

**UNIVERSIDADE FEDERAL DE MINAS GERAIS
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**AVALIAÇÃO DA ATIVIDADE BIOLÓGICA DO PEPTÍDEO 3
SEMELHANTE À INSULINA (INSL3) NOS TESTÍCULOS DE ZEBRAFISH
(*Danio rerio*) ADULTOS**

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**Belo Horizonte
Julho 2015**

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Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas
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**ATA DA DEFESA DE TESE DE DOUTORADO DE
LUIZ HENRIQUE DE CASTRO ASSIS**

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Às **treze horas** do dia **17 de julho de 2015**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado de Programa, para julgar, em exame final, o trabalho final intitulado: "**AVALIAÇÃO DA ATIVIDADE BIOLÓGICA DO PEPTÍDEO 3 SEMELHANTE Á INSULINA (INSL3) NOS TESTÍCULOS DE ZEBRAFISH (DANIO RERIO) ADULTOS**", requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, o Presidente da Comissão, **Dr. Luiz Renato de França**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dr. Luiz Renato de França	UFMG	APROVADO
Dr. Sergio Ricardo Batlouni	CAUNESP	APROVADO
Dr. José Enemir dos Santos	PUC/MG	APROVADO
Dr. José Dias Corrêa Júnior	UFMG	APROVADO
Dr. José Carlos Nogueira	UFMG	APROVADO

Pelas indicações, o candidato foi considerado: APROVADO

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 17 de julho de 2015.**

Dr. Luiz Renato de França (Orientador) _____

Dr. Sergio Ricardo Batlouni _____

Dr. José Enemir dos Santos _____

Dr. José Dias Corrêa Júnior _____

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Cristina Guacimosa Pinheiro
Coordenadora do Programa de Pós-Graduação em Biologia Celular / UFMG

Dedico este trabalho aos meus pais, Vera e Sirlei, e às
minhas irmãs, Marina e Maria Lúcia, por todo o
suporte e amor!

“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota.”

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Lista de abreviaturas

- 11-KT** – 11-cetotestosterona
- A_{diff}** – Espermatogônias do tipo A diferenciadas
- Amh** – Hormônio anti-mülleriano
- Arg** – Arginina (*Arginine*)
- Asp** – Ácido aspártico (*Aspartate*)
- A_{und}** – Espermatogônias do tipo A indiferenciadas
- BMP4** – Proteína morfogenética óssea 4 (*bone morphogenetic protein 4*)
- BrdU** – Deóxi-bromo uridina (*5-bromo-2'-deoxyuridine*)
- CSF1** – Fator estimulador de colônia 1 (*colony stimulating factor 1*)
- E2** – 17 β -estradiol
- Ef1 α** - Fator de alongamento eucariótico alfa 1 (*eukaryotic translation elongation factor 1 alpha*)
- Fsh/FSH** – Hormônio folículo estimulante
- GDNF** – Fator neurotrófico derivado das células da glia (*glial cell line-derived neurotrophic factor*)
- GFR α 1** – Receptor α 1 para fator neurotrófico derivado das células da glia (*glial cell line-derived neurotrophic Family receptor α 1*)
- Gln** – Glutamina (*Glutamine*)
- Glu** – Ácido glutâmico (*Glutamate*)
- Gsdf** – Fator derivado de célula somática gonadal (*gonadal somatic cell derived factor*)
- hINSL3** – INSL3 humano
- His** – Histidina (*Histidine*)
- Igf3** - Fator de crescimento semelhante a insulina 3 (*insulin-like growth factor 3*)
- Ile** – Isoleucina (*Isoleucine*)
- Insl3/ INSL3** – Peptídeo 3 semelhante a insulina (*insulin-like 3*)
- Lh/LH** – Hormônio luteinizante
- Nanos2/NANOS2** – Fator intrínseco de células germinativas (*germ cell-intrinsic factor*)
- OA** – 11-cetoandrostenediona
- Odf3b** – Fibra densa exterior 3b de calda de espermatozoide (*Outer Dense Fiber Of Sperm Tails 3b*)
- OHA** – 11 β -hidroxiandrostenediona
- Phe** – Fenilalanina (*Phenylalanine*)
- Piwil1** – Elemento P semelhante dos testículos de Drosófila 1 (*P-element induced wimpy testis in Drosophila1*)
- Rxfp/RXFP** – Receptores para relaxinas (*Relaxin Family peptide receptors*)
- SSC** – Células tronco espermatogoniais
- Sycp3** – Proteína do complexo sinaptonêmico 3 (*synaptonemal complex protein 3*)
- Trp** – Triptofano (*Tryptophan*)
- Val** – Valina (*Valine*)

RESUMO

A espermatogênese é um processo cíclico e organizado que resulta na formação do gameta masculino, o espermatozoide, a partir de células espermatogoniais indiferenciadas. Estas espermatogônias indiferenciadas podem sofrer divisões que resultarão na produção de células comprometidas com o desenvolvimento da espermatogênese (diferenciação) ou na produção de novas espermatogônias indiferenciadas (auto renovação), sendo o balanço entre estes dois tipos de divisão crucial para a manutenção da espermatogênese ao longo da vida reprodutiva do animal. A regulação do equilíbrio entre a auto renovação e a diferenciação das espermatogônias indiferenciadas em peixes adultos é um processo ainda pouco conhecido, particularmente nos aspectos relacionados à influência de fatores parácrinos. O INSL3 (peptídeo 3 semelhante à insulina) é membro da família relaxina de peptídeos expresso pelas células de Leydig nos testículos dos vertebrados. Em mamíferos, é sabido que o INSL3 é importante no processo de descenso testicular durante o desenvolvimento e para a sobrevivência de células germinativas durante a vida adulta. Em peixes, embora não ocorra processo de descenso testicular, o gene *insl3* é altamente expresso pelas células de Leydig, não somente durante o desenvolvimento mas também durante a vida adulta, indicando a existência de potenciais funções biológicas ainda não identificadas conservadas ao longo do processo evolutivo. Estudos anteriores demonstraram que o hormônio anti-mülleriano (Amh), além de inibir a diferenciação espermatogonial e a produção de andrógenos, foi capaz de reduzir o efeito estimulatório do hormônio Folículo estimulante (Fsh) sobre a transcrição do gene *insl3* nos testículos de zebrafish. Neste contexto, decidimos investigar se o Insl3 em zebrafish teria uma função antagonista àquela observada para o Amh. Assim, nossos estudos envolvendo cultura de testículo de zebrafish demonstraram que o INSL3 humano (hINSL3) aumentou o índice de proliferação de espermatogônias do tipo A indiferenciadas (A_{und}), ao mesmo tempo em que reduziu o índice de proliferação das células de Sertoli associadas a este tipo espermatogonial quando estas células germinativas apresentavam-se em proliferação. Ainda, experimentos em cultura utilizando-se testículos de zebrafish com espermatogônias pré-marcadas com BRdU *in vivo* mostraram que o hINSL3 reduziu a área e o número de cistos de espermatogônias do tipo A_{und} no parênquima testicular, enquanto a área ocupada por cistos de espermatogônias do tipo A diferenciadas (A_{diff}) mostrou-se aumentada. Estas análises morfométricas foram confirmadas pelas análises moleculares, nas quais o PCR quantitativo demonstrou que o hINSL3 reduziu de forma significativa a transcrição do gene *nanos2*, que é um marcador de espermatogônias indiferenciadas em zebrafish e outros vertebrados. Desta forma, concluímos que o hINSL3 promoveu a diferenciação das espermatogônias do tipo A_{und} em cultura de testículos de zebrafish. No entanto, nossos estudos não demonstraram ação moduladora do hINSL3 na produção e liberação de andrógenos. Finalmente, estudo utilizando-se animais com deficiência androgênica e de gonadotrofinas, em decorrência do tratamento *in vivo* com o hormônio 17 β -estradiol, mostrou que a transcrição do gene *insl3* é drasticamente reduzida em condições favoráveis a auto renovação e, ainda, que a recuperação parcial da espermatogênese observada após a introdução do hormônio 11-cetoandrostenediona no tratamento *in vivo* foi independente da elevação dos níveis de transcrição do gene *insl3*. Neste contexto, nossos resultados indicam que o hINSL3 estimula a proliferação e diferenciação de espermatogônias do tipo A_{und} nos testículos de zebrafish sexualmente maduros independentemente da participação de andrógenos, potencialmente como um mediador dos efeitos do Fsh na espermatogênese.

ABSTRACT

Spermatogenesis is a cyclical and organized process that leads to the production of male gamete, the spermatozoa, from undifferentiated spermatogonia. Undifferentiated spermatogonia have the capacity to produce, by cell division, either more undifferentiated spermatogonia (self-renewal) or germ cells committed to the development of spermatogenesis (differentiation). Therefore, the balance between self-renewal and differentiation is crucial for the maintenance of spermatogenesis during the reproductive life. Nevertheless, the regulation of this balance in adult fish still needs to be elucidated, particularly the aspects involving paracrine regulation. INSL3 (insulin-like peptide 3) is a relaxin peptide family member produced by Leydig cells in vertebrate testes. In mammals, it is known that INSL3 has a crucial role on testicular descent during development and also on germ cell survival during adult life. In teleosts, although the testes remain inside the body cavity, the gene *insl3* is highly expressed during development and also during the adult life, potentially indicating evolutionary older functions yet undiscovered. Previous observations showed that Amh (anti-müllerian hormone), in addition to inhibiting spermatogonial differentiation and androgen release, inhibited Fsh (follicle-stimulating hormone)-induced increase in *insl3* transcript levels in zebrafish testis. Therefore, we investigated if the two growth factors might have antagonistic effects. *Ex vivo* studies indicated that hINSL3 increased the proliferation of type A undifferentiated (A_{und}) spermatogonia, while reducing proliferation of Sertoli cells associated with proliferating A_{und} . Moreover, *ex vivo* experiments using zebrafish testes with *in vivo* pre-labeled type A_{und} spermatogonia showed that hINSL3 decreased the number and area of testicular parenchyma occupied by cysts of type A_{und} spermatogonia, and increased the area occupied by cysts of type A differentiating (A_{diff}) spermatogonia. Corroborating the morphometric data, quantitative PCR analyses showed that hINSL3 decreased the transcription of *nanos2* gene, a marker for type A_{und} spermatogonia in zebrafish and other vertebrates. Hence, we conclude that hINSL3 promoted differentiation of type A_{und} spermatogonia in zebrafish testes culture. However, our investigations did not indicate effects of hINSL3 on androgen release. Finally, the investigations using animals with androgens and gonadotropins deficiency, due to an *in vivo* exposure to 17β -estradiol, showed that mRNA levels for *insl3* gene were drastically reduced in this pro self-renewal condition. Additionally, the partial recovery of spermatogenesis observed after the introduction of 11-ketoandrostenedione on the *in vivo* treatment was not related to the recovery of *insl3* transcription. In this context, our data corroborate the hypothesis that hINSL3 recruited A_{und} spermatogonia into differentiation without androgen participation, potentially mediating an Fsh effect on spermatogenesis.

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Introdução

1. Introdução

1.1 Peixes teleósteos

O grupo dos peixes teleósteos representa cerca de 50% do total de ~50 mil espécies de vertebrados atualmente catalogadas (Nelson, 2006). Seu sucesso adaptativo pode ser atribuído a variadas especializações nos mecanismos de locomoção, habitats, comportamento e, especialmente, reprodução, caracterizadas pela ampla diversidade de estratégias reprodutivas (Le Gac & Loir, 1999; Nakatani *et al.*, 2001; Moyle & Cech, 2003). Neste contexto, a liberação dos gametas para a fertilização externa; o desenvolvimento de órgãos especializados para fertilização interna; os mais variados níveis de cuidado parental; e a migração reprodutiva, seja na forma de “piracema” ou naquelas acompanhadas por grandes alterações osmóticas, são exemplos da complexidade reprodutiva dos teleósteos.

Além da inegável relevância das investigações envolvendo a biologia comparada dos vários sistemas constituintes dos organismos vertebrados, é importante que se conheça as peculiaridades relacionadas à biologia reprodutiva dos peixes devido, por exemplo, ao grande potencial econômico da piscicultura no mercado mundial de alimentos. Além disto, algumas espécies de teleósteos são modelos biológicos experimentais amplamente utilizados nas áreas de reprodução, genética e melhoramento, como por exemplo o zebrafish (*Danio rerio*), o medaka (*Oryzias latipes*), a truta arco-íris (*Oncorhynchus mykiss*) e a tilápia (*Oreochromis niloticus*) (Feitsma *et al.*, 2007; Nakamura *et al.*, 2012; Lacerda *et al.*, 2013; Rolland *et al.*, 2013).

1.2 Zebrafish (*Danio rerio*) como modelo experimental

O zebrafish (Figura 1 – página 58), no Brasil também conhecido como “paulistinha”, é um teleósteo de água doce originário da Índia, pertencente à família Cyprinidae (Engeszer *et al.*, 2007; Nasiadka & Clark, 2012). Este teleósteo possui vida média de três anos, atingindo a maturidade sexual aos três meses de vida. Sua reprodução ocorre em temperaturas médias de 26-28°C, por cerca de 18 meses. A fêmea deste teleósteo pode desovar aproximadamente 100-200 ovos por semana (McGonnell & Fowkes, 2006).

Esta espécie é amplamente utilizada como modelo experimental em pesquisas biomédicas, no estudo de genética evolutiva, comportamento e fisiologia. A ampla e crescente popularidade do zebrafish como modelo experimental pode ser atribuída a características como condições simples de manuseio em laboratório, prole numerosa, fertilização externa (de fácil manipulação), fácil observação do desenvolvimento embrionário propiciado pelo aspecto transparente de seus embriões, além do genoma totalmente sequenciado e disponibilizado, facilitando assim a geração de diversas linhagens de animais transgênicos (Grunwald & Eisen, 2002; Nasisdka & Clark, 2012). Neste contexto bastante favorável, o zebrafish vem sendo também utilizado com sucesso em pesquisas envolvendo regulação da função testicular (Leal *et al.*, 2009a; Leal *et al.*, 2009b; Nóbrega, *et al.*, 2010; Morais *et al.*, 2013).

1.3 Estrutura dos testículos

À exemplo das demais espécies de vertebrados, os testículos em teleósteos desempenham funções gametogênica (produção do gameta masculino, o espermatozoide) e endócrina (produção hormonal) e são constituídos por compartimentos tubular e intersticial (Figura 2 – página 59) (Schulz *et al.*, 2010; Schulz

& Nóbrega, 2011). O compartimento tubular é formado, da periferia para o centro, pelas células peritubulares mióides (de atividade contrátil), membrana basal, epitélio seminífero e o lume tubular. As células de Sertoli são as únicas células somáticas no epitélio seminífero. Elas desempenham papel crucial no processo de formação dos gametas masculinos, sendo essenciais para a sobrevivência e desenvolvimento das células germinativas durante a espermatogênese (Schulz & Miura, 2002; Schulz *et al.*, 2010). O epitélio seminífero em testículos de teleósteos se organiza em estruturas denominadas “cistos”, os quais são formados por células de Sertoli que envolvem as células germinativas que se diferenciam / progridem de maneira sincrônica (Figura 3 – página 59). Ao receberem estímulos hormonais as células de Sertoli produzem fatores que irão influenciar e direcionar a espermatogênese (Schulz *et al.*, 2010). Neste contexto, atribuem-se às células de Sertoli as funções de sustentação e nutrição das células germinativas, secreção de fluido para a formação do lúmen tubular, produção de proteínas e de vários fatores de crescimento, liberação de espermátides maduras (espermição) e intermediação hormonal da espermatogênese através de receptores para esteroides (andrógenos e estrógenos) e para gonadotrofinas e fatores de crescimento (Schulz *et al.*, 2010; França *et al.*, 2015). Outra propriedade importante das células de Sertoli é sua eficiente capacidade fagocitária, retirando do epitélio seminífero produtos/corpos residuais, bem como células germinativas em apoptose e eventuais espermatozoides que permanecem no lume tubular após a espermição (Almeida *et al.*, 2008; França *et al.*, 2015).

No compartimento intersticial, além de vasos sanguíneos, células e fibras do tecido conjuntivo, encontram-se as células responsáveis pela produção de hormônios esteroides nos testículos, as células de Leydig (Figuras 2 e 3 – páginas 59 e 60) (Schulz *et al.*, 2010; Schulz & Nóbrega, 2011). Em teleósteos, sob o estímulo tanto do hormônio

luteinizante (Lh) quanto do folículo estimulante (Fsh), estas células produzem e secretam a 11-cetotestosterona (11-KT), que atua na regulação da espermatogênese (de Waal *et al.*, 2008).

1.4 Espermatogênese

A espermatogênese é um processo cíclico, complexo, coordenado e filogeneticamente conservado, no qual as espermatogônias-tronco podem sofrer dois tipos de divisão: *i*) divisão para auto-renovação, garantindo assim a fertilidade em longo prazo; e *ii*) divisão para prover duas células filhas, conectadas por uma ponte citoplasmática, as quais formam, após número de divisões mitóticas espécie-específico, os espermatócitos primários. Estes espermatócitos passarão então pelo processo de divisão meiótica produzindo células haploides (espermátides), as quais, posteriormente, darão origem aos espermatozoides (Figura 3 – página 60) (Schulz *et al.*, 2010). O balanço entre esses dois tipos de divisão espermatogonial é altamente coordenado. Assim, quando o mesmo é alterado rumo à auto-renovação, a formação de seminomas pode ser desencadeada. Por outro lado, se este balanço é desequilibrado rumo à diferenciação, a população de espermatogônias-tronco pode ser depletada e a espermatogênese interrompida (de Rooij & Grootegoed, 1998).

Morfofuncionalmente, a diferenciação das células germinativas em espermatozoides pode ser dividida em três fases: *i*) fase mitótica ou espermatogonial, durante a qual uma série de divisões mitóticas, as primeiras lentas e as demais rápidas, expandem exponencialmente o número de células germinativas; *ii*) fase meiótica ou espermatocitária, na qual o material genético é duplicado, recombinado e segregado durante as duas divisões meióticas, resultando em células haploides; e *iii*) fase espermiogênica, onde as células germinativas haploides sofrem transformações drásticas,

tais como alta compactação do material genético, redução do volume celular e formação do flagelo, dando assim origem aos espermatozoides (Russell *et al.*, 1990; Sharpe, 1994; Eddy, 1999).

Durante a fase mitótica, o número de divisões / gerações espermatogoniais pode variar bastante entre as diversas espécies de peixes (Schulz *et al.*, 2010). Pelo fato da denominação das diferentes gerações de espermatogônias ser bastante variada neste grupo de vertebrados, o uso de nomenclatura semelhante para o mesmo tipo celular tornou-se necessário. Assim, recentemente, tentativa foi feita no sentido de se classificar / caracterizar as células germinativas de teleósteos empregando-se a mesma terminologia utilizada para vertebrados superiores (Schulz *et al.*, 2010). Desta forma, baseado em critérios morfológicos, as células espermatogoniais em peixes podem ser classificadas em dois tipos: tipo A e tipo B (Figura 4 – página 61). Funcionalmente, as espermatogônias do tipo A podem ser subdivididas em espermatogônias indiferenciadas (A_{und}) (Figura 4A), incluindo-se as células tronco espermatogoniais (SSC); e espermatogônias diferenciadas (A_{diff}) (Figura 4B). As espermatogônias do tipo A_{und} originam as espermatogônias do tipo A_{diff} , que compartilham algumas características morfológicas com sua célula precursora. O comprometimento irreversível com a diferenciação/maturação e alterações morfofuncionais, tais como menor tamanho celular e maior quantidade de heterocromatina, resulta na formação de espermatogônias do tipo B (Figura 4B) que, caracteristicamente em peixes, passam por várias e rápidas divisões. Neste sentido, o melhor critério para discriminar espermatogônias do tipo B inicial do tipo tardia baseia-se no tamanho celular/nuclear e no número de células por cisto (Leal *et al.*, 2009a; Schulz *et al.*, 2010).

1.5 Proliferação das células de Sertoli

Diferentemente de répteis, aves e mamíferos, nos quais é considerado que as células de Sertoli não proliferam após a puberdade, a espermatogênese em teleósteos e anfíbios está intimamente associada com a proliferação desse elemento somático. O número de células de Sertoli presente nos testículos é o principal fator responsável pelo número de cistos germinativos, o que, em última análise, determina o tamanho deste órgão e a magnitude da produção espermática (Schulz *et al.*, 2005; Petersen & Söder, 2006; Leal *et al.*, 2009a; Schulz *et al.*, 2010; França *et al.*, 2015). Estudos em teleósteos mostraram que a proliferação das células de Sertoli ocorre principalmente na fase mitótica ou espermatogonial da espermatogênese, quando o número de células germinativas e, conseqüentemente, o tamanho dos cistos espermatogênicos aumentam rapidamente (Matta *et al.*, 2002; Schulz *et al.*, 2005; Leal *et al.*, 2009a; Schulz *et al.*, 2012). Em tilápia, esta atividade proliferativa persiste até o estabelecimento das junções oclusivas (*tight junctions*) entre as células de Sertoli no final da meiose (Batlouni *et al.*, 2009). Pelo fato de ser considerado que as células de Sertoli em peixes não sofrem diferenciação terminal, retendo assim a sua capacidade mitótica ao longo da vida reprodutiva do animal, cada novo cisto é, em tese, composto por novas células de Sertoli originadas de divisões mitóticas (Schulz *et al.*, 2005), as quais, a semelhança de mamíferos, parecem também sofrer influência do Fsh para a sua proliferação (Schulz *et al.*, 2012).

Além da proliferação que acompanha o crescimento dos cistos espermatogênicos já existentes, outra modalidade de proliferação das células de Sertoli acontece nos testículos de teleósteos no contexto de formação de novos cistos, a partir da auto-renovação das SSC. Esta premissa parte do princípio de que, nesta fase da espermatogênese a sobrevivência das células germinativas depende do seu íntimo

contato com essa célula somática. Assim, durante a formação de novos cistos o primeiro passo seria a formação de mais nichos (ver próximo item) através da produção de outras células de Sertoli, aspecto este crucial para a acomodação de novas células tronco provenientes da auto-renovação das células tronco espermatogoniais (Schulz *et al.*, 2012).

1.6 Nicho e regulação das células tronco espermatogoniais

Em praticamente todos os órgãos e sistemas, a função das células tronco adultas é mantida por microambiente especializado, denominado nicho (Voog & Jones, 2010). No testículo, este microambiente é composto pelas próprias SSC, células de Sertoli e outros elementos somáticos adjacentes, além de matriz extracelular (Hofmann, 2008; Nóbrega *et al.*, 2010; Oatley & Brinster, 2012; Yoshida, 2012). Por estar em íntimo contato com as SSC, a célula de Sertoli é considerada como o principal componente do nicho espermatogonial (de Rooij & Griswold, 2012).

Na última década, estudos em mamíferos identificaram diversos fatores de crescimento que são considerados importantes na regulação do nicho das SSC, ou seja, envolvidos na auto-renovação ou diferenciação destas células (de Rooij & Griswold, 2012; Oatley & Brinster, 2012). Dentre eles, podem ser citados o fator estimulador de colônia 1 (CSF1), promotor da auto-renovação das SSC e produzido pelas células de Leydig e peritubulares mióide (Oatley *et al.*, 2009; Campos-Junior *et al.*, 2012), o fator neurotrófico derivado de células da glia (GDNF), a activina A e a BMP4 (*bone morphogenetic protein 4*), produzidos pelas células de Sertoli (Meng *et al.*, 2000; Nagano *et al.*, 2003; Yomogida *et al.*, 2003; Loveland & Robertson, 2005; Kokkinaki *et al.*, 2009; Savitt *et al.*, 2012). Estudos em roedores sugerem fortemente que o FSH regula a produção de GDNF (Tadokoro *et al.*, 2002), que atua nas SSC através de receptores

RET (ret proto-oncogene) e GFR α 1 (*glial cell line-derived neurotropic Family receptor alfa 1*) desempenhando papel importante na auto-renovação destas células tronco espermatogoniais, enquanto a activina A e a BMP4 promovem a diferenciação das mesmas (Buageaw *et al.*, 2005; Hofmann, 2008; Jijiwa *et al.*, 2008; Johnston *et al.*, 2011; Campos-Junior *et al.*, 2012).

Em teleósteos, o nicho de SSC é ainda pouco investigado (Nóbrega *et al.*, 2010). Porém, alguns marcadores em potencial para SSC tem sido descritos, podendo ser citados, por exemplo, o Gfra1 e o Nanos2 (Skaar *et al.*, 2011; Lacerda *et al.*, 2013; Bellaiche *et al.*, 2014). Além disso, estudo recente em zebrafish mostrou que o hormônio anti-mülleriano Amh (durante a vida adulta do animal, expresso pelas células de Sertoli em cistos contendo espermatogônias do tipo A_{und}) inibe a proliferação/diferenciação espermatogonial induzida por andrógenos (Skaar *et al.*, 2011).

Diferentemente de mamíferos, para os quais não existem indicativos de que hormônios esteroides influenciam no equilíbrio entre auto-renovação e diferenciação das SSC, estudos em teleósteos mostraram que o hormônio 17 β -estradiol (E2) foi capaz de estimular a auto-renovação das SSC em enguias, ao passo que a 11-KT estimulou a diferenciação espermatogonial (Miura *et al.*, 2002). Ainda em teleósteos, estudo em zebrafish mostrou que a insuficiência androgênica em decorrência ao bloqueio de produção de gonadotrofinas devido a exposição *in vivo* ao E2, possui marcante efeito na dinâmica da espermatogênese, promovendo drástica redução no número de células germinativas em diferenciação, ao mesmo tempo em que promove o acúmulo de espermatogônias do tipo A_{und} nos testículos (de Waal *et al.*, 2009).

1.7 Peptídeo 3 semelhante a insulina (*insulin-like 3*) INSL3

Os peptídeos que constituem a família das relaxinas e seus respectivos receptores são expressos em grande variedade de órgãos e sistemas nas mais diversas espécies de vertebrados. A relaxina foi descoberta por Frederick Hisaw na década de 1920, após estudos acerca da endocrinologia reprodutiva em suínos, como fator responsável pela expansão e relaxamento do ligamento púbico, pouco antes do parto em fêmeas gestantes. Posteriormente, a relaxina foi identificada em diversas espécies e classificada como hormônio peptídeo (Bathgate *et al.*, 2013). Evolutivamente, acredita-se que nos vertebrados as relaxinas tenham surgido a partir da família insulina de peptídeos, com a qual apresentam grande similaridade em sua estrutura química (Bathgate *et al.*, 2006a). A forma ativa melhor estudada para os peptídeos da família das relaxinas possui duas cadeias peptídicas (A e B) estabilizadas por três pontes dissulfídicas; duas intermoleculares entre as cadeias A e B, e uma intramolecular na cadeia A (Bathgate *et al.*, 2013). Através de região bem conservada entre as relaxinas até então conhecidas, estes peptídeos se ligam e ativam seus receptores RXFP (*Relaxin Family Peptide Receptors*), acoplados a proteína G (Hsu *et al.*, 2002; Bathgate *et al.*, 2013).

O peptídeo 3 semelhante a insulina (*insulin-like 3* - INSL3) é um membro da família relaxina de peptídeos que é altamente expresso pelas células de Leydig nas gônadas masculinas, tanto durante o desenvolvimento embrionário quanto durante a vida adulta, em diversas espécies de vertebrados (Bathgate *et al.*, 2013; Good-Ávila *et al.*, 2009). Embora os testículos não sejam o único sítio de expressão para essa relaxina, sua expressão em outros órgãos é drasticamente menor quando comparada a gônada (Bathgate *et al.*, 2013). Durante o desenvolvimento em mamíferos, o INSL3 apresenta papel crucial no processo de descenso testicular, que é fundamental para sucesso da espermatogênese durante a vida reprodutiva na grande maioria das espécies destes

vertebrados. Animais *knockout* para o gene *INSL3* bem como seu receptor *RXFP2*, apresentam o fenótipo criptorquídico, no qual as gônadas permanecem na cavidade abdominal, devido ao não desenvolvimento do gubernáculo (Zimmermann *et al.*, 1999; Nef & Parada, 1999; Kumagai *et al.*, 2002). Apesar da marcante expressão do *INSL3* nas células de Leydig em testículos de animais sexualmente maduros (Bathgate *et al.*, 2013), pouco se sabe a respeito de seu papel sobre a função testicular. Recentemente, foi demonstrado que o *INSL3* é capaz de aumentar a capacidade esteroidogênica das células de Leydig de camundongos *in vitro* (Pathirana *et al.*, 2012), reforçando a hipótese de função autocrina para este peptídeo, levantada com o descobrimento da expressão de seu receptor pelas células de Leydig nesta espécie (Anand-Ivell *et al.*, 2002). Ainda, embora nenhum efeito tenha sido detectado na espermatogênese em camundongos (Huang *et al.*, 2012), efeito anti-apoptótico do *INSL3* nas células germinativas em ratos e em suínos já foi descrito na literatura (Kawamura *et al.*, 2004; Sagata *et al.*, 2015).

Similar a mamíferos, o gene *insl3* é intensamente expresso nas células de Leydig em zebrafish sexualmente maduros, sugerindo a participação deste peptídeo na regulação da espermatogênese nesta espécie de teleosteo (Good *et al.* 2012). Estudos recentes sobre regulação do processo espermatogênico em zebrafish mostraram que o Fsh é capaz de elevar os níveis basais da expressão do *insl3* nesta espécie, tanto em abordagens *in vivo* quanto em abordagens *in vitro*, sendo este efeito não mediado pela ação esteroidogênica desta gonadotrofina (García-López *et al.*, 2010). Por outro lado, foi demonstrado que o Amh, expresso predominantemente em células de Sertoli associadas com espermatogônias do tipo A_{und}, inibe a ação estimuladora do Fsh sobre a expressão do *insl3* (Skaar *et al.*, 2011). Uma vez que, juntamente com o efeito inibitório na expressão do *insl3*, o Amh é conhecido na literatura como agente bloqueador da diferenciação das espermatogônias do tipo A_{und}, bem como da síntese de hormônios

esteroides mediada pelo Fsh (Skaar *et al.*, 2011), surge a hipótese de que -
antagonicamente - o Insl3 em zebrafish pode estar relacionado com a diferenciação
espermatogonial e com a esteroidogênese.

**Justificativa
e
objetivos**

2. Justificativa e objetivos

2.1 Justificativa e objetivo geral

Diversos estudos em mamíferos envolvendo peptídeos membros da família relaxina, incluindo-se o INSL3, têm demonstrado que estes peptídeos exercem importantes funções nos mais diversos órgãos e sistemas. Especialmente em estudos envolvendo a biologia da reprodução, o INSL3 ocupou considerável espaço nas últimas décadas, tendo sido demonstradas importantes funções no sistema genital masculino nos aspectos referentes ao desenvolvimento testicular e estabelecimento e evolução da espermatogênese. Entretanto, embora o interesse acerca desta relaxina tenha crescido recentemente, até o presente momento dados relevantes acerca da biologia do INSL3 no trato reprodutivo foram gerados somente a partir de estudos realizados em mamíferos, sendo os estudos em vertebrados não mamíferos (e.g peixes e anfíbios) limitados a investigações evolutivas e de padrão de expressão gênica.

Em peixes teleósteos, onde o processo de desenvolvimento da gônada masculina não envolve descenso testicular, as células de Leydig produzem *Insl3* durante o desenvolvimento embrionário e durante a vida adulta desses animais. Ainda, estudos recentes em zebrafish detectaram a produção de RNA mensageiro (RNAm) para 2 subtipos de receptores para o *Insl3* nos testículos (Good-Ávila *et al.* 2009; Good *et al.* 2012), o que sugere fortemente a existência da participação dessa relaxina no controle da função testicular nessa espécie de teleósteo.

