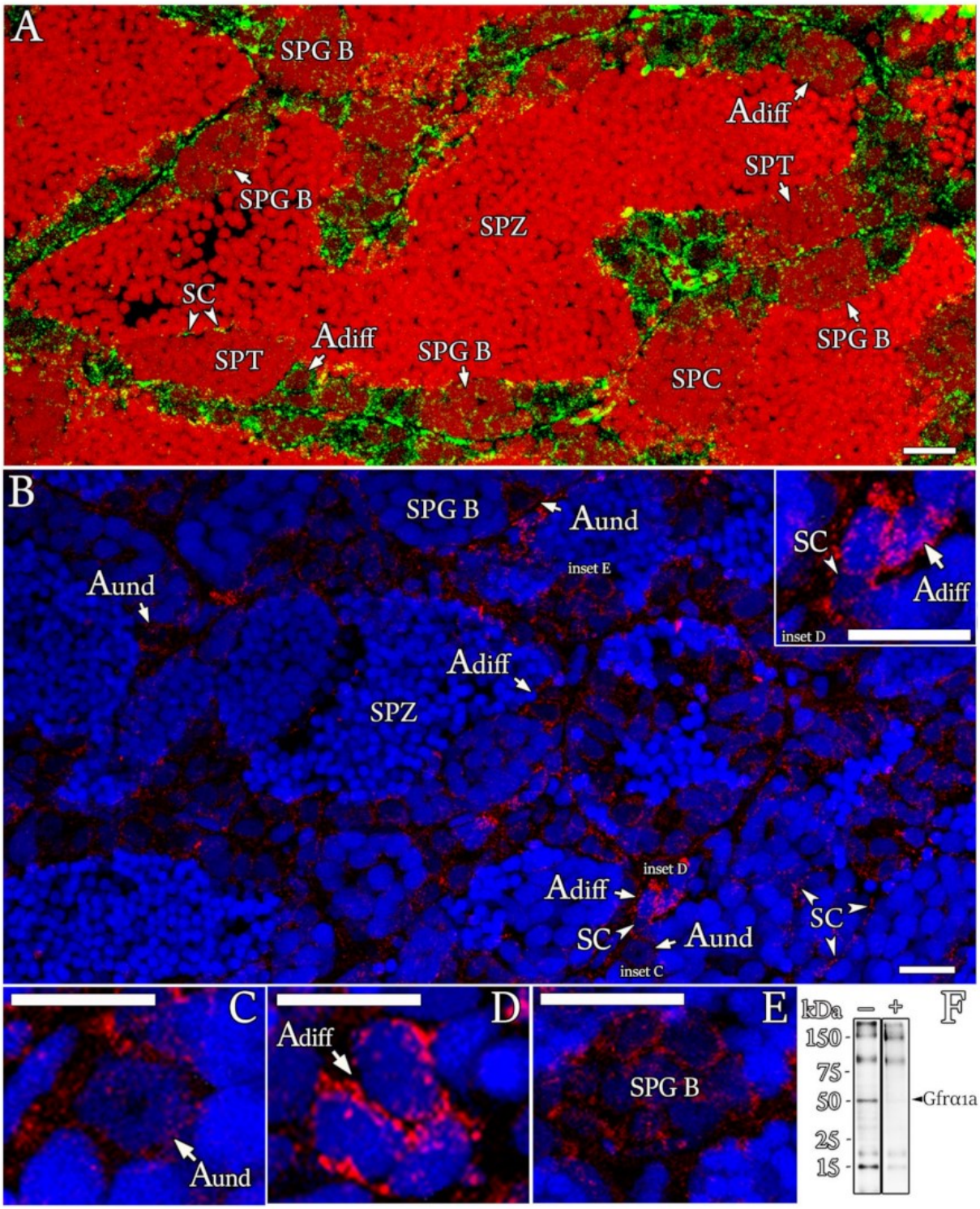


Figure 5. Cellular localization of Gfrα1a in zebrafish testis. (**A**–**E**) Immunofluorescence for Gfrα1a (green—**A**; red—**B**–**E**) in testis sections of sexually mature zebrafish. The spermatogonial generations, including type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}) and type B spermatogonia (SPG B), were immunoreactive to Gfra1a, although staining patterns among them varied according to developmental stage. The signal was not found in spermatocytes (SPCs), spermatids (SPTs) and spermatozoa (SPZ). Note that Sertoli cells (SCs) contacting germ cells at different stages of development were also immunoreactive to Gfrα1a. Cell nuclei were counterstained with propidium iodide (A) or Hoechst (B–E). Scale bars: 15 µm. (F) Gfrα1a (approximately 52 kDa

(kilodaltons)) immunoblots of whole testes with (+) or without (−) preadsorbed antibodies, confirming the presence of the protein in the zebrafish testes and antibody specificity.

3.4. Three-Dimensional Model for Predicting the Interaction between rhGDNF and Zebrafish Gfrα1a

In this study, we used a recombinant human hormone because the recombinant zebrafish Gdnf is not commercially available. Therefore, to investigate whether rhGDNF could have effects on zebrafish spermatogenesis, we first generated a 3D structure model to predict the possible interaction sites between human GDNF and zebrafish Gfrα1a (Figure 6A, box 2, box 3). The 3D structure (hetero-2-2-mer) was built according to the homology of the 4ux8.1 template and showed a GMQE value of 0.63 with 74% of identity and a resolution of 24Å (method: Electron Microscopy) when compared to human GDNF-GFRA1 interaction (merged in the 3D structure) (Figure 6A, box 2, box 3). Moreover, the predictive model demonstrated that 89.8% of the amino acid residues were in the most favorable regions, 7% of residues were situated in allowed regions (~2% expected) and 3.1% in the outlier regions according to Ramachandran plots. The 3D structures of the hetero-2-2-mer (GDNF-zebrafish Gfrα1a) were based on the homology modeling templates and are shown in Figure 6A (box 2, box 3). More detailed information regarding the predictive interaction model between GDNF and zebrafish Gfrα1a can be found in the Supplementary Materials (Figure S3, Video S1). In agreement with the 3D model, the alignment of zebrafish Gdnfa with rhGDNF showed conserved regions, particularly in the binding sites to human GFRA1 or zebrafish Gfrα1a (Figure 6B).

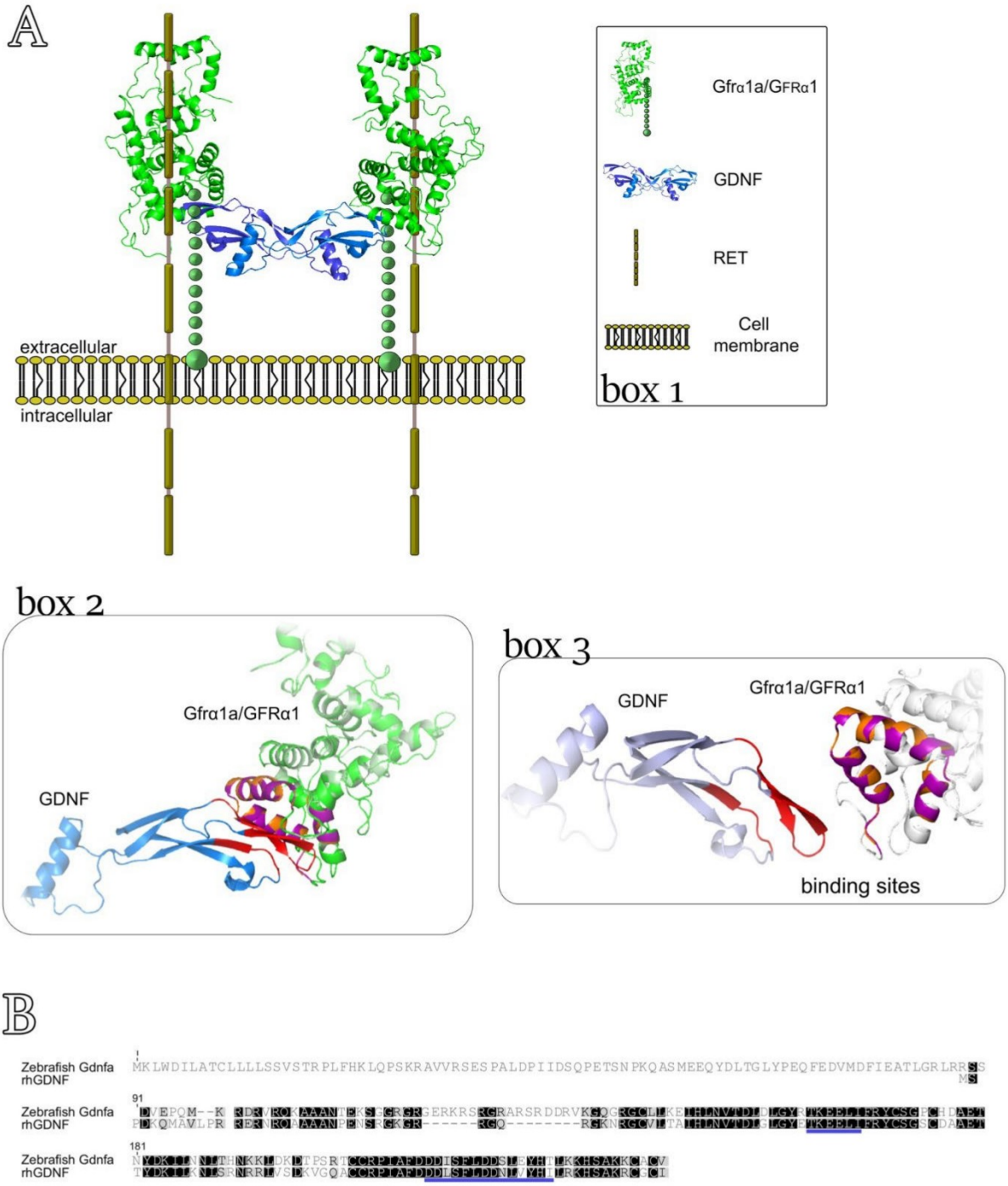


Figure 6. A 3D model to predict the interaction between rhGDNF and zebrafish Gfrα1a. (**A**) Box 1 depicts the molecular components of the complex GDNF-GFRα1-RET. Boxes 2 and 3 show the predictive 3D model (template 4ux8.1) in which the structural similarities between zebrafish Gfrα1a and human GFRA1 are represented by orange and purple coloring and the identity of the structure formed at the binding sites is indicated in red. In box 2, green is used to indicate the conserved amino acid sequences between zebrafish Gfrα1a and human GFRA1 and blue indicates the GNDF protein. In box 3, we highlighted the interaction sites between human GDNF and zebrafish Gfrα1a/human GFRA1. (**B**) Alignment of zebrafish Gdnfa with rhGDNF. The blue lines indicate the conserved binding sites to zebrafish Gfrα1a or human GFRA1.

3.5. Biological Effects of rhGDNF

To investigate the roles of Gdnf in zebrafish spermatogenesis, we first examined whether rhGDNF could affect germ cell composition and cellular proliferation, using a previously established primary testis tissue culture system (Figure 7A–D). The results showed that rhGDNF (100 ng/mL) increased the abundance of types A_{und} and A_{diff} spermatogonia as compared to basal conditions (Figure 7C). These data are also consistent with the proliferation activity of these cells, showing that treatment with rhGDNF (100 ng/mL) augmented the mitotic index of both types of spermatogonia (A_{und} and A_{diff}) as compared to their basal mitotic index (approximately 1,5-fold increase for A_{und} and A_{diff}, with $p < 0.001$ and $p < 0.01$, respectively) (Figure 7A,B,D). Moreover, histomorphometrical analysis showed that rhGDNF decreased the frequency of type B spermatogonia, whereas no effects were observed for meiotic and post-meiotic germ cells (Figure 7C). In this study, we also quantified Sertoli cell proliferation (Figure 7E), reasoning that change in the proliferation of Sertoli cells associated with types A_{und} or A_{diff} spermatogonia would indicate the creation of new niche space or support the development of differentiating spermatogonial cysts, respectively [62]. Our results then demonstrated that treatment with rhGDNF stimulated Sertoli cell proliferation (1,5-fold increase, $p < 0.050$), particularly if the Sertoli cells associated with proliferating types A_{und} and A_{diff} spermatogonia (Figure 7E).

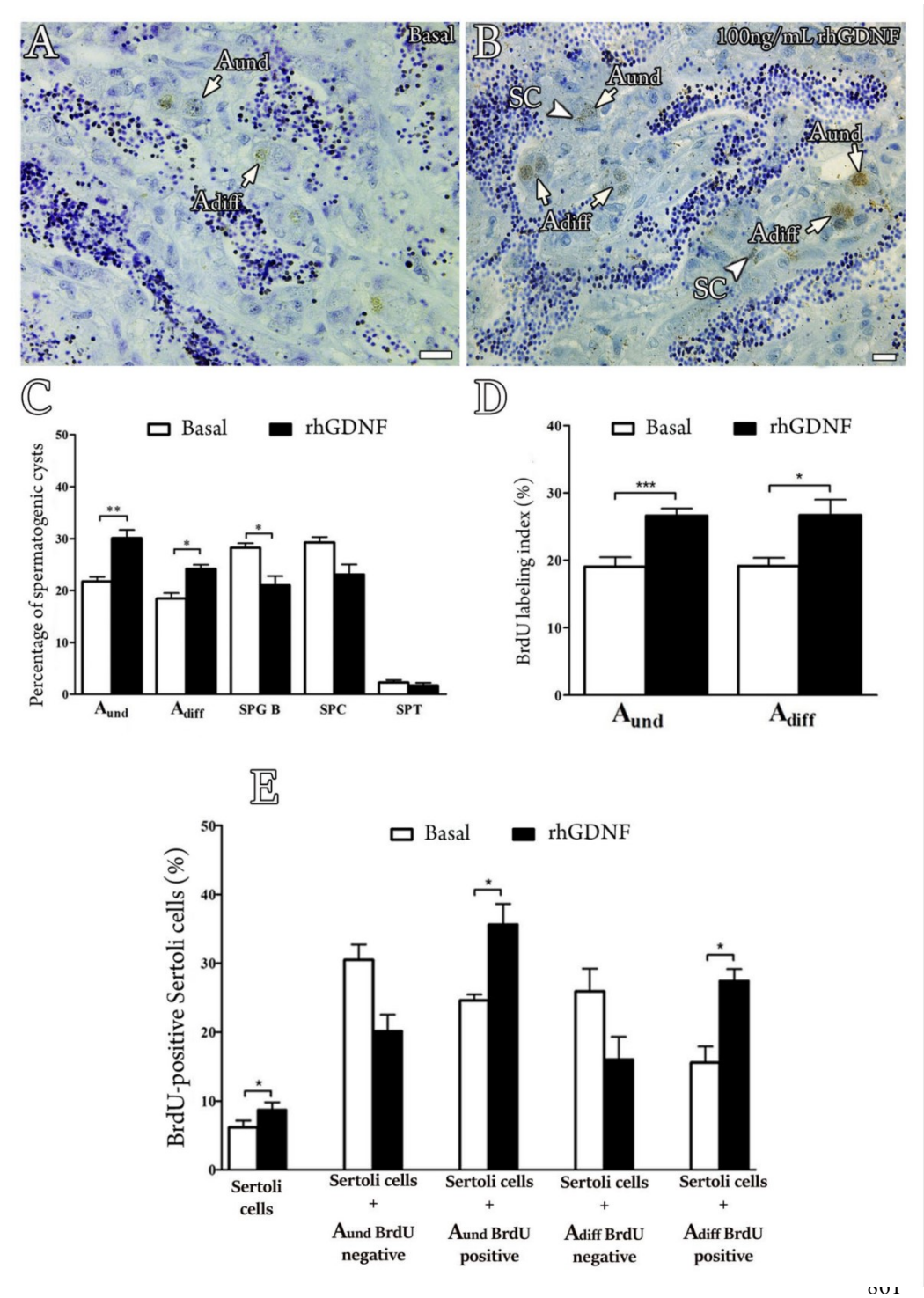


Figure 7. Effects of Gdnf on germ cell composition and cellular proliferation, using a previously established primary testis tissue culture system. (**A**, **B**) BrdU immunodetection from zebrafish testicular explants incubated for 7 days in the absence (Basal) or presence of rhGDNF (100 ng/mL), demonstrating a higher proliferation activity for type A undifferentiated spermatogonia (A_{und}) and type A differentiated spermatogonia (A_{diff}) in the presence of rhGDNF. (**C**) Frequency of different germ cell cysts after 7 days of incubation in the absence (Basal) or presence of rhGDNF (100 ng/mL). Types A_{und}, A_{diff} and B spermatogonia (SPG B), spermatocytes (SPCs) and spermatids (SPTs) were identified according to morphological characteristics, as described by Leal and collaborators [55]. (**D**) Mitotic indices of type

A_{und} and A_{diff} spermatogonia after incubation in the absence (Basal) or presence of rhGDNF (100 ng/mL) for 7 days. (**E**) Mitotic indices of Sertoli cells in association with BrdU-negative or BrdU-positive type A_{und} and A_{diff} spermatogonia in the absence (Basal) or presence of rhGDNF (100 ng/mL) for 7 days. Sertoli cells were identified according to morphological characteristics, as described previously [55]. In fish, Sertoli cells (SCs) have a triangular nuclear shape, dark chromatin and usually they appear surrounding spermatogenic cysts, as shown in Figure S1. Bars represent the mean ± SEM (n = 10). Paired t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bars: 15 µm.

