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ESTUDO COMPARADO DA FUNÇÃO TESTICULAR EM DIFERENTES LINHAGENS DE CAMUNDONGOS NA MATURIDADE SEXUAL E AO LONGO DO DESENVOLVIMENTO PÓS-NATAL

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Às **quatorze horas** do dia **20 de fevereiro de 2020**, 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: **"ESTUDO COMPARADO DA FUNÇÃO TESTICULAR EM DIFERENTES LINHAGENS DE CAMUNDONGOS NA MATURIDADE SEXUAL E AO LONGO DO DESENVOLVIMENTO PÓS-NATAL"**, requisito final para obtenção do grau de Doutora em Biologia Celular. Abrindo a sessão, a Presidente da Comissão, **Dra. Gleide Fernandes de Avelar**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Gleide Fernandes de Avelar	UFMG	APROVADA
Dra. Mariana Machado Neves	UFV	APROVADA
Dr. Ralph Gruppi Thomé	UFSJ	APROVADA
Dr. Hélio Chiarini-Garcia	UFMG	Aprovada
Dra. Amanda Maria Sena Reis	UFMG	Aprovada

Pelas indicações, a candidata foi considerada: APROVADA
O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 20 de fevereiro de 2020.**

Drª. Gleide Fernandes de Avelar (Orientadora) Gleide Fernandes Avelar.

Drª. Mariana Machado Neves

Dr. Ralph Gruppi Thomé

Dr. Hélio Chiarini-Garcia

Drª. Amanda Maria Sena Reis Amanda Maria Sena Reis

Prof. Erika Cristina Jorge
Coordenadora do Programa de Pós Graduação em Biologia Celular ICB/UFMG

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

Erika Cristina Jorge

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“O real não está na saída nem na chegada:
ele se dispõe para a gente é
no meio da travessia.”

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RESUMO

Camundongos são importantes modelos experimentais para o estudo de aspectos reprodutivos em mamíferos, principalmente devido ao seu baixo custo de manutenção, alta taxa proliferativa, rápido ciclo reprodutivo, além da possibilidade de geração de transgênicos para fins específicos através de manipulação genética. Dessa forma, o estudo comparado das linhagens fornece subsídio para o entendimento das peculiaridades testiculares de cada uma delas, de maneira a propiciar que sejam selecionadas de acordo com suas características específicas para abordagens experimentais aplicadas em reprodução. Além disso, camundongos geneticamente mutados permitem o estudo da função de genes específicos nos testículos. Nesse contexto, na presente tese, tivemos como objetivos principais avaliar, comparativamente, aspectos reprodutivos em diferentes linhagens de camundongos durante o desenvolvimento pós-natal e na maturidade sexual (70 dias). No primeiro artigo, avaliamos, através de morfometria, imunohistoquímica, dosagem hormonal e injeção de azul de tripan, diversos parâmetros reprodutivos em três linhagens de camundongos adultos frequentemente utilizadas na literatura (C57BL6, *Swiss* e BALB/c). Nesse trabalho, nós observamos que a linhagem *outbred Swiss* foi a que apresentou mais diferenças em relação às demais, especialmente quando comparada à linhagem C57BL6. Ademais, ao quantificarmos os macrófagos testiculares em cada uma das linhagens investigadas, observamos uma proporção muito maior dessas células do que aquela até então descrita na literatura para camundongos (entre 0,63 e 1,26 células de Leydig/macrófago), reforçando a importância desses tipos celulares no testículo. Interessantemente, tal proporção de macrófagos alterou entre as linhagens, demonstrando que o *background* genético impacta neste parâmetro. Além disso, identificamos que os testículos das diferentes linhagens de camundongos apresentam composições celulares, dosagem hormonal e morfologias distintas, o que reforça a importância do conhecimento acerca das diferenças e perculiaridades de cada linhagem para a correta seleção e uso em estudos envolvendo parâmetros específicos ou tratamentos em reprodução. No segundo artigo, investigamos, por meio de morfometria, imunohistoquímica e qPCR, a função dos genes *Foxn1* e *Prkdc* nos testículos de camundongos adultos. Utilizando linhagens que apresentam mutação nesses genes (*nude* e *scid*), identificamos que o fator de transcrição Foxn1 parece envolvido na regulação da população e função das células de Leydig (CL) no testículo, já que os camundongos *nude* apresentaram CL menores, mas em maior população e com expressões mais altas de genes relacionados à esteroidogênese. Já a

subunidade catalítica da proteína de reparo de quebra de fita dupla de DNA (DNA-PKc) parece ter um papel relevante na regulação das células de Sertoli e da espermatogênese, já que observamos, em camundongos *scid*, um aumento no número dessas células somáticas, bem como na produção espermática diária, apesar das maiores perdas celulares observadas ao longo do processo espermatogênico, especialmente na meiose. Diante das alterações observadas nos testículos de camundongos *nude* na maturidade sexual, especialmente quanto aos parâmetros de CL, no terceiro artigo desta tese, investigamos, através de morfometria, imunohistoquímica e dosagem hormonal, a função testicular de camundongos portadores de mutação no gene *Foxn1* ao longo do desenvolvimento pós-natal (1 a 25 dias pós-natal - DPN) e, em algumas análises, também na maturidade sexual (70DPN). Nesse estudo, nós observamos que, em geral, as alterações testiculares encontradas para os camundongos *nude* no desenvolvimento pós-natal não seguiram o mesmo padrão visto para a maturidade sexual. Além disso, os resultados demonstram que Foxn1 parece afetar de maneiras distintas as diferentes populações de CL e que os efeitos da expressão prejudicada de Foxn1 são mais expressivos no final do desenvolvimento (a partir de 10DPN) e na maturidade sexual, coincidindo com o período em que a maior parte das CL são do tipo adulta. O padrão de expressão de Foxn1, que aumenta sua frequência e intensidade ao longo do desenvolvimento pós-natal, bem como a dupla marcação de Foxn1 e Hsd17b3, evidenciam a possibilidade desse fator de transcrição ser um marcador específico das CL adultas. Por outro lado, a identificação de que algumas CL expressam apenas Foxn1 ou Hsd17b3, isoladamente, reforça a existência de distintas populações de CL, de acordo com seu padrão de expressão de proteínas.

Palavras-chave: camundongos C57BL6, *Swiss* e BALB/c, genes *Foxn1* e *Prkdc*, espermatogênese, células de Sertoli, células de Leydig, desenvolvimento testicular.

ABSTRACT

Mice are important experimental models for studies involving reproductive aspects in mammals, mainly due to its low maintenance cost, high prolificacy, fast reproductive cycle, besides the possibility of genetic manipulation generating transgenic mice for specific objectives. Thus, strain comparative study provides background for the understanding of their testicular peculiarities, which enables their selection according to their own characteristics for applied experimental approaches in reproduction. Moreover, genetically mutated mice allow the study of specific gene functions in the testis. In this sense, our main goals in this thesis were to comparatively evaluate reproductive aspects in different mice strains along post-natal development and in sexual maturity (70 days). In the first paper, we evaluated, using morphometry, immunohistochemistry, hormone plasmatic levels and trypan blue injection, several reproductive parameters in three adult mice strains frequently used in the literature (C57BL6, Swiss and BALB/c). In this work we observed that the outbred Swiss strain was the one that presented most of the differences in comparison to the others, especially C57BL6. Furthermore, we quantified the testicular macrophages in each investigated strain and found a much higher proportion of these cells than the one so far described in the literature (between 0.63 and 1.26 Leydig cell/macrophage), reinforcing the importance of these cells in the testis. Interestingly, this macrophage proportion altered between the strains, indicating that genetic background impacts this parameter. Moreover, we identified that the testis from the different mice strains present distinct cell compositions, hormone levels and morphology, reinforcing the importance of the knowledge about the differences and peculiarities that each strain possesses in order to properly select and use them in studies involving specific parameters or treatments in reproduction. In the second paper, we investigated, through morphometry, immunohistochemistry and qPCR, *Foxn1* and *Prkdc* genes functions in adult mice testis. Using mice strains that present mutation in these genes (nude and scid), we identified that Foxn1 transcription factor seem to be involved in the regulation of Leydig cells (LC) population and function, since nude mice present smaller LC, but with a higher population and higher expression of steroidogenesis-related genes. On the other hand, the catalytic subunit of the DNA double-strand break repair protein (DNA-PKc) seem to have a relevant role on the regulation of Sertoli cells and spermatogenesis, once we observed, in scid mice, a higher number of these somatic cells, followed by a higher daily sperm production, even though they have higher germ cell loss along spermatogenesis, especially in meiosis. Once we

observed several testicular alterations in nude mice in sexual maturity, especially regarding LC parameters, in the third paper from this thesis, we investigated, using morphometry, immunohistochemistry and hormone plasmatic levels, the testis function of *Foxn1* mutated mice along post-natal development (1 to 25 post-natal days – Pnd) and, in some analysis, also in sexual maturity (70Pnd). In this study, we observed that, in general, the testicular alterations found for nude mice along post-natal development did not follow the same pattern seen in sexual maturity. Furthermore, the results show that Foxn1 seem to affect distinctively the different LC populations and the effect of impaired Foxn1 expression are more significant at late development (from 10Pnd onwards) and at sexual maturity, coinciding with the period when the majority of the LC are the adult type. Foxn1 expression pattern, which increases its frequency and intensity along post-natal development, as well as the double staining of Foxn1 and Hsd17b3, evidence that this transcription factor might be a specific adult LC marker. On the other hand, the identification that some LC express only Foxn1 or Hsd17b3, separately, reinforces the existence of distinct LC populations, according to its protein expression pattern.

Key words: C57BL6, Swiss and BALB/c mice, *Foxn1* and *Prkdc* genes, spermatogenesis Sertoli cell, Leydig cell, testis development.

LISTA DE ABREVIATURAS

3β-HSD ou Hsd3b = 3β-hydroxisteroide desidrogenase (*3β-hydroxysteroid dehydrogenase*)

17β-HSD3 ou Hsd17b3 = 17β-hydroxisteroide desidrogenase tipo 3 (*17β-hydroxysteroid dehydrogenase type 3*)

AR = receptor de andrógeno (*androgen receptor*)

cDNA = acido desoxirribonucléico complementar (*complementary deoxyribonucleic acid*)

CES ou SEC = ciclo do epitélio seminífero (*seminiferous epithelium cycle*)

CGP ou PGC = célula germinativa primordial (*primordial germ cells*)

CL ou LC = célula de Leydig (*Leydig cell*)

CLA ou ALC = célula de Leydig adulta (*adult Leydig cell*)

CLF ou FLC = célula de Leydig fetal (*fetal Leydig cell*)

COUP-TFII = fator de transcrição II do promotor da ovoalbumina de galinha (*chicken ovalbumin upstream promoter-transcription factor II*)

CS ou SC = célula de Sertoli (*Sertoli cell*)

DAB = diaminobenzidina (*diaminobenzidine*)

Dhh = *desert hedgehog*

DHT = diidrotestosterona (*dihydrotestosterone*)

DNA-PK = proteína quinase ativada por DNA (*DNA-dependent protein kinase*)

DNA-PKc = subunidade catalítica da proteína quinase ativada por DNA (*catalytic subunit of the DNA-dependent protein kinase*)

DPC = dias pós-coito

DPN ou Pnd = dias pós-natal (*postnatal days*)

DSP = produção espermática diária (*daily sperm production*)

EDS = sulfonato dimetano etano (*ethane dimethane sulfonate*)

EPM ou SEM = erro padrão da média (*standard error mean*)

Fshb = subunidade beta do hormônio folículo estimulante (*follicle-stimulating hormone beta subunit*)

Foxn1 = *forkhead box protein N1*

Foxl2 = *forkhead box protein L2*

Foxo1 = *forkhead box protein O1*

Foxp3 = *forkhead box protein P3*

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

GH = hormônio do crescimento (*growth hormone*)

GnRH = hormônio liberador de gonadotrofinas (*gonadotropin-releasing hormone*)

IGF1 = fator de crescimento semelhante à insulina tipo-1 (*insulin-like growth factor 1*)

IGS = índice gonadossomático (*gonadosomatic index*)

iNOS = óxido nítrico sintase induzível (*inducible nitric oxide synthase*)

i.p. = injeção intraperitoneal (*intraperitoneal injection*)

LH = hormônio luteinizante (*luteinizing hormone*)

MPW = janela de masculinização (*masculinization programming window*)

NHEJ = junção de extremidades não-homólogas (*non-homologous end joining*)

PCR = reação em cadeia da polimerase (*polymerase chain reaction*)

PDGF-A = fator de crescimento derivado de plaqueta (*platelet-derived growth factor*)

PS = espermatócito em paquíteno (*pachytene spermatocyte*)

PTMC = célula peritubular mioide (*peritubular myoid cell*)

qPCR = PCR quantitativa ou PCR em tempo real (*quantitative or real time PCR*)

RNAm ou mRNA = ácido ribonucléico mensageiro (*messenger ribonucleic acid*)

RPM = rotações por minuto (*rotations per minute*)

SCID = imunodeficiência severa combinada (*severe combined immunodeficiency*)

SF-1 = fator esteroidogênico 1 (*steroidogenic factor 1*)

StAR = proteína reguladora aguda da esteroidogênese (*steroidogenic acute regulatory protein*)

TGF = fator de crescimento e transformação (*transforming growth factor*)

WT = selvagem (*wild type*)

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1. INTRODUÇÃO

1.1. DESENVOLVIMENTO DO TESTÍCULO

Em camundongos, a crista urogenital origina-se do mesoderma intermediário cerca de 9,5 dias pós-coito (dpc) e, aos 10 dpc, as gônadas começam a se desenvolver a partir da superfície ventromedial do mesonefro (Tilmann & Capel, 2002). Nesta fase, a gonada é bipotencial, com capacidade para se diferenciar em testículo ou em ovário, a depender da expressão de genes específicos durante o desenvolvimento (Greenfield, 2015). A célula de Sertoli é o primeiro elemento somático a se diferenciar no testículo, originando-se de células do epitélio celomático. Suas precursoras expressam o gene determinante sexual *Sry*, aos 10,5 dpc, o qual é um fator de transcrição responsável por iniciar uma cascata de ativação de genes específicos do testículo e repressão do desenvolvimento ovariano, direcionando a gônada à via de diferenciação masculina (Karl & Capel, 1998; Capel, 2000). Durante a determinação sexual, *Sry* é importante para estimular a expressão do gene *Sox9*, outro fator de transcrição fundamental para a diferenciação das células de Sertoli e formação dos testículos (Barrionuevo *et al.* 2006; Sekido and Lovell-Badge 2008; Greenfield, 2015).

Por outro lado, as células germinativas primordiais (CGP) se originam externamente à crista urogenital, antes da formação das gônadas. As CGP têm origem no epiblasto adjacente ao mesoderma extra-embrionário cerca de 7,25 dpc (Bendel-Stenzel *et al.*, 1998; Ross & Capel, 2005) e migram da base do alantoide ao longo do intestino posterior para as cristas genitais, proliferando ativamente nesta fase. Quando alcançam a crista genital, cerca de 10 a 11 dpc (Ross & Capel, 2005), são envolvidas pelas células de Sertoli, formando assim os cordões seminíferos, que também têm a participação das células peritubulares mioides. A formação dos cordões seminíferos é considerada o primeiro indicador morfológico da diferenciação sexual (Bendel-Stenzel *et al*., 1998; França & Chiarini-Garcia, 2005; Ross & Capel, 2005).

Em relação às células de Leydig (CL), foram descritas duas populações deste tipo celular – uma fetal e outra adulta. A fetal se origina de uma população progenitora esteroidogênica aos 12,5 dpc em camundongos (Byskov, 1986; Barsoum & Yao, 2010). Tal tipo celular é importante para a secreção de andrógenos, requeridos para a diferenciação do sistema urogenital masculino durante a gestação (Huhtaniemi & Pelliniemi, 1992; Zimmermann *et al.*, 1999). A diferenciação das CL fetais é induzida através da produção de Desert hedgehog (Dhh) pela célula de Sertoli, a qual também tem o papel de estimular a

atividade esteroidogênica das CL fetais, que ainda é independente de gonadotrofinas (Pierucci-Alves *et al*., 2001; Yao *et al*., 2002; Griswold & Behringer, 2009).

Apesar de a população de CL fetal ainda estar presente no testículo adulto (Shima *et al*., 2015), 7 dias após o nascimento, uma segunda população de CL começa a se diferenciar (Baker *et al.*, 1999 ; Nef *et al*., 2000). Essa população corresponde às CL adultas, que produzem esteroides importantes para o desenvolvimento e função do sistema genital (Robaire *et al*., 2006; Risbridger & Taylor, 2006). Recentemente, Qin e colaboradores (2008) demonstraram a presença de precursoras das CL adultas no interstício testicular ainda durante o desenvolvimento fetal, o que comprova a presença de subtipos de CL adultas. Além disso, em 2018, Shima e colaboradores demonstraram que as CL fetais têm o potencial de se desdiferenciar na fase fetal e posteriormente formar populações de células de Leydig adultas, de células peritubulares mioides e de pericitos. Sendo que tanto as células peritubulares mioides quanto os pericitos, no testículo pós-natal, podem se rediferenciar em células de Leydig adultas, servindo como potenciais células tronco das CL adultas.

Durante sua diferenciação, as CL adultas passam por quatro fases diferentes: tronco, progenitora, imatura e adulta (Chen *et al*., 2010). Fatores tais como hormônio luteinizante (LH) (Huhtaniemi *et al*., 1981; Benton *et al*., 1995) e andrógenos (Buzek & Sanborn, 1988) são considerados reguladores da diferenciação dessas células. Por outro lado, os estrógenos produzidos pelas CL adultas são fatores importantes na inibição da diferenciação das precursoras (Jegou & Sharpe 1993; Sharpe 1993).

De maneira interessante, a produção de testosterona pelas populações fetal e adulta de células de Leydig apresenta particularidades. Enquanto as células adultas realizam todas as etapas da via esteroidogênica, as fetais não conseguem sintetizar testosterona devido à ausência da enzima 17βHSD3, que medeia a conversão de androstenediona em testosterona. No testículo fetal, tal enzima é expressa nas células de Sertoli e, desssa forma, a produção de andrógeno se dá por cooperação entre as células de Leydig fetais e as células de Sertoli (O'Shaughnessy *et al*., 2000; Shima *et al*., 2013).

1.2. ESTRUTURA TESTICULAR EM MAMÍFEROS

O testículo dos mamíferos eutérios é um órgão com forma arredondada ou ovoide, localizado no escroto, reúne funções endócrinas e exócrinas e está envolvido por uma cápsula de tecido conjuntivo fibroso, a túnica albugínea. O parênquima testicular possui dois compartimentos: o compartimento intersticial (intertubular) e o compartimento tubular. No primeiro estão localizadas as células de Leydig, vasos sanguíneos e linfáticos, células do tecido conjuntivo e outros tipos celulares como macrófagos (Russell *et al*., 1990). O

compartimento tubular constitui a maior parte do testículo, sendo os túbulos seminíferos compostos por túnica própria, epitélio seminífero e lume. A túnica própria é constituída de membrana basal e pelas células peritubulares mioides, que são contráteis e auxiliam na propulsão dos espermatozoides e de fluido ao longo dos túbulos (Maekawa *et al*., 1996; Losinno *et al*., 2016). As células peritubulares mioides, em conjunto com as células de Sertoli, produzem os componentes da membrana basal que formam a túnica própria, garantindo a integridade estrutural dos túbulos seminíferos (Thompson *et al*., 1995; Zhou *et al*., 2019). O epitélio seminífero é composto pelas células germinativas em diferentes estágios de diferenciação e pelas células de Sertoli, responsáveis pela secreção do fluido presente no lume tubular (Russell *et al*., 1990; Hess & França, 2008) .

