UNIVERSIDADE FEDERAL DE MINAS GERAIS
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DEPARTAMENTO DE MORFOLOGIA

PADRONIZAÇÃO DAS TÉCNICAS DE TRANSPLANTE DE ESPERMATOGÔNIAS EM ZEBRAFISH (*DANIO RERIO*) E UTILIZAÇÃO DESTA ESPÉCIE COMO MODELO EXPERIMENTAL PARA SE INVESTIGAR A BIOLOGIA DAS ESPERMATOGÔNIAS TRONCO EM TELEÓSTEOS

DOUTORANDO: Rafael Henrique Nóbrega
ORIENTADOR: Prof. Dr. Luiz Renato de França

BELO HORIZONTE
2011

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PADRONIZAÇÃO DAS TÉCNICAS DE TRANSPLANTE DE ESPERMATOGÔNIAS EM ZEBRAFISH (*DANIO RERIO*) E UTILIZAÇÃO DESTA ESPÉCIE COMO MODELO EXPERIMENTAL PARA SE INVESTIGAR A BIOLOGIA DAS ESPERMATOGÔNIAS TRONCO EM TELEÓSTEOS.

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Lista de Abreviaturas

11-KT - *11-ketotestosterone* (11-cetotestosterona)

Amh - *anti-Müllerian hormone* (hormônio anti-Mülleriano)

Bcl6b - *B-cell CLL/lymphoma 6 member B protein* (proteína do membro B de linfoma 6)

BrdU - *bromodeoxyuridine* (5-bromo-2'-deoxiuridina)

Cadherin-1 - *also known as CAM 120/80 or epithelial cadherin (E-cadherin)* – (Caderina-1)

CCL9 - *chemokine (C-C motif) ligand 9* (quimiocina com motivo C-C para ligante 9)

CD24 - *cluster of differentiation 24 or heat stable antigen (HSA)* (cluster de diferenciação 24)

CD9 - *CD9 molecule* (molécula CD9)

CDH1 - *cadherin 1, type 1, E-cadherin (epithelial)* (caderina 1 ou E-caderina)

c-kit - *mast/stem cell growth factor receptor (SCFR) also known as proto-oncogene c-Kit or tyrosine-protein kinase Kit or CD117* (proteína tirosina-quinase Kit)

DAZL - *deleted in azoospermia-like* (deleção semelhante à azoospermia)

E2 - *estradiol*

Egr3 - *early growth response protein 3* (proteína 3 de resposta ao crescimento inicial)

EPCAM - *epithelial cell adhesion molecule* (molécula de adesão de célula epitelial)

ERM/ETV5 - *ets-related molecule* (molécula relacionada à ets)

eSRS21 - *eel spermatogenesis-related substance 21* (substância 21 relacionada à espermatogênese de enguia)

eSRS34 - *eel spermatogenesis-related substance 34* (substância 34 relacionada à espermatogênese de enguia)

FACS - *fluorescence-activated cell sorting*

FSH - *follicle-stimulating hormone* (hormônio folículo-estimulante)

Gcna1 - *germ cell nuclear antigen 1* (antígeno nuclear 1 de célula germinativa)

GDNF – *glial cell line-derived neurotrophic factor* (fator neurotrófico derivado de linhagem de célula da Glia)

GFP - *green fluorescent protein* (proteína fluorescente verde)

GPR125 - *G protein coupled receptor 125* (receptor 125 acoplado à proteína G)

GRFα1 - *GDNF family receptor alpha-1* (família de receptores alfa para GDNF)

IGF - *insulin-like growth factor* (fator de crescimento semelhante à insulina)

Lacz/Rosa26 - *generalized lacZ expression with the ROSA26 Cre reporter strain*

LH - *luteinizing hormone* (hormônio luteinizante)

Lin-28 - *Lin-28 homolog A is a protein that in humans is encoded by the LIN28 gene.* (Lin-28 é uma proteína que em humanos é codificada pelo gene LIN28)

LPR4 - *lipid phosphate phosphatase-related*

MACS - *magnetic-activated cell sorting*

MHC1 - *major histocompatibility complex class I (*complexo principal de histocompatibilidade)

Nanos2,3 - *nanos homolog 2,3. The mouse Nanos proteins, Nanos2 and Nanos3, are required for germ cell development and share a highly conserved zinc-finger domain.* (proteína Nanos 2 e 3 - homólogos ao Nanos de *Drosophila* - são requeridas para o desenvolvimento de células germinativas e compartilham domínios de dedo de zinco altamente conservados.

NEUROG3 (Ngn3) - *neurogenin 3* (neurogenina 3)

NS1 - *nucleostemin 1* (nucleoestemina)

Numb - *numb gene homolog (Drosophila)* (gene homólogo ao *numb* de *Drosophila*)

Oct-4 - *octamer-binding transcription factor 4* (fator de transcrição 4 ligador de octâmero)

PD-ECGF - *platelet-derived endothelial cell growth factor* (fator de crescimento de célula endotelial derivado de plaqueta)

PKH26 - *red fluorescent cell linker* (marcador fluorescente vermelho de membrana celular)

PKH67GL - *green fluorescent cell linker* (marcador fluorescente verde de membrana celular)

PLZF - *promyelocytic leukemia zinc finger* (motivos dedos de zinco de leucemia promielocítica)

PTU - *6-n-propyl-2-thiouracil*

RBM - *RNA binding motif protein* (proteína de motivo com ligação para RNA)

Ret - *receptor tyrosine kinase* (receptor tirosiona-quinase para GDNF)

sall4 - *sal-like 4*

Sohlh1,2 - *spermatogenesis- and oogenesis-specific basic helix–loop–helix (bHLH) transcription factor* (fator de transcrição hélice-alça-hélice básico específico de espermatogênese e oogênese)

SOX - *SRY-related HMG-box*

Stra8 - *stimulated by retinoic acid gene 8 protein* (proteína estimulada pelo gene 8 de ácido retinóico)

Taf4b - *TAF4B RNA polymerase II, TATA box binding*

Thy1 (CD90) - *thymus cell antigen 1, theta* (antígeno 1 de célula de timo)

UTF1 - *undifferentiated embryonic cell transcription factor 1* (fator de transcrição 1 de célula embrionária indiferenciada)

Vasa (**Vas**) - *protein which is localized in polar granules and essential for the development of germ cells* (proteína localizada nos grânulos polares e essencial para o desenvolvimento das células germinativas)

W - *white spotting*

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Resumo

A espermatogênese é um complexo processo de desenvolvimento celular no qual um pequeno número de espermatogônias tronco (SPGTs) origina um grande número de espermatozóides, os quais, como vetores gênicos, são capazes de propagar as o genoma da espécie. Este processo ocorre continuamente durante toda a vida reprodutiva do animal, devido ao potencial destas células de se auto-renovarem para manter seu número, ou de se diferenciarem em células-filhas que originarão espermatozóides. O balanço adequado entre estes dois processos é fundamental para a homeostase da espermatogênese, e sua regulação ocorre em locais específicos dos túbulos seminíferos denominados nichos espermatogoniais. Uma vez que a biologia da SPGT é desconhecida para um grande número de espécies, o presente estudo teve como objetivo inicial caracterizar, tanto do ponto de vista morfológico quanto funcional, as SPGTs em zebrafish (*Danio rerio*), o nicho espermatogonial, e por fim padronizar as técnicas de transplante de SPGTs para esta espécie. Duas populações de espermatogônias isoladas (A_{und*} e A_{und}) foram identificadas morfologicamente como potenciais candidatos a células tronco. Utilizando zebrafish *vasa::egfp* e a capacidade das células tronco em reter BrdU (5-bromo-2'-deoxiuridina) por longo período de tempo, observamos que o nicho espermatogonial se localiza próximo ou adjacente ao compartimento intersticial, sugerindo que a regulação deste nicho em zebrafish depende, pelo menos em parte, de hormônios e fatores parácrinos provenientes das células do compartimento intertubular. Através das técnicas de transplante de espermatogônias, as quais foram padronizadas com sucesso no presente estudo para o zebrafish, demonstramos que a fração celular enriquecida de A_{und*} e A_{und} foi capaz de colonizar os túbulos seminíferos, cuja espermatogênese endógena foi depletada com busulfan, e originar cistos de espermatogônias do tipo B e espermatócitos, 3 semanas pós-transplante. Além disso, quando transplantadas em fêmeas, estas espermatogônias foram capazes de se diferenciarem em oócitos. Desta forma, comprovamos o potencial tronco e a plasticidade destas espermatogônias. Os perfis hormonais e de expressão gênica dos testículos depletados antes do transplante sugerem que os elevados níveis de andrógenos, associados à baixa expressão do *amh* e elevada do *igf3* (*igf1b*), podem ter contribuído para a criação de microambiente no nicho do animal receptor que propicia a diferenciação, ao invés de colonização/auto-renovação. Esta condição de pró-diferenciação pode ter interferido no comportamento das SPGTs transplantadas. Em síntese, o presente estudo mostra que o zebrafish representa um interessante modelo experimental para se estudar a biologia da SPGT em peixes.

Palavras-chave: *espermatogônias-tronco, transplante de espermatogônias tronco, espermatogênese, peixes teleósteos, zebrafish*

Abstract

Spermatogenesis is a complex cellular developmental process, in which a small population of spermatogonial stem cells (SSCs) generates a large number of spermatozoa that act as genome vectors by propagating a species. In many species, the spermatogenic process occurs continuously or repeatedly during the adult male lifespan due to the capability of the SSCs to either self-renewal to maintain their number, or to differentiate into daughter cells that eventually will form spermatozoa. The proper balance between these two pathways is crucial for the spermatogenic homeostasis, and this balance is tightly regulated in the specific regions of the seminiferous tubules that house the SSCs, which are referred to as stem cell niche. Since the biological properties of SSCs are unknown for several species, the present study first aimed at characterizing the SSCs and their niche in zebrafish (*Danio rerio*) by morphological and functional means. Subsequently, we aimed at developing a SSC transplantation technique for this species. Two populations of single spermatogonia (A_{und*} and A_{und}) were identified in zebrafish testis as candidate SSCs. Using *vasa::egfp* transgenic zebrafish and stem cell properties, such as to retain a BrdU-label for extended periods of time, we have localized the SSC niche in zebrafish as being preferentially located close to the interstitial compartment. This suggests that hormones and/or paracrine factors from interstitial cells or molecules derived from the interstitial vasculature, might play important roles in the regulation of the SSC niche. Using SSC transplantation techniques, which were successfully developed and standardized in zebrafish, we have shown that germ cell fractions enriched in A_{und*} and A_{und} were able to colonize busulfan-depleted seminiferous tubules of recipient males and develop into cysts of type B spermatogonia and spermatocytes three weeks after transplantation. Moreover, type A_{und} spermatogonia differentiated into oocytes when transplanted into zebrafish ovaries. Therefore, we have shown both the stemness and the sexual plasticity of type A_{und} spermatogonia, and hence the presence of SSCs in this population of germ cells in the zebrafish testes. Hormonal and gene expression profiles from busulfan-depleted testes before transplantation indicate that higher androgen levels combined with the down-regulation of *amh* and up-regulation of *igf3* (*igf1b*) might have created a particular environment (niche), in which the balance of signaling molecules was shifted towards favoring SSC differentiation over their self-renewal. This pro-differentiation condition might have influenced the fate of the donor SSC after transplantation. In summary, the present work suggests that zebrafish might represent an interesting model for studying the SSC cell biology in teleost fish.

Keywords: *spermatogonial stem cell, spermatogonial stem cell transplantation, spermatogenesis, teleost fish, zebrafish*

1. INTRODUÇÃO E REVISÃO DA LITERATURA

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1.1. Espermatogênese em mamíferos

A espermatogênese é um processo que compreende uma série de eventos precisos e altamente coordenados, no qual, dependendo da espécie, uma espermatogônia tronco pode formar diariamente milhares de espermatozóides. Este processo pode ser dividido em três grandes fases (Russell et al., 1990; Sharpe, 1994; França & Chiarini-Garcia, 2005): *(1) fase espermatogonial ou proliferativa*, caracterizada por sucessivas divisões mitóticas das espermatogônias; *(2) fase espermatocitária ou meiótica*, em que o material genético dos espermatócitos é duplicado, recombinado e segregado, formando células haplóides denominadas de espermátides; e *(3) fase espermiogênica ou de diferenciação*, na qual as espermátides passam por modificações estruturais e funcionais altamente complexas para originar os espermatozóides que, após sofrerem o processo de maturação final no epidídimo, estarão aptos para a fecundação. Uma característica única e marcante deste processo é que o desenvolvimento das células germinativas é acompanhado por divisões celulares incompletas, resultando em grupos, "clones" ou "cistos" de células germinativas conectadas por pontes citoplasmáticas (Hunckins 1971; Russell et al., 1990; De Rooij & Russell, 2000). Desta forma, as espermatogônias que não estão conectadas por pontes citoplasmáticas, ou seja, isoladas (A_{is}), são consideradas as mais primitivas do processo e foram denominadas de espermatogônias tronco por diversos pesquisadores (Hunckins 1971; Russell et al., 1990; De Rooij & Russell, 2000). O esquema abaixo (Figura 1) ilustra o "modelo A_{is}", no qual a seqüência de desenvolvimento linear das células espermatogênicas no rato e no camundongo, desde a espermatogônia isolada (A_{is}) até a formação de espermátides, estão mostrados: A_{is} (isolada) $\rightarrow$ A_{pr} (pareada) $\rightarrow$ A_{al} (alinhadas com várias gerações) $\rightarrow$ A_1 (não estão mostradas na figura 1$\rightarrow$ A_2 $\rightarrow$ A_3 $\rightarrow$ A_4 $\rightarrow$ In $\rightarrow$ B $\rightarrow$ espermatócitos primários $\rightarrow$ espermatócitos secundários $\rightarrow$ espermátides). As espermatogônias A do

tipo isolada, pareada e alinhada são funcionalmente consideradas indiferenciadas, enquanto as do tipo A1-4, intermediária (In) e do tipo B são diferenciadas. Também merece ser mencionado que a última geração de A_{al} se diferencia em $A_{1,}$ não sofrendo, portanto, divisão mitótica. Já o "modelo A_{is}", desenvolvido em 1971 (Hunckins 1971), propõe que as espermatogônias isoladas (A_{is}) atuam como células tronco, e as espermatogônias conectadas por pontes citoplasmáticas (A_{pr}, A_{al}, A_1) são terminalmente já diferenciadas e comprometidas com a formação de espermatozóides (Figura 1). Este modelo tem servido de base conceitual para compreender a biologia das espermatogônias tronco em diversas espécies de mamíferos. No entanto, estudos recentes têm demonstrado que a diferenciação espermatogonial proposta pelo "modelo A_{is}" não é unidirecional e irreversível como se pensava (Barroca et al., 2009; Nakagawa et al., 2010; Yoshida, 2010), mas sim de caráter multidirecional e reversível dependendo das necessidades fisiológicas do tecido (ver abaixo).

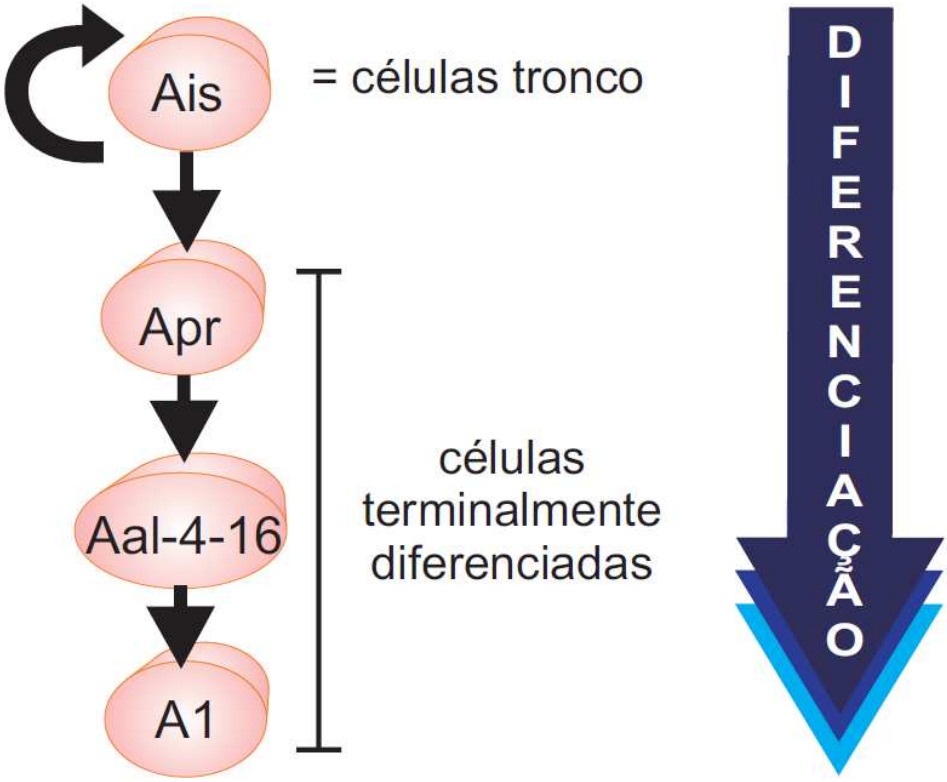


Figura 1. Ilustração esquemática do "modelo A_{is}". As espermatogônias A isoladas (A_{is}) atuam como células tronco, e as gerações subseqüentes conectadas por pontes citoplasmáticas; espermatogônias pareadas, alinhadas e A1 (A_{pr}, A_{al}, A_1), são

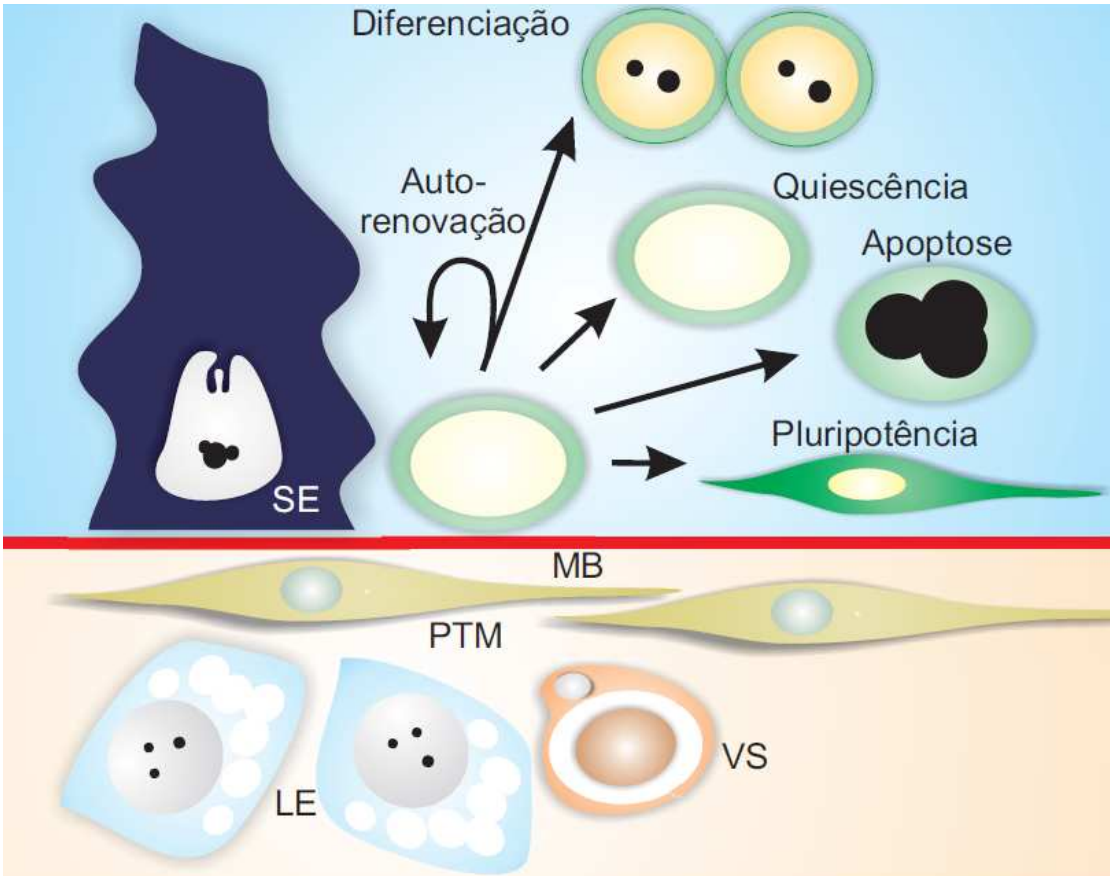
espermatogônias terminalmente já diferenciadas. Neste modelo, o potencial tronco é único e exclusivo das espermatogônias isoladas, e a diferenciação é sempre unidirecional e irreversível.

À semelhança das demais células tronco do corpo, a espermatogônia tronco tem capacidade de se auto-renovar e, ao mesmo tempo, originar células-filhas diferenciadas que desempenharão papel importante na funcionalidade tecidual. No caso das espermatogônias tronco, as células-filhas diferenciadas originarão espermatozóides que estarão aptos para fertilização. Assim, as espermatogônias tronco são as únicas células tronco do corpo que contribuem com material gênico para a formação de novos organismo. (De Rooij & Russell, 2000; De Rooij, 2001 e 2006a,b, Ehmcke et al., 2006a; Yan, 2006; Hofmann, 2008). Desta forma, as espermatogônias tronco têm atraído interesse de pesquisadores como alternativa para a conservação de espécies e transgênese.

O balanço adequado entre a auto-renovação e a diferenciação das espermatogônias tronco é essencial para garantir a continuidade e a homeostase do processo espermatogênico, sendo sugerido na literatura que a localização destas espermatogônias pode ocorrer em regiões especializadas do túbulo seminífero denominadas de nichos (De Rooij, 2001 e 2006a,b; Yan, 2006; De Rooij, 2009).

Por definição, nicho é o microambiente molecular e celular responsável por manter as células tronco em seu estado indiferenciado. Desta forma, o nicho regula as propriedades das células tronco em se auto-renovar, diferenciar, entrar em quiescência, apoptose, ou pluripotência (Hofmann, 2008), conforme ilustrado na Figura 2 na página seguinte. Ao que parece, o nicho é composto por no mínimo três elementos: 1) *células de suporte*; 2) *células tronco*; e 3) *matriz extracelular adjacente* (Spradling et al., 2001; Fuchs et al., 2004; Smith, 2006). Nos testículos de roedores, apesar do diâmetro dos túbulos seminíferos ser uniforme ao longo de sua extensão e comprimento, as espermatogônias tronco localizam-se preferencialmente nas áreas dos túbulos seminíferos próximas do interstício, em especial de células de Leydig e vasos sangüíneos (Chiarini-Garcia et al., 2001; 2003; Yoshida et al., 2007). Estas evidências sugerem que o nicho das espermatogônias

tronco seja formado pelas células de Sertoli, membrana basal na qual estas espermatogônias estão apoiadas, células peritubulares mióides, e



pelos elementos intersticiais como as células de Leydig e vasos sangüíneos (Figura 2) (McLean 2005; De Rooij, 2006b; Yan, 2006; Hess et al., 2006; Cooke et al., 2006; De Rooij, 2009; Yoshida, 2010).

Figura 2. Representação esquemática do nicho das espermatogônias tronco em roedores. Apesar da membrana basal ser uniforme ao longo da circunferência dos túbulos seminíferos, as espermatogônias tronco concentram-se preferencialmente nas áreas próximas do interstício, em especial de células de Leydig e vasos sangüíneos. O nicho das espermatogônias tronco, portanto, é definido pelas células de Sertoli (SE), membrana basal (MB), células peritubulares mióides (PTM) subjacentes e pelos elementos intersticiais, como células de Leydig (LE) e vasos sangüíneos (VS). Através de sua composição celular e molecular, o nicho regula as propriedades das espermatogônias tronco como ilustrado na Figura 2.

A sinalização endócrina e parácrina gerada por estes tipos celulares favorece a permanência e auto-renovação das espermatogônias tronco neste local, enquanto que condições diferentes à estas estimulam a

diferenciação, quiescência, apoptose, ou até mesmo pluripotência das espermatogônias tronco (De Rooij, 2009).

A exata localização dos nichos espermatogoniais, bem como a sinalização endócrina e parácrina envolvida na manutenção, auto-renovação e diferenciação das espermatogônias tronco, têm sido objeto de pesquisas recentes. Por exemplo, foi demonstrado que níveis elevados de testosterona inibem a diferenciação das espermatogônias tronco em roedores (Meistrich & Shetty, 2003), enquanto, ao propiciar maior proliferação das espermatogônias tronco, a diminuição dos níveis deste andrógeno promove maior eficiência no transplante de espermatogônias (Dobrinski et al., 2001). Assim como a testosterona, a citocina Csf-1 (fator estimulatório de colônia 1) produzida pelas células de Leydig e por pelo menos parte da população das células peritubulares mióides, também foi caracterizada como fator estimulatório da auto-renovação das espermatogônias tronco em camundongos (Oatley et al., 2009). Tais achados uma vez mais sugerem a localização preferencial das espermatogônias tronco próximas das células intersticiais. Ainda neste contexto, Yoshida e colaboradores (2007) elegantemente demonstraram que as espermatogônias indiferenciadas (A_{is}, A_{pr} e A_{al}), incluindo-se as espermatogônias tronco, concentram-se nas áreas dos túbulos seminíferos adjacentes aos vasos sangüíneos do interstício, e que quando as mesmas se diferenciam em A_1, movimentam-se para fora da área vascular. Tal proximidade com os vasos sangüíneos sugere que o nicho germinativo em mamíferos é estabelecido em conseqüência da organização vascular gonadal. Apesar do papel dos vasos sangüíneos na sobrevivência das espermatogônias tronco ainda ser desconhecido, estudos recentes mostram que as células endoteliais estabelecem um "nicho vascular" não apenas por fornecerem oxigênio ou nutrientes para as células tronco, mas também por secretar fatores, denominados fatores angiócrinos, que regulam a atividade das células tronco hematopoiéticas (Butler et al., 2010; Kobayashi et al., 2010).

Embora as células intersticiais pareçam desempenhar importante função na regulação dos nichos espermatogoniais, as células de Sertoli são ainda consideradas como os principais elementos orquestradores do nicho espermatogonial (Oatley et al., 2010). Por exemplo, em modelos

experimentais que aumentam o número de células de Sertoli, como o hipotireoidismo neonatal transitório, a disponibilidade de nichos é maior, e conseqüentemente o número de espermatogônias indiferenciadas também (Auharek & França, 2010). Além disso, a eficiência do transplante de espermatogônias tronco é significativamente maior quando estes modelos são empregados como doadores ou receptores do transplante (Oatley et al., 2010). Oatley e colaboradores (2010) demonstram que a maior disponibilidade de nichos/espermatogônias tronco não é acompanhada pelo aumento de vasos sangüíneos e células intersticiais, mas apenas pelo número de células de Sertoli, sugerindo, portanto, que as células de Sertoli são os elementos somáticos chave que ditam o número de espermatogônias tronco e nichos em testículos de mamíferos.

É inquestionável que mais estudos são necessários para compreender a natureza celular do nicho espermatogonial, porém não há dúvidas de que os fatores extracelulares secretados pelas células do nicho são essenciais na regulação da atividade das espermatogônias tronco. Dentre os fatores extracelulares, o GDNF é considerado um dos mais importantes na manutenção das espermatogônias tronco em mamíferos (Meng et al., 2000; Yomogida et al., 2003; Naughton et al., 2006). Sob influência do FSH, este fator secretado pelas células de Sertoli atua por meio do complexo de receptores GFRα1/c-RET localizado na superfície das espermatogônias tronco (De Rooij, 2006b; Hess et al., 2006; Cooke et al., 2006). Estudos demonstram que camundongos deficientes para GDNF (heterozigotos) (Meng et al., 2000) e camundongos *knockouts* para GDNF/GFRα1/c-RET (Naughton et al., 2006) perdem progressivamente suas espermatogônias tronco devido à incapacidade das mesmas de se auto-renovarem e manterem seu estado indiferenciado. Por outro lado, camundongos com superexpressão de GDNF possuem acúmulo de espermatogônias indiferenciadas nos testículos, lembrando um seminoma (Meng et al., 2001; Yomogida et al., 2003). Assim, estes resultados sugerem fortemente a importância do GDNF na sobrevivência e auto-renovação das espermatogônias tronco nos testículos de mamíferos. Ainda neste contexto, técnicas de *microarray* empregadas em culturas de espermatogônias tronco têm sido utilizadas recentemente para se identificar os possíveis genes envolvidos na sinalização do GDNF (Oatley

et al., 2006). Neste estudo foi demonstrado que seis genes são altamente regulados pelo GDNF, dentre eles os que codificam os fatores de transcrição Bcl6b (*B cell CLL/lymphoma 6, member B*) e o ERM (relacionado à família ETS, também conhecido como ETV5) (Oatley et al., 2006). Camundongos *knockouts* para Bcl6b (Oatley et al., 2006) e ERM (Chen et al., 2005) confirmam a importância desses fatores na sobrevivência das espermatogônias tronco, uma vez que o epitélio seminífero desses animais encontra-se desprovido de espermatogônias, sendo portanto constituído somente por células de Sertoli.

Particularmente sobre o ERM, estudos recentes têm demonstrado que camundongos *knockouts* para este fator ($ERM^{-/-}$) apresentam a primeira onda espermatogênica normal, mostrando perda gradual das espermatogônias após a mesma (Hess et al., 2006). Uma vez que os níveis de GDNF são normais nesse modelo ($ERM^{-/-}$), sugere-se que o GDNF é responsável por manter as espermatogônias na primeira onda espermatogênica. Portanto, o ERM seria essencial para a sobrevivência das espermatogônias durante a puberdade e a fase adulta (Hess et al., 2006). De maneira interessante, o início da expressão do ERM coincide com o término do período de proliferação das células de Sertoli, com conseqüente diferenciação/maturação das mesmas. Esta mudança de status funcional das células de Sertoli deve ser a principal razão para que outros fatores, além do GDNF, sejam requeridos para a regulação dos nichos espermatogoniais na fase adulta (Hess et al., 2006). Essas observações sugerem que os nichos espermatogoniais são dinâmicos, isto é, passam por modificações estruturais e funcionais conforme a idade/período de desenvolvimento do animal. Além disso, estudos recentes utilizando *microarray* de cultura primárias de células de Sertoli de animais $ERM^{-/-}$ (ou $Etv5^{-/-}$) demonstram significativa redução na expressão de quimiocinas em comparação com as células de Sertoli de animais controle (Simon et al., 2010). Este estudo também demonstra que as quimiocinas com motivos cisteínicos CCL9 são reguladas pelo ETV5, e promove a quimioatração entre células de Sertoli $Etv5^{-/}$ e as espermatogônias tronco, sugerindo pela primeira vez que as células de Sertoli produzem fatores quimiotáxicos para as espermatogônias tronco (*homing*) (Simon et al., 2010).

A tabela na página seguinte sumariza os principais genes/proteínas expressos nos diferentes tipos de espermatogônias de roedores (ver revisão em Phillips et al., 2010). *Oct4*, *Gfra1*, CD24, *Nanos2,3*, *Egr3*, *Plzf*, *Sox-3*, *Bcl6b*, *Ret*, *Sohlh2*, *Cdh1*, *Gpr125*, *Utf1* e *Lin28* são exclusivos das espermatogônias indiferenciadas (A_{is}, A_{pr}, A_{al}), e têm sido apontados na última década como potenciais marcadores das células tronco da linhagem germinativa masculina (Phillips et al., 2010).

	células germinativas						
Marcadores moleculares	**Ais**	**Apr**	**Aal**	**A1-4**	**In**	**B**	**SPC**
c-kit			X	X	X	X	X
GCNA1	X	X	X	X	X	X	X
VASA	X	X	X	X	X	X	X
DAZL	X	X	X	X	X	X	X
Stra8	X	X	X	X	X	X	X
α6-integrina	X	X	X	X	X	X	X
β1-integrina	X	X	X	X	X	X	X
Epcam	X	X	X	X	X	X	X
Pouf5f1 (Oct4)	X	X	X				
GFR-α1	X	X	X				
CD24	X	X	X				
Thy1 (CD90)	X	X	X				
Nanos 2	X	X					
Nanos 3	X	X	X				
CD9	X	X	X	X	X	X	
EGR3	X	X					
Ngn3	X	X	X				X
PLZF	X	X	X				
RBM	X	X	X	X			
Sox-3	X	X	X				
TAF4B	X	X	X	X	X	X	X
Bcl6b	X	X	X				
Numb	X	X	X	X	X	X	X
Lrp4	X	X	X	X	X	X	X
Ret	X	X	X				
Sohlh1			X	X	X	X	X
Sohlh2	X	X	X				
CDH1	X	X	X				
GPR125	X	X	X				
Nucleostemin	X	X	X	X	X	X	X
UTF1	X	X	X				
Lin28 (Text17)	X	X	X				

Tabela 1. Tabela ilustrando os marcadores moleculares identificados nos diferentes tipos de células germinativas tais como: espermatogônias do tipo A isolada (Ais), pareada (Apr), alinhada (Aal), e do tipo A diferenciadas (A1-4); espermatogônias intermediárias (In); espermatogônias do tipo B (B); e espermatócitos (SPC). Os genes específicos e

exclusivamente expressos nas espermatogônias indiferenciadas têm sido considerados como potencias marcadores das espermatogônias tronco (Modificado de Phillips et al., 2010).

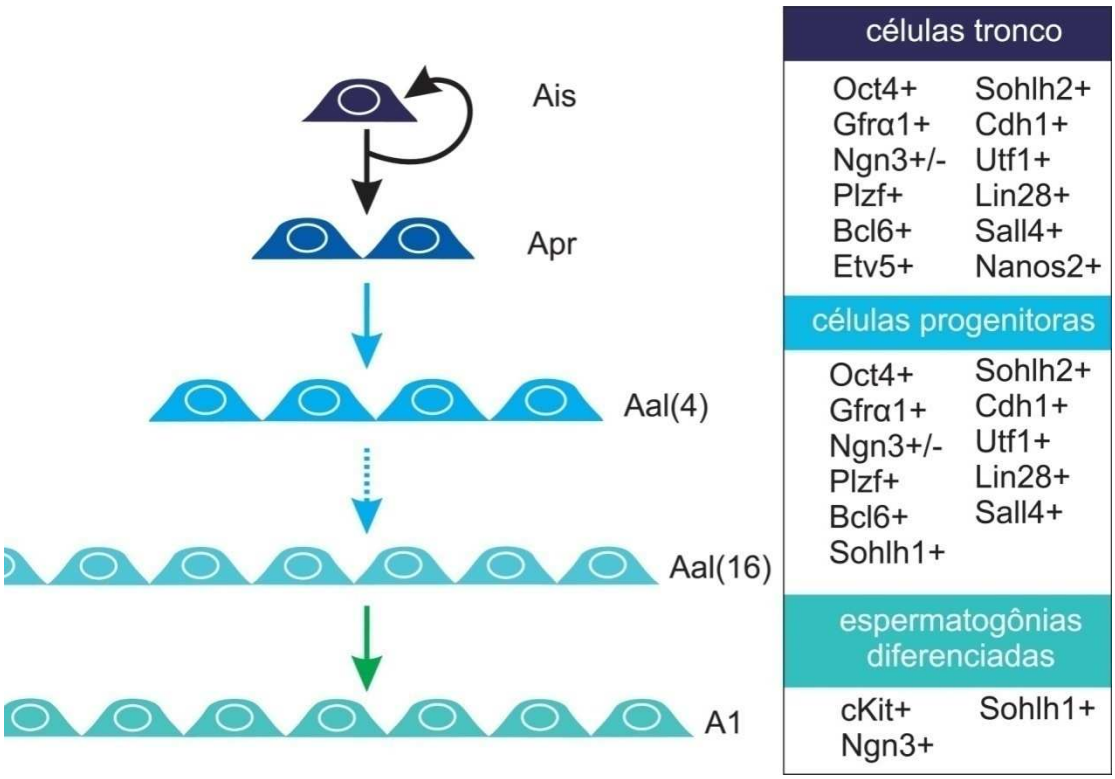


Figura 3. Perfil gênico expresso nos diferentes tipos de espermatogônias de roedores; espermatogônia do tipo isolada (Ais), pareada (Apr), alinhada (Aal4-16), e diferenciadas do tipo 1 (A1) (Modificado de Phillips et al., 2010).

A Figura 3 acima sumariza esquematicamente os principais marcadores moleculares encontrados nas diferentes gerações espermatogoniais, de acordo com seu nível hierárquico de diferenciação. Dentre eles, vale a pena mencionar o gene *Nanos2*, que é a forma homóloga do gene Nanos de *Drosophila*, altamente conservado durante a evolução, e que em mamíferos, de acordo com Sada e colaboradores (2009), atua como importante fator intrínseco da auto-renovação das espermatogônias tronco (Sada et al., 2009). Estes mesmos autores demonstraram que *Nanos2* é expresso preferencialmente nas espermatogônias A_{is} e A_{pr}, e estudos de expressão constitutiva ou deleção de *Nanos2* resultam em acúmulo de espermatogônias no testículo e

depleção da espermatogênese, respectivamente. Estes resultados sugerem, uma vez mais, que as espermatogônias que expressam *Nanos2* possuem atividade tronco, e que *Nanos2* tem importante papel na atividade das espermatogônias tronco (Sada et al., 2009). Em medaka (*Oryzias latipes*) (Adrianichthyidae, Beloniformes), uma espécie de peixe teleósteo, a expressão de *Nanos2* também ocorre em células germinativas indiferenciadas (Aoki et al., 2009), sugerindo que o papel de *Nanos2* na manutenção das células germinativas tronco é altamente conservado.

Por outro lado, estudos recentes têm demonstrado que a expressão dos diversos genes citados acima, e listados na Tabela 1 e Figura 3, não é homogênea para um mesmo tipo de espermatogônia. Nakagawa e colaboradores (2010) mostraram que as espermatogônias A_{is} diferem entre si em relação à expressão de GFRα1 e Ngn3, existindo A_{is} que são GFRα1+/Ngn3- (~87,5%), GFRα1+/Ngn3+ (~6,25%), ou GFRα1-/Ngn3+ (~6,25%). O mesmo também se aplica para as espermatogônias do tipo A_{pr} e A_{al}, porém com maior freqüência de GFRα1-/Ngn3+. De modo interessante, nas espermatogônias alinhadas (A_{al}) há um aumento gradual da fração GFRα1-/Ngn3+ conforme estas células expandem seu número, ocorrendo, por exemplo, 100% de GFRα1-/Ngn3+ nas A_{al-16} (Nakagawa et al., 2010). Desta forma, os autores sugerem uma correlação entre o tamanho do clone/cisto das espermatogônias indiferenciadas e a expressão de GFRα1 e Ngn3; onde clones pequenos têm maior probabilidade de serem GFRα1+/Ngn3-, enquanto que cistos/clone maiores GFRα1-/Ngn3+. Além disso, a reversibilidade entre as populações GFRα1+/Ngn3- e GFRα1-/Ngn3+ também foi demonstrada por Nakagawa e colaboradores (2010). Espermatogônias isoladas GFRα1+ dão origem à espermatogônias isoladas ou pareadas Ngn3+, e o inverso também foi comprovado; ou seja, espermatogônias isoladas, pareadas e alinhadas Ngn3+ dão origem à espermatogônias isoladas GFRα1+.

Interessantemente, a reversibilidade das espermatogônias pareadas ou alinhadas Ngn3+ em espermatogônias isoladas GFRα1+ pode ocorrer acompanhado da fragmentação dos clones/cistos (Nakagawa et al., 2010). Estas evidências demonstram que o "modelo A_{is}" é mais dinâmico e reversível do que se imaginava antes. E também menos linear uma vez que os mesmos autores (Nakagawa et al., 2010) mostram que

espermatogônias isoladas podem dar origem diretamente à espermatogônias A_1. O diagrama na página seguinte (Figura 4) ilustra a possível reinterpretação e extensão do "modelo A_{is}".

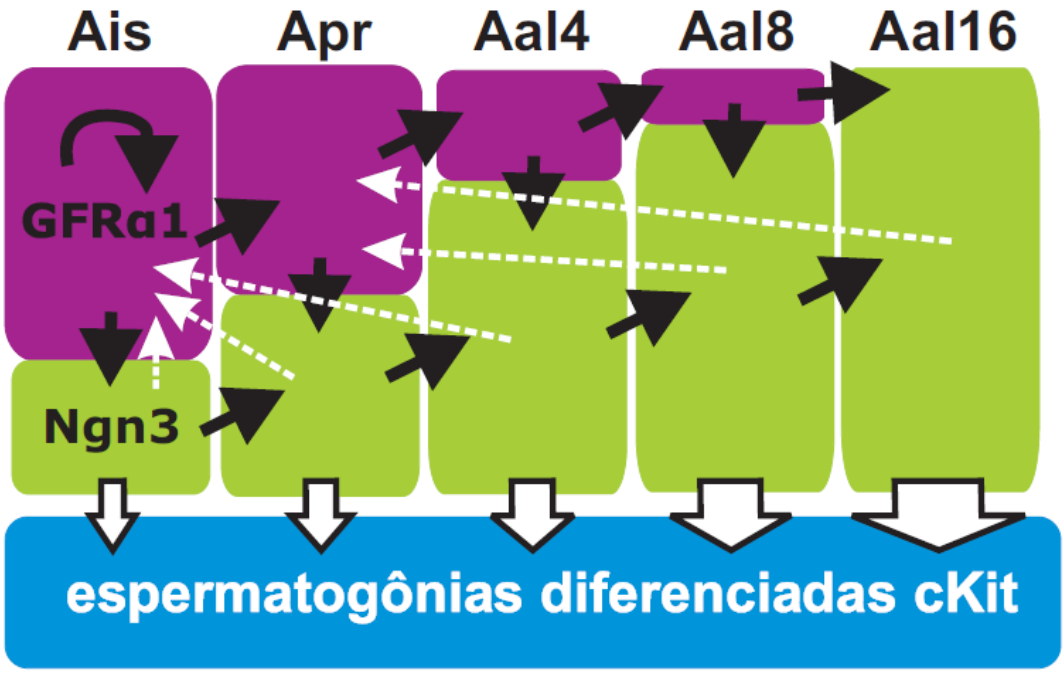


Figura 4. Reinterpretação e extensão do "modelo Ais" de acordo com os achados recentes de Nakagawa e colaboradores (2010). O diagrama ilustra a expressão heterogênea de GFRα1 e Ngn3 dentro de um mesmo tipo espermatogonial (espermatogônia do tipo isolada, A_{is}; pareada, A_{pr}; e alinhada A_{al4}, A_{al8}, A_{al16}); bem como o caráter dinâmico e reversível entre os diversos tipos de espermatogônia, como pode ser observado pelas diferentes setas (setas pretas, fluxo comumente observado; setas pontilhadas em branco, reversão; e setas brancas abertas, diferenciação em espermatogônias cKit+ onde a largura da seta indica a probabilidade de diferenciação. (Modificado de Nakagawa et al., 2010).

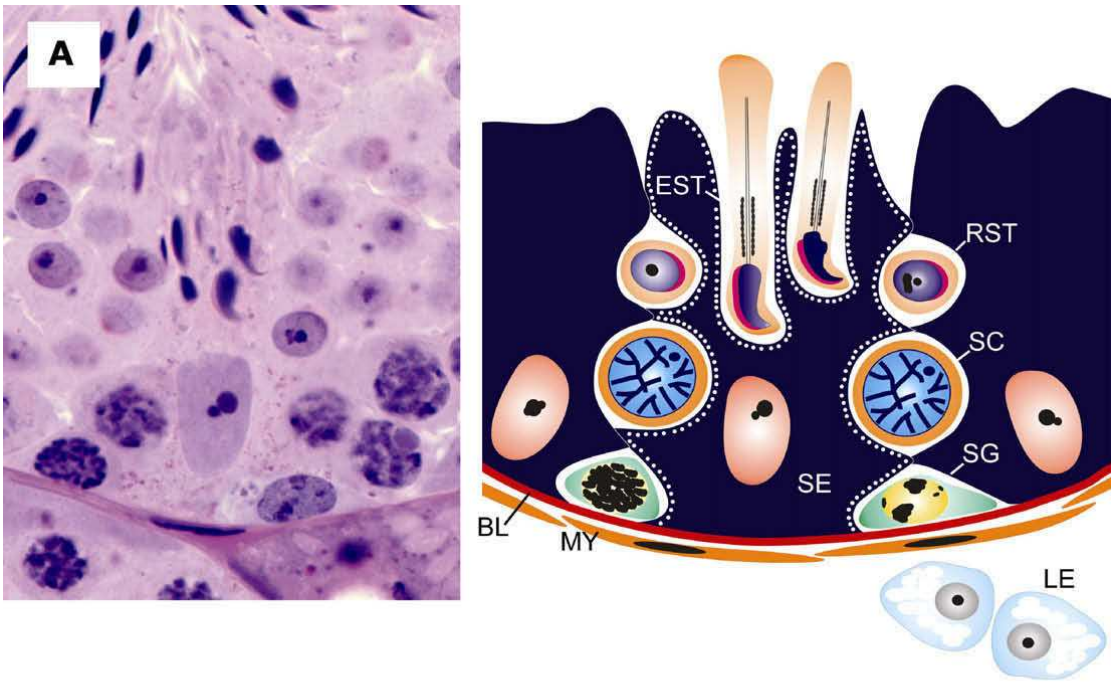
Este modelo sugere que a fração que contém as espermatogônias tronco não se restringe apenas ao compartimentos das espermatogônias do tipo isolada, mas também a outros tipos de espermatogônias, consideradas terminalmente diferenciadas pelo "modelo A_{is}", como as espermatogônias pareadas e alinhadas. O que justifica perfeitamente os achados anteriores de Nakagawa e colaboradores (2007) que demonstraram que em situações de injúria testicular (quimioterapia, raio X e outros) ou após transplante, as espermatogônias pareadas e alinhadas

apresentam potencial de se auto-renovar, sendo consideradas como "células tronco potencial" em contraste com as células tronco reais, denominadas "células tronco atuais" (A_{is}). No entanto, a presente reinterpretação do "modelo A_{is}", sugere que as "células tronco potencial" pertençam à fração GFRα1+ das espermatogônias pareadas e alinhadas, e que ao invés de "potencial" estas células sejam realmente "atuais". De modo geral, o conceito de espermatogônia tronco precisa ser revisto, e o compartimento das espermatogônias tronco precisa ser identificado, levando-se em consideração a expressão gênica heterogênea dos clones/cistos espermatogoniais, bem como, aspectos funcionais e comportamentais dos diversos tipos de espermatogônias. Portanto, não resta dúvida de que o sistema tronco espermatogênico é muito mais plástico, reversível e adaptável do que se imaginava antes nas últimas décadas.

1.2. Espermatogênese em peixes

Os peixes teleósteos constituem um dos grupos mais abundantes e diversificados dentre os vertebrados (Nelson, 2006). Conforme mostra a Figura 5 a seguir, a espermatogênese nos peixes ocorre no interior de estruturas denominadas espermatocistos, ou cistos, que se formam quando uma espermatogônia primária ou do tipo A é completamente envolvida pelos prolongamentos das células de Sertoli (Grier, 1993; Pudney, 1993; 1995; Schulz et al., 2010). As células germinativas derivadas desta espermatogônia dividem-se sincronicamente para constituir um clone de células germinativas que é envolvido por um número variado de células de Sertoli, dependendo do tipo de cisto (Vilela et al., 2003). Portanto, diferentemente de mamíferos (Russell et al., 1990), na espermatogênese cística as células de Sertoli normalmente estão em contato com apenas um tipo específico de célula germinativa durante a evolução do processo espermatogênico (Nóbrega et al., 2009; Schulz et al., 2010). Estes cistos encontram-se apoiados na túnica própria dos túbulos seminíferos, que é formada por camada acelular denominada

de membrana basal e pelas células peritubulares mióides (Koulish et al., 2002).



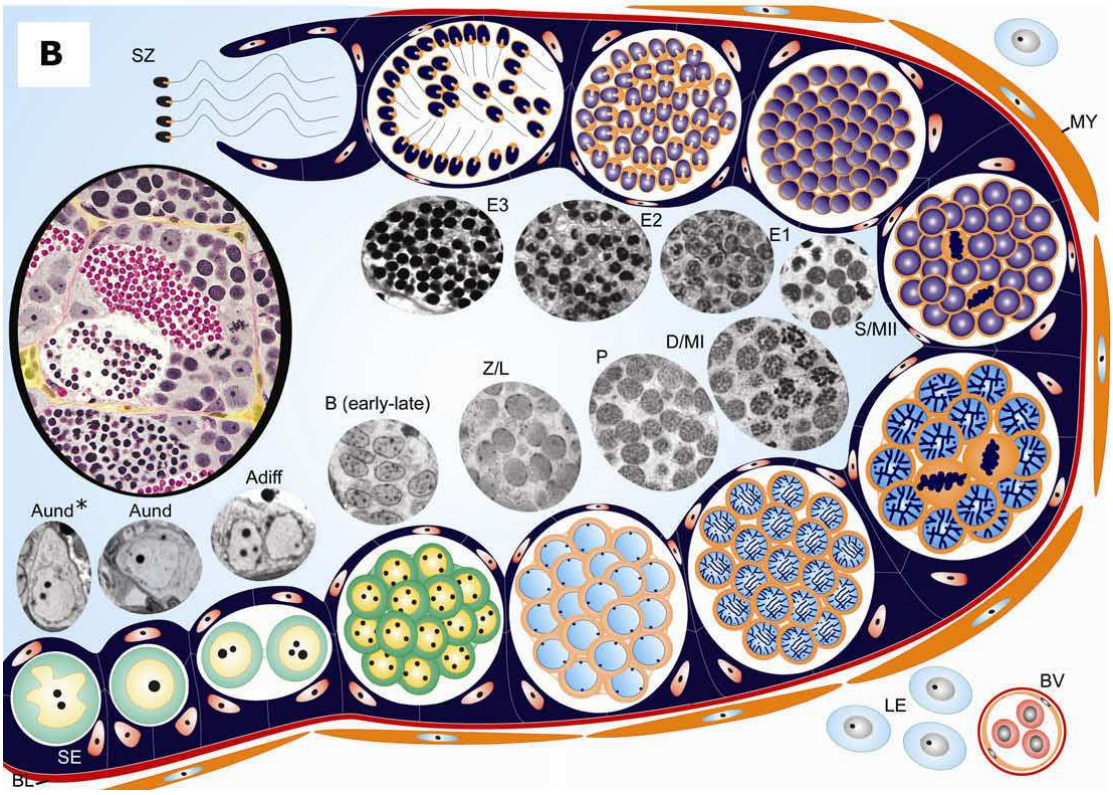


Figura 5. Comparação entre a espermatogênese não-cística de amniotas (répteis, aves, mamíferos) (A) e anamniotas (peixes, anfíbios) (B). Assim, esta figura ilustra as diferenças entre a relação célula de Sertoli/célula germinativa na espermatogênese não-cística e cística. Em A, a célula de Sertoli suporta ao mesmo tempo diferentes "clones" de células germinativas em diferentes fases de desenvolvimento. Enquanto em B a célula de Sertoli suporta ao mesmo tempo apenas um "clone" em uma mesma fase de desenvolvimento. Legendas: células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig (LE), espematogônia (SG); espermatócito (SC); espermátide arredondada (RST); espermátide alongada (EST); espermatogônia do tipo A indiferenciada* (Aund*) (célula tronco?); espermatogônia do tipo A indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); espermatogônia do tipo B (B early-late); espermatócitos primários em leptóteno/zigóteno (L/Z), paquíteno (P), diplóteno/metáfase I (D/MI); espermatócitos secundários/metáfase II (S/MII); espermátides iniciais (E1); intermediárias (E2); finais (E3); espermatozóides (SZ); e vasos sangüíneo (BV) (Retirado de Schulz et al., 2010).