In order to elucidate the molecular mechanisms mediated by rhGDNF on basal or Fsh-induced spermatogenesis, we performed gene expression analyses of selected genes related to Gdnf signaling (*gdnfa, gfrα1a and gfrα1b*), Sertoli cell growth factors (*igf3* and *amh*), Fsh signaling (*fshr*) and germ cell markers (undifferentiated spermatogonia—*pou5f3*; differentiated spermatogonia and preleptotene spermatocytes—*dazl*; and primary spermatocytes—*scyp3l*) (Figure 8).

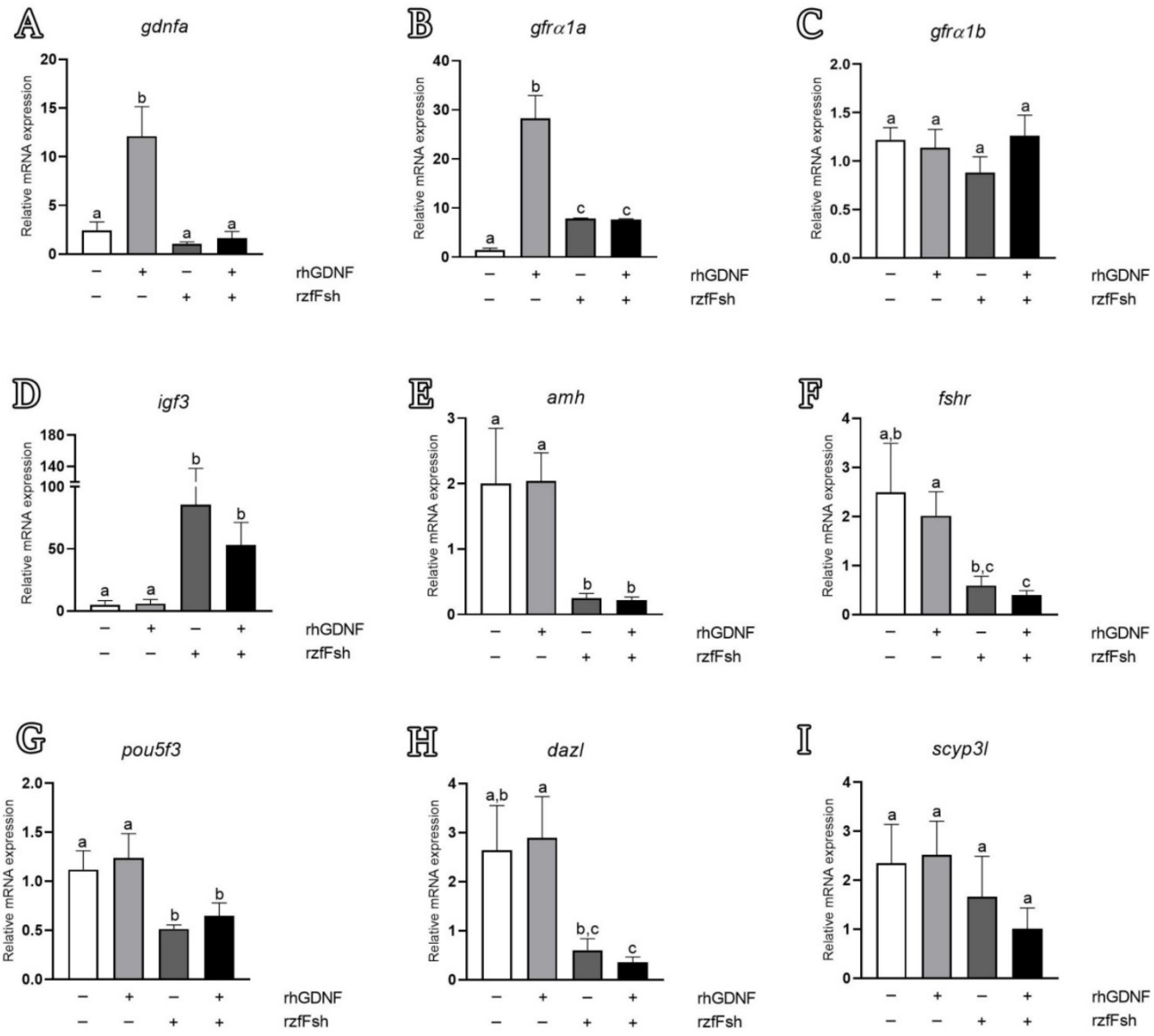


Figure 8. Relative mRNA levels of genes related to Gdnf signaling (*gdnfa*, *gfrα1a* and *gfrα1b*) (**A–C**), Sertoli cell growth factors (*igf3* and *amh*) (**D–E**), Fsh signaling (*fshr*) (**F**) and germ cell markers (undifferentiated spermatogonia—*pou5f3* (**G**); differentiated spermatogonia and preleptotene spermatocytes—*dazl* (**H**); and primary spermatocytes—*scyp3l* (**I**)). Testicular explants were cultivated for 7 days with rhGDNF, rzfFsh or both (rhGDNF + rzfFsh). The relative mRNA levels were normalized with the β-actin levels. Bars represent the mean ± SEM (n = 20). One-way ANOVA followed by the Student–Newman–Keuls test, in which different letters denote significant differences ($p < 0.05$) among treatment conditions.

RT-qPCR analysis revealed that rhGDNF increased the transcript levels of *gdnfa* and *gfrα1a*, whereas *gfrα1b* mRNA levels remained unaltered when compared with basal condition levels (Figure 8A–C). The transcript abundance for the other genes (Sertoli cell growth factors, Fsh signaling and germ cell markers) did not change following rhGDNF treatment (Figure 8D–I). We further investigated whether rhGDNF could affect the Fsh-induced changes in testicular gene

expression, since Fsh is considered the major endocrine player regulating the zebrafish spermatogonial phase [48,54,63]. We first showed that Fsh did not modulate the transcript levels of gdnfa, gfrα1a or gfrα1b in the zebrafish testes (Figure 8A–C). However, Fsh was able to nullify the rhGDNF-increased *gdnfa* and *gfrα1a* mRNA levels following co-treatment (Figure 8A, B). With respect to Sertoli cell growth factors, we demonstrated that rhGDNF did not change Fsh-mediated expression on *igf3* (Figure 8D) or *amh* mRNA levels (Figure 8E). As expected, and in agreement with previous studies [54,64], Fsh increased *igf3* mRNA levels (Figure 8D) and down-regulated amh transcription (Figure 8E). The other evaluated genes were not responsive to Fsh or co-treatment (Figure 8F–I). Nevertheless, it is worth mentioning that transcript levels of *fshr*, *pou5f3* and *dazl* were significantly higher following rhGDNF treatment than in the co-treatment with Fsh (Figure 8F–H).

3.6. In Silico Analysis of Putative Regulatory Sequences Upstream of Human GDNF, Mouse Gdnf and Zebrafish Gdnfa

To support our expression analysis, we investigated the putative regulatory sequences upstream of the transcriptional start site (TSS) of human GDNF (NM_000514.4), mouse Gdnf (NM_010275.3) and zebrafish gdnfa (NM_131732.2) (Figure 9). The in silico analysis showed three different types of cAMP response elements (CRE), several N-box and E-box motifs, one NF-kB binding site and a TATA-Box within the 2000 bp upstream of human GDNF (Figure 9, Table S2). The upstream sequence of the Gdnf mouse gene showed similar regulatory binding sites to the human GDNF (Figure 9, Table S2). For zebrafish, we predicted a non-canonical TATA-Box, one CRE close to a GC-Box, one N-Box, four E-Boxes and two androgen receptor (AR) half binding sites within the 2000 bp upstream of gdnfa (Figure 9, Table S2).

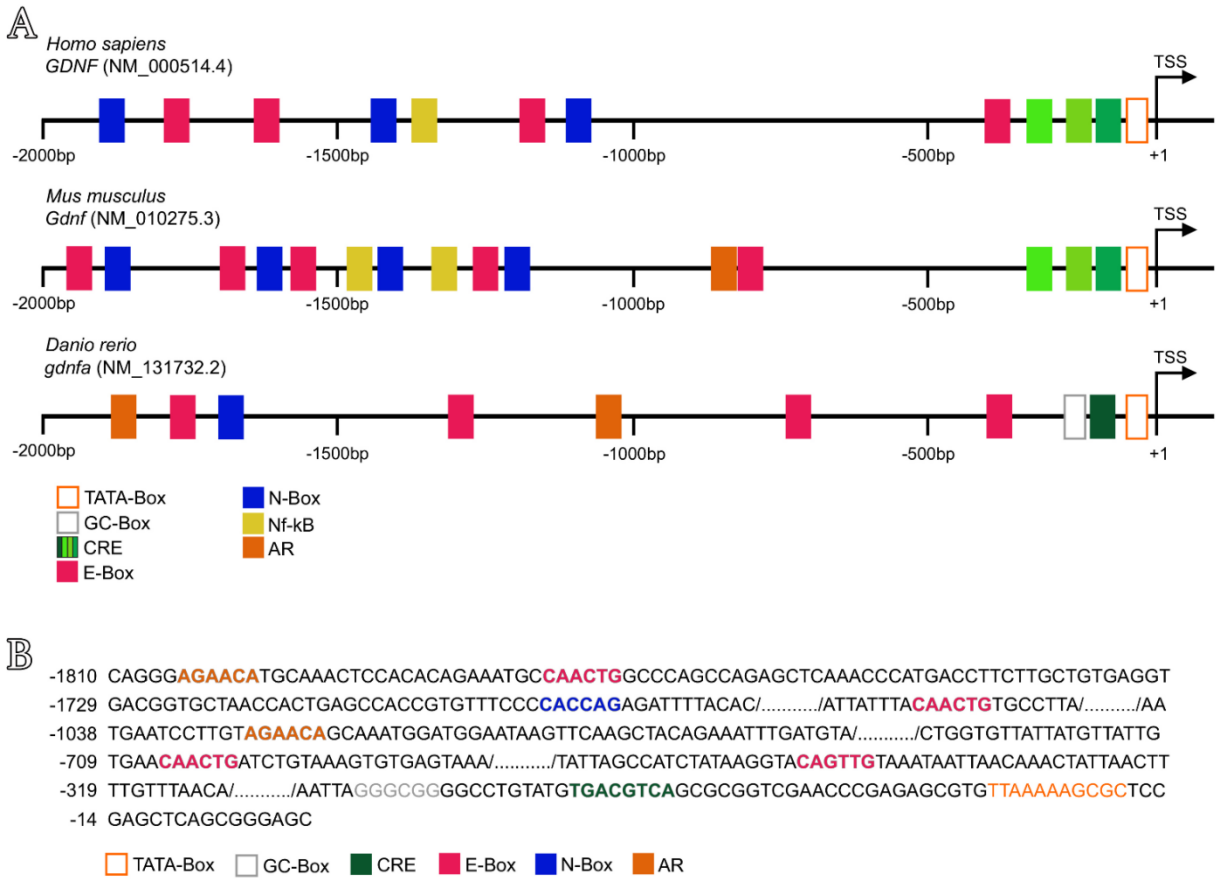


Figure 9. Predicted regulatory sequences upstream of human GDNF, mouse Gdnf and zebrafish gdnfa. (**A**) The upstream region (2000 bp) of human GDNF contains three different sequences of cAMP response elements (CRE), four E-box sequences, three N-box sequences and one Nf-kB binding site. The upstream region (2000 bp) of mouse Gdnf contains an N-box/E-box-rich region at bp −1300 to −1900 and additional E-boxes downstream, one androgen receptor binding site (AR), two Nf-kB binding sites and three different sequences of CRE close to the TSS (transcriptional start site). The upstream region (2000 bp) of zebrafish gdnfa contains four E-box sequences and one N-box sequence, two AR half sequences and only one CRE close to a GC-box and the TSS. TSS is the transcription start site (position +1). (B) Sequences of putative binding sites upstream of zebrafish gdnfa. In the open orange box is shown the TATA-box sequence, in the open gray box the GC-box, in the dark green box the CRE, in the pink box the E-box, in the blue box the N-box, and in the filled orange box the AR half binding site.

4. Discussion

This study demonstrated the involvement of the Gdnf/Gfrα1 signaling pathway in the regulation of the spermatogonial phase in zebrafish. Our first analysis identified two zebrafish paralogs for the Gfrα1-encoding gene, named zebrafish gfrα1a and gfrα1b. The predicted amino acid sequences of zebrafish Gfrα1a and Gfrα1b revealed high identity to GFRα1 from other mammalian species investigated in this study (>60% and >57% sequence identity for Gfrα1a and Gfrα1b, respectively). Moreover, both paralogs have conserved domains and residues which are typical of GFRα1 family members, such as 3 cysteine-rich domains (D1, D2 and D3), 28 cysteine residues (plus 2 in the terminal region) and 2 triplets (MLF and RRR) [65,66,67]. Studies in mice using site-directed mutagenesis have shown that some of these conserved regions (e.g., two triplets—MLF and RRR—in the D2 domain) are critical for Gfrα1 binding to Gdnf, activation of the receptor complex and elicitation of downstream signal transduction [65,67]. This evidence suggested that, theoretically, both zebrafish Gfrα1a and Gfrα1b could bind and elicit a response to Gdnf/GDNF (e.g., rhGDNF). Moreover, in agreement with previous studies [33,68], phylogenetic analysis demonstrated that zebrafish Gfrα1a and 1b are clustered with other fish Gfrα1a and 1b sequences; the paralogs diverged, forming two distinct sub-clades within the fish clade. Additional analysis of chromosome neighboring genes revealed that both zebrafish gfrα1a- and gfrα1b-containing regions are syntenic to human GFRA1- and rodent Gfrα1-containing regions. Altogether, this evidence confirmed that zebrafish gfrα1a and gfrα1b are duplicated genes that diverged from each other after the teleost-specific whole genome duplication. It is well established that, around 320 million years ago, the common ancestor of the teleosts experienced a third round of whole genome duplication [69,70]. This event was responsible for the generation of a large number of duplicated genes that could follow different evolutionary paths, such as co-expression (both copies retain the ancestral function), non-functionalization (function loss or complete deletion of one copy), sub-functionalization (specialization of each copy, sub-function partition), or neo-functionalization (acquisition of a novel function) [69,70]. In this study, we could not determine the specific roles of Gfrα1a and Gfrα1b. Additional studies (e.g., specific knockouts of each copy) are required to confirm this hypothesis and to unravel the specific roles for each Gfra1 paralog in zebrafish spermatogenesis.

