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Regulation of spermatogonial development by Fsh: The complementary roles of locally produced Igf and Wnt signaling molecules in adult zebrafish testis

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ABSTRACT

Spermatogenesis is a cellular developmental process characterized by the coordinated proliferation and differentiation activities of somatic and germ cells in order to produce a large number of spermatozoa, the cellular basis of male fertility. Somatic cells in the testis, such as Leydig, peritubular myoid and Sertoli cells, provide structural and metabolic support and contribute to the regulatory microenvironment required for proper germ cell survival and development. The pituitary follicle-stimulating hormone (Fsh) is a major endocrine regulator of vertebrate spermatogenesis, targeting somatic cell functions in the testes. In fish, Fsh regulates Leydig and Sertoli cell functions, such as sex steroid and growth factor production, processes that also control the development of spermatogonia, the germ cell stages at the basis of the spermatogenic process. Here, we summarize recent advances in our understanding of mechanisms used by Fsh to regulate the development of spermatogonia. This involves discussing the roles of insulin-like growth factor (Igf) 3 and canonical and non-canonical Wnt signaling pathways. We will also discuss how these locally active regulatory systems interact to maintain testis tissue homeostasis.

1. Topic and content

Spermatogenesis is a cellular differentiation process providing haploid sperm from diploid spermatogonial stem cells (SSC). Spermatogenesis is controlled by, and germ cell survival depends on, testicular somatic cells. They receive signals from the endocrine system. The somatic cells, in turn, produce local signals that modulate germ cell development. The review deals with local signaling molecules produced by Leydig and Sertoli cells, and how pituitary Fsh engages these local regulators to control zebrafish spermatogenesis.

Spermatogenesis takes place in the testis, a tissue consisting of the interstitial or intertubular compartment, embedding and surrounding the seminiferous or spermatogenic compartment. The former contains different somatic cell types such as Leydig, myoid and mast cells, macrophages, connective and nervous tissue elements, and blood/lymphatic vessels. The spermatogenic compartment contains only two cell types: the somatic Sertoli cells and the germ cells in different stages of development, jointly forming the germinal epithelium. In this epithelium, the Sertoli cells take care of the germ cells, supporting their survival and development. In the cystic type of spermatogenesis found in anamniote vertebrates (fishes and amphibia), cytoplasmic extensions of dynamic (i.e. proliferating) Sertoli cells form cysts that envelope a developing germ cell clone all derived from a single SSC (Schulz et al., 2015; França et al., 2015). This review will discuss signaling systems that are involved in regulating the proliferation activity of SSCs. When dividing, they can either form additional stem cells (self-renewal), or they can produce differentiating offspring destined to form spermatozoa. We will discuss how Fsh regulates different signaling routes involved in this self-renewal/differentiation balance, focusing on Leydig cell-derived, non-canonical signaling to stimulate self-renewal, and on Sertoli cell-derived Igf signaling that uses canonical Wnt signaling components to promote differentiation of spermatogonia.

1.1. Spermatogonial stem cells and spermatogenesis

Spermatogenesis relies on a population of SSCs, undifferentiated

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diploid cells, usually present as single cells that are in zebrafish in close contact with one or two Sertoli cells (Leal et al., 2009a). Depending on the genetically determined number of mitotic cell cycles, a given SSC is able to produce hundreds of spermatozoa during a single wave of spermatogenesis. Spermatozoa carry a haploid set of genes for fusion with the haploid counterpart within the egg during fertilization. SSCs reside in a specific microenvironment, referred to as the SSC niche. The SSC niche is formed physically by Sertoli cells, but also Leydig and myoid cells provide signals relevant to modulate SSC behavior (Oatley et al., 2009; Ding et al., 2011; Chen et al., 2016a). In mammals, initial studies reported that SSCs are located along the basement membrane of the seminiferous tubules, with a preference close to capillaries just outside the tubular walls (Chiarini-Garcia et al., 2003; Yoshida et al., 2007). However, this concept was challenged later, showing that SSCs are randomly distributed over the basement membrane, preferring areas where two adjacent tubuli directly contact each other, while avoiding areas adjacent to interstitial tissue (Hara et al., 2014; Chan et al., 2014). In zebrafish, morphological analysis of the localization of single type A_{und} spermatogonia, the cell population that contain the SSC in fish, showed that ~75% of these cells are found close to the interstitial tissue in zebrafish (Nóbrega et al., 2010), i.e. similar to the first but not the most recent concept in mammals.

SSC activity can differ depending on the reproductive strategy of a given species. A recent study in rainbow trout has shown that although the stem cell capacity of A_{und} spermatogonia is detectable during the entire year, the level of activity differs depending on the season (Sato et al., 2017). This is probably different in fish species that do not show seasonal reproductive cycles. In zebrafish and medaka, two species that can show continuous reproductive activity after puberty, one would expect a more stable stem cell activity to safeguard continuous sperm production. A detailed comparison of seasonal versus continuous reproduction as regards SSC activity is still missing in fish.

When proliferating, SSCs can either produce more SSCs (self-renew) or produce differentiating daughter cells. Maintaining the correct balance between self-renewal and differentiation of A_{und} is critical for long-term fertility (De Rooij 2015, 2017). When a given SSC undergoes self-renewal, the newly produced A_{und} daughter cell will have to find an empty/new niche to act as stem cell and establish a new spermatogenic cyst (Fig. 1). SSCs that undergo a differentiating proliferation, on the other hand, give rise to type A differentiating spermatogonia (A_{diff}), which form in zebrafish, together with a slowly increasing number of Sertoli cells, cysts containing first 2, then 4 and 8 A_{diff} spermatogonia (Leal et al., 2009a). Differentiating germ cells remain interconnected by cytoplasmic bridges until the end of spermatogenesis, allowing a synchronized development of the germ cells derived from the same SSC. A_{diff} in cysts containing 8 germ cells continue differentiating mitotic cell cycles, resulting in 6 subsequent generations (producing 16, 32, 64, 128, 256 and 512 cells, respectively) of type B spermatogonia (type B). The last generation of B spermatogonia differentiate (without proliferating) into primary spermatocytes and then proceed through the two meiotic divisions. Theoretically, this results in 2048 spermatids. Counting of the actual cell numbers, however, showed that ~1300 spermatids are found, suggesting that during the different stages of spermatogenesis, ~40% of all formed germ cells are lost (Leal et al., 2009a). Spermatids emerge from the second meiotic division as haploid cells. Finally, during a differentiation process known as spermiogenesis, the DNA is maximally condensed, superfluous cytoplasm and organelles are resorbed by Sertoli cells and a flagellum is formed. To prepare spermiation, the cytoplasmic bridges are broken to individualize spermatozoa that are released by the Sertoli cells through opening of the cyst, providing the spermatozoa access to the lumen of the spermatogenic tubule (spermiation).

