ANDRÉ FELIPE DE FIGUEIREDO ALMEIDA

PROLIFERAÇÃO DAS CÉLULAS DE SERTOLI NA REGIÃO DE TRANSIÇÃO ENTRE OS TÚBULOS SEMINÍFEROS E A *RETE TESTIS* EM RATOS WISTAR

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Ciências.

Área de Concentração: Biologia Celular

Orientador: Prof. Guilherme Mattos Jardim Costa

Co-Orientador: Prof. Luiz Renato de França

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Esta tese foi desenvolvida no Laboratório de Biologia Celular do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação do Prof. Dr. Guilherme Mattos Jardim Costa e co-orientação do Prof. Dr. Luiz Renato de França, e com o auxílio das seguintes instituições:

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ATA DA DEFESA DE TESE DE DOUTORADO DE

ANDRÉ FELIPE DE FIGUEIREDO ALMEIDA

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Às **quatorze horas** do dia **28 de junho de 2019**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"PROLIFERAÇÃO DAS CÉLULAS DE SERTOLI NA REGIÃO DE TRANSIÇÃO ENTRE OS TÚBULOS SEMINÍFEROS E A RETE TESTIS EM RATOS WISTAR"**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, o Presidente da Comissão, **Dr. Guilherme Mattos Jardim Costa**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dr. Guilherme Mattos Jardim Costa	UFMG	APROVADO
Dr. José Carlos Nogueira	FASEH	Aprovado
Dr. Rafael Henrique Nóbrega	UNESP/Botucatu	APROVADO
Dr. Marc Roger Jean Marie Henry	UFMG	APROVADO
Dr. Augusto Barbosa Reis	Medicina - UFMG	APROVADO
Dr. Luiz Renato de França	UFMG	APROVADO

Pelas indicações, o candidato foi considerado: APROVADO

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 28 de junho de 2019.**

Dr. Guilherme Mattos Jardim Costa (Orientador) _____

Dr. José Carlos Nogueira _____

Dr. Rafael Henrique Nóbrega _____

Dr. Marc Roger Jean Marie Henry _____

Dr. Augusto Barbosa Reis _____

Dr. Luiz Renato de França _____

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador

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"Durante muito tempo achei que o

problema do mundo era o modelo,

Mas demorei a entender que a mudança

nunca seria possível vinda de fora.

A resposta sempre foi muito mais simples!

Seja como quer, mas seja verdadeiro;

Faça o que quiser, mas faça com amor;

Seja a diferença que você quer ver primeiro"

(Lucas Mazoni Guerra)

LISTA DE ABREVIATURAS

AR: Receptor de Andrógeno (*androgen receptor*)

CDKS: Complexos de Quinases Dependentes de Ciclina (c*yclin-dependent kinases*)

FSH: Hormônio Folículo Estimulante (*follicle stimulating hormone*)

GATA-4: Fator de Transcrição; Quarto membro da família de proteínas de ligação GATA

GDNF: Fator Neurotrófico Derivado de Células da Glia (*glial cell line-derived neurotrophic factor*)

Ki-67: Marcador de Proliferação Celular MKI67

LC: Célula de Leydig (*Leydig cell*)

P21: Inibidor de Quinase Dependente de Ciclina p21

P27: Inibidor de Quinase Dependente de Ciclina p27

PAS: Ácido Periódico de Schiff (*periodic acid-Schiff*)

PTU: 6-n-Propil-2-Tiouracil (*propylthiouracil*)

SC: Célula de Sertoli (*Sertoli cell*)

ST: Túbulo Seminífero (*Seminiferous tubule*)

T3: Triiodotironina (*Triiodothyronine*)

T4: Tiroxina (*Thyroxine*)

TH: Hormônios Tireoidianos (*Thyroid hormones*)

TR: Região de Transição (*Transition region*)

TRSC: Célula de Sertoli da Região de Transição (*Transition region Sertoli cell*)

RESUMO

A região de transição corresponde ao segmento dos túbulos seminíferos que se conecta à *rete testis*. Recentemente, especial atenção tem sido dada aos estudos referentes a esta singular área do parênquima testicular, em particular devido à presença de um pool de células indiferenciadas nesta região e às interações destas células, por exemplo, com aquelas do sistema imunológico. Desta forma, o presente estudo foi desenvolvido com o objetivo de melhor caracterizar morfofuncionalmente a região de transição, tendo como foco principal diversas análises sobre as células de Sertoli. Assim, utilizando-se de importantes marcadores, a caracterização fenotípica das células de Sertoli da região de transição em ratos Wistar foi realizada. Diferentemente do paradigma até então estabelecido na literatura, os resultados encontrados mostraram que as células de Sertoli da região de transição exibem distinto fenótipo funcional, retendo seu potencial proliferativo mesmo em ratos Wistar adultos. Em estudos subsequentes, a avaliação da dinâmica de proliferação/diferenciação dessas importantes células somáticas foi também realizada após a indução do hipotireoidismo transiente via tratamento com PTU. Por esta abordagem, e baseado na capacidade mitótica das células de Sertoli da região de transição, foi possível estimular a proliferação dessas células, aumentando significativamente diversos parâmetros-chave relacionados com a produção espermática, tais como número total de células de Sertoli por testículo e peso testicular. Em conclusão, a região de transição parece apresentar pools de células de Sertoli progenitoras e emerge como uma importante área para se estimular o crescimento em comprimento dos túbulos seminíferos e maximizar a função testicular.

ABSTRACT

The transition region corresponds to the segment of the seminiferous tubules that connects to the *rete testis*. Recently, special attention has been focused on this unique testicular parenchyma area taking into account the presence of undifferentiated cells and singular interactions with immune system cells. The present thesis was elaborated in order to better characterize morpho-functionally the transition region, specially related to its Sertoli cell population. Then, using several important functional markers, a phenotypic evaluation was performed on Sertoli cells located in the transition region in Wistar rats. Unlike the established paradigm in literature, our results showed that rat Sertoli cells located in the transition region exhibit a distinct functional phenotype and retain the proliferative capacity into adulthood. In subsequent studies, a pre-pubertal transient hypothyroidism was induced via PTU-treatment in order to evaluate the effects on the Sertoli cells proliferation/differentiation dynamics. Using this approach and based on the known mitotic capacity of the Sertoli cells located in the transition region, it was possible to stimulate the proliferation of these cells and significantly increase several key testis parameters related to sperm output, such as the total number of Sertoli cells per testis and testis weight. In conclusion, the transition region seems to harbor pools of progenitor Sertoli cells and emerges as an important area to stimulate growth in length of the seminiferous tubules and maximize testis function.

SUMÁRIO

1. INTRODUÇÃO E JUSTIFICATIVA

O presente estudo foi desenvolvido com base em estudos morfofuncionais sobre as células de Sertoli localizadas na região de transição (TRSCs) dos túbulos seminíferos (ST), particularmente nos aspectos que envolvem o potencial proliferativo destas células. Para tal finalidade, uma sucinta introdução geral acerca da estrutura e função testiculares, espermatogênese e células de Sertoli (SCs) foi inicialmente desenvolvida. Duas abordagens principais foram elaboradas com o objetivo de se investigar esta população de SCs. A primeira envolveu a avaliação e caracterização fenotípica destas importantes células somáticas por meio de marcadores moleculares, cujos achados se encontram no primeiro artigo intitulado "Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the *rete testis* in Wistar rats", artigo este publicado no periódico científico Cell Cycle. Já no segundo artigo, avaliação da dinâmica de proliferação/diferenciação das TRSCs após a indução de hipotireoidismo transiente via PTU foi realizada. Os dados obtidos neste estudo compõem o manuscrito intitulado "Prepubertal PTU-treatment in rat increases Sertoli cell number and sperm production", que se encontra publicado na revista científica Reproduction. Finalmente, atualizada e minuciosa revisão de literatura foi realizada a fim de se reunir o máximo de dados e informações disponíveis sobre esta específica região testicular que é ainda relativamente pouco estudada.

1.1 Estrutura testicular em mamíferos e espermatogênese

O testículo de mamíferos é responsável pela produção de gametas e pela biossíntese de andrógenos e está localizado no escroto na maioria das espécies. O escroto propicia um microambiente capaz de regular a temperatura do testículo, cuja termorregulação ocorre por meio de vários mecanismos envolvendo vasos sanguíneos, músculo liso e esquelético, glândulas sudoríparas e pelos

na grande maioria das espécies. Entretanto, este órgão pode se apresentar no canal inguinal e na cavidade abdominal próximo à bexiga ou aos rins em algumas espécies (Setchell & Breed, 2006).

O testículo é envolvido por uma cápsula de tecido conjuntivo fibroso de espessura variada, a túnica albugínea, que emite trabéculas/septos para o interior do órgão delimitando os lóbulos testiculares. O parênquima testicular pode ser morfofuncionalmente dividido em dois compartimentos: compartimento dos ST (tubular) e compartimento intersticial (intertubular). O compartimento tubular ocupa, na grande maioria das espécies de mamíferos já investigadas, entre 70 a 95% do parênquima testicular (Russell *et al*., 1990; França & Russell, 1998; Hess & França, 2007). Os ST se estendem numa rede convoluta que se conecta por meio da região de transição (TR) à *rete testis* (França *et al*., 2005), e são compostos por túnica própria, epitélio seminífero e lúmen tubular.

A túnica própria reveste externamente os ST e é constituída de matriz extracelular e células peritubulares mioides – células contráteis que auxiliam na propulsão dos espermatozoides e fluido testicular. Já o epitélio seminífero é composto por células germinativas em diferentes estágios de diferenciação e pelas SCs, enquanto o lúmen tubular é resultante da secreção de fluido pelas SCs sob estímulos do hormônio folículo estimulante (FSH) e, principalmente, andrógenos. Localizado na região central dos túbulos, esse fluido é responsável por carrear os espermatozoides recém-formados/espermiados, ainda imóveis, através dos ductos excurrentes (*rete testis*, dúctulos eferentes e ducto epididimário) (Russell *et al*., 1990; Hess & França, 2007).

Em relação ao compartimento intertubular, seus componentes são as células de Leydig (LCs), vasos sanguíneos e linfáticos, nervos e uma população celular variável constituída principalmente de macrófagos, linfócitos, fibroblastos e mastócitos (Russell *et al*., 1990). A LC é a maior fonte de andrógenos (principalmente testosterona) e de fonte variada de outros esteroides e fatores (Russell *et al*., 1990; Smith & Walker, 2014). Embora exista uma grande variação quanto à densidade volumétrica (%) dos diferentes componentes do compartimento intertubular entre as diversas espécies, comumente

a LC é o tipo celular mais frequente neste compartimento (Fawcett *et al.*, 1973; França & Russell, 1998; Hess & França, 2007; Lara *et al*., 2018a).

A espermatogênese é um processo cíclico e bem coordenado, no qual espermatogônias diploides se dividem e se diferenciam para formar o espermatozoide (gameta masculino haploide). Este processo pode ser morfofuncionalmente dividido em três fases ou etapas: i) proliferativa ou espermatogonial, na qual ocorrem sucessivas divisões mitóticas das espermatogônias; ii) meiótica ou espermatocitária, caracterizada pela duplicação de DNA, recombinação gênica e segregação dos cromossomos homólogos; e iii) fase de diferenciação ou espermiogênica, na qual as células haploides sofrem complexo processo de diferenciação, com a formação do flagelo e compactação do DNA, resultando em espermatozoides, que passam por processo de maturação no epidídimo tornando-se assim aptos a fertilizarem o ovócito (Russell *et al*., 1990; Hess & França, 2007).

1.2 Células de Sertoli

As SCs exercem papel crucial na espermatogênese e na estrutura dos ST e, juntamente com as células peritubulares mioides, sintetizam a membrana basal que é fundamental para a integridade e função tubular (Dym, 1994). Ademais, devido a suas junções de oclusão, as SCs dividem o epitélio seminífero em compartimentos basal e adluminal (Russell & Griswold, 1993). No compartimento basal estão localizadas as espermatogônias e espermatócitos primários iniciais, enquanto no adluminal encontram-se os espermatócitos primários a partir de zigóteno, espermatócitos secundários e espermátides. Funcionalmente, as junções de oclusão compõem a barreira de SCs/hematotesticular que propicia um microambiente particular e considerado imunoprivilegiado essencial para o desenvolvimento do processo espermatogênico (Russell *et al.*, 1990; Russell & Griswold, 1993; Yazama, 2008; Meinhardt & Hedger, 2011; Mital *et al*., 2011; Domke *et al*., 2014; Kaur *et al*., 2014; Jiang *et al*., 2014; França *et al*., 2016; Stammler *et al*., 2016).

Durante o processo espermatogênico, as SCs e as células germinativas interagem física e funcionalmente de maneira bastante complexa (Kyrönlahti *et al*, 2011; Sugimoto *et al.*, 2012; Chen & Liu, 2015; Lara *et al*., 2018b). Existem distintas formas de junções intercelulares entre esses dois tipos celulares, incluindo-se os desmossomos, complexos túbulos-bulbares, junções do tipo "gap" e junções à base de actina, conhecidas como especializações ectoplasmáticas (Russell & Griswold, 1993; Kopera et *al*., 2010; Li *et al*., 2017; Lara *et al*., 2018b). Estas últimas são fundamentais para impedir a descamação (*sloughing*) de células germinativas do epitélio seminífero, evitando assim a infertilidade. Em particular, as junções semelhantes a desmossomos e as especializações ectoplasmáticas da porção basal do epitélio seminífero coexistem ao nível da barreira hematotesticular, onde auxiliam as junções de oclusão na manutenção da barreira imunológica (Kopera *et al*., 2010; França *et al*., 2011; França *et al*., 2012; França *et al*., 2016; Lara *et al*., 2018b). De modo geral, evidencia-se a necessidade da interação parácrina das células germinativas com os componentes somáticos do testículo, principalmente SCs, LCs e células peritubulares mioides, para que o processo espermatogênico transcorra de maneira normal e eficiente (Russell *et al.*, 1994; França & Russell, 1998; Welsh *et al.*, 2009; Rossi & Dolci, 2013; Rebourcet *et al*., 2014; Lara *et al*., 2018b). A integridade funcional da membrana basal elaborada pelas SCs e peritubulares mioides é também fundamental para o processo espermatogênico (Dym, 1994; Richardson *et al*., 1998; Siu & Cheng, 2004; Wen *et al*., 2016; Lara *et al*., 2018b).

A considerável variação na forma e estrutura das SCs durante o ciclo do epitélio seminífero demonstra o alto grau de plasticidade desta célula, o que reflete as alterações morfofuncionais que ocorrem nas células germinativas (Russell *et al.*, 1993; Sugimoto *et al*., 2012; França *et al*., 2016; Yoshida, 2016). Além da composição da barreira de SCs, anteriormente citada, as SCs realizam outras funções essenciais para o desenvolvimento das células germinativas, dentre as quais podem ser mencionadas: fornecimento de nutrientes e diversos outros fatores para as células germinativas, bem como a própria sustentação para essas células espermatogênicas; participação ativa no processo de

liberação (espermiação) das espermátides para o lúmen tubular; e fagocitose tanto do excesso de citoplasma (corpos residuais) resultante da liberação das células espermiadas, quanto de células germinativas que sofrem apoptose (Griswold, 1998; Hess & França, 2007, Sofikitis *et al*., 2008; França *et al*., 2016). Conforme também já foi mencionado, as SCs secretam ainda fluido em direção ao lúmen tubular, o qual possui substâncias e inúmeros fatores importantes para a função epididimária e maturação espermática, servindo também de veículo para o transporte dos espermatozoides (Griswold, 1988; Robaire & Viger, 1995). Embora em menor quantidade, a secreção de fluido também ocorre em direção ao compartimento intertubular, estando assim envolvida em elaborados mecanismos de regulação parácrina de outros tipos celulares do testículo, tais como as LCs, células peritubulares mioides e musculares lisas dos vasos.

As SCs fazem ainda a intermediação hormonal do processo espermatogênico apresentando, dentre outros, receptores de andrógenos (AR) e de FSH (Russell *et al*., 1990; Russell & Griswold, 1993; Sharpe, 1994; França & Russell, 1998; Hess & França, 2007; França *et al*., 2016). Além disso, as SCs são consideradas como o principal componente somático do nicho espermatogonial, que é definido como o microambiente específico que regula o controle da auto-renovação, diferenciação e mesmo apoptose das espermatogônias-tronco (Voog & Jones, 2010; Oatley & Brinster, 2012; De Rooij & Griswold, 2012; Yoshida, 2012; Iwamori, 2014; De Rooij, 2017), sendo composto pelas próprias células-tronco germinativas, células somáticas adjacentes e matriz extracelular (Hofmann, 2008; Caires *et al.*, 2010; Spinnler *et al.,* 2010; Costa *et al*., 2012; Oatley & Brinster, 2012; França *et al*., 2016). Por estarem em íntimo contato com as células germinativas, as SCs, além de oferecer o suporte cito-arquitetural importante na manutenção do nicho, atuariam fundamentalmente através da sinalização por GDNF - que é um fator essencial para a proliferação e auto-renovação espermatogonial (Hofmann *et al*, 2005; Lee *et al.*, 2007; Hofmann, 2008; Chiarini-Garcia *et al*., 2009; Kokkinaki *et al.*, 2009; Nakagawa *et al.*, 2010; Campos-Júnior *et al*., 2012; Song & Wilkinson, 2014; Chen & Liu, 2015; França *et al*., 2016; Singh *et al*., 2017).

Diversos estudos demonstram que o número total de SCs por testículo é o principal fator na determinação do tamanho do testículo e da magnitude da produção espermática (Hess *et al.*, 1993; Sharpe, 1994; França *et al.*, 1995; Hess & França, 2007), tendo por fundamento o fato de que as SCs tem capacidade de suporte de células germinativas relativamente fixa para cada espécie (França & Russell, 1998; Hayrabedyan *et al.*, 2012; França *et al.*, 2016). Assim, uma maior população de SCs propicia maior número de nichos espermatogoniais, resultando em aumento do número das células germinativas (França *et al.*, 1995; Auharek & França, 2010). Desta forma, conforme considerado na literatura, o número de SCs estabelecido durante o desenvolvimento testicular, no período que antecede a puberdade, determina, em última análise, o tamanho do testículo e a magnitude da produção espermática no indivíduo adulto (Hess *et al.*, 1993; Sharpe, 1994; França & Russell, 1998; Hess & França, 2007; Lara *et al.*, 2018a).

Embora esta premissa esteja sendo revista para mamíferos em geral (Chui *et al.*, 2011; Hayrabedyan *et al.*, 2012; Tarulli *et al.*, 2012; Tarulli *et al.*, 2013), considera-se, em ratos e camundongos, que as SCs proliferam mais ativamente durante a fase fetal, após a diferenciação sexual, ocorrendo um pico de proliferação antes do nascimento (Orth, 1984; Orth *et al.*, 1988; Orth, 1993; McCoard *et al.*, 2003; França *et al.*, 2016). Após o nascimento, é estabelecido para esses roedores que a proliferação das SCs se estende por 2 a 3 semanas, reduzindo de forma gradual até o início da proliferação de espermatócitos primários. Assim, por volta do 21º dia pós-natal em ratos, divisões das SCs não mais ocorrem (Steinberger & Steinberger, 1971; Orth, 1982; Auharek & França, 2010; Picut *et al.*, 2015), permanecendo o número de SCs por testículo estável por toda a vida reprodutiva do animal (França *et al.*, 2005; Hayrabedyan *et al.*, 2012). Embora os mecanismos que regulam a proliferação das SCs não estejam ainda completamente elucidados, sabe-se que o FSH desempenha importante papel na regulação da atividade mitótica das SCs (Orth, 1984, 1993; Heckert & Griswold, 2002; Meachem *et al.*, 2005; Nascimento *et al.*, 2016). Dentre outros fatores (Fragale *et al.*, 2001*;* Kazutaka *et al.*, 2011; Nicholls *et al.*, 2012), é sugerido que andrógenos e estrógenos também

influenciam a atividade mitótica dessas células (Orth, 1984, 1993; Sharpe *et al.*, 2003; Johnston *et al.*, 2004; Atanassova *et al.*, 2005; Scott *et al.*, 2007, 2008; Auharek *et al.*, 2010, 2011, 2012; Lucas *et al.*, 2014; Mital *et al.*, 2014).

Em contrapartida, os hormônios tireoidianos (THs) atuam (principalmente via p21 e p27 e ativação do receptor TRalpha1) de forma marcante na transição do estado mitótico para o não-mitótico (maturação/diferenciação) das SCs localizadas ao longo dos ST, no período que antecede a puberdade (Cooke *et al.*, 2005; Holsberger *et al.*, 2005; Wagner *et al.*, 2008; Auharek & França, 2010; Fumel *et al.*, 2012; Chatonnet *et al.*, 2014; Gao *et al.*, 2014; Sun *et al.*, 2015). Neste contexto, devido à redução do período proliferativo e à aceleração do processo de maturação dessas células, altas concentrações de triiodotironina (T3) durante o período neonatal levam a uma diminuição do peso testicular, do número de SCs e da produção espermática nos animais adultos (França *et al.*, 1995; Cooke, 1996; Auharek & França, 2010). De modo inverso, a diminuição da concentração de THs torna mais lenta a transição da fase proliferativa para a fase madura das SCs, prolongando assim seu período mitogênico e, por consequência, aumentando o peso testicular, a população adulta de SCs e a produção espermática (Van Haaster *et al.*, 1992; Joyce *et al.*, 1993; Cooke, 1996; Auharek & França, 2010). Por inibir a síntese de THs e a conversão da tiroxina (T4) em T3, o PTU (6-n-propil-2-tiouracil) é uma droga bociogênica comumente empregada para tratar casos de hipertireoidismo (Cooke & Meisami, 1991). Assim, esta droga tem sido também bastante utilizada em estudos envolvendo os efeitos do hipotireoidismo na espermatogênese em várias espécies de mamíferos (Cooke, 1991; Cooke *et al.*, 1992, 1993; Joyce *et al.*, 1993; Hess *et al.*, 1993; França *et al.*, 1995; Kirby *et al.*, 1996; Jansen *et al.*, 2007; Auharek & França, 2010; Kobayashi *et al.*, 2014; Lara & França, 2017).

1.3 Região de Transição

A TR corresponde à porção dos ST que se conecta à *rete testis* (Perey *et al.*, 1961), representando uma particular e muito importante região de união entre duas áreas de origens

embriológicas distintas, onde elementos dos ST são provenientes da gônada primitiva enquanto a *rete testis* é derivada dos remanescentes dos túbulos mesonéfricos (Roosen-Runge, 1961; Svingen & Koopman, 2013). Anatomicamente, a TR se distingue por apresentar um epitélio seminífero composto por SCs consideradas fenotipicamente modificadas, cujos citoplasmas se projetam distalmente em direção à *rete testis*, formando estrutura similar a uma válvula (Dym, 1974; Osman, 1978; Nykänen, 1979; Osman, 1980; Lindner & Holstein, 1982; Hermo & Dworkin, 1988). Ademais, a população de células germinativas na TR é composta por espermatogônias-tronco indiferenciadas (Aiyama *et al*., 2015) e, do ponto de vista imunológico, em comparação com as demais regiões dos ST, é observada uma maior prevalência de linfócitos e macrófagos circundando a TR (Takahashi *et al*., 2007).

Os principais estudos disponíveis na literatura até o presente momento sobre a TR estão pormenorizadamente descritos em revisão intitulada *"Insights into transition region of the seminiferous tubules"* (ver **Anexo**).

1.4 Justificativa

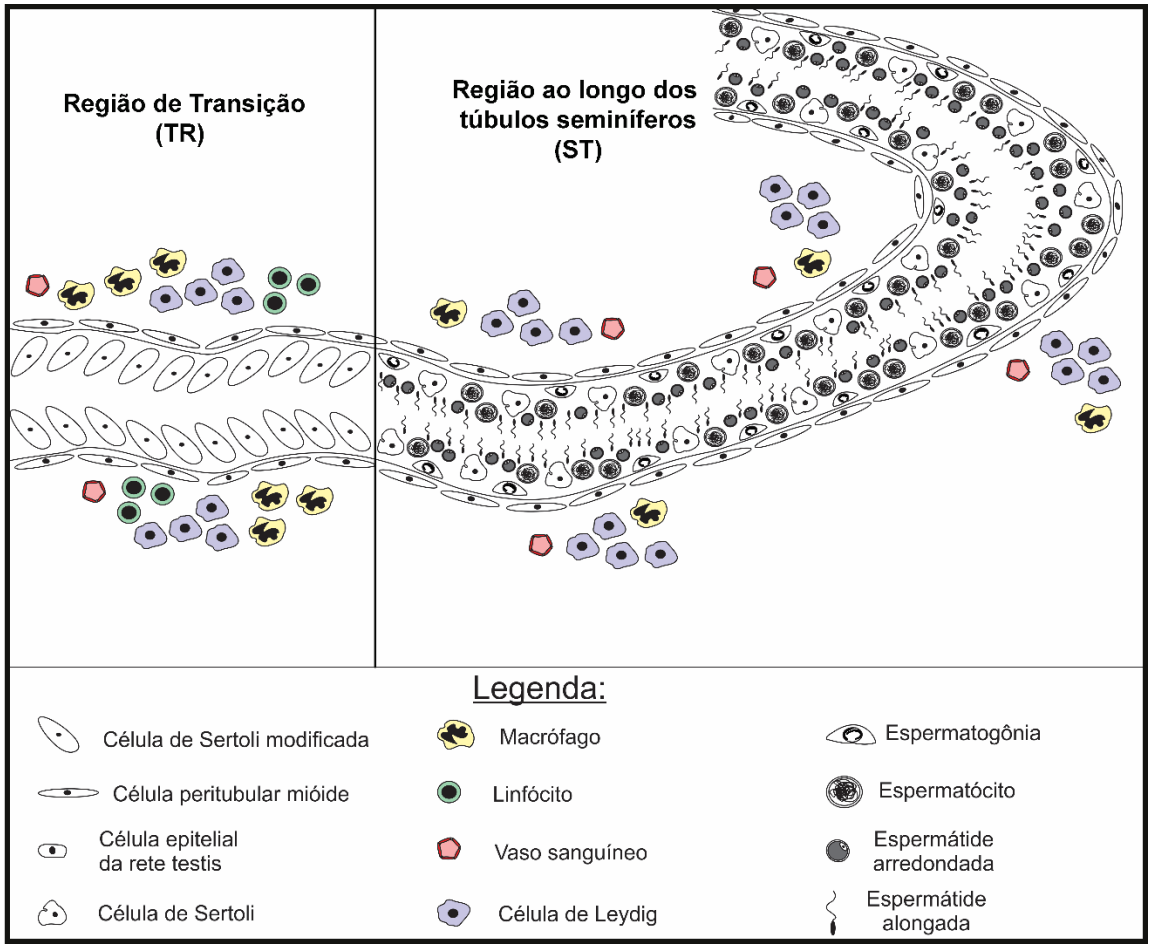
Devido à presença de um pool de SCs imaturas e de células-tronco espermatogoniais, a TR tem sido recentemente alvo de interessantes estudos envolvendo a biologia reprodutiva, podendo ser citado, como exemplo, a formação de novos nichos de células germinativas e investigações referentes às interações das células somáticas da TR com as do sistema imune. Portanto, a TR desponta como um microambiente testicular funcionalmente bastante singular. No entanto, existem muitas questões ainda não elucidadas acerca da morfofisiologia da TR e suas implicações para a função testicular. Neste sentido, o presente estudo foi delineado com a finalidade de melhor compreender a dinâmica de proliferação/diferenciação das TRSCs em ratos Wistar pré-púberes e sexualmente maduros, bem como a regulação exercida por THs sobre essas células. Esperamos que os resultados eventualmente obtidos possam fornecer importantes subsídios que auxiliem nos mecanismos responsáveis pelo desenvolvimento e crescimento dos ST a partir das SCs.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar as características morfofuncionais das células de Sertoli presentes na região de transição entre os túbulos seminíferos e a *rete testis* (Fig. 1) por meio de análises morfométricas, estereológicas, imuno-histoquímicas, moleculares e hormonais.