Em teleósteos, a seqüência das células espermatogênicas, a partir de espermatogônias até a formação de espermatozóides, segue o seguinte esquema (Figura 6): espermatogônias indiferenciadas → espermatogônia primária (do tipo A ou diferenciada) → espermatogônias do tipo B (número variado de gerações) → espermatócitos primários (pré-leptóteno, leptóteno/zigóteno, paquíteno, diplóteno) → espermatócitos secundários → espermátides (iniciais, E1; intermediárias, E2; finais, E3) → espermatozóides.

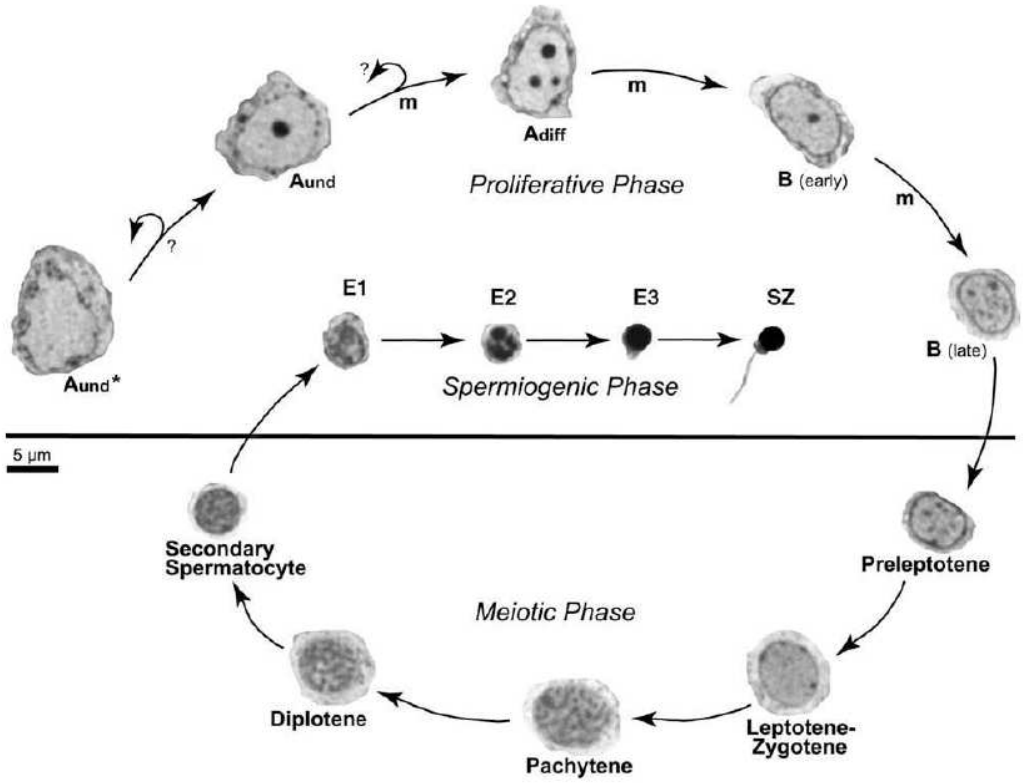


Figura 6. Diagrama ilustrando a seqüencia dos tipos celulares em peixes a partir da espermatogônia indiferenciada (célula tronco?) até a formação de espermatozóides, através das três fases da espermatogênese; ou seja, proliferativa, meiótica, e espermiogênica. Legendas: Espermatogônia do tipo A indiferenciada* (Aund*); espermatogônia do tipo A indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); espermatogônia do tipo B inicial (B early); espermatogônia do tipo B final (B late); espermatócitos primários em pré-leptóteno, leptóteno/zigóteno, paquíteno, diplóteno; espermatócitos secundários; espermátides iniciais (E1); intermediárias (E2); finais (E3); e espermatozóides (SZ). Na fase proliferativa, a auto-renovação das espermatogônias tronco é indicada pela seta curvada, e nos estágios posteriores a letra m indica que os tipos celulares estão separados por divisões mitóticas, cujo número varia de acordo com a espécie. O primeiro sinal de interrogação entre Aund* e Aund questiona se estas células estão separadas por mitose ou se correspondem à um mesmo tipo celular em diferentes fases do ciclo celular. O segundo sinal de interrogação é utilizado para indicar incerteza em relação ao potencial tronco das espermatogônias do tipo indiferenciado (Aund) (Retirado de Schulz et al., 2010).

Ao final do processo espermatogênico, os espermatozóides recém-formados são liberados no lúmen dos túbulos seminíferos após a abertura das junções entre as células de Sertoli, (Miura, 1999; Le Gac & Loir, 1999; Schulz & Miura, 2002; Schulz, 2003).

Usualmente, a disposição da cromatina nuclear e o tamanho do núcleo, aliado ao número de células germinativas por cisto, são parâmetros utilizados como referências para se distinguirem os diferentes tipos espermatogoniais (Matta et al., 2002; Vilela et al., 2003). No entanto, merece ser mencionado que, à semelhança de mamíferos, existem consideráveis diferenças em relação ao número de gerações de espermatogônias diferenciadas em peixes (Miura, 1999; Schulz & Miura, 2002; Schulz et al., 2010).

Diferentemente de mamíferos, a duração total do processo espermatogênico nos peixes é normalmente mais rápida (Silva, 1987; Koulish et al., 2002; Vilela et al., 2003), e também influenciada pela temperatura, como nos demais vertebrados. Por exemplo, a duração conjunta das fases meiótica e espermiogênica é de aproximadamente 5-6 dias à 35ºC e de 10-11 dias à 25ºC em tilápias (*Oreochromis niloticus*) (Cichlidae, Perciformes) (Vilela et al., 2003; Lacerda et al., 2006a). Além

disso, estudos morfométricos em tilápias têm demonstrado que, em relação ao número teórico esperado, a espermatogênese dos peixes é mais eficiente que a dos mamíferos já investigados. Esta maior eficiência do arranjo cístico (clonal) provavelmente ocorre devido a maior disponibilidade de fatores produzidos pelas células de Sertoli e ao menor número de apoptoses das células germinativas, resultando em maior capacidade de suporte (eficiência) das células de Sertoli. Em tilápias, uma célula de Sertoli suporta ~100 espermátides, número este cerca de 10 vezes superior ao da maioria dos mamíferos estudados (Matta et al., 2002; Vilela et al., 2003; Schulz et al., 2005).

Outro aspecto peculiar da função testicular de peixes é a proliferação das células de Sertoli. Nos mamíferos, é considerado que a proliferação destas células ocorre somente no período fetal e pré-pubere, sendo portanto o fator determinante na produção espermática final, uma vez que cada célula de Sertoli suporta um número relativamente constante de células germinativas (França & Russell, 1998; França & Chiarini-Garcia, 2005). Em peixes teleósteos que apresentam crescimento contínuo, a proliferação das células de Sertoli foi reportada em tilápias, (*Oreochromis niloticus*) e bagres africanos (*Clarias gariepinus*) (Clariidae, Siluriformes) adultos (Schulz et al., 2005). Nestas espécies, a divisão das células de Sertoli ocorre principalmente quando as mesmas estão associadas com espermatogônias, de forma a garantir um número adequado destas células somáticas para suportar o desenvolvimento das células germinativas durante a espermatogênese. Portanto, a cada período reprodutivo dos teleósteos, a proliferação das células de Sertoli provavelmente é o fator primário responsável pelo aumento do testículo e da produção espermática (Schulz et al., 2005; Nóbrega et al., 2009; Schulz et al., 2010).

Semelhantemente aos mamíferos, pouco se sabe a respeito das espermatogônias tronco em peixes. Alguns autores consideram que as espermatogônias primárias ou do tipo A são as espermatogônias tronco (Miura, 1999; Schulz & Miura, 2002). Nos peixes, estas espermatogônias são as maiores células da linhagem germinativa (Miura, 1999; Schulz & Miura, 2002), apresentando as seguintes características morfológicas: núcleo volumoso contendo cromatina finamente granular, um ou dois

nucléolos, e citoplasma com grande quantidade de "*nuages*", que é um material elétron-denso formado por ribonucleoproteínas e RNAs (Miura, 1999; Quagio-Grassiotto & Carvalho, 1999; Schulz & Miura, 2002).

Com exceção de alguns dados ainda incipientes disponíveis para a enguia japonesa (*Anguilla japonica*) (Anguillidae, Anguilliformes) (Miura et al., 2002; Miura et al., 2003; Miura & Miura, 2003; Miura et al., 2006), praticamente não existem estudos sobre a regulação dos nichos espermatogoniais em peixes. Conforme mostra a Figura 7 abaixo, na enguia japonesa dois fatores são considerados como fortes candidatos na regulação da auto-renovação e diferenciação das espermatogônias tronco: 1) o PD-ECGF (*Platelet-derived endothelial cell growth factor*), fator de crescimento de célula endotelial derivado de plaqueta, também conhecido como eSRS34 (eel Spermatogenesis Related Substance 34) ou "fator de renovação das espermatogônias tronco"; e 2) o AMH que é o hormônio anti-Mülleriano, também conhecido como eSRS21 (eel Spermatogenesis Related Substance 21) ou "substância inibidora da espermatogênese". A Figura 7 mostra ainda que, sob a influência do estradiol-17β (E2), as células de Sertoli produzem o PD-ECGF, que estimula a renovação das espermatogônias tronco. No entanto, quando se adiciona anticorpo anti-PD-ECGF à co-cultura de células de Sertoli/células germinativas, a auto-renovação das espermatogônias tronco induzida pelo E2 é bloqueada (Miura et al., 2003).

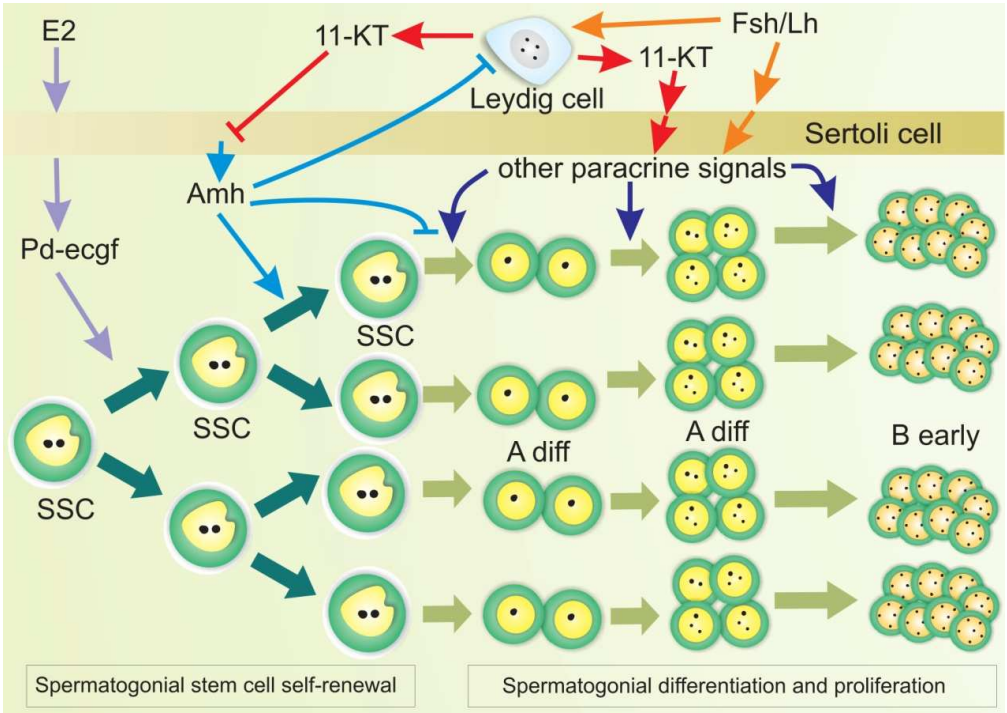


Figura 7. Diagrama esquemático ilustrando a possível regulação das espermatogônias tronco (SSC) em peixes teleósteos. Legendas: estradiol-17β (E2); fator de crescimento de célula endotelial derivado de plaqueta (Pd-ecgf); hormônio anti-Mülleriano (Amh); 11-cetotestosterona (11-KT); hormônios gonadotrópicos, folículo estimulante (Fsh) e luteinizante (Lh); espermatogônia do tipo A diferenciada (A diff); e espermatogônia do tipo B inicial (B early). Fatores estimulatórios da auto-renovação das espermatogônias tronco, ou inibitórios de diferenciação espermatogonial: Pd-ecgf e Amh, respectivamente; fatores que contribuem para a diferenciação espermatogonial: Fsh/Lh e 11-KT que atuariam sinergeticamente para inibir os efeitos do Amh e produzir outros fatores parácrinos, como por exemplo, fator de crescimento semelhante à insulina (Igf) que estimulam a diferenciação e proliferação espermatogonial.

Outros experimentos ilustrados na Figura 7 mostram que o hormônio anti-Mülleriano (AMH), produzido pelas células de Sertoli, é responsável por inibir a diferenciação e proliferação espermatogonial (Miura et al., 2002; Miura et al., 2006). A expressão deste hormônio é reprimida pela 11-KT (11-cetotestosterona), que em peixes é o principal andrógeno sintetizado pelas células de Leydig. Outros fatores como o Igf1 e a activina B e outros fatores parácrinos parecem também atuar na diferenciação e proliferação espermatogonial. Na enguia japonesa, por exemplo, sob influência da 11-KT as células de Sertoli produzem a activina B que estimula a proliferação das espermatogônias B, porém sem iniciar a meiose. Já o Igf1 induz a síntese de DNA nas espermatogônias de truta arco-íris (*Onchorhynchus mykiss*) (Salmonidae, Salmoniformes), e permite a continuidade da espermatogênese induzida pela 11-KT na enguia japonesa (Miura & Miura, 2003).

Em síntese, os dados disponíveis para a enguia japonesa sugerem que a sobrevivência das espermatogônias tronco dos peixes ocorre em função do balanço adequado de fatores provenientes dos nichos. Pelo fato dos nichos espermatogoniais serem dinâmicos e com elevada plasticidade ao longo do desenvolvimento do indivíduo, os mesmos devem sofrer influência de diversos fatores (externos e internos). Dentre os quais podem ser citados, por exemplo, o fotoperíodo, a temperatura, o pH e a concentração iônica da água que provavelmente resultam na determinação da sazonalidade reprodutiva em espécies de clima temperado e tropical. Portanto, a melhor compreensão dos mecanismos

regulatórios dos nichos requer a investigação dos mesmos em várias espécies de teleósteos. Talvez, uma abordagem que poderá ajudar na elucidação deste aspecto é a investigação de desreguladores endócrinos tais como estrógenos e anti-andrógenos, que afetam a reprodução dos peixes (McGonnell & Fowkes, 2006).

1.3. Transplante de espermatogônias em mamíferos

O transplante de espermatogônias é uma importante técnica que foi desenvolvida inicialmente por Brinster e colaboradores (Brinster & Zimmermann, 1994; Brinster & Avarbock, 1994). Esta técnica consiste na transferência de espermatogônias tronco de um doador fértil para o testículo de um receptor infértil, onde estas células se desenvolvem e formam espermatozóides férteis com características genéticas do doador (ver Figura 8 na próxima página).

Esta metodologia tem se tornado uma valiosa abordagem para se investigar a biologia da espermatogônia tronco no testículo, bem como caracterizar os nichos espermatogoniais, e verificar os efeitos da manipulação *in vitro* na função das espermatogônias tronco (Orwig & Schlatt, 2005; McLean, 2005). O transplante de espermatogônias também tem proporcionado enormes avanços no estudo da espermatogênese em si, das interações entre células de Sertoli e células germinativas, além de pesquisas em potencial na área de produção animal, preservação de espécies ameaçadas de extinção, cultura de células, medicina reprodutiva e produção de animais transgênicos (McLean, 2005; Orwig & Schlatt, 2005; Hill & Dobrinski, 2006; Dobrinski, 2006).

Outro aspecto importante do transplante é a utilização com sucesso de doadores e receptores de diferentes espécies (transplante interespecífico ou xenogênico), como por exemplo, o transplante de espermatogônias de rato para camundongo (Clouthier et al., 1996), e de hamster para camundongo (Ogawa et al., 1999). No entanto, conforme será visto adiante, ao que parece o êxito do transplante xenogênico depende do grau de parentesco entre o doador e o receptor.

Nesse sentido, Dobrinski e colaboradores (1999; 2000) demonstraram que a transferência de espermatogônias de não-roedores (coelhos, cães, animais domésticos de grande porte e primatas) para camundongos imunodeficientes resulta apenas na colonização do testículo, porém sem passar da fase espermatogonial. Esses resultados sugerem que algumas etapas da espermatogênese parecem depender de interações parácrinas espécie-específicas, o que explicaria a incapacidade do ambiente testicular do camundongo de suportar a espermatogênese de espécies doadoras filogeneticamente distantes (McLean, 2005; Hill & Dobrinski, 2006; Dobrinski, 2006).

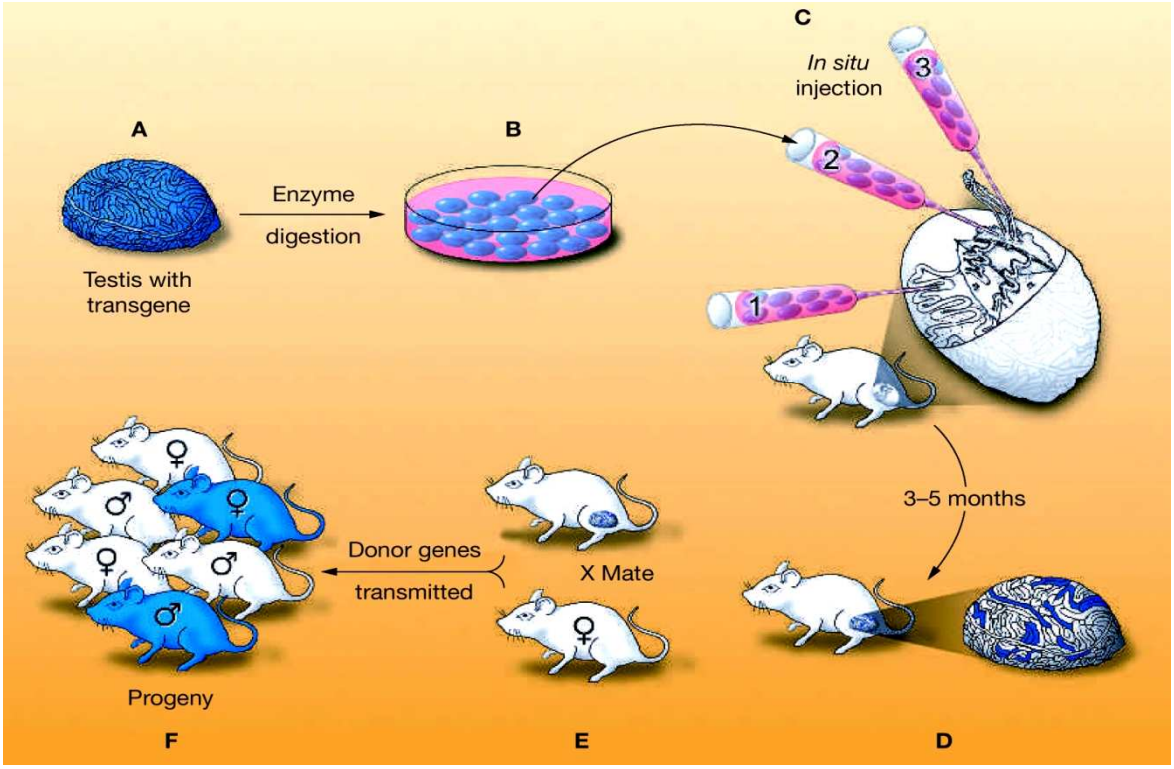


Figura 8. Principais fases do transplante de espermatogônias em camundongos. Retirado de Kubota & Brinster (2006). A = testículo do animal transgênico que expressa o gene da β-galactosidase de *Escherichia coli* (lacZ); B = suspensão de células germinativas obtidas através de digestão enzimática; C = transplante das células germinativas através dos túbulos seminíferos (1), *rete testis* (2), e dúctulos eferentes (3); D = testículo transplantado com células germinativas do doador; E = cruzamento do animal transplantado com fêmea normal; F = progênie do receptor portando características genéticas do camundongo doador transgênico.

Da mesma forma que nos roedores, o transplante singênico de espermatogônias também tem sido realizado com sucesso em outros animais como suínos, ruminantes, aves e gatos domésticos (Honaramooz et al., 2002; 2003; Hill & Dobrinski, 2006; Trefil et al., 2006; Silva, 2009). No entanto, algumas modificações no que diz respeito à marcação e a injeção das células do doador se fizeram necessárias. Assim, em função da ausência de marcadores transgênicos como lacZ/ROSA26 (Brinster & Zimmermann, 1994) e GFP (*green fluorescent protein*) (Brinster et al., 2003), a visualização das células transplantadas nesses animais é feita através do corante fluorescente PKH26 (*Red Fluorescent Cell Linker*) (Honaramooz et al., 2002; 2003). Esse corante lipofílico se liga de forma estável à membrana das células e tem sido utilizado para marcar células antes do transplante com a finalidade de se acompanhar o destino das mesmas no receptor, através de microscopia de fluorescência. As células coradas são visualizadas em vermelho, uma vez que o PKH26 tem excitação (551nm) e emissão (567nm) semelhantes ao da rodamina. Por não apresentar efeito citotóxico aparente, o PKH26 permite a identificação das células transplantadas no testículo do receptor por período relativamente longo (cerca de 3-4 meses) (Honaramooz et al., 2002). Em aves, o PKH-67 GL (de fluorescência verde), pertencente à mesma família de fluorocromos do PKH26, também foi empregado com sucesso na marcação das células do doador (Trefil et al., 2006).

Nos animais de grande porte (suínos e ruminantes), limitações anatômicas dificultam a injeção direta das células do doador nos túbulos seminíferos do receptor (Honaramooz et al., 2002; 2003). Dessa forma, utiliza-se a ultra-sonografia guiada para introduzir um cateter na *rete testis* desses animais, onde as células do doador serão transplantadas (Honaramooz et al., 2002; 2003). Da mesma forma que nos roedores, o azul de tripan (0,4%) também é utilizado nesses animais para se monitorar o preenchimento dos túbulos seminíferos, além de servir para avaliar a viabilidade das células do doador antes do transplante (Brinster & Zimmermann, 1994; Honaramooz et al., 2002; 2003).

De maneira geral, tanto em espécies de roedores como em outras espécies, a eficiência do transplante depende da quantidade de células tronco a serem transplantadas e do microambiente testicular do receptor,

que deve ter nichos acessíveis para a colonização das células do doador. Por essa razão, os melhores receptores são aqueles que possuem poucas ou nenhuma espermatogônia endógena. Ou seja, com epitélio seminífero constituído somente por células de Sertoli funcionais (Brinster et al., 2003). Nos roedores, os camundongos estéreis W/W^v constituem excelentes receptores para o transplante (Brinster & Zimmermann, 1994). O locus dominante *white spotting* (W) codifica o receptor c-kit que juntamente com o seu ligante são responsáveis pela proliferação e migração das células germinativas primordiais durante o desenvolvimento embrionário (Loveland & Schlatt, 1997). Por essa razão, o mutante W/W^v é praticamente desprovido de espermatogônias, sendo um dos modelos de receptor de eleição para o transplante em camundongos (Brinster & Zimmermann, 1994).

Uma alternativa aos mutantes estéreis, que nem sempre estão disponíveis para outras espécies, é o uso de receptores normais cuja espermatogênese endógena foi depletada antes do transplante (Brinster & Zimmermann, 1994; Brinster et al., 2003; Honaramooz et al., 2005). Os principais métodos de depleção da espermatogênese endógena consistem no pré-tratamento com drogas quimioterápicas, como o busulfan (1,4-dimetanosulfonoxibutano), irradiação local dos testículos e condições que elevam a temperatura do testículo (criptorquidismo e insulação) (Brinster et al., 2003; Honaramooz et al., 2005). Dentre eles, o busulfan é a abordagem mais utilizada para se destruir a espermatogênese endógena (Honaramooz et al., 2005). Esta droga quimioterápica é um agente alquilante de DNA, que transfere radicais alquil aos nucleotídeos e forma ligações cruzadas no DNA, as quais levam à morte massiva das células com grande capacidade proliferativa como, por exemplo, as espermatogônias. A dose efetiva de busulfan varia entre as espécies e até mesmo entre linhagens, podendo ser letal em função de severa depressão funcional da medula óssea (Brinster et al., 2003; Honaramooz et al., 2005; Hill & Dobrinski; Dobrinski, 2006). A depleção da espermatogênese pelo busulfan não é totalmente irreversível, uma vez que a espermatogênese endógena, mesmo que lentamente, pode se recuperar após o tratamento. No entanto, foi demonstrado que o tratamento com

busulfan durante a gestação resultou em prole permanentemente inférteis (Honaramooz et al., 2005).

Outro importante aspecto sobre o transplante que deve ser levado em consideração é a preparação das células do doador. O número de espermatogônias tronco no testículo é muito pequeno, sendo seu valor estimado de 0,03% do total de células germinativas (~35000) ou 1,3% de todas as espermatogônias no camundongo (Tegelenbosch & De Rooij, 1993). Em ratos, o número estimado de espermatogônias tronco é 830.000 (Orwig et al., 2002). No entanto, as propriedades funcionais e o uso de marcadores das espermatogônias tronco têm permitido o desenvolvimento de abordagens para se aumentar o número de espermatogônias tronco, tanto *in vivo* como *in vitro,* antes das mesmas serem transplantadas (McLean, 2005; Hill & Dobrinski, 2006; Dobrinski, 2006). Além de aumentar a proporção de espermatogônias, esta seleção também diminui a quantidade de "células contaminantes", como hemácias, células somáticas do testículo e outras células germinativas (Hill & Dobrinski, 2006).

As estratégias de enriquecimento *in vivo* consistem de abordagens que resultam em doadores com grande quantidade de espermatogônias tronco, como por exemplo animais imaturos; animais com deficiência em vitamina A que é um dos fatores responsáveis pela diferenciação espermatogonial, animais com criptorquidismo experimental ou tratados com PTU (6-n-Propyl-2- thiouracil) que provoca um hipotireoidismo neonatal transitório levando a um aumento de células de Sertoli e espermatogônias tronco (McLean, 2005; Hill & Dobrinski, 2006; De Rooij, 2006; Dobrinski, 2006; Luo et al., 2006; Oatley et al., 2010). Já o enriquecimento *in vitro* é feito a partir de suspensão de células proveniente da digestão enzimática do testículo do doador, e pode ser realizado através de: 1) seleção por fluorescência (FACS) ou campo magnético (MACS) com base nos marcadores (positivos e negativos) das espermatogônias tronco, como scatter^{low}, α6-integrina^{+}, β1-integrina^{+}, CD24^{+}, Thy-1^{+}, αv-integrina^{-}, Kit^{-}, MHC-I^{-}, CD9^{+}, Rho^{low}, Ep-CAM^{+}, EE2^{+} (McLean, 2005); 2) velocidade de sedimentação seguido de plaqueamento diferencial com componentes da matriz extracelular como laminina (Luo et al., 2006); e 3) gradiente de percoll (Lacerda et al., 2006a). O maior

número de espermatogônias tronco gerado pelo enriquecimento *in vitro* tem propiciado novas e auspiciosas dimensões ao transplante de espermatogônias, como por exemplo: o cultivo das espermatogônias tronco com a finalidade de avaliar os fatores que atuam na sua auto-renovação/diferenciação; o congelamento das espermatogônias por um determinado tempo e seu descongelamento seguido de transplante, conforme já observado com sucesso em camundongos; a possibilidade de preservação do genótipo de espécies ameaçadas de extinção ou de interesse comercial; a manipulação gênica das espermatogônias tronco, através de transdução lentiviral, retroviral ou por um vírus adeno-associado recombinante, com perspectivas de produção de transgênicos ou melhoramento genético, dentre outras aplicações (McLean, 2005; Ehmcke et al., 2006b; Hill & Dobrinski, 2006; Dobrinski, 2006).

A seguir serão mencionadas algumas abordagens ilustrando como o transplante de espermatogônias tem propiciado respostas a diversos aspectos funcionais da espermatogênese e seus mecanismos de regulação, até então desconhecidos. Por exemplo, o transplante xenogênico (ratos para camundongos) mostrou que o genótipo da própria célula germinativa é responsável pela determinação da duração da espermatogênese (França et al., 1998). Conforme é sabido na literatura, esta duração é rigidamente estabelecida para cada espécie (França & Russell, 1998). Assim, as células somáticas, particularmente a célula de Sertoli, não influenciam o ritmo de desenvolvimento das células germinativas. Esta importante descoberta serve como paradigma para outros sistemas de auto-renovação do corpo (Weglarz & Sandgren, 2000). Ainda ilustrando a adequacidade do transplante de células germinativas em estudos funcionais, a formação de espermatozóides férteis em camundongos normais, a partir do transplante de células germinativas de camundongos *knockout* para receptor de estrógeno do tipo α (Mahato et al., 2000) e receptores de andrógenos (Johnston et al., 2001), mostrou que estes receptores não são necessários para o desenvolvimento do processo espermatogênico. Em outra abordagem bastante interessante, foi demonstrado que o transplante de espermatogônias de camundongo doador infértil (Sl), devido à ausência do ligante para c-kit (Stem Cell Factor) produzido pelas células de Sertoli, resultou no desenvolvimento do

processo espermatogênico em camundongos receptores W/W (Ohta et al., 2000), cujas células germinativas não apresentam o receptor c-kit. Vários outros aspectos importantes para a função testicular têm sido investigados nos últimos anos (McLean, 2005; Dobrinski, 2006). Merece também ser citado que, através da utilização da técnica de transplante de espermatogônias tronco isoladas de camundongos com leucemia, a fertilidade foi restaurada sem induzir a leucemia, após os camundongos terem sofrido tratamento quimioterápico (Fujita et al., 2005).

1.4. Transplante de espermatogônias em peixes

Na mesma linha dos estudos desenvolvidos em mamíferos por Brinster e colaboradores, o transplante de espermatogônias foi também realizado com sucesso no Laboratório de Biologia Celular do ICB/UFMG em peixes teleósteos, a partir de estudos feitos por Lacerda e colaboradores (2006a; 2010). Essa metodologia, inédita para esse grupo de vertebrados, foi padronizada e desenvolvida utilizando-se a tilápia-nilótica como modelo experimental. A Figura 9 na próxima página ilustra as principais fases do transplante de espermatogônias em tilápias.

Um aspecto interessante dessa metodologia desenvolvida para tilápias foi a preparação dos receptores que tiveram sua espermatogênese endógena suprimida antes do transplante. Estudos anteriores demonstraram que em temperaturas elevadas (30-35ºC) a espermatogênese das tilápias é mais acelerada, apresentando provavelmente maior atividade mitótica das espermatogônias (Vilela et al., 2003; Lacerda et al., 2006a; 2010). Esta estratégia facilitou a depleção da espermatogênese em tilápias tratadas com duas injeções de busulfan (18mg e 15mg por kg de peso corporal) e mantidas à 35ºC (Lacerda et al., 2006a; 2010), cujos túbulos seminíferos apresentavam em sua quase totalidade somente células de Sertoli. Além do busulfan, existem potenciais abordagens que podem suprimir a espermatogênese endógena em peixes, como, por exemplo, a irradiação gama, que é capaz de promover apoptoses de espermatogônias em *Oryzias latipes*

(Kuwahara et al., 2002). No entanto, merece ser citado que a irradiação do animal *in toto* pode causar graves problemas ou comprometimento em outros órgãos cujas funções são vitais.

Outro modelo interessante para ser utilizado como receptor em teleósteos é o morpholino *dead end* (*dnd*) desenvolvido em zebrafish (*Danio rerio*) (Cyprinidae, Cypriniformes) (Slanchev et al., 2005). O gene *dnd* é responsável pela migração e sobrevivência das células germinativas primordiais durante a embriogênese do zebrafish. Todos os embriões morpholinos *dnd* desenvolvem-se como machos, que são, no entanto, desprovidos de células germinativas em seus testículos (Slanchev et al., 2005). Esse testículo assim formado permanece até os 90 dias pós-fertilização e depois é reabsorvido. Merece ser ressaltado que, em condições normais, em zebrafish todas as larvas inicialmente se diferenciam como fêmeas e, posteriormente, cerca de 70% delas se tornam machos (Uchida et al., 2002; Slanchev et al., 2005).

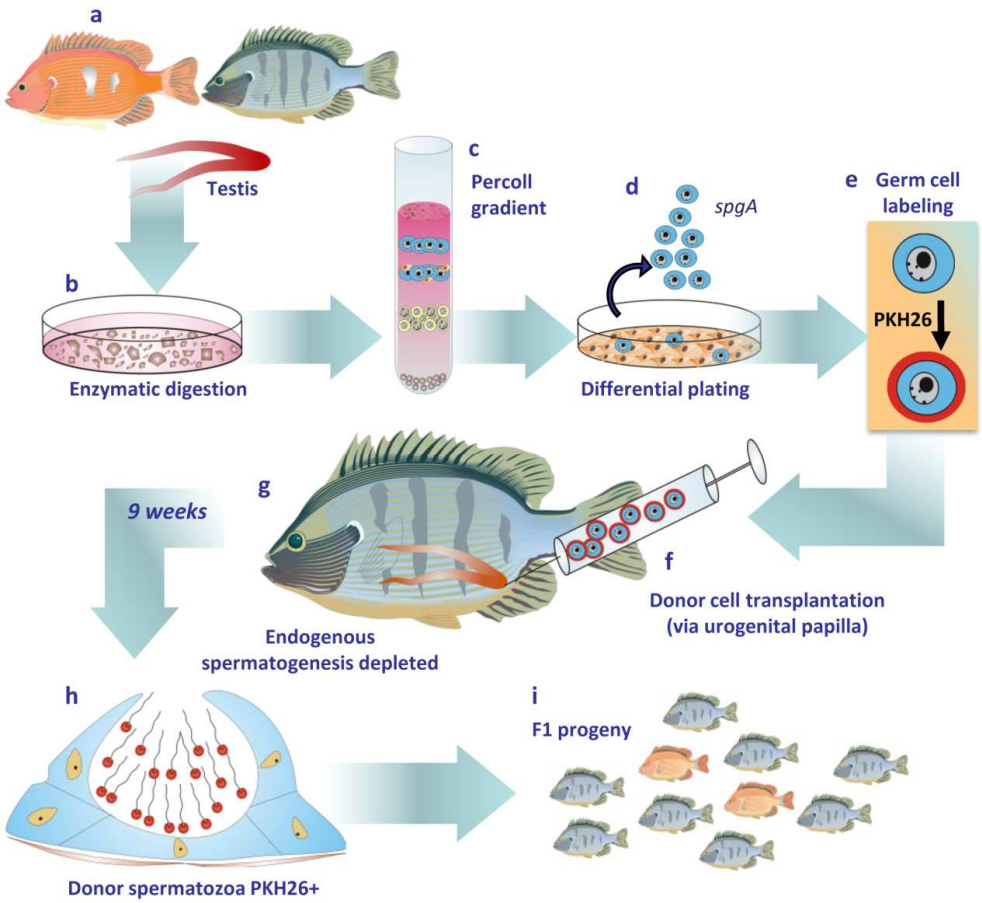


Figura 9. Diagrama ilustrativo das principais etapas do transplante de espermatogônias em tilápias-nilótica (*Oreochromis niloticus*), conforme estudos desenvolvidos por Lacerda e colaboradores (2006a, 2010). Resumidamente, as etapas do transplante na tilápia consistem na remoção dos testículos dos doadores (a), os quais são fragmentados e submetidos à digestão enzimática (b) para se obter a suspensão celular. Esta suspensão é centrifugada em gradiente de Percoll (c) com a finalidade de se obter um *pool* de espermatogônias (d). Após plaqueamento diferencial (para eliminar as células somáticas), as espermatogônias a serem transplantadas são coradas pelo marcador fluorescente PKH26 (vermelho) (e). A via de transplante é a papila urogenital (f) dos receptores que tiveram sua espermatogênese endógena suprimida com busulfan + temperatura de 35ºC (g). Cada tilápia recebeu 2.10^6 de espermatogônias. Para se avaliar a presença das células marcadas com PKH26, as tilápias transplantadas foram sacrificadas em diversos intervalos de tempo pós-transplante. Algumas semanas após o transplante, cistos espermatogênicos marcados foram observados, sendo possível encontrar células marcadas com características de espermatozóides (h), 9 semanas pós-transplante. Estes espermatozóides foram aptos a fertilizar oócitos e transmitir as características gênicas do doador para a progênie F1 (i). Retirado de Lacerda e colaboradores (2010).

Técnica envolvendo o transplante de células germinativas primordiais GFP (*green fluorescent protein*) na cavidade celomática de larvas de trutas arco-íris foi também desenvolvida recentemente (Takeuchi et al., 2003; Yoshizaki et al., 2011). Estas células, assim transplantadas, migram e colonizam as cristas genitais, podendo se diferenciar tanto em células germinativas masculinas quanto femininas, dependendo do sexo genético do indivíduo. Os machos adultos resultantes desse tipo de transplante são, portanto, capazes de produzirem espermatozóides com características genéticas do doador. Assim, ao fertilizarem oócitos de fêmeas normais, indivíduos com células germinativas primordiais GFP+ também são produzidos (Takeuchi et al., 2003; Yoshizaki et al., 2011).

Em continuação à esta linha de pesquisa acima mencionada, outros estudos demonstraram o sucesso do transplante xenogênico em outras espécies, como truta arco-íris e salmão masu (*Oncorhynchus masou*) (Salmonidae, Salmoniformes) (Takeuchi et al., 2004), e pearl danio (*Danio albolineatus*) (Cyprinidae, Cypriniformes) e zebrafish (*Danio rerio*) (Saito et al., 2008).

Em estudos mais recentes, conforme ilustra a Figura 10 na página seguinte, foi observada a produção de oócitos e espermatozóides a partir

de espermatogônias provenientes de trutas arco-íris adultas transplantadas na cavidade celomática de larvas da mesma espécie (Okutsu et al., 2006a) ou de espécies diferentes, como espermatogônias de yellow tail, *Seriola quinqueradiata* (Carangidae, Perciformes), em larvas de nibe croaker, *Nibea mitsukurii* (Sciaenidae, Perciformes) (Higuchi et al., 2011).

Finalmente, a produção de trutas viáveis a partir do transplante de células germinativas primordiais criopreservadas também foi obtida (Kobayashi et al., 2006; Okutsu et al., 2006b; Yoshizaki et al., 2011).

Mais recentemente ainda, foram produzidas trutas a partir do transplante de células germinativas primordiais na cavidade celomática de salmões triplóides que apresentam naturalmente poucas células germinativas endógenas em suas gônadas (Okutsu et al., 2007; Yoshizaki et al., 2011). Esta mesma metodologia também foi empregada por Wong e colaboradores (2011), que usaram larvas de zebrafish híbridos e inférteis do cruzamento entre pearl danio (*Danio albolineatus*) e zebrafish (*Danio rerio*).

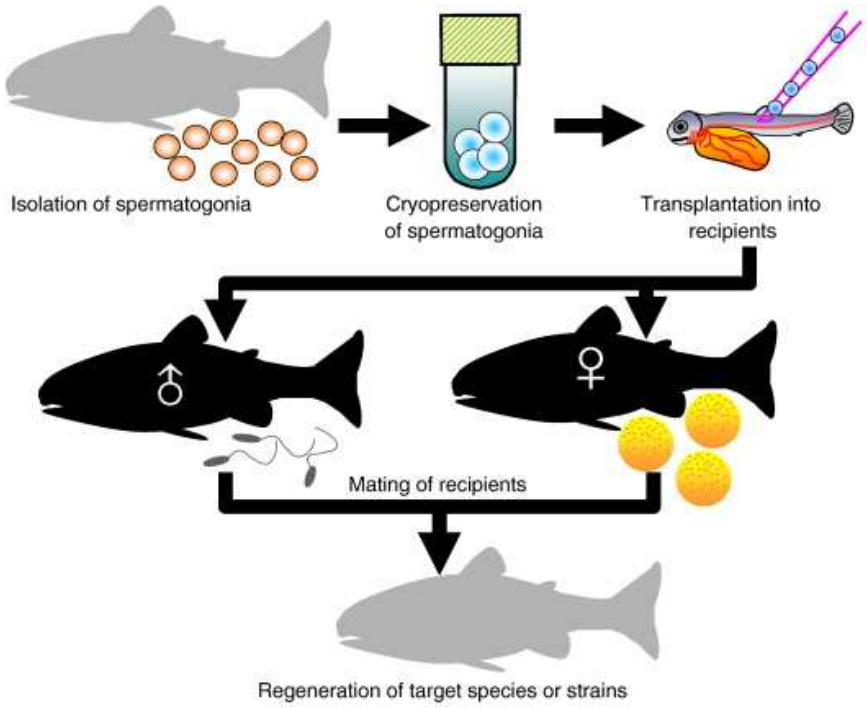


Figura 10. Diagrama ilustrativo dos principais passos do transplante de espermatogônias que foram obtidas de espécies ameaçadas de extinção ou de interesse econômico, criopreservadas e transplantadas na cavidade celomática de larvas da mesma espécie ou de espécies filogeneticamente relacionadas. As espermatogônias transplantadas são

capazes de colonizar o primórdio gonadal, e originar espermatozóides e oócitos, que resgatam o genótipo da espécie doadora. Retirado de Yoshizaki e colaboradores (2011).

Com base nos estudos mencionados acima pode ser inferido que o transplante de células germinativas em peixes, seja de espermatogônias ou de células germinativas primordiais, representa uma importante abordagem experimental para o estudo das espermatogônias tronco e da função testicular, além de propiciar um cenário bastante promissor nas áreas de biotecnologia e produção em aqüicultura, conforme ilustra a Figura 11 abaixo.

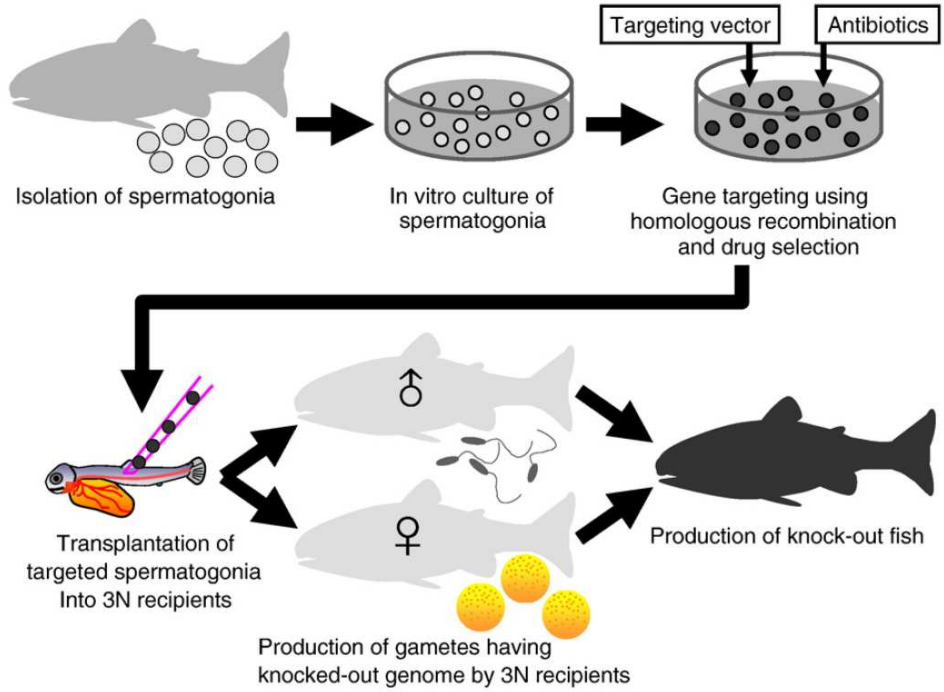


Figura 11. Uma das potenciais abordagens do transplante de espermatogônias tronco em peixes teleósteos é a formação de animais geneticamente modificados. O diagrama mostrado ilustra a manipulação gênica das espermatogônias tronco *in vitro*, e o seu posterior transplante na cavidade celomática de larvas triplóides. As espermatogônias transplantadas são capazes de colonizar o primórdio gonadal, e originar gametas que formarão indivíduos portando as características genéticas modificadas, no caso do esquema o knock-out de um determinado gene de interesse. Retirado de Yoshizaki e colaboradores (2011).

1.5. O zebrafish (*Danio rerio*) como modelo experimental

O *Danio rerio* (zebrafish), originário da Ásia (principalmente Índia), é também conhecido como paulistinha ou peixe-bandeira, devido às suas listras horizontais que lembram a bandeira do Estado de São Paulo.

Este pequeno teleósteo de água doce (~5cm de comprimento) pertence à família Cyprinidae e à ordem Cypriniformes. Em função de seu fácil manejo, poder ser mantido em alta densidade populacional, apresentar fertilização externa com geração de alto número de indivíduos, possuir embriões transparentes que permitem fácil visualização e monitoramento de seu desenvolvimento, o zebrafish tem se tornado excelente modelo experimental para pesquisas científicas experimentais e biomédicas em vertebrados. Este teleósteo possui vida média em torno de 3 anos, sua primeira maturação ocorre com 3 meses de vida e se reproduz na temperatura média de 27-28ºC, por cerca de 18 meses (McGonnell & Fowkes, 2006). A fêmea desta espécie pode desovar cerca de 100-200 ovos semanalmente (McGonnell & Fowkes, 2006).

O seqüenciamento recente do genoma do zebrafish, disponibilizado no site ZFIN, abriu enormes possibilidades para que estudos na área de genética sejam desenvolvidos neste teleósteo, propiciando inclusive a geração de excelentes modelos transgênicos na área de biologia da reprodução. Especificamente nesta área, o zebrafish já vem sendo utilizado como modelo experimental em estudos envolvendo a função testicular e sua regulação endócrina (McGonnell & Fowkes, 2006). No entanto, não existem ainda dados sólidos referentes à função testicular desta espécie, particularmente àqueles relacionados com o processo espermatogênico e sua regulação.

1.6. Justificativa e objetivos

Conforme já foi mencionado, o transplante de espermatogônias é uma excelente abordagem experimental para se investigar a biologia das espermatogônias tronco. Além disso, esta técnica tem proporcionado enormes avanços no estudo da espermatogênese em si, nas interações entre células de Sertoli e células germinativas, apresentando ainda potencial para pesquisas no campo da agropecuária, preservação de

espécies ameaçadas de extinção e de animais valiosos do ponto de vista zootécnico, medicina reprodutiva e produção de animais transgênicos, dentre outras. Em peixes teleósteos, esta técnica foi desenvolvida recentemente com sucesso no Laboratório de Biologia Celular/ICB-UFMG, utilizando-se a tilápia-nilótica como modelo experimental (Lacerda et al., 2006a, 2010). Desta forma, esta abordagem propiciou um cenário novo e bastante promissor nas áreas consideradas mais aplicadas como biotecnologia e produção em aqüicultura. Com base nessa logística bastante favorável e desenvolvida na UFMG, para estudos envolvendo o transplante de espermatogônias em peixes, e considerando-se o zebrafish como modelo experimental, nossos objetivos consistiram-se em caracterizar as gerações espermatogonias em zebrafish, assim como nicho espermatogonial, e for fim, padronizar as técnicas de transplante de espermatogônias tronco para este teleósteo. Parte desta padronização foi desenvolvida na Holanda (CNPq – doutorado sanduíche) sob supervisão e logística do Prof. Dr. Rüdiger W Schulz (University of Utrecht, Holanda).

Portanto, como objetivos principais, no **Capítulo 2** desta tese são apresentados os aspectos gerais da espermatogênese de zebrafish, assim como nova proposta de caracterização dos diversos tipos espermatogoniais de acordo com a morfologia nuclear, semelhantemente ao realizado em mamíferos. Já o **Capítulo 3** caracteriza, pela primeira vez, o nicho espermatogonial em zebrafish. A presença de espermatogônias tronco no testículo desta espécie pode ser demonstrada através de técnicas tais como: incorporação e retenção de bromodeoxiuridina (BrdU; 5-bromo-2'-deoxiuridina) por longo período de tempo (*Label Retaining Cell*); recuperação da espermatogênese após transplante em testículos depletados; ou diferenciação em oócitos após transplante em ovários. No **Capítulo 3** também é apresentada a padronização das técnicas de transplante de espermatogônias para zebrafish machos cuja espermatogênese foi depletada com busulfan, e fêmeas induzidas à desova. Finalmente, utilizando análises hormonais e gênicas dos testículos receptores antes do transplante, ainda neste capítulo é sugerido que os níveis elevados de andrógenos (11-KT), a expressão diminuída do *amh* (hormônio anti-Mülleriano) e a elevada do *igf1b* (*igf3*) (fator de crescimento semelhante à insulina 1b ou 3), criam

um nicho testicular que favorece a diferenciação espermatogonial, ao invés da auto-renovação. Esta condição de pró-diferenciação do ambiente receptor pode ter interferido no comportamento das espermatogônias transplantadas.

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2. Histological and Stereological Evaluation of Zebrafish (*Danio rerio*) Spermatogenesis with an Emphasis on Spermatogonial Generations

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Histological and Stereological Evaluation of Zebrafish (*Danio rerio*) Spermatogenesis with an Emphasis on Spermatogonial Generations[1]

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ABSTRACT

The zebrafish has become an important vertebrate model for basic and biomedical research, including the research field of the biology of reproduction. However, very few morphological and stereological data are available regarding zebrafish testis structure and spermatogenesis. In this careful histomorphometric evaluation of the testis, we studied spermatogonial cells using molecular markers, determined the combined duration of meiotic and spermiogenic phases, and examined the formation of the Sertoli cell barrier (tight junctions). We found at least nine spermatogonial generations and propose a morphology-based nomenclature for spermatogonial generations that is compatible with the one used in higher vertebrates. The number of germ cells per cyst increased dramatically (1 to ~1360 cells) from undifferentiated spermatogonia type A to early spermatids. The combined duration of meiotic and spermiogenic phases is approximately 6 days, one of the shorter periods among the teleost fish investigated to date. The number of Sertoli cells per cyst increased 9-fold during the maturational cycle of spermatogenic cysts and stabilized in the meiotic phase at a ratio of approximately 100 early spermatids per Sertoli cell (Sertoli cell efficiency). Similarly to mammals, Sertoli cell proliferation ceased in the meiotic phase, coinciding with the formation of tight junctions between Sertoli cells. Hence, the events taking place during puberty in the germinal epithelium of mammals seem to recapitulate the "life history" of each individual spermatogenic cyst in zebrafish.