1.2. Regulation of spermatogenesis by pituitary hormones: scenarios in mouse and fish

The brain-pituitary axis is the major regulator of spermatogenesis in vertebrates. Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and regulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the gonadotropic cells of the pituitary (Stamatiades and Kaiser, 2018). These two pituitary hormones in turn modulate somatic cell functions in the testis, which then communicate with germ cells via short-range signaling molecules. In mammals, FSH regulates Sertoli cell functions, such as the production of growth factors inducing spermatogonial proliferation and differentiation, e.g. fibroblast growth factor 2 or insulin-like growth factor (IGF) 1 (Mullaney and Skinner, 1992; Pitetti et al., 2013). On the other hand, LH regulates Leydig cell functions, in particular the production of androgens, which in turn modulate germ cell development at several stages: Leydig cell-derived androgens promote SSC self-renewal by increasing the production of GDNF by myoid cells (Chen et al., 2014, 2016a), and by decreasing the production of WNT5A by Sertoli cells (Tanaka et al., 2016) while also stimulating the development of meiotic and post-meiotic germ cells (Holdcraft and Braun, 2004; De Gendt et al., 2014).

Knockout studies in both mice and zebrafish advanced our understanding of gonadotropin functions. *FSH receptor* (*Fshr*) knockout mice showed smaller testes and reduced fertility (Dierich et al., 1998; Abel et al., 2000), while in *LH receptor* (*Lhcgr*) knockout mice, spermatogenesis is blocked (Lei et al., 2001), underlining the relevance of androgens in regulating mammalian spermatogenesis. In general, androgen effects on germ cells are mediated by somatic cells in the testis, which do express the *androgen receptor* (*Ar*) gene, in contrast to germ cells (Johnston et al., 2001; Zhou et al., 2002). Androgens also stimulate spermatogenesis in fish (Ohta et al., 2007; Leal et al., 2009b; García-García et al., 2017; De Castro Assis et al., 2018), but androgen receptor knock-out zebrafish are still producing spermatozoa, although mutant testes reached only 1/5th of the weight of wild-type testes (Tang et al., 2018; Crowder et al., 2018). Moreover, loss of *cyp17a1*, an enzyme required for androgen production, in zebrafish or medaka is compatible with normal spermatogenesis while secondary sex characters and reproductive behavior disappeared (Sato et al., 2008; Zhai et al., 2018). Clearly, spermatogenesis in fish is less dependent on androgens than in mammals. However, also in mice a recent report showed that a constitutively active FSHR expressed by Sertoli cells in *Lhcgr* mutant mice restored nearly-normal spermatogenesis by activating the expression of usually androgen-regulated genes in Sertoli cells (Oduwole et al., 2018a). In zebrafish, *fshr* knockout animals present smaller testes and delayed spermatogenesis, while *lhcgr* knockout males did not show a change in the reproductive phenotype (Zhang et al., 2015). The latter report suggested that Fsh can drive spermatogenesis in the absence of Lh signaling, which is probably explained by the steroidogenic activity of Fsh in fish (Ohta et al., 2007; García-López et al., 2010), but may also be related to the steroid-independent effects of Fsh (see below). A recent study in zebrafish reported that loss of *fshb* and *lhcgr* (i.e. Lh and Fshr are present) does not compromise spermatogenesis, leading to the hypothesis that Lh maintains spermatogenesis by interacting with the Fshr (Chu et al., 2015). However, this hypothesis requires further discussion since the capacity of Lh to cross-activate the Fshr under physiological conditions is debatable, in particular since a number of studies reported Fsh-specific effects that were not elicited by Lh. For example, hormone receptor activation studies in zebrafish have shown a cross-activation of the Lhcgr by Fsh, while Lh did not activate the Fshr using defined amounts of recombinant zebrafish Fsh or Lh and Chinese hamster ovary (CHO) cells expressing Fshr or Lhr (So et al., 2005). A more recent report indicates, on the other hand, that co-injecting constructs expressing *lh* and *fshr* in CHO or African green monkey kidney fibroblast-like (COS-7) cells increased luciferase activity, suggesting that Lh activated the Fshr (Xie et al., 2017). However,

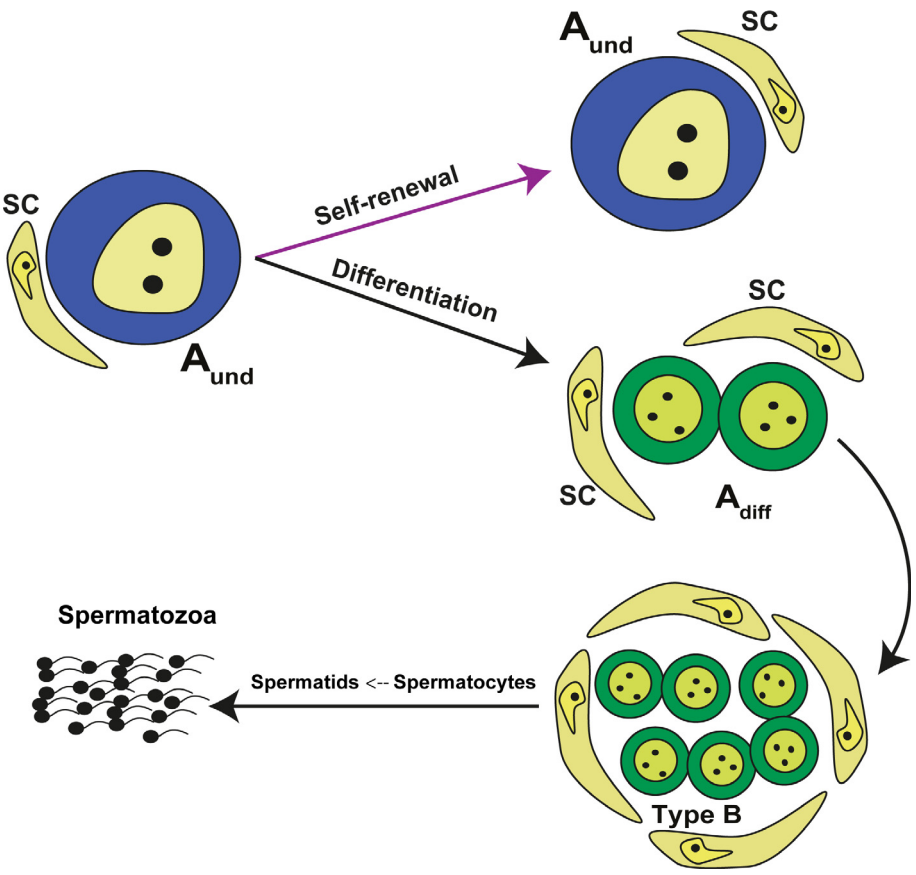

Fig. 1. Schematic representation of self-renewal proliferation of type A undifferentiated spermatogonia (A_{und}) to produce another A_{und} that forms a new spermatogenic cyst upon associating with Sertoli cells (SC), or of differentiating proliferation via type A differentiating spermatogonia (A_{diff}) and type B spermatogonia, before entering meiosis and then spermiogenesis to finally produce spermatozoa.

the actual Lh concentration was not known/determined. Moreover, *in vivo* studies did not provide evidence that Lh activated the Fshr. For instance, both Fsh and Lh elevated circulating androgen levels, but only Fsh changed the levels of a number of Leydig (García-López et al., 2010; Assis et al., 2015; Crespo et al., 2016; Safian et al., 2018a) and Sertoli cell transcripts (Nóbrega et al., 2015; Crespo et al., 2016) in zebrafish. Similarly, in rainbow trout, both Fsh and Lh regulated the expression of several genes, but the two gonadotropins also showed specific effects on the expression of 82 (Fsh) and 175 (Lh) genes (Sambroni et al., 2013a). More work is needed to fully understand the specific gonadotropin functions in fish. It seems clear that the gonadotropins share some functions while also exerting specific effects on their target cells. The mechanism(s) implementing these effects await further elucidation.

