



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



Caracterização de marcadores de espermatogônias tronco e sua
regulação endócrina e parácrina em zebrafish (*Danio rerio*)

Lucas Benites Doretto

Botucatu, São Paulo

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INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

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Orientador: Prof. Dr. Rafael Henrique Nóbrega

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“O amor ao estudo deve ser um traço característico de cada jovem. Isto é, que o jovem deve ampliar constantemente seu horizonte espiritual, aprofundar seus conhecimentos da teoria socialista, estudar história, a cultura de seu povo e finalmente, amar sua profissão, e melhorar constantemente sua qualificação técnica. Este é o dever de nossos jovens operários, estudantes e empregados”

As Tarefas da Juventude

Klement Gottwald, 25 de Abril de 1947

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Resumo

1 As células tronco são classificadas em dois grandes grupos de acordo com sua origem
2 e capacidade de diferenciação. Células tronco embrionárias (CTE) são originadas do zigoto,
3 e podem ser classificadas como totipotentes, isto é, capazes de originar um
4 indivíduo inteiro, ou pluripotentes, quando originam os três folhetos embrionários (ecto,
5 meso e endoderme). As células tronco adultas (CTA) são as células tronco encontradas nos
6 tecidos fetais e adultos; classificadas como uni, oligo ou multipotentes dependendo da
7 variedade de tecidos originados a partir delas. Marcadores de células tronco, como antígenos
8 de superfície específicos, fatores de transcrição como OCT4 e NANOG são expressos em
9 CTE e algumas CTA, mas são rapidamente reprimidos à medida que as células se
10 diferenciam. O presente trabalho tem como objetivo identificar marcadores de células tronco
11 e com isso, os efeitos do hormônio endócrino Fsh e do fator parácrino GDNF na atividade
12 proliferativa e gênica dessas populações de células e também de células de Sertoli. Foi
13 observado que os marcadores Pou5f3 e Gfra1a são principalmente expressos em
14 espermatogônias tronco indiferenciadas e que sua expressão reduz significativamente sob
15 efeito do recombinante zebrafish Fsh. Por outro lado, genes como o *igf3*, *nanos3* e *nanog*
16 tiveram sua expressão aumentada significativamente. O recombinante humano GDNF não
17 altera significativamente a expressão desses genes, porém estimula a proliferação de
18 espermatogônias tipo Aund e Adiff e células de Sertoli associadas. Logo, conclui-se que o
19 rzfFsh atua de maneira endócrina na diferenciação de espermatogônias tronco Pou5f3+ e
20 Gfra1a+ via células de Sertoli, visto que seu receptor é principalmente expresso em cistos
21 indiferenciados. O rhGDNF, que por sua vez é expresso em células germinativas, estimula a
22 proliferação de Aund e Adiff e células de Sertoli associadas através de seu receptor Gfra1a,
23 expresso em ambas populações.

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Abstract

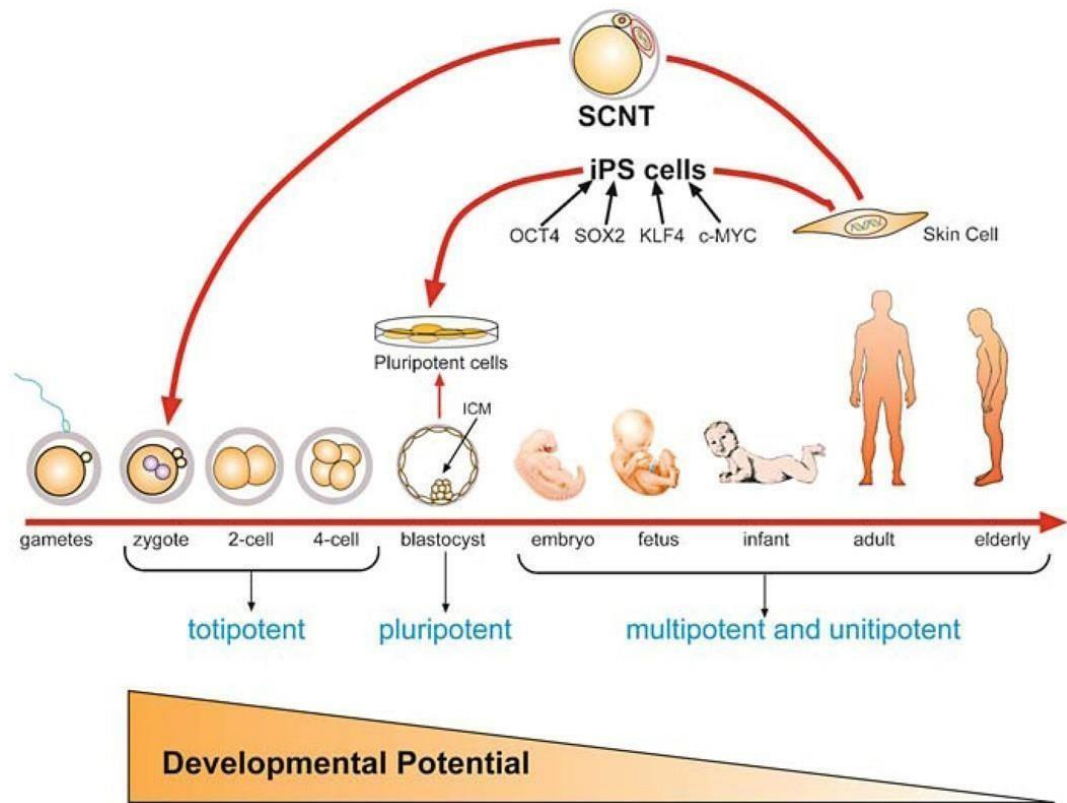
Stem cells are classified into two major groups according to their origin and capacity for differentiation. Embryonic stem cells (ESCs) originate from the zygote, and can be classified as totipotent, i.e., capable of originate whole individuals, or pluripotent, when they originate the three embryonic leaflets (ecto, meso and endoderm). Adult stem cells (ASC) are the cells found in fetal and adult tissues; classified as uni, oligo or multipotentes depending on the variety of tissues originated from them. Markers of stem cells, such as specific surface antigens and transcription factors such as OCT4 and NANOG are expressed in ESCs and some ASCs, but are rapidly repressed as the cells differentiate. The present work aims to identify stem cell markers and the effects of the endocrine hormone Fsh and paracrine factor GDNF on the proliferative activity of these cell populations and Sertoli cells as well as gene expression. It has been observed that the Pou5f3 and Gfra1a markers are mainly expressed in undifferentiated spermatogonia stem cell and their expression is significantly reduced by the recombinant zebrafish Fsh. On the other hand, genes like *igf3*, *nanos3* and *nanog* had their expression significantly increased. The human recombinant GDNF does not significantly alter the expression of these genes, but it stimulates the proliferation of Aund and Adiff spermatogonia and associated Sertoli cells. Therefore, it is concluded that rzfFsh acts as an endocrine factor in the differentiation of the spermatogonia stem cell Pou5f3+ and Gfra1a+ via Sertoli cells, since its receptor is mainly expressed in undifferentiated cysts. rhGDNF, which in turn is expressed in germ cells, stimulates the proliferation of Aund and Adiff and associated Sertoli cells through its Gfra1a receptor, expressed in both populations.

1. Introdução Geral

1.1 Células tronco e seu nicho

As células tronco são consideradas por definição biológica como as únicas células dos organismos multicelulares capazes de se autorrenovar como também de se diferenciarem em um ou mais tipos celulares específicos (Weissman, 2000; Fuchs *et al.*, 2004; Mitalipov & Wolf, 2008). O balanço entre estes dois processos é essencial para garantir o funcionamento dos tecidos do organismo (de Rooij *et al.*, 2009). Para exemplificar, se o processo de autorrenovação for favorecido, as células tronco se multiplicam e formam uma espécie de hiperplasia. Por outro lado, se a diferenciação é favorecida, o tecido gradativamente perde sua função devido a exaustão da população de células tronco e, conseqüentemente, da produção de células diferenciadas. Tais células podem ser classificadas de acordo com sua origem e capacidade de diferenciação; as células tronco embrionárias (CTE) e as células tronco adultas (CTA) (Smith, 2006; Mitalipov & Wolf, 2008). As CTE são originadas a partir do zigoto que se divide e forma blastômeros totipotentes até o estágio de 4 células. Totipotência é aqui definido como a capacidade de uma única célula originar um indivíduo inteiro (Figura 1) (Mitalipov & Wolf, 2008).

Com a progressão do desenvolvimento embrionário (estágio de 8 células), os blastômeros perdem gradativamente sua totipotência a qual se encerra irreversivelmente quando os mesmos se diferenciam para formar o maciço celular interno e o trofoblasto (Figura 1) (Mitalipov & Wolf, 2008). Nesta etapa, as CTE podem ser consideradas pluripotentes, pois originam os diversos tipos de tecidos do corpo (Figura 1) (Mitalipov & Wolf, 2008). No entanto, estas células perdem a capacidade de se organizar e formar um embrião propriamente dito. As células tronco pluripotentes dão origem às CTA que estão presentes nos diversos tecidos do corpo (epitelial, conjuntivo, ósseo, cartilaginoso, adiposo, muscular e nervoso) podendo se diferenciar em um (unipotente), poucos (oligopotente) ou vários (multipotente) tipos celulares (Figura 1). A Figura 1 retirada de Mitalipov & Wolf (2008), ilustra bem as diferenças entre as CTE e CTA assim como a definição de totipotência, pluripotência, multi, oligo e unipotência.



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Figura 1. O desenvolvimento embrionário se inicia com o zigoto o qual se divide para formar blastômeros. O zigoto e os blastômeros (até 4 células) são totipotentes, isto é, são capazes de formar um indivíduo por si só. Esta capacidade diminui gradativamente durante o desenvolvimento, originando células tronco pluripotentes (capazes de originar diversos tecidos do corpo através dos três folhetos embrionários), multipotente (vários tipos, porém em número limitado), oligopotente (poucos tipos celulares), unipotente (um único tipo celular) ou células somáticas terminalmente diferenciadas. As células somáticas terminalmente diferenciadas podem readquirir pluripotência a partir da introdução de genes de pluripotência (iPS = induced pluripotent stem cell), ou podem readquirir a totipotência pela transferência nuclear somática em citoplasma de oócitos (SCNT = somatic cell nuclear transfer). Retirado de Mitalipov & Wolf (2008).

Em geral, as CTA constituem uma população rara e de pequeno número e estão distribuídas em lugares específicos (nichos) nos mais diversos tecidos do corpo (Hsu & Fuchs, 2012). A atividade das CTA depende de cada tecido em função de seu *turnover* celular (Hsu & Fuchs, 2012). Por exemplo, tecidos como pele, intestino e sangue onde o *turnover* celular ocorre de forma diário, a demanda e a atividade das células tronco é elevada e constante. No folículo capilar, as células tronco são recrutadas periodicamente em função do ciclo periódico de crescimento capilar. Por outro lado, existem tecidos de baixo *turnover* como o tecido muscular esquelético e nervoso, nos quais as células tronco estão quiescentes ou raramente se dividem em condições homeostáticas normais. No entanto, em casos de injúrias, estas células são ativadas e começam a se proliferar e diferenciar para regenerar o tecido danificado (Hsu & Fuchs, 2012). Com base na dinâmica tecidual acima mencionada e no ciclo celular, alguns autores classificam as células tronco como dormentes, quiescentes, reserva ou *long-term stem cells* para se referirem às células que

117 raramente se dividem; ou de ativas, amplificação transitória ou *short-term stem cells* para as
118 células tronco que se dividem rapidamente ou de forma transitória (Figura 2) (Schulze
119 1979,1988; Weissman, 2000; Nakamura *et al.*, 2010; Li & Clevers, 2010).

120 As células tronco quiescentes (*long-term stem cells*) dariam origem às células tronco
121 ativas (*short-term stem cells*) as quais originam precursores multipotentes (Weissman, 2000).
122 As células tronco quiescentes possuem um longo ciclo celular (demonstrado pela seta
123 curva contínua), enquanto que as células tronco ativas têm ciclos celulares curtos (seta
124 curva descontínua) com renovação rápida (Weissman, 2000).

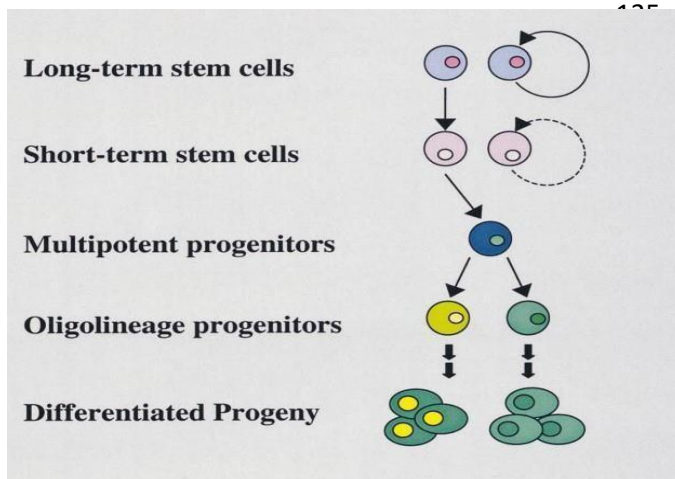
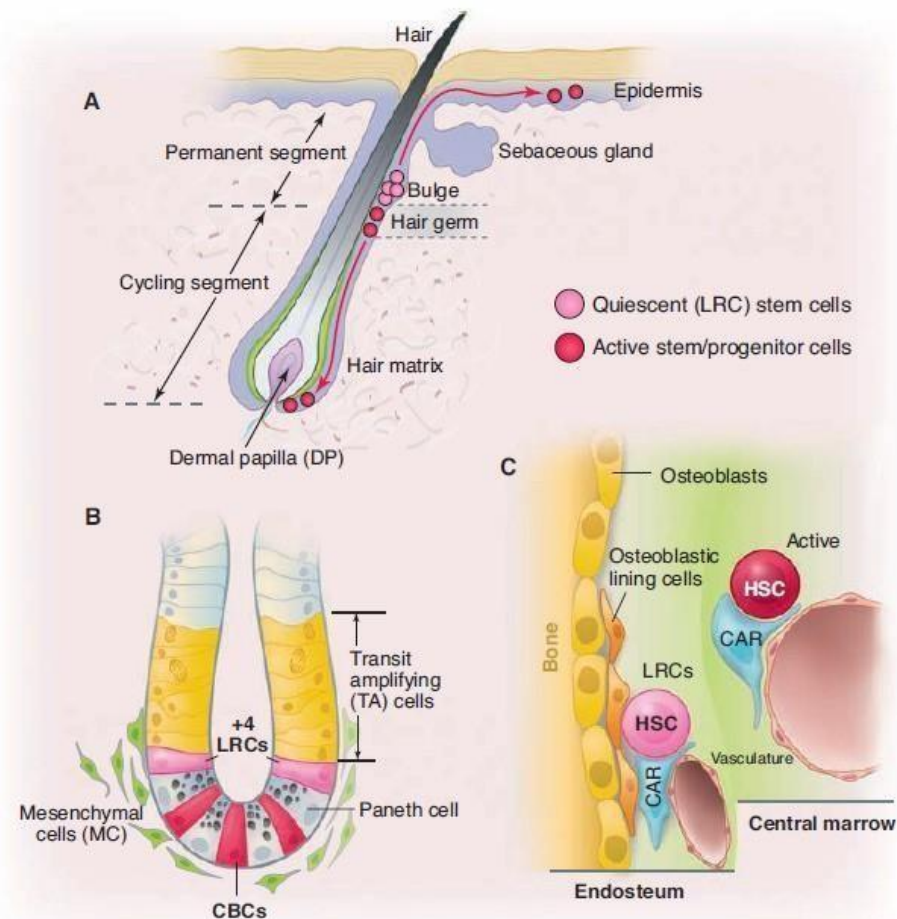


Figura 2. Classificação das células tronco de acordo com seu ciclo celular. “*Longterm stem cells*” para designar as células tronco quiescentes de baixa renovação, e “*short-term stem cells*” para as células tronco de rápido ciclo celular. Retirado de Weissman (2000).