When evaluating the expression profiling of Gfrα1a and Gfrα1b, we found that both paralogs are expressed in zebrafish testes. Considering the greater homology with the mammalian

GFRA1 and the modulation by rhGDNF, we developed an antibody for zebrafish Gfrα1a (although it could be able to recognize zebrafish Gfrα1b). Our data revealed that Gfrα1a was found in all types of zebrafish spermatogonia, although the staining pattern varied among the different generations of spermatogonia. Gfrα1a was mainly detected in early types of spermatogonia (A_{und} and A_{diff}), and immunostaining decreased as spermatogonial clones became larger and more differentiated. Likewise, accumulating evidence has shown that GFRA1 is a conserved marker for mammalian type A undifferentiated spermatogonia [22,23,24,25,26,27,28,71,72,73] and the frequency of GFRA1+ spermatogonia decreases as spermatogonia progress from A_s to Aal [72,73]. Similarly, in other fish species, mRNA or protein levels of Gfrα1a were found mainly in type A_{und} spermatogonia of dogfish (Scyliorhinus canicula) [29,53], rainbow trout (Oncorhynchus mykiss) [30,31,32], medaka (Oryzias latipes) [33] and tilapia (Oreochromis niloticus) [73]. In rainbow trout, Nakajima et al. [30] reported that gfrα1 transcripts decreased throughout spermatogonial development and became undetectable in spermatids and spermatozoa. In medaka, Zhao et al. [33] showed a moderate signal for gfrα1a and gfrα1b mRNA in spermatocytes, but no expression was found in spermatids and spermatozoa. Altogether this evidence is in agreement with our results and supports our hypothesis that the Gndf-Gfrα1a signaling pathway is important for the regulation of the zebrafish spermatogonial phase but is not required for meiotic and post-meiotic phases. Strikingly, our study also detected the Gfrα1a protein among Sertoli cells associated with different types of germ cells. In rainbow trout, Maouche et al. [68] demonstrated that gfrα1a1 transcripts were mainly expressed in somatic testicular cells, while gfrα1a2 was restricted to type A_{und} spermatogonia. To our knowledge, our study and the one of rainbow trout [68] were the first to show that Gndf-Gfrα1a is not only involved in the control of type A_{und} spermatogonia but can also modulate the functions of Sertoli cells.

Investigation of the Gdnf ligands (Gdnfa and Gdnfb) revealed that both are expressed in zebrafish testes, although gdnfb has shown a Ct value greater than 30. These data suggest that Gdnfa might be the main ligand in zebrafish testes. Further in situ hybridization and RT-qPCR analysis demonstrated that gdnfa is mainly expressed in the germ cells. gdnfa was not expressed in somatic testicular cells. In both analyses, we were not able to identify the germ cell types expressing gdnfa in zebrafish testes. Nakajima and collaborators [30], on the other hand, demonstrated that gdnf mRNA and protein were expressed in type A_{und} spermatogonia of immature rainbow trout. Moreover, the same authors showed that gdnf and gfrα1 were co-expressed and that their expression changed synchronously during germ cell development [30]. Altogether, this evidence supports our findings that zebrafish Gdnfa is a germ cell-derived factor

that exerts autocrine and paracrine functions on spermatogonia and Sertoli cells, respectively, in zebrafish testes. Moreover, these data provide new insights into the Gndf-Gfrα1a signaling pathway in fish as compared to mammals. In mammals, GDNF is secreted by testicular somatic cells (Sertoli cells [2,9,10], peritubular myoid cells [11,12] and testicular endothelial cells [14]), acting only as a paracrine factor for GFRA1-expressing undifferentiated spermatogonia [21,22,23,24,25,26,27,28,71,72]. This difference is likely related to the events that took place after the teleost-specific whole genome duplication, involving, for example, non- and neo-functionalization of the Gdnf paralogs. Moreover, these findings suggest that the common vertebrate ancestor expressed Gdnf in testicular somatic cells, while Gdnf expression in germ cells is considered an evolutionary novelty which is exclusive to fish.

To assess the biological roles of Gdnf in zebrafish spermatogenesis, we used a rhGDNF. There is strong evidence that rhGDNF can bind to zebrafish Gfrα1a and elicit a downstream signal transduction in zebrafish testes. The first item of evidence is the predictive 3D model which examined the interaction sites between human GDNF and zebrafish Gfrα1a based on the binding interaction with human GFRA1. This analysis revealed structural similarities between zebrafish Gfrα1a and human GFRA1 (Figure 6A, box 2), and higher identity of the structure formed at the binding sites between human GDNF and human GFRA1, and with Gfrα1a zebrafish (Figure 6A, box 2). Moreover, this analysis also showed that most of the amino acid residues identified as crucial for ligand–receptor interactions are conserved in the zebrafish Gfrα1a, with exceptions for the residues Gly155 and Ile175, which were replaced by Glu and Thr, respectively. The predictive 3D model was also supported by Ramachandran plots which showed that 89.8% of the amino acid residues were in the most favorable regions, 7% of residues situated in allowed regions (~2% expected) and 3.1% in outlier regions. The second item of evidence is the sequence alignment demonstrating conserved regions between rhGDNF and zebrafish Gdnfa, such as the binding sites to GFRA1/Gfrα1a. The last item of evidence is the capability of rhGDNF to induce proliferation and modulate gene expression in zebrafish testes (see below), indicating that rhGDNF not only can bind to zebrafish Gfrα1a but also can trans-activate the receptor complex and trigger molecular and cellular responses.

With regard to biological functions, our results demonstrated that rhGDNF (100 ng/mL) increased the mitotic index of types A_{und} and A_{diff} spermatogonia when compared to basal conditions. Consistently, histomorphometric analysis revealed that both types A_{und} and A_{diff} became more abundant, while type B significantly decreased following rhGDNF treatment. Altogether, these results indicated not only that Gdnf stimulates proliferation of the most

undifferentiated spermatogonia (A_{und} and A_{diff}) but that it is also involved in blocking late differentiation into type B spermatogonia. Similar functions have been described in mammalian and non-mammalian species. In mammalian species, particularly rodents, GDNF promotes self-renewing proliferation of SSCs ([2]; see reviews in Parekh et al. [7] and Mäkelä and Hobbs [8]), although a recent study in mice has shown that GDNF could be more associated with blocking differentiation rather than actively stimulating SSC proliferation [4]. In dogfish, rhGDNF promoted in vitro proliferation and long-term maintenance of spermatogonia with stem characteristics [53]. In medaka, Wei et al. [34] demonstrated that recombinant medaka Gdnfa and Gdnfb were involved in the proliferation and survival of medaka SSCs. Furthermore, the knockdown of medaka gfrα1a and gfrα1b subsequently confirmed that both receptors mediated the proliferation and survival of medaka SSCs [33]. In this study, Zhao et al. [33] also showed that genes related to differentiation (e.g., c-kit) were up-regulated when the expression of both receptors was lowered. Altogether, this evidence from different species sustains our conclusion that the Gndf-Gfrα1 signaling pathway is associated with maintaining the pool of undifferentiated spermatogonia (A_{und} and A_{diff}) through promoting their proliferation and also by inhibiting their differentiation. Moreover, as zebrafish Gdnfa and its receptor (Gfrα1a) are co-expressed, it is important to highlight that the above-mentioned function is an autocrine loop of Gdnf on types A_{und} and A_{diff} spermatogonia.

In this study, we also quantified Sertoli cell proliferation because change in the proliferation of Sertoli cells associated with types A_{und} or A_{diff} spermatogonia would indicate the creation of new niche space or support for the development of spermatogonial cysts, respectively [62]. In fish, in contrast to mammals, Sertoli cells are not terminally differentiated and continue to proliferate during spermatogenesis in adult males of different species, including zebrafish [52,71,74]. Strikingly, our results demonstrated that Gndf promotes proliferation of Sertoli cells that are particularly associated with types A_{und} and A_{diff} spermatogonia which are also undergoing mitosis (BrdU-positive cells). These data indicate for the first time that a germ cell-derived factor is involved in the creation of new spermatogenic cysts, i.e., new available niches, in addition to supporting the development of early differentiating spermatogonial cysts. In the first case, as Gdnf stimulates the proliferation of type A_{und}, the newly formed, single spermatogonium must recruit its own Sertoli cells to form a new spermatogenic cyst. Therefore, it is reasonable that new Sertoli cells would be produced to create a niche into which the newly formed, single type A_{und} can be recruited or attracted (germ cell homing). Consistently, in mice, Gdnf has been shown to be important for germ stem cell homing as it acts as a SSC chemotactic factor [75]. In the second case

(supporting the development of differentiating spermatogonial cysts), Gdnf-induced Sertoli proliferation would provide structural and nutritional support for the development of early differentiating spermatogonia. In both cases, Gdnf effects on Sertoli cells might be mediated directly through Gfrα1a, which is also expressed in Sertoli cells of zebrafish. In agreement with our observation, a study in rodents has shown that Gdnf promoted the proliferation of immature Sertoli cells through its interaction with Gfrα1 and neural cell adhesion molecules (NCAMs), both co-expressed in Sertoli cells [76,77]. Although there is evidence of Gfrα1a expression in Sertoli cells, we cannot exclude that Gdnf-induced Sertoli cell proliferation may be mediated by other growth factor(s) produced by type A undifferentiated spermatogonia.

We further evaluated whether Gdnf could modulate testicular gene expression or affect Fsh-induced gene expression in zebrafish explants. Previous studies have shown that Fsh is the major endocrine player regulating zebrafish spermatogonial development through targeting Sertoli and Leydig cell functions, such as sex steroid and growth factor production [47,54,63,78,79]. Our results showed that Gdnf positively modulates its own regulatory pathway (Gdnfa-Gfrα1a). This would be the first demonstration that a germ cell factor can affect the spermatogonial niche through an autocrine and paracrine loop. It seems that Gdnf signaling would enhance its own production and sensitivity to favor the creation of new spermatogonial niches (type A_{und} spermatogonia and Sertoli cells). Notably, gfrα1b was not modulated by any treatment, which indicates that zebrafish Gfrα1a may be the mammalian GFRα1 homologous form. Moreover, we showed that Fsh did not modulate gdnfa expression in zebrafish testis explants. Similarly, Bellaiche et al. [31] demonstrated that Fsh did not modulate the expression of gdnfb in immature and early maturing rainbow trout testicular explants either. This regulation in fish is different from the one reported in mammals, where Fsh has been shown to stimulate the expression of Gdnf in the testes [79]. One possible explanation for this different regulation would be the distinct cellular sites expressing Gdnf in mammalian and fish testes. In zebrafish, Gdnf is mainly secreted by germ cells, which are not the direct targets of Fsh, while in mammals, Gdnf is secreted by somatic cells, including Sertoli cells, which are known to express Fsh receptors. Additionally, to support our data, we performed an in silico analysis within regions −2000 to +1 bp upstream of the zebrafish gdnfa gene to search cAMP response elements (CREs). As is well known, Fsh stimulates the cAMP-dependent protein kinase A signaling pathway, leading to phosphorylation of the cAMP response element-binding protein (CREB), which is necessary to transactivate several genes containing CREs [80,81]. Lamberti and Vicini [59] demonstrated that three CRE binding sites in the murine Gdnf promoter are directly involved in basal and cAMP-induced expression of Gdnf in Sertoli cells. In our in

silico analysis, we demonstrated that the zebrafish gdnfa promoter (−2000 to +1 bp) has fewer conserved DNA binding sites compared with human and mouse GDNF/Gdnf promoters. Moreover, our analysis showed only one CRE site near to the zebrafish gdnfa transcription start site, instead of three CREs, as reported in human and mouse. The difference in the promoter region and the lower number of CRE binding sites could be the reason that Fsh could not stimulate gdnfa expression in zebrafish testes.

The GDNF/Gdnf promoter region also contains several E- and N-boxes that allow the binding of basic helix–loop–helix proteins with potential repressor activity through Notch signaling [82]. Activation of the Notch receptor cleaves and releases the Notch intracellular domain which migrates to the nucleus to form a transcriptional complex with the DNA-binding protein RBPJ (recombining binding protein suppressor of hairless) [83,84]. The canonical targets of RBPJ include the HES and HEY families of transcriptional repressors, which are basic helix–loop–helix proteins [85,86,87]. Transcriptional repressors of the HES family (HES1–7) bind to N-box promoter regions of their target genes, while repressors from the HEY family (HEY1, HEY2 and HEYL) are associated with E-box promoter regions [86]. In zebrafish, it is known that Fsh stimulates Notch signaling [63]. Therefore, we speculate that Fsh nullified the Gdnf-increased gdnfa expression through the Notch pathway and transcription repressors HES and HEY, which would bind to E- and N-boxes within the zebrafish gdnfa promoter region. Functional studies of the gdnfa promoter region are required to elucidate how Fsh and Gdnf regulate the expression of gdnfa in zebrafish testes.

In this study, we demonstrated that gfrα1a transcripts were up-regulated by Fsh but not with the same intensity as observed in the Gdnf treatment (a three-fold increase as compared to Fsh). In immature rainbow trout, Bellaiche et al. [31] reported that gfra1a mRNA levels were increased following in vitro treatment with Fsh (100 ng/mL—the same concentration as was used in our work). Moreover, the same authors reported that testicular gfra1a levels increased towards the end of the reproductive cycle, which coincides with the natural elevation of plasma Fsh levels in rainbow trout [31]. Therefore, in contrast to mammalian species, in which Fsh up-regulated GDNF, we have evidence from two teleost species that Fsh modulates the Gdnf-Gfrα1 pathway through stimulating, not ligand, but receptor (gfra1a) mRNA levels. However, there are some questions that remain. The first concerns whether the Fsh-induced expression of gfra1a is mediated by Sertoli cells, germ cells or both. In this work, we have demonstrated that gfra1a is expressed by Sertoli and germ cells, while the Fsh receptor is exclusively expressed by somatic cells (Sertoli and Leydig cells) [55]. Therefore, if Fsh-induced gfra1a expression is mediated by germ cells, this

indicates that the regulation occurs indirectly through growth factors or androgens released by somatic cells (Sertoli and Leydig cells). Moreover, we cannot exclude that the increase in gfra1a could also be a consequence of the proliferation of spermatogonia or/and Sertoli cells stimulated by Fsh. More studies are necessary to address the nature of Fsh regulation on gfra1a expression in zebrafish testes. Although Gdnf or Fsh independently stimulated gfra1a mRNA levels in zebrafish testes, we observed that co-treatment affected negatively the Gdnf-induced expression of gfra1a. This is also noted for other genes such as pou5f3 or dazl, whose expressions were higher in the Gdnf treatment as compared to co-treatment with Fsh. For pou5f3, a stem cell marker, this observation suggested that Gdnf could be more involved in the maintenance of stemness than in increasing the number of stem cells in zebrafish testes. On the contrary, Fsh would be more associated with proliferation towards differentiation, as pou5f3 was significantly decreased following Fsh co-treatment. Therefore, our data indicate that the pro-differentiating effects of Fsh seemed to be more potent over the stem cell maintenance properties of Gdnf. On the other hand, at the level of differentiation, Gdnf decreased the Fsh effects on spermatogonial differentiation, as the expression of dazl, a marker of spermatogonial differentiation, was significantly down-regulated. Altogether, these observations suggest that Gdnf could promote stem cell maintenance through blocking spermatogonial differentiation. This conclusion is also supported by histomorphometrical data showing that Gdnf decreased the frequency of type B spermatogonia and accords with the higher expression of Gfrα1a in type A_{diff} spermatogonia.