1.3. Androgen-independent effect of Fsh on zebrafish spermatogenesis

In zebrafish and rainbow trout, hundreds of testicular transcripts respond to Fsh but not to sex steroids (Crespo et al., 2016; Sambroni et al., 2013b). In zebrafish, these genes belong to several pathways known to regulate cell proliferation and differentiation in testis (Igf3 and insulin-like peptide 3 [Insl3]) and other tissues such as Tgf-β, Wnt, Notch, and Hedgehog signaling. In the following section, we summarize recent advances in our understanding of Fsh effects that do not involve sex steroid action on both, Leydig and Sertoli cells, and their consequences on zebrafish spermatogenesis (Fig. 2). Additional and yet to be characterized mechanisms mediating sex steroid-independent Fsh effects will probably exist.

1.3.1. Regulation of Leydig cells functions

Next to stimulating androgen production in Leydig cells, zebrafish Fsh also stimulated the expression of the relaxin peptide family member *insl3* (García-López et al., 2010). In mammals, INSL3 is required for the testicular descent into the scrotum during fetal development (Kumagai

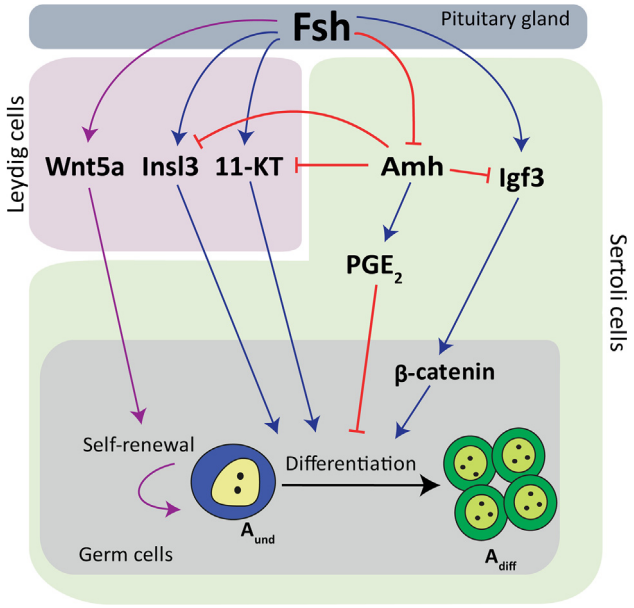

Fig. 2. Schematic representation of the effects of Fsh in zebrafish testis. Fsh stimulates the production of A_{und} by increasing Wnt5a production in Leydig cells (Safian et al., 2018a) while also inducing A_{und} differentiation via activation of several pathways: 11-KT (García-López et al., 2010) and Insl3 (Assis et al., 2015; Crespo et al., 2016) production in Leydig cells. In Sertoli cells, Fsh stimulates Igf3 production (Nóbrega et al., 2015), which activates the β-catenin pathway in A spermatogonia (Safian et al., 2018b). Fsh also down-regulates Amh, which otherwise would inhibit Insl3, 11-KT and Igf3 signaling, or promote PGE_2 signaling, in turn inhibiting germ cell differentiation (Skaar et al., 2011; Morais et al., 2017). Receptors for androgens and Amh are not expressed by germ cells and will therefore develop biological activity in indirect manners via the receptor-expressing cell types.

et al., 2002). Potential INSL3 functions in adult testis remain under investigation in mammals and include modulation of androgen production (Pathirana et al., 2012) and germ cell apoptosis (Kawamura et al., 2004). In zebrafish, human INSL3 and recombinant zebrafish Insl3 promoted spermatogenesis without changing androgen production, by stimulating the differentiating proliferation of A_{und} into A_{diff} spermatogonia (Assis et al., 2015; Crespo et al., 2016). Moreover, we have found recently that Fsh modulates the expression and production of the growth factor Wnt5a in Leydig cells, which induced the proliferation and accumulation of A_{und} while also stimulating the generation of new Sertoli cells (Safian et al., 2018a). The function of WNT5A to stimulate self-renewal proliferation of A_{und} is conserved between mice and zebrafish but the hormonal regulation and cellular source differ. While zebrafish *wnt5a* expression is up-regulated in an androgen-independent manner by Fsh in Leydig cells (Safian et al., 2018a), mice *Wnt5a* transcript is down-regulated in Sertoli cells by androgens upon LH stimulation (Tanaka et al., 2016).

1.3.2. Regulation of Sertoli cells functions

In Sertoli cells, Fsh down-regulated the transcript levels of *anti-müllerian hormone* (*amh*). This Tgf-β family member is known for inducing Müllerian duct regression during male sex differentiation in tetrapod vertebrates (Adolfi et al., 2018). Still, over-expression of human AMH reduced the number of mature Leydig cells, decreasing the levels of androgen production and the expression of androgen-regulated genes in rodents (Racine et al., 1998; Rouiller-Fabre et al., 1998). In zebrafish, Amh reduced the mitotic activity of A_{und} spermatogonia and reduced their differentiation into A_{diff} spermatogonia but this occurred in an androgen-independent manner (Skaar et al., 2011; Morais et al., 2017). The molecular mechanism used by Amh to inhibit spermatogenesis are under investigation. We have recently found that Amh inhibited the effects of a new member of the Igf family, Igf3; Amh moreover down-regulated the expression of steroidogenic genes while stimulating prostaglandin E2 signaling, in turn inhibiting spermatogonial differentiation (Morais et al., 2017) (Fig. 2). Down-regulation of testicular *amh* transcript levels has been observed in different fishes when entering pubertal development (eel, Miura et al., 2002; salmon parr, Maugars and Schmitz, 2008; sea bass, Mazón et al., 2014; rainbow trout, Sambroni et al., 2013a), suggesting that control of the expression of this potent inhibitory Tgf-beta family member is relevant in fish in general.