1.3. ESPERMATOGÊNESE EM MAMÍFEROS

A espermatogênese é um processo cíclico que ocorre nos túbulos seminíferos, no qual espermatogônias diploides se dividem e se diferenciam para dar origem a espermatozoides maduros. Baseado em características morfológicas e funcionais, o processo espermatogênico pode ser dividido em três fases: fase proliferativa ou espermatogonial, caracterizada por várias e sucessivas divisões mitóticas dos diferentes tipos de espermatogônias; fase meiótica/espermatocitária, na qual ocorre a duplicação do DNA, a recombinação gênica e duas divisões meióticas (reducional/equacional) que resultam na formação de uma célula haploide denominada espermátide; e fase de diferenciação ou espermiogênica, na qual as espermátides arredondadas passam por drásticas alterações morfológicas e funcionais tais como a formação do acrossoma, do flagelo e a condensação nuclear, resultando na formação do espermatozoide (Russell *et al*., 1990; Sharpe, 1994; Hess & França 2008; Lara *et al*., 2018a).

Durante o processo espermatogênico em mamíferos, diferentes associações celulares, denominadas estádios, sucedem-se de forma ordenada formando o ciclo do epitélio seminífero (CES). Tais estádios podem ser classificados de acordo com o sistema acrossômico, o qual leva em consideração o desenvolvimento do acrossoma e a morfologia das espermátides, além das alterações no núcleo destas células e a ocorrência de divisões meióticas. Através do sistema acrossômico, foram definidos doze estádios para camundongos (Russell *et al*., 1990; Hess & França, 2008). Os estádios do CES são considerados relativamente constantes para uma mesma espécie, apesar de terem sido observadas, na literatura, variações em sua frequência em diferentes raças ou linhagens (França & Russell, 1998; Soares *et al*., 2009). A duração total da espermatogênese está sob controle do genótipo da célula germinativa (França *et al*., 1998) e é baseada em 4,5 ciclos espermatogênicos. O processo completo ocorre entre

30 e 78 dias na maioria dos mamíferos já investigados (França & Russell, 1998; Hess & França, 2008; Costa *et al*., 2010; Figueiredo *et al*., 2017).

1.4. REGULAÇÃO HORMONAL DA FUNÇÃO TESTICULAR

Os testículos são orgãos de função anfícrina, já que são responsáveis tanto pela produção e diferenciação das células da linhagem germinativa, quanto pela secreção de hormônios (Russell *et al*., 1990). A produção dos hormônios esteroides no testículo adulto ocorre através de estímulos do LH em receptores localizados na membrana citoplasmática das células de Leydig. O LH é uma glicoproteína sintetizada e secretada na adenohipófise, sob a influência do hormônio liberador de gonadotrofinas (GnRH) proveniente do hipotálamo. O controle de retroalimentação negativo do LH é exercido pela testosterona tanto na adenohipófise quanto no hipotálamo (Shupnik & Schreihofer, 1997). Os andrógenos atuam na diferenciação do trato genital masculino e da genitália externa na fase fetal (Pelliniemi *et al*., 1996), além de serem responsáveis pelo aparecimento dos caracteres sexuais secundários e manutenção quantitativa da espermatogênese (Sharpe, 1994; Zirkin *et al*., 1994; De Gendt *et al*., 2004). Na fase adulta, os andrógenos são importantes para diversos processos fisiológicos do testículo, como a manutenção das junções de oclusão da barreira de células de Sertoli, a continuidade da meiose e a espermiação das espermátides no lume tubular (Yeh *et al*., 2002; Holdcraft &Braun, 2004; Meng *et al*., 2005).

Assim como o LH, o hormônio folículo estimulante (FSH) é sintetizado na adenohipófise sob o estímulo do GnRH e atua nas células de Sertoli maduras estimulando a secreção de nutrientes e substâncias importantes para o desenvolvimento da espermatogênese, como o ácido retinoico (Santos & Kim, 2010). Além disso, as células de Sertoli interagem com as células germinativas e produzem inibina, que atua diminuindo a produção de GnRH no hipotálamo e de FSH na adenohipófise (Pineau *et al*., 1990).

1.5. CÉLULAS DE LEYDIG

De maneira geral, a célula de Leydig é o componente mais abundante do compartimento intertubular (Russell *et al*., 1990), e é fundamental para a produção de andrógenos (Bardin, 1996). A sua ultraestrutura evidencia a atividade esteroidogênica deste tipo celular, o qual apresenta retículo endoplasmático liso bem desenvolvido, numerosas gotículas lipídicas e mitocôndrias com cristas tubulares (Russell *et al*., 1996). A produção dos hormônios esteroides ocorre através de estímulos do LH em receptores localizados na membrana citoplasmática das células de Leydig. A síntese de testosterona se dá a partir do colesterol, captado da corrente sanguínea, que é translocado para a membrana interna da mitocôndria pela proteína reguladora da esteroidogênese aguda (StAR). Essa proteína é

expressa nas adrenais e gônadas e sua produção é induzida pelo LH. A via esteroidogênica (figura 1) se inicia na mitocôndria com a conversão do colesterol em pregnenolona envolvendo o citocromo P450. Os passos seguintes da via esteroidogênica ocorrem no retículo endoplasmático liso. A partir da formação da pregnenolona, existem duas vias paralelas. Na primeira, a enzima 3βHSD converte a pregnenolona em progesterona. Na segunda, a enzima 17α-hidroxilase converte a pregnenolona em 17-OH-pregnenolona. As duas vias levam à produção de androstenediona, que é convertida pela enzima 17βHSD em testosterona. Após sua síntese, a testosterona é liberada, seguindo para os órgãos periféricos por meio da circulação sanguínea. Na maioria das células-alvo a testosterona é convertida em diidrotestosterona (DHT) pela 5α-redutase. A DHT apresenta maior afinidade pelo receptor que a testosterona, sendo assim um potente andrógeno. Além disso, é possível a formação de estrógenos a partir da testosterona ou de seu precursor androstenediona por meio da ação da enzima aromatase (Colleta & Carvalho, 2005).

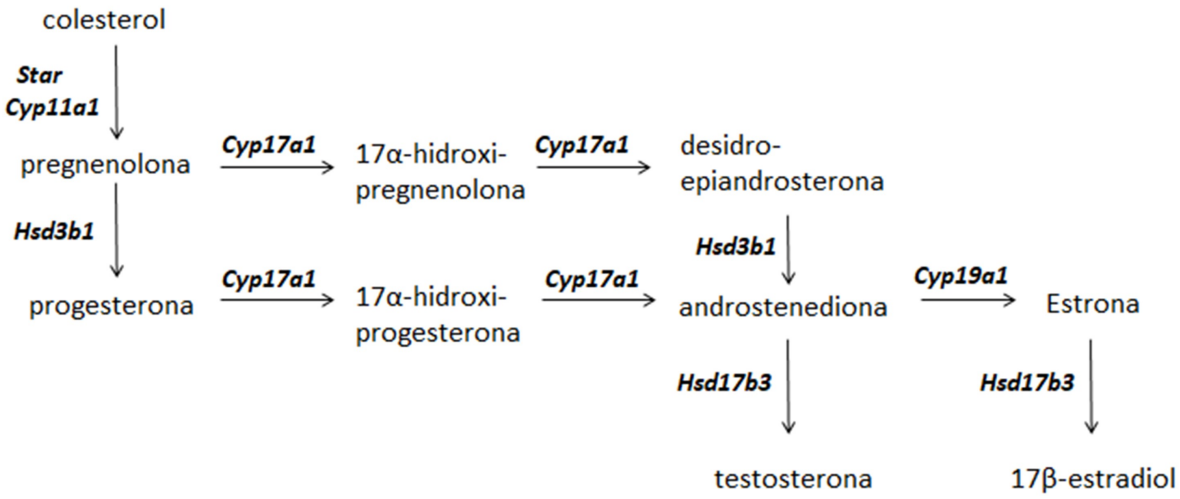

Figura 1: Via esteroidogênica nas gônadas, com indicação dos genes envolvidos em cada etapa biossintética e seus produtos finais. Fonte: adaptado de Miyabayashi *et al*., 2017.

Além da produção de esteroides sexuais, as células de Leydig também apresentam funções na homeostase testicular, principalmente na manutenção do ambiente testicular imunoprivilegiado. Tem sido sugerido que as CL podem mediar a regulação da população de leucócitos testiculares e assim, garantir a imunoregulação. As células de Leydig apresentam receptores específicos em sua membrana citoplasmática que as permite interagir com os linfócitos, sendo as únicas células endócrinas diferenciadas que espontâneamente formam

rosetas com os linfócitos (Rivenzon *et al*., 1974). Essa relação complexa entre tais tipos celulares está envolvida na manutenção da condição testicular como um ambiente imunologicamente protegido, onde as respostas imunes são limitadas em condições fisiológicas. Isso ocorre devido aos fatores imunomoduladores secretados pelas células de Leydig, como os andrógenos, que atuam como imunossupressores (Diemer *et al*., 2003; Lara *et al*., 2018b).

1.6. MACRÓFAGOS TESTICULARES

Os macrófagos são células do sistema imune inato presentes em basicamente todos os orgãos do corpo, apresentando funções específicas. No testículo da maior parte das espécies já investigadas, os macrófagos são o segundo tipo celular mais abundante no interstício, sendo as células de Leydig as mais prevalentes neste compartimento testicular. A partir de estudos realizados em ratos, estima-se uma razão em torno de um macrófago para cada três a cinco células de Leydig no testículo (Ewing *et al*., 1979; Miller, 1982; Bergh, 1985; Niemi *et al*., 1986; Diemer *et al*., 2003).

Recentemente, duas populações distintas de macrófagos testiculares foram descritas baseado em sua localização, morfologia, padrão de expressão gênica, origem embrionária e desenvolvimento pós-natal: macrófagos peritubulares, na região adjacente aos túbulos seminíferos; e macrófagos intersticiais, próximos às células de Leydig no intertúbulo (DeFalco *et al*., 2015; Mossadegh-Keller *et al*., 2017). Além de sua localização diferencial, as duas populações também podem ser distinguidas por sua morfologia e expressão gênica. Os macrófagos intersticiais são arredondados e apresentam um padrão $MCSFR^{+}MHCII^{-}$, enquanto os macrófagos peritubulares são células alongadas e expressam os marcadores $M\text{-}CSFR^{lo}MHCII^{+}$ (Mossadegh-Keller *et al.*, 2017).

Durante o desenvolvimento testicular, os macrófagos intersticiais são os primeiros a se diferenciarem, derivando de células progenitoras do saco vitelino e sendo identificados na crista urogenital aos 10,5 dpc (DeFalco *et al*., 2014). Já os macrófagos peritubulares aparecem apenas 2 semanas após o nascimento e derivam exclusivamente de progenitores da medula óssea (Mossadegh-Keller *et al*., 2017). Apesar das altas taxas proliferativas durante o desenvolvimento; no testículo adulto os macrófagos residentes apresentam uma baixa taxa de mitose em condições de homestase.

Os macrófagos apresentam diversas funções no testículo, como na regulação do nicho de espermatogônias tronco (DeFalco *et al*., 2015), esteroidogênese e proliferação de células de Leydig (Hutson *et al*., 2006) e na organogênese e vascularização do testículo durante o desenvolvimento (DeFalco *et al*., 2014). Interessantemente, a depleção dos macrófagos

testiculares no período embrionário demonstrou que estes tipos celulares são fundamentais para o estabelecimento da vascularização normal e formação dos cordões testiculares, uma vez que os testículos nos quais os macrófagos estavam ausentes exibiram uma rede vascular desorganizada, indicando que a remodelação de vasos mediada por macrófagos é crítica para a organogênese normal do testículo (DeFalco *et al*., 2014).

Os macrófagos intersticiais apresentam uma íntima interação celular com as células de Leydig adjacentes através de associações de membrana chamadas digitações, as quais funcionam como locais de ancoragem para a troca de fatores entre as duas células (Miller *et al*., 1983; Hutson, 2006). Devido a esta associação, os macrófagos intersticiais são capazes de influenciar a proliferação, diferenciação e esteroidogênese nas células de Leydig (Cohen *et al*., 1997; Nes *et al*., 2000; Hutson *et al*, 2006; DeFalco *et al*., 2014). Os macrófagos intersticiais, por exemplo, secretam 25-hidroxicolesterol, o qual pode ser utilizado pelas células de Leydig para a síntese de testosterona (Nes *et al*., 2000; Hutson, 2006). Uma vez que o colesterol é uma molécula apolar, durante a esteroidogênese, a enzima StAR realiza sua hidroxilação para que possa ser translocado para a membrana interna da mitocôndria. Dessa forma, ao secretar 25-hidroxicolesterol, os macrófagos fornecem uma via alternativa para a esteroidogênese, independente da StAR. Corroborando com este achado, após depleção transiente dos macrófagos no testículo de camundongos adultos, DeFalco e colaboradores, 2014, observaram uma redução de 50% nos níveis de testosterona intratesticular.

Além disso, sabe-se que os macrófagos têm um papel na regulação da proliferação das CL. Gaytan e colaboradores, 1994, observaram que, após depleção seletiva dos macrófagos, pela injeção de liposomas de difosfonato de diclorometileno nos testículos de ratos ao longo do desenvolvimento pós-natal, houve uma concomitante e significativa redução no número de células de Leydig, demonstrando a importância dos macrófagos na homeostase da população destas células esteroidogênicas.

1.7. CÉLULAS DE SERTOLI

A célula de Sertoli é o tipo celular somático presente nos túbulos seminíferos, interagindo com as células germinativas de maneira bastante complexa, tanto física quanto bioquimicamente. As junções de oclusão entre células de Sertoli adjacentes dividem o epitélio seminífero em compartimentos basal e adluminal (Hess & França, 2008; Lara *et al*., 2018b). No compartimento basal estão localizadas as espermatogônias e espermatócitos primários iniciais (pré-leptótenos/leptótenos), enquanto no adluminal encontram-se os espermatócitos primários a partir de zigóteno, espermatócitos secundários e espermátides. Funcionalmente, as junções de oclusão, formadas por ocludinas e claudinas, compõem a barreira de células de

Sertoli que propicia um microambiente específico e imunoprivilegiado, essencial para o desenvolvimento do processo espermatogênico (Russell *et al*., 1990; Hess & França, 2008; Lara *et al*., 2018b). Especialmente durante a meiose, as células germinativas passam a expressar diferentes antígenos de superfície, que podem ser detectados pelo sistema imune e levar a uma reação autoimune. Por isso, a barreira de células de Sertoli ajuda a criar um ambiente imunoprivilegiado, no qual antígenos autoimunogênicos podem ser tolerados, garantindo o desenvolvimento das células germinativas e a continuidade do processo espermatogênico (Cheng & Mruk, 2012; Lara *et al*., 2018b).

Além da formação da barreira, as células de Sertoli desempenham outras funções essenciais para o desenvolvimento das células germinativas, como o fornecimento de nutrientes e inúmeros outros fatores importantes, suporte físico (sustentação) para as células espermatogênicas, fagocitose do excesso de citoplasma (corpos residuais) resultante da liberação das células espermiadas, bem como das células germinativas que sofrem apoptose, além de importante função de intermediação hormonal na espermatogênese através de receptores para esteroides (andrógenos e estrógenos) e para FSH (Griswold, 1993; Zirkin *et al*.,1994; O'Shaughnessy, 2014; Lara *et al*., 2018a). Ademais, as células de Sertoli participam ativamente no processo de espermiação das espermátides para o lume tubular, além de serem responsáveis pela secreção de fluido, o qual possui substâncias importantes para a função epididimária e maturação espermática, servindo também de veículo para o transporte dos espermatozoides.

Em camundongos, assim como na maioria dos mamíferos já investigados, as células de Sertoli proliferam ativamente durante o período perinatal e pré-púbere (Orth, 1984; Orth, 1993; McCoard *et al*., 2003; Lara *et al*., 2018a). Os mecanismos regulatórios responsáveis por esta proliferação das células de Sertoli envolvem estímulos de FSH, andrógenos e, recentemente foi identificado o papel dos receptores de IGF1 e de insulina para a determinação do tamanho do testículo (Orth, 1984; Heckert & Griswold, 2002, Johnston *et al*., 2004; Scott *et al*., 2007, 2008; Auharek *et al*., 2012; Pitetti *et al*., 2013). Devido ao fato de cada célula de Sertoli suportar um número limitado de células germinativas, o número total destas células, que é estabelecido durante o período proliferativo, determina o tamanho do testículo e o número final de espermatozoides produzidos e, de maneira geral, a eficiência reprodutiva nos machos (França *et al*., 2000; Leal e França, 2006). Embora tenha sido observado recentemente uma pequena população de células de Sertoli localizadas na região de transição dos túbulos seminíferos com a rede testicular e que retêm a capacidade de proliferar

no testículo adulto (Meachem *et al*., 2005; Figueiredo *et al*., 2016), nos testículos dos mamíferos sexualmente maduros, as células de Sertoli estão completamente diferenciadas.

1.8. CAMUNDONGOS PORTADORES DE MUTAÇÃO NO GENE *Foxn1*

A descrição da mutação no gene *Foxn1* foi feita pela primeira vez por Flanagan (1966), que relatou a descoberta de camundongo mutante espontâneo cujo fenótipo não possuía pelos no corpo (*nude*). Com intuito de se estudar as características desses camundongos, as matrizes heterozigotas que deram origem ao mutante foram mantidas e acasaladas, o que permitiu a criação de um estoque deste fenótipo *nude*. Flanagan ainda observou que havia maior mortalidade nos animais sem pelos, o que foi posteriormente corroborado pela constatação de que esses animais eram atímicos, apresentando deficiência de linfócitos T (Pantelouris, 1968). Outros estudos demonstraram que o fenótipo dos camundongos *nude* é resultado de mutação no mesmo gene (*Foxn1*), que apresenta efeitos pleiotrópicos (Eaton, 1976; Nehls *et al*., 1996).

O gene *Foxn1*, cujo nome oficial é *forkhead box N1*, está localizado no cromossomo 11 em camundongos (Byrd, 1993; Shultz *et al*., 1978; Lisitsyn *et al*., 1994; Segre *et al*., 1995; Schorpp *et al*., 1997) e codifica o fator de transcrição Foxn1, da família winged helix/forkhead (Takahashi *et al*., 1992; Byrd, 1993; Nehls *et al*., 1994). A proteína Foxn1 compreende um domínio de ativação transcricional C-terminal e um domínio de ligação ao DNA (Brissette *et al*., 1996; Schlake, 2001). A perda da função de um (ou dos dois) domínio(s) leva ao fenótipo *nude*. Os camundongos *nude* são portadores de mutação autossômica recessiva no gene *Foxn1*, sendo, portanto, animais homozigotos recessivos *(Foxn1*-/-). Essa mutação consiste numa deleção de um par de bases (G) no exon 3, que leva à introdução de um *stop* códon prematuro. Sendo assim, é produzida uma proteína inativa (Schlake, 2001).