Portanto, diante da necessidade de melhor compreender e de se ampliar os conhecimentos acerca das funções do *Insl3* na biologia da reprodução em vertebrados não mamíferos, e utilizando-se como base os resultados relativamente recentes acerca da regulação da transcrição do gene *insl3* nos testículos de zebrafish (García-Lopez *et al.*,

2010; Skaar *et al.*, 2011), o presente estudo teve como objetivo geral a utilização de ferramentas moleculares e abordagens morfofisiológicas e morfométricas para investigar o potencial papel modulador do *Insl3* na dinâmica de divisão das espermatogônias e na liberação de andrógenos nos testículos de zebrafish adultos.

2.2 Objetivos específicos

Com a finalidade de se investigar potenciais efeitos moduladores do *Insl3* na dinâmica de divisão das espermatogônias e na liberação de andrógenos nos testículos de zebrafish adultos, os objetivos específicos do presente estudo foram:

- avaliar se o tratamento *ex vivo* com o INSL3 humano (hINSL3) altera o status de proliferação das gerações/tipos espermatogoniais iniciais;
- avaliar se o tratamento *ex vivo* com o hINSL3 altera o status de proliferação das células de Sertoli e Leydig;
- investigar a influência do hINSL3 na dinâmica de proliferação das espermatogônias do tipo A_{und} , utilizando-se explantes testiculares;
- investigar os níveis de transcrição de potenciais genes modulados pelo hINSL3 através de estudos em explantes testiculares, na tentativa de elucidar possíveis mecanismos genéticos usados pelo *Insl3* na modulação da função testicular;
- avaliar se o tratamento *ex vivo* com o hINSL3, sozinho ou em associação com o Fsh, modula a liberação *in vitro* de andrógenos pelas células de Leydig;
- avaliar os níveis de transcrição dos genes *insl3* e de seus receptores *rxfp2a* e *rxfp2b* em resposta a diferentes tratamentos hormonais *in vivo*;
- analisar morfometricamente os testículos de zebrafish após a exposição *in vivo* a diferentes tratamentos hormonais, com o intuito de se comparar potenciais

mudanças histológicas com o padrão de transcrição observado para o *insl3* e receptores nas mesmas condições.

Materiais e métodos

3. Materiais e métodos

3.1 Animais

Zebrafish machos sexualmente maduros (123 animais) do tipo selvagem foram utilizados no presente estudo. Os animais foram mantidos e tratados nas dependências da Faculdade de Ciências da Universidade de Utrecht – Holanda, de acordo com o regulamento nacional da Holanda aprovado pelo Comitê Holandês de Uso e Cuidado Animal.

3.2 INSL3 humano (hINSL3)

O hINSL3, sintetizado de acordo com metodologia previamente publicada na literatura (Bathgate *et al.*, 2006b), foi gentilmente doado pelo Prof. Dr. John D. Wade, da Universidade de Melbourne, Victoria, Austrália. Este peptídeo foi diluído em PBS estéril e a solução foi dividida em alíquotas na concentração de 100 µg/mL. Estas alíquotas foram então congeladas em nitrogênio líquido e mantidas a temperatura de -80 °C.

Devido aos seguintes aspectos considerados a seguir, o hINSL3 se apresenta como excelente candidato para o estudo da atividade biológica desta relaxina nos testículos de zebrafish. Dois genes para os receptores de Insl3 (*rxfp2a* e *rxfp2b*), parálogos ao gene *RXFP2* humano, são abundantemente expressos nos testículos de zebrafish (Good *et al.*, 2012; Yegorov *et al.*, 2014). A especificidade da interação entre o ligante e o receptor é principalmente determinada pelos resíduos Phe¹³¹ e Gln¹³³ presentes no receptor RXFP2 com resíduos Trp²⁷ presente na cadeia B do ligante hINSL3; resíduos Trp¹⁷⁷ e Ile¹⁷⁹ presentes no receptor RXFP2 com os resíduos His¹² e Val¹⁹ presentes na

cadeia B do ligante hINSL3; e resíduos Asp¹⁸¹ e Glu²²⁹ presentes no receptor RXFP2 com resíduos Arg¹⁶, Arg²⁰ e Asp²²⁷ presentes na cadeia B do ligante hINSL3 (Büllesbach & Schwabe, 1999; Büllesbach & Schwabe, 2004; Büllesbach & Schwabe, 2005; Büllesbach & Schwabe, 2006; Rosengren *et al.*, 2006; Scott *et al.*, 2007). O alinhamento da sequência dos receptores para Insl3 do zebrafish Rxfp2a e Rxfp2b com o receptor humano RXFP2 mostrou que estes receptores contêm resíduos idênticos nos sítios de interação ligante-receptor, exceto para os resíduos Ile¹⁷⁹ e Glu²²⁹ no receptor RXFP2, que são substituídos pelos resíduos Val e Ala nos receptores Rxfp2a e Rxfp2b, sem potencialmente comprometer a interação entre o hINSL3 e os receptores em zebrafish.

3.3 Explantes testiculares

Para se analisar o potencial efeito do hINSL3 na proliferação de células germinativas e somáticas, bem como na liberação de andrógenos e nos níveis de mRNA testicular, o sistema de cultura para testículos de zebrafish foi utilizado de acordo com protocolo previamente publicado na literatura (Leal *et al.*, 2009b), sendo 8 zebrafish utilizados para análises de proliferação e cinética espermatogonial e 12 utilizados para análises de níveis de RNAm. A concentração ideal de 100 ng/mL para o hINSL3 foi escolhida para a realização dos experimentos de acordo com análises preliminares e dados na literatura sobre o uso de hINSL3 em estudos *in vitro* (Pathirana *et al.*, 2012).

Com o intuito de se investigar a proliferação de células germinativas e somáticas, bem como os níveis de RNAm testicular, os peixes foram sacrificados e tiveram os testículos coletados. Os dois testículos de cada animal foram então submetidos a um período de 7 dias de incubação, um (escolhido de forma aleatória) servindo como controle para o seu contralateral. Cada testículo foi colocado sobre uma membrana de nitrocelulose de 0,25 cm² (25 µm de espessura e poros de 0,22 µm; Millipore, Billerica,

MA, EUA) no topo de um cilindro de agarose de 750 μ L [1.5% w/v, Ringer's solution (pH 7,4)] em placas de cultura de 24 *wells* (Corning Inc., New York, USA), contendo 1mL de meio de cultura (Figura 5 – página 62). Para as análises de liberação de andrógenos, os testículos foram incubados diretamente em 200 μ L de meio de cultura em placas de 96 *wells*, durante um período de ~18 horas. Todos os componentes utilizados para a preparação da cultura de tecido foram preparados a fresco, de acordo com protocolo publicado (Leal *et al.*, 2009b).

3.4 Análises morfométricas

3.4.1 Incorporação de BrdU *ex vivo*

Para avaliar se o hINSL3 afeta a atividade de proliferação de diferentes gerações/tipos espermatogoniais, bem como das células de Sertoli e de Leydig em zebrafish, 50 μ g/mL do marcador de proliferação celular *5-bromo-2'-deoxyuridine* (BrdU) (Sigma-Aldrich, St. Louis, MO, EUA) foram adicionados ao meio de cultura durante as últimas 6 horas do período de 7 dias de incubação, de acordo com protocolo publicado para este tipo de abordagem em zebrafish (Leal *et al.*, 2009b).

3.4.2 Incorporação de BrdU *in vivo*

Neste experimento, 12 zebrafish machos foram expostos ao BrdU (4 mg/mL) dissolvido na água do aquário durante período de ~12 horas/dia por 3 dias consecutivos, de forma a permitir que este marcador de proliferação se incorporasse ao DNA das espermatogônias do tipo A_{und}, que apresentam ciclo celular mais lento quando comparado as outras células germinativas (Nóbrega *et al.*, 2010). Posteriormente a exposição *in vivo* ao BrdU, os peixes foram mantidos em água normal durante 4 dias, período suficiente para que as demais espermatogônias de ciclo celular mais acelerado

avancem no processo espermatogênico e também de modo a diluir a marcação do BrdU nestes tipos celulares (Nóbrega *et al.*, 2010). Subsequentemente, os testículos foram coletados e colocados em cultura, conforme descrito no item 3.3, durante 4 dias na presença (100 ng/mL) ou ausência do hINSL3. Este tipo de abordagem nos permite analisar de modo confiável os efeitos do hINSL3, particularmente na dinâmica de proliferação de espermatogônias do tipo A_{und} pré marcadas *in vivo* com o BrdU.

3.4.3 Preparação histológica e análises em microscopia de luz

Após o período de cultura, os testículos destinados à análises morfológicas e de proliferação celular foram fixados por 16 horas a 4°C em *methacarn* [(v/v) 60% álcool absoluto, 30% clorofórmio e 10% de ácido acético glacial] preparado a fresco. Em seguida, o material foi desidratado, incluído em Technovit 7100 (Heraeus Kulzer), seccionados em 4 µm de espessura. As amostras destinadas às análises de proliferação celular foram submetidas à imunohistoquímica (IHC) para a detecção das células marcadas com BrdU, conforme protocolo publicado na literatura (Leal *et al.*, 2009b). Os cistos/células germinativas foram identificados de acordo com critérios morfológicos já bem estabelecidos na literatura para células germinativas de zebrafish, tais como tamanho nuclear, proporção entre eucromatina e heterocromatina e número de células germinativas por cisto (Leal *et al.*, 2009a; Schulz *et al.*, 2010).

Com o intuito de se avaliar os efeitos do hINSL3 sobre a proliferação e dinâmica das células espermatogoniais/somáticas, 100 cistos/células foram avaliados de forma aleatória para cada tipo celular investigado e o índice de proliferação/marcação com BrdU foi calculado com base na porcentagem de células BrdU positivas encontradas. Com base no número de pontos obtidos sobre os diferentes tipos espermatogoniais, a proporção da área de parênquima testicular ocupada por cistos de espermatogônias do tipo A_{und} , A_{diff} , e do tipo B, foi também calculada. Para tal finalidade, 30 campos

aleatórios foram fotografados em aumento de 400x utilizando-se microscópio de luz convencional com câmera digital acoplada e analisados no software ImageJ (*Image Processing and Analysis in Java*), utilizando-se retículo com 540 pontos.

3.5 Análise dos níveis de RNAm testicular

A capacidade do hINSL3 em modular os níveis de RNAm testicular foi investigada após 7 dias de cultura de testículo, na ausência ou presença de hINSL3, conforme descrito no item 3.3, utilizando-se PCR quantitativo (qPCR). O RNA total foi extraído das amostras utilizando-se o micro-kit RNAqueous® (Ambion, Austin, TX, EUA), seguindo as instruções do fabricante. Para a quantificação dos níveis de mRNA de genes selecionados [*amh*, *gsdf*, *sycp3*, *piwi11* (García-López *et al.*, 2010), *igf3* (Morais *et al.*, 2013) e *odfb3* (Leal *et al.*, 2009b) e *nanos2*], o cDNA foi sintetizado utilizando-se 2 µg de RNA para cada uma das amostras, conforme protocolo publicado na literatura (de Waal *et al.*, 2008). Os níveis de RNAm do fator de alongamento 1 alfa (*ef1α*), que permaneceram estáveis sob as diferentes condições experimentais investigadas, serviram como controle endógeno (inset Figura 10 – página 67). Todas as reações de qPCR foram realizadas com volume de 20 µL de reação e os valores quantitativos dos ciclos (Cq) foram determinados utilizando-se o sistema 7900HT de PCR em tempo real (*Applied Biosystems*), mantendo-se as configurações padrão. A Tabela 1 apresenta os *primers* utilizados nas reações de qPCR.

3.6 Análise da liberação de andrógeno *in vitro*

Para investigar se o hINSL3 modula a liberação de andrógenos (basal ou estimulada pelo Fsh), os níveis de 11β-hidroxiandrostenediona (OHA), precursor da 11-KT, foram medidos no meio de cultura utilizando-se bioensaio adaptado para análises

de liberação de andrógenos em zebrafish (García-López *et al.*, 2010). Os resultados foram expressos como nanograma (ng) de OHA liberado por miligrama (mg) de testículo. Os testículos foram colocados em cultura conforme descrito no item 3.3, na presença ou ausência de 25 ng/ml de Fsh e/ou 100 ng/mL de hINSL3, respectivamente.

3.7 Tratamento hormonal *in vivo*

Zebrafish adultos foram separados em três grupos de 25 animais e mantidos separadamente em 3 aquários, todos contendo igualmente 13 litros de água e devido sistema de aeração. Para se efetuar o tratamento, soluções estoque para os hormônios 17 β -estradiol (E2) e 11-cetoandrostenediona (OA), que é precursor da 11-KT, foram preparadas em água Milli-Q nas concentrações de 10 μ M e 100 μ M, respectivamente, para posterior diluição na água dos aquários. Após 2 dias de aclimatação, diferentes tanques receberam diferentes tratamentos hormonais (Figura 6 – página 63). No tanque número 1, os animais foram expostos a dose de 10nM de E2 durante 5 semanas consecutivas; no tanque número 2, os animais foram expostos a dose de 10nM de E2 durante as 5 semanas consecutivas, sendo adicionada dose de 100nM de OA durante as últimas duas semanas; no tanque número 3 os animais não receberam nenhum tratamento hormonal e foram utilizados como grupo controle. Durante o período de exposição, todos os tanques tiveram a água renovada diariamente. Os procedimentos de preparo das soluções estoque, a escolha das concentrações utilizadas, bem como o manuseio dos animais durante o período de tratamento foram feitos de acordo com especificações já descritas na literatura (Van der Ven *et al.*, 2003; de Waal *et al.*, 2009).

Após o período de 5 semanas, os animais foram sacrificados para dissecação e coleta dos testículos. Foram então obtidos os pesos corporal (Pc) e testicular (Pt) para cada um dos animais dos três grupos para o cálculo do índice gonadosomático (IGS),

utilizando-se a fórmula $100x[Pt/Pc]$. Em cada um dos grupos, testículos de 8 animais foram processados para análises histológicas, conforme descrito no item 3.4.2. A frequência de cistos nas três diferentes fases da espermatogênese para os três diferentes grupos foi calculada com base no número de cistos encontrados no total de 500 cistos aleatoriamente avaliados por animal. Ainda, testículos de 12 animais de cada um dos três grupos investigados foram processados para análises de qPCR para os genes *insl3*, e seus receptores *rxfp2a* e *rxfp2b* (Good *et al.*, 2012), conforme descrito no item 3.5. Os níveis de RNAm do fator de alongamento 1 alfa (*ef1α*), que permaneceram estáveis sob as diferentes condições experimentais investigadas, serviram como controle endógeno (inset Figura 14 – página 69)

3.8 Análise estatística

Para a realização de análises estatísticas foi utilizado o software *GraphPad Prism 5.0* (GraphPad Software, Inc., San Diego, California, USA). Os grupos controle e tratado foram testados para diferenças significativas utilizando-se os testes “*t*” de *Student’s* para as observações pareadas e ANOVA (Newman-Keuls) para comparação de múltiplos grupos. O nível de significância $p < 0,05$ foi considerado em todas as análises. Os dados obtidos foram apresentados como média \pm erro padrão.

Resultados

4. Resultados

4.1 Proliferação de células germinativas e somáticas

Após 7 dias de incubação na ausência (basal) ou presença de 100 ng/mL de hINSL3, e com base nas contagens de cistos/células positivas para marcação de BrdU (Figura 7A-B, e Figura 8A-C), foram calculados os índices de proliferação para espermatogônias do tipo A_{und} e A_{diff} (Figura 7C), células de Leydig, células de Sertoli e células de Sertoli especificamente associadas a espermatogônias do tipo A_{und} BrdU negativas ou positivas (Figura 8D). Os resultados deste experimento indicaram que, em cultura, o hINSL3 foi capaz de aumentar de forma significativa a proliferação de espermatogônias do tipo A_{und} , ao passo que nenhum efeito foi observado para cistos de espermatogônias do tipo A_{diff} (Figura 7C), ou para as células de Leydig, células de Sertoli e células de Sertoli especificamente associadas a espermatogônias do tipo A_{und} BrdU negativas (Figura 8D). No entanto, foi possível observar decréscimo ($p < 0,05$) no índice de proliferação das células de Sertoli que se encontravam associadas à espermatogônias do tipo A_{und} , positivas para BrdU (Figura 8D).

Ainda que os resultados indiquem que o hINSL3 foi capaz de aumentar a taxa de proliferação das espermatogônias do tipo A_{und} , esta observação por si só não traduz a natureza desta divisão celular (auto-renovação *versus* diferenciação). No entanto, considerando-se que a atividade proliferativa das células de Sertoli associadas à espermatogônias do tipo A_{und} BrdU positivas, ou seja, em proliferação, foi parcialmente inibida em resposta ao hINSL3, é improvável que novos nichos espermatogoniais e, conseqüentemente, novos cistos, estejam sendo produzidos para a acomodação das novas células. Portanto, é plausível inferir que as duas células filhas provenientes da

divisão de uma espermatogônia do tipo A_{und} permaneceriam no mesmo cisto espermatogênico, caracterizando um processo de diferenciação espermatogonial.

4.2 Análise da cinética espermatogonial

Para averiguar a hipótese de que a atividade proliferativa observada para as espermatogônias do tipo A_{und} após o tratamento com o hINSL3 levaria a diferenciação espermatogonial (resultando na formação de clones de espermatogônias em um mesmo cisto espermatogênico) e não a auto-renovação (resultando na formação de dois cistos individuais), espermatogônias A_{und} foram marcadas *in vivo* com BrdU, através da exposição dos peixes a BrdU dissolvido na água do aquário, conforme descrito no item 3.4.2. Considerando-se que o BrdU foi incorporado ao DNA das espermatogônias do tipo A_{und} antes do início do período de incubação com o hINSL3, é possível inferir se o efeito proliferativo promovido pelo tratamento *ex vivo* com este peptídeo resultaria na auto-renovação ou diferenciação espermatogonial, através da observação das mudanças nos índices de marcação para BrdU para estas células e através das gerações espermatogoniais subsequentes, após o tratamento em cultura com hINSL3.

As análises mostraram que o tratamento em cultura com o hINSL3 resultou num decréscimo de ~ 3 e ~ 2 vezes para os índices de marcação com BrdU para as espermatogônias do tipo A_{und} e A_{diff} , respectivamente, enquanto nenhum efeito foi observado para este parâmetro em cistos de espermatogônias do tipo B (Figura 9A). Interessantemente, em análises morfométricas no mesmo experimento foi possível observar decréscimo significativo na proporção da área de parênquima testicular ocupado por cistos de espermatogônias do tipo A_{und} , ao passo que aumento significativo para o mesmo parâmetro foi observado para espermatogônias do tipo A_{diff} (Figura 9B). Em conjunto, essas observações indicam que o hINSL3 provocou o declínio da

população de espermatogônias do tipo A_{und} através de estímulo proliferativo, culminando na diferenciação destas células germinativas e na consequente formação de cistos contendo espermatogônias do tipo A_{diff} .

4.3 Análises dos níveis de RNAm testicular

Objetivando-se elucidar os mecanismos moleculares usados pelo hINSL3 na modulação da proliferação de células germinativas, foram realizadas por meio de reações de qPCR quantificações dos níveis de RNAm de genes específicos selecionados. Assim, foram analisados os níveis de RNAm de três genes expressos pelas células de Sertoli (*amh*, *gsdf* e *igf3*), além dos genes *piwi1*, *sycp3* e *odf3b*, expressos respectivamente por células espermatogoniais, espermatócitos e espermátides. Nossos resultados não indicaram diferenças significativas nos níveis de RNAm para nenhum dos genes acima citados (Figura 10). No entanto, o hINSL3 foi capaz de reduzir em ~6 vezes ($p < 0,05$) os níveis de transcrição para o gene marcador de espermatogônias tronco, *nanos2*.

4.4 Análise da liberação de andrógeno *in vitro*

Com o objetivo de se avaliar se os efeitos observados após o tratamento em cultura com hINSL3 estariam associados com a modulação da liberação de andrógenos, basal ou estimulada pelo Fsh, os níveis de OHA no meio de cultura após ~18 horas de incubação foram medidos. Este precursor do principal andrógeno produzido nos testículos de teleósteos, a 11-KT, é apontado na literatura como excelente indicador da atividade esteroideogênica das células de Leydig em testículos de zebrafish (García-López *et al.*, 2010). No entanto, nossos resultados indicaram que o hINSL3 não apresenta atividade regulatória na liberação de andrógenos, tanto basal quanto na liberação de

andrógenos estimulada pelo Fsh, sugerindo que os efeitos observados na proliferação espermatogonial não estariam relacionados com a produção de andrógenos (Figura 11).

4.5 Tratamento hormonal *in vivo*

Estudos em zebrafish demonstraram que a insuficiência androgênica provocada pela exposição *in vivo* ao hormônio E2 resultou no bloqueio da diferenciação espermatogonial e no acúmulo das espermatogônias do tipo A_{und} no testículo, bem como na depleção de células germinativas em diferenciação (i.e. espermatogônias do tipo A_{diff}, espermatogônias do tipo B, espermatócitos e espermatídes) (de Waal *et al.*, 2009). Ainda, a exposição associada ao andrógeno OA durante as duas últimas semanas de tratamento foi capaz de recuperar parcialmente a espermatogênese nestes animais (González, 2012). Utilizando-se a mesma metodologia, foram avaliados os níveis de RNAm dos genes *insl3* e seus receptores *rxfp2a* e *rxfp2b* em testículos em quadro de insuficiência androgênica em decorrência do bloqueio da produção de gonadotrofinas promovida pela exposição *in vivo* ao E2, e em testículos em recuperação após a exposição *in vivo* ao OA. Conforme esperado, após 5 semanas de exposição ao E2 diluído na água do aquário, foi possível observar decréscimo significativo no índice gonadosomático para os animais tratados (Figura 12), em relação aos animais do grupo controle. Este parâmetro foi parcialmente recuperado no grupo dos animais adicionalmente expostos ao hormônio OA durante as duas últimas semanas do tratamento (Figura 12). A análise morfométrica demonstrou que a exposição em longo prazo ao hormônio E2 reduziu significativamente a frequência de cistos de espermatogônias do tipo A_{diff}, do tipo B, espermatócitos e espermatídes, ao mesmo tempo em que aumentou drasticamente a frequência para cistos de espermatogônias do tipo A_{und} (Figura 13). Ainda, recuperação deste parâmetro foi observada para cistos de espermatogônias do tipo A_{diff} e do tipo B no grupo de animais

adicionalmente expostos ao hormônio OA durante as duas últimas semanas do tratamento (Figura 13).

Os resultados das reações de qPCR para o gene *insl3* e os receptores *rxfp2a* e *rxfp2b*, mostraram que o tratamento *in vivo* com E2 possui forte efeito inibitório na transcrição do gene *insl3*, ao mesmo tempo em que nenhuma alteração significativa em relação ao grupo controle foi observada para os genes dos receptores *rxfp2a* e *rxfp2b*, nos diferentes grupos tratados analisados (Figura 14). Ainda, o tratamento adicional com OA não foi capaz de recuperar os níveis de transcrição do gene *insl3* para os níveis observados para o grupo controle (Figura 14).

Discussão

5. Discussão

Embora informações acerca da evolução e expressão de relaxinas em peixes teleósteos já tenham sido publicadas na literatura (Good-Ávila *et al.*, 2009; Yegorov *et al.*, 2009; Good *et al.*, 2012), no nosso conhecimento não existem estudos que reportem atividades biológicas destes peptídeos neste grupo de vertebrados. Devido ao fato de que resíduos identificados como importantes na interação INSL3-RXFP2 (ligante-receptor) em humanos são idênticos nos receptores Rxfp2a e Rxfp2b em zebrafish (com exceção dos resíduos Ile¹⁷⁹ e Glu²²⁹, que são substituídos pelos aminoácidos Val e Ala), o hINSL3 se apresenta como promissora ferramenta para o estudo do papel fisiológico desta relaxina em peixes teleósteos. Tal afirmativa é corroborada pelos interessantes resultados obtidos nos nossos experimentos, sendo os mesmos o primeiro indicativo de que os receptores Rxfp2 retiveram a sua capacidade de responder ao INSL3 durante a evolução dos vertebrados, ainda que promovendo a regulação de processos biológicos diferentes (e.g. descenso testicular em mamíferos, e proliferação espermatogonial em zebrafish).

Ainda, embora estudo relativamente recente tenha demonstrado que o processo espermatogênico em camundongos adultos é independente da participação INSL3 (Huang *et al.*, 2012), estudos em outras espécies de mamíferos apontaram a participação desta relaxina na sobrevivência de células germinativas bem como na quantidade e qualidade de espermatozoides produzidos (Kawamura *et al.*, 2004; Sagata *et al.*, 2015). Assim, devido ao fato de que em algumas espécies de mamíferos os receptores para o INSL3 são expressos no testículo, exclusivamente nas células germinativas (Sagata *et al.*, 2015), e de que o INSL3 produzido pelas células de Leydig é transportado para o interior dos túbulos seminíferos (Minagawa *et al.*, 2014), é provável que esta relaxina

desempenhe outros papéis fisiológicos ainda desconhecidos durante o desenvolvimento da espermatogênese em animais adultos.

Através da utilização da cultura de testículos, técnica esta já bem estabelecida no estudo da fisiologia reprodutiva de zebrafish (Leal *et al.*, 2009b), foi possível demonstrar que o hINSL3 aumentou de forma significativa a atividade proliferativa das espermatogônias dos tipo A_{und} . Além disso, investigações mais detalhadas indicaram que a proliferação deste tipo espermatogonial, quando estimulada pelo hINSL3, levaria a diferenciação e não a auto-renovação espermatogonial.

As espermatogônias do tipo A_{und} são células isoladas, envolvidas por uma ou duas células de Sertoli (Leal *et al.*, 2009a), sendo as SSC consideradas parte integrante da população deste tipo celular (Nóbrega *et al.*, 2010). Como células indiferenciadas, as SSC têm a capacidade de produzir através de divisão celular tanto novas SSC (auto-renovação), quanto células comprometidas com o processo espermatogênico e a formação final dos espermatozoides, sendo o primeiro passo nesta direção, a formação de pares de espermatogônias do tipo A_{diff} (Schulz *et al.*, 2010). Estas últimas apresentam volume celular consideravelmente menor quando comparadas as espermatogônias do tipo A_{und} (~44%), o que facilita a sua identificação em cortes histológicos, permanecendo ainda no mesmo cisto espermatogênico conectadas por ponte citoplasmática (Leal *et al.*, 2009a; Schulz *et al.*, 2010). Em contrapartida, o processo de auto-renovação resulta na produção de duas novas SSC completamente separadas devido à citocinese completa, sendo necessária a formação novas de células Sertoli para a produção de dois novos cistos espermatogênicos independentes, cada um contendo uma das duas células filhas produzidas após a divisão mitótica (França *et al.*, 2015). Nossos resultados indicaram decréscimo no índice de proliferação das células de Sertoli quando associadas à espermatogônias do tipo A_{und} em divisão (positivas para BrdU), após a exposição do

testículo ao hINSL3 em cultura. Uma vez que o aumento da proliferação observado para as espermatogônias do tipo A_{und} não é acompanhada da formação de novas células de Sertoli e, conseqüentemente, novos nichos disponíveis para a formação de novos cistos espermatogênicos, é plausível interpretar este resultado como indicativo circunstancial de que as novas células espermatogoniais formadas permanecem no mesmo cisto espermatogênico, caracterizando assim o início do processo de diferenciação. Para se obter evidências diretas para esta hipótese, foram utilizadas abordagens morfométricas e moleculares adicionais.

Desta forma, através das análises de PCR quantitativo foi demonstrado que os níveis de RNAm para 3 dos 4 genes marcadores de células germinativas investigados se mantiveram estáveis após a exposição dos testículos ao hINSL3 em cultura. Interessantemente, foi possível observar decréscimo significativo nos níveis de transcrição para o gene *nanos2* após o tratamento. Em camundongos, a manutenção da população das SSC está relacionada à produção da proteína NANOS2 (Sada *et al.*, 2009). A deficiência induzida dessa proteína em animais adultos leva ao desequilíbrio entre os processos de auto renovação e diferenciação das SSC e a conseqüente depleção da população deste grupo de células germinativas, enquanto a sua superprodução leva ao acúmulo de SSC nos testículos (Sada *et al.*, 2009; Yu *et al.*, 2014). Em teleósteos, o gene ortólogo *nanos2* parece desempenhar papel semelhante ao encontrado em mamíferos, considerando-se que a perda induzida de sua função resultou em testículos com número células germinativas drasticamente reduzido em tilápias (Li *et al.*, 2014) e, que tanto seu RNAm e quanto sua proteína foram detectados em espermatogônias do tipo A_{und} nesta espécie de teleósteo (Lacerda *et al.*, 2013). Ainda em teleósteos, estudo recente em truta (*O. mykiss*) aponta o *nanos2* como excelente candidato a marcador molecular para as SSC nesta espécie, sendo expresso apenas por uma subpopulação de

espermatogônias do tipo A_{und} (Bellaiche *et al.*, 2014). Apesar de ainda não existirem informações publicadas sobre o *nanos2* em testículos de zebrafish adultos, estudo recente demonstrou que este gene é importante para a manutenção de células tronco germinativas em ovários nesta espécie (Beer & Drapper, 2013). Portanto, neste cenário, a diminuição dos níveis de RNAm para *nanos2* em testículos de zebrafish que foram expostos ao hINSL3 é compatível com a hipótese de que esta relaxina participa do processo de diferenciação das espermatogônias do tipo A_{und} em testículos de animais adultos desta espécie.

Num outro contexto, estudo publicado na literatura demonstrou que o *Amh* é um fator inibitório da diferenciação espermatogonial, além de reduzir significativamente a produção de RNAm para *insl3* em testículos de zebrafish (Skaar *et al.*, 2011). Uma vez que nossos dados sugerem que o hINSL3 estimula a diferenciação espermatogonial, nós investigamos se este efeito incluiria a regulação negativa da transcrição do gene *amh*. No entanto, nenhum efeito significativo foi observado. Ainda, nenhum efeito foi observado para os genes *gsdf* e *igf3*, que estão envolvidos na proliferação espermatogonial em truta e zebrafish respectivamente (Sawatari *et al.*, 2007; Morais *et al.*, 2013), demonstrando que a ação observada em nossos estudos para o hINSL3 não envolvem a regulação da transcrição destes fatores de crescimento.

Em consonância com os baixos níveis de RNAm para o *nanos2* encontrado nas análises moleculares, abordagens morfométricas demonstraram que o tratamento com o hINSL3 levou ao decréscimo significativo da área de parênquima testicular ocupado por cistos de espermatogônias do tipo A_{und} , ao passo que este mesmo parâmetro aumentou significativamente para espermatogônias do tipo A_{diff} . Tal resultado sugere que o hINSL3 promoveu o declínio da população de espermatogônias do tipo A_{und} através de estímulo proliferativo, demonstrado pelo aumento no índice de proliferação para essa

população espermatogonial, resultando na diferenciação destas células germinativas e na consequente formação de cistos contendo espermatogônias do tipo A_{diff} .

Ainda no contexto morfológico, os resultados das análises do efeito do hINSL3 na dinâmica de proliferação de populações espermatogoniais previamente marcadas com BrdU reforçam a hipótese sugerida no presente estudo. Considerando-se que o índice de marcação, ou seja, o número de células marcadas para BrdU seja semelhante em ambos os grupos (controle e tratado) no início do período de incubação com o hINSL3, o declínio observado na população de espermatogônias do tipo A_{und} BrdU-positivas, associado ao aumento no seu índice de proliferação e diminuição da área de parênquima testicular ocupado por este tipo celular e, ainda, a diminuição nos níveis de RNAm para *nanos2* e o aumento da área representada por espermatogônias do tipo A_{diff} no parênquima testicular, reflete diretamente a ação pró diferenciação deste peptídeo.