One of the prominent effects of Fsh was to strongly stimulate the expression and production of Igf3 (Nóbrega et al., 2015; Crespo et al., 2016). The *igf3* sequence was first characterized in fish as a gene expressed exclusively in gonadal tissue (Wang et al., 2008). After a decade, Igf3 has been identified in several more fish species (Li et al., 2011; Sambroni et al., 2013a; Melo et al., 2015; Song et al., 2016), in some of them also showing extragonadal expression (Fig. 3). However, the *igf3* gene has not been reported yet in tetrapods, although it was detected in the spotted gar (Braasch et al., 2016), suggesting that *igf3* gene was lost in tetrapod vertebrates (Figs. 3 and 4). Functional studies showed that Igf3 is the first Fsh-regulated, Sertoli cell-derived growth factor identified in vertebrates that stimulates spermatogenesis (Nóbrega et al., 2015).

1.4. Igf signaling system and its role in regulating spermatogenesis

1.4.1. Igf signaling system

The IGF signaling system in teleost fish consists of three Igfs ligands (Igf1, 2 and 3), two Igf1 receptors (Igfr1a and 1b), two Igf2 receptors (Igfr2a and 2b), and nine Igf binding proteins (Igfbps) (Duan et al., 2010). Remarkably, Igfr1 interacts with all Igf ligands while Igf2r did not responded to Igf2 in zebrafish (Williams et al., 2012). Igfs are produced in the liver under the control of growth hormone (Gh), but also locally in many tissues in response to Gh and other hormones (Duan et al., 2010). In the blood, close to 90% of the total IGF is bound

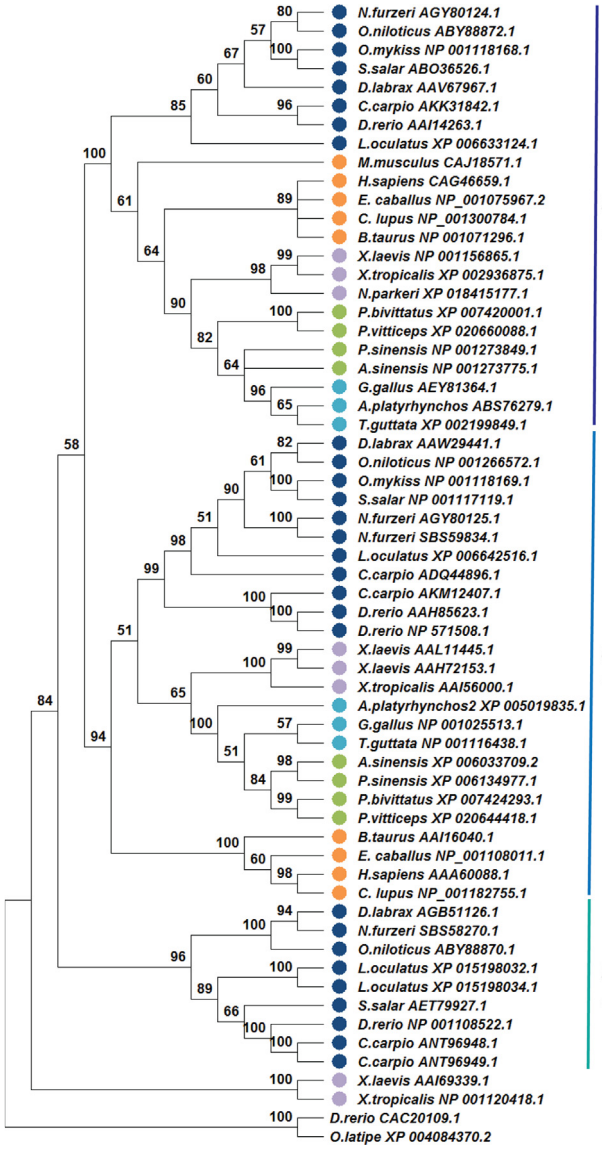

Fig. 3. Distribution of Igf ligands in different animal groups. Alignment and construction of the Neighbor-joining (NJ) tree was performed using ClustalW and MEGA 7 (Kumar et al., 2016). For NJ analysis, the p-distance method and bootstraping of 1000 replicates (cut-off value 50%) were used. Insulin sequences of zebrafish and medaka were used as out groups. All sequences were obtained from NCBI and their respective accession numbers are noted after the name of each specie in the tree. Different taxonomic groups are showed in color code: fish (blue), amphibians (purple), reptiles (green), birds (light blue) and mammals (orange). *Xenopus leavis* sequence AAI69339.1 and *X. tropicalis* sequence NP_001120418.1 were noted as paralogous genes of Igf3 in NCBI. While a protein-protein BLAST analysis showed that both sequences shared homology with Igf ligands (data not shown), our analysis suggests that the two sequences formed an independent cluster, so that their initial identification may have to be revised.

to IGFBPs (Baxter, 2000), with IGFBP3 and Igfbp2b as most abundant ones in mammals and fish, respectively (Shimizu and Dickhoff, 2017; García de la Serrana and Macqueen, 2018). The IGF-IGFBP complexes increase the half-life of the IGFs by preventing their proteolytic degradation, but also regulate IGF bioavailability by limiting or facilitating their interaction with the IGF1 receptors (Fig. 5) (Jones and Clemmons, 1995; Firth and Baxter, 2002; Duan and Xu, 2005). Similar to the Igf ligands, Igfbps are produced in different tissues (Maures and Duan 2002; Chen et al., 2004; Kamangar et al., 2006; Kamei et al., 2008; Safian et al., 2012). Igfbps have a higher affinity for Igfs than the