Uma vez que os camundongos homozigotos são altamente susceptíveis a infecções por um amplo espectro de patógenos, eles devem se mantidos isolados, num ambiente livre de contaminações. Sob essas condições, sua vida é prolongada, equivalendo às linhagens normais (Rygaard e Friis, 1974). Por outro lado, a ausência do timo e o consequente comprometimento do sistema imune, fazem desses animais importantes modelos experimentais usados em pesquisas em oncologia, crescimento de tumores, transplantes e imunologia, especialmente devido à não rejeição (Zeng *et al*., 2006; Ding & Tredget, 2017).

Existem poucas observações acerca da histologia testicular de camundongos *nude* na literatura. Shire e Pantelouris (1974) observaram a ocorrência de distúrbio na maturação das

espermátides, enquanto Ruitenberg e Berkvens (1977) e Rebar e colaboradores (1982) encontraram morfologia anormal do epitélio germinativo e concentrações séricas de gonadotrofinas e testosterona reduzidas. Além disso, Masahiro e colaboradores (1997) também observaram degeneração de células germinativas. Apesar da escassez de estudos na literatura, outras proteínas da família winged helix/forkhead já foram reportadas como reguladoras de genes envolvidos com a reprodução e com a síntese de gonadotropinas (FSH e LH) (Uhlenhaut & Treier, 2011; Thackray, 2014). O Foxl2, por exemplo, tem um papel importante na determinação sexual da via feminina e é requerido para a expressão da subunidade beta do FSH (Justice *et al*., 2011). Além disso, Foxo1 é importante para a auto-renovação espermatogonial, além de modular a expressão da subunidade beta do FSH (Goertz *et al*., 2011; Skarra *et al*., 2013) enquanto o Foxp3 é relevante para a espermatogênese e fertilidade (Jasurda *et al*., 2014). Logo, uma vez que alterações reprodutivas em camundongos *nude* já foram relatadas e seguindo a tendência das demais proteínas da família, Foxn1 tem o potencial de atuar regulando a produção hormonal e a reprodução.

1.9. CAMUNDONGOS PORTADORES DE MUTAÇÃO NO GENE *Prkdc*

Imunodeficiência severa combinada (SCID) é uma síndrome que resulta na perda da imunidade por linfócitos T e B. Tal síndrome é observada em diversas espécies, como cavalos, cães e humanos (Buckley, 2004; Perryman, 2004). Em camundongos, foi identificada pela primeira vez por Bosma e colaboradores em 1983, a partir de um grupo de animais de linhagem BALB/c congênica (C.B-17). Posteriormente, observou-se que esses camundongos eram severamente deficientes em linfócitos B e T e altamente susceptíveis a infecções por causa da sua inabilidade de gerar resposta imune antígeno-específica (Schuler *et al*., 1986; Malynn *et al*., 1988; Blackwell *et al*., 1989; Lieber *et al*., 1989; Bosma & Carroll, 1991).

Em 1989, Bosma e colaboradores concluíram que a condição observada nesses camundongos ocorria devido a uma mutação recessiva no cromossomo 16, responsável pela produção de uma subunidade da enzima envolvida no reparo de quebra de fita dupla de DNA (DNA-PK - proteína quinase ativada por DNA). A DNA-PK é composta por duas subunidades, uma catalítica (DNA-PKc), codificada pelo gene *Prkdc*, e outra heterodimérica (Ku70/80) responsável pelo reconhecimento do DNA. Para que possa atuar no reparo de quebra de fita dupla de DNA, a proteína DNA-PK necessita da ação das duas subunidades, sendo que a Ku70/80 atua reconhecendo as extremidades de DNA livres e recrutando a subunidade catalítica (DNA-PKc) que, após fosforilada, se torna ativa. Em seguida, as extremidades livres de DNA são processadas e ligadas novamente por um complexo proteico

de reparo e a integridade do DNA é restaurada. Esse tipo de reparo é denominado junção de extremidades não-homólogas (NHEJ) (Chang *et al*., 2017). A enzima DNA-PK apresenta importante papel na recombinação dos segmentos variável (V), diversidade (D) e junção (J) dos genes de imunoglobulinas e de receptores de células T. Essa recombinação V(D)J é um processo que utiliza NHEJ para promover a diversidade do sistema imune. Uma vez que os camundongos *scid* não completam a recombinação gênica V(D)J, as suas células T e B não se tornam maduras (Lieber *et al*., 1989).

Pelo fato de apresentarem imunodeficiência mais severa que os camundongos *nude*, os camundongos *scid* suportam melhor enxertos e transplantes de células, tecidos e tumores, funcionando como importantes modelos experimentais (Campos-Junior *et al*., 2014; Rodriguez-Sosa *et al*., 2014) . Devido à sua imunodeficiência, esses camundongos devem ser mantidos em ambiente livre de patógenos e de infecções para ter sua expectativa de vida semelhante aos camundongos normais.

Apesar do fenótipo apresentado pelos camundongos *scid* representar potencial impacto sobre a gametogênese, haja vista a intensa atividade proliferativa das células no processo espermatogênico, a literatura disponível acerca dos aspectos reprodutivos deste genótipo é ainda escassa. Mesmo sendo considerados férteis, Hamer e colaboradores (2003) observaram que camundongos *scid* machos apresentam aumento na ocorrência de apoptose de espermatócitos em paquíteno no estádio IV do CES. Nesta fase ocorre um ponto de checagem da meiose, no qual possíveis danos ao DNA são identificados e podem ser reparados pela maquinaria de reparo de DNA. Caso os erros não sejam eficientemente corrigidos, as células são induzidas a sofrer apoptose a fim de evitar a propagação dos danos às células filhas. Por isso, o aumento de apoptose observado nos camundongos *scid* nesta fase do CES parece ocorrer em função da inativação da subunidade catalítica da proteína de reparo de quebra de fita dupla de DNA, DNA-PK, nesta linhagem (Hamer *et al*., 2003; De Rooij & De Boer, 2003).

2. OBJETIVOS

2.1. OBJETIVOS GERAIS

Caracterizar a estrutura e a função testiculares em camundongos de diferentes linhagens selvagens (BALB/c, C57BL6, *Swiss*) e portadoras de mutação (Foxn1$^{-/-}$ *nude* e Prkdc$^{-/-}$ *scid*), durante o desenvolvimento pós-natal e na maturidade sexual.

2.2. OBJETIVOS ESPECÍFICOS

Artigo 1 - Comparative testis structure and function in three representative mice strains – Analisar comparativamente a função testicular em camundongos selvagens das linhagens BALB/c, C57BL6 e *Swiss* na maturidade sexual (70 dias pós-natal - DPN), em particular quanto aos seguintes aspectos:

- Obter dados biométricos (peso corporal, peso testicular, peso da vesícula seminal e do epidídimo) dos camundongos avaliados, para subsequente determinação do índice gonadossomático, bem como obter a distância anogenital para determinação do índice anogenital;
- Avaliar, por meio de morfometria, os seguintes parâmetros testiculares de camundongos das linhagens investigadas: diâmetro tubular e do lume e altura do epitélio seminífero; proporção volumétrica dos componentes testiculares; proporção núcleo citoplasma e diâmetro das células de Leydig; número de células de Sertoli e de Leydig por testículo e por grama de testículo; volume celular das células de Leydig; números celulares, obtidos por contagem no estádio VII do CES, para o cálculo da eficiência de células de Sertoli e do índice meiótico; comprimento total de túbulos seminíferos por testículo e por grama de testículo, bem como a produção espermática diária por testículo e por grama de testículo;
- Avaliar a frequência dos estádios do CES, e determinar, por meio da injeção de timidina triciada, a duração do CES e da espermatogênese, bem como a eficiência espermatogênica;
- Avaliar os volumes nuclear e celular bem como quantificar o número de macrófagos testiculares nas três linhagens avaliadas;
- Quantificar a intensidade da marcação obtida para a enzima 3βHSD nas células de Leydig por meio da avaliação da intensidade de pixels;
- Determinar a concentração plasmática dos esteroides sexuais testosterona e estradiol.

Artigo 2 – *Foxn1* and *Prkdc* genes are important for testis function: evidence from nude and scid adult mice – Analisar a função testicular em camundongos portadores de mutação nos genes *Foxn1* e *Prkdc*, em comparação ao BALB/c selvagem, na maturidade sexual (70DPN), em particular quanto aos seguintes aspectos:

- Obter dados biométricos (peso corporal e peso testicular) dos camundongos avaliados, para subsequente determinação do índice gonadossomático;
- Avaliar, por meio de morfometria, os seguintes parâmetros testiculares: diâmetro tubular e do lume e altura do epitélio seminífero; proporção volumétrica dos componentes testiculares; proporção núcleo citoplasma e diâmetro das células de Leydig; número de células de Sertoli e de Leydig por testículo e por grama de testículo; volumes nuclear, citoplasmático e celular das células de Leydig; números celulares, obtidos por contagem no estádio VII do CES, para o cálculo da eficiência de células de Sertoli e índice meiótico; comprimento total de túbulos seminíferos por testículo e por grama de testículo, bem como a produção espermática diária por testículo e por grama de testículo;
- Investigar a ocorrência e localização das proteínas Foxn1 e DNA-PKc nos testículos de camundongos BALB/c selvagens e portadores de mutação nesses genes;
- Avaliar a expressão gênica e ocorrência das proteínas receptor de andrógeno (AR), aromatase e 3βHSD nos testículos dos animais portadores de mutação;
- Quantificar a intensidade de marcação obtida para as proteínas AR, 3βHSD e aromatase por meio da avaliação da intensidade de pixels.

Artigo 3 - Impaired *Foxn1* gene expression affects normal testis post-natal development – Analisar a função testicular em camundongos portadores de mutação no gene *Foxn1*, em comparação ao BALB/c selvagem, em idades marcadas por eventos significativos do desenvolvimento pós-natal (1, 5, 10, 15, 20 e 25DPN) e, em algumas análises, também na maturidade sexual (70DPN), em particular quanto aos seguintes aspectos:

- Obter dados biométricos (peso corporal e peso testicular) dos camudongos avaliados, para subsequente determinação do índice gonadossomático, bem como obter a distância anogenital para determinação do índice anogenital;

- Avaliar, por meio de morfometria, os seguintes parâmetros: diâmetro tubular/cordonal e percentual de túbulos seminíferos/cordões com lume; proporção volumétrica dos componentes testiculares; proporção núcleo citoplasma e diâmetro das células de Leydig; número e volume de células de Sertoli e de Leydig; comprimento total de tubulos seminíferos por testículo e por grama de testículo;
- Avaliar a expressão da proteína Foxn1 ao longo do desenvolvimento testicular pós-natal;
- Definir o fenótipo da população de célula de Leydig Foxn1-positivas, por meio da co-localização com a enzima Hsd17b3 ao longo do desenvolvimento pós-natal;
- Determinar a concentração plasmática de testosterona em todas as idades avaliadas.

3. RESULTADOS

ARTIGO 1 – Submetido para publicação

Cell and Tissue Research

COMPARATIVE TESTIS STRUCTURE AND FUNCTION IN THREE REPRESENTATIVE MICE STRAINS

--Manuscript Draft--

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Abstract:	Mice are widely used as experimental models due to several positive characteristics and in particular their suitability for studies involving molecular biology and transgenesis. Despite the large number of mice strains currently available, the literature regarding their basic reproductive biology is still relatively scarce. Herein, we comparatively evaluated several important and correlated parameters related to testis structure and function in sexually mature male mice of inbred (C57BL/6, n = 19; BALB/c, n = 17) and outbred (Swiss, n = 17) strains, frequently utilized in research. Swiss mice presented significant variation for many parameters evaluated, including higher sperm production, mainly when compared to the C57BL/6 strain. However, some key parameters such as the duration of spermatogenesis, the Sertoli cell number per testis, and the spermatogenic efficiency were similar among the different strains. Although presenting significantly higher Leydig cell (LC) occupancy and numbers per testis gram and per testis, the anogenital index was smaller in Swiss mice, whereas testosterone levels and 3β-HSD expression were similar among strains. Regarding the LC/macrophages relationship, in comparison to the literature, we reported a much higher contribution of macrophages to the mouse intertubule. Thus, we estimated that there are around 1.6 macrophages per LC in BALB/c mice and this intriguing finding could be relevant to testis function in overall and spermatogonial biology in particular. Taken together, our results highlight the importance of knowing more accurately the testis structure and function in the different mice strains available for research, particularly when a specific testis parameter is being investigated.
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Author Comments:	I would like to reiterate that the present MS has two authors sharing the first authorship as well as two corresponding authors.
Suggested Reviewers:	Rex Hess rexhess@illinois.edu Dr. Hess has expertise in this field
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	Marie-Claude Hofmann MHofmann@mdanderson.org Dr. Hofmann has recognized expertise in this field.
	Stefan Schlatt Stefan.Schlatt@ukmuenster.de Dr. Schlatt has large experience in the field.
Opposed Reviewers:	

To the Editor-in-Chief

Cell and Tissue Research

Belo Horizonte, Brazil, February 1st 2020

Dear Editor,

Please find enclosed the original manuscript entitled "Comparative testis structure and function in three representative mice strains" by Oliveira, Lara et al., which is being submitted for publication in Journal of Anatomy. This original MS gathers a thorough comparative evaluation of several important parameters related to testis structure and function in sexually mature male mice of inbred (C57BL/6, BALB/c) and outbred (Swiss) strains frequently utilized in research. Our results showed the existence of meaningful differences among these mice strains, mainly regarding the intertubular compartment, particularly related to Leydig cells and macrophages, as well as their possible functional interactions. In overall, our findings add valuable data to literature regarding mice testis function, somehow providing a pattern for three strains largely utilized worldwide. Therefore, we hope that our study will attempt the standards required by Cell and Tissue Research.

Finally, we confirm that this original paper was not submitted to any other journal, and that all authors have approved the manuscript and its submission.

Sincerely yours,

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COMPARATIVE TESTIS STRUCTURE AND FUNCTION IN THREE REPRESENTATIVE MICE STRAINS

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Abstract

Mice are widely used as experimental models due to several positive characteristics and in particular their suitability for studies involving molecular biology and transgenesis. Despite the large number of mice strains currently available, the literature regarding their basic reproductive biology is still relatively scarce. Herein, we comparatively evaluated several important and correlated parameters related to testis structure and function in sexually mature male mice of inbred (C57BL/6, n = 19; BALB/c, n = 17) and outbred (Swiss, n = 17) strains, frequently utilized in research. Swiss mice presented significant variation for many parameters evaluated, including higher sperm production, mainly when compared to the C57BL/6 strain. However, some key parameters such as the duration of spermatogenesis, the Sertoli cell number per testis, and the spermatogenic efficiency were similar among the different strains. Although presenting significantly higher Leydig cell (LC) occupancy and numbers per testis gram and per testis, the anogenital index was smaller in Swiss mice, whereas testosterone levels and 3β-HSD expression were similar among strains. Regarding the LC/macrophages relationship, in comparison to the literature, we reported a much higher contribution of macrophages to the mouse intertubule. Thus, we estimated that there are around 1.6 macrophages per LC in BALB/c mice and this intriguing finding could be relevant to testis function in overall and spermatogonial biology in particular. Taken together, our results highlight the importance of knowing more accurately the testis structure and function in the different mice strains available for research, particularly when a specific testis parameter is being investigated.

Key words: Mice strains, Leydig cell, Sertoli cell, macrophages, sperm production

Introduction

Particularly because they have many positive attributes, such as low maintenance cost associated with high prolificacy, and fast reproductive cycle, since puberty and sexual maturity are reached, respectively, few weeks or months after birth, mice are considered a very useful experimental laboratory model. Among other important aspects, these attributes allow the relatively easy obtention of transgenic and genetically modified mice strains that are largely used, for instance, in studies involving molecular biology, physiology, biomedicine, and developmental investigations, including ageing (Plum et al., 2005; Lloyd, 2007; Enríquez, 2019; Portela et al., 2019).

In general, there are two types of mice strains: outbred and inbred. The first present some genetic variation, whereas the second one is considered genetically identical after 20 generations of blood related mating (Davisson, 1999). Since they can mimic human populations due to the genetic diversity, outbred animals are widely used in biomedical research. On the other hand, as the variation is limited, experiments with inbred strains require fewer animals that could be used, for example, to address more specific goals and objectives such as toxicological and drug development studies (Festing, 2010). From the above brief description it can be inferred that extensive inter- and intra-strains phenotypic variations could occur. This is observed, for instance, in studies quantifying plasma proteins (Michaud et al., 2018), and analyzing specific immune responses related to inflammatory angiogenesis (Marques et al., 2011; Enríquez, 2019), showing that genetic background influences many biological processes.

Even though the testis structure and organization are quite similar within a species, each strain/breed may present particular features. For instance, different

dog breeds have significant variations in the frequencies of the stages of the seminiferous epithelium cycle and in the duration of spermatogenesis (Soares et al., 2009), whereas several key testis parameters are different among boar breeds (Okwun et al., 1996). Moreover, it was also observed that different rat strains show distinct sensitivity to radiation in the testis, which led to great variations in spermatogenesis recovery after exposure (Abuelhija et al., 2012). These aforementioned findings indicate that genetic variations present in laboratory rodent colonies have important implications for the design, outcome, and reproducibility of biological experiments (Justice & Dhillon, 2016). Thus, considering the great importance of mice as experimental model in basic and applied research and the gaps in the literature regarding some key aspects related to their reproduction, in the present study we aimed to compare testis structure and function among the three most frequently employed wild type mice, seeking to provide background for further studies involving this species.

Material and Methods

Animals and tissue preparation

Fifty three sexually mature male mice (70 days of age), belonging to three different strains, were used in the present study as follows: C57BL/6 (inbred; n = 19), Swiss (outbred; n = 17) and BALB/c (inbred; n = 17). These mice were housed in a standard animal facility, under controlled temperature and photoperiod (12L:12D), with access to water and rodent food *ad libitum.*

In the afternoon before sacrifice, 4 to 6 mice from each strain received intraperitoneal (i.p.) injection of trypan blue solution (1% w/v), a vital dye that enables histological identification of testicular resident macrophages (Otto et al., 1996; Hutson, 2006). On the day of euthanasia, the mice received i.p. injection of heparin

(125 IU/g BW; Hepamax-s®, Blau, Brazil) and, 15 minutes later, i.p. injection of pentobarbital (150 mg/kg BW; Thiopentax®, Cristalia, Brazil). Body weight and anogenital distance were recorded and blood was collected by heart puncture before the mice were perfused-fixed with either 4% buffered glutaraldehyde for histomorphometric analyses, or Bouin's solution for the immunohistochemistry and macrophage analyses. After fixation, the testes, epididymis and seminal vesicle were trimmed out, weighed and testis fragments measuring 1-3 mm thickness were routinely processed. Glutaraldehyde-fixed fragments were embedded in glycol methacrylate (Leica Biosystems, Germany) and stained with toluidine blue and 1% sodium borate for histological and morphometric analyses (n = 9-12 mice per strain), whereas Bouin's fragments were embedded in paraffin for immunohistochemistry and macrophage visualization (n = 4-6 mice per strain).

Thymidine injections and autoradiographic analysis

In order to estimate the duration of the seminiferous epithelium cycle and, therefore, to calculate the spermatogenic efficiency, six animals (two per strain) received i.p. injection of tritiated thymidine (50 µCi in 0.2 mL; thymidine [methyl-^{3}H], specific activity 82.0 Ci/mmol, Amersham Life Science, England), a specific marker for cells that are synthesizing DNA. These mice were sacrificed at 1 hour or 21 days after injection.