As Gdnf is a member of the TGF-β superfamily, its role in inhibiting spermatogonial differentiation is likely consistent with other TGF-β superfamily members, such as Amh. Amh is a Sertoli cell growth factor which has been characterized as an inhibitor of spermatogonial differentiation in zebrafish [48,64,87] (see the review in Adolfi et al. [88]). In this regard, we also examined whether Gdnf's role could be modulated through Amh or by inhibiting Igf3, a pro-differentiation growth factor produced by Sertoli cells [48,54,78,79]. Our data showed that rhGDNF did not modulate either amh or igf3 mRNA levels in the zebrafish testicular explants. Therefore, Gdnf's role in inhibiting spermatogonial differentiation is not mediated by Amh or Igf3 and it could occur by acting directly on germ cells (autocrine) or indirectly through a different growth factor released by somatic cells (paracrine).

In summary, Figure 10 depicts our main findings regarding Gdnf actions in zebrafish testis. Gdnf is a germ cell growth factor that acts on type A spermatogonia and Sertoli cells in autocrine- and paracrine-dependent manners, respectively. The Gdnf receptor, named Gfrα1a, is expressed in type A spermatogonia (highly expressed in types A_{und} and A_{diff}) and Sertoli cells. The main

actions of Gdnf are: (1) the creation of new available niches by stimulating proliferation of both type A_{und} spermatogonia and their surrounding Sertoli cells. In this context, we highlight that Gdnf stimulates the proliferation of Sertoli cells, which are associated with type A_{und} undergoing mitosis. As a consequence, Gdnf increases the number of available niches and maintains the stemness pool in the zebrafish testes; (2) support of the development of differentiating spermatogonial cysts through proliferation of type A_{diff} and their surrounding Sertoli cells; and finally, (3) inhibition of late spermatogonial differentiation, as shown by the decrease in type B spermatogonia and down-regulation of dazl in the co-treatment with Fsh. Altogether, our data indicate that the autocrine and paracrine roles of Gdnf are evolutionary novelties in fish, although some paracrine functions are conserved, being similar to those observed for mammalian GDNF.

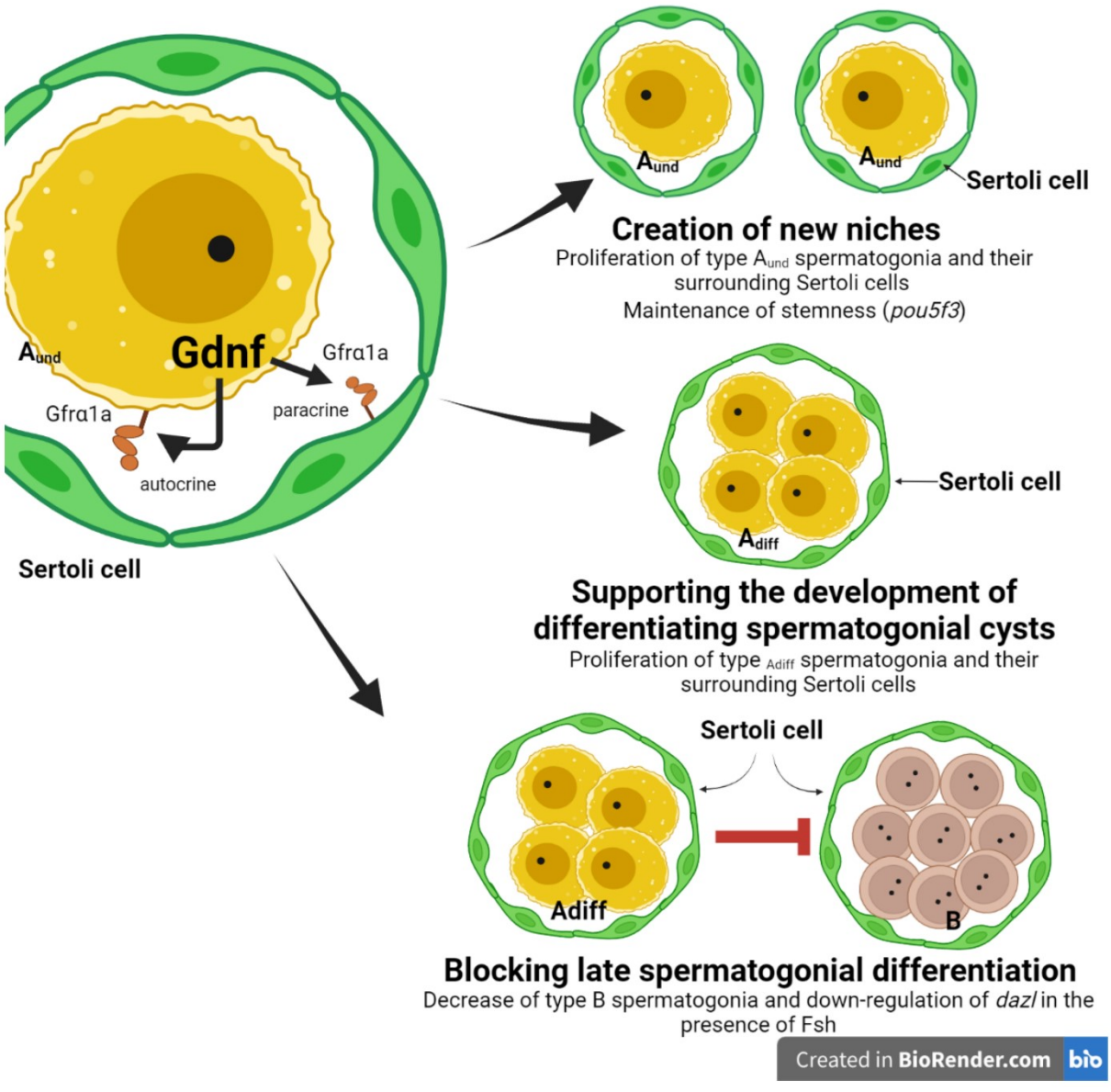


Figure 10. Summary of the effects of Gdnf in the zebrafish spermatogonial niche. Gdnf is a germ cell growth factor which acts on type A spermatogonia and their surrounding Sertoli cells in autocrine- and paracrine-dependent manners, respectively. The Gdnf receptor, named Gfrα1a, is expressed in type A spermatogonia (early spermatogonia, with higher expression in types A_{und} and A_{diff}) and Sertoli cells. The main actions of Gdnf are: (1) the creation of new available niches; (2) support of the development of early differentiating spermatogonial cysts; and (3) blocking of late spermatogonial differentiation.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/cells11081295/s1, Figure S1: Morphological characteristics of zebrafish germ cells and gdnfa in situ hybridization, Figure S2: Immunofluorescence control using either preadsorbed antibody or omitting the primary antibody, Figure S3: Predicted protein complex models between Danio rerio Gfrα1 and rhGNDF (hetero-2-2-mer)., Table S1: Parameters set to reconstruct the phylogeny tree., Table S2: Predicted regulatory binding sites of the GDNF promoter in Homo sapiens, Mus musculus and Danio rerio,Video S1: Interaction between rhGDNF and zebrafish Gfrα1a.

Author Contributions

Conception and design of experiments, R.H.N., L.B.D., A.J.B. and E.R.M.M.; performance of experiments, L.B.D., A.J.B., E.R.M.M., R.T.N., B.M.d.S., J.M.B.R., I.F.R., M.d.S.R., A.T.-N. and D.F.C.; data analysis, L.B.D., A.J.B., B.M.d.S. and R.T.N.; contribution of reagents/materials/analysis tools, R.H.N.; writing of the manuscript, L.B.D., G.M., C.S. and R.H.N. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal study protocol was approved by the National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research of São Paulo State University (Protocol n. 666-CEUA).

Informed Consent Statement

Not applicable.

Data Availability Statement

Data is contained within the article or supplementary material. The data presented in this study are available in this manuscript and supplemental material.

Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 2

Identification and characterization of long non-coding RNAs (lncRNAs) in zebrafish (*Danio rerio*) testis

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ABSTRACT

Non-coding RNAs are associated with the regulation of important biological processes, including spermatogenesis. Among them, long non-coding RNAs (lncRNAs) emerge as regulatory elements in the processes of self-renewal and differentiation of mammalian stem cells, such as neuronal, epidermal, cardiac, and hematopoietic stem cells. Moreover, lncRNAs are abundant in mammalian testis, and some of them are exclusively expressed in spermatogonia. However, the role of these transcripts in the regulation of testicular function and in the biology of spermatogonial stem cells (SSCs) is barely understood. Furthermore, information regarding lncRNAs in fish testis is scarce to date. Considering this background and the lack of knowledge about lncRNAs signaling in fish testis, this study aimed to unravel the modulation of lncRNAs under Fsh, a major endocrine player that regulates spermatogonial development through the targeting of Sertoli and Leydig cells, impacting sex steroid and growth factor production without the influence of androgens. Firstly, we identified 5161 novel lncRNAs and 76 differentially expressed genes DEGs, of which 46 were up and 30 were down-regulated. Further, GO and WEGO analysis of DEGs demonstrated that these transcripts are largely related to signal transduction and organismal systems, such as the endocrine system. Focusing on mRNA, we found 270 DEGs, with 174 being up and 96 being down-regulated. Interestingly, the most enriched pathways were related to thyroid hormone signaling, and steroidogenesis. Altogether, our data reveals a significant number of new lncRNAs and their relation with important pathways that modulate SSCs biology. Furthermore, important genes responsible for maintaining SSCs pluripotent state were modulated, indicating a probable activity of Fsh in creating a more favorable environment for the differentiation of early spermatogonia.

Key-words: fish, spermatogenesis, spermatogonia, ncRNAs, lncRNAs

1. Introduction

Long non-coding RNAs (lncRNAs) are RNA molecules longer than 200 nucleotides that lack protein-coding function (Mercer et al., 2009). LncRNAs are known to play important regulatory roles in many biological processes, such as stem cell differentiation and development. Although the molecular mechanisms are still not fully understood, some lncRNAs have been shown to regulate the transcription of key genes that trigger mammalian stem cell differentiation (Perry and Ulitsky, 2016), while others are involved in maintaining stem cells as undifferentiated cells in various mammalian systems (Kretz et al., 2012; Jiang et al., 2015; Deng et al., 2016). Some studies have identified a large number of lncRNAs specifically expressed in testis, suggesting that they play a key role in spermatogenesis and in the maintenance of fertility (Cabili et al., 2011; Hong et al., 2018; Wen et al., 2016). Furthermore, studies have suggested that dysregulation of non-coding RNAs, such as lncRNAs, are associated with male infertility (Wichman et al., 2017; Zhang et al., 2019). A previous study showed up-regulation of some key sperm/testis-specific/enriched lncRNAs from asthenozoospermic patients, suggesting the relevance of lncRNA in disrupted sperm motility (Zhang et al., 2019).

In fish species, several studies have described relevant roles for lncRNAs in fundamental biological processes such as growth and development in rainbow trout (Ali et al., 2018) and Japanese flounder (Wu et al., 2020); stress responses in the red cusk-eel (Dettlett et al., 2020), rainbow trout (Quan et al., 2020), and Atlantic salmon (Valenzuela-Muñoz et al., 2021); and immune responses in Japanese flounder (Xiu et al., 2021), grouper (He et al., 2022), miiuy croaker (Pan et al., 2022), snakehead fish (Zhang et al., 2023), and common carp (Liu et al., 2021). Nevertheless, the regulatory roles of lncRNAs in fish reproduction have not been extensively investigated. Efforts to identify and characterize lncRNAs involved in fish sex differentiation have been reported for the Chinese tongue sole (Feng, B et al., 2021), tNile tilapia (Zhong, H et a., 2022), and tiger pufferfish (Yan et al., 2021), and also during spermatogenesis for the Atlantic halibut (Yan, H et al., 2021), yellow catfish (Jing et al., 2014), and the Japanese flounder (Cheng et al., 2022). However, information about the relevance of lncRNAs in fish testis, specially in the regulation of spermatogonial stem cells (SSC), is quite sparse.

Song and collaborators (2019) have demonstrated that the knockdown of *igf3* (insulin-like growth factor 3) – a gene responsible for controlling reproduction (Li et al., 2021) and spermatogonial differentiation in fish (Nóbrega et al., 2015) – alters the expression of 124 lncRNAs in the common carp (*Cyprinus carpio*). *igf3* is a Sertoli cell-derived growth factor that is stimulated by the follicle stimulating hormone (Fsh), a major hormone responsible for the

maintenance of the spermatogenic process in mammals (Recchia et al., 2021; Khanehzad et al., 2021) and fish (Ramos-Júdez et al., 2022; Nóbrega et al., 2015, Sambroni et al., 2013). Therefore, here we aimed to understand the biological effects of rzFsh (recombinant zebrafish Fsh) on the modulation of lncRNAs in zebrafish adult testis using a deep transcriptome analysis, to identify new lncRNAs involved in fish spermatogenesis, as well as to propose molecular mechanisms by which the identified lncRNAs contribute to the formation and maturation of spermatozoa in zebrafish.

2. Materials and Methods

2.1 Animals

Adult male zebrafish were outbreed and raised in the aquarium facility of the Department of Structural and Functional Biology, Institute of Biosciences, São Paulo State University, Botucatu, São Paulo, Brazil. Fish were kept in 10-L tanks in a water recirculating system (28°C; pH 7.6) under a 14h:10h (light, dark) photoperiod. Salinity, pH, dissolved oxygen, and ammonia levels were monitored daily. Handling and experimentation were consistent with Brazilian legislation regulated by the National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 666-CEUA) of Sao Paulo State University, Campus Botucatu (Botucatu, Brazil).

2.2 *ex vivo* testis exposure

In order to investigate the effects of Fsh on lncRNAs in an androgen-independent manner, zebrafish testess (n = 8 per replica, three replicates in total) were exposed to recombinant zebrafish Fsh (rzf Fsh) (U-Protein Express B.V; Utrecht, the Netherlands) for 7 days in an *ex vivo* culture system. After dissecting out the testes, each testis (left or right) was placed on a nitrocellulose membrane (0.25 cm^2 area; 25 µm thickness; 0.22 µm porosity) on top of an agarose cylinder (1.5% w/v, Ringer's solution pH 7.4) surrounded with 1 mL of culture medium into a 24-well plate, as described by Leal et al. (2009). In this system, one testis was incubated in the presence of rzf Fsh (L-15 basal medium supplemented with 100 ng/mL rzf Fsh and 25 ng/mL trilostane), while its contra-lateral one was incubated with L-15 and 25 ng/mL trilostane to prevent the production of biologically active steroids (Nóbrega et al., 2015). The medium was changed every 3 days of culture. After 7 days, testes were collected for total RNA extraction.