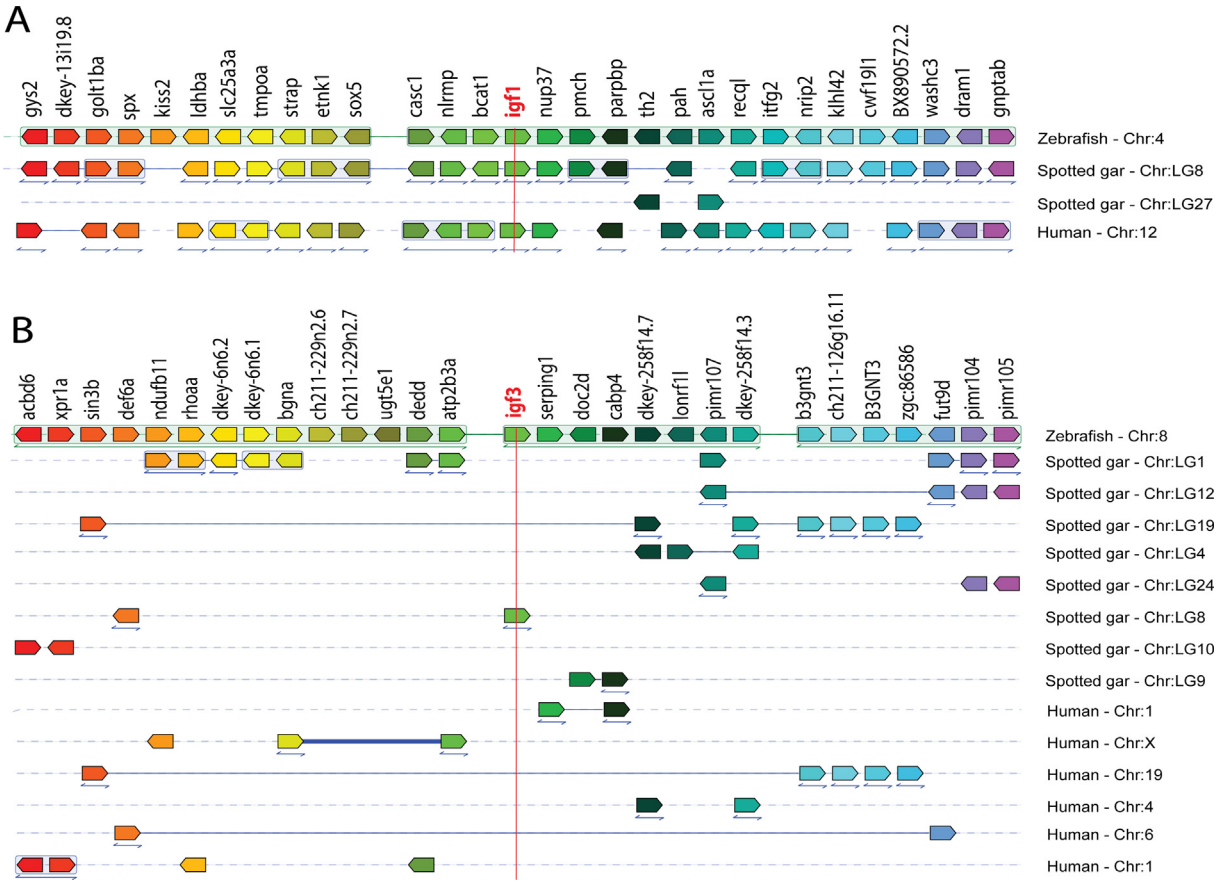

Fig. 4. Synteny analysis of Igf1 (A) and Igf3 (B) in zebrafish, spotted gar and human. The analyses were performed using the website Genomicus v69.01, option PhyloView (Louis et al., 2012; Louis et al., 2014; Nguyen et al., 2017; http://www.genomicus.biologie.ens.fr/genomicus-95.01/cgi-bin/search.pl). The reference gene (in red) is surrounded by their neighbouring genes (top) in their respective genome location (right). Genes with the same color are homologs while a line between two genes represent a break in the continuity of the alignment. A double headed arrow under a gene indicates that the order of the gene shown was reversed.

Igf1r and most of the circulating Igfs is bound to an Igfbp, so that locally produced Igfbps appear primarily involved in modulating the bioactivity of also locally produced Igf ligands (Fig. 5) (Allard and Duan, 2018). This modulation can enhance or reduce Igf activity (Duan and Xu, 2005; Duan et al., 2010).

The IGF1R consist of two extracellular α subunits and two intracellular β subunits. IGF ligands interact with the α subunits, triggering the autophosphorylation and activation of the β subunits, which each contain tyrosine kinase activity (Hakuno and Takahashi, 2018). The activation of IGF1R triggers two signaling pathways, the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway (Fig. 5) (Hakuno and Takahashi, 2018). The MAPK pathway consists of three kinases, the RAF proto-oncogene serine/threonine-protein kinase (RAF), the mitogen-activated protein kinase (MEK) and the extracellular signal-regulated kinases (ERK). MAPK pathway activation leads to the translocation of ERK from the cytosol into the nucleus to regulate gene expression and eventually stimulate cell proliferation and differentiation (Zhang and Liu, 2002). PI3K, on the other hand, activates the 3′-phosphoinositide-dependent kinase-1 (PDK1)/protein kinase B (AKT), which promotes cell division and survival targeting multiple proteins (Fig. 5).

1.4.2. Role of Igf signaling in spermatogenesis

IGF/insulin signaling regulates early male germ cell development in several taxonomic groups, ranging from nematoda (Michaelson et al., 2010; Hubbard, 2011; McLeod et al., 2010) to mammals (Pitetti et al., 2013). Most functional studies have been done in mammals. A double knock-out of both the *insulin receptor* and the *IGF1 receptor* (*IGF1R*) strongly decreased mouse testis size as a result of reduced Sertoli cell proliferation and hence reduced germ cell-supporting capacity (Pitetti et al., 2013). Blocking the IGF1R of mouse or bovine SSCs in primary cell culture reduced their proliferation and stemness (Wang et al., 2015; Lei et al., 2018). In addition, Yao et al. (2017) reported that the IGF1R protein was found on round and elongated spermatids and that exposure to IGF1 increased the percentage of spermatids 31 days after incubating neonatal (5.5 days post-partum) mouse testicular fragments in tissue culture.

In rainbow trout, *igf1* and its receptor were expressed in a cell fraction enriched in Sertoli cells and also in spermatogonia and spermatocytes (Le Gac et al., 1996). Moreover, recombinant human IGF1 was required as permissive factor for the androgen-stimulated differentiation of spermatogonia in primary tissue culture of prepubertal eel testis (Nader et al., 1999). More recently, Igf3 has raised interest since *Igf3* transcripts were detected during gonadal development of common carp (Song et al., 2016), in immature rainbow trout testis (Sambroni et al., 2013b) and in zebrafish ovarian (Li et al., 2011; Irwin and Van Der Kraak, 2012) and testis tissue (Baudiffier et al., 2012). Remarkably, *igf3,* but not *igf1, igf2a* and *b,* transcript levels increased strongly in response to Fsh in zebrafish testis (Nóbrega et al., 2015; Crespo et al., 2016). In addition, an earlier report indicated that *igf3* transcripts were up-regulated by T_3 (the biologically most active thyroid hormone (Nelson and Habibi, 2009)) and that T_3-stimulated Sertoli cell and A_{und} proliferation was completely and partially blocked, respectively, by an Igf1r inhibitor in zebrafish (Morais et al., 2013). A more direct study showed that recombinant zebrafish Igf3 promoted, in an androgen-independent manner, the proliferation of both type A_{und} and type A_{diff} spermatogonia and up-regulated the expression of genes related to

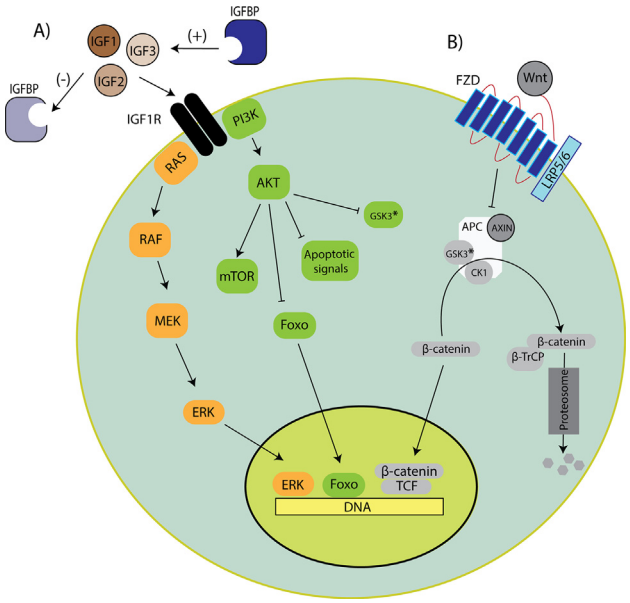

Fig. 5. Schematic representation of part of the Igf and the canonical Wnt signaling pathways. Igf activity is modulated by Igfbps, which support or inhibit Igf activity. (A) Igf/Igf1r interaction activates MAPK and PI3K/AKT pathways. One of the targets of AKT is glycogen synthase kinase 3 (GSK3), which is inhibited by Igf signaling. GSK3 is also part of the β-catenin destruction complex. This cross-talk between Igf and canonical Wnt signaling is indicated by the asterisks associated with the two GSK3 molecules, shown in their functional context, i.e. downstream of AKT or part of the β-catenin destruction complex. (B) Canonical Wnt ligands interact with the Frizzled (FZD) receptors and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to block the activity of the β-catenin destruction complex. The resulting accumulation of β-catenin and its translocation to the nucleus then allow β-catenin-dependent changes in gene expression.