Figura 1. Ilustração esquemática de diferentes regiões do parênquima testicular compreendendo a região de transição (TR) e ao longo dos túbulos seminíferos (ST).



2.2 Objetivos Específicos

Referentes ao artigo I intitulado "As células de Sertoli da região de transição dos túbulos seminíferos com a *rete testis* apresentam capacidade proliferativa até a fase adulta em ratos Wistar":

- Analisar, por imuno-histoquímicas e imunofluorescências, a dinâmica proliferativa e de diferenciação das TRSCs envolvendo os fatores Ki-67, BrdU, p27, GATA-4, AR e Sox-9.

Referentes ao artigo II intitulado "O tratamento de ratos Wistar pré-púberes com PTU aumenta o número de células de Sertoli e a produção espermática":

- Avaliar o crescimento ponderal e a biometria testicular ao longo do período experimental;
- Obter o número total de células de Sertoli, bem como a produção espermática diária por testículo, utilizando-se de técnicas histomorfométricas;
- Avaliar o *status* histológico da glândula tireoide e a produção de T4;
- Analisar a dinâmica proliferativa e o status de diferenciação das TRSCs por imuno-histoquímicas e imunofluorescência para Ki-67, p21 e Sox-9.

3. ARTIGO 1

Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the *rete testis* in Wistar rats (Figueiredo *et al*., 2016)

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REPORT

Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the *rete testis* in Wistar rats

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ABSTRACT

Sertoli cells (SCs) play a crucial role in testis differentiation, development and function, determining the magnitude of sperm production in sexually mature animals. For over 40 years, it has been considered that these key testis somatic cells stop dividing during early pre-pubertal phase, between around 10 to 20 days after birth respectively in mice and rats, being after that under physiological conditions a stable and terminally differentiated population. However, evidences from the literature are challenging this dogma. In the present study, using several important functional markers (Ki-67, BrdU, p27, GATA-4, Androgen Receptor), we investigated the SC differentiation status in 36 days old and adult Wistar rats, focusing mainly in the transition region (TR) between the seminiferous tubules (ST) and the *rete testis*. Our results showed that SCs in TR remain undifferentiated for a longer period and, although at a lesser degree, even in adult rats proliferating SCs were observed in this region. Therefore, these findings suggest that, different from the other ST regions investigated, SCs residing in the TR exhibit a distinct functional phenotype. These undifferentiated SCs may compose a subpopulation of SC progenitors that reside in a specific microenvironment capable of growing the ST length if needed from this particular testis region. Moreover, our findings demonstrate an important aspect of testis function in mammals and opens new venues for other experimental approaches to the investigation of SC physiology, spermatogenesis progression and testis growth. Besides that, the TR may represent an important site for pathophysiological investigations and cellular interactions in the testis.

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Androgen Receptor; BrdU; GATA-4; Ki-67; p27; seminiferous tubules; Sertoli cell; transition region

Introduction

Sertoli cells (SCs) play a crucial role in testis differentiation, development, and function, particularly on the aspects related to spermatogenesis.[1-3] Furthermore, SCs are one of the key elements of the spermatogonial stem cells microenvironment (niche), providing the former with all the necessary substances regulating their proliferation and differentiation.[4-8]

The number of SCs in the testis established before puberty determines the magnitude of sperm production in sexually mature animals.[2,9-11] In this regard, it is well known that SCs in laboratory rodents proliferate during the fetal and early postnatal period of testis development.[12-14] For over 40 years, the accepted paradigm has been that SCs cease to divide and become an adult, terminally differentiated cell population.[3,15,16] However, suggesting that SCs may still proliferate in some areas of the testis, even after the stabilization of the tubular diameter and SC efficiency (number of germ cells per SC) in early puberty, the testis continues to grow, with significant increases in weigh, sperm production and tubular length during the post-puberty period in several mammalian species investigated.[16-24] Although challenging the dogma that SCs do not proliferate in pre-pubertal and sexually mature mammals has been controversial, some mammalian seasonal breeders have been reported to show season-dependent variations in SC proliferation activity.[24-26]

In all vertebrates spermatogenesis occurs in the seminiferous tubules (ST).[27] Particularly in mammals, the region that connects the seminiferous tubules to the *rete testis* is known as the transition region or transitional zone (TR)[28,29] and very few studies have been devoted to this particular area of the testis.[30-34] Anatomically, it is considered that this region is composed of morphologically modified SCs that form a plug-like valve structure on the luminal aspect of the seminiferous tubules.[33,35] Furthermore, few advanced germ cells are present in the TR,[30,31] and according to a recently published study,[36] this region emerges as a potential site or niche for spermatogonial stem cells. In their study, the stable and selective maintenance of A_{single} GFRα1-positive spermatogonia was observed in the TR, as well as the expression of high levels of GDNF (the GFRα1 ligand), a major niche factor produced by SCs.[36]

In order to better understand SC proliferation/differentiation dynamics in pre-pubertal and adult Wistar rats, in the present study we investigated several important factors related to this key testis somatic cell function, particularly in the TR (Fig. 1). Our findings suggest a distinct behavior/function of a SCs subset in this region.

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kccy.

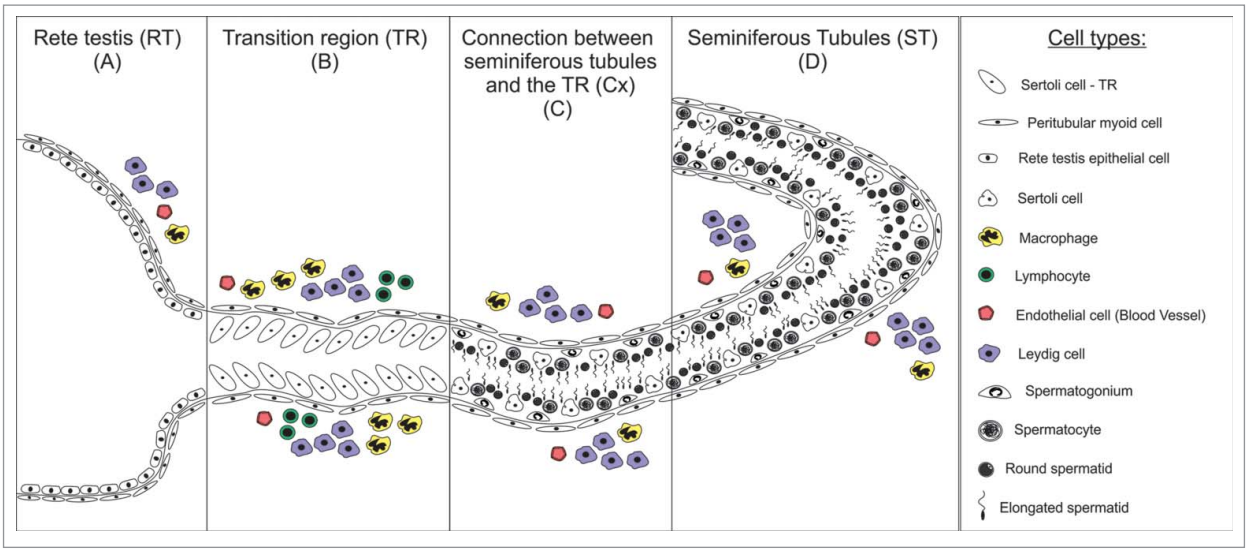

Figure 1. Schematic illustration of the different testicular parenchyma areas investigated in pre-pubertal and adult Wistar rats. The morphological and functional characteristics of Sertoli cells in the transition region (TR) (B), in the area adjacent to the transition region (Cx) (C), and along the other areas of seminiferous tubules (ST) (D) were evaluated. The Cx area was arbitrarily defined as a region of the ST corresponding to approximately 250 micrometers from the beginning of TR. Each cell type represented in the above scheme is depicted in the box located at the right side.

Results

Sertoli cell proliferation markers

As shown in Figure 2, proliferative SCs were found only in TR in both pre-pubertal and adult rats. The analysis for Ki-67 revealed that approximately 4% of SCs were proliferating in this region in pre-pubertal rats on day 36. Although still proliferating, this activity was significantly reduced (less than 1%; $p < 0.05$) in adult rats. The above pattern was qualitatively confirmed by BrdU immunolabeling (Fig. 3).

Sertoli cell differentiation markers

1P27

In contrast to the proliferative markers, p27 is a protein that promotes cell-cycle inhibition and is characteristically expressed in non-proliferative cells. Immunostaining for p27 showed an opposite pattern (Fig. 4), as p27-negative SCs were found only in the TR in both pre-pubertal and adult ages. The vast majority of SCs observed was p27-positive.

GATA-4

In both ages investigated, SCs not expressing the transcription factor GATA-4 were found mainly in the TR (Fig. 5). Approximately 8% of the GATA-4 negative SCs were found in pre-pubertal rats, while in adults this figure was significantly reduced (~4%; $p < 0.05$). In the Cx (connection between seminiferous tubules and transition region; please see section 4.3), very few GATA-4 negative SCs were noted (less than 1%) in young and in adult rats. All SCs in the other tubular areas expressed GATA-4 (Fig. 5).

Androgen receptor (AR)

AR expression in SC is associated with the onset of puberty, but first appears postnatally after all major SC proliferation has begun to decline significantly.[37] AR-negative SCs located in the TR in pre-pubertal rats were observed frequently, as approximately one fifth (~17%) of the SCs did not express AR. In adult rats its expression was halved (around 8%; $p < 0.05$) (Fig. 6). Regarding the Cx, very few negative SCs (less than 1%) were observed in both investigated ages. As expected, all SCs expressed AR in the other seminiferous tubular areas (Fig. 6).

Immunofluorescence

Double-staining for Ki-67 and AR in the 3 regions showed a distinct immunolabeling pattern in both pre-pubertal and adult rats, as all Ki-67-positive SCs were AR-negative (Fig. 7 and 8). A particularly interesting pattern was exhibited by some peritubular myoid cells in the TR and epithelial cells from the *rete testis*, which were positive for the proliferation marker Ki-67 as well as for AR (Figs. 7 and 8).

Double-staining for Ki-67 and Sox-9 confirmed that the somatic cells located in the TR epithelium were positive for both markers (Fig. 9). Considering their nuclear morphology, location and Sox-9 expression, these somatic cells might be considered as putative Sertoli cells.

Discussion

This study is the first to report that Sertoli cell proliferation in a rodent species can occur well beyond the perinatal period and even into pre-pubertal and adult life. For over 40 years, it has been reported that Sertoli cell proliferation in the laboratory rodent peaks just prior to birth and that SCs stop dividing before puberty, between 15-21 days of age in the rat.[12,13,38-40] The potential for SC proliferation beyond the perinatal period

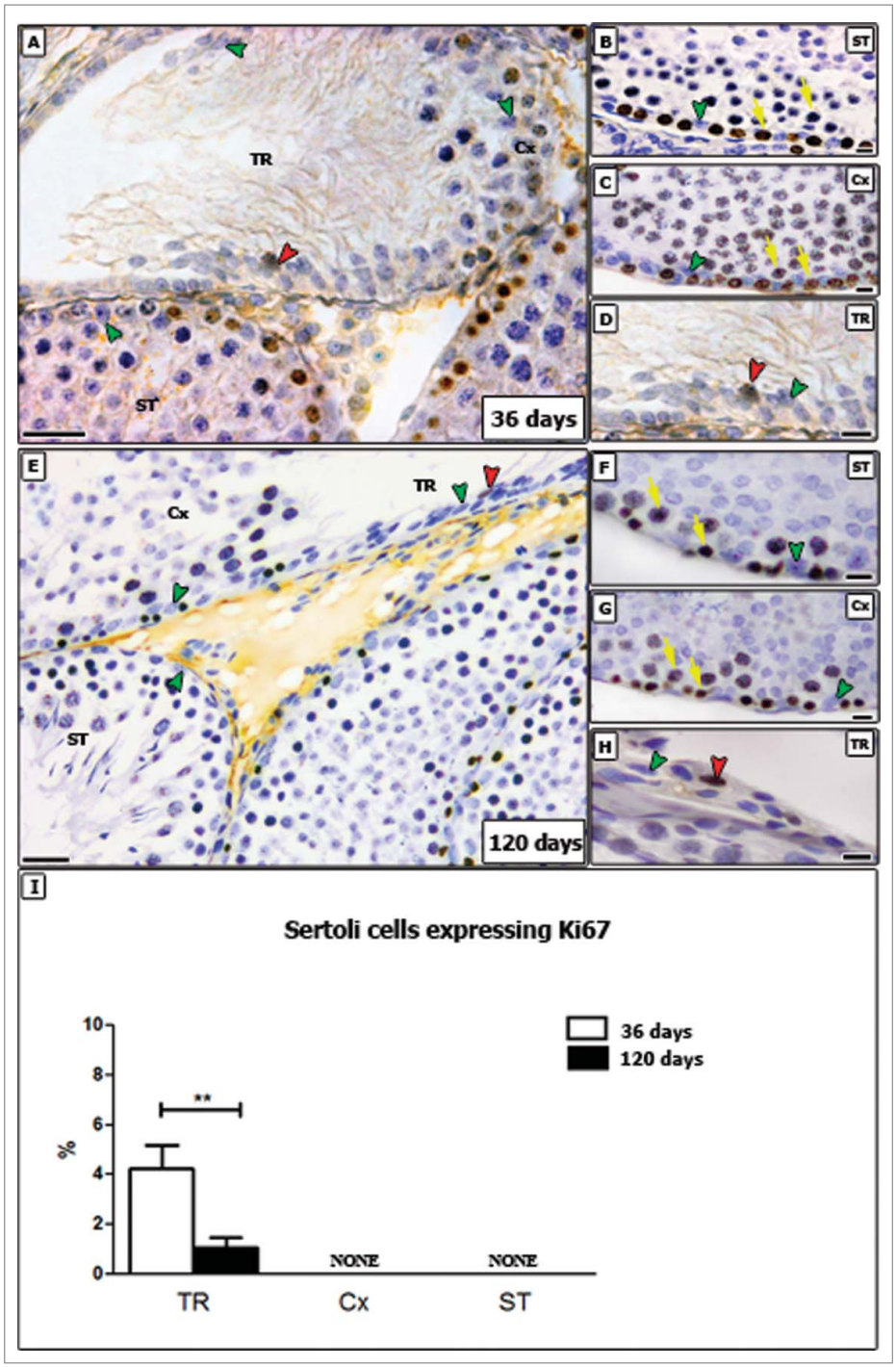

Figure 2. Ki-67 immunostaining in different testicular areas of pre-pubertal (A-D) and adult (E-H) Wistar rats. Images showing the seminiferous tubules (ST; B and F), the area adjacent to the transition region (Cx; C and G) and the transition region (TR; D and H). Positive and negative Sertoli cells are indicated respectively by red and green arrowheads. In TR, Sertoli cells were observed proliferating at 36 and 120 days. The number of proliferating Sertoli cells observed in adults (~1%) are significantly lower than those found in pre-pubertal rats (~4%) ($p < 0.05$) (I). As expected, proliferating germ cells were observed in the seminiferous epithelium (yellow arrows). Bar: 50 μm (A and E); 10 μm (B-D, F-H).

was thought to occur only under specific experimental conditions.[24-26,41,42] Nevertheless, it is well known that the testis increases in size well into adulthood, with a significant increase in total lengths of the seminiferous tubules, but without a further increase in tubular diameter and SC efficiency.[19,43] However, a satisfactory explanation for this tubular lengthening has not been presented until now. Immature Sox-9 positive SCs, capable of proliferation, were found in the transition region of the seminiferous tubules, adjacent to the *rete testis*. These cells would permit a slow but continuous growth of the tubules, as well as the preservation of progenitor SCs that are capable of extending the period of mitotic activity.

Prior studies of SC proliferation appear to have overlooked the *rete testis* junction, as they did not comment on the transition region.[12,13,38] Therefore, it was reasonable for those studies to conclude that SCs no longer divide after

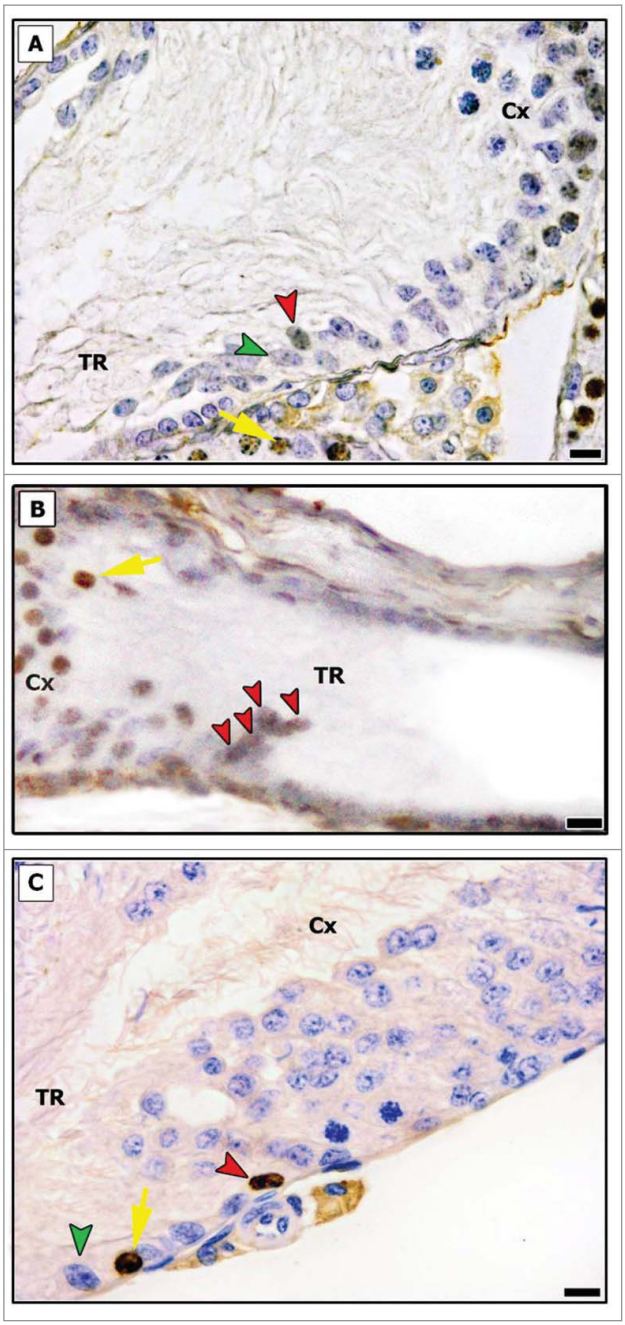

Figure 3. BrdU immunostaining in the transition region (TR) and in the area adjacent to the TR (Cx) in pre-pubertal (A and B) and adult (C) Wistar rats. Proliferating Sertoli cells were found in TR (red arrowheads), and a cluster of BrdU labeled Sertoli cells was found in pre-pubertal rat (B). Negative Sertoli cells are indicated by green arrowhead (A and C). Proliferating germ cells are shown by yellow arrows. Bar: 10 μm.

postnatal day 21. Other observations also supported this traditional view. In the rat, SC differentiation begins between 14 and 21 days postnatal and is marked by at least 3 key morphological events: an intense proliferation of primary spermatocytes, formation of the blood-testis barrier (SCs barrier) and the opening of the seminiferous tubule lumen due to SC secretions.[14,44] Our results also found that SCs outside the transition region were positive only for differentiation markers and at 36 and 120 days of age, only within the *rete testis* connection did SCs exhibit markers for proliferation.

A small portion of SCs within the transition region displayed features of being undifferentiated and negative for p27, GATA-4 and AR. GATA-4 staining of the SC nucleus is an accepted marker for the differentiating SC.[45,46] This transcription factor is mainly expressed in Sertoli and Leydig cells and has been implicated in the development and function of the mammalian testis, particularly in the regulation of gene expression and cell differentiation.[47,48]

In the current study, 5-10% of the SCs in the transition region did not express GATA-4 at both ages investigated, while in other regions of the testis, all SCs were positive for this factor. In the testis, GATA-4 regulates SC differentiation and function and is required for proper interaction between these somatic cells and germ cells.[45,49,50] In the conditional knockout of GATA-4, the testis was atrophic, with impairment of spermatogenesis and loss of fertility. Most importantly, SCs exhibited altered morphology and had increased permeability at the blood-testis barrier,[45] consistent with an immature phenotype. *Dmrt1* is also under GATA-4 regulation and in the *Dmrt1* knockout mouse, SCs failed to complete differentiation and exhibited over-proliferation.[51-53]

AR is an inducible transcription factor that regulates gene expression in response to androgens,[54-56] and also serves as a marker for SC maturation.[37] An increase in AR expression in SC is associated with the general termination of SC proliferation.[57] In contrast, the absence of AR inhibits SC maturation.[58] A recent report found that hormonal suppression in men resulted in an increased SC expression of Ki-67 and PCNA, which was coincident with a decrease in AR, suggesting that SC are capable of de-differentiation.[24] In the current study, SCs within the transition region appear to remain undifferentiated from the perinatal period.

Thyroid hormones (TH) are considered the main regulators of SC proliferation and differentiation.[22,23,59-61] TH acts through the cell-cycle inhibitors p21 and p27,[14,62-64] and serves as an important regulator of AR, increasing its expression in the rat SC.[65] Thus, the presence of a pool of AR- and p27-negative SCs in the transition region also indicates a small pool of undifferentiated SCs reside in this unique region.

In conclusion, although it has been accepted for many years that SCs stop dividing by day 21 in the rodent species,[12,13,44] data obtained in the present study indicate that SCs at the seminiferous tubule/*rete testis* junction, known as the transition region, express markers for immature and proliferating cells at 36 and 120 days of age (Fig. 10). It is possible that this discovery has been delayed due to the fact that most rodent samples collected for histology are taken from transverse sections of the testis at the midline, often overlooking the *rete testis* region that is located off-center and more cephalic.[66] The current study highlights the need to include samples from the transition region when testing for potential effects on SC proliferation. Proliferation beyond the early postnatal period was thought to occur only under certain experimental conditions such as hemicastration,[67] transient hypothyroidism,[61] hormonal suppression,[24] SC transplantation,[68] and during recrudescence in hibernating mammals.[23,69] However, the data presented here suggests that SCs within the transition region exhibit a

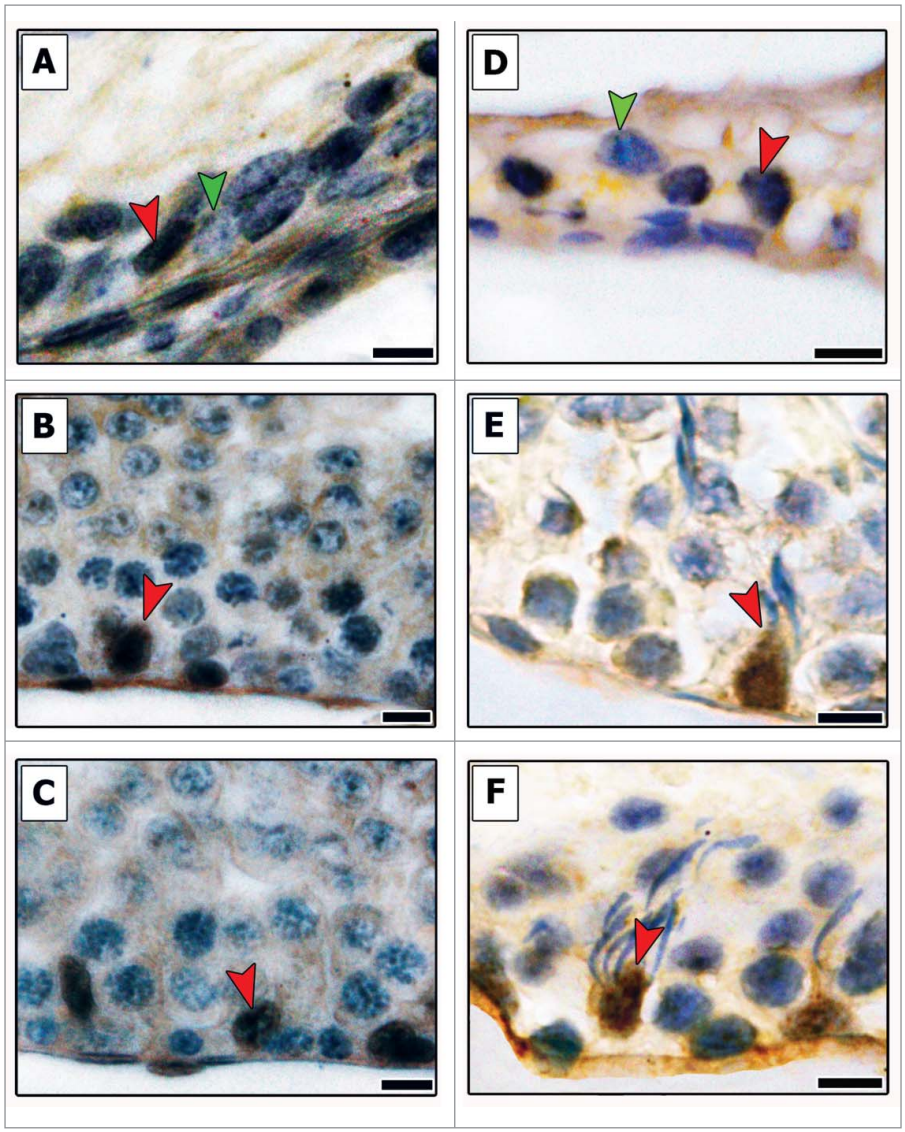

Figure 4. Evaluation of p27 immunostaining in different areas of testicular parenchyma in pre-pubertal (A–C) and adult (D–F) Wistar rats. The transition region (TR) (A and D), the area adjacent to the transition region (B and E) and along the seminiferous tubules (C and F) were investigated for this cell-cycle inhibitor. All Sertoli cells present in Cx and ST were positive for p27 (red arrowheads, B–F), whereas negative Sertoli cells (green arrowheads) were observed only in the TR. Bars: 10 μm.

distinct functional phenotype, long after the differentiation of SCs located in other regions of the seminiferous tubules. These undifferentiated SCs may compose a subpopulation of SCs progenitors that are capable of growing the seminiferous tubules if necessary. In this context, several questions are raised. Is the transition region a potential site for chemical toxicity or the onset of specific diseases? Could these immature SCs be a site for antigen leakage into *rete testis* fluid? Do new germ cell clonal units fill new stem cell niches that would form after SC division?