139 Estudos têm demonstrado que as células tronco quiescentes e as células tronco
140 ativas constituem diferentes subpopulações que coexistem em diferentes regiões de um
141 mesmo tecido (ver revisão em Li & Clevers, 2010) (Figura 3).

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 144 **Figura 3.** Coexistência das células tronco quiescentes (células coloridas em rosa) e células tronco ativas
 145 (células representadas pela cor vermelha). **A** é um folículo capilar, **B** uma cripta intestinal e **C** medula óssea. Retirado
 146 de Li & Clevers (2010).

147
 148 As células tronco residem em regiões anatômicas específicas, conhecidas como
 149 nichos. O termo nicho foi cunhado inicialmente por Schofield em 1978, mas o conceito
 150 nicho permaneceu vago até sua identificação e caracterização em gônadas de *Drosophila*
 151 *melanogaster* (Onczy *et al.*, 1996; Fuller, 1998; Hardy *et al.*, 1979; Kiger & Fuller, 2001).
 152 Funcionalmente, o nicho é entendido como o microambiente tecidual que abriga as
 153 células tronco, e através de uma rede complexa de sinalização celular influencia as
 154 características e sua atividade de autorrenovação e diferenciação (ver revisão em Hsu &
 155 Fuchs, 2012). Todo nicho, além das células tronco, é constituído por células
 156 somáticas/estromais, vasos sanguíneos e matriz extracelular (Figura 4) (Fuchs *et al.*, 2004;
 157 Jones & Wagers, 2008). As células somáticas/estromais além de fornecer suporte
 158 estrutural para as células tronco são responsáveis por secretar uma série de fatores de
 159 crescimento solúveis locais que regulam de forma parácrina a atividade das células tronco
 160 (Figura 4).

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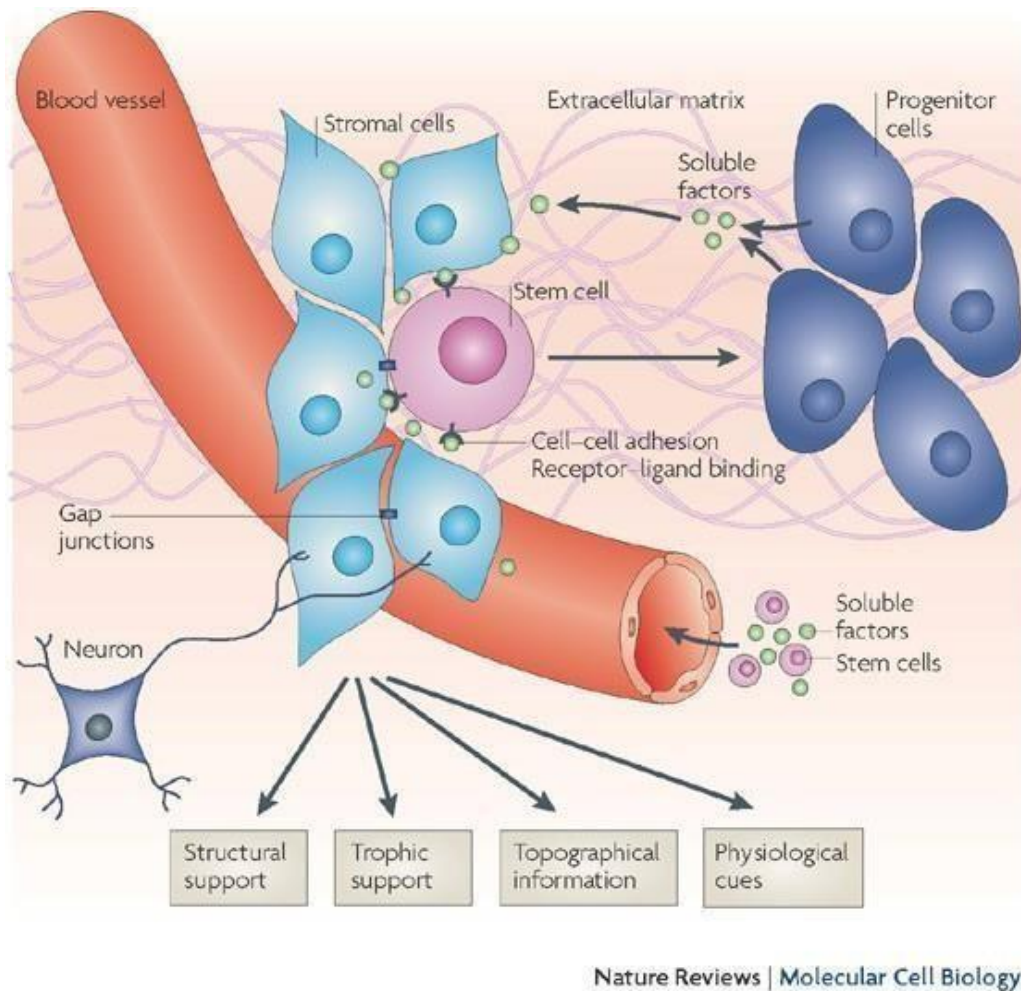


Figura 4. Elementos do nicho das células tronco. Note células de suporte/estromais/somáticas, célula tronco, matriz extracelular, vasos sanguíneos dentre outros. Retirado de Jones & Wagers (2008)

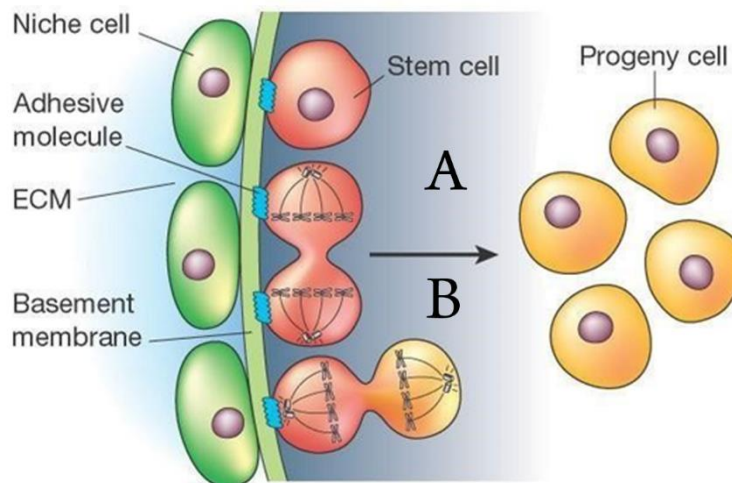
Estes fatores de crescimento também são produzidos localmente por outros tipos celulares que também influenciam o destino das células tronco (Figura 4) (Jones & Wagers, 2008).

Estudos têm demonstrado o papel dos vasos sanguíneos no nicho. Evidências recentes mostram que além de ser um aporte de oxigênio, hormônios e outras substâncias vindas do sangue, as células endoteliais produzem fatores de crescimento, conhecidos como fatores angiócrinos, que também regulam a atividade das células tronco (Fuchs *et al.*, 2004; Jones & Wagers, 2008; Butler *et al.*, 2010). Além das células somáticas/estromais, a matriz extracelular também constitui uma fonte de fatores de crescimento solúveis que estão envolvidos na regulação parácrina do nicho (Fuchs *et al.*, 2004; Jones & Wagers, 2008). Estes fatores de crescimento estão associados a diversos elementos da matriz extracelular e podem ser liberados quando a matriz é

213 degradada ou remodelada dependendo da condição fisiológica (Fuchs *et al.*, 2004; Jones
214 & Wagers, 2008).

215 Também vale mencionar o papel das junções celulares de adesão e das
216 integrinas em reter as células tronco em seus nichos (Fuchs *et al.*, 2004). As primeiras
217 se estabelecem entre as células tronco e as células de suporte enquanto que as segundas
218 prendem estas células à matriz (Figura 4) (Jones & Wagers, 2008).

219 A influência do nicho na atividade das células tronco pode ser compreendida
220 nos testículos de *Drosophila melanogaster* (Sprandling *et al.*, 2001). Neste modelo é
221 proposto que quanto mais distante do nicho, maior é a probabilidade das células tronco
222 se diferenciarem, uma vez que estas estão afastadas das condições moleculares e
223 estruturais que as mantêm no seu estado indiferenciado (Figura 5) (Sprandling *et al.*,
224 2001). Isso também depende muito do tipo de divisão assumido pelas células tronco;
225 se o fuso mitótico se encontra paralelo ao maior eixo da célula, as células-filhas
226 resultantes serão iguais e indiferenciadas (divisão simétrica) (Figura 5A) (Sprandling *et*
227 *al.*, 2001). No entanto, se o fuso mitótico for perpendicular ao maior eixo da célula, as
228 células-filhas resultantes serão diferentes; uma se mantém indiferenciada e a outra se
229 diferencia por estar longe do nicho (divisão assimétrica) (Figura 5) (Sprandling *et al.*,
230 2001).



231 **Figura 5.** Influência do nicho no destino das células tronco. Quanto mais distante do nicho, as
232 células tronco tendem a se diferenciar, uma vez que ficam distantes dos fatores que as mantêm
233 indiferenciadas. Dois tipos de divisões são conhecidas: simétrica (A) e assimétrica (B). A simétrica gera duas
234 células tronco iguais, enquanto que a assimétrica gera uma célula tronco indiferenciada e outra célula que se
235 diferencia. Figura retirada de Sprandling e colaboradores (2001).

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1.2 Pluripotência

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Por definição, o termo pluripotência é usado para se referir ao potencial das células tronco em se diferenciar nos três folhetos embrionários; endoderma, mesoderma e ectoderma (ver revisão em Mitalipov & Wolf, 2008). Assim sendo, as células do maciço celular interno (MCI) do blastocisto são pluripotentes e virtualmente capazes de originar todas as células somáticas e também as da linhagem germinativa do corpo (Mitalipov & Wolf, 2008). A pluripotência do MCI diminui gradualmente até a formação da gástrula.

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As CTE expressam marcadores específicos que vão desde antígenos, atividade enzimática específica como da fosfatase alcalina e telomerase a fatores de transcrição, como OCT4 e NANOG, que são rapidamente reprimidos a medida que as células se diferenciam (Mitalipov & Wolf, 2008). Dentre os fatores de transcrição relacionados à pluripotência, estudos mostraram que tal estado depende de uma tríade composta pelos fatores de transcrição OCT4, NANOG e SOX2 (ver revisão em Wang *et al.*, 2012). O OCT4 (*octamer-binding transcription factor 4*) é codificado pelo gene *Pou5f1* e trata-se de um fator de transcrição da família POU. O NANOG por sua vez, é codificado pelo gene *Nanog* (Wang *et al.*, 2012).

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OCT4 e NANOG são proteínas chaves na manutenção da pluripotência das CTE, atuando como parceiros na autorrenovação das mesmas (Wang *et al.*, 2012; Sánchez-Sánchez *et al.*, 2011). Os dois fatores de transcrição são expressos no MCI, epiblasto e nas células germinativas primordiais durante o desenvolvimento embrionário, e nas espermatogônias e oócitos na vida adulta (Wang *et al.*, 2012; Sánchez-Sánchez *et al.*, 2011). Grande parte dos estudos até então disponíveis foram feitos em camundongos e humanos devido o desconhecimento das formas ortólogas em outros vertebrados. Essas formas também foram identificadas em aves, *Xenopus* (somente Oct4), axolote (*Ambystomamexicanum*), zebrafish (*Danio rerio*) e em medaka (*Oryzias latipes*), demonstrando que esses fatores não são exclusivos de mamíferos (Tapia *et al.*, 2012; Wang *et al.*, 2011).

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Em relação ao OCT4, vale mencionar que o gene ancestral foi duplicado durante a evolução dos vertebrados originando duas formas; *oct4* e *pou2* (Tapia *et al.*, 2012), entretanto o *oct4* foi perdido nos peixes teleósteos e *Pou2* perdida nos mamíferos. Atualmente, o nome dado a zebrafish é *pou5f3* (Frankenberg *et al.*, 2014). Estudos em modelos de peixes teleósteos, como zebrafish e medaka, têm demonstrado que a

270 função do Oct4 relacionado à pluripotência foi mantida na cópia presente nos
271 teleósteos. Além disso, o padrão de expressão também é o mesmo, sendo expresso do
272 zigoto até a gástrula e também nas células germinativas primordiais (ver revisão em
273 Sánchez-Sánchez *et al.*, 2011). No entanto, o Oct4 (*spg/pou2*) parece ter adquirido
274 outras funções em zebrafish, como na regionalização do cérebro durante o
275 desenvolvimento embrionário, dentre outras (Lunde *et al.*, 2004). Por outro lado, em
276 medaka, o Oct4 não desempenha nenhum papel na regionalização do encéfalo, mas é
277 expresso nas células germinativas primordiais e nas espermatogônias tronco dos
278 indivíduos adultos (Sánchez-Sánchez *et al.*, 2010).

