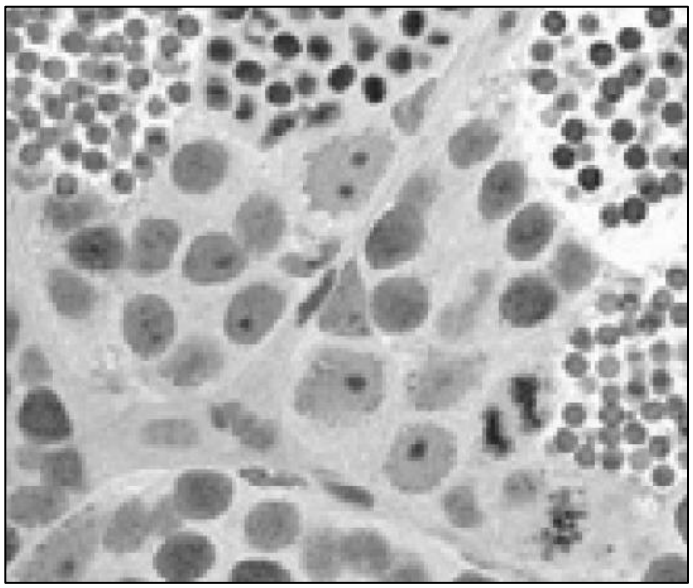
The role of IGF3 in zebrafish spermatogenesis

Research project



Marina Buitelaar
Supervisor: Rüdiger W. Schulz
Daily supervisors: Roberto Morais and Rafael H. Nóbrega
Utrecht University

Research project

Author: Marina Buitelaar

Supervisor: Rüdiger W. Schulz

The experiments described in this report were performed at the group developmental biology at Utrecht University, the Netherlands, and at the group cellular biology at UNESP, Botucatu, Brazil.

Image cover: adapted from Schulz, R.W. and Nóbrega R.H. (2011). Regulation of spermatogenesis. In: Farrel A.P. (ed.), *Encyclopedia of fish physiology: from genome to environment,* vol 1: 627-634. San Diego: Academic Press.

Table of contents

Introduction

Spermatogenesis is the process in which haploid flagellated spermatozoa arise from undifferentiated spermatogonial stem cells. Spermatogenesis takes place in the testis, the male reproductive organ. The testis consists of two compartments: the interstitial tissue and the germ epithelium (fig. 1). The interstitial tissue consists of blood vessels, connective tissue, myoid cells and the steroid producing Leydig cells. The germ epithelium is organized in tubuli and consists of germ cells surrounded by Sertoli cells. The Sertoli cells are in close contact with the germ cells and support them during a sequence of developmental stages until spermatozoa are produced and released in the tubular lumen. In fish, the Sertoli cells fully surround a clone of germ cells, forming a cyst until the end of the spermatogenesis. Therefore, the Sertoli cells are only in contact with germ cells at the same stage of spermatogenesis, different from the mammalian Sertoli cells that are in contact with germ cells at different developmental stages at the same time. The developmental stages of the germ cells can be divided into three main phases: the spermatogonial phase, meiosis and the spermiogenesis (Schulz *et al.*, 2010). The spermatogonial phase starts with the spermatogonial stem cells, which are part of the population of type A undifferentiated spermatogonia. In a transplantation assay, some of the type A undifferentiated spermatogonia cells were shown to be able to self-renew and to produce germ cells in recipient testes and ovaries (Nobrega *et al.*, 2010). A characteristic of spermatogonial stem cells is that they occur as single cells in a cyst. Once they proliferate, they can give rise to two new single spermatogonial stem cells or to two type A differentiating spermatogonia, connected by a cytoplasmic bridge.

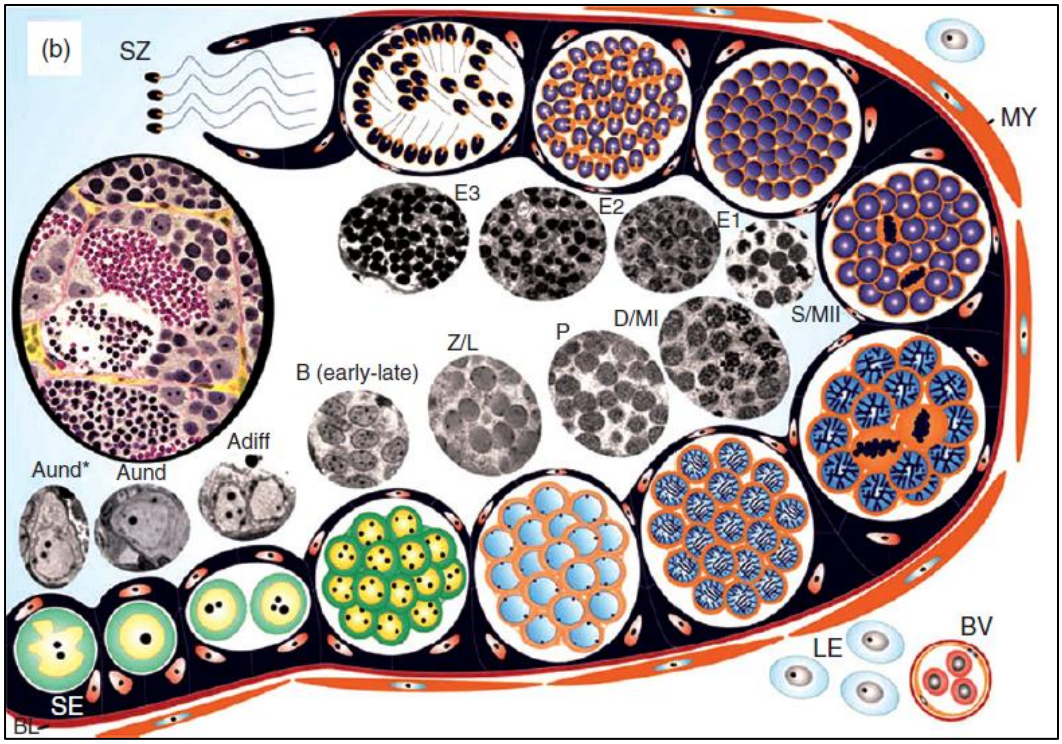


Figure 1: Schematic picture of a zebrafish testis. *The figure shows the interstitial tissue, containing Leydig cells (LE) and blood vessels (BV), and the germ epithelium that exists of germ cells and Sertoli cells (SE). The germ epithelium is delineated by the basal lamina (BL) and myoid cells (MY). The germ cells are shown in the different developmental stages: type A undifferentiated spermatogonia (Aund), A differentiated spermatogonia (Adiff), type B spermatogonia (B), leptotenic/zygotenic primary spermatocytes (Z/L), pachytenic primary spermatocytes (P), diplotenic primary spermatocytes/metaphase I (D/MI), secondary spermatocytes/metaphase II, early (E1), intermediated (E2) or late (E3) spermatids and spermatozoa (SZ). From Schulz* et al.*, 2010.*

The differentiating division is the first step towards the formation of sperm. After this division, the germ cells proliferate and differentiate, thereby proceeding into type B spermatogonia and subsequently into germ cells in meiosis, called spermatocytes. Once meiosis is finished, the haploid spermatids undergo differentiation into flagellated spermatozoa to finish the process of spermatogenesis (Schulz *et al.*, 2010).