Methods

Animals

Twenty Wistar rats (*Rattus novergicus*; aged 36 and 120 days) were used in this study. All animal experiments were performed in strict accordance with the Guidelines for Animal Use and Experimentation as set forth by the Animal Experimentation Ethics Committees from the Federal University of Minas Gerais (Belo Horizonte, Brazil; CEUA 398/2013).

Histology and immunostaining

Rats were sacrificed by pentobarbital overdose (50 mg/Kg BW). For immunohistochemical staining and according to the specific antibodies used, testes samples from 16 rats were fixed for 24h in Bouin's solution or 4% paraformaldehyde (PFA) or 10% formalin. The samples were then dehydrated in ethanol and routinely embedded in paraplast. Serial sections (5 μm thick) from the chosen area were incubated overnight at 4°C with the following antibodies and dilutions: anti-AR (androgen receptor; 1:50; Santa Cruz Biotechnology, sc-816), anti-GATA4 (1:100; Santa Cruz Biotechnology, sc-25310), anti-Ki-67 (1:100; PharMingen, #558615) or anti-p27 (1:50; PharMingen, #554069). Reactions were visualized using biotin-conjugated secondary antibodies in combination with the Elite ABC Kit (Vector Laboratories, CA) or using Alexa-488 anti-rabbit/633 anti-mouse

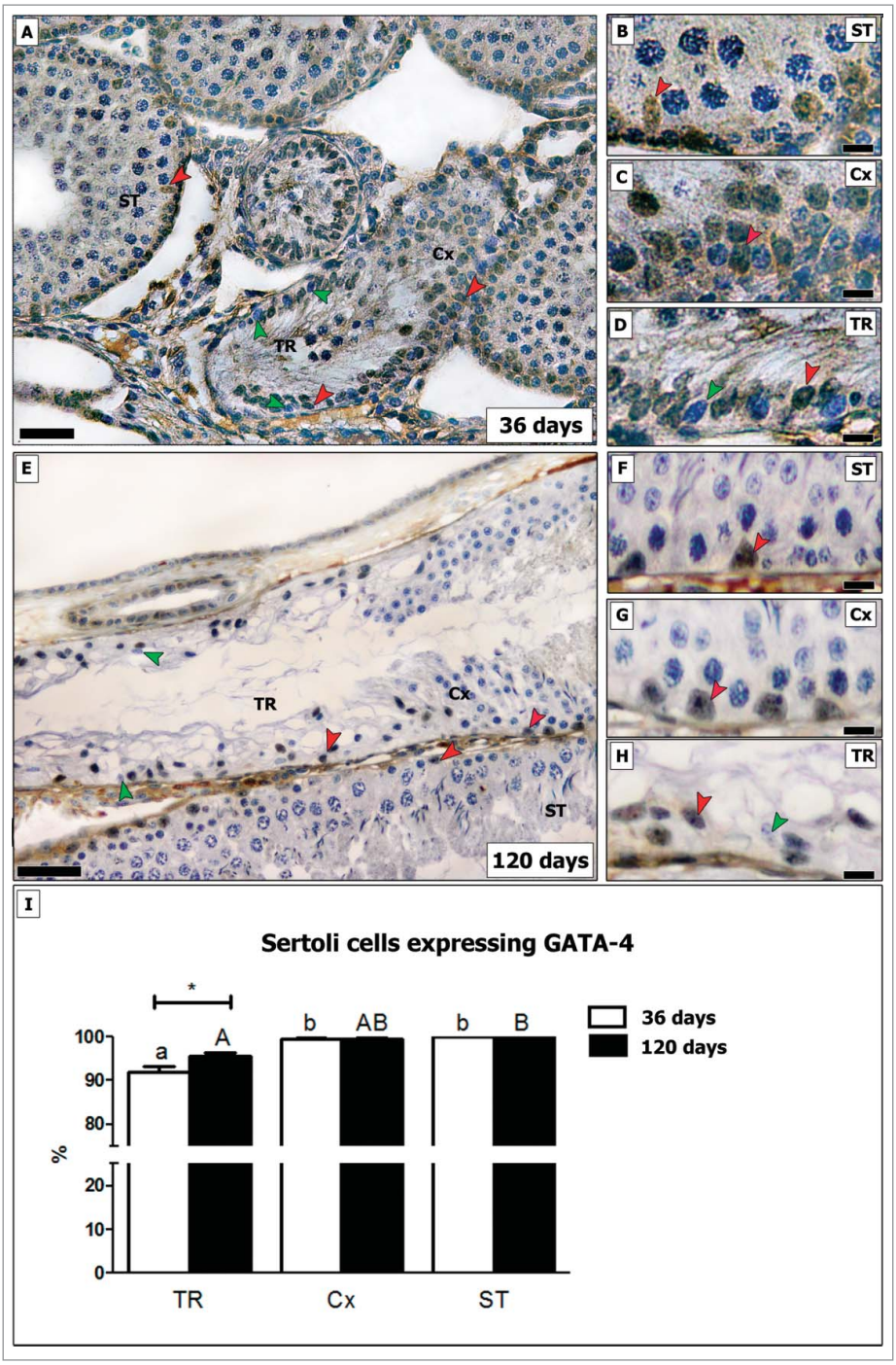

Figure 5. Evaluation of GATA-4 immunostaining in different testicular areas of pre-pubertal (A–D) and adult (E–H) Wistar rats. Images showing the seminiferous tubules (ST; B and F), the area adjacent to the transition region (Cx; C and G) and the transition region (TR; D and H). Positive and negative Sertoli cells are indicated respectively by red and green arrowheads. In TR, about 8% of Sertoli cells do not express GATA-4 in pre-pubertal rats. In adults, the percentage of GATA-4 negative Sertoli cells are reduced by half (4%; $p < 0.05$) (I). In the Cx (B, G), few GATA-4 negative Sertoli cells were found in both young and adult rats (less than 1%). Bar: 50 μm (A and E); 10 μm (B-D, F-H). Different small and capital letters represent statistically significant differences between regions (TR, ST or Cx) respectively of young and adult rats ($p < 0.05$). Considering the same region, statistically significant differences ($p < 0.05$) were observed only for TR.

conjugated secondary antibodies (1:200 dilution; Thermo Fisher Scientific) - visualized using a Nikon fluorescence microscope (Eclipse Ti). Aiming to simultaneously evaluate the SCs regarding their proliferative and differentiation status, a double-staining for Ki-67 and AR in pre-pubertal and adult rats was also performed. A double-staining for Ki-67 and Sox-9 (anti-Sox-9; 1:50 dilution; Santa Cruz Biotechnology, sc-20095) was also performed to demonstrate that Sertoli cells are indeed able to proliferate in the TR. Additionally, in order to investigate qualitatively SC proliferation activity focusing exclusively on the S phase of the cell cycle (Ki-67 protein is present during all active phases of the cell cycle; G1, S, G2, and mitosis), BrdU (150 mg/Kg BW) was intraperitoneally injected into 4 rats (2 pre-pubertals and 2 adults) 2h before their sacrifice, and this evaluation was performed using anti-BrdU (1:100 dilution; PharMingen, #347580).

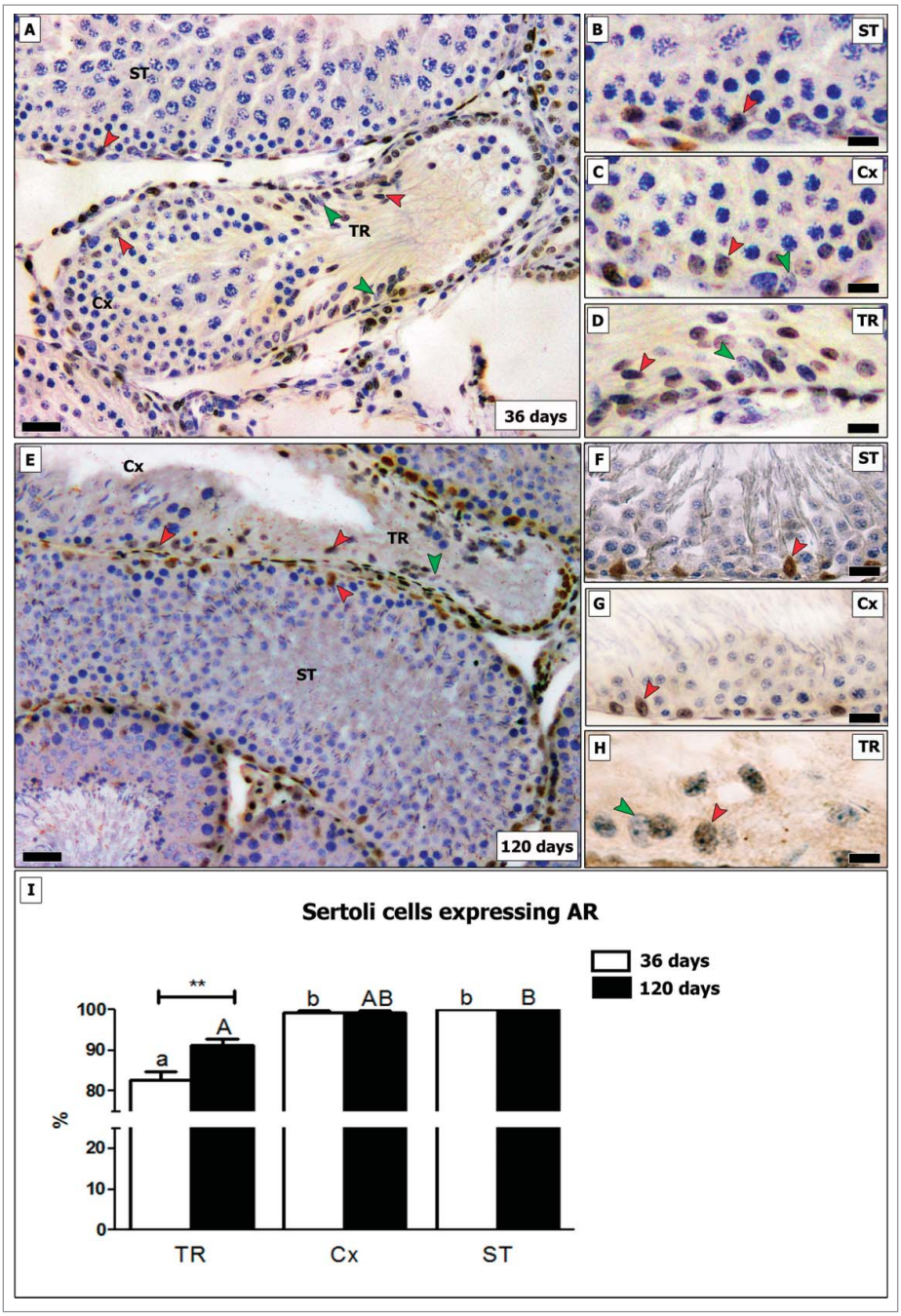

Figure 6. Evaluation of AR immunostaining in different testicular areas of pre-pubertal (A-D) and adult (E-H) Wistar rats. Images showing the seminiferous tubules (ST; B and F), the area adjacent to the transition region (Cx; C and G) and the transition region (TR; D and H). Positive and negative Sertoli cells are indicated respectively by red and green arrowheads. In TR, approximately 17% of Sertoli cells do not express AR in pre-pubertal rats. In adults, the percentage of AR negative Sertoli cells are reduced by half (8%; $p < 0.05$) (I). In the Cx (B, G), few AR negative Sertoli cells were observed in both young and adult rats (less than 1%). Bar: 50 μm (A and E); 10 μm (B-D, F-H). Different small and capital letters represent statistically significant differences between regions (TR, ST or Cx) respectively for young and adult rats ($p < 0.05$). Considering the same region, statistically significant differences ($p < 0.05$) were observed only for TR.

Quantitative analyses

After immunolabeling following standardized protocols,[70,71] the evaluated samples were photographed at 200x and, according to the illustration shown in Figure 1, the SC functional markers were quantified in the TR, in the connection between seminiferous tubules and the TR (Cx; arbitrarily defined as 250 μm of extension) and along the other ST areas. For Ki-67, AR and GATA4, the percentage of positive/negative SCs was calculated in each of the 3 investigated regions and, according to a pilot study and statistical analysis, at least an area of 50.000μm^2 per region was evaluated. All the stained samples were analyzed using the image analysis software Image J v.1.45s (Image Processing and Analysis, in Java) and an Olympus microscope (BX60).

Statistical analyses

All data were tested for normality and homoscedasticity of the variances. Quantitative data are represented as the mean ±

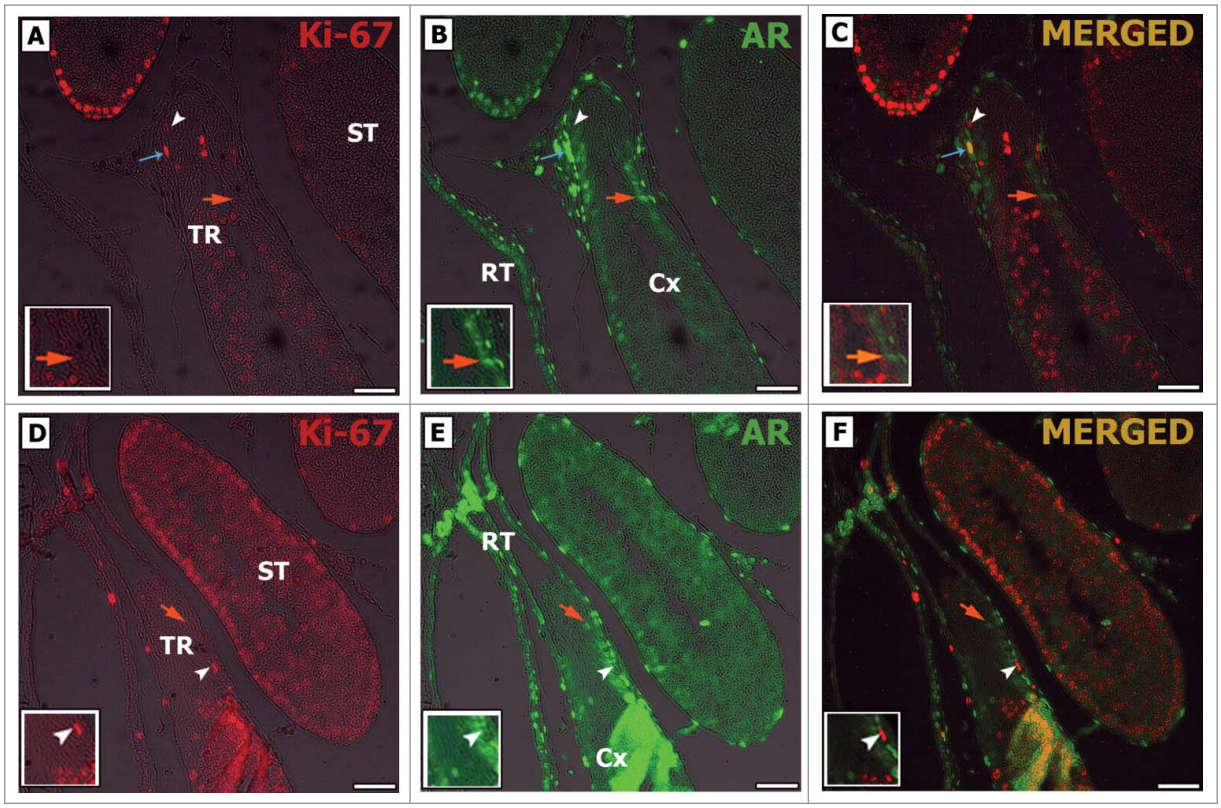

Figure 7. Double-label immunofluorescence for Ki-67 (A, D) and AR (B, E) in pre-pubertal Wistar rats. As it can be observed, AR positive Sertoli cells are Ki-67 negative (orange arrow C, F), while Ki-67 positive Sertoli cells are AR negative (white arrowhead C, F). Peritubular myoid cells were observed in TR expressing Ki-67 and AR (blue arrow C). TR: transition region; Cx: area adjacent to the TR; ST: seminiferous tubules. Bar: 50 μm.

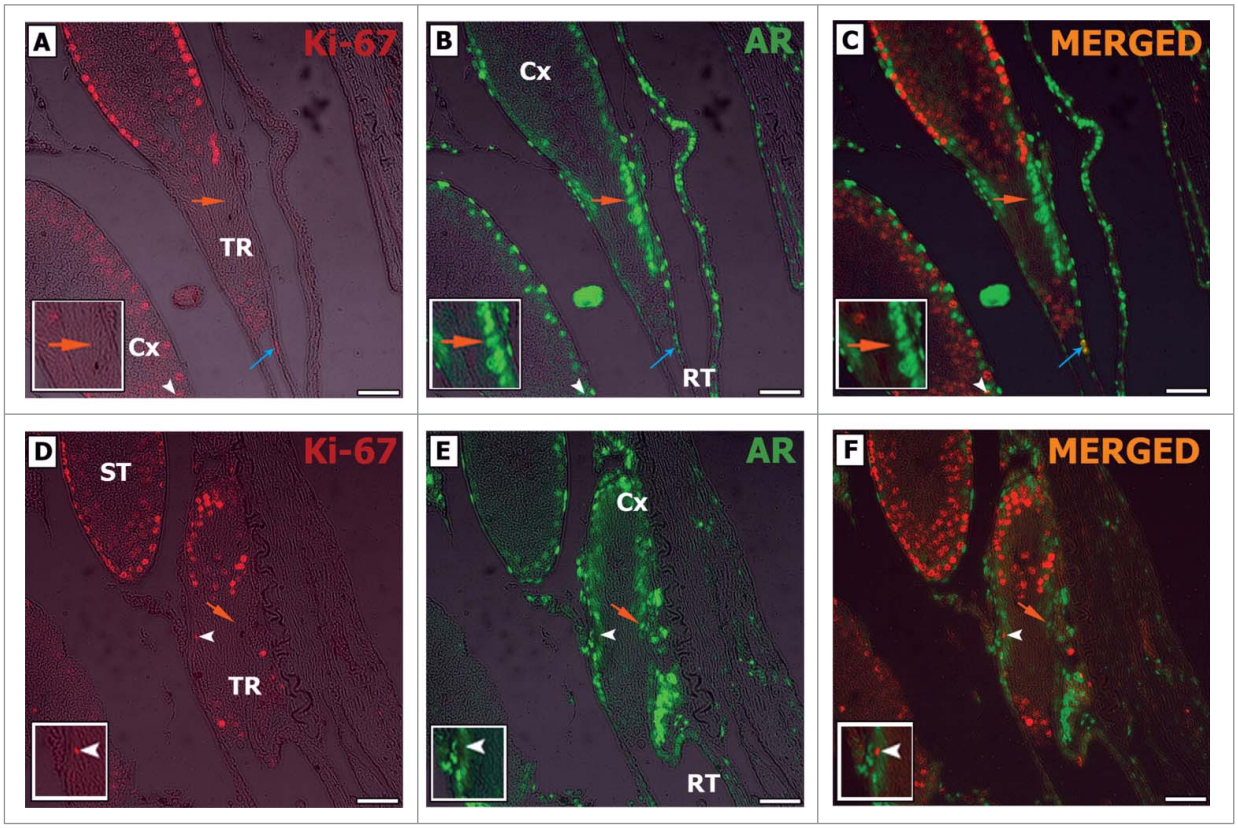

Figure 8. Double-label immunofluorescence for Ki-67 (A, D) and AR (B, E) in adult Wistar rats. As it can be noted, AR positive Sertoli cells are Ki-67 negative (orange arrow C, F), while Ki-67 positive Sertoli cells are AR negative (white arrowhead C, F). *Rete testis* epithelial cells were also observed expressing Ki-67 and AR (RT blue arrow C). TR: transition region; Cx: area adjacent to the TR; ST: seminiferous tubules. Bar: 50 μm.

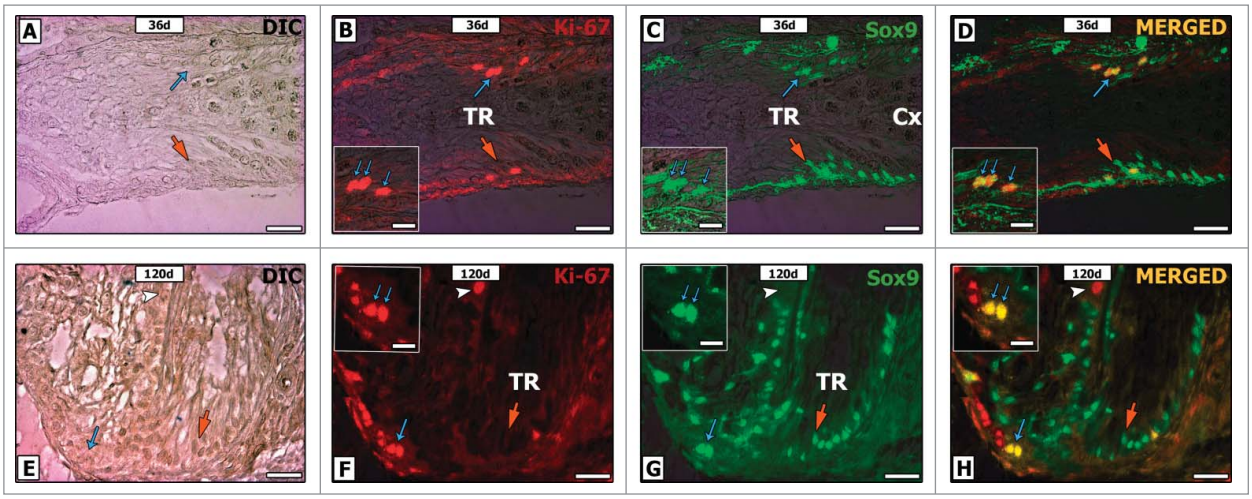

Figure 9. Double-label immunofluorescence for Ki-67 (B, F) and Sox-9 (C, G) in pre-pubertal (A-D) and adult (E-H) Wistar rats. As it can be seen, some Sox-9 positive Sertoli cells was also Ki-67 positive (blue arrow D, H). Otherwise, other Sox-9 positive Sertoli cells were negative for Ki-67 (orange arrow D, H). Few proliferative germ cells were also observed in the TR (white arrowhead H). TR: transition region; Cx: area adjacent to the TR. Bar: 50 μm.

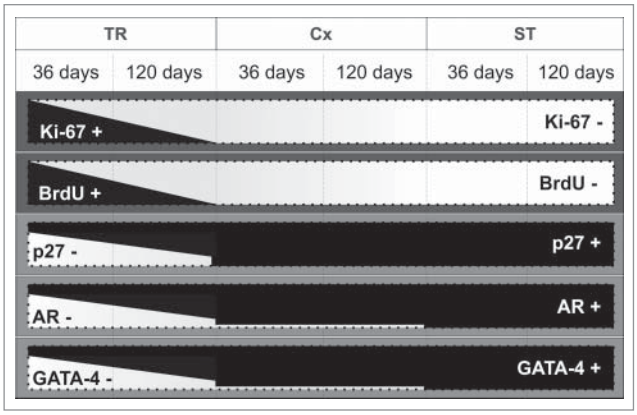

Figure 10. Diagram summarizing the results found in the present study. Representation of positive (+; black areas) and negative (-; white areas) Sertoli cells for each functional marker considered (Ki67, BrdU, p27, AR, and GATA-4) in the 3 testis parenchyma regions evaluated in 36 and 120 days-old Wistar rats. As shown schematically, the relative expression of these markers in TR is clearly distinct when compared to the other 2 investigated regions (Cx and ST). TR: transition region; Cx: area adjacent to the TR; ST: seminiferous tubules.

SEM (standard error of mean). Analyses were conducted using the graphics and statistics program PRISM v5.0 (GraphPad Software, Inc.). Data were assessed by one-way ANOVA for comparisons within groups followed by Newman-Keuls test in case of normal distribution, or by Kruskal-Wallis followed by Dunn's test in case of nonparametric data. Student's unpaired t-test was performed for single comparisons between groups. Differences were considered statistically significant at $p < 0.05$.

Abbreviations

AR Androgen receptor
Cx Connection between seminiferous tubules and transition region
SC Sertoli cell
ST Seminiferous tubules
TR Transition region

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

Conceived and designed the experiments: AFAF, LRF, GMJC. Performed the experiments: AFAF, GMJC. Analyzed the data: AFAF, GMJC. Contributed reagents/materials/analysis tools: AFAF, GMJC. Wrote the paper: AFAF, LRF, RAH, GMJC.

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4. ARTIGO 2

Prepubertal PTU-treatment in rat increases Sertoli cell number and sperm production (Figueiredo *et al*., 2019)

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REPRODUCTION

RESEARCH

Prepubertal PTU treatment in rat increases Sertoli cell number and sperm production

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Abstract

The number of Sertoli cells (SCs) ultimately determines the upper limit of sperm production in the testis. Previous studies have shown that thyroid hormones (TH) receptors are abundantly expressed in developing SCs; therefore, it was highly significant to discover that transient neonatal hypothyroidism induced by the goitrogen 6-n-propyl-2-thiouracil (PTU) can extend SCs proliferation beyond the first 2 weeks postnatal and increase testis weight and sperm production. Further studies concluded that treatment must begin before day 8 post birth in rats. Recent studies, however, showed that SCs present in the transition region at the *rete testis* exhibit a more immature phenotype and have prolonged mitotic activity, which led to the hypothesis that SCs in this region will retain the capacity to respond to PTU treatment over a longer period of time. In the present study, male Wistar rats were treated with PTU from days 21 to 40 and were evaluated at 40 and 160 days of age. Similar to neonatal rat SCs, it was demonstrated that prepubertal SCs in the transition region have a high mitotic activity and are highly sensitive to TH levels. This delayed, transient hypothyroidism resulted in significantly increased testis weight, SCs number and daily sperm production. The results demonstrate for the first time that Sertoli cells showing plasticity in the transition region can be stimulated to increase proliferation and contribute to a late stage surge in testis weight and sperm output.

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Introduction

Sertoli cells (SCs) play a crucial role in testis physiology, from the fetal period of organ differentiation to the adult (Magre & Jost 1991), controlling general growth and development of the seminiferous tubules and providing support required for survival and evolution of germ cells into sperm (França *et al.* 1995, 2016, Rotgers *et al.* 2018). The importance of this key somatic testicular cell is best exemplified by the fact that the magnitude of sperm production in sexually mature animals is ultimately determined by the total number of Sertoli cells per testis (Orth *et al.* 1988, Sharpe 1994, Hess & França 2007, França *et al.* 2016).

Although it is well established that SCs become terminally differentiated before puberty, recent studies have shown that a subset of rodent SCs are still capable of proliferation in the transition region after this period, having mitotic activity even in adults (Figueiredo *et al.* 2016, Kulibin & Malolina 2016, Malolina & Kulibin 2017). This particular region, which is a border between two embryologically distinct areas (Roosen-Runge 1961, Svingen & Koopman 2013), is where seminiferous tubules (STs) connect to the *rete testis*, representing a unique functional environment that exhibits substantial plasticity. Therefore, the transition region may serve as an area capable of forming a new spermatogonial stem cell niche (Aiyama *et al.* 2015), given that new SCs are continually available for an extended period of time (Figueiredo *et al.* 2016). Therefore, we hypothesized that the transition region represents an area where the ST could grow in length after puberty.