279 Até o presente momento, o Oct4 não foi demonstrado em espermatogônias
280 tronco de zebrafish, mas trabalhos usando anticorpo anti-Oct4 de roedores
281 demonstram a presença da proteína nas espermatogônias tronco de *Labeo rohita*, que
282 também é membro da família Cyprinidae (Panda *et al.*, 2011).

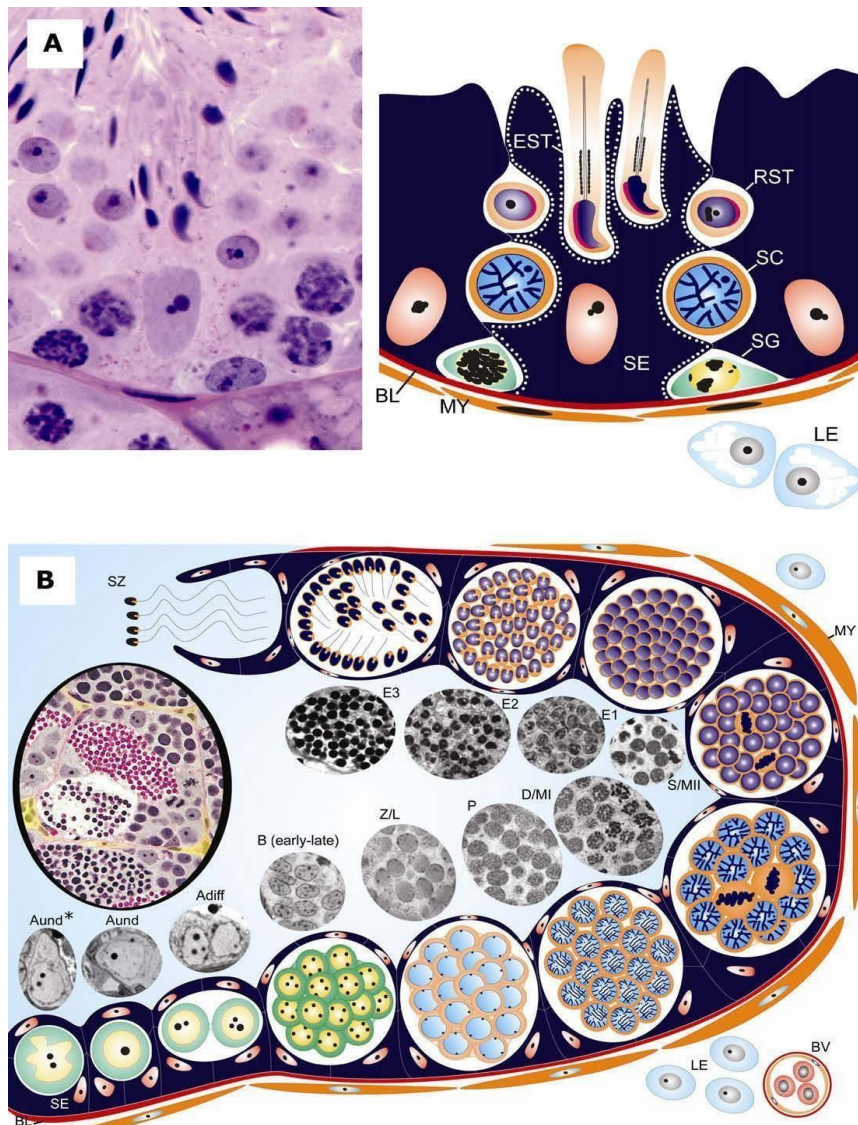
283 O NANOG por sua vez é considerado um fator crucial na manutenção da
284 pluripotência embrionária em mamíferos (Kuijk *et al.*, 2010) e também na regulação de
285 grupos de genes responsáveis pelo controle da pluripotência celular (Chambers *et al.*
286 2003; Cavaleri e Schöler 2003; Sun *et al.* 2014; Mitsui *et al.* 2003 Zhang *et al.*, 2009). Em
287 peixes, sabe-se que tal gene é importante para o desenvolvimento embrionário (Camp
288 *et al.*, 2009), uma vez que sua depleção leva a problemas no desenvolvimento da
289 gástrula e consequente morte em zebrafish (Wang *et al.*, 2016) e também em medaka
290 (Sánchez-Sánchez *et al.*, 2010).

291 O terceiro elemento chave da pluripotência é o SOX2 que atua como co-fator
292 do OCT4/NANOG para manter as células tronco em seu estado indiferenciado (ver
293 revisão em Sánchez-Sánchez *et al.*, 2011). Outros fatores relacionados à pluripotência
294 das CTE têm sido investigados, como por exemplo, o KLF4 (kruppel-like factor 4),
295 TCF3 (transcription factor 3) e STAT3 (signal transducer and activator of transcription
296 3) responsáveis por exemplo, a induzir formação das chamadas iPS (*induced pluripotent
297 stem cells*) a partir de células já diferenciadas, no caso, fibroblastos (Takahashi e
298 Yamanaka, 2006). Embora o papel desses fatores durante o desenvolvimento
299 embrionário seja bem caracterizado em mamíferos, pouco se conhece sobre a função
300 e regulação dos mesmos em peixes teleósteos. A identificação e a caracterização
301 funcional dos fatores que mantém a pluripotência nos peixes são, portanto, um grande

302 desafio para compreender o papel e evolução destas moléculas em diversas classes de
303 vertebrados.

304 **1.3 Espermatogênese de peixes teleósteos e sua regulação endócrina**

305 A espermatogênese é um processo altamente conservado entre os cordados e
306 compreende uma série de eventos altamente precisos e coordenados, nos quais uma
307 única espermatogônia tronco se diferencia para originar milhares de espermatozóides.
308 Este processo é dividido em três grandes fases (Russell *et al.*, 1990; Sharpe, 1994;
309 França & Chiarini-Garcia, 2005; Nóbrega *et al.*, 2009; Schulz *et al.*, 2010): (1) fase
310 espermatogonial ou proliferativa, caracterizada por sucessivas divisões mitóticas das
311 espermatogônias; (2) fase espermatocitária ou meiótica, em que o material genético
312 dos espermatócitos é duplicado, recombinado e segregado, formando células haplóides
313 denominadas de espermátides; e (3) fase espermiogênica ou de diferenciação, na qual
314 as espermátides passam por modificações estruturais e funcionais altamente complexas
315 para originar os espermatozóides, que estarão aptos para a fecundação. Embora
316 conservada, a espermatogênese apresenta certas peculiaridades dependendo do grupo
317 estudado. Em peixes teleósteos, por exemplo, a espermatogênese ocorre no interior
318 de estruturas denominadas espermatocistos, ou cistos, que se formam quando uma
319 única espermatogônia primária ou do tipo A é completamente envolvida pelos
320 prolongamentos das células de Sertoli (Figura 6) (Grier, 1993; Pudney, 1993; 1995;
321 Schulz *et al.*, 2010).



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323 **Figura 6.** Comparação entre a espermatogênese em estádios de amniotas (répteis, aves, mamíferos) (A) e
 324 cística dos anamniotas (peixes, anfíbios) (B). A Figura ilustra as diferenças entre a relação célula de
 325 Sertoli/célula germinativa na espermatogênese não-cística e cística. Em A, a célula de Sertoli suporta ao
 326 mesmo tempodiferentes clones de células germinativas em diferentes fases de desenvolvimento. Enquanto
 327 que em B, a célula de Sertoli suporta apenas um clone em uma mesma fase de desenvolvimento por vez.
 328 Legendas: células de Sertoli (SE); lâmina basal (BL); células peritubulares mióides (MY), células de Leydig
 329 (LE), espermatogônia (SG); espermatócito (SC); espermátide arredondada (RST); espermátide alongada
 330 (EST); espermatogônia do tipo A indiferenciada* (Aund*) (célula tronco?); espermatogônia do tipo A
 331 indiferenciada (Aund); espermatogônia do tipo A diferenciada (Adiff); espermatogônia do tipo B (B early-
 332 late); espermatócitos primários em leptóteno/zigóteno (L/Z), paquíteno (P), diplóteno/metáfase I (D/MI);
 333 espermatócitos secundários/metáfase II (S/MII); espermátides iniciais (E1); intermediárias (E2); finais (E3);
 334 espermatozoides (SZ); e vasos sanguíneos (BV). Retirado de Nóbrega, 2014.

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As células germinativas derivadas desta espermatogônia dividem-se
 sincronicamente e constituem 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).

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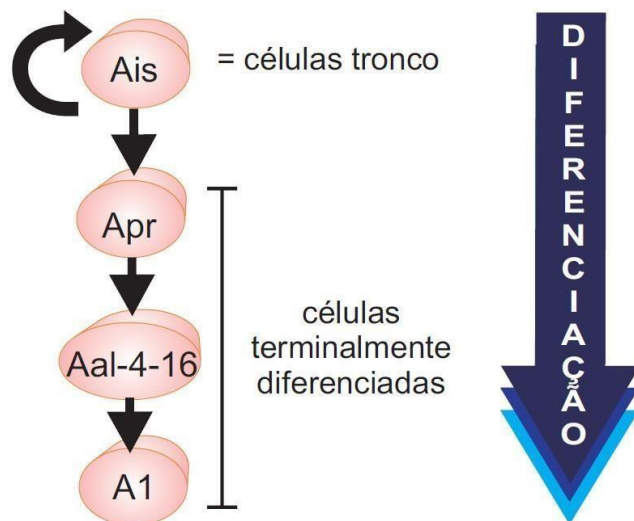
Diferentemente dos mamíferos, onde as células de Sertoli estão em contato
 com várias gerações de células germinativas (Russell *et al.*, 1990), na espermatogênese

342 cística as células de Sertoli normalmente estão em contato com apenas um tipo
343 específico de célula germinativa durante a evolução do processo espermatogênico
344 (Figura 6) (Nóbrega *et al.*, 2009; Schulz *et al.*, 2010). Estes cistos encontram-se apoiados
345 na túnica própria dos túbulos seminíferos, que é formada por camada acelular
346 denominada de membrana basal e pelas células peritubulares mióides (Figura 6)
347 (Koulish *et al.*, 2002).

348 A continuidade da espermatogênese é fundamental para manter a fertilidade
349 masculina, uma vez que, diariamente, milhões de espermatozóides são produzidos por
350 grama de testículo. Em humanos, por exemplo, cerca de 13×10^7 espermatozóides são
351 produzidos por dia. Ou seja, de forma mais ilustrativa, pouco mais de mil
352 espermatozóides são formados a cada batimento cardíaco (Russell *et al.*, 1990; Sharpe,
353 1994; França & Chiarini-Garcia, 2005). Por essa razão, quando comparada com outros
354 sistemas de autorrenovação do corpo, tais como pele e intestino, a espermatogênese é
355 considerada um dos processos de reposição celular mais eficientes (Russell *et al.*, 1990).
356 A elevada e constante demanda de espermatozóides durante a vida reprodutiva
357 masculina se em função das espermatogônias tronco, que são consideradas a base do
358 processo espermatogênico. À semelhança das demais células tronco do corpo, a
359 espermatogônia tronco tem capacidade de se autorrenovar e ao mesmo tempo originar
360 células-filhas diferenciadas que irão formar os espermatozoides. Assim, as
361 espermatogônias tronco são as únicas células tronco do corpo que contribuem com
362 material gênico para a formação de novos organismos. (de Rooij & Russell, 2000; de
363 Rooij, 2001 e 2006a,b, Ehmcke e Schlatt, 2006; Yan, 2006; Hofmann, 2008).

364 Uma característica única do processo espermatogênico é a divisão incompleta
365 das células germinativas, o que resulta em células conectadas por pontes
366 citoplasmáticas (Hunckins 1971; Russell *et al.*, 1990; de Rooij & Russell, 2000). O
367 modelo Ais (espermatogônias isoladas), desenvolvido em 1971 por Hunckins, propõe
368 que as Ais atuam como células tronco, e as espermatogônias conectadas por pontes
369 citoplasmáticas são terminalmente já diferenciadas e comprometidas com a formação
370 de espermatozóides (Figura 7).

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Figura 7. Ilustração esquemática do “modelo Ais”. As espermatogônias A isoladas (Ais) atuam como células tronco, e as gerações subsequentes conectadas por pontes citoplasmáticas; espermatogônias pareadas (Apr), alinhadas (Aal) e A1, 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 (Huckins, 1971).

Genes como *Oct4*, *Gfra1*, *Cd24*, *Nanos2,3*, *Egr3*, *Plzf*, *Sox-3*, *Taf4b*, *Bcl6b*, *Ret*, *Soblb2*, *Cdb1*, *Gpr125*, *Utf1* e *Lin28* são expressos exclusivamente nas espermatogônias indiferenciadas (Ais, Apr, Aal), e têm sido apontados na última década como potenciais marcadores espermatogônias tronco (Phillips *et al.*, 2010; de Rooij & Griswold, 2012).

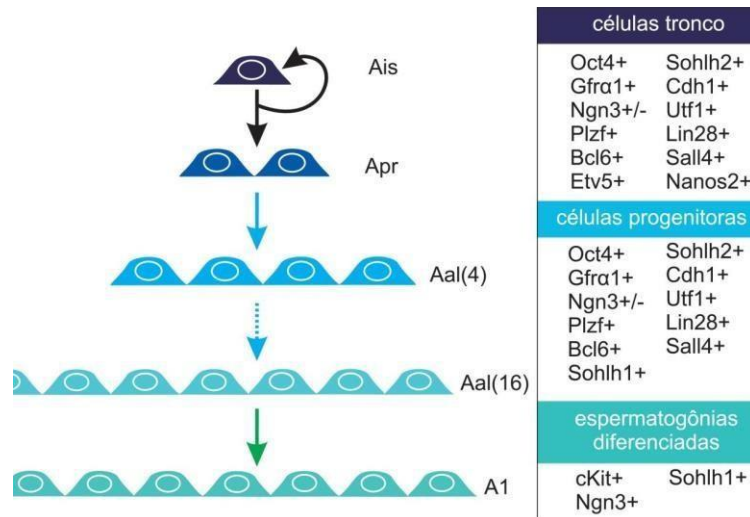


Figura 8. Perfil gênico expresso nos diferentes tipos de espermatogônias de roedores; espermatogônia do tipo isolada (Ais), pareada (Apr), alinhada (Aal4-16), e diferenciadas do tipo 1 (A1). Presente (+), ausente (-), transitoriamente expresso (+/-). (Modificado de Phillips *et al.*, 2010).