The process of spermatogenesis is well regulated by pituitary hormones. Two main pituitary hormones regulating spermatogenesis are the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones are produced in the anterior pituitary in response to gonadotropin-releasing hormone (GnRH). This hormone is secreted from neurons in the hypothalamus and stimulates the endocrine cells in the pituitary to secrete FSH and LH (Schulz and Nóbrega, 2011). In mammals, FSH targets the Sertoli cells and LH the Leydig cells (Silverthorn *et al.*, 2010). In fish, this division is less specific because the Leydig cells express both the FSHR and the LHR, as found in African catfish (García-Lopez *et al.*, 2009), Japanese eel (Ohta *et al.*, 2007) and zebrafish (García-Lopez *et al.*, 2010). Further, the FSHR can be activated by LH as well. Yet, this activation needs a high concentration of LH and therefore only takes place during peaks of LH plasma levels. Hence, the steroid producing Leydig cells can be regulated by both LH and FSH, but the Sertoli cells in general only by FSH (Schulz *et al.*, 2010). The main steroids produced by the Leydig cells in response to LH and FSH are androgens, but other steroids like estrogen (E2) and progesterone can be produced too (Schulz and Nóbrega, 2011). The steroids produced by the Leydig cells feedback to the brain and pituitary. When the blood levels of steroids are high, the release of gonadotropins is down-regulated and in this way, a negative feedback system exists that keeps the blood levels of the gonadotropins in homeostasis (Silverthorn *et al.*, 2010). Manipulation of this feedback system induces a gonadotropin insufficiency. Gonadotropin insufficiency induced by E2 causes disruption of the spermatogenic process in adult zebrafish (Waal *et al.*, 2008). The effect of gonadotropins on spermatogenesis can also be seen directly in tissue culture. FSH was shown to induce the spermatogenesis in tissue culture of testes of Japanese eel (Ohta *et al.*, 2007) and zebrafish (Nobrega, unpublished results). Because LH is not found in the blood during the start of spermatogenesis, it is suspected that FSH is the gonadotropin that stimulates early stages of spermatogenesis (Schulz *et al.*, 2010).

FSH influences spermatogenesis in several ways. A very important action of FSH in fish is the stimulation of androgen production. The steroid 11-ketotestosterone (11-KT) is the main androgen in most fish, including zebrafish. It shows a high binding affinity to the androgen receptor (Miura *et al.*, 1991; Waal *et al.*, 2008). In zebrafish, it was demonstrated that FSH increases the release of 11-KT both ex *vivo* and *in vivo* (García-López *et al.*, 2010, Skaar *et al.*, 2011). The FSH-stimulated release of 11-KT was caused by an up-regulation of genes important for androgen production, like *steroidogenic acute regulatory protein* (*star*) and *cytochrome P450 family 17 subfamily A polypeptide 1* (*cyp17α*) (García-López *et al.*, 2010). 11-KT supported the entire process of spermatogenesis in *ex vivo* tissue culture with zebrafish testes (Leal *et al.*, 2009). The supporting function of 11-KT was also found in juvenile Japanese eel, where also all stages of spermatogenesis could be induced *ex vivo* by adding 11-KT to the medium. The addition of 11-KT induced the mitosis of type A and early type B spermatogonia, the only germ cells existing in the juvenile eel testis (Miura *et al.*, 1991). The production of 11-KT can be down-regulated by E2 *in vivo*,

because E2 induces gonadotropin insufficiency by the negative feedback system. This down-regulation of 11-KT interrupted spermatogenesis in zebrafish: the proliferation of type A spermatogonia was reduced and the differentiation of type A into type B spermatogonia was blocked (Waal *et al.*, 2009). Germ cells do not have androgen receptors, but Sertoli cells do (Waal *et al.*, 2008). Androgens therefore stimulate spermatogenesis indirectly, for example via the Sertoli cells. In agreement with this, Sertoli cell activation was found in response to 11-KT in tissue culture of juvenile Japanese eel testes (Miura *et al.*, 1991). In conclusion, FSH stimulates the Leydig cells to produce androgens, which in turn stimulate the proliferation and differentiation of the spermatogonia via the Sertoli cells.

FSH is also capable of regulating the Sertoli cells directly, because the Sertoli cells express the FSHR (García-López *et al.*, 2010). More specifically, the FSHR was mostly detected in Sertoli cells surrounding type A and early type B spermatogonia (Ohta *et al.*, 2007). FSH regulates the release of a number of growth factors in mammalian Sertoli cells, but in fish, not much is known about the role of FSH in Sertoli cell functioning. However, recently it was found that FSH regulates the release of a Sertoli cell derived growth factor, the TGFβ family member anti-Müllerian hormone (AMH) (Miura *et al.,* 2002; Skaar *et al.*, 2011). In mammals, the main function of this hormone is to cause the degradation of the Müllerian ducts in male gonads, but AMH is also involved in steroidogenesis and in follicular development (Josso *et al.*, 2006). The function of AMH in fish was not known, but with adult zebrafish testes culture it was demonstrated that AMH has an inhibitory effect on spermatogenesis. Firstly, AMH reduces or completely blocks the FSH stimulated release or 11-KT, by reducing the up-regulation of genes related to androgen production. Moreover, AMH inhibited 11-KT stimulated spermatogenesis in testes tissue culture. After a tissue culture with AMH and 11-KT, the number of cysts with type A spermatogonia was increased and the number of cysts containing other stages was reduced. BrdU analysis revealed that AMH blocks the differentiation and reduces the proliferation of type A undifferentiated spermatogonia. In line with this, the AMH protein was detected mostly in Sertoli cells surrounding type A spermatogonia. FSH was found to down-regulate the expression of AMH in adult zebrafish testes (Skaar *et al.*, 2011). Thus, FSH down-regulates AMH, an inhibitor of spermatogenesis and androgen production, thereby stimulating spermatogenesis (fig. 2).

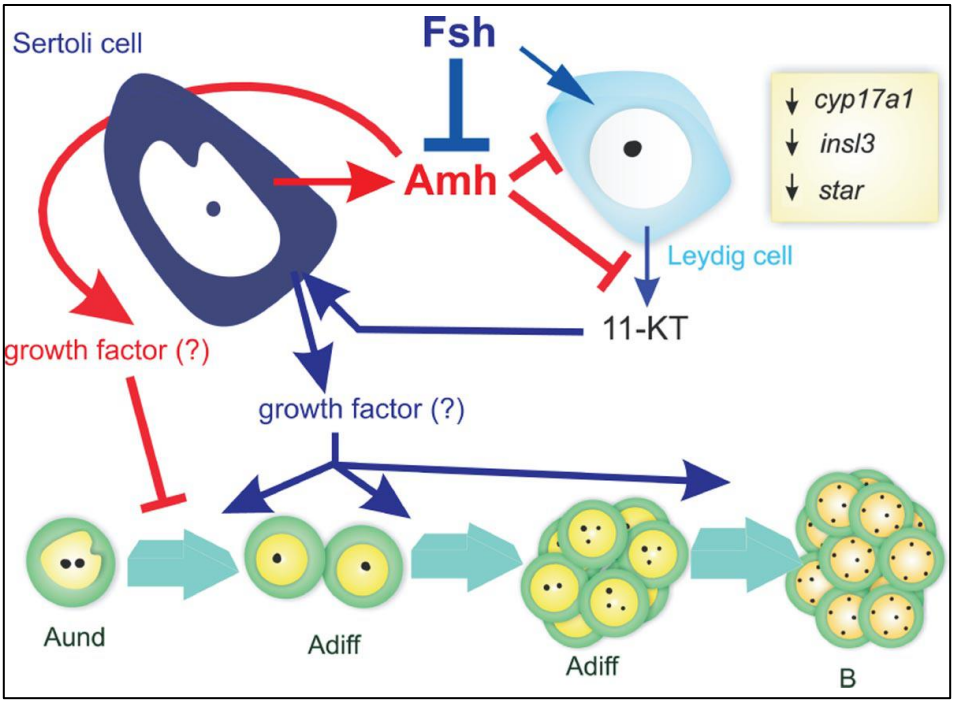


Figure 2: Amh effects on zebrafish testis functions. *FSH stimulates Leydig cells to produce the androgen 11-KT, by increasing the expression of genes related to androgen production. In their turn, the androgens stimulate spermatogenesis via the Sertoli cells. FSH also down-regulates the expression of* amh *mRNA levels. Amh reduces the production of 11-KT and the 11-KT stimulated spermatogenesis. Amh might functions via an autocrine loop, because germ cells do not have Amh receptors. From: Skaar* et al*, 2011.*