For the autoradiographic analysis, unstained testis sections were dipped into the autoradiography emulsion (Kodak NTP-2; Eastman Kodak Company, Rochester, NY, USA) at 45ºC. After drying for about one hour in the dark at 25ºC, sections were placed in sealed black boxes and stored at 4ºC for approximately four weeks. Subsequently, the slides were developed in Kodak D-19 solution (Eastman Kodak Company) at 15ºC and stained with toluidine blue. The analysis of these sections

was performed under light microscopy to identify the most advanced germ cell type labeled at the two different intervals post thymidine injections. Cells were considered labeled when five or more grains were observed over the nucleus, in the presence of low background (Costa et al., 2010; Figueiredo et al., 2017).

Testis stereology

All morphometric analyses were performed following described methods published elsewhere (Auharek et al., 2011; Costa et al., 2017; Lara & França, 2017; Oliveira et al 2020). Briefly, the volume densities (%) of various testicular components were determined using a 441-intersection grid placed in the microscope's ocular at 400x magnification. Fifteen fields randomly chosen (6,615 points) were counted for each animal and points were classified as one of the following: seminiferous tubules, including tunica propria, seminiferous epithelium and lumen; intertubular compartment, comprising Leydig cells, macrophages, blood and lymphatic vessels. The volume of each component was determined as the product of its volume density and testis volume. The specific density of the testis was considered to be 1.0 (Leal & França, 2006) and the testis capsule (~3.5%) was excluded from the testis weight.

The tubular diameter and the seminiferous tubules epithelium height were measured in thirty round cross sections, chosen randomly for each animal, using an ocular micrometer calibrated with a stage micrometer, at 400x magnification. The total length of the seminiferous tubules was obtained by dividing the seminiferous tubules volume by the square radius of the tubule multiplied by the π value (Attal & Courot, 1963; Dorst & Sajonski, 1974).

Stages and duration of the seminiferous epithelium cycle

The characterization of the stages of the seminiferous epithelium cycle was performed based on the acrosomic system, as described by Russell et al. (1990). The relative stage frequencies were determined from the analysis of approximately 300 rounded seminiferous tubules cross-sections per animal, at 400x and 1,000x magnifications (Leal & França, 2006).

The duration of the spermatogenic cycle was estimated based on the stage frequencies and the most advanced germ cell type labeled at the two different intervals after thymidine injections. The calculation of total duration of spermatogenesis took into account that approximately 4.5 cycles are necessary for the completion of this process, from type A spermatogonia to spermiation (Amann & Schanbacher, 1983).

Cell counts and ratios

Ten randomly chosen round seminiferous tubules cross-sections per animal at stage VII of the seminiferous epithelium cycle were used for the cell counts. All germ cells nuclei and Sertoli cell nucleoli present at the cross-sections were counted and then corrected for section thickness and nucleus/nucleolus diameter according to Abercrombie (1946), as modified by Amann & Almquist (1962). For that, ten nuclei or nucleoli diameters were measured per animal for each cell type analyzed and cell ratios were obtained from the corrected counts. The total number of Sertoli cells was also determined from the corrected counts of Sertoli cell nucleoli per seminiferous tubule cross-sections and the total length of seminiferous tubules, according to Hochereau-de-Reviers & Lincoln (1978). The daily sperm production (DSP) per testis and per testis gram (spermatogenic efficiency) were determined according to the formula developed by França (1992), as follows: DSP = total number of Sertoli cells

per testis x ratio of round spermatids to Sertoli cells at stage VII x stage VII relative frequency (%) / stage VII duration (days).

Leydig cell and macrophage individual volumes were calculated from their nuclear and cytoplasmic volumes. The nuclear volume was obtained from the mean nuclear diameter using the formula $4/3\pi r^3$, were r = nuclear diameter/2. For this purpose, thirty nuclei were measured for each animal. In order to calculate the nucleus/cytoplasm proportion, a 441-point grid was placed over the histological section at 1,000x magnification and 1,000 points over each cell type were counted per animal. The estimation of the number of Leydig cells and macrophages in the testis took into account the cellular individual volume and the volume occupied by each cell in the testis parenchyma, and the data obtained was used to calculate the Leydig cell/macrophage ratio.

Immunohistochemistry

Immunohistochemical staining of 3β-HSD in Leydig cells was performed using standardized protocols. Briefly, paraffin sections at 5µm thickness were mounted on coated slides, dewaxed and rehydrated. Subsequently, antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 5 min in a microwave oven and endogenous peroxidase was blocked by incubating slides in 30% hydrogen peroxide (Sigma Aldrich) for 30 min at room temperature. Nonspecific binding sites were blocked with 10% normal rabbit serum (R9133, Sigma Aldrich) in PBS, before the addition of primary antibody anti-3β-HSD (1:100, sc-30820, Santa Cruz Biotechnology) and incubation at 4°C overnight. In the next day, the slides were exposed to the appropriate secondary antibody (1:100, ab6740, Abcam) for 60 min at room temperature. Detection of the signal was achieved by incubating the slides in streptavidin (TS-125-HR, Thermo Scientific) for 30 min, followed by the reaction with

peroxidase substrate 3,3'-diaminobenzidine (DAB, Sigma Aldrich) and counterstaining with hematoxylin (Merck). After dehydration, sections were mounted and analyzed by light microscopy.

In order to better evaluate the expression of 3β-HSD, we quantified the level of protein expression after the immunohistochemistry staining, as previously described by Oliveira et al. (2020). For this analysis, ten digital images from the testicular parenchyma were randomly captured using an Olympus BX60 microscope coupled to a camera. These images were converted to grayscale in Photoshop CS6 v13.0, and the pixel intensity of thirty labeled Leydig cells per animal was measured using ImageJ 1.51j8, normalizing by the background of each image (pixel intensity of the lumen of seminiferous tubules or blood vessels).

Hormone measurements

For the hormonal evaluation, blood samples collected at the time of euthanasia were centrifuged (2000 rpm for 10 min at 4ºC) and the plasma obtained was stored at −20°C. The samples were processed in the automated Cobas 8000 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone and estradiol through electrochemiluminescence assay. Testosterone coefficients of variation (CV) intra- and inter-assay were, respectively, 1.1% and 1.5%; whereas estradiol intra- and inter-assay's CVs were 1.7% and 1.9%. The procedures were performed by Tecsa – Technology in Animal Health, Belo Horizonte, MG, Brazil.

Statistical Analysis

All data are presented as mean ± standard error mean (SEM) and were tested for normality (D'Agostino & Pearson) and homoscedasticity; analyzed accordingly, using one-way ANOVA followed by the Tukey post-test or Kruskall-Wallis followed by

Dunn's multiple comparisons test. To evaluate the relationship between two parameters, Pearson's correlation was performed. All analyses were done using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). The significance level considered was $p < 0.05$.

Results

Biometric data and testis morphometry

Biometric and testis morphometric data are presented in Table 1. The body weight was significantly higher in Swiss mice, whereas, in comparison to C57BL/6, the testis weight showed higher values in Swiss and BALB/c. Therefore, gonadosomatic and anogenital indexes were smaller in Swiss mice, when compared to the other two strains. Epididymis and seminal vesicle weights were significantly increased in Swiss mice.

Regarding the testicular components volume densities, for most parameters evaluated, significant differences were found when C57BL/6 was compared to Swiss mice, whereas, in overall, BALB/c showed intermediate values (Table 1). Of note, Swiss mice had significantly higher percentage of intertubular compartment, particularly for Leydig cells. In addition, macrophage occupancy in the intertubular compartment presented higher value in BALB/c, being the same pattern observed for lymphatic vessels (Table 1; Fig 5A). The tubular diameter in Swiss mice was increased when compared to BALB/c ($p<0.05$); however, seminiferous epithelium height and the total length of seminiferous tubules per testis gram and per testis were not different among the three strains (Table 1).

Stages frequencies and seminiferous epithelium cycle length

Table 2 shows the frequencies of the twelve stages of spermatogenesis, characterized according to the acrosomic system. As it can be observed, except for

stages VIII and IX, the relative stage frequencies were very similar among the three strains, in particular when these stages were grouped in the pre-meiotic (stages VII–XI), meiotic (stage XII) and post-meiotic (stages I–VI) phases of the cycle (Table 2).

Differences regarding the most advanced germ cell type labeled after thymidine injections were not observed among strains (Fig. 1). Thus, 1h after injection preleptotene/leptotene primary spermatocytes at stage VIII (Fig 1A) were the most advanced germ cells labeled, whereas the nuclei of elongated spermatids at stage I containing typical thymidine grains were observed at 21 days (Fig 1B). Based on the most advanced germ cells type labeled at each time point post-thymidine injections and on the stages frequencies obtained for each strain, the mean duration of the seminiferous epithelium cycle was estimated to be 8.7 days for C57BL/6, 8.8 days for Swiss and 8.9 days for BALB/c mice (Table 2). Considering that approximately 4.5 cycles are necessary for completion of the spermatogenic process, the total duration of spermatogenesis was similar among strains and 39.2 days in C57BL/6, 39.6 days in Swiss and 40.1 days in BALB/c (Table 2).

Cell counts and daily sperm production

The cell counts, ratios and daily sperm production are presented in Table 3 and Figure 2. The number of Sertoli cell nucleoli per stage VII cross-section was not different among the strains, whereas, in overall, the cells numbers, from type A spermatogonia to spermatids, were significantly higher in Swiss mice, particularly when compared to C57BL/6. The Sertoli cell efficiency, estimated from the number of round spermatids per each Sertoli cell at stage VII, followed a similar trend and was increased in Swiss mice in comparison to the C57BL/6 strain (Fig 2A). The meiotic index, measured as the number of round spermatids produced per pachytene primary spermatocytes, was quite similar among strains and around three

haploid cells were observed, meaning that about 25% of germ cell losses occurred along the meiotic phase of spermatogenesis (Table 3).

The Sertoli cell numbers per gram of testis and per testis were not significantly different across the three mice strains, and the same pattern was observed for spermatogenic efficiency (daily sperm production per gram of testis) (Fig 2B-D). However, the daily sperm production per testis was significantly increased in Swiss when compared to C57BL/6 mice (Fig 2E). Leydig cell parameters are shown in Figure 3. Although in comparison to the other two mice strains the Leydig cell individual volume was significantly higher in BALB/c (Fig 3A), Leydig cell number per testis and per gram of testis were significantly increased in Swiss mice (Fig 3 B-C).

Hormone measurements and 3β-HSD expression

The plasmatic hormone data are depicted in Figure 4. Although the testosterone levels were numerically increased in Swiss mice, no significant differences were observed among the three strains herein evaluated (Fig 4A). However, regarding estradiol our results showed that their levels were about four-fold higher in Swiss and BALB/c mice (Fig 4B). Based on the pixel intensity, the expression of the steroidogenic enzyme 3β-HSD in Leydig cells cytoplasm did not change among the three strains (Fig 4C-F).

Testicular macrophages evaluation

Figure 5 shows the results obtained for the gonad macrophages. In comparison to the C57BL/6, BALB/c mice presented significantly higher macrophage occupancy in the testis (Fig 5A and Table 1). However, regarding macrophage nuclear volume an opposite trend was observed and the mean value observed for this parameter was significantly increased in C57BL/6 mice (Fig 5B), whereas no

differences were found for the individual cellular volume among strains (Fig 5C). Similar to the macrophage occupancy (%), when compared to the C57BL/6 strain, the total number of this cell per gram of testis (not shown) and per testis (Fig 5D) was significantly higher in BALB/c mice.

Figure 5E-G and the inserts represent the identification of testicular macrophages using the trypan blue vital stain, as well as the Leydig cell/macrophage ratios in the three strains herein evaluated. As expected, only macrophages were stained by this vital dye (Fig 4D-F and 5E-G). In this evaluation, approximately 1.3 Leydig cell for each macrophage was observed in the testis intertubular compartment of Swiss mice, whereas this proportion was approximately 1.0 to 1.0 for C57BL/6. Interestingly, with a Leydig/macrophage ratio of 0.63, the BALB/c mice presented much more macrophages than Leydig cells in the testis interstitium.

Discussion

In spite of the great importance of mice as a laboratory experimental model, to our knowledge, this is the first study to perform a more comprehensive comparative morphometric and functional investigation of the testis and spermatogenesis in three frequently used mice strains. Our results showed the existence of relevant differences among C57BL/6, Swiss and BALB/c mice, which should be taken into account when a specific study is being planned or developed. In overall, the Swiss mice, an outbred strain, was the one presenting most of the observed significant differences. As illustrated in Table 4, the lack of enough and consistent data in literature using similar methodologies makes it very challenging to comparatively discuss the results herein obtained with those found for other mice strains. However, we expect that our findings may provide a better background for further studies involving mice.

The spermatogenic cycle length is considered species specific (Clermont, 1972; Amann & Schanbacher, 1983; Hess & França, 2008) and is controlled by the germ cell genotype (França et al., 1998). However, breed or strain differences can be observed (Amann & Schanbacher, 1983; Hess & França, 2008; Soares et al., 2009) and, more recently, a study from our laboratory has shown that the duration of spermatogenesis in mice can be accelerated by higher environmental temperature (Costa et al., 2018), an important finding already observed in fish (Alvarenga & França, 2009). Besides its biological importance that allows, for instance, a better comprehension of reproductive strategies, the determination of the spermatogenic cycle length is a key parameter for the estimation of spermatogenic efficiency (daily sperm production per testis gram), which is also very useful for species comparisons (França et al., 2005; Hess & França, 2008; Lara et al., 2018a). In the present study, we did not observe significant differences for the duration of spermatogenesis among C57BL/6, Swiss and BALB/c mice, which lasted approximately 40 days and was relatively fast in comparison to other mammalian species already investigated (França et al., 2005; Costa et al., 2010; Lara et al., 2016; Lara et al., 2018a). This duration was quite similar to that found in other mice studies, including results from C57BL/6, Swiss and C3H (Clermont & Trott, 1969; Auharek et al., 2011; Costa et al., 2018). To our knowledge, the cycle length herein obtained for BALB/c has not been previously estimated. In overall, the stage frequencies obtained in our study along the spermatogenic cycle were similar to the other investigations in mice (Auharek et al., 2011; Costa et al., 2018). The same occurred with the stage frequencies combined as pre- and post-meiotic stages/phases, which seems to be phylogenetically determined among members of the same family (França et al.,

2005; Lara et al., 2018a), that were, respectively, one-quarter and two-thirds of one spermatogenic cycle.

In the present study, significant differences were found for several biometric and morphometric parameters investigated, such as body, testis, epididymis and seminal vesicle weights, as well as gonadosomatic index, the percentages of tubular and intertubular compartments and tubular diameter. In spite of that, the seminiferous epithelium height, the total length of seminiferous tubules, the numbers of Sertoli cells per gram of testis and per testis, the meiotic index (germ cells loss during meiosis), and the daily sperm production per gram of testis, were similar among the three different mice strains. However, in comparison to C57BL/6 mice, due to significantly higher testis weight and Sertoli cell efficiency (number of round spermatids per Sertoli cell) found for Swiss mice, the daily sperm production per testis presented higher values in this strain.

Similar to the observed in most studies reported in the literature for mammals (França et al., 2005; Hess & França, 2008; Lara et al., 2016), the germ cells loss that occurred during meiosis (~25%) was not different between the inbred (C57BL/6 and BALB/c) and outbred (Swiss) mice strains herein investigated. However, in overall, the corrected numbers of spermatocytes and spermatids per seminiferous tubules cross sections obtained at stage VII were higher (~10-15%) in Swiss mice and, as the number of Sertoli cells per tubule cross section did not change significantly among strains, this somatic cell efficiency was also higher in Swiss mice. An intriguing result observed in the present study was the significantly lower number (less than 50%) of spermatogonia per tubule cross section found in C57BL/6 mice. According to the literature (Mäkelä & Toppari, 2018), the vast majority of spermatogonia present at stage VII are type A1 (differentiated) spermatogonia and

few spermatogonial stem cells are also observed in this stage. This result suggests that the differentiated spermatogonial divisions (A1→A2→A3→A4→In→B) are much more efficient along spermatogenesis in C57BL/6, an important aspect that surely deserves further investigation. Besides many factors, such as retinoic acid, GDNF and the proximity to blood vessels, a specific subpopulation of testicular macrophages may also be involved in the spermatogonial stem cell niche regulation (Yoshida et al., 2007; DeFalco et al., 2015; Mäkelä & Hobbs, 2019). Therefore, C57BL/6 mice seems to be an interesting model to further investigate the possible interactions of intertubular and tubular factors and its effects on niche regulation and spermatogonial divisions.

The most relevant results found in this study were probably those associated to the intertubular compartment, specially the findings related to the Leydig cells and macrophages and their possible functional interactions. Regarding the Leydig cells, the highest occupancy values, as well as the numbers of this steroidogenic cell per gram of testis and per testis, were observed in Swiss mice, whereas the Leydig cell individual size was more prominent in BALB/c. These variations are very difficult to interpret and are frequently observed in the literature among different species and strains/breeds (França et al., 2005; França, 2007; Costa et al., 2010; Lara et al., 2018a). For instance, the Leydig cell volume density in mammals varies from ~1% in rams to ~35% in capybaras (França et al., 2005; Costa et al., 2010), whereas the variation for Leydig cell individual volume is from ~400µm^3 in rams to ~5,000µm^3 in horses (Johnson & Neaves, 1981; Lunstra & Schanbacher, 1988). Regarding the Leydig cell number per gram of testis, it ranges from ~6 million in guinea pigs to ~160 million in wild boars (França & Russell, 1998; Russell, 1996; Almeida, 2006). The mechanisms that regulate proliferation and function of Leydig cell populations

are still not fully understood and are under constant debate (Ye et al., 2017; Shima et al., 2018). However, it is known that hormones such as LH, testosterone and estrogens are somehow involved with Leydig cell regulation, as well as some factors like IGF-1, TGFs, COUP-TFII, interleukins and PDGF-A (Haider, 2004; Qin et al., 2008; Kilcoyne et al., 2014).

The anogenital index is a well-known marker of androgen exposure during the late fetal masculinization programming window (MPW) (Welsh et al., 2008; van den Driesche et al., 2011; Dean & Sharpe, 2013; Auharek et al., 2011, 2012), and there is no linear correlation between body weight and anogenital distance (Mitchell et al., 2015). In the present study, we observed that the Swiss mice showed significantly reduced anogenital index. However, the Leydig cell numbers and percentage were higher in Swiss mice that, together with BALB/c, presented significantly higher estradiol levels. In this regard, the Swiss mice reduced anogenital index is an intriguing finding that is difficult to interpret, requiring further studies aiming to investigate if this mice strain is less exposed to androgens during the MPW. Regarding the seminal vesicle, which is also an androgen-dependent organ, although significantly heavier in Swiss mice, its ratio in relation to the body weight was similar among the three strains, representing around 1% of the total body mass. Therefore, a narrow, positive and significant correlation between seminal vesicle and body weight was detected for all strains herein investigated.

Since the 3β-HSD is an enzyme that plays its role at a very early phase of the steroidogenic process, the absence of differences regarding its expression between the three mice strains indicates that, at this phase of the steroidogenic pathway, the enzyme expression is similar among the strains. The balance between androgens and estrogens is essential for normal development of male sexual organs, brain's

androgenization as well as the establishment of the secondary sexual characteristics and maintenance of the reproductive status during adulthood (Schulster et al., 2016).