Leydig cells, Sertoli cells, spermatogenesis, teleost fish, testis

INTRODUCTION

The zebrafish (*Danio rerio*; family Cyprinidae, order Cypriniformes) is a small freshwater teleost fish (~5 cm in length) originally from Central Asia [1]. Easy handling, high number of progeny, transparent embryos, and forward and reverse genetics approaches have contributed to the importance of this model species in basic and biomedical research; reproductive physiology [2], integrative physiology in general [3], and more applied research fields, such as ecotoxicology [4] or aquaculture [5], increasingly make use of the zebrafish model.

Spermatogenesis is a complex and highly coordinated process by which diploid spermatogonia produce millions of spermatozoa daily [6]. This process is fueled by spermatogonial stem cells, which have the potential for both self-renewal and for differentiating into spermatogonia committed to sperm development [7–9]. In fish, as in other anamniote vertebrates, spermatogenesis occurs in cysts that are formed when a single spermatogonium is completely surrounded by the cytoplasmic projections of one or two Sertoli cells [10]. As in all animals, the cells resulting from differentiating mitotic divisions of single spermatogonia remain interconnected by cytoplasmic bridges that synchronize developmental processes among the members of the same germ cell clone [11, 12]. Thus, in this cystic type of spermatogenesis, a given Sertoli cell is in contact with only one germ cell clone. This is the main difference in relation to noncystic spermatogenesis, as occurs in amniote vertebrates (reptiles, birds, and mammals), where several clones at different stages of development are distributed along basal, lateral, and adluminal surfaces of a Sertoli cell [6, 13, 14]. Apart from the cystic arrangement, the spermatogenic process in teleost fish is very similar to that of mammals [15].

With regard to the biology of male reproduction, zebrafish were studied to assess the effects of endocrine disruptors [2, 16] or the production of reproductive pheromones [17]. However, few quantitative data are available regarding the basic aspects of testis morphology and spermatogenesis in zebrafish. Histological and stereological investigations are adequate approaches to a better understanding of the spermatogenic process and testis function in fish [18, 19]. Such an evaluation allows the determination of the dynamics in the numbers of germ cells and Sertoli cells for each type of spermatogenic cyst, and it provides information on the number of spermatogonial generations, on the magnitude of germ cell loss occurring during spermatogenesis, and on Sertoli cell efficiency.

Our long-term aim is to further our understanding of the regulation of spermatogenesis. We hypothesize that in the cystic mode of spermatogenesis, Sertoli cell functions related to supporting a given stage of spermatogenesis can be studied undisturbed by germ cells in other stages of development, in contrast to noncystic spermatogenesis, where several germ cell clones are in contact with a given Sertoli cell. A basis required for such studies, as well as for other fields, like reproductive toxicology, is a thorough description of zebrafish spermatogenesis, including quantitative data that allow a better

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understanding of spermatogenesis. Such data have not yet been published, and the main objectives of the present study were to perform a comprehensive stereological study of the different types of spermatogenic cysts (the number of germ cells and Sertoli cells per spermatogonial, spermatocyte, and spermatid cyst). Although the different stages of meiosis and spermiogenesis are relatively easy to differentiate, the successive spermatogonial generations are not. Therefore, we devoted particular attention to the mitotic phase of spermatogenesis, propose a nomenclature for the different spermatogonial generations, and have started with a molecular characterization of the spermatogonia. Based on these data, we are able to estimate the number of spermatogonial generations, the magnitude of germ cell loss, and Sertoli cell number and efficiency. We also determined the duration of the combined meiotic and spermiogenic phases at 27°C through the incorporation of 5′bromo-2′-deoxyuridine (BrdU). Finally, the permeability of the Sertoli cell barrier was assessed during the spermatogenic phases by using an electron-dense tracer, lanthanum nitrate.

MATERIALS AND METHODS

Animals, Sampling, Biometry, and Testis Structure

Thirty sexually mature zebrafish—outbred animals and Tübingen AB strain (TABs)—were used for histology and stereology (TABs), analysis of the duration of spermatogenesis (TABs), and Sertoli cell barrier analysis (outbred). The animals were anesthetized and weighed, and the testis was dissected out, weighed, and fixed by immersion (see below). The gonadosomatic index (GSI) was obtained by the formula GSI = (testes weight/body weight) × 100. All procedures used followed approved guidelines for the ethical treatment of animals and national laws. Experimental protocols were submitted to, and approved by, the Utrecht University and Federal University of Minas Gerais (CETEA) committees for animal experimentation and care.

Testis Stereology

For histological and stereological analysis, testes were fixed in 4% buffered (PBS) glutaraldehyde at 4°C overnight. The tissue was dehydrated and embedded in 2-hydroxyethyl methacrylate. In a pilot study, we determined the nuclear diameter of the different germ cell types to determine the section thickness that avoids counting the same nucleus twice, using the procedure described previously [20]. According to these results, testes were serially sectioned at 2 and 3 μm thickness; histological sections were stained with 1% toluidine blue.

The total number of germ cells and Sertoli cells per spermatogonial, spermatocyte, and spermatid cyst was counted on serial sections using a total of 13 zebrafish. This evaluation was performed after selecting cysts entirely encompassed by serial sections. At least five cysts were analyzed for each germ cell type in each fish. The Sertoli cell efficiency was estimated from the ratio of germ cells:Sertoli cells for each cyst type.

For a high-resolution light microscopy analysis of the spermatogonial compartment and to differentiate morphologically the spermatogonial generations, small fragments of zebrafish testes (n = 5) were fixed by immersion in 5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2–7.4) for at least 24 h. After fixation, the material was rinsed three times in the same buffer, postfixed in osmium-ferrocyanide, dehydrated, embedded in araldite, sectioned for light microscopy (1 μm thick), and stained with toluidine blue.

The individual Leydig and germ cell volumes (μm^3) were obtained from the nuclear volume and the proportion (%) between nucleus and cytoplasm of each cell type evaluated, which were determined as described previously [20].

Germ cell loss (apoptosis) was analyzed qualitatively and quantitatively and was expressed as a percentage of apoptotic cells per cyst. These apoptotic cells were morphologically characterized as darker-stained cells, as is typical in this condition for cells embedded in plastic and stained with toluidine blue [19].

Immunocytochemical and In Situ Hybridization Experiments

Testis tissue from adult males (outbred or TABs) was fixed in 4% paraformaldehyde in PBS overnight at 4°C. For immunocytochemistry, the tissue was rinsed briefly in PBS before being immersed in PBS containing 20% (w/v) sucrose for 1 day at 4°C. Tissue was then frozen in Neg50 (Richard-Allan Scientific, Kalamazoo, MI). Cryosections (10 μm thick) were used to detect Vasa [21] or Piwil1 (also known as Ziwi) [22] protein using previously described antisera raised in rabbit.

Rabbit anti-Vasa was used at a dilution of 1:200 and incubated overnight at room temperature before incubation with a goat anti-rabbit antiserum labeled with fluorescein isothiocyanate (FITC; 1:200; A11008; Invitrogen, Carlsbad, CA) for 90 min at room temperature. The buffer also contained 1 μg/ml propidium iodide to label DNA. The sections were analyzed with a confocal laser scanning microscope using 488 and 536 nm as excitation wavelengths for FITC and propidium iodide, respectively. Detection of Piwil1 protein and staining of DNA by 4′,6′-diamidino-2-phenylindole were done as described by Houwing et al. [22].

For in situ hybridization, paraformaldehyde-fixed tissue was dehydrated and embedded in paraffin according to conventional techniques. Sections of 5 μm thickness were used for the detection of mRNA encoding proliferating cell nuclear antigen (pcna), a protein involved in DNA synthesis and repair [23] and shown to be predominantly expressed in rapidly proliferating spermatogonia in Japanese eel [24]. The zebrafish *pcna* mRNA sequence (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=18859222) was used to design specific primers. A zebrafish *pcna* PCR product (634 bp) was generated using primers 1039 and 1040 (Supplemental Table S1 available online at www.biolreprod.org) and zebrafish testis cDNA as template, and it was gel purified and served as template for digoxigenin (DIG)-labeled cRNA probe synthesis by in vitro transcription. For cRNA synthesis, 300 ng of PCR product was incubated at 37°C for 2.5 h in a 20-μl reaction volume containing 4 μl of 5× T3/T7 RNA buffer (Invitrogen), 2 μl of 0.1 M dithiothreitol, 1 μl (29.7 U/μl) of RNAguard RNase inhibitor (GE Healthcare, Fairfield, CT), 2 μl of 10× DIG RNA labeling mix (Roche, Mannheim, Germany), and either 2 μl (50 U/μl) of T3 RNA polymerase (for sense probe; Epicentre, Madison, WI) or 2 μl (50 U/μl) of T7 RNA polymerase (for antisense probe; Epicentre).

Duration of Spermatogenesis (Meiotic and Spermiogenic Phases)

To estimate the combined duration of meiotic and spermiogenic phases at 27°C, 16 animals (TABs) were exposed to BrdU dissolved in water (3 mg/ml) for approximately 15 h. The animals (n = 2 per time point investigated) were sampled at 1 h and at 1, 2, 3, 4, 5, 6, and 7 days after the exposure was terminated.

BrdU incorporation was detected following a protocol modified from Van de Kant and de Rooij [25]. Zebrafish testes were fixed for 5 h at room temperature in freshly prepared methacarn (60% [v/v] absolute ethanol, 30% chloroform, and 10% glacial acetic acid) and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Five-micrometer-thick sections were subjected to antigen retrieval (1% [v/v] periodic acid in water at 60°C for 30 min) and peroxidase blocking (1% [v/v] H_2O_2 in PBS for 10 min). Thereafter, slides were incubated at room temperature for 1 h with mouse anti-BrdU (1:80 [BD Biosciences, San Jose, CA] diluted in PBS containing 1% [w/v] bovine serum albumin [BSA; Sigma-Aldrich, St. Louis, MO]), and then for an additional hour with biotinylated horse anti-mouse (1:100 [Vector Laboratories, Burlingame, CA] diluted in PBS containing 1% [w/v] BSA). Revelation of immunostaining was done using avidin-biotin complex incubation for 1 h (Vector Laboratories) followed by diaminobenzidine (Dako, Glostrup, Denmark) substrate development for 20 sec. Nuclei were counterstained with hematoxylin Gill no. 3 (Sigma-Aldrich) for 30 sec. For a negative control, the primary antibody (mouse anti-BrdU) was replaced with the same concentration of normal mouse immunoglobulin G (BD Biosciences).

Sertoli Cell Barrier

To investigate the presence and timing of the formation of the Sertoli cell barrier (tight junctions between Sertoli cells) during spermatogenesis, lanthanum nitrate ($La(NO_3)_3$) was prepared by slowly adding drops of 0.01 N NaOH to 2% $La(NO_3)_3$ (Vetec, Rio de Janeiro, Brazil) until the solution reached pH 7.8. This opalescent solution was mixed with an equal volume of cacodylate-buffered glutaraldehyde to make a fixative containing 1% lanthanum and 2% glutaraldehyde in 0.1 M cacodylate at pH 7.8 or 7.3.

The testes from outbred animals were prefixed for 2 min in the fixative containing lanthanum, and then small pieces (2–3 mm^3) were fixed by immersion in the same fixative for 5 h at room temperature. The material was rinsed overnight in the same lanthanum solution without glutaraldehyde at 4°C (pH 7.8 or 7.3 according to the fixative pH). The samples were postfixed for 8 h in 1% OsO_4 in 0.1 M cacodylate buffer containing 1% lanthanum at pH 7.8 or 7.3 (according to the fixative pH). After overnight staining in 0.5% aqueous uranyl acetate, the material was dehydrated in alcohol, embedded in araldite, and documented using a JEOL 100 CX-II transmission electron microscope (80 kV).

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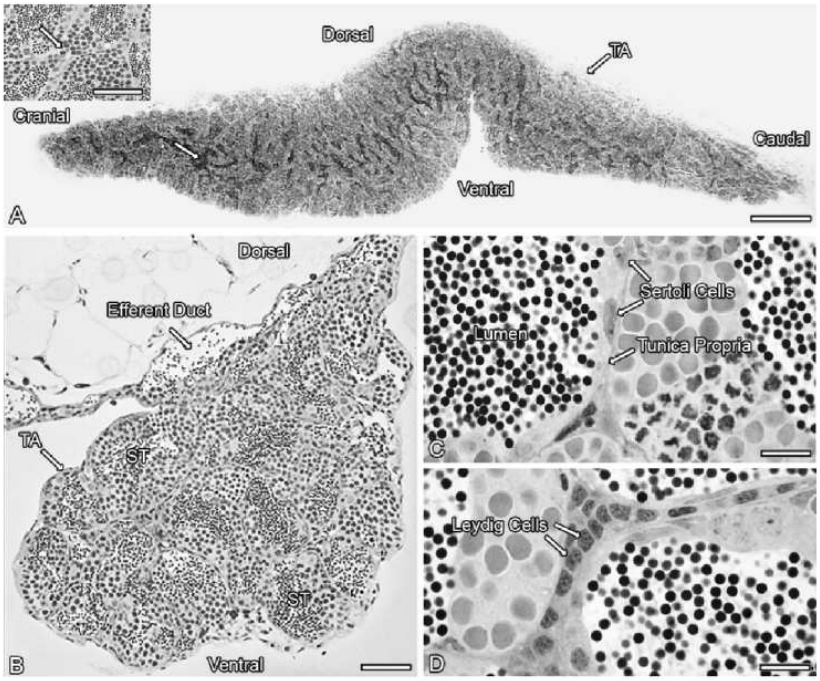

FIG. 1. **A**) Longitudinal section of a zebrafish testis showing the dorsal-ventral and caudal-cranial axis. The tunica albuginea (TA) externally surrounds the testis. The seminiferous tubules sometimes show an anastomosing pattern (arrow in **A** and the inset). **B**) Cross-section of zebrafish testis showing the tunica albuginea (TA), the efferent duct, and seminiferous tubules (ST). **C**) Tubular compartment of the testis showing tunica propria surrounding the seminiferous tubule, Sertoli cells often in the basal region of spermatogenic cysts, and lumen containing spermatozoa. **D**) Intertubular compartment showing a cluster of Leydig cells. Bars = 400 μm (**A**), 50 μm (inset and **B**), and 10 μm (**C** and **D**).

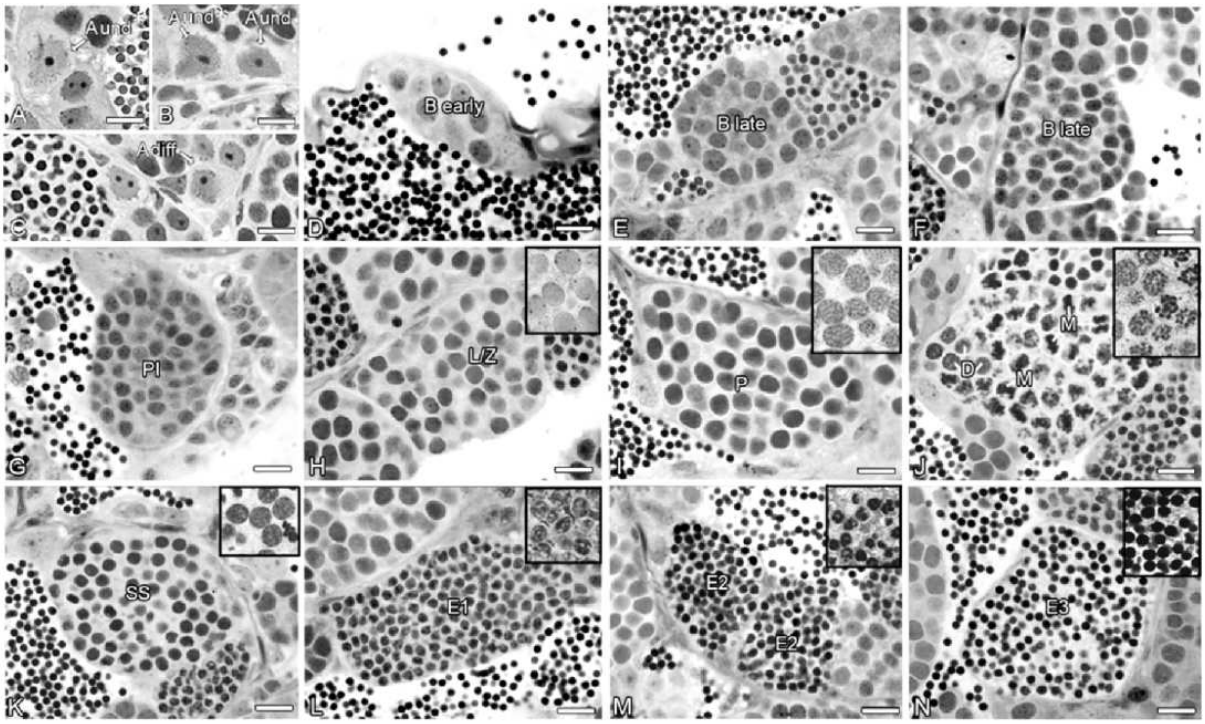

FIG. 2. Different germ cell cysts observed during the spermatogenic process in zebrafish from a type A undifferentiated* spermatogonium to spermatids before spermiation. **A**) The most undifferentiated type A spermatogonium (A_{und}*; probable stem cell; refer to Fig. 3 for a more detailed account on spermatogonia). **B**) Type A undifferentiated spermatogonium (A_{und}*) and type A undifferentiated spermatogonium (A_{und}; possibly limited stem cell capacity). **C**) Type A differentiated spermatogonia (A_{diff}). **D**) Early type B spermatogonia (B_{early}). **E** and **F**) Late type B spermatogonia (B_{late}). **G**–**J**) Primary spermatocytes in preleptotene (Pl), in the transition of leptotene to zygotene (L/Z), pachytene (P), diplotene (D), and meiotic figures from the first meiotic division (M). **K**) Secondary spermatocytes (SS). **L**–**N**) Spermatids at initial (E1), intermediate (E2), and final (E3) steps of development. Bars = 10 μm (or 5 μm for the insets in **H**–**N**).

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TABLE 1. Stereology of the different germ cell cysts in zebrafish (n = 8; mean ± SEM).

Cyst type*	No. of Sertoli cells per cyst	No. of germ cells per cyst†	Germ cells: Nuclear diameter (μm)	Nuclear volume (μm³)‡	Cytoplasm volume (μm³)§	Cell volume (μm³)	Cyst volume (μm³)¶	No. of germ cells (cyst) per Sertoli cell
SPG_1–A_{und}	1.2 ± 0.1[a]	1.0 ± 0[a]	8.6 ± 0.1[a]	336 ± 16 (0.50)[a]	342 ± 18[a] (0.50)	677 ± 34[a]	677 ± 34[a]	0.8 ± 0.1[a]
SPG_2–$A_{diff}1$	2.1 ± 0.1[b]	2.0 ± 0[b]	6.6 ± 0.1[b]	150 ± 4 (0.51)[b]	147 ± 6[b] (0.49)	297 ± 9[b]	593 ± 18[a]	1.0 ± 0.1[a]
SPG_3–$A_{diff}2$	2.7 ± 0.1[b]	3.7 ± 0[c] [4]	6.0 ± 0.1[c]	112 ± 1 (0.52)[c]	105 ± 3[c] (0.48)	216 ± 4[c]	790 ± 14[a]	1.4 ± 0.1[a]
SPG_4–$A_{diff}3$	3.9 ± 0.2[c]	7.2 ± 0.1[c] [8]	5.7 ± 0.1[d]	99 ± 1 (0.55)[c]	81 ± 3[c,d] (0.45)	180 ± 3[c,d]	1318 ± 24[a]	1.8 ± 0.1[a]
SPG_5–B1	5.1 ± 0.2[d]	14 ± 0.3[c] [16]	5.6 ± 0.1[d]	92 ± 1 (0.58)[c,d]	66 ± 4[d,e] (0.42)	158 ± 5[d,e]	2159 ± 69[a]	2.7 ± 0.1[a]
SPG_6–B2	5.4 ± 0.1[d]	28 ± 1[c] [32]	5.3 ± 0.1[e]	76 ± 1 (0.60)[d,e]	51 ± 2[e,f] (0.40)	128 ± 2[e,f]	3624 ± 90[a]	5.3 ± 0.1[a,b]
SPG_7–B3	6.2 ± 0.1[e]	55 ± 1[c,d] [64]	5.1 ± 0.1[e,f]	71 ± 0.5 (0.61)[d,f]	45 ± 1[e,g] (0.39)	116 ± 2[f,g]	6473 ± 190[a,b]	9 ± 0.3[b,c]
SPG_8–B4	9.3 ± 0.2[f]	113 ± 3[d] [128]	5.0 ± 0.1[f]	64 ± 1 (0.61)[e,f,g]	41 ± 2[e,h] (0.39)	105 ± 2[f,g]	11 949 ± 429[b,c]	12 ± 0.2[c]
SPG_9–B5	11.2 ± 0.4[g]	208 ± 8[e] [256]	4.7 ± 0.1[g]	53 ± 1 (0.64)[f,h]	29 ± 1[f,g,h,i] (0.36)	82 ± 1[f,g,h]	16 945 ± 755[c,d]	19 ± 1.1[d]
Pl	11.9 ± 0.2[h]	423 ± 9[f] [512]	4.4 ± 0.1[h]	45 ± 1 (0.61)[g,h,i]	29 ± 2[f,g,h,j] (0.39)	74 ± 3[g,h,i]	31 325 ± 1283[e]	36 ± 0.8[e]
L/Z	12.0 ± 0.3[g,h]	395 ± 12[f]	5.1 ± 0.1[e,f]	70 ± 1 (0.59)[e,f,h]	48 ± 3[e,i,j] (0.41)	117 ± 4[e,f,g,h,i]	46 087 ± 1163[f]	33 ± 0.6[e,f]
P	12.8 ± 0.2[i]	377 ± 19[f]	6.0 ± 0.1[c]	112 ± 2 (0.60)[c,d]	76 ± 1[d,e] (0.40)	188 ± 2[c,e]	71 585 ± 3602[g]	30 ± 1.5[f]
D	13.0 ± 0.4[i,j]	397 ± 8[f]	5.2 ± 0.1[e,f]	74 ± 2 (0.61)[d,g,h]	48 ± 3[e,i,j] (0.39)	122 ± 4[e,f,g,h]	48 608 ± 1539[f]	31 ± 1.2[e,f]
S	13.8 ± 0.4[j,k,l]	768 ± 26[g] [1024]	3.9 ± 0.1[i]	31 ± 1 (0.59)[i,j]	21 ± 1[g,h,i] (0.41)	52 ± 2[h,i,j]	38 808 ± 1362[h]	56 ± 1.7[g]
E1	14.3 ± 0.4[k]	1353 ± 45[h] [2048]	3.0 ± 0.1[j]	14 ± 0.2 (0.60)[j,k]	9 ± 1[i,j] (0.40)#	22 ± 1[j]	29 465 ± 1183[e]	94 ± 2.34[h]
E2	14.1 ± 0.3[k]	1299 ± 23[h,i]	2.5 ± 0.1[k]	8 ± 0.3 (0.61)[k]	5 ± 0.3[i,j] (0.39)	14 ± 1[j]	17 616 ± 773[c,d]	92 ± 2.3[h]
E3	13.1 ± 0.2[i,l]	1262 ± 13[i]	2.1 ± 0.1[l]	5 ± 0.1 (0.60)[k]	3 ± 0.1[i,j] (0.40)	8 ± 0.1[j]	10 771 ± 185[b,c]	96 ± 1.6[h]

* SPG_1–A_{und}, primary spermatogonia; SPG_2–$A_{diff}1$, SPG_3–$A_{diff}2$, SPG_4–$A_{diff}3$, different generations of type A differentiated spermatogonia; SPG_5–B1, SPG_6–B2, different generations of type B early spermatogonia; SPG_7–B3, SPG_8–B4, SPG_9–B5, different generations of type B late spermatogonia; Pl, preleptotene primary spermatocytes; L/Z, leptotene/zygotene primary spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; S, secondary spermatocytes; E1, initial spermatids; E2, intermediate spermatids; E3, final (mature) spermatids.

† Values in brackets indicate the theoretical number of germ cells formed after mitotic and meiotic divisions.

‡ Values in parentheses are the percentage of nucleus in the cell.

§ Values in parentheses are the percentage of cytoplasm in the cell.

¶ Sertoli cells not included.

Flagellum included.

[a-l] Different superscript letters indicate that mean values differ significantly ($P < 0.05$).

Statistical Analysis

All quantitative data are presented as the mean ± SEM and analyzed via ANOVA (Student-Newman-Keuls test). The analysis was performed using the software STATISTICA 3.11 for Windows (StatSoft Inc., Tulsa, OK). The significance level in comparisons was considered to be $P < 0.05$.

RESULTS

Biometry and Testis Structure

The mean testis weight of zebrafish was 4.7 ± 0.2 mg, resulting in a GSI of 1.0% ± 0.05%. The testes are paired and elongated organs that stretch dorsally through the length of the body cavity and are connected to the dorsal body wall via the mesorchium. The testes join caudally to form a single spermatic duct that terminates on the urogenital papilla. The anastomosing seminiferous tubules (Fig. 1A and inset, arrows) empty dorsally into the main testicular efferent duct (Fig. 1B) that continues caudally as a single spermatic duct. Different types of germ cell cysts (from spermatogonia to spermatids) are distributed along the seminiferous epithelium (Fig. 2 and Supplemental Fig. S1).

Number of Germ Cells and Sertoli Cells per Cyst

All stereological data are presented in Table 1. The total number of Sertoli cells per cyst increased gradually and significantly from type A undifferentiated spermatogonium to pachytene spermatocyte cysts, and it shows a strong trend toward stabilization from diplotene spermatocytes to mature spermatids (Table 1). The number of germ cells per cyst increased geometrically, as expected, from a single type A undifferentiated spermatogonium to early spermatids, showing a reduction of 7% of the expected theoretical number from intermediate to final spermatid cysts (Table 1).

The data obtained for the number of spermatogonia and early spermatocytes per cyst allowed us to estimate that nine generations of spermatogonia are present in zebrafish—one generation of type A undifferentiated spermatogonia (see below for morphological details on two forms of type A undifferentiated spermatogonia that are both found as single germ cells in early cysts), three generations of type A differentiating spermatogonia, and five generations of type B (early: B1–B3; late: B4 and B5) spermatogonia. Because the nuclear morphology among the different generations of type B spermatogonia was very similar, the criteria used to distinguish each generation of this cell type were the decreasing cell size and the increasing number of spermatogonia per cyst. Regarding the Sertoli cell efficiency for spermatids, approximately 100 spermatids were found per Sertoli cell. Considering the theoretical number expected and the number obtained, we can estimate that the total germ cell loss during spermatogenesis was ~38%. Using the deviation from theoretical number of germ cells for each generation, germ cell loss (by apoptosis) occurs in two steps—in the final spermatogonial generation and during the first meiotic prophase (B5 to diplotene), and then during the second meiotic division (secondary spermatocytes to early spermatids; Table 1). Although only ~62% of the germ cells complete spermatogenesis in zebrafish, apoptotic germ cells are rarely detected in adult zebrafish.

Germ Cell and Leydig Cell Stereology and Cyst Volume

Although the total number of germ cells per cyst increased during spermatogenesis, the opposite was observed for the nuclear diameter and for the nuclear, cytoplasmic, and cellular volumes (Table 1). The trend was broken by a gradual but transient increase of these parameters in primary spermatocytes (from preleptotene to pachytene spermatocytes). Based on the

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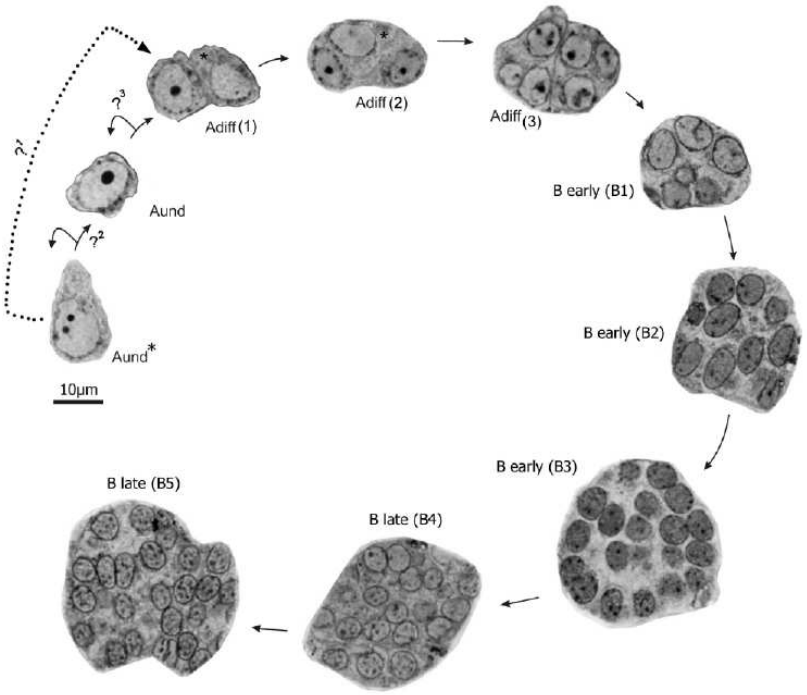

FIG. 3. Nine spermatogonial generations in zebrafish. The terminology addressing the different spermatogonial generations was based on morphological features, such as nuclear shape, heterochromatin amount, nucleolar characteristics, and number of germ cells in a cyst. Using high-resolution light microscopy applied to 1-μm-thick, araldite-embedded sections, we propose to differentiate five types of spermatogonia: type A undifferentiated* (A_{und}*; irregular outline of nuclear envelope and high ratio between cytoplasm and nucleus); type A undifferentiated (A_{und}; smooth outline of nuclear envelope and low ratio between cytoplasm and nucleus); type A differentiated (A_{diff} 1, 2, and 3), type B early (B1–B3), and type B late (B4 and B5). The first question mark ($?^1$) indicates a doubt regarding a possible asymmetric division of A_{und}* (dotted line leading to a pair of spermatogonia). The second question mark ($?^2$) indicates the doubt as to whether or not type A_{und}* is separated from type A_{und} by a mitosis. The third question mark ($?^3$) indicates uncertainty regarding the "stemness" of A_{und}. The asterisk inside the cells indicates spermatogonia showing the cytological charcateristics of type A_{und}* cells in small cysts with otherwise type A_{diff} spermatogonia, an observation possibly indicating asymmetrical division of A_{und}* (dotted line).

total number of germ cells per cyst and the individual volume of each germ cell type, we determined the total cyst volume, which showed a strong and steady increase from type A undifferentiated spermatogonium to pachytene spermatocytes, decreasing from this maximum to 85% in late spermatid cysts (Table 1).

The Leydig cell nuclear, cytoplasmic, and cell volumes were 53 ± 0.9 μm^3, 52 ± 0.7 μm^3, and 105 ± 1.6 μm^3, respectively. Leydig cells are not randomly distributed in the interstitial space but often form clusters, sometimes in the form of ringlike structures around blood vessels (Supplemental Fig. S2). Although not properly quantified, these clusters appeared to be larger in the periphery of the testis.

Spermatogonia Morphology, Spermatocytes, and Spermiogenesis

Two types of type A undifferentiated spermatogonia can be differentiated in zebrafish testes based on morphological criteria. They are referred to as type A undifferentiated* (A_{und}*) and type A undifferentiated (A_{und}; Fig. 3).

Both cell types differ from all later stages in that they are completely enveloped as a single cell by Sertoli cells and are the largest germ cells (~677 μm^3), with a large nucleus (diameter, 8.6 μm) containing poorly condensed chromatin and one or two compact nucleoli. Specific for type A_{und}* spermatogonia is that their nuclear envelope has an irregular outline that is not found in type A_{und} spermatogonia, and that the ratio between cytoplasm and nucleus is higher in type A_{und}* than in all other spermatogonia, including type A_{und} (Fig. 3).

The type A differentiated (A_{diff}) spermatogonia are very similar to the undifferentiated type. However, because these cells result from mitoses with incomplete cytokinesis, they are grouped (two, four, or eight) within the cyst and are smaller than the type A_{und} spermatogonia. The nucleus of type A_{diff} spermatogonia is denser and smaller, the nuclear envelope acquires a regular round shape, and two or three small nucleoli are found that assume eccentric positions (Fig. 3 and Supplemental Fig. S1). Moreover, heterochromatin starts to appear as flecks along the nuclear envelope (Fig. 3). When performing analysis by high-resolution light microscopy, we have sometimes observed small cysts of type A spermatogonia in which one of the germ cells showed morphological characteristics of type A_{und}* spermatogonia, and the other cells showed the characteristics of type A_{diff} spermatogonia (Fig. 3, asterisk in cysts with $A_{diff}1$ and $A_{diff}2$).

Type B early spermatogonia have an elongated/round nucleus with one or two small nucleoli. A major difference from type A spermatogonia is the increased amount of heterochromatin, which is distributed as round clumps throughout the nucleus or associated with the nuclear envelope (Fig. 3 and Supplemental Fig. S1).

In type B late spermatogonia, the number of germ cells inside the cyst increases (Table 1). The nucleus is round, smaller than in the early type B spermatogonia, and dense, and the heterochromatin (round clumps and associated with the nuclear envelope) reaches maximum density (Fig. 3 and Supplemental Fig. S1).

Spermatocytes in the different phases of meiosis can be identified by nuclear characteristics, such as size, chromosome condensation, and meiotic figures of the chromosomes (e.g., metaphase I or II; Fig. 2, G–K and Table 1). Leptotene/zygotene spermatocytes have a larger, rounder nucleus compared with the last generation of type B spermatogonia, showing a clear chromatin with small spots of heterochromatin bordering the nuclear envelope (Fig. 2H). Pachytene spermatocytes are the largest cell type among the spermatocytes. Their nucleus is denser and contains chromosomes as bold

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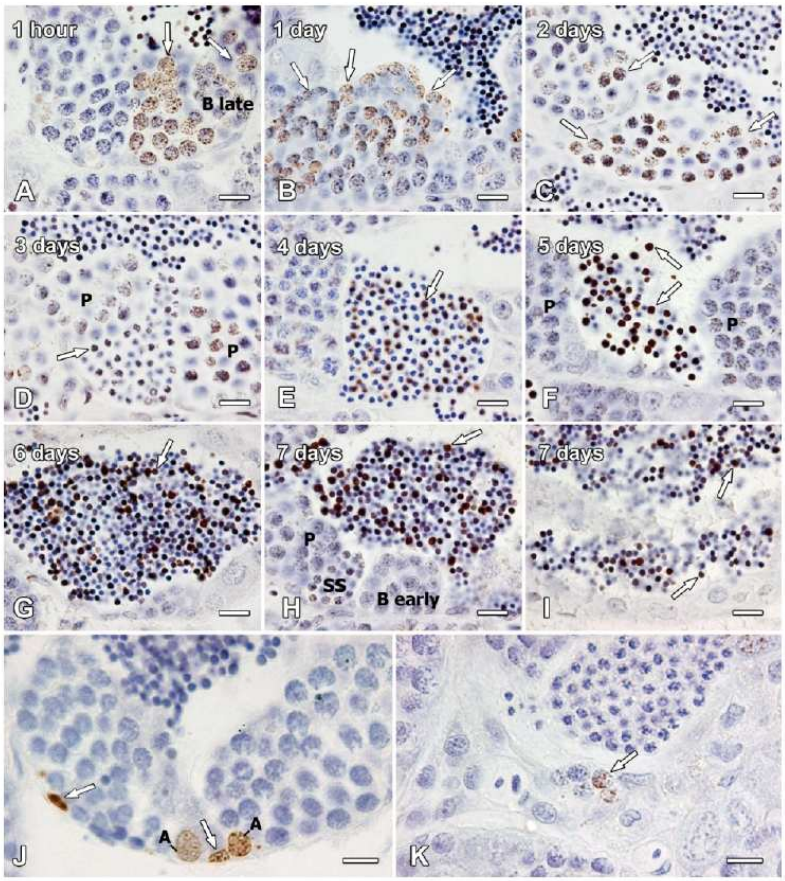

FIG. 4. Proliferating (BrdU-positive) somatic and germ cells in adult zebrafish testis. The following labeled cells were observed at different time periods after BrdU exposure: (**A**–**C**) at 1 h, and at 1 and 2 days, primary spermatocytes (pachytene, arrows); (**D** and **E**) at 3 and 4 days, spermatids (arrows); (**F**) at 5 days, spermatozoa (arrows) during spermiation; (**G** and **H**) at 6 and 7 days, spermatozoa (arrows) in the lumen of the seminiferous tubules; (**I**) at 7 days, spermatozoa (arrows) in the lumen of the efferent duct; (**J**) at 1 h, Sertoli (arrows); and (**K**) Leydig cell (arrow). A, type A spermatogonium; B early, early type B spermatogonia; B late, late type B spermatogonia; P, pachytene primary spermatocytes; SS, secondary spermatocytes. Bars = 10 μm (**A**–**I**) or 20 μm (**J** and **K**).

lines from the periphery to the central part of the nucleus (Fig. 2I). Diplotene spermatocytes are always found together with metaphasic figures (metaphase I; Fig. 2J). In this cell type, the chromosomes reach their maximum degree of condensation. Secondary spermatocytes are rare because they quickly enter into meiosis II. They have a round nucleus with a dense chromatin (Fig. 2K).

Spermiogenesis in zebrafish is marked by a striking reduction in the cellular volume (Table 1). We propose to differentiate three types of spermatids in zebrafish spermiogenesis: early (Fig. 2L), intermediate (Fig. 2M), and final spermatids (Fig. 2N). This classification is based on the increasing nuclear compaction and space between the spermatids (reflecting the loss of cytoplasmic bridges and the flagellum formation; Fig. 2, L–N).

Immunocytochemical and In Situ Hybridization Experiments

Our studies on molecular markers showed that antibodies against zebrafish Vasa and Piwil1 intensely labeled single type A undifferentiated spermatogonia and, in the case of Piwil1, also small cysts with type A differentiated spermatogonia (Supplemental Fig. S3, A and B). Vasa protein was also present at intermediate levels in differentiating spermatogonia and at low levels in spermatocytes, whereas spermatids and spermatozoa remained unlabeled. In the case of Piwil1, the labeling became very weak in late type B spermatogonia and spermatocytes, and it was not detected in haploid germ cells. The in situ hybridization for *pcna* mRNA, on the other hand, provided an intense labeling of more differentiated spermatogonia (Supplemental Fig. S3, C and D).

Duration of Spermatogenesis (Meiotic and Spermiogenic Phases)

The most advanced germ cells that carried a BrdU label after different periods following BrdU exposure at 27°C are shown in Figure 4. One hour after the termination of exposure (i.e., ~16 h after the start of contact with BrdU), the most advanced germ cells labeled were identified as early pachytene spermatocytes (Fig. 4A). At 1, 2, and 3 days after BrdU exposure, the most advanced cells labeled were late pachytene spermatocytes (Fig. 4, B–D), implying that it took less than 1 day to form pachytene from preleptotene spermatocytes. Four days after exposure, labeled spermatids were identified, whereas after 5 days we noticed the first labeled spermatozoa in the tubular lumen (Fig. 4, E and F). After 6 and 7 days of exposure, we regularly found labeled spermatozoa in the lumen of the seminiferous tubules (Fig. 4, G and H). Also, after 7 days, spermatozoa were found in the lumen of the efferent duct system (Fig. 4I). Based on these observations, we can estimate

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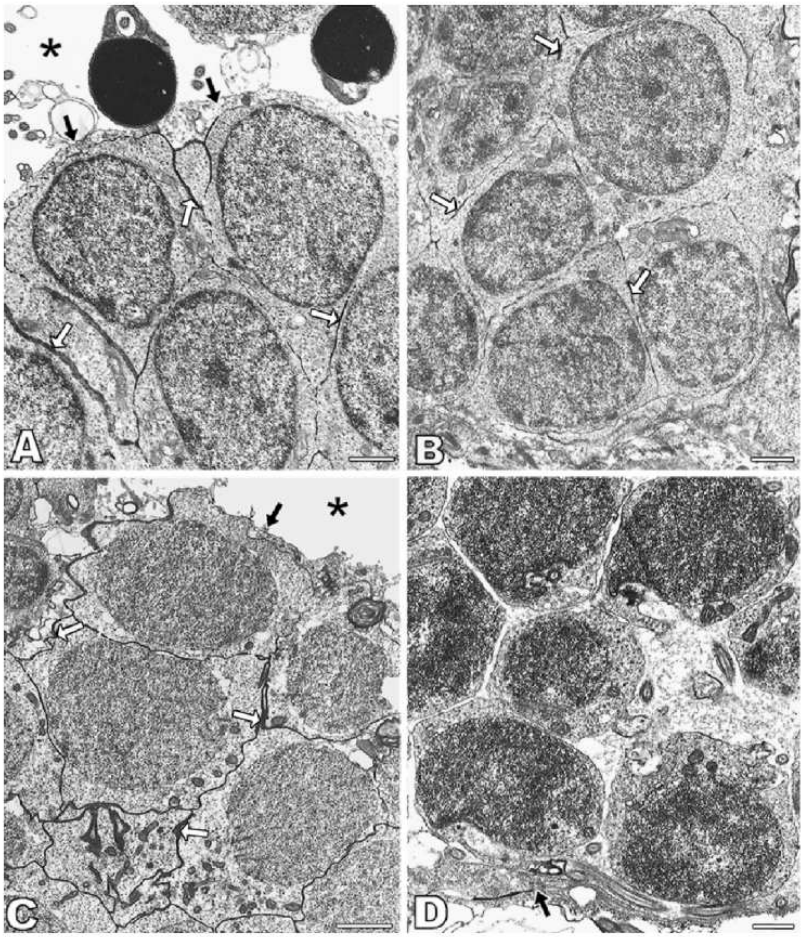

FIG. 5. Transmission electron microscopy of zebrafish testis sections using lanthanum as a tracer to investigate the permeability of the Sertoli cell barrier at different stages of spermatogenesis. The barrier is not observed in cysts containing type B spermatogonia (**A**) or primary spermatocyte cysts at the initial stages of meiosis (leptotene/zygotene; **B** and **C**), because lanthanum is present between the germ cells (white arrows). **D**) Lanthanum is found at the level of tight junctions between Sertoli cells in spermatid cysts (arrow). Note that the tracer is not present between the germ cells. The asterisks in the panels indicate the lumina of spermatogenic tubules, where lanthanum has never been found. The black arrows in **A** and **C** indicate the Sertoli cell cytoplasm. Bars = 1 μm (**A** and **B**), 2 μm (**C**), or 0.5 μm (**D**).

that the combined duration of meiotic and spermiogenic phases is very short in this species and lasts approximately 6 days (15 h of incubation plus 5 days after incubation).

Sertoli and Leydig cells were also labeled (Fig. 4, J and K), indicating that both cell types are able to proliferate in sexually mature animals.

Sertoli Cell Barrier

During spermatogenesis, lanthanum can be found inside spermatogonial and early meiotic cysts (leptotene/zygotene spermatocytes; Fig. 5, A and B). On the other hand, lanthanum was not found inside early spermatid cysts (Fig. 5, C and D), demonstrating that the Sertoli cell barrier (tight junctions) is established at the beginning of spermiogenesis, therefore obstructing the entry of lanthanum into the cyst lumen. Lanthanum has never been found in the lumen of spermatogenic tubules.

DISCUSSION

Testis Structure and Distribution of Spermatogonia

The zebrafish testicular parenchyma shows the typical vertebrate pattern with the germinal and interstitial compartments. As in other fishes and amphibians, the seminiferous tubules contain spermatogenic cysts formed by Sertoli cells that envelop a group of synchronously developing germ cells, which are derived in a clonal manner from one stem cell. The morphology of the different germ cell types observed for zebrafish is generally similar to what is described for most species of teleost fish [18, 19, 26–29], including the absence of acrosomes in spermatozoa [15].

Regarding the distribution of spermatogonia along the germinal compartment, two testis types have been described in fish: restricted and unrestricted [27–29]. In the restricted type (a derived character), undifferentiated type A spermatogonia are confined to the distal end of seminiferous tubules, near the tunica albuginea. In the unrestricted type (a primitive character), these spermatogonia are found at random sites along the seminiferous tubule throughout the testis. An intermediate type appears to exist in a number of orders (e.g., Gadiformes [30], Perciformes [18], Pleuronectiformes [31]), where undifferentiated type A spermatogonia show a preferential, but not exclusive, location close to the tunica albuginea. We have found that undifferentiated type A spermatogonia in zebrafish were distributed along the entire germinal compartment. The zebrafish testis belongs, therefore, to the unrestricted type. Moreover, zebrafish testes contain anastomosing tubules, which is another primitive feature

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previously described in the orders Cypriniformes, Characiformes, Salmoniformes, and others [29].

Sertoli Cell Number

In the present study, the number of Sertoli cells per cyst was quantified using serial sections. The final number of Sertoli cells in mammals is established at puberty, when Sertoli cells cease proliferating and differentiate terminally [6]. Because a Sertoli cell's capacity to support germ cells is limited, Sertoli cell number limits testis size and determines the spermatogenic capacity [32]. Data on the Sertoli cell number for teleosts with external fertilization are only available for *Oreochromis niloticus* [18–20] and zebrafish (this study). In both species, the number of Sertoli cells per cyst increased (4-fold in tilapia, 9-fold in zebrafish) and tended to stabilize in the meiotic phase, attaining a ratio of ~92 and ~95 spermatids per Sertoli cell in tilapia and zebrafish, respectively. During spermatogenesis in guppies (*Poecilia reticulata*), a species with internal fertilization, it was observed that the number of Sertoli cells per cyst also increased considerably (~30-fold, from ~4 to ~130 Sertoli cells per cyst) from a cyst with a single, undifferentiated spermatogonium until the initial phase of the spermiogenesis, when nearly 24 000 spermatids per cyst were found (i.e., ~170 spermatids per Sertoli cell [33]). We conclude that it is a typical feature of adult teleost spermatogenesis that Sertoli cell number increases per cyst during its development. Detailed studies in tilapia and African catfish [18, 19] showed that the vast majority of dividing (i.e., incorporating ^{3}H-thymidine or BrdU) Sertoli cells were associated with expanding spermatogonial cysts. It may not seem surprising, therefore, that we have found proliferating Sertoli cells in the testes of young adult zebrafish. However, tilapia and catfish show life-long somatic and testis growth, unlike birds or mammals and unlike zebrafish. However, a certain growth of both body and testis takes place after the zebrafish have completed puberty (first reproduction) at approximately 12 wk of age. At this point, we are unable to determine whether the observed Sertoli cell proliferation represents remaining growth until attainment of final testis size, or whether during cystic spermatogenesis a certain loss of Sertoli cells occurs that requires compensatory proliferation. The scarce literature available on Sertoli cells in teleosts speculates that after spermiation, these cells can degenerate or become integrated into the epithelium of the spermatic ducts [10, 28, 34]. Similar to what was observed in tilapia [18, 19], and despite analyzing several hundreds of cysts in zebrafish, Sertoli cells with the morphological characteristics of apoptosis were not observed. One explanation may be that the very high phagocytotic activity of Sertoli cells [35] removes apoptotic Sertoli cells quickly, so that they do not accumulate to a noticeable degree. Alternatively, Sertoli cells may be "recycled" after spermiation to contribute to the formation/growth of a new/still developing cyst.

Sertoli Cell Efficiency and Germ Cell Loss

Considering the number of spermatids per Sertoli cell in zebrafish, tilapia, and guppies (90–170) [18–20, 33], the Sertoli cell efficiency is quite high in fish, with their cystic arrangement of the spermatogenic process—10 to 20 times higher than in mammals [6, 36, 37]. Despite the great range in the number of spermatids per cyst in tilapia, zebrafish, and guppies (~650, ~1300, and ~24 000, respectively), the efficiency of the Sertoli cells in these three species of teleost fish is relatively similar. Moreover, experimentally induced changes in Sertoli cell numbers led to corresponding changes in germ cell numbers in tilapia [20], suggesting that, like in mammals [37], the number of Sertoli cells is the main factor determining the magnitude of the sperm production.

Germ cell apoptosis constitutes a normal process during spermatogenesis [38] and can occur in different developmental phases. In mammals, it is considered mainly to function in density regulation of spermatogonia and to eliminate cells with chromosomal damage (meiotic phase), whereas cell loss during spermiogenesis is less prominent [37]. The quantitative significance of germ cell loss becomes clear when considering that in mammals, only two to three spermatozoa of 10 theoretically possible cells are produced from type A1 spermatogonia [7, 37]. In the present study, we found that instead of the theoretically expected 2048 mature spermatids, approximately 1300 were formed. Hence, 6 of 10 possible cells reached the final stages of development, which contributes to the high efficiency of fish spermatogenesis. We speculate that the tailored proliferation of Sertoli cells is a relevant factor for the high survival rate of germ cells in the fish testis.

Apoptosis is mainly found during spermiogenesis in tilapia (~25% vs. ~10%), whereas in zebrafish there is a greater loss (~20% vs. 10%) during the spermatogonial phase. In both species, about 10% apoptosis occurred during meiosis. In guppies [33] and Atlantic cod [30], the main loss of germ cells occurred in the spermatogonial phase. These findings suggest that although the total losses are quite similar among fish species, apoptosis can occur during different stages along the spermatogenic process, which may reflect differences in the fine regulation of this process. The frequency with which apoptotic cells were observed in sections was surprisingly low, despite the overall loss of ~40% of germ cells. We ascribe this apparent discrepancy to the high phagocytotic efficiency of zebrafish Sertoli cells [35]. However, our understanding of the process of apoptosis and its regulation in fish is still incipient, and only a few species of teleost fish have been investigated in this regard.

Spermatogonial Generations

Knowing the precise number of spermatogonial divisions is fundamental to understanding the regulation of spermatogenesis. Previous reports stated that the number of spermatogonial generations in teleosts varies from four to six in zebrafish [39, 40] up to 14 in guppy [33], and intermediate values are observed in other teleost species [18, 26, 40]. Stereological analysis of spermatogenic cysts, as done by serial sections in the present study, is a laborious but powerful approach to establish the number of cells per cyst, which allows deduction of the number of spermatogonial generations. This permitted the calculation that nine generations of spermatogonia (type A undifferentiated spermatogonia, three generations of type A differentiating spermatogonia, and five generations of type B spermatogonia) are present in zebrafish. Ando and collaborators [40] reported four or five generations of spermatogonia in zebrafish, but the conclusion was based on counting spermatids in single sections where the cysts showed the maximum diameter. The difference in results may be based on the fact that this approach assumes a spherical shape of spermatid cysts and would lead to an underestimation of cell numbers if the actual shape was irregular or elongated, which is not unusual in zebrafish according to our observations.