spermatogonial differentiation (Nóbrega et al., 2015).

The biological activity of Igf ligands can be modulated by Igf binding proteins (Igfbp) that are produced locally in Igf target tissues (Duan et al., 2010; García de la Serrana and Macqueen, 2018). This also applies to the zebrafish testis, expressing all 9 *ifgpb* genes present in the zebrafish genome (Safian et al., 2016). In this species, Fsh usually activates both self-renewal and differentiating divisions of type A spermatogonia (Safian et al., 2016; Safian et al., 2018a). However, pharmacological inhibition of Igfbps in the presence of Fsh shifted the balance, favored the differentiation of A_{und}, which became partially depleted, and led to the accumulation of differentiating spermatogonia in testis tissue culture. This indicates that Igfbps protect A_{und} from excessively strong pro-differentiation signals of Fsh-induced Igf3 (Safian et al., 2016). A subsequent study then showed that the Igf3 signaling system and the roles of Igfbps therein is organized in a somewhat complex manner to achieve this protection. First, transcript levels of *igfbp* mRNAs are modulated by Fsh and its down-stream mediators (11-ketotestosterone and Igf3); second, certain *igfbp* transcripts are up- others are down-regulated by Fsh, androgen or Igf3 and this can occur quickly (1–3 days) or with a certain delay (3–7 days); finally, some Igfbps restrict while others support Igf activity (Safian et al., 2017). These findings are summarized in Fig. 6.

Once the Igf receptor is activated, recent work shed light on one of the intracellular mechanisms used by Igf3 to stimulate spermatogenesis. This mechanism involves cross-activating β-catenin signaling to stimulate the differentiation of type A spermatogonia in zebrafish testis (Safian et al., 2018b); β-catenin is part of a pathway known as canonical Wnt signaling, and the molecule that is probably responsible for the cross-activation is glycogen synthase kinase 3 (GSK3; see Fig. 5).

1.5. Wnt signaling system and its role in regulating spermatogenesis

1.5.1. Wnt signaling system

The Wnt signaling system is a conserved cell-to-cell communication system that consists of two intracellular branches. The first one is referred to as the canonical or β-catenin-dependent pathway and relies on the levels of cytoplasmic β-catenin (Fig. 5). Sufficiently high levels of cytoplasmic β-catenin allow its translocation into the nucleus, where it interacts with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family, modulating the expression of Wnt-related genes (Salic et al., 2000). The levels of cytoplasmic β-catenin remain low in the absence of WNT ligand due to the tight control of the β-catenin destruction complex. This complex is a dynamic structure formed by the GSK3 and casein kinase 1 (CK1), the scaffolding protein Axin and the adenomatous polyposis coli (APC) protein (Stamos and Weis, 2013) (Fig. 5). In the absence of Wnt ligands, the complex is active and GSK3 and CK1 phosphorylate β-catenin, which allows its ubiquitination by the E3-ubiquitin ligase β-TrCP, targeting it for proteasome degradation (Fig. 5). However, binding of Wnt ligands to a membrane receptor of the Frizzled (FZD) family and its co-receptor, the low-density lipoprotein receptor-related protein 5/6 (LRP5/6), blocks the activity of the β-catenin destruction complex, thereby increasing the levels of cytoplasmic β-catenin (Fig. 5) (Nusse, 1999). Activation of FZD/LRP5/6 by Wnt recruits the β-catenin destruction complex and Dishevelled (DVL) to the receptor complex, inactivating AXIN, GSK3 and CK1, leading to the release of β-catenin from the complex (Clevers and Nusse, 2012).

The second signaling branch, the non-canonical Wnt pathway, also involves Wnt ligands and frizzled receptors. However, the non-canonical pathway activates other intracellular systems in a β-catenin-independent manner (Van Amerongen, 2012). Although the non-canonical pathway is not fully characterized yet, it was proposed that DVL directly activates intracellular pathways such as Jun N-terminal kinases (JKNs) and the planar cell polarity pathway upon WNT/FZD (LRP5/6 is not involved) interaction (Gao and Chen, 2010). In addition, non-canonical Wnt ligand can also interact with alternatives receptors (Winkel et al., 2008; Mikels et al., 2009; Lin et al., 2010).

Several Wnt ligands and receptors have been described and many of them are usually expressed in the same tissue at the same time. It is not clear how some Wnt ligands selectively activate the β-catenin-dependent pathway while others induce the β-catenin-independent pathway. Typically, WNT3A and WNT6 have been associated to the canonical pathway (Yeh et al., 2012; Takase and Nusse, 2016) while WNT5A and B seem to activate the non-canonical pathway specifically (Yeh et al., 2011) in mice testis. Notably, despite the large number of Wnt ligands expressed in cerebral endothelial cells, two co-receptors (Reck and GPR124) are involved in the specific response to WNT7 in these cells, suggesting that cells can express specific proteins to increase their responsiveness to specific ligands when required (Eubelen et al., 2018).

1.5.2. Role of Wnt signaling system in spermatogenesis

The WNT signaling system is relevant in the development and regulation of several cell types in multiple tissues. In the gonads, this signaling system was initially linked to the sex differentiation process. Down-regulation of canonical WNT signaling is required for testis differentiation in mammals and fish (Colvin et al., 2001; Kim et al., 2006; Jameson et al., 2012; Sreenivasan et al., 2014). WNT signaling also regulates spermatogenesis in adults (Yeh et al., 2011; Yeh et al., 2012; Kerr et al., 2014; Takase and Nusse, 2016; Tokue et al., 2017), but the results were somewhat inconsistent. Kerr et al. (2014) reported that loss of *β-catenin* in all germ cells depleted spermatocytes and spermatids without affecting the number of PLZF-positive undifferentiated spermatogonia. However, the canonical WNT reporter gene *Axin2* is expressed specifically in undifferentiated spermatogonia and *β-catenin* knock-out in *Axin2*-positive spermatogonia reduced the proliferation activity and number of PLZF-positive spermatogonia in mice, resulting