There are clear indications that FSH stimulates spermatogenesis in other ways, next to the stimulation of androgen production and the down-regulation of the inhibitory AMH. Trilostane is an inhibitor of 3β-hydroxy steroid dehydrogenase (3β-HSD), an enzyme necessary to produce biological active steroids. In tissue culture where estrogen pretreated zebrafish testes were incubated with this inhibitor and FSH, spermatogenesis still proceeded (Nobrega and Schulz, unpublished data). Hence, the proliferation and differentiation of spermatogonia is stimulated by FSH in an androgen-independent manner. Because FSH cannot regulate the germ cells directly, there has to be a mediator of the androgen-independent stimulation of FSH. A good candidate for this stimulation is a fish-specific insulin-like growth factor (IGF), IGF3. The IGF system is involved in many processes related to proliferation and differentiation. IGF signaling is involved in primordial germ cell survival and migration (Schlueter *et al.*, 2007), fin regeneration (Chablais & Jaźwiński, 2010) and notochord development (Zou *et al.*, 2009). In mammals and birds, there are only two IGFs. In zebrafish, four distinct IGF genes were identified, possibly as a result of a genome duplication (Wang *et al.*, 2007; Zou *et al.*, 2009). It is plausible that all IGFs mediate their effects by binding to the IGF-1R. The IGF subtypes all maintain a similar overall tertiary structure and all the IGFs are capable of interacting with this receptor and of activating the intracellular signaling (Wang *et al.*, 2007; Zou *et al.*, 2009). Both *igf-1Ra* and *igf-1Rb* were found to be expressed in the gonads of zebrafish (Maures *et al.*, 2002) and gilthead seabream (Perrot *et al.*, 2002).

Out the IGFs, IGF3 is the best candidate to mediate the stimulation of spermatogenesis by FSH. IGF3 is only expressed in the gonads, which suggests that it fulfills an important role in the gonads (Wang *et al.*, 2007; Zou *et al.*, 2009). In the ovary, *igf3* expression is regulated by gonadotropins and IGF3 is involved in oocyte maturation and in follicle cell survival (Irwin & van der Kraak, 2012; Li *et al.*, 2010, Nelson & van der Kraak, 2010). Not much is known yet about the role of IGF3 in the testes, but with immunohistochemistry it is already found that IGF3 protein is present in the Sertoli cells, presumably the Sertoli cells surrounding type A spermatogonia (Nobrega, unpublished results). Furthermore, injection of FSH was shown to cause an 20-fold up-regulation of *igf3* mRNA. Also, tissue culture with seabream IGF1 demonstrated that IGF1 is able to support spermatogenesis by stimulating both spermatogonial proliferation and the entry of spermatogonia into meiosis and spermatogenesis (dissertation Waal, 2009). It is possible that IGF1 and IGF3 are using the same receptor to exert their effect and therefore, these results suggest that IGF3 can stimulate spermatogenesis as well. Given that gonadotropins stimulate *igf3* expression and that IGF3 is produced in Sertoli cells, it is quite possible that IGF3 is a mediator of the effect of FSH on spermatogenesis. Different techniques were used in this master project to test this hypothesis on different levels and to find out more about the role and regulation of IGF3 in the testis. A gene expression analysis was done to examine the response of *igf3* and *amh* expression to different doses of FSH and a fish injection experiment with FSH was done to confirm the gene expression results on the protein level. Further, an inhibitor of the IGF1R was tested. The IGF1R kinase inhibitor NVP-AEW541, originally used as anti-cancer agent (Garcia-Echeverría *et al.*, 2004), was tested for its capability to inhibit the IGF3-mediated stimulation of spermatogenesis. In case it turns out that this inhibitor works, the inhibitor can be used in combination with trilostane to block both the IGF3 and the androgen actions, to see whether FSH can still stimulate the spermatogenesis in tissue culture. This experiment would confirm or reject the hypothesis of IGF3 mediating FSH stimulation of spermatogenesis. The regulation of zebrafish spermatogenesis is an important research subject. Knowledge about the regulation of fish spermatogenesis can give insight in the mammalian regulation of spermatogenesis as well, but more importantly, this information can be used to improve the production of fish for human consumption.

Material and methods

Animals

Sexually mature male and female zebrafish were obtained from the Biology department at Utrecht University, the Netherlands, and from UNESP University in Botucatu, Brasil. The fish were kept in water of 26 °C with constant aeration and were fed twice a day. Sexually mature male zebrafish were selected for pretreatment with estradiol. A stock solution of 10 mM estradiol was prepared with 4.05 mg of 17β-estradiol (E2, Sigma) and 1.5 L of MQ water. The solution was kept stirring at 40 °C and was used only 3 days after preparation. Further dilution to a concentration of 10 nM took place in the aquarium. The water was changed daily and the fish were kept under constant aeration and were fed at least once per day.

Tissue culture

Isolation of the testes took place in an laminar flow cabinet, using equipment sterilized in 70% ethanol each time used. Fish were anesthetized on ice water and killed by means of piercing the fish with a needle between the eyes. The body cavity was opened and the intestines were removed in order to isolate the testes. The testes were taken out of the fish carefully with a different set of equipment and were cleaned in phosphate buffered saline (PBS, PAA) in order to avoid contamination of the testes. Incubation was carried out in tissue culture-treated 24 wells plates (Costar) containing 1 mL of medium. The tissue was placed on sterilized pieces of white GSW filter paper (with nitrocellulose, 0.22 µm, Millipore), which were lying on top of agar blocks made with 700 µl of 1.5% agar dissolved in ringer solution (containing Mg^{2+} and Ca^{2+}) (fig. 3). Incubation took place at 25 °C in Leibovitz-15 medium, containing 15 g/l of Leibovitz-15 (Gibco), 0.25 µg/mL of fungizone (Gibco), 200 U/mL of penicillin (Gibco), 200 µg/mL of streptomycin (Gibco), 0.5% of bovine serum albumin (BSA Boehringer-Mannheim fraction, Sigma) and 0.01 M of HEPES (Merck). The pH of the medium was adjusted to 7.4 and the medium was sterilized with a 0.22 µm filter unit (PVDF membrane, Millopore). From each fish, one testis was incubated in basal medium and the other in experimental medium to adjust for interindividual differences.

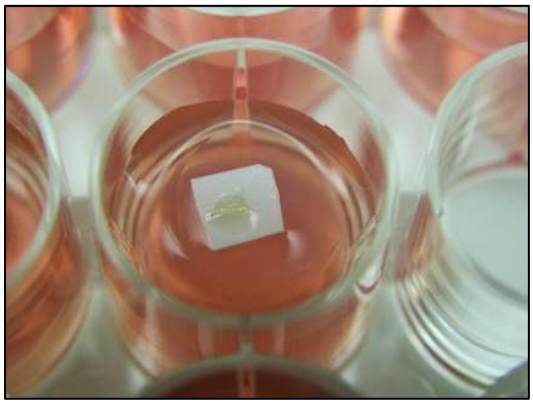


Figure 3: tissue culture with zebrafish testes. *Zebrafish testes were placed on GSW filter paper, lying on top of agar blocks, preventing the testes to sink in the medium.*

Testing the inhibitor

Tissue culture with the inhibitor

Tissue culture was performed as described above with untreated zebrafish (n=8) and with E2 treated zebrafish (n=8). A pre-incubation with NVP-AEW541 (Selleckchem) was done for 6 hours in the described medium containing in addition 0.01 nM of retinoic acid (RA, Sigma) and 5 µM of NVP-AEW541, dissolved in DMSO. Control medium contained 0.01 nM of RA, 100 ng/ml of rzfIGF3 and 0.05% DMSO. After the 6 hours of pre-incubation, both media were changed and then the experimental medium also included 100 ng/ml of rzfIGF3 as well. Before incubation, the medium was sterilized using syringe driven filters units of 0.22 µm (Millipore). The incubation lasted 7 days and meanwhile the medium was changed once. At the 7th day of tissue culture, BrdU incorporation was done in a tissue culture-treated 96 wells plate (Costar) for 6 hours. Testes were placed in 200 µl of the same medium as before, containing in addition 50 µg/ml of BrdU (Sigma) dissolved in a 0.9% NaCl solution. After 6 hours of incubation, testes were fixed in methacarn at 4 °C for at least 15 hours.