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Em vertebrados, as gonadotropinas hipofisárias hormônio Folículo Estimulante (Fsh) e hormônio Luteinizante (Lh) controlam o desenvolvimento gonadal através de sinais locais, como os esteroides sexuais, fatores de crescimento (Pierce and Parson, 1981; McLachlan *et al.*, 1996), small RNAs (sRNA) (van den Driesche *et al.*, 2014; Panneerdoss *et al.*, 2012) e mudanças epigenéticas (Skaar *et al.*, 2011). As gonadotropinas são glicoproteínas heterodiméricas com estrutura complexa consistindo em duas subunidades; α estrutura comum e β , relacionada a especificidade

405 hormonal. Ambas estruturas se ligam para formar uma estrutura dimérica
406 biologicamente ativa (Pierce, 1988). Em teleósteos sazonais, o Fsh está envolvido no
407 desenvolvimento e crescimento da gônada imatura enquanto que o Lh participa da
408 regulação da espermatogênese tardia, incluindo a maturação final e liberação dos
409 gametas (oocitação e espermição) (Ogiwara *et al.*, 2013; Chauvigne *et al.*, 2014). Porém,
410 suas funções ainda diferem das encontradas nos mamíferos (Zhang *et al.*, 2015),
411 evidenciando assim, a grande robustez evolutiva encontrada no sistema
412 endócrino/reprodutivo de peixes. Um exemplo para tal situação é a capacidade de
413 próprio Fsh estimular células de Leydig a liberarem andrógenos de forma mais potente
414 que o Lh (García-Lopez *et al.*, 2010). Além do mais, sabe-se que o Fsh é ainda capaz
415 de induzir a produção do fator de crescimento semelhante a insulina 3 (Igf3) pelas
416 células de Sertoli, estimulando consequentemente a diferenciação das espermatogônias
417 tronco e também a entrada na meiose (Nóbrega *et al.*, 2015) (Figura 9). Além do mais,
418 ainda em teleósteos, o Fsh também é responsável por inibir a liberação do hormônio
419 anti-Mülleriano (Amh), hormônio este responsável por inibir a diferenciação das
420 espermatogônias tronco e também o processo de esteroidogênese (Skaar *et al.*, 2011)
421 (Figura 9). Vale ainda ressaltar que o Fsh estimula a produção de fatores de
422 crescimento nas células de Sertoli e também regula uma série de genes em testículos
423 de zebrafish, como demonstrado recentemente por estudos de RNA seq (Crespo *et al.*,
424 2016). Em conjunto, esses resultados mostram que o Fsh é tido como um fator crucial
425 na regulação do nicho espermatogonial em peixes teleósteos.

426 Outro fator importante para a regulação de espermatogônias em mamíferos é
427 o GDNF (Glial cell line-derived neurotrophic fator) (Meng *et al.*, 2000; Yomogida *et al.*
428 *et al.*, 2003; Naughton *et al.*, 2006). Sob influência do FSH, o GDNF é secretado pelas
429 células de Sertoli e atua por meio de seu receptor GDNF *family receptor alpha-1* (GFR α 1)
430 localizado na superfície das espermatogônias tronco (De Rooij, 2006b; Hess *et al.*,
431 2006; Cooke *et al.*, 2006). Estudos demonstram que camundongos deficientes para
432 *Gdnf* (heterozigotos) (Meng *et al.*, 2000) e camundongos *knockouts* para *Gdnf/Gfra1/c-*
433 *Ret* (Naughton *et al.*, 2006) perdem progressivamente suas espermatogônias tronco
434 devido à incapacidade das mesmas de se autorrenovarem e manterem seu estado
435 indiferenciado. Sabe-se que em peixes que seu homólogo *gfra1a*, é expresso em
436 espermatogônias indiferenciadas de tilápia do Nilo (Lacerda *et al.*, 2013), *dogfish*
437 (Bosseboeuf *et al.*, 2013) e truta Arco-íris (Nakajima *et al.*, 2014). No entanto, nessa

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classe de vertebrados pouco se sabe a respeito da função do Gdnf na regulação de células germinativas e somáticas.

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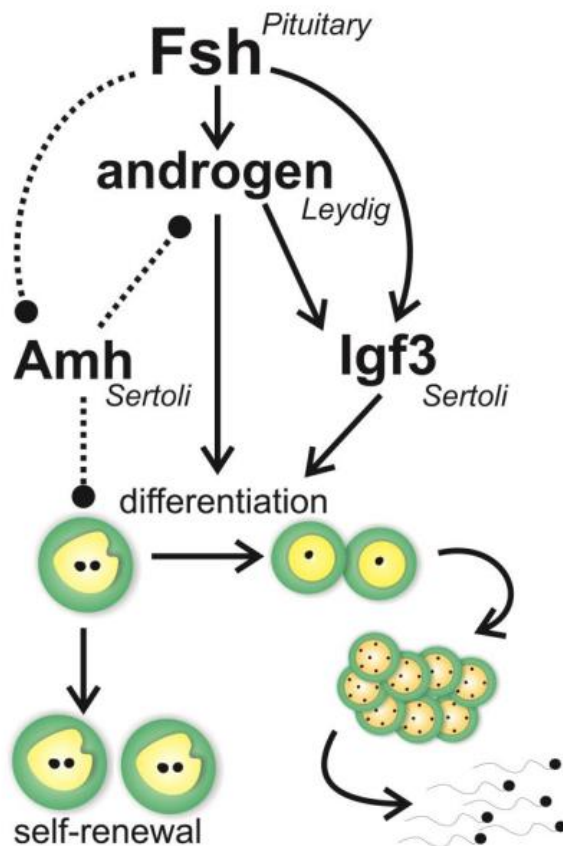


Figura 9. Representação esquemática da atividade biológica do Fsh na espermatogênese em zebrafish (Nóbrega *et al.*, 2015). O Fsh estimula a produção de andrógenos nas células de Leydig, que por sua vez promove a diferenciação das células germinativas. Ao mesmo tempo, o Fsh reduz (linha pontilhada) a expressão do Amh nas células de Sertoli, caso contrário, poderia inibir a produção de andrógenos e a diferenciação das espermatogônias. O Fsh também estimula a expressão de Igf3 nas células de Sertoli, que promove a diferenciação das células germinativas; Andrógenos também (fracamente) estimulam a produção de Igf3. Imagem retirada de Nóbrega *et al.*, 2015.

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Ainda recentemente, Zhang e colaboradores (2015) demonstraram que na ausência de Fsh e/ou Lh, machos de zebrafish são completamente férteis, apesar do atraso no crescimento do testículo em ambos heterozigotos e homozigotos para esta mutação. Este resultado sugere uma visão diferente da regulação da espermatogênese em teleostes, sugerindo que tal processo é complementar a outras vias de indução de sinalização. Semelhantemente, em mamíferos, o FSH tem capacidade de iniciar a espermatogênese, mas não em mantê-la nos indivíduos adultos (Kumar *et al.*, 1997; Plant & Marchall., 2001; Tapanainen *et al.*, 1997). Sendo assim, análises mais detalhadas das vias de regulação do nicho espermatogonial são cruciais e necessárias para um entendimento mais robusto da biologia das espermatogônias tronco.

478 **2. Justificativa**

479 As células germinativas são as únicas células de organismos metazoários capazes de
480 transmitir o material genético de uma determinada população de indivíduos para as
481 conseqüentes gerações. Logo, tal população de células garante a sobrevivência de
482 espécies ao longo do tempo evolutivo. Tendo isso em vista, este trabalho fornece
483 informações a respeito dos efeitos de fatores de regulação endócrino e parácrino na
484 atividade proliferativa e gênica de espermatogônias tronco. Tais fatores, que direta ou
485 indiretamente são responsáveis pelo controle de diferenciação e autorrenovação dessas
486 células, vão por fim, contribuir para a homeostase do processo espermatogênico.

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488 **3. Objetivos**

489 O objetivo geral desse trabalho foi caracterizar populações de espermatogônias
490 tronco de zebrafish (*Danio rerio*) a partir de marcadores moleculares e analisar os efeitos
491 do Fsh e do GDNF nessas células e também em células de Sertoli, no que diz respeito
492 a proliferação conjunta entre ambas populações e análise de expressão gênica.

493 Objetivos específicos:

494 1 – Identificar marcadores de espermatogônias tronco presentes no interior e na
495 membrana celular dessas células e;

496 2 – Analisar os efeitos dos hormônios Fsh e GDNF nessas populações de células.

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Capítulo 1

**Cross-talk between Sertoli and Spermatogonial Stem cells
via Fsh (Follicle stimulating hormone) and Gdnf (Glial cell-
derived neurotrophic factor) in zebrafish (*Danio rerio*) testis**

Artigo em preparação

825 **Introduction**

826 Spermatogenesis is a biological process in which a single spermatogonia stem cell
827 (SSC) is able to produce a large number of haploid cells (Hess & Franca, 2008; Rüdiger
828 *et al.*, 2010). To maintain this process throughout life, SSC self-renew to produce more
829 stem cells, and/or produce differentiated daughter cells ultimately committed with the
830 sperm formation (De Rooij and Russell, 2000; De Rooij, 2001 and 2006a, b, Ehmcke *et*
831 *al.*, 2006; Yan, 2006). The balance between these two processes (self-renewal and
832 differentiation) is finely and precisely coordinated in the so-called spermatogonial stem
833 cell niche (De Rooij, 2001 and 2006a, b; Yan, 2006). The niche is composed by the SSC
834 itself and the surrounding Sertoli cells and the nearby elements from the interstitial
835 compartment, such as Leydig cells, peritubular myoid cells, blood vessels and the
836 extracellular matrix (Spradling *et al.*, 2001; Fuchs *et al.*, 2004). There is a remarkable
837 difference between amniotes (reptiles, birds and mammals) and anamniotes (fish and
838 amphibians) with regards the spermatogonial niche (Schulz *et al.*, 2005). In the
839 amniotes, SSC is located at the basal compartment of the seminiferous epithelium, lying
840 directly on the basal lamina (extracellular matrix) of the epithelium (Schulz *et al.*, 2010)
841 which is nearby to the interstitial cells. While in the anamniote group, SSC are separated
842 from the basal lamina and the interstitium throughout Sertoli cells, which completely
843 surrounded a single SSC, forming the spermatocyst or cyst (Callard, 1996). Therefore,
844 anamniote Sertoli cells are considered important elements which mediate and integrate
845 signals in the niche (Yan, 2006; Hess *et al.*, 2006; Cooke *et al.*, 2006; Yoshida, 2015).

846 Pituitary gonadotropin Fsh (Follicle-stimulating hormone) seems to be an
847 important endocrine signal that regulates spermatogonial niche in fish (Pierce and Parson,
848 1981; McLachlan *et al.*, 1996; Huhtaniemi and Themmen, 2005; Ohta *et al.*, 2007; García-
849 López *et al.*, 2009 and 2010; De Rooij and Griswold, 2012). In zebrafish, Fsh stimulates

850 spermatogonial proliferation and differentiation in an androgen independent manner
851 (Nóbrega *et al.*, 2015; de Castro Assis *et al.*, 2018). Studies have shown that in the
852 zebrafish testis, Sertoli cells transduce signals from Fsh into the production of growth
853 factors that are required by spermatogonia proliferation and differentiation (Meng *et al.*,
854 2000; Yomogida *et al.*, 2003; Nagano *et al.*, 2003; Loveland and Robertson, 2005; De
855 Rooij and Griswold, 2012; Savitt *et al.*, 2012). In general Fsh modulates the balance
856 between stimulatory and inhibitory growth factors; increasing Igf3 (Insulin-like growth
857 factor 3) that promotes spermatogonial proliferation and differentiation (Nóbrega *et al.*,
858 2015; de Castro Assis *et al.*, 2018), while decrease Amh (Anti-Müllerian hormone) which
859 is involved on blocking spermatogonial differentiation and mantaining cells at their
860 quiescence state (Skaar *et al.*, 2011). Recent studies showed that Fsh-stimulated
861 spermatogonial proliferation modulated several signaling system (i.e. Tgf-b, Hedgehog,
862 Wnt, Notch and β -catenin pathways) (Crespo *et al.*, 2016; Safian *et al.*, 2018). In
863 mammals, FSH also regulates Sertoli cell growth factor production involved on
864 spermatogonia development [e.g. Activin, Amh, Inhibin, BMPs, CSF (colony-stimulating
865 factor)] (Oatley *et al.*, 2009; Skaar *et al.*, 2011; Barakat *et al.*, 2008; Zhao *et al.*, 2001;
866 Neumann *et al.*, 2011; Loveland and Robertson, 2005). Among these factors, FSH
867 induced the Sertoli cell release of glial cell line-derived neurotrophic factor (GDNF)
868 (Tadokoro *et al.*, 2002) which has a remarkable role on SSC self-renewal and
869 maintenance (Meng *et al.*, 2000; Gautier *et al.*, 2014; de Castro Assis *et al.*, 2018). GDNF
870 is a member of the transforming growth factor- β superfamily and was originally identified
871 as a survival factor for midbrain dopaminergic neurons (Lin *et al.*, 1993) and an important
872 factor for SSC in rodents (Meng *et al.*, 2000), dogfish (Gautier *et al.*, 2014) and zebrafish
873 (de Castro Assis *et al.*, 2018) so far. Knockout studies in mice with either GDNF or its
874 receptor GFR α 1/c-RET showed a progressively loss of SSCs due their inability to self-

875 renew and maintenance (Naughton *et al.*, 2006). Interestingly, *gdnf* is expressed in trout
876 germ cells (from spermatogonia to spermatocyte) (Nakajima *et al.*, 2014) but not in
877 Sertoli cells, as found in mouse (Meng *et al.*, 2000).

878 In this study, we evaluated the effects of Fsh into SSC gene expression and how
879 Fsh affected Sertoli and spermatogonial proliferation. To first address this question, we
880 characterized SSC transcripts/protein in the zebrafish testes and also Fsh receptor
881 localization. Further, we examined whether Gdnf is involved in SSC niche by evaluating
882 the expression sites of Gdnf and its receptor in the testes and the biological effects of this
883 same ligand on Sertoli and spermatogonial proliferation. We found a bilateral cooperation
884 between Sertoli and spermatogonial cells to regulate the spermatogonial niche in
885 zebrafish.