2.3 Transcriptome analysis (RNA-Seq) of zebrafish testis

To prepare the Illumina RNAseq libraries, 2µg of total RNA from the control and Fsh exposed group (n = 5 pairs) were extracted with TRIzol® according to the manufacturer's information (Invitrogen, Carlsbad, CA, EUA). RNA integrity was evaluated using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The samples with RNA Integrity Number (RIN) ≥ 7 were subjected to further library construction. The libraries were obtained by sequencing on the Illumina HiSeq2500 sequencer system (Illumina, Inc.) with individual readings of 2x125 nucleotides.

The raw RNA-Seq data will be deposited in the NCBI GEO database, under accession numbers. The reads mapped to the reference genome (*Danio rerio*: GRCz11) with HISAT (Kim, D et al., 2015) were used to reconstruct the transcripts with StringTie (Pertea, M et al., 2015). To remove transcripts replicated in different samples, we compared and merged the reconstructed transcripts under different conditions using cuffcompare (Trapnell, C. et al 2010). The reconstructed transcripts were subjected to a series of filtering steps to identify the most reliable transcripts, following the methodology outlined by Prensner and colleagues (2021). Initially, transcripts with a total length less than 200 nucleotides were excluded. Subsequently, "background" transcripts were eliminated if their maximum expression value across all samples fell below the specified expression threshold (e.g., 2 FPKM), and if they were present in only one sample. Furthermore, transcripts overlapping with known mRNA and lncRNA on the correct strand were filtered out. To evaluate the protein-coding potential of the remaining transcripts, we employed three tools: Coding-Non-Coding Index (CNCI, Sun et al., 2013), Coding Potential Calculator (CPC, Kong et al., 2007), and Pfam (Mistry et al., 2020) for protein domain analysis. Finally, transcripts identified by all three lncRNA prediction software tools were categorized as novel lncRNA (see Figure 1).

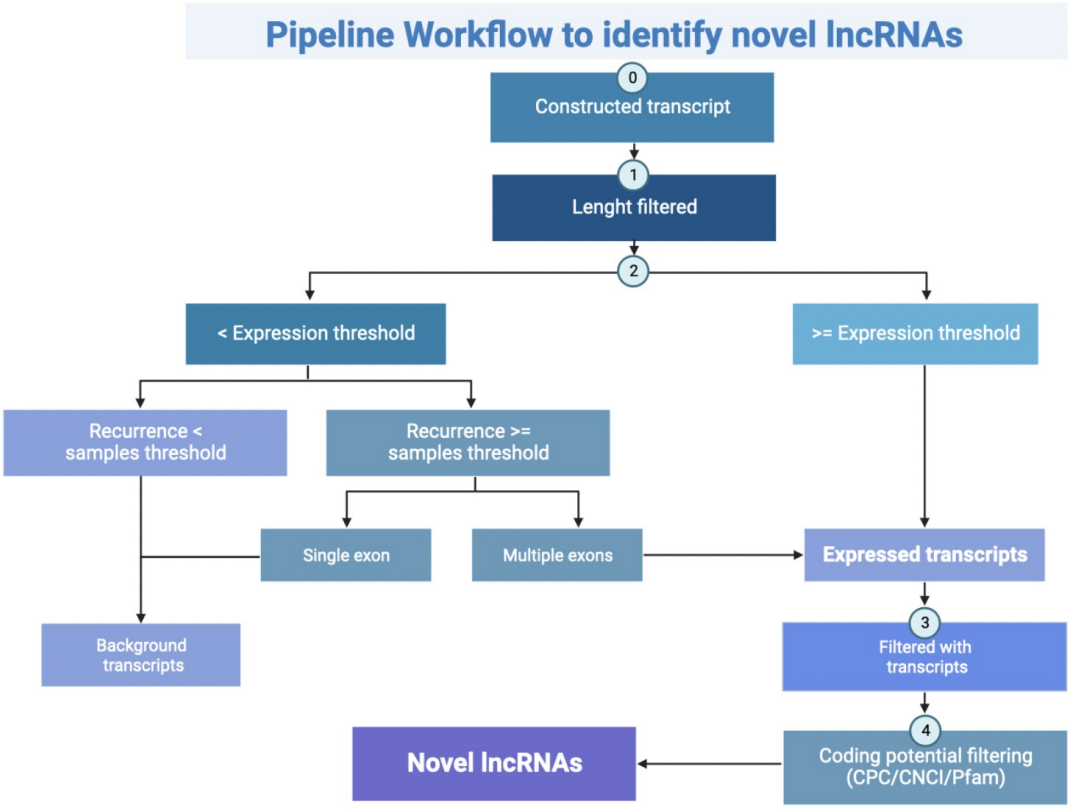


Figure 1. Pipeline implemented to identify novel long non-coding RNAs (lncRNAs) in this study. Firstly, it was excluded transcripts shorter than 200 nucleotides. Subsequently, we eliminated "background" transcripts that showed maximum expression levels below a pre-defined threshold (e.g. 2 FPKM) and were present in only one sample. Thirdly, we filtered out transcripts that overlapped with known messenger RNAs (mRNAs) and lncRNAs on the correct strand. Next, we assessed the protein-coding potential of the remaining transcripts using established tools, including the Coding-Non-Coding Index (CNCI), the Coding Potential Calculator (CPC), and Pfam for protein domain analysis. Lastly, we considered only those transcripts predicted as lncRNA by all of the lncRNA prediction software used in our study to classify them as novel lncRNAs.

2.4 Differential expression and functional enrichment analysis

The count Matrix was generated by HTSeq with the parameter *intersection_nonempty* methods (Anders et al., 2015). Transcript normalization, library expression analysis, and detection of differentially expressed genes (DEG) between the treated and control groups was performed in the R/Bioconductor environment using the DESeq2 method (Love et al., 2014). It was established the following conditions: genes with a log2 Fold Change > 0.5 or log2 Fold Change < −0.5 and adjusted p-value < 0.03 were considered as differentially expressed. Once the DEG for both mRNAs and lncRNAs were determined, they were analyzed from the functional point of view using the functional enrichment analysis of the genetic ontology terms (Gene Ontology - GO)

(Altschul et al., 1990). GO classifies genes based on their involvement in biological processes, molecular functions, and cellular components. To summarize the information obtained from functional enrichment we applied to use the WEGO analysis (Ye J et al., 2018) (platform available in https://wego.genomics.cn/) (visited in december, 2022) to visualize, compare, and plott GO annotation results.

2.5 mRNA/protein-lncRNA interaction prediction and network construction

We selected 34 DEGs from the mRNA pipeline (18 down and 13 up-regulated) and 5 genes (*pou5f3*, *nanos3*, *nanog, fshr*, and *amh*) mainly related to spermatogenesis, and all the lncRNA (novel lncRNA and lncRNA from ZFLNC, accessed 03/23/2023 - Hu, X et al., 2018).

To predict the interaction between mRNA and lncRNA it was used LncTar (Li, J et al., 2015) with default parameters, and considered interaction when ndG < -0.1. Additionally, it was retrieved the amino acid sequence of the selected DEG from BIOMART (Smedley, D et al., 2009), and predicted the interaction between protein and lncRNA using the predictors RPICool (Akbaripour-Elahabad, M et al., 2016) and RPIseq (Mupiralla et al., 2011) with default parameters, considering positive interaction with probability > 0.5.

For the differently expressed lncRNA, the same methodology was performed, however using the zebrafish cds, and peptide sequences allocated in the Ensembl database (GRCz11, accessed 05/17/2023).

The network interaction of mRNA/protein-lncRNA was constructed from both interaction predictions (mRNA-lncRNA and protein-lncRNA) to visualize the possible regulation of genes related to germinative cells and lncRNA. The sub networks for specific genes were extracted and visualized using CytoScape (Shannon et al., 2003). Additionally, the selected genes and lncRNAs were mapped into the genome of *D. rerio* (GRCz11, accessed 05/17/2023) with gmap mapping (parameters -k 15, -B 5, - -no-chimeras, - -nofails, - -min-trimmed-coverage, and - -min-identity 0.80) (Wu & Watanabe., 2009). We used IGV viewer (James et al., 2005) to visualize the position of the lncRNAs in the genome and design primers for the obtained validation.

2.6 Quantitative real-time PCR analysis

It was validated the RNA-Seq results examining 19 DEG (11 genes and 8 lncRNA) using quantitative real-time PCR (RT-qPCR). Total RNA was obtained from control testis (n = 5, 3 replicates) and Fsh exposed testis (n = 5, 3 replicates) using TRizol® reagent (Invitrogen,

Carlsbad, CA, EUA), and cDNA was synthesized for all samples using reverse transcriptase according to the manufacturer's protocols (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR reactions were carried out for biological replicates with three technical replicates each. The number of amplification cycles (Ct-cycle threshold) were determined through a StepOnePlus™ Real-Time PCR System (Thermo fisher, Waltham, MA, USA, EUA). The mRNA levels of the targets (Cts) were normalized by the reference gene β-actin, expressed as relative values of control group (as fold induction), according to the 2−(ΔΔCT) method. Primers (Table 1) were designed based on zebrafish sequences available from the Genbank database.

Target Genes	Primer Sequences (5′–3′)	References
b-actin	AGACATCAGGGAGTGATGGT (F)	Tovo-Neto et al.
	CAATACCGTGCTCAATGGGG (R)	
igf3	TGTGCGGAGACAGAGGCTTT (F)	Morais et al
	CGCCGCACTTTCTTGGATT (R)	
bmp16	CACCCCAACCCCAGTACAAA (F)	This paper
	TGGGTCCTCGATGAGATGGT (R)	
dmrt1	TGCCCAGGTGGCGTTACGG (F)	Griffin et al.
	CGGGTGATGGCGGTCCTGAG (R)	
inhbab	CCTCAAAGTAGCCAAGGGCA (F)	This paper
	TGGTCCTCTGGTCTATCGGT (R)	
cyp17a1	GGGAGGCCACGGACTGTTA (F)	Morais et al
	CCATGTGGAACTGTAGTCAGCAA (R)	
gpx3	GAGAGCAGGCCCTTTTCACA (F)	This paper
	TTGACTTTCAGCGGTTCCCA (R)	
il34	AACCTCCATACATCCACCCG (F)	This paper
	CCTCCATAAAAGCACACGCAC (R)	
ccl25b	TTCCCGCTGTTGTTTTCAAGA (F)	This paper
	GCTCCTTTATTATCGCCTGGAC (R)	

camk1gb	GCGCACTTGCCTCTAGAAAT (F)	This paper
	TCCTGGATGTTATCCGTGCT (R)	
fkbp5	CGCCGGTGAGACTAAACAGA (F)	This paper
	ACATGCCCTTGTTCCCAAAA (R)	
nanos1	CGTCCGCAGGCTATTCTTCT (F)	This paper
	AGAGAGGCACCCATAGGACT (R)	
T162	GCAGCAACAGGAGAAGAGATG (F)	This paper
	TTCCCAGCTTGGTCTTTCTACC (R)	
T350	ACACATATCGAGACGGACTGC (F)	This paper
	ACGTGCTGATGGTCTTTGGA (R)	
T111	ACTGCCAAGAATTTGAGTGTGT (F)	This paper
	TTGTTTGGTCGCCACATGGA (R)	
T572	TGTGGGCTGGACTTAGGAAG (F)	This paper
	AGGGCTGGTGCACTGTAATC (R)	
T738	GAGGGGGTCCATTAGAAGCC (F)	This paper
	CGTAGATACTGGTGCAGGGG (R)	
C487	AGGCTTTTTGGAGACTGAAGGA (F)	This paper
	TGTATTCAGGGCATCCAGCC (R)	
C207	TGGCAGTGTCTTAGCTGGTT (F)	This paper
	GACTGGATGGATGAATGGGCT (R)	
C963	ACGACGACAAGAAAAGCTGAGA (F)	This paper
	GTATGCTGGGATTTCTGTTGCC (R)	

Table 1. Primers used for the gene expression analysis (RT-qPCR) of selected mRNA and lncRNA for validation of RNA-Seq results.

2.7 Statistical analysis

Data were initially checked for deviations from the normal distribution and the homogeneity of variances through the Shapiro–Wilk and Bartlett's tests, respectively. Results

are expressed as means ± standard deviation (SD). Data were analyzed using a paired Student's t-test. p-values ≤ 0.05 were considered statistically significant (*p <0.05, ** p <0.01, *** p <0.001).

3. Results

3.1 Transcriptome data analysis

RNA sequencing of 10 samples (5 paired-testes incubated with 100 ng/mLFsh + 25 ng/mL trilostane and their contra-lateral ones incubated with 25 ng/mL) generated 566.518,788 raw reads. After filtering, approximately 85% of the mapping ratio of the reads on the zebrafish genome were used to assemble and generate 668.672 transcripts with more than 200 bp (Figure 2A). From these, 12.444 transcripts overlap with known mRNA and lncRNA (Figure 2A). Then it was used for a prediction pipeline and quantification of mRNA, and another for lncRNA.

The long-non-coding pipeline identified 2,069 known and 5.161 novel lncRNA (Figure 2B). Annotated lncRNA found were classified in 4 types according to the Ensembl database. 655 antisense lncRNA, 1394 lincRNA, 52 sense intronic and 936 processed transcripts. However novel lncRNA were classified into 5 classes (Figure 2C), i: transfrag falling entirely within a reference intron (2.223); j: potentially novel isoform (476); s: intron of the transfrag overlaps a reference intron on the opposite strand (6); x: exonic overlap with reference on the opposite strand (1.204); and u: intergenic transcript (1.252) (Figure 2C).