Spermatogonial morphology in fish has been described on the light [19, 28, 30, 31, 33, 41, 42] and electron [41–43] microscopy levels. However, there is no consensus about the terminology used to address the different spermatogonial generations in this group of vertebrates. This difficulty is in part due to the cystic mode of spermatogenesis, so that—unlike

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the situation in reptiles, birds, and mammals—there are no stages (specific cellular associations) in the seminiferous epithelium of fish. Two types of spermatogonia have been described in fish considering basically the size and number of cells within the cysts. For example, single, larger spermatogonia were designated primary spermatogonia or type A spermatogonia; smaller and grouped spermatogonia (in pairs or more), on the other hand, were referred to as secondary or type B spermatogonia [44, 45]. However, close morphofunctional analysis reveals cells with significant differences in morphology and kinetics among the secondary or type B spermatogonia, with some generations being more similar to the type A undifferentiated spermatogonia and others more similar to the more differentiated types of smaller spermatogonia in medium-sized or large groups. Hence, the first generations of grouped spermatogonia in small cysts share a number of characteristics with the single spermatogonia in fish as well as with the type A spermatogonia in rodents. Therefore, based on similar criteria (e.g., nuclear and nucleolar features, and the presence, amount, and distribution of heterochromatin) used to discriminate between different types of spermatogonia in rodents [46], we evaluated zebrafish testes with high-resolution light microscopy and proposed a nomenclature for spermatogonial generations that is in line with established systems in higher vertebrates. In this way, we have described the following sequence of spermatogonial generations: undifferentiated type A spermatogonia (A_{und}* and A_{und}) → type A differentiated (A_{diff}) → type B early (B_{early}) → type B late (B_{late}). A_{und}* and A_{und} are single cells with a large nucleus containing poorly condensed chromatin and one or two nucleoli. A_{und}* spermatogonia have an irregular nuclear envelope that is not found in type A_{und}. A_{diff} are grouped (approximately two, four, or eight) within the cyst, and their nucleus is denser than in the previous spermatogonial generation, with heterochromatin as flecks along the nuclear envelope. The type B early spermatogonia (theoretically 16–32 cells; we counted 14–28 cells) have an elongated/round nucleus with one or two small nucleoli. A major difference from type A spermatogonia is the increased amount of heterochromatin that is distributed as round clumps throughout the nucleus or associated with the nuclear envelope. In the type B late spermatogonia (~55 to 208 cells), the nucleus is round, smaller, and more dense, and the heterochromatin forms round clumps associated with the nuclear envelope.

This morphological classification is standardized and reproducible for the zebrafish, and it can be applied to other fish species as well, except perhaps for the absolute values of the cell/nuclear sizes, whereas the relative changes of these parameters are found also in other species [20, 33]. Therefore, this categorization may provide a basis for standardizing the nomenclature used for spermatogonia in fish. Moreover, this classification parallels the nomenclature in mammals, mainly regarding the amount of heterochromatin, which is expected to simplify comparative approaches. Especially in mice, this approach has been used extensively [46, 47]. Finally, the present classification has the flexibility to allow for adjustments if required by future results—for example, regarding the molecular and/or functional characterization of the different developmental stages of germ cells, such as indicated by the expression analysis of stage-specific proteins/mRNAs (Supplemental Fig. S3) or by the capacity of germ cells to colonize a recipient's testis after transplantation (stemness) [48, 49].

The coexistence of undifferentiated and differentiated spermatogonia type A in the same cyst is intriguing in the context of stemness of spermatogonia. We were able to make this observation because the high-resolution approach allowed identifying cell membranes and cyst borders on the light microscopy level. Future work will have to evaluate the ratio of equal:unequal division of type A_{und}* spermatogonia (e.g., stem cell self-renewal vs. differentiation fate) and the regulation of this balance—for instance, regarding the orientation of the mitotic spindle pole, such as that described in *Drosophila* testis [50], or regarding factors (e.g., glial cell line-derived neurotrophic factor) secreted by Sertoli cells in rodents (see De Rooij [51] for review). In addition, an intriguing question to be investigated in fish refers to the "stemness" of a subset of differentiating spermatogonia, as has been described recently for mouse testis under certain injury conditions [52].

Meiotic and Postmeiotic Cells

Compared with the studies on tilapia [18, 19], where the same methods were used as here, the size of germ cells in zebrafish is ~3-fold smaller in practically all of the spermatogenic phases, notwithstanding that a similar pattern was found regarding the morphological changes during spermatogenesis in zebrafish, tilapia, and guppies [33]. A noticeable exception in guppies is that the maximum diameter among primary spermatocytes was observed in zygotene cells.

In general, volume changes of meiotic and postmeiotic germ cells in zebrafish and tilapia were similar to those observed in mammals [37]. However, in mammals, diplotene spermatocytes show the maximum volume [37]. We do not have an explanation for the shift to pachytene in teleosts (zygotene in guppies) at present. It seems reasonable to expect the maximum cell volume in diplotene spermatocytes that have accumulated the genetic and cytoplasmic material required for the two upcoming fast meiotic divisions and the formation of spermatids [37]. Clearly, this aspect of teleost spermatogenesis deserves further study. With pachytene spermatocytes being the biggest cell type in the meiotic phase, it is not surprising that pachytene cysts also show the maximum average volume of spermatogenic cysts. Cyst volume decreased progressively during spermiogenesis. This may reflect the remarkable nuclear condensation and elimination of cell organelles and cytoplasm during spermiogenesis [18, 19, 37, 53, 54]. Moreover, apoptotic loss of germ cells also occurs during this stage (see above).

Leydig Cells

We are not aware of studies on the individual volume of Leydig cells in teleosts other than tilapia [20, 48]. Similarly to the germ cells, Leydig cells in tilapia were 3- to 4-fold larger than observed here in zebrafish. In mammals, Leydig cell volume can vary from ~400 μm^3 in ovine and wild boars [55, 56] to ~5000 μm^3 in horses [57], with most species ranging between 1000 and 2000 μm^3 [56, 58]. Hence, the volume of Leydig cell ranges at the low end of this comparative scale. As in other fish [12], Leydig cells formed groups or clusters with extensive plasma membrane contact zones. Although we did not quantify this aspect, it seems that Leydig cell clusters were larger/contained more cells close to the tunica albuginea than in central areas of the testis.

Duration of Spermatogenesis (Meiotic and Spermiogenic Phases)

A number of studies have dealt with the duration of spermatogenesis in teleosts [18, 39, 59–63]. Determining the duration of spermatogenesis in mammals is classically based on the fact that the different germ cell generations form specific, recurrent cell associations, known as stages of the seminiferous epithelium. The determination of their relative frequencies

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enables the total duration of the differentiation process from stem cells to spermatozoa to be deduced [6, 37, 56, 58, 64]. In cystic spermatogenesis, however, this approach is not feasible. It is possible, however, to determine the duration of the meiotic and spermiogenic phases (i.e., from preleptotene/leptotene up to spermatozoa). This period is identical to the time required for an S-phase marker, such as BrdU, which is incorporated during the final round of DNA synthesis, to appear in spermatozoa. Using this approach, we found that the combined duration of the meiotic and spermiogenic phases is about 6 days in zebrafish. This is similar to the duration found in an Indian freshwater perch, *Colisa fasciata* [65], whereas a period of 3 wk was the longest found among teleosts in black mollies *Poecilia sphenops* [61]. In general, fish germ cells proceed faster through meiosis/spermiogenesis than mammalian germ cells, which take 3–7 wk [37, 56].

Different from the homoeothermic mammals, the duration of spermatogenesis in fish varies depending on the water/body temperature [66]. Studies describing the effects of temperature on the duration of spermatogenesis in fish [59, 60] or reptiles [67] showed that in general, elevated temperatures accelerate germ cell development. The fact that zebrafish usually reproduce in temperatures close to 30°C can explain, at least in part, the short duration of the meiotic and spermiogenic phases. The short duration, a high number of spermatogonial generations, and a high efficiency of the Sertoli cell jointly allow the prediction that the daily sperm production per volume unit of the zebrafish testicular parenchyma will be rather high.

Sertoli Cell Barrier

Experimental evidence of the Sertoli cell barrier ("blood-testis barrier") became available when certain dyes were incapable of reaching meiotic and postmeiotic germ cells in the mammalian seminiferous epithelium [68, 69]. The use of electron-dense tracers, such as lanthanum, showed in mice [70, 71] that tight junctions between neighboring Sertoli cells in the seminiferous epithelium prevented the passage of the tracer. The Sertoli cell barrier has been demonstrated in all other classes of vertebrates (birds [72], reptiles [73], amphibians [74], and fish [75, 76]). It appears that the effective seclusion of the genetically and immunologically distinct meiotic and postmeiotic cells is an important, evolutionarily conserved principle in spermatogenesis.

In fish, depending on the species, the establishment of the tight junctions between Sertoli cells occurs in cysts containing primary spermatocytes [75] or spermatids [12, 77, 78]. In zebrafish, lanthanum was detected among spermatogenic cells until the beginning of the meiotic prophase but was excluded from cysts containing early spermatids, demonstrating that the Sertoli cell barrier is established at the end of meiotic phase. Interestingly, we have never observed lanthanum in the lumen of spermatogenic tubules, even though it was able to enter cycts containing germ cells up to the stage of leptotene/zygotene spermatocytes. Therefore, we consider it possible that future analysis will provide evidence for tight junctions among adluminal Sertoli cells (i.e., separating the lumen of spermatogenic tubules from the lumen of spermatogenic cysts) already being established shortly after cyst formation. The formation of tight junctions among Sertoli cells at the basal part of the germinal epithelium, on the other hand, appears to depend on the stage of development of the germ cells. This situation suggests that not only in mammals but also in zebrafish, the germinal epithelium is polarized. Regarding the establishment of the microenvironment required for germ cell development, other structures besides the Sertoli cell barrier may be involved, such as the capillary endothelium, the basal lamina, and peritubular myoid cells [78].

In conclusion, this is the first detailed study using morphological, stereological, and molecular approaches on spermatogenesis in zebrafish. Also, we are proposing a new nomenclature for the spermatogonial generations in zebrafish that will allow further comparisons with other fishes but also with higher vertebrates, such as mammals. The new results obtained for the zebrafish in the present study contribute to the characterization of the testicular function in this important teleost model species, which may be of great value in future studies on the regulation of spermatogenesis.

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Supplemental Table S1: Primers used to generate a DNA template for DIG-labelled cRNA probe synthesis

Primer	Nucleotide sequence (5' → 3')
1039[a]	T3Rpps-CAGGAGAAAGTGTCCGACTATGAGATGAAA
1040[b]	T7Rpps-AACCACAGCAGACACTGAGTAATAAAATGCAT

[a]Primer 1039 contains the T3 RNA polymerase promoter sequence (underlined) at its 5'-end (T3Rpps; 5'-TTTACCTGTTATTAACCCTCACTAAAGGG-3').

[b]Primer 1040 contains the T7 RNA polymerase promoter sequence (underlined) at its 5'-end (T7Rpps; 5'-TTTACCTGTAATACGACTCACTATAGGG-3').

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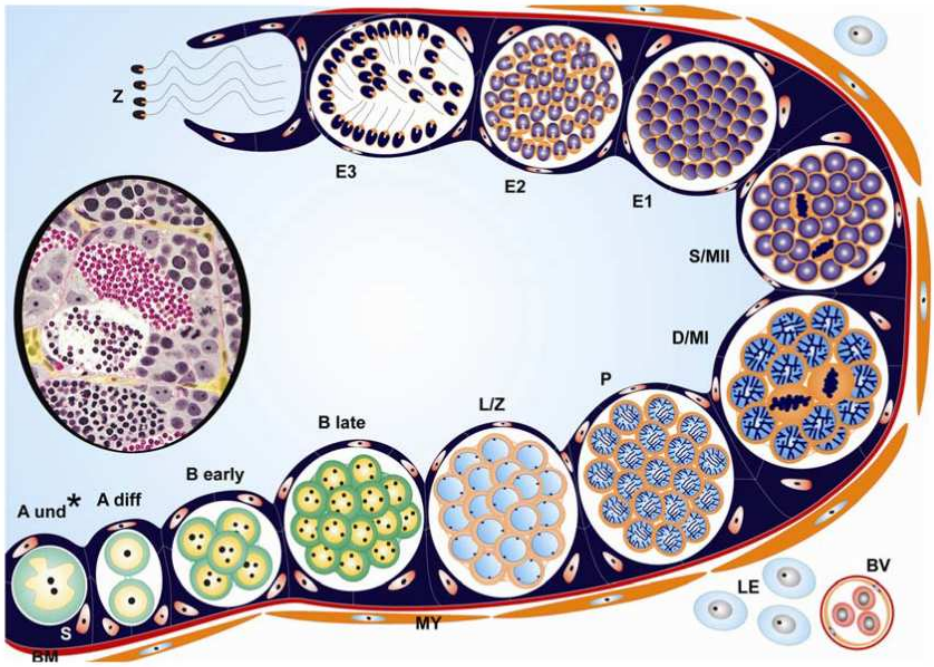

Supplemental Figure S1

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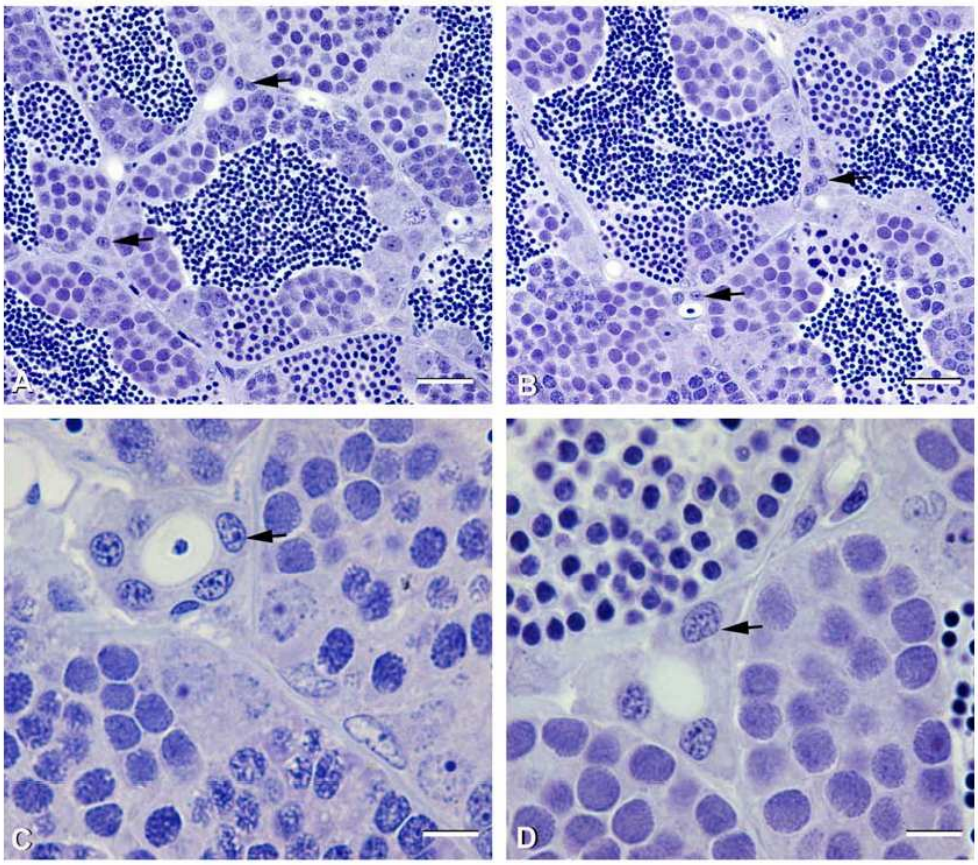

Supplemental Figure S2

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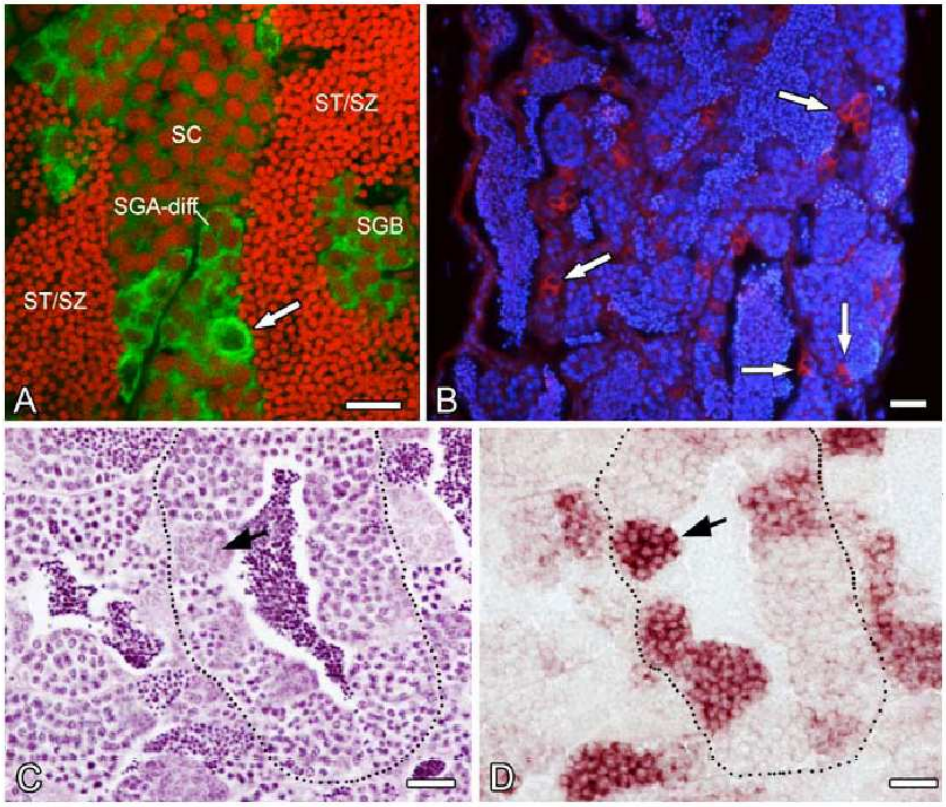

Supplemental Figure S3

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3. Spermatogonial Stem Cell Niche and Spermatogonial Stem Cell Transplantation in Zebrafish

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Spermatogonial Stem Cell Niche and Spermatogonial Stem Cell Transplantation in Zebrafish

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Abstract

Background: Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis, and reside within a specific microenvironment in the testes called "niche" which regulates stem cell properties, such as, self-renewal, pluripotency, quiescence and their ability to differentiate.

Methodology/Principal Findings: Here, we introduce zebrafish as a new model for the study of SSCs in vertebrates. Using 5′-bromo-2′-deoxyuridine (BrdU), we identified long term BrdU-retaining germ cells, type A undifferentiated spermatogonia as putative stem cells in zebrafish testes. Similar to rodents, these cells were preferentially located near the interstitium, suggesting that the SSC niche is related to interstitial elements and might be conserved across vertebrates. This localization was also confirmed by analyzing the topographical distribution of type A undifferentiated spermatogonia in normal, *vasa::egfp* and *fli::egfp* zebrafish testes. In the latter one, the topographical arrangement suggested that the vasculature is important for the SSC niche, perhaps as a supplier of nutrients, oxygen and/or signaling molecules. We also developed an SSC transplantation technique for both male and female recipients as an assay to evaluate the presence, biological activity, and plasticity of the SSC candidates in zebrafish.

Conclusions/Significance: We demonstrated donor-derived spermato- and oogenesis in male and female recipients, respectively, indicating the stemness of type A undifferentiated spermatogonia and their plasticity when placed into an environment different from their original niche. Similar to other vertebrates, the transplantation efficiency was low. This might be attributed to the testicular microenvironment created after busulfan depletion in the recipients, which may have caused an imbalance between factors regulating self-renewal or differentiation of the transplanted SSCs.

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Introduction

Spermatogenesis is a cellular developmental process by which self-renewing spermatogonial stem cells (SSCs) differentiate into millions of sperm daily [1,2]. To sustain this process continuously throughout the male reproductive life span, SSCs reside within a specific microenvironment in the testes called "niche" which regulates their properties, such as, self-renewal, pluripotency, quiescence and their ability to differentiate [3–5]. Despite of its crucial importance on SSC fate, the cellular and molecular composition of SSC niche remain unknown for several species of vertebrates. In rodents, the SSC niche has recently been identified within regions of the seminiferous tubules which are adjacent to the interstitial compartment [6,7], preferentially along the branches of the interstitial blood vessels [8]. It has been hypothesized that the cellular and molecular environment near the interstitial compartment promotes SSC renewal, and when SSCs leave these areas, the associated changes in their environment promote SSC differentiation [5]. The proximity of SSC niche to the interstitium perhaps reflects the vascular supply of oxygen, nutrients, or hormones, such as follicle-stimulating hormone (FSH) or luteinizing hormone (LH) which influence Leydig and Sertoli cell functions on SSC self-renewal and also on SSC retention and homing in the niche [5,9–11]. For example, FSH induces the secretion of GDNF (glial cell-line derived neurotrophic factor), an extrinsic stimulator of SSC self-renewal, produced by Sertoli cells [3,12].

Currently, the only means to study SSCs and their niche is by exploiting the stem cells' functional properties, such as slow-cycling and quiescent nature through the label-retaining cell (LRC) approach [13], or by studying SSC functionality and plasticity by transplantation assays. In this context, transplantation techniques developed by Brinster and collaborators [14,15] has enabled tremendous progress in the phenotypic and functional investigations of SSCs. Nowadays, SSC transplantation approaches have

been developed for a number of species including also teleost fish [16,17]. These data have broad implications for understanding the regulation of spermatogenesis, stem cell biology, etiology of male infertility [18–22], and also for advancing biotechnologies such as conservation of valuable genetic stocks, preservation of endangered species, and also as new option for transgenesis [16,17,19].

In anamniote vertebrates (fishes and amphibians), we find the cystic type of spermatogenesis [2]. There are two main differences compared to higher vertebrates. First, within the spermatogenic tubules, cytoplasmic extensions of Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium. Second, the cyst-forming Sertoli cells retain their capacity to proliferate also in adult fish [23,24]. Hence, the basic functional unit of the spermatogenic epithelium in fish is a spermatogenic cyst formed by a dynamic group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone. Different clones being in different stages of development generate the typical histological picture of fish testes, where the tubular compartment contains cysts of different sizes with groups of germ cells in different stages of spermatogenesis (see Figures 1, 2 and 3). Detailed histological studies in zebrafish (*Danio rerio*) have described two subtypes of type A undifferentiated spermatogonia in the testes, designated as A_{und*} and A_{und} [23]. It is not known if these two subtypes are separated by mitosis, or represent different stages of the same cell cycle, and moreover, there is no information on the spermatogonial stem cell niche in the zebrafish testis, or on the stemness of A_{und*} and A_{und}.

In the current study, we identified the putative SSCs and their niche by identifying the LRCs in zebrafish testes and using a transgenic zebrafish expressing enhanced green fluorescent protein under the control of the germ cell specific *vasa* promoter (*vasa::egfp*) [25]. Furthermore, we evaluated the spatial relationship between blood vessels and the SSC niche, using testes expressing enhanced green fluorescent protein under the control of the endothelial cell specific *fli* promoter (*fli::egfp*) [26]. To confirm the biological activity (re-establishment of function and plasticity) of the potential SSCs, we developed a transplantation assay in zebrafish. Finally, we studied the recipient's testicular microenvironment prior to transplantation as it might influence the behavior of transplanted SSCs.

Materials and Methods

Animals

Sexually mature zebrafish males and females, and sexually mature transgenic zebrafish males expressing enhanced green fluorescent protein under the control of the germ cell-specific *vasa* promoter (*vasa::egfp*) [25] or the endothelial cell-specific *fli* promoter (*fli::egfp*) [26] were used. Animal housing and experimentation were consistent with Dutch and Brazilian national regulations and were approved by the Utrecht University and Federal University of Minas Gerais animal use and care committees, respectively.

Topographical distribution of type A undifferentiated spermatogonia in zebrafish seminiferous tubules

Testes from males (n = 5) were fixed in 4% buffered glutaraldehyde at 4°C overnight, dehydrated, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany, http://www.kulzer-technik.de), sectioned and stained according to conventional histological procedures [23,27]. The topographical distribution of A spermatogonia was recorded by examining if type A undifferentiated spermatogonia (A_{und*} or A_{und}) were adjacent to the interstitial compartment, or contacted one or more tubules (intertubule). The position of 500 type A undifferentiated spermatogonia was counted per animal and expressed as percentage of the total number evaluated; to determine if the distribution of these cells follows a random pattern, the tubular perimeters adjacent to the interstitium, or intertubule were measured using Image J software (National Institutes of Health, Bethesda, Maryland, USA, http://rsbweb.nih.gov/ij), and the values were expressed as percentage of the total tubular perimeter (n = 50 tubules/animal).

Identification and quantification of label retaining cells (LRCs) in zebrafish testes

To estimate the cell cycle duration of type A undifferentiated spermatogonia (A_{und*} and A_{und}), 10 males were exposed to BrdU (Sigma-Aldrich, St. Louis, MO, USA, http://www.sigmaaldrich.com) dissolved in water (4 mg/ml) for approximately 4 and 10 h. To identify the LRC population, 25 males were pulsed with BrdU dissolved in water (4 mg/ml) for 10 h/day during 3 consecutive days. Animals (n = 5) were sacrificed immediately after the third BrdU pulse, and after 4, 11, 18 and 25 days of chase. Testes were fixed, embedded, and sectioned as described above. BrdU incorporation was detected by immunohistochemistry as described previously [28]. The labeling index of both types of A undifferentiated spermatogonia (A_{und*} or A_{und}) was determined by counting the total number of labeled cells out of 300 cells (labeled and non-labeled) during the different periods after chase. After 18 and 25 days of chase, the spatial distribution of labeled A_{und*} or A_{und} (LRCs) was determined by counting the number of labeled cells situated near the interstitium, or in the intertubule, or near the testicular capsule. The values were expressed as percentage of positive-BrdU cells in the mentioned regions.

Whole-mount analysis of *vasa::egfp* testes under confocal laser scanning microscopy (CLSM) and examination of *fli::egfp* testes

Testes from transgenic *vasa::egfp* [25] or *fli::egfp* [26] zebrafish were fixed in 2% buffered paraformaldehyde for 2 h. For whole-mount examination, *vasa::egfp* testes were permeabilized with 0.2% PBT (0.2% Triton X-100 in PBS) for 10 min, and subsequently stained in DAPI (Invitrogen Molecular Probes, Carlsbad, CA, USA, www.invitrogen.com) for 5 min, followed by two rinses in PBS for 10 min. *vasa::egfp* testes (n = 5) were analyzed by CLSM 510 Meta (Zeiss, Jena, Germany, www.zeiss.de/lsm) using 358 nm and 488 nm as excitation wavelengths for DAPI and GFP, respectively. *fli::egfp* testes were frozen in Tissue-Tek (Sakura Finetek Europe B.V., Leiden, Netherlands, http://www.sakura.eu) and cryosectioned at 10 µm, permeabilized in 0.2% PBT for 10 min, stained with DAPI (Invitrogen) for 5 min, and mounted with a coverslip using an anti-fading Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA, http://www.vectorlabs.com). Sections were examined under fluorescence microscopy using filters for DAPI and FITC visualization.

Depletion of endogenous spermatogenesis in zebrafish male recipients for SSC transplantation

To deplete endogenous spermatogenesis, we first examined the effect of different temperatures. Overall, the speed of spermatogenesis in teleost fish is significantly influenced by temperature [29]. Hence, males were kept in water at 20°C (n = 15), 27°C (n = 12), 30°C (n = 7), or 35°C (n = 12) for at least one week. Then, animals received one single intraperitoneal injection of ^{3}H-thymidine (Amersham/GE Healthcare, Piscataway, NJ, USA,

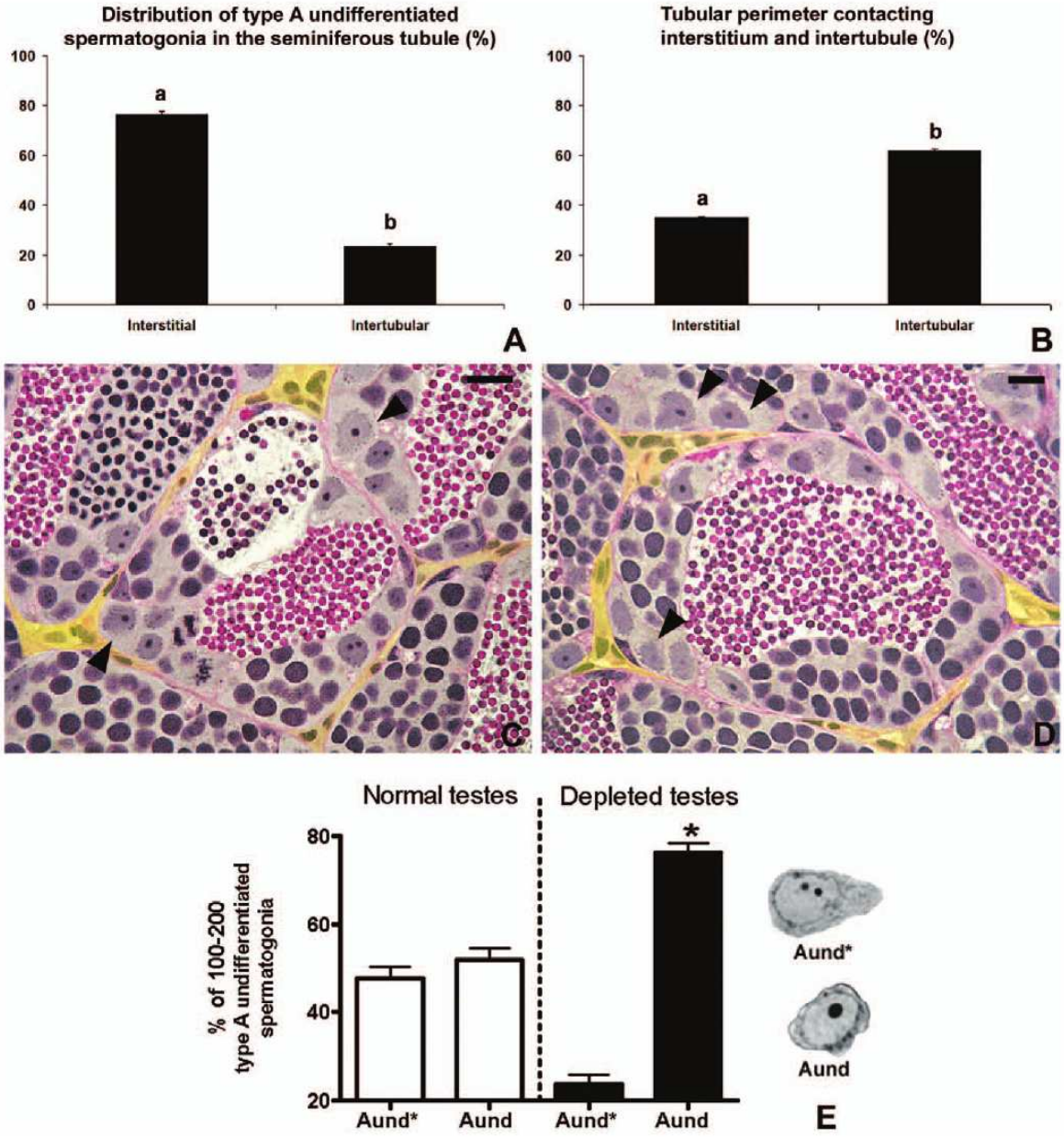

Figure 1. Topographical distribution of type A undifferentiated spermatogonia in zebrafish testes. A. Quantification of type A undifferentiated spermatogonia as located near the interstitium or in the intertubular area. Note that ~76% of type A undifferentiated spermatogonia are preferentially located near the interstitium. **B.** Tubular perimeter of the regions contacting the interstitium or intertubular areas. **A,B.** Bars represent the mean ± SE which are expressed as percentage. Different letters mean significant differences among the groups. **C,D.** Histological sections of the zebrafish seminiferous tubules. Note that most of type A undifferentiated spermatogonia (arrowheads) are distributed near the (yellow) interstitium. Staining: PAS (Periodic Acid Schiff)/Ferric Hematoxylin/Metanil Yellow. Scale bar = 10 μm. **E.** Relative number of type A_{und*} and A_{und} in normal (n = 7) and busulfan-depleted testes (n = 10). Bars represent the percentage mean ± SE of 100–200 type A spermatogonia, significant differences among the groups ($p<0.05$) are indicated by an asterisk.
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http://www.gehealthcare.com) (2 μCi/g/BW), and were sacrificed at 2 h, 12 h, 1, 2, 3, 4, 5 and 6 days after thymidine injection. Testes were weighed for calculating the gonadosomatic index (GSI = testes weight/body weight ×100), fixed, embedded and sectioned as above, and prepared for autoradiographic analysis to estimate the duration of meiosis and spermiogenesis as described previously [16,29]. Based on these results (Figure S1), 35°C was chosen as optimal temperature to deplete endogenous zebrafish

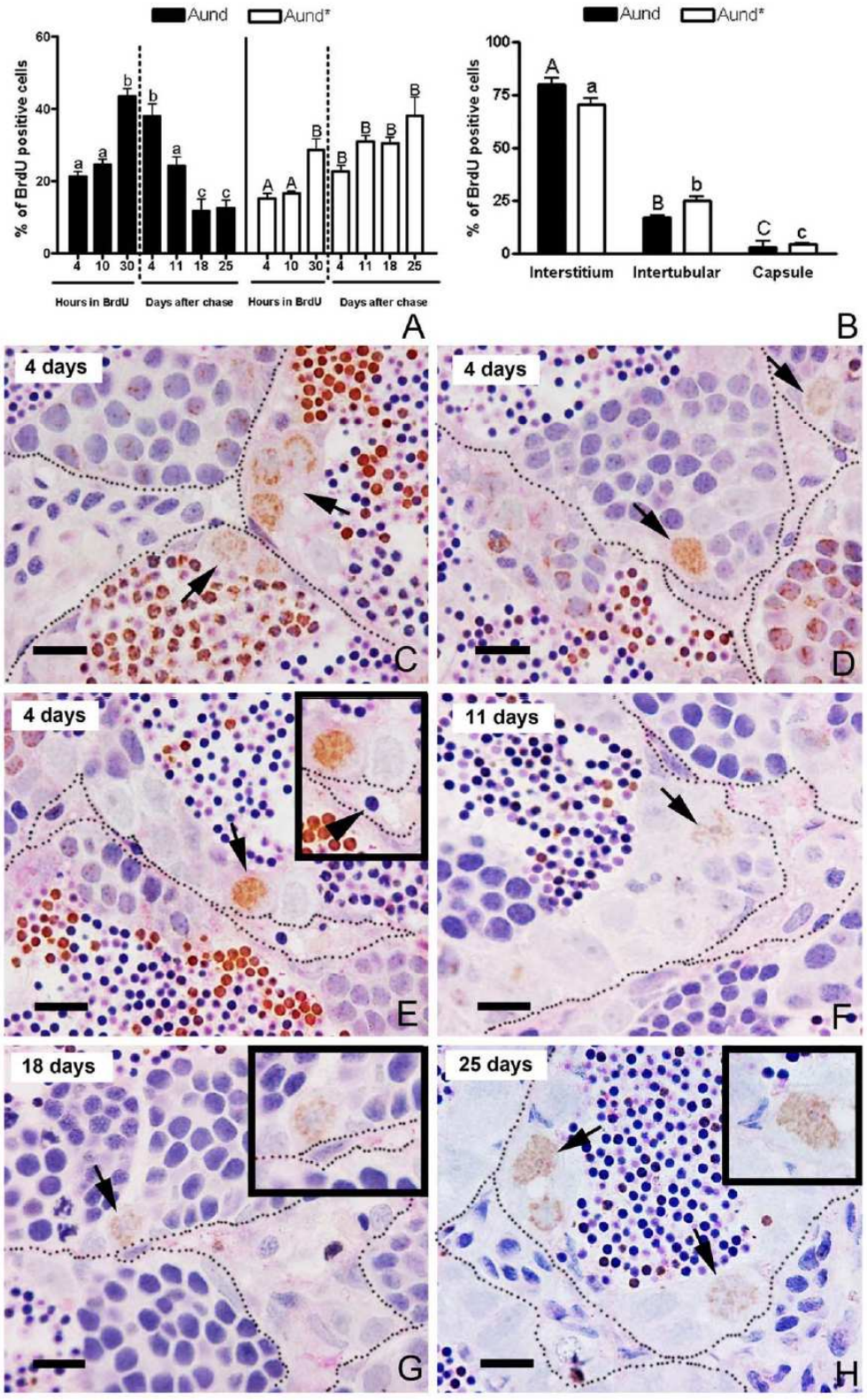
Aund
Aund*
% of BrdU positive cells
Hours in BrdU
Days after chase
Interstitium
Intertubular
Capsule
A
B
4 days
C
4 days
D
4 days
E
11 days
F
18 days
G
25 days
H

Figure 2. Label retaining cell (LRC) approach in zebrafish testes. A. BrdU labeling index of type A_{und*} and A_{und} during BrdU exposure (4, 10, and 30 h) and after 4, 11, 18 and 25 days of chase. Bars represent the percentage mean ± SE (n = 5); different letters denote significant differences ($p<0.05$) in time. **B.** Distribution of BrdU-positive A_{und*} and A_{und} spermatogonia after 18 and 25 days of chase. Bars represent the mean ± SE which are expressed as percentage, and different letters indicate significant differences ($p<0.05$) among the groups. **C–H.** BrdU immunodetection with PAS staining after 4 (**C–E**), 11 (**F**), 18 (**G**), and 25 (**H**) days of chase. Note that BrdU immunostaining is diluted as a consequence of the progression of spermatogenesis. Only the slow-cycling cells (stem cells candidates) are able to retain the BrdU label for long periods of time. LRCs are indicated by arrows. Some of LRC are near to blood vessels (**arrowhead**) (**E**). Insets are high magnification of the LRCs (type A undifferentiated spermatogonia). The interstitium is delimited by spotted lines. Scale bars = 10 μm.
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spermatogenesis. Then, 60 zebrafish males were kept at 35°C for one week, and received a single intraperitoneal dose of 30 or 40 mg/Kg/BW of busulfan (Sigma). Testes from males (n = 6) were sampled 2, 4, 6, 10 and 12 days after the injection, weighed, fixed, embedded, sectioned and stained as above. As control group, males (n = 30) received a single intraperitoneal injection of dimethyl sulfoxide and sampled at the same reported periods. To evaluate the optimal dose and the best window of depletion, frequency of spermatogenic cysts, germ cell apoptosis, and Sertoli cell only phenotype were determined for each sampled period. Results were expressed as percentage of the total number of counted structures. To address the composition of type A undifferentiated spermatogonia (A_{und*} or A_{und}) in busulfan-depleted testes (n = 10), the percentage of both subtypes was determined by counting the number of A_{und*} and A_{und} out of 100–200 type A undifferentiated spermatogonia in each male. As control, the percentage of both subtypes was also determined in normal testes (n = 7).

Preparation of zebrafish female recipients for SSC transplantation

Ovoposition was induced according to usual procedures (http://zfin.org). To evaluate if females after ovoposition may be suitable recipients for SSC transplantation, ovaries (n = 5) were fixed, embedded, and sectioned as above, and stained with PAS to determine the number of postovulatory follicles (POFs)/mm^2 of tissue. POFs consisting for a great part of granulosa cells remaining after ovulation were considered as a space potentially available for transplanted SSCs. The number of POFs/mm^2 from preovulatory females was also evaluated as a control.

Donor cell preparation and SSC transplantation into male and female zebrafish recipients

Testes from males (n = 10) were digested with 0.2% collagenase and 0.12% dispase [30]. The obtained cell suspension was immediately submitted to FACS (Fluorescence Activated Cell Sorting) using an inFlux cell sorter (BD Bioscience, San Jose, CA, USA, www.bdbiosciences.com). *Vasa* is highly expressed in type A undifferentiated spermatogonia, but expression decreases during meiosis and spermiogenesis [23]. Since type A undifferentiated spermatogonia are the largest germ cell type in zebrafish testes (~10 μm nuclear diameter [23]), FACS settings were adjusted to sort a cell population displaying large size and high intensity of fluorescence, which should enrich type A undifferentiated spermatogonia. To validate the enrichment, the unsorted and sorted cell fractions obtained by FACS was analyzed under fluorescence microscopy, or fixed, embedded, sectioned, and stained as above to determine the percentage of germ cell type. For SSC transplantation, the FACS-enriched cell fraction was suspended in L-15 medium (Sigma), 5% trypan blue and 10% calf serum. SSCs were transplanted into testes or ovaries through the genital pores, using a glass capillary needle coupled to a peristaltic pump (Figure S2). To optimize this procedure, zebrafish male and female genital pores were analyzed morphologically (diameter and angle) to adjust the settings for the glass capillary needle (Figure S2). The transplantation route was standardized and tested by injecting trypan blue (Figure S2).

SSC transplantation analysis

Recipients were sacrificed 2 and 3 weeks (males) or 3 and 4 weeks (females) after transplantation. The gonads were fixed in 2% buffered paraformaldehyde for 2 h, permeabilized, and stained with DAPI before analysis by CLSM, as described above. As positive control, Vasa protein expression was examined by GFP immunodection in *vasa::egfp* gonad sections or by Vasa immunocytochemistry in wild-type males [31]. The PCR detection of *gfp* DNA from donor-derived germ cells in male and female recipients was carried out as described previously [32].

11-Ketotestosterone (11-KT) plasma levels in busulfan-depleted male zebrafish and 11-KT release by depleted testes *in vitro*

Males (n = 19) were sampled 10 days after busulfan (40 mg/kg) treatment. A blood sample was collected for quantification of 11-ketotestosterone (11-KT) plasma levels, as described previously [33]. As controls, 11-KT plasma levels were also quantified in zebrafish kept at 27°C (n = 7), or at 35°C (n = 11). In other experiments, carried out at the same time, but published separately [33], 11-KT plasma levels were measured 2 h after a single injection of recombinant zebrafish Fsh or hCG. Results are expressed as ng 11-KT/ml of plasma. To evaluate testicular 11-KT release in tissue culture, testes were collected from adult zebrafish kept at 27°C (control) (n = 7), or from busulfan-treated zebrafish (n = 7). The two testes of a given fish were incubated in parallel, such that one of them (randomly chosen left or right) served as control (basal) for the contralateral one, which was incubated in the presence of 1 μM of the adenylate cyclase activator forskolin [33]. After incubation the medium was processed for the quantification of 11-KT [34]. Results were expressed as ng 11-KT/mg of tissue.

Gene expression in spermatogenesis-depleted testes in busulfan-treated zebrafish

Testes from males kept at 27°C (control) (n = 7), or at 35°C (n = 5), or treated with busulfan (n = 12) were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was extracted from testes using the RNAqueous®-Micro Kit (Ambion, Austin, TX, USA, http://www.ambion.com). Further processing to determine the threshold cycle (Cq) values of the reference endogenous control gene *elongation factor 1-alpha* (*ef1α*) and *β-actin1*, as well as of *insulin-like 3 (insl3)* [35], *steroidogenic acute regulatory protein* (*star*), and *cytochrome P450, family 17, subfamily A, polypeptide 1* (*cyp17a1*), *androgen receptor* (*ar*), *anti-Müllerian hormone* (*amh*), *gonadal soma-derived growth factor (gsdf), insulin growth factor 1a (igf1a)* and *1b (igf1b)* [36], and germ cell genes *piwil1* (spermatogonia), and *synaptonemal complex protein 3 (sycp3l)* (spermatocytes) by qPCR analysis was performed as reported [33,37,38]. No significant differences ($P>0.05$) were found among the mean *β-*

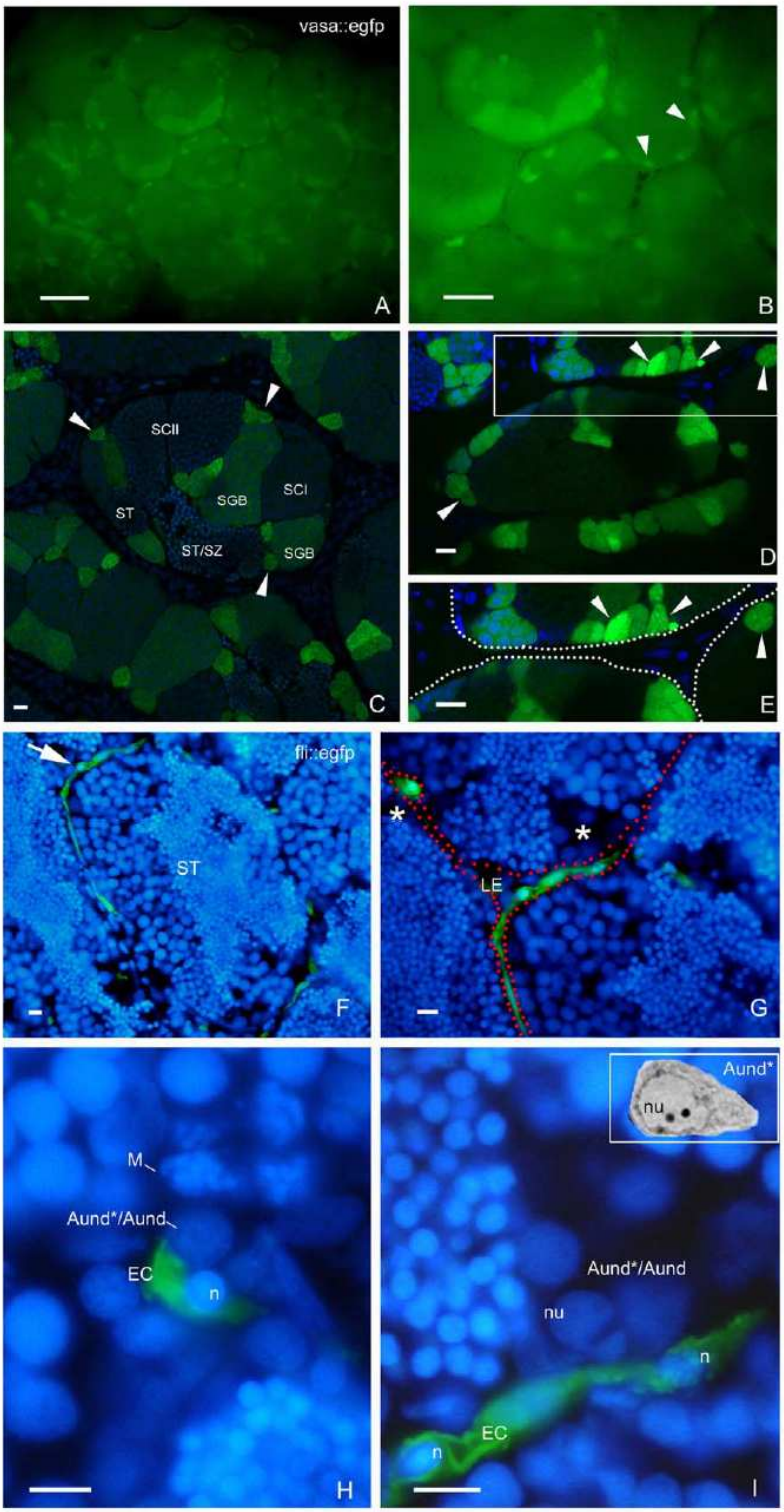

Figure 3. SSC niche in zebrafish testes. Whole-mount of *vasa::egfp* testes analyzed under fluorescence (**A,B**) and CLSM (**C**–**E**). **A,B.** Most of the brightest and small spots (type A undifferentiated spermatogonia) (arrowheads) are adjacent the triangle/lozenge dark areas (interstitium). Scale bars = 0.1 mm and 0.5 mm, respectively. **C**–**E.** Vasa is highly expressed in type A undifferentiated spermatogonia, and is gradually decreased during the spermatogenesis. A_{und*}/A_{und} (arrowheads), type B spermatogonia (SGB), primary spermatocytes (SCI), secondary spermatocytes (SCII), spermatids (ST), spermatids and spermatozoa (ST/SZ). Note that most of A_{und*}/A_{und} (arrowheads) are situated near the interstitium (**D**, dark areas; **E**, delimited by

spotted lines). **E** is a high magnification of the square in **D**. Scale bars = 10 μm. **F–I:** Cryosections of *fli::egfp* testes stained with DAPI (nuclear staining) and analyzed under fluorescence microscopy. The arrow in **F** shows a blood vessel (green) surrounding the circumference of a seminiferous tubule (ST). Interstitium (delimited with red dotted lines), Leydig cells (LE) and group of type A spermatogonia (asterisks) are shown in **G**. **H,I.** A_{und*}/A_{und} are near the endothelial cell (EC) nuclei (n). Note a metaphase figure (**M**) in **H**. Nucleolus (nu) of type A undifferentiated spermatogonia is shown in **I**. Compare the similar morphology of A_{und*} (inset) with the cells found near the endothelial cell. Scale bars = 10 μm.
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actin1 and *ef1α* Cq values in the different groups (Figure S3) thus validating *β-actin1* and *ef1α* as suitable references for the current experiments. Then, relative mRNA levels of the selected genes were normalized to *β-actin1* and *ef1α*, and expressed as fold of relative control (27°C) mRNA levels. Nomenclature of zebrafish proteins, mRNAs and genes are according to ZFIN (http://zfin.org) rules.

Statistical analysis

Significant differences between two groups were identified using Student test (paired and unpaired) ($P<0.05$). Comparisons of more than two groups were performed with one-way ANOVA followed by Student-Newman-Keuls test ($P<0.05$). Graph Pad Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, http://www.graphpad.com) was used for all statistical analysis.

Results

Characterization of SSC candidates and their niche in zebrafish testes

Analyzing the topographical distribution of type A spermatogonia showed that 75% of these cells are situated adjacent to the interstitial compartment (Figure 1A,C,D) although this compartment represents only $1/3^{rd}$ of the total perimeter of the spermatogenic tubules (Figure 1B). Quantification of the mitotic index of these cells was achieved by determining the BrdU labeling index during three successive 10 h long exposures to BrdU during three consecutive days. The results suggested that both subtypes of type A undifferentiated spermatogonia (A_{und*} and A_{und}) have a long cell cycle lasting at least 10 h but less than 30 h (Figure 2A). To confirm the existence of slow-cycling cells (stem cell candidates), testes were examined for BrdU-positive type A_{und*} and A_{und} spermatogonia at different periods (up to 25 days) after the final BrdU exposure; putative stem cells are considered to be part of the LRC population. During this chase period, two distinct patterns of BrdU staining were found among the two subtypes of type A spermatogonia (Figure 2A). A_{und} rapidly lost the label until the 18^{th} day of chase, when the labeling index stabilized at ~10% for this cell type (Figure 2A). On the other hand, the percentage of BrdU-positive type A_{und*} spermatogonia varied in a statistically not significant manner around 30% during the complete chase period (Figure 2A). Apart from some somatic elements, type A undifferentiated spermatogonia (A_{und*} and A_{und}) were the only BrdU retaining germ cells after 18 and 25 days of chase. Intriguingly, most of the labeled spermatogonia (71% of A_{und*} and 80% of A_{und}) were situated adjacent to the interstitium (Figure 2B,C–H). A similar pattern was found in *vasa::egfp* testes examined under CLSM where the strongest expression of *vasa* was observed adjacent to the interstitium (Figure 3 and Video S1). To further study the spatial relation between the vasculature and undifferentiated spermatogonia, testes of transgenic zebrafish expressing GFP in endothelial cells (*fli::egfp*) were analyzed. Capillaries surround the seminiferous tubules (Figure 3F), and type A undifferentiated spermatogonia were often found in close association with endothelial cells (Figures 2E-inset; 3G–I). A combination of the above results is illustrated schematically in Figure S4, showing a hypothetical spermatogonial stem cell niche in zebrafish.