886

887 **Results**

888 **Identification of SSC transcripts and protein in the zebrafish testes**

889 We have analyzed the presence of selected mRNA (*pou5f3*, *nanog* and *nanos3*)
890 considered to be related with SSC pluripotency in mammals (Table 1). In order to identify
891 their pluripotency in zebrafish, expression analysis in embryos at different stages of
892 development, early (blastula - undifferentiated state) and late stage (long-pec - more
893 differentiated stage), were evaluated. The mRNA levels of *pou5f3*, *nanog* and *nanos3*
894 decreased in the long-pec stage (Fig. 1), suggesting that these genes might be involved in
895 the pluripotency state of the embryo. Further, we have identified their expression (mRNA
896 and protein) sites in the adult testes by qPCR, *in situ* hybridization and
897 immunofluorescence. The transcripts of *pou5f3*, *nanog* and *nanos3* are expressed in both
898 adult gonads (Fig. 1), and their sites of expression were identified in early spermatogonia

899 from type A undifferentiated spermatogonia (A_{und}) to type A differentiated spermatogonia
900 (A_{diff}) (Supplemental Fig. 1). The immunofluorescence has shown that Pou5f3 protein
901 was localized preferentially in type A_{und} (Fig. 2). Less or no signal could be found in
902 differentiated germ cells (type B, spermatocytes and spermatids) (Fig. 2). The
903 immunodetection for Nanos3 and Nanog have not been optimized yet, although the
904 protein has been detected in the testes (Data not shown).

905

906 **Effects of rzf Fsh on SSC gene expression and Sertoli and germ cell proliferation**

907 Testicular explants treated with 100ng/mL rzf Fsh showed differential expression
908 for the SSC transcripts (Fig. 3). Interestingly, *pou5f3* mRNA levels decreased while
909 *nanog* and *nanos3* were up-regulated with the Fsh treatment (Fig. 3A). Evaluating the
910 Gdnf (*gdnfa*; *gdnfb* was not evaluated because it is not expressed in the testis) and its
911 receptor (*gfra1a* and *gfra1b*), we found that rzf Fsh did not stimulate *gdnfa* and *gfra1b*
912 expression, but decreased *gfra1a* mRNA levels (Fig. 3A). No changes were detected for
913 *dmrt*, and as expected, *igf3* was highly expressed in the testes stimulated with rzf Fsh
914 (Fig. 3A). When examining the Sertoli and spermatogonial proliferation, rzf Fsh
915 stimulated proliferation of type A_{und} and Sertoli cells belonging to the same cyst (Fig. 3
916 B). Interestingly, immunofluorescence showed a strong and concentrated signal for Fsh
917 receptor in Sertoli cells associated with type A_{und} and A_{diff} (Fig 3C). Fshr could also be
918 detected in Sertoli cells associated with other germ cell types and in Leydig cells, as
919 expected (Fig. 3C).

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923 **Localization of Gdnfa/Gfra1a in the zebrafish testes**

924 During embryonic development, *gdnfa* and *gfra1a* transcript levels increased
925 significantly from blastula to long-pec stage (Fig. 4A). *gdnfa* and *gfra1a* are also
926 expressed in both adult gonads (Fig. 4A). To determine the *gdnfa* expressing cells in the
927 zebrafish testes, two approaches were employed; *in situ* hybridization (Fig. 4B) and qPCR
928 expression analysis in the somatic and germ cell enriched fractions obtained from a
929 differential plating method (Fig. 4C). While the *in situ* hybridization showed signal that
930 could be either in Sertoli or germ cell (Fig. 4B), qPCR analysis showed higher *gdnfa*
931 transcript levels in the germ cell enriched fraction (Fig. 4C). The Gdnf receptor, Gfra1a
932 was found in the cell surface of type A_{und} and A_{diff}, and also in the membrane of Sertoli
933 cells (Fig. 4D).

934

935 **Biological effects of rh GDNF on zebrafish spermatogenesis**

936 Testicular explants treated with 100 ng/ml rh GDNF for 7 days of culture showed
937 an increased proportion of types A_{und} and A_{diff} in the zebrafish testes (Fig. 5A). A
938 reduction of type B spermatogonia is seen in the GDNF treatment (Fig. 5A). In agreement
939 with the morphometrical analysis, the spermatogonial mitotic index showed an increased
940 of BrdU incorporation by types A_{und} and A_{diff} in the zebrafish testes treated with rh GDNF
941 (Fig. 5B). Interestingly, the mitotic index for Sertoli cells was also elevated (Fig. 5C),
942 showing that the dividing Sertoli cells were found in association with cysts of A_{und} and
943 A_{diff} which were also BrdU-positive (Fig. 5C). Expression analysis, on the other hand,
944 showed no changes on the selected transcripts, including the SSC mRNAs (Fig. 5D).

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946

947 **Discussion**

948 Sertoli cell acts as a paracrine relay station for different endocrine or paracrine
949 signals (e.g., gonadotropins, sexual steroids, growth factors, among others), transducing
950 these different signals into growth factors that are required for germ cell development
951 (Tadokoro *et al.*, 2002; Miura *et al.*, 2002; Skaar *et al.*, 2011; Meng *et al.*, 2000; Yomogida
952 *et al.*, 2003; Savitt *et al.*, 2012; Loveland and Robertson, 2005; Nagano *et al.*, 2003;
953 Yoshida *et al.*, 2015). In the cystic spermatogenesis, this function is more evident, once
954 all germ cells, from a single SSC until late spermatids, are completely surrounded by
955 cytoplasmic extensions of Sertoli cells (Schulz *et al.*, 2010). Studies in zebrafish have
956 shown that Fsh is a major regulator of spermatogonial proliferation and differentiation
957 through production of stimulatory growth factors in Sertoli cells, such as Igf3 (Nóbrega
958 *et al.*, 2015). Fsh also down-regulated inhibitory factors in Sertoli cells creating a
959 permissive condition for spermatogonial proliferation in the zebrafish testes (Miura *et al.*,
960 2002; Skaar *et al.*, 2011). In this work, we have studied how Fsh regulates SSC genes and
961 affects Sertoli cell proliferation and spermatogonial proliferation. To address this
962 question, we first characterized some selected SSC transcripts in zebrafish testes. For this
963 selection, we found orthologues of stem cell pluripotent transcripts (*pou5f3*, *nanog* and
964 *nanos3*) of mammals in zebrafish. These transcripts showed higher expression at early
965 stages of development (blastula), decreasing their levels in a more differentiated stage
966 (long-pec stage) which confirms that they are involved in pluripotency.

967 Pou5f3 is expressed in zebrafish testes, preferentially located in type A_{und}
968 spermatogonia. Similar results were found in medaka (Sanchez-Sanchez *et al.*, 2010b) in
969 which *pou5f1* was expressed in type A_{und}. *Rhandia quelen* also showed *pou5f3* expression
970 in types A_{und} and A_{diff} spermatogonia (Lacerda *et al.*, 2018). Therefore, *pou5f3* can be
971 considered a SSC marker in *D. rerio*.

972 In zebrafish, *nanos3* was expressed in early spermatogonia, although we could not
973 show its localization by immunofluorescence. In rainbow, *nanos2* (an isoform of *nanos3*)
974 was found restricted to subpopulations of type A spermatogonia (Bellaiche *et al.*, 2014).
975 Moreover, these authors showed that both *nanos2* and *nanos3* are highly expressed in
976 gonads composed by type A spermatogonia, while lower levels of expression were found
977 when these cells enter into differentiation (Bellaiche *et al.*, 2014). Although it is known
978 that *nanos3* transcripts are present in rainbow trout and zebrafish gonads, the specific role
979 of Nanos2 and Nanos3 are still unknown.

980 Nanog is considered to be a crucial factor for the maintenance of embryonic
981 pluripotency (Kuijk *et al.*, 2010) and germ cell development (Theunissen and Jaenisch,
982 2014). In zebrafish, it has been shown that Nanog play an important role during early
983 embryonic development (Camp *et al.*, 2009). Studies in zebrafish (Wang *et al.*, 2016) and
984 medaka (Sánchez-Sánchez *et al.*, 2010) have demonstrated that Nanog deficient embryos
985 had problems in the gastrula development and are lethal. Little is known about the role of
986 Nanog in fish gonads. In the present study, we detected *nanog* expression in adult testes
987 and showed its transcripts restricted to early spermatogonia. We could not demonstrated
988 yet the localization of the expressing protein, although Western Blot analysis confirmed
989 the presence of Nanog in the zebrafish testes. Although not yet conclusive for Nanos3
990 and Nanog, we believe that these genes are expressed in SSCs in zebrafish testes, based
991 on data from literature and for our expression analysis during embryonic development.
992 Further studies showing the protein localization in the testes will answer this question.
993 Another interesting issue to be addressed is whether these proteins are co-localized or not
994 in the same cell.

995 Once characterized the SSC transcripts in the zebrafish testes, we evaluated their
996 expression under Fsh stimulation. Interestingly, we found two different patterns of

997 expression among them; a decreased expression for *pou5f3*, while *nanog* and *nanos3*
998 transcripts were up-regulated by Fsh. Considering that previous studies have show that
999 Fsh stimulates both proliferation and differentiation, increasing type A_{und} and A_{diff} in
1000 zebrafish testes (Nóbrega *et al.*, 2015), our results indicate that higher expression of
1001 *nanog* and *nanos3* could indicate the formation of new cysts of A_{und} . However, this
1002 observation did not matched with *pou5f3* expression. Previous studies in zebrafish have
1003 shown two populations of type A_{und} ; one with long S-phase, named as slow-dividing cells
1004 (quiescent stem cells), and another population with short S-phase, named as active-
1005 dividing cells (active stem cells) (Nóbrega *et al.*, 2010). We believe that *pou5f3* would be
1006 related to the slow dividing cells (quiescent stem cells), that under Fsh stimulation is
1007 decreasing its expression to originate the active stem cells. Further studies will make an
1008 effort to co-localize markers of S-phase and these proteins (Pou5f3, Nanog and Nanos3)
1009 in the zebrafish testes.

1010 In this study, immunofluorescence for Fsh receptor showed a strong signal in
1011 Sertoli cells surrounding type A_{und} , where most of the SSC transcripts were found. When
1012 evaluating the proliferation of Sertoli and spermatogonia, we found that Fsh stimulates
1013 both Sertoli and type A_{und} proliferation from the same cyst. In agreement with *nanog* and
1014 *nanos3* expression, this data indicates the formation of new cysts. In zebrafish, as in other
1015 vertebrates, Fsh modulates the production of growth factors in the Sertoli cells (Barakat
1016 *et al.*, 2008; Mullaney and Skinner, 1992; Nicholls *et al.*, 2012; Pitetti *et al.*, 2013;
1017 Tadokoro *et al.*, 2002). Among the Fsh-induced stimulatory growth factors produced by
1018 Sertoli cell, Igf3 role has been well described in zebrafish testes in the last years (Nóbrega
1019 *et al.*, 2015; Safian *et al.*, 2018). Igf3 promotes spermatogonial proliferation and
1020 differentiation and antagonizes inhibitory factors, such as *Amh* (Nóbrega *et al.*, 2015).
1021 Therefore, we can conclude that Fsh acts in Sertoli cells surrounding type A_{und} , increasing

1022 and releasing stimulatory factors (e.g. Igf3), which promote type A_{und} proliferation. At
1023 the same manner, Fsh also stimulates Sertoli cell proliferation, which together with type
1024 A_{und}, lead to the formation of new cysts. Based on this findings, we suggest that Fsh
1025 orchestrates and integrates the functions of both somatic and germ cell in the SSC niche.

1026 In rodents, FSH stimulates the expression and release of GDNF (Meng *et al.*,
1027 2000) which is crucial factor for SSC self-renewal and maintenance in the testis. GDNF
1028 is produced by Sertoli cells and its receptor, Gfra1/c-Ret, are found in SSCs (Naughton
1029 *et al.*, 2006). In teleosts, there are two isoforms of Gdnf, Gdnfa and Gdnfb (Bellaiche *et*
1030 *al.*, 2014). Gdnfb is expressed in the brain, while Gdnfa in the gonads. The receptors,
1031 Gfra1a and Gfra1b are both expressed in the adult testes of zebrafish. In this study, we
1032 showed that Fsh did not modulate the expression of Gdnfa, different from mammals
1033 (Simon *et al.*, 2007; Takodoro *et al.*, 2002; Ding *et al.*, 2011). Conversely, in rainbow
1034 trout, Fsh had a negative effect on *gdnfb*, which is the isoform present in the gonads
1035 (Bellaiche *et al.*, 2014). In our study, we showed using a differential plate method that
1036 *gdnfa* is expressed in germ cell enriched fraction. This result is in agreement with
1037 Nakajima and collaborators (2014) who demonstrated that *gdnf* is expressed in type A_{und}
1038 of rainbow trout. The presence of Gdnf in germ cells and not in Sertoli cells explain the
1039 non-modulation of *gdnfa* expression by Fsh in zebrafish testes. With regards to its
1040 receptor, Gfra1a, immunofluorescence has demonstrated that the receptor is present in
1041 the cell membrane of Sertoli cells and also in early spermatogonia, such as type A_{und}.
1042 Such transcripts were also found in type A_{und} of different fish species, as in Nile-tilapia
1043 (Lacerda *et al.*, 2013), dogfish (Bosseboeuf *et al.*, 2013) and rainbow trout (Nakajima *et*
1044 *al.*, 2014). Therefore, we demonstrated here that Gdnf is a germ cell paracrine factor and
1045 the Gdnf/Gfra1a signaling occurs in an autocrine fashion in germ cells, while in Sertoli
1046 cells, Gdnf acts through a paracrine manner. With regards to the biological effects of rh

1047 GDNF (high homology with zebrafish *Gdnfa*), we showed an increase of cysts of type
1048 A_{und} and A_{diff} , but no changes in the SSC gene expression. The higher frequency of A_{und}
1049 and A_{diff} is in line with the higher mitotic index of these cells when zebrafish testes were
1050 treated with GDNF. Similar results were seen in dogfish, where rh GDNF promoted
1051 expansion of SSC colonies *in vitro* (Gautier *et al.*, 2014). Interestingly, we also found that
1052 GDNF increased the number of BrdU-positive Sertoli cells which were found in
1053 association with cysts of A_{und} and A_{diff} , also positive for BrdU. We conclude that GDNF
1054 promoted spermatogonial proliferation, increasing cysts of types A_{und} and A_{diff} but did
1055 not change SSC gene expression. This result suggests that GDNF might be involved in
1056 SSC maintenance. On the other hand, we showed for the first time that a germ cell growth
1057 factor affected *gfra1a* expressing Sertoli cells. As germ cell divides, the secreted GDNF
1058 might stimulate Sertoli cell proliferation as well aiming to form new cysts or
1059 accommodate the newly-formed germ cells in the cyst.