Among many of the cell-to-cell interactions in the testis, it is well established the relationship between Leydig cells and macrophages. In this regard, Leydig cells and macrophages are usually present in large numbers in the testis intertubular compartment and it is already known that testicular macrophages can influence Leydig cells proliferation, differentiation and function, even promoting steroidogenesis (Gaytan et al., 1994; Cohen et al., 1997; Hales, 2002; Hutson, 2006; DeFalco et al., 2015). Additionally, it has already been shown that Leydig cell and macrophage numbers increase proportionally during development (Gaytan et al., 1994; Wang et al., 1994; Smith et al., 2015), and several studies in rats estimated that there is 1 macrophage for every 3-5 Leydig cells (Ewing et al., 1979; Miller, 1982; Bergh, 1985; Niemi et al., 1986; Diemer et al., 2003).

Although the mechanisms related to the interactions between Leydig cells and macrophages are still not totally understood, the presence of membrane associations between these two testis somatic cells, called interdigitations, reinforces their intimate morphofunctional relationship (Itoh et al., 1995; Hutson, 2006; DeFalco et al., 2015; Lara et al., 2018b). Most of the investigations above cited were made in rats and, to our knowledge, there are very few quantitative studies related to macrophages in the mouse testis. In the present study, we found significant differences for several parameters evaluated in testis macrophages, particularly for BALB/c mice that presented higher percentage and number of this cell in the testis. More importantly, based on the ratios obtained and in contrast to Swiss mice (~1.3 Leydig cell per macrophage), we estimated that there are much more macrophages than Leydig cells (~1.6 macrophage per Leydig cell) in the BALB/c mice intertubular

compartment. Using F4/80, a macrophage-specific antigen, it was reported that approximately 20% of mice testis interstitial cells were macrophages (Hume et al., 1984), whereas Li et al. (1998) found that macrophage density was around 600 cells per mm² of the intertubular compartment in this species. Testicular macrophages are phenotypically highly heterogeneous and different subpopulations of these cells are present in the testis (Itoh et al., 1995; Winnall & Hedger, 2013; DeFalco et al., 2015; Mossadegh-Keller et al., 2017; Mossadegh-Keller & Sieweke, 2018). Therefore, we should be cautious when interpreting data based on a single antigen, since it might not be able to identify the totality of resident macrophages. In comparison to the literature, in the present study we reported a much higher contribution of macrophages to the mouse interstitial compartment, which, in overall, represent almost half of the cell population present in the testis interstitium. Most importantly, we showed, for the first time, that this contribution is highly variable among different mice strains. Therefore, in order to better comprehend the intricate relationship between Leydig cells and macrophages during development and adulthood, more accurate investigations are necessary, preferentially involving different mice strains. Particularly taking into consideration recent data showing that, besides their important role in the regulation of testis immune privilege, a specific subpopulation of testicular macrophages may also be involved in the spermatogonial stem cell niche (Winnall & Hedger, 2013; DeFalco et al., 2015; Potter & DeFalco, 2017; Mossadegh-Keller et al., 2017).

In summary, several important differences in testicular morphology, cellular composition and function were observed in the present study for the three mice strains herein investigated. Among them, it could be highlighted the results found for the intertubular compartment, in particular those related to Leydig cells and

macrophages and their possible functional interactions. However, in order to better comprehend and advance our knowledge in these relevant aspects, further mechanistic and/or functional studies using different mice strains should be developed.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures and protocols followed approved guidelines for the ethical treatment of animals according to the ethics committee in animal experimentation of the Federal University of Minas Gerais, Brazil.

Author contributions

CFAO and NLML contributed equally to this manuscript by performing experiments, analyzing the data and writing the paper. BRLC performed experiments and analyzed the data. LRF conceived the study and wrote and revised the paper final version. GFA conceived the study, performed experiments, analyzed the data and wrote the paper.

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Table 1: Biometric and morphometric data in three different mice strains (mean ± SEM).

	C57BL/6	Swiss	BALB/c
Body weight (g)	25 ± 0.6[a]	39 ± 0.5[b]	27 ± 0.4[a]
Testis weight (mg)	92 ± 2[a]	111 ± 3.2[b]	105 ± 2[b]
Gonadosomatic index (%)*	0.73 ± 0.01[a]	0.57 ± 0.02[b]	0.78 ± 0.01[c]
Epididymis weight (mg)	42 ± 2[a]	54 ± 2[b]	41 ± 3[a]
Seminal vesicle weight (mg)	217 ± 13[a]	430 ± 20[b]	292 ± 19[c]
Anogenital index**	4.5 ± 0.07[a]	3.6 ± 0.14[b]	4.2 ± 0.13[a]
Volumetric density (%)			
Tubular compartment	93.3 ± 0.5[a]	91.1 ± 0.7[b]	92.1 ± 0.4[ab]
Tunica propria	2.8 ± 0.1[a]	3.2 ± 0.1[b]	3.0 ± 0.1[ab]
Seminiferous epithelium	81.5 ± 0.6[a]	77.5 ± 0.5[b]	79.7 ± 0.7[ab]
Lumen	9.0 ± 0.5	10.4 ± 0.5	9.4 ± 0.7
Intertubular compartment	6.5 ± 0.4[a]	8.9 ± 0.7[b]	7.9 ± 0.4[ab]
Leydig cell	3.7 ± 0.2[a]	5.3 ± 0.4[b]	4.0 ± 0.4[ab]
Blood vessels	2.1 ± 0.2	2.4 ± 0.1	2.6 ± 0.3
Lymphatic space	0.5 ± 0.1[a]	1.1 ± 0.3[ab]	0.9 ± 0.1[b]
Macrophages	0.96 ± 0.03[a]	1.10 ± 0.09[ab]	1.17 ± 0.05[b]
Tubular diameter (µm)	205 ± 2[ab]	211 ± 3[a]	199 ± 3[b]
Seminiferous epithelium height (µm)	79 ± 1.0	77 ± 1	76 ± 1.0
Total length of seminiferous tubule per testis (m)	2.7 ± 0.1	2.9 ± 0.1	2.9 ± 0.1
Length of seminiferous tubule per testis gram (m)	29.5 ± 0.9	26.5 ± 1.2	29.5 ± 0.9

Different letters in the same line indicate significant differences ($p < 0.05$) among strains.
*Gonadosomatic index: [Total testicular weight (g) / Body weight (g)] x 100
**Anogenital index: Anogenital distance (mm) / Body weight cubic root (g)

Table 2: Stage frequencies (%) and duration of spermatogenesis (days) in three different mice strains (mean ± SEM).

	C57BL/6	Swiss	BALB/c
I	13.3 ± 0.7	14.5 ± 0.6	15.4 ± 0.6
II-III	6.7 ± 0.8	7.2 ± 0.6	4.6 ± 0.7
IV	4.7 ± 0.4	4.8 ± 0.4	3.9 ± 0.4
V	4.8 ± 0.3	4.9 ± 0.3	4.2 ± 0.5
VI	7.6 ± 0.7	7.4 ± 0.6	8.6 ± 0.9
VII	30 ± 0.9	25.9 ± 1.4	28.9 ± 1.4
VIII	4.5 ± 0.3[a]	6.3 ± 0.5[b]	4.7 ± 0.5[a]
IX	4.9 ± 0.4[ab]	3.6 ± 0.3[a]	5.1 ± 0.4[b]
X	5.4 ± 0.5	4.4 ± 0.3	5 ± 0.5
XI	9 ± 0.6	9.7 ± 0.5	8.4 ± 0.6
XII	9.1 ± 0.8	11.3 ± 0.4	11.2 ± 0.9
Pre-meiotic (VIII-XI)	23.8 ± 1	24 ± 1.3	23.1 ± 0.9
Meiotic (XII)	9.1 ± 0.8	11.3 ± 0.4	11.2 ± 0.9
Post-meiotic (I-VII)	67.1 ± 4	64.7 ± 3.3	65.7 ± 4
Duration of one cycle	8.7	8.8	8.9
Duration of spermatogenesis	39.2	39.6	40.1

Different letters in the same line indicate significant differences ($p < 0.05$) among strains.

Table 3: Cell counts and ratios per seminiferous tubule cross sections at stage VII of the seminiferous epithelium cycle in three different mice strains (mean ± SEM).

	C57BL/6	Swiss	BALB/c
Sertoli cell nucleoli	8.6 ± 0.3	8.4 ± 0.4	7.7 ± 0.2
Spermatogonia	0.7 ± 0.1[a]	1.5 ± 0.1[b]	1.5 ± 0.1[b]
Pre-leptotene spermatocytes	27 ± 1[a]	33 ± 1[b]	31 ± 1[ab]
Pachytene spermatocytes	30 ± 1[a]	34 ± 1[b]	29 ± 1[a]
Round spermatids	88 ± 3[a]	100 ± 4[b]	89 ± 3[a]
Meiotic index	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.1

Different letters in the same line indicate significant differences ($p < 0.05$) among strains.

Table 4: Comparative data related to biometry, testis stereology and spermatogenesis in mice strains already investigated.

Parameters	C57BL/6[a] (Inbred)	Swiss[b] (Outbred)	BALB/c[c] (Inbred)	C3H[d] (Inbred)	ICR[e] (Outbred)	NMRI[f] (Outbred)	Parkes[g] (Outbred)	CF-1[h] (Outbred)
Body weight (g)	25	39	27	25	37	25-31	33	35
Testis weight (mg)	92	111	105	78	125	140	100	120
Gonadosomatic Index (%)	0.7	0.6	0.8	0.6	0.7	1.0	0.6	0.7
Seminiferous tubules (%)	93	91	92	-*	93	-	-	-
Leydig cell (%)	3.7	5.3	3.9	-	3.9	-	-	-
Tubular diameter (µm)	205	211	199	-	202	211-217	200	199
Sertoli cells per gram of testis ($x10^6$)	64	55	57	-	-	-	-	-
Sertoli cells per testis ($x10^6$)	5.8	5.9	5.6	4.1	-	-	-	-
Sertoli cell efficiency	10	12	12	-	-	-	-	-
Pre-meiotic phase[i] (%)	24	24	23	29-33	25	-	-	-
Meiotic phase[j] (%)	9	11	11	10-11	10	-	-	-
Post-meiotic phase[k] (%)	67	65	66	57-61	65	-	-	-
Meiotic index[l]	3.0	3.0	3.1	-	-	2.1	-	-
Spermatogenic cycle length (days)	8.7	8.8	8.9	8.6	-	-	-	-
Spermatogenesis total duration (days)	39.2	39.6	40.1	38.7	-	-	-	-
DSP per gram of testis ($x10^6$)	75	75	74	-	-	-	-	29
DSP per testis ($x10^6$)	6.8	8.1	7.3	-	-	-	-	-

[a, b, c] Present paper.
[d] Oakberg, 1956; Clermont & Trott, 1969; Allan et al., 2004.
[e] Avelar et al., 2000; Korejo et al., 2016.
[f] Jafari et al., 2018; Khorsandi & Oroojan et al., 2018, Fisher et al., 2019.
[g] Joshi & Singh, 2018.
[h] Cagen et al., 1999; Bustos-Obregon et al., 2007.
[i] Combined stages frequencies after spermiation and prior to methaphase.
[j] Meiotic division I through meiosis II.
[k] Combined stages frequencies after completion of meiosis until spermiation.
[l] Measured as the number of round spermatids produced per pachytene primary spermatocyte.
* = not found or available in the literature.

Figure Legends

Fig. 1 Representative illustration in C57BL/6 mice of the most advanced germ cell type labeling after tritiated thymidine injections. One hour after injection (A) labeled preleptotene spermatocytes (arrows) were observed at stage VIII of the seminiferous epithelium cycle, whereas on twenty-one days after injection (B) elongated spermatids (arrowheads) at stage I were the most advanced labeled germ cell. The same pattern described above was observed in the two other mice strains investigated. Bars = 10µm

Fig. 2 Sertoli cell and sperm production parameters in the three mice strains investigated (A-E). As it can be noted, in comparison to the C57BL/6, Sertoli cell efficiency (A) and daily sperm production per testis (E) were significantly increased in Swiss mice. Different letters indicate significant differences ($p < 0.05$) among strains

Fig. 3 Leydig cells number and size in the three mice strains investigated (A-C). Observe that the Leydig cell volume (A) is higher in the BALB/c strain, whereas Leydig cell numbers per gram of testis (B) and per testis (C) are significantly increased in Swiss mice. Different letters indicate significant differences ($p < 0.05$) among strains

Fig. 4 Plasmatic testosterone and estradiol levels (A-B) and 3β-HSD evaluation of Leydig cells (C-F) present in the intertubular testis compartment, in the three mice strains investigated. Note that, in comparison to Swiss and BALB/c mice strains, estradiol levels are significantly reduced in C57BL/6 mice (B). By both qualitative and quantitative methodologies, the 3β-HSD localization and intensity of staining were similar in all three mice strains. Different letters indicate significant

differences ($p < 0.05$) among strains. White arrows indicate macrophage in the interstitial compartment. Bars = 30µm

Fig. 5 Macrophage parameters evaluated in the intertubular testis compartment in the three mice strains investigated (A-G). Observe that, in comparison to the C57BL/6 mice, the macrophage occupancy was higher in BALB/c mice (A). In contrast, macrophage nuclear volume (B) was significantly increased in C57BL/6 mice, when compared to the other two investigated mice strains, while no difference is observed for the macrophage individual volume (C). The number of macrophages per testis (D) is significantly increased in BALB/c mice, when compared to the C57BL/6. Figures 5 E-G show the Leydig cells with no trypan blue staining (arrows), whereas the macrophages cytoplasm is stained with trypan blue (arrowheads), and the inserts in the left bottom corner depict a macrophage higher magnification. The Leydig cell/macrophage ratios presented above these inserts varies substantially among the three evaluated strains, being much higher in Swiss and lower in BALB/c mice. Different letters indicate significant differences ($p < 0.05$) among strains. Bars = 30µm