Apesar da análise do índice mitótico não ter indicado efeito do hINSL3 na proliferação das espermatogônias do tipo A_{diff} , foi observado decréscimo significativo no índice de marcação para BrdU para este tipo celular, nos experimentos envolvendo a dinâmica de proliferação de populações espermatogoniais previamente marcadas com BrdU. Este resultado pode ser explicado tomando-se por base as considerações apresentadas a seguir. Nóbrega e colaboradores, utilizando-se de metodologia semelhante para o estudo da dinâmica de divisão de subpopulações espermatogoniais em zebrafish (Nóbrega *et al.*, 2010) demonstrou que o ciclo celular das espermatogônias iniciais nesta espécie de teleósteo possui duração aproximada de 30 horas. Neste caso, os 4 dias (~96 horas) de incubação utilizados neste experimento são suficientes para que ocorram cerca de 3-4 ciclos celulares destes tipos espermatogoniais, representando uma diluição de 8-16 vezes no número de espermatogônias do tipo A_{und} BrdU-positivas presente no início do período de incubação, e também uma diluição de 8-16 vezes na

concentração de BrdU detectável nas gerações espermatogoniais posteriores. Além disso, o tratamento em cultura com o hINSL3 resulta no recrutamento tanto de espermatogônias do tipo A_{und} BrdU-positivas (~55% no início do período de incubação, Figura 9A) quanto BrdU-negativas (~45% no início do período de incubação, Figura 9A). Conseqüentemente, o tratamento com hINSL3 em cultura leva ao aumento do número de espermatogônias do tipo A_{diff} BrdU-negativas, originadas a partir de espermatogônias do tipo A_{und} BrdU-negativas.

Portanto, uma vez observado aumento na proporção da área de parênquima ocupada por cisto de espermatogônias do tipo A_{diff} , ao passo que este mesmo parâmetro se apresentou estável para espermatogônias do tipo B após o período de incubação com hINSL3, no nosso entendimento, a redução do número de espermatogônias do tipo A_{diff} BrdU-positivas, mesmo que seu índice mitótico não se encontre alterado após o tratamento com hINSL3, é reflexo da formação deste tipo de célula germinativa a partir do recrutamento de espermatogônias do tipo A_{und} BrdU-negativas, associada a perda da capacidade de se detectar o BrdU por meio de técnicas de imunohistoquímica devido sua diluição após repetidos ciclos celulares.

A participação de andrógenos na diferenciação de espermatogônias em teleósteos já foi demonstrada através de estudos *in vitro* (Miura *et al.*, 1991; Amer *et al.*, 2001; Leal *et al.*, 2009b) e *in vivo* (Cavaco *et al.*, 1998). Estudo relativamente recente demonstrou que o INSL3 possui efeito autócrino sobre as células de Leydig em camundongos, estimulando a produção e liberação de andrógenos *in vitro* (Pathirana *et al.* 2012). Uma vez que o Fsh é um potente fator estimulador da produção tanto de andrógenos quanto de Insl3 em zebrafish e, ainda, considerando-se o fato de que o Fsh recombinante de zebrafish foi capaz de induzir a diferenciação das espermatogônias do tipo A_{und} em testículos de zebrafish em cultura (Skaar *et al.* 2011; Moraes, 2013), decidimos investigar

se os efeitos observados para o hINSL3 nos nossos estudos estariam associados à uma potencial ação pró esteroidogênese desta relaxina. Nossos experimentos *in vitro* não apontaram efeitos significativos do hINSL3 na liberação de andrógenos, tanto na basal quanto naquela estimulada pelo Fsh. Desta forma, no presente estudo, os resultados indicam que os efeitos observados para essa relaxina não seriam dependentes da modulação da produção e liberação de andrógenos, sendo portanto uma via adicional de estímulo da diferenciação das espermatogônias do tipo A_{und}.

Finalmente, os dados obtidos nas análises morfométricas e para os níveis de mRNA referentes aos genes *insl3*, *rxfp2a* e *rxfp2b* nos animais submetidos aos tratamentos hormonais *in vivo* reforçam nossos resultados encontrados nos estudos em cultura de testículo. A criação de um contexto fisiológico favorável à auto-renovação através da exposição *in vivo* dos animais ao hormônio E2 resultou no aumento significativo da frequência de cistos de espermatogônias do tipo A_{und} (~85% dos cistos avaliados). Interessantemente, o acúmulo deste tipo espermatogonial após exposição prolongada ao E2 foi concomitante à forte inibição da transcrição do gene *insl3*. Desta forma este resultado pode ser considerado mais um indicativo da participação do Insl3 na diferenciação deste tipo espermatogonial em zebrafish. Ainda, a recuperação parcial da espermatogênese independente da recuperação dos níveis de transcrição do *insl3*, observada nos animais expostos adicionalmente ao OA durante as duas semanas finais de tratamento, associado aos resultados encontrados nas análises de liberação *in vitro* de andrógenos sob estímulo do hINSL3, demonstram que as vias pró-diferenciação ativadas pelo Insl3 e por andrógenos em testículos de zebrafish, embora possuam o Fsh como gatilho inicial, atuam de forma independente.

Conclusões

6. Conclusões

Embora o processo de descenso testicular não ocorra em zebrafish, o *Insl3* é produzido em larga escala pelas células de Leydig, não somente durante o desenvolvimento, mas também durante a sua vida adulta. No nosso conhecimento, o presente estudo foi o primeiro a investigar a atividade do *Insl3* na espermatogênese em peixes teleósteos. Os resultados das abordagens morfológicas e moleculares em estudos *ex vivo* e *in vivo* utilizadas nas nossas investigações demonstraram que:

1. o hINSL3 foi capaz de aumentar significativamente o índice de proliferação das espermatogônias do tipo A_{und} , ao mesmo tempo em que diminuiu o índice de proliferação para células de Sertoli associadas a espermatogônias do tipo A_{und} em divisão, em cultura de testículos de zebrafish, indicando a não formação de novos cistos espermatogênicos em decorrência da divisão destas espermatogônias;
2. o tratamento *ex vivo* com hINSL3 diminuiu a população de espermatogônias do tipo A_{und} , ao mesmo tempo em que aumentou a população das espermatogônias do tipo A_{diff} ;
3. a exposição ao hINSL3 em cultura de testículo promoveu o decréscimo significativo da transcrição do gene marcador para espermatogônias indiferenciadas, *nanos2*;
4. os níveis de transcrição do gene *insl3* foram drasticamente reduzidos em um quadro favorável à auto renovação das espermatogônias do tipo A_{und} , promovido pela deficiência androgênica em decorrência à exposição *in vivo* ao hormônio E2;
5. a recuperação parcial da espermatogênese observada após a inclusão do andrógeno OA (precursor da 11-KT) no tratamento *in vivo*, ocorre independentemente da recuperação dos níveis de transcrição do gene *insl3*.

Assim, no contexto acima mencionado, nossos resultados indicam que o Insl3 estimula a proliferação e diferenciação de espermatogônias do tipo A indiferenciadas nos testículos de zebrafish sexualmente maduros, efeito este independente da participação de andrógenos.

Perspectivas

7. Perspectivas

O estudo desenvolvido no trabalho ora apresentado fornece bons subsídios para a melhor compreensão das funções do *Insl3* na espermatogênese em teleósteos. A partir dos resultados encontrados até o presente momento, novas investigações que complementam as informações até então obtidas encontram-se atualmente em desenvolvimento, nos propiciando aprofundar o entendimento acerca das funções desse peptídeo nos testículos de zebrafish.

Neste sentido, já iniciamos experimentos em cultura de testículos utilizando-se o *Insl3* recombinante de zebrafish (*zfrInsl3*), que foi recentemente adquirido pelo nosso grupo de pesquisa, com o intuito de se avaliar e comparar os efeitos desta relaxina na proliferação e cinética espermatogonial e níveis de mRNA testicular com os efeitos observados para o *hINSL3*. Ainda, experimentos envolvendo transfecção de células HEK-293 com o objetivo de se verificar quais dos dois subtipos de receptores para *Insl3* expressos nos testículos de zebrafish são estimulados pelo *hINSL3* e pelo *zfrInsl3*, também encontram-se em andamento.

Embora até o momento não tenha sido possível localizar o sítio específico de expressão dos receptores para *Insl3* nos testículos de zebrafish, novos protocolos para hibridização *in situ* e imunohistoquímica (Sagata *et al.*, 2015) estão em fase de teste em nosso laboratório, com o intuito de se verificar quais os tipos de células testiculares expressam os receptores para essa relaxina.

Finalmente, o fácil acesso ao genoma do zebrafish, em combinação com o rápido surgimento de ferramentas de edição de genoma e as melhorias na eficiência desta ferramenta para o estudo desta espécie, fornece aos pesquisadores excelente oportunidade para se investigar as funções de genes de interesse. Neste contexto, o tipo

II do sistema CRISPR (Repetições Palíndromas Curtos Regularmente Aglomeradas de Interspaced), conhecido por sistema CRISPR-Cas, foi ajustado por Hwang e colaboradores para a edição do genoma do zebrafish (Hwang *et al.*, 2013). Desta forma, a Divisão de Pesquisa em Biologia da Reprodução da Universidade de Utrecht, Holanda, está utilizando o sistema de CRISPR/Cas9 para gerar zebrafish *knockouts* pra o gene *insl3*. No nosso entendimento, estes novos experimentos representam um passo crucial para o avanço nos estudos da atividade biológica do Insl3 na espermatogênese nessa espécie de teleósteo.

Tabelas e figuras

8. Tabelas e figuras

Tabela 1 – Primers utilizados nas análises dos níveis de transcrição para genes testiculares.

Genes	Primers	Sequência (5'-3')	Referências
<i>ef1α</i>	AH (Fw)	GCCGTCCCACCGACAAG	(Morais <i>et al.</i> , 2013)
	AG (Rv)	CCACACGACCCACAGGTACAG	
<i>amh</i>	AD (Fw)	CTCTGACCTTGATGAGCCTCATTT	(García-López <i>et al.</i> , 2010)
	AE (Rv)	GGATGTCCCTTAAGAACTTTTGCA	
<i>igf3</i>	2680 (Fw)	TGTGCGGAGACAGAGGCTTT	(Morais <i>et al.</i> , 2013)
	2681 (Rv)	CGCCGCACTTTCTTGGATT	
<i>gsdf</i>	2366 (Fw)	CATCTGCGGGAGTCATTGAAA	(García-López <i>et al.</i> , 2010)
	2367 (Rv)	CAGAGTCCTCCGGCAAGCT	
<i>piwi11</i>	2542 (Fw)	GATACCGCTGCTGAAAAAAGG	(García-López <i>et al.</i> , 2010)
	2543 (Rv)	TGGTTCTCCAAGTGTGTCTTGC	
<i>sypc3</i>	2730 (Fw)	AGAAGCTGACCCAAGATCATTCC	(García-López <i>et al.</i> , 2010)
	2731 (Rv)	AGCTTCAGTTGCTGGCGAAA	
<i>odf3b</i>	2791 (Fw)	GATGCCTGGAGACATGACCAA	(Leal <i>et al.</i> , 2009b)
	2792 (Rv)	CAAAGGAGAAGCTGGGAGCTTT	
<i>nanos2</i>	4817 (Fw)	AAACGGAGAGACTGCGCAGAT	Presente trabalho
	4818 (Rv)	CGTCCGTCCCTTGCCTTT	
<i>insl3</i>	2466 (Fw)	TCGCATCGTGTGGGAGTTT	(Good <i>et al.</i> , 2012)
	2467 (Rv)	TGCACAACGAGGTCTCTATCCA	
<i>rxfp2a</i>	3258 (Fw)	CAATTCCAGTCTCTGTCAGCACAT	(Good <i>et al.</i> , 2012)
	3259 (Rv)	CTCAACGTCATTCTCCGCAA	
<i>rxfp2b</i>	3262 (Fw)	CTGCCAGACTCTGTGCCATA	(Good <i>et al.</i> , 2012)
	3263 (Rv)	AGTCGTGATGCTATTACCCTCGAA	

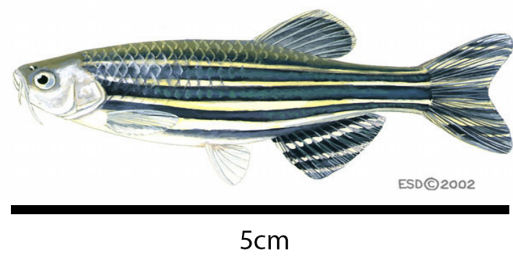


Figura 1 – Aspecto corporal externo do zebrafish (*Danio rerio*).

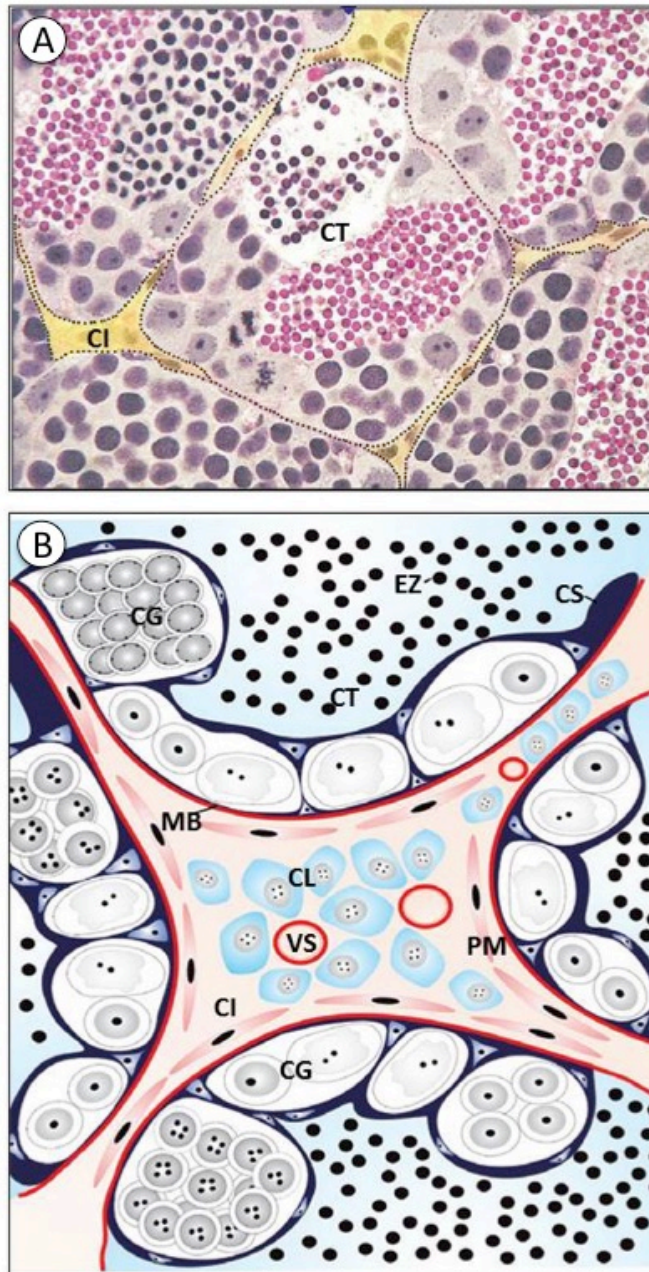


Figura 2 – Corte histológico (A) e figura esquemática (B) ilustrando a estrutura testicular em teleósteos. Conforme mostrado, os testículos em teleósteos são divididos em compartimento intersticial (CI), onde são encontradas as células de Leydig (CL) e vasos sanguíneos (VS), e compartimento tubular (CT), formado pelas células peritubulares mióides (PM), membrana basal (MB), células germinativas (CG) e células de Sertoli (CS) compondo o epitélio seminífero. Adaptado de Schulz & Nóbrega, 2011.

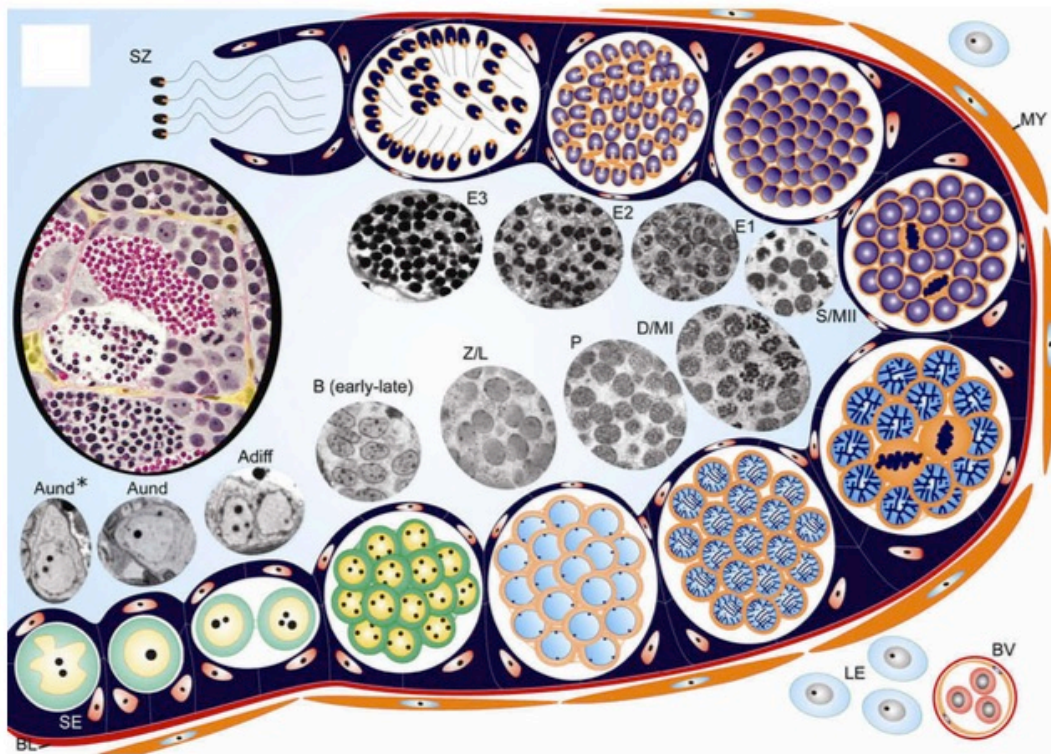


Figura 3 – Figura esquemática ilustrando a estrutura cística e a progressão da espermatogênese em teleostes (zebrafish). Os cistos espermatogênicos são formados por células de Sertoli que envolvem as células germinativas que se diferenciam/progridem de maneira sincrônica. (MY) células peritubulares mióides, (BL) membrana basal, (SE) células de Sertoli, (A_{und*} e A_{und}) espermatogônias do tipo A indiferenciadas, (A_{diff}) espermatogônias do tipo A diferenciada, ($B_{early-late}$) espermatogônias do tipo B iniciais e tardias, espermátocitos primários [(L/Z) leptóteno/zigóteno, (P)paquíteno, (D/MI) diplóteno/meiose I], (S/MII) espermátocitos secundários/meiose II, (E1-3) espermátides iniciais e tardias, (SZ) espermatozoides, (BV) vasos sanguíneos e (LE) células de Leydig. Esquema retirado de Schulz *et al.*, 2010.

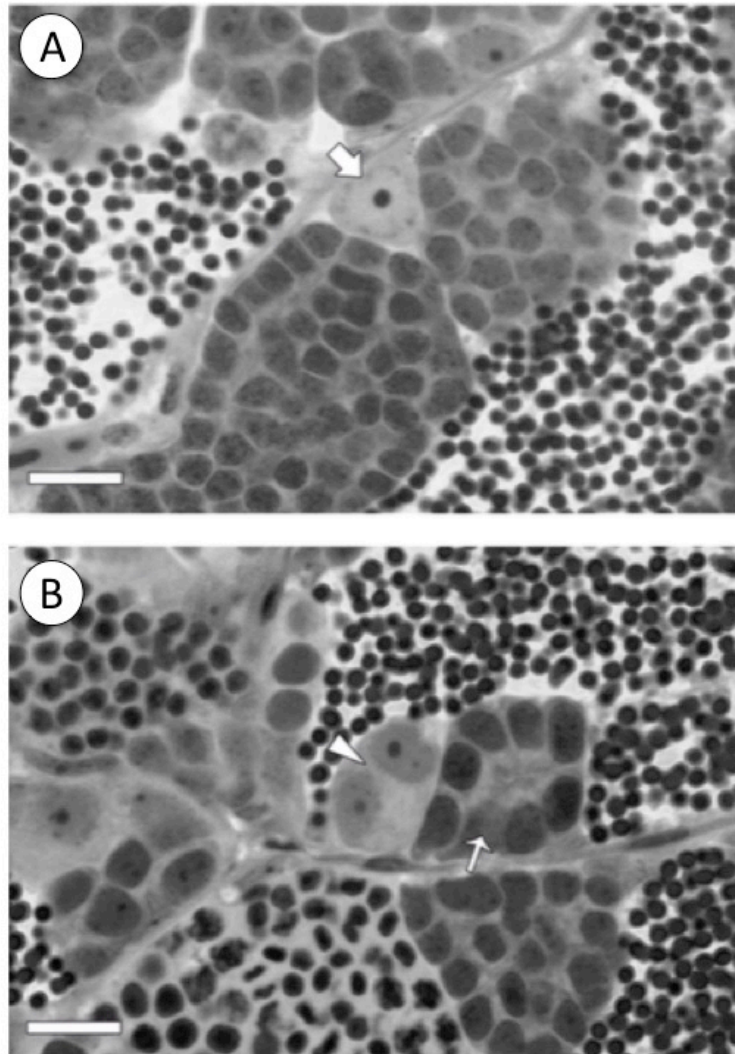


Figura 4 – Características morfológicas das espermatogônias do tipo A_{und} , A_{diff} e do tipo B de zebrafish. (A) As espermatogônias do tipo A_{und} (seta) são células germinativas isoladas em seus cistos espermatogênicos e apresentam o maior diâmetro nuclear dentre as células germinativas nos testículos de zebrafish ($\sim 9 \mu\text{m}$), além de apresentarem núcleo rico em eucromatina, com um ou dois nucléolos. (B) Apesar de compartilhar semelhanças morfológicas com as espermatogônias do tipo A_{und} , as espermatogônias do tipo A_{diff} (cabeça de seta) possuem menor diâmetro nuclear ($\sim 6 \mu\text{m}$) e ocorrem em clones de 2-8 células por cisto espermatogênico, enquanto as espermatogônias do tipo B (seta menor), apresentam núcleo denso, ovalado e menor diâmetro ($\sim 5 \mu\text{m}$) quando comparado as espermatogônias do tipo A. As secções foram preparadas para análises histológicas de acordo com Leal *et al.*, 2009a. Aumento 1000x, barras de escala = $10 \mu\text{m}$.

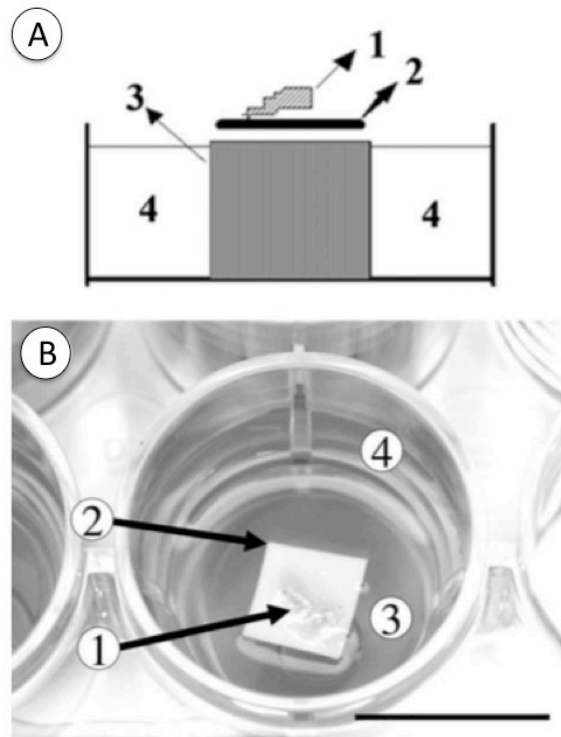


Figura 5 – (A) Figura esquemática e (B) fotografia do sistema de cultura de testículo de zebrafish. 1: testículo, 2: membrana de nitrocelulose, 3: cilindro de agarose, 4: meio de cultura. Extraído de Leal *et al.*, 2009b.

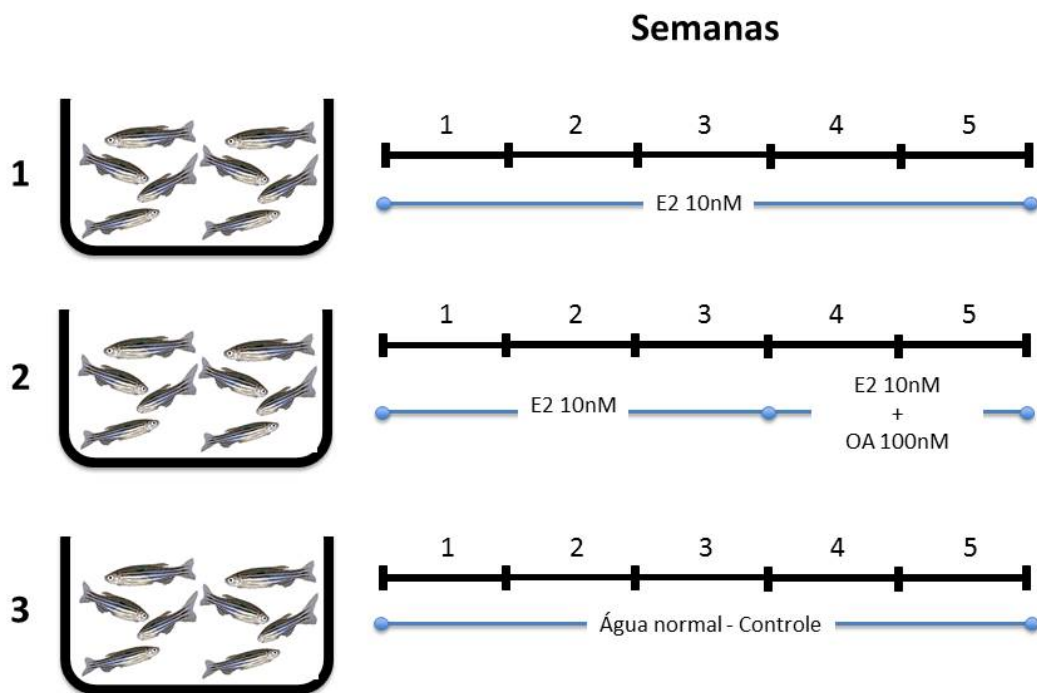


Figura 6 – Esquema ilustrativo da abordagem utilizada para os tratamentos hormonais *in vivo*. No tanque número 1, os animais foram expostos a dose de 10nM de E2 durante 5 semanas consecutivas; no tanque número 2, os animais foram expostos a dose de 10nM de E2 durante as 5 semanas consecutivas, sendo adicionada dose de 100nM de OA durante as últimas duas semanas; no tanque número 3 os animais não receberam nenhum tratamento hormonal e foram utilizados como grupo controle.

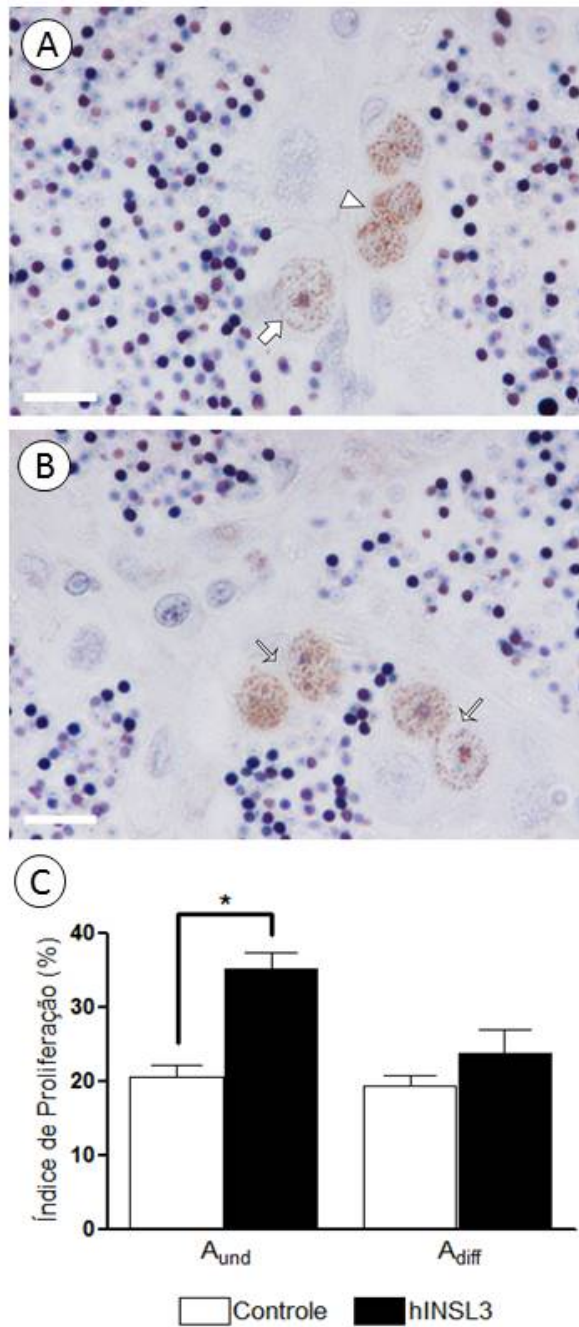


Figura 7 – (A e B) Secções testiculares mostrando células germinativas BrdU positivas: espermatogônia do tipo A_{und} (seta), espermatogônias do tipo A_{diff} (setas menores) e espermatogônias do tipo B (cabeça de seta). (C) Índice de proliferação (mitótico) de espermatogônias do tipo A_{und} e A_{diff} após 7 dias de incubação na ausência (Controle) ou presença de 100ng/mL de hINSL3. Diferença estatística significativa (p<0,05) representada pelo asterisco (*). Os resultados estão apresentados na forma de média ± erro padrão (n=8). Barras = 10µm.

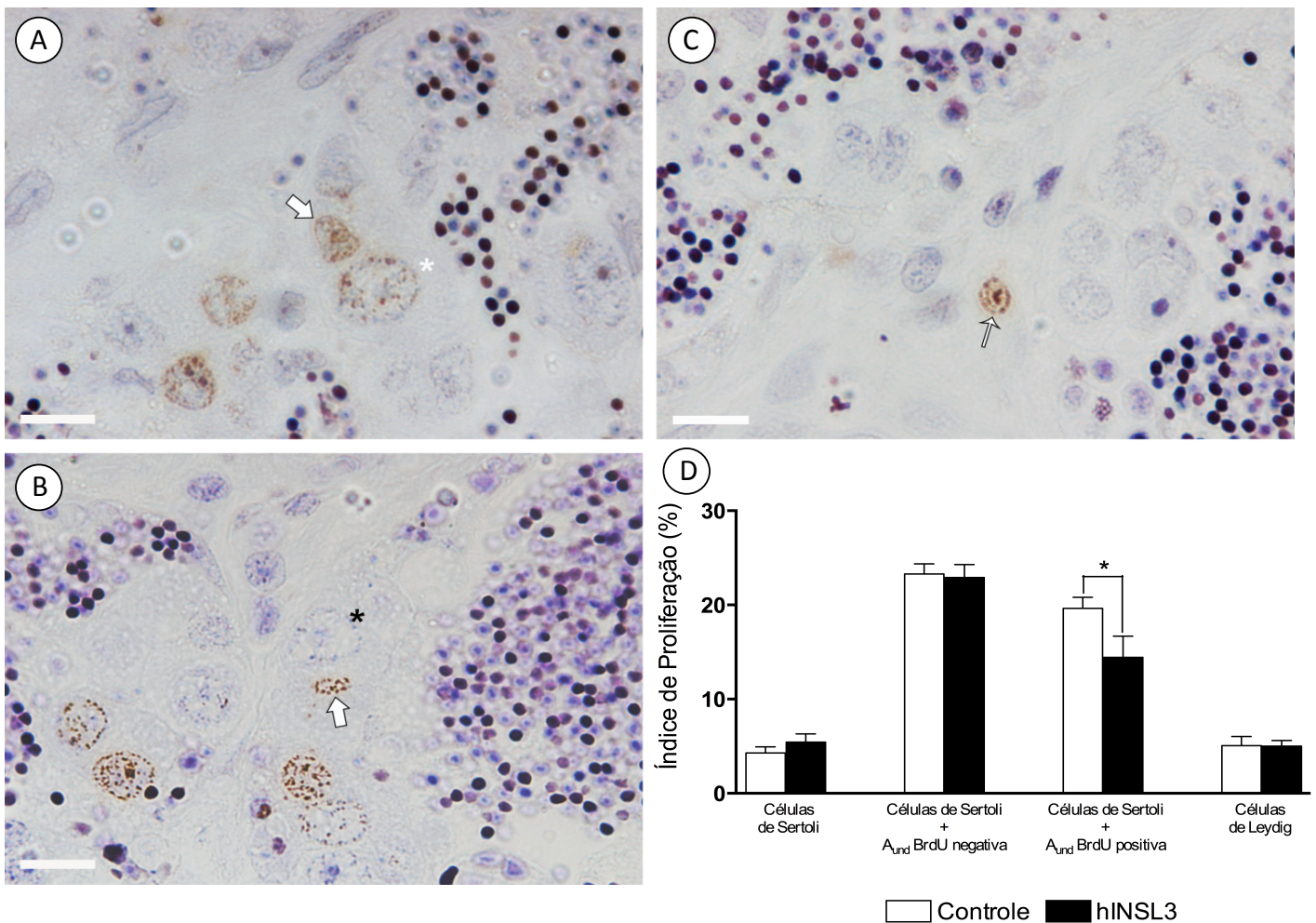


Figura 8 – (A, B e C) Secções testiculares mostrando células de Sertoli BrdU positiva (seta maior) em associação com espermatogônia do tipo A_{und} BrdU-positiva (asterisco branco) e BrdU-negativa (asterisco preto), e célula de Leydig BrdU positiva (seta menor). (D) Índice de proliferação (mitótico) de células de Leydig, células de Sertoli e células de Sertoli especificamente associadas à cistos de espermatogônias do tipo A_{und} BrdU positivas ou negativas após 7 dias de incubação na ausência (Controle) ou presença de 100ng/mL de hINSL3. Diferença estatística significativa ($p < 0,05$) representada pelo asterisco (*). Os resultados estão apresentados na forma de média \pm erro padrão ($n=8$). Barras = 10 μ m.