1060 As conclusion, we showed that endocrine (Fsh) and paracrine signals integrate
1061 and coordinate both Sertoli and germ cell functions in the SSC niche (Figure 6). Sertoli
1062 cells transduce the pituitary gonadotropin signal, Fsh, into growth factor production
1063 which affect SSC proliferation. As consequence of SSC proliferation, Fsh also stimulates
1064 Sertoli cell mitotic division in order to create new cysts (Figure 6). On the other side,
1065 GDNF, a germ cell paracrine signal, acts in the maintenance of SSC, but also stimulates
1066 Sertoli cell proliferation in order to create cysts or accommodate the newly-formed germ
1067 cells in the cyst (Figure 6). The cross-talk between SSCs and the surrounding Sertoli cells
1068 through endocrine and paracrine factors assure the proper development of both cells and
1069 spermatogenesis *per se* along the entire reproductive life.

1070

1071

1072 **Material and Methods**

1073 **Animals, sampling and ethics statement**

1074 The animals were kept in facility system under photothermal and water controlled
1075 conditions. For experimentation, they were euthanized by overdose with benzocaine
1076 hydrochloride ($\geq 250\text{mg}$) previously dissolved in ethanol and then mixed in appropriated
1077 volume of water. The animals were immersed in the benzocaine solution until death.

1078 A number of about 100 animals (zebrafish) were used in this project. This project
1079 (protocol 666-CEUA) is in accordance with the current legislation (Law 11.794/2008 and
1080 Decree 6.899/2009) and with the normative resolutions applicable by the Ethical
1081 Principles in Animal Experimentation elaborated by the Brazilian Society of Science in
1082 Laboratory Animals (SBCAL/COBEA) and approved by the Committee of Ethics in
1083 animal use (CEUA) of the Institute of Biosciences of Botucatu on October 14, 2014.

1084

1085 **Pluripotency genes expression: RT-qPCR and RT-PCR**

1086 To evaluate gene expression, RNA from samples was obtained using PureLink®
1087 RNA Mini Kit Kit (Ambion®) following the manufacturer's protocol. DNase treatment
1088 using DNase I, RNase-free kit (Invitrogen, Carlsbad, CA, USA) was performed and
1089 subsequently, cDNA was synthesized using SuperScript® II Reverse Transcriptase kit
1090 (Invitrogen™, Carlsbad, CA, USA) using random hexamers according to standard
1091 protocols (Nobrega., 2010). RT-PCR and qPCR reactions were conducted using specific
1092 primers for zebrafish *pou5f3*, *nanos3*, *nanog*, and *gdnf* (Table 1 and 2). Zebrafish β -actin
1093 (NCBI: AF057040.1) (Table 1) was used as endogenous reference gene for RT-PCR
1094 reactions, while elongation factor 1 α (*ef1 α*) and β -actin were used as housekeeping genes
1095 for RT-qPCR. The RT-PCR products were separated on a 1-1,5% agarose gel and
1096 visualized over a UV transilluminator. For RT-qPCR, the quantification cycle (cq) values

1097 of those genes were determined in a StepOne system (Life Technologies) using SYBR
1098 Green (Invitrogen) and specific primers (Table 1). All RT-qPCR reactions (10-20 μ l) used
1099 900 nM for each primer and 300 ng of total cDNA. Each reaction was performed in
1100 duplicate. Relative gene expression levels were calculated according to the $\Delta\Delta$ Ct method
1101 as described previously (Vischer, Teves, Ackermans *et al.*, 2003). Expression levels for
1102 each gene were normalized with two endogenous reference genes (see above) and
1103 subsequently calibrated to the Cts of the proper group of genes ($\Delta\Delta$ Ct) for each
1104 experiment.

1105

1106 **Protein localization for the pluripotency genes**

1107 For immunohistochemistry, zebrafish anti bodies were synthesized using specific
1108 antigens. Testis (n = 5 animals) were fixed 2 hours in 4% paraformaldehyde and
1109 incorporated in paraplast (Paraplast®, Sigma Aldrich). Cuttings of 5 μ m thickness were
1110 mounted on silanized slides. After deparaffinization and hydration, the sections were
1111 submitted to antigenic recovery in a humid chamber sodium citrate (10nM; pH 6.0) in
1112 microwave for 10 minutes. For blocking, BSA 1% was used for 1 hour. Subsequently,
1113 the slides were incubated overnight at 4°C with the anti-Pou5f3 (dilution 1: 200). After
1114 washing, the slides were incubated for 60 minutes at 37°C with the secondary antibody
1115 corresponding to their primer (all diluted 1:200). Subsequently, the sections were
1116 counterstained and mounted with ProLong (DAPI). Germ cells were classified based on
1117 morphological criteria (Leal *et al.*, 2009b). The secondary antibody only control was done
1118 without the primary antibody. Secondary antibody was let 1 hour at 37°C (figure S2).

1119 **Testis tissue cultures and differential plating method**

1120 The effect of recombinant zebrafish Fsh (rzfFsh) (100ng/ml) on pluripotency genes
1121 expression was analyzed using a previously described organ culture system for Japanese

1122 eel (Miura *et al.*, 1991) and zebrafish testes (Leal *et al.*, 2009). The morphology of the
1123 testis and the populations identification was done by toluidine blue immersed for 10
1124 minutes, then washed under tap water and mounted for light microscope. rzfFsh was
1125 purchased from U-Protein Express B.V; Utrecht, the Netherlands, and detailed
1126 information about rzf Fsh synthesis was provided by García-López and collaborators
1127 (2010). For expression analysis, testes from 15 animals collected and placed on
1128 nitrocellulose membrane on top of agar blocks, which were incubated in Leibovitz (L-15)
1129 (Sigma) culture medium containing or not rzf Fsh (100ng/ml). After 7 days (medium was
1130 refreshed every 3 days), testis were collected for RT-qPCR analysis as described above.
1131 BrdU (100 µg/ml; 5-bromo-2-deoxyuridine; Sigma Aldrich) was added in the last 6 hours
1132 of incubation, and samples were collected for BrdU immunodetection as described
1133 previously (Nóbrega *et al.*, 2015). The mitotic index was determined by counting the
1134 number of Aund-BrdU positive cells in 50 randomly chosen optical fields (100x) between
1135 basal and Fsh conditions. Also, in order to separate somatic from germ cells, testes from
1136 10 animals were submitted to the differential plating method as described by Luo and
1137 collaborators (2006). By this technique, somatic cells firmly adhere to the bottom of the
1138 plate while germ cells remain in suspension for 2-3 days of culture or are weakly
1139 associated with the somatic cells (Figure 5).

1140 **Statistical analyses**

1141 Results were expressed as mean values \pm SEM. Significant differences between two
1142 groups were identified using paired Student's t-test ($p < 0.05$) for Fsh treatment and
1143 unpaired for the others treatments. Comparisons of more than two groups were performed
1144 with one-way ANOVA followed by Student-Newman-Keuls test ($p < 0.05$). Graph Pad
1145 Prism 4.0 (Graph Pad Software, Inc., San Diego, CA, USA, <http://www.graphpad.com>)
1146 was used for all statistical analysis.

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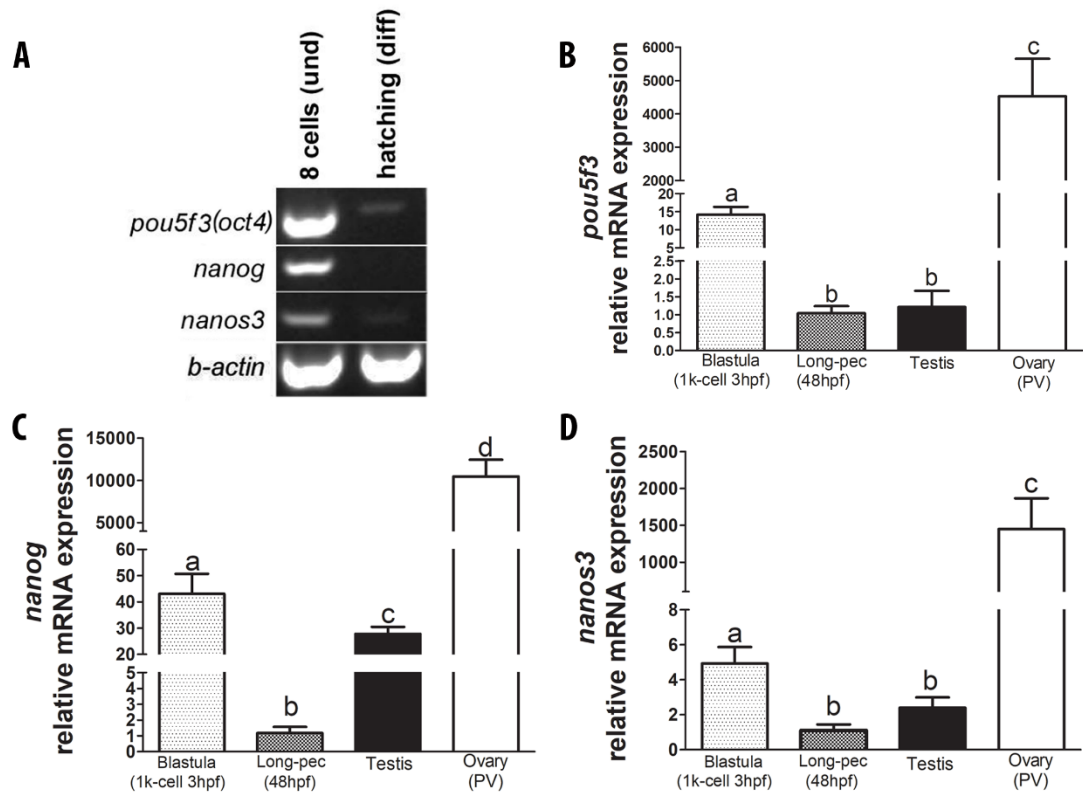
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1349 Figure 1. A. RT-PCR for 8-cell stage and hatching on zebrafish embryos. RT-PCR shows differential
 1350 expression for pluripotency genes candidates in two different stages of development. B, C and D. Relative
 1351 expression of mammalian orthologue genes present in gonads and zebrafish embryos. A. *b-actin* was used
 1352 as positive control. Gene expression in B, C and D were normalized with reference gene (*ef1a*) and
 1353 expressed as relative values of the lower expression (somite). Different letters indicate significant
 1354 differences among the groups ($P < 0.05$).

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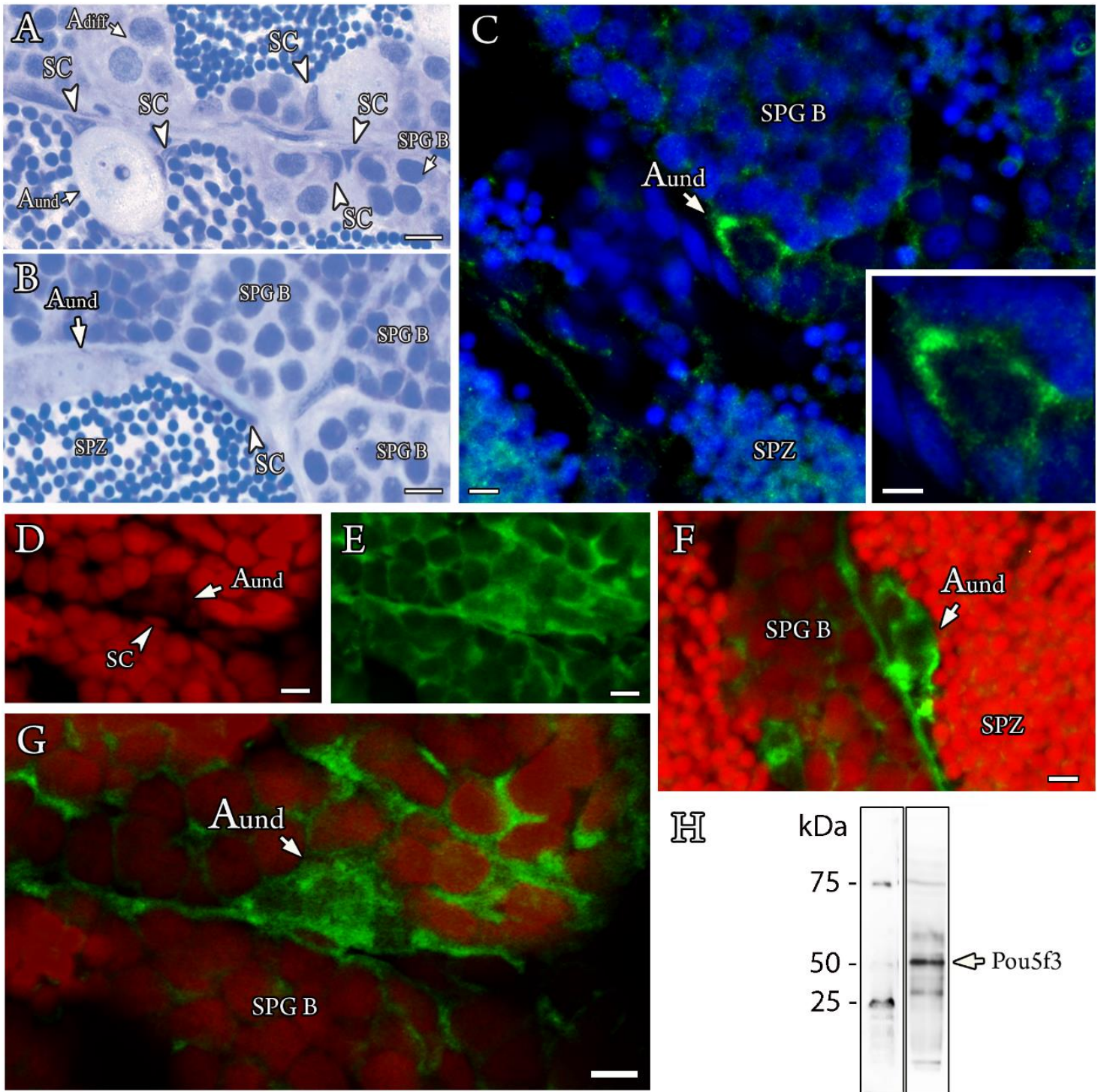
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1364 Figure 2. (A-B) Toluidine blue staining of testes showing the different populations of spermatogonial cells.
 1365 (C, G and F) Immunostaining of Pou5f3 [C – DAPI counterstaining and D, F and G propidium iodide (PI)
 1366 counterstain]. (E) Pou5f3 staining without PI. The proteins are concentrated in undifferentiated
 1367 spermatogonia stem cells (A_{und}) as indicated by arrows in C, F and G. A_{und} , type A undifferentiated
 1368 spermatogonia; A_{diff} , type A differentiated spermatogonia; SPG B, spermatogonia type B; SPZ,
 1369 spermatozoa; SC, Sertoli cells. Bars – 5 μ M; (H) Pou5f3 immunoblot of whole testis.