Figure 1

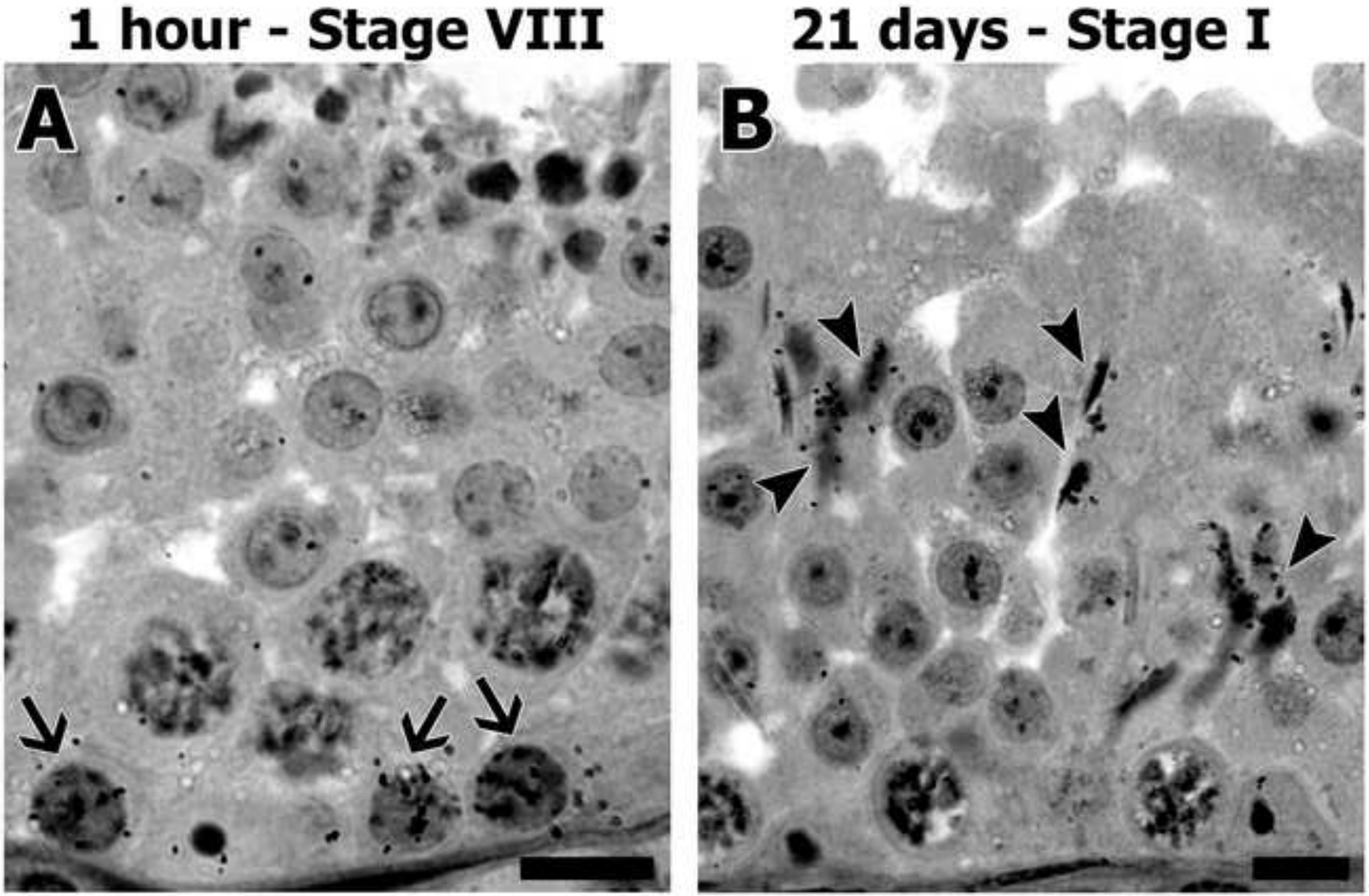


Figure 2

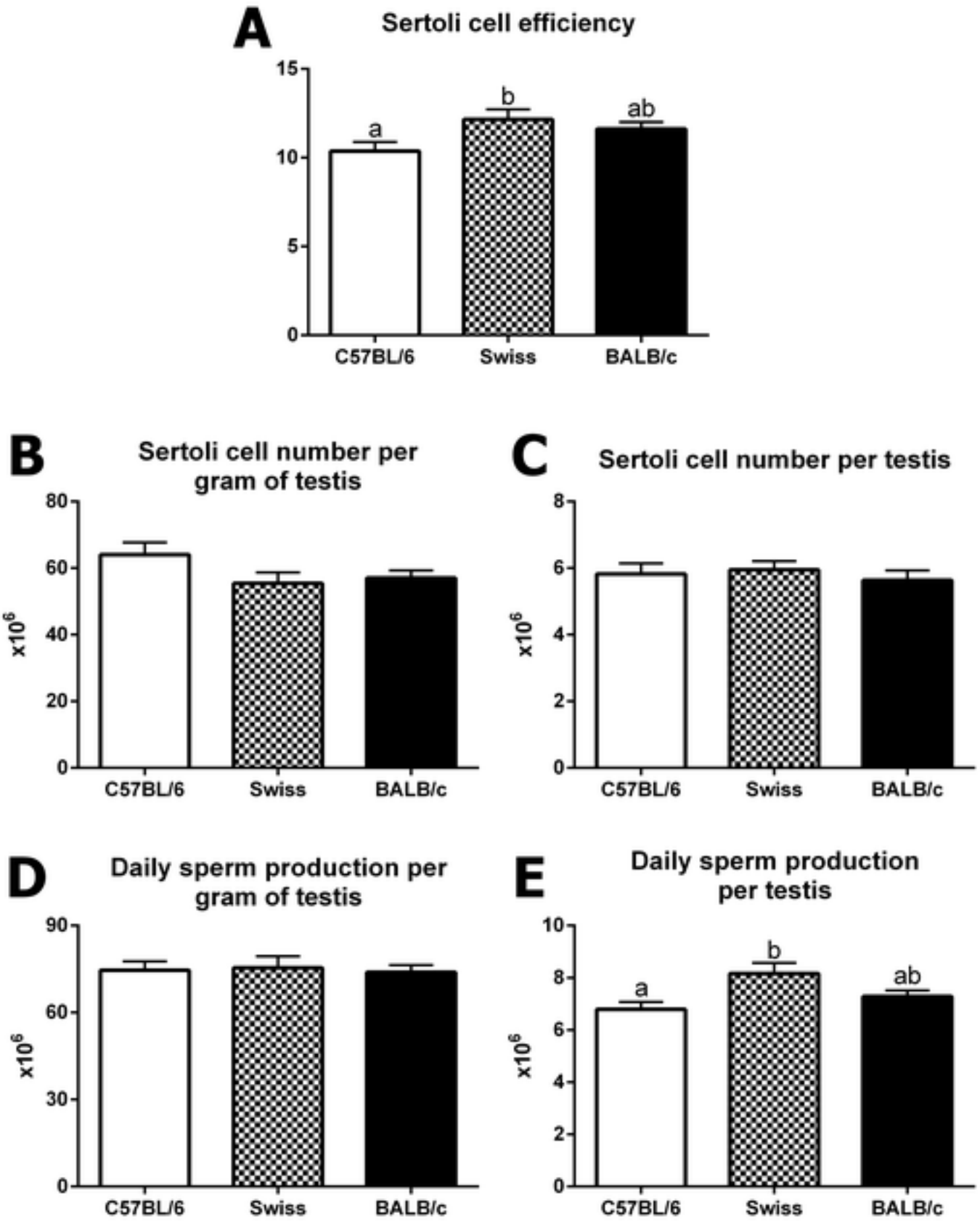


Figure 3

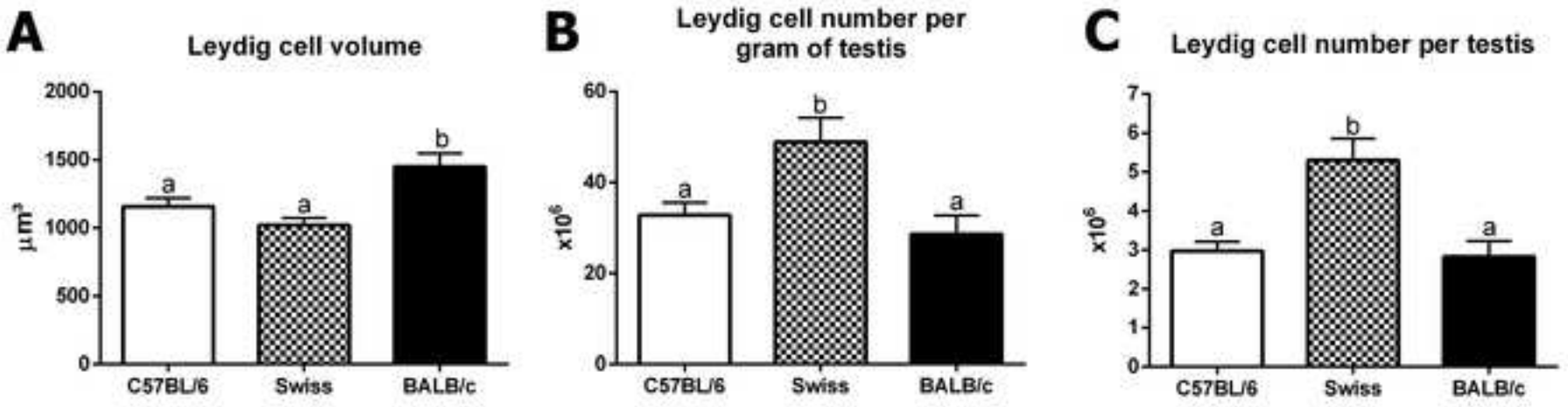


Figure 4

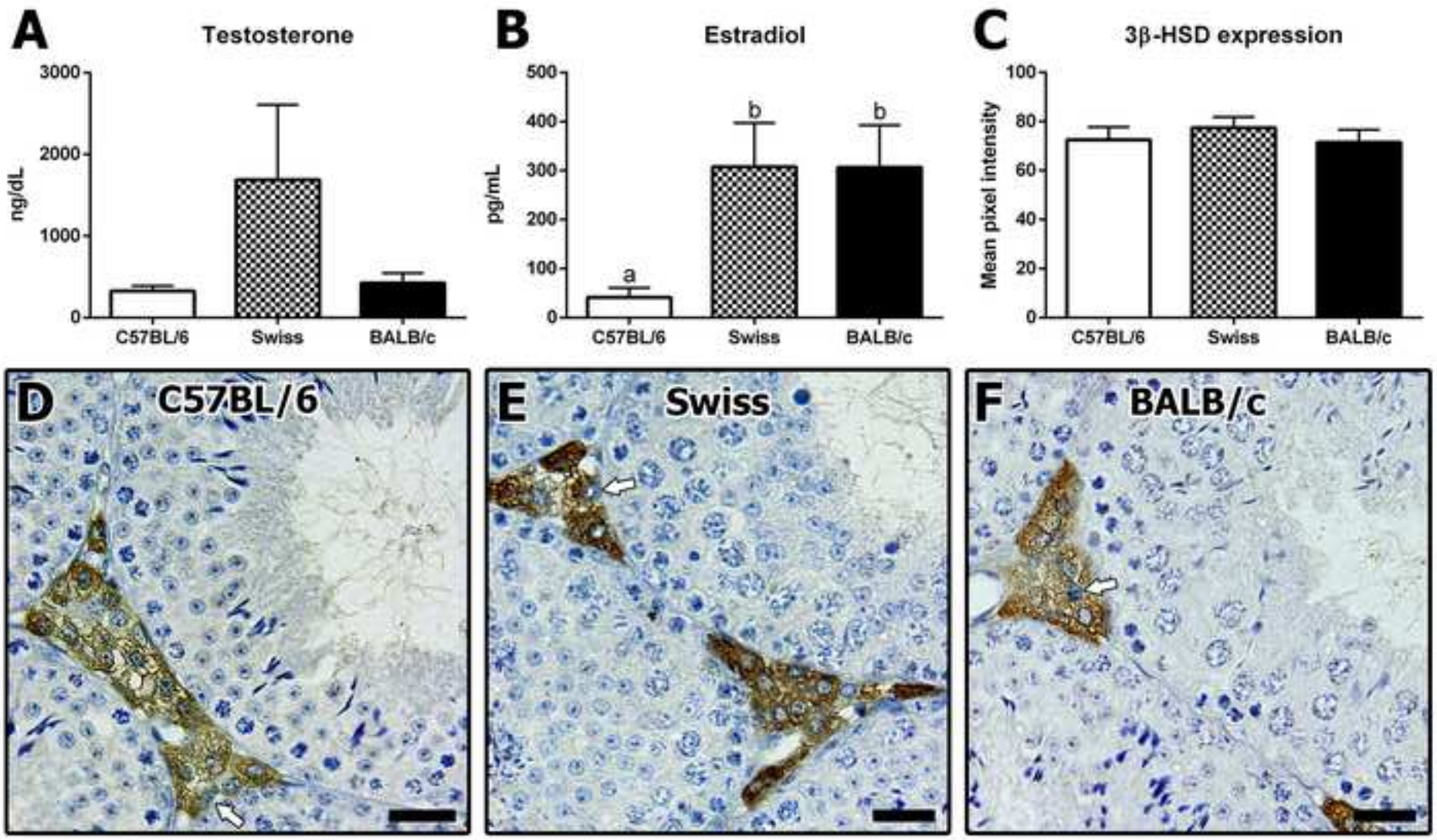
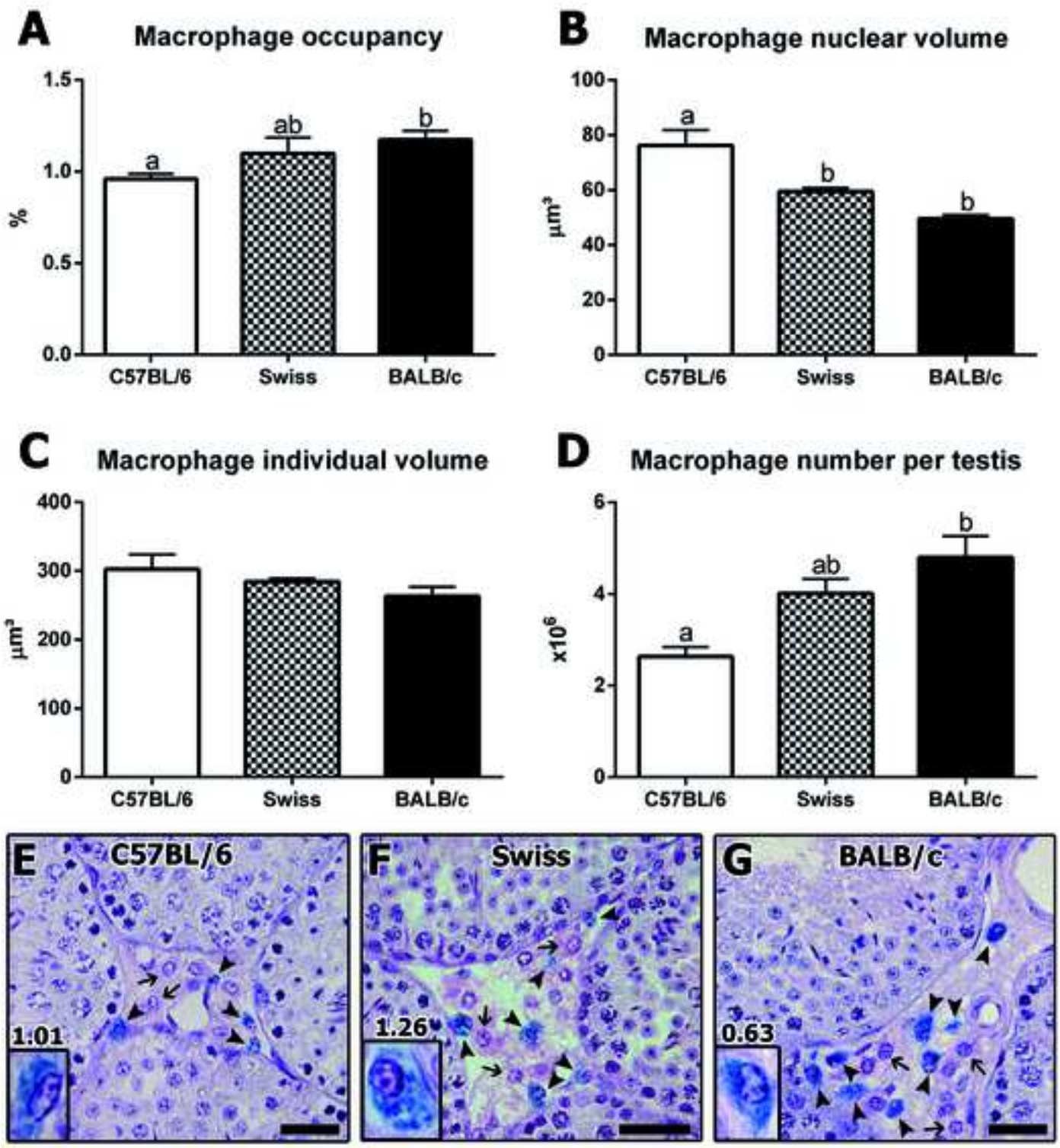


Figure 5



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REGULAR ARTICLE

Foxn1 and *Prkdc* genes are important for testis function: evidence from nude and scid adult mice

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Abstract

Mutations in *Foxn1* and *Prkdc* genes lead to nude and severe combined immunodeficiency (scid) phenotypes, respectively. Besides being immunodeficient, previous reports have shown that nude mice have lower gonadotropins and testosterone levels, while scid mice present increased pachytene spermatocyte (PS) apoptosis. Therefore, these specific features make them important experimental models for understanding *Foxn1* and *Prkdc* roles in reproduction. Hence, we conducted an investigation of the testicular function in nude and scid BALB/c adult male mice and significant differences were observed, especially in Leydig cell (LC) parameters. Although the differences were more pronounced in nude mice, both immunodeficient strains presented a larger number of LC, whereas its cellular volume was smaller in comparison to the wild type. Besides these alterations in LC, we also observed differences in androgen receptor and steroidogenic enzyme expression in nude and scid mice, suggesting the importance of *Foxn1* and *Prkdc* genes in androgen synthesis. Specifically in scid mice, we found a smaller meiotic index, which represents the number of round spermatids per PS, indicating a greater cell loss during meiosis, as previously described in the literature. In addition and for the first time, Foxn1 was identified in the testis, being expressed in LC, whereas DNA-PKc (the protein produced by *Prkdc*) was observed in LC and Sertoli cells. Taken together, our results show that the changes in LC composition added to the higher expression of steroidogenesis-related genes in nude mice and imply that Foxn1 transcription factor may be associated to androgen production regulation, while *Prkdc* expression is also important for the meiotic process.

Keywords Nude mice · Scid mice · Leydig cell · *Foxn1* · *Prkdc*

Introduction

A recessive autosomal mutation in the *Foxn1* gene (*Foxn1*$^{-/-}$) leads to the nude mouse phenotype, characterized by a lack of skin fur development and agenesis of the thymus (Mecklenburg et al. 2005). The *Foxn1* gene encodes a transcription factor (Foxn1) from the winged helix/forkhead family (Byrd 1993; Nehls et al. 1994) that consists of over 100 proteins, several of which are reported to regulate reproduction (Uhlenhaut and Treier 2011) and gonadotropin (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) gene expression (Thackray 2014). Foxl2, for instance, has a central role in female sex determination and is required for normal *Fshb* expression (Justice et al. 2011). Moreover, Foxo1 is important for spermatogonial stem cell self-renewal and modulates gonadotropin beta-subunit gene expression (Goertz et al. 2011; Skarra et al. 2013), while Foxp3 is relevant for spermatogenesis and male fertility (Jasurda et al. 2014). Even though the studies regarding testis function in nude mice are scarce, it is known that they have lower serum levels of testosterone and gonadotropins in comparison to the wild type (Rebar et al. 1982). Therefore, as other proteins of the family, Foxn1 might have a potential role in hormonal regulation and reproduction.

Mutations in the *Prkdc* gene lead to the severe combined immunodeficiency (scid) phenotype. The DNA-dependent protein kinase (DNA-PK) is an enzyme important for repairing DNA double-strand breaks. This enzyme comprises two subunits: one is the catalytic (DNA-PKc), encoded by the *Prkdc* gene, while the other is the heterodimer Ku70/80,

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responsible for DNA recognition (Lees-Miller and Meek 2003). Despite being considered fertile, Hamer et al. (2003) observed that scid male mice have an increase of pachytene spermatocyte apoptosis at stage IV of the seminiferous epithelium cycle (SEC), when there is a meiotic checkpoint. Therefore, the susceptibility of this cell type to apoptosis is related to a failure in repairing DNA double-strand breaks that occur during genetic recombination (Hamer et al. 2003; De Rooij and De Boer 2003).

Due to their peculiar reproductive aspects, both nude and scid mice are potential experimental models for the understanding of *Foxn1* and *Prkdc* gene roles in reproduction. Therefore, the aim of the present study is to perform a detailed investigation of testicular function in nude and scid mice, seeking to provide further insights into the importance of these genes in the regulation of spermatogenesis.

Material and methods

Animals, testis sample collection and processing

The nude mice used in this study were purchased from Taconic, Germantown, USA and the scid mice from Fiocruz, Bahia, Brazil. Sixteen sexually mature (70 days) male mice, from each of the investigated strains (BALB/c wild type, BALB/c nude and BALB/c scid), were used in the following distribution for each group: nine animals were used for light microscopy and histomorphometry, four were used for immunohistochemistry and three had their testis collected for qPCR gene expression analyses. The wild-type mice were housed in a standard animal facility and the immunodeficient mice were housed in filtered microisolator cages, with a ventilated rack system (Alesco®), under a controlled photoperiod (12 h light, 12 h dark), with access to autoclaved water and rodent food ad libitum.

In accordance with the animal experimentation guidelines, in the present study, we sought to use the minimum necessary number of animals to obtain a biological response. Therefore, by determining the sample size based on the most instable variable that was established as the Leydig cell number per gram of testis (Auharek et al. 2011), the experimental number of animals needed was obtained from a calculation made according to Sampaio (2002). Hence, our histomorphometric analysis was statistically validated using nine animals per group.

The mice used for histomorphometry ($n = 9$ per group) and immunohistochemistry ($n = 4$ per group) were weighed, anesthetized with an intraperitoneal injection of pentobarbital (150 mg/kg, Thiopentax®; Cristalia, Brazil) and perfusion-fixed with glutaraldehyde 4% in 0.05 M phosphate buffer (for histomorphometric analysis) or Bouin's solution (for immunohistochemistry), following the procedure described by Sprando et al. (1990). After fixation, testes were weighed and sectioned in smaller fragments with a razor blade. The testis fragments were dehydrated in increasing alcohol concentrations and embedded in glycol methacrylate (Leica Biosystems, Germany) for histomorphometric analysis or Paraplast® (Sigma-Aldrich, Germany) for immunohistochemistry.

For the gene expression analysis, the mice ($n = 3$ per group) were weighed, euthanized and orchiectomized; had their testis frozen in liquid nitrogen; and were stored at − 80 °C. The messenger RNA (mRNA) relative levels of androgen receptor, 3-beta-hydroxysteroid dehydrogenase (3BHSD) and aromatase were analyzed. Beta-actin expression was used as an internal control.

Histomorphometric analysis

Seminiferous tubular diameter and height of the seminiferous epithelium were measured from 20 tubules per animal at 400x magnification using an ocular micrometer calibrated with a stage micrometer. The volume densities of testis tissue components were determined from 15 randomly chosen fields per animal using a 441-point intersection grid placed in the eyepiece of a light microscope (6615 points) at 400x magnification. Points were classified as one of the following: seminiferous tubules (comprising tunica propria, seminiferous epithelium and lumen), Leydig cells, connective tissue, blood and lymphatic vessels. The volume of each testis component was determined as the product of its volume density and testis volume (the testis capsule was excluded from the testis weight). The total length of the seminiferous tubule was obtained by dividing the seminiferous tubule volume by the square radius of the tubule times π (Attal and Courot 1963; Dorst and Sajonski 1974).

For the cell counts, spermatogonia, pre-leptotene/leptotene spermatocytes, pachytene spermatocytes, round spermatids and Sertoli cell (SC) nucleoli present at stage VII of the seminiferous epithelium cycle were counted in 10 round or nearly round seminiferous tubule cross-sections, chosen at random, for each animal. These counts were corrected for section thickness (4 μm) and nuclear or nucleolar diameter according to Abercrombie (1946), as modified by Amann and Almquist (1962). For this purpose, the diameters of 10 nuclei or nucleoli were measured per animal for each cell type analyzed. Cell ratios were obtained from the corrected counts obtained at stage VII. The total number of SC was determined from the corrected counts of SC nucleoli per seminiferous tubule cross-section and the total length of seminiferous tubules according to Hochereau-de Reviers and Lincoln (1978). Daily sperm production (spermatogenic efficiency) was determined according to the formula developed by Franca (1992), as follows: DSP = total number of SC per testis × ratio of round

spermatids to SC at stage VII × stage VII relative frequency (%) / stage VII duration (days).