A second tissue culture was done for untreated zebrafish, using a concentration of 10 µM for NVP-AEW541, because this concentration was found to significantly affect apoptosis in zebrafish follicle culture (Irwin & van der Kraak, 2012). Also, the concentration of DMSO in the basal condition was adjusted to 0.1%.

BrdU detection

The tissue was dehydrated by incubating it in subsequently 70%, 80%, 95% and 3 times 100% ethanol for 30 minutes. Then the tissue was pre-embedded overnight and embedded in resin (Technovit 7100). The blocks of resin were attached to histoblocks (Kulzer) with Technovit 3040 and Technovit universal liquid. Sections of 3 µm thickness were made and incubated in periodic acid at 60 °C (1%, Merck) for half an hour. Thereafter, perioxidase was blocked by immersing the sections with 1% H_2O_2 dissolved in PBS for 10 minutes. Unspecific binding sites were blocked for 10 minutes with 5% normal goat serum (Vector) dissolved in PBS containing 1% BSA. Then, slides were incubated with anti-BrdU (1:80, BD biosciences) dissolved in PBS containing 1% of BSA. To prepare the slides for immunostaining, the slides were incubated with Bright vision poly-anti Ms/Rb/Ra IgG one component (Immunologic) for half an hour. For detection, slides were incubated for 3 till 7 minutes with DAB powder (0.5 mg/ml, Sigma) dissolved in 150 µl of PBS containing 0.01% H_2O_2. In case there was no clear BrdU signal visible after 7 minutes, $CoCl_2$ and Ni (both 0.005%) were added to the solution and the slides were incubate for 1 minute more. The nuclei were counterstained with hematoxuline gill no. 3 (EMS) for 3 minutes. After each step in the BrdU detection process, the slides were washed well in order to remove residual sufficiently.

BrdU labeling index

The effect of the tissue culture was analyzed by determining the BrdU labeling index for type A undifferentiated spermatogonia. The BrdU labeled and unlabeled type A undifferentiated spermatogonia were counted at a 1000x magnification under the light microscope. At least 100 cells were counted in maximal three sections per treatment. Type A undifferentiated spermatogonia were identified based on some or all of the following characteristics: single cell in a cyst, large volume, large amount of cytoplasm, irregular nuclear membrane and containing one or two nucleoli (Leal *et al.*, 2009;

Schulz *et al.*, 2010). The BrdU labeling index was defined as the percentage of BrdU labeled type A undifferentiated spermatogonia of the total amount of counted type A undifferentiated spermatogonia.

FSH dose response

Tissue culture for FSH

For the FSH dose response a tissue culture was done as described under 'tissue culture'. The testes were either incubated in the basal medium (as described) or in medium containing rzfFSH. Again, one testis of each fish was incubated in the control medium and the other one at experimental conditions. Different concentrations of FSH were used, namely 25, 50, 75, 100 and 1000 ng/ml. For each concentration 6 fish were used. The tissue was incubated at 25 °C for 5 days and then weighted and snap frozen in liquid nitrogen.

RNA extraction

RNA extraction was done with the RNAqeous Micro-kit (Ambion) according to the instructions of the producer. Briefly, 100 µl of lysate buffer was added to the tube and tissue was ruptured using sterile blue pestles (Invitrogen) and a rotator. After adding 50 µl of 100% ethanol, the solution was briefly centrifuged and placed on top of a micro filter. This was centrifuged in a precooled centrifuge until all the liquid passed through (30 seconds or more). After several wash steps, the residual was discarded and the RNA was dissolved in 3 times 10 µl of elution buffer at 75 °C. This solution was treated with DNase for 20 minutes at 37 °C. After incubation with DNase inactivating reagent, the supernatant was collected and measured with a Nanodrop spectrophotometer for purity and RNA concentration. Samples that turned out to have a low concentration of RNA were excluded from further processing, together with the pair sample.

cDNA synthesis

Single strand cDNA was synthesized from 2 µg of RNA diluted in DEPC-treated water (PAA). Synthesis was performed with 750 ng of random hexamers (Invitrogen), dNTPs (0.05 mM each, Invitrogen), 1st strand cDNA synthesis buffer, DTT (0.01 M), and 2.5 U/µl superscript II RNase H- reverse transcriptase (Superscript II RNase H- reverse transcriptase kit, invitrogen). After adding random hexamers, the solution was heated at 70 °C for ten minutes and then rapidly cooled to 4 °C. Incubation with the buffer, DTT and dNTPs took place at 25 °C for ten minutes. After adding the superscript II RNase H- reverse transcriptase the tubes were incubated at 42 °C for 50 minutes, then at 70 °C for 10 minutes, followed by rapidly cooling to 4 °C. In order to store the samples at -80 °C, 100 µL of DEPC-treated water was added to the sample.

qPCR

The cDNA was subjected to a qPCR using primers for the following genes of interest: *amh*, *cyp19a1a* (coding for an aromatase), *igf3* and *insl3* (a leydig specific gene, function unknown). The nuclear housekeeping genes *elongation factor 1α* and *18s* (rDNA) were used to standardize the samples. The appropriate primers, probes and detection assay for each gene are shown in table 1. For the Taqman essay, 10 µl of taqman assay, 0.4 µl of probe, 0.67 µl of both primers and 3.26 µl of DEPC treated water were added to each well. Then 5 µl of cDNA was added making a total volume of 20 µl in each well. For

the Sybergreen assay also 5 µl of cDNA was used, together with 10 µl of sybergreen assay and 2.5 µl of both primers. For the 18s assay , 5 µl of 200 times diluted cDNA, 10 µl of taqman assay, 1 µl of 18s assay and 4 µl of DEPC treated water were added to each well. The qPCR was performed in duplo in a 96 wells plate using a AB qPCR machine.

Table 1: *Primers, probes and detection assay for the genes analysed with qPCR. The detection assays were TaqMan universal PCR mastermix (Applied biosystems) and PowerSYBR Green PCR mastermix (applied biosystems).*

Gene	Primer	Probe	Detection assay
Amh	AD-AE	EF	Taqman
Cyp19	3403-3405		Sybergreen
Igf3	2680-2681		Sybergreen
Insl3	2466-2467		Sybergreen
ef1α	AG-AH	AI	Taqman
18s	18s assay		Taqman

Fish injection experiment

Fish injection

Mature zebrafish were injected with rzf FSH protein to test the effect of FSH on the IGF3 protein amount. The zebrafish (n=9), with an average weight of 0.5 g, were injected with 100 ng/gBW FSH diluted in water. Injection was carried out in the body cavity of the fish, using 1 mL insulin syringe U-100 and a small needle of size 23G. The fish were placed back in tanks with water of 26 °C under constant aeration for 6 hours and thereafter they were euthanized in ice water and killed by piercing a needle through the head. Both testes were collected and snap frozen in liquid nitrogen and stored at – 80 °C. Testes from non-injected zebrafish (n=5) from the same batch were used as a control.

Protein extraction

The frozen testes were pooled with testes from 3 animals per sample, because the weight of the testes was very low. The testes in the samples were disrupted in 100 µl of lysis buffer, containing ripa buffer and protease inhibitor in a ratio of 10:1. The tissue was disrupted by stirring and by taking up the tissue with a syringe with a big needle in the beginning and a small needle at the end. The samples were vortexed several times for more than a minute during disruption to enhance the process. The tissue was cooled on ice as much as possible by performing the disruption in steps and with multiple samples in order. After disruption, the samples were incubated on ice for at least one hour and vortexed every 15 minutes. Thereafter, the samples were centrifuged at 4000 rpm at 4 °C for 20 minutes. The supernatant was saved and measured for protein amount using a Nanodrop spectrophotometer. Samples were stored at -80 °C until processing.