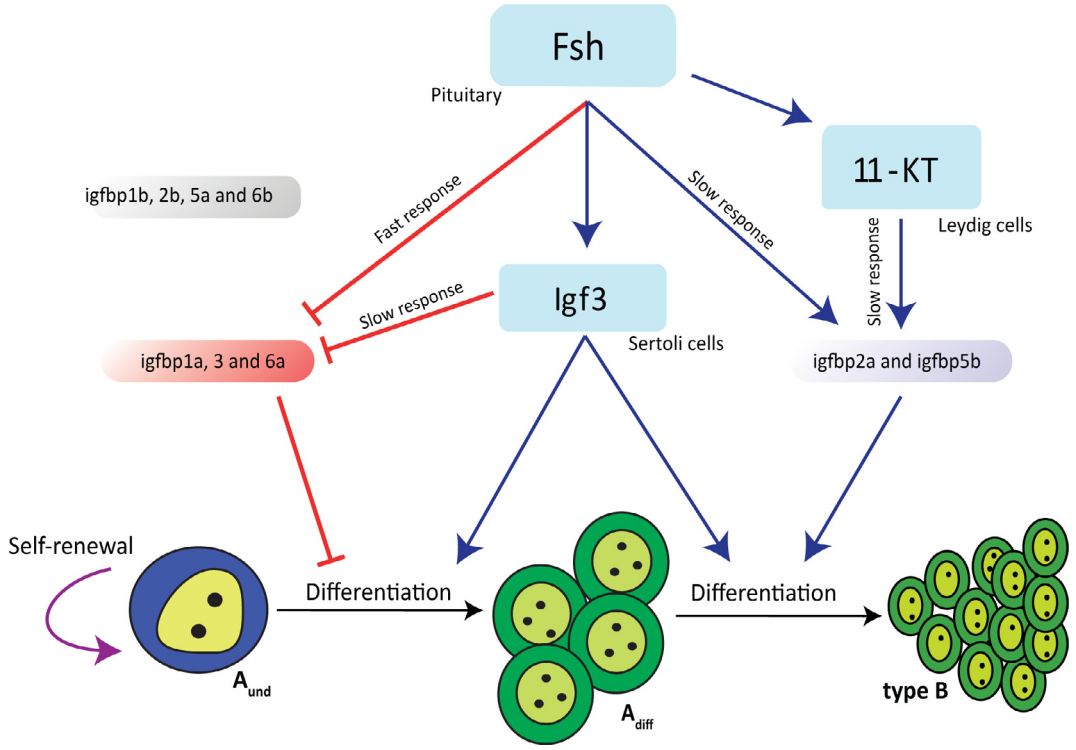

Fig. 6. Schematic representation of the effects of Fsh and two downstream mediators, Igf3 and 11-KT, on *igf-binding protein* (*igfbp*) transcript levels and proposed roles of Igfbps in adult zebrafish testis (adapted from Safian et al., 2017).

in the loss of differentiating spermatogonia (Takase and Nusse, 2016). In the same line, Tokue et al. (2017) showed that activation of β-catenin signaling promoted spermatogonial differentiation in mice, which can be inhibited by SHISA6, an endogenous antagonist of canonical Wnt signaling in a subpopulaton of GFRα-positive, undifferentiated spermatogonia. Irrespective of the exact identity of the cells responding to β-catenin signaling, it seems clear that activation of this pathways commits spermatogonia to differentiation. Regarding the non-canonical Wnts signaling, few studies have addressed its role on spermatogenesis. Yeh et al. (2011) showed that the non-canonical ligand WNT5A maintained the number of SSCs by reducing their apoptosis in culture. Further work on WNT5A in mice testis *in vivo* showed that LH-dependent androgen production down-regulated *Wnt5a* transcript levels in Sertoli cells, resulting in SSC differentiation (Tanaka et al., 2016). These results highlight the relevance of Wnt signaling for regulating the fate of spermatogonia in the adult mouse testis. However, a recent report challenged this view. After the loss of Gpr177 (porcupine), a protein required for the intracellular transport and release of Wnt ligands, either in germ cells or in SCs, mice showed normal spermatogenesis and it was only at 8 months of age that the germ cell-specific loss of Gpr177 was reflected in smaller testis and a reduced fertility (Chen et al., 2016b). It appears that β-catenin driven gene expression is required for the differentiation of spermatogonia but that this is not linked to the availability of porcupine-dependent Wnt ligands.

1.6. Interplay between Igf and Wnt signaling in zebrafish testis

In zebrafish, Fsh stimulates the proliferation of all types of spermatogonia, maintaining the proportion of A_{und} while increasing the one of A_{diff} and B spermatogonia (Safian et al., 2016). These results suggest that Fsh triggered a balanced activation of both self-renewal and differentiating divisions of type A spermatogonia in zebrafish testis, i.e. new cysts with A_{und} are formed (self-renewal) while some A_{und} undergo differentiating proliferation upon Fsh stimulation. In rodents, signals stimulating self-renewal are GDNF (Meng et al., 2000), CSF1 (Oatley et al., 2009) and WNT5A (Tanaka et al., 2016). Among these factors, only GDNF production has been linked to FSH (Tadokoro et al., 2002), although more recent work showed that androgen-regulated GDNF production by myoid cells is more relevant for self-renewal than FSH-regulated GDNF production by Sertoli cells (Chen et al., 2016c). The production of WNT5A by Sertoli cells is indirectly reduced by LH-stimulated androgen production (Tanaka et al., 2016), while a pituitary hormone effect on CSF1 regulation has not been reported yet. In fish, studies in juvenile eel identified the estradiol-stimulated platelet-derived endothelial cell growth factor (Miura et al., 2003) and the androgen-suppressed Amh (Miura et al., 2002) as signals promoting SSC self-renewal. In zebrafish, Fsh but not androgens suppressed testicular *amh* transcript levels (Skaar et al., 2011; Crespo et al., 2016). Moreover, Amh inhibited the differentiation of A_{und} spermatogonia in zebrafish, resulting in their accumulation despite the also slightly reduced proliferation activity of A_{und} spermatogonia in the presence of Amh (Skaar et al., 2011; Lin et al., 2017). On the other hand, estradiol treatment *in vivo* reduced spermatogonia proliferation, nevertheless leading to an accumulation of type A spermatogonia as a result of blocking Fsh and androgen release in zebrafish (De Waal et al., 2009). T_3 (via Igf3) was the first hormone shown to expand the population of A_{und} spermatogonia without blocking their differentiation in adult fish (Morais et al., 2013). However, none of these mechanisms explain the balanced effect that Fsh exerts on spermatogonia by promoting both self-renewal and differentiation. In this regard, we propose that Fsh uses the non-canonical Wnt pathway to maintain the population of A_{und} spermatogonia, while promoting their differentiation via Igf3-stimulated activation of β-catenin signaling. This model is summarized in Fig. 7 and discussed in the following paragraphs.