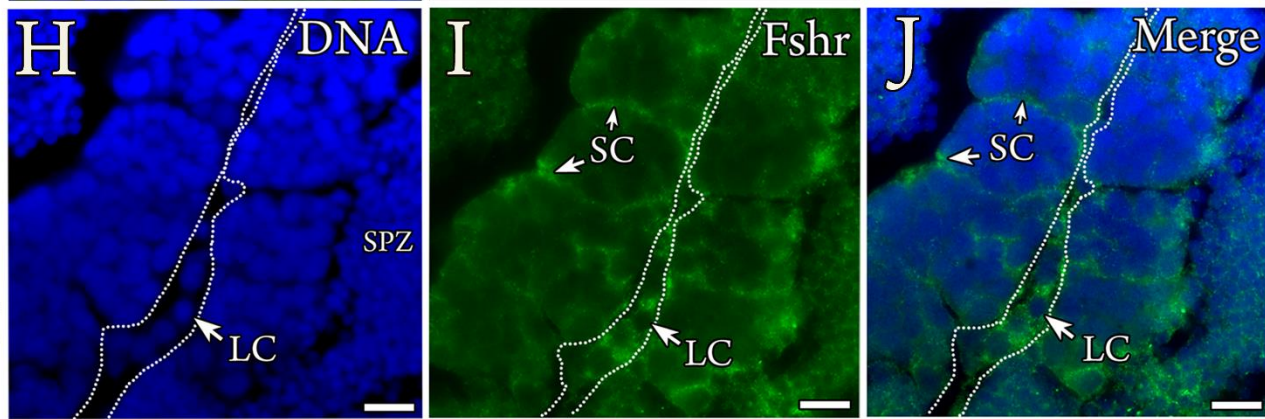
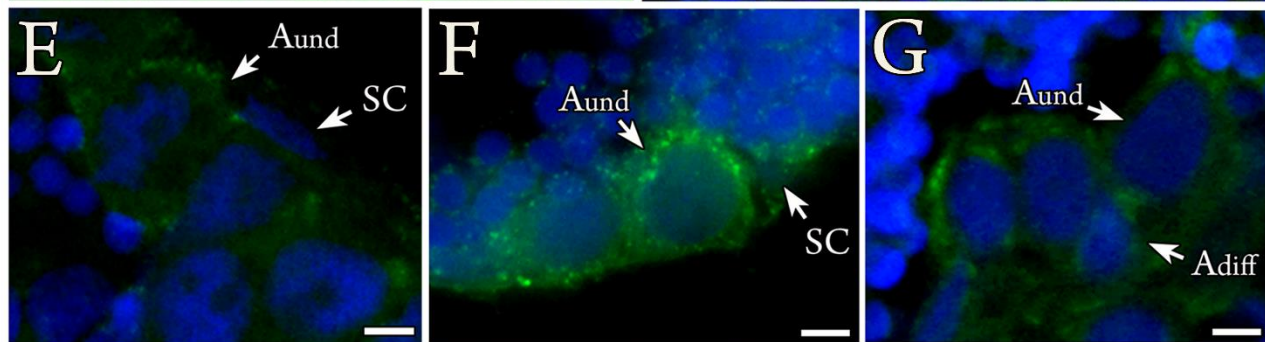
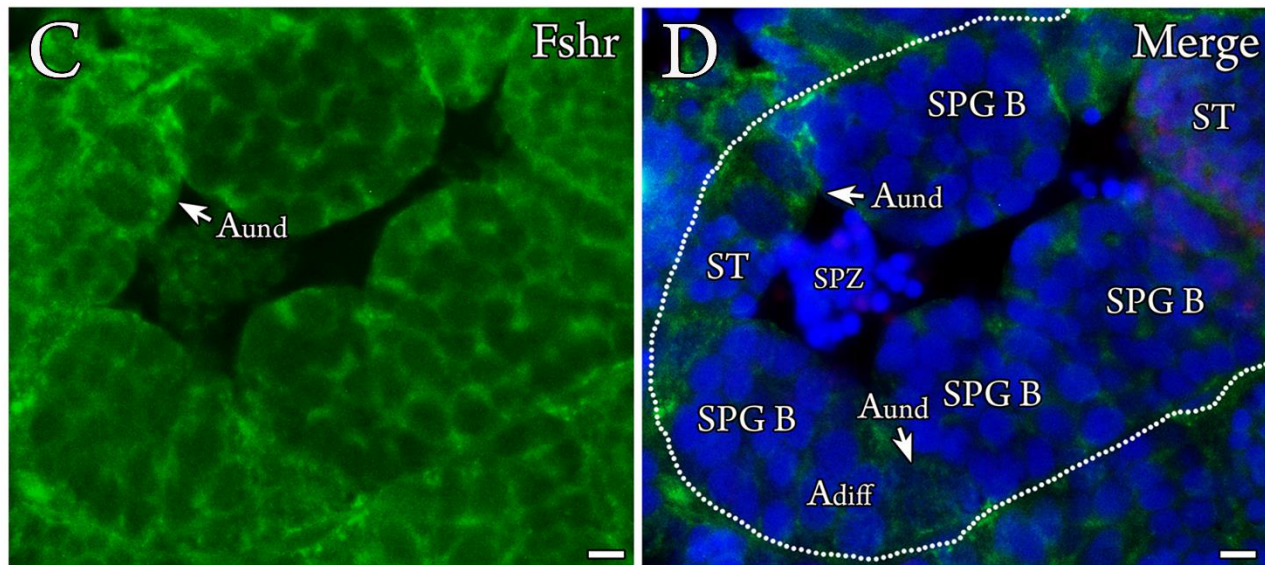
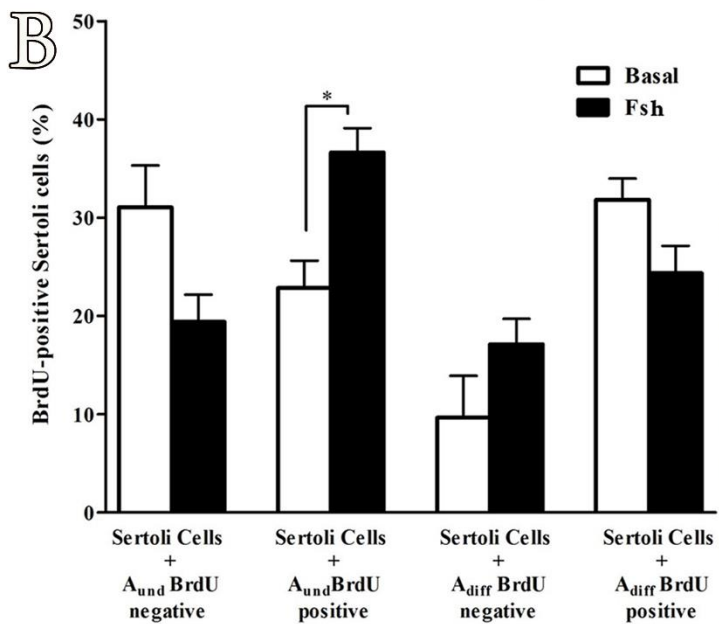
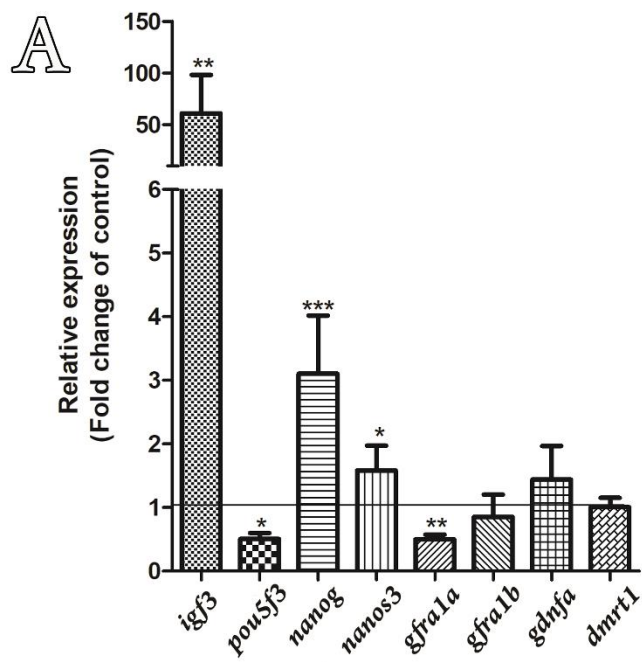


Figure 3 – rzfFsh effects on germ and somatic cells proliferation and fluorescence immunohistochemistry for Fshr in zebrafish testis of adult individuals. (A) Relative expression of selected genes in adult zebrafish testis under influence of 100ng/uL of rzf Fsh culture for 7 days. (B) Sertoli cells proliferation in association with undifferentiated (Aund) or differentiated (Adiff) spermatogonia BrdU-positive or negative. Results are presented as mean \pm standard error (n = 8). (A, B) Significant statistical differences ($p < 0.05$) related to control (horizontal line) are denoted by asterisks (*). (C-D) Immunofluorescence detection for Fshr in zebrafish testis. (E-G) Sertoli cells associated with type A undifferentiated (Aund) positive for Fshr. (H-J) Fshr expressing Sertoli cells belonging to different germ cell cysts. Note that Leydig cells are also positive for Fshr. Aund, type A undifferentiated spermatogonia; Adiff, type A differentiated spermatogonia; SPG B, spermatogonia type B; ST, spermatids; SPZ, spermatozoa; SC, Sertoli cells; LC, Leydig cells. Bars A-E 5uM and F-H 10uM.

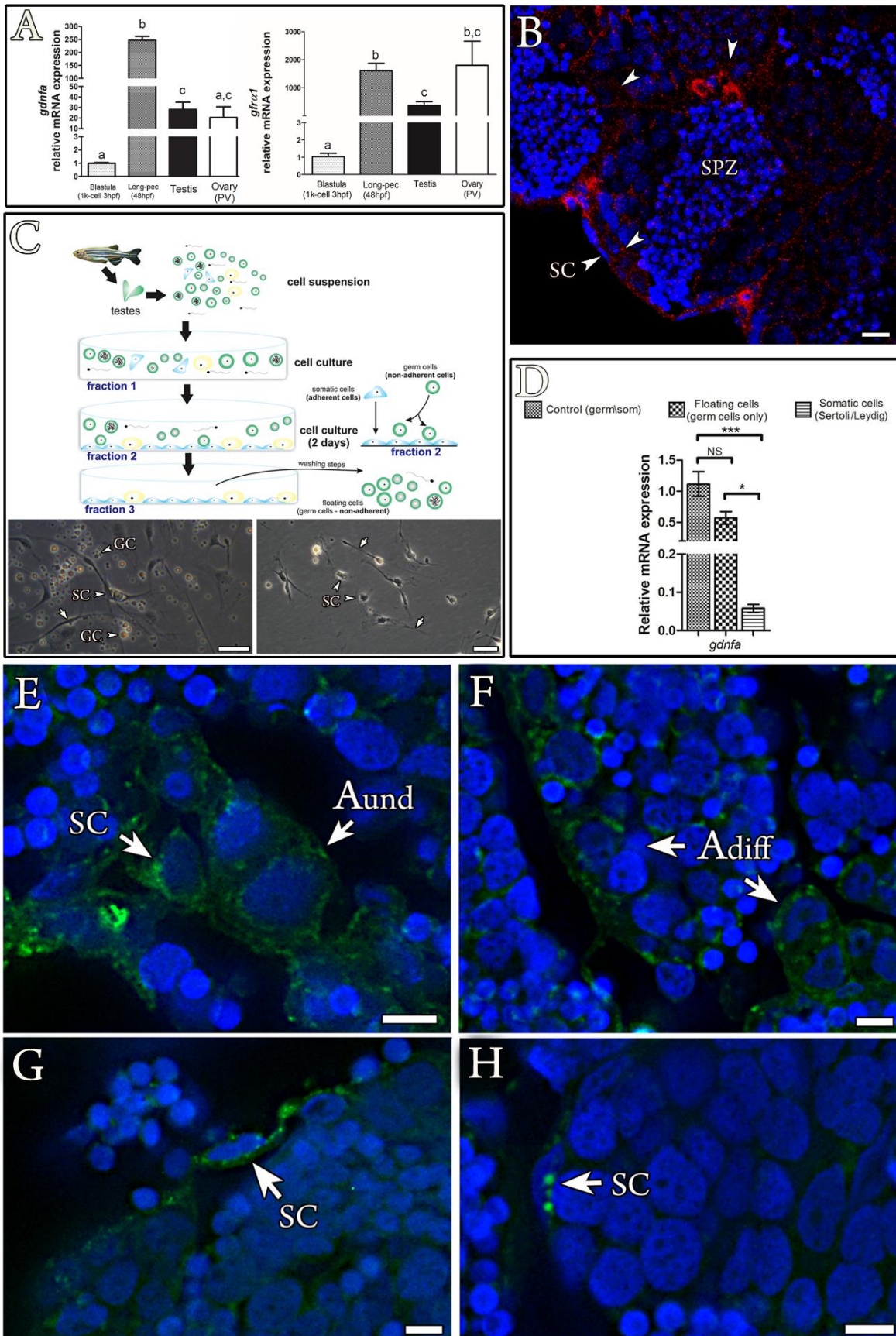


Figure 04 – *gdnfa/gfra1* expression in embryonic and adult cells and its sites of expression in testes of adult zebrafish. (A) Expression of *gdnfa/gfra1* present in gonads and zebrafish embryos. Different letters indicate significant differences among the groups ($P < 0.05$). Genes were normalized with reference gene (*ef1 α*) and calibrated with the presented lower expression (somite). (B) mRNA sites of expression for *gdnfa* transcripts. We were unable to determine if the expression is concentrated either in germinative or Sertoli cells. (C) Differential plating technique. GC – germinative cells. SC – Sertoli cells. (E-H) Colocalization of Fshr and DAPI. (D) *gdnfa* is expressed in germ cells. Control has both germinative and Sertoli cells. Different letters indicate significant differences among the groups ($P < 0.05$). (E) Aund positive staining in the membrane of both Sertoli cell and Aund. (F) Adiff cysts positive for Gfra1a (G-H) Membrane of Sertoli cells expressing Gfra1a. DAPI counter staining. Aund, type A undifferentiated spermatogonia; Adiff, type A differentiated spermatogonia; SC, Sertoli cells. Bars - 5 μ M.