Western blot

The proteins obtained from the fish were subjected to a western blot analysis to determine the amount of IGF3 protein in the samples. The average amount of protein in those samples was 37.7 mg/ml. For each sample a volume with a total amount of 400 µg protein was loaded on the gels. The samples were prepared for western blot by adding the loading buffer (Biorad), which included 2-mercaptoethanol (Biorad) in a 20 times dilution. The loading buffer was added in a ratio of 1:2, because a high volume of

each sample was necessary to obtain 400 µg of proteins. After adding the loading buffer, the proteins were delinearized by heating the samples until 95 °C for 5 minutes. Thereafter, the samples and 1.28 µg of pure rzfIGF3 were loaded on a 15% SDS gel (Biorad). After running the gel for 1-2 hours on 120 V, the proteins were transferred to a membrane at 300 mA for 75 minutes. After transfer, the membrane was placed in ponceau 0.1% for 1-2 minutes and washed in basal solution, containing trisma base (1 M), NaCl (5 M), 1 ml of tween 20 and deionized water until 2 L. Then, the aspecific binding sites were blocked with 3% non-fat milk for 1 hour and the membrane was placed in the antibody against IGF3 (2 µg/ml, rabbit) diluted in BSA 1% overnight at 4 °C. The membrane was washed in basal solution for 5x5 minutes and incubated with goat-anti-rabbit diluted in BSA 1% for 1 hour, also followed by 5x5 minutes rinsing in basal solution. The bands were detected using the chemiluminescent substrate kit (Thermo scientific).

Statistical analysis

The data obtained after tissue culture were analyzed by means of a paired student t-test. Data for the FSH dose response were log transformed. Results were represented as significant when p=0.05 or smaller.

Results

FSH dose response

Zebrafish testes were incubated with various concentrations of FSH to test the response in gene expression. This response was determined by gene expression analysis with extracted RNA after tissue culture. The results are expressed in relative q-values, which show the relative amount of RNA present in the sample, normalized with the geometric mean between the reference genes *18S* and *EF1α*. The gene expression analysis was done for *amh* (fig. 4A) and *igf3* (fig. 4B). A clear dose response curve is visible for *amh* expression, but the *amh* expression only differs significantly from the basal at high doses of FSH. The testes treated with 100 and 1000 ng/ml of FSH show a lower amount of *amh* RNA than the testes incubated in basal conditions. For *igf3* the opposite is true, because testes incubated with FSH show a higher amount of *igf3* RNA. Only incubation with 25 ng/ml of FSH did not give a significant increase, the other doses did. Also for *igf3*, a clear dose response curve is visible.

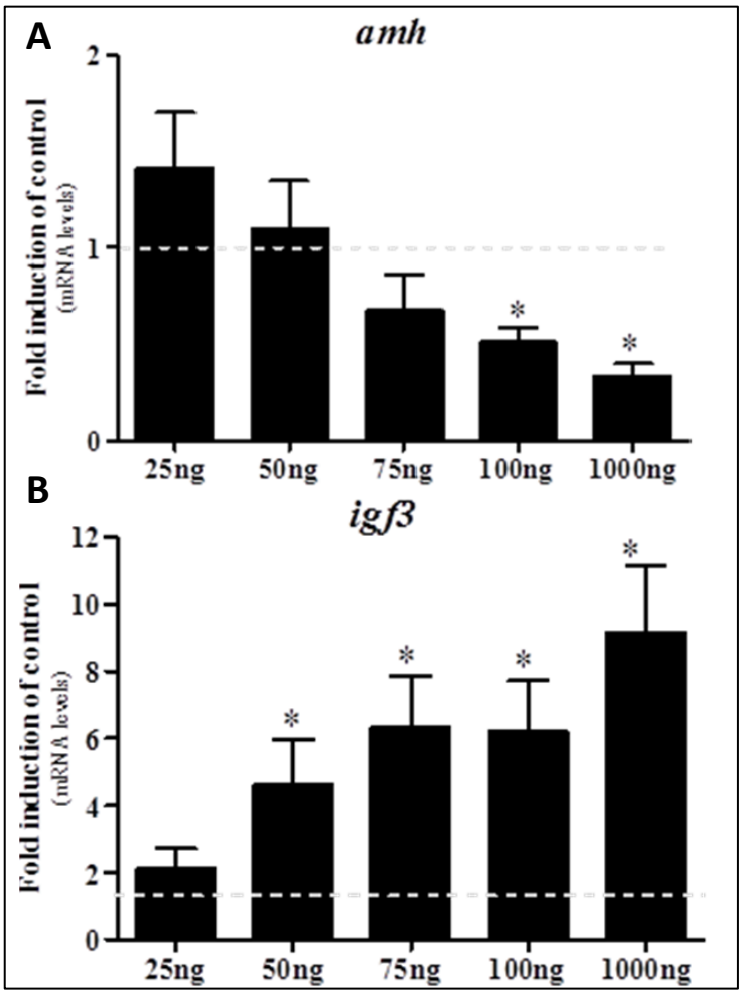


Figure 4: Gene expression in zebrafish testes after 5 days of basal or FSH treatment. *The relative amount of RNA of* amh *(A) and* igf3 *(B), determined by RNA extraction, cDNA synthesis and qPCR. Ct values were normalized by dividing by the geometric mean between the reference genes* 18S *and* EF1α*.*

FSH injection up-regulates IGF3 protein

FSH up-regulates the *igf3* expression in zebrafish testes. An *in vivo* experiment was done to test the effect of FSH on the IGF3 protein amount as well. Mature zebrafish were injected with rzf FSH protein and were killed 6 hours later to collect the testes. The proteins in those testes were extracted and subjected to a western blot analysis in order to determine the amount of IGF3 protein (fig. 5). The bands in the figure appear around 16 kD, which fits the length of the IGF3 protein (17 kD). According to the darkness and thickness of the bands, it seems that the testes form the injected fish contain a higher amount of IGF3 protein than the testes from non-injected fish. A loading control for β-actin was done, but this did not worked out. It is necessary to repeat the whole experiment to confirm the results obtained in this project.

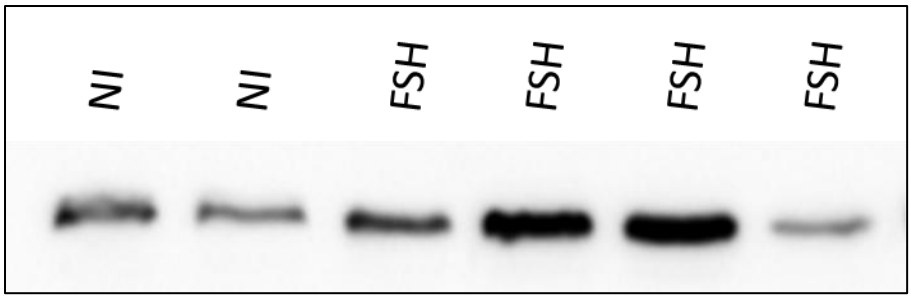


Figure 5: western blot for IGF3. *Proteins extracted from testes from non-injected fish (NI) and from fish injected with 100 ng/gBW FSH (FSH) were subjected to a western blot on a 15% SDS gel. The antibodies used were rabbit-anti-IGF3 and goat-anti-rabbit.*

IGFR1 inhibitor blocks IGF3 mediated stimulation of spermatogenesis

The IGFR inhibitor NVP-AEW541 was tested for its capability to block the IGF3 stimulated increase in spermatogenesis. This was checked by incubating zebrafish testes for 7 days with IGF3 or with IGF3 and NVP-AEW541. At the last day of incubation BrdU was incorporated. After dehydration, embedding and detection of BrdU, the non-proliferating and proliferating type A undifferentiated spermatogonia were quantified. The tissue culture was done with testes of untreated fish (fig. 6A) and of fish pretreated with E2 for 21 days (fig. 6B). The results are represented in the BrdU index of the type A undifferentiated spermatogonia, which is the percentage of BrdU labeled cells with respect to the total amount of type A undifferentiated spermatogonia. BrdU labeling is an indication that the cell is proliferating. For the tissue culture with testes from the untreated fish, there is a clear difference in the BrdU index between the IGF3 and the IGF3 and NVP-AEW541 treatment. In the testes treated only with IGF3, on average 40% of the type A undifferentiated spermatogonia are dividing. In the testes treated with IGF3 and NVP-AEW541 solely 24% of the type A undifferentiated spermatogonia is dividing. For the tissue culture with testes from E2 pretreated fish no significant difference in the BrdU index for type A undifferentiated spermatogonia was found.