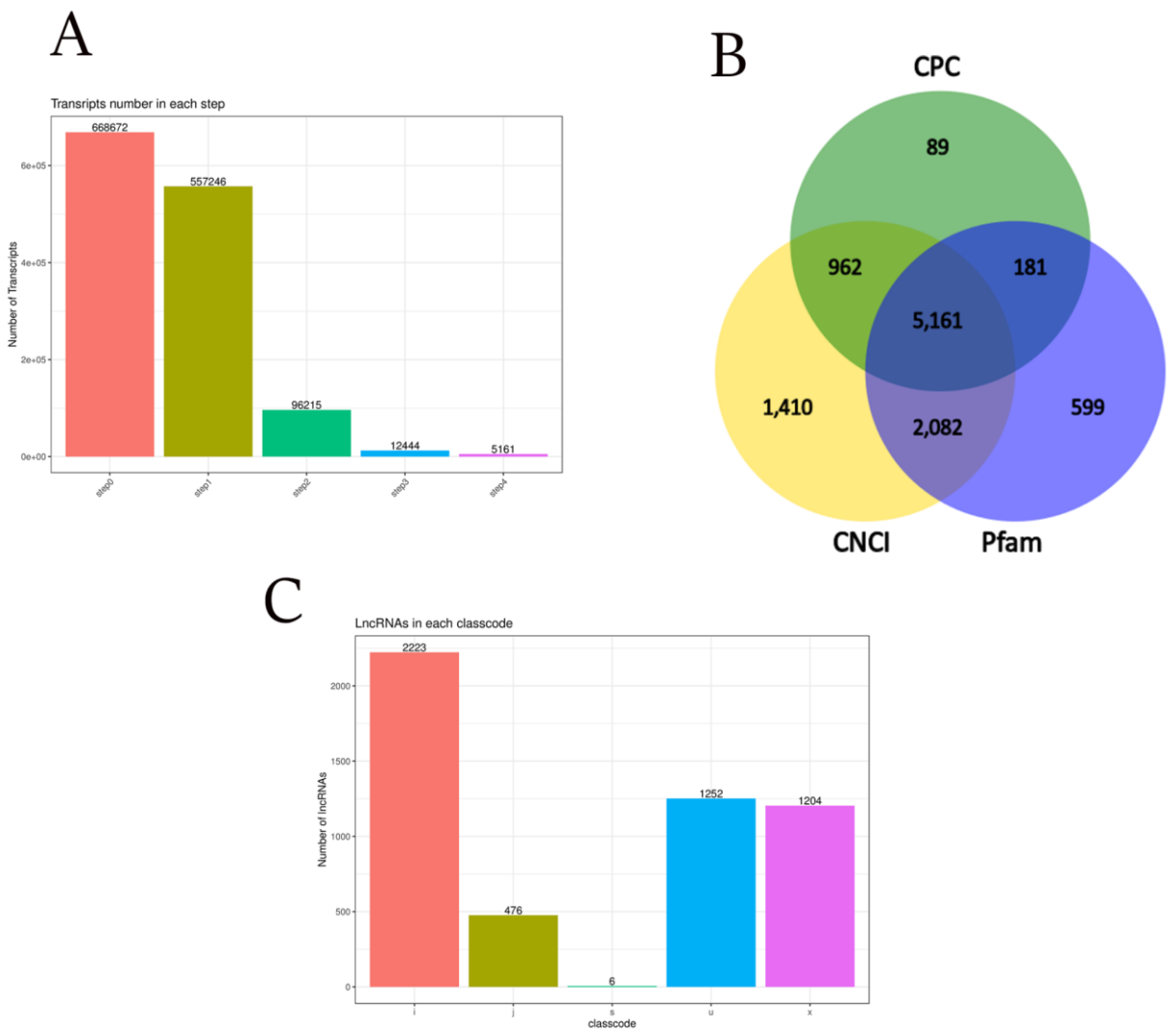


Figure 2. Overview of the transcripts identified by the novel lncRNA prediction pipeline. **a)** number of transcripts in each filtering step. X axis represents 4 filtering steps (step 0 depicts all the assembled transcripts), Y axis represents transcript number. **b)** Venn diagram representing the number of transcripts in the prediction of novel lncRNA according to CNCI, CPC and Pfam for protein domain analysis. **c)** Bar graph representing the number of lncRNA in each novel lncRNA class code type (class *i*: transfrag falling entirely within a reference intron; class *j*: potentially novel isoform; class *s*: an intron of the transfrag overlaps a reference intron on the opposite strand; class *u*: intergenic transcript; class *x*: exonic overlap with reference on the opposite strand.). Y axis depicts the corresponding lncRNA number.

3.2 Differential expression and functional enrichment analysis

3.2.1 lncRNA: DEG, GO, and WEGO analyzes

Expression density distribution showed the expression profile of each sample that reflects the low variation in the expression of genes (Supp Figure 1A). It was also observed the clustering of libraries separating control and treatment according to their expression profile (Supp Figure

1B). When compared different expressed lncRNAs between control (trilostane) and Fsh + trilostane groups, we found 76 differentially expressed transcripts, of which 46 were up and 30 were down-regulated (Figure 3A and Supp Table 1: C-vs-T_lncRNA). To gain insight into this set of DEG, we conducted the GO (Gene Ontology) analysis for the transcripts, which classifies genes based on their involvement in biological processes, molecular functions, and cellular components, and used the WEGO (Web Gene Ontology Annotation Plot) tool to visualize these results.

From this analysis, we obtained a comprehensive list of terms and selected the top ten terms from each category that contained the highest number of genes. The enrichment analysis of up-regulated DEG in the Fsh + trilostane group compared to the control (trilostane) showed that proteolysis and other cellular components, such as membrane, cytoplasm, and nucleus, were the most significantly enriched terms (Figure 3B). WEGO analysis identified that the most enriched categories include "transcription signaling" and "membrane modulation" (Figure 3C).

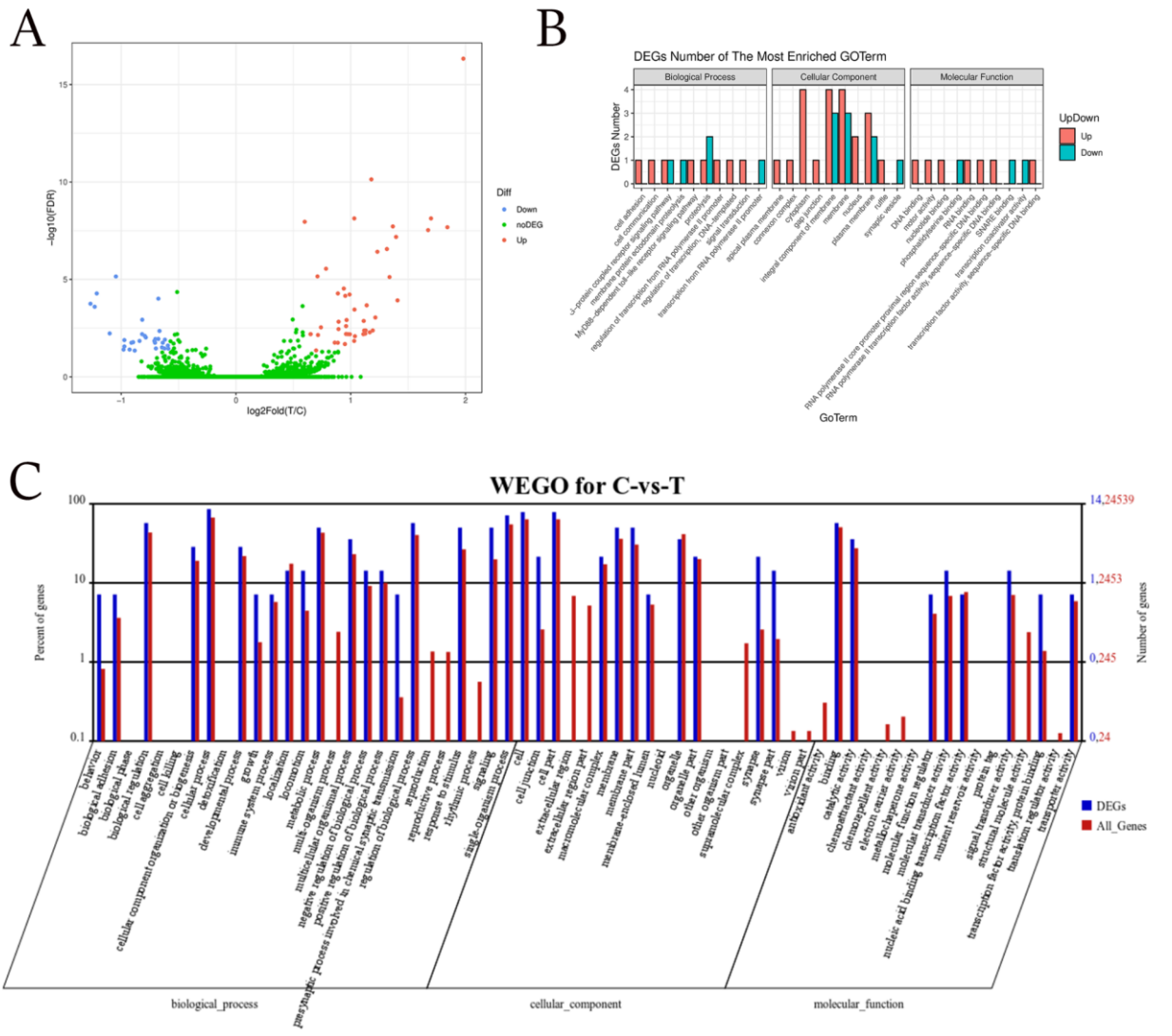


Figure 3. Differentially expressed lncRNA and GO terms enriched after Fsh treatment. **a)** Volcano plot of differentially expressed lncRNA. X axis represents different compared conditions, Y axis represents differentially expressed lncRNA number, red color indicates up-regulated genes, and blue color indicates down-regulated genes. **b)** Distribution of Gene Ontology categories for the set of differentially expressed lncRNA after Fsh treatment. **c)** WEGO plot shows the categories of biological processes, molecular functions, and cellular components with their respective percentages of associated genes. Data were analyzed using Gene Ontology software and plotted with WEGO.

3.2.2 mRNA: DEG, GO, and WEGO analysis

With respect to mRNA, Fsh + trilostane modulates 270 genes, 174 up and 96 down-regulated (Figure 4a and Supp table 2). Among the up-regulated genes it was observed insl3 and igf3, that were known to be responsive to Fsh treatment (Nóbrega et al., 2015; Morais et al., 2017; Crespo et al, 2018) (Supp table 1: C-vs-T_mRNA). Interesting genes from steroidogenic pathways, such as *cyp17a1*, *cyp11c1*, *hsd3b1, hsd11b2*, and *star* were up-regulated (Table 2). Also, both

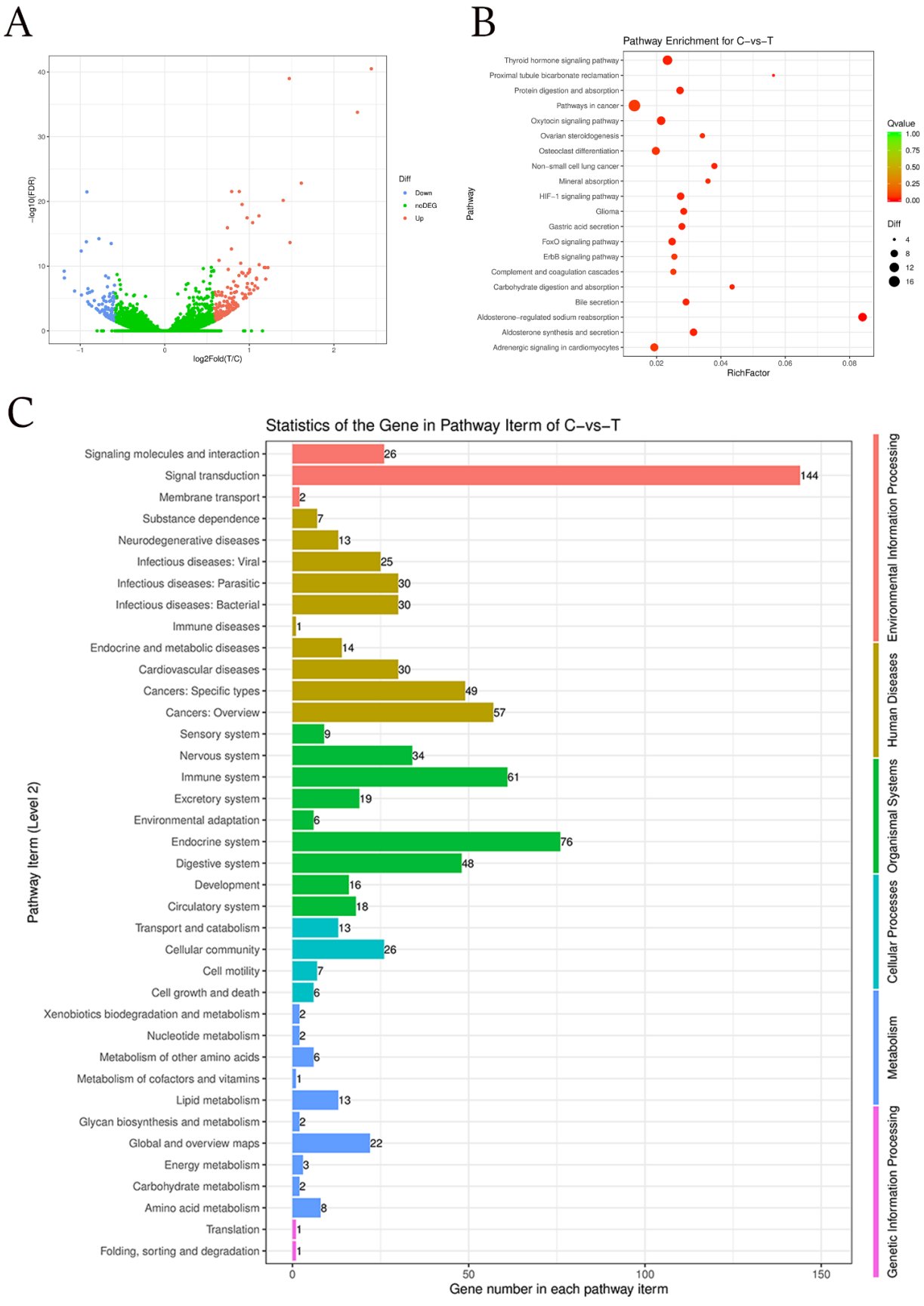
germ and Sertoli cell-specific (Webster et al., 2017) genes such as *dmrt1*, *dmrt2a*, *dmrt3a* and *igf3* (Moraes et al., 2017) (Sertoli cells), and the Tgf-β signaling genes *bmp7b*, *bmp16*, and *inhbab* were also up-regulated in the presence of Fsh + trilostane (Table 2). The Leydig cell-specific gene *insl3* was also up-regulated, as well as the thyroid pathway genes *cth* and *cthr* (Table 2). Moreover, the spermatogonia stem cell marker *nanos1* was down-regulated in four samples when compared individually (Table 2).

Table 2. Genes related to spermatogenesis modulated by Fsh + trilostane.

	genes	condition
Steroidogenic pathway	*cyp17a1*; *cyp11c1*; *hsd3b1*; *hsd11b2; star*	up
Thyroid pathway	*cth; cthr*	up
Sertoli cell/Germ cell	*igf3*; *dmrt1*; *dmrt2a*; *dmrt3a*	up
Leydig cell	*insl3; star*	up
Tgf-□ signaling	*bmp7b*; *bmp16; inhbab*	up
Spermatogonia stem cell	*nanos1*	down*

*comparison of individual samples: testis pair - left *vs*. right (see methods).

The enrichment analysis of up-regulated genes (Figure 4A) showed that biological processes related to environmental information management – such as signal transduction – and to organismal systems – such as the immune system and the endocrine system – are the most enriched processes in the treated group compared to control (Figure 4C). As expected, it was observed the enrichment of the thyroid hormone signaling and the thyroid hormone synthesis pathways as a result of Fsh treatment (Figure 4B).



1823

1824 **Figure 4**. Differentially expressed mRNA and enriched pathways after Fsh treatment. **a)** Volcano plot of

differentially expressed mRNA. X axis represents different comparison conditions, Y axis represents DEG number, red color indicates up-regulated genes, and blue color indicates down-regulated genes. b) Statistics of the Gene in Pathway Iterm of C−vs−T DEGs. c) Pathways enrichment. Y axis represents pathway term, X axis represents Rich factor. The dots sizes represent the number of DEG: the bigger the dots, the more DEG. Different colors represent different q-values.

3.3 mRNA/protein-lncRNA interaction prediction and network construction

It was obtained 49, 106, and 46 interactions between mRNA or protein and lncRNA according to the predictors LncTar, RPICool, and RPIseq, respectively (Table 3). Network was constructed removing the redundancy and retrieved 133 interactions (Supp Table 2). We extracted the sub-networks selecting genes of interest and the first neighbor of each of them (Table 3, Figure 5).

Table 3. Number of predicted interactions between mRNA or proteins and lncRNA.

tools	n. of interactions (up)*	n. of interactions (down)
LncTar	23	26
RPICool	13	93
RPIseq	08	38

* *pou5f3* and *amh* genes were grouped together with up-regulated genes.
tools: tools used to predict the interactions between mRNA and lncRNA, and between proteins and lncRNA, **n. of interactions (up)**: number of predicted interactions of up-regulated genes and lncRNA, **n. of interactions (down)**: number of predicted interactions of down-regulated genes and lncRNA.