Endocrine factors regulate reproductive physiology (Wagner *et al.* 2008), including the capacity of SCs to proliferate (Cooke *et al.* 2005, Holsberger & Cooke 2005). Several hormones, such as the follicle-stimulating hormone (FSH), activin A, androgens and estrogens, among others, are involved in SCs proliferation (Orth 1984, Meachem *et al.* 2005, Nicholls *et al.* 2013, Lucas *et al.* 2014). Thyroid hormones (THs) are a key factor in stimulating their differentiation (Cooke *et al.* 1994, França *et al.* 1995, Auharek & França 2010, Sun *et al.* 2015). The drug 6-n-propyl-2-thiouracil (PTU) is used to

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treat hyperthyroidism and thus an antithyroid medication that decreases TH by blocking the conversion of thyroxin (T4) to triiodothyronine (T3) (Taurog *et al.* 1976, Shiroozu *et al.* 1983, Crofton & Zoeller 2005, Dong 2006). Interestingly, PTU has been used experimentally in animals to modulate SC proliferation and differentiation, by creating a transient hypothyroidism (Cooke *et al.* 1992, França *et al.* 1995, Auharek & França 2010, Waqas *et al.* 2019). In 1991, Cooke and colleagues demonstrated that PTU treatment in rats, from birth to weaning, increased testis size by 80% and sperm production by nearly 140%. This surge in sperm output was explained by an increase of 157% in the number of Sertoli cells (Hess *et al.* 1993), which was eventually shown to be due to a delay in differentiation, allowing the cells to continue dividing beyond the normal period after birth (Cooke *et al.* 1994, França *et al.* 1995). Further studies showed that TH inhibits SCs proliferation, while transient hypothyroidism does the opposite (Van Haaster *et al.* 1992); however, the increase in testis weight and SC proliferation depends on the timing of a return to the euthyroid condition (Rijntjes *et al.* 2017).

After testing several different treatment periods, it was concluded that if treatment began on postnatal day 8 or later PTU would not increase testis weight (Cooke *et al.* 1992). However, our research group, having demonstrated that SCs in the transition region (TRSCs) have a prolonged mitotic activity (Figueiredo *et al.* 2016), hypothesized that this unique region may retain SCs capable of responding to PTU over a longer period of time. Therefore, the present study investigated the effects of PTU treatment on TRSCs proliferation, by creating a transient hypothyroidism during an alternate window of prepubertal development, but after the accepted age when SC have ceased to divide (21–40 days old). The results demonstrate for the first time that Sertoli cells in the transition region at the *rete testis* can be stimulated by late stage transient hypothyroidism to increase proliferation, which contributes to a significant increase in testis weight and sperm output at 160 days of age. Because hypothyroidism remains an ongoing concern during pregnancy (Gutvirtz *et al.* 2019), these data provide new awareness regarding testis growth, niche formation and spermatogenesis progression in experimental animals and young boys.

Materials and methods

Animals

In this study, 37 male Wistar rats (*Rattus novergicus*) were used. All experiments were performed in strict accordance with the Guidelines for Animal Use and Experimentation as set by the Animal Experimentation Ethics Committees from the Federal University of Minas Gerais (Belo Horizonte, Brazil; CEUA 398/2013).

Experimental design

Four experimental groups, with eight male rats each, were evaluated. The animals were killed at different ages (40 and 160 days old) and distributed into control and PTU-treated groups. Each group was kept in separate cages and the PTU treatment (6-propil-2-tiouracil, Sigma) was given from 21 to 40 days of age. Day 21 was chosen to begin treatment, because it is well established that rat SCs ceased dividing at this age (Orth 1982, Almirón & Chemes 1988). PTU was added to the drinking water at 0.03% (w/v) and, to improve PTU-palatability, ten drops of sweetener (Stevia Linea) were added per liter of solution. Five young male rats were used as starting controls for histological evaluation of the testes and thyroid glands, as well as for the initial measurements of free thyroxine (T4) (see below). All groups were kept in an average temperature of 22°C and 70% average humidity with 12-h photoperiod. Water and pelleted food were available *ad libitum*. The experimental design is shown in Fig. 1. For all groups, body weights (BW) were measured weekly from day 21 to day 160.

Testis weight and gonadosomatic index

All rats were killed by pentobarbital overdose (100 mg/kg BW). Testis weight (TW) was obtained after the testis was separated from the epididymis. The net TW was estimated by subtracting weights of the tunica albuginea and the mediastinum (Johnson *et al.* 1981). Because testicular density is very close to 1 (França 1991), testicular volume was considered equal to its weight. The gonadosomatic index (testes mass divided by body weight) was estimated for all investigated animals.

Histomorphometric analysis

For histological and morphometric analyses, testis and thyroid gland fragments were collected, weighed and fixed by immersion for 24 h in 4% glutaraldehyde in a 0.05 M phosphate buffer, pH 7.2 at 4°C. The specimens were dehydrated in ethanol and routinely embedded in glycol-methacrylate (Historesin, Leica). Histological sections, 4 µm thick, were obtained and then stained with toluidine blue solution in 1% sodium borate.

Volume densities of the testis parenchyma components

The volume densities (%) of the ST were obtained using a graticule containing 441 intersections. All histomorphometric analyses used the Image J v.1.45s software (Image Processing and Analysis, in Java). The intersections that coincided with tunica propria, seminiferous epithelium and tubular lumen were counted in 15 randomly chosen fields by horizontal scanning of the histological sections. Five images per histological section and spaced 200 µm apart were evaluated at 400× magnification.

Seminiferous tubules and thyroid follicular lumen diameters

The mean diameter of the ST was obtained from cross-sections of tubules that were round or nearly round, regardless of

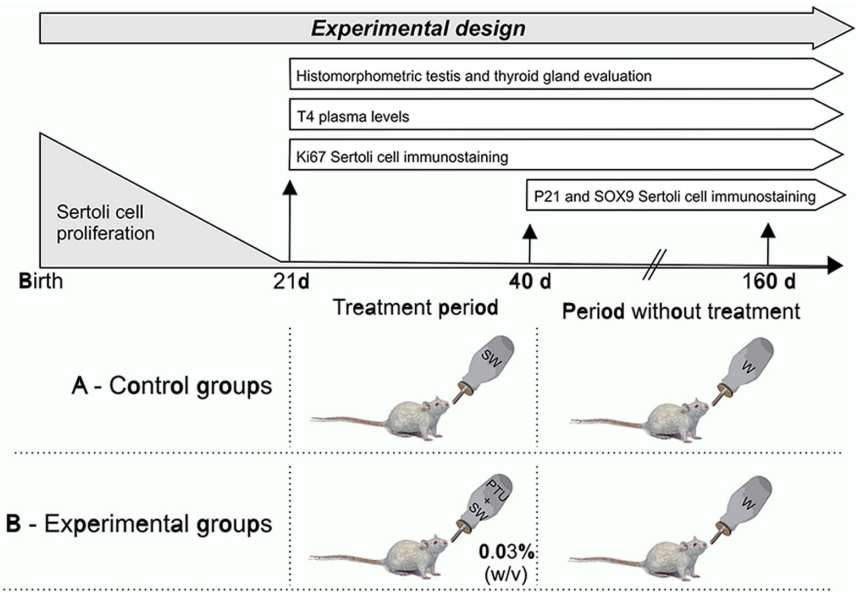

Figure 1 Schematic illustration of the experimental design. Five 21-day-old male rats were used for the immunostaining evaluation of the Sertoli cells proliferation (SCs), the testis and thyroid gland histological status, as well as for thyroid hormone (T4) plasma level. Because SCs are not expected to proliferate after 3 weeks of age (Orth 1982, Almirón & Chemes 1988), except in the transition region (Figueiredo *et al.* 2016), PTU treatment began on day 21 post-partum and extended until day 40. The animals were killed on 40 and 160 days post birth. At these two latter ages, 32 male rats were evaluated (eight per each experimental group). PTU, 6-propil-2-thiouracil; S, sweetener; W, water.

the stage of the seminiferous epithelium cycle (SEC). Thirty ST cross-sections were measured at 100× of magnification per animal. Regarding thyroid follicular lumen, their mean diameters were estimated according to the following formula: (major diameter + minor diameter)/2 (Rajab *et al.* 2017). Thirty thyroid follicles, randomly chosen, were measured at 200× of magnification per animal.

Seminiferous tubules and transition region lengths

The length of the ST, expressed in meters, was estimated according to the method of Attal and Courot (1963), considering the values for the total volume of the ST and the mean tubular diameter obtained for each animal. Thus, the total length of the ST was determined by dividing ST volume by the squared radius of the tubule (tubular diameter divided per two) times the *pi* value (Johnson & Neaves 1981). The individual length of the transition region, expressed in micrometers, was obtained from regions presenting a clear and continuous connection between the ST and the *rete testis*.

Evaluation of the most advanced germ cell type

In order to verify if the PTU treatment affects the germ cell progression and differentiation, particularly in 40-day-old rats, the most advanced germ cell type was evaluated. This evaluation was performed also based on the germ cell associations and, when deemed pertinent, the development of the acrosomal system and the morphology of the developing spermatid nucleus were considered.

Cell counting

In order to estimate some key testis parameters, SCs nucleoli and round spermatids nuclei were counted in ten round or nearly round ST cross-sections, chosen randomly, for each animal at 200× magnification. In this evaluation, only ST in stage VII, according the acrosomic system (Russell *et al.* 1993), were considered. The cell counts were corrected for section thickness and nucleus or nucleolus diameter according to Abercrombie (1946) as modified by Amann (1962).

The total number of SCs per testis was estimated from the corrected counts of SC nucleoli per tubule cross-section and the total length of ST, according to Hochereau-de Reviers and Lincoln (1978). Following this calculation, and considering the testis net weight, the number of SCs per gram of testis was also determined. Regarding the spermatogenic and SC efficiencies, the ratio between corrected counts of round spermatids and SCs was calculated in 160-day-old rats. Since the duration of the SEC in Wistar rats is 13.3 days and the frequency of stage VII is around 18% (Russell *et al.* 1993), the daily sperm production (DSP) per testis was calculated according to the following formula developed by França (1992): DSP = (total number of SCs per testis) × (the ratio of round spermatids per SCs at stage VII) × (stage VII relative frequency (%))/(stage VII duration (days)).

Quantification of apoptotic germ cells

Germ cell apoptosis, which is easily recognized in histological sections embedded in plastic, was quantified in 40- and 160-day-old rats according to their typical morphological characteristics, such as cell shrinkage, formation of apoptotic bodies and chromatin condensation (Russell *et al.* 1993, Elmore 2007, de Alvarenga & de França 2009). For all rats, germ cell apoptosis was determined in ten random ST cross-sections, at stages VII–VIII of the SEC, and expressed as the total number of apoptotic cells per ST cross-section.

Hormonal analyses and immunostaining

In order to perform hormonal analysis for each animal, the blood samples (0.3 mL) were collected via cardiac puncture at 21, 40 and 160 days of age, when the animals were still anesthetized. The plasma was separated by centrifugation at 10,000 ***g*** for 10 min and stored at −80°C. Aiming to assess thyroid function and to check hypothyroidism treatment, the

concentration of free T4 was measured by radioimmunoassay (RIA) kit from TECSA® (Belo Horizonte, MG, Brazil), with sensitivity of 0.02 ng/dL and intra-assay and inter-assay coefficients of variation of 4.1 and 7.3%, respectively.

For immunohistochemical staining, testicular samples were fixed for 24 h in methacarn at 4°C, dehydrated in ethanol and routinely embedded in paraplast. Serial sections (5 µm thick) were incubated overnight at 4°C with anti-KI67 (1:100 dilution; Pharmingen, #550609, lot number 82421) and anti-P21 (1:100 dilution; Pharmingen, #550827, lot number 54131). Reactions were visualized using biotin-conjugated secondary antibodies (1:200 dilution; Imuny, IC1M02, lot number 16160) in combination with Elite ABC Kit (mixture of reagent A (100 µL) and reagent B (100 µL) into 5 mL of buffer; Vector Laboratories, CA, USA). Detection of signal was obtained via peroxidase substrate 3,39-diaminobenzidine (DAB, Sigma Aldrich) reaction and counterstaining with hematoxylin (Merck). In order to demonstrate the SC proliferation dynamics in the transition region after PTU treatment, a double immunofluorescence for KI67 (1:100 dilution; Pharmingen, #550609, lot number 82421) and SOX9 (1:50 dilution; Santa Cruz Biotechnology, sc-20095, lot number H0715) was also performed. Reactions were visualized using Alexa-488 (anti-rabbit) and Alexa-633 (anti-mouse) conjugated secondary antibodies (1:200 dilution; Thermo Fisher Scientific) using a Nikon fluorescence microscope (Eclipse Ti).

After immunolabeling following standardized protocols (Ramos-Vara 2005), samples were photographed at 200× using Olympus microscope (BX60). SCs proliferation or differentiation were analyzed in the transition region and along the ST. The percentage of KI67 positive/negative SCs (evidence of proliferation) and of P21 positive/negative SCs (evidence of differentiation) were calculated per animal according to a previous study (Figueiredo *et al.* 2016). At least an area of 50,000 µm^2 (ranging from 5 to 10 tubular longitudinal sections) and 30 ST cross-sections were analyzed in the TR and along the ST, respectively. All the stained samples were analyzed for area and number of cells using the 'freehand' and 'cell counter' tools, respectively, in the image analysis software Image J v.1.45s (Image Processing and Analysis, in Java).

Statistical analyses

All data were tested for normality and homoscedasticity of the variances. Data were assessed by Student's *t*-test and were represented as the mean ± S.E.M. (standard error of the mean). Data analyses were performed using the graphics and statistics program PRISM v5.0 (GraphPad Software, Inc). Differences were considered statistically significant at $P < 0.05$.

Results

Biometric data

The BW measured throughout the experiment are shown in Fig. 2. As it can be observed, the BW in both groups was similar during the first 2 weeks of PTU treatment, but were significantly lower ($P < 0.05$) in treated rats only when they reached 40 days of age (Table 1), i.e. at the end of the treatment period. This significant difference lasted until 3 weeks (~140 days of age) before the animals were killed and, after that, in comparison to the controls, the BW of treated rats recovered totally (Table 2).

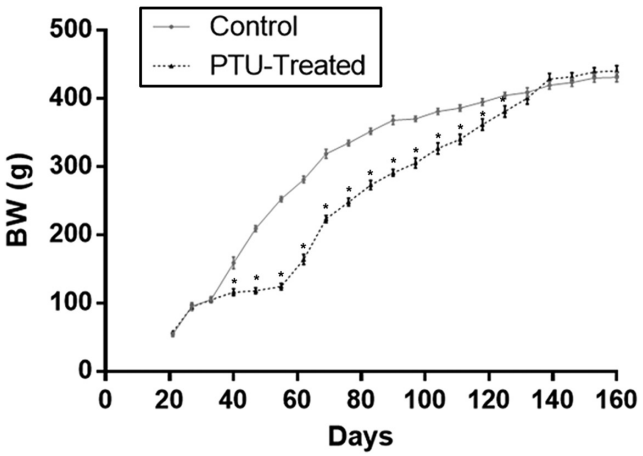

Figure 2 Evaluation of body weight (BW) throughout the experimental period. Significant differences (Student's *t*-test, $P < 0.05$) were observed at the end of PTU treatment (40 days) and continued until the animals were almost 20 weeks old.

Thyroid gland and hormonal analyses

At 21 days of age, as expected, the thyroid gland was normal, with variable sized follicles filled with thyroid colloid and having a mean lumen diameter of 55 µm (Fig. 3A and G). At the end of PTU treatment, most thyroid glands follicles in treated rats were devoid of colloid (Fig. 3C) and, in comparison to the controls, the follicular lumen diameter was significantly reduced (16 µm vs 60 µm, Fig. 3G). On day 160, both groups exhibited normal and similar thyroid gland histology with follicular lumen diameter measuring in average 70 µm (Fig. 3D, E and G). Confirming the efficacy of the treatment and the histological evaluation, on day 40 the free T4 level was drastically reduced ($P < 0.05$) in the treated group (Fig. 3F). As expected because thyroid gland histology was normal, adult rat T4 plasmatic levels were quite similar in both investigated groups ($P < 0.05$).

Quantitative and qualitative testis evaluation

Regarding the key testicular parameters quantitatively evaluated, in comparison to the controls at 40 days of age, PTU-treated rats showed reduced ($p < 0.05$) TW, tubular diameter and seminiferous tubule volume. In contrast, the results obtained for several parameters such as the number of SCs per testis and testis gram, the number of apoptotic germ cells per ST cross-sections (Supplementary Fig. 1, see section on supplementary data given at the end of this article) and the transition region individual length were increased ($p < 0.05$)

Table 1 Biometric and morphometric data from control and PTU-treated Wistar rats at 40 days of age (mean ± S.E.M.).

Parameter	Control ($n=8$)	PTU ($n=8$)
Body weight (g)	159 ± 4	116 ± 2*
Testis weight (mg)	702 ± 53	471 ± 40*
Gonadosomatic index (%)	0.88 ± 0.05	0.81 ± 0.11
Tubular diameter (μm)	266 ± 16	221 ± 7*
Percentage of seminiferous tubules (%)	90.5 ± 0.9	92.1 ± 1.4
Volume of seminiferous tubules (mL)	0.61 ± 0.06	0.42 ± 0.04*
Individual length of transition region (μm)	226 ± 9.8	260 ± 3.6*
Total length of seminiferous tubules (m)	11.1 ± 0.6	10.9 ± 0.6
Sertoli cell per testis ($\times 10^6$)	34.2 ± 0.6	39.9 ± 0.9*
Sertoli cell/gram/testis ($\times 10^6$)	53.5 ± 6.4	91.3 ± 3.5*
Round spermatids per Sertoli cell	5.8 ± 0.6	4.9 ± 0.4
Apoptotic germ cells per tubular cross-section	0.8 ± 0.2	1.8 ± 0.3*

*Statistically significant ($P<0.05$).

in treated animals. Also, at this age, no significant differences were observed for the gonadosomatic index, the ST total length and the number of round spermatids per SC (Table 1). However, the qualitative evaluation showed that, at the end of treatment, spermatogenesis was delayed in treated rats (Fig. 4G and H).

Confirming that the treated rats recovered totally from the hypothyroidism by the end of the experimental period, the data found for most parameters evaluated were similar in both groups at 160 days of age (Table 2). However, the results obtained for several very important parameters related to sperm production, such as TW, the ST volume, the ST total length and SCs per testis, were significantly higher ($P<0.05$) in rats treated with PTU, which reflected a much higher DSP per testis (~30%) in these animals (Table 2). As a result of similar BW and augmented TW, the GSI was increased ($P<0.05$) in PTU-treated rats (Table 2). Histologically, no differences were observed in the testicular parenchyma, including the transition region in both adult groups evaluated (Fig. 4). It is worth mentioning that at both 40 and 160 days of age, the number of SCs was increased significantly ($P<0.05$) in the treated males compared to the control rats.

Immunostaining evaluation

To assess the potential effects of transient hypothyroidism on SCs proliferation the number of KI67 staining cells were evaluated (Fig. 5). SCs in the transition region showed nearly 5-fold greater KI67 positive SCs in the PTU-treated compared to controls (18.4% versus 3.7%, respectively). However, on day 160, there was no difference between the groups in SC proliferation (Fig. 5). Most importantly, seminiferous tubule areas outside the TR, in both controls and PTU-treated testes, showed no proliferative SCs at 21, 40 or 160 days of age (Supplementary Fig. 2A, B, C, D and E). To estimate the number of differentiated SCs in the transition region, the number of SCs with P21 labeling were assessed (Fig. 6). On day 40, at the end of the treatment period, TRSCs in the PTU-treated males had a significant reduction in P21 staining (80.9%) compared to controls (88.3%), indicating that a higher population of immature SCs was present in the treated group. However, on day 160, there was no difference in SC differentiation, with both groups showing approximately 90% labeling for P21 (Fig. 6). To confirm the specific labeling of SCs in the transition region, SOX9, a specific marker for SCs, was used in

Table 2 Biometric and morphometric data from control and PTU-treated Wistar rats at 160 days of age (mean ± S.E.M.).

Parameter	Control ($n=8$)	PTU ($n=8$)
Body weight (g)	431 ± 3	440 ± 3
Testis weight (g)	1.58 ± 0.03	1.81 ± 0.03*
Gonadosomatic index (%)	0.73 ± 0.02	0.82 ± 0.02*
Tubular diameter (μm)	334 ± 5	336 ± 2
Percentage of seminiferous tubules (%)	89.6 ± 1.4	89.5 ± 0.7
Volume of seminiferous tubules (mL)	1.32 ± 0.02	1.51 ± 0.02*
Individual length of transition region (μm)	317 ± 13	329 ± 15
Total length of seminiferous tubules (m)	15.2 ± 0.4	17.1 ± 0.3*
Sertoli cell per testis ($\times 10^6$)	36.5 ± 1.5	42.7 ± 1.8*
Sertoli cell/gram/testis ($\times 10^6$)	24.7 ± 0.9	25.2 ± 0.9
Round spermatids per Sertoli cell	9.2 ± 0.3	10.0 ± 0.3
Apoptotic germ cells per tubular cross-section	1.0 ± 0.2	1.0 ± 0.3
Daily sperm production per testis ($\times 10^6$)	25.6 ± 1.1	32.8 ± 1.2*
Daily sperm production/gram/testis ($\times 10^6$)	17.3 ± 0.6	19.4 ± 0.6

*Statistically significant ($P<0.05$).
N = number of rats.

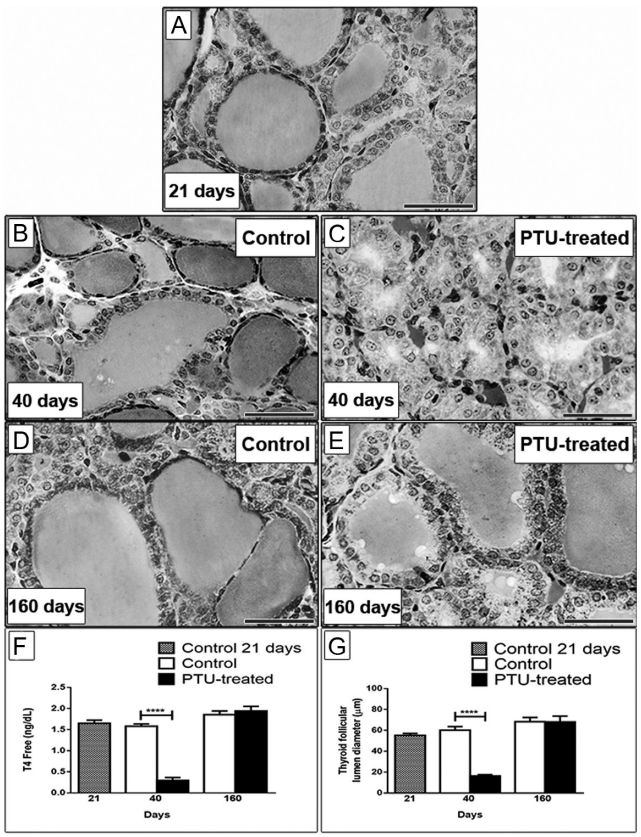

Figure 3 Free T4 blood plasma levels and thyroid gland histomorphometric evaluation (A, B, C, D, E, F and G). In 21-day-old rats, thyroid glands exhibited characteristic histology, having variable-sized follicles filled with thyroid colloid (A). At the end of PTU treatment (on day 40), thyroid gland follicles in controls (B) remained filled with colloid, but in treated rats the follicles differed significantly, as most follicles had no colloid (C). At the end of the experimental period (on day 160), the thyroid gland histology was normal in both groups (D and E). T4 levels (F) and thyroid follicular lumen diameters (G) were significantly reduced (Student's *t*-test, $P < 0.05$) in treated rats only on day 40. Bar: 50 µm.

double-staining with KI67, the marker for proliferation (Fig. 7).

Discussion

The present study uncovered two major findings regarding Sertoli cell proliferation in the rat: (a) transient hypothyroidism during the prepubertal period (21–40 days of age) induced SC proliferation by day 160 and (b) this stimulation of SC proliferation occurred only in the transitional regions, which are the terminal ends of the seminiferous tubule, connecting to the rete testis (Perey *et al.* 1961, Hermo & Dworkin 1988). Previous studies concluded that to increase testis weight, transient hypothyroidism could not begin after day 8 post birth, as treatment during the prepubertal period (days 24–48) had no effect on testis weight by day 90 (Cooke *et al.* 1992). However, based on data from the present study,

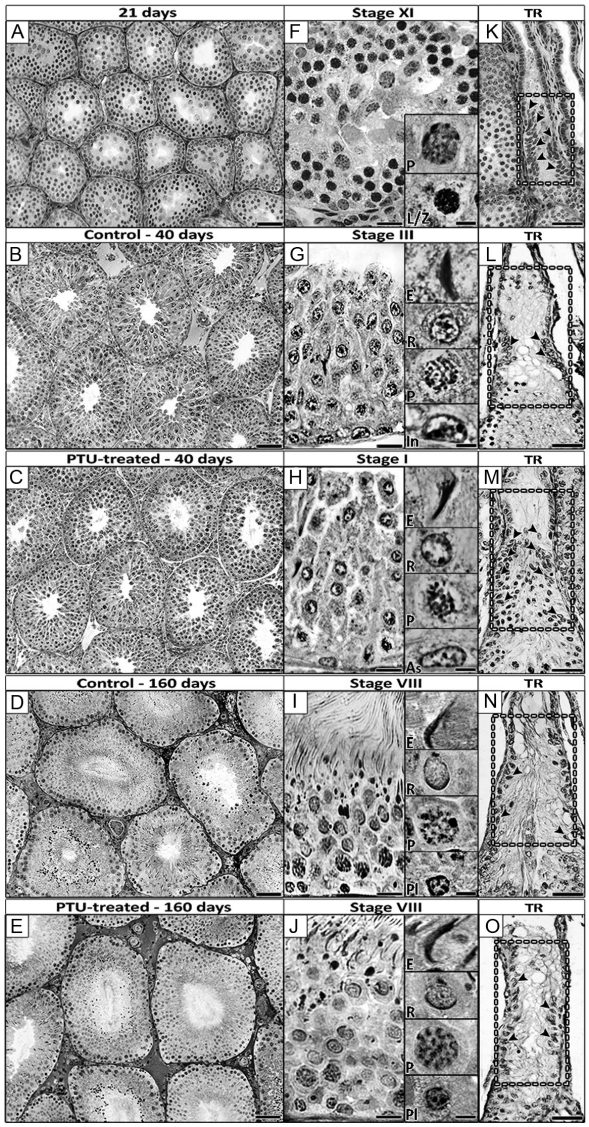

Figure 4 Histological appearance of the seminiferous tubules (ST) at different magnifications (A, B, C, D, E, F, G, H, I and J) and its transition region (K, L, M, N and O) in all three investigated ages. The inserts represent the most advanced germ cell type/germ cells association. At 21 days of age, the ST diameters were much smaller (A) and pachytene spermatocyte were in association with leptotene/zygotene cells (F), presumably in stage XI of the seminiferous epithelium cycle (SEC). On day 40, in comparison with treated rats (C) and as expected, spermatogenesis was more advanced in the control testis (G), in which elongated spermatids in stage III of the SEC were present, and the ST diameters were slightly larger (B). At 160 days, spermatogenesis was complete in both groups and all cellular associations were similar in control (D and I) and treated rats (E and J). In the transition region (dotted white line rectangle), Sertoli cell (black arrowheads) density in 21-day-old control rats (K) was similar to that seen in the 40-day-old PTU-treated rats (M), whereas the controls showed a reduced density on day 40 (L). As, type A spermatogonia; E, elongated spermatids; In, type intermediate spermatogonia; L/Z, leptotene in transition to zygotene spermatocytes; P, pachytene spermatocytes; PI, pre-leptotene spermatocytes; R, round spermatids. Bar: 50 µm (A, B, C, D and E; K, L, M, N and O); 20 µm (F, G, H, I and J); 5 µm (Inserts).