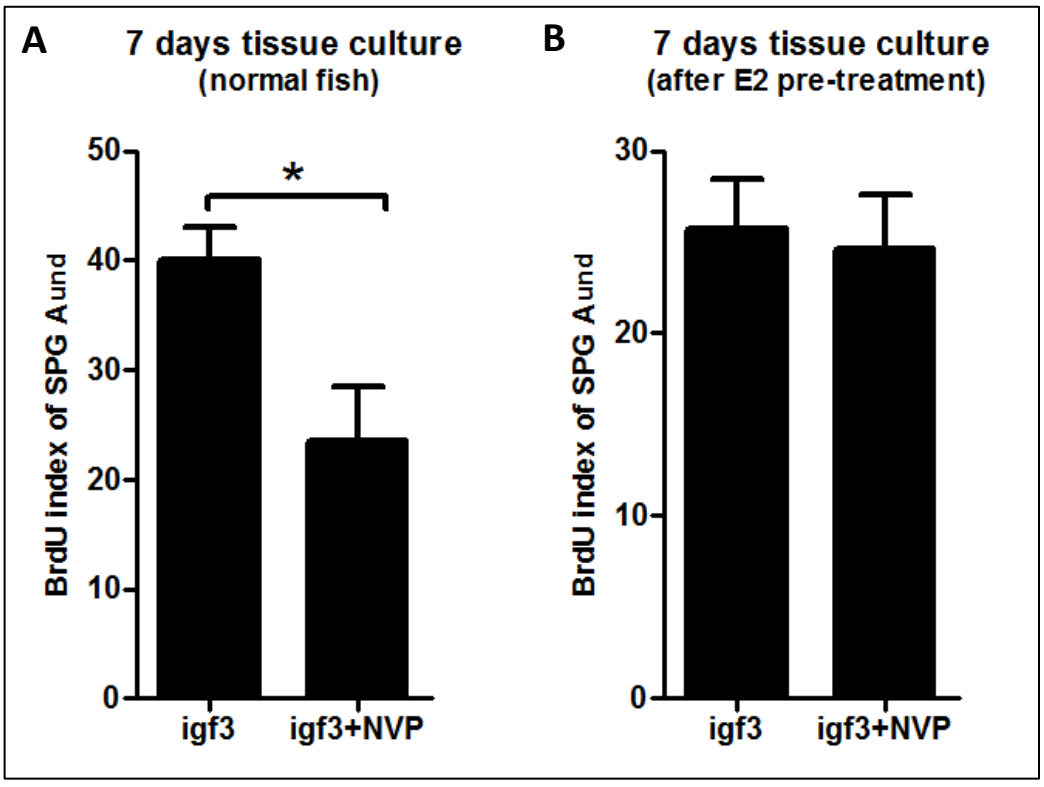


Figure 6: BrdU index of type A undifferentiated spermatogonia after tissue culture with IGF3 or IGF3 and NVP-AEW541. *The percentage of BrdU labeled type A undifferentiated spermatogonia with respect to the total amount of type A undifferentiated spermatogonia in zebrafish testes after tissue culture with untreated fish (A) or fish pretreated with E2 for 21 days (B). One testes of each fish was incubated only with 100 ng/ml of IGF3 and the other was treated with 5 µM NVP-AEW541 as well. For NVP-AEW541 a pre-incubation of 6 hours was used.*

Discussion

FSH dose response

A gene expression analysis was done to examine the response of the testis to different doses of FSH. This hormone plays a key role in regulating fish spermatogenesis. FSH is able to induce spermatogenesis in tissue culture with testes of the Japanese Eel (Ohta *et al.*, 2007) and zebrafish (Nobrega, unpublished results). Unlike in mammals, FSH is able to stimulate both the Leydig cells and the Sertoli cells in fish testis (Schulz *et al.*, 2010). Therefore, FSH can influence spermatogenesis in several ways. Firstly, it stimulates the Leydig cells to release the androgen 11-KT, which is able to support the entire process of spermatogenesis in zebrafish testes (García-López *et al.*, 2010; Leal *et al.*, 2009; Skaar *et al.*, 2011). FSH also influences the expression of the growth factor AMH (Skaar *et al.*, 2011). This growth factor is expressed in Sertoli cells and inhibits spermatogenesis. AMH also reduces or blocks the FSH stimulated release of 11-KT and also inhibits the 11-KT stimulated spermatogenesis, by blocking the differentiation and reducing the proliferation of type A undifferentiated spermatogonia. It was found that the *amh* mRNA levels were down-regulated by FSH (Skaar *et al.*, 2011). This was also found in the gene expression analysis in this study. Testes of adult zebrafish were incubated with different doses of FSH in tissue culture. Two doses of FSH resulted in a significant down-regulation of *amh* mRNA. This confirms the findings of Skaar *et al.* (2011). The goal of the tissue culture was to find the dose response curve of *amh* expression for FSH. The two doses, which resulted in a significant down-regulation, were the highest doses used. A minimum of 100 ng/ml of FSH for five days was necessary in order to reduce the *amh* expression. Thus, a clear dose response curve was found for the *amh* expression. Next to the stimulation of androgen release and the down-regulation of *amh* expression, FSH still affects spermatogenesis in another, androgen-independent manner. A candidate for mediating the other way in which FSH stimulates spermatogenesis is IGF3. The IGF3 protein is present in Sertoli cells, presumably the Sertoli cells surrounding type A spermatogonia (Nobrega, unpublished results). An injection of FSH in zebrafish caused an up-regulation of *igf3* mRNA (dissertation Waal, 2009). In the gene expression analysis in this study, this was also found. Incubation of zebrafish testes with FSH in tissue culture resulted in up-regulation of *igf3* expression. Only incubation with 25 ng/ml of FSH did not give a significant increase, the other doses did. Also for *igf3* a dose response curve for FSH is visible. Thus, the gene expression analysis following the tissue culture with FSH confirmed earlier results about the up-regulation of *igf3* and the down-regulation of *amh*. Clear dose response curves were found for both genes.

Fish injection experiment

Igf3 expression is up-regulated by FSH. A fish injection experiment was done to check the response to FSH on protein level. Fish were injected with FSH and killed 6 hours later in order to extract the protein from the testes. The proteins were subjected to a western blot analysis for the IGF3 protein. It seemed that the injected fish had a higher amount of IGF3 in the testes than the non-injected fish. A standardization was still required, because it is not certain that the exact same amount of protein was

loaded in each well. A loading control for β-actin was done, but this did not worked out. It is necessary to repeat the whole experiment to confirm the results obtained in this project.

IGF1R inhibitor

Tissue culture experiments were used to test the capability of the IGF1R inhibitor NVP-AEW541 to block the IGF3 mediated stimulation of spermatogenesis. NVP-AEW541 inhibits the autophosphorylation of the IGF1R by blocking the ATP binding site and was originally introduced as anti-cancer agent (Garcia-Echeverria *et al.*, 2004). Although NVP-AEW541 was designed to inhibit mammalian IGF1R, it was shown that NVP-AEW541 also prevents autophosphorylation of the IGF1R in zebrafish. Immunofluorescence showed that exposure to the inhibitor resulted in lower levels of p-IGF1R (Chablais & Jaźwiński, 2010). The inhibitor was already used in research on follicular apoptosis and fin regeneration in zebrafish, where NVP-AEW541 treatment resulted in impaired fin regeneration or a reduced follicular apoptosis (Chablais & Jaźwiński, 2010; Irwin & van der Kraak, 2012). In this study, NVP-AEW541 was used to block the stimulatory effect of IGF3 on zebrafish spermatogenesis. Although treatment with NVP-AEW541 did not completely block the stimulatory effect, it caused a clear reduction in spermatogonial proliferation in the testes. The number of BrdU labeled type A undifferentiated spermatogonia was much lower in NVP-AEW541 treated testes in comparison with testes only treated with IGF3. A basal activity of spermatogonia could be the effect of other stimulatory factors. This indicates that NVP-AEW541 is able to reduce the stimulatory effect of IGF3 on the type A spermatogonial proliferation. However, the tissue culture with testes form E2 pretreated fish did not show a difference between the control and NVP-AEW541 treated at all. An explanation could be that the E2 treatment already results in a down-regulation of IGF1R in the testes and therefore, there is no difference in NVP-AEW541 treatment and control. However, this explanation does not seem plausible because Filby *et al.* (2006) found that *igf1r* expression in the gonads did not change after 14 days of a 35 ng/l E2 treatment in fathead minnow. Interestingly, the treatment with 10 µM of NVP-AEW541 resulted in a reduction in spermatogonial proliferation for both untreated and E2 pretreated fish, in contrast with the 5 µM treatment (Morais, unpublished results). A possibility is that the E2-treatment causes a higher sensitivity for IGF3, so that only a higher concentration of NVP-AEW541 causes a reduction of spermatogonial proliferation. It is necessary to repeat the tissue cultures to elucidate and confirm the previous results.