Recently, we have shown that Fsh recruits non-canonical Wnt signaling to increase the proportion of A_{und} spermatogonia. This involved Fsh-mediated increases in the transcript levels of *wnt5a* in Leydig cells, elevated Wnt5a protein production, and the requirement for the release of Wnt ligands (Safian et al., 2018a). Moreover, an inhibitor of Wnt5a blocks Fsh-stimulated self-renewal division of A_{und}, which was

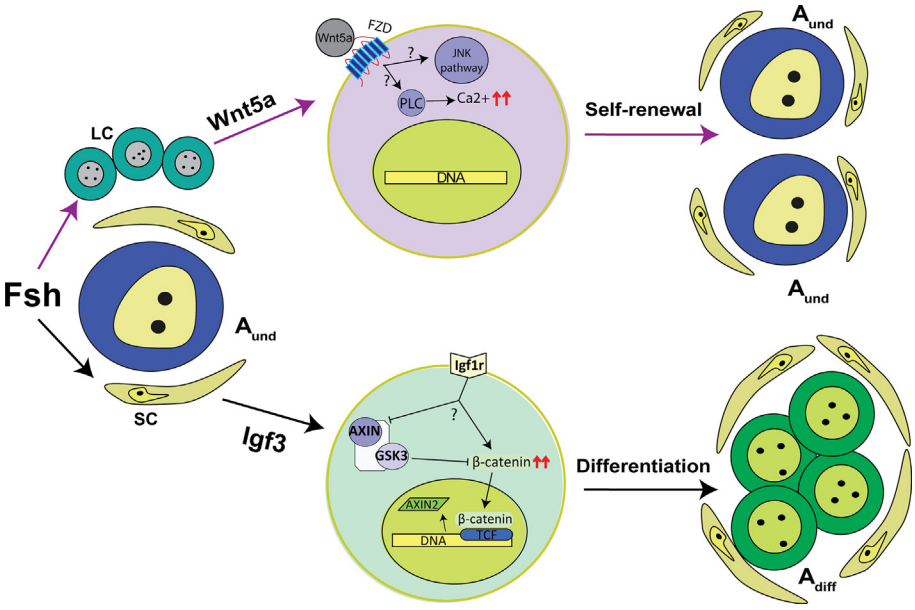

Fig. 7. Fsh makes use of different components of the Wnt signaling system to exert a balanced stimulatory effect on self-renewal and differentiation of A_{und} spermatogonia in zebrafish testis. On the one hand, Fsh stimulates self-renewal by stimulating the production of Wnt5a in Leydig cells (LC). Using Frizzled (FZD) receptors, non-canonical Wnt ligands, like Wnt5a, activate JNK and PLC pathways in a β-catenin-independent manner. However, it is not known yet how exactly Wnt5a develops biological activity in zebrafish A_{und} spermatogonia. On the other hand, Fsh promotes differentiation via Sertoli cell (SC)-derived Igf3, which in turn activates the β-catenin pathway to stimulate differentiation of type A spermatogonia.

stimulated by an agonist of Wnt5a in the absence of Fsh (Safian et al., 2018a). Collectively, these results indicate that Fsh stimulated Leydig cell Wnt5a protein production and release to expand the population of type A_{und} spermatogonia; zebrafish Lh did not have these effects. We have no direct information considering the receptor used by Wnt5a to modulate A_{und} behavior in zebrafish testis. However, an *in vitro* study reported that WNT5A maintained the number of murine SSCs by activating the JNK pathway and that SSCs and progenitors expressed *Fzd5*, *Fzd7* and *Ror2* receptors (Yeh et al., 2011). All these receptors are also expressed in zebrafish testis tissue (Crespo et al., 2016).

The canonical pathway, on the other hand, turned out to be relevant for another aspect of Fsh-stimulated spermatogenesis. Using compounds that block β-catenin-mediated signaling and a β-catenin-sensitive reporter line, we found that Fsh-stimulated Igf3 release activates β-catenin signaling in type A spermatogonia to stimulate their differentiation. Importantly, this occurs without the need to release Wnt ligands (Safian et al., 2018b), suggesting that Igf3 activates β-catenin signaling in a manner not involving Fzd receptors. Cross-activation of β-catenin signaling by Igf/insulin receptors have been described previously (Playford, et al., 2000; Desbois-Mouthon et al., 2001; Ye et al., 2010; Wang et al., 2010; Hu et al., 2012) and may be relevant in the zebrafish testis as well. The Igf3-mediated but Wnt-independent effects reconcile studies reporting the relevance of canonical Wnt singling in adult testis (Takase and Nusse, 2016; Tokue et al., 2017) and a study showing that loss of Gpr177 (preventing the release of Wnt ligands) is compatible with normal spermatogenesis in young adult mice (Chen et al., 2016b). It appears that Fsh stimulates the differentiation of type A spermatogonia by activating β-catenin signaling via Igf3, involving the destabilization of the β-catenin destruction complex via GSK3β. At the same time, Fsh promotes the generation of new A_{und} spermatogonia through Wnt5a and the non-canonical Wnt pathway (Fig. 7). These balanced effects of Fsh help to avoid depletion of A_{und} spermatogonia while promoting spermatogonial differentiation. The support of both activities safeguards long-term fertility and testis tissue homeostasis, supported by fine-tuning Igf3 effects through Igfbps (Safian et al., 2016, 2017). Other Fsh effects of relevance for spermatogenesis include the stimulation of steroidogenesis (García-López et al., 2010), modulation of the production of other growth factors and signaling molecules such as Insl3 (Assis et al., 2015; Crespo et al., 2016), Amh (Skaar et al., 2011), PGE_2 (Morais et al., 2017), Gsdf (Sawatari et al., 2007), Gdnf (Bellaïche et al., 2014), and others still awaiting further characterization.

2. Conclusions

Our understanding of the multiple functions of Fsh in vertebrates has increased considerably in the last years in mammals (Das and Kumar, 2018; Oduwole et al., 2018b) but also in piscine models like trout and zebrafish. Still, several questions remain to be investigated. For example, results obtained by gene knockout studies targeting gonadotropins and/or their receptors require further attention in order to fully understand specific and overlapping Fsh and Lh functions in fish. Cell type-specific and/or inducible knockouts may help to resolve these issues. Irrespective of this point, it is clear that fish Fsh stimulates spermatogenesis by inducing sex steroid production but also by regulating the production of a range of growth factors and other signaling molecules in Sertoli and Leydig cells. Among these factors, the interplay between Wnt and Igf signaling is relevant to maintain testis tissue homeostasis in adult male zebrafish. It will be interesting to test the function of other members of the Wnt signaling system such as canonical Wnt ligands and proteins that modulate their activity (secreted frizzled-related proteins, R-spondins, Dickkopf-related protein) and how they interact with Igf3, but also with sex steroids and other growth factors such as Amh and Insl3. In addition, although advances have been made to understand the function of Igf3, much more is to be learnt about this growth factor. For example, a report in common carp showed that knock down of gonadal *igf3* modulated the transcript levels of 124 long non-coding RNAs and 353 mRNAs (Song et al., 2019).

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