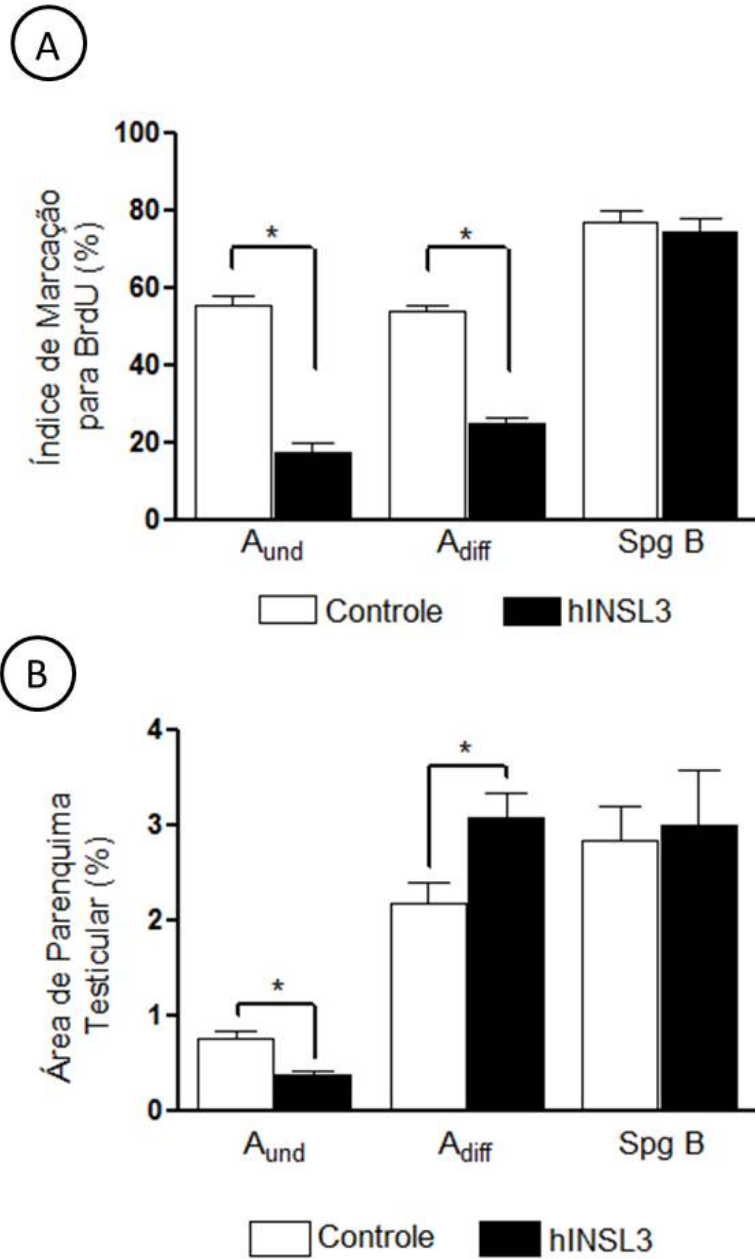


Figura 9 – Índices de marcação com BrdU (A) e área de parênquima testicular (B) para espermatogônias do tipo A_{und}, A_{diff} e do tipo B (Spg B) após 4 dias de incubação na ausência (Controle) ou presença de 100ng/mL de hINSL3. Diferenças estatísticas significativas ($p < 0,05$) representadas pelos asteriscos (*). Os resultados estão apresentados na forma de média \pm erro padrão ($n=8$).

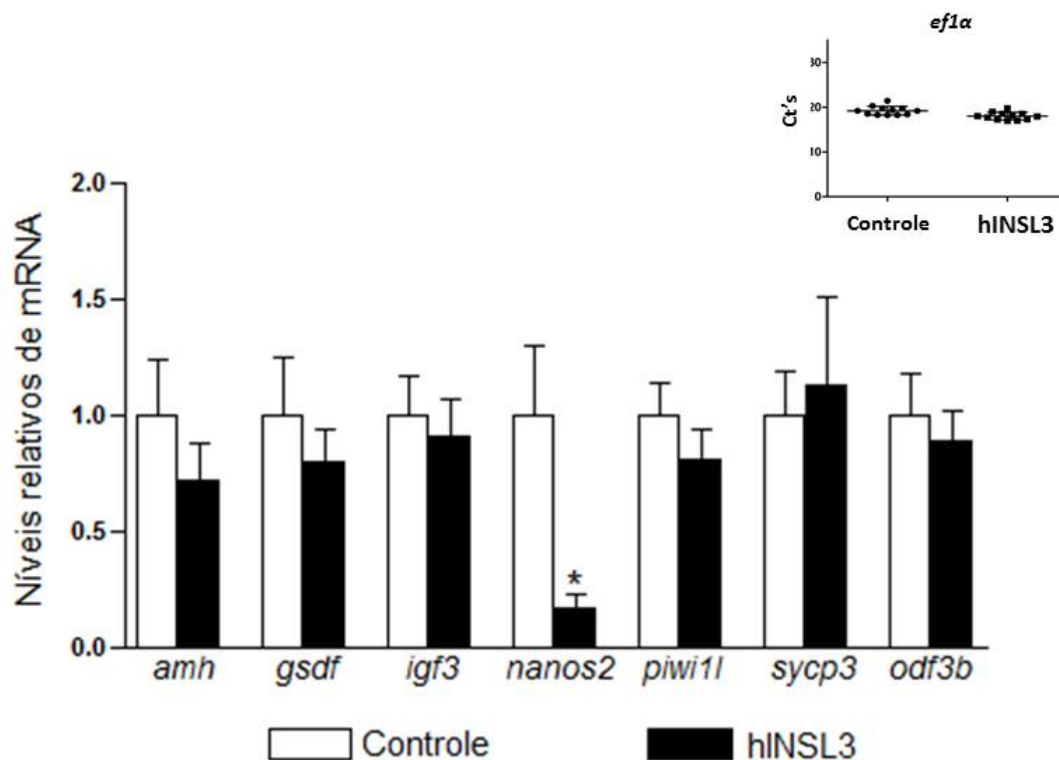


Figura 10 – Níveis de transcrição (mRNA) para vários genes selecionados (células de Sertoli: *amh*, *gsdf*, *igf3*; e células germinativas: *nanos2*, *piwi11*, *sycp3* e *odf3b*). O inset mostra os níveis de transcrição (Ct's values) para o gene *ef1a* nas condições controle (ausência de hINSL3) e presença de 100ng/mL de hINSL3. Diferenças estatísticas significativas ($p < 0,05$) representadas pelos asteriscos (*). Os resultados estão apresentados na forma de média \pm erro padrão ($n=12$).

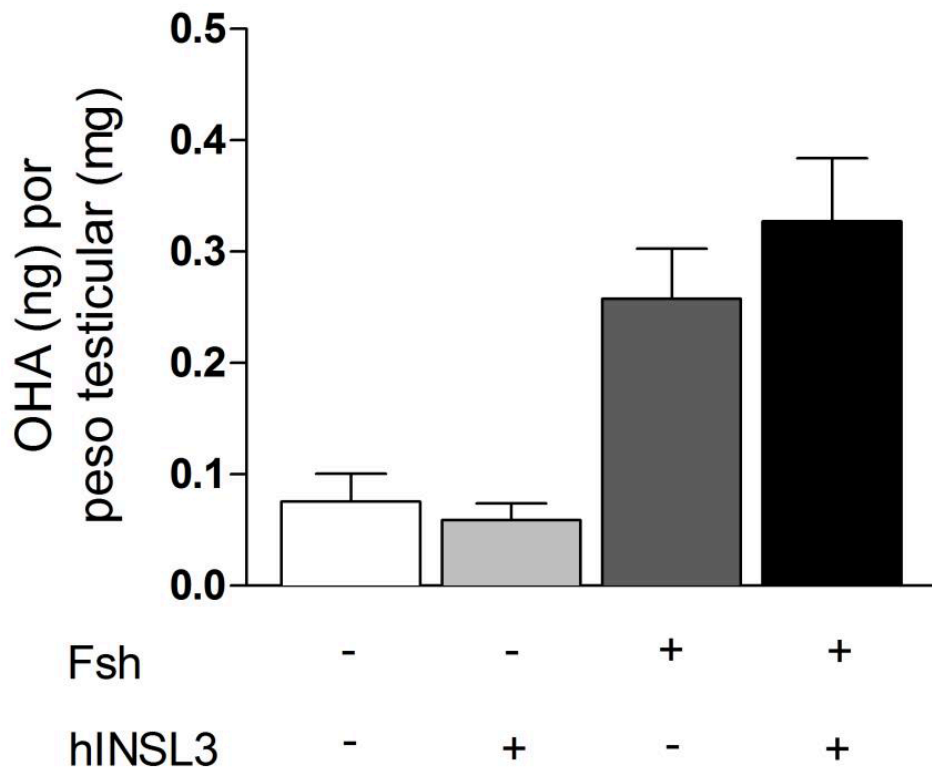


Figura 11 – Análise do efeito do hINSL3 (100ng/mL) na liberação de andrógeno *in vitro*, basal ou estimulada pelo Fsh. Os resultados estão expressos em nanograma (ng) de OHA por unidade (mg) de peso testicular e apresentados na forma de média \pm erro padrão (n=8).

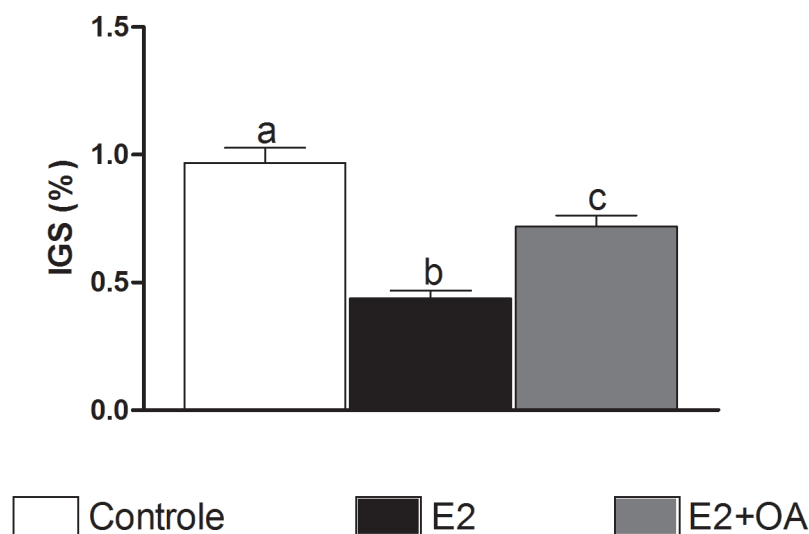


Figura 12 – Índice gonadosomático (IGS) para zebrafish após os tratamentos hormonais *in vivo*. As letras representam diferenças estatísticas ($p < 0,05$) entre os grupos. Os resultados estão expressos na forma de média \pm erro padrão ($n=20$).

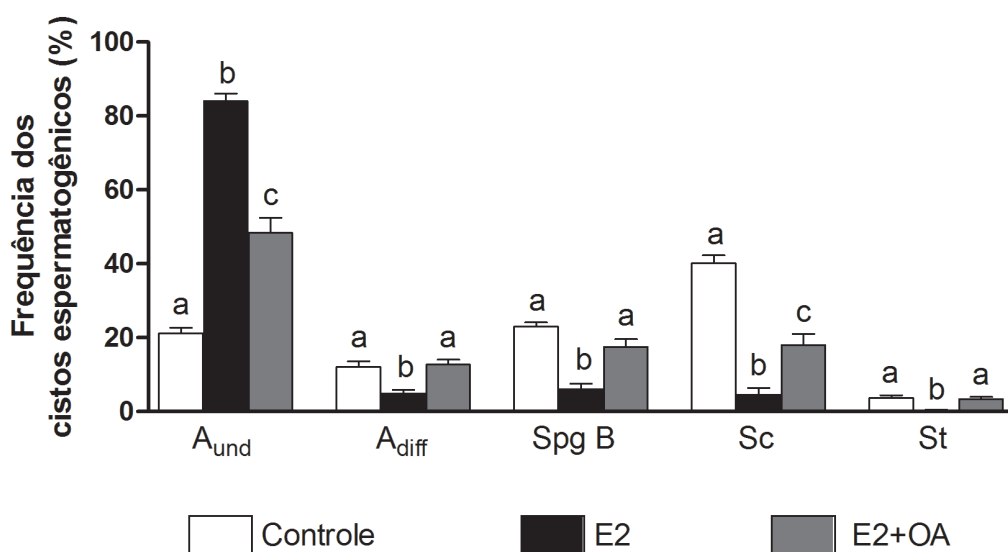


Figura 13 – Frequência dos cistos nas três diferentes fases da espermatogênese nos testículos de zebrafish, após os tratamentos hormonais *in vivo*. As letras representam diferenças estatísticas ($p < 0,05$) observadas entre os grupos para cada um dos diferentes tipos de cistos investigados. Os resultados estão expressos na forma de média \pm erro padrão ($n=8$). A_{und} = cistos de espermatogônias do tipo A indiferenciadas, A_{diff} = cistos de espermatogônias do tipo A diferenciadas, Spg B = cistos de espermatogônias do tipo B, Sc = cistos de espermatócitos, St = cistos de espermátides.

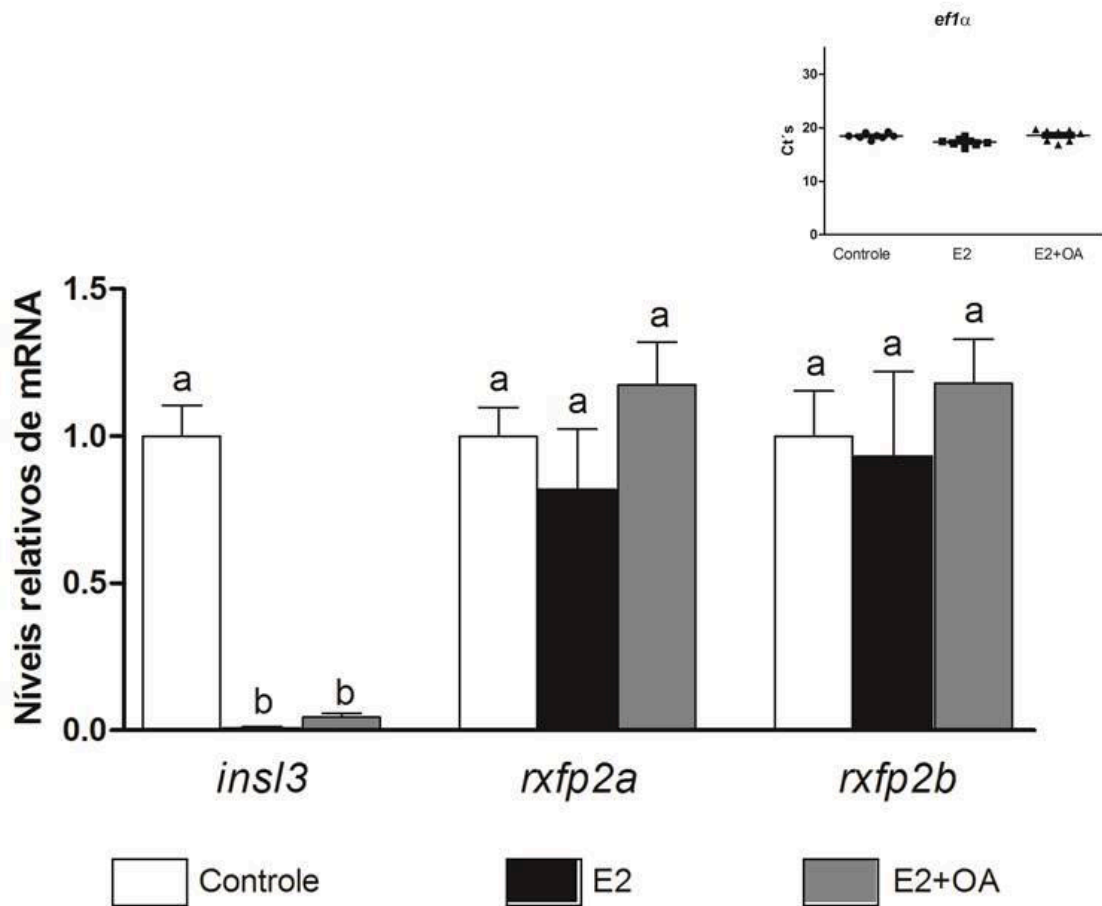


Figura 14 – Análise do efeito dos diferentes tratamentos hormonais *in vivo* na transcrição dos genes *insl3* e seus receptores *rxfp2a* e *rxfp2b*. O inset mostra os níveis de transcrição (Ct's values) para o gene *eflα* para os diferentes tratamentos e para o grupo controle. As letras representam diferenças estatísticas observadas entre os grupos para cada um dos genes investigados. Os resultados estão apresentados na forma de média \pm erro padrão (n=12).

Referências

Referências

Almeida FF, Kristoffersen C, Taranger GL, Schulz RW (2008) Spermatogenesis in atlantic cod (*Gadus morhua*): a novel model of cystic germ cell development. *Biol Reprod* 78(1):27-34

Amer MA, Miura T, Miura C, Yamauchi K (2001) Involvement of sex steroids hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). *Bio Reprod* 65:1057-1066

Anand-Ivell RJ, Relan V, Balvers M, coiffec-Dorval I, Fritsch M, Bathgate RA, Ivell R (2002) Expression of the insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biol Reprod* 74(5):945-953

Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, Summers RJ (2006a) International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol Rev* 58(1):7-31

Bathgate RA, Lin F, Hanson NF, Otvos LJr, Guidolin A, Giannakis C, Bastiras S, Layfield SL, Ferraro T, Ma S, Zhao C, Gundlach AL, Samuel CS, Tregear GW, Wade JD (2006b) Relaxin-3: Improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both *in vivo* and *in vitro*. *Biochemistry* 45:1043-1053

Bathgate RAD, Halls ML, van der Westhuizen ET, Callander GE, Kocan M, Summers RJ (2013) Relaxin Family peptides and their receptors. *Physiol Rev* 93:405-480

Batlouni SR, Nóbrega RH, França LR (2009) Cell junctions in fish seminiferous epithelium. *Fish Physiol Biochem* 35(1):207-217

Beer RL, Draper BW (2013) Nanos3 maintains germline stem cells and expression of the conserved germline stem cell gene nanos2 in the zebrafish ovary. *Dev Biol* 374:308-318

Bellaiche J, Lareyre J, Cauty C, Yano A, Allemand I, Le Gac F (2014) Spermatogonial stem cell quest: nanos2, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. *Biol Reprod* 90(4):791-14

Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S (2005) GDNF family receptor alpha 1 phenotype of spermatogonial stem cells in immature mouse testis. *Biol Reprod* 73(5):1011-1016

Büllesbach EE, Schwabe C (1999) Tryptophan B27 in the relaxin-like factor (RLF) is crucial for RLF receptor-binding. *Biochemistry* 38:3073-3078

Büllesbach EE, Schwabe C (2004) Synthetic cross-links arrest the C-terminal region of relaxin-like factor in an active conformation. *Biochemistry* 43:8021-8028

Büllesbach EE, Schwabe C (2005) LGR8 signal activation by the relaxin-like factor. *J Biol Chem* 280:14586-14590

Büllesbach EE, Schwabe C (2006) The mode of interaction of the relaxin-like factor (RLF) with the leucine-rich repeat G protein-activated receptor 8. *J Biol Chem* 281:26136-26143

Campos-Junior PH, Costa GM, Lacerda SM, Rezende-Neto JV, de Paula AM, Hofmann MC, França LR (2012) The spermatogonial stem cell niche in the collared peccary (*Tayassu tajacu*). *Biol Reprod* 86(5):155,1-10

Cavaco JE, Vilrocx C, Trudeau VL, Schulz RW, Goos HJ (1998) Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). *American J Physiol* 275:1793-1802

de Rooij DG, Griswold JA (2012) Question about spermatogonia posed and answered since 2000. *J Androl* 33(6):1085-1095

de Rooij DG, Grootegoed JA (1998) Spermatogonial stem cells. *Curr Opin Cell Biol* 10(6):694-701

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

de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J (2008) Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* 136:225-234

Eddy EM (1999) Role of heat shock protein HSP70-2 in spermatogenesis. *Rev Reprod* 4(1):23-30

Engeszer RE, Patterson LB, Rao AA, Parichy DM (2007) Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* 4(1):21-40

Feitsma H, Leal MC, Moens PB, Cuppen E, Schulz RW (2007) Mlh1 deficiency in zebrafish results in male sterility and aneuploid as well as triploid progeny in females. *Gen Society of America* 175(4):1561-1569

França LR, Nóbrega RH, Morais RDVS, Assis LHC, Schulz RW (2015) Sertoli cell structure and function in anamniote vertebrates In: Griswold MD (ed) *Sertoli cell biology*, Second edition. Elsevier Inc, pp:385-407

García-López A, de Jonge H, Nóbrega RH, de Waal PP, van Dijk W, Hemrika W, Taranger GL, Bogerd J, Schulz RW (2010) Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* 151(5):2349-2360

González NEG (2012) Efecto de los esteroides sobre la dinámica de las células germinales testiculares de pez cebra. Universidad de Murcia 121pp.

Good S, Yegorov S, Martijn J, Franck J, Bogerd J (2012) New insights into ligand-receptor pairing and coevolution of relaxin family peptides and their receptors in teleosts. *Intern Journ Evol Biol* 2012:14

Good-Ávila SV, Yegorov S, Harron S, Bogerd J, Glen P, Ozon J, Wilson BC (2009) Relaxin gene family in teleosts: phylogeny, syntenic mapping, selective constraint, and expression analysis. *BMC Evo Biol* 9:293-311

Grunwald DJ, Eisen JS (2002) Headwaters of the zebrafish – emergence of a new model vertebrate. *Nature Rev Gen* 3(9):717-724

Hoffmann MC (2008) Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Mol Cell Endocrinol* 288(1-2):95-103

Hsu SY, Nakabayash K, Nishi S, Kumagai J, Kudo M, Sherwood OD, Hsueh AJ (2002) Activation of orphan receptors by the hormone relaxin. *Science* 295:637-638 and 671-674 (comment)

Huang Z, Rivas B, Agoulnik AL (2012) Insulin-like 3 signaling is important for testicular descent but dispensable for spermatogenesis and cell survival in adult mice. *Biol Reprod* 87(6):143

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnol* 31:227-229

Jijiwa M, Kawai K, Fukihara J, Nakamura A, Hasegawa M, Susuki C, Sato T, Enomoto A, Asai N, Murakumo Y, Takahashi M (2008) GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. *Genes Cells* 13(4):365-374

Johnston DS, Olivas E, DiCandeloro P, Wright WW (2011) Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. *Biol Reprod* 85(4):763-769

Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ (2004) Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *PNAS* 101(19):7323-7328

Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY, Dym M (2009) The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. *Biol Reprod* 80(4):707-717

Kumagai J, Hsu SY, Matsumi H, Roh J, Fu P, Wade JD, Bathgate RAD, Hsueh AJW (2002) INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J Biol Chem* 277(35):31283-31286

Lacerda SM, Costa GM, Araújo M, Campos-Junior PHA, Segatelli TM, Peixoto MTD, Resende RR, França LR (2013) Phenotypic characterization and *in vitro* propagation and transplantation of the Nile tilapia (*Oreochromis niloticus*) spermatogonial stem cells. *Gen Comp Endocrinol* 192:95-106

Le Gac F, Loir M (1999) Male reproductive system in fish. In: Knobil, E. & Neill, J.D. (Eds.). *Encyclopedia of Reproduction*. San Diego: Academic Press, v. 3, pp. 20-30.

Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW (2009a) Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biol Reprod* 81(1):177-187

Leal MC, de Waal PP, García-López A, Chen SX, Bogerd J, Schulz RW (2009b) Zebrafish primary testis tissue culture: an approach to study testis function *ex vivo*. *Gen Comp Endocrinol* 162:134-138

Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, Wang D (2014) Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics* 197:591-599

Loveland KL, Robertson DM (2005) The TGF β superfamily in Sertoli cell biology. In: Griswold M, Skinner M (eds.) *Sertoli cell biology*. Maryland Heights, Missouri: Elsevier Science: 227-247

Matta SLP, Vilela DAR, Godinho HP, França LR (2002) The goitrogen 6-npropyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. *Endocrinology* 143:970-978

McGonell IM, Fowkes RC (2006) Fishing for gene function – endocrine modelling in the zebrafish. *J Endocrinol* 189(3):425-439

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 287(5457):1489-1493

Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proc Natl Acad Sci USA* 88:5774-5778

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

Minagawa I, Sagata D, Pitia AM, Kohriki H, Shibata M, Sasada H, Hasegawa Y, Kohsaka T (2014) Dynamics of insulin-like 3 and its receptor expression in boar testes. *J Endocrinol* 220:247-261

Morais RDVS, Nóbrega RH, Gómez-González NE, Schmidt R, Bogerd J, França LR, Schulz RW (2013) Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. *Endocrinology* 154:4365-4376

Morais, RDVS (2013) Regulação funcional dos testículos de zebrafish (*Danio rerio*) adultos através do T3 e do Igf3. Universidade Federal de Minas Gerais 108pp

Moyle PB, Cech Jr JJ (2003) *Fishes: An Introduction to Ichthyology*. Fifth Edition, 2003

Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL (2003) Maintenance of mouse male germ line stem cells *in vitro*. *Biol Reprod* 68:2207-2214

Nakamura S, Watakabe I, Nishimura T, Toyoda A, Tanigushi Y, Tanaka M (2012) Analysis of medaka *sox9* orthologue reveals a conserved role in germ cell maintenance. *PloS One* 7(1): e29982

Nakatani K, Agostinho AA, Baumgartner G, Bialetzki A, Sanches PV, Makrakis MC, Pavanelli CS (2001) *Ovos e larvas de peixes de água doce: Desenvolvimento e manual de identificação*. Maringá: EDUEM

Nasiadka A, Clark MD (2012) Zebrafish breeding in the laboratory environment. *ILAR J*, 53(2):161-168

Nef S, Parada LF (1999) Cryptorchidism in mice mutant for *INSL3*. *Nat Genet* 22(3):295-299

Nelson JS (2006) *Fishes of the world*. 4th edition, Wiley, New Jersey, p622

Nóbrega RH, Greebe CD, van de Kant H, Bogerd J, França LR, Schulz RW (2010) Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS ONE* 5(9):e12808

Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testis. *Physiol Rev* 92(2):577-595

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* 136(7):1191-1199

Pathirana IN, Kawate N, Büllesbach EE, Takahashi M, Hatoya S, Inaba T, Tamada H (2012) Insulin-like peptide 3 stimulates testosterone secretion in mouse Leydig cells via cAMP pathway. *Reg Peptides* 178(1):102-106

Petersen C, Söder O (2006) The Sertoli cell – A hormonal target and ‘super’ nurse for germ cells that determines testicular size. *Horm Research* 66(4):153-161

Rolland AD, Lardenois A, Goupil A, Lareyre J, Houlgatte R, Chalmel F, Le Gac F (2013) Profiling of androgen response in rainbow trout pubertal testis: relevance to male gonad development and spermatogenesis. *Plos One* 8(1):e53302

Rosengren KJ, Zhang S, Lin F, Daly NL, Scott DJ, Hughes RA, Bathgate RAD, Craik DJ, Wade JD (2006) Solution structure and characterization of the LGR8 receptor binding surface of insulin-like peptide 3. *J Biol Chem* 281:28287-28295

Russell LD, Ettlin RA, Sinha-Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis. In: Russell LD, Ettlin RA, Sinha-Hikim AP, Clegg ED (ed) *Mammalian spermatogenesis*. Clearwater: Cache River Press 1:1-40

Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science* 325:1394-1398

Sagata D, Minagawa I, Kohriki H, Pitia AM, Uera N, Katakura Y, Sukigara H, Terada K, Shibata M, Park EY, Hasegawa Y, Sasada H, Kohsaka T (2015) The insulin-like factor 3 (INSL3)-receptor (RXFP2) network functions as a germ cell survival/anti-apoptotic factor in boar testis. *Endocrinol* 156:1523:1539

Savitt J, Singh D, Zhang C, Chen LC, Folmer J, Shokat KM, Wright WW (2012) The *in vivo* response of stem and other undifferentiated spermatogonia to the reversible inhibition of glial cell line-derived neurotrophic factor signaling in the adult. *Stem Cells* 30(4):732-740

Sawatari E, Shikina S, Takeuchi T, Yoshizaki G (2007) A novel transforming growth factor- β superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). *Dev Biol* 301:266-275

Schulz RW, França LR, Lareyre J, LeGac F, Chiarini-Garcia H, Nóbrega RH, Miura T (2010) Spermatogenesis in fish. *Gen Comp Endocrinol* 165(3):390-411

Schulz RW, Nóbrega RH (2011) Anatomy and histology of fish testis In: Farrell AP (ed) *Encyclopedia of fish physiology: from genome to environment*, Volume 1, pp. 616-626. Academic Press: San Diego

Schulz RW, Menting S, Bogerd J, França LR, Vilela DAR, Godinho HP (2005) Sertoli cell proliferation in the adult testis – Evidence from two fish species belonging to different orders. *Biol Reprod* 73(6):891-898

Schulz RW, Miura T (2002) Spermatogenesis and its endocrine regulation. *Fish Physiol Biochem* 26:43-56

Schulz RW, van Dijk W, Chaves-Pozo E, García-López A, França LR, Bogerd J (2012) Sertoli cell proliferation in the adult testis induced by unilateral gonadectomy in African catfish. *Gen Comp Endo* 177:160-167

Scott DJ, Wilkinson TN, Zhang S, Ferraro T, Wade JD, Tregear GW, Bathgate RAD (2007) Defining the LGR8 residues involved in binding insulin-like peptide 3. *Mol Endocrinol* 21:1699-1712

Sharpe RM (1994) Regulation of spermatogenesis. In: Knobil E, Neill JD (ed) The physiology of reproduction. New York: Raven Press 2:1363-1434

Skaar KS, Nóbrega RH, Magaraki A, Olsen LC, Schulz RW, Male R (2011) Proteolytically activated, recombinant anti-mullerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* 152(9):3527-3540

Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y (2002) Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mech Dev* 113(1):29-39

Van der Ven LT, Wester PW, Vos JG (2003) Histopathology as a tool for evaluation of endocrine disruption in zebrafish (*Danio rerio*). *Environ Toxicol Chem* 22:908-913

Voog J, Jones DL (2010) Stem cell and the niche: a dynamic duo. *Cell Stem Cell* 6(2):103-115

Yegorov S, Good-Ávila SV, Parry L, Wilson BC (2009) Relaxin family genes in humans and teleosts. *Ann NY Acad Sci* 1160:42-44

Yegorov S, Bogerd J, Good SV (2014) The relaxin peptide receptors and their ligands: new developments and paradigms in the evolution from jawless fish to mammals. *Gen Comp Endocrinol* 209:93-105

Yomogida K, Yagura Y, Tadokoro Y, Nishimune Y (2003) Dramatic expansion of germinal stem cell by ectopically expressed human glial cell line-derived neurotropic factor in mouse Sertoli cells. *Biol Reprod* 69(4):1303-1307

Yoshida S (2012) Elucidating the identity and behavior of spermatogenic stem cell in the mouse testis. *Reproduction* 144(3):293-302

Yu M, Mu H, Niu Z, Chu Z, Zhu H, Hua J (2014) miR-34c enhances mouse spermatogonial stem cells differentiation by targeting Nanos2. *J Cell Biochem* 115:232-242

Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, Adham IM (1999) Targeted disruption of *INSL3* gene causes bilateral cryptorchidism. *Mol Endocrinol* 13(5):681-691

Anexos

INSL3 stimulates spermatogonial differentiation in testis of adult zebrafish (*Danio rerio*)

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Abstract INSL3 (insulin-like peptide 3) is a relaxin peptide family member expressed by Leydig cells in the vertebrate testis. In mammals, INSL3 mediates testicular descent during embryogenesis but information on its function in adults is limited. In fish, the testes remain in the body cavity, although the *insl3* gene is still expressed, suggesting yet undiscovered, evolutionary older functions. Anti-Müllerian hormone (Amh), in addition to inhibiting spermatogonial differentiation and androgen release, inhibits the Fsh (follicle-stimulating hormone)-induced increase in *insl3* transcript levels in zebrafish testis. Therefore, the two growth factors might have antagonistic effects. We examine human INSL3 (hINSL3) effects on zebrafish germ cell proliferation/differentiation and androgen release by using a testis tissue culture system. hINSL3 increases the proliferation of type A undifferentiated (A_{und}) but not of type A differentiating (A_{diff}) spermatogonia, while reducing the proliferation of Sertoli cells associated with proliferating A_{und} . Since the area occupied by A_{und} decreases and

that of A_{diff} increases, we conclude that hINSL3 recruits A_{und} into differentiation; this is supported by the hINSL3-induced down-regulation of *nanos2* transcript levels, a marker of single A_{und} spermatogonia in zebrafish and other vertebrates. Pulse-chase experiments with a mitosis marker also indicate that hINSL3 promotes spermatogonial differentiation. However, hINSL3 does not modulate basal or Fsh-stimulated androgen release or growth factor transcript levels, including those of *amh*. Thus, hINSL3 seems to recruit A_{und} spermatogonia into differentiation, potentially mediating an Fsh effect on spermatogenesis.