These experiments were repeated and recently, NVP-AEW541 was used in tissue culture in combination with trilostane and FSH. The results of this tissue culture showed that FSH was still able to stimulate the spermatogenesis. This shows that IGF3 does not mediate the androgen-independent stimulation by FSH alone, but the possibility that IGF3 mediates the androgen-independent stimulation together with other stimulatory factors cannot be excluded. Moreover, the experiments were repeated and there might be a partial inhibition in spermatogonial proliferation as a result of NVP-AEW541. Hence, IGF3 could be mediating the androgen-independent stimulation by FSH together with other factors. Those factors should be produced by the Sertoli cells and are likely factors that do not operate via the IGF1-R. Factors that are produced by Sertoli cells and regulate spermatogenesis are gonadal soma derived factor (GSDF) and an orthologue of platelet-derived endothelial cell growth factor (PD-ECGF) (Schulz *et al.*, 2010). These factors are possible other mediators of the FSH androgen-independent stimulation of spermatogenesis.

IGF3 effect on spermatogenesis

The results from the tissue culture with NVP-AEW541 demonstrated that IGF signaling stimulates spermatogenesis. Blocking the IGF1R receptor resulted in a decrease in proliferation of type A spermatogonia, which shows IGF involvement in spermatogenesis. The IGF system was already shown to be involved in a lot of processes related to proliferation and differentiation. In zebrafish it was found that IGF signaling is involved in primordial germ cell survival and migration, fin regeneration and midline and notochord development (Chablais & Jaźwiński, 2010; Schlueter *et al*., 2007; Zou *et al.*, 2009). The involvement of the IGF system in spermatogenesis is not surprising, because this developmental process is all about proliferation and differentiation. In addition, a lot of studies have proved that IGF signaling is involved in the mammalian spermatogenesis as well. In testes from IGF1 null mutant mice, only a small amount of the spermatogenesis sustained, due to low testosterone levels (Baker *et al.*, 1996). Later studies also showed that IGF1 is involved in testosterone production by the leydig cells. Wang *et al.* (2003) found that in IGF1 null mice, there was an imbalance between testosterone biosynthetic and metabolic enzymes, resulting in low levels of testosterone in the blood (Wang *et al.*, 2003). Also in other parts of the animal kingdom, IGFs were found to stimulate spermatogenesis. In the newt, an aquatic amphibian, it was found that IGF1 and IGF2 promote the differentiation from spermatogonia into spermatocytes (Nakayama *et al.*, 1999). It appears that IGF signaling is involved in the male reproduction process throughout the animal kingdom. In fish, IGF1 en IGF2 are also expressed in the gonad, but the fish genome contains two more IGF genes, probably by a genome duplication (Zou *et al.*, 2009). Until recently, not much was known about the role of the new IGFs in fish. In this study, it was shown that IGF3 stimulates spermatogenesis by promoting the type A spermatogonial proliferation. This was also found in an earlier tissue cultures at our lab (Nobrega, unpublished results) and it also agrees with the stimulation of spermatogonial proliferation with seabream IGF1 (dissertation Waal, 2009), because both IGFs are able to activate the IGF-1R (Zou *et al.*, 2009). Here, recombinant zebrafish IGF3 was used, which simulates the natural situation better. The IGF3 stimulated proliferation of type A spermatogonia accords well with the fact that *igf3* expression and IGF3 protein is mostly found in Sertoli cells surrounding type A spermatogonia (Nobrega, unpublished results; dissertation Waal, 2009). Because all the IGFs are capable of interacting with the IGF1R and activating the intracellular signaling, it could be that another IGF is the biologic functional stimulator of spermatogenesis (Zou *et al.*, 2009). For several reasons, IGF3 is the most obvious candidate. Firstly, *igf3* is only expressed in the gonads, which suggest that it fulfills an important role in the gonadal processes. This is in contrast with the other *igfs* that are expressed widely in various tissues including the brains, the gills and the liver (Wang *et al.*, 2007). Second, *igf3* mRNA is found in Sertoli cells, which are the main executors of supporting spermatogenesis (dissertation Waal, 2009). Furthermore, *igf3* expression highly increases following FSH injection in zebrafish (dissertation Waal, 2009). Less certain is the way in which IGF3 exerts its function. An autrocrine effect of IGF1 was found in mouse by Froment *et al.*, 2007. Inactivation of the IGF-1R caused a reduced proliferation and an increased cell death of Sertoli cells. Also, the bulk of the IGF1R was found to be present on somatic cells in the zebrafish testes (dissertation Waal, 2009). However, in gilthead seabream and in rainbow trout the IGF1R was also found on spermatogonia and primary spermatocytes (Le Gac *et al.*, 1996; Perrot *et al.*, 2000). In seabream it was found specifically on the membranes of type A spermatogonia (Le Gac *et al.*, 1996). Also in newt a binding assay for IGF1 showed that both Sertoli

cells and germ cells (spermatogonia and primary spermatocytes) possess the IGF1R (Nakayama *et al.*, 1999). On basis of these facts, IGF3 could operate by autocrine effects, paracrine effects or by a combination of both.

Conclusion

Gene expression analysis showed that FSH up-regulates *igf3* expression and the western blot indicates that FHS increases IGF3 protein amount in the testes. Tissue culture with NVP-AEW541 shows that this inhibitor is able to block the IGF3 stimulated increase in type A spermatogonia and confirms that IGF3 exerts an important function in spermatogenesis. Recent experiments with the inhibitor indicate that IGF3 is not the sole mediator of the androgen-independent stimulation by FSH, but might be mediating the FSH stimulation together with other factors. Furthers research could be aimed at clarifying the exact role of IGF3 in the testes and at discovering the way in which IGF3 exerts its function. Further, other possible mediators could be investigated for their role in the testes in order to gain more insight in the regulation of fish spermatogenesis.

Summary

Spermatogenesis is the process in which haploid flagellated spermatozoa arise from undifferentiated spermatogonial stem cells. This process takes place in the testes and is well regulated by the pituitary hormones, luteinizing hormone (LH) and follicle-stimulation hormone (FSH). In mammals, FSH targets the Sertoli cells, which support the germ cells during development, and LH targets the steroid producing Leydig cells. In fish, FSH is able to stimulate the production of steroids by the Leydig cells too. FSH is suspected to be the gonadotropin that regulates the early stages of spermatogenesis. It stimulates the spermatogenesis in several ways. Firstly, FSH stimulates the production is 11-KT, the main androgen in fish. This androgen stimulates the proliferation and differentiation of the spermatogonia via the Sertoli cells. FSH further down-regulates the release of anti-Müllerian hormone (AMH), an Sertoli cells derived growth factor that inhibits spermatogenesis. Next to the stimulation of androgen production and the down-regulated of AMH, FSH stimulates spermatogenesis in another way. A possible mediator of this stimulation is insulin-like growth factor 3 (IGF3). This factor is a fish-specific IGF and is only expressed in the gonads, which suggests an important role in spermatogenesis. It was already found that *igf3* expression is stimulated by gonadotropins and that *igf3* is expressed in Sertoli cells. Further, it was found that IGF1 stimulates the spermatogenesis, which indicates that IGF3 can stimulate spermatogenesis too. Therefore, it is quite possible that IGF3 is a mediator of the effect of FSH on spermatogenesis. Different techniques were used in this master project to test this hypothesis on different levels and to find out more about the role and regulation of IGF3 in the testis. Firstly, a gene expression analysis was done to examine the response in *igf3* and *amh* expression to different doses of FSH. This analysis confirms that FSH stimulates *igf3* expression and down-regulates *amh* expression. Clear dose response curves were found for both genes. Second, a fish injection experiment was done in order to determine the effect of FSH on IGF3 protein level. This experiment indicates that FSH injection causes an increase in IGF3 protein level in the testes, but the experiment needs to be repeated. Lastly, NVP-AEW541, an inhibitor of the IGF1R, was tested for its capability to inhibit the IGF3-mediated stimulation of spermatogenesis. This inhibitor caused a clear reduction in spermatogonial proliferation in the testes. Therefore, this inhibitor can be used to test whether IGF3 is mediating the androgen-independent stimulation by FSH.