Preparation of male and female recipients for SSC transplantation

Zebrafish were exposed to different temperatures to optimize the treatment with busulfan (Figure 4A, Figure S1). Higher temperatures accelerated spermatogenesis (Figure S1), whereas at 35°C, spermatogenesis did not progress beyond metaphase I and showed abnormalities, such as massive apoptosis in particular amongst spermatocytes, absence of sperm, and a significant decrease in the GSI (Figure 4A,E; Figure S1). Both doses of busulfan (30 or 40 mg/Kg/BW) tested at 35°C induced further and a more general germ cell apoptosis (Figure 4D–H) and a progressive GSI decrease, which reached its lowest value 10 days after injection (Figure 4B,C). However, only the higher dose suppressed efficiently endogenous spermatogenesis, resulting in 88% of the spermatogenic tubules showing a Sertoli cell only appearance, i.e. all germ cells were missing (Figure 4D,I). Analyzing the composition of type A spermatogonia in the 12% of tubules where some germ cells remained and comparing this to normal testes demonstrated that the ratio between A_{und*} and A_{und} shifted from 1:1 to 1:3 in busulfan-depleted testes (Figure 1E). The preponderance of A_{und} might be associated with the observation that 12 days after injection, most tubules showed the first type B spermatogonia and/or spermatocytes again, i.e. showed a fast recovery of endogenous spermatogenesis after treatment with 40 mg/Kg/BW of busulfan at 35°C (Figure 4D).

To prepare female zebrafish recipients for SSC transplantation, ovoposition was induced; a pilot study indicated a high mortality among females when applying the combination of high temperature and busulfan. The increased number of POFs showed that ovoposition created spaces and "free" somatic cells (follicle cells from POFs), potentially suitable for receiving injected germ cells, and to support transplanted SSCs development, respectively (Figure S5).

Donor cell isolation and male and female transplantation

Using *vasa::egfp* transgenic zebrafish as donors, a testicular cell suspension was obtained and subsequently submitted to FACS, in order to enrich transplantable SSC (Figure 4J,K) by sorting for big cells carrying high fluorescence (Figure 4L,M). Indeed, after sorting, we observed an enrichment of type A undifferentiated spermatogonia (Figure 4K–N). The sorted cells ($8.10^3-4.10^4$ cells/μl) were injected through the genital pore (Figure 4O, Figure S2). Since females have a prominent belly, direct injections into ovaries (via lateral body wall) were also successfully performed (Figure S2). After transplantation, zebrafish recipient males and females were placed in water of 27°C.

Male and female transplantation analysis

After two weeks of transplantation, donor cells colonized the recipient's seminiferous epithelium, and formed clusters, which were situated near the interstitial compartment (Figure 5A,B). Using CLSM, we found that these clusters were composed of ~8 cells/cyst (Figure 5C,D; Video S2). Three weeks after transplantation, donor-derived cysts had increased in number and size, and were found at different stages of spermatogenesis (e.g. differentiating type A spermatogonia, type B spermatogonia and spermatocytes) along the recipient's seminiferous epithelium (Figure 5E,F; Video S3). Donor type A undifferentiated spermatogonia (A_{und*} and A_{und}) were also

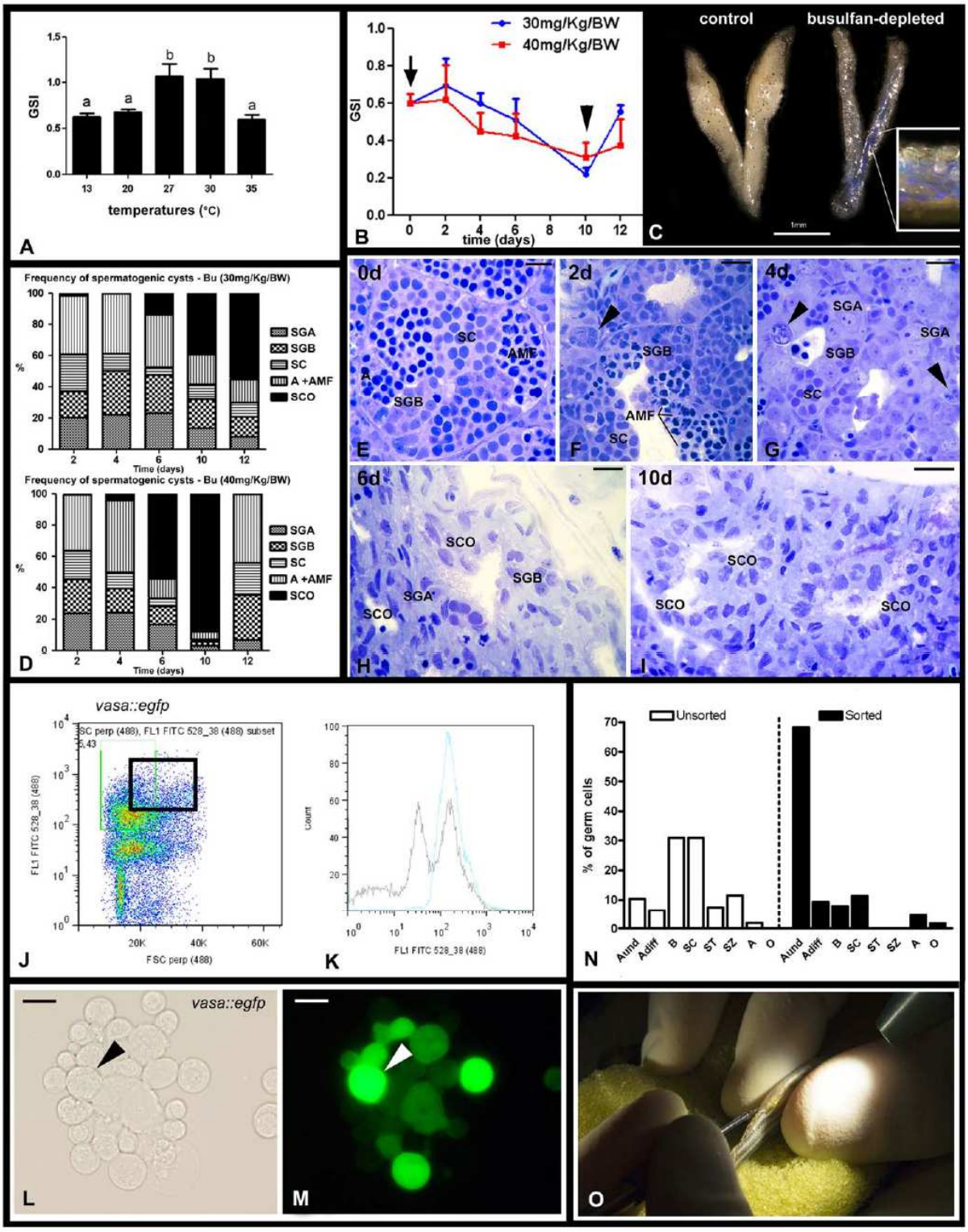

Figure 4. Depletion of endogenous spermatogenesis in male recipients for SSC transplantation. A. Effects of different temperatures on zebrafish GSI (gonadosomatic index). Bars represent mean ± SE (n = 15, 20°C), (n = 12, 27°C), (n = 7, 30°C), and (n = 12, 35°C). Different letters indicate significant differences ($p<0.05$) among groups. **B.** Effects of two single doses of busulfan (30 or 40 mg/Kg) on zebrafish GSI. The arrow indicates the

day of injection (day 0), the arrowhead shows that the lowest GSI is observed 10 days after injection. Dots represent mean ± SE (n = 6). **C.** Testes from control (27°C, left) and busulfan-depleted animals. Inset shows a high magnification of the depleted testis after in vivo injection via the urogenital pore of a solution containing trypan blue. **D.** Frequency of spermatogenic cysts after a single dose of 30 or 40 mg/Kg busulfan. Type A spermatogonia (SGA), type B spermatogonia (SGB), spermatocytes (SC), apoptosis and abnormal metaphase I figures (A+AMF), Sertoli cell only (SCO). Bars represent means expressed as percentage. **E–I.** Histological sections of testes collected at 0 (**E**), 2 (**F**), 4 (**G**), 6 (**H**), and 10 (**I**) days after a single injection of 40 mg/Kg busulfan at 35°C. Note that busulfan induced spermatogonial apoptosis (arrowheads) after 4 days of injection. Scale bars = 10 μm. **J.** Testicular cell suspensions were obtained from *vasa::egfp* testes and subjected to FACS. Dot plot shows the total testicular suspension from which a population of large cells (forward scatter, FSC; abscissa), showing an intense fluorescence (FL1 FITC; ordinate) was sorted (black square). **K.** Histogram shows an enrichment of the sorted cells after FACS. Blue line (sorted cells), black line (total testicular cell suspension). FL1 FITC (x axis) means intensity of fluorescence, and counts (y axis), the number of events. **L,M.** Fraction of sorted cells under normal light (**L**) and under fluorescence (**M**). Arrow indicates a large cell carrying high fluorescence. Scale bars = 10 μm. **N.** Histogram showing the percentage of germ cells in the unsorted and sorted fractions. Despite of the contamination with other germ cell types (cell clumping before FACS), there is an enrichment of type A undifferentiated spermatogonia (A_{und*}/A_{und}) population, which might contain SSC candidates. Undifferentiated type A spermatogonia (A_{und*} and A_{und}), differentiating type A spermatogonia (A_{diff}), type B spermatogonia, spermatocytes (SC), spermatids (ST), spermatozoa (SZ), apoptosis (A) and others (O). Bars represent means expressed as percentage. **O.** Germ cell transplantation into zebrafish genital pore using a glass capillary needle.
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found in the recipient seminiferous epithelium (Figure 5E,F). The *vasa* expression pattern in donor-derived germ cells was the same as observed in *vasa::egfp* testes immunostained for GFP, or in wild-type testes immunostained for Vasa (Figure S6). The transplantation efficiency was considered not high (30%) as colonization and donor-derived spermatogenesis was observed in about 3 out of 10 recipients. With regard to SSC transplantation into females, cell clusters derived from transplanted SSC were found in recipient ovaries after three weeks of transplantation (Figure 6A, Video S4). Small GFP-positive oocytes at an early stage of oocyte development were also found in recipient females (Figure 6B,C). Surprisingly, these male-derived germ cells progressed into oocyte development, and gave rise to advanced oocytes after one month of transplantation (Figure 6D–F; Video S5). Clusters of GFP-positive cells, possibly clones of oogonia, were also found near male-derived oocytes (Figure 6G,H). The presence of GFP-positive cells in both transplanted males (three weeks after transplantation) and females (one month after transplantation) was also confirmed by PCR analysis (Figure S7).

Hormonal characterization and gene expression in busulfan-depleted zebrafish

The relatively low efficiency of colonization after transplantation triggered studies on the recipient's testicular microenvironment prior to transplantation. The plasma levels of 11-KT were ~3 times higher in fish kept at 35°C (with or without busulfan injection) than in non-treated zebrafish kept at 27°C (Figure 7A). The temperature-induced elevation of 11-KT plasma levels was similar to the stimulatory effect of hCG (10 IU/g) or recombinant zebrafish Fsh (100 ng/g) [33]. Also, testicular 11-KT release in primary culture was higher from tissue of males exposed to 35°C and busulfan than from testis tissue of control males kept at 27°C, in particular with regard to basal release (7-fold higher), while the difference in forskolin-stimulated androgen did not reach statistical significance (Figure 7B). Quantifying the expression of selected genes revealed that transcript levels of steroidogenesis and androgen signaling genes (*star, ar, cyp17a1*) had increased 3-fold in busulfan-depleted testes (Figure 7C). In most cases, exposure to 35°C alone did not change gene expression. There are two interesting exceptions. The transcript levels of *amh* were significantly down-regulated at 35°C and in busulfan-depleted testes, while *igf1b* transcript levels were strongly up-regulated. Expression of germ cell-specific genes, such as, *piwil1* and *sycp3l* significantly decreased only on busulfan-treated animals (Figure 7C), while *gsdf* and *insl3* mRNA levels did not change among the groups (Figure 7C).

Discussion

Using a BrdU pulse-chase approach, we identified the LRC population in zebrafish testis. Within this category of testicular cells, somatic cells were found (e.g. Sertoli and interstitial cells), but type A undifferentiated spermatogonia (A_{und*} and A_{und}) were the only germ cells able to retain the label after 18 and 25 days of chase, constituting therefore the putative SSCs. Two distinct patterns of BrdU dilution were found among the two subtypes of type A spermatogonia in zebrafish. The BrdU dilution kinetics for type A_{und} showed a rapid decline that stabilized at the 18th day after chase, while the percentage of BrdU-positive A_{und*} spermatogonia remained constant. Applying mathematical modeling analysis to hematopoietic stem cells (HSCs) [39], a similar biphasic profile of BrdU dilution was described. The initial, rapid decline was attributed to the faster turnover of activated cells, while the subsequent, decelerated decline resulted from the slow turnover of quiescent cells. This suggested the presence of a heterogeneous population of HSCs which can be activated or deactivated according to the systemic needs [39]. Likewise, the biphasic profile found in zebrafish testes might be attributed to functional differences between the two subtypes of spermatogonia, in which A_{und} would be the more rapidly dividing, "active" population (related to differentiation and rapid proliferation, as indicated by the more rapid loss of BrdU), and A_{und*}, the slow-cycling, "reserve" population (related to slow self-renewal proliferation, as indicated by the relatively stable BrdU labeling index). Also in the human testis, two types of single A spermatogonia (pale and dark) are present, and might play distinct roles as "reserve" versus "active" stem cell, respectively [40,41]. In another teleost fish, in female medaka (*Oryzias latipes*), a BrdU pulse-chase experiment also revealed two distinct populations of oogonial stem cell, one being fast and the other a slow-dividing cell population [42]. Across the vertebrates, it seems that tissues with constant demand for stem cells to proliferate and generate differentiated cells, two populations of stem cells are required; one to rapidly expand and produce differentiated cells, and another which rarely divides and functions as "reserve" in case of insults.

According to the "immortal strand hypothesis" [43], stem cells divide asymmetrically, since a hypothetical mechanism would sort chromatids containing the original template DNA strands in one daughter cell, passing therefore the new strands to the other daughter cell [44]. Hence, in a stem cell self-renewal division, the new stem cell would inherit the original templates, the other daughter cell, destined to differentiate, would inherit the new strands. Recently, it has been demonstrated that the "immortal strand hypothesis" is not exclusive of self-renewing cells, but also of divisions in which daughter cells adopt divergent fates [45]. In this case, daughter cells inheriting the original templates would also retain the more immature phenotype, whereas daughter cells inheriting the newer templates acquired a more differentiated phenotype [45]. The BrdU dilution for A_{und} suggests the existence

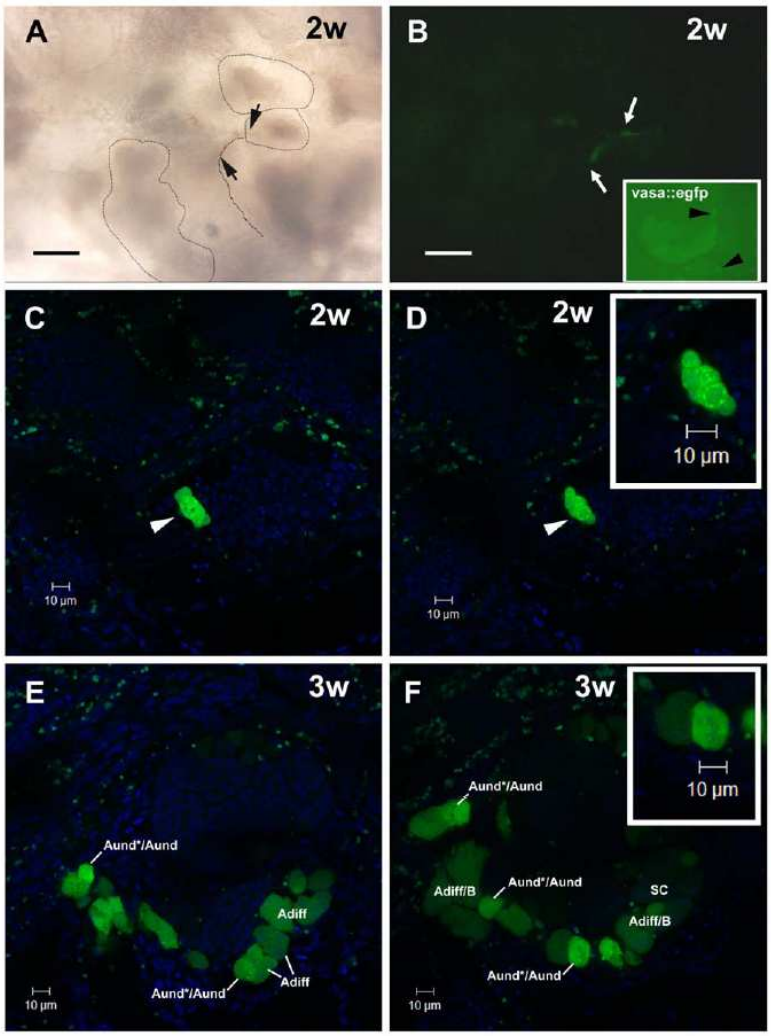

Figure 5. SSC transplantation into male zebrafish recipients. A,B. Recipient testes after two weeks (2w) of transplantation analyzed by light (**A**) and fluorescence (**B**) microscopies. Seminiferous tubules are delimited by stippled lines. Arrows indicate the same area in **A** and **B**. Donor cells formed clusters situated near the interstitium in a similar way as observed in *vasa::egfp* testes (see arrowheads in the inset). Nuclei (blue) are stained with DAPI. Scale bars = 50 µm. **C,D.** CLSM analysis of recipient testes after two weeks (2w) of transplantation. Arrowheads indicate a donor-derived cyst composed of ~8 cells. **Inset.** High magnification of donor-derived cyst. Nuclei (blue) are stained with DAPI. **E,F.** Recipient testes after 3 weeks (3w) of transplantation analyzed under CLSM. Donor-derived cysts increased their number and size, being found at different stages of spermatogenesis. Type A undifferentiated spermatogonia (A_{und*}/A_{und}), type A differentiating spermatogonia (A_{diff}), type B spermatogonia (B), and spermatocytes (SC). **Inset** shows a high magnification of type A undifferentiated spermatogonia. Nuclei (blue) are stained with DAPI.
doi:10.1371/journal.pone.0012808.g005

of two types of template DNA strand segregation, one random type, possibly reflecting the rapid loss of the label up to the 18th day of chase, and one non-random type (asymmetrical division), which could explain the retention of BrdU after 18 and 25 days of chase. We speculate that the asymmetrical division of A_{und} might prevent its exhaustion during spermatogenesis, maintaining, therefore, a subset of immature A_{und}. An asymmetrical division of type A undifferentiated spermatogonia was suggested previously in zebrafish based on morphological evidence [2].

The LRC assay also revealed the precise location of stem cells, and consequently the characterization of the neighboring cells that form the stem cell niche [46–48]. As in rodents [6,7], we found that slow-cycling BrdU-labeled type A undifferentiated spermatogonia (A_{und*} and A_{und}) were preferentially situated in regions of the seminiferous tubules adjacent to the interstitial compartment. This observation suggests that elements of the SSC niche are probably conserved across vertebrates. The preferential location of SSC close to the interstitial compartment in rodents may be related to a locally high concentration of androgens which inhibit spermatogonial differentiation [49]. 11-KT is the main androgen in zebrafish [37] and other teleost fish, and is able to support full spermatogenesis in testis tissue culture of juvenile Japanese eel (*Anguilla japonica*) [50] or adult zebrafish [38]. Moreover, induction of androgen insufficiency in adult male zebrafish inhibited the differentiation of type A to type B spermatogonia [51]. Although the possible role(s) of androgen signaling in regulating zebrafish

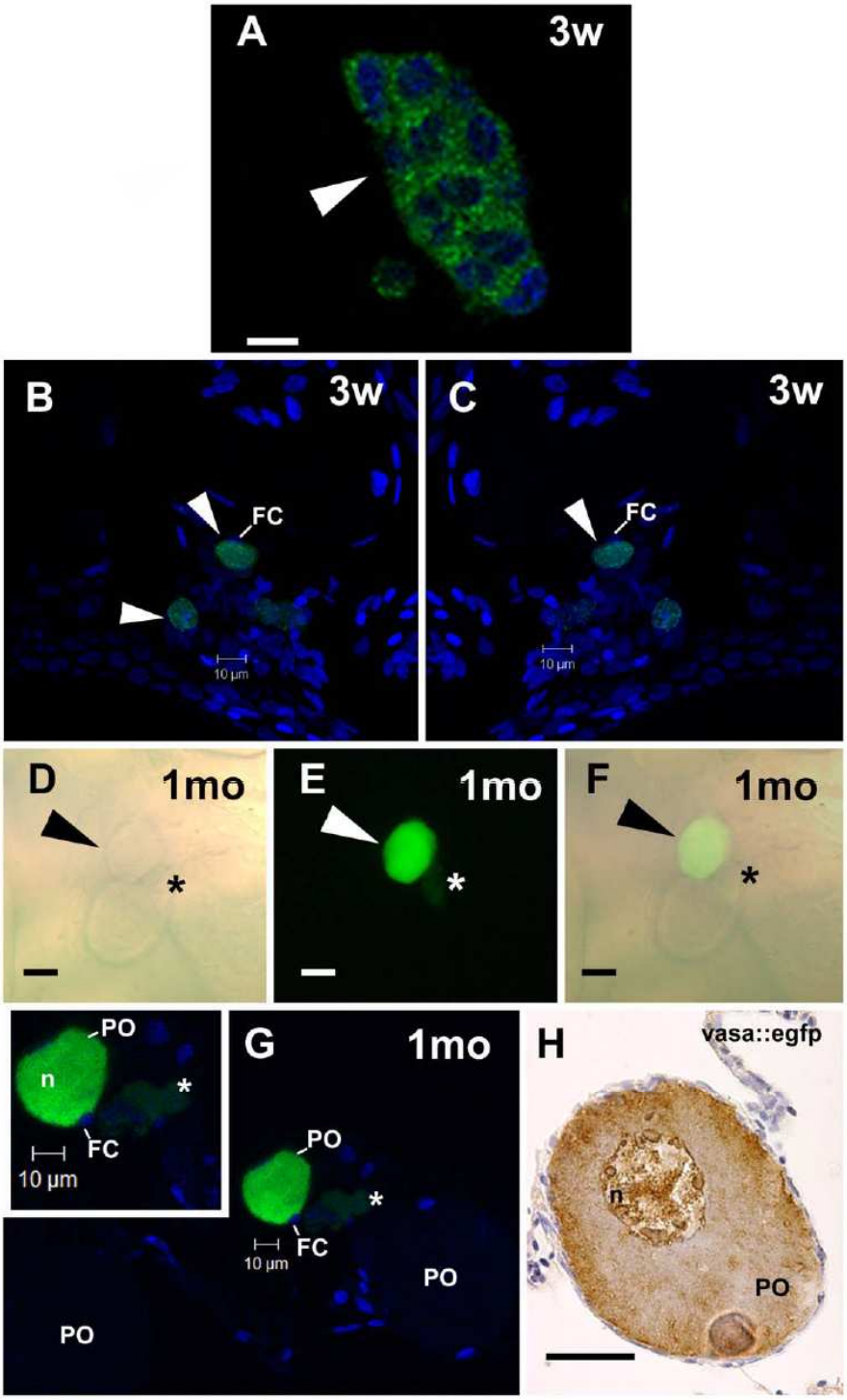

Figure 6. SSC transplantation into female zebrafish recipients analyzed under CLSM. A. A GFP cell cluster-derived from transplanted SSC after three weeks (3w) of transplantation. Nuclei (blue) are stained with DAPI. Scale bar = 10 µm. **B,C.** Arrowhead indicates an early donor-derived oocytes surrounded by follicle cells (FC) after three weeks (3w) of transplantation. Nuclei (blue) are stained with DAPI. **D–F.** Arrowhead indicates an advanced GFP oocyte, which was originated from transplanted SSC into zebrafish ovaries after one month (1mo) of transplantation. Note a small GFP cell cluster (asterisk) near the donor-derived oocyte. Light (**D**) and fluorescence (**E**) microscopies, and overlay of both (**F**). Scale bars = 25 µm. **G.** The same oocyte in **D–F** examined under CLSM. A green donor-derived perinucleolar oocyte (PO), endogenous perinucleolar oocyte (PO), small GFP cell cluster (asterisk), and nucleus (n) are shown. **Inset.** High magnification of advanced donor-derived oocyte. Nuclei (blue) are stained with DAPI. **H.**

Perinucleolar oocyte from *vasa::egfp* ovaries immunostained for gfp. Compare similar *vasa* expression pattern between donor-derived oocyte and perinucleolar oocyte from *vasa::egfp* ovaries. Scale bar = 25 μm.
doi:10.1371/journal.pone.0012808.g006

SSC activity still have to be clarified, the available evidence in fish strongly suggests that, different from mammals, androgens stimulate spermatogonial differentiation [2]. On the other hand, estrogens stimulated SSC self-renewal in Japanese eel [52], so that Leydig cells may be relevant as a source for aromatizeable androgens, thereby contributing directly and/or indirectly to the zebrafish SSC niche. Moreover, Leydig cell paracrine signaling might influence SSC behavior, since colony stimulating factor 1 (Csf1) produced by Leydig and myoid cells is an extrinsic stimulator of SSC self-renewal in mice [10].

Endothelial cells support the expansion of normal and malignant stem cells not only by delivering oxygen and nutrients, but also by the paracrine release of endothelial cell growth factors and trophogens, which are referred to as "*angiocrine factors*" [53]. Thus, endothelial cells have been pointed out as an important element of the stem cell niche in several systems, including the rodent SSC niche [8,54]. In the zebrafish model, the distribution of SSC candidates (A_{und*} and A_{und}) close to blood vessels might indicate an involvement of endothelial cells in regulating SSC function. While there is no information on angiocrine signaling in the zebrafish testis, it has been shown that platelet-derived endothelial cell growth factor (PD-ECGF) induced SSC self-renewal in Japanese eel [55,56].

The functional capacity of the identified SSC candidates in zebrafish was investigated by transplantation assays. In this assay, SSC candidates were transplanted into zebrafish in which endogenous spermatogenesis had been depleted by busulfan, similar to the technique developed for rodents and others species [14–17,19]. It seems that similar to tilapia [16], zebrafish spermatogenesis is more sensitive to busulfan at elevated temperatures (35°C), resulting in 88% of spermatogenic tubules showing Sertoli cells only after 10 days of treatment. Interestingly, spermatogenesis recovered quickly after having passed the nadir of busulfan-induced depletion. It has been shown previously that proliferation of surviving, undifferentiated spermatogonia is greatly enhanced when the number of differentiating spermatogonia is reduced following busulfan exposure [57,58]. In addition, differentiated spermatogonia can act as "potential stem cells", shifting their nature of transit-amplifying cells to self-renewal in order to rapidly recover spermatogenesis in depleted testes [59]. Analyzing the composition of type A spermatogonia in normal versus busulfan-depleted testes in zebrafish, we have demonstrated a relative increase in the population of A_{und}, and a relative decrease in the population of A_{und*} spermatogonia in busulfan-depleted testes. Then, considering A_{und} as the "active" stem cell, our results suggest that the spermatogenic recovery after busulfan treatment might be associated with a preponderance of the active stem cell population A_{und}.

The treatment with busulfan induced an increase in androgen plasma levels as well as an increase in testicular androgen release. This might reflect a suppression of the negative feedback on gonadotropin release. In rat, the negative feedback on FSH release exerted by germ cells via Sertoli cell-derived inhibin is transiently eliminated after busulfan treatment [60], explaining the increase in circulating FSH levels between 6 and 10 weeks after busulfan injection [61,62]. In mammals, androgen plasma levels were not elevated in busulfan-treated animals [60–62]. However, in fish, also Leydig cells express the receptor for Fsh, which is a strongly steroidogenic gonadotropin in fish (e.g. [33,63,64]). Hence, we attribute the increased androgen levels after busulfan treatment to higher Fsh plasma levels. Consistent with the elevated androgen production, busulfan treatment increased the mRNA levels of transcripts encoding proteins involved in steroidogenesis, such as *star* and *cyp17a1*. The down-regulation of *amh* mRNA expression in busulfan-depleted testes also seems coherent with higher androgen levels, since androgens down-regulated *amh* expression in juvenile Japanese eel testis and stimulated spermatogonial differentiation towards meiosis [65]. Moreover, since Sertoli cells also proliferate in the adult fish testis, in particular those associated with expanding spermatogonial cysts [23,24], the busulfan treatment may have depleted the proliferating Sertoli cell population as well, thereby contributing to lower levels of *amh* mRNA, and possibly also to the arrest in spermatogenesis. With regard to the expression of *igf* gene family members, a strong up-regulation in particular of *igf1b* mRNA levels has been observed. This is interesting in the context of ongoing work showing that recombinant zebrafish Fsh increases *igf1b* levels in adult testis tissue culture (unpublished data). Spermatogonial proliferation was stimulated in newt testis in primary tissue culture by exposure to Igf1 [66]. Since Sertoli cells also express a factor (similar to platelet-derived endothelial cell growth factor) that stimulates SSC self-renewal in Japanese eel [55], busulfan-induced changes in the number and/or physiological state of Sertoli cells might have resulted in a more generalized imbalance of factors released by Sertoli cells and relevant for SSC self-renewal and differentiation. Taken together, our data suggest that busulfan treatment might create a microenvironment in zebrafish testes where the balance of somatic (mainly Sertoli) cell-derived factors has shifted to a reduced level of factors supporting self-renewal and/or an elevated level of factors stimulating differentiation. While this might facilitate the fast recovery after the severe loss of germ cells induced by busulfan, it may also influence the behavior of transplanted cells. Thus, transplanted SSCs may differentiate rather than self-renew, possibly limiting the efficiency of donor-colonization (30%) in the recipient seminiferous tubules; however, a relatively low efficiency of donor-derived colonization is not exceptional for zebrafish and has been observed in other vertebrates as well [67]. Future studies will aim at weakening the pro-differentiation or strengthening the self-renewal environment, for example by an estrogen treatment [51] of the recipients.

Despite of the limited efficiency, we have taken important steps towards the development and standardization of SSC transplantation assay to confirm the "stemness" of SSC candidates in zebrafish. Transplantation of a FACS-enriched fraction of A_{und*} and A_{und} into busulfan-depleted testes showed that stem cell candidates were able to colonize, self-renew, and to differentiate in the recipient seminiferous tubules. This confirms the presence of stem cells in the transplanted pool of type A undifferentiated spermatogonia. Moreover, spermatogonial colonization started from regions within the recipient's seminiferous tubules which are adjacent to the interstitial compartment, the putative SSC niche in zebrafish testes. This might indicate that the SSC niche creates a cellular and molecular environment which is optimal for colonization and development of transplanted SSCs. Since zebrafish have been used as a model for understanding the mechanisms of testicular germ cell tumors [68], and most of these tumors have an unknown etiology, a standard experimental design including reciprocal transplantation of germ cells from affected donors to wild-type testes and vice versa could be applied to evaluate whether the defect is on Sertoli cells or intrinsic to the

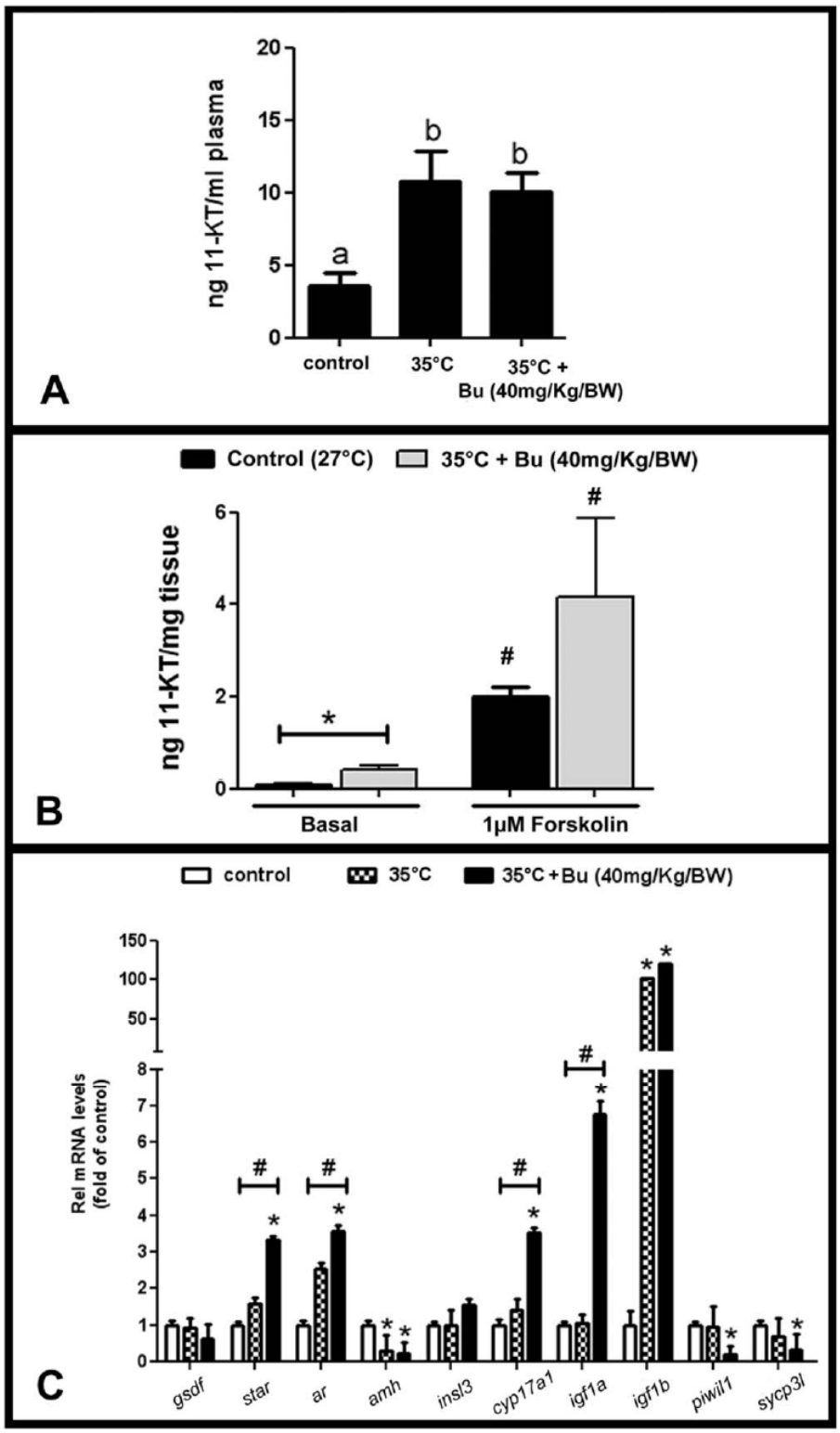

Figure 7. Characterization of male recipients prior SSC transplantation. A. 11-KT plasma levels (ng/ml plasma) from zebrafish kept at 27°C (control) (n = 7), 35°C (n = 11), and busulfan-depleted animals (n = 19). Bars represent the mean±SE. Different letters mean significant differences ($p<0.05$) among groups. **B.** In vitro 11-KT (ng/mg testis) release from control (27°C) (n = 7) and busulfan-depleted testes [35°C + Bu (40 mg/Kg/BW)] (n = 7) in basal and 1 µM forskolin-induced. Bars represent the mean±SE. * means significant differences ($p<0.05$) between control and depleted in the same experimental condition (Student unpaired t-test). # indicates significantly higher ($p<0.05$) than the respective basal release (Student paired

t-test). **C.** Relative mRNA levels of *gsdf, star, amh, insl3, cyp17a, igf1a, igf1b, piwil1* and *sycp3l* from control (27°C) (n = 7), 35°C (n = 4), and busulfan-depleted testes [35°C + Bu (40 mg/Kg/BW)] (n = 12). Bars represent the mean±SE of relative mRNA levels normalized to *β-actin1* or *ef1α*, and expressed as fold of relative control (27°C) mRNA levels. * and # are significantly different ($p<0.05$) from control levels (Student unpaired t-test) and among the groups (ANOVA), respectively.
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germ cells. Another important application of germ cell transplantation is transgenesis through the male germ line using transplantation of transfected germ cells [3,19]. This might be an option to create transgenic animals in a shorter time than is possible with conventional methods [19].

Finally, we have demonstrated the plasticity of the transplanted spermatogonia by placing the SSC candidates into a different microenvironment, i.e. into an ovary. SSC candidates were able to colonize recipient ovaries and differentiate into female germ line cells.

We present in the current work characteristics of zebrafish SSC candidates (A_{und*} and A_{und}), their niche, and their functional capability to both self-renew and differentiate in recipient testes, as well as their remarkable plasticity in recipient ovaries. To our knowledge, this is the first work to report on a SSC niche in fish. As in rodents, SSC location in zebrafish was characterized within areas of the seminiferous tubules which are opposite to the interstitial compartment, suggesting the influence of interstitial elements on SSC self-renewal and maintenance. We developed and standardized SSC transplantation techniques in male and female zebrafish, showing donor-derived spermatogenesis and male-derived oocytes, respectively, after transplantation. Thus, we introduced SSC transplantation in zebrafish as a promising technique for the study of SSCs.

Supporting Information

Figure S1 Effects of different temperatures on zebrafish spermatogenesis. A. More advanced labeled germ cell after 2 h, 12 h, 1d, 2d, 3d, 4d, 5d, 6d of 3H-thymidine injection in zebrafish males kept at 20°C, 27°C, 30°C, and 35°C. Leptotene/zygotene (L/Z) and pachytene (P) spermatocytes, metaphase I (MI), initial spermatids (E1), intermediate spermatids (E2), final spermatids (E3) and spermatozoa (Z). Scale bars = 10 μm. Cells were considered labeled when four to five or more grains were present over the nucleus in the presence of low-to-moderate background (i.e., very few grains per histological field observed under oil immersion). Black squares indicate the time in which labeled spermatozoa were found in the lumen of zebrafish testes at different temperatures. B. Histogram showing the combined duration of meiotic and spermiogenic phases at different temperatures. Spermatogenesis did not progress beyond the first meiotic division at 35°C. X axis represents the time, whereas y axis the 3H-thymidine labeled germ cell [Leptotene/zygotene (L/Z), pachytene (P) and diplotene (D) spermatocytes, metaphase I (MI), initial spermatids (E1), intermediate spermatids (E2), final spermatids (E3) and spermatozoa (Z)]. C–E. Histological sections of zebrafish testes at 27°C, 30°C, and 35°C. Sperm free and a massive germ cell apoptosis is seen in zebrafish seminiferous tubules at 35°C. Scale bars = 50 μm.
Found at: doi:10.1371/journal.pone.0012808.s001 (4.05 MB TIF)

Figure S2 Standardization of SSC transplantation techniques. A. Transplantation device, in which a glass capillary needle is coupled to a peristaltic pump. B,C. Standardization of transplantation via using trypan blue to monitor the efficiency of the injections. Note trypan blue inside the testis (B) and ovaries (C). D,E. Genital pore histological sections stained with toluidine blue (male - D, female - E). Genital pore measurements: 120×80 μm dimensions, 63° angle (female); 75×50 μm dimensions, 60° angle (male). Anus (A), genital pore (G, arrow), and urethra (U). Scale bars = 100 μm. F. Ovary cell transplantation throughout injections into the lateral body wall (indicated by stippled line). G. Trypan blue was used to monitor the specificity of the injection. Note trypan blue inside the ovaries.
Found at: doi:10.1371/journal.pone.0012808.s002 (2.98 MB TIF)

Figure S3 Scatter plot to check the stability of 18S rRNA, β-actin1 and ef1α mRNAs as a housekeeping gene in 35°C (n = 4), and depleted testes (35°C + busulfan 40 mg/Kg/BW) (n = 12). Each dot in the scatter plot represents the average Cq-value of duplicate measurements for each fish in the different experimental condition. Stability was seen only between β-actin1 and ef1α. Control (27°C) was not shown, but followed the same pattern.
Found at: doi:10.1371/journal.pone.0012808.s003 (0.26 MB TIF)

Figure S4 The hypothetical SSC niche in zebrafish testes. SSC niche is indicated by a red circled line. The niche is constituted by elements of the tubular and interstitial compartments (IC) such as: Sertoli cells (SE); basement membrane (BM); peritubular myoid cells (PM); Leydig cells (LE), blood vessels (BV); and other interstitial elements. Type A undifferentiated spermatogonia (Aund*/Aund), type A differentiated spermatogonia (Adiff), type B early spermatogonia (B early) and type B late spermatogonia (B late) and spermatozoa (SZ) are illustrated.
Found at: doi:10.1371/journal.pone.0012808.s004 (2.57 MB TIF)

Figure S5 Preparation of female zebrafish recipients for SSC transplantation. A–C. Ovaries histological sections of ovoposition-induced females stained with PAS (Periodic acid Schiff). Arrows indicate postovulatory follicles after ovoposition. Note in C the available follicle cells which can support the transplanted SSC development. Scales bars = 250 μm (A), 100 μm (B), 25 μm (C). D. Number of postovulatory follicles (POFs)/mm2 of ovary in control (n = 5) and ovopositioned females (n = 5). Bars represent the mean± SE. * means significant differences ($p<0.05$) between the groups (Student unpaired t-test).
Found at: doi:10.1371/journal.pone.0012808.s005 (1.53 MB TIF)

Figure S6 Vasa expression pattern during male germ cell development using gfp immunodetection in sections of vasa::egfp testes (A), or vasa immunodetection in cryosections of wild-type testes (B). Type A undifferentiated spermatogonia (Aund*/Aund), type A differentiating spermatogonia (Adiff), type B spermatogonia (B), spermatocytes (SC), spermatids (ST), spermatozoa (SZ). Scale bars = 25 μm (A) and 10 μm (B).
Found at: doi:10.1371/journal.pone.0012808.s006 (2.83 MB TIF)

Figure S7 PCR analysis for GFP detection using primers located in the YFP gene, 2% agarose gel containing ethidium bromide, showing the detection of a faint background band. A. vasa::egfp testes were used as a positive control. Transplanted male after 3 weeks of transplantation, transplanted female after 1 month of transplantation, H2O water as negative control. Bands at left side are DNA markers from SMART ladder (Eurogentec). B. PCR detection using primers located in the PD-ECGF (plated-derived endothelial cell growth factor) gene as positive control for genomic DNA in the different individuals, 2% agarose gel containing ethidium bromide, showing the detection of a faint background band. Bands at left side are DNA markers from SMART ladder.

Found at: doi:10.1371/journal.pone.0012808.s007 (0.82 MB TIF)

Video S1 Z stack from vasa::egfp testis showing the distribution of vasa positive cells in the zebrafish seminiferous tubules.
Found at: doi:10.1371/journal.pone.0012808.s008 (1.63 MB WMV)

Video S2 Z stack from wild-type zebrafish testis 2 weeks after SSC transplantation. Note a donor-derived cyst composed of ~8 cells in the seminiferous epithelium.
Found at: doi:10.1371/journal.pone.0012808.s009 (0.83 MB WMV)

Video S3 Z stack from wild-type zebrafish testis 3 weeks after SSC transplantation. SSCs were able to colonize recipient testis and differentiate into daughter cells committed with the spermatogenic process. Donor-derived cysts at different stages of zebrafish spermatogenesis were found in recipient seminiferous epithelium.
Found at: doi:10.1371/journal.pone.0012808.s010 (0.81 MB WMV)

Video S4 Z stack from ovaries fragments 3 weeks after SSC transplantation into female zebrafish ovaries. Small cell clusters-derived from SSC were seen in female ovaries.

Found at: doi:10.1371/journal.pone.0012808.s011 (1.10 MB WMV)

Video S5 Z stack from ovaries fragments 1 month after SSC transplantation. SSCs were able to colonize female ovaries and differentiate into female germ cell line. Note a male-derived oocyte in the female recipient ovary.
Found at: doi:10.1371/journal.pone.0012808.s012 (0.60 MB WMV)

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Author Contributions

Conceived and designed the experiments: RHN CDG JB LRdF RWS. Performed the experiments: RHN CDG HvdK. Analyzed the data: RHN CDG HvdK JB LRdF RWS. Contributed reagents/materials/analysis tools: RHN LRdF RWS. Wrote the paper: RHN JB LRdF RWS. Molecular biology, primers design, qPCR, PCR: JB.

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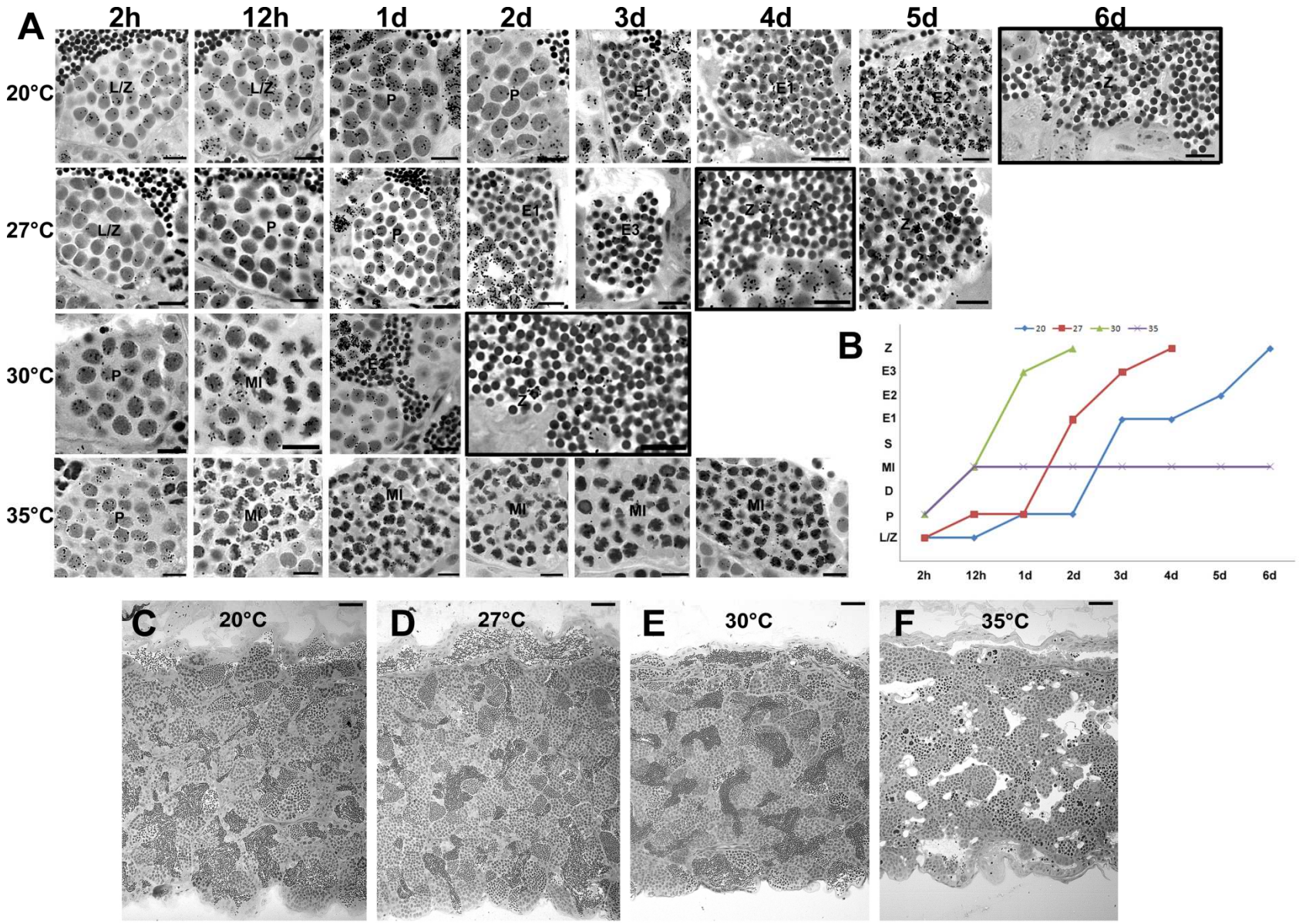
A
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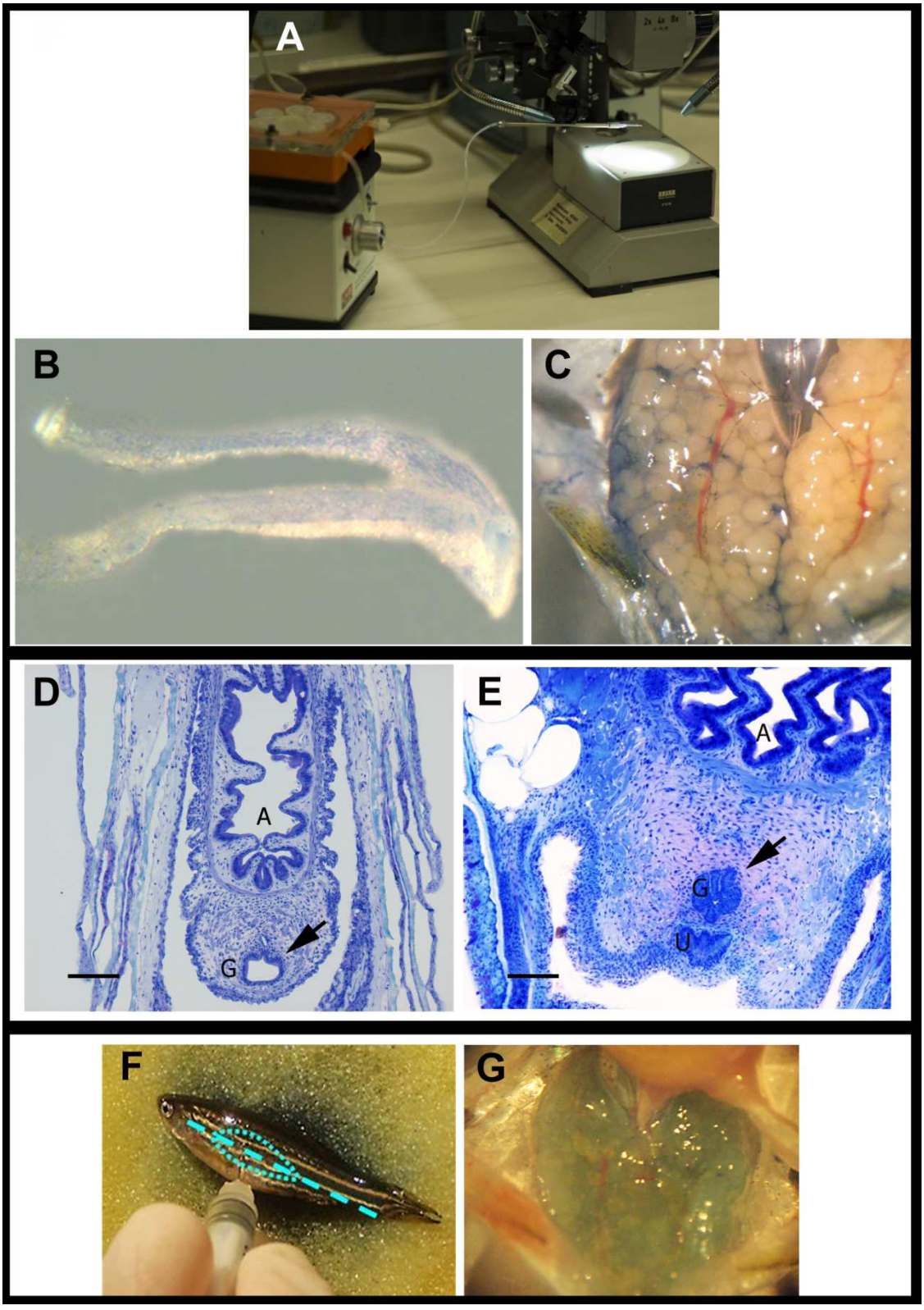
Figure S1.

Figure S2.