Keywords INSL3 · Spermatogonial differentiation · Androgen release · Gene expression · Adult testis · Zebrafish

Introduction

Insulin-like peptide 3 (INSL3) is a relaxin peptide family member expressed in the male reproductive system by Leydig cells during fetal development and adult life. During embryonic development in mammals, INSL3 has a role that is crucial for the success of spermatogenesis in adult life (Kumagai et al. 2002). Knockout mice for INSL3 or its receptor RXFP2 show a cryptorchid phenotype, in which the testes remain inside the body cavity and fail to descend into the scrotum, since the gubernacula do not develop properly (Zimmermann et al. 1999; Nef and Parada 1999; Kumagai et al. 2002). However, although INSL3/RXFP2 are also expressed in the adult testis, information on potential functions in mature testis is incomplete. During the last decade, some studies have suggested a role for INSL3 in spermatogenesis (Kawamura et al. 2004; Pathirana et al. 2012). The effect of INSL3 on germ cell survival has been recorded in rat (Kawamura et al. 2004), whereas a more recent study

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reported no effects on germ cell apoptosis following RXFP2 ablation in mice (Huang et al. 2012). Nevertheless, testosterone release by cultured mouse Leydig cells increases in response to INSL3 (Pathirana et al. 2012), suggesting an autocrine role of this peptide as previously proposed after the localization of RXFP2 to Leydig cells (Anand-Ivell et al. 2006).

Although more information concerning relaxins has been obtained in the past few years, knowledge of their biological activity mostly comes from studies in mammals and little is known about their role in submammalian vertebrates such as teleost fish, a taxonomic group including nearly 50 % of all vertebrate species (Yegorov et al. 2014). Studies regarding Insl3, other relaxins and their receptors in teleost fish are limited to gene expression data (Good-Ávila et al. 2009; Yegorov et al. 2009; Good et al. 2012). Evidently, no testicular descent occurs in fish and studies directed to finding other, evolutionary older biological activities might also provide new leads for the respective activities of INSL3 in higher vertebrates.

In zebrafish (*Danio rerio*), for example, *insl3* gene expression has been localized to Leydig cells (Good-Ávila et al. 2009). Recent studies with recombinant zebrafish follicle-stimulating hormone (Fsh) have shown that the stimulation of zebrafish testis explants with Fsh increases testicular *insl3* mRNA levels, an effect not mediated by the steroidogenic activity of Fsh (García-López et al. 2010) but, instead, by a direct effect on Leydig cells that express both gonadotropins receptors in fish (García-López et al. 2010; Ohta et al. 2007; Chauvigné et al. 2012). On the other hand, recombinant anti-Müllerian hormone (Amh) inhibits the stimulatory effect of Fsh on *insl3* mRNA levels in zebrafish (Skaar et al. 2011). This opens up the possibility that, at sites with high levels of Amh, Fsh is less efficient at increasing levels of *insl3* mRNA in Leydig cells. Since, in addition to suppressing *insl3* mRNA expression, Amh inhibits the differentiation of type A undifferentiated (A_{und}) spermatogonia and Fsh-stimulated steroidogenesis, we hypothesize that Insl3 stimulates germ cell differentiation and steroidogenesis. This hypothesis is tested as part of our broader goal to understand the effect of Insl3 on testis function.

Materials and methods

Animals

Adult male zebrafish were bred and raised in the aquarium facility of the Department Biology, Utrecht University. The experiments followed the Dutch National regulations for animal use in experimentation. For morphometric/androgen release and mRNA analyses, 8 and 12 animals were used per experiment, respectively.

Human INSL3

Human INSL3 (hINSL3) was synthesized by using the continuous flow Fmoc (N-(9-fluorenyl)methoxycarbonyl)-solid phase methodology together with regioselective disulfide bond formation as previously described (Bathgate et al. 2006) and was obtained as a kind gift from Prof. John D. Wade, University of Melbourne, Victoria, Australia. The peptide was dissolved at a concentration of 100 $\mu\text{g/ml}$ in sterile phosphate-buffered saline (PBS) and aliquots were flash-frozen in liquid N_2 and stored at -80°C . We reasoned that hINSL3 would be biologically active in zebrafish testis because of the following considerations. Two *rxfp2* genes (*rxfp2a* and *rxfp2b*), paralogous to the human *RXFP2* gene, are abundantly expressed in zebrafish testis (Good et al. 2012; Yegorov et al. 2014). The specificity of the interaction of hINSL3 with RXFP2 is mainly determined by RXFP2 residues Phe¹³¹ and Gln¹³³ interacting with hINSL3 B-chain residue Trp²⁷, RXFP2 residue Trp¹⁷⁷ with hINSL3 B-chain residue His¹², RXFP2 residue Ile¹⁷⁹ with hINSL3 B-chain residue Val¹⁹, RXFP2 residues Asp¹⁸¹ and Glu²²⁹ with hINSL3 B-chain residue Arg²⁰ and Asp²²⁷ with hINSL3 residue Arg¹⁶ (Büllesbach and Schwabe 1999, 2004, 2005, 2006; Rosengren et al. 2006; Scott et al. 2007). Alignment of the zebrafish *Rxfp2a* and *Rxfp2b* receptor sequences with the human RXFP2 receptor sequence revealed that the zebrafish receptor contains identical residues at the ligand-receptor interaction sites, except for RXFP2 residues Ile¹⁷⁹ and Glu²²⁹, which are replaced by Val and Ala in the zebrafish *Rxfp2a* receptor. Human INSL3 should therefore be able to interact with both zebrafish *Rxfp2* receptors, as Val¹⁷⁹ and Ala²²⁹ would not hinder hINSL3 interacting with the *Rxfp2a* receptor.

Tissue culture

A primary testis tissue culture system was used to study the effects of hINSL3 on germ and somatic cell proliferation, androgen release and testicular mRNA levels, according to protocols previously established (Leal et al. 2009b). The concentration of 100 ng hINSL3/ml was chosen based on a pilot experiment and data published in mammals (Pathirana et al. 2012). To study proliferation and transcript levels, the two testes from each fish were dissected and incubated for 7 days, one under stimulatory conditions (receiving medium containing hINSL3) and the other under basal conditions (receiving only tissue culture medium). The testes were placed on a 0.25- cm^2 piece of nitrocellulose membrane (25 μm thickness, 0.22 μm pore size; Millipore, Billerica, Mass., USA), on top of a 700- μl agarose cylinder (1.5 % [w/v] in Ringer's solution, pH 7.4) that was placed in a 24-well flat-bottom plate (Corning, New York, USA). Medium (1 ml) was added such that the agar cylinder was just not submerged; the medium

was refreshed after 4 days. To study androgen release, testes were submerged in 200 μ l tissue culture medium in a 96-well plate for \sim 18 h. All components for the tissue culture studies were freshly prepared according to published protocols (Leal et al. 2009b).

Germ and Sertoli cell proliferation analysis

To evaluate the capacity of hINSL3 to modulate the proliferation activity of various spermatogonial generations and of Leydig and Sertoli cells, 50 μ g/ml proliferation marker 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) was added to the tissue culture medium of the testes during the last 6 h of the 7-day incubation period. During this final period of 6 h, the tissue was submerged in the medium.

In a pulse-chase set-up, zebrafish were exposed *in vivo* to BrdU dissolved in water (4 mg/ml) for \sim 12 h per day on 3 consecutive days to allow BrdU incorporation into the DNA of all dividing cells, including slowly dividing, single type A_{und} spermatogonia, followed by a 4-day chase period, during which the BrdU labeling was cleared from rapidly dividing cell types, including many of the rapidly proliferating spermatogonia (Nóbrega et al. 2010). Subsequently, the testes were dissected and incubated for 4 days *ex vivo* in the absence or presence of hINSL3 (100 ng/ml), as described above. Importantly, BrdU was not present in the medium during this tissue culture period of 4 days, so that the localization of the proliferation marker at the end of the tissue culture period allowed hINSL3 effects on the dynamics of BrdU to be examined in the germ cells that had taken up the marker previously *in vivo*.

After the tissue culture period, the testes were fixed at 4 °C overnight in freshly prepared methacarn (60 % [v/v] absolute ethanol, 30 % chloroform and 10 % acetic acid), dehydrated, embedded in Technovit 7100 (Heraeus Kulzer), sectioned at a thickness of 4 μ m and used to localize BrdU, as described previously (Leal et al. 2009a). The germ cells/cysts were identified according to previously published morphological criteria (Leal et al. 2009a). In brief (see Fig. 1a), the type A_{und} spermatogonium is a single germ cell and the largest spermatogonial cell type in zebrafish, with a large nucleus (\sim 9 μ m diameter), poorly condensed chromatin and one or two compact nucleoli; it is enveloped by a Sertoli cell, thus forming the initial stage of a spermatogenic cyst. The type A_{diff} spermatogonia, although morphologically not very different from type A_{und} , show a smaller (\sim 6 μ m diameter) and denser nucleus and occur in groups of 2, 4, or 8 cells (1st, 2nd and 3rd/final generation of type A_{diff} spermatogonia) in the same stage of development inside a cyst (Fig. 1b), because of the synchronized development among the members of the same germ cell clone based on cytoplasmic bridges remaining from an incomplete cytokinesis during differentiating mitoses. The five generations of type B spermatogonia (16 to 256 cells)

show a further reduced nuclear size (\sim 5 μ m diameter); the nucleus is slightly elongated/ovoid and clearly contains more heterochromatin than in type A spermatogonia (Fig. 1b).

To quantify proliferation, the mitotic index was determined by examining 100 randomly chosen germ cells/cysts or somatic cells and by discriminating between BrdU-labeled and unlabeled cells. To evaluate the proportion of area occupied by type A_{und} , A_{diff} and B spermatogonia, 30 randomly chosen fields were photographed at \times 400 magnification by using a conventional microscope equipped with a digital camera. The images were analyzed quantitatively by using ImageJ software (Image Processing and Analysis in Java). With a specific plug-in, a 540-point grid was made to quantify the proportion of the area for the various germ cell types, based on the number of points counted over those germ cell types.

Relative quantification of testicular mRNA levels

The effects of hINSL3 on testicular mRNA levels were investigated after 7 days of testis tissue culture. Total RNA was isolated from the tissue by using an RNAqueous Micro kit (Ambion), according to the manufacturer's protocol, in order to quantify the mRNA levels of selected testicular genes. The selection included *insl3* (García-López et al. 2010), Sertoli cell genes known to modulate spermatogonial proliferation and differentiation behavior, such as *amh* (Miura et al. 2002; Skaar et al. 2011), *gsdf* (Sawatari et al. 2007) and *igf3* (Morais et al. 2013) and four germ cell markers, namely *nanos2* (expressed in single type A_{und} spermatogonia; Beer and Draper 2013), *piwill* (expressed in all generations of type A spermatogonia; Houwing et al. 2007), *sycp3* (expressed in spermatocytes; Chen et al. 2013) and *odf3b* (expressed in spermatids; Yano et al. 2008). Two micrograms of isolated RNA was taken from each sample to synthesize cDNA as described previously (de Waal et al. 2008). The relative mRNA levels were determined by using real time, quantitative polymerase chain reaction (qPCR) assays, according to published protocols for all genes analyzed (see Table 1), except for *nanos2*. For detecting *nanos2* mRNA (Beer and Draper 2013), primers were designed (Table 1) and validated for specificity and amplification efficiency on serial dilutions of testis cDNA (Bogerd et al. 2001). All qPCRs and calculations were performed as described previously (Bogerd et al. 2001; de Waal et al. 2008; García-López et al. 2010) in 20- μ l reaction volumes and quantification cycle (Cq) values were obtained by a Step One Plus Real-Time PCR System (Applied Biosystems) by using default settings. Elongation factor 1 α (*ef1 α*) mRNA was used as the endogenous control, since its expression remained stable under both basal and treated conditions.

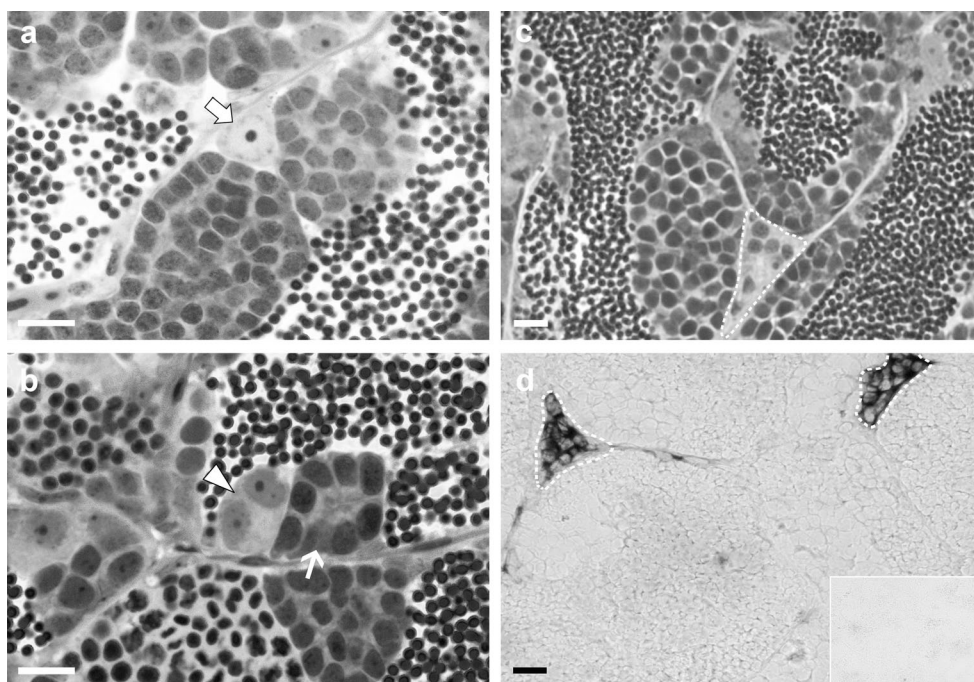


Fig. 1 Morphological characteristics of zebrafish type A undifferentiated (A_{und}) and type A differentiating (A_{diff}) and type B spermatogonia and Leydig cells and in situ hybridization for *insl3* mRNA in adult zebrafish testis. **a** Type A_{und} spermatogonia (arrow) are single germ cells showing a large and clear nucleus, with poorly condensed chromatin and one or two compact nucleoli. **b** A cyst containing two type A_{diff} spermatogonia (arrowhead) that show smaller nuclei stained more intensely by toluidine-blue. Type B spermatogonia (thin arrow) have still smaller,

slightly elongated/ovoid nuclei that moreover show a high amount of heterochromatin. **c** Interstitial space (dashed line) with a group of Leydig cells. **d** Adult zebrafish testis section showing the detection of *insl3* mRNA by in situ hybridization in Leydig cells cluster (dashed lines). Inset in **d** non-specific staining obtained with the sense probe. **a–c** Sections were prepared for morphological analysis according to Leal et al. (2009b). Magnification $\times 1000$ (**a**, **b**), $\times 600$ (**c**, **d**). Bars $10\ \mu\text{m}$

Cellular localization of *insl3* mRNA

To localize the cellular expression of *insl3* mRNA, in situ hybridization was performed by using digoxigenin (DIG)-labeled cRNA probes previously designed and validated (Good-

Ávila et al. 2009). Adult zebrafish testes were fixed in 4 % paraformaldehyde (PFA) in PBS (pH 7.4) at 4 °C overnight and subsequently transferred to 20 % sucrose in PBS until the tissue remained submerged. In situ hybridization was carried out on 10- μm -thick cryosections (Leica cryostat), which were

Table 1 Primers used for mRNA levels measurement of *efl α* (elongation factor 1 α), *insl3* (insulin-like peptide 3), *amh* (anti-Müllerian hormone), *gsdf*, *igf3* (two Sertoli cell genes known to modulate spermatogonial proliferation and differentiation), *nanos2*, *piwill*, *sycp3*, *odf3b* (four germ cell markers); Fw, forward; Rv, reverse

Target genes	Primers	Sequence (5'-3')	Reference
<i>eflα</i>	AG (Fw)	GCCGTCCACCGACAAG	Morais et al. 2013
	AH (Rv)	CCACACGACCCACAGGTACAG	
<i>insl3</i>	2466 (Fw)	TCGCATCGTGTGGGAGTTT	Good et al. 2012
	2467 (Rv)	TGCACAACGAGGTCTCTATCCA	
<i>amh</i>	AD (Fw)	CTCTGACCTTGATGAGCCTCATTT	García-López et al. 2010
	AE (Rv)	GGATGTCCCTTAAGAACTTTTGCA	
<i>igf3</i>	2680 (Fw)	TGTGCGGAGACAGAGGCTTT	Morais et al. 2013
	2681 (Rv)	CGCCGCACTTCTTGGATT	
<i>gsdf</i>	2366 (Fw)	CATCTGCGGGAGTCATTGAAA	García-López et al. 2010
	2367 (Rv)	CAGAGTCCTCCGGCAAGCT	
<i>piwill</i>	2542 (Fw)	GATACCGCTGCTGGAAAAAGG	García-López et al. 2010
	2543 (Rv)	TGGTTCTCCAAGTGTGCTTGC	
<i>sycp3</i>	2730 (Fw)	AGAAGCTGACCCAAGATCATTC	García-López et al. 2010
	2731 (Rv)	AGTTCAGTTGCTGGCGAAA	
<i>odf3b</i>	2791 (Fw)	GATGCCTGGAGACATGACCAA	Leal et al. 2009b
	2792 (Rv)	CAAAGGAGAAGCTGGGAGCTTT	
<i>nanos2</i>	4817 (Fw)	AAACGGAGAGACTGCGCAGAT	This paper
	4818 (Rv)	CGTCCGTCCCTTGCCTTT	

post-fixed with 4 % PFA and then treated with proteinase K (5 $\mu\text{g}/\text{mL}$; Sigma-Aldrich) at room temperature for 5 min. Hybridization, with 500 ng riboprobe per milliliter, was carried out in hybridization buffer (5 \times standard sodium citrate, 50 % deionized formamide, 10 % dextran sulfate, 5 \times Denhardt's, 250 $\mu\text{g}/\text{ml}$ yeast tRNA, 500 μg calf thymus DNA) at 65 $^{\circ}\text{C}$ overnight. The slides were then washed in 5 \times and 0.2 \times standard sodium citrate at 55 $^{\circ}\text{C}$, each for 30 min and blocking was performed with 1 % heat-inactivated goat serum (Vector) in blocking buffer (0.1 M TRIS-HCl, 0.15 M NaCl, pH 7.5) at room temperature for 1 h. Subsequently, the slides were incubated with alkaline-phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:1000 in blocking solution at 4 $^{\circ}\text{C}$ overnight. Color development was conducted by incubating the sections in Nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Roche) in the dark. After being air-dried, the slides were mounted using Aquamount (Thermo Scientific) and micrographs were taken with an Olympus AX70 microscope.

Androgen release analysis

To determine whether basal or Fsh-stimulated testicular androgen release was modulated by hINSL3, the levels of 11 β -hydroxyandrostenedione (OHA), a known precursor of 11-ketotestosterone (11-KT), were measured in testis tissue culture medium using a steroid release bioassay previously adapted for zebrafish testis (García-López et al. 2010). The results were calculated as nanograms of OHA released per milligram of testis tissue when incubated in the absence or presence of 25 ng/ml Fsh and/or 100 ng/ml hINSL3, respectively.

Statistics

The statistical analyses were performed by using the GraphPad Prism 5 software package (GraphPad Software, San Diego, Calif., USA). Differences between control and experimental groups were tested for statistical significance by using the Student's *t*-test for paired observation and analysis of variance (post-test Newman-Keuls) for multiple group comparisons. A significance level of $P < 0.05$ was applied in all analyses. Data are presented as means \pm standard error of mean (SEM).

Results

Spermatogonial and somatic cell proliferation

Based on counting BrdU-positive cells, we calculated the mitotic index of type A_{und} and A_{diff} spermatogonia (Fig. 2a, b), Leydig cells and Sertoli cells associated with BrdU-positive or BrdU-negative type A_{und} spermatogonia (Fig. 3a-c). In testis tissue culture, hINSL3 significantly stimulated the

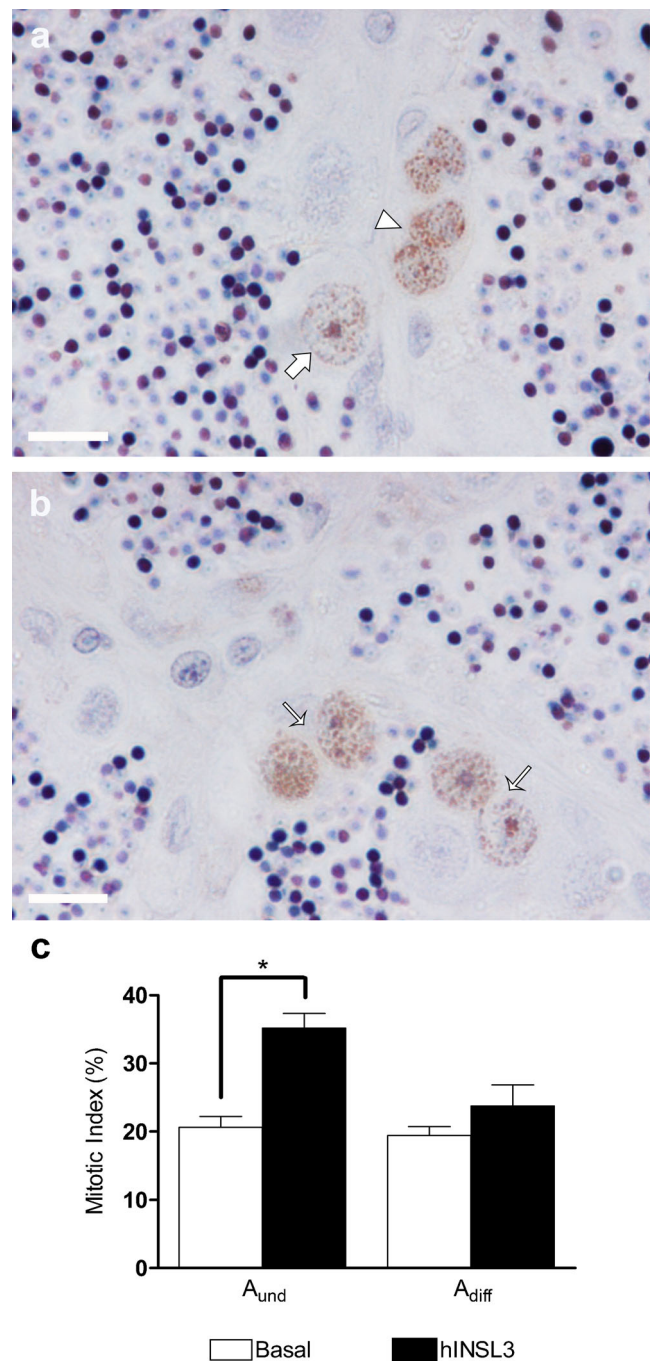
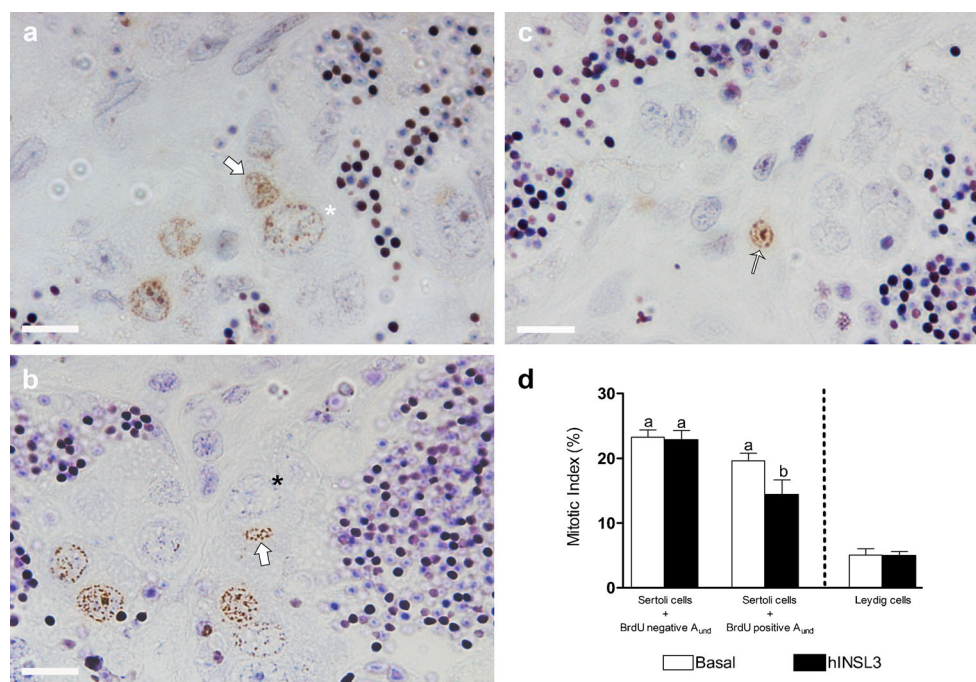


Fig. 2 a, b Testis tissue sections showing BrdU-positive germ cells: type A_{und} (arrow), type A_{diff} (thin arrows) and type B (arrowhead) spermatogonia. Magnification $\times 1000$. Bars 10 μm . c Mitotic indices of type A_{und} and A_{diff} spermatogonia after incubation for 7 days in the absence (Basal) and presence (hINSL3) of 100 ng hINSL3/ml. *Significant difference ($P < 0.05$) between treated and control. Results are presented as means \pm SEM ($n = 8$)

proliferation of type A_{und} spermatogonia, whereas no significant changes were observed for type A_{diff} spermatogonia (Fig. 2c). Moreover, no effects were observed for Leydig cells or for Sertoli cells associated with BrdU-negative type A_{und} spermatogonia, whereas a significant decrease in proliferation

Fig. 3 **a, b** Testis tissue sections showing BrdU-positive Sertoli cells nuclei (arrows) in association with BrdU-positive (white star) and BrdU-negative (black star) type A_{und} spermatogonia. **c** BrdU-positive Leydig cell nucleus (thin arrow). **d** Mitotic indices of Sertoli cells in association with BrdU-negative or BrdU-positive type A_{und} spermatogonia and of Leydig cells after incubation for 7 days in the absence (Basal) or presence (hINSL3) of 100 ng hINSL3/ml. Different letters indicate significant differences ($P < 0.05$) between the absence and presence of hINSL3. Magnification $\times 1000$ (a–c). Bars 10 μm . Results are presented as means \pm SEM ($n = 8$)



was found for Sertoli cells associated with BrdU-positive type A_{und} spermatogonia (Fig. 3d).

In order to determine whether the increased proliferation activity of type A_{und} spermatogonia was associated with self-renewal (leading to two single A_{und}) or differentiation (leading to a pair of A_{diff}), we labeled these cells with BrdU in vivo via a 3-day pulse and 4-day chase protocol, before the exposure of testis tissue to hINSL3 ex vivo. This allowed the study of the dynamics of spermatogonial proliferation by examining the way that exposure to hINSL3 ex vivo influenced the BrdU indices of type A_{und} , type A_{diff} and type B spermatogonia that previously had taken up the BrdU label in vivo. Quantification of the BrdU indices in this type of experiment for the various spermatogonial cell types showed that hINSL3 induced a decrease of approximately three- to two-fold, respectively, for type A_{und} and A_{diff} spermatogonia, whereas no change was recorded for type B spermatogonia (Fig. 4a). At the same time, the proportion of the area occupied by type A_{und} spermatogonia was significantly reduced, whereas an increase was observed in the proportion of type A_{diff} spermatogonia (Fig. 4b).

Androgen release

To evaluate whether the effects of hINSL3 on germ cell proliferation and differentiation were associated with a modulation of basal or gonadotropin-stimulated androgen release, we used (recombinant zebrafish) Fsh, which is a potent steroidogenic hormone in fish (reviewed by Schulz et al. 2010). Our results showed that hINSL3 neither modulated basal nor Fsh-stimulated androgen release (Fig. 5).

Relative testicular transcript levels and cellular localization of *insl3* mRNA

The quantification of the relative transcript levels of selected genes was intended to start an elucidation of the molecular mechanism used by hINSL3 to modulate germ cell proliferation. We analyzed the transcript levels of *insl3*, three Sertoli cell genes (*amh*, *gsdf* and *igf3*) and a spermatogonial (*piwil1*), a spermatocyte (*sycp3*) and a spermatid (*odf3b*) gene. The qPCR results showed no significant difference between basal and hINSL3-stimulated conditions for these transcripts (Fig. 6). However, transcript levels of *nanos2*, a marker for single type A_{und} spermatogonia, was down-regulated six-fold in testis tissue exposed to hINSL3 (Fig. 6).