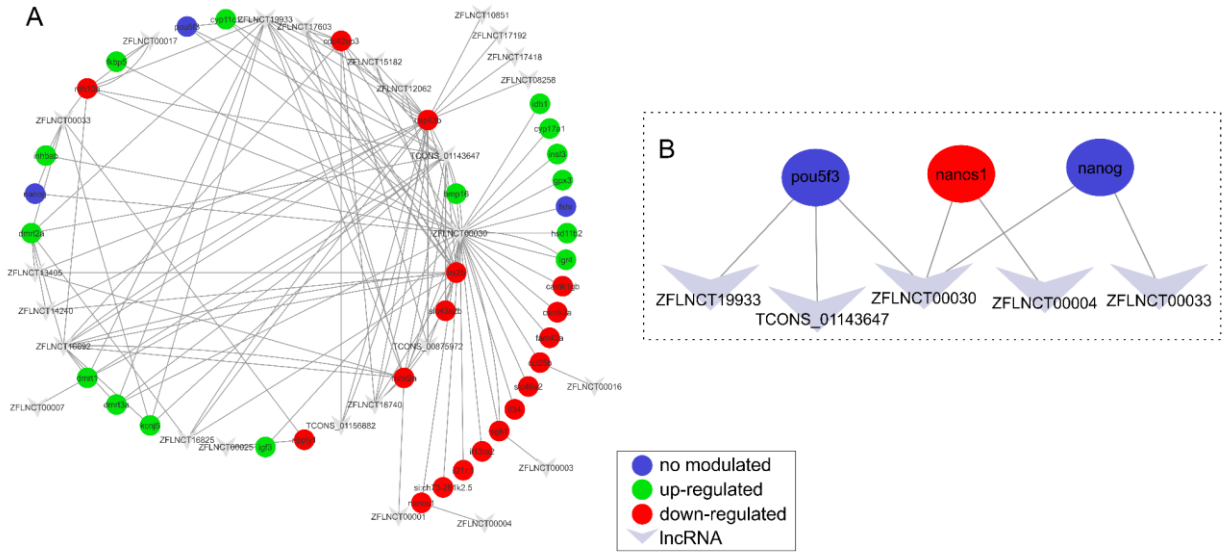


Figure 5. Interaction of spermatogenesis-related genes and lncRNA. Subnetworks extracted according to *pou5f3*,

nanog, and *nanos1* and their first neighbor, using CytoScape.

3.4 RNA-Seq results validation

To corroborate RNA-Seq quality and validate the reliability of genes and lncRNAs identified to be differentially expressed under Fsh + trilostane treatment, 19 differentially expressed transcripts (11 DEG and 8 lncRNA) were selected (specific and randomily) and their expression were evaluated by RT-qPCR using total RNA from zebrafish testes exposed in trilostane in the presence or absence of rzf Fsh (Figures 14 and 15). Fold changes from RT-qPCR analyzes were then compared with the RNA-Seq expression profiles. The same trend in expression levels of the selected genes and lncRNAs from the RNA-Seq was observed in the validation assays using RT-qPCR. For example, *igf3, dmrt1*, *inhbab*, *cyp17a1*, and *gpx3* were up-regulated, while *il34, ccl25b*, *camk1gb*, and *nanos1* were down-regulated following Fsh + trilostane (Figure 14). Moreover, the analysis for the differentially expressed lncRNA showed that T111, T572, T738 were up-regulated, while C487, C207, and C963 were down-regulated, regardless of the methodology. Additionally, the lncRNAs T162 and T350 were not differentially modulated neither in the RNA-Seq dataset, nor in RT-qPCR results. These results indicate that our dataset from RNA-Seq analysis for both mRNA and lncRNA is robust.

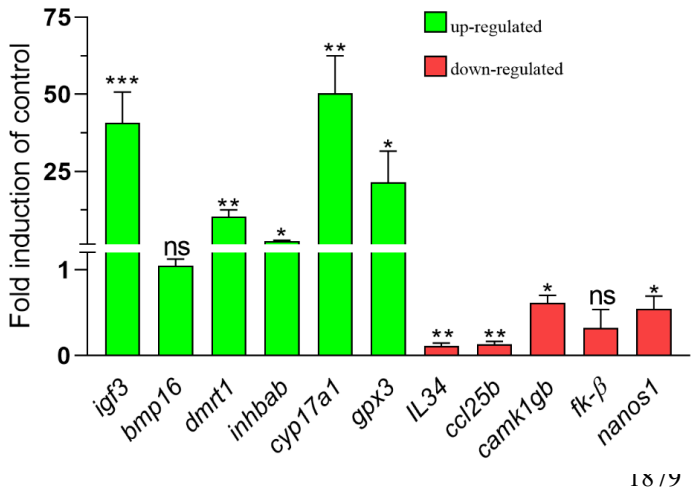


Figure 6. Expression analysis (RT-qPCR) for 11 mRNA DEGs. *igf3*: insulin-like growth factor 3; *bmp16*: bone morphogenetic protein 16; *dmrt1*: doublesex and mab-3 related transcription factor 1; *inhbab*: inhibin subunit beta a; *cyp17a1:* cytochrome P450 family 17 subfamily A member; *gpx3*: glutathione peroxidase 3; *il34*: interleukin 34; *ccl25b*: chemokine (C-C motif) ligand 25b; *camk1gb*: calcium/calmodulin-dependent protein kinase ig; *fk-β*: fkbp prolyl isomerase 5; *nanos1*: nanos c2hc-type zinc finger 1. The * indicates statistically significant differences between transcript abundance (T-test; $p < 0.05$). Bars represent mean ± SEM fold change (n = 8) relative to control, which is set at 1, NS: not significant.

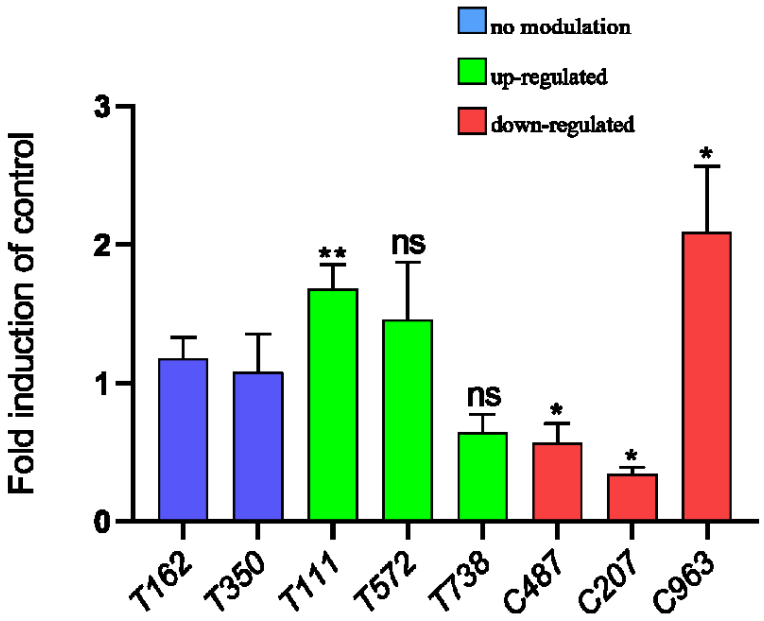


Figure 7. Expression analysis (RT-qPCR) for 6 differentially expressed and 2 not modulated lncRNA. The * indicates statistically significant differences between transcript abundances (T-test; $p < 0.05$). Bars represent mean ± SEM fold change (n = 8) relative to control, which is set at 1, NS: not significant.

3.4 Fsh modulates no-DEG antisense lncRNA CT025854

In this study, it was observed that Fsh modulates a no-DEG lncRNA named CT025854, which is located on the opposite strand of *igf3*, and may act as antisense regulator of this gene.

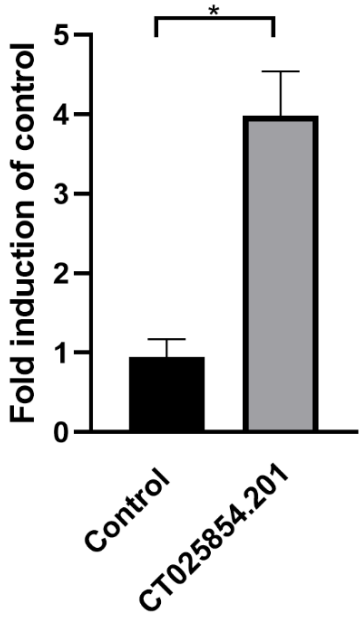


Figure 8. Expression analysis of the lncRNA isoform CT025854.201 in the absence (control) or in the presence of rzfFsh (100 ng/mL) for 7 days (RT-qPCR). The * indicates statistically significant differences between the transcript abundances (T-test; $p < 0.05$). Bars represent the mean ± SEM fold change (n = 8) relative to control.

4. Discussion

This study demonstrated the involvement of the Fsh signaling pathway in the modulation of lncRNA in adult zebrafish testis. Although lncRNA are key regulators of numerous biological processes (Mattick et al., 2023), a systematic identification of lncRNA involved in adult zebrafish testis is currently lacking. Therefore, in this study, we aimed to identify and characterize lncRNA in zebrafish testis and explore their potential roles in regulating zebrafish spermatogenesis through a germ cell perspective. Our analysis identified 76 lncRNA differentially expressed, of which 46 were up and 30 were down-regulated (Figure 3). Moreover, we also identified 5,161 novel lncRNAs specifically expressed in adult zebrafish testis (Figure 2, Supp Table 1) and categorized these transcripts into five models: transfrags falling entirely within reference introns, potentially novel isoforms, introns of the transfrags overlapping reference introns on the opposite strand, intergenic transcripts, and exonic overlapping with the reference on the opposite strand (Figure 2). Differentially expressed mRNA and enriched pathways of transcripts were also analysed to corroborate our data (Figure 4). We also predicted interactions between the differentially expressed mRNA or proteins and lncRNA (Figure 5), providing insights into the regulatory networks involved in Fsh-induced spermatogenesis. Finally, we analyzed the expression profiles of specific transcripts through RT-qPCR, which corroborated the expression patterns seen on RNA-Seq data (6-7), thus validating our transcriptome results.

A total of 7,230 lncRNA (2,069 annotated and 5,161 novel), and 24,719 protein-coding transcripts were identified in adult zebrafish testes (Supp Figure 1). Cabili et al. (2011) suggest that human large intergenic noncoding RNAs (lincRNA) display a greater degree of tissue specificity when compared to mRNAs, and approximately one-third of the 8,000 human lincRNA are specifically expressed in the testis. Here, we observed that lncRNA have shorter open reading frames (ORF) lengths and fewer exons than mRNA. On the other hand, mRNA has a higher number of transcripts and genes compared to lncRNAs (Supp Figure 2). These findings highlight distinct features of lncRNA and mRNA in terms of their coding potential and structural complexity, suggesting potential functional divergence between the two RNA classes.

In mammals, due to the abundance of lncRNA in the testis, it is believed that they play fundamental roles in the spermatogenic process and, consequently, in the maintenance of fertility (Wichman et al., 2017). Certain lncRNA have a significant role in the development of male germ cells, such as Spga-lncRNA1 and Spga-lncRNA2, two spermatogonia-specific lncRNA essential

for preserving SSC stemness (Luk et al., 2014). Additionally, lncRNA-033862 has been identified as a molecular marker for SSC maintenance (Bie et al., 2018; Robles et al., 2019). This lncRNA is highly expressed in mouse SSC and is regulated by GDNF (Glial cell line-derived neurotrophic factor) signaling, and it can potentially regulate the self-renewal, survival, and maintenance of SSC (Hu et al., 2017). Doretto et al. (2022) showed that, in zebrafish, a germ cell derived facter, gdnfa, was able to promote the proliferation of undifferentiated spermatogonial cysts. In our prediction analysis (Figure 5), a new lncRNA named TCONS_01143647 directly interacted with *pou5f3* transcript also following Fsh treatment. Despicted the fact that we need further validation, the new lncRNA may be involved in the modulation of *pou5f3* expression, and consequently, promoting the progression of spermatogenesis. Both results corroborate with the idea that Fsh may act directly in germ cells by modulating lncRNAs expression thus, forming new differentiated cysts.

Here, Fsh was also able to modulate coding genes related to cell pluripotency maintenance, such as *nanos1*, as well as the no-DEG lncRNA CT025854 (CT025854.1-201 - ensembl.org/Danio_rerio accessed on 27/03/2023) (Figure 8A), present on the opposite strand of *igf3* (Figure 8B). It is well known that Fsh, through *igf3* (Nóbrega et al., 2015), plays a crucial role in regulating the functions of Sertoli cells, and thereby modulating the fate of SSC (Recchia et al., 2021). In the initial stages of spermatogenesis in fish, Fsh regulates both Leydig and Sertoli cells activities, making it the main gonadotropin driving these stages of spermatogenesis in fish, according to previous studies (Garcia-López et., 2010; Gomez et al., 1998; Campbell et al., 2003; Ohta et al., 2007; Chauvigne et al., 2012). In zebrafish, in a cAMP-dependent manner, Fsh stimulates the production of *igf3* in Sertoli cells. In turn, *igf3* stimulates the proliferation and differentiation of spermatogonia and their entry into meiosis via Igf receptor signaling, independent of androgen release, in the adult zebrafish testis (Nóbrega et a., 2015). These findings suggest that androgens have a moderate stimulatory effect on *igf3* transcript levels, although Fsh could still effectively stimulate *igf3* transcript levels when androgen production was blocked (Nóbrega et al., 2015). Based on the evidence presented, it is possible that the lncRNA CT025854, modulated by Fsh (Figure 8B), could influence the *igf3* expression and consequently, the SSC fate. Prediction analysis demonstrated that *igf3* transcript is also able to interact with two know lncRNAs, ZFLNCT00025 and ZFLNCT00030 (Figure 5, Supp Table 2). These results highlight the potential of non-coding RNAs, specifically antisense and trans-acting lncRNAs, in the modulation of gene expression in the testis. In this sense, further research is needed to elucidate the precise mechanism by which those lncRNAs affects SSC fate.

During evolution, each of the three *Nanos* paralogues (*Nanos1*, *Nanos2*, and *Nanos3*) present in mammals acquired a different expression pattern and a distinct role in germline development (Haraguchi et al., 2003; Jaruzelska et al., 2003, Tsuda et al., 2003). In fact, when we analyzed the enrichment of functional-related terms for differentially expressed mRNAs, it was observed that the signal transduction pathway (Figure 4), where *nanos1* is present, is one of the most enriched pathways. As far as we know, this work is the first one to show the modulation of *nanos1* by Fsh. In humans, *NANOS1* has important roles in mRNA translation within the chromatoid body and in apoptosis repression in germ cells (Ginter-Matuszewska et al., 2011; Janecki et al., 2020). In a recent study that used single-cell RNA sequencing (scRNA-seq), Li et al. (2023) identified a high expression of *nanos3* in primordial germ cells (PGC), which give rise to gonocytes and, ultimately, to SSC. Therefore, *nanos3* is a notable molecular marker for early cells. In the same article, the authors show that this gene is capable of interacting with a testis-specific lncRNA in zebrafish.