Regarding Leydig cells (LC), as their nucleus in mice is round or nearly round, the nuclear volume was easily determined from its mean nuclear diameter. For this purpose, 30 nuclei showing an evident nucleolus were measured for each animal. LC nuclear volume was obtained by the formula 4 / 3πr3, where r is nuclear diameter / 2. To calculate the proportion between nucleus and cytoplasm and the cytoplasmic volume, a 441-point intersection grid was placed over the sectioned material at × 1000 magnification and one thousand points over LC per testis were counted for each animal. The volume of each individual LC was obtained as the sum of its nuclear and cytoplasmic volumes. The number of LC per testis was estimated from the LC individual volume and the volume density occupied by LC in the testis parenchyma.

Quantitative qPCR gene expression

The primers sequences chosen for the testicular mRNA relative level analysis (Table 1) were obtained from validated sequences at the PrimerBank (http://pga.mgh.harvard.edu/primerbank) and synthetized by Integrated DNA Technologies (IDT®). Total RNA was extracted using TRIzol® (Thermo Fisher Scientific, USA) according to the manufacturer's instructions and its concentration was determined by spectrophotometry in NanoDrop 2000 (NanoDrop Inc., Wilmington, DE, USA). After that, the volume corresponding to 2 μg of RNA was subjected to reverse transcription followed by a polymerase chain reaction. Using the cDNA generated, the qPCR was performed using SYBR Green Supermix 240 (Bio-Rad, Marnes la Coquette, France) and each assay was replicated with the same parameters. Data were normalized to beta actin, as a reference gene, which showed a constant expression pattern under the conditions analyzed. All procedures were carried out as described by Morais et al. (2017).

Table 1 Primers used for gene expression

Primer	Sequence
Androgen receptor forward	5′-CTGGGAAGGGTCTACCCAC-3′
Androgen receptor reverse	5′-GGTGCTATGTTAGCGGCCTC-3′
Aromatase forward	5′-ATGTTCTTGGAAATGCTGAA CCC-3′
Aromatase reverse	5′-AGGACCTGGTATTGAAGACGAG-3′
3-Beta-HSD forward	5′-TGGACAAAGTATTCCGACCAGA-3′
3-Beta-HSD reverse	5′-GGCACACTTGCTTGAACACAG-3′
Beta-actin forward	5′-GGCTGTATTCCCCTCCATCG-3′
Beta-actin reverse	5′-CCAGTTGGTAACAATGCCATGT-3′

Immunohistochemistry

For the immunohistochemistry analyses, the procedures were as follows: testis sections were mounted on coated slides, dewaxed and rehydrated in decreasing alcohol concentrations. Then, antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min after boiling in a microwave oven. Slides were incubated in 30% hydrogen peroxide (Sigma-Aldrich) to block endogenous peroxidase activity. Nonspecific binding sites were blocked with 10% normal serum for 30 min, before the addition of primary antibodies and incubation overnight at 4 °C. After this procedure, the slides were exposed to the appropriate secondary antibodies for 60 min at room temperature. Detection of the signal was performed by incubating the sections in streptavidin (TS-125-HR, Thermo Scientific) for 30 min, followed by a reaction with peroxidase substrate 3,3-diaminobenzidine (DAB) (Sigma-Aldrich) and counterstaining with hematoxylin (Merck).

We used primary antibodies against androgen receptor (1:150, cat no. SC-815; Santa Cruz Biotechnology, USA), the steroidogenic enzyme aromatase (1:300, cat no. PA5-19633; Thermo Fisher Scientific, UK) and 3BHSD (1:750, cat no. SC-30820; Santa Cruz Biotechnology, USA), as well as antibodies anti-Foxn1 (1:400, cat no. SC-30195; Santa Cruz Biotechnology, USA) and anti-DNA-PKc (1:250, cat no. SC-9051; Santa Cruz Biotechnology, USA). The slides were qualitatively analyzed in order to evaluate which cell types were labeled as well as their location in the testicular parenchyma.

In order to better evaluate the expression of the androgen receptor, aromatase and 3BHSD, we decided to perform a second method of analysis. Thus, in addition to mRNA quantification by qPCR, we also performed a semiquantitative analysis of the intensity of labeling obtained for those proteins assessed through immunohistochemistry, as previously described elsewhere (Dornas et al. 2007, 2008; Oliveira et al. 2013). For this analysis, six random images were captured from the testicular parenchyma of each animal using an Olympus BX60 microscope with a coupled camera. The images were treated to convert to gray scale in Photoshop CS3 v10.0 and the pixel intensity was measured from 30 to 60 labeled cells per animal using ImageJ 1.47t, normalized by the pixel intensity obtained in the background of the image (lumen of seminiferous tubules or blood vessels).

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM) and were tested for normality and homoscedasticity of the variances. Data were analyzed accordingly using ANOVA and the means were compared using the Student-Newman-Keuls test. Pearson's correlation was used to evaluate the relationship between two variables. All analyses were

performed using GraphPad Prism 6 (GraphPad Software, Inc.) and the significance level considered was $p < 0.05$.

Results

Biometric data

The body and testicular weights and gonadosomatic index are shown in Table 2. No difference was observed in the body weight among the three groups. On the other hand, in comparison to the wild type, the testis weight and gonadosomatic index (ratio between total testis mass and body mass) were significantly smaller in the nude mice.

Histomorphometric analysis

As observed in Table 2, although the tubular diameter and seminiferous epithelium height were higher in the nude mice in comparison to the other groups, the total length of seminiferous tubules per testis was significantly smaller in this strain.

The volume density (%) and absolute volume (μl) of each testicular component are summarized in Table 3. The intertubular compartment and LC volume densities were different among the three groups and higher values were observed in the immunodeficient strains, particularly for the scid mice. Because the higher values were found for the scid mice intertubular compartment and LC, this pattern was also followed for absolute volumes. Therefore, a positive and significant correlation between intertubular compartment and LC occupancy was found for nude ($r = 0.82$) and scid ($r = 0.91$) mice. Additionally, significant differences were observed for blood vessels, lymphatic space and connective tissue occupancies. The absolute volume and the percentage of blood vessels were significantly reduced, while connective tissue volume density and the absolute volume were significantly increased in scid mice, when compared to the other groups. The lymphatic space volume density, as well as the absolute volume, were smaller in nude mice and showed an intermediate value in scid mice when compared to the wild type. On the other hand, as expected, the tubular compartment volume density and the absolute volume showed an opposite pattern when compared to the intertubular space, and the smallest values were found for the immunodeficient strains. In the tubular compartment, significant differences were observed for lumen and tunica propria absolute volumes and the volume densities. Hence, when compared to the wild type, the smallest lumen occupancy was observed for scid mice, while nude mice presented an intermediate value. An opposite pattern was found for tunica propria, once the highest value was found for scid mice.

Cell counts

The cell counts and ratios obtained at stage VII of the seminiferous epithelium cycle are shown in Table 4. The lowest SC efficiency was observed in scid mice, with approximately 8 round spermatids supported by each SC. The meiotic index, which expresses the number of round spermatids produced per pachytene spermatocyte and represents the cell loss during meiosis, was lower for scid, in comparison with nude and wild-type mice.

Still focusing on SC, its numbers per testis and per gram of testis were significantly higher in the scid mice than in the other two strains. Furthermore, as shown in Table 4, daily sperm production per testis was significantly smaller in nude mice, whereas this parameter per gram of testis was higher in the scid mice. Therefore, as expected, daily sperm production per testis showed a positive and significant correlation with the SC number per testis in wild-type, nude and scid strains ($r = 0.84$, $r = 0.86$ and $r = 0.73$, respectively).

Substantial differences were observed in the LC parameters (Table 5). In this regard, the nuclear, cytoplasmic and individual volumes of LC in nude mice were the lowest volumes found in this study. Interestingly, the number of LC per testis and per gram of testis were also significantly different among the strains investigated, showing an opposite trend when compared to the cellular volume. In this regard, LC number was

Table 2 Biometric and morphometric data in BALB/c wild-type, nude and scid mice (mean ± SEM)

	Wild type	Nude	scid
Body weight (g)	26 ± 0.5	28 ± 0.8	25 ± 1
Testis weight (mg)	102 ± 2^{a}	83 ± 5^{b}	89 ± 6^{ab}
Gonadosomatic index (%)	0.8 ± 0.02^{a}	0.6 ± 0.03^{b}	0.7 ± 0.03^{a}
Tubular diameter (μm)	224 ± 2^{a}	243 ± 3^{b}	212 ± 3^{c}
Seminiferous epithelium height (μm)	77 ± 1^{a}	84 ± 2^{b}	76 ± 1^{a}
TLST/testis (m)	2.3 ± 0.07^{a}	1.5 ± 0.09^{b}	2.1 ± 0.15^{a}
TLST/testis g (m)	23 ± 0.4^{a}	19 ± 0.4^{b}	25 ± 0.6^{c}

Different letters in the same line indicate significant differences ($p < 0.05$) among groups

TLST total length of seminiferous tubules

Table 3 Volume density (%) and absolute volumes (μl) of testis components in BALB/C wild type, nude and scid mice (mean ± SEM)

	Wild type Volume density % (μl)	Nude	scid
Seminiferous tubules	92 ± 0.004^{a} $(91 \pm 2)^{a}$	90 ± 0.01^{b} $(72 \pm 5)^{b}$	88 ± 0.01^{c} $(76 \pm 6)^{b}$
Seminiferous epithelium	80 ± 0.01 $(79 \pm 2)^{a}$	78 ± 0.005 $(62 \pm 4)^{b}$	78 ± 0.01 $(67 \pm 6)^{ab}$
Lumen	9.4 ± 0.01^{a} $(9.3 \pm 0.8)^{a}$	7.5 ± 0.005^{b} $(6 \pm 0.6)^{b}$	4.1 ± 0.001^{c} $(3.5 \pm 0.3)^{c}$
Tunica propria	3 ± 0.001^{a} $(2.9 \pm 0.1)^{a}$	4.5 ± 0.002^{b} $(3.5 \pm 0.2)^{a}$	6.2 ± 0.004^{c} $(5.3 \pm 0.6)^{b}$
Intertubular compartment	8 ± 0.004^{a} $(7.8 \pm 0.4)^{a}$	10 ± 0.01^{b} $(7.9 \pm 0.5)^{a}$	12 ± 0.01^{c} $(10.2 \pm 0.5)^{b}$
Leydig cells	4 ± 0.004^{a} $(3.9 \pm 0.4)^{a}$	7 ± 0.006^{b} $(5.6 \pm 0.6)^{b}$	9 ± 0.01^{c} $(7.3 \pm 0.3)^{c}$
Blood vessels	2.6 ± 0.003^{a} $(2.5 \pm 0.3)^{a}$	2.6 ± 0.004^{a} $(2 \pm 0.3)^{a}$	1.5 ± 0.002^{b} $(1.2 \pm 0.2)^{b}$
Lymphatic space	0.93 ± 0.001^{a} $(0.9 \pm 0.1)^{a}$	0.13 ± 0.0004^{b} $(0.1 \pm 0.03)^{b}$	0.45 ± 0.001^{c} $(0.4 \pm 0.1)^{c}$
Connective tissue	0.5 ± 0.0003^{a} $(0.5 \pm 0.04)^{a}$	0.3 ± 0.001^{a} $(0.2 \pm 0.08)^{a}$	1.5 ± 0.002^{b} $(1.3 \pm 0.2)^{b}$

Different letters in the same line represent significant statistical difference ($p < 0.05$)

highly increased in nude mice in comparison with the other two groups.

Immunohistochemistry and qPCR gene expression

The protein localization, pixel intensity quantification and relative gene expression levels of the androgen receptor, 3BHSD and aromatase are shown in Figs. 1, 2, and 3, respectively. In general, the results from qPCR and pixel intensity measurement followed a similar pattern. As expected, androgen receptor expression was restricted to the nuclear compartment of the peritubular myoid cell, endothelial cell, SC and LC (Fig. 1). The pixel intensity analysis showed stronger labeling intensity of this receptor in the LC of immunodeficient mice (nude and scid) when compared to the wild type ($p < 0.05$). The evaluation of mRNA indicated that, when compared to the baseline (wild type), the androgen receptor levels were 25-fold higher for both immunodeficient mice strains.

The LC cytoplasmic localization of the steroidogenic enzymes 3BHSD (Fig. 2) and aromatase (Fig. 3) was conserved among strains. However, the labeling levels of 3BHSD, as demonstrated by the evaluation of pixel intensity, were higher in nude mice whereas aromatase was equally expressed among strains. A similar pattern was observed from qPCR analyses. 3BHSD mRNA levels were about 25-fold and 50-fold higher in scid and nude mice, respectively. In addition, an increase of approximately 15-fold in aromatase mRNA levels was observed in nude testis when compared to the wild type. On the other hand, the aromatase mRNA levels were similar between scid and wild-type mice.

Foxn1 expression was observed specifically in LC regardless of mice strains (Fig. 4). However, the intracellular distribution of this protein was found to be different, depending on the strain; hence, in wild-type and scid mice, Foxn1 presented both cytoplasmic and nuclear localization, whereas in nude mice, the nuclear labeling was rarely observed. Regarding

Table 4 Cell counts and ratios per seminiferous tubule cross-section at stage VII of the seminiferous epithelium cycle in BALB/c wild type, nude and scid mice (mean ± SEM)

	Wild type	Nude	scid
Sertoli cell (SC) nucleoli	6 ± 0.2^{a}	7.2 ± 0.3^{b}	8.1 ± 0.4^{b}
Type A spermatogonia	1.4 ± 0.1^{a}	1.2 ± 0.1^{b}	1.9 ± 0.1^{c}
Pre-leptotene/leptotene spermatocytes	30 ± 1^{a}	25 ± 1^{b}	30 ± 1^{a}
Pachytene spermatocytes (P)	27 ± 1	28 ± 1	30 ± 1
Round spermatids (R)	63 ± 2	69 ± 4	62 ± 2
Sertoli cell efficiency (R/SC)	10.5 ± 0.2^{a}	9.8 ± 0.5^{a}	7.9 ± 0.5^{b}
Meiotic index (R/P)	2.3 ± 0.004^{a}	2.5 ± 0.1^{a}	2.1 ± 0.04^{b}
Sertoli cell per testis ($\times 10^{6}$)	3.5 ± 0.1^{a}	2.8 ± 0.2^{a}	4.3 ± 0.3^{b}
Sertoli cell per g of testis ($\times 10^{6}$)	35 ± 1^{a}	35 ± 2^{a}	51 ± 3^{b}
Daily sperm production per testis ($\times 10^{6}$)	4.1 ± 0.2^{a}	3.1 ± 0.3^{b}	3.8 ± 0.3^{ab}
Daily sperm production per g of testis ($\times 10^{6}$)	41 ± 1^{ab}	38 ± 2^{a}	44 ± 2^{b}

Different letters in the same line represent significant statistical difference ($p < 0.05$)

Table 5 Leydig cell parameters in BALB/c wild-type, nude and scid mice (mean ± SEM)

	Wild type	Nude	scid
Nuclear diameter (μm)	7.6 ± 0.1[a]	6.6 ± 0.1[b]	7.3 ± 0.1[a]
Leydig cell individual volume (μm^3)	1444 ± 99[a]	619 ± 63[b]	967 ± 43[c]
Nuclear volume (μm^3)	228 ± 5[a]	155 ± 8[b]	207 ± 10[ab]
Cytoplasmic volume (μm^3)	1216 ± 95[a]	465 ± 57[b]	760 ± 35[c]
Leydig cell number per testis ($\times 10^6$)	2.8 ± 0.4[a]	9.5 ± 1.1[b]	7.6 ± 0.2[c]
Leydig cell number per g of testis ($\times 10^6$)	28.7 ± 4[a]	121.9 ± 14[b]	92.3 ± 7[c]

Different letters in the same line represent significant statistical difference ($p < 0.05$)

DNA-PKc, its expression followed a cytoplasmic distribution in both LC and SC, in wild-type, nude and scid mice (Fig. 4).

Discussion

Although nude and scid mice are important experimental models, there are few reports in the literature regarding their reproductive biology. Therefore, analysis of their testicular structure and function is highly relevant. We comparatively evaluated several important testis morphofunctional parameters in sexually mature nude, scid and wild-type mice. Our main findings strongly suggest that the *Foxn1* gene may be involved in testis endocrine regulation, since substantial differences were observed in nude mice regarding LC, such as number and size, as well as the expression of steroidogenic enzymes. As related to scid mice, the results indicate a relevant role for the *Prkdc* gene in SC and germ cells, as significant increases were observed on SC number and daily sperm production.