The cellular localization of *insl3* transcripts in zebrafish testis tissue was analyzed by in situ hybridization on cryosections. Transcripts were exclusively observed in the interstitial compartment (Fig. 1d), with a specific hybridization signal exclusively on Leydig cells that often formed clusters in the interstitium (Fig. 1c, d). No signal was observed with the sense *insl3* riboprobe (negative control; inset Fig. 1d).

Discussion

Although information is available concerning the evolution and expression of relaxin peptide family members in teleost fish (Good-Ávila et al. 2009; Yegorov et al. 2009; Good et al. 2012), no previous reports exist, to our knowledge, on the

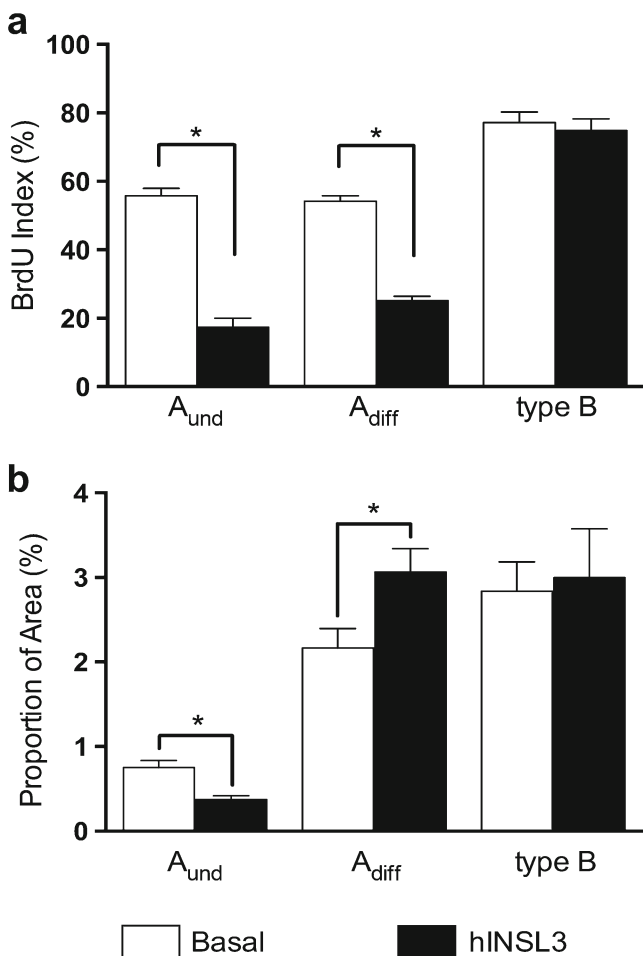


Fig. 4 BrdU indices of type A_{und}, type A_{diff} and type B spermatogonia after BrdU exposure in vivo and subsequent tissue culture in the absence (*Basal*) and presence (*hINSL3*) of 100 ng hINSL3/ml (**a**) and volumetric proportion of cysts of type A_{und}, type A_{diff} and type B spermatogonia after tissue culture in the absence (*Basal*) and presence (*hINSL3*) of hINSL3 (**b**). *Significant differences ($P < 0.05$) between the absence and presence of hINSL3. Results are presented as means±SEM ($n=8$)

biological activity of these peptides in submammalian vertebrates. We reasoned that hINSL3 would be able to interact with both zebrafish Rxfp2 receptors, since the residues identified as important for ligand-receptor interaction with the human receptor for hINSL3 (i.e., RXFP2) are identical in the zebrafish Rxfp2a and Rxfp2b receptors, except for RXFP2 residues Ile¹⁷⁹ and Glu²²⁹, which are replaced by Val and Ala and most likely do not hinder the interaction of hINSL3 with the zebrafish Rxfp2a receptor. This is supported by our finding that hINSL3 stimulates the differentiation of type A_{und} spermatogonia (further discussed below). Hence, despite the biological differences in the action of INSL3 in teleosts vs. mammals and their evolutionary distance, paralogous teleost receptors, Rxfp2a and Rxfp2b, are still able to respond to mammalian hINSL3. This suggests that, although the neofunctionalization of certain aspects of INSL3 occurs in mammals (i.e., testicular descent), older conserved functions

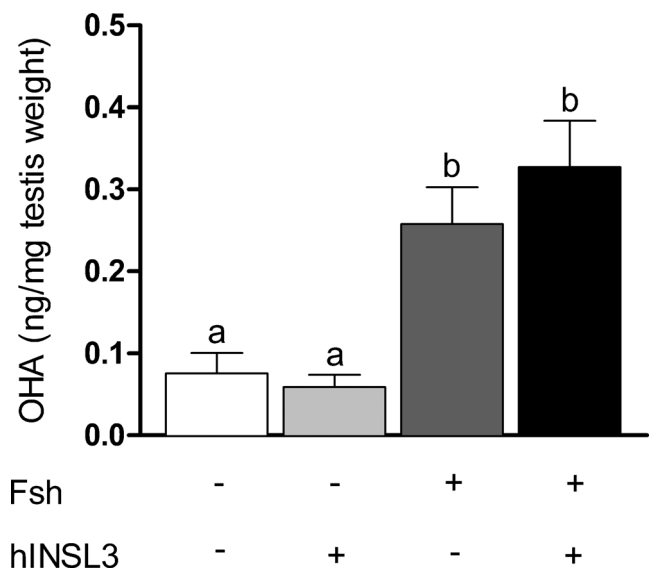


Fig. 5 Effects of hINSL3 (100 ng/ml) on basal or follicle-stimulating hormone (*Fsh*; 25 ng/ml)-stimulated androgen release ex vivo. Results (means±SEM; $n=8$) are presented as nanograms of 11β-hydroxyandrostenedione (*OHA*) released per milligram (mg) of testis weight

of INSL3 presumably take place across vertebrates. Whereas spermatogenesis is known not to be dependent on INSL3 function in adult mice (Huang et al. 2012), INSL3 might have as yet to be identified effects on germ cell development in higher vertebrates and the detection of RXFP2 in mammalian germ cells is compatible with the notion that INSL3 modulates germ cell development in a paracrine manner (Huang et al. 2012; Minagawa et al. 2014; Sagata et al. 2015), notwithstanding the known (neo-functionalized) role of INSL3 in mammalian testicular descent.

Using a testis tissue culture approach, we showed that hINSL3 significantly increases the mitotic index of type A_{und}

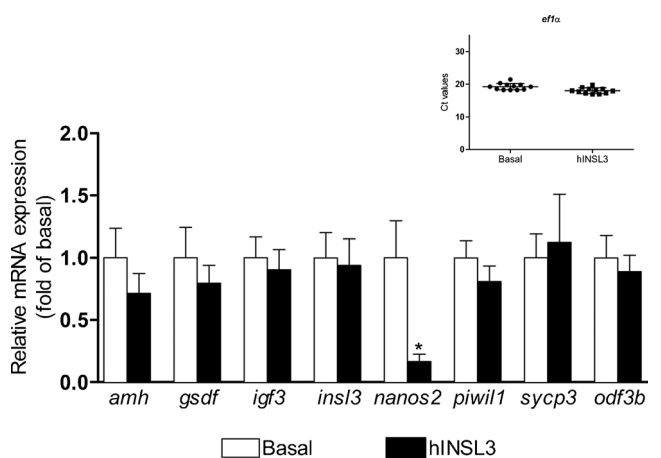


Fig. 6 Relative mRNA levels of *insl3* and selected Sertoli (*amh*, *gsdf* and *igf3*) and germ cell (*nanos2*, *piwil1*, *sycp3* and *odf3b*) genes. *Inset* Transcript levels of *ef1α* in the absence (*Basal*) and presence (*hINSL3*) of hINSL3; Ct values for the reference gene *ef1α*. Results are presented as means±SEM ($n=12$)

spermatogonia. Moreover, we propose that these proliferating type A_{und} spermatogonia are preferentially recruited into differentiation by hINSL3. Type A_{und} spermatogonia are single germ cells enveloped by one or sometimes two Sertoli cells in zebrafish (Leal et al. 2009a); spermatogonial stem cells (SSC) are part of this germ cell population (Nóbrega et al. 2010). As undifferentiated cells, SSCs have the capacity to produce either more SSCs (self-renewal proliferation) or germ cells committed to the spermatogenic process (differentiating proliferation), the first step being to form a pair of considerably smaller type A_{diff} spermatogonia. The latter cells represent ~44 % of the cellular volume of A_{und} , such that a pair of A_{diff} are slightly smaller than a single A_{und} (Leal et al. 2009a) and remain connected via a cytoplasmic bridge inside a single cyst, one of the hallmarks of differentiating germ cell divisions. On the other hand, SSC undergoing self-renewal produce two completely separated type A_{und} daughter cells and the newly generated germ cell needs to recruit its own Sertoli cell to create a new spermatogenic cyst. Consequently, to produce new spermatogenic cysts, Sertoli cell proliferation is also required (França et al. 2015). Here, we found that Sertoli cells contacting BrdU-positive type A_{und} spermatogonia show reduced proliferation activity when incubated with hINSL3 (Fig. 3d), which we interpret as circumstantial evidence that the A_{und} spermatogonia are undergoing germ cell differentiation not self-renewal. To obtain more direct evidence for this hypothesis, we used additional molecular and morphological approaches.

Quantifying selected germ cell marker genes show that three out of the four transcripts remain stable, whereas *nanos2* mRNA levels are down-regulated in testis tissue exposed to hINSL3. In male mice, SSC maintenance depends on the RNA binding protein NANOS2; its conditional loss in adults results in a loss of SSCs to differentiation, whereas overexpression increases testicular SSC numbers (Sada et al. 2009). Furthermore, miR-34c-mediated down-regulation of the NANOS2 protein enhances murine SSC differentiation (Yu et al. 2014). In teleost fish, Nanos2 appears to play a similar role, as the loss of *nanos2* function in tilapia results in germ-cell-deficient testes (Li et al. 2014) and as both protein and transcript are detected in single type A_{und} spermatogonia (Lacerda et al. 2013; Bellaiche et al. 2014). In the adult zebrafish testis, *nanos2* mRNA is expressed exclusively in single, *vasa*-positive germ cells that are considered to have germ line stem cell-like characteristics in both sexes (Beer and Draper 2013). Hence, a decrease in *nanos2* transcript levels in testis tissue incubated with hINSL3 is compatible with a hINSL3-induced differentiation of single type A_{und} spermatogonia, such as the SSCs in zebrafish.

A second approach that we employed is based on quantitative histology. In agreement with the decreased *nanos2* transcript levels, we found a decrease in the area occupied by type A_{und} spermatogonia and an increased area occupied by A_{diff} spermatogonia (Fig. 4b). Hence, this

increase in type A_{diff} cells directly suggests that hINSL3 stimulation increases the proliferation of A_{und} spermatogonia and their differentiation into A_{diff} spermatogonia but does not change the mitotic activity of A_{diff} spermatogonia.

We observed that A_{und} and A_{diff} spermatogonia pre-labeled with BrdU in vivo lose the BrdU label faster during a subsequent tissue culture period of 4 days when incubated in the presence but not absence of hINSL3 (Fig. 4a). We consider this to be evidence that hINSL3 is involved in the differentiation of type A_{und} spermatogonia. To explain this, we need to refer to spermatogonial dynamics in zebrafish.

Tracing a BrdU pulse through time, Leal et al. (2009a, 2009b) demonstrated that meiosis and spermiogenesis in zebrafish take 6 days, both in vivo and in tissue culture. The only information available for the duration of the mitotic phase of zebrafish germ cell differentiation indicates that one cell cycle of type A_{und} spermatogonia takes place within 30 h (Nóbrega et al. 2010). Therefore, we estimate that, during the 96 h of tissue culture for the experiments shown in Fig. 4, three to four spermatogonial divisions might have taken place. Since BrdU is incorporated into the A_{und} spermatogonia at the beginning of the tissue culture, this means that, in the case of differentiating divisions, the BrdU would have shifted into the A_{diff} cell pool, leading to a decrease of the BrdU index for type A_{und} cells. Thus, in conjunction with the reduction of *nanos2* transcript levels, the surface area occupied by A_{und} spermatogonia (Fig. 4b) and the loss of BrdU from the A_{und} cell pool is the third line of evidence showing that hINSL3 induces the differentiation of single type A_{und} spermatogonia in zebrafish.

However, the BrdU index of the A_{diff} spermatogonia is also significantly reduced following exposure to hINSL3 (Fig. 4a). This is unexpected, since their proliferation activity is not changed by hINSL3 (Fig. 2c) but might be explained by the following considerations. The hINSL3-mediated recruitment of type A_{und} cells into differentiation probably occurs irrespective of their BrdU-labeling status. About 45 % of the A_{und} spermatogonia do not contain BrdU under control conditions (Fig. 4a: 55 % BrdU-labeled, i.e., 45 % unlabeled) and hINSL3 will have stimulated the production of BrdU-negative A_{diff} from the initially BrdU-negative A_{und} spermatogonia. Moreover, initially, BrdU-positive type A_{und} spermatogonia recruited by hINSL3 into differentiation potentially undergo three or four cell cycles (three cycles from A_{und} to the third and last generation of A_{diff} , or four cycles to the first generation of type B spermatogonia), which is associated with an 8- to 16-fold dilution of BrdU that thereby might have become undetectable. We propose that the reduced BrdU index of type A_{diff} spermatogonia, despite their unchanged mitotic activity, reflects the combined effects of an increased production of BrdU-negative A_{diff} cells from initially BrdU-negative A_{und} cells, a loss of BrdU detectability in A_{diff} cells by dilution attributable to repeated cell cycling and finally a shift of the BrdU label into the B spermatogonia population

for the cells that undergo four differentiating cell cycles. Since the proportion of area occupied by A_{diff} spermatogonia becomes more prominent, although no change is observed for type B spermatogonia (Fig. 4b), we assume that the increased production of type A_{diff} with undetectable levels of BrdU is the main factor in this regard.

Since type B spermatogonia do not show changes after hINSL3 treatment for the parameters investigated (Fig. 4a, b), other experiments need to be performed to determine whether type B spermatogonia do not respond to hINSL3 or whether potential changes among type B spermatogonia are compensated for by the dynamics of “neighboring” germ cell generations (i.e., type A_{diff} spermatogonia and primary spermatocytes). However, major changes in the meiotic or post-meiotic germ cell generations do not seem likely considering the relatively stable expression levels of the marker genes *syce3* and *odf3b*, respectively (Fig. 6).

Previous work has shown that Sertoli cell-derived Amh inhibits spermatogonial differentiation and also reduces Leydig cell *insl3* mRNA levels (Skaar et al. 2011). Since our present data suggest that hINSL3 stimulates spermatogonial differentiation, we hypothesize that hINSL3 action might include a down-regulation of *amh* transcript levels. However, this is not the case. Moreover, transcript levels of *gsdf* and *igf3*, coding for growth factors stimulating spermatogonial proliferation in trout (Sawatari et al. 2007) or zebrafish (Morais et al. 2013), are also unaffected by hINSL3, suggesting that a change in the transcript levels of these growth factors is not directly involved in mediating hINSL3 action.

Recent work in mice has shown that INSL3 has an autocrine stimulatory effect on androgen release from Leydig cells (Pathirana et al. 2012) and androgens are known to stimulate spermatogenesis in tissue culture in various fish species (e.g., Miura et al. 1991; Leal et al. 2009b). However, our results show no effect of hINSL3 on basal or Fsh-stimulated androgen release, indicating that hINSL3 effects on zebrafish spermatogonia are not mediated by acutely modulating androgen production.

In summary, in this work, we investigated the potential role of hINSL3 on spermatogenesis in zebrafish, a species in which *insl3* gene expression is also found in Leydig cells but in which testicular descent does not occur. Morphometric studies and gene analysis of germ cell markers support the hypothesis that hINSL3 stimulates the differentiating proliferation of type A_{und} to type A_{diff} spermatogonia. To our knowledge, this is the first study of the biological activity of a relaxin family member in fish reproduction. Future studies should be directed (1) at investigating whether zebrafish *Insl3* shows comparable biological activities and (2) at identifying the testicular cell types expressing receptors for *Insl3*, as these aspects will lead us towards our larger goal of understanding the effects of *Insl3* on testis function in teleosts.

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References

- Anand-Ivell RJ, Relan V, Balvers M, Coiffec-Dorval I, Fritsch M, Bathgate RA, Ivell R (2006) Expression of insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biol Reprod* 74:945–953
- Bathgate RA, Lin F, Hanson NF, Otvos L Jr, Guidolin A, Giannakis C, Bastiras S, Layfield SL, Ferraro T, Ma S, Zhao C, Gundlach AL, Samuel CS, Tregear GW, Wade JD (2006) Relaxin-3: improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo. *Biochemistry* 45:1043–1053
- Beer RL, Draper BW (2013) *nanos3* maintains germline stem cells and expression of the conserved germline stem cell gene *nanos2* in the zebrafish ovary. *Dev Biol* 374:308–318
- Bellaiche J, Lareyre J, Cauty C, Yano A, Allemand I, Le Gac F (2014) Spermatogonial stem cell quest: *nanos2*, marker of a subpopulation of undifferentiated A spermatogonia in trout testis. *Biol Reprod* 90:79
- Bogerd J, Blomenröhr M, Andersson E, Putten HHAGM van der, Tensen CP, Vischer HF, Granneman JCM, Janssen-Dommerholt C, Goos HJT, Schulz RW (2001) Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). *Biol Reprod* 64:1633–1643
- Büllesbach EE, Schwabe C (1999) Tryptophan B27 in the relaxin-like factor (RLF) is crucial for RLF receptor-binding. *Biochemistry* 38:3073–3078
- Büllesbach EE, Schwabe C (2004) Synthetic cross-links arrest the C-terminal region of relaxin-like factor in an active conformation. *Biochemistry* 43:8021–8028
- Büllesbach EE, Schwabe C (2005) LGR8 signal activation by the relaxin-like factor. *J Biol Chem* 280:14586–14590
- Büllesbach EE, Schwabe C (2006) The mode of interaction of the relaxin-like factor (RLF) with the leucine-rich repeat G protein-activated receptor 8. *J Biol Chem* 281:26136–26143
- Chauvigné F, Verdura S, Mazón MJ, Duncan N, Zanuy S, Gómez A, Cerda J (2012) Follicle-stimulating hormone and luteinizing hormone mediate the androgenic pathway in Leydig cells of an evolutionary advanced teleost. *Biol Reprod* 87:35
- Chen SX, Bogerd J, Schoonen NE, Martijn J, Waal PP de, Schulz RW (2013) A progestin (17 α ,20 β -dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *Gen Comp Endocrinol* 185:1–9
- França LR, Nóbrega RH, Morais RDVS, Assis LHC, Schulz RW (2015) Sertoli cell structure and function in anamniote vertebrates. In: Griswold MD (ed) *Sertoli cell biology*, 2nd edn. Elsevier, Amsterdam, pp 385–407
- García-López A, Jong H de, Nóbrega RH, Waal PP de, Dijk W van, Hemrika W, Taranger GL, Bogerd J, Schulz RW (2010) Studies in zebrafish reveal unusual cellular expression patterns of gonadotropin receptor messenger ribonucleic acids in the testis and unexpected functional differentiation of the gonadotropins. *Endocrinology* 151:2349–2360

- Good S, Yegorov S, Martijn J, Franck J, Bogerd J (2012) New insights into ligand-receptor pairing and coevolution of relaxin family peptides and their receptors in teleosts. *Int J Evol Biol* 2012:310278
- Good-Ávila SV, Yegorov S, Harron S, Bogerd J, Glen P, Ozon J, Wilson BC (2009) Relaxin gene family in teleosts: phylogeny, syntenic mapping, selective constraint, and expression analysis. *BMC Evol Biol* 9:293–311
- Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, Elst H, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RHA, Hannon GJ, Draper BW, Ketting RF (2007) A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell* 129:69–82
- Huang Z, Rivas B, Agoulnik AL (2012) Insulin-like peptide 3 signaling is important for testicular descent but dispensable for spermatogenesis and cell survival in adult mice. *Biol Reprod* 87:1–8
- Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ (2004) Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci U S A* 101:7323–7328
- Kumagai J, Hsu SY, Matsumi H, Roh JS, Fu P, Wade JD, Bathgate RA, Hsueh AJ (2002) INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J Biol Chem* 277:31283–31286
- Lacerda SMSN, Costa GMJ, Silva MA, Campos-Junior PHA, Segatelli TM, Peixoto MTD, Resende RR, França LR (2013) Phenotypic characterization and in vitro propagation and transplantation of Nile tilapia (*Oreochromis niloticus*) spermatogonial stem cells. *Gen Comp Endocrinol* 192:95–106
- Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, França LR, Schulz RW (2009a) Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with emphasis on spermatogonial generations. *Biol Reprod* 81:177–187
- Leal MC, Waal PP de, García-López A, Chen SX, Bogerd J, Schulz RW (2009b) Zebrafish primary testis tissue culture: an approach to study testis function ex vivo. *Gen Comp Endocrinol* 162:134–138
- Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, Wang D (2014) Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. *Genetics* 197:591–599
- Minagawa I, Sagata D, Pitia AM, Kohriki H, Shibata M, Sasada H, Hasegawa Y, Kohsaka T (2014) Dynamics of insulin-like 3 and its receptor expression in boar testes. *J Endocrinol* 220:247–261
- Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991) Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proc Natl Acad Sci U S A* 88:5774–5778
- Miura T, Miura C, Konda Y, Yamauchi K (2002) Spermatogenesis-preventing substance in Japanese eel. *Development* 129:2689–2697
- Morais RDVS, Nóbrega RH, Gómez-González NE, Schmidt R, Bogerd J, França LR, Schulz RW (2013) Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. *Endocrinology* 154:4365–4376
- Nef S, Parada LF (1999) Cryptorchidism in mice mutant for INSL3. *Nat Genet* 22:295–299
- Nóbrega RH, Greebe CD, Kant H van de, Bogerd J, França LR, Schulz RW (2010) 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, Miura T (2007) Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. *Biol Reprod* 77:970–977
- Pathirana IN, Kawate N, Büllsbach EE, Takahashi M, Hatoya S, Inaba T, Tamada H (2012) Insulin-like peptide 3 stimulates testosterone secretion in mouse Leydig cell via cAMP pathway. *Regul Pept* 178:102–106
- Rosengren KJ, Zhang S, Lin F, Daly NL, Scott DJ, Hughes RA, Bathgate RAD, Craik DJ, Wade JD (2006) Solution structure and characterization of the LGR8 receptor binding surface of insulin-like peptide 3. *J Biol Chem* 281:28287–28295
- Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science* 325:1394–1398
- Sagata D, Minagawa I, Kohriki H, Pitia AM, Uera N, Katakura Y, Sukigara H, Terada K, Shibata M, Park EY, Hasegawa Y, Sasada H, Kohsaka T (2015) The insulin-like factor 3 (INSL3)-receptor (RXFP2) network functions as a germ cell survival/anti-apoptotic factor in boar testes. *Endocrinology* 156:1523–1539
- Sawatari E, Shikina S, Takeuchi T, Yoshizaki G (2007) A novel transforming growth factor- β superfamily member expressed in gonadal somatic cells enhances primordial germ cell and spermatogonial proliferation in rainbow trout (*Oncorhynchus mykiss*). *Dev Biol* 301:266–275
- Schulz RW, França LR, Lareyre J, Le Gac F, Chiarini-Garcia H, Nóbrega RH, Miura T (2010) Spermatogenesis in fish. *Gen Comp Endocrinol* 165:390–411
- Scott DJ, Wilkinson TN, Zhang S, Ferraro T, Wade JD, Tregear GW, Bathgate RAD (2007) Defining the LGR8 residues involved in binding insulin-like peptide 3. *Mol Endocrinol* 21:1699–1712
- Skaar KS, Nóbrega RH, Magaraki A, Olsen LC, Schulz RW, Male R (2011) Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* 152:3527–3540
- Waal PP de, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J (2008) Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* 136:225–234
- Yano A, Suzuki K, Yoshizaki G (2008) Flow-cytometric isolation of testicular germ cells from rainbow trout (*Oncorhynchus mykiss*) carrying the green fluorescent protein gene driven by trout *vasa* regulatory regions. *Biol Reprod* 78:151–158
- Yegorov S, Good-Ávila SV, Parry L, Wilson BC (2009) Relaxin family genes in humans and teleosts. *Ann N Y Acad Sci* 1160:42–44
- Yegorov S, Bogerd J, Good SV (2014) The Relaxin family peptide receptors and their ligands: new developments and paradigms in the evolution from jawless fish to mammals. *Gen Comp Endocrinol* 209:93–105
- Yu M, Mu H, Niu Z, Chu Z, Zhu H, Hua J (2014) miR-34c enhances mouse spermatogonial stem cells differentiation by targeting Nanos2. *J Cell Biochem* 115:232–242
- Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayerina K, Holstein AF, Engel W, Adham IM (1999) Targeted disruption of INSL3 gene causes bilateral cryptorchidism. *Mol Endocrinol* 13:681–691

Sertoli cell structure and function in anamniote vertebrates

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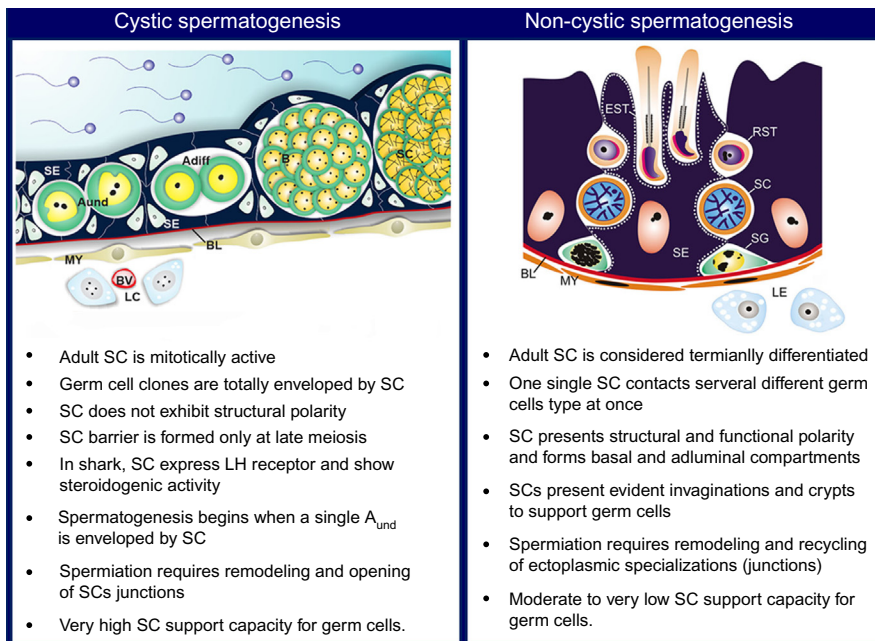
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s0010 I. Introduction

p0010 The “raison d’être” of Sertoli cells in the adult testis is to facilitate the survival and development of male germ cells so that spermatogenesis can provide spermatozoa in sufficient numbers to guarantee fertility. This function is conserved across vertebrates. However, taxonomic groups at the basis of the phylum—fish and amphibians collectively referred to as anamniote vertebrates—differ from the amniote vertebrates in having the cystic type of spermatogenesis. The main differences between cystic and noncystic spermatogenesis are summarized in [Figure 13.1](#). In cystic spermatogenesis, Sertoli cell structure and function have a number of characteristics that are very interesting from a comparative point of view and provide new insights into Sertoli cell physiology. This chapter focuses on specific aspects of Sertoli cells in cystic spermatogenesis that are relevant for understanding important Sertoli cell functions in vertebrates in general.

p0015 Testicular anatomy in amphibians and fish (except for sharks, which do not have steroidogenic Leydig cells in the interstitial compartment) follows the general vertebrate pattern of two compartments, germinative and interstitial, that are separated from each other by a basement membrane and peritubular myoid cells [1]. The interstitial compartment contains steroid hormone-producing Leydig cells, blood vessels, macrophages and mast cells, neural and connective tissue elements that are continuous with the tunica albuginea, and the testis organ wall. The (intra)tubular compartment houses the germinal epithelium that holds two cell types, the somatic Sertoli cells and the germ cells. Via the pituitary gonadotropic hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), interstitial steroidogenic Leydig cells as well as Sertoli cells, both expressing the receptors for FSH and LH, are integrated into endocrine regulatory circuits governing puberty and adult functioning of the testis. The survival and development of germ cells *in vivo* depends on their close and continuous interaction with Sertoli cells so that the Sertoli cell number limits the spermatogenic capacity of a testis [2]. This limitation renders determination of Sertoli cell number one of the most important aspects of testis physiology,



f0010 **Figure 13.1** Schematic representation of the main differences between Sertoli cells in cystic and noncystic spermatogenesis. A_{diff} , type A differentiated spermatogonia; A_{und} , type A undifferentiated spermatogonia; B, type B spermatogonia; BL, basal lamina; BV, blood vessel; EST, elongated spermatid; LE or LC, Leydig cells; MY, peritubular myoid cells; RST, round spermatid; SC, spermatocytes; SE, Sertoli cell; SG, spermatogonia. Part of the figure was published previously [1].

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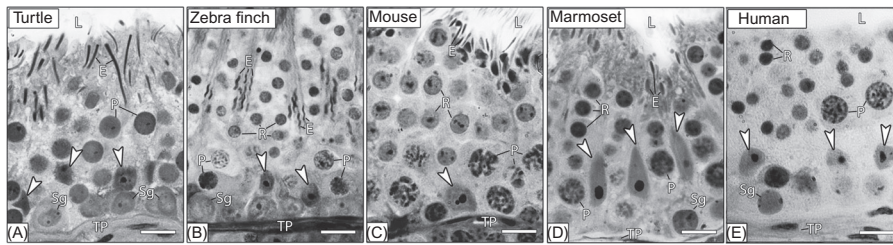
and we discuss the occurrence and regulation of Sertoli cell proliferation in cystic spermatogenesis. Sertoli cell proliferation is accompanied by progressive morphological and functional differentiation that is discussed, along with information on the regulation of these processes.

s0015 **II. Sertoli cell proliferation**

s0020 **A. Development of existing spermatogenic cysts**

p0020 The manners of Sertoli cell proliferation differ between amniote and anamniote vertebrates, reflecting a major difference in the Sertoli cell/germ cell relationship.

p0025 In birds and mammals, Sertoli cells proliferate until puberty so that in the adult testis, a given number of resident, postmitotic, and terminally differentiated Sertoli cells support successive waves of spermatogenesis. During these waves, a given Sertoli cell supports several different developmental stages of germ cells at a time. Typically, at the basolateral surface (basal compartment), mammalian Sertoli cells



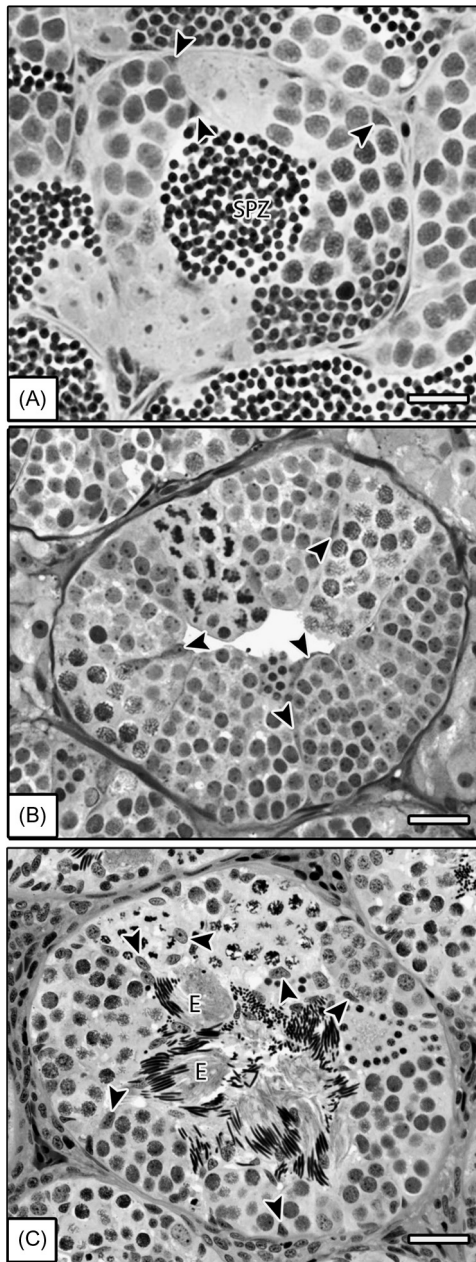
f0015 **Figure 13.2** Seminiferous tubule cross sections of noncystic spermatogenesis in a turtle (A), bird, zebra finch (B), mouse (C), marmoset (D), and human (E), showing Sertoli cell nuclei (arrowhead), spermatogonia (Sg), pachytene spermatocytes (P), and round (R) and elongated (E) spermatids. Also shown are the tunica propria (TP) and lumen (L). In this mode of spermatogenesis, one single Sertoli cell contacts several different germ cell types at the same time, and the Sertoli cell barrier delineates two compartments in the seminiferous epithelium: the basal compartment in contact with the tunica propria, where spermatogonia and early spermatocytes are located; and the adluminal compartment containing spermatocytes and spermatids. Bars = 20 μ m.

contact spermatogonia and early spermatocytes. Inside the blood–testis barrier, lateral parts contact spermatocytes and early (round) spermatids, while apical/adluminal parts contact late (elongating) spermatids (Figure 13.2 and Chapter 1).