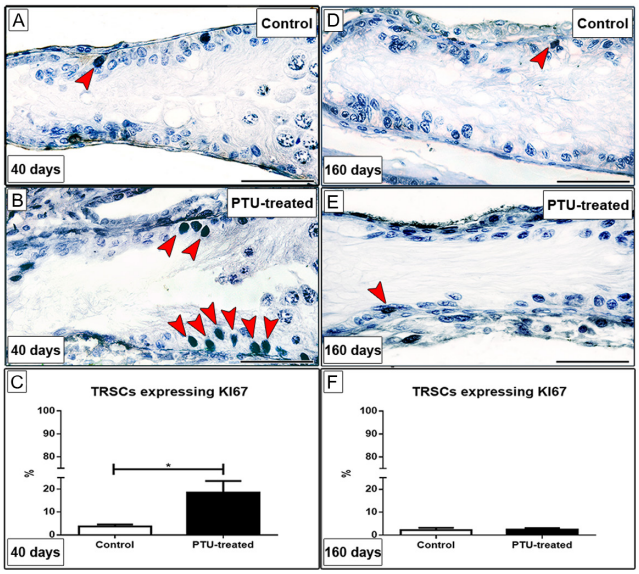

Figure 5 Immunostaining for proliferation of transition region Sertoli cells (TRSCs), using anti-KI67, in control and PTU-treated rats on days 40 (A, B and C) and 160 (D, E and F). The number of mitotic SCs (red arrowheads) was approximately five-fold higher (Student's *t*-test, $P<0.05$) at the end of PTU treatment (C). In adult rats, proliferation was quite low and similar in both groups (F). Bar: 50 µm.

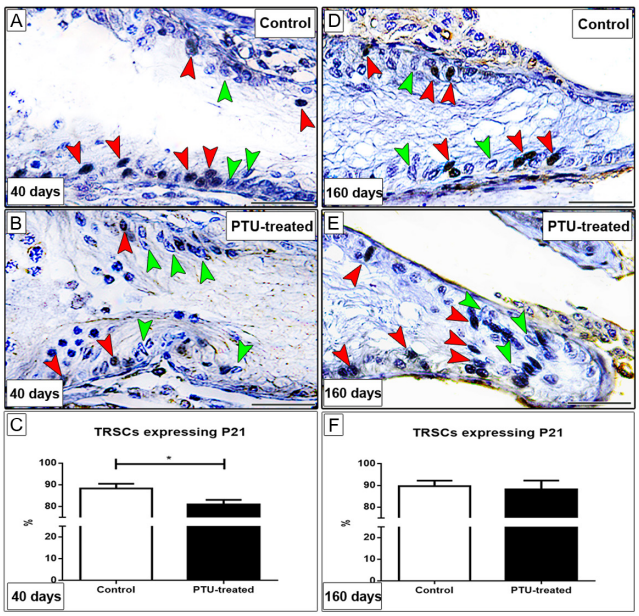

Figure 6 Immunostaining for differentiation of transition region Sertoli cells (TRSCs), using anti-P21 in control and PTU-treated rats on days 40 (A, B and C) and 160 (D, E and F). The number of mature SCs (red arrowheads) was significantly lower (Student's *t*-test, $P<0.05$) at the end of PTU treatment (C). In adult rats, the number of SCs expressing this cell-cycle inhibitor was approximately 90% and similar in both experimental groups. Bar: 50 µm.

it is possible that the previous study would have found an increase, if the study had observed weights out to day 160. Transient neonatal PTU treatment from birth to day 25 showed continuous increases in testis weight and DSP from day 90 to 160 (Cooke *et al.* 1991).

In comparison to former studies involving PTU treatment (Cooke *et al.* 1991, 1992, Joyce *et al.* 1993), low dosages of PTU (<0.1% w/v) induce faster testis maturation due to the more rapid return to euthyroidic status, which is critical to enhance SC proliferation and testis growth (Cooke *et al.* 1993, Rijntjes *et al.* 2017). Cooke and colleagues (1993) previously demonstrated that PTU doses, ranging from 0.006 to 0.1% (w/v), are quite effective in significantly suppressing T4 concentrations. The 0.03% (w/v) PTU dose administered in the present work was 5-fold higher than the lowest effective dose used previously (Cooke *et al.* 1993) and treatment was direct via water in the present study, versus the mother's milk in the Cooke study. Efficacy of the 0.03% (w/v) PTU dosage was confirmed as a sensitive method to increase testis mass by the observed reduction in thyroxin levels at the end of the treatment period. Just after PTU treatment, although an initial reduction in testis mass was observed, the number of Sertoli cells per testis was increased at day 40. Similar findings were obtained by Rijntjes *et al.* (2009), treating dams with an iodide-poor diet and evaluating pups that maintained the hypothyroid condition until euthanasia. In PTU-treated rats evaluated on day 160, the increased testis size and function are attributed to the timing of return to the euthyroid condition and also to the lower dosage of PTU which would allow more rapid maturation of the testis, due to a more rapid return to euthyroidism after cessation of treatment (Cooke *et al.* 1993, Rijntjes *et al.* 2017).

The transition region is only a small area of the seminiferous tubule terminal ends, but the epithelium is packed with modified, immature SCs that are mitotically active even in the adult (Hermo & Dworkin 1988, Figueiredo *et al.* 2016, Kulibin & Malolina 2016, Malolina & Kulibin 2017). Early studies on the proliferation of rat SCs concluded that SC division stopped between day 16 and 21 post birth (Orth 1982, Van Haaster *et al.* 1993). This supposition was of course correct, but only if the observations were made within the parenchyma or body of the testis, which would occur in routine sectioning for histology, as it has always been common to use a midline, transverse plane of section. However, in rodents, the exit of the transition region and *rete testis* does not occur in the mid-region, but rather leaves the testis off center and more cephalic (La *et al.* 2012, Ford *et al.* 2014, Nakata 2019), requiring specific and careful sectioning to study. Thus, the discovery that SCs in this region are capable of proliferation was rather significant, in light of the past and present studies of transient hypothyroidism, as only in this unique region were SCs found to be proliferating in adult testes, in both controls and treated males, but significantly increased after PTU treatment.

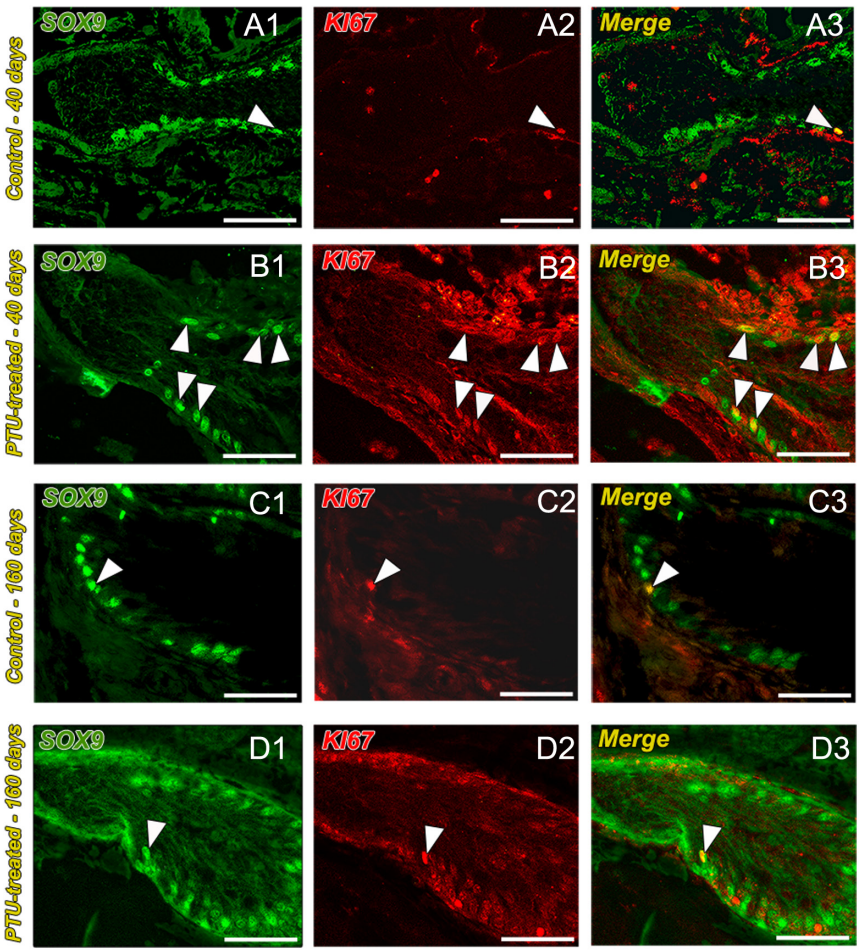

Figure 7 Double immunofluorescence staining for SOX9, a Sertoli cell marker (A1-D1) and KI67, a proliferation marker (A2-D2) in the transition region of control and PTU-treated rats on days 40 (A1-B3) and 160 (C1-D3). The number of mitotic Sertoli cells (SCs; white arrowheads) were noticeably increased (B3) at the end of PTU treatment. In adult rats, few SCs (blue arrowheads) were seen proliferating in both experimental groups (C3, D3). Bar: 50 µm.

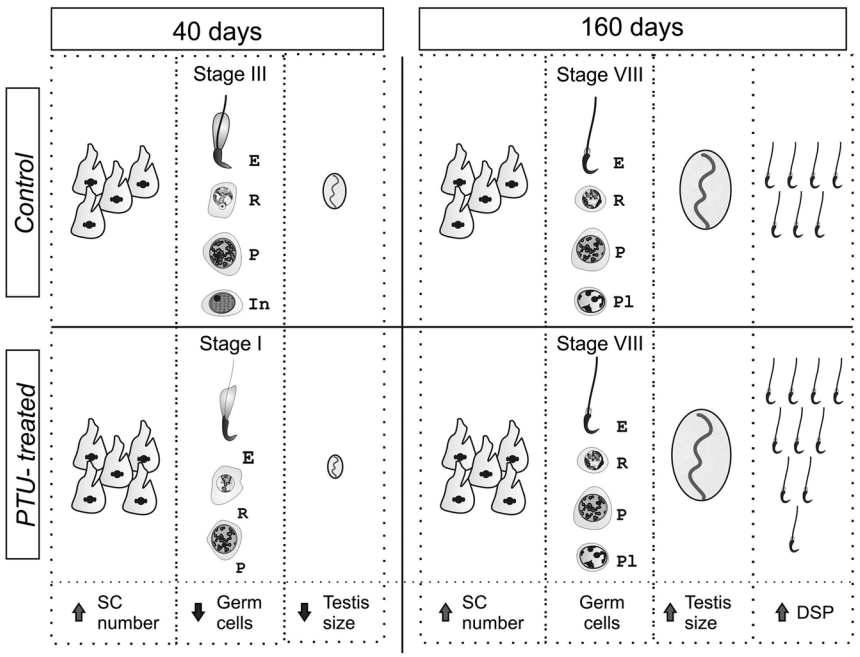

Figure 8 Schematic illustration summarizing the main results. On day 40, although delayed spermatogenesis was observed in PTU-treated rats, in comparison to the control group, the number of Sertoli cells (SC) was significantly higher. On day 160, all the key testis parameters evaluated, such as testis weight, SC number and the daily sperm production per testis (DSP), were significantly higher in rats that underwent the PTU treatment.

This study corroborates our recent data showing that TRSCs have considerable mitotic activity from 36 to 120 days of age, even in control rats (Figueiredo *et al.* 2016). It is interesting that SCs away from the transition region did not show evidence of proliferation in adult rats, but in normal men, after gonadotropin suppression and in tubules from men with seminoma, SCs do show an induced capacity to proliferate (Tarulli *et al.* 2012, 2013). However, the major difference between the present study in rats and those showing SC proliferation in men is the immature status of SCs in the transition region only in adult rats (Figueiredo *et al.* 2016), with or without the PTU treatment, while in men SCs in the testis parenchyma appear not to be terminally differentiated and thus are capable of responding to gonadotropin suppression with de-differentiation and proliferation (Tarulli *et al.* 2012, 2013). TW and sperm production increase markedly in pigs after puberty (around four months and extending up to four years of age), without any significant increase in tubular diameter and SC efficiency (Swierstra 1973, França *et al.* 2000, Avelar *et al.* 2010), which suggests that testicular growth after puberty may involve primarily the terminal regions of the seminiferous tubules. The importance of SC plasticity in the transition region of men and large mammals remains to be determined.

In conclusion, based on the already known proliferative capacity of TRSCs (Figueiredo *et al.* 2016, Kulibin & Malolina 2016) and using prepubertal PTU treatment, we were able to significantly increase several key testis parameters related to sperm production in adult rats such as TW, SCs number and DSP (Fig. 8). Thus, the transition region is a unique testis site for investigating both spermatogonial niche formation (Aiyama *et al.* 2015), as well as SC proliferation in the adult male, the potential interactions and regulation of these two niche cell populations. Possible implications of these findings for clinical and therapeutic applications in male infertility should be investigated.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0127.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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5. DISCUSSÃO

O primeiro artigo representa a quebra de um paradigma estabelecido na literatura por várias décadas, segundo o qual a proliferação de SCs cessaria antes da puberdade, particularmente entre 15-21 dias de idade em ratos (Steinberger & Steinberger, 1971; Russell & Clermont, 1977; Orth, 1982; Almirón & Chemes, 1988; Matthiesson *et al*., 2006). Assim, o presente estudo é o primeiro a reportar que a proliferação das SCs se estende até a idade adulta. No entanto, esta proliferação ocorre somente na TR. Este importante achado contribui para explicar o aumento em tamanho do testículo e da produção espermática após a puberdade em algumas espécies já investigadas acarretando significativo aumento no comprimento dos ST, sem contudo alterar o diâmetro tubular e a eficiência das SCs (Swierstra, 1973; França *et al*., 2000; Avelar *et al*., 2010). Portanto, estas TRSCs permitem um contínuo, embora lento, crescimento tubular, constituindo em tese uma população de SCs progenitoras.

Em nosso estudo, as SCs situadas ao longo dos ST expressaram marcadores clássicos de diferenciação (GATA-4, p27 e AR), enquanto apenas na TR foram observadas SCs expressando marcadores de proliferação (Ki-67 e BrdU). Ademais, corroborando o fato de exibirem características indiferenciadas, uma subpopulação de SCs na TR não expressa tais marcadores de diferenciação. Conforme é sabido, os THs são considerados os principais reguladores da diferenciação das SCs (Orth, 1984; Cooke *et al*., 1994; França *et al*., 2000; Holsberger *et al*., 2003; Tarulli *et al*., 2012). Mais especificamente, os THs induzem a expressão de p21 e p27 - que atuam inibindo a função dos complexos de quinases dependentes de ciclina (Cdks), que por sua vez levam à progressão do ciclo celular (Van Haaster *et al*., 1993; Arambepola *et al*., 1998; Cooke *et al*., 2005; Holsberger & Cooke, 2005), bem como a expressão de AR no parênquima testicular (Arambepola *et al*., 1998; Hazra *et al*., 2013). Tendo em vista que o aumento da expressão de AR nas SCs é associado com a cessação geral do período proliferativo, a ocorrência de SCs negativas para AR e p27 na TR corrobora a presença de uma subpopulação de SCs indiferenciadas nesta região.

Com base nos importantes resultados obtidos no primeiro artigo publicado, investigamos se as TRSCs seriam ainda responsivas à regulação por THs por um tempo mais prolongado. Portanto, no segundo artigo procuramos avaliar os efeitos do hipotireoidismo transiente induzido por tratamento com PTU na proliferação das TRSCs. Para tal finalidade e diferentemente de outros trabalhos da literatura (Cooke *et al*., 1991, 1992, 1993; Hess *et al*., 1993; Joyce *et al*., 1993), iniciamos o tratamento três semanas após o nascimento, ou seja, na fase pré-púbere. Assim, o tratamento nesse período reforçaria a atividade proliferativa das TRSCs e comprovaríamos que os possíveis efeitos testiculares seriam exclusivamente decorrentes das TRSCs. Conforme hipotetizado, os resultados obtidos mostraram que o hipotireoidismo induzido durante o período pré-púbere foi capaz de aumentar significativamente a proliferação das TRSCs nos ratos Wistar tratados, sendo responsável, portanto, pelo aumento significativo encontrado ao final do tratamento para o número total de SCs por testículo, bem como para o tamanho testicular e da produção espermática diária observados nos ratos Wistar adultos.

Além de sua relevância biológica *per se*, estes sólidos resultados alteram o conceito estabelecido em estudos prévios, segundo o qual para se promover aumento testicular o hipotireoidismo transiente deveria ser implementado no período neonatal (Cooke *et al*., 1992). Já é sabido que o aumento do peso testicular e da proliferação das SCs depende do tempo de retorno ao estado eutireoideo, sendo maximizado quanto mais rápido for este retorno (Cooke *et al*., 1993; Rijntjes *et al*., 2017). O fato de termos utilizado uma dose de PTU relativamente baixa (0,03% p/v; comumente é empregada a dose de 0,1% p/v) permitiu maturação testicular mais acelerada devido ao retorno também mais rápido ao estado eutireoideo. De acordo com a literatura (Cooke *et al*., 1993), doses de PTU tendo como mínimo 0,006% p/v são bastante efetivas em suprimir as concentrações de T4. Portanto, a dose de PTU usada no presente estudo se mostrou adequada para proporcionar os efeitos por nós desejados no que se refere à dinâmica proliferativa das TRSCs.

A evidenciação de que a TR abriga uma população de SCs indiferenciadas e proliferativas torna-se bastante relevante pelo fato desta pequena área dos ST corresponder à única região testicular em que SCs proliferam em testículos de animais adultos (Hermo & Dworkin, 1988; Figueiredo *et al*., 2016; Kulibin & Malolina, 2016; Malolina & Kulibin, 2017). Neste importante cenário, a TR emerge como uma região singular onde os ST podem ser originalmente formados (Malolina & Kulibin, 2017) e por onde continuam a crescer ao longo do desenvolvimento pós-natal, tornando-se bastante atrativa para se investigar tanto a formação de nicho espermatogonial (Aiyama *et al*., 2015), quanto a proliferação de SCs em machos adultos e suas possíveis interações. Ademais, não seria totalmente descabido se especular que este pool de células somáticas da TR possa eventualmente contribuir para a repopulação de SCs após injúria testicular, quando novas SCs seriam eventualmente necessárias.

6. CONCLUSÕES

A partir dos resultados obtidos no presente estudo, em que foi realizada a investigação morfofuncional das TRSCs e dos efeitos testiculares do hipotireoidismo transiente nessas células, as seguintes conclusões podem ser ressaltadas:

- Diferentemente do paradigma até então estabelecido na literatura, segundo o qual as SCs não mais proliferariam após a terceira semana pós-parto em ratos, existe uma subpopulação de SCs localizadas na TR dos ST apresentando distinto fenótipo funcional, que retém o potencial proliferativo mesmo em indivíduos adultos;

- Baseado na capacidade proliferativa de TRSCs e utilizando-se o tratamento com PTU na fase pré-púbere, é possível aumentar significativamente diversos parâmetros testiculares correlacionados em indivíduos adultos, tais como peso testicular, número total de SCs por testículo e produção espermática.

- As SCs imaturas da TR representam uma subpopulação progenitora de SCs capaz de estimular o crescimento em comprimento dos ST, emergindo como um importante pool de SCs responsável pela maximização da função testicular em animais adultos.

7. PERSPECTIVAS

Os resultados apresentados neste estudo propiciam uma nova perspectiva acerca do crescimento testicular e da progressão da espermatogênese, onde a TR representa uma atrativa região para se investigar a formação dos nichos espermatogoniais, bem como suas potenciais interações com o parênquima testicular circundante. Neste contexto, diversas questões podem ser levantadas em abordagens futuras, como por exemplo: i) em situações de injúria testicular, desencadeadas por agentes químicos tóxicos ou inflamatórios, seria potencialmente a TR o local por onde o acometimento inicial ocorre? ii) Como se apresenta a barreira de células de Sertoli/hematotesticular na TR dada a presença de SCs imaturas? iii) Seria a TR uma área vulnerável para o escape de auto-antígenos diante situações não homeostáticas? iv) A formação de novos nichos de células-tronco germinativas estaria correlacionada com as divisões de SCs em locais específicos da TR? v) As SCs localizadas em outras áreas dos ST se desdiferenciariam caso colocadas em um microambiente que mimetizasse as condições da TR? vi) Quais fatores estariam envolvidos na manutenção de SCs imaturas? vii) Os tumores testiculares tais como sertoliomas e seminomas poderiam ser iniciados nesta região específica do parênquima testicular?

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9. ANEXOS

- **Revisão em preparação:**

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INSIGHTS INTO TRANSITION REGION OF THE SEMINIFEROUS TUBULES

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Abstract

The transition region corresponds to the segment of the seminiferous tubules that connects to the *rete testis*. The present review raised refined data at the cellular, histochemical and molecular levels regarding this very particular region of testicular parenchyma, as well as its potential functions for testis physiology. The transition region stands out as a niche for both undifferentiated Sertoli and spermatogonial cells and as the site where seminiferous tubules are originally formed and continue to grow into adulthood. In addition, the transition region exhibits particular interactions with immune system cells. In this context, the homeostatic micromilieu in the transition region is fundamental for normal testis function. Several morphofunctional aspects of transition region are discussed in this review, including the particular subset of undifferentiated Sertoli cells, the dynamics of Sertoli cell proliferation and differentiation, the blood-testis-barrier features, the immunological interaction among cells and the comparative morphology among species. The concepts and information herein presented may stimulate further investigations over the transition region and the development of new medical approaches for treatments of infertility and other disorders of testis function.

1. Introduction

The transition region (TR) is a specific area of the testis that corresponds to the junction between the seminiferous tubules (ST) and the *rete testis* (Fig. 1) (Perey *et al*., 1961). This tubular segment exhibits a seminiferous epithelial lining composed of modified Sertoli cells (SCs) (Dym, 1974; Osman 1978, 1979; Osman and Plöen, 1978, 1979; Nykänen, 1979; Lindner & Holstein, 1982; Wrobel *et al*., 1986; Hermo and Dworkin, 1988). Germinative cells are rarely noticed and the germ cell population mainly consists of single spermatogonia and eventual germ cells in early spermatogenic stages (Fig. 1) (Dym, 1974; Nykänen, 1979; Hermo and Dworkin, 1988; Aiyama *et al*., 2015).

Recently, beyond the available morphological descriptive data, several ultrastructural, molecular and functional studies have brought new evidences that this particular testicular area has important and specific functions for testis physiology (Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016; Malolina and Kulibin, 2017). Among them, it can be cited the establishment and maintenance of undifferentiated Sertoli and Spermatogonial cells, the growing of ST and the particular interaction with the immune system (Takahashi *et al*., 2007; Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016; Malolina and Kulibin, 2017; Nagasawa *et al*., 2018; Figueiredo *et al*., 2019). The detailed understanding on the TR area may provide new concepts related to the testis germ and somatic cells functions, as well as their regulation and interactions, allowing the development of new physiological approaches that could help to deal with specific

fertility problems. Based on this scenario, the present review updates the knowledge of the transition region, providing new data at the cellular, histochemical and molecular levels. Furthermore, the balance of cell differentiation and proliferation, the BTB, the immunological aspects and a brief comparative morphology of this region among animals are herein discussed.

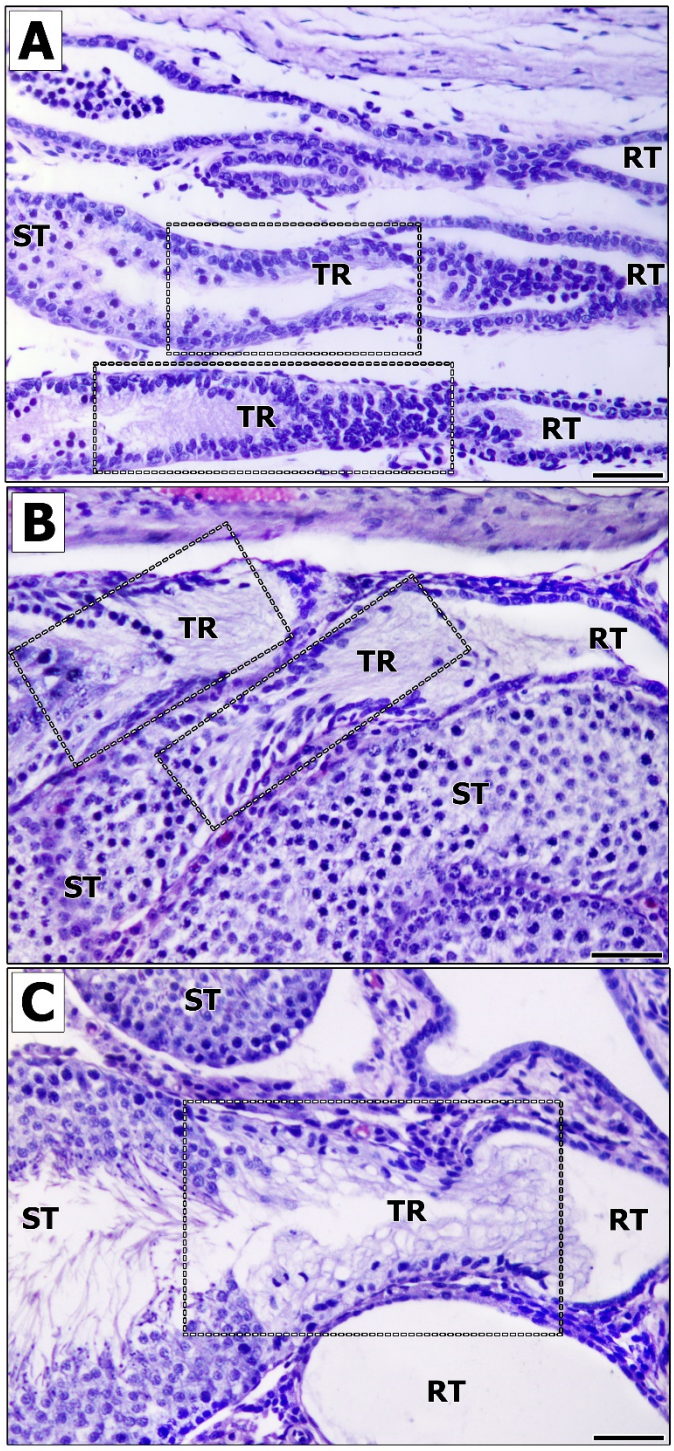

Figure 1. Histological images of development in the transition region of Wistar rats at early prepubertal (A), late prepubertal (B) and adult (C) stages. This area is composed by a particular subset of Sertoli cells (TRSCs), which project distally forming a characteristic valve-like structure. At

early stages of development, the TR (dotted white line rectangle) epithelium is composed by high density of packed TRSCs confined in a very small area. As sexual maturation occurs, there is a gradual reduction in TR cell density as the TR enlarges both in length and diameter. Bar: 50μm.