To deeply explore the biological functions of lncRNA and mRNA in zebrafish testis, GO and WEGO analyses were performed for target transcripts of differentially expressed lncRNA and mRNA. Through these analyses, we found various candidate genes – some of which have been demonstrated to be involved with male reproduction. Genes from the steroidogenic pathway, such as *cyp17a1*, *cyp11c1*, *hsd3b1, hsd11b2*, and *star* were up-regulated. Also, the Sertoli cell/Germ cell genes *igf3*, *dmrt1*, *dmrt2a*, and *dmrt3a*, as well as the Tgf-β signaling genes *bmp7b*, *bmp16*, and *inhbab* were also up-regulated in the presence of Fsh without androgen stimuli (Table 2). The Leydig cell gene *insl3* was also up-regulated as well as the thyroid pathway. Surprisingly, the thyroid hormone signaling pathway was the most enriched by Fsh (Figure 4B). In fact, Rodrigues and collaborators (2022) showed that the isolated testis from methimazole-induced hypothyroidism zebrafish was completely unresponsive to Fsh. However, the co-treatment with T4 restored the Fsh-induced response by increasing the 11-Ketotestosterone (11-KT) concentration to the levels observed following treatment of the control group with Fsh alone. It shows the Fsh dependency from T3 to restore 11-KT plasma levels to normality and the crosstalk between the Hypothalamic-Pituitary-Thyroid and the Gonadal Axes in zebrafish.

In this study, we also show the modulation of *dmrt1*, *dmrt2a*, and *dmrt3a* genes. Among these genes, *dmrt1* have been reported to be involved in gonad development, testis sex determination, and testis differentiation in several fish species (Herpin & Schartl, 2011, Kopp et al., 2012), including medaka (Nanda et al., 2002, Hattori et al., 2007, Martinez-Bengochea et al., 2022), Southern catfish (Zheng S et al., 2022) and African scat (Peng et al., 2023). Additionally,

dmrt1 expression was also detected in rainbow trouts (Marchand et al., 2000) as well as in Siberian sturgeons during early and advanced stages of gonadal development (Berbejillo et al., 2012).

Moreover, the *dmrt2* gene has very important roles in embryonic development and also in testicular development, and sex reversal (Kim et al., 2003; Yoshizawa et al., 2011; Zhou et al., 2008). For example, in zebrafish, *dmrt2a* contributes to the regulation of the left-right patterning of the mesoderm (Lourenço et al., 2011), while *dmrt2b* regulates somite differentiation (Zhou et al., 2008). Moreover, Feng and collaborators (2021) showed by prediction analisys that the lncRNA DMRT2-AS can directly regulate *dmrt2* in the Chinese tongue sole, and this lncRNA plays an important role in the male gonadal differentiation. However, in zebrafish, the importance of Fsh for the modulation of the *dmrt* gene family is still unclear. Our prediction analysis also demonstrated that some lncRNAs can interact with genes from the *dmrt* family (Figure 5, Supp Table 2). Further, we found 5 lncRNAs that possibly interact with germ cell line pluripotency genes, *pou5f3, nanos1* and *nanog* (Figure 5). Also, genes from the steroidogenic pathway like *cyp17a1, cyp11c1* and *insl3* interact with know and novel lncRNAs (Figure 5, Supp Table 2). It is know that lncRNAs may act in these pathway, like the lncRNA *Start* (*Steroidogenesis activating lncRNA in testis*), which is a probably regulator of steroidogenesis in mouse Leydig cells (Otsuka, K et al., 2021. In fish, steroidogenic modulation by ncRNAs is also demonstrated (Cheng, J et al., 2022).

In conclusion, this study provided the first high coverage sequencing analysis of mRNA and lncRNA expression profiles in adult zebrafish testis under rzfFsh in an androgen-independent manner and that probably, these lncRNAs have the potencial to directly interact with spermatogonia stem cells. Also, the list of lncRNAs generated in our study is a valuable resource for understanding their regulatory roles in zebrafish testes development and spermatogenesis. In addition, the useful insights could be provided in further zebrafish reproductive performance regulation research involving these lncRNAs and genes from germinative stem cell niche.

5. References

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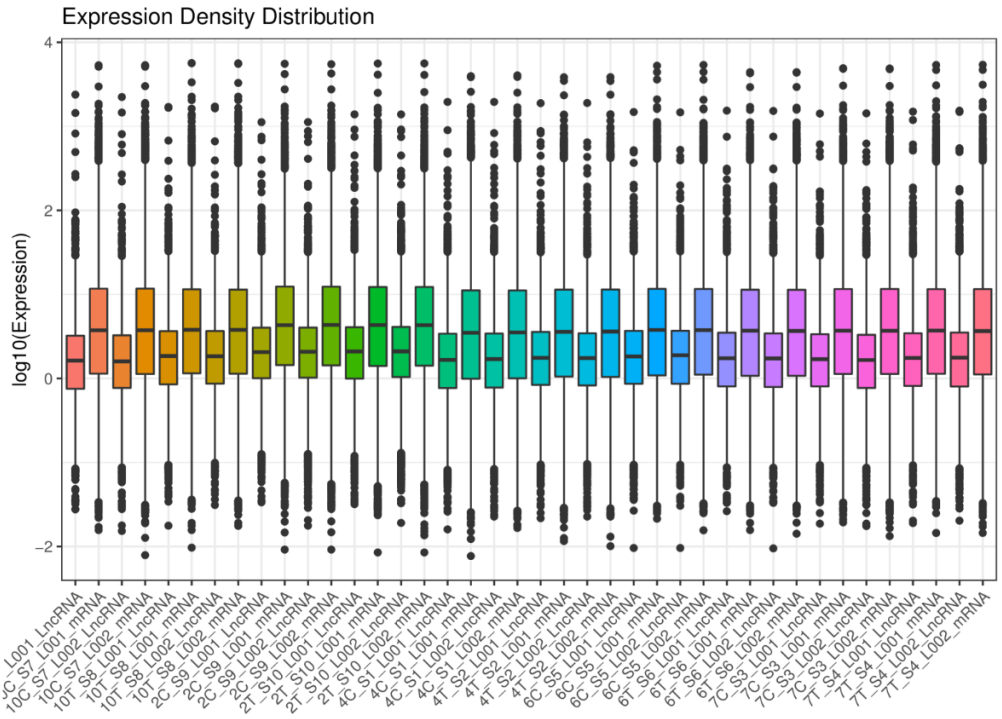
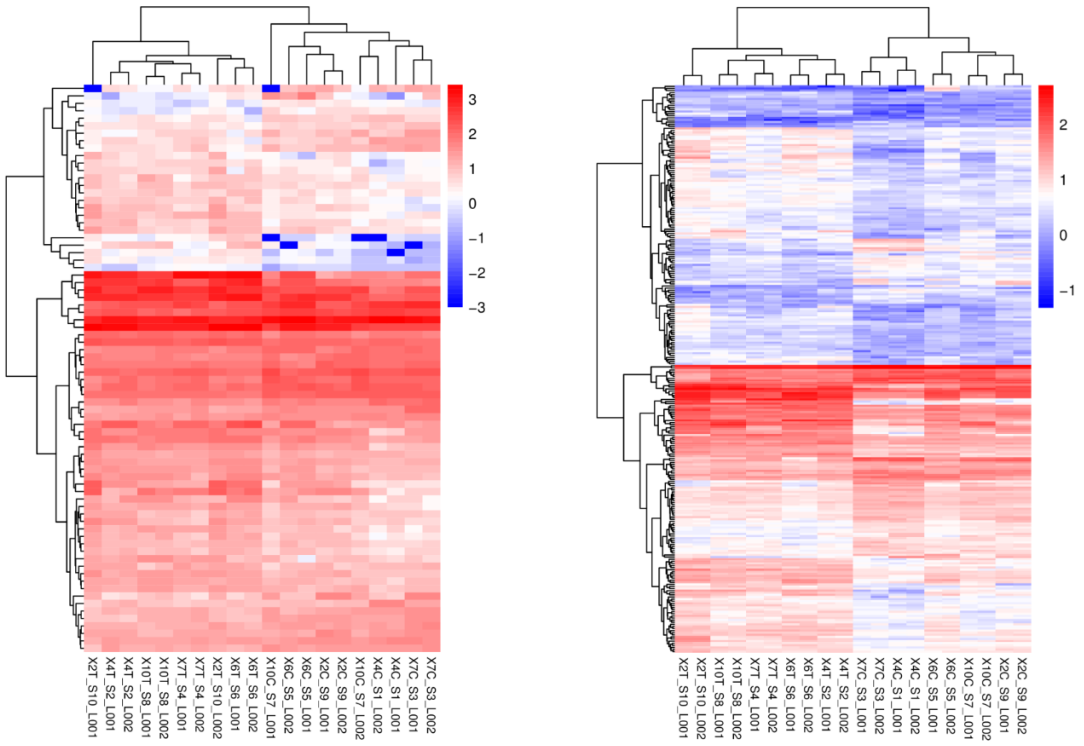
5. Supplemental material

A

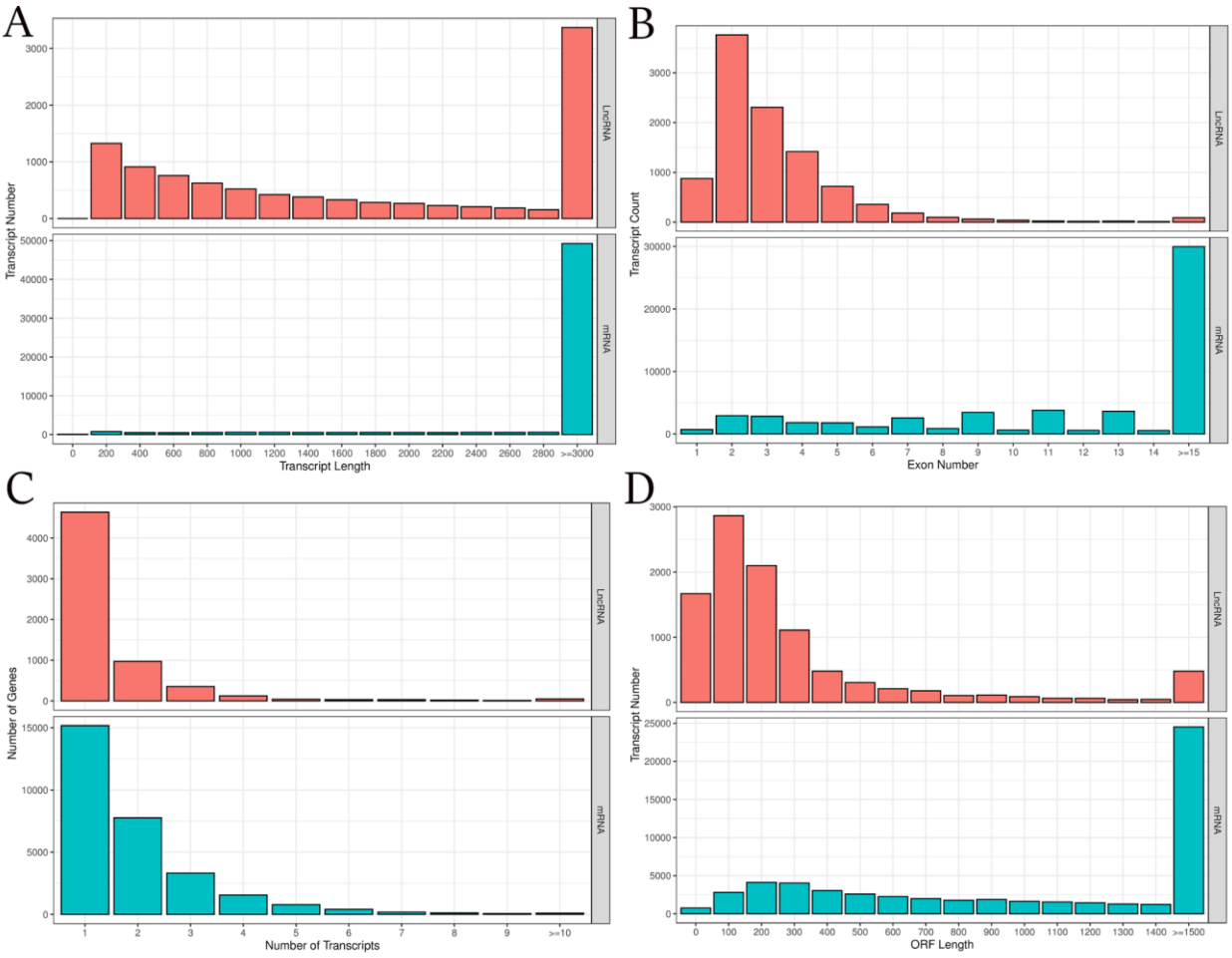

Expression Density Distribution
log10(Expression)
4
2
0
−2

B


lncRNA
mRNA

2332

Supplementary Figure 1. Libraries analysis from RNASeq to different expressions. A) Expression density distribution of libraries from lncRNA, and mRNA pipeline. This plot shows the samples have very similar gene expression profiles which makes them comparable for the differential expression analysis. B) Heat map of the hierarchical clustering of expression data. Each column represents an experimental condition (e.g. Control-vs-Treat), each row represents a gene. Expression differences are shown in different colors. Red means up-regulation and blue means down-regulation.



Supplementary Figure 2. Comparison of characteristics between lncRNA and mRNA. The bar chart illustrates the differences in open reading frame (ORF) lengths (A) and exon numbers (B) between long non-coding RNA (lncRNA) and messenger RNA (mRNA). Our analysis revealed that lncRNA exhibits shorter ORF lengths and fewer exons compared to mRNA (D). Conversely, mRNA demonstrates a higher abundance of transcripts and genes when compared to lncRNAs (C).

CONCLUSION

The regulation of spermatogenesis in vertebrates by germinal players and the role of ncRNAs, particularly lncRNAs in modulating germ and somatic mRNAs and others ncRNAs expression, represent a novel and unexplored field in molecular and fisiological studies. Our study is the first, to the best of our knowledge, to identify germinal players that acts by both paracrine and autocrine manner, regulating Sertoli and germinative cell proliferation. In the first chapter of this thesis, we found that rhGdnf stimulates the proliferation of type Aund spermatogonia and their surrounding Sertoli cells, creating new available niches and maintaining the stemness pool in the zebrafish testes. It also supports the development of differentiating spermatogonial cysts and inhibits late spermatogonial differentiation. Our data also reveal that the autocrine and paracrine roles of Gdnf are evolutionary novelties in fish, although some paracrine functions are conserved and similar to those observed for mammalian GDNF. In chapter 2, *ex vivo* testis explants of adult zebrafish under Fsh in an androgen independent manner, revealed 5161 novel lncRNAs in the adult gonad and intrinsic characteristics of this ncRNA class. lncRNA exhibits shorter ORF lengths and fewer exons compared to mRNA. Conversely, mRNA demonstrates a higher abundance of transcripts and genes when compared to lncRNAs. This high number of transcripts provides a great opportunity to study the modulation of these lncRNAs in zebrafish testis. Further, analysis of DEGs showed that biological processes related to cellular components, such as membrane, cytoplasm, and nucleus, were the most significantly modulated. When analyzed the WEGO results, we identified that the most enriched categories included "transcription signaling" and "membrane modulation". Regarding our prediction analysis of lncRNA-protein and a possible modulation of the lncRNA CT025854, an antisense lncRNA of the gene *igf3* by rzfFsh, we suggest the potential importance of non-coding RNAs, as the aforementioned lncRNA, in the regulation of crucial genes from germinative cell line. In conclusion, our study sheds light on the regulation of spermatogenesis in zebrafish by germinative players and the role of lncRNAs in modulating germinative gene expression. We have identified the function of gdnfa that acts in both a paracrine and autocrine manner, maintaining spermatogenesis homeostasis. Furthermore, our high coverage sequencing total RNA in adult zebrafish gonad has provided a great opportunity to study the modulation of novel lncRNAs in zebrafish testis.