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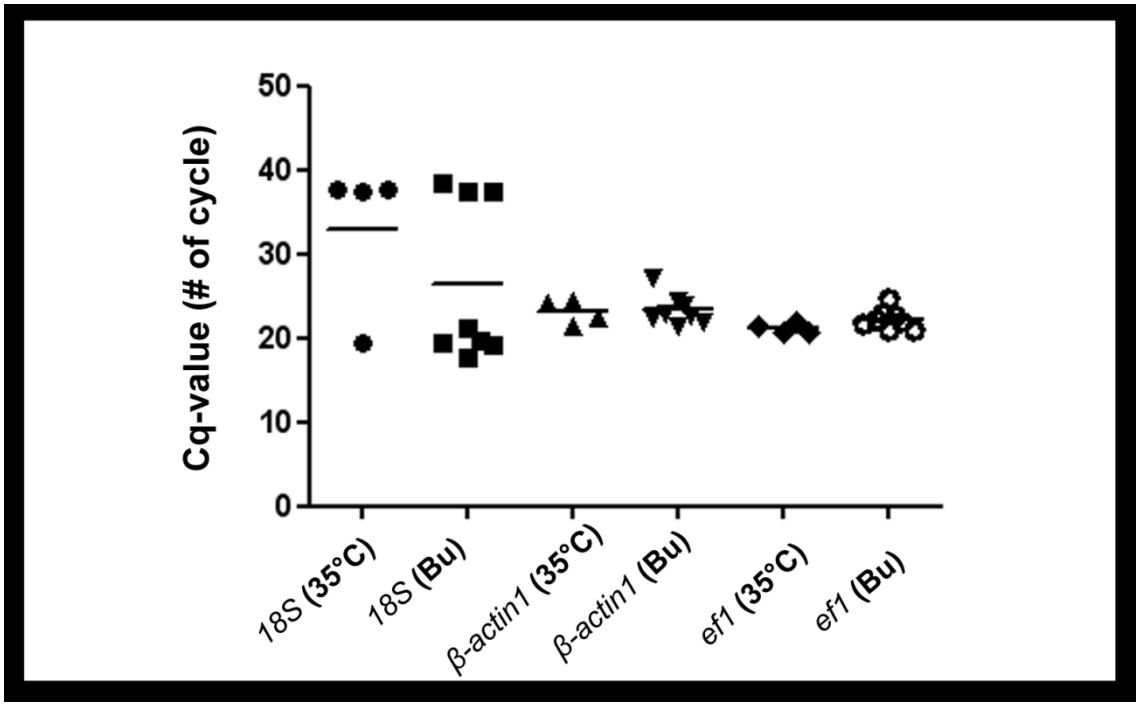
Cq-value (# of cycle)
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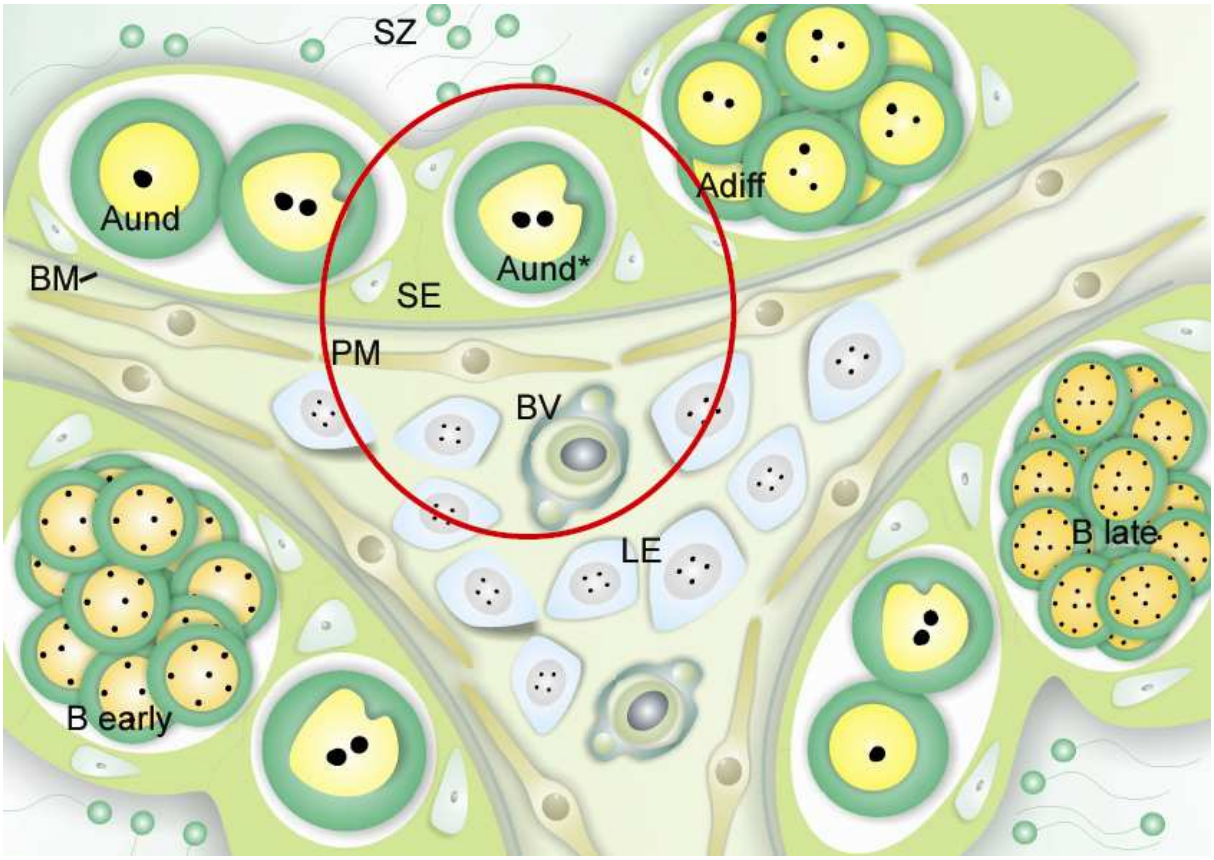
β-actin1 (Bu)
ef1 (35°C)
ef1 (Bu)

Figure S4.



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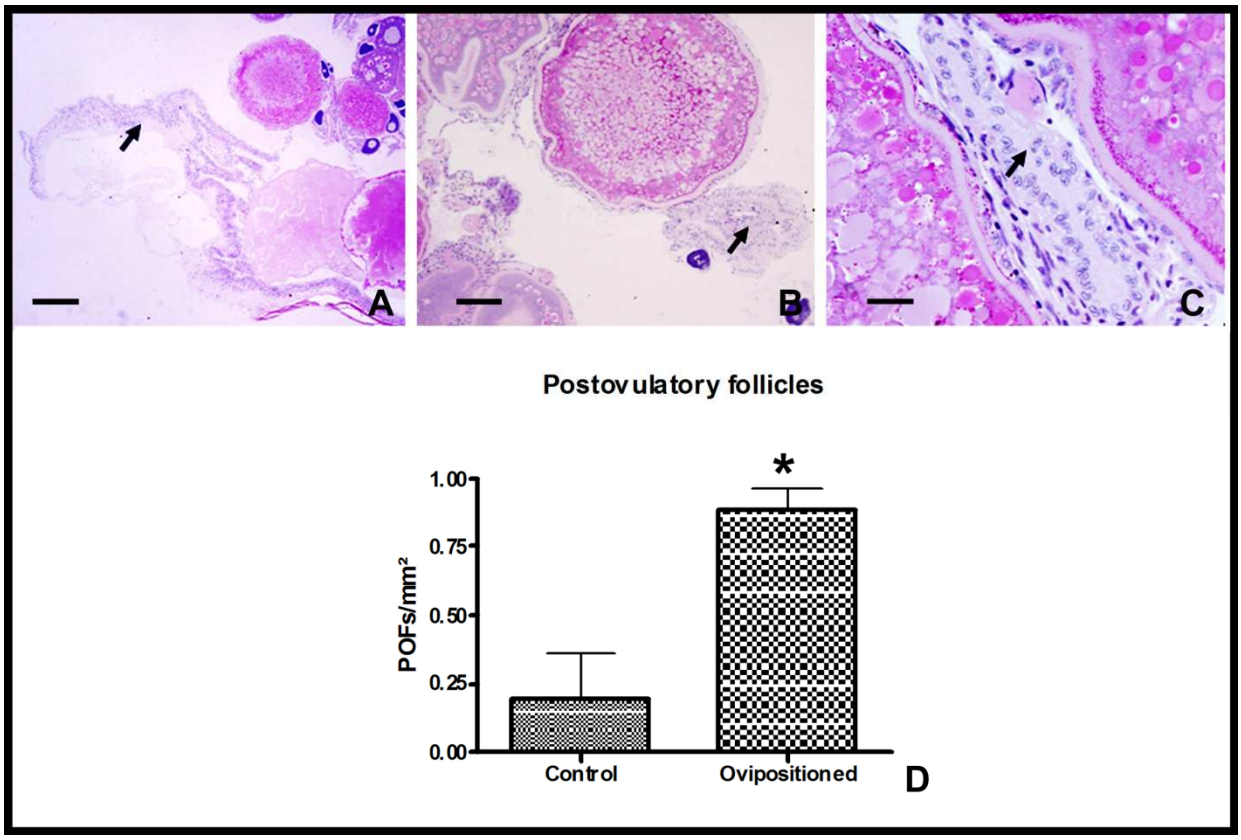
A
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Postovulatory follicles
POFs/mm²
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Control
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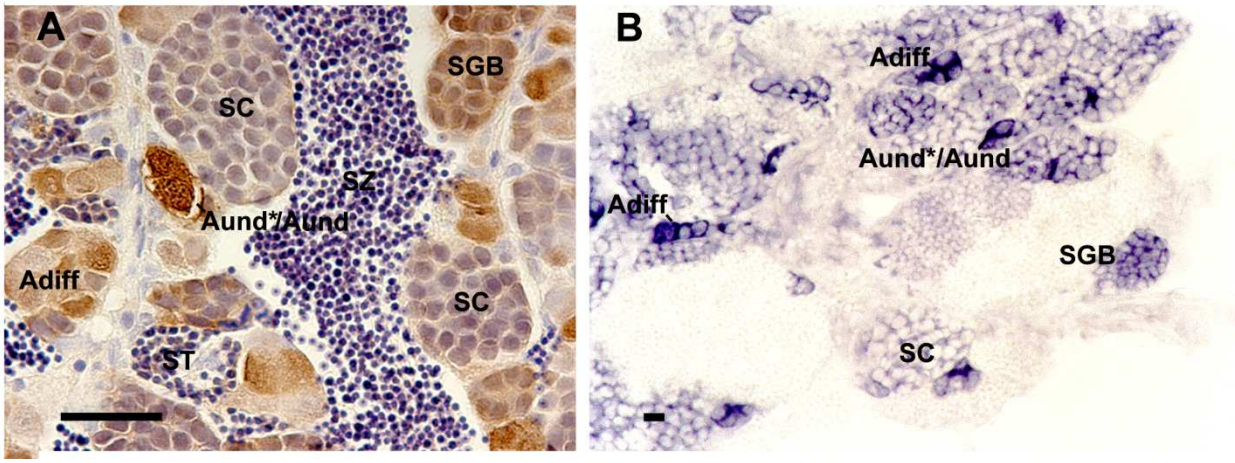
D

Figure S6.



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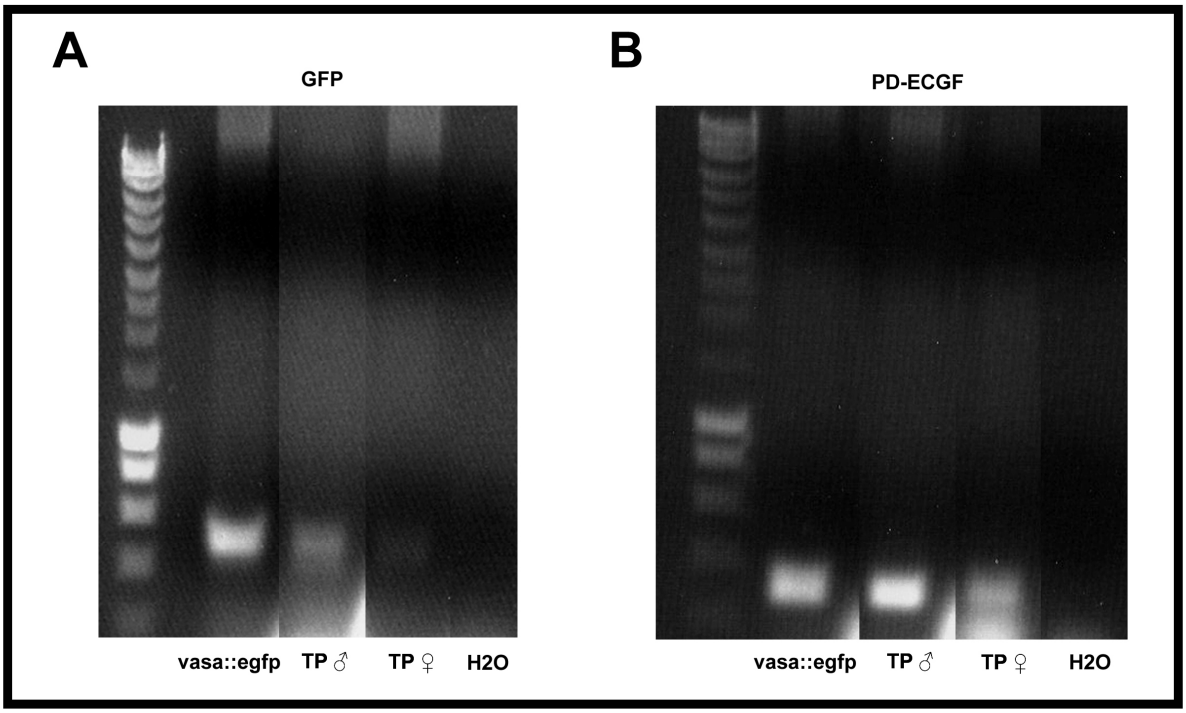

4. Perspectivas e Conclusões

No nosso entendimento, os resultados obtidos no presente estudo propiciam novos conhecimentos ao cenário da biologia espermatogonial em peixes teleósteos, particularmente no que diz respeito às caracterizações dos diferentes tipos de espermatogônias e do nicho espermatogonial, bem como nos aspectos referentes à padronização das técnicas de transplante de espermatogônias tronco em zebrafish.

Nas últimas décadas, a nomenclatura das espermatogônias em peixes teleósteos tem sido utilizada de forma inconsistente. Classicamente, dois tipos de espermatogônias são caracterizadas e descritas em peixes: espermatogônias do tipo A, também consideradas isoladas ou primárias; e espermatogônias do tipo B ou secundárias, que são espermatogônias resultantes da primeira divisão da espermatogônia primária. Esta classificação, além de não mencionar a existência de células tronco, também agrupa distintos tipos espermatogoniais no grupo das espermatogônia do tipo B. Desta forma, propusemos uma nova terminologia espermatogonial, com base no número de células por cisto, utilizando ainda os critérios tradicionalmente empregados para roedores tais como morfologia nuclear, número e disposição de nucléolos, e tipo e padrão de distribuição de cromatina. Assim, as espermatogônias de zebrafish foram classificadas em: A_{und*} (isolada - célula tronco?) → A_{und} (isolada – célula tronco?) → A_{diff} (2-7 células por cisto) → B_{early} (14-28 células por cisto) → B_{late} (55-208 células por cisto). Embora as espermatogônias isoladas A_{und*} e A_{und} apresentem diferentes morfologias nucleares, não foi possível demonstrar se A_{und*} e A_{und} estão separadas por divisão mitótica ou se correspondem ao mesmo tipo celular em diferentes fases do ciclo celular. Experimentos que melhor caracterizem as espermatogônias isoladas em zebrafish, seja através de marcadores moleculares, ou mesmo através de técnicas de microarray, poderão futuramente elucidar esta questão. Além disso, o padrão de divisão das espermatogônias isoladas, se simétrico ou assimétrico, também é

desconhecido e merece, portanto, ser melhor investigado em peixes teleósteos.

Utilizando zebrafish transgênicos que expressam Gfp sob o promotor do gene específico de célula germinativa, *vasa*, e sabendo da propriedade das células tronco em reter BrdU (ou outros marcadores de proliferação) por longo período de tempo, o nicho espermatogonial em zebrafish pode também ser caracterizado. Nossos resultados mostraram que, semelhantemente ao nicho espermatogonial em mamíferos, as células tronco espermatogoniais em zebrafish localizam-se preferencialmente próximas ou adjacentes ao compartimento intersticial. Neste sentido, nossas observações sugerem que a atividade do nicho espermatogonial nesta espécie de teleósteo depende de fatores externos aos túbulos seminíferos tais como, por exemplo, gonadotropinas (Fsh, Lh) e andrógenos, ou ainda fatores parácrinos possivelmente secretados pelas células de Leydig, peritubulares mióides ou endoteliais, dentre outros tipos celulares. No entanto, assim como em mamíferos, a caracterização exata dos elementos celulares envolvidos neste processo, bem como a possível interação entre eles na regulação do destino das espermatogônias tronco, é ainda um grande desafio para os pesquisadores nesta área do conhecimento.

Através das técnicas de transplante de espermatogônias, padronizadas no presente estudo para zebrafish, demonstramos que frações enriquecidas de espermatogônias A_{und*} e A_{und} de doadores transgênicos *vasa::egfp* foram capazes de colonizar testículos de receptores selvagens (wild-type) cuja espermatogênese endógena havia sido previamente depletada com busulfan. As células transplantadas nos testículos dos peixes receptores desenvolveram-se em cistos de espermatogônias do tipo B e espermatócitos, 3 semanas após o transplante. Não foi possível observar cistos de espermátides, e tampouco espermatozóides, uma vez que a expressão de *vasa* diminui com o desenvolvimento das células germinativas. Também demonstramos que espermatogônias A_{und*} e A_{und} transplantadas em ovários de zebrafish se diferenciam em oócitos, sugerindo que o nicho receptor é fundamental para determinar o destino das espermatogônias tronco transplantadas. Assim, através das técnicas de transplante de espermatogônias tronco,

demonstramos que as espermatogônias A_{und*} e A_{und} possuem plasticidade e potencial tronco. Pois, além de colonizarem e resgatarem a espermatogênese após o transplante em testículos com espermatogênese endógena depletada, também foram capazes de se diferenciarem em oócitos em ovários, cujas células gametogênicas finais (oócitos) foram naturalmente desovadas.

Entretanto, deve ser salientado que a eficiência do transplante foi de 30%, o que de certa forma limita o emprego desta técnica para se estudar a biologia das espermatogônias tronco, ou mesmo, por exemplo, para gerar animais transgênicos. Meios que otimizem a eficiência da técnica de transplante merecem ser investigados. Uma das alternativas vislumbradas para se melhorar esta eficiência seria utilizar receptores naturalmente inférteis, como, por exemplo, animais híbridos resultantes do cruzamento entre *Danio rerio* e *Danio malabaricus*. Pois, ao que parece, o tratamento com busulfan cria um microambiente no testículo receptor que favorece diferenciação ao invés de colonização/auto-renovação, conforme demonstrado pelo perfil hormonal e gênico citados a seguir.

As análises hormonais e gênicas dos testículos depletados com busulfan antes do transplante nos permitiram ter uma melhor compreensão do comportamento das células transplantadas neste tipo de ambiente testicular. Assim, níveis elevados de andrógenos, associados à diminuição da expressão gênica do *amh* (hormônio anti-Mülleriano), além de níveis drasticamente elevados de *igf3* (*igf1b*) (fator de crescimento semelhante à insulina 3), sugerem a existência de ambiente (nicho) no animal receptor que favorece a diferenciação espermatogonial, ao invés da auto-renovação. Esta condição de pró-diferenciação do ambiente receptor provavelmente deve ter interferido no comportamento das espermatogônias tronco transplantadas. Neste sentido, estes importantes resultados nos levaram a desenvolver estudos que se encontram em andamento, e que se referem ao efeito biológico de determinados fatores de crescimento recombinante (Amh e Igf3) na regulação do nicho espermatogonial em zebrafish. Nestes estudos, observamos efeito "yin-yang" destes fatores de crescimento. Pois, enquanto o Amh bloqueia a diferenciação espermatogonial sem promover auto-renovação das

espermatogônias tronco, o Igf3, cuja expressão é estimulada por Fsh e andrógenos, estimula a diferenciação e proliferação espermatogonial. Ainda em relação aos peixes receptores que tiveram sua espermatogênese endógena depletada com busulfan, seria interessante investigar o efeito do estradiol nos níveis androgênicos e na expressão do *amh* e do *igf3*. Se o tratamento estrogênico minimizar os efeitos de pró-diferenciação observado no ambiente testicular dos peixes receptores, este método poderia ser utilizado como alternativa para se otimizar a colonização/auto-renovação das espermatogônias tronco após o transplante.

Em síntese, no presente estudo o zebrafish é apresentado como modelo experimental para se investigar a biologia da espermatogônia tronco em peixes teleósteos. Neste sentido, além de avançarmos o conhecimento sobre a caracterização das células germinativas espermatogoniais, particularmente acerca das espermatogônias tronco e sua localização (nicho), todas as técnicas necessárias ao transplante de espermatogônias em zebrafish foram padronizadas, permitindo, assim, através da utilização das mesmas, demonstrar funcionalmente o potencial de células tronco destas espermatogônias, tanto nos túbulos seminíferos quanto nos ovários de zebrafish. No entanto, a eficiência das técnicas de transplante, particularmente no que se refere à produção de número viável e contínuo de gametas, necessita ser ainda otimizada. Alternativas para se alcançarem estes objetivos seriam, por exemplo, minimizar os efeitos pró-diferenciação do ambiente testicular do animal receptor. Neste contexto, os resultados obtidos através das análises hormonais e gênicas nos forneceram importantes indícios de como alguns fatores de crescimento regulam o nicho espermatogonial. Estas valiosas informações poderão ser úteis no futuro para se melhor compreender a regulação das espermatogônias tronco em zebrafish, podendo ainda ser estendidas para outras espécies de peixes teleósteos, em especial àquelas de interesse econômico, ou mesmo ameaçadas de extinção. Abordagens visando controlar a puberdade ou expandir o número de espermatogônias tronco *in vitro* antes do transplante, com finalidades comerciais ou de preservação de espécies, bem como geração de peixes transgênicos, também poderão ser implementadas.

5. Anexos

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An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish

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Abstract Although there are almost thirty-thousand species of fish living in a great variety of habitats and utilizing vast reproductive strategies, our knowledge of morphofunctional and quantitative aspects of testis structure and spermatogenesis is still incipient for this group of vertebrates. In this review, we discuss aspects that are important to better understanding of testis structure and function, and of the development of germ cells (GC) during spermatogenesis. To achieve this, we have recently completed a number of studies presenting morphometric and functional data related to the numbers of GC and Sertoli cells (SC) per each type of spermatogenic cyst, the number of spermatogonial generations, the SC efficiency, and the magnitude of GC loss that normally occurs during spermatogenesis. We also investigated SC proliferation and the relationship of this important event to early spermatogenic cysts. The available data strongly suggest that SC proliferation in sexually mature tilapia is the primary factor responsible for the increase in testis size and for determination of the magnitude of sperm production. The influence of temperature on the duration of spermatogenesis in tilapia was also evaluated and we have used this knowledge to deplete endogenous spermatogenesis in this teleost, in order to develop an experimental system for GC transplantation. This exciting technique results in new possibilities for investigation of spermatogenesis and spermatogonial stem cell biology, creating also an entirely new and promising scenario in biotechnology—transgenic animal production and the preservation of the genetic stocks of valuable animals or endangered species.

Keywords Testis · Sertoli cells · Spermatogenesis · Spermatogonial stem cells · Germ cells transplantation

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Introduction

Fish are the most numerous and diverse group of vertebrates and are well adapted to live in a great variety of aquatic habitats, ranging from the Himalayas or Andes at 4,000 m altitude to abyssal zones at depths of 4,000–6,000 m (Moyle and Cech 2004). The diversity of fish is reflected in their gonad structure and reproductive strategies that, for instance, range from external to internal fertilization, with different modes of parental care (Hoar 1969; Nagahama 1983; Pudney 1995, 1996; Vazzoler 1996; Nakatani et al. 2001). In

addition, fish display different patterns of breeding influenced seasonally by environmental factors such as temperature, salinity, photoperiod, and others (Grier and Taylor 1998; Cinquetti and Dramis 2003; Alvarenga et al. 2006; Batlouni et al. 2006; Nóbrega 2006; Godinho 2007). Despite this extraordinary diversity in reproduction, spermatogenesis is highly conserved among the different fish species. Structural and functional aspects of this biological process are, in general, similar to those of other vertebrate classes, for example mammals (Pudney 1995; Schulz and Miura 2002; Miura and Miura 2003; Schulz 2003). However, there are relatively few data from systematic morphofunctional evaluation of the testis in fish, particularly for the seminiferous epithelium.

In order to provide a more comprehensive understanding of testis function in fish, this review will focus on some aspects related to the spermatogenic process. In particular, we will discuss the few quantitative data available regarding spermatogenic cysts and proliferation of Sertoli cells. Some of the data presented and discussed here were generated by research recently conducted in our laboratory. In the final section, we will discuss the experimental approach of germ cell transplantation in fish, with especial attention to the technique recently developed in our laboratory using the Nile tilapia as model. This fascinating technique is based on mammalian germ cell transplantation studies (Brinster and Zimmermann 1994; Brinster and Avarbock 1994). It consists of isolation of spermatogonia from a donor animal and transplantation of these cells into an infertile recipient, where they colonize the testis and form mature sperm with the donor's genetic characteristics. Germ cell transplantation in fish, and in other vertebrates, has paved the way for investigating spermatogenesis and the biology of spermatogonial stem cells and testis function, and has led to a new option for biotechnology—production of transgenic animals or the preservation of genetic stocks of valuable animals or endangered species.

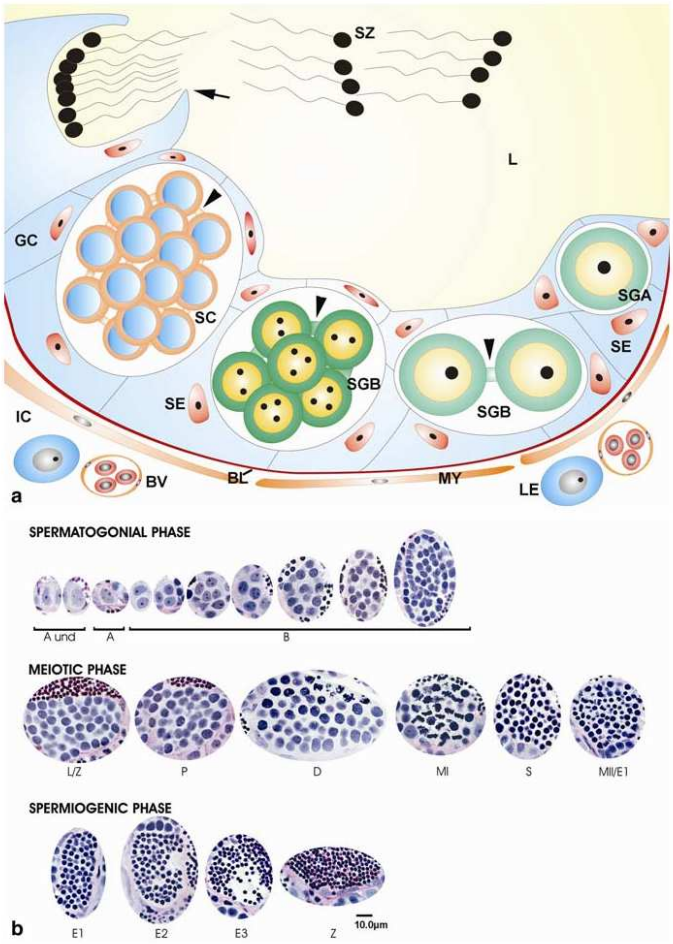
Testis structure and spermatogenesis

Similar to other vertebrates, the basic organization of the testis is highly conserved in fish and this organ is divided into two major compartments (Fig. 1a):

Fig. 1 (**a**) This figure illustrates the basic organization of the fish testis. This organ is usually divided into two compartments: the tubular (germinal) compartment (*GC*) and the intertubular (interstitial) compartment (*IC*). In the intertubular compartment, Leydig cells (*LE*) are present, and are the major source of testicular steroids in fish. These cells are located near the blood vessels (*BV*). Other elements that are present in the interstitium, for example nerves, collagen fibres, fibroblasts, macrophages and granulocytes are not represented in this figure. The tubular compartment is formed by the tunica propria, the seminiferous epithelium, and the lumen. The tunica propria consists of a layer of myoid cells (*MY*), collagen fibres, and the basal lamina (*BL*) of the germinal epithelium. In fish, the germinal epithelium is organized in cysts; these are formed when a single spermatogonia (primary or type A spermatogonia, *SGA*) is completely enveloped by Sertoli cells (*SE*). The cells resulting from the mitotic divisions of a single primary spermatogonia remain connected by cytoplasmic bridges (*arrowheads*) due to the incomplete cytokinesis. The cytoplasmic bridges are responsible for the synchronized germ cell development, forming a clone of germ cells. Cysts of secondary or type B spermatogonia (*SGB*) and spermatocytes (*SC*) are also represented. Spermiation (*arrow*) involves several changes in the Sertoli cells junctions, leading to the cyst opening and consequently to sperm (*SZ*) release into the seminiferous tubule lumen (*L*). (**b**) Illustration of zebrafish (*Danio rerio*) testis showing the three different phases of spermatogenesis in fish: spermatogonial or proliferative, meiotic or spermatocytary, and spermiogenic or differentiation phase. The cellular progression of germ cells during spermatogenesis is: primitive/type A undifferentiated spermatogonia (*A und*) → primary/type A differentiated spermatogonia (*A*) → secondary/type B spermatogonia (*B*) → primary spermatocytes at leptotene/zygotene (*L/Z*), pachytene (*P*), diplotene (*D*), metaphase I (*MI*) → secondary spermatocytes (*S*) → metaphase II/early spermatids (*MII/E1*) → early spermatids (*E1*) → intermediate spermatids (*E2*) → late spermatids (*E3*) → spermatozoa (*Z*). *Scale bar* = 10 μm

1. the tubular compartment comprising the seminiferous epithelium, where the Sertoli/germ cells units called cysts are located, the tunica propria, composed of basal lamina and peritubular myoid cells, and the tubular lumen filled with fluid secreted by Sertoli cells; and
2. the intertubular compartment containing the steroidogenic Leydig cells, connective tissue cells, and blood vessels (Grier 1993; Pudney 1995, 1996, 1999; Loir et al. 1995; Le Gac and Loir 1999; Nóbrega and Quagio-Grassiotto 2007).

In the seminiferous epithelium, spermatogenesis takes place within cysts or spermatocysts that are formed when a single spermatogonia (primary or type A spermatogonia) is completely enveloped by Sertoli cells (Pudney 1995, 1996; Loir et al. 1995; Le Gac and Loir 1999). Due to the incomplete cytokinesis,



the cells resulting from the mitotic divisions of the single spermatogonia remain interconnected by cytoplasmic bridges (Pudney 1995, 1996; Loir et al. 1995; Le Gac and Loir 1999); these bridges are responsible for the synchronous development of germ cells in a clonal fashion (Pudney 1995, 1996; Loir et al. 1995; Le Gac and Loir 1999). For this reason, it is believed that in the cystic arrangement of spermatogenesis, Sertoli cells are usually in contact with a single germ cell clone; this is the main difference from non-cystic spermatogenesis (reptiles, birds, and mammals) in which Sertoli cells are in contact with several germ cell clones at the same time (Pudney 1995, 1996; Loir et al. 1995; Le Gac and Loir 1999).

Spermatogenesis is a complex biological process of cellular transformation that produces male haploid germ cells from diploid spermatogonial stem cells (Russell et al. 1990; Hess and França 2007). Similar

to mammals, spermatogenesis in fish is divided into three major phases as can be observed in Fig. 1b:

1 the *proliferative* or *spermatogonial*, in which spermatogonia undergo rapid successive mitotic divisions;
2 the *meiotic* or *spermatocytary*, in which the genetic material is duplicated, recombined, and segregated; and
3 the *differentiation* or *spermiogenic* in which spermatids undergo drastic morphological and functional changes, such as nuclear condensation and flagellum formation, giving rise to spermatozoa that are structurally equipped to reach and fertilize the egg (Russell et al. 1990; Hess and França 2007).

As an illustrative aspect of the spermiogenic phase, it should be mentioned that the ultrastructural characteristics of spermiogenesis and spermatozoa show high variation among different fish species, being a useful tool for systematic and phylogenetic studies in these vertebrates. It should be mentioned that in fish, sperm penetrates the egg through the micropyle, and the acrosome is not formed during spermiogenesis in most of species (Jamieson 1991).

Although there has been no conclusive study of the spermatogonial phase and the number of spermatogonial generations, it is believed that in fish the progression of germ cells during spermatogenesis is as follows (Fig. 1b): primitive/undifferentiated type A spermatogonia (stem cell?) → primary/type A differentiated spermatogonia → secondary/type B spermatogonia (several generations; generation numbers depending on the species) → leptotene/zygotene spermatocytes → pachytene spermatocytes → diplotene spermatocytes → metaphase I → secondary spermatocytes → metaphase II → early spermatids → intermediate spermatids → final spermatids → spermatozoa (Fig. 1b). Functionally, differentiated spermatogonia are those cells committed to forming mature sperm in a specific time period, according to the duration of spermatogenesis for each species (França et al. 1998; Hess and França 2007).

It is well characterized for mammals that the foundation of spermatogenesis is the spermatogonial stem cell, which has the potential for both self-renewal and for differentiation into spermatogonia committed to sperm development (De Rooij and Russell 2000; De Rooij 2006a, b). Thus, the proper balance between the processes of self-renewal and differentiation is essential to ensure the continuous homeostasis of spermatogenesis.

There are, currently, no reliable morphological and molecular markers enabling identification of spermatogonial stem cells in fish, or in well-studied rodents such as rat and mice. However, for many authors, a primary or type A spermatogonium is regarded as being the spermatogenic stem cell in fish. A primary spermatogonium is a single cell (not connected to other cells by cytoplasmic bridges), and is regarded as the largest cell of the spermatogenenic process (with a nuclear diameter of approximately 10 μm in various species) (Miura 1999; Pudney 1995). Morphologically, these primary or type A spermatogonia have a clear and large nucleus with one or two evident nucleoli (Miura 1999; Quagio-Grassiotto and Carvalho 1999; Negrão et al. 2002; Gusmão et al. 2002; Lo Nostro et al. 2003; Fishelson 2003; Fishelson et al. 2006, 2007). Their cytoplasm is considered poorly differentiated and contains mitochondria associated with a large amount of electron-dense material, the nuages. These nuages, or dense germinal bodies, contain ribonucleoproteins and RNAs with long half-life times, including, for example, *vasa* mRNA, a specific product of the germ cell lineage (see reviews in Quagio-Grassiotto and Carvalho 1999; Braat et al. 1999).

Although there is no consensus concerning the terminology used to address the different spermatogonial generations in fish, some criteria such as the cell and nuclear size, nuclear morphology, and cell number per cyst have been used to differentiate early spermatogonia from the more differentiated ones (illustrated in Fig. 1b); see, for instance, studies of tilapias *Oreochromis niloticus* (Schulz et al. 2005) and Atlantic cod *Gadus morhua* (Almeida et al. 2008). In addition, it has been suggested that the cell cycle in early spermatogonia is longer than in the more differentiated spermatogonia. For example, in African catfish (*Clarias gariepinus*), BrdU (5-bromo-2′-deoxyuridine) incorporation in early spermatogonia was one fifth that in late spermatogonia (see review in Schulz and Miura 2002). The availability of fish models expressing green fluorescent protein gene, under the promoter region of the *vasa* gene, will probably provide new possibilities of characterizing the different spermatogonial generations (Yoshizaki et al. 2000; Krovel and Olsen 2002;

Takeuchi et al. 2002; Okutsu et al. 2006a, b; Shikina et al. 2008). This is possible because *vasa* is strongly expressed in early spermatogonia, and the association with FACS (fluorescent activated cell sorting) enables isolation of undifferentiated spermatogonia (Yano et al. 2008).

As occurs in mammals (Russell et al. 1990; De Rooij and Russell 2000), it seems that the number of mitotic divisions of spermatogonial cells before they form spermatocytes changes with the phylogenetic position of a series, particularly in relation to order level. In this regard, stereological investigations have shown there are ten spermatogonial divisions in Anguilliformes, eight in Characiformes, eight or nine in Cypriniformes, six or eight in Salmoniformes, eleven in Gadiformes, seven to ten in Perciformes, ten in Beloniformes, and fourteen in Cyprinodontiformes (Miura 1999; Ando et al. 2000; Fishelson 2003; Nóbrega 2003; Vilela et al. 2003; Fishelson et al. 2006, 2007; Bizzotto and Godinho 2007; Almeida et al. 2008).

Precise knowledge of the number of mitotic divisions that spermatogonia undergo is essential for analyzing the regulatory mechanisms underlying spermatogonial fate. In this context, recent findings from the Japanese eel (*Anguilla japonica*) have elucidated some regulatory mechanisms of self-renewal/differentiation of spermatogonial stem cells for this species. From these studies, two factors have emerged as potential candidates for the self-renewal/differentiation of spermatogonial stem cells:

1 PD-ECGF (platelet-derived endothelial cell growth factor) also known as "spermatogonial stem cell renewal factor"; and
2 AMH (anti-Müllerian hormone) also known as "spermatogenesis preventing substance" (see reviews in Schulz and Miura 2002; Miura and Miura 2003; Miura et al. 2006).

Knowledge of the duration of spermatogenesis is very useful for understanding reproductive biology, in particular the functional and regulatory mechanisms of spermatogenesis and reproductive strategies. In mammals, the duration of spermatogenesis is determined by the fact that the different germ cell generations form specific cell associations, known as stages of the seminiferous epithelium, so that determining their relative frequencies enables estimation of the total length of spermatogenesis from stem cells to spermatozoa (França and Russell 1998; Leal and França 2006). In fish, this approach is not feasible but it is possible to determine the duration of the meiotic and spermiogenic phases combined, i.e., from preleptotene/leptotene to spermatozoa.

The length of spermatogenesis is species-specific (França et al. 1998) and has only been determined for a few fish species. According to the available literature, the duration of spermatogenesis, from preleptotene to spermatozoa, is 12 days for medaka, *Oryzias latipes*, 14.5 days for guppy, *Poecilia reticulata*, 21 days for *Poecilia shenops*, and 14 days for *O. niloticus* (see reviews in Nagahama 1983; Vilela et al. 2003). In relation to mammalian species investigated up to date, the duration of spermatogenesis in fish is shorter (Silva 1987; Koulish et al. 2002; Vilela et al. 2003) and different from homeothermic animals the length of fish spermatogenesis is usually influenced by the temperature; higher temperatures accelerate this process, whereas at lower temperatures its duration is longer (Nagahama 1983; Vilela et al. 2003; Lacerda et al. 2006).

The development of germ cells, from spermatogonia to spermatozoa, is strictly regulated, and requires a specific microenvironment that is created by Sertoli cells (Schulz and Miura 2002). Germ cell survival depends ultimately on Sertoli cell–Sertoli cell and Sertoli cell–germ cell interactions. Thus, these interactions are crucial to providing the structural and physiological support for germ cell development during spermatogenesis in fish (Pudney 1993; Loir et al. 1995). In mammals, it is well established that each Sertoli cell is able to support a relatively fixed number of germ cells in a species-specific manner. Therefore, considering that Sertoli cells in mammals proliferate only during the fetal and prepubertal periods, the total number of Sertoli cells in the testis is the primary factor determining the magnitude of sperm production and testis size (Cooke et al. 1994; Hess and França 2007). In fish, however, Sertoli cells are able to proliferate in adult males as found for several species (Billard 1969; Grier 1993; Koulish et al. 2002; Matta et al. 2002; Vilela et al. 2003; Schulz et al. 2003; Lo Nostro et al. 2003; Chaves-Pozo et al. 2005; Schulz et al. 2005; Nóbrega 2006; Almeida et al. 2008). In these species, as shown for tilapias (*O. niloticus*) and African catfish (*C. gariepinus*), Sertoli cells proliferate mainly during the early spermatogonial phase in order to provide space and factors required to support germ

cell development during spermatogenesis. It should be mentioned that the spermatogenic cysts reach their maximum volume at late meiosis (Schulz et al. 2005) as illustrated in Fig. 1b, and it is probably not a coincidence that the number of Sertoli cells per cyst stabilizes also in this phase of spermatogenesis, as found for tilapias (*O. niloticus*) (Schulz et al. 2005) and guppy (*P. reticulata*) (Billard 1969). In this regard, as most fish species exhibit continuous body growth, Sertoli cell proliferation could be considered the primary factor responsible for the increase of testis size and sperm production during each reproductive cycle in these vertebrates (Schulz et al. 2005). Thus, in contrast with mammals, it could be suggested that Sertoli cells in fish are not fully differentiated, entering in a new mitotic cell cycle each time they are recruited. In tilapias, studies performed in our laboratory have demonstrated that Sertoli cells located near the blind ending of seminiferous tubules, in the area close to the tunica albuginea, are more immature and have higher proliferative capacity (Schulz et al. 2005). The regulatory mechanisms of Sertoli cell proliferation in fish remain unclear, but recent data have demonstrated that, similar to mammals (Cooke et al. 1994), their division is influenced by thyroid hormones (Matta et al. 2002).

Another peculiar characteristic of fish Sertoli cells, in comparison with mammals, is related to the capacity of Sertoli cells to support germ cells (Sertoli cell efficiency). In the few fish species in which this has been investigated, one Sertoli cell is able to support around 100 spermatids, which is approximately ten times higher than in most mammalian species already evaluated (Matta et al. 2002; Vilela et al. 2003; Schulz et al. 2005; Bizzotto and Godinho 2007). This high efficiency is attributed to the ready availability of regulatory factors, and the lower percentage of germ cell apoptosis during spermatogenesis.

Apoptosis is an integral part of spermatogenesis in vertebrates (Roosen-Runge 1977; Shaha 2007) and occurs in response to different factors, such as to temperature (Setchell 2006), cell density, and elimination of abnormal germ cells, often at so-called checkpoints (Baum et al. 2005; Yu and Wu 2008). In this regard, apoptosis plays an important functional role during spermatogenesis, influencing directly the number of sperm produced for each species, because it reduces drastically the expected theoretical number of spermatics cells. In mammals, it is assumed that only two or three spermatozoa are formed from ten spermatic cells that are theoretically expected. There are relatively few data in the literature regarding apoptosis in fish and the mechanisms involved in this important homeostatic process (Schulz and Miura 2002). Although high variation might occur, depending on species, apoptosis is observed in all three phases of spermatogenesis. To illustrate this aspect, a few examples are mentioned showing that germ cell loss through apoptosis can occur predominantly in spermatogonia in *P. reticulata, Sparus aurata, Sparus acanthias*, and *G. morhua* (Billard 1969; Chaves-Pozo et al. 2005; McClusky 2005; Almeida et al. 2008); during meiosis and spermiogenesis in *Torpedo marmorata*, *Serrasalmus spilopleura* (Prisco et al. 2003; Nóbrega 2006); or during spermiogenesis in tilapias *O. niloticus* (Schulz et al. 2005).

Germ cell transplantation in fish

Germ cell transplantation is the only functional approach available for investigation of spermatogonial stem cell biology. In this powerful and fascinating technique developed by Brinster and collaborators (Brinster and Zimmermann 1994; Brinster and Avarbock 1994), transplanted mice spermatogonia were able to colonize and develop in recipient mice testes and form fertile sperm with the donor genotype characteristics. Recently, a similar approach was developed in fish using transplantation of primordial germ cells carrying GFP (green fluorescent protein) into the coelomatic cavity of rainbow trout (*Onchorhynchus mykiss*) larvae (Takeuchi et al. 2003). After transplantation, these cells were able to migrate to and to colonize the undifferentiated gonad, giving rise to female or male germ cells, after the gonad had differentiated into an ovary or a testis, depending on the individual's genetic sex. In addition, males derived from this transplantation were also capable of producing sperm with donor genetic characteristics (Takeuchi et al. 2003). Using the same methodology, xenogenic transplantation between rainbow (*O. mykiss*) and masu (*Onchorhynchus masou*) trouts were also successfully performed (Takeuchi et al. 2004). Okutsu et al. (2006a) have also shown oocyte production from spermatogonia transplanted into the coelomatic cavity of rainbow trout larvae, and this result illustrates the plasticity

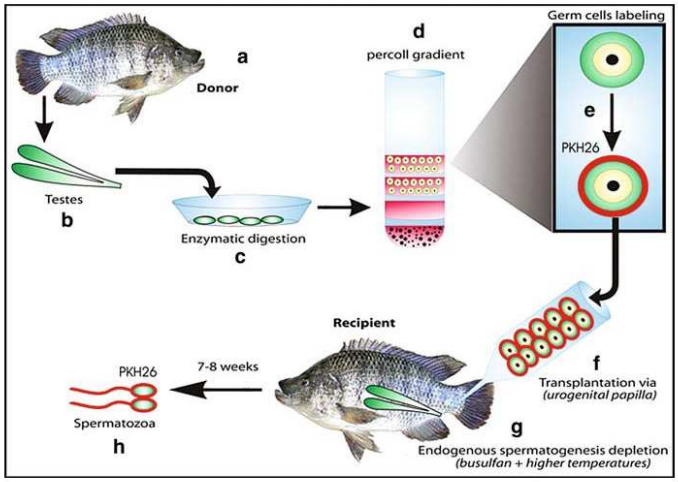


Fig. 2 This figure illustrates the main steps utilized for transplantation of germ cells in *Oreochromis niloticus*. Donor testes were removed (*a*, *b*), fragmented and digested using proteolytic enzymes (*c*), such as trypsin and collagenase. The cell suspension obtained was submitted to percoll gradient centrifugation (*d*), aiming at selecting the most undifferentiated spermatogonia. Because there are no genetic markers available for tilapias, the red fluorescent cell linker (PKH26), a lipophilic dye for the plama membrane, was used to label the germ cells (*e*). The germ cells were transplanted via the common spermatic duct located in the urogenital papilla (*f*) to recipient fish in which endogenous spermatogenesis was first depleted by treatment with busulfan combined with higher temperatures (*g*). Each recipient tilapia received 1 ml cell suspension containing 10^7 germ cells per ml. The fish were sacrificed at different times post-transplantation in order to follow the development of PKH26-labeled germ cells. Transplanted germ cells colonized the recipient testis and generated spermatozoa 7–8 weeks post-transplantation (*h*)

and bipotential capability of spermatogonial cells (early germ cells) in fish.

Several other findings using this methodology have recently been published, for example the production of viable rainbow trout derived from the transplantation of cryopreserved primordial germ cells, production of trout offspring after germ cell transplantation to triploid salmon parents, and xenogenic transplantation using goldfish and zebrafish (Kobayashi et al. 2006; Okutsu et al. 2006b, 2007; Saito et al. 2008). To improve this very promising scenario even further, an in-vitro system enabling spermatogonia to expand their population before these cells are transplanted has been developed (Shikina et al. 2008).

Based on mammalian studies, in the past five years our laboratory has conducted studies in this very important and exciting field for fish reproduction. In this regard, we were the first research group to be able to transplant viable PKH26-labelled germ cells directly into the testis via the genital papillae and the common spermatic duct, as shown in Fig. 2. In these studies, using the Nile tilapia (*O. niloticus*) as recipient, endogenous spermatogenesis was depleted before transplantation using the chemotherapeutic drug busulfan in association with higher (~35°C) water temperature (Lacerda et al. 2006, 2008). Therefore, similar to mammals, a very useful experimental approach was developed in fish in order to evaluate spermatogonial stem cell biology, offering also a unique opportunity for studies related to biotechnology in aquaculture, besides providing interesting possibilities for transgenesis, and the preservation of endangered species and the genetic stock of zootechnically valuable animals.

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Spermatogenesis in fish

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ABSTRACT

Spermatogenesis is a developmental process during which a small number of diploid spermatogonial stem cells produce a large number of highly differentiated spermatozoa carrying a haploid, recombined genome. We characterise morphologically the different germ cell stages with particular attention for the spermatogonial generations, including the stem cells and their specific capacity to colonise a recipient's testis after transplantation. We propose a nomenclature for fish germ cells to improve the comparability among different teleost fish but also to higher vertebrates. Survival and development of germ cells depends on their continuous and close contact to Sertoli cells, and we review their multiple roles in the cystic mode of spermatogenesis seen in fish. We then discuss gene expression patterns associated with testis maturation. The endocrine system of vertebrates has evolved as master control system over spermatogenesis. In fish, both pituitary gonadotropins LH and FSH stimulate gonadal sex steroid hormone production directly by activating Leydig cells. Information is reviewed on the effects of progestin, androgens, and estrogens on global testicular gene expression patterns (microarray analysis), and on the molecular mechanisms by which steroids regulate specific candidate genes (identified by subtractive hybridization approaches) during early stages of testis maturation. Moreover, progestin and androgen effects on spermiation and milt hydration are discussed. Sex steroids mainly act via receptors expressed by Sertoli cells. One type of response is that Sertoli cells change growth factor expression, which subsequently modulates germ cell proliferation/differentiation via mechanisms yet to be characterised. Finally, we review data on germ cell autonomous processes, mainly derived from loss-of-function mutant fish lines, before identifying a number of focus areas for future research activities.
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1. Introduction

The cellular basis of sexual reproduction in animals are haploid gametes, one sex producing a relatively small number of large gametes, eggs that are rich in reserves, the other sex producing a much larger number of small gametes, spermatozoa, which evolved as highly specialized, motile genome vectors. Although the development of eggs and sperm show common principles, many aspects of gametogenesis differ between sexes. With regard to oocyte development, the reader is referred to Lubzens et al. (2010). The present chapter will first deal with morphological aspects of male germ cell development, will then turn to gene expression patterns accompanying spermatogenesis, before discussing extrinsic (hormones and growth factors) and intrinsic (cell autonomous) regulation of germ cell development.

Fish are the most diverse and numerous group of vertebrates. Our knowledge on spermatogenesis in fish, however, is limited to a few species used in basic research and/or in aquaculture biotechnology (e.g. catfish, cod, eel, guppy, medaka, salmon, tilapia, trout, and zebrafish). While trying to distil general principles from the available information, we will also indicate knowledge caveats that require more research efforts, and be cautious with too detailed speculations that might melt soon in the light of new data.

An important aim of the morphological section is to characterise teleost spermatogenic cells. Considering the existing literature, we feel that in particular the identification and naming of the different spermatogonial generations needs clarification and standardization; also the different meiotic and post-meiotic stages will be discussed. We hope that this exercise will contribute to forming a basis necessary for comparative and applied research on spermatogenesis in fish, and allowing intra- and inter-species comparisons among fish, but also with higher vertebrates, where

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generally accepted classification systems for spermatogenic cells exist (Hess and França, 2007).

Attention will then be directed towards the transcriptomic characterization of germ cells, based on the recent publication of large-scale EST sequences, and using the rainbow trout as example (Govoroun et al., 2006; Mazurais et al., 2005).

In the following section, we will review the regulation of spermatogenesis via pituitary hormones (FSH, LH, GH), sex steroids, and locally produced growth factors. The reader is referred to Kah et al. and Levavi et al. (both this volume) as regards the brain and pituitary parts of the BPG axis.