2. TRSC structural morphology

The SCs of the TR (TRSCs) are modified in a distinct manner, differing from the SCs of the ST (Schulze, 1974; Fawcett, 1975). The TRSCs are columnar and present an oval-shaped nuclei, smaller nucleolus, more peripheral heterochromatin and commonly does not show indentations of the nuclear envelope (Dym, 1974; Osman, 1978; Nykänen, 1979; Osman, 1980; Lindner & Holstein, 1982; Hermo and Dworkin, 1988). These modified SCs project distally into the *rete testis*, forming a structure which resembles a valve, with narrow lumen and thicken lamina propria (Fig. 1), that functions to prevent a reflux of spermatozoa and *rete testis* fluid entering into the tubular lumen under normal conditions (Roosen-Runge, 1961; Lindner & Holstein, 1982). The expressive narrowed lumen may exert an increased luminal pressure in this area, resulting in structural strengthening of the TR basement membrane (Hermo and Dworkin, 1988). Interestingly, while the TR epithelium present high density of TRSCs in a very small area in prepubertal rats, a gradual reduction in their density are observed in adult animals (Fig 1). This phenomenon may be explained by the ST growing and enlargement.

The apical region of TRSCs exhibits many cytoplasmic inclusions, considerable amount of lipid droplets and concentrically arranged membrane formations of endoplasmic reticulum (Dym, 1974; Osman, 1978; Nykänen, 1979; Lindner & Holstein, 1982). Moreover, increased number of microfilaments and microtubules is dispersed throughout the cytoplasm but preferentially abundant in the apical regions (Osman and Plöen, 1978; Lindner & Holstein, 1982; Wrobel *et al*., 1982). TRSCs commonly lack tight junctions (black arrows) in the SC ectoplasmic specialization (Fig. 2).

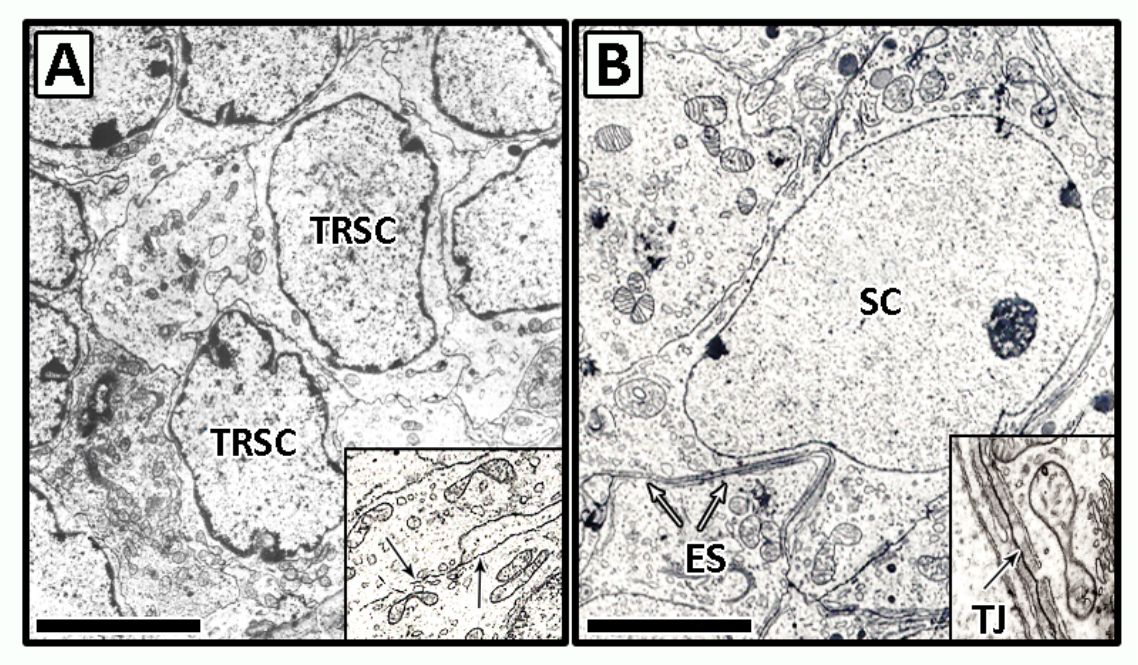

Figure 2. Transmission electron micrographs of Sertoli cells located in the transition region (TR) (A) and in other seminiferous tubules area (B) from a prepubertal Wistar rat. Sertoli cells located in the TR (A), in comparison to more differentiated Sertoli cells from other areas of seminiferous tubules (B), do not present conspicuous nucleolus and tight junctions (black arrows) in their ectoplasmic specialization (white arrows). Observe that Sertoli cells from the TR exhibit a more immature phenotype such as, for instance, peripheral nuclear heterochromatin. ES: ectoplasmic specialization; SC: Sertoli cell; TRSC: transition region Sertoli cell; TJ: tight junctions. Bar: 5µm.

There is a thickening of the tunica propria (observed by the PAS staining), which consists of a multilayered basal lamina and peritubular myoid cells irregularly dispersed among thick bundles of collagen fibrils (Fig. 3) (Nykänen, 1979; Wrobel *et al*., 1979; Nykänen, 1980; Lindner & Holstein, 1982; Tainosho *et al*., 2011). The basement membrane complex provide support to the overlapping TRSCs (Hermo and Dworkin, 1988). It is also mentioned that the multilayered basal lamina might control the opening/closing of the valve-like structure of the TR (Tainosho *et al*., 2011).

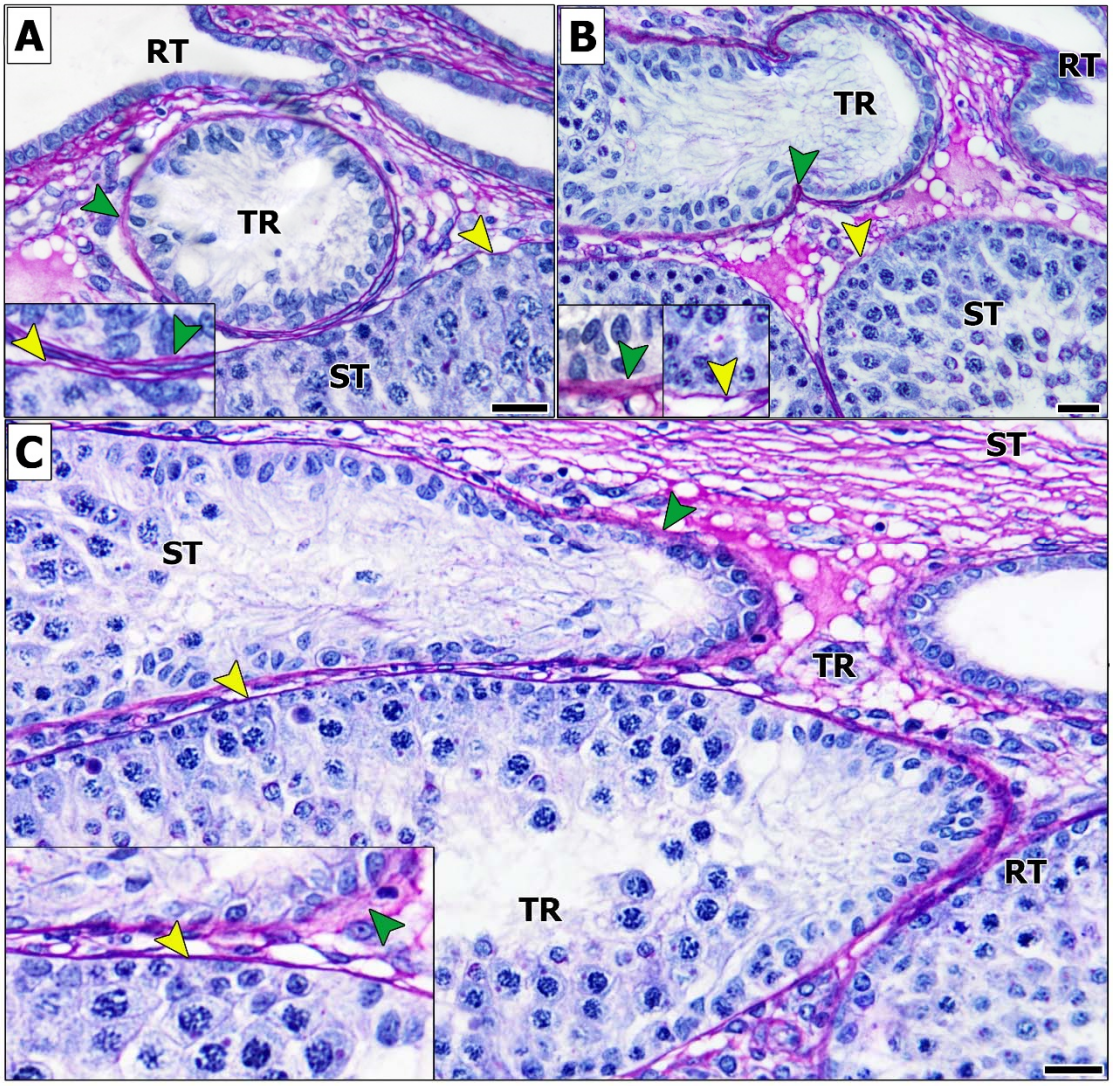

Figure 3. Periodic acid-Shiff (PAS) staining in testis from a prepubertal Wistar rat. Transversal (A) and longitudinal (B-C) sections of the transition region (TR) are represented. Note the presence of a thick tunica propria PAS-positive (green arrowheads) in the transition region (TR) in comparison to a thin tunica propria (yellow arrowheads) in other areas of seminiferous tubules (ST). The thick tunica propria may facilitate opening/closing of the valve-like structure of the TR. RT: *rete testis*. Bar: 20µm.

3. Histochemical and molecular characteristics

Histochemical differences have also been demonstrated between the ST and the TR. Osman (1984) reported a higher concentration of alkaline phosphatase and glycogen in the TR, and speculated that it could be associated to a high energy storage since no full spermatogenesis occurs in this area or to a secretion of fluid rich in carbohydrate for the passing spermatozoa. It is also mentioned that TRSCs present intense endocytic and phagocytotic activities involved in the regulation and modification of fluid composition as well as the selective elimination of abnormal spermatozoa in this testicular region

(Dym, 1974, 1976; Osman, 1978; Osman and Plöen, 1978; Osman, 1979; Sinowatz *et al*., 1979; Osman, 1980; Lindner & Holstein, 1982; Wrobel *et al*., 1982; Hermo and Dworkin, 1988). Amselgruber and colleagues (1994) later demonstrated a pronounced expression of S-100 protein in TRSCs that may act in the microtubule assembly-disassembly system, which would be associated to secretory and absorptive functions in the intratesticular excurrent duct system.

By means of immunofluorescence and qRT-PCR, it was shown that the TR harbors a population of SCs that express key transcriptional factors such as Wt1, Sox9 and Nr5a1, which are necessary and responsible for efficient reprogramming of mouse embryonic fibroblasts into Sertoli-like cells (Buganim *et al*., 2012). Currently, it is observed that a subpopulation of SCs are mitotically active within the TR of sexually mature laboratory rodent models (BrdU-, Cyclin-D1- and Ki-67-positive) (Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016; Figueiredo *et al*., 2019). These cells do not express typical differentiated SCs markers such as the androgen receptor (AR), the transcription factor GATA-4 and the cell cycle inhibitor p21 (Tarulli *et al*., 2013; Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016). Moreover, Malolina and Kulibin (2017) showed a temporal variation of the expression of Dmrt1 (a key factor for SC differentiation - Raymond *et al*., 2000; Minkina *et al*., 2014) in TRSCs, which could not be detected after the 25th day of mice post-natal development. All the data presented above corroborate the idea that adult SCs are not a homogeneous and terminally differentiated population as previously thought.

4. TRSC proliferative capacity

Unlike the SCs from ST that lose the capability to proliferate during the differentiation in the post-natal development (Cupp and Skinner, 2005), an extended proliferation of TRSCs have been demonstrated by various approaches *in vitro* and *in vivo* in sexually mature animals (Naito *et al*., 2009; Aiyama *et al*., 2015; Zakhidova and Marshak, 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016; Malolina and Kulibin, 2017; Figueiredo *et al*., 2019). Naito and colleagues (2009) showed that some TRSCs incorporated BrdU after experimental autoimmune orchitis induction. In culture, TRSCs exhibit high proliferative activity forming colonies of epithelial-like cells (Kulibin and Malolina, 2016). Under normal conditions, Aiyama *et al*. (2015) observed that some SCs proliferate (BrdU and Cyclin-D1 positive labelling) in TR during the active sexual period in adult hamsters, a seasonal species. Interestingly, some of the BrdU-positive TRSCs were able to move into the regions of ST with active spermatogenesis, contributing to the SCs population within the seminiferous epithelia. Lately, our group confirmed proliferation (BrdU and Ki-67 positive labelling) of a TRSCs subset in rats, a nonseasonal species and important laboratory model (Figueiredo *et al*., 2016). In this study,

proliferative SCs were observed without expressing AR. This observation is in accordance with the data collected in men after hormonal suppression. It was observed that the SC expression of Ki-67 and PCNA coincided with decreased expression of AR (Tarulli *et al*., 2013; Haverfield *et al*., 2015). Furthermore, only a small population of proliferative human SCs isolated from adult human testes is able to expand *in vitro* (Chui *et al*., 2011). Summing these data from different species, it can be said that a progenitor SCs subset exists specifically established in the TR.

Interesting information recently raised by Malolina and Kulibin (2017) is that TRSCs share a common origin with *rete testis* cells and both represent the source of actively proliferating somatic cells capable of forming ST under 3D culture conditions, suggesting that the TR represents the site where ST are originally formed. This data supports other speculations regarding the TR as the specific place where the ST continue to grow in sexually mature individuals (Tarulli *et al*., 2013; Aiyama *et al*., 2015; Figueiredo *et al*., 2016).

5. Blood-testis barrier and TRSC junctions

The BTB in the TR has not yet been well characterized so the available data were obtained from some descriptive morphological studies in a few mammalian species. It is reported that neighboring cells are commonly connected by desmosome-like junctions, whereas tight junctions and typical Sertoli-Sertoli junctions are not described in the TR (Osman, 1978; Lindner & Holstein, 1982; Wrobel *et al*., 1986; Hermo and Dworkin, 1988). Another distinctive feature compared to other SCs is the presence of luminal occlusion junctions (Fig. 4A-B, black arrows) (Nykänen, 1979; Osman *et al*., 1979; Hermo and Dworkin, 1988). In this regard, an ultrastructural study performed by our research group also revealed that TRSCs exhibit few actin filaments and endoplasmic reticulum cisternae. Furthermore, the poorly developed junction among TRSCs can be attributed to their immature phenotype (Fig. 4). A subluminal/apical junctional complex is absent between the connected apical portions of TRSCs, emerging lateral intercellular spaces where TRSCs might release their vesicular contents (Wrobel *et al*., 1982).

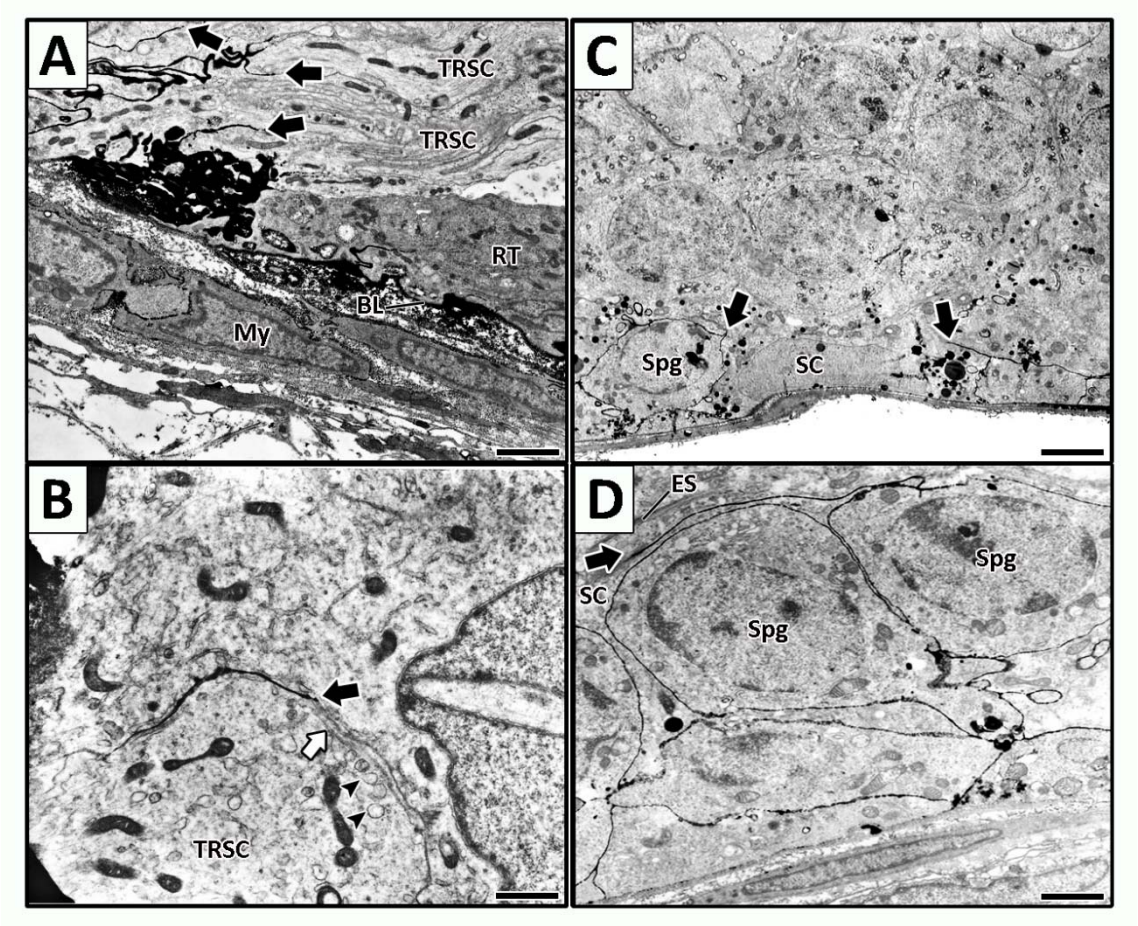

Figure 4. Ultrastructural evaluation of the Sertoli cell barrier in the transition region and along seminiferous tubules in adult Wistar rat. Using the lanthanum-tracer method, different patterns were observed for the transition region (TR) (A-B) and along the seminiferous tubules (ST) (C-D). A) Lanthanum passes through peritubular myoid cells, penetrates into the seminiferous epithelia basal lamina, then among Sertoli cells (SCs), and stops in the luminal occlusive junctions (black arrows) of the TR. B) High magnification of the lanthanum blockage by luminal occlusion junctions (black arrow), showing the presence of few actin filaments (white arrow) and few endoplasmic reticulum cisternae (black arrowheads), resembling an immature junction. C) Along other areas of ST, tight junctions among SCs interrupted lanthanum progression (black arrows) prior the adluminal portion of ST. D) High magnification of lanthanum blockage by basal occlusion junctions (black arrow) located within ectoplasmic specializations, preventing the entry of tracer into the adluminal compartment of ST. BL: basal lamina; ES: ectoplasmic specialization; My: peritubular myoid cell; RT: *rete testis*; SC: Sertoli cell; Spg: spermatogonial cell; ST: seminiferous tubules; TR: transition region; TRSC: transition region Sertoli cell. Bar: 5µm.

Corroborating these data and using hypertonic fixative technique as an experimental approach, our group observed that TRSCs normally protect the apical regions, while several basolateral damages are usually noticed along the TR (Fig. 5). However, artifacts also occurred in some apical portions but not in the lumen, corroborating that TRSCs have effective apical occlusive junctions. Probably, this particular localization of TRSCs occlusive junctions is related to distinct profile of gene expression and other unknown functions. These aspects might be further investigated in order to reach definitive conclusions regarding apical occlusive junction's activity.

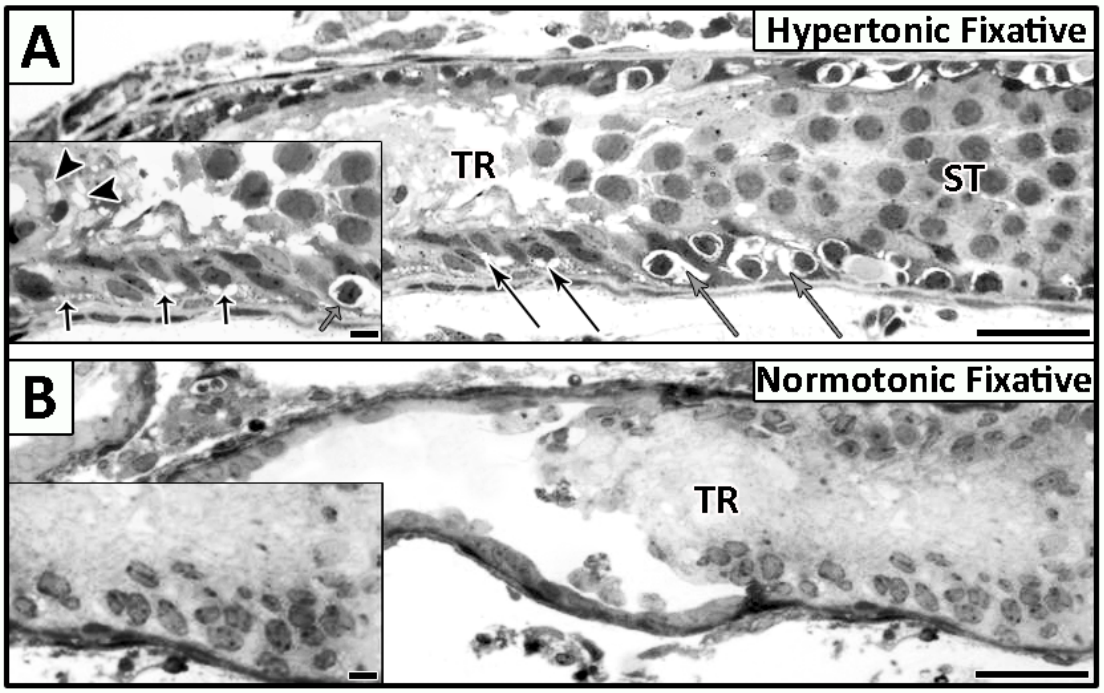

Figure 5. Evaluation of the transition region after hypertonic fixative exposition in prepubertal Wistar rat. A) The basal compartment of the seminiferous epithelium presents several artifacts caused by the hypertonic fixative both in transition region (TR; black arrows) and along the seminiferous tubules (ST; grey arrows). Artifacts are also seen in adluminal compartment only in the TR (black arrowheads). B) No artifacts are seen in the control testis using normotonic fixative. Inserts represent a high magnification of the TR epithelia. Bar: 50µm; Inserts: 10µm.

In general, the existence of typical wide gaps among TRSCs, the basal lamina of the epithelial layer and the myoid cell layer (Tainosho *et al*., 2011) would contribute to an altered SC/BTB in the TR. Differing from the ST, the basal lamina, which is a key element for a selective filtration barrier (Paulsson, 1992), exhibits distinct structure in the TR being wavy and multilayered. The idea of an altered BTB is corroborated by the fact that meiosis and spermiogenesis do not occur in this particular area, events that require a favorable micromilieu in the adluminal tubular compartment created by an

effective SC/BTB (Dym and Romrell, 1975; Wrobel *et al*., 1982). Besides that, alterations of the junctions between TRSCs are considered key factors for the occurrence of spermatogonia without contact with the basal lamina as usually seen in this region (Holstein and Hubmann, 1981; Wrobel *et al*., 1982). In men, these cells do not exhibit specialized intercellular junctions, although they display slender invaginations in a basolateral position, which may serve as anchoring devices in the epithelium and as increased exchange area among TR cells (Wrobel *et al*., 1982).

Furthermore, our research group have observed that TRSCs express less connexin 43 (Cx43) than SC from other ST areas in prepubertal and adult rats (Fig. 6). It is known that this gap junction protein plays an important role in SC development, being essential for the normal SC maturation and cessation of mitotic activity (Sridharan *et al*., 2007).