Acknowledgements

Rüdiger, I would like to thank you for your feedback on this report and for your patience in your explanations. Whenever I wanted your advice, I could walk into your room and step out of it much wiser half an hour later. Roberto, I would like to thank you for your daily supervising, the patience you took to explain the different techniques to me and all the nice conversations we had during lunch time and work. Furthermore, I would like to thank Rafael for the opportunity to go to Brazil. It was a great experience and I learned a lot about doing research and about another culture.

References

Baker, J., Hardy, M.P., Zhou, J., Bondy, C., Lupu, F., Bellvé, A.R. and Efstratiadis, A. (1996). Effects of an IGF1 gene null mutation on mouse reproduction. *Molecular endocrinology* 10: 903-918.

Chablais, F. and Jaźwiński, A. (2010) IGF signaling between blastema and wound epidermis is required for fin regeneration. *Development* 137: 871-879.

Filby, A.L., Karen, L.T. and Tyler, C.R. (2006) Multiple molecular effect pathways of an environmental oestrogen in fish. *Journal of molecular endocrinology* 37: 121-134.

Froment, P., Vigier, M., Nègre, D., Fontaine, I., Beghelli, J., Cosset, F.L., Holzenberger, M. and Durand, P. (2007) Inactivation of the *IGF-1* receptor gene in primary Sertoli cells highlight the autocrine effects of IGF-1. *Journal of endocrinology* 194: 557-568.

Garcia-Echeverría, C., Pearson, M.A., Marti, A., Meyer, T., Mestan, J., Zimmermann, J., Gao, J., Brueggen, J., Capraro, H.G., Cozens, R., Evans, D.B., Fabbro, D., Furet, P., Prota, D.G., Liebetanz, J., Martiny-Baron, G., Ruetz, S. and Hofmann, F. (2004) *In vivo* antitumor activity of NVP-AEW541 – a novel, potent, and selective inhibitor of the IGF-1R kinase. *Cancer cell* 5: 231-239.

García-López, A., Bogerd, J., Grenneman, J.C., van Dijk, W., Trant, J.M., Taranger, G.L. and Schulz, R.W. (2009) Leydig cells express follicle-stimulation hormone receptors in African catfish. *Endocrinology* 150: 357-365.

García-López, A., de Jonge, H., Nobrega, R.H., de Waal, P.P., van Dijk, W., Hemrika, W., Tranger, G.L., Bogerd, J. and Schulz, R.W. (2010) Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropin. *Endocrinology* 151: 2349-2360.

Irwin, D.A. and van der Kraak, G. (2012) Regulations and actions of insulin-like growth factors in the ovary of zebrafish (*Danio Rerio)*. *General and comparative endocrinology reviews:* 177: 187-194.

Josso, N., Picard, J.Y., Rey, R. and di Clemente, N. (2006) Testicular anti-Müllerian hormone: history, genetics, regulation and clinical applications. *Pediatric endocrinology* 3; 347-358.

Leal, M.C., Waal, de P.P., García-López, Chen, S.X., Bogerd, J. and Schulz, R.W. (2009) Zebrafish primary testis tissue culture: an approach to study testis function *ex vivo*. *General and comparative endocrinology* 162: 134-138.

Li, J., Liu, Z., Wang, D. and Cheng, C.H.K. (2010) Insulin-like growth factor 3 is involved in oocyte maturation in zebrafish. *Biology of reproduction* 84: 476-486.

Maures, T., Chan, S.J., Xu, B., Sun, H., Ding, J. and Duan, C. (2002) Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish. *Endocrinology* 143: 1858-1871.

Miura, T., Yamouchi, K., Takahashi, H. and Nagahama, Y. (1991) Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *Proceedings of the national academy of sciences* 88: 5774-5778.

Miura, T., Miura, C., Konda, Y., Yamouchi, K. (2002). Spermatogenesis-preventing substance in Japanese eel. *Development* 129: 2689-2697.

Nakayama, Y., Yamamoto, T.and Abé, S. (1999). IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. *Integrative journal of developmental biology* 43: 343-347.

Nelson, S.N. and van der Kraak, G. (2010) Characterization and regulation of the insulin-like growth factor (IGF) system in zebrafish (*Danio rerio*) ovary. *General and comparative endocrinology* 168: 111-120.

Nobrega, R.H., Greebe, C.D., van der Kant, H., Bogerd, J., França, L.R. and Schulz, R.W. (2010) Spermatogonial stem cell niche and spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *Plos one* 5: e12808.

Ohta, T., Miyake, H., Miura, C., Kamei, H., Aida, K. and Miura, T. (2007) Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. *Biology of reproduction* 77: 970-977.

Perrot, V., Moiseeva, E.B., Gozes, Y., Chan, S.J. and Funkenstein, B. (2000) Insulin-like growth factor receptors and their ligands in gonads of a hermaphroditic species, the gilthead seabream (*Sparus aurata*): expression and cellular localization. *Biology of reproduction* 63: 229-241.

Schlueter, P.J., Peng, G., Westerfield, M. and Duan, C. (2007) Insulin-like growth factor receptor 1b is required for zebrafish primordial germ cell migration and survival. *Cell death and differentiation* 14: 1095–1105.

Schulz, R.W., de Franca, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H. and Miura, T. (2010) Spermatogenesis in fish. *General and comparative endocrinology* 165: 390–411.

Schulz, R.W. and Nóbrega R.H. (2011). Regulation of spermatogenesis. In: Farrel A.P., (ed.), *Encyclopdia of fish physiology: from genome to environment,* volume 1: 627-634. San Diego: Academic Press.

Skaar, K.S., Nobrega, R.H., Magaraki, A., Olsen, L.C., Schulz, R.W. and Male, R. (2011) Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* 152: 225-234.

Silverthorn, D.E., Johnson, B.R., Ober, W.C., Garrison, C.W., Silverthorn, A.C. (2010). Reproduction and development. In: Silverthorn, D.U. *Human physiology: an integrated approach*. San Francisco, Pearson Education: 828-862.

de Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W. and Bogerd, J. (2008) Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio)* testis. *Reproduction* 136: 225-234.

de Waal, P.P., Leal, M.O., García-López, A., Liarte, S., Jonge, H. de, Hinfray, N., Brion, F., Schulz, R.W. and Bogerd, J. (2009) Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis. *Journal of endocrinology* 202: 287-297.

de Waal, P.P., Leal, M.C., García-López, A., Nobrega, R.H., Skaar, K.S., van Dijk, W., Bogerd, J. and Schulz, R. Paracrine effects of insulin-like growth factors on adult zebrafish testis. Manuscript in preparation.

Wang, D.S., Jiao, B., Hu, C., Huang, X., Liu, Z. and Cheng, C.H.K. (2008) Discovery of a gonad-specific IGF subtype in teleost. *Biochemical and biophysical research communication* 367: 336-341.

Wang, G.M., O'Shaughnessy, P.J., Chubb, C., Robaire, B. and Hardy, M.P. (2003) Effects of insulin-like growth factor I on steroidogenic enzyme expression levels in mouse leydig cells. *Endocrinology* 144: 5058-5064.

Zou, S., Kamei, H., Modi, Z. and Duan, C. (2009) Zebrafish IGF genes: gene duplication, conservation and divergence, and novel roles in midline and notochord development. *Plos one 9:* e7026.