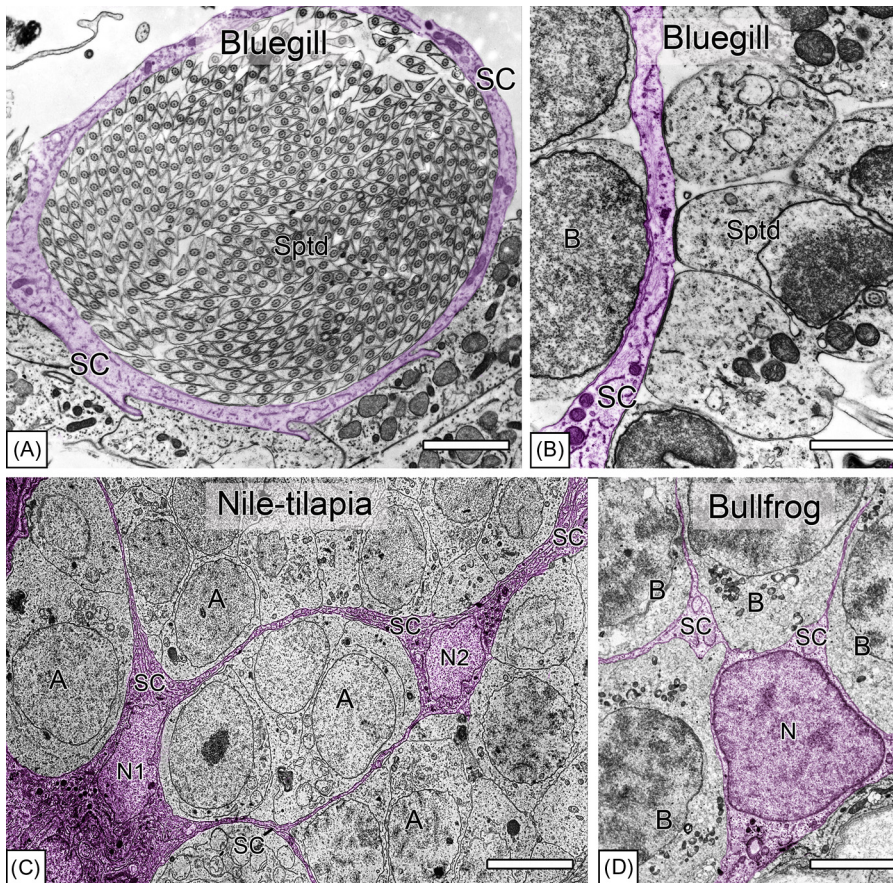
p0030 In anamniote vertebrates (fishes and amphibians), in contrast, the functional unit of the germinal epithelium is the spermatogenic cyst (Figure 13.3). This cyst consists initially of a single spermatogonium enveloped by the cytoplasmic extensions of Sertoli cells. When this initial cyst enters the spermatogenic process, both the cyst-forming Sertoli cells and germ cells proliferate and differentiate. A species-specific number of Sertoli cells is associated with cysts at specific stages of spermatogenesis, thereby establishing predictable Sertoli cell/germ cell ratios for a given stage of germ cell development [2–5]. Because Sertoli cells associated with growing cysts are positive in proliferation assays, at least part of the increase in Sertoli cell number per cyst is attributed to the division of these cells [4]. The major increase in cyst volume and Sertoli cell number per cyst occurs during the mitotic expansion of spermatogonia [2,4,5]. Therefore, unlike in amniote vertebrates, Sertoli cells are not terminally differentiated and do not proliferate in the anamniote testis. The situation is complicated somewhat by the fact that a Sertoli cell can contact more than one germ cell clone on the two sides of a cytoplasmic extension (Figure 13.4), although this ability is less complex than the typical situation of Sertoli cells in adult birds and mammals (Figure 13.2).

p0035 It seems that in all vertebrate species, testis function requires a functional Sertoli cell barrier and the interaction between the morphological and physiological barrier components to efficiently regulate the entry and exit of molecules [6]. This immunological barrier leads to an immune-privileged site that protects germ cells from immunological destruction [6]. The Sertoli cell barrier function is relatively well studied in mammals but still requires careful investigation in lower vertebrates. In fish, Sertoli cell proliferation stops when the germ cell clone completes meiosis and

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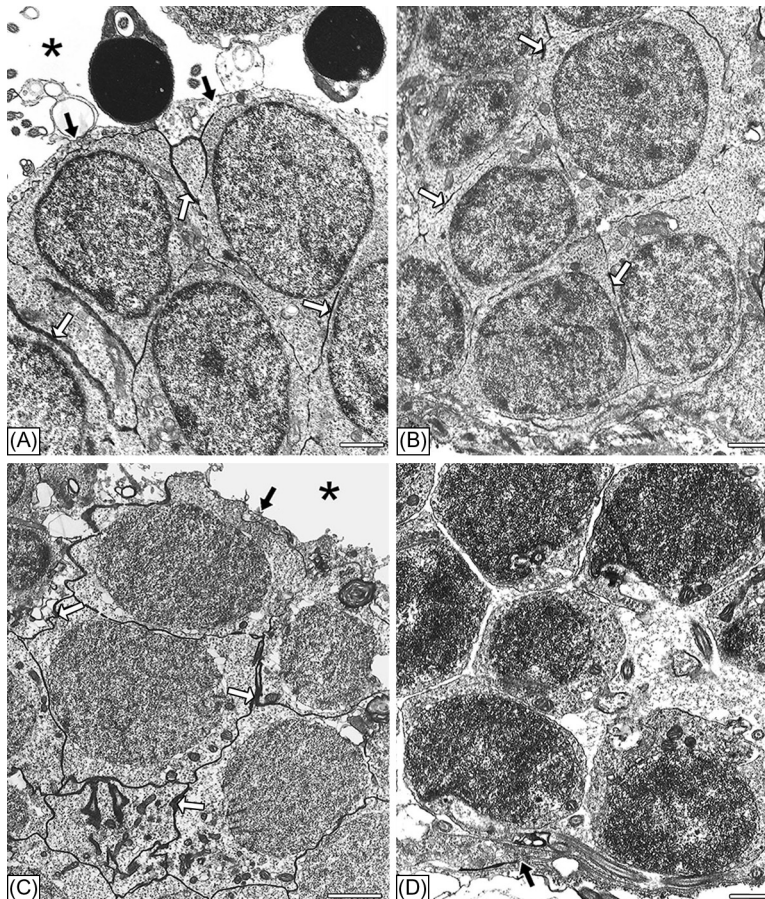


f0020 **Figure 13.3** Seminiferous tubule cross sections showing cystic spermatogenesis in different phases of development and enveloped by Sertoli cells (arrowhead) in zebrafish (A), Nile tilapia (B), and amphibian bullfrogs (C). The zebrafish tubular lumen is usually filled with sperm (SPZ), while the elongate spermatids (EL) cysts are open in bullfrogs but are still contacting SCs. Bars = 100 μ m.



f0025 **Figure 13.4** Seminiferous epithelium ultrastructure in fish and amphibians showing Sertoli cells (in purple) and germ cells. Strikingly, in the bluegill fish (A), a Sertoli cell is enveloping hundreds of spermatids (Sptds). This figure also shows that one Sertoli cell is able to contact different cysts, which can be observed in the bluegill (B), where Sertoli cell cytoplasm is facing type B spermatogonia (B) and Sptds. In the Nile tilapia (C), one Sertoli cell is apparently facing different type A spermatogonial (A) cysts. Sertoli cell cytoplasmic projections (D) are also observed in different germ cells in a bullfrog type B spermatogonial cyst. N, Sertoli cell nucleus. Bars: A = 2 μ m; B = 5 μ m; C = 5 μ m; D = 3 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this book.)

enters spermiogenesis, at which time tight junctions are also established among the Sertoli cells [2,4,5] (Figure 13.5). A similar trend has been observed in studies in bullfrogs in our laboratory. Because meiotic germ cells in fish are apparently not shielded from the vascular system, a tight Sertoli cell barrier seems not to be necessary for meiosis in fish. However, our knowledge of the barrier structure and function in fish is rather fragmentary. For instance, as shown for zebrafish

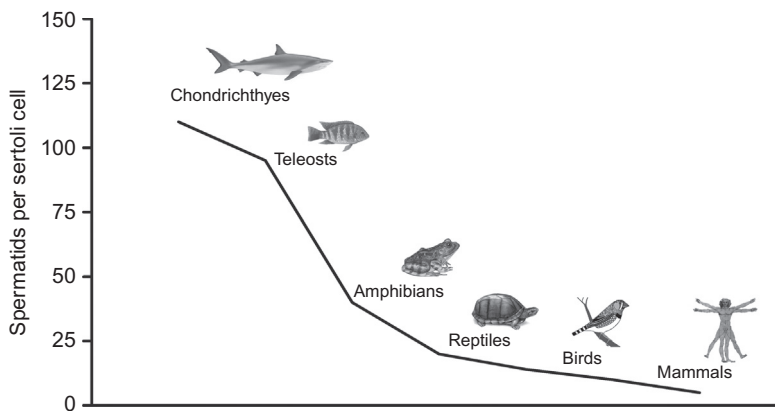


f0030 **Figure 13.5** Ultrastructure of zebrafish testis using lanthanum as a tracer to investigate the Sertoli cell barrier. In cystic spermatogenesis, the barrier is not observed in cysts containing type B spermatogonia (A) or primary spermatocytes cysts at the initial stages of meiosis (leptotene/zygotene) (B and C) because lanthanum is present between the germ cells (white arrows in A–C). (D) Lanthanum is found at the level of tight junctions between Sertoli cells in spermatid cysts (black arrow) and are not present between the germ cells and in the lumen (asterisks in A and C). The black arrows in A and C indicate the Sertoli cell cytoplasm enveloping the cyst. Bars in A and B = 1 μm , C = 2 μm , D = 0.5 μm . The figure was published previously [5].

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(Figure 13.5), even without a functional barrier, lanthanum, a tracer used to investigate the barrier efficacy, is never found in the tubular lumen. With respect to proliferation activity, however, Sertoli cells seem to behave in a similar way throughout vertebrates, given that Sertoli cells stop proliferating when tight junctions have been established. In this way, a predictable spermatid/Sertoli cell ratio is established. Interestingly, this ratio decreases during vertebrate evolution, being around

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f0035 **Figure 13.6** Number of spermatids per Sertoli cell, based on the available literature, for different vertebrate groups. This figure illustrates a progressive decrease in Sertoli cell efficiency during evolution.

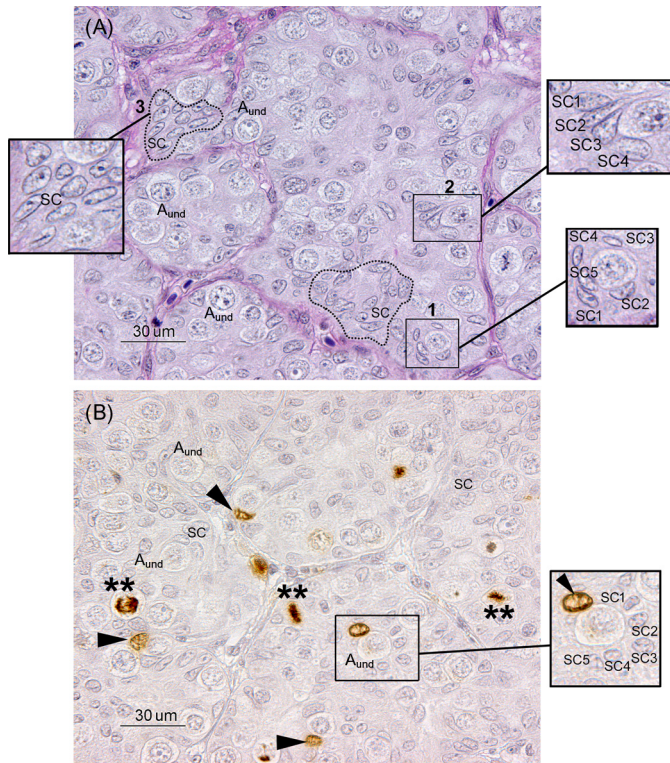
100 in fish but decreasing to <10 in most mammalian species, reaching about 4 in humans (Figure 13.6).

p0040 After completion of spermiogenesis, the cyst opens to release sperm into the tubular lumen (spermiation). The seminiferous epithelium in anamniote vertebrates is composed of spermatogenic cysts in which different germ cell clones (i.e., descendants of different stem cells, usually in different stages of development) are taken care of by Sertoli cells enveloping the clone until spermiation. This finding seems to imply that an individual Sertoli cell is not a resident but rather a transient cell in the anamniote testis (see the following section).

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s0025 **B. Generation of new spermatogenic cysts—Sertoli cell progenitors**

p0045 In addition to the Sertoli cell proliferation that accompanies the development of spermatogenic cysts, another mode of Sertoli cell proliferation takes place in the fish testis in the context of the production of new spermatogenic cysts. Because germ cells cannot survive unless they are in close contact with Sertoli cells, we hypothesize that the first new Sertoli cells are produced, thereby creating a niche into which a newly formed, single type A_{und} (type A undifferentiated) spermatogonium, a spermatogonial stem cell (SSC) candidate can be recruited. This assumption explains the observation made in the seasonally reproducing Atlantic salmon, for example, that at the beginning of the testis growth phase, groups of Sertoli cells appear that are not (yet) in contact with germ cells or several Sertoli cells are grouped around a single germ cell (Figure 13.7). In this setting, it seems possible that similar to recent observations in mice [7], Sertoli cells produce a paracrine factor, glial cell-lined derived neurotrophic factor (GDNF) that increases the number of SSCs in the vicinity of these Sertoli cells, for example, by stimulating SSC



f0040 **Figure 13.7** Sertoli cell groups in Atlantic salmon testis at the beginning of the seasonal testis growth phase. (A) **Squares (1,2)** show several Sertoli cells, indicated by numbered Sertoli cell nuclei that are grouped around a single type A undifferentiated spermatogonium (A_{und}). Areas delineated by **dashed lines** (e.g., **3**) show Sertoli cells apparently not (yet) in contact with germ cells (possibly containing Sertoli cell progenitor cells). Staining: Hematoxylin and eosin. (B) Immunocytochemical detection of a proliferation marker [phosphorylated histone H3 (pH3)] on another section of the same testis shown in A. pH3-positive Sertoli cell nuclei (**arrowheads**) indicate proliferation of Sertoli cells at the beginning of testis growth. The square highlights several Sertoli cells (**SC1–SC5**) that are already associated with a single spermatogonium type A_{und} . Sertoli cells continue proliferating, potentially providing niche space for new, single type A_{und} spermatogonia (cyst formation). Double asterisks indicate pH3-positive single type A_{und} spermatogonia.

self-renewal divisions or by attracting SSCs from other areas. Interestingly, in rainbow trout, *Gdnf* and its receptor have been detected in spermatogonia type A_{und} , suggesting that this factor can also function in an autocrine loop [8]. Studies in adult zebrafish have revealed a regulatory link (via thyroid hormone and a growth factor of the Igf family; see below) between the proliferation of Sertoli cells and of single type A_{und} spermatogonia [9]. Therefore, our current model of cyst formation assumes that Sertoli cells are first generated to provide new niche space that can then be occupied by single type A_{und} spermatogonia.

- p0050 New cysts are produced at different stages of the life cycle, and the intensity of cyst production varies with the reproductive strategy of a species, in particular in seasonally reproducing species. Regardless of the reproductive strategy, cyst generation takes place in all species between completion of male sex differentiation and puberty. This slow growth populates the prepubertal testis that often grows not more than allometrically with cysts containing mainly type A_{und} spermatogonia. Because many fish species display lifelong growth, a certain level of (allometric) testis growth associated with the generation of new cysts and Sertoli cells continues after puberty.
- p0055 In seasonally reproducing species, large (e.g., 50-fold) changes in testis weight are recorded [10,11]. These changes reflect the production of new spermatogenic cysts associated with a relatively slow growth of the testis at the beginning of a seasonal cycle. This growth becomes rapid when the cysts go through spermatogenesis, and germ cells and the associated Sertoli cells proliferate and differentiate until the cysts open to release spermatozoa into the tubular lumen. At a certain point in time, the generation of new cysts ceases and existing cysts continue to develop and complete spermatogenesis, such that the fully mature testis in these species typically shows spermatogenic tubuli with lumina filled with spermatozoa and a small number of quiescent cysts with a single type A_{und} spermatogonium, representing the start-up reservoir for the next season's spermatogenic wave.
- p0060 The testis weight built up during spermatogenic activity declines in a stepwise way. The first step occurs during completion of spermiogenesis, when Sertoli cells remove residual bodies while the production of new cysts stops. The second step of testis weight decrease is due to the use of sperm during the spawning season. Testis weight finally returns to low levels when residual spermatozoa are phagocytized by Sertoli cells after completion of the spawning season in preparation for the start of the next seasonal growth period. Thus, in seasonally reproducing species, Sertoli cell proliferation for the formation of new cysts is activated at the beginning of the seasonal testicular growth phase and is turned off to initiate the completion of the ongoing spermatogenic wave. In contrast, many species living in (sub)tropical zones display continuous spermatogenesis after puberty; prominent examples used as experimental models are medaka, tilapia, and zebrafish. In these species, Sertoli cell proliferation in the context of the formation of new cysts is observed continuously after puberty.
- p0065 We hypothesize that the new Sertoli cells required for the generation of new spermatogenic cysts are derived from a Sertoli cell precursor population that potentially has stem cell characteristics. There is no formal proof for this hypothesis yet, but circumstantial evidence suggests the presence of an undifferentiated population of somatic cells in the testis. One line of evidence is the long-term capacity to produce new Sertoli cells during successive reproductive cycles. Another line of evidence is the fully functional sex reversal in adults, for example, in female goldfish with androgen-induced female-to-male sex change [12], in estrogen-induced male-to-female sex change in medaka [13], or in the natural sexual plasticity in sequentially hermaphroditic species [14]. These observations suggest that an undifferentiated somatic precursor cell population can produce cells that have a male (Sertoli) or

female (granulosa) cell fate, depending on the environment in which they are situated. Clearly, germ cell-supporting somatic cells (or their precursors) in fish show an astonishing developmental plasticity in terms of sexual fate and proliferation capacity.

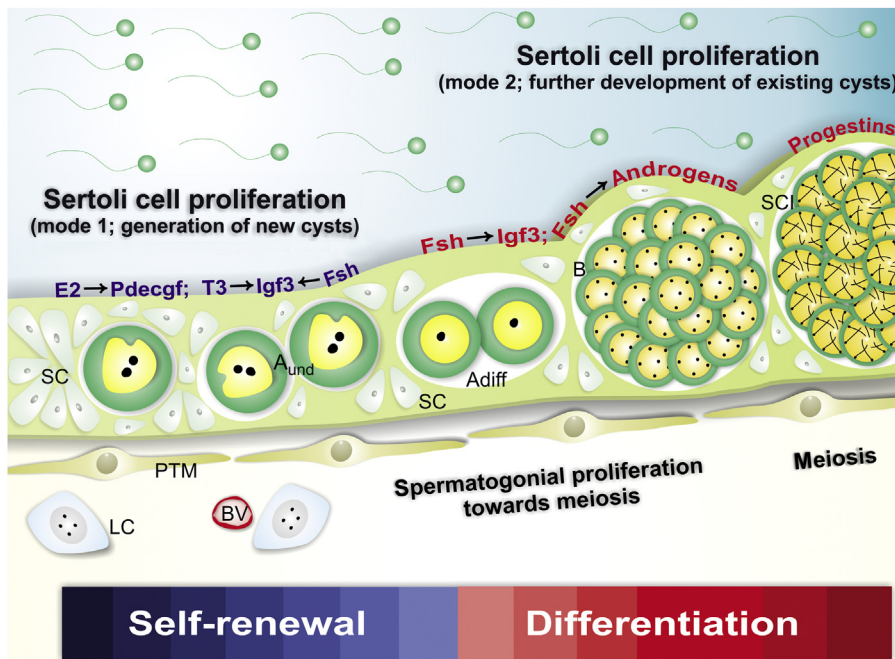
s0030 **C. Intratesticular sites of Sertoli cell proliferation**

p0070 Where does Sertoli cell proliferation in the context of cyst production take place in the testis? Depending on the species, cysts with a single type A_{und} spermatogonium, among which are SSCs, are distributed apparently at random throughout the germinal epithelium or are restricted to the periphery of the testis near the tunica albuginea [15]. In the first case, the cysts show little mobility during spermatogenesis. In the latter case, the cysts move away from the tunica as the germ cells divide and differentiate toward the region of the spermatic duct located centrally in the testis, where spermiation occurs and the cysts open to release spermatozoa. This arrangement is typical for teleost orders that are considered more evolved (atheriniformes, cyprinodontiformes, and beloniformes), while the former arrangement is found in less evolved taxonomic groups, such as in characiformes, cypriniformes, and salmoniformes [16]. Intermediate situations exist in perciformes (e.g., tilapia, *Oreochromis niloticus* [17]) or some pleuronectiformes (e.g. sole, *Solea senegalensis* [18]), where cysts with single type A_{und} spermatogonia have a preferred, but not exclusive, location close to the tunica albuginea. Close inspection of the Atlantic cod *Gadus morhua* revealed that new cysts are formed in a germinative zone in the periphery of the testicular parenchyma, which is divided into several lobes arranged around an efferent duct [11]. This structure results in a zonation of the lobes because early stages of development take place in the periphery, while advanced stages occur close to the efferent duct. The setting is based on appositional growth that is fuelled by the cyst-generating activity of the germinative zone rather than through movement of developing cysts. Hence, positional cues for the formation of new cysts exist in certain species, such that progenitor germ and Sertoli cells are likely to be found close to the tunica in the periphery of the testis. In the bluehad wrasse *Thalassoma bifasciatum*, it is possible that cellular elements from the tunica can differentiate into Sertoli cell progenitors [19].

p0075 However, in species in which the cysts with single type A_{und} spermatogonia (i.e., the potential site for the production of new cysts) are distributed throughout the testis, such as zebrafish, the intratubular location of these cysts is not random. As in rodents [20–22] and other mammals, such as horses [23] and peccaries [24], single type A_{und} spermatogonia in zebrafish preferentially locate to areas of the seminiferous tubules near blood vessels and the interstitium [25].

s0035 **D. Regulation of Sertoli cell proliferation**

p0080 The two modes of Sertoli cell proliferation, one in the context of the production of new spermatogenic cysts (mode 1) and the other in the context of the growth and development of existing cysts (mode 2), are regulated differently (Figure 13.8). This conclusion is based on the observation that these modes can take place



f0045 **Figure 13.8** Schematic representation of Sertoli cell proliferation in relation to endocrine and paracrine regulation of fish spermatogenesis. Generation of new cysts occurs by **mode 1** of Sertoli cell proliferation, while **mode 2** accompanies the development of existing cysts. Proliferation in **mode 1** can occur when Sertoli cells are not (yet) in contact with germ cells or when multiple Sertoli cells contact a single type A undifferentiated spermatogonium (A_{und}). In zebrafish, cysts with single type A_{und} spermatogonia are preferentially found close to the interstitial area (Leydig cells [LC]) and blood vessels [25] (BVs). We hypothesize that mode 1 of Sertoli cell proliferation increases niche space into which single type A_{und} spermatogonia can be recruited for the generation of new cysts. **Mode 1** is stimulated by thyroid hormone, in part by increases in the production and release of Sertoli cell-derived insulin-like growth factor 3 [9] (**Igf3**). Because *igf3* gene expression is strongly stimulated by follicle-stimulating hormone, this gonadotropin may also be relevant to mode 1, as is the case for estrogens via stimulation of Sertoli cell production of Pdecgf [26]. **Mode 2** of Sertoli cell proliferation accompanies the development of existing cysts from the expansion of the spermatogonial population until the end of meiosis/beginning of spermiogenesis and stops when tight junctions are formed between Sertoli cells in teleost testes [2,4,5]. During spermatogonial proliferation toward meiosis, FSH stimulates Sertoli cell Igf3 and LC androgen production, which both promote cyst development. In the meiotic phase, progestins might be involved in **mode 2**. Sertoli cell (SC), type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B spermatogonia (B), primary spermatocyte (SCI), Leydig cell (LC), peritubular myoid cell (PTM), and blood vessel (BV).

independently of each other. In seasonally reproducing species, such as the Atlantic salmon, Sertoli cell proliferation for cyst formation (mode 1) starts earlier than the second mode of Sertoli cell proliferation. The latter mode commences once cysts that have started to differentiate reach a size that requires increasing the Sertoli cell number to accommodate the growing germ cell clones. When mode 2 of Sertoli cell proliferation starts, mode 1 is still ongoing. Later in the season, when mode 1 of Sertoli cell proliferation stops at around the time that the testis reaches its maximum weight, mode 2 of Sertoli cell proliferation continues in the context of completing the development of existing cysts.

p0085 The production of new cysts also requires the production of single type A_{und} spermatogonia derived from self-renewal divisions of SSCs. The self-renewal of SSCs is observed when they divide in a specific niche environment, while displacement from the niche would result in the tipping of the signaling balance toward differentiating cell division (see Chapter 4). SSCs are among the single type A_{und} spermatogonia that are present in all vertebrates; however, in mice, paired or aligned undifferentiated spermatogonia can also retain stem cell potency [27]. In the cystic type of spermatogenesis, SSCs are not in contact with the basement membrane and are enveloped by Sertoli cells, which are thought to contribute importantly to the niche characteristics. To survive, a newly formed, single type A_{und} spermatogonium must recruit its own Sertoli cell support to form a new spermatogenic cyst. As shown in Figure 13.7, Sertoli cells proliferate excessively at the beginning of the testicular growth phase in the seasonally reproducing Atlantic salmon, which transiently leads to a situation in which a single type A_{und} spermatogonium is either surrounded by several Sertoli cells already that nevertheless keep proliferating, or in which Sertoli cells form groups that are not in contact with germ cells (yet).

p0090 What is the available information regarding the regulation of mode 1 of Sertoli cell proliferation? Studies in different fish species have shown that a low dose of estrogen stimulates SSC self-renewal (eel [26]; huchen [28]; medaka [29]). In eel, this effect involved estrogen receptor-dependent expression of a Sertoli cell-derived growth factor [26]. Although it has not been studied directly, we believe that this stimulatory effect of low estrogen doses is associated with the production of new spermatogenic cysts which must involve mode 1 of Sertoli cell proliferation.

p0095 Thyroid hormones exert well-known (inhibitory) effects on Sertoli cell proliferation in mammals [30]. Studies in zebrafish showed, however, that in contrast to findings in rodents, triiodothyronine (T3) increased the mitotic index of Sertoli cells, in particular of Sertoli cells not associated with germ cells (yet) or associated with type A spermatogonia. Moreover, proliferation of type A_{und} spermatogonia was stimulated as well. Jointly, these observations suggest that T3 increases the available niche space by stimulating mode 1 of Sertoli cell proliferation, resulting in the production of new spermatogenic cysts containing a single type A_{und} spermatogonium [9]. T3-stimulated proliferation involves the Igf signaling system because the T3 effect on proliferation is abrogated fully (A_{und}) or partially (Sertoli cell) by an Igf1 receptor inhibitor. Interestingly, fish gonads express the *igf3* gene, a new Igf family member [31] that may have arisen from the *igf1* gene in a neofunctionalization event in the context of the teleost-specific whole genome duplication.

Thyroid hormone receptors are expressed by Sertoli cells in zebrafish [9], as is Igf3 protein/mRNA (see below), and exposure to T3 increased testicular *igf3* mRNA levels so that T3-stimulated proliferation of spermatogonia and (part of) Sertoli cell proliferation are mediated by Sertoli cell-derived Igf3.

p0100 A point not studied yet in this concept is that, as mentioned above, we believe that mode 1 of Sertoli cell proliferation is fueled by a Sertoli cell progenitor or stem cell population. Therefore, we must postulate a niche for Sertoli cell progenitor cells as well. Signaling molecules that are potentially relevant to the SSC niche and are derived from other somatic elements (e.g., myoid, Leydig, or endothelial cells) but also from Sertoli cell progenitors themselves might be relevant to a potential Sertoli cell niche in the fish testis.

p0105 The *igf3* gene provides a link to the next aspect of regulation of Sertoli cell proliferation, the role of gonadotropins, given that FSH is a more potent stimulator of *igf3* gene expression than T3 in the zebrafish testis [32]; stimulatory effects of FSH on *igf3* mRNA have also been described for other species (e.g., rainbow trout [33]).

p0110 With regard to the biological activity of piscine gonadotropins, cellular localization data show that Leydig cells express the receptors for FSH and LH, which both stimulate steroidogenesis directly, while Sertoli cell functions are predominantly regulated by FSH [1]. Consequently, an important difference for the situation in higher vertebrates is that FSH in fish is also a potent steroidogenic hormone, next to regulating Sertoli cell activities. Most information on circulating FSH levels in male fish is available from salmonid species [34–36]; these studies typically show that these species have annual reproductive cycles or their cycles occur only once in a lifetime. Transiently elevated FSH blood levels are associated with the period of spermatogonial proliferation at the beginning of the testis growth phase. FSH blood levels increase again when spermiation becomes increasingly prominent, but they decrease before the actual spawning season commences, at which time prominent increases in plasma LH levels occur.