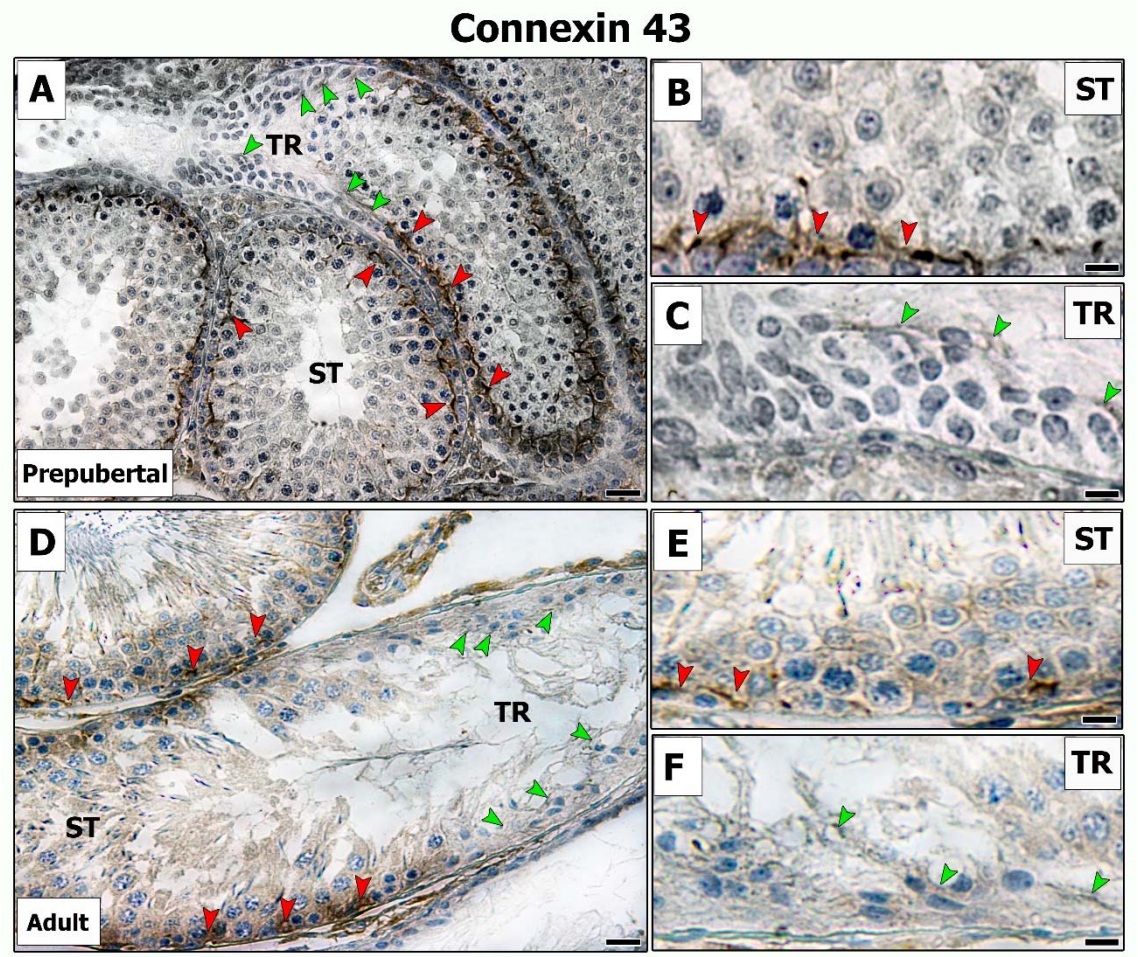

Figure 6. Connexin 43 (Cx43) immunostaining. The labeling was performed in the transition region (TR) and along the seminiferous tubules (ST) areas in prepubertal (A-C) and adult (D-F) Wistar rats. Note that Sertoli cells (SCs) located in the TR (C, F) do not typically express Cx43 near the basal compartment (green arrowheads) as it is intensely observed in SCs along the ST (red arrowheads). Bar: 50µm (A, D); 10µm (B-C; E-F).

Interestingly, in some cases of infertility and testicular cancer, disruption in the hormonal microenvironment is reported especially related to the androgenic signaling. This loss results in disruption of BTB functionality, disorganization of junctions (claudin-11, ZO-1), downregulation of Cx43 and the presence of SCs exhibiting immature characteristics (Brehm *et al*., 2002; Maga and Hubscher, 2003; Roger *et al*., 2004; Brehm *et al*., 2006; Fink *et al*., 2006), generally resembling the aspects of TR.

6. Immunological aspects of TR

Immunologically, in comparison with other regions of ST, a higher prevalence of immune cells, such as lymphocytes and macrophages, is observed surrounding the TR (Itoh *et al*., 1995, 1999; Takahashi *et al*., 2007). The observation that more lymphocytes are present around TR suggests that this specific area may be a place where autoreactive lymphocytes might eventually access germ cell antigens, especially considering the indications that the BTB in the TR is functionally distinct (Osman, 1984; Tainosho *et al*., 2011). In this regard, systemic immunoglobulins and exogenously administered bloodborne horseradish peroxidase have been shown to infiltrate into the TR and the *rete testis* but not into the ST (Johnson, 1970; Koskimies *et al*., 1971; Tung *et al*., 1971; Itoh *et al*., 1998), demonstrating that the BTB in the TR is definitely distinct compared with other testicular regions.

Other important clue is that, although rarely seen, few macrophages and lymphocytes are observed into the TR epithelia in normal conditions (Fig. 7A) (Dym and Romrell, 1975; Holstein, 1978; Sinowatz *et al*., 1979; Hees *et al*., 1989; Yakirevich *et al*., 2002; Takahashi *et al*., 2007; Naito *et al*., 2008). Many macrophages located around the TR might be actively endocytosing germ cell remnants (Holstein, 1978; Takahashi *et al*., 2007). Through a similar perspective, the presence of a possible resident population of intra-epithelial lymphocytes might be playing a key role in segregating sperm antigens from the general circulation in a testicular segment where luminal contents are absorbed and where the BTB is altered (Dym and Romrell, 1975). It is worth considering that protruding cytoplasmic strings from TRSCs occlude the TR lumen and may serve as sensors, contacting the spermatozoa and monitoring their presence and antigenic expression (Takahashi *et al*., 2007). Besides that, the presence of phagocytosed spermatozoa has been also found within TRSCs in many mammalian species already investigated (Dym, 1974; Osman and Plöen, 1978; Nykänen, 1979; Wrobel *et al*., 1982). This observation might be attributed to the fact that the basal lamina and the cytoplasm of TRSCs is intimately interconnect in the TR, providing a wide surface area where some germ cell autoantigens may be excreted and absorbed (Tainosho *et al*., 2011).

Therefore, the TR is considered to be immunologically vulnerable and probably the weakest site for some testicular injuries (Tung *et al*., 1987; Itoh *et al*., 1995). There is a possibility that, in some

cases, germ cell autoantigens leak into the TR easily allowing accumulation of macrophages and lymphocytes. Actually, a rapid accumulation of macrophages in the TR is mentioned since the prepubertal period, which may imply that these cells contribute somehow to testicular development (DeFalco *et al*., 2015). Furthermore, Takahashi *et al*. (2007) indicated that testicular macrophages can take up germ cell autoantigens from the TR epithelium under normal conditions. A primary macrophage function is in the host defense system against exogeneous agents (Hutson, 1994), so that it is hypothesized that macrophages accumulate in the TR in order to protect the seminiferous epithelium from genital tract infections.

In testicular pathological contexts, it seems that lymphocytic infiltration initiates around the TR (Itoh *et al*., 1995; Naito *et al*., 2009). During the course of experimental autoimmune orchitis (EAO), for instance, lymphocytes preferably accumulate around the TR and might gain access to testicular autoantigens, probably facilitated by the intrinsic TR microenvironment (Itoh *et al*., 1995, 1998; Takahashi *et al*., 2007). The proper accumulation of macrophages may be the reason why lymphocytes first gather around the TR during EAO (Itoh *et al*., 1995, 1998; Takahashi *et al*., 2007). Naito and colleagues (2009) demonstrated that, after the onset of EAO, lymphocytes can migrate into the TR wall, secrete several cytokines such as interleukin IL-1, IL-2, IL-6, IL-10, interferon-γ and tumor necrosis factor-α, leading to degeneration of the TR epithelium before spreading spermatogenic disturbances. Interestingly, after lipopolysaccharide (LPS) treatment, our research group observed that degeneration of the seminiferous epithelium starts from the TR (Fig. 7B). Then, massive degeneration of germ cells, appearance of multinucleate giant cells and vacuolization occur along the ST (Fig. 7C). The combination of several deleterious effects posteriorly lead to the severe SC only phenotype (Fig. 7D). Therefore, the micromilieu of an incomplete BTB provides a higher susceptibility for the establishment of testicular inflammation in the TR, which arises as an onset of some pathological conditions.

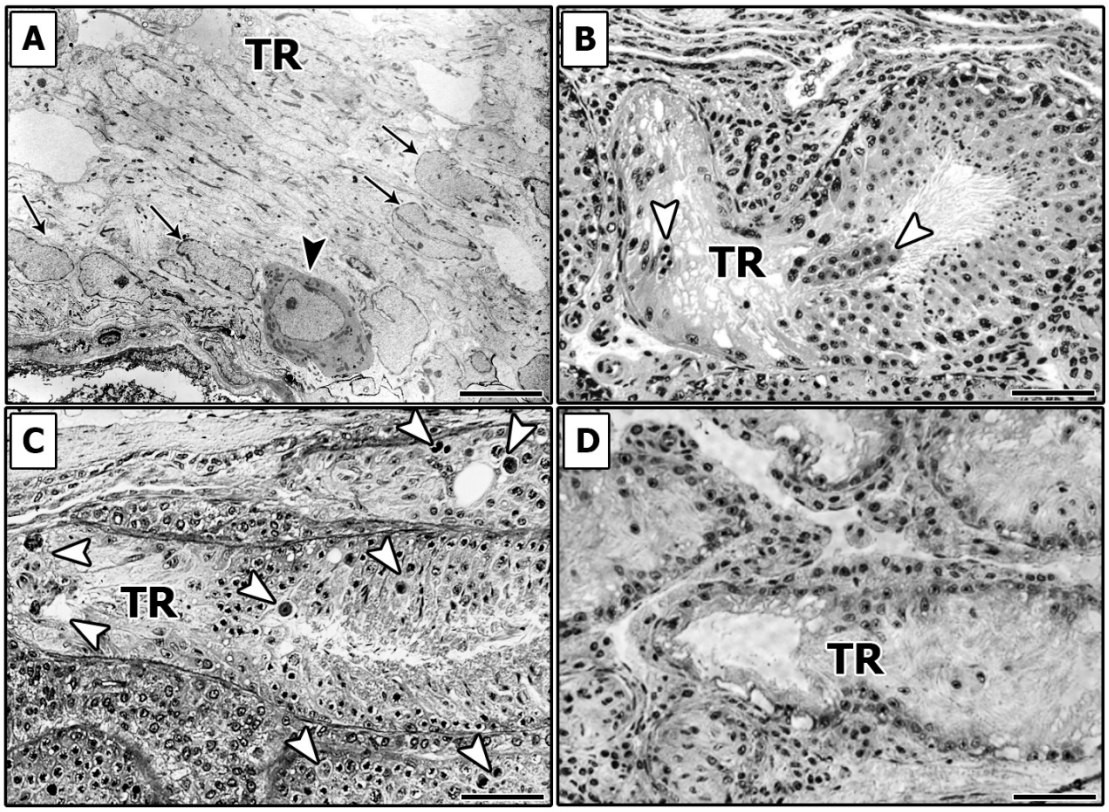

Figure 7. Immunological aspects of the transition region (TR). A) Transmission electron micrograph showing the presence of a macrophage (black arrowhead) among Sertoli cells (black arrows) in sexually mature Wistar rat. B-D) After testicular inflammatory insult via lipopolysaccharide treatment in sexually mature mice, alterations of the seminiferous epithelium (white arrowheads) start from the TR then spread along the seminiferous tubule epithelia, generating the SC only phenotype. Bar: 10µm (A); 50µm (B-D).

Recently, Mesa *et al*. (2018) characterized immunohistochemically the pathogenesis of adenomatous hyperplasia of the *rete testis*. One of the most interesting findings is that the lesion affects primarily the septal *rete testis*, i.e., the portion facing the TR. Curiously, this *rete testis* area shares similar features with the TR, such as reduced expression of androgen receptors and higher proliferative activity. The authors speculated that this testicular segment is intrinsically more susceptible to toxic or immune agents mainly due to a significant influence related to the altered BTB. This idea was primarily explored by Dym and Romrell (1975), who speculated that initial injury of germinal epithelium may be caused by lymphocytes present in the terminal portions of the ST (i.e., TR), then spread the damage along the tubules. On the other hand, an active immunosuppression exerted by human SCs limiting the proliferation of lymphocytes was reported by Chui and colleagues (2011). Several proteins were also identified in the *rete testis* fluid, potentially associated with sperm protection, such as albumin,

clusterin and serotransferrin (van Tilburg *et al*., 2017). The protein composition of this fluid might be crucial to the regulation of a healthy spermatogenesis.

7. TRSC and Spermatogonial Stem Cells (SSCS)

There are also evidences that associate the TR as a potential niche for SSCs. Aiyama *et al*. (2015) demonstrated a constitutively strong expression of GDNF, a major factor for SSCs niche, supporting a significant high proliferation and selective maintenance of Asingle subpopulation of GFRα1-positive spermatogonial cells in the TR. *I.e*,, the high levels of GDNF increase the population of undifferentiated spermatogonia while repress the differentiation of these cells into more advanced spermatogenic cells (Meng *et al*., 2000). This characteristic may explain the lack of advanced spermatogenic cells in the TR. This configuration is analogous to the germline stem cell (GSC) niche described in some invertebrate species. In *Drosophila* and nematodes, for instance, undifferentiated spermatogonial cells are maintained in a niche environment restricted to the terminal segment of the tubular structure of the gonads. This region is populated by specialized somatic cell structures that repress the germline cells differentiation and provide the necessary factors for the stem cell maintenance (Dansereau and Lasko, 2008; Morrison and Spradling, 2008; White-Cooper and Bausek, 2010; Yuan and Yamashita, 2010; de Cuevas and Matunis, 2011; Spradling *et al*., 2011). Curiously, the GSC niche in these invertebrates also contains a subset of somatic cyst progenitor cells (TRSCs-like) that are proliferative and contribute distally to the post-mitotic supporting cyst cells (de Cuevas and Matunis, 2011), strengthening the idea of the existence of a stem SC subset. In mouse testes, Nagasawa *et al*. (2018) recently demonstrated a frequent STAT3-activated (p-STAT3) signals in TRSCs. This protein is an important cytokine transducer that plays crucial role in the maintenance of both GSCs and somatic cyst progenitor cells in Drosophila testis niche (de Cuevas and Matunis, 2011). Interestingly, p-STAT3 signals positively correlate with the high levels of GDNF and may be associated with the TRSCs niche function to maintain GFRα1-positive undifferentiated spermatogonia in the TR.

8. Comparative morphology of TR

Despite some species-specific differences in the morphological pattern of the TR, the general features were collected mainly by means of light and electron microscopies in many mammalian species, including laboratory animals and men (Roosen-Runge, 1961; Marin-Padilla, 1964; Vitale-Calpe and Aolu, 1969; Dym, 1974; Cavicchia and Burgos, 1977; Wrobel *et al*., 1978; Nykänen, 1979; Osman *et al*., 1979; Lindner & Holstein, 1982; Ezeasor, 1986; Wrobel *et al*., 1986).

In men, particular attention is drawn to the presence of ectopic modified germinal epithelium arranged around a center of connective tissue, a particular structure with unknown function located in the proximal part near the *rete testis* (Lindner & Holstein, 1982). In this preceding detailed study in human specimens, Lindner and Holstein (1982) identified different morphological types of TRSCs possibly related to different stages of function - later, a mixed phenotype was also characterized in culture of mice TRSCs (Kulibin and Malolina, 2016; Malolina and Kulibin, 2017). Among human TRSCs, A-pale type spermatogonia are seen either adjacent or having no contact with the basal lamina along the TR (Lindner & Holstein, 1982). In the transitional region of pigs, Avelar and colleagues (2010) reported the presence of an intense mitotically active SCs population, where primarily ST growth in length and the start of the testicular parenchyma maturation probably takes place.

To our knowledge, the only available descriptive data found in literature on the TR in a non-mammalian species is for the domestic fowl (Lake, 1957; Aire and Malmqvist, 1979; Osman, 1980). Similar to laboratory rodents and other mammals already investigated, TRSCs interspersed with spermatogonial cells line the terminal segment (Osman, 1980). The TRSCs of domestic fowl present cytoplasmic protrusions and globules near the luminal surface (Osman, 1980), supporting a secretory function originally suggested by Lake (1957). Intraepithelial lymphocytes and free macrophages were also found throughout the excurrent ducts in this bird species (Aire and Malmqvist, 1979). Although no further detailed studies have been done so far, these morphological data closely resemble what is described in better investigated mammalian species. To date, there are no reports regarding the TR (or some analogous structure) in amphibian or reptilian species. In perciform fish, the distal region of the ST near the tunica albuginea, recognized as the blind end region, is pointed out as the source of new spermatogenic cysts formation (Schulz *et al*., 2005). This specific region exhibits confined distribution of mitotic SCs associated with undifferentiated spermatogonia, which consist in analogous features to the TR. Although further studies are still required, all above-mentioned data evinces a well-conserved feature during evolution so that it can be hypothesized the existence of stem SCs in the TR. Moreover, it can be suggested that the TR provide factors to function as a niche for progenitor SCs.

Finally, laboratory rodents, including mice, rats and hamsters, are the best models that have had so far their TR better investigated and characterized (Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin & Malolina, 2016; Malolina & Kulibin, 2017; Nagasawa *et al*., 2018; Figueiredo *et al*., 2019). Recent studies have indicated an important plasticity of TR in the testis function, since this particular area is both a potential niche for spermatogonial stem cells (Aiyama *et al*., 2015; Nagasawa *et al*., 2018) and a possible harbor of progenitor SCs (Figueiredo *et al*., 2016; Kulibin & Malolina, 2016; Malolina & Kulibin, 2017; Figueiredo *et al*., 2019). This configuration allows speculations that the TR might be a specific site where ST continue to grow after puberty. In summary, the TR emerges as a

unique testis area for investigations related to the interactions of Sertoli and spermatogonial niche cells. Another important aspect that must be further investigated is the presence of the primary cilium in TRSCs (Ou *et al*., 2014; Dores *et al*., 2016), structure that has been reported to exert control over miRNAs regulating SC proliferation and maturation (Procópio *et al*., 2017). In this context, detailed investigations are required in order to better understand the molecular mechanisms involved in this niche dynamics.

9. Conclusion

The TR presents many unexplored aspects and still requires the performance of several experiments and analyses. Besides the available data showing distinct morphological and histochemical features between TRSCs and SCs from other ST areas, recent studies have shown other molecular differences between them and also among TRSCs. Summarizing, the TR is composed by TRSCs that present particular structural morphology and distinct functional phenotype. They present poorly developed junctional complexes and an altered SC/BTB. A subset of these cells retains a considerably high proliferative potential and display immature morphological features. Their proliferative capacity in the adult stage might contribute to the SC population within the seminiferous epithelia. In this context, the TR also emerges as a potential SSC niche considering the high levels of GDNF and p-STAT3 expression. Moreover, immune cells surrounding the TR, probably attracted by chemotactic factors, are important to prevent antigen from escaping into the systemic circulation. It is also believed that these cells may release factors, under non-homeostatic circumstances, that may spread testicular damages along the ST. Thus, the microenvironment of the TR, which seems to be well-conserved during evolution, dictates important parameters and conditions for the normal testis function. The deeper the knowledge dig into this amazing testicular region, certainly more important applications for the medical science will be raised in a near future.

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The Sertoli cell: what can we learn from different vertebrate models?

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Abstract

Besides having medical applications, comparative studies on reproductive biology are very useful, providing, for instance, essential knowledge for basic, conservation and biotechnological research. In order to maintain the reproductive potential and the survival of all vertebrate species, both sperm and steroid production need to occur inside the testis. From the approximately fifty thousand vertebrate species still alive, very few species are already investigated; however, our knowledge regarding Sertoli cell biology is quite good. In this regard, it is already known that since testis differentiation the Sertoli cells are the somatic cells in charge of supporting and orchestrating germ cells during development and full spermatogenesis in adult animals. In the present review, we highlight key aspects related to Sertoli cell biology in vertebrates and show that this key testis somatic cell presents huge and intrinsic plasticity, particularly when cystic (fish and amphibians) and non-cystic (reptiles, birds and mammals) spermatogenesis is compared. In particular, we briefly discuss the main aspects related to Sertoli cells functions, interactions with germ cells, Sertoli cells proliferation and efficiency, as well as those regarding spermatogonial stem cell niche regulation, which are crucial aspects responsible for the magnitude of sperm production. Most importantly, we show that we could greatly benefit from investigations using different vertebrate experimental models, mainly now that there is a big concern regarding the decline in human sperm counts caused by a multitude of factors.

Keywords: Sertoli cell; vertebrates; amniotes; anamniotes; spermatogenesis.

Introduction

Since testis differentiation, in all vertebrate species so far investigated the Sertoli cells are the somatic cells in charge of supporting and orchestrating germ cells during their development and full spermatogenesis in adult animals (Pudney, 1993; Hess and França, 2005; Oatley *et al*., 2011; Griswold, 2015; França *et al*., 2016). Therefore, the total number of Sertoli cells per testis, as well as their proper interactions with germ cells and the number of these cells per Sertoli cell (Sertoli cell efficiency), are the key qualitative and quantitative determinants of sperm production (Sharpe, 1994; Hess and França, 2007; Griswold, 2015; França *et al*., 2016). In the present review, we concisely discuss and compare the key aspects related to Sertoli cell biology in different vertebrate groups, such as anamniotes (fish and amphibians) and amniotes (reptiles, birds and mammals), where respectively cystic and non-cystic spermatogenesis are observed (França *et al*., 2015, 2016).

Sertoli cell discovery and morphology

The testis is the male gonad, where steroidogenesis and sperm production take place. Spermatogenesis is a highly organized process in which the germ cells go through several divisions and intricate differentiation steps, resulting in the production of the spermatozoa. This process is orchestrated mainly by the Sertoli cell that were named after Enrico Sertoli, author of the first publication reporting their existence (Sertoli, 1865; Ebner, 1888; Hess and França, 2007). In this publication, Enrico Sertoli describes their morphology "not unlike trees", including details such as their bifurcated branches of cytoplasm, the niches where germ cells fit and the large nucleolus, apart from calling them "mother cells", anticipating the comprehension of their crucial functions (Sertoli, 1865; Hess and Vogl, 2015).

Using advanced staining techniques and electron microscopy, many decades later the details of the Sertoli cells morphology and function were shown and the understanding of this complex cell's role in the testis morphophysiology have been a major topic in male reproduction research. Therefore, numerous reviews (Regaud, 1899; Elftman, 1963; Fawcett, 1975; Clermont *et al*., 1987; Sharpe, 1988; de Kretser, 1990; Clermont, 1993; Russell, 1993a,b; Vogl *et al*., 1993; Kerr, 1995; Griswold, 1998; Walker, 2003a,b; Wong and Cheng, 2009; Vogl *et al*., 2013; Ramaiah and Wilkinson, 2015; Griswold, 2016; França *et al*., 2016) and books (Russell and Griswold, 1993; Skinner and Griswold, 2005; Griswold, 2015) have been published describing the Sertoli cell morphology and functions, mostly focusing on mammals. However, due to the cystic versus non-cystic arrangement of spermatogenesis, distinct Sertoli cell characteristics are observed when comparing different vertebrate species [anamniotes (fish and amphibians) and amniotes (reptiles, birds and mammals)] (Please see Section 4 and Figure 1) (Russell and Griswold, 1993; Griswold, 1998; Hess and França, 2007; Schulz *et al*., 2010; França *et al*., 2015, 2016).

Reflecting their relationship with germ cells, in overall the Sertoli cell shape may vary according to the species and the progression of spermatogenesis.

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Considerable variations are also observed for the expression of proteins and growth factors, which also change according to the age of development and seasonality (Russell, 1993a,b; Rothbarth *et al*., 2001; França and Chiarini-Garcia, 2005; Hess and Vogl, 2015; França *et al*., 2016). Additionally, as the germ cell requirements, interactions and metabolic needs change substantially, high variations are observed on the Sertoli cell cytoplasm extension, the amount of nuclear pores, the presence and translocation of organelles and the protein expression pattern and location across the different phases of spermatogenesis (Toppari *et al*., 1991; França *et al*., 1993; Cavicchia *et al*., 1998; Johnston *et al*., 2008; Wright, 2015). To illustrate the above-mentioned variation on Sertoli cells characteristics, due to their endocytic activity in the elimination of residual bodies, an increase in the amount of lipid and lysosomes is usually observed in the Sertoli cells cytoplasm after spermiation (Ye *et al*., 1993; França *et al*., 1995; Hess and França, 2005). Structural characteristics of the Sertoli cells also varies among species, such as the heavily vacuolated nucleolus present in some ruminants (Pawar and Wrobel, 1991; Steger and Wrobel, 1994), the nucleus localization in the middle of the seminiferous epithelium in monkeys (Hess and França, 2005), the presence of Charcot-Bottcher cristaloids in men (Czaker, 1994; França and Chiarini-Garcia, 2005), and the presence and amount of lipid droplets and glycogen in the Sertoli cell cytoplasm (Fouquet, 1968; Russell, 1993a,b; Tedde *et al*., 1993; Erkan and Sousa, 2002).

Proliferation and maturation

It is widely accepted that the number of Sertoli cells per testis and the Sertoli cell efficiency (which is measured as the number of germ cells per Sertoli cell) are the main determinants of the sperm production of a given species (Sharpe, 1994; Hess and França, 2007; Griswold, 2015; França *et al*., 2016). In this regard, the Sertoli cell proliferation that usually ends before puberty is a crucial event for testis physiology (Lara *et al*., 2018b). In laboratory rodents (such as rats and mice), this proliferation occurs mainly during fetal life, reaching its maximum activity just before birth (Orth, 1982, 1993; McCoard *et al*., 2003; França *et al*., 2016). However, the postnatal Sertoli cell mitotic activity is highly variable according to the species, lasting for instance 2-3 weeks in laboratory rodents. In humans, this somatic cell proliferate during the perinatal and neonatal period, becoming quiescent for several years and having a second peak of proliferation just before puberty (Cortes *et al*., 1987; Sharpe *et al*., 2003; Tarulli *et al*., 2012). In a scale of months or few years, a similar pattern is also observed in pigs, primates and cattle (Gondos and Berndston, 1993; França *et al*., 2000; Cooke *et al*., 2005).

In most studied species, FSH and androgens are considered important factors that regulate Sertoli cell proliferation (Skinner and Griswold, 2005; Lara *et al*., 2018b). Other factors usually associated with this process are estrogens, activins, TGF-beta, BMPs, interleukins and TNFalpha (Puglisi *et al*., 2004; Tarulli *et al*., 2012; Lucas *et al*., 2014a,b). After the fetal and postnatal periods of mitotic divisions, which are rather variable among different species, Sertoli cells stop proliferating and start to differentiate around puberty, being therefore able to support full spermatogenesis. This maturation process correlates for instance with the initiation of meiosis, the establishment of the Sertoli cell barrier and fluid secretion/lumen formation, which are clear signs of Sertoli cells maturation (Griswold, 2015; França *et al*., 2016).

Based mainly in studies developed in laboratory rodents (rats and mice), thyroid hormones are considered a key factor in the regulation of Sertoli cell differentiation/maturation (Cooke *et al*., 2005). In these studies, it has been shown that neonatally induced hypothyroidism prolong the proliferative period and delay Sertoli cell maturation, increasing therefore their final number and, consequently, the testis size and the magnitude of sperm production (Van Haaster *et al*., 1992; Joyce *et al*. 1993; Auharek and França 2010; Lara and França, 2017). In an opposite way, hyperthyroidism accelerates Sertoli cell maturation, resulting in a smaller population of this cell in the testis, as well as smaller testis size and lower sperm production (Van Haaster *et al*., 1993; Cooke *et al*., 2005; Auharek and França, 2010). Similar results regarding thyroid hormones effects on testis size and sperm production were observed in chicken and the Nile tilapia (Kirby *et al*., 1996; Matta *et al*., 2002). However, paradoxically, in pigs thyroid hormones seem to regulate Sertoli cells in an opposite way. In this regard, postnatally induced hypothyroidism significantly decreased the number of Sertoli cells, testis size and sperm production, whereas a higher dose of thyroid hormone (T3) augmented the number of Sertoli cells per testis (Tarn *et al*., 1998; Silva-Jr, 2000; Klobucar *et al*., 2003). In bulls, no effects on Sertoli cells number were observed after the induction of neonatal hypothyroidism, whereas human studies suggested that decreased thyroid hormones might be associated to testicular enlargement (Jannini *et al*., 1995; Cooke *et al*., 2005). There is still no explanation regarding the observed inconsistencies of thyroid hormones effects among the different species of amniotes (reptiles, birds and mammals). One of the possibilities might be related to the aforementioned differences on Sertoli cell proliferation pattern. The expression of thyroid hormone receptors and/or interactions with other testis somatic cell types (i.e Leydig and peritubular myoid cells) should also not be excluded.