The subsequent section will summarize the information in fish on processes operating in a germ cell-autonomous manner at specific stages of spermatogenesis, as opposed to the endocrine regulation of spermatogenesis via signalling molecules derived from sources outside germ cells.

Finally, we will discuss points that we consider as major knowledge caveats requiring significant research attention in the future. Here, we will encounter key-points of spermatogenic development, such as the equilibrium between stem cell self-renewal and differentiation. Some of these key-events are located at the cross-roads of basic and applied research (e.g. aquaculture biotechnology and ecotoxicology), therefore justifying particular attention.

2. Testis structure, spermatogenesis, and germ cell morphology

2.1. General structure of the teleost testis

In all vertebrates, from fish to mammals, the testis is composed of two main compartments, the intertubular (or interstitial) and the tubular compartment. The intertubular compartment contains steroidogenic Leydig cells, blood/lymphatic vessels, macrophages and mast cells, neural and connective tissue cells, the latter being continuous with the tunica albuginea (Koulish et al., 2002), i.e. the testis organ wall. The tubular compartment is delineated by a basement membrane and peritubular myoid cells and houses the germinal epithelium. This epithelium contains only two cell types, the somatic Sertoli cells and the germ cells, which are found at different stages of development. In vivo, germ cells can only survive in close and continuous interaction with Sertoli cells, so that Sertoli cell number determines the spermatogenic capacity of a testis and is an important target of signalling systems regulating spermatogenesis (Matta et al. 2002).

In amniote vertebrates (reptiles, birds, mammals) it is considered that Sertoli cells proliferate until puberty when only spermatogonia and a few early spermatocytes are present in the germinal epithelium. Hence, the adult amniote testis contains a fixed number of "immortal" Sertoli cells supporting successive waves of spermatogenesis. During these waves, a given Sertoli cell supports at the same time different developmental stages of germ cells (i.e. cells belonging to different clones; see below). For example, the Sertoli cell basis contacts spermatogonia, whereas lateral parts contact spermatocytes and early spermatids, and adluminal parts late spermatids (Fig. 1A).

In anamniote vertebrates (fishes and amphibians), on the other hand, we find the cystic type of spermatogenesis (Callard 1996). There are two main differences compared to the testis of higher vertebrates. First, within the spermatogenic tubules, cytoplasmic extensions of Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium (Fig. 1B). Second, the cyst-forming Sertoli cells retain their capacity to proliferate also in adult fish (Schulz et al., 2005). Hence, the basic functional unit of the spermatogenic epithelium in fish is a spermatogenic cyst formed by a dynamic group of Sertoli cells surrounding and nursing one synchronously developing germ cell clone. Different clones being in different stages of development generate the typical histological picture of fish testes, where the tubular compartment contains differently sized groups of germ cells in different stages of spermatogenesis (Fig. 1B, inset). Comparing cystic and non-cystic spermatogenesis, cystic Sertoli cells might be more efficient in supporting germ cell development, e.g. by concentrating specific growth factors required for each developmental phase, resulting in a low percentage of germ cell apoptosis, and consequently high spermatogenic yield. The non-cystic Sertoli cells, on the contrary, are more diversified or regionalized in context with the complex requirements for nursing different germ cell clones at the same time.

Two types of spatial arrangements have been described in the literature for the teleost testes based on the distribution of spermatogonia in the germinal compartment (Grier, 1981). In the first type (restricted spermatogonial distribution), the distal regions of the germinal compartment, near the tunica albuginea, are occupied by Sertoli cells surrounding early, undifferentiated spermatogonia (see below for a more detailed description of spermatogonial generations). As the cells divide and enter in meiosis, the cysts migrate towards the region of the spermatic ducts located centrally in the testis, where spermiation occurs, i.e. the cysts open to release spermatozoa. This type of arrangement is found in the higher teleosts, such as in the order Atheriniformes, Cyprinodontiformes and Beloniformes (Parenti and Grier, 2004). In the second type (unrestricted spermatogonial distribution), spermatogonia are spread along the germinal compartment throughout the testis. The cysts do not migrate or get displaced during their development (Grier, 1981). The unrestricted distribution of spermatogonia is considered a more primitive pattern found in less evolved taxonomic groups, such as in the order Cypriniformes, Characiformes, and Salmoniformes (Parenti and Grier, 2004). However, also intermediate forms appear to exist between restricted and unrestricted spermatogonial distribution, such as found in Perciformes, tilapia *Oreochromis niloticus* (Vilela et al., 2003); Pleuronectiformes, *Solea senegalensis* (García-López et al., 2005) or Gadiformes, *Gadus morhua* (Almeida et al., 2008). In these species, undifferentiated spermatogonia show a preferred, but not exclusive, location close to the tunica albuginea. In cod, spermatogonia are derived from a germinative zone in the periphery of the testicular parenchyma, which produces new spermatogonial cysts (Almeida et al., 2008). This results in a zonation of the testis: early stages of development reside in the periphery and advanced stages are found close to the collecting duct, a situation arising through appositional growth that is fuelled by the cyst-generating activity of the germinative zone rather than through movement of developing cysts.

Some tropical species do not display apparent seasonal variations in spermatogenic activity. However, in many species with habitats at higher latitudes, reproduction is a seasonal or cyclic event related to environmental cues (Billard and Breton, 1978; Nash, 1998). Active spermatogenesis may take place in summer (trout, carp, pike), in spring (tench, bream, whiting, sea bream), or may begin in autumn and finish in spring (killifish, stickleback, roach) (Billard and Breton, 1978; Billard, 1986). For species with a clear-cut seasonal reproduction such as salmonids, pike, or Atlantic cod, cysts with rapidly proliferating spermatogonia can be observed only at the beginning of testicular recrudescence. In these species, tubules are filled after completion of spermatogenesis with only two types of germ cells, large numbers of spermatozoa and some scattered, quiescent type A spermatogonia (Billard, 1986). In other cyclic species, germ cells remaining in the regressed testis may be spermatogonia and spermatocytes (tench), or even all germ cell types, such as in goldfish, where the seasonal regression is of a quantitative rather than of a qualitative nature (Billard, 1986).

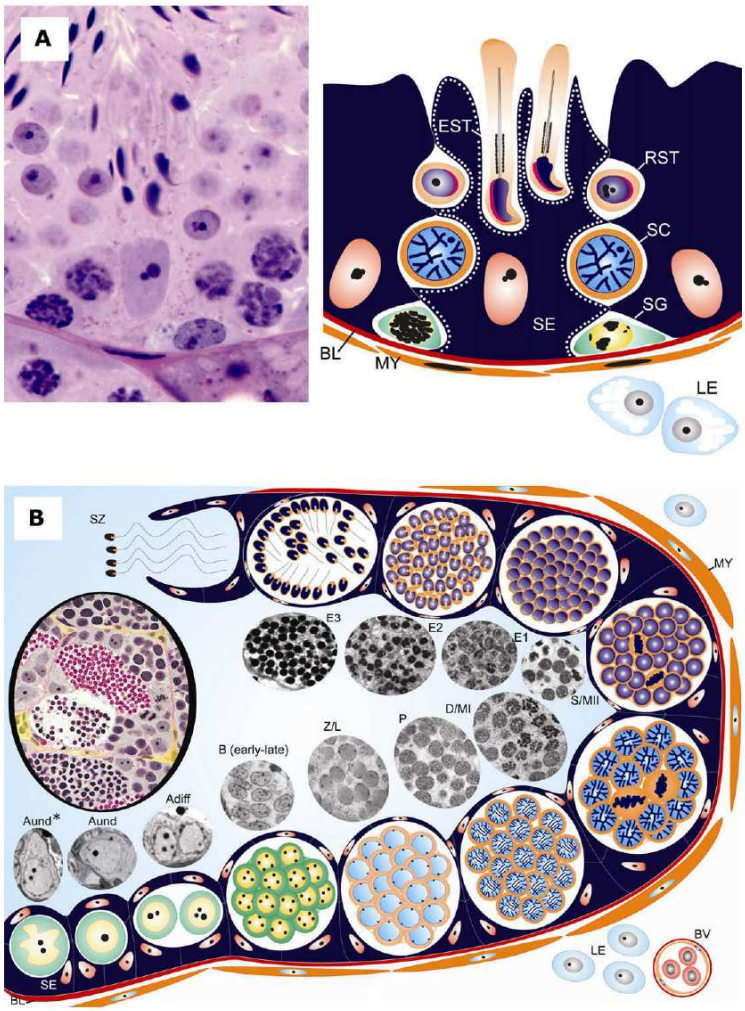

Fig. 1. Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between cystic (B) and non-cystic (A) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). The interstitial Leydig cells (LE) and blood vessels (BV) are shown. (A) spermatogonia (SG); spermatocyte (SC); round spermatid (RST); and elongated spermatid (EST). (B) Type A undifferentiated spermatogonia (A_{und}) (stem cell?); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); spermatogonia type B [B (early–late)]; leptotenic/zygotenic primary spermatocytes (L/Z); pachytenic primary spermatocytes (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ).

2.2. Sertoli cells

The main Sertoli cell functions are to support germ cell survival, development, and physiological functioning. Moreover, Sertoli cells secrete fluid that generates the tubular lumen, and they phagocytise apoptotic germ cells, residual bodies discarded by spermatids during spermiogenesis, and residual sperm. Therefore, the development of spermatogenic cells strictly depends on their interaction with the somatic elements of the testis, amongst which Sertoli cells play a crucial role in animals in general. In invertebrates like in fruitfly, for example, Sertoli cells (also called cyst cells) are derived from somatic stem cells (hub cells). When cyst cells contact differ-

entiating germ cells, they form cysts similar to those in the testes of anamniote vertebrates (Decotto and Spradling, 2005). Sertoli cells are the first somatic cell type to differentiate in the vertebrate testis and this cell type plays a crucial role in directing testis differentiation and development (DiNapoli and Capel, 2008). Also, as already mentioned Sertoli cell number per testis quantitatively limits (spermatogenic ceiling) sperm production in sexually mature animals throughout the vertebrate phylum (Hess and França, 2007; Nóbrega et al., 2009).

In the cystic mode of spermatogenesis in fish, germ cell number and volume increases greatly per cyst during the spermatogenic process. Therefore, it is not surprising that the number of Sertoli cells per cyst also increases, which occurs in a predictable manner. A specific number of Sertoli cells is observed per cyst in a given species and for a given stage of germ cell development (Billard, 1969b; Matta et al., 2002). At least part of the increase in Sertoli cell number per cyst is based on Sertoli cell proliferation (Schulz et al., 2005). The major increase in cyst volume occurs during the period of rapid mitotic expansion of the spermatogonia, which may explain why proliferating Sertoli cells are mainly found associated with spermatogonial cysts. The increases in cyst volume and Sertoli cell number per cyst both level off during meiosis/start of spermiogenesis (Matta et al., 2002; Schulz et al., 2005). Intriguingly, this coincides with the formation of occluding or tight junctions between neighbouring Sertoli cells in fish (Silva and Godinho, 1989; Batlouni et al., 2009), establishing a privileged space for the late meiotic/spermiogenic germ cells. The reason for sealing off meiotic and post-meiotic cells may reside in the large number of meiosis- and spermiogenesis-specific germ cell gene products that may have to be shielded from the immune system on the one hand, and may require a specific microenvironment for proper functioning on the other hand.

It seems that in fish spermatogenesis, Sertoli cells are formed by mitosis just in time and in exactly the number required. This tailored Sertoli cell proliferation, first described in the guppy (Billard, 1969b), may be (part of) the background for the observation that cystic spermatogenesis is more efficient than the non-cystic spermatogenesis in mammals, as indicated by parameters like apoptotic germ cell loss (~30% in fish, Matta et al., 2002; approximately 60–80% in rat or mice; Hess and França, 2007), or the number of spermatids formed per Sertoli cell (~100 in guppy, tilapia, or zebrafish; Billard, 1969b; Nóbrega et al., 2009; 8–10 in rats and mice, Hess and França, 2007). Many fish species grow continuously during adulthood, including growth of the testis, so that part of the Sertoli cell proliferation may reflect postpubertal organ growth. Moreover, many species show a seasonally recurring growth-shrinkage cycle of testis weight and volume. In these species, it is possible that also Sertoli cells are lost during the post-spawning involution of the testes. Hence, Sertoli cells would have to be formed anew during the next reproductive season when spermatogonial proliferation is resumed. We speculate that fish testis contains, next to spermatogonial stem cells, a second precursor or stem cell population giving rise to somatic Sertoli cells. Considering that natural and steroid-induced male-to-female sex change is possible in fish (Guiguen et al., 2010), it moreover seems that the somatic precursor cell population can assume male (Sertoli) or female (granulosa) cell fate. Hence, the germ cell-supporting somatic cells in the fish testis show an astonishing developmental plasticity in terms of sexual fate and cell number. However, experimental proof for the existence of a somatic stem cell type, for example by cell lineage tracing experiments (Goenczy and DiNardo, 1996), is not available yet in fish.

Fish Sertoli cells are also astonishing with regard to their efficiency as phagocytotic cells. Usually, macrophages or other phagocytotically active cell types are not present in the tubular compartment of the testis, and phagocytosis is part of the physiological function of Sertoli cells. During spermiogenesis, spermatids shed the so-called residual bodies that contain superfluous cytoplasmic material that is discarded during the 'metamorphosis' to flagellated sperm. A certain level of apoptosis is normal and although fish spermatogenesis is comparatively efficient in this regard, still 30–40% of all germ cells that could be produced theoretically become apoptotic before differentiating to spermatozoa (Billard, 1969b; Vilela et al., 2003). Sertoli cells remove and recycle this material very efficiently, so that cellular debris rarely appears in spermatogenic tubules. Indeed, even when a loss-of-function of a protein required during meiosis leads to apoptotic loss of all spermatocytes in zebrafish (Feitsma et al., 2007), the fraction of apoptotic spermatocytes visible in histological sections from mutant males is only ~10% of the total number of spermatocytes, suggesting a rapid clearance of material phagocytised by Sertoli cells. In addition, in many seasonal breeders, Sertoli cells play an important role in the phagocytosis of residual sperm after the spawning season (Grier and Taylor, 1998; Almeida et al., 2008).

In mammals, formation of occluding junctions between Sertoli cells marks the end of the period of Sertoli cell proliferation and their terminal differentiation, so that Sertoli cells can now support meiotic and post-meiotic germ cell stages in the adluminal part of the germinal epithelium, segregated by occluding junctions from the basal compartment containing spermatogonia. An alike development can be observed in the teleost testis, although adapted to three specific conditions of cystic spermatogenesis. First, maximum cyst volume is attained when germ cells have progressed well into meiosis (pachytene spermatocyte stage) and it is therefore not surprising that Sertoli cell proliferation is observed – although levelling off from more intense proliferation during the spermatogonial phase – until the end of meiosis/beginning of spermiogenesis (Schulz et al., 2005). Strikingly, formation of occluding junctions and termination of Sertoli cell proliferation are temporally related also in different fish species (e.g. Bergmann et al., 1984). Second, terminal differentiation takes place in an asynchronous manner in the fish testis accompanying each spermatogenic wave and reflecting the fact that each cyst functions as an independent Sertoli/germ cell unit that follows its own developmental timing. This is in contrast to a general wave of Sertoli cell differentiation in a relatively short time window in the germinal epithelium of amniote vertebrates. Third, unlike mammals (Setchell, 1986), the Sertoli cell barrier in fish is established at the end of or after meiosis and only haploid germ cells are shielded from the vascular compartment and from the immune system. In a number of species, these junctions restrict the entry of large molecules into the cysts (Abraham et al., 1980; Bergmann et al., 1984; Leal et al., 2009) and in trout, experimental breaching of the barrier leads to the appearance of anti-sperm antibodies (Secombes et al., 1985). However, before occluding junctions are established, Sertoli cells are connected to each other and to germ cells by other types of intercellular junctions (Loir et al., 1995; Pudney, 1993; Grier, 1993; Batlouni et al., 2009).

Finally, the close contact between developing germ cells and Sertoli cells is broken by opening of the cysts, such that the lumen of spermatogenic cysts becomes continuous with the lumen of the spermatogenic tubules. This process is called spermiation and is homologous to the androgen-stimulated (O'Donnell et al., 2000) disintegration of the ectoplasmic specializations that connected late spermatids and Sertoli cells in higher vertebrates. In the literature, the term spermiation is used in two ways in fish: *Sensu strictu* to describe the termination of Sertoli-germ cell contact; in a broader sense to indicate that strippable sperm (milt) is present in the spermatic duct system. In the present paper, the term spermiation is used in the strict sense, while processes related to the hydration/capacitation of sperm refer to the "final maturation" of spermatozoa that takes place, at least in part, outside the testis

in the spermatic duct system are discussed further below (see Section 4.2: steroid hormones).

Considering spermiation *sensu strictu*, disintegration of occluding junctions at the adluminal pole of Sertoli cells may be a mechanism to release sperm into the tubular lumen (Billard et al., 1972). It is not clear yet if a certain loss of Sertoli cells occurs in association with spermiation in fish. In any case, following spermiation Sertoli cells can establish a single-layered epithelium lining the seminiferous tubule, and then were referred to as "lobule boundary cell". However, this was associated with an erroneous identification as Leydig cell homologue, as clarified by Grier and Linton (1977), and the term is no longer used. The epithelium of the spermatic duct system seems to be continuous with the Sertoli cell epithelium lining the lumen in fully mature testis (Billard et al., 1972; van den Hurk et al., 1978).

If at the end of a spawning season residual spermatozoa are left in the testis or spermatic duct system, they are removed by Sertoli cells via phagocytosis (Billard et al., 1972; van den Hurk et al., 1978; Almeida et al., 2008). Neighbouring tubules can show different activities during this period, removal of residual sperm while spermatogonial proliferation is quiescent, or start of spermatogonial proliferation while clearance of residual sperm had been completed. It seems possible that Sertoli cells removing residual sperm produce paracrine factors that prevent resumption of spermatogonial proliferation in the direct vicinity (rainbow trout: Schulz, 1984; Atlantic cod: Almeida et al., 2008).

In summary, piscine Sertoli cells pass sequentially through different stages of activity/differentiation that reflect the developmental stage of the single germ cell clone they take care off. At any given point in time, a fish Sertoli cell is less "busy" than a mammalian Sertoli cell, which is in contact with ~6 different germ cell clones in different stages of development.

2.3. Spermatogenic process – an overview

Spermatogenesis is a highly organized and coordinated process, in which diploid spermatogonia proliferate and differentiate to form mature spermatozoa. The duration of this process is usually shorter in fish than in mammals and is also influenced by the water temperature (Nóbrega et al., 2009). In principle, however, the process is conserved in vertebrates and morpho-functionally can be divided in three different phases: the mitotic or spermatogonial phase with the different generations of spermatogonia (i.e., undifferentiated spermatogonia including the stem cells, and differentiated or differentiating spermatogonia); the meiotic phase with the primary and secondary spermatocytes; and the spermiogenic phase with the haploid spermatids emerging from meiosis and differentiating – without further proliferation – into motile, flagellated genome vectors, the spermatozoa. Except for the mitotic phase, in which the number of spermatogonial generations is quite variable between, but determined genetically within species, the other two phases present striking similarities among different vertebrate species (see review by Nóbrega et al., 2009).

In mammals, all somatic and germ cells of the foetus originate from the inner-cell-mass cells of the blastocyst (Kubota and Brinster, 2006). The embryonic or primordial germ cells (PCG) migrate to the genital ridge, colonize the undifferentiated gonads, and are then called gonocytes, which might contain different subpopulations (Ohmura et al., 2004). Gonocytes generate male or female germ cells depending on the sex of the gonad. In an ovary the gonocytes will form oogonial stem cells that eventually all differentiate, and enter the first meiotic division before birth. Hence gonial stem cells are considered to be absent from the mammalian ovary at birth. In the foetal testis, however, after a brief period of proliferation the gonocytes are prevented from entering meiosis by the action of retinoid-degrading enzyme CYP26B1 expressed by Sertoli cells (Bowles et al., 2006). Instead, gonocytes enter a mitotic arrest and become spermatogonial stem cells (SSCs) soon after birth. Throughout postnatal life, SSCs will self-renew themselves or produce daughter cells committed to differentiate into spermatozoa. In adult mammals, recent findings suggest the presence of an SSC subpopulation that is totipotent and able to form different cell types (Guan et al., 2006; Conrad et al. 2008).

Although several candidates are emerging (e.g. RET, OCT4, PLZF, GPR125), at present there are no unique molecular or morphological markers to identify spermatogonial stem cells in vertebrates. Investigations on stem cell biology rely on a functional approach, the spermatogonial transplantation assay that is based on the capacity of transplanted SSCs to populate a recipient's testis.

Based on their morphology (mainly the amount and distribution of heterochromatin) and their presence in the different stages of the seminiferous epithelium cycle, in well studied laboratory rodents (mice, rats, and hamsters) the spermatogonial cells located in the basal compartment of the seminiferous tubules of adult animals can be classified in three different types (De Rooij and Russell, 2000): type A, intermediate, and type B.

Functionally, type A spermatogonia are divided in undifferentiated (A single, A_s; A paired, A_{pr}; A aligned, A_{al}; the latter usually clones of 4, 8, or 16 cells) that are continuously present; and in differentiated spermatogonia (A_1–A_4) that are present at specific stages of the epithelial cycle and committed to form mature sperm following a genetically determined schedule (De Rooij and Russell, 2000). Within the type A spermatogonia, the A_s is classically considered as stem cell. The A_s cells comprise only 0.3% of all germ cells and have a preferential location (niche) in the seminiferous tubules areas adjacent to the interstitium (Chiarini-Garcia and Russell, 2002), often in the vicinity of blood vessels (Yoshida et al., 2007). Due to incomplete cytokinesis, spermatogonial cells resulting from the mitotic (non-self renewal) divisions of A_s cells remain interconnected by cytoplasmic bridges until spermiogenesis/spermiation in all animals. The bridges are responsible for the synchronized development of germ cells derived as clonal cell group from the same stem cell (De Rooij and Russell, 2000). However, the concept of A_s cells being the only type of spermatogonia with stem cell capacity has been challenged recently because undifferentiated spermatogonia type A_{pr} and A_{al} may have the potential to revert to the stem cell fate under certain conditions (Nakagawa et al., 2007). In any case, adequate regulation of the balance between self-renewal and differentiation of SSCs is essential to assure the continuous homeostasis of spermatogenesis in species with continuous spermatogenesis, or to implement the start and end of seasonal reproductive activity.

The number of spermatogonial generations is genetically determined. For instance, mice and rats have six generations of differentiated spermatogonia (A_1–A_4, In, and B), whereas stallions and donkeys have five (A_1–A_3, B_1–B_2). Thus, different species can have different numbers of type A or type B generations, or can lack the intermediate spermatogonia. Within a species, also the number of generations of undifferentiated A_{al} spermatogonia can vary slightly and two or three A_{al} generations can be observed in rodents (De Rooij and Russell, 2000). In laboratory rodents the typical progression from A_S to preleptotene (Pl) primary spermatocytes is as follows, with the number in brackets giving the theoretical cell number that disregards germ cell loss: $A_s \rightarrow A_{pr}$ (2) $\rightarrow A_{al4}$ (4) $\rightarrow A_{al8}$ (8) $\supset A_1$ (8) $\rightarrow A_2$ (16) $\rightarrow A_3$ (32) $\rightarrow A_4$ (64) $\rightarrow$ In (128) $\rightarrow$ B (256) $\rightarrow$ Pl (512). The step from A_{al8} (8) $\supset A_1$ (8) is particular since it marks the retinoic acid-dependent differentiation without mitotic division of the last A_{al} generation to A1 spermatogonia. In this way, usually nine mitotic divisions occur in laboratory rodents.

The last S-phase in germ cells occurs in preleptotene spermatocytes, after which they enter the very long prophase of the first

meiotic division. The first meiotic prophase is subdivided into stages that carry names describing morphological aspects of the condensing/moving chromosomes: leptonema, zygonema, pachynema, diplonema, diakinesis, and the respective cells are specified as leptotene, zygotene, pachytene, and diplotene spermatocytes, respectively (Cobb and Handel, 1998). During the first meiotic prophase, paternal and maternal chromosomes are aligned. This involves a specific set of meiotic proteins, the synaptonemal complex proteins (Costa and Cooke, 2007), functioning as molecular "zippers" to facilitate the correct positioning of the parental chromosomes for the DNA double strand breaks and their subsequent healing in the context of meiotic cross-overs. Another reason for the long duration of the first meiotic prophase is the intense gene expression required for meiosis-specific proteins on the one hand (Eddy, 1998), and for the mRNAs required during later stages (spermiogenesis) on the other hand, when replacement of histones by protamins renders the germ cell genome inaccessible. Consequently, the mRNAs for many 'late' spermatogenic genes are expressed already in primary spermatocytes, and are stored and protected until use by association with RNA binding proteins (Braun, 1998). Primary spermatocytes are frequently found in testis sections, which reflect the long duration of the first meiotic prophase. They can be identified easily by their relatively large size, and by the characteristic nuclear staining patterns, representing the progressively condensing chromosomes. During the first meiotic division, homologous (maternal and paternal) chromosomes become separated into the nuclei of secondary spermatocytes. These cells are short-lived since they immediately enter the second meiotic cell cycle, skipping DNA-synthesis and leaving the chromosomes largely condensed. During the subsequent M-phase, the sister chromatids become separated into a haploid set in the spermatid nuclei.

Spermatids undergo a final differentiation period known as spermiogenesis, during which they develop a flagellum, the DNA is maximally compacted into a small nucleus, and superfluous cellular material is discarded into the so-called residual body. In higher vertebrates, the Golgi system gives rise to the acrosome that is important during fertilization. In most teleost fish, however, spermatids do not develop an acrosome. In species with distinct sex chromosomes, spermatogenesis-specific genes have the tendency to accumulate on the male-specific chromosome, for example a number of the structural genes required for the formation of the flagellum are on the mammalian Y chromosome (Burgoyne, 1998). The respective mRNAs are required when the germ cell genome has become inaccessible due to maximal chromatin condensation; also, these genes are not available in the nucleus in those 50% of the cells that received the X chromosome during meiosis. In this context, the RNA-binding proteins are relevant that distribute the required mRNAs among clone members, while the cytoplasmic bridges serve as intercellular transport routes for the RNA-protein complexes.

In amniote vertebrates, germ cell clones move during development from the basal compartment of the tubulus where spermatogonia are in contact with the basement membrane, through the tight-junctional barrier between neighbouring Sertoli cells (early spermatocytes), towards the adluminal part (spermatocytes and spermatids) until they are released (spermiation) by Sertoli cells. During spermiogenesis, specific junctions (ectoplasmic specializations) are formed between spermatids and Sertoli cells, which later disintegrate together with the cytoplasmic bridges among spermatids in preparation for spermiation.

2.4. Fish spermatogenesis

In this section, we will discuss the morpho-functional characteristics of the different developmental stages of fish germ cells, from stem cells to spermatozoa. Also, we will provide criteria for the identification of the different germ cell stages and we will propose a nomenclature; in this context, comparisons with the yet better investigated rodent models will be used as well.

We propose to use the same terminology used in higher vertebrates (see above) for fish germ cells, since the available information indicates that similar developmental processes are taking place during spermatogenesis in all vertebrates. For the purpose of comparability between different fish species and between different classes of vertebrates, the use of the same nomenclature for the same cell types is required. Undifferentiated type A spermatogonia (A_{und}) give rise to differentiated type A spermatogonia (A_{diff}), still sharing some morphological characteristics with A_{und} but with a greatly reduced potential for self-renewal. Irreversible commitment to further maturation and a change in a number of morphological aspects gives rise to the more rapidly dividing type B spermatogonia, of which there are usually several generations. The number of generations varies between species (Ando et al., 2000) and is genetically determined. For example, 14 generations were found in the guppy (Billard, 1969b), 10–12 in mosquito fish (Geiser, 1924), 9 in zebrafish (Leal et al., 2009) 6 in mudminnow (Foley, 1927), redfish (Moser, 1967), and rainbow trout (Loir, 1999), or 3 in mullet (Van der Horst, 1978). Cytoplasmic bridges, present between germ cells from the first spermatogonial division, interconnect them to a clonal syncytium in one cyst (Loir et al., 1995).

In species showing several spermatogonial generations, like guppy, zebrafish, or trout, a differentiation can be made based on cell/nuclear size and number of cells per cysts into early and late type B spermatogonia (B_{early} and B_{late}, respectively; see Figs. 2 and 4). When information is available on the exact number of generations, the generations of type B spermatogonia can be numbered (Schulz et al. 2005; Leal et al., 2009). Both in mammals (De Rooij and Russell, 2000) and fish, the type B spermatogonia are dividing more rapidly than the type A spermatogonia, as indicated by the ~5-fold higher mitotic index of B type over A type spermatogonia in African catfish (Schulz et al. 2005). After the final mitosis, type B spermatogonia differentiate into primary (preleptotene) spermatocytes, from where the ensuing developmental stages are: primary spermatocytes (1st meiotic division) → secondary spermatocytes (2nd meiotic division) → spermatids (differentiation without proliferation) → spermatozoa.

The different spermatogonial generations often pose problems as regards their identification and therefore receive particular attention. In general, stem cells are defined as the most undifferentiated cells of a particular lineage. They reside in a niche created by a supporting cell type, Sertoli cells, and the surrounding extracellular matrix. The niche is defined by its specific microenvironment that allows the stem cell to retain its stemness (Hofmann, 2008; Li and Xie, 2005). For different stem cell systems, both symmetric and asymmetric divisions have been described, i.e. the two daughter cells assume similar of different fates, respectively. Although there is no formal proof yet, vertebrate SSCs are considered to divide symmetrically in both self-renewal or differentiating divisions, while the fate assumed would be determined by the somatic environment: if a SSC is exposed to signalling favouring self-renewal, i.e. is in a stem cell niche, a differentiating outcome is unlikely while displacement from the niche, or change of signalling within the niche, would favour differentiation (Kostereva and Hofmann 2008; Oatley and Brinster 2008). Information on specific morpho-functional or molecular characteristics of the SSC niche in the teleost testis is still missing.

One of the undifferentiated traits of SSCs is that they are single cells, i.e. not connected via cytoplasmic bridges to clone members. Accordingly, a stem cell self-renewal division provides single daughter cells, while a pair of spermatogonia joined via a cytoplas-

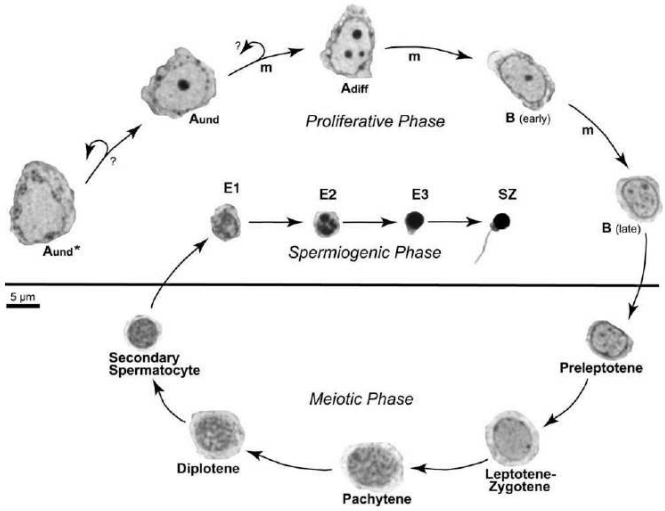

Fig. 2. Step-by-step representation of zebrafish spermatogenesis from type A undifferentiated spermatogonia to spermatozoa, throughout the three phases of the spermatogenic process: proliferative or spermatogonial, meiotic or spermatocytary and spermiogenic. The spermatogonial micrographs show the most characteristic features of each cell type, although there is a range of their morphology and size. Type A undifferentiated spermatogonia (A_{und}*) (stem cell?); type A undifferentiated spermatogonia (A_{und}); type A differentiated spermatogonia (A_{diff}); type B spermatogonia (B); self-renewal (curved arrows); mitosis (m); early spermatids (E1); intermediate spermatids (E2); final spermatids (E3); and spermatozoa (SZ). A_{und}* and A_{und} are single cells, whereas A_{diff}, B (early) and B (late) are grouped. The first question mark (?) indicates a doubt if A_{und}* and A_{und} are separated by a mitosis or represent different stages of the same cell cycle (see also Fig. 4), while the second question mark indicates uncertainty as regards the "stemness" of A_{und}.

mic bridge emerges from a differentiating mitosis (Oatley and Brinster, 2008). In different species, morphologically and/or functionally different types of single spermatogonia have been described, and it is possible that they also differ in the degree of stemness and/or activity. In the human testis, for example, two types (pale and dark) of single A spermatogonia are present and might play distinct roles as "reserve" versus "active" stem cell, respectively (Schulze, 1979, 1988). Also, recent experiments suggested the existence of "true" and "potential" spermatogonial stem cells in mice testes, since after severe testicular damage (e.g. after chemo- or radiotherapy), undifferentiated spermatogonia (A_{pr} and A_{al}) can act as stem cells (Nakagawa et al., 2007). Finally, a so-called side population among SSCs was described in mice testis (Lassalle et al., 2004; Shimizu et al., 2006), characterized by a differential efflux of a fluorescent dye (Hoechst 33342). However, the properties of the testicular side population are not undisputed (Kubota et al., 2003).

Also in fish unique molecular markers for SSCs are not known yet, although the first candidate has been proposed recently (notch1; Yano et al., 2009). Functional data, such as derived from SSC transplantation studies, are scarce in fish. Transplantable germ cells are present in adult rainbow trout testis (Yano et al., 2009), and primordial germ cells (PGCs) collected at very early stages of ontogenesis from different fish species can be transplanted (e.g. Saito et al., 2008; Yoshizaki et al., 2005), but the recipients have always been fish at early stages of ontogenesis, so that transplanted germ cells joined the recipient's PGCs in their migration to the genital ridges. At present, there is only one proof-of-principle publication reporting the transplantation of SSCs to an adult male recipient (Lacerda et al., 2006). Clearly, this is a very promising approach but at the same time a clear caveat requiring significant research attention since transplantation models are urgently needed for studies on stem cell biology in fish.

A (partially) restricted distribution of spermatogonia provides positional clues for stemness, since at the end of spermatogenic tubules close to the tunica albuginea a very high (e.g. Billard, 1969b), or an elevated (e.g. Matta et al., 2002; Schulz et al., 2005) density of type A_{und} undifferentiated spermatogonia is observed. Exploiting this feature in tilapia, we have analysed tubules close to the tunica albuginea on the ultrastructural level (Fig. 3).

Different from mammals, spermatogonia in teleosts are not in direct contact with the basal lamina, and are always completely surrounded by Sertoli cells. Morphometric studies have shown a striking and steady decrease in germ cell volume in tilapia (approximately 2300 to 160 μm^3) from A_{und} spermatogonia to the last generation of B spermatogonia (Schulz et al. 2005). Simultaneously, germ cell number (1 to ~120 cells) as well as Sertoli cell number increased (1.4 to 3.6 cells) per spermatogonial cyst. We can therefore conclude that tilapia spermatogonia go through 7 rounds of mitosis before differentiating into spermatocytes, i.e. there are eight generations of spermatogonia. These results are a good illustration of the dynamics of spermatogenic cysts in fish and how morphometric investigations can help our understanding about the functional aspects of spermatogenesis.

Besides the nuclear size, that also decreases dramatically (~550 to 80 μm^3) from type A_{und} to type B spermatogonia, the amount and distribution of chromatin and cytoplasmic structure/organelles also changes in tilapias. In this regard, the two types of A_{und} spermatogonia differ in tilapia. The most undifferentiated type is most frequently found close to the tunica albuginea (Fig. 3A) while another type of A_{und} (Fig. 3B) can also be found at a certain distance from the tunica; both cell types are large single cells surrounded by

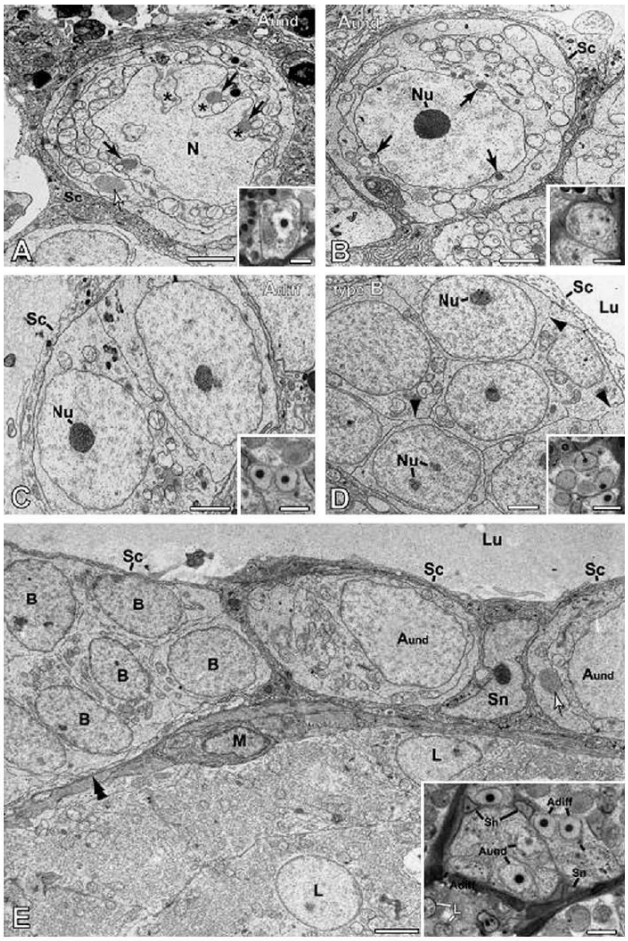

Fig. 3. Characterization of tilapia spermatogonial types under electron and light (insets) microscopy. A_{und}, type A undifferentiated spermatogonia; A_{diff}, type A differentiated spermatogonia; B, type B spermatogonia; Sc, Sertoli cell cytoplasm; Sn, Sertoli cell nucleus; N, nucleus; Nu, nucleoli; asterisks, nuclear folds; black arrows, "*nuages*"; white arrow, low electron dense "*nuage*" far from the nucleus; Lu, lumen; arrowheads, spermatogonial membrane opening; L, Leydig cell nucleus; M, myoid cell nucleus; double arrowhead, basal membrane of the seminiferous tubules. Bar, 1.5 μm; insets bar, 7 μm.

Sertoli cells. However, it seems that the cell with an exclusive location close to the tunica has a slightly smaller nucleus, less heterochromatin, a more convoluted nuclear envelope, and more mitochondria that are closer to the nucleus and surrounded by smooth endoplasmic reticulum (Fig. 3A). Particularly interesting is that these cells present large amounts of electron-dense material called "nuage", usually located between the nuclear envelope and the mitochondria. It is assumed that this dense material is composed of ribonucleoproteins and mRNA species showing a long half-life time, including *vasa* or *piwi* mRNA, specific products of the germ cell lineage (Knaut et al., 2000; Houwing et al. 2007). Intriguingly, "nuage" is one of the cellular characteristics of PGCs, so that this cell type may be closest to the ontogenetically earliest germ cell type. Apparently, the second type A_{und} (Fig. 3B) spermatogonium is the second largest germ cell type in tilapia. The main differences to the previous cell type is a reduced amount of "nuage", a more smooth outline of the nuclear membrane, an increased amount of heterochromatin, and a prominent nucleolus (Fig. 3B). It is not known if these two types represent different phenotypes (e.g. during different phases of the cell cycle) of the same cell type, or if they are separated by a mitotic division/differentiation. The somewhat broader distribution of the larger type A_{und}

spermatogonia suggests that in any case a displacement from the tunica takes place. Future studies will have to show if both cell types have (similar?) stem cell capacity, or if one of them can be considered as a reserve stem cell, or even a more primitive germ cell (PGC/gonocyte?).

A similar situation appears to exist in zebrafish (Leal et al., in press) although due to the unrestricted distribution of spermatogonia, there are no positional clues. However, careful analysis of the morphology of spermatogonia revealed that there are two similar types of single A_{und} spermatogonia (Figs. 2 and 4) as in tilapia, although in zebrafish there seems to be no size difference between these cells, both being larger than all other germ cell types.

Type A_{diff} spermatogonia are found in cysts with two to eight germ cells (Figs. 3C and 4). Their nuclei are round to oval, they contain no or very little nuage-like material, show one or more nucleoli, and are somewhat smaller than the A_{und} spermatogonia. With the latter cells, they share a low amount of heterochromatin in the nucleus, and a relatively large cytoplasmic volume (Figs. 1B, 2, and 4).

Type B spermatogonia are present in several generations. These cells occur in cysts with 16 or more germ cells, the number depending on the species-specific number of mitotic cell cycles that are completed before the germ cells differentiate into primary spermatocytes. The B spermatogonia show a progressively decreasing cell and nuclear volume, with a small rim of cytoplasm surrounding an oval to round nucleus that gradually shows more heterochromatin (Fig. 4).

After geometrical expansion of the spermatogonia number, the last type B spermatogonia differentiate into spermatocytes to enter meiosis, a sequence of two specialized cell cycles. Meiosis I—reductional, where the homologous chromosomes are segregate into secondary spermatocytes; and the meiosis II—equational, where the sister chromatids segregate, resulting in four haploid cells harboring one copy of each chromosome. One of the aims of meiosis is to generate genetic diversity through two events, first the recombination (crossing-over) and then the segregation of homologous chromosomes.

Under light microscopy, the criteria used to identify the meiotic cells are based mainly on nuclear characteristics, such as, size (diameter), shape, degree of chromosome condensation, and associated metaphasic figures (Figs. 1B and 2). In leptotene/zygotene zebrafish spermatocytes, the nucleus is larger (~5.5 μm) and more round in comparison with the last type B spermatogonia, the chromatin is clear and shows small dots of heterochromatin outlining the nuclear membrane (Figs. 1B and 2). Pachytene spermatocytes are the most frequent meiotic germ cells (the approximate duration of pachytene in zebrafish is 12 h—unpublished result), and has a large (~6.5 μm) and dense nucleus, with the chromosomes appearing as bold lines from the nuclear membrane to the center of the nucleus (Figs. 1B and 2). Diplotenic primary spermatocytes are often found together with metaphasic figures (metaphase I); the chromosomes reach their maximum degree of condensation and are found close to the nuclear membrane (Figs. 1B and 2). Sometimes, in the diplotenic primary spermatocytes, the outline of the nuclear membrane is not clear, or has disappeared already.

The secondary spermatocytes, resulting from meiosis I, are rare because they quickly enter meiosis II. They are small cells with a dense nucleus (~ 4 μm), usually found together with metaphasic figures (metaphase II) (Fig. 1B, 2). A fine description of meiotic germ cells using light and electron microscopy can be found for

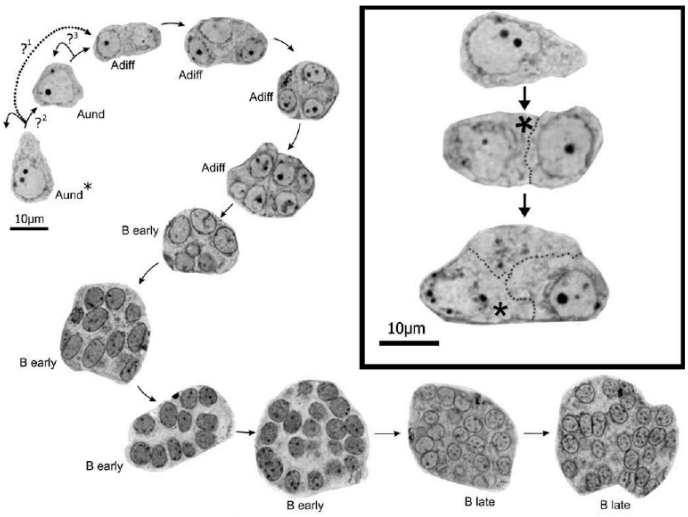

Fig. 4. Spermatogonial generations in zebrafish. The terminology to address the spermatogonial generations in zebrafish was based mainly on nuclear (heterochromatin amount) and nucleolar characteristics, and the number of germ cells inside the cyst. Using high resolution light microscopy applied to araldite-embedded sections, we propose five types of spermatogonia: type A undifferentiated* (A_{und}*), type A undifferentiated (A_{und}), type A differentiated (A_{diff}), type B early (B_{early}) and type B late (B_{late}). The curved arrows indicate self-renewal while the simple arrows indicate mitosis. The first question mark ($?^1$) indicates a doubt as regards a possible asymmetric division of A_{und}* (in the inset, note the stippled line towards a pair of spermatogonia). The second question mark ($?^2$) refers to the uncertainty if type A_{und}* is separated from A_{und} by mitosis or if they represent different stages of the same cell cycle. The third question mark ($?^3$) indicates a doubt as regards the "stemness"of A_{und}. Inset: The asterisks show undifferentiated spermatogonia belonging to cysts with other types of spermatogonia. This observation might indicate that asymmetrical divisions can occur but requires further studies in teleosts.

several species of fish (see Pudney, 1995; Quagio-Grassiotto and Carvalho, 1999; Schulz et al., 2005). Histomorphometric analyses from tilapia testis (Schulz et al., 2005) have demonstrated that volume changes of meiotic germ cells were similar to those observed in mammals (França and Russell, 1998). However, in mammals, diplotene spermatocytes show the maximum volume (França and Russell, 1998) whereas in tilapia, pachytene spermatocytes are the biggest cell. We do not have an explanation for the shift to pachytene, or even zygotene in guppies, in teleosts at present.

Spermiogenesis consists of series of morphological changes that lead to the differentiation of spermatids into spermatozoa. The changes include nuclear condensation, elimination of organelles and cytoplasm, flagellum formation, and the rearrangement of cellular organelles along the spermatozoon cytoplasm (Jamieson, 1991). These remarkable modifications during spermiogenesis may explain the cyst volume decrease during this phase in tilapia (Schulz et al., 2005). Moreover, apoptotic loss of germ cell may occur in this phase.

It is possible to characterize three types of spermatids with respect to the nuclear condensation: E1 (early spermatids), E2 (intermediate spermatids), and E3 (final spermatids; Fig. 1B, 2; Schulz et al., 2005). Also, in fish, three types of spermiogenesis (type I, II, and III) have been described (Mattei, 1970; Quagio-Grassiotto and Oliveira 2008) based on the orientation of the flagellum to the nucleus, and on whether or not a nuclear rotation occurs. Type I is characterized by a perpendicular flagellum in relation to the nucleus with nuclear rotation; in type II, the flagellum develops parallel to the nucleus without nuclear rotation, and in type III, the flagellum is central without nuclear rotation (Mattei, 1970; Quagio-Grassiotto and Oliveira 2008). These patterns are reflected in the spermatozoa structure, and highly conserved within taxonomic units, and are, therefore, a powerful tool for phylogenetic analyses in fish (Jamieson, 1991; Quagio-Grassiotto and Oliveira 2008).

In teleost fish spermatozoa generally have no acrosome, and the impenetrable chorion is pierced by a micropyle that gives access to the membrane of the oocyte. Spermatozoa often show a spherical nucleus with homogenous, highly condensed chromatin, a nuclear fossa, a midpiece of variable size with or without a cytoplasmic channel, and one or two long flagella (Jamieson 1991). Moreover, fish spermatozoa can be classified into two forms, aquasperm and introsperm, according the external or internal mode of fertilization, respectively (Jamieson, 1991).

At the end of spermiogenesis, when intercellular bridges are broken and spermatozoa are individualized, the junctional complex between the cyst-forming Sertoli cells undergoes a dynamic remodeling that culminates into the cyst opening and hence the release of spermatozoa into the tubular lumen (Fig. 1B). It is not clear if in fish the release of spermatozoa is facilitated by myoid cells or/and the Sertoli cell cytoskeleton.

In some species, the release of germ cells occurs already at the spermatid (or spermatocyte) stage, and spermiogenesis (and meiosis) is (are) completed in the tubular lumen. This type of spermatogenesis is called semicystic spermatogenesis (Mattei, 1993) and is found in different taxonomic groups, such as Opheliidae (Mattei, 1993), Scorpaenidae (Muñoz et al., 2002), Bleniidae (Lahnsteiner and Patzner, 1990), Corydoradinae (Spadella et al., 2007), and Soleidae (García-López et al., 2005 and Kamstra and Schulz, unpublished observation). In semicystic spermatogenesis, Sertoli cells form earlier than in other species an epithelial lining of the spermatogenic tubules. However, the fact that individualized spermatids from different germ cell clones proceed through spermiogenesis in the tubular lumen seems but a variation of the timing of germ cell individualization.

There are cases, however, where spermatozoa are not "individualised" during release. Instead, sperm bundles are formed within the cyst when Sertoli cells secrete a capsule around a group of spermatids, thereby creating spermatophores (Grier, 1984). In the case of spermatozeugmata, on the other hand, Sertoli cells enlarge and send projections around the gametes to anchor them at the luminal margin of the cyst (Grier, 1993; Pudney, 1993). Also junctions between differentiating spermatids and Sertoli cells have been described (mosquito fish; Arenas et al. 1995). Interestingly, in this species spermatids undergo an elongation process, which is atypical for most teleosts but typically seen in mammals. Since also in those cartilaginous fish showing elongated spermatids ectoplasmic specializations were found (Stanley and Lambert 1985), the two features may be functionally connected.

The fluid, in which spermatozoa are suspended in the tubular lumen after spermiation, is produced by Sertoli cells (Billard et al. 1972; Setchell, 1986). In some species, like catfish (Loir et al., 1989), seminal vesicles are present as differentiation of the spermatic duct. In African catfish, the seminal vesicles are tubular structures that develop from caudal part of immature testis where spermatogenic tissue merges into spermatic duct system (van den Hurk et al., 1987); seminal vesicle tissue develops under the influence of rising androgen levels during puberty (Cavaco et al., 2001). Their columnar, single-layered epithelium is homologous to Sertoli cells (Loir et al., 1989) and expresses high levels of FSHR (Bogerd et al., 2001) while the thin lamina propria, on which they reside, contains some cells that show morphological characteristics of Leydig cells (van den Hurk et al., 1987). The seminal vesicles produce a fluid characterised by an acid pH value; the Leydig cell-like population may be a source for pheromones (Resink et al., 1987).

Data dealing with the timing of spermatogenetic events are scarce. The duration of meiosis plus spermiogenesis varies from 7 to 21 days in 4 tropical species (Silva and Godinho, 1983, Sinha et al., 1983) to 1–3 months in species living in temperate and cold zones (Louie and Dixon, 1972; Billard, 1983). At 25 °C, the total duration of spermatogenesis in guppy is 36 days (Billard, 1969b).

3. A complex and changing testicular transcriptome during maturation

Different approaches, such as analysis of candidate gene expression or suppressive subtractive hybridization (SSH) cDNA libraries served to study the dynamic gene expression patterns associated with the spermatogenic process. More recently, global testicular transcriptome analyses have been carried out using arrays that allow a high throughput and cost effective description of the temporal changes in expression of hundreds or thousands of genes simultaneously. LeGac and collaborators (Mazurais et al., 2005) analyzed gene expression in trout testes assigned to histologically defined stages of gonadal maturation, from stage I (immature) to stage IV (spermiogenesis). The initial data, obtained using 1152 trout cDNAs spotted on macroarrays, supported the view that the transition from prepubertal to maturing testis was associated with changes in the expression of numerous genes. In addition, gene expression profiles analysis showed that differentially expressed genes were segregating into five distinct clusters, some of them being enriched in genes involved in the same biological process. Cluster A included up-regulated genes linked to protein modification and cell organisation and biogenesis. Cluster B grouped genes transiently over-expressed at stage II as spermatogonia type B actively proliferate; different biological processes were present but none seemed significantly over-represented in this cluster. Cluster C included genes up-regulated from stage II onwards; the genes are involved in proliferation, protein modification and biosynthesis. Clusters D and E were up regulated during the meiotic and post-meiotic stages III and IV, respectively. As expected, gene annotation was associated with mitotic/meiotic progression and with

protein synthesis/catabolism, protein modification, and cell organisation and biogenesis.