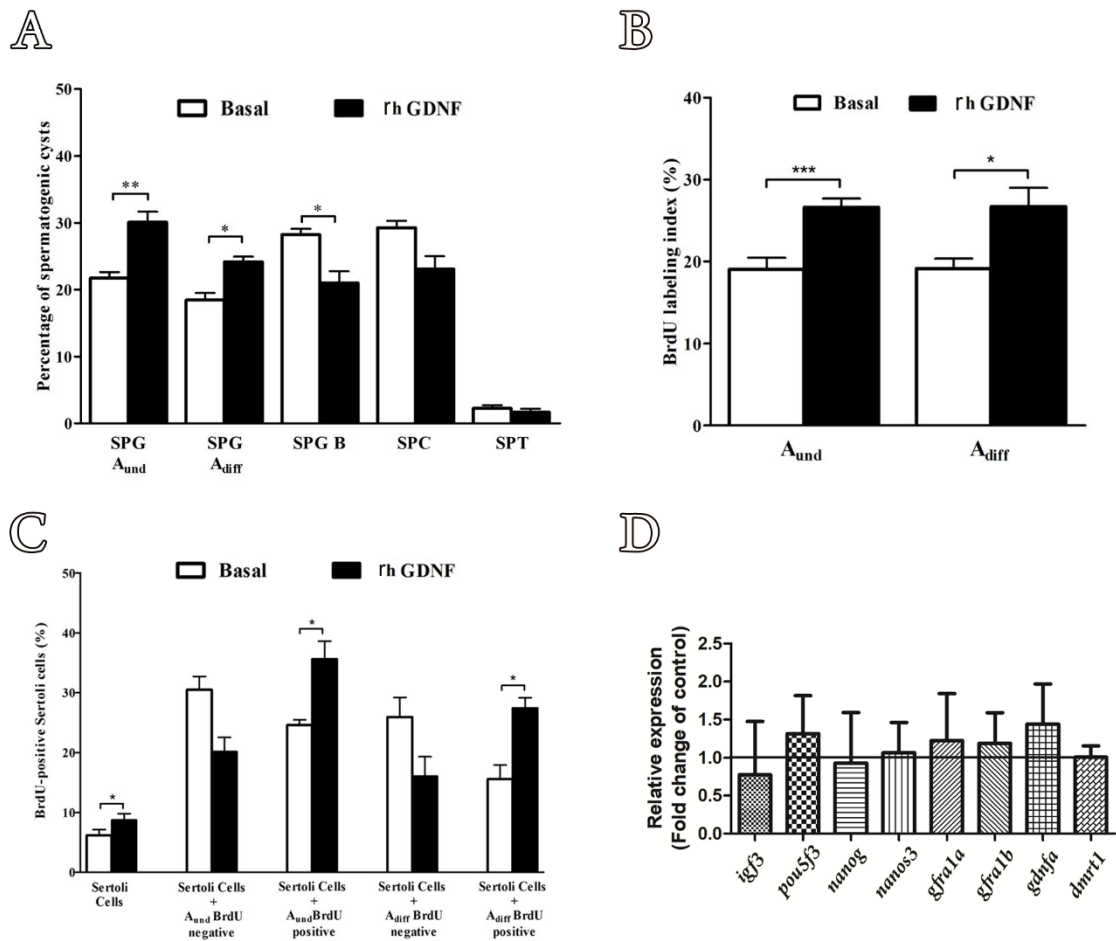


Figure 05 –Proliferation frequency for cysts and germinative cells index, associated or not with Sertoli cells and gene expression analysis for selected. (A) The recombinant were able to stimulate cysts frequency countaining both SPG A_{und} and A_{diff} and reduce SPG type B frequency. (B) rhGDNF was also capable of stimulating A_{und} and A_{diff} not associated with Sertoli cells. (C) BrdU labeling indices for Sertoli cells associated and not associated (free) with germ cell cysts located in the interstitial or intertubular region of the germinal compartment after 7 days of incubation in the absence (control/basal) or presence of rhGDNF. Both A_{diff} and A_{und} Sertoli cell associated were stimulated by rhGDNF. (A, B and C) Significant statistical difference ($p < 0.05$) represented by the different symbols (*). Results are presented as mean \pm standard error ($n = 8$). (D) Relative gene transcription analyses of selected genes and somatic factors *igf3* and *dmrt1* under influence of 100ng/ml of rhGDNF in adult zebrafish testis. No significant statistical differences ($p < 0.05$) were found.

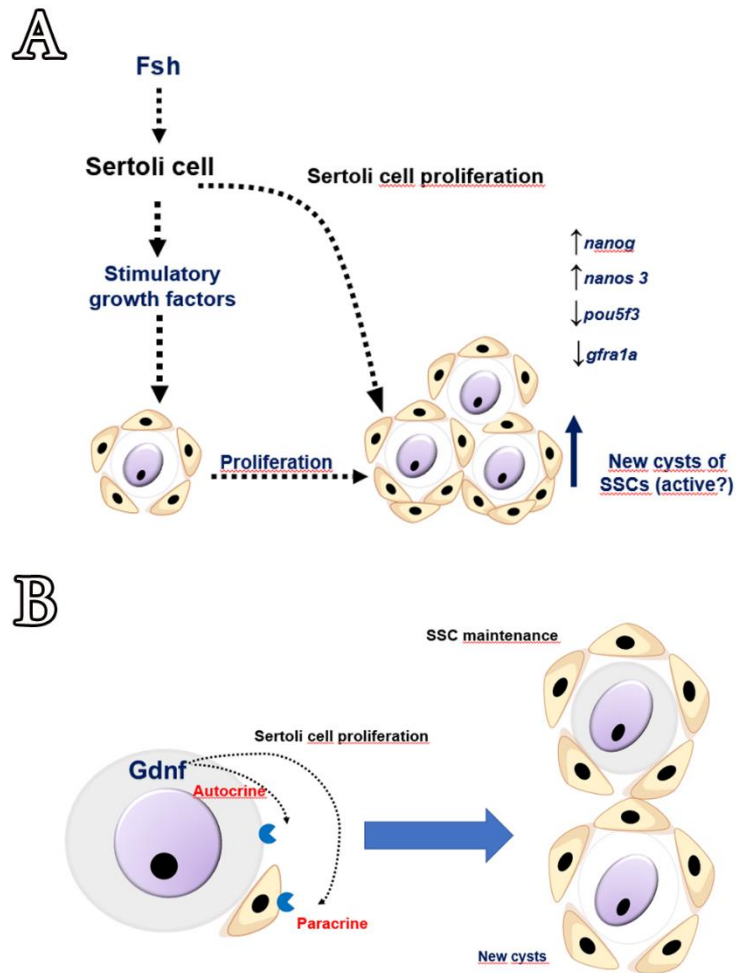


Figure 06. Endocrine effects of Fsh (A) and paracrine/autocrine effects of GDNF (B) on germ and Sertoli cells proliferation. (A) Fsh induces Sertoli cell to produce stimulatory factors and to proliferate. Such factors will act mainly undifferentiated cysts by up-regulating *nanog* and *nanos3* transcripts expression and down-regulating *gfra1a* and a true pluripotency gene, *pou5f3*. The cysts will have then a permissive pathway through proliferation. (B) Gdnf by its way, will act through its receptor, Gfra1a, present in germ and Sertoli cells stimulating cyst proliferation.

Table 1. Primers used for RTq-PCR analysis of gene mRNA expression		
Gene	Primers sequences (5'-3')	Accession
<i>b-actin</i>	CGAGCTGTCTTCCCATCCA TCACCAACGTAGCTGTCTTCTG	AF025305.1
<i>dmrt1</i>	ATGGCAGAGCAGAACGATTT TCCATGCTGTTGTGGGACTA	NM_205628.2
<i>ef1a</i>	GCCGTCCCACCGACAAG CCACACGACCCACAGGTACAG	FJ915061.1
<i>gdnfa</i>	CCGCAGTGAGAGCCCCG TCCCATTAGGTCATATTGTTCTC	NM_131732.2
<i>gfra1a</i>	TCGACTGGCTCCCATCTATTTC AGGTGTCATTTCAGGTTGCAGG	NM_131730.1
<i>gfra1b</i>	CCTGTGCTTGATTTAGTGCA GCATCCGTACTTTCCCAAAC	NM_131731.1
<i>igf3</i>	TGTGCGGAGACAGAGGCTTT CGCCGCACTTTCTTGGATT	HQ241070.1
<i>nanog</i>	TGTCCTACAACAAGACTGAGCC CAGGAATCTGGCGTGTGGG	NM_001098392.1
<i>nanos3</i>	GTCATGGATCTATGGAGAC GTCTGAATCCGCGTCAGATT	NM_131878.1
<i>pou5f3(oct4)</i>	GAGAGATGTAGTGCGTGTAT GCTCGTAATACTGTGCTTCA	NM_131112.1
<i>shippo</i>	GATGCCITGGAGACATGACCAA CAAAGGAGAAGCTGGGAGCTT	NM_199958.1

Supplemental material

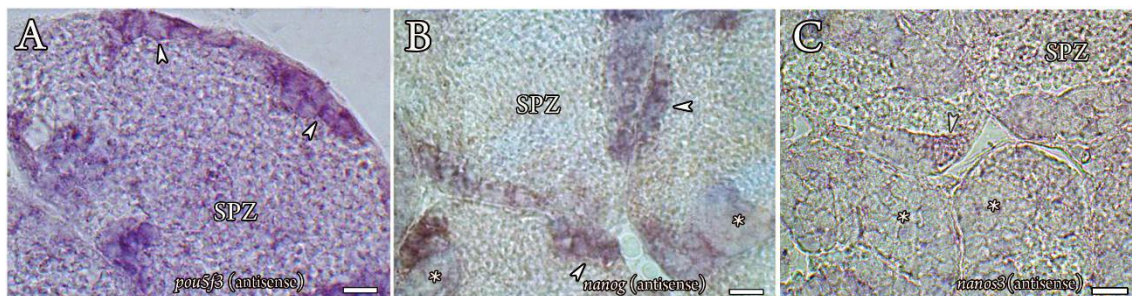


Figure S1. Chromogenic in situ hybridization for cellular localization of *pou5f3* (A), *nanog* (B) and *nanos3* (C) in zebrafish testis. (A-C) transcripts localization in testis. Transcripts are concentrated in undifferentiated spermatogonia cysts as indicated by arrowheads.

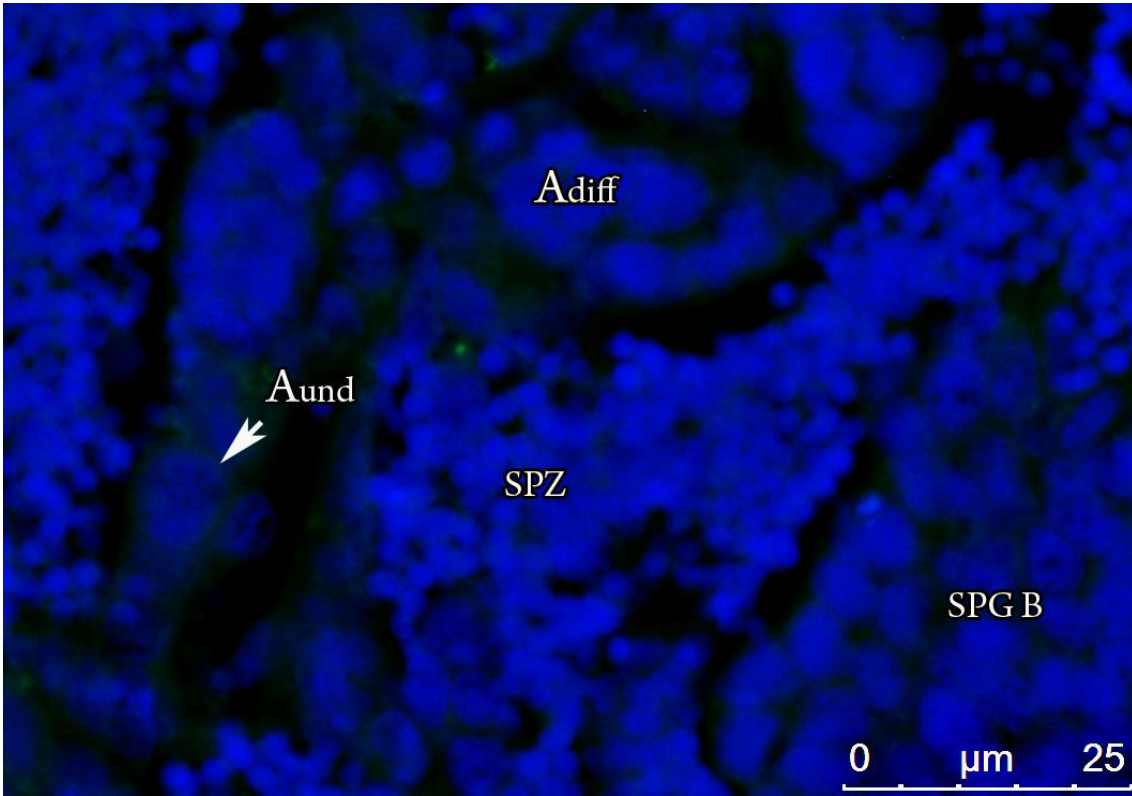


Figure S2. Fluorescence staining without the zebrafish primary antibody. No secondary antibody background.

6. Conclusões finais

Conclui-se que o Fsh, através de fatores de crescimento liberados pelas células de Sertoli estimula a formação de novos cistos de espermatogônias tronco, como pode ser observado pelo aumento de expressão de *nanos3* e *nanog*, aumento de cistos de Aund e proliferação de células de Sertoli associadas a estes cistos iniciais. Interessantemente, o Fsh diminuiu a expressão de *pou5f3* e *gfra1a*, considerados também marcadores de espermatogônias tronco. Embora especulativo, este resultado sugere que tais genes estariam associados a espermatogônias de longo ciclo celular (células tronco quiescentes), que dariam origem às células tronco ativas *Nanog* e *Nanos3* positivas sob estimulação do Fsh.

Por outro lado, demonstramos que o *Gdnf* é um fator de crescimento presente em células germinativas e atua de forma autócrina na manutenção das espermatogônias tronco. Este fator também atua de forma parácrina nas células de Sertoli estimulando sua proliferação. De forma geral, os resultados desta tese demonstram que fatores endócrinos e parácrinos são necessários para integrar as funções das células de Sertoli e germinativas no nicho espermatogonial.