In relation to anamniotes, mainly due to continuous body growth, it has been shown that the Sertoli cell population is more dynamic, and that these cells remain mitotically active even after sexual maturity (Bouma and Cloud, 2005). Particularly, during spermatogenesis progression the number of Sertoli cells enveloping an individual spermatogenic cyst increases along with the germ cell number within that cyst. Interestingly, coincident with the formation of tight junctions between Sertoli cells, and similar to the establishment of puberty in mammals, usually the

Sertoli cell number per cyst stabilizes after meiosis is complete and spermiogenesis initiates (Matta *et al*., 2002; Vilela *et al*., 2003; Schulz *et al*., 2005; Leal *et al*., 2009; França *et al*., 2016). As it occurs in mammals, FSH seems to be the main factor involved in Sertoli cell proliferation in anamniotes (Schulz *et al*., 2010, 2012; França *et al*., 2016). Also, as hypothyroidism increase Sertoli cell number in tilapia (Matta *et al*., 2002), thyroid hormones may also be important for Sertoli cell proliferation in fish (Matta *et al*., 2002; Morais *et al*., 2013). However, in contrast to mammals, thyroid hormones stimulate Sertoli cell proliferation in zebrafish (Morais *et al*., 2013). Recently, França *et al*. (2015) proposed two modes of Sertoli cell proliferation in fish. In the first, Sertoli cell proliferate to provide new niches for spermatogonial stem cells and to form new spermatogenic cysts (Morais *et al*., 2013; França *et al*., 2015). In the second mode, Sertoli cells already enveloping an existing cyst would divide, in order to accommodate the increase in the germ cell number until meiosis is complete and Sertoli cell maturation occurs (Billard and Breton, 1978; Almeida *et al*., 2008; França *et al*., 2015). An interesting aspect that could contribute to advance our understanding on Sertoli cell proliferation/maturation in fish is the observation that one Sertoli cell may be in contact with different cysts of germ cells in different phases of spermatogenesis (França *et al*., 2016).

In mammals, indicating the final maturation of this cell type and its capability to support full spermatogenesis is the establishment of Sertoli cell barrier, a remarkable feature that occurs around puberty (Cheng and Mruk, 2012; Griswold, 2015; Lara *et al*., 2018a). This barrier, observed close to the basement membrane, is formed by specialized junctional complexes between adjacent Sertoli cells and helps to protect the germ cells undergoing meiosis from an autoimmune reaction; creating therefore an immune privileged environment within the seminiferous tubules (Tung and Fritz, 1978; Francavilla *et al*., 2007; França *et al*., 2012; França *et al*., 2016). The main component of this barrier are tight junctions that divide the seminiferous epithelium in two compartments: basal and adluminal, where respectively early (spermatogonia and young spermatocytes) and late (more advanced spermatocytes and spermatids) germ cells are located. Due to the complex cyclic dynamics of the Sertoli cells barrier and the formation of a transient intermediate compartment, young primary spermatocytes move across these compartments without causing any damage to the junctional structure and testis physiology (Cheng and Mruk, 2012; França *et al*., 2012). Other components of this important barrier include gap junctions, desmosomes, and two types of adherens junctions that are testis-specific (tubulobulbar complexes and ectoplasmic specialization) (Lee and Cheng, 2004; Vogl *et al*., 2008; Cheng *et al*., 2011; Vogl *et al*., 2013; Lara *et al*., 2018a). Overall, these structural components make the Sertoli cell barrier one of the tightest in mammals (Mital *et al*., 2011; Cheng and Mruk, 2012).

Regarding other vertebrate groups, there are relatively few studies related to the Sertoli cell barrier, and it is already known that the junctional complexes constituents are highly variable across vertebrates (Bergmann *et al*., 1984; Grier, 1993, França *et al*., 2012). Tight junctions and desmosomes are usually observed between adjacent Sertoli cells in anamniotes (fish and amphibians), and the barrier is present after meiosis is complete and the cyst is composed by early spermatids, the haploid cells (Pudney, 1993; McClusky, 2006; Batlouni *et al*., 2009; Schulz *et al*., 2010; França *et al*. 2012). However, a study in zebrafish showed that lanthanum, a tracer widely used to investigate barrier effectiveness, is never observed in the seminiferous tubule lumen, even when there is no functional barrier present (Leal *et al*., 2009). Similar to mammals, in reptiles and birds the barrier seems to be formed immediately after the onset of meiosis; however, there is still little information regarding its composition and structure (Bergmann *et al*., 1984; Bergmann and Schindelmeiser, 1987; Gribbins, 2011; Cheng and Mruk, 2012; Ahmed *et al*., 2016).

The transition region

The dogma that the adult Sertoli cells population constitutes a terminally differentiated population in mammals has been challenged by several recent studies (Hayrabedyan *et al*., 2012; Haverfield *et al*., 2015; Figueiredo *et al*., 2016; França *et al*., 2016). Therefore, the possible existence of a progenitor Sertoli cell has been strengthened by new evidences raised from sexually mature laboratory rodent models, indicating that the transition region, where the seminiferous tubules connect to the *rete testis*, could be a specific area for immature Sertoli cells (Tarulli *et al*., 2013; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016). This assumption is supported mainly by current molecular observations showing that a subpopulation of Sertoli cells within the transitional region are mitotically active (BrdU-positive; Cyclin-D1-positive; Ki-67-positive) and that they do not express typical differentiated Sertoli cells markers such as the transcription factor GATA-4 and the androgen receptor (Tarulli *et al*., 2013; Aiyama *et al*., 2015; Figueiredo *et al*., 2016; Kulibin and Malolina, 2016). Moreover, it has already been described that the adult Sertoli cells population is not morphologically homogeneous. In this particular aspect, the transition region presents modified Sertoli cells that exhibit features that resemble undifferentiated Sertoli cells, such as the presence of more ovoid nucleus, with less indentations, smaller nucleolus and more peripheral heterochromatin (Dym, 1974; Osman, 1978; Nykänen, 1979). More recently, it has been shown that these modified Sertoli cells divide in culture and are able to form colonies and generate cord-like structures (Kulibin and Malolina, 2016). Because this particular area of mammalian testis also contains spermatogonial stem cells, it has been suggested that the transition region might be an area where the seminiferous tubules continues to grow in sexually mature individuals (Tarulli *et al*., 2013; Aiyama *et al*., 2015; Figueiredo *et al*., 2016). Moreover, this assumption is corroborated by new findings

indicating that the transition region is a site where seminiferous tubules are originally formed (Malolina and Kulibin, 2017).

Similar to horses and showing a testis gradient during postnatal testis development, in pigs the testis parenchyma grows asynchronously, starting its maturation nearby the intermediate and the central (transitional) area, where the testis mediastinum and the *rete testis* are located (Avelar *et al*., 2010). Moreover, resembling sexually mature laboratory rodents, Sertoli cell mitotic activity in pubertal pigs was higher in the transitional region, which is probably a primary site of seminiferous tubules growth in length. In some teleost orders, there are also evidences of the existence of functionally different testicular regions. For instance, once this specific area presents restricted distribution of proliferative Sertoli cells associated with immature spermatogonia, in perciform fish it was demonstrated that the distal region of the seminiferous tubules, nearby the tunica albuginea (blind end region), might be the source of new spermatogenic cysts formation (Schulz *et al*., 2005). Considering that the above-mentioned region present immature Sertoli cells exhibiting high proliferative potential, one could hypothesize the existence of stem Sertoli cells, an important aspects that still needs further investigation.

It is interesting to mention that in some invertebrate species, such as flies and nematodes, the germline stem cell niche is located at the terminal end of the tubular aspect of the gonads. Therefore, in this location, undifferentiated spermatogonial cells are steadily maintained (de Cuevas and Matunis, 2011; Aiyama *et al*., 2015). Another important aspect regarding the germline stem cell niche in these invertebrates is the presence of proliferative somatic cyst progenitor cells (similar to the transition region in mammals?), which produce distally somatic cyst cells that support the development and differentiation of germ cells (Dansereau and Lasko, 2008). Therefore, there are some evidences indicating the existence of a stem Sertoli cell pool, which could be a well-conserved feature during evolution.

Relationship between Sertoli and germ cells

Cystic vs Non-Cystic spermatogenesis

The maintenance of continuous sperm production throughout life in males is very complex and the fine regulation (protein synthesis and signaling) of spermatogenesis is under constant investigation (Wong and Cheng, 2009; Ramaiah and Wilkinson, 2015; Yang and Oatley, 2015; França *et al*., 2016; Griswold, 2016). In this context, although showing particularities among different vertebrate groups, the interactions between Sertoli and germ cells is crucial for the development and completion of spermatogenesis (Hess and França, 2007). In amniotes (reptiles, birds and mammals), a single and non-dividing Sertoli cell supports the development of different germ cells at the same time (non-cystic pattern of spermatogenesis) (Fig. 1; Russell and Griswold, 1993; França *et al*., 2015). In particular, at their different areas/regions the Sertoli cells present the following contacts/functions: i) regulate spermatogonia self-renew or differentiation in the basal compartment of the seminiferous epithelium (de Rooij, 2001); ii) create contact with spermatocytes on its lateral side, regulating the meiotic process from the duplication of DNA content to the formation of spermatids (Russell, 1977); iii) interact specifically with spermatids in the adluminal/apical portions, regulating their morphology, the reabsorption of residual bodies and controlling spermiation (Meistrich and Hess, 2013).

Different from the seminiferous epithelium organization cited above, in fish and amphibians (anamniotes) the cystic type of spermatogenesis is observed (Figure 1; França *et al*., 2015). In these vertebrate groups, spermatogenic cysts are formed when Sertoli cells surround the type A spermatogonia. From this point, Sertoli cells synchronously coordinate the development of these cells until they differentiate into sperm (Schulz *et al*., 2010, França *et al*., 2015). Thus, in this arrangement, a histological testis section is composed of different spermatogenic cysts, i.e., distinct units of germ cells in the same stage of development enveloped by groups of Sertoli cells (Leal *et al*., 2009). It is believed that in this cystic organization the Sertoli cells are more efficient, meaning that more germ cells can be supported by Sertoli cells during their development, allowing a higher sperm production (França *et al*., 2015). Moreover, it is hypothesized that this cystic structure allows the concentration of specific factors required for each phase of spermatogenesis (spermatogonial, spermatocitary, and spermiogenic), resulting in lower germ cell apoptosis (Vilela *et al*., 2003; França *et al*., 2015).

As it was already mentioned, due to continuous testis growth during adulthood, another characteristic already described in cystic spermatogenesis is the capacity of Sertoli cells to divide in adult animals, where Sertoli cells are observed proliferating mainly when they are in contact with mitotically active spermatogonia and in association with spermatocytes (França *et al*., 2015). Although there is still no proof regarding the existence of Sertoli stem cell in fish testis, some authors believe in the existence of a stem cell population, giving rise to somatic Sertoli cells (Morais *et al*., 2013; França *et al*., 2015).

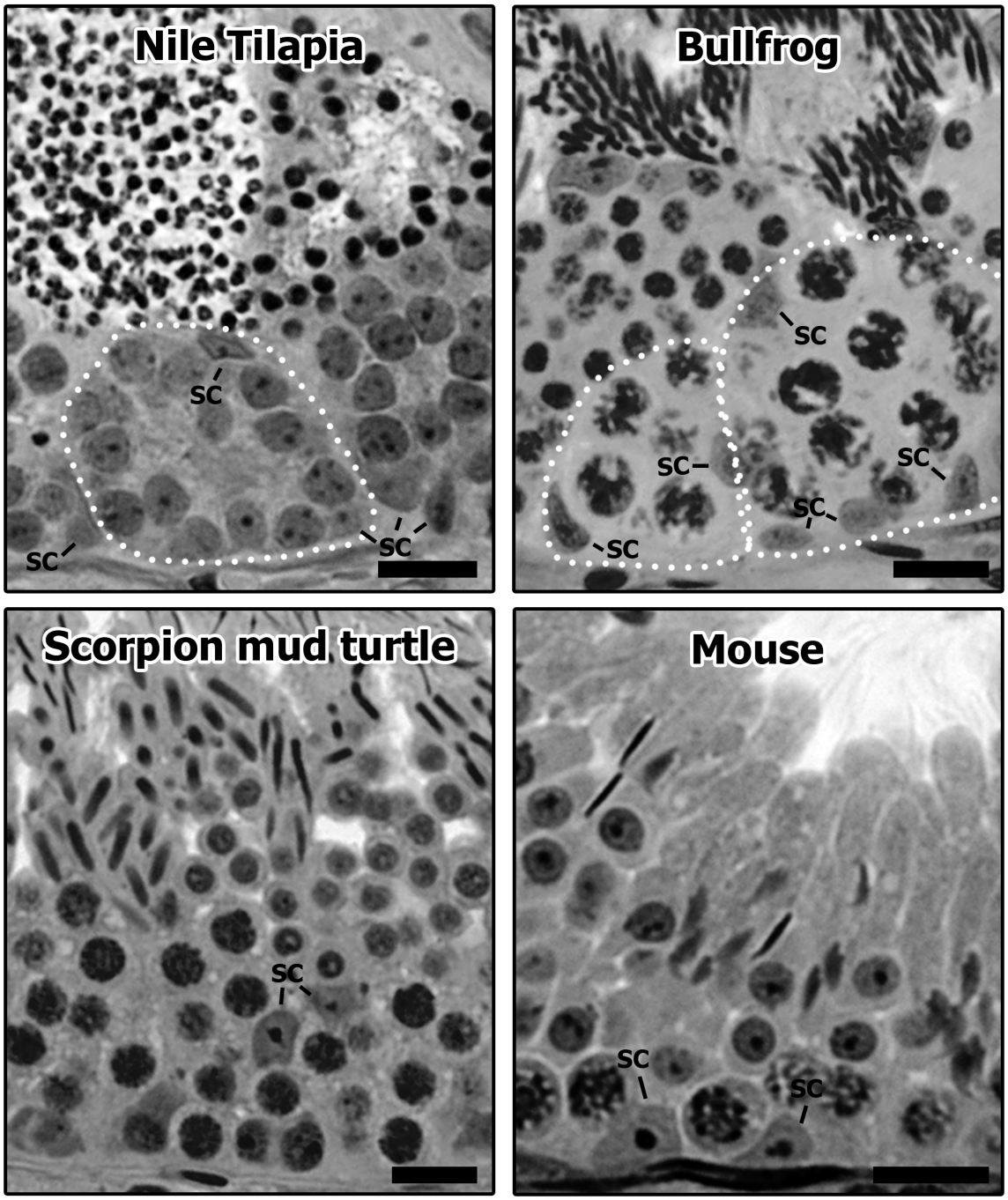

Figure 1. Histological sections from the seminiferous epithelium in different vertebrate species presenting cystic [Nile Tilapia (*Oreochromis niloticus*) and bullfrog (*Lithobates catesbeianus*)] or non-cystic [Scorpion mud turtle (*Kinosternon scorpioides*) and mouse (*Mus musculus*)] spermatogenesis arrangements. As it can be observed, the germ cell clones in the cystic arrangement are completely enveloped by the Sertoli cells (SC; white dotted lines), whereas in non-cystic arrangement one single Sertoli cell contacts several different germ cell types, despite exhibiting conspicuous structural polarity. Bars = 15 µm.

Spermatogonial stem cell's niche

The Sertoli cells play a key role in the functional regulation of spermatogonial stem cells niche, where other somatic testicular cells (Leydig, peritubular myoid cells and macrophages), extracellular matrices and soluble factors actively participate in the complex interaction/signaling with these stem germ cells (Chiarini-Garcia *et al*., 2001; Oatley and Brinster, 2012). In this microenvironment, depending on the stimulus, a balance between self-renewal and differentiation factors regulates the fate of these cells that are capable of self-renewal, differentiation and/or entering into apoptosis (Oatley *et al*., 2011). Acting through Sertoli cells, the glial cell-line derived neutrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) are among the several factors considered important for the regulation of spermatogonial stem cells niche (Tadokoro *et al*., 2002; El Ramy *et al*., 2005; Simon *et al*., 2007; Chen and Liu, 2015; Chen *et al*.,

2016; Potter and DeFalco, 2017). In this particular aspect, the total number of Sertoli cells per testis determine the number of available spermatogonial stem cell niches and, consequently, these somatic cells dictates the magnitude of sperm production capacity (Oatley *et al*., 2011).

Nowadays, two types of spermatogonial stem cell niche are being proposed. In the first type, called closed niche, these undifferentiated cells are concentrated in a particular testicular parenchyma region; whereas in the second type, called opened niche, spermatogonia present a specific distribution inside the seminiferous tubules (Aiyama *et al*., 2015; Yoshida, 2016). In mammals, recent data have demonstrated that the transition region between seminiferous tubules and the *rete testis* is the closed niche area. The Sertoli cells in this region produce high amount of GDNF, maintaining the neighboring spermatogonia in an undifferentiated state (Aiyama *et al*., 2015). Using different species models, several studies have demonstrated that spermatogonial stem cells are usually located in the seminiferous tubules area facing blood vessels of the testis interstitial compartment. It is speculated that FSH, coming from the blood vessels, stimulates the GDNF synthesis of surrounding Sertoli cells (de Rooij, 2009).

In fish, the distribution of spermatogonia along the testis parenchyma present a very high variation. For example, similar to mammals, in tilapias the closed and opened niche are normally observed and a restricted spermatogonial distribution (closed niche) is characterized by the presence of spermatogonial stem cells that are located in the distal blind end area of the seminiferous tubules nearby the tunica albuginea (Vilela *et al*., 2003; Lacerda *et al*., 2014). However, further studies are still necessary to investigate whether the secretion of GDNF is increased in this region. Also, as it occurs in mammals, in tilapias an unrestricted distribution of undifferentiated/stem spermatogonial cells is observed and these cells are frequently observed in regions of the seminiferous tubules that are facing the blood vessels located in the intertubular compartment **(**Lacerda *et al*., 2014**).**

Because it allows a broad view of testis function, comparative reproductive biology studies are a powerful tool. In these comparative investigations, it was found that typical proteins that are expressed by undifferentiated spermatogonia in mammals, such as OCT4, NANOS2, PLZF and GFRA1, are also expressed in some fish species already investigated (Lacerda *et al*., 2014). The GFRA1 is a membrane receptor involved in spermatogonial self-renewal and its ligand (GDNF), the most extensively studied niche factor, is produced by Sertoli cells. Recent studies have demonstrated that GDNF can promote high proliferation of spermatogonial stem cells *in vitro* in mammals and fish (Aponte *et al*., 2008; Gautier *et al*., 2014). The secretion of GDNF by Sertoli cells is cyclic and, in mammals, coincident with the differentiation of spermatogonial stem cells to type A differentiated spermatogonia that are committed to spermatogenesis, the lowest values of this peptide are found in stages near spermiation (Johnston *et al*., 2011). Therefore, this Sertoli cell regulation ensures a proper gem cell homeostasis and regulates the germ cell density observed in the seminiferous epithelium. Other important factors produced by Sertoli cells are leukemia inhibitory factor (LIF) and WNT5A, essential peptides that promote spermatogonial stem cell survival (Oatley and Brinster, 2012; França *et al*., 2016).

The possible role of different cells in regulating spermatogonial stem cell niche can be observed investigating different animal models, with peculiar testis parenchyma cytoarchitecture. For instance, because differentiated spermatogonia was found preferentially facing Leydig cells cords, studies in the collared peccary allowed to demonstrate that products from Leydig cells, probably androgens, act as a spermatogonial stem cells pro-differentiation factor (Campos-Junior *et al*., 2012). In the scorpion mud turtle testis, spermatogonial stem cells were located close to lymphatic vessels and blood vessels (Costa *et al*., 2018). In horses, these stem cells were located far from the connective tissue (Costa *et al*., 2012), whereas in chinchilla more spermatogonial stem cells are produced after the establishment of puberty, leading to a gradual and striking increase in Sertoli cell efficiency and sperm production after puberty (Leal and França, 2009).

Spermatogenic efficiency

The relative mass of tubular compartment in the testis determines the space devoted to sperm production (Hess and França, 2007). Thus, in general, species with high proportion of seminiferous tubules present high sperm production and, besides the influence of Sertoli and germ cell factors, the number of Sertoli cells per testis is considered one of the most important determinant of the magnitude of sperm production (Cooke *et al*., 2005; Hess and França, 2007; Lara *et al*., 2018b). In another important aspect, Sertoli cells show distinct capacities to support germ cell development and each Sertoli cell is able to support a relatively fixed, species-specific, number of germ cells. For instance, whereas chinchilla Sertoli cell can support 14 spermatids, each human Sertoli cell is able to support only 3 spermatids, resulting respectively in a huge difference in daily sperm production per testis gram (~60 vs 4-4.5 million) between these species (Hess and França, 2007; Lara *et al*., 2018b). The size of the Sertoli cells and, as a consequence, the space that they occupy in the seminiferous epithelium is another important factor to be considered. Species with reduced Sertoli cells occupancy in the seminiferous epithelium, such as mice (~15%), present higher Sertoli cell and spermatogenic efficiencies when compared to humans, whose Sertoli cells show high occupancy (~40%) in the seminiferous epithelium (Hess and França, 2007).

In relation to the germ cells, the number of differentiated spermatogonial generations, which is phylogenetically determined, is also crucial in determining the magnitude of sperm production. For example, in vertebrates the number of spermatogonial generations varies from around ten in fish to two in humans (França *et al*., 2016). Additionally, particularly

in mammals, germ cell loss, which is quite frequent during the spermatogonial (density-dependent regulation) and meiotic (DNA damage) phases of spermatogenesis, also significantly influences the total sperm output (Russell *et al*., 2002; Shaha *et al*., 2010; Aitken *et al*., 2011; Murphy and Richburg, 2014). The spermatogenic cycle length, which is controlled by the germ cell genotype (França *et al*., 1998), is another key factor in determining the efficiency of spermatogenesis (Hess and França, 2007). Considering the majority of the mammalian species already investigated, each spermatogenic cycle lasts about 9 to 12 days, whereas the total duration of spermatogenesis (that takes approximately ~4.5 cycles) lasts approximately 40 to 54 days. The faster the cell differentiation occurs from spermatogonia to spermatozoa, the higher the daily sperm production is. Particularly in humans, another factor that contributes to the lower sperm production is the quite long duration (~70 days) of spermatogenesis (Hess and França, 2007). In fish, the duration of spermatogenesis is very short and is influenced by the water temperature (Egami and Hyodo-Taguchi, 1967; Billard, 1990; Shimizu, 2003; Vilela *et al*., 2003; Nóbrega *et al*., 2009), meaning that higher temperature accelerates germ cells pace during spermatogenesis (Vilela *et al*., 2003; Lacerda *et al*., 2006; Alvarenga and França, 2009; Nóbrega *et al*., 2009).

During evolution, considering the different vertebrates groups, spermatogenic efficiency continually reduces and this characteristic is highly associated with the Sertoli cell support capacity, which decreases from around 100-150 (in fish) to 3 (in humans) spermatids for each Sertoli cell (França *et al*., 2015). In general, the support capacity of this cell in anamniotes is at least 10 times higher than that observed in mammals (França *et al*., 2015). Once more, these findings reinforce that Sertoli cell efficiency is critically important in determining the magnitude of sperm production (Hess and França, 2007), and claim our attention to the fact that perhaps in a near future humans will not produce sperm anymore.

Sertoli-Germ cell junctions

Interactions among testicular cells, in particular between Sertoli and germ cells, are crucial to maintain and regulate spermatogenesis in a very coordinated and organized manner, providing all the necessary structural and nutritional support for the developing germ cells (França *et al*., 2016; Cheng and Mruk, 2015; Lara *et al*., 2018a). Therefore, on its basal side Sertoli cells contact spermatogonia through adherens junctions, guiding their homing, niche and colonization (Lara *et al*., 2018a). In Chinese soft-shelled turtle, extensive adherens junctions were also observed between Sertoli and germ cells during active spermatogenesis, preventing the sloughing of germ cells from the epithelium (Ahmed *et al*., 2016). Desmosomes and gap junctions are also observed contacting adjacent germ cells, such as spermatocytes and early spermatids. At their adluminal aspect, Sertoli cells contact elongated spermatids through ectoplasmatic specialization, organizing the movement of these haploid cells as well as their release during spermiation (Cheng and Mruk, 2012; Lara *et al*., 2018a). Different from mammals, ectoplasmic specializations in fish seem to occur only in species with elongated spermatozoa, and this observation lead us to believe that this specialization has a role in the process of spermatid elongation (Batlouni *et al*., 2009). Probably being involved in the maturation of spermatids and similar to mammals, tubulobulbar complexes were described in repiles (Ahmed *et al*., 2016).

Present between Sertoli cell and germ cells, intercellular channels composed of gap junctions are essential to maintain the metabolic coupling and cell signaling. One of the most studied constitutive protein of the gap junction is connexin 43 (Kidder and Cyr, 2016). In humans (Defamie *et al*. 2003) and rats (Batias *et al*. 2000), connexin 43 is observed in Sertoli, spermatogonia and spermatocytes cells, which suggests an accurate communication among these cells. Similarly, in catfish (*Pseudoplatystoma fasciatum*) connexin 43 was observed in Sertoli cells and inside the germinal cysts containing spermatogonia and primary spermatocytes (Batlouni *et al*. 2005). In both fish and mammals, the expression of connexin 43 in Sertoli cells varies according to the stage of germ cell development, suggesting that a particular group of germ cells can modulate this protein expression in somatic cells (Pointis *et al*., 2010). However, in rainbow trout testis connexin 43 is temporally expressed in some specific spermatogenic cysts (de Montgolfier *et al*., 2007), whereas in guinea pig and mink this protein seems to play a role during the translocation of early spermatocytes into the adluminal compartment (Pelletier, 1995).

Concluding remarks

Although most available studies are focused on few mammalian species, particularly laboratory rodents, in this review we attempted to highlight and discuss several key aspects related to Sertoli cell biology in vertebrates. From the several topics and parameters evaluated, we can observe that, in order to accomplish their key functions in supporting the development of full spermatogenesis across vertebrates, Sertoli cells present huge and intrinsic plasticity, especially when cystic and non-cystic spermatogenesis is compared. In particular, besides the importance of knowing the regulation of Sertoli cells functions, the better understanding of the fine mechanisms related to Sertoli cell proliferation and efficiency, as well as for spermatogonial stem cell niche regulation, would significantly improve our knowledge that could be applied for instance to conservation and biotechnological approaches. Importantly, we would also greatly benefit from investigations using different experimental models, in which we could address for instance the intricate relationship between Sertoli and germ cells, mainly now that there is a big concern regarding the decline in human sperm counts caused by a multitude of factors.

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