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p0115 In mammals, FSH is an important regulator of Sertoli cell proliferation and adult Sertoli cell function. Many of these functions were uncovered by studies that analyzed experimentally induced or spontaneous FSHR loss-of-function mutations in animal models or humans [37].

p0120 What do we know about the possible roles of FSH as a direct regulator of Sertoli cell activities in fish? With respect to Sertoli cell proliferation, it is tempting to speculate that elevated FSH plasma levels at the start of spermatogonial proliferation are important. In support of this concept, we found that incubating adult zebrafish testis tissue with recombinant zebrafish FSH in the presence of trilostane (which inhibits the production of biologically active steroid hormones) stimulated the proliferation of Sertoli cells and of type A_{und} and type A_{diff} spermatogonia (Nóbrega, Morais, França, Schulz, Bogerd; unpublished results), suggesting that not only can new cysts be formed but also that the further differentiation of existing cysts is stimulated. Experiments in prepubertal male sea bass injected with recombinant FSH point in the same direction. Next to plasma androgen levels, testis growth was stimulated, along with Sertoli cell and germ cell proliferation; postmeiotic stages of development were reached 23 days after injection [38]. In adult African

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catfish, surgical removal of one testis induced increased androgen release and *fshr* mRNA levels in the contralateral, remaining testis, which were associated with increased proliferation activity of Sertoli cells and spermatogonia type A_{und}, that is compatible with activated FSH signaling [39].

p0125 As mentioned above, FSH is a potent steroidogenic hormone in fish and induces the testicular release of 11-ketotestosterone (11KT), the main androgen in fish [40,41]. In a tissue culture system with prepubertal eel testis (which is rich in type A spermatogonia), incubation with 11KT induced full spermatogenesis, such that all stages of germ cell development were present [42]. This stimulatory effect of 11KT has been observed in other species as well (e.g., Japanese huchen [28] and zebrafish [43]). Although the effects of FSH/androgen on mode 2 of Sertoli cell proliferation, which accompanies the growth of differentiating cysts, have not been studied directly yet, we predict that FSH/androgen stimulation of germ cell proliferation toward advanced spermatogonial generations will also stimulate mode 2 of Sertoli cell proliferation.

p0130 A progestin is relevant to spermatogenesis in fish. There are two peaks of circulating 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) during the reproductive cycle of salmonid fish—a prominent peak in the spawning season and a small peak during the progression of spermatogonial proliferation and entry into meiosis [44–46]. Studies in Japanese eel showed that DHP stimulated spermatogonial DNA synthesis and was required to initiate meiosis [47]. A strong, stimulatory effect of DHP on spermatogenesis was also reported in adult zebrafish [48]. In this study, spermatogenesis was first interrupted by estrogen-induced androgen insufficiency and was then restarted by exposure to DHP, which induced proliferation of type A and B spermatogonia and entry into meiosis.

p0135 In an individual spermatogenic cyst, the Sertoli cell number increases steadily during the mitotic phase and levels off during meiosis to reach its maximum value upon completion of meiosis and the start of spermiogenesis, when tight junctions are also established between neighboring Sertoli cells [2,5]. We have explained that estrogens, thyroid hormones, and FSH can stimulate mode 1 of Sertoli cell proliferation, while FSH, androgens, and progestins can stimulate mode 2 of Sertoli cell proliferation (Figure 13.8), which stops when spermiogenesis starts. Switching from the level of a single cyst to the tissue level in seasonally reproducing species, mode 1 of Sertoli cell proliferation for the production of new spermatogenic cysts stops at a certain moment, after which mode 2 continues until all cysts have entered spermiogenesis. When spermiation follows, these species typically show spermatogenic tubules filled with spermatozoa and a limited number of quiescent cysts with single type A_{und} spermatogonia scattered along the basement membrane and awaiting the start of the subsequent spermatogenic wave [1]. Studies in Atlantic salmon showed that the cessation of the production of new cysts was associated with a decrease in the expression of *fshbeta* mRNA in the pituitary gland [49]. Because FSH plasma levels decrease in salmonids at this stage [35], one possible explanation for the cessation of the production of new cysts is reduced FSH stimulation. However, it is also possible that FSH receptor expression decreases in Sertoli cells in association with type A_{und} spermatogonia in testes that have reached their seasonal maximum weight,

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which might bear some similarity to the stage-dependent (i.e., induced by the specific germ cell composition) changes occurring in FSH receptor mRNA levels in adult rat testis [50].

s0040 III. Sertoli cell functions

p0140 After discussing different aspects of Sertoli cell proliferation, we now review Sertoli cell functions, starting with the function of these cells as paracrine communicators that translate endocrine signals into locally acting signals, and potentially also respond to signals from the germ cells.

s0045 A. Paracrine relay station

p0145 With respect to germ cell signaling to Sertoli cells, experimental evidence has yet to be published in fish. However, ongoing ultrastructural work in our laboratory showed that a single Sertoli cell can be in contact with different germ cell clones in quite different stages of spermatogenesis (Figure 13.5). We believe that Sertoli cells in this situation receive signals from the two different germ cell clones and respond appropriately. In rats, the density of undifferentiated spermatogonia modulates growth factor production by Sertoli cells [51], and the changing germ cell composition during the epithelial cycle is associated with changes in FSH receptor expression [50]. In zebrafish, the transforming growth factor (Tgf) β family member anti-Müllerian hormone (Amh) is produced by Sertoli cells in contact with type A spermatogonia. However, Amh production is downregulated when Sertoli cells contact type B spermatogonia or at later stages of germ cell development [43]. As mentioned above, there is no direct experimental evidence available yet to show that germ cells modulate Sertoli cell functions in fish, so we turn to the opposite direction of information flow, Sertoli cell signaling to germ cells.

p0150 When we discussed the regulation of Sertoli cell proliferation, we introduced the concept of the major endocrine input received by Sertoli cells through FSH and via the steroidogenic activity of (in fish) FSH and LH. All three types of sex steroids modulate Sertoli cell functions. In some selected cases, (part of) the molecular mechanisms involved have been elucidated, and these findings are summarized below.

p0155 With respect to the early stages of spermatogenesis, we referred to the stimulatory effects of low estrogen levels and thyroid hormone on the production of single type A_{und} spermatogonia. For thyroid hormone, this also involved Igf3. The effect of low estrogen levels is mediated by a nuclear estrogen receptor located in Sertoli cells that triggers the expression of platelet-derived endothelial cell growth factor (Pdecgf); recombinant Pdecgf mimics estrogen's effects and was designated in eel as an SSC renewal factor [26].

p0160 Although thyroid hormone is stimulatory, FSH has a much stronger effect on upregulating *igf3* mRNA in zebrafish Sertoli cells (Nobrega, Morais, de Waal, França, Schulz, Bogerd, unpublished results). These ongoing studies also show that zebrafish Igf3 stimulates the mitotic indices of A_{und} and A_{diff} spermatogonia

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without modulating androgen production. Still, FSH does have clear steroidogenic potency, and sex steroids have clear effects on Sertoli cell functions. As in other vertebrates, the androgen receptor is expressed by Sertoli cells but not by germ cells, and in zebrafish in particular, by Sertoli cells in contact with type A spermatogonia [52]. In general, the stimulatory effect of androgens on spermatogenesis and Sertoli cell proliferation has already been discussed, while little is known yet about the molecular mechanisms involved in fish. One exception is a finding in eel that androgen-stimulated *Inhba* (activin) production is involved in the stimulation of spermatogonial proliferation toward meiosis [53].

p0165 FSH-driven androgen production also provides a cross-link to thyroid hormone effects. In zebrafish, thyroid hormone receptors are not only expressed by Sertoli cells but also by Leydig cells, and thyroid hormone potentiates the stimulatory effect of FSH on androgen production [9]. In this way, thyroid hormone in the presence of low FSH levels stimulates the production of new spermatogenic cysts (as discussed earlier), while in combination with FSH, differentiation-inducing signals prevail in the context of the upregulation of *Igf3* production and the potentiation of steroidogenic effects.

p0170 Also relevant to FSH is the *Tgf β* family member *Amh*. Fish do not have Müllerian ducts, and *Amh* probably has evolutionarily older functions in teleosts. *Amh* inhibited the onset of gonadotropin- or androgen-stimulated spermatogenesis in prepubertal Japanese eel [54]. Later work in adult zebrafish showed that *Amh* inhibited FSH-stimulated androgen production by downregulating the expression of steroidogenesis-related genes, resulting in reduced androgen release. *Amh* also inhibited androgen-supported spermatogenesis by blocking the differentiation of type A spermatogonia, resulting in an accumulation of type *A_{und}* spermatogonia [43]. Finally, FSH downregulated *amh* mRNA levels in adult zebrafish testis. Taken together, these observations place FSH in a central position with respect to the switch to differentiation—FSH downregulates *Amh* expression in Sertoli cells, which would otherwise inhibit differentiation of type *A_{und}* spermatogonia and compromise steroidogenesis. At the same time, FSH directly stimulates Leydig cell androgen production, which is potentiated by thyroid hormone. FSH also stimulates the expression of *Igf3*, which promotes proliferation of Sertoli cells and of type A spermatogonia. Thus, the biological activities of FSH coordinate signaling systems to increase the number of spermatogenic cysts and then support their progress through the mitotic phase of spermatogenesis.

p0175 Other research, again based on the eel model, revealed a signaling system that is regulated by the fish-specific progestin that becomes relevant when the fish approaches meiosis. DHP, which is typically also found in male fish, induced trypsin expression [55] and taurine biosynthesis [56] in Sertoli cells. Trypsin then stimulated germ cells to express a solute carrier gene (*slc6a6*) that functions as a taurine transporter so that elevated taurine levels in germ cells could trigger germ cell entry into meiosis [57].

s0050 **B. Spermiation**

p0180 In most fish species, spermatogenic cysts open after completion of spermiogenesis, so that the lumen of the opening cyst becomes continuous with the lumen of the

spermatogenic tubule. The close contact between germ cells and Sertoli cells is broken and spermatozoa are released. This process is called “spermiation.” In mammals, spermiation takes place at a specific stage of the cycle of the germinal epithelium that is known to be particularly dependent on androgens, and androgen signaling is required for the disintegration of the ectoplasmic specializations that connect late spermatids and Sertoli cells [58,59].

p0185 Experimental data on cellular and molecular mechanisms operating in Sertoli cells during spermiation in fish are limited. Data in Atlantic salmon indicate that spermiation is associated with elevated plasma androgen levels; the mitotic, meiotic, and most of the spermiogenic phases take place in the presence of plasma androgen levels (15–20 ng/ml) that are clearly higher than the levels found in immature fish (<2 ng/ml). However, the transition to large-scale spermiation, which leads to the typical histological picture of mature testes, in which the tubular lumen is filled with (and serves as a storage place for) spermatozoa, is associated with a second step of plasma androgen level increase to >40 ng/ml [49]. However, a direct role of androgens in spermiation has not been demonstrated yet. In fish, the highest levels of circulating progestins are observed in fully mature males in seasonally reproducing species. Progestins like DHP or 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -S) are believed to induce or advance spermiation in salmonid and cyprinid fishes [60], increasing testicular fluid production [61,62], and stimulating spermatozoa motility [63,64] by altering the pH and fluidity of the seminal fluid [65].

p0190 In a number of taxonomic groups of fish (e.g., opheliidae [66], scorpaenidae [67], bleniidae [68], and soleidae [18]), spermiation takes place shortly after the completion of meiosis and is referred to as “semicyclic” [66]. In bullfrogs, cysts also open early (Figure 13.3), but spermatids remain in close contact with Sertoli cells until the completion of spermiogenesis; in this species, opening of the cyst and spermiation are separate events. In fishes with semicyclic spermatogenesis, round spermatids complete spermiogenesis while in a free state in the tubular lumen. The Sertoli cells that formed the cysts are transformed into a single-layered epithelium lining the tubular lumen, and these Sertoli cells may still provide support to spermatids. Spermiogenesis in fish is less complex than in tetrapod vertebrates, as suggested by the absence of an acrosome in teleost spermatozoa and by its short duration (e.g., 2 days in zebrafish [5]). Direct, individual contact between Sertoli cells and spermatids during spermiogenesis seems to be limited to the peripheral cell layers of spermatid clones in teleosts in general because the clone size usually amounts to hundreds of cells while only the peripheral layer(s) seem to have the chance of direct Sertoli cell contact.

s0055 **C. Phagocytosis of apoptotic germ cells and removal of residual sperm**

p0195 Fish Sertoli cells are astonishing with regard to their efficiency as phagocytotic cells, which is part of the normal physiological function of Sertoli cells. Electron microscopy studies have demonstrated Sertoli cell phagocytic activity and shown

vesicles with hydrolytic enzymes, lysosomes, or phagosomes with degenerating spermatids and spermatozoa. In addition, residual bodies were observed in the cytoplasm of Sertoli cells. Acid phosphatase in Sertoli cell lysosomes is a marker of phagocytic activity in fish and rodents [69,70]. Altogether, these morphological features indicate the capability of Sertoli cells to remove residual bodies during spermiogenesis, apoptotic germ cells (30–40% loss compared to the expected number [3,5,17]), and residual sperm after spermiation. These characteristics have been reported for the Sertoli cells of several teleost species, especially after spermiation and at the end of a spawning season [71–73].

p0200 In many fishes, the tubular lumen is an important sperm storage site. At the end of a spawning season, residual spermatozoa can remain in the tubular lumen. These spermatozoa are phagocytized by the Sertoli cells that form the epithelial lining of spermatogenic tubules, an aspect of Sertoli cell physiology that has been lost in higher vertebrates in which derivatives of mesonephric tissue form efferent duct and sperm storage tissues. Interestingly, the clearing of residual sperm is completed before the next wave of spermatogenesis can start. The timing of these processes differs locally because neighboring tubules can have rather different activities. For example, phagocytosis of residual sperm was ongoing in one tubule in which spermatogonia seemed to be quiescent, while spermatogonial proliferation resumed in an adjacent tubule in which residual sperm were not visible [11,74]. This finding suggests that local signaling, potentially originating from Sertoli cells still occupied with phagocytosis, can suppress the resumption of spermatogonial proliferation in the vicinity. No information is available about the endocrine regulation (if it does exist) of the phagocytotic activity of Sertoli cells in fish. In rats, phagocytosis mediated by AcP in Sertoli cells is not hormone dependent [69]. In humans, however, testicular acid phosphatase gene expression is upregulated by androgens and downregulated by estrogens [75].

s0060 **D. Fate after completion of cyst development**

p0205 The lifelong generation of new Sertoli cells along with the production and differentiation of spermatogenic cysts allows assuming that Sertoli cells are at least partially lost when a cyst has completed its development, in particular because seasonally reproducing species show large increases and then similarly large decreases in testis weight after the spawning season before embarking on the next season's growth phase. AU:8

p0210 So far, clear evidence for Sertoli cell apoptosis is only available for sharks. In the shark testis, developing cysts migrate during development through the testis from the dorsal to the ventral surface, where cysts open to release sperm into an efferent duct system while the cyst-forming Sertoli cells become apoptotic [76]. There are no results available in teleosts or amphibians yet. However, it is conceivable that during spermiation, some Sertoli cells are lost, perhaps especially those Sertoli cells that had a mainly adluminal position and did not contact another germ cell clone. Other Sertoli cells—perhaps preferentially those Sertoli cells that had extended contact areas with the basement membrane—can become integrated into the epithelial lining of the spermatogenic tubule, while Sertoli cells that do contact another germ cell clone may continue to support this clone [19].

s0065 **IV. Concluding remarks**

p0215 From a comparative point of view, there are, in our opinion, two main differences worth pinpointing between anamniote and amniote Sertoli cells. One difference is the astonishing plasticity of the numbers and stages of differentiation of Sertoli cells in the adult anamniote testis. The other difference is the considerably higher germ cell-supporting capacity. With respect to the first point, it will be exciting to investigate further the presence, characteristics, and regulation of Sertoli cell precursor cells and to search for formal proof for the presence of a true somatic stem cell population in the anamniote testis. The potential to provide additional Sertoli cells via mode 1 and the tailored increase in Sertoli cell number during the progress of cystic spermatogenesis via mode 2 of Sertoli cell proliferation may be part of the “secret” of the high capacity of anamniote Sertoli cells to support germ cells. However, several other structural and functional aspects of Sertoli cell/germ cell interaction probably contribute to this high efficiency and await clarification and comparative analysis in higher vertebrates. For both issues, we wish to stress the equal importance of developing technical approaches on the molecular, physiological, and morphological levels.

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References

- [1] Schulz RW, de França LR, Lareyre JJ, LeGac F, Chiarini-Garcia H, Nóbrega RH, et al. Spermatogenesis in fish. *Gen Comp Endocrinol* 2010;165:390–411.
- [2] Matta SL, Vilela DA, Godinho HP, França LR. The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increases Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*) model. *Endocrinology* 2002;143:970–8.
- [3] Billard R. La spermatogenèse de *Poecilia reticulata* I. Estimation du nombre de générations goniales et rendement de la spermatogenèse. *Ann Biol Anim Bioch Biophys* 1969;9:251–71.
- [4] Schulz RW, Menting S, Bogerd J, de França LR, Vilela DAR, Godinho HP. Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. *Biol Reprod* 2005;73:891–8.
- [5] Leal MC, Cardoso ER, Nóbrega RH, Batlouni SR, Bogerd J, de França LR, et al. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biol Reprod* 2009;81:177–87.
- [6] França LR, Auharek SA, Hess RA, Dufour JM, Hinton BT. Morphofunctional and immunological aspects of the blood–testis and blood–epididymal barriers. In: Cheng CY, editor. *Biology and regulation of blood–tissue barriers*. Landes Bioscience and Springer Science+Business Media; 2012. p. 237–59.
- [7] Dovere L, Fera S, Grasso M, Lamberti D, Gargioli C, Muciaccia B, et al. The niche-derived glial cell line-derived neurotrophic factor (GDNF) induces migration of mouse spermatogonial stem/progenitor cells. *PLoS One* 2013;8:e59431.

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- [8] Nakajima S, Hayashi M, Kouguchi T, Yamaguchi K, Miwa M, Yoshizaki G. Expression patterns of *gdnf* and *gfra1* in rainbow trout testis. *Gene Expr Patterns* 2014;14:111–20.
- [9] Morais RD, Nóbrega RH, Gómez-González NE, Schmidt R, Bogerd J, de França LR, et al. Thyroid hormone stimulates the proliferation of Sertoli cells and single type A spermatogonia in adult zebrafish (*Danio rerio*) testis. *Endocrinology* 2013;154:4365–76.
- [10] Billard R, Breton B. Rhythm of reproduction in teleost fish. In: Thorpe JE, editor. *Rhythmic activity of fishes*. New York, NY: Academic Press; 1978. p. 31–53.
- [11] Almeida FFL, Kristoffersen C, Taranger GL, Schulz RW. Spermatogenesis in Atlantic cod (*Gadus morhua*): a novel model of cystic germ cell development. *Biol Reprod* 2008;78:27–34.
- [12] Kobayashi M, Aida K, Stacey NE. Induction of testis development by implantation of 11-ketotestosterone in female goldfish. *Zool Sci* 1991;8:389–93.
- [13] Shibata N, Hamaguchi S. Evidence for the sexual bipotentiality of spermatogonia in the fish, *Oryzias latipes*. *J Exp Zool* 1988;245:71–7.
- [14] Kobayashi Y, Nakamura M, Sunobe T, Usami T, Kobayashi T, Manabe H, et al. Sex change in the Gobiid fish is mediated through rapid switching of gonadotropin receptors from ovarian to testicular portion or vice versa. *Endocrinology* 2009;150:1503–11.
- [15] Grier HJ. Cellular organization of the testis and spermatogenesis in fishes. *Am Zool* 1981;21:345–57.
- [16] Parenti LR, Grier HJ. Evolution and phylogeny of gonad morphology in bony fishes. *Integr Comp Biol* 2004;44:333–48.
- [17] Vilela DAR, Silva SGB, Peixoto MTD, Godinho HP, de França LR. Spermatogenesis in teleost: insights from the Nile tilapia (*Oreochromis niloticus*) model. *Fish Physiol Biochem* 2003;28:187–90.
- [18] García-López Á, Martínez-Rodríguez G, Sarasquete C. Male reproductive system in Senegalese sole *Solea senegalensis* (Kaup): anatomy, histology and histochemistry. *Histol Histopathol* 2005;20:1179–89.
- [19] Koulisch S, Kramer CR, Grier HJ. Organization of the male gonad in a protogynous fish, *Thalassoma bifasciatum* (Teleostei: Labridae). *J Morphol* 2002;254:292–311.
- [20] Chiarini-Garcia H, Raymer AM, Russell LD. Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. *Reproduction* 2003;126:669–80.
- [21] Yoshida S, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* 2007;317:1722–6.
- [22] de Rooij DG, Griswold MD. Questions about spermatogonia posed and answered since 2000. *J Androl* 2012;33:1085–95.
- [23] Costa GM, Avelar GF, Rezende-Neto JV, Campos-Junior PH, Lacerda SM, Andrade BS, et al. Spermatogonial stem cell markers and niche in equids. *PLoS One* 2012;7(8):e44091.
- [24] Campos-Junior PH, Costa GM, Lacerda SM, Rezende-Neto JV, de Paula AM, Hofmann MC, et al. The spermatogonial stem cell niche in the collared peccary (*Tayassu tajacu*). *Biol Reprod* 2012;86(5):155 1–10.
- [25] Nóbrega RH, Greebe CD, van de Kant H, Bogerd J, de França LR, Schulz RW. Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. *PLoS One* 2010;5(9):e12808.
- [26] Miura T, Ohta T, Miura CI, Yamauchi K. Complementary deoxyribonucleic acid cloning of spermatogonial stem cell renewal factor. *Endocrinology* 2003;144:5504–10.
- [27] Yoshida S. Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. *Reproduction* 2012;144:293–302.

- [28] Amer MA, Miura T, Miura C, Yamauchi K. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen (*Hucho perryi*). *Biol Reprod* 2001;65:1057–66.
- [29] Song M, Gutzeit HO. Effect of 17 α -ethynylestradiol on germ cell proliferation in organ and primary culture of medaka (*Oryzias latipes*) testis. *Dev Growth Differ* 2003;45:327–37.
- [30] Cooke PS, Holsberger DR, França LR. Thyroid hormone regulation of Sertoli cell development. In: Skinner MK, Griswold MD, editors. *The Sertoli cell biology*. Elsevier Science; 2005. p. 217–26. AU:10
- [31] Wang DS, Jiao B, Hu C, Huang X, Liu Z, Cheng CH. Discovery of a gonad-specific IGF subtype in teleost. *Biochem Biophys Res Commun* 2008;367:336–41.
- [32] de Waal PP. Hormonal regulation of spermatogenesis in zebrafish [Ph.D. thesis]. Utrecht University, the Netherlands; 2009. ISBN: 978.90.393.5143.7.
- [33] Sambroni E, Lareyre JJ, Le Gac F. Fsh controls gene expression in fish both independently of and through steroid mediation. *PLoS One* 2013;8(10):e76684.
- [34] Campbell B, Dickey JT, Swanson P. Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. *Biol Reprod* 2003;69:2109–17.
- [35] Gomez JM, Weil C, Ollitrault M, Lebail PY, Breton B, LeGac F. Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* 1999;113:413–28.
- [36] Prat F, Sumpter JP, Tyler CR. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). *Biol Reprod* 1996;54:1375–82.
- [37] Huhtaniemi IT, Themmen AP. Mutations in human gonadotropin and gonadotropin receptor genes. *Endocrine* 2005;26:207–17.
- [38] Mazón MJ, Gómez A, Yilmaz O, Carrillo M, Zanuy S. Administration of follicle-stimulating hormone *in vivo* triggers testicular recrudescence of juvenile European sea bass (*Dicentrarchus labrax*). *Biol Reprod* 2014;90(1):6 1–10
- [39] Schulz RW, van Dijk W, Chaves-Pozo E, García-López A, de França LR, Bogerd J. Sertoli cell proliferation in the adult testis is induced by unilateral gonadectomy in African catfish. *Gen Comp Endocrinol* 2012;177:160–7.
- [40] Idler DR, Bitners II, Schmidt PJ. 11-Ketotestosterone: an androgen for sockeye salmon. *Can J Biochem Physiol* 1961;39:1737–42.
- [41] Borg B. Androgens in teleost fish. *Comp Biochem Physiol* 1994;109C:219–45.
- [42] Miura T, Yamauchi K, Takahashi H, Nagahama Y. Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). *Proc Natl Acad Sci USA* 1991;88:5774–8.
- [43] Skaar KS, Nóbrega RH, Magaraki A, Olsen LC, Schulz RW, Male R. Proteolytically activated, recombinant anti-Müllerian hormone inhibits androgen secretion, proliferation, and differentiation of spermatogonia in adult zebrafish testis organ cultures. *Endocrinology* 2011;152:3527–40.
- [44] Dépêche J, Sire O. *In vitro* metabolism of progesterone and 17 α -hydroxyprogesterone in the testis of the rainbow trout, *Salmo gairdneri* Rich., at different stages of spermatogenesis. *Reprod Nutr Develop* 1982;22:427–38.
- [45] Scott AP, Sumpter JP. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. *Gen Comp Endocrinol* 1989;73:46–58.

- [46] Vizziano D, Le Gac F, Fostier A. Effect of 17β -estradiol, testosterone, and 11 -ketotestosterone on $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one production in the rainbow trout testis. *Gen Comp Endocrinol* 1996;104:179–88.
- [47] Miura T, Higuchi M, Ozaki Y, Ohta T, Miura C. Progesterin is an essential factor for the initiation of the meiosis in spermatogenic cells of the eel. *Proc Natl Acad Sci USA* 2006;103:7333–8.
- [48] Chen SX, Bogerd J, Schoonen NE, Martijn J, de Waal PP, Schulz RW. A progesterin ($17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one) stimulates early stages of spermatogenesis in zebrafish. *Gen Comp Endocrinol* 2013;185(1–9).
- [49] Melo MC, Andersson E, Fjellidal PG, Bogerd J, de França LR, Taranger GL, et al. Salinity and photoperiod modulate pubertal development in Atlantic salmon (*Salmo salar*). *J Endocrinol* 2014;220:319–32.
- [50] Kliesch S, Penttila TL, Gromoll J, Saunders PTK, Nieschlag E, Parvinen M. FSH receptor messenger-RNA is expressed stage-dependently during rat spermatogenesis. *Mol Cell Endocrinol* 1992;84:R45–9.
- [51] Johnston DS, Olivas E, DiCandeloro P, Wright WW. Stage-specific changes in GDNF expression by rat Sertoli cells: a possible regulator of the replication and differentiation of stem spermatogonia. *Biol Reprod* 2011;85:763–9.
- [52] de Waal PP, Wang DS, Nijenhuis WA, Schulz RW, Bogerd J. Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis. *Reproduction* 2008;136:225–34.
- [53] Miura T, Miura C, Yamauchi K, Nagahama Y. Human recombinant activin induces proliferation of spermatogonia *in vitro* in the Japanese eel (*Anguilla japonica*). *Fisheries Sci* 1995;61:434–7.
- [54] Miura T, Miura C, Konda Y, Yamauchi K. Spermatogenesis-preventing substance in Japanese eel. *Development* 2002;129:2689–97.
- [55] Miura C, Ohta T, Ozaki Y, Tanaka H, Miura T. Trypsin is a multifunctional factor in spermatogenesis. *Proc Natl Acad Sci USA* 2009;106:20972–7.
- [56] Higuchi M, Celino FT, Tamai A, Miura C, Miura T. The synthesis and role of taurine in the Japanese eel testis. *Amino Acids* 2012;43:773–81.
- [57] Higuchi M, Miura C, Iwai T, Miura T. Trypsin regulates meiotic initiation in the Japanese eel (*Anguilla japonica*) by promoting the uptake of taurine into germ cells during spermatogenesis. *Biol Reprod* 2013;89(3):58 1–9
- [58] O'Donnell L, Stanton PG, Bartles JR, Robertson DM. Sertoli cell ectoplasmic specializations in the seminiferous epithelium of the testosterone-suppressed adult rat. *Biol Reprod* 2000;63:99–108.
- [59] Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol* 2010;315:49–56.
- [60] Ueda H, Kambegawa A, Nagahama Y. Involvement of gonadotrophin and steroid hormones in spermiation in the amago salmon, *Oncorhynchus rhodurus*, and goldfish, *Carassius auratus*. *Gen Comp Endocrinol* 1985;59:24–30.
- [61] Baynes SM, Scott AP. Seasonal variations in parameters of milt production and in plasma concentration of sex steroids of male rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol* 1985;57:150–60.
- [62] Yueh WS, Chang CF. $17\alpha,20\beta,21$ -trihydroxy-4-pregnen-3-one and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one stimulated spermiation in protandrous black porgy, *Acanthopagrus schlegelii*. *Fish Physiol Biochem* 1997;17:187–93.

- [63] Miura T, Yamauchi K, Takahashi H, Nagahama Y. The role of hormones in the acquisition of sperm motility in salmonid fish. *J Exp Zool* 1992;261:359–63.
- [64] Tubbs C, Thomas P. Functional characteristics of membrane progesterin receptor alpha (mPRalpha) subtypes: a review with new data showing mPRalpha expression in sear-out sperm and its association with sperm motility. *Steroids* 2008;73:935–41.
- [65] Scott AP, Sumpter JP, Stacey N. The role of the maturation-inducing steroid, 17,20 β -dihydroxypregn-4-en-3-one, in male fishes: a review. *J Fish Biol* 2010;76: 183–224.
- [66] Mattei X. Peculiarities in the organization of testis of *Ophidion sp.* (Pisces: Teleostei). Evidence for two types of spermatogenesis in teleost fish. *J Fish Biol* 1993;43:931–7.
- [67] Muñoz M, Casadevall M, Bonet S. Testicular structure and semicyclic spermatogenesis in a specialized ovuliparous species: *Scorpaena notata* (Pisces, Scorpaenidae). *Acta Zool* 2002;83:213–9.
- [68] Lahnsteiner F, Patzner RA. Spermiogenesis and structure of mature spermatozoa in blennioid fishes (Pisces, Blenniidae). *J Submicr Cytol Pathol* 1990;22:565–76.
- [69] Chemes H. The phagocytic function of Sertoli cells: a morphological, biochemical and endocrinological study of lysosomes and acid phosphatase localization in the rat testis. *Endocrinology* 1986;119:1673–81.
- [70] Porowski M, Wassermann GF, Achaval M. Localization of acid phosphatase activity in the testis of two teleostean species (*Oreochromis niloticus* and *Odonthestes perugiae*). *Braz J Biol* 2004;64:853–8.
- [71] van Vuren JHJ, Soley JT. Some ultrastructural observations of Leydig and Sertoli cells in the testis of *Tilapia rendalli* following induced testicular recrudescence. *J Morphol* 1990;206:57–63.
- [72] Lo Nostro FL, Grier H, Mejjide FJ, Guerrero GA. Ultrastructure of the testis in *Synbranchus marmoratus* (Teleostei, Synbranchidae): the germinal compartment. *Tissue Cell* 2003;35:121–32.
- [73] Chung EY, Yang YC, Kang HW, Choi KH, Jun JC, Lee KY. Ultrastructure of germ cells and the functions of Leydig cells and Sertoli cells associated with spermatogenesis in *Pampus argenteus* (Teleostei: Perciformes: Stromateidae). *Zool Studies* 2010;49:39–50.
- [74] Schulz R. Serum levels of 11-oxotestosterone in male and 17 β -estradiol in female rainbow trout (*Salmo gairdneri*) during the first reproductive cycle. *Gen Comp Endocrinol* 1984;56:111–20.
- [75] Yousef GM, Diamandis M, Jung K, Diamandis EP. Molecular cloning of a novel human acid phosphatase gene (ACPT) that is highly expressed in the testis. *Genomics* 2001;74:385–95.
- [76] McClusky LM. The caspase-dependent apoptosis gradient in the testis of the blue shark, *Prionace glauca*. *Reproduction* 2013;145:297–310.

NON-PRINT ITEM

Abstract

Anamniote vertebrates (fishes and amphibians) show cystic spermatogenesis, in which Sertoli cells present important characteristics that provide new insights into Sertoli cell physiology. Unlike amniotes (in reptiles, birds, and mammals), anamniote Sertoli cells are mitotically active, and this activity can be differentiated into two modes: (i) Sertoli cell progenitors proliferate to form new niche space that then can be occupied by single A_{und} spermatogonia to form new spermatogenic cysts that are regulated by estrogens, thyroid hormone, follicle-stimulating hormone (FSH), and insulin-like growth factor 3; and (ii) Sertoli cells already associated with existing cysts proliferate to accommodate the growing germ cell clone, which is modulated by FSH, androgens, and progestins. Sertoli cells' terminal differentiation occurs when meiosis is completed by the accompanied germ cells and, simultaneously, tight junctions among Sertoli cells are formed. Because anamniote Sertoli cells show very high support capacity for germ cells, comprehensive studies of Sertoli cells may provide important clues to the regulatory mechanisms of these cells in vertebrates.

Keywords: Fish; amphibian; testis; cystic spermatogenesis; plasticity; Sertoli cell proliferation; Sertoli cell efficiency