Hence, changes in gene expression detected by global transcriptome analysis required further investigations to determine if changes in gene expression occurred within a numerically stable cell population and/or were the result of the proliferation of specific cell populations. To determine the cellular origin of gene expression, transcriptome data were collected from spermiating trout and from isolated germ cell populations enriched in spermatogonia, spermatocytes, or haploid cells (Rolland et al., submitted for publication). This study used a cDNA microarray (GEO datasets platform GPL3650) designed by the AGENAE consortium and carrying more gene representatives (9152 contigs with a UniGene annotation). The classification of about 8000 expression values, obtained for each of the 50 testicular samples, again clearly displayed distinct transcriptomes in relation to the cell types or stages of gonadal maturation. In addition, cDNA microarrays data were supplemented by Q-PCR and in situ hybridisation (ISH) approaches to address the interactions within and between complex gene networks.

Genes could be classified in at least 8 main clusters that reflect major expression profiles (A–H; see Fig. 5). For instance, the transition from the gonadal stages I to VIII was marked by changes of the expression pattern of genes encoding extra-cellular matrix proteins (cluster C). This observation is in agreement with the tubular reorganization observed within the testis during the reproductive cycle. In addition, the expression levels of cell cycle and mitotic spindle regulatory genes increased in relation to the rapid mitotic activity of the type B spermatogonia (cluster F and G). Genes encoding growth factors were differently expressed throughout the spermatogenetic process (cluster B). For instance, two members of the TGFβ superfamily, anti-Müllerian hormone (AMH) and gonadal soma derived factor (GSDF), were dramatically repressed in the Sertoli cells as germ cell differentiation occurred. A very interesting observation was that genes involved in the repression of DNA recombination were up-regulated during the same period, suggesting that meiotic processes are postponed in spermatogonia (cluster F). Transcripts encoding meiotic and post-meiotic proteins (sycp1, sycp3, dyneins) were significantly over-represented in clusters G and H and their levels of expression increased simultaneously with the appearance of large numbers of spermatocytes and spermatids. Another cluster in C contained genes that were highly expressed only at spermiation (Fig. 5).

4. Extrinsic regulation of spermatogenesis

4.1. Pituitary hormones and growth factors

The gonadotropins, LH and FSH, are the most important pituitary hormones regulating testicular physiology. Two points are of great relevance for their biological activity, the specificity, with which the gonadotropins interact with their receptors and the cellular site(s) of receptor expression. In mammals, FSH and LH interact with their receptors (FSHR and LHR, respectively) in a highly specific manner with little overlap in biological activities at physiological hormone concentrations. LH regulates Leydig cell sex steroid production; FSH regulates Sertoli cell activities, such as the structural, nutritional, and regulatory (paracrine) support of germ cell development (Huhtaniemi and Themmen, 2005). Both, receptor specificity and site of expression, contribute to the impression that the biological activities of fish gonadotropins seem less clearly separated. In most cases where the binding characteristics of FSHR and LHR have been characterized using hormones from the same (or very closely related) species (African catfish: Garcia-Lopez et al., 2008; Channel catfish: Sampath Kumar et al., 2000; Japanese eel: Kazeto et al., 2008; zebrafish: So et al., 2005; but with the possible exception of rainbow trout: Sambroni et al., 2007), the FSHR showed a preference for FSH, but was also activated by LH. However, cross-activation of the FSHR requires high LH concentrations and may be restricted to periods with peak LH plasma levels, such as during the spawning season. From an evolutionary point of view, it is interesting to note that an avian FSHR behaved in a

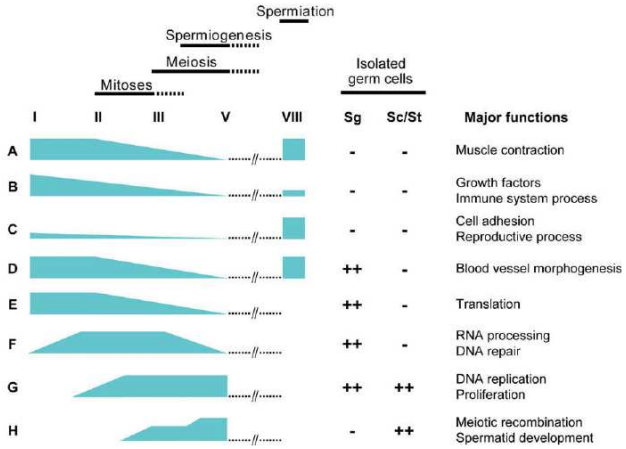

Fig. 5. Gene expression clusters of male gonad development in the rainbow trout. Different development stages of male gonad (I–VIII) together with isolated germ cells (Sg, spermatogonia; Sc, primary spermatocytes; St, spermatids) were used to establish a comprehensive data set of gene expression during trout spermatogenesis. More than 3000 differentially expressed genes were grouped according to their expression profiles in both developing testes and isolated germ cells (expression clusters A–H). A representative expression pattern of each cluster is schematized during gonad maturation whereas – and ++ indicate a low and a high expression in isolated germ cells, respectively. Major functions, as evidenced through a GeneOntology term enrichment analysis, are also indicated for each cluster.

similar manner as regards the cross-activation by LH (Wakabayashi et al., 1997). The LHR too can be cross-activated by FSH, but the required FSH concentrations (close to 1 μg/ml in rainbow trout and African catfish) are far above maximum plasma levels, so that the LHR can be considered as LH-specific physiologically.

Still, piscine FSHs are potent steroidogenic hormones (Planas et al., 1993). One possibility to explain FSH-mediated stimulation of Leydig cell steroidogenesis is to assume that Leydig cells in fish, different from their mammalian counterparts, express the FSHR gene. An antibody raised against the eel FSHR detected immunoreactive material on both, Leydig and Sertoli cells (Ohta et al., 2007). In African catfish, both Leydig and Sertoli cells expressed the FSHR gene, while LHR gene expression was restricted to Leydig cells (Garcia-Lopez et al., 2008). Taken together, the data on receptor pharmacology and localization suggest that Leydig cell steroidogenesis is directly regulated by LH and by FSH, while Sertoli cell functions are predominantly regulated by FSH, although high LH concentrations, such as during the spawning season, might cross-activate the FSHR.

Data on LH plasma levels are available from a number of fish species, but much less data has been published on circulating FSH levels, and as regards males, information is restricted to salmonid species only (Campbell et al., 2003; Gomez et al., 1999; Prat et al., 1996). In these species, plasma LH levels were very low or undetectable during the start of testis development (slow and rapid proliferation of spermatogonia), became detectable when germ cells entered meiosis, but did not increase clearly until close to the spawning season. FSH levels, on the other hand, showed a transient increase in association with spermatogonial proliferation, then re-increased with spermiation *sensu strictu*, and decreased before the spawning season started. Campbell et al. (2003) reported that elevated circulating levels of androgens and FSH coincided in male Chinook salmon with active spermatogonial proliferation and Ohta et al. (2007) demonstrated that suppressing the steroidogenic activity of FSH abolished the stimulatory effect of FSH on spermatogonial proliferation in Japanese eel. Hence, the present knowledge in fish indicates that FSH-mediated steroidogenesis stimulates early stages of spermatogenesis.

What do we know about the possible roles of FSH as a direct regulator of Sertoli cells? Prominent FSH activities in mammals are: stimulation of Sertoli cell proliferation during the prepubertal period; induction of terminal differentiation of Sertoli cells together with androgens and thyroid hormones during puberty; regulation of growth factor release, which in turn modulates spermatogenesis; or support of terminal phases of spermiogenesis and spermiation. Many of these functions were uncovered by analysing the effects of experimentally induced or pathologically characterized FSHR loss-of-function animal models or patients (Huhtaniemi and Themmen, 2005). However, information on FSH effects on fish spermatogenesis that is not related to steroidogenesis is not available so far. It is tempting to speculate that elevated FSH plasma levels during the start of spermatogonial proliferation may be functionally related to proliferation of Sertoli cells, considering that the number of these cells increases in association with the mitotic expansion of spermatogonial cysts in tilapia and African catfish (Schulz et al., 2005).

A few growth factors have been identified that are produced by Sertoli cells and that regulate germ cell proliferation behaviour (see Section 4.2 below), such as the TGFβ family members activin (Miura and Miura, 2001), GSDF (Sawatari et al., 2007), AMH (Miura et al., 2002) and its receptor (Morinaga et al., 2007), or an orthologue of platelet-derived endothelial cell growth factor (PD-ECGF; Miura et al., 2003). While in some cases information is available on the modulation of expression of these growth factor genes by androgens (activin, AMH) or estrogens (PD-ECGF), no information is available as regards the role of FSH in regulating growth factor expression in fish. In mammals, for example, FSH stimulates AMH expression in the prepubertal but not in the adult testis (Lukas-Croisier et al., 2003) and AMH exerts an inhibitory effect on Leydig cell differentiation (Salva et al., 2004) and androgen production (Fynn-Thompson et al., 2003). It will be interesting to study in fish these endo- and paracrine mechanisms. We anticipate that technical advances (e.g. production of recombinant FSH and establishment of testis cell/tissue culture approaches for more species) will allow significant progress in the near future considering the role of FSH in fish spermatogenesis.

Other pituitary hormones next to LH and/or FSH can modulate testicular steroidogenesis, such as growth hormone (Singh et al., 1988), and cross-talk between the reproductive and growth axes occurs via sex steroids that modulate pituitary GH release (Melamed et al., 1995). In pubertal Chinook salmon, the start of spermatogonial proliferation coincided with elevated levels of FSH, androgens, and of insulin-like growth factor 1 (IGF1; Campbell et al., 2003), a growth factor released by the liver in response to GH stimulation. In addition to a possibly systemic action, binding studies revealed the presence of GH receptors in trout testis and also enriched Sertoli cell population (LeGac et al., 1993; Gomez et al., 1998). Also IGF1 mRNA expression and IGF-I receptors were demonstrated on germ and Sertoli cells in trout (LeGac et al., 1996) and tilapia testis (Berishvili et al., 2006), and experimental evidence supports the role of IGF1 in proliferation and/or differentiation of spermatogonia (Loir and LeGac, 1994; Vinas and Piferrer, 2008). Also, it has been proposed that GH, directly and/or through locally produced IGF1, can modulate testicular steroidogenesis (LeGac et al., 1996) and/or germ cell proliferation (Loir, 1999). Finally, it is interesting to note that IGF1 plays a permissive role as regards the stimulatory effects of androgens on spermatogenesis in Japanese eel (Miura and Miura, 2001). In general it appears that regulatory input to spermatogenesis via gonadotropins and sex steroids can be modulated or fine-tuned by other pituitary hormones and local signalling in the testis. The mechanisms involved are largely unknown but may involve the differential expression, per spermatogenic cyst, of hormone and growth factor receptors.

4.2. Steroid hormones

The sex steroids, progestagens, androgens, and estrogens are mainly produced in the gonads. Plasma levels of steroid hormones show important variations during male gonad maturation. In general, estrogens are considered 'female' hormones but are formed in male vertebrates as well (Schlinger and Arnold 1992; Hess et al. 1995; Betka and Callard 1998). In male teleosts, 17β-estradiol (E2) is present in blood serum in rather low concentrations (Miura et al. 1999; Amer et al. 2001; Chaves-Pozo et al., 2007). E2 plasma levels show a transitory elevation at the beginning of the reproductive cycle in trout (Gomez et al., 1999). Androgens (testosterone, T; 11-ketotestosterone, 11-KT) increase gradually as spermatogenesis proceeds and decrease at spermiation. During the reproductive cycle of salmonid fish, there are two peaks of 17α,20β-dihydroxy-4-pregnen-3-one (DHP) blood plasma levels. A big peak is observed in the spawning season, another small peak during the progression of spermatogonial proliferation. The small peak of DHP was known in salmonid fish for some time (Depeche and Sire, 1982; Scott and Sumpter, 1989; Vizziano et al., 1996). Taken together, these data suggest that steroid hormones have important and distinct roles in controlling fish spermatogenesis. These roles will be discussed below.

4.2.1. Estrogens

Estrogens bind to nuclear receptors that act as ligand inducible transcription factors. Three estrogen receptor subtypes (alpha, beta1 and beta2) are expressed in fish and the male gonad is a

major site of expression. Estrogen receptors expression has been reported in the somatic testicular cells and in haploid male germ cells (Miura et al., 1999; Chang et al. 1999; Bouma and Nagler, 2001; Wu et al., 2001; Menuet et al., 2002; Ito et al., 2007).

These findings suggest that estrogens have an important role in regulating gene expression in the testis. Administration of E2 to maturing sea bream males resulted in the identification of numerous estrogen dependent genes in the testis (Pinto et al., 2006). For many of these genes, there is no evidence that they are relevant for testicular functions, although they could be considered as biomarkers of estrogen exposure. Another group of estrogen dependent genes may be more relevant since they were involved in different biological processes including cell proliferation (PCNA, fibrinogen B and G), lipid metabolism, protein metabolism and folding, transport, and cell communication (Pinto et al. 2006).

In another study, LeGac and collaborators (unpublished results) have administrated E2 implants to prepubertal male trout (stage I/II with spermatogonia only), resulting in E2 plasma levels of 1–8 ng/ml, and analysed global transcriptome changes in the testis. Although the treatment significantly up-regulated 80 transcripts and down-regulated 33 other transcripts, gene expression variations did not exceed 1.5- to 3-fold with the exception of the vitellogenin encoding gene (up to 10- to 30-fold induction), a well known biomarker for oestrogen exposition. E2 had significant regulatory effects (direct or indirect) on the expression of genes important for the regulation of the steroidogenesis (i.e. Star, 3βHSD, aromatase A and B) and for spermatogenesis: increased expression of genes encoding for retinol binding proteins I and II suggest that estrogens control the homeostasis of retinoic acid within the testis. Retinoic acid availability is required for the proliferation and differentiation of the undifferentiated spermatogonia in mouse (Zhou et al., 2008), although it seems less relevant in zebrafish (Alsop et al., 2008). In addition, the AMH gene was down-regulated in oestrogen treated trout, which supports the involvement of estrogens in the stimulation of the spermatogonial stem cell renewal in male as initially proposed in eel (Miura et al., 1999). In medaka, administration of low doses of ethinyl estradiol resulted in a similar stimulatory effect of estrogens on spermatogonial stem cell renewal, whereas high doses had an inhibitory effect (Song and Gutzeit, 2003). In gilthead seabream, supraphysiological doses (>100 ng/ml) of estrogens inhibited proliferation, induced apoptosis of undifferentiated spermatogonia, accelerated late events in spermatogenesis and induced post-spawning processes such as infiltration of acidophilic granulocytes (Chaves-Pozo et al., 2007). In maturing fish, high oestrogen doses reduced the seminal fluid volume, increased sperm density, and may lead to sterility (Lahnsteiner et al., 2006).

4.2.2. Androgens

Two androgen receptor subtypes (α and β) have been described in fish and they are all predominantly expressed in the gonad (Takeo and Yamashita, 1999; Todo et al., 1999; Ikeuchi et al., 2001). The AR was expressed in Sertoli and interstitial cells but not in germ cells (Ikeuchi et al., 2001), suggesting that androgens develop biological activity via the testicular somatic cells.

Androgens strongly influence testicular gene expression. In prepubertal trout, both T and 11-KT treatments (inducing blood plasma levels of 15–30 ng/ml) have similar effects on a common group of 155 up-regulated and 20 down-regulated genes (LeGac et al., 2008; Rolland et al., submitted for publication). Some of these genes are expressed in Sertoli cells and have been previously demonstrated to regulate spermatogenesis and steroidogenesis. For instance, expression of the AMH gene that inhibits differentiation of spermatogonia is significantly suppressed by T and 11-KT. Numerous transcription factors of known significance for testis development were regulated by T or 11-KT treatment (see Table 1 for selected examples). Of note is that androgen treatment also increased numerous transcripts involved in muscle development and function. In view of the known myogenic action of androgens, it is therefore interesting to note that the peritubular cells in the testis are myoid cells with contractile characteristics, known androgen-responsive target cells in higher vertebrates.

Beside similar responses, T and 11-KT also affected a subset of genes differentially. LeGac and colleagues (unpublished results) observed that 56 genes were up-regulated by 11-KT but not by T, and 260 genes were up-regulated by T but not by 11-KT. This finding indicates the possibly specific effects of each of these androgens. In mice, different classes of androgen receptor binding DNA response elements exist which might be an essential feature of differential androgen target gene regulation (McPhaul and Young, 2001).

Many transcripts that changed after sex steroid treatment displayed an interesting pattern of expression during testis maturation (Rolland et al., submitted for publication). For example, 3 subgroups of androgen-responsive genes are up-regulated respectively, in the prepubertal stage I, at the initiation of rapid spermatogonial proliferation (stage II), and in meiotic/post meiotic stages (stages III–V). This suggests that these particular steroid-regulated genes play a significant role during testis maturation and spermatogenesis. Furthermore, most of these maturational changes in gene expression appear highly correlated with the changes in circulating androgen levels. For instance, some genes are up-regulated when androgen levels increased moderately during the initiation of spermatogenesis; others are over-expressed in parallel to the large increase in androgen levels occurring around spawning time (Rolland et al., submitted for publication). Indeed, a large proportion of genes up-regulated by androgen treatment is found over-expressed at stage VIII (cluster C, Fig. 5). Since several of these genes code for ion or water transporters (Atp1b1a, Aqp4, Kcnk15, Slc26a4, Slc27a11), androgens might be of particular importance for milt hydration or for providing a proper environment for sperm maturation/conservation. This study may serve as a basis for hypothesis-driven research and provide insight as regards gene networks and pathways under sex steroid control in the trout testis. Combined with the in situ localization of gene expression and the search for steroid responsive elements in their regulatory sequences, such approaches will progressively allow for the comprehension of gene regulations involved at the key steps of spermatogenesis.

Androgens are effective in supporting either the whole process of spermatogenesis, or at least some steps such as spermatogonial multiplication and spermatocyte formation (guppy) or maturation (killifish) (Remacle, 1976; Billard et al., 1982; Fostier et al., 1983; Billard, 1986; Nagahama, 1994; Borg, 1994). They may also participate in the initiation of puberty (Miura et al., 1991a,b; Cavaco et al., 1998). In addition, androgens induces spermiation in some species but were clearly less effective than progestins (Ueda et al., 1985).

4.2.3. Progestins

Nuclear and membrane-bound progestin receptors types are highly expressed in fish gonads (Todo et al., 2000; Zhu et al., 2003). The highest levels of circulating progestins are observed during the entire spermiation process and these hormones have been involved in the regulation of several testicular functions. Progestins as DHP or 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) advance and induce spermiation in Salmonidae and Cyprinidae (Ueda et al., 1985), increase milt production (Baynes and Scott, 1985; Yueh and Chang, 1997), and stimulate spermatozoa motility (Miura et al., 1992; Tubbs and Thomas, 2008). Besides, the involvement of DHP in meiosis was recently reported in male eel (Miura et al., 2006).

Table 1
Transcription factors relevant for testis development were up- or down-regulated in immature rainbow trout testis following androgen treatment in vivo.

Up regulated
Doublesex- and mab-3-related transcription factor 1: DMRT1 is known as an important factor in sex differentiation. DMRT1 is essential for testicular development in mice and might play a role in medaka spermatogenesis. DMRT1 expression was inhibited by E2 and stimulated by T and 11KT, while DHP had no significant effect. When testis maturation proceeded until after the initiation of meiosis, DMRT1 expression was under-expressed in germ cells
Transcription factor AP-1 (Proto-oncogene c-jun): c-jun and c-fos (components of the transcription factor AP1) regulate cellular growth and differentiation and also exert regulatory roles in steroidogenesis and spermatogenesis. In trout, c-jun transcript was under-expressed in germ cell populations as compared to total testis, and to decrease in stage III compared to stage I-II of testis development; it is up regulated after 11KT, T, or DHP treatment but not modified by E2
Transcription factor E2F4 (E2F-4): The E2F family plays a crucial role in cell cycle control and suppresses proliferation-associated genes. In trout, this transcript was over expressed in germ cells, increased in stage III (start of meiosis), and was up-regulated after T or 11KT, but not after DHP treatment
LIM domain transcription factor LMO4 interacts with Smad proteins and modulates TGFp signalling in epithelial cells; it takes part in the negative regulation of estrogen receptor alpha transactivation functions. In our experiments, its expression is very stable during testis maturation, but is up regulated after T or 11KT treatment
Nuclear receptor OB1 (DAX-1) functions as a negative regulator of steroidogenesis during the development and adult function of the reproductive axis; it is also suggested as a negative co-regulator of nuclear receptors, including androgen and progesterone receptor.
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1): PPAR are ligand-inducible transcription factors implicated in lipid transport and metabolism; in mammals PPARγ could play a major role in testicular somatic cells. In trout testis we found that the PPAR co-activator is under expressed in germ cells, compared to whole testis. It is up-regulated after T, 11KT, and DHP treatments.
Transcription factor SOX-8: this SRY-related HMG-box protein may be involved in male sex determination and AMH regulation but its role during spermatogenesis is not known. It shows only limited variation in expression during testis maturation but is up-regulated after androgen and DHP treatment
Nuclear matrix transcription factor 4 (Cas-associated zinc finger protein) regulates transcription from matrix metalloproteinase promoters and from the type-I collagen promoter. It may suppress BMP2 signalling, and impaired spermatogenesis was found in Nmp4-disrupted mice. We found that this factor is over-expressed in testis in stage III–V, compared to stage I and to germ cell populations. It is up-regulated after T or11KT, but not after E2 or DHP treatment
Down regulated
T-box transcription factor TBX1 (Testis-specific T-box protein): Involved in early developmental gene regulation, TBX1 has a second phase of expression in adult testis. In our experiments, TBX1 was under-expressed in isolated germ cells compared to the whole testis, and its expression decreased from stage III to VIII. It is down-regulated after T and 11KT treatments, also after DHP treatment but to a lesser extent

4.3. Molecular regulation of spermatogenesis

4.3.1. Spermatogonial stem cell renewal

As mentioned earlier, spermatogonial mitosis can be categorized in slow spermatogonial renewal and rapid proliferation of differentiated spermatogonia towards meiosis (Clermont, 1972). Both kinds of spermatogonial mitosis are regulated by different mechanisms by steroid hormones.

Evidence obtained in different vertebrate classes indicates that spermatogonial renewal is regulated by E2. In Japanese eel, the role of E2 in spermatogenesis was investigated using in vitro and in vivo experimental approaches (Miura et al., 1999). Slow renewal mitosis of eel spermatogonia was promoted by implanting E2, but was suppressed by tamoxifen (an oestrogen receptor antagonist). In vitro, 10 pg/ml of E2 was sufficient to induce spermatogonial renewal divisions in cultured testicular tissue, confirming the in vivo observations. E2 treatments did not, however, promote rapid spermatogonial proliferation and meiosis. In Japanese huchen (*Hucho perryi*), E2 similarly promoted spermatogonial stem cell renewal in vitro (Amer et al. 2001). In medaka, administration of low doses of estrogenic compounds resulted in a similar effect whereas high doses had an inhibitory effect (Song and Gutzeit, 2003). These findings clearly indicate that oestrogen is also a 'male' hormone, and plays an important role in SSC renewal. This function of estrogens is not only known from teleost fish, but also from other vertebrates, such as mammals (Kula 1988), amphibians (Minucci et al., 1997), and reptiles (Chieffi et al., 2002). Furthermore, in female teleosts oogonial renewal proliferation is regulated by E2 (Miura et al., 2007).

Generally, E2 induces target gene expression via its nuclear receptor, and the factors translated from oestrogen target genes affect biological processes. The molecular mechanisms of oestrogen-induced spermatogonial renewal were analyzed in Japanese eel (Miura et al., 2003, 2007). Eel spermatogenesis related substance 34 (eSRS34), a homologue of mammalian platelet derived-endothelial cell growth factor (PD-ECGF), was identified by expression screening as gene up-regulated by E2 and functionally analysed using eel testis tissue and cell culture systems. Recombinant eSRS34 induced spermatogonial stem cell renewal, while addition of a specific anti-eSRS34 antibody prevented spermatogonial stem cell renewal induced by E2. These results indicate that eSRS34 is a 'spermatogonial stem cell renewal factor' (Fig. 6). Fibrinogens (FgB and FgG) are known as positive regulators of cell proliferation in wound healing and in tumours (Staton et al., 2003). In sea bream testis, FgB and FgG are up-regulated by E2 stimulation (Pinto et al., 2006), and also may be regulators of spermatogonial renewal like eSRS34.

Other genes have been involved in the proliferation of fish spermatogonial stem cells. A new member of the TGFβ family, GSDF, increases PGC numbers and promotes the mitotic activity of the spermatogonia in prepubertal trout (Sawatari et al., 2007). However, a duplicated copy of the GSDF gene has been identified recently in salmonids (GSDF2). The GSDF2 transcripts are restricted to the testis and the Sertoli cells. Contrary to the first gene copy, GSDF2 encodes a protein containing all the conserved cysteine residues known to be important for the function of the TGFβ family members (Lareyre et al., 2008). To date, it is not known whether the two GSDF factors have distinct or redundant functions in trout.

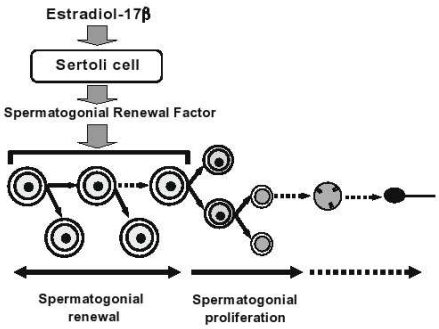

Fig. 6. A schematic summary of possible oestrogen-dependent control mechanisms of spermatogonial renewal in fish.

4.3.2. Spermatogonial proliferation toward meiosis

Upon gonadotropic stimulation, spermatogonial mitosis switches from the slow self-renewal pathway to rapid proliferation toward meiosis. We refer to this point as the initiation of spermatogenesis. As discussed above, FSH plays a major regulatory role during early stages of spermatogenesis, while LH is mainly involved in the final stages of maturation. In agreement with this notion, FSH was the key gonadotropin for initiation of spermatogenesis using eel testicular organ culture (Ohta et al., 2007) (Fig. 7).

The eel Fsh receptor is expressed by Leydig cells and by Sertoli cells surrounding type A and early type B spermatogonia. In an in vitro organ culture, recombinant eel Fsh (r-eFsh) induced complete spermatogenesis from the proliferation of spermatogonia to spermiogenesis during 36 days of culture; also spermatozoa were observed in the testicular fragments. Spermatogenesis induced by r-eFsh was inhibited by trilostane, a specific inhibitor of 3β-hydroxysteroid dehydrogenase. Thus, the main function of FSH during the initiation of spermatogonial proliferation is stimulating the production of spermatogenesis-inducing steroids, such as 11-ketotestosterone (Miura et al., 1991a), the major androgen of teleost fish. In fact, FSH can induce the production of 11-ketotestosterone in testis in vitro (Kamei et al., 2005, Ohta et al., 2007). Future work will have to show if FSH has additional functions next to stimulating Leydig cell steroid production, for example in relation to FSH receptor expressing Sertoli cells.

11-Ketotestostrone was first identified by Idler et al. (1961) as a major androgenic steroid in the male sockeye salmon (*Oncorhynchus nerka*). In various teleost fish, this steroid has since then been shown to be synthesized in the testis following GTHs stimulation and high levels were detected in the serum during spermatogenesis (Billard et al. 1982). The function of 11-ketotestosterone for spermatogenesis became clear for the first time using an eel testicular organ culture system (Miura et al., 1991a). When 11-KT was added to the organ culture, spermatogenesis was induced from the proliferation of spermatogonia to spermiogenesis. This action of 11-KT is not limited to the Japanese eel but has been recognized also in goldfish (Kobayashi et al., 1991) and Japanese huchen (Amer et al., 2001). These findings indicate that 11-KT is one of the factors involved in the initiation of spermatogonial proliferation toward meiosis. However, it is believed that the action of 11-KT is mediated by other factors produced by Sertoli cells that, in contrast to germ cells, express androgen receptor protein (Ikeuchi et al., 2001). It is possible that some of these factors are growth factors, such as IGF1 and activin B. IGFs are known to be mediators of growth hormone action in vertebrates. Although IGF1 is also necessary for the regulation of eel spermatogenesis, its role is to support the action of 11-KT. More specifically, in Japanese eel 11-KT is necessary for the induction of spermatogenesis, whereas IGF1 is necessary for the continuation of the process (Nader et al. 1999).

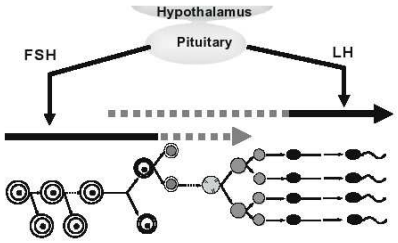

Fig. 7. A schematic summary of the roles of FSH, mainly regulating processes during the mitotic phase, and of LH, mainly regulating processes during the spermiogenic phase, on fish spermatogenesis.

How does 11-KT initiate spermatogonial proliferation in fish? In Japanese eel, two members of the TGFβ superfamily, AMH (Miura et al., 2002) and activin B (Miura et al. 1995a), have important roles during the initiation of spermatogenesis induced by 11-KT.

Activin B is a dimeric growth factor composed of two activinβ B subunits. In the Japanese eel, activin B was found in the testis at the initiation of spermatogenesis after hCG stimulation, with its expression site restricted to Sertoli cells. Both transcription and translation of eel activin B were induced by 11-KT stimulation in vitro. Further, activin B induced proliferation of spermatogonia, but was unable to induce meiosis or further developmental steps (Miura et al., 1995a).

AMH, also known as Müllerian inhibiting substance, is secreted as intercellular signalling protein. It was identified and later purified on the basis of its ability to induce regression of the female genitalia primordium, the Müllerian duct, in mammalian embryos (Balanchard and Josso, 1974). In mammals, AMH is produced by Sertoli cells of the foetal and prepubertal testis and by ovarian granulosa cells after birth (Balanchard and Josso, 1974; Vigier et al., 1984). AMH was isolated from eel testis as gene down-regulated by human chorionic gonadotropin (hCG) treatment using gene expression screening. AMH was expressed in Sertoli cells of immature testes, but was strongly down-regulated after gonadotropin stimulation. Expression of AMH mRNA was suppressed by 11-KT in vitro. To examine the function of AMH in spermatogenesis, recombinant eel AMH (eAMH) was added to a testicular organ culture system. Spermatogonial proliferation induced by 11-KT in vitro was suppressed by recombinant eAMH. Furthermore, addition of a specific anti-eAMH antibody induced spermatogonial proliferation in a germ cell/somatic cell co-culture system. This data set indicates that eAMH prevents the initiation of spermatogenesis and, therefore, suppression of AMH expression is necessary to initiate spermatogenesis. Hence, AMH is a spermatogenesis-preventing substance. Down-regulation of AMH mRNA during the progression of spermatogenesis has been reported in some teleost species (Rodríguez-Marí et al., 2005; Halm et al., 2007; Pala et al., 2008), which supports our hypothesis as regards the role of AMH. Taken together, it seems possible that the two TGFβ family members AMH and activin play opposing roles with regard to the differentiation of rapidly proliferating spermatogonia, activin B being a stimulatory, and AMH an inhibitory factor (Fig. 8).

AMH is also related to the control of gamete development before initiation of spermatogenesis in fish. In medaka, AMH stimulated germ cell proliferation already before hatching just after primordial germ cells had reached the gonadal primordium (Shiraishi et al., 2008). In medaka, a loss-of-function mutation of the AMH receptor not only resulted in excessive proliferation of the PGCs before hatching but also a premature initiation of meiosis, and prevented germ cell proliferation during male sex differentiation after hatching (Morinaga et al., 2007), so that AMH may also act as a meiosis preventing substance. Thus, AMH is multi-functional factor for reproduction in fish. It would be interesting to investigate in higher vertebrates if the phylogenetically old functions of AMH have been conserved, at least to some extent.

4.3.3. Initiation of meiosis

Following mitotic proliferation, type B spermatogonia enter the meiotic prophase and differentiate into primary spermatocytes. DHP, a major progestin in teleost fish, is an indispensable hormone for the initiation of meiosis in spermatogenesis.

Progestins are sex steroid hormones important for female reproduction. In mammals, the principal physiological action of progestin is to prepare the reproductive tract for pregnancy and

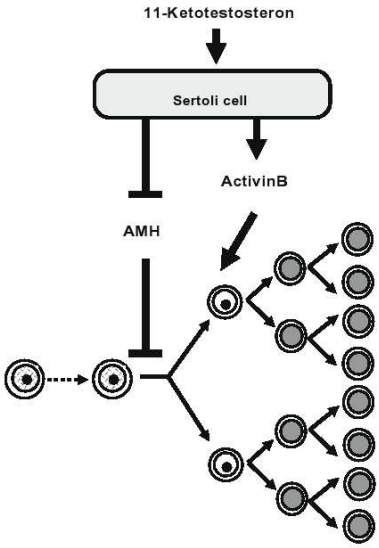

Fig. 8. A schematic summary of the possible control mechanisms at the initiation of spermatogonial proliferation toward meiosis in fish. 11-KT; 11-ketotestosterone, AMH; anti-Müllerian hormone.

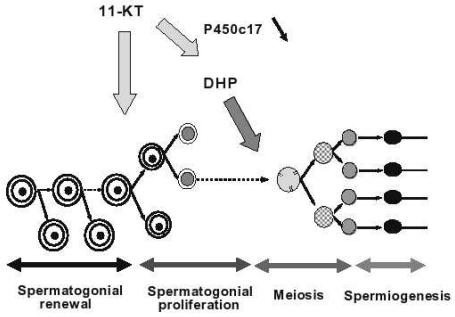

Fig. 9. A schematic summary of the relationship between 11-ketotestosterone (11-KT) and DHP (17α,20β-dihydroxy-4-pregnen-3-one) in regulating fish spermatogenesis. 11-KT can inhibit the expression of P450c17, facilitating DHP production via an increased availability of substrate for DHP synthesis.

to provide nutritive support for the embryo during gestation (Thomas, 1998). In all vertebrates, progestin also plays important roles in gametogenesis. In females, progestins regulate oocyte maturation by binding to an oocyte plasma membrane receptor. Progestin binding inhibits oocyte adenylate cyclase, resulting in reduced cyclic AMP dependent protein kinase activity. This induces the activation of "maturation promoting factor" (MPF) via Cdc25, and thereby the resumption of division I of meiosis (Nagahama, 1997).

During the reproductive cycle of male salmonid fish, there are two peaks of DHP blood plasma levels. A big peak is observed in the spawning season, another small peak during the progression of spermatogonial proliferation; the spawning-associated peak will be discussed later. The small peak of DHP was known in salmonid for some time (Depeche and Sire, 1982; Scott and Sumpter, 1989), but its role had not been clarified clearly yet. Recently, a role for DHP in early spermatogenesis became clear using the eel testis tissue/cell culture systems (Miura et al., 2007).

DHP and its nuclear receptors were present in the testis at an early stage of spermatogenesis, and DHP was shown to induce DNA replication in spermatogonia. Although 11-ketotestosterone also stimulated DNA synthesis in spermatogonia, as mentioned above, antibodies against DHP prevented DNA replication when added during the period when meiosis was initiated. DHP treatment also induced the expression of meiosis specific markers, such as Dmc1 and Spo11. Furthermore, Spo11 expression and synaptonemal complexes, specific features of the meiotic prophase, were detected in testicular fragments cultured with DHP in some germ cells that showed morphological characteristics of undifferentiated spermatogonia. These data indicate that DHP induces spermatogonia to enter the meiotic prophase (Fig. 9).

Recently, DHP was shown to induce meiosis not only in spermatogenesis, but also during early oogenesis in Japanese huchen, *Hucho perryi*, and common carp, *Cyprinus carpio* (Miura et al., 2007). In huchen and carp ovarian organ culture, DHP induced DNA synthesis of oogonia, and the expression of Spo11 and synaptonemal complex proteins in some germ cells. Therefore, DHP is also implicated in the regulation of early oogenesis from oogonial proliferation to initiation of the first meiotic division. These data from male and female gonad tissue and cell culture systems suggest that DHP, a progestin, is an essential factor for the initiation of meiosis in both spermatogenesis and oogenesis.

What are the molecular mechanisms by which DHP initiates meiosis in fish? Although we attempted to clone key factors regulated by DHP stimulation (Ozaki et al., 2006), the mechanism has not been clarified yet. The zebrafish loss-of-function mutant for a DNA-repair enzyme, the Mlh1 mutant male, is completely infertile due to a block in meiosis I (Feitsma et al., 2007). Such mutant lines may be suitable models to further study DHP-regulated control mechanisms of meiosis.

4.3.4. Final maturation of male gametes

During the breeding season of male teleost fish, numerous plasma hormone levels show remarkable changes which are initiated by an increase in LH secretion (Prat et al., 1996). LH secretion induces an increase in the production of the testicular steroids, such as 11-KT and DHP or 20β-S (the latter mostly in marine species). 11-KT injections induced spermiation in goldfish and some salmonids, and DHP injections had similar effects in several salmonids (Ueda et al., 1985; Milla et al., 2008) and eel (Miura et al., 1991b). Recently, it has been proposed that 11-deoxycorticosterone participates in the control of milt fluidity as well (Milla et al., 2008). While LH and these steroids clearly are involved in regulating spermiation in fish, the mechanism of action of these hormones on milt hydration, sperm migration to the sperm duct, or increase in milt volume, are still unclear.

In some species, spermatozoa released from Sertoli cells after completion of spermiogenesis are not yet capable of fertilizing eggs. In salmonids, spermatozoa in the testis and in the sperm duct are immotile. Dilution with fresh water induces motility of spermatozoa collected from the sperm duct while testicular spermatozoa remain immotile after dilution. Thus, spermatozoa acquire the ability of motility during their passage through the sperm duct.

Sperm maturation, the phase during which non-functional gametes develop into mature spermatozoa (fully capable of vigorous motility and fertilization) involves physiological but no morphological changes. In salmonids, sperm maturation (the acquisition

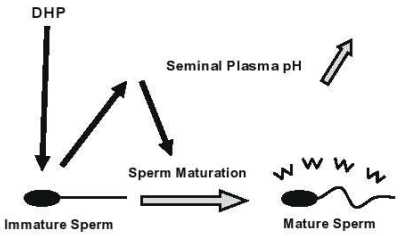

Fig. 10. A schematic summary of the possible control mechanisms of sperm maturation in fish.

of sperm motility) has been induced by increasing the seminal plasma pH (approximately to pH 8.0) in the sperm duct, which results in elevation of intra-sperm cAMP levels (Morisawa and Morisawa, 1988; Miura et al. ,1992). Similar results have been reported for Japanese eel spermatozoa by Miura et al. (1995b) and Ohta et al. (1997).

Sperm maturation is also regulated by the endocrine system. In some teleosts including Japanese eel, it has been suggested that DHP regulates sperm maturation (Miura et al., 1991b, 1992). It seems that DHP action is mediated through an increase in the seminal plasma pH, which in turn increases the cAMP content in sperm, thereby allowing the acquisition of sperm motility (Miura et al., 1991b, 1992, 1995b; Fig. 10). However, progestins may have a direct action on sperm motility since 20β-S binds to sperm plasma membrane and stimulates sperm hypermotility in Atlantic croaker and seatrout (Tubbs and Thomas, 2008). A membrane bound progestins receptor (mPRα) has been localized on the plasma membrane of the spermatozoon mid piece in Atlantic croaker (Thomas et al., 2005).

Thus, estrogenic, androgenic, and progestagenic sex steroids, are important regulators for the progression of spermatogenesis from spermatogonial stem cell renewal to sperm maturation.

5. Intrinsic regulation of spermatogenesis

Experimental approaches depriving the testis from crucial extrinsic input, e.g. hypophysectomy (Billard, 1969a; Khan et al., 1986), or testis cell/tissue culture in the absence of hormones/growth factors demonstrated that the development beyond the slow self-renewal of undifferentiated type A spermatogonia requires extrinsic regulatory input in fish. Similar conclusions can be drawn from experimental models in mammals, where loss of the pituitary gonadotropins for different reasons (e.g. Kendall et al., 1995; Seminara et al., 2003) resulted in a failure of pubertal maturation. Still, interspecies germ cell transplantation experiments showed that the timing of the different developmental steps constituting spermatogenesis is determined by the germ cell genome (França et al., 1998), i.e. reflects cell autonomous processes. It appears therefore that hormones/growth factors do not implement a step-wise regulation of germ cell development, but rather are responsible for regulating the balance and transition between the different micro-environments that are required for the germ cells to execute their autonomous developmental program.

The complexity of this developmental program can be exemplified through a short side-step to yeast. From the nearly 6200 genes in the genome of *Saccharomyces cerevisiae* (Goffeau et al., 1996), 1600 are expressed differentially during meiosis. When deleting 300 of these genes one by one (Rabitsch et al., 2001), ~10% were found to be required for proper segregation of the meiotic chromosomes only. Using the relation of 10% of 1600 meiosis-related genes being required for meiosis, 160 genes represent ~2.5% of the yeast genome. Calculating with the estimated 37,460 genes in the zebrafish genome, 2.5% represented more than 900 potentially meiosis-specific genes in zebrafish; additional spermatogenesis-specific genes may function during the mitotic and the spermiogenic phases in multicellular organisms. Some of the spermatogenesis-specific genes seem to be highly conserved, as exemplified by the rescue of a meiotic defect in a *Drosophila* mutant by the homologous human gene (Xu et al., 2003). In mammals, several cases of compromised gene functions derived from mutagenesis screens, targeted disruption of candidate genes, or from the genetic analysis of infertility patients, were characterised with regard to their spermatogenesis phenotypes. These germ cell-autonomous processes are often based on germ cell-specific (variants of) cell cycle regulators, transcription factors, or enzymes sometimes referred to as chauvinist genes that, when suffering a loss-of-function mutation, lead to an arrest of spermatogenesis at a specific stage, thereby identifying the essential function(s) of the gene product. The respective information has been reviewed comprehensively (De Rooij and de Boer, 2003). The information available in fish is reviewed below.

The gene *dead end* (*dnd*) encodes an RNA binding protein that is a component of the germ plasm in zebrafish, and is specifically expressed in primordial germ cells (PGCs). Dnd protein is localized to perinuclear germ granules (nuage) within PGCs. Knockdown of *dnd* mRNA results in failure of PGCs to migrate towards the genital ridges and subsequent PGC apoptosis, resulting in a germ cell-free, sterile gonad where the somatic compartment differentiates as a testis (Slanchev et al., 2005; Weidinger et al., 2003). A similar phenotype as regards the loss of all PGCs has been observed in mice where a point mutation resulted in a premature stop codon of the murine *dnd* orthologue gene (Youngren et al., 2005).

At a later stage in zebrafish ontogenesis, loss-of-function mutations of the two germ cell-specific members of the Piwi subclass of the Argonaut family of proteins result in complete germ cell loss as well (Houwing et al., 2008; Houwing et al., 2007). Akin phenotypes have been described in mice, although loss of these proteins (called MIWI and MILI, respectively) still allows survival of spermatogonia and apoptosis occurs at the spermatocyte stage (Deng and Lin, 2002). However, ablation of an additional mouse variant of the Argonaut family, MIWI2, also affects SSC maintenance (Carmell et al., 2007). In zebrafish, the *piwi* orthologues are *ziwi* and *zili*, coding for enzymes involved in the metabolism of a germ line-specific class of non-coding, small RNAs, the Piwi-interacting (pi) RNAs. Most piRNAs are involved in transposon silencing, possibly by modulating the DNA methylation pattern and/or by piRNA-guided breakdown of transposon-derived sequences, while some of them might also target non-repetitive, exonic sequences (Houwing et al., 2008). Loss of Ziwi protein results in the apoptotic loss of all germ cells at 3–4 weeks of age and, in contrast to wild-type siblings, mutant germ cells were not able to enter differentiation (spermatogenesis) until they became apoptotic. The present model assumes that Ziwi and Zili collaborate to amplify the piRNAs required for transposon silencing; still, Ziwi alone seems capable to generate low levels of piRNAs (Houwing et al., 2008). Therefore, it may not be surprising that germ cell loss in *zili* occurs at a later stage (6–7 weeks of age) than in *ziwi* loss-of-function mutants. Similar to Ziwi, germ cells cannot enter the differentiation path (spermatogenesis) in the absence of Zili until entering apoptosis.

The last example discussed in the context of germ cell autonomous regulation of spermatogenesis refers to a gene, *mlh1*, operating during meiosis. In leptotene spermatocytes, a large number of double-strand breaks are generated in the chromosomes (Marcon and Moens, 2005). The homologues are still apart, but begin to search for homology. Synapsis, close association of homologues

by binding of synaptonemal complex proteins, starts in zygotene and is completed in pachytene. During synapsis the double-strand breaks are repaired. A fraction of these, on average one or two breaks per pair, is repaired via homologous recombination with a nonsister chromatid of the homolog. These crossing-over sites become visible in diplotene, when desynapsis takes place, and are stabilized as chiasmata, the only sites that keep the chromosome pair together. The mutL complex involved in meiosis consists of Mlh1 and Mlh3 and is present in distinct foci on the synapsed elements during pachytene. In mice, the MLH1/MLH3 foci coincide in timing, number, and position with the presumptive sites of crossing over (Marcon and Moens 2005). The idea is therefore that the complex stabilizes a limited number of recombination sites for repair by crossing over, while the other sites will be repaired via different mechanisms. *Mlh1* and *mlh3* knockout mice have very similar phenotypes in respect to meiosis, males are sterile and lack spermatozoa completely. A very similar phenotype has been found in zebrafish lacking a functional Mlh1 protein (Feitsma et al., 2007). The arrest in spermatogenesis was localized to metaphase I and primary spermatocytes accumulated at this arrest point, showing irregular meiotic figures in the nuclei and massive apoptosis. Surprisingly, females were fully fertile, but their progeny shows high rates of dysmorphology and mortality within the first days of development caused by aneuploidy, resulting from meiosis I chromosomal missegregation.

6. Future aspects and research directions

Spermatogenesis provides motile cellular vectors through an evolutionary conserved process that has been placed under the control of the brain-pituitary system in vertebrates. The master control exerted by the vertebrate endocrine system over testis maturation (puberty) and adult spermatogenesis, whether continuous or intermittent in seasonally reproducing species, provides numerous evolutionary advantages, such as silencing of the reproductive system until somatic development and/or seasonal conditions are sufficiently favourable for an individual to "afford" reproduction. Moreover, integrating (re-)activation of spermatogenesis, via its dependency on sex steroids, with reproductive behaviour and secondary sexual characters, allows for sexual selection to have an impact. Seemingly wasteful (ornamental) competitive traits, often present in males, reflect the individual's competence to recruit resources from the environment, involve several genes spread over the genome, and hence can serve as genome quality indicators that are used during mate choice. In this way deleterious alleles are efficiently removed, and advantageous ones rapidly fixed in a population (Clutton-Brock, 2007; Siller, 2001).

What are the most important steps in spermatogenesis under control of the endocrine system? With the information available at present, there seem to be three points where reproductive hormones play a critical regulatory role: (i) the balance between self-renewal and differentiation of SSCs; (ii) the transition of type A spermatogonia to rapidly proliferating type B spermatogonia, and (iii) the entry into meiosis. During later developmental stages, on the other hand, the endocrine system seems to assume a permissive rather than stimulatory role, enabling Sertoli cells and possibly other somatic cells to generate the microenvironment germ cells require to proceed through meiosis and spermiogenesis.

The sensitivity of the earlier stages for stimulatory input via the endocrine system is a characteristic feature for vertebrates and makes this period very interesting from the point of view of basic biology. Knowledge on the regulatory mechanisms is only beginning to emerge, and respective information will contribute significantly to our understanding of (spermatogonial) stem cell biology. Moreover, the recent finding that SSCs are pluripotent cells in mice and men will further boost research into understanding the balance between SSC self-renewal and differentiation, from a basic biology, comparative, but also from an applied (e.g. medical, veterinary, conservation biology) point of view.

In addition, the early stages of spermatogenesis are of great interest for research fields, such as aquaculture biotechnology and ecotoxicology. Surface freshwater bodies, for example, contain biologically significant amounts of estrogens, oestrogen-like compounds, anti-androgens, and other agonists/antagonists of nuclear receptors, and detrimental effects on spermatogenesis of these compounds, termed endocrine disrupting chemicals, have been described in wild populations of fish and in experimental models (Hutchinson et al., 2006). In aquaculture biotechnology, precocious male maturation is commonly observed problem in different species, with adverse effects on growth and flesh composition, for example (see Taranger et al., 2010). Detailed knowledge about the underlying processes triggering and sustaining testis maturation will be of great relevance for developing approaches to control male puberty, and for estimating the impact of, and the requirement for monitoring/regulating the presence of endocrine disrupting chemicals in surface freshwater bodies.

Improving our knowledge on the early stages of spermatogenesis sensitive to exogenous stimulatory input, we need experimental systems suitable to investigate, for example, SSC biology in fish. Until now, the SSC transplantation assay is the only system available in vertebrates, has been adapted for tilapia already, and should be developed for other species as well. For example, we known that 11-KT suppresses Sertoli cell AMH expression and thereby facilitates the start of spermatogenesis, but since also the receptor for AMH is expressed by Sertoli cells, we in fact only know about the initial autocrine loop of this signalling system that will probably consist of at least one other signalling molecule produced by Sertoli cells, a respective receptor on germ cells, and the germ cell's response system to this signalling. Considering that in the fruitfly, different regulatory systems function in parallel to control SSC behaviour (Li and Xie, 2005), including signalling from SSCs to the somatic cells, and assuming an at least as complex regulatory network in the vertebrate stem cell niche in the testis, we can safely predict that there is a lot to learn.

In order to learn more about SSCs and other stages of germ cell development as well as their interaction with somatic cells of the testis, we need to enrich or purify these cell types. This is not only required for transplantation assays to investigate SSC biology, but also for transcriptomic or proteomic profiling of the different cell types. Possible approaches include the generation of transgenic lines expressing fluorescent proteins under the control of promoter/enhancer sequences that are specific for the cell types or developmental stages of interest. Also, double or triple transgenic lines expressing distinct reporter genes will be excellently suited for cell lineage tracing studies, or for investigating the interaction of different cell types during testis differentiation and adult functioning. Finally, the complete process of spermatogenesis can be studied in fish using ex vivo tissue culture systems (eel: Miura et al., 1991; goldfish: Remacle 1976; tilapia: Tokalov and Gutzeit, 2005). Similar systems should be developed for model species like medaka and zebrafish, in order to study the direct effects of hormones/growth factors on spermatogenesis, possibly including the live-imaging of developmental processes with tissue from transgenic animals expressing fluorescent proteins and modern imaging devices.

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