It is noteworthy that both mutated genotypes did not induce alterations in testicular cytoarchitecture, since the basic structure of the seminiferous epithelium was preserved. Despite that, differences were observed in several histomorphometric parameters. Interestingly, even though they do not have an increase in Sertoli and germ cell numbers, nude mice

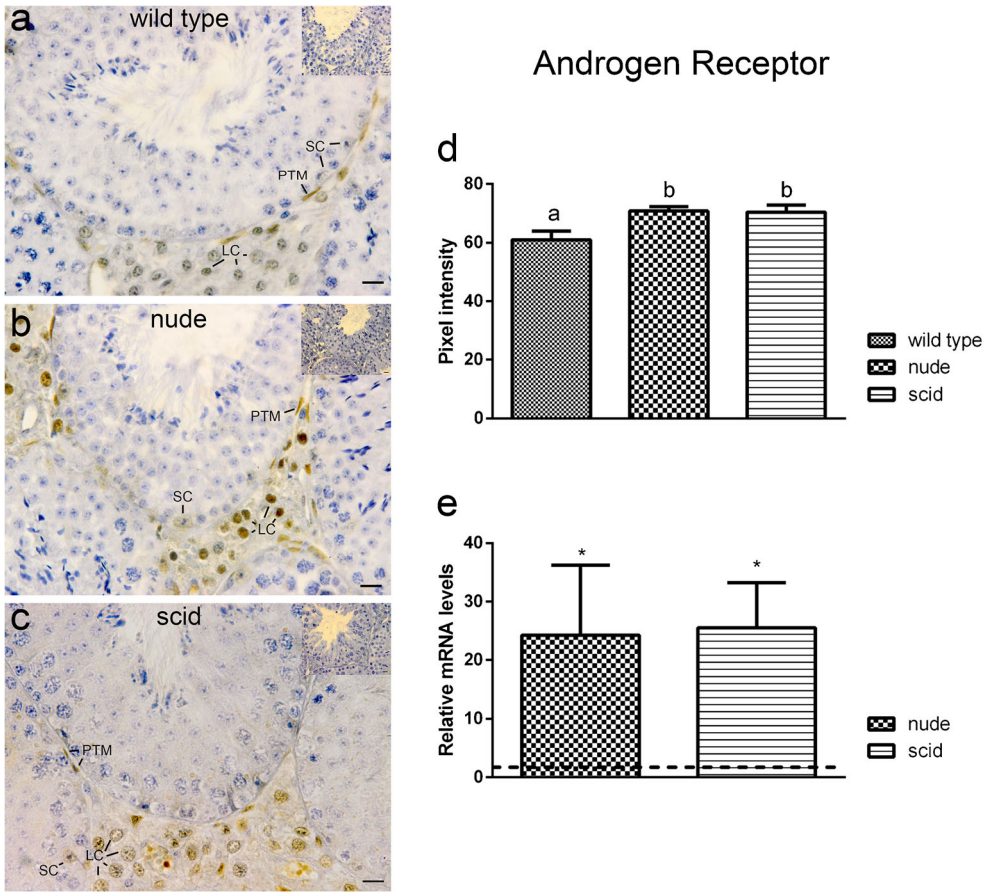

Fig. 1 Androgen receptor expression. Immunolabeling in the Leydig, peritubular myoid and Sertoli cell nucleus in BALB/c wild-type (**a**), nude (**b**) and scid (**c**) mice. Inserts show the negative control. **d** Pixel intensity quantification. **e** Gene expression by qPCR. The dashed line in **e** represents the value of 1, determined for the wild type, and the columns refer to the relative amount of mRNA in the two groups evaluated. LC Leydig cell; PTM peritubular myoid cell; SC Sertoli cell. Different letters and asterisks represent significant statistical difference ($p < 0.05$). Bar = 14 μm

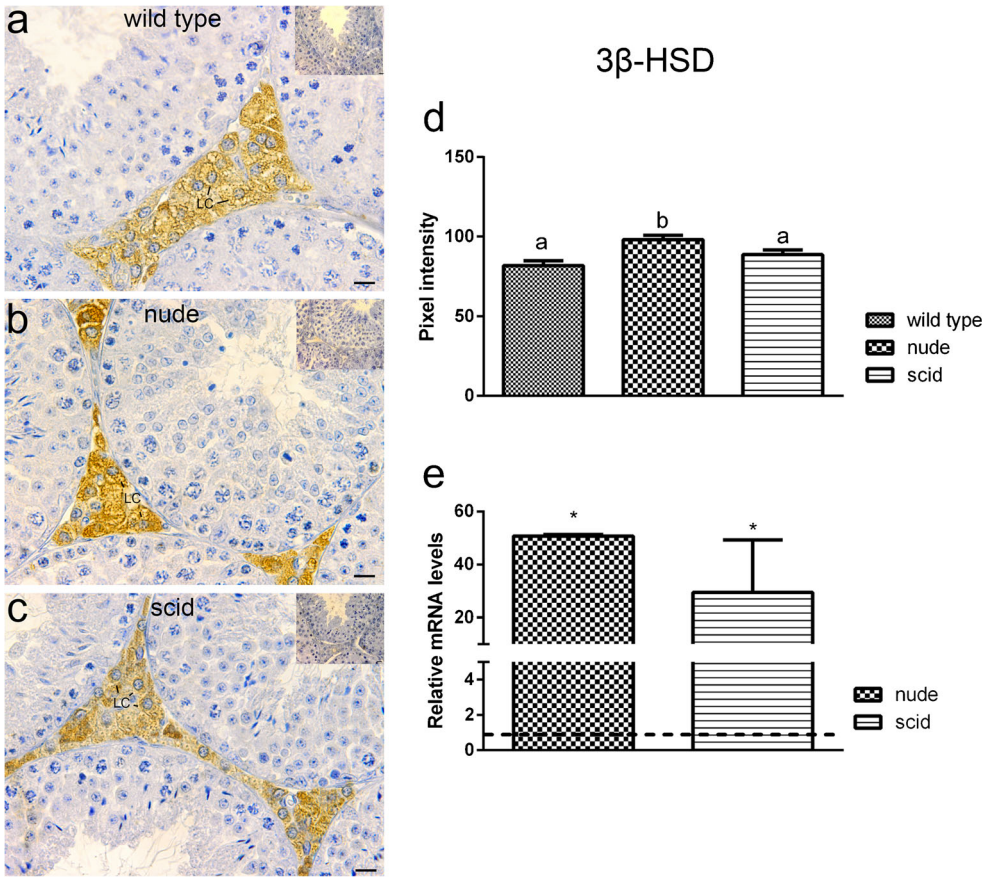

Fig. 2 3β-HSD expression. Immunolabeling in the Leydig cell cytoplasm in BALB/c wild-type (**a**), nude (**b**) and scid (**c**) mice. Inserts show the negative control. **d** Pixel intensity quantification. **e** Gene expression by qPCR. The dashed line in **e** represents the value of 1, determined for the wild type and the columns refer to the relative amount of mRNA in the two groups evaluated. LC Leydig cell. Different letters and asterisks represent significant statistical difference ($p < 0.05$). Bar = 14 μm

showed a higher tubular diameter and a taller seminiferous epithelium height. Although these are related parameters, a decrease in the amount of cells does not necessarily result in a lower epithelium height. This is a very complex and intricate issue that surely deserves further investigation using other methodologies. For instance, Gholami et al. (2015) considered that in some particular conditions, disordered arrangements in the cells or structural adjustments may compensate the epithelium height by increasing the distance between cells.

Furthermore, some differences were also observed in the absolute volume and volume density of several parameters. In the immunodeficient strains, the volume of seminiferous tubules was lower than that in the wild type, while the proportion of the intertubular compartment was increased, mainly due to the higher LC occupancy. In fact, many interesting results were observed for those cells, especially in nude mice. So, since LCs have a smaller individual volume, it seems that their increased total number may be a compensatory mechanism to ensure normal androgen levels and therefore maintain homeostasis. Corroborating these data, a similar LC pattern was also observed in iNOS-deficient mice (Auharek et al. 2011). In this regard, it was important to evaluate the function of these steroidogenic cells, especially because it is already known that testosterone levels are reduced in adult nude mice (Rebar et al. 1982).

Nevertheless, in the present study, we found that, in comparison to the wild type, LC from both immunodeficient strains presented stronger labeling and higher mRNA levels of androgen receptor. It is already documented in the literature that castrated rats have increased androgen receptor mRNA levels as a response to lower levels of circulating testosterone (Tan et al. 1988). Therefore, the same type of receptor upregulation could be happening as a compensatory mechanism, once nude mice have a lower level of circulating androgens, leading to a higher expression of its receptor. Furthermore, some studies already showed that LH is essential for the expression of the P450c17 steroidogenic enzyme but not for 3BHSD (Keeney et al. 1988). Since *Foxn1* mutant mice have lower gonadotropin levels (Rebar et al. 1982), the higher expression of 3BHSD may indicate an attempt to compensate lower levels of LH, in order to better use the available substrate (pregnenolone), once its synthesis could be downregulated. Another important steroidogenic enzyme is aromatase, which converts androgen into estrogen (Genissel et al. 2001). Once there are less androgens in nude mice (Rebar et al. 1982), the increase in aromatase mRNA levels in this strain also suggests a compensatory mechanism to ensure suitable

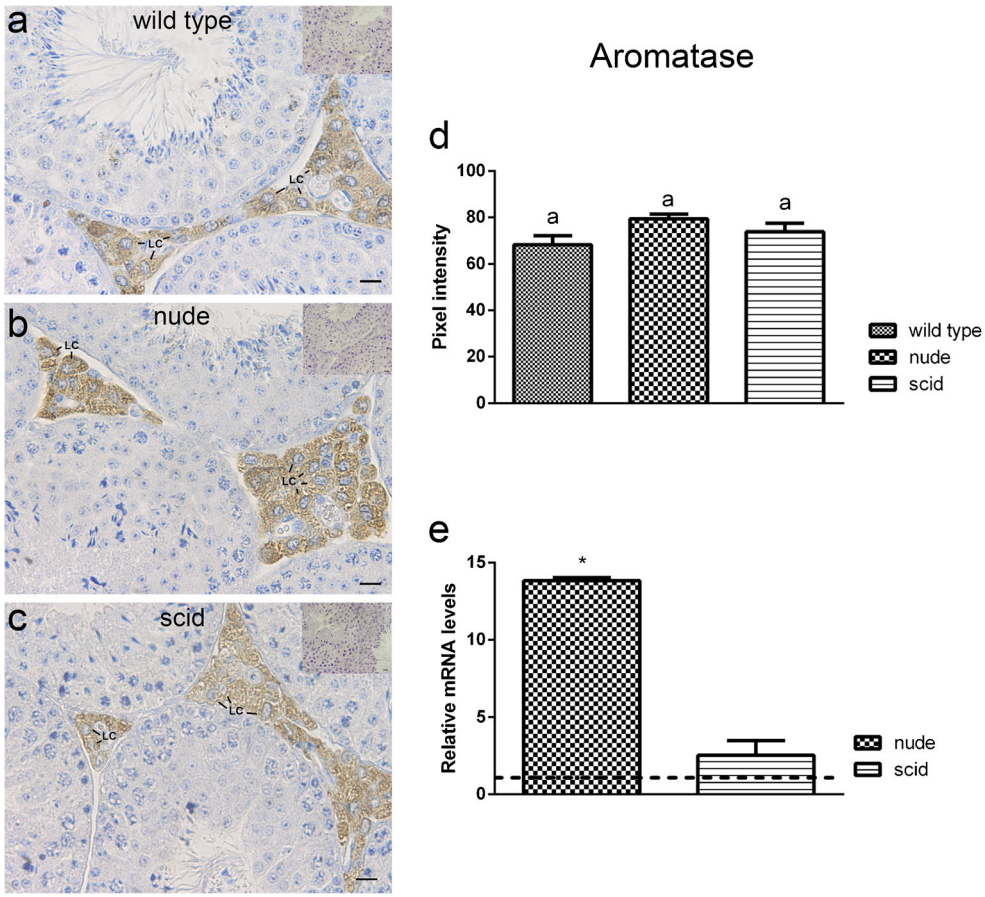

Fig. 3 Aromatase expression. Immunolabeling in the Leydig cell cytoplasm in BALB/c wild-type (**a**), nude (**b**) and scid (**c**) mice. Inserts show the negative control. **d** Pixel intensity quantification. **e** Gene expression by qPCR. The dashed line in **e** represents the value of 1, determined for the wild type and the columns refer to the relative amount of mRNA in the two groups evaluated. LC Leydig cell. Different letters and asterisks represent significant statistical difference ($p < 0.05$). Bar = 14 μm

estrogen levels, in order to maintain testicular physiological function.

Thus, the evaluation of localization and grade of protein expression in Leydig cell was enabled, once immunohistochemistry preserves the tissue cytoarchitecture. Therefore, the measurement of pixel intensity, observed for many authors (Dornas et al. 2007, 2008; Oliveira et al. 2013) as a suitable methodology for evaluating protein occurrence, was considered in the present study as a qPCR complementary tool that provided some important information regarding differential expression of steroidogenic enzymes and androgen receptor across the strains.

Spermatogenesis requires a balance between proliferation and apoptosis, which regulates the number of germ cells supported by each SC, ensuring the homeostasis of sperm production. The highest germ cell loss in meiosis was found in the scid mice and it is consistent with previous reports describing more apoptosis in pachytene spermatocytes at stage IV in mice carrying the *Prkdc* gene mutation (Hamer et al. 2003; De Rooij and De Boer 2003). Consequently, an important parameter that ultimately defines daily sperm production, the SC efficiency, was found to be reduced in scid mice (7.9 round spermatids per SC), a value comparable to the less efficient species already investigated, such as dog and marmoset (Lara et al. 2018).

In rodents, SC proliferation occurs during fetal and postnatal periods and is mainly regulated not only by FSH and androgens but also by the insulin family of growth factors, activin and cytokines (Desjardins and Ewing 1993; Meachem et al. 2005; Scott et al. 2008; Lucas et al. 2014; Meroni et al. 2019). Interestingly, in the Sertoli cell–selective androgen receptor knockout (SCARKO) mouse, the final number of Sertoli cells is unaltered, while the androgen receptor knockout (ARKO) mouse exhibits a progressive reduction in SC number/testis (Tan et al. 2005). This happens because androgens do not modulate SC proliferation via direct action on SC, since SC starts expressing androgen receptor when they become mature and stop proliferating. Therefore, androgen receptor expression in other testicular cell types (especially peritubular myoid cells (PTMCs)) is considered an important mediator for the stimulatory effect of testosterone on Sertoli cell proliferation (Buzzard et al. 2003; Tan et al. 2005).

The higher SC number observed in scid mice may suggest a higher proliferative activity or an extension of its proliferative period. Although there are no reports concerning the hormonal profile of scid mice during postnatal development, our

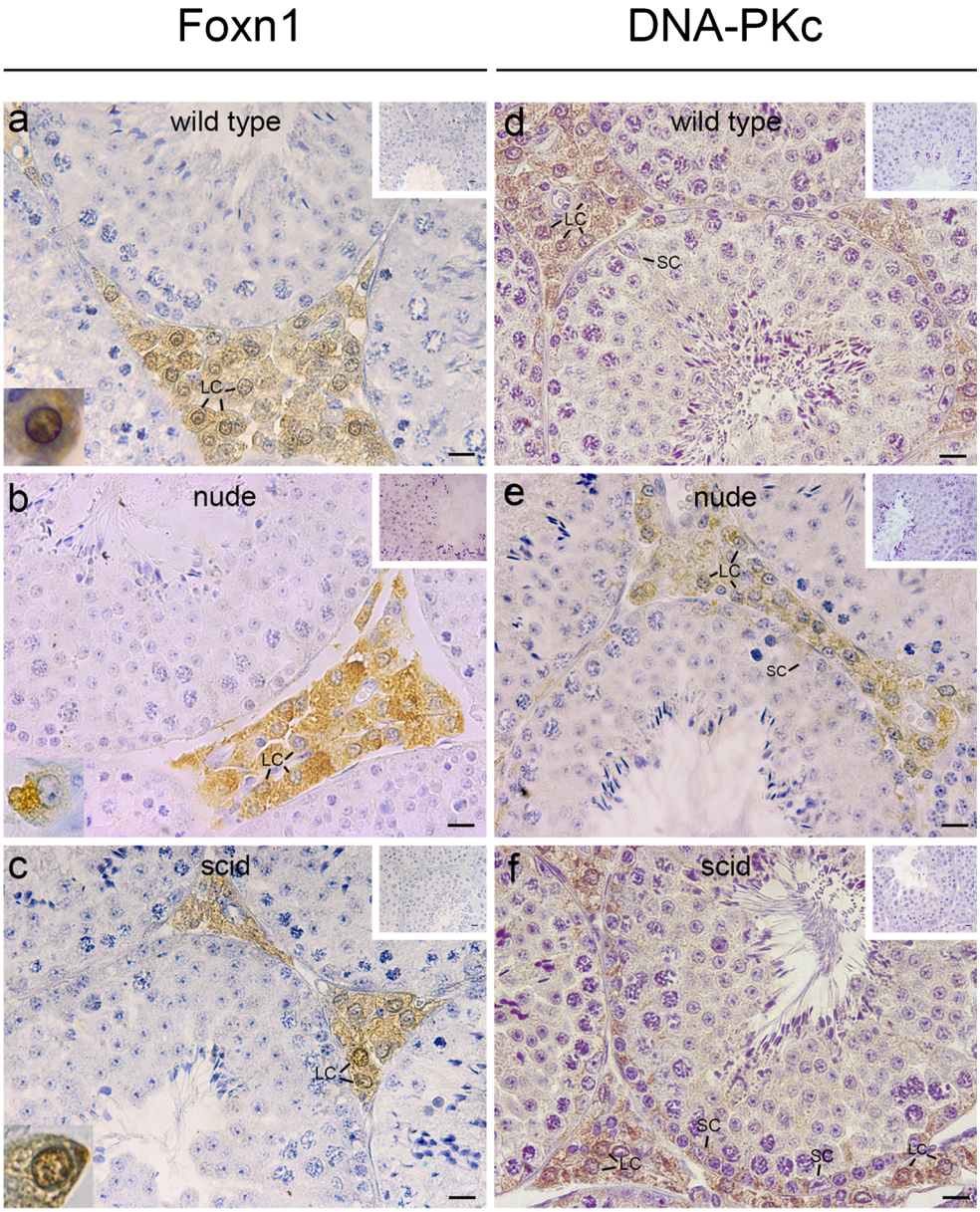

Fig. 4 Distribution of Foxn1 and DNA-PKc in the testis. Expression of the transcription factor Foxn1 in the testis of BALB/c wild type (**a**), nude (**b**) and scid (**c**) mice. **a**, **c** The expression of the protein in the cytoplasm and nucleus of the Leydig cells. **b** The nuclear labeling is absent in the Leydig cells. Distribution of the protein DNA-PKc in the testis of BALB/c wild-type (**d**), nude (**e**) and scid (**f**) mice. Note the cytoplasmic labeling in the Leydig and Sertoli cells. LC Leydig cell; SC Sertoli cell. Bar = 14 μm

indirect evaluation of androgen action by androgen receptor and 3BHSD analyses indicated a higher expression of these proteins in the testis of adult scid mice. Therefore, it is reasonable to hypothesize that androgens' indirect effect, possibly exerted through the secretion of paracrine factors (such as activin A, produced by PTMC), stimulates SC proliferation in scid mice. Due to the observed increase in SC number, total seminiferous tubule length and daily sperm production, which are highly correlated parameters, the scid mice showed the highest spermatogenic efficiency (daily sperm production per gram of testis) among the three groups investigated, despite its lowest SC efficiency.

The transcription factor Foxn1 is translocated to the cell nucleus after phosphorylation, where it regulates the expression of several genes (Mecklenburg et al. 2005). In the present study, we observed that Foxn1 was equally expressed in the LC cytoplasmic compartment across the strains, whereas its nuclear expression was rarely observed in nude mice. The difference regarding intracellular distribution of Foxn1 protein is probably explained by the fact that nude mice mutation generates a premature stop codon, producing an unstable inactive protein that lacks the DNA binding domain (Nehls et al. 1994; Brissette et al. 1996). Therefore, although still producing the protein, as observed by its presence in the LC cytoplasm, Foxn1 expression was rarely seen in the nucleus, being inactive in nude mice. In this context, the cytoplasmatic/nuclear distribution of the Foxn1 transcription factor in the wild-type LC strongly suggests its participation in the genetic regulation of the function of these cells, possibly in the steroidogenic pathway. This type of steroidogenic regulation is

already observed for another protein of the forkhead family, Foxl2, which is a transcription factor involved in the modulation of the expression of steroidogenic acute regulatory protein (*StAR*) and aromatase (*CYP19A1*) genes (Pisarska et al. 2004; Pannetier et al. 2006).

Regarding the DNA-PK enzyme, its presence was already described in the germ cell nucleus and in the cytoplasm of SC and LC (Hamer et al. 2003). Therefore, once this protein was observed in the cytoplasm of SC and LC in the present study, our results indicate the involvement of these somatic cells in the synthesis of the catalytic subunit of DNA-PK repair enzyme. The *Prkdc* gene mutation also leads to a premature stop codon, synthesizing an unstable protein that accumulates in the cytoplasm (Blunt et al. 1996), which explains the expression of this protein also in scid mice testis.

It is important to mention that even though nude and scid mice strains share an immunodeficient phenotype, our results indicate that testicular alterations in these mice are probably more related to their genotypes than to the immunodeficiency itself. Corroborating that, we observed that many parameters differed between nude and scid mice, which indicates that several and different mechanisms led to the distinct observed testicular alterations. Moreover, suggesting a particular role for these proteins in testis function, we identified the expression of DNA-PKc and Foxn1 in the testis.

Taken together, our present findings provide a better understanding of nude and scid mice adult testis function. For the first time, we showed that Foxn1 and DNA-PKc proteins might play a role in maintaining testis physiology. Considering these quite relevant results and, in order to better understand the involvement of *Foxn1* in testis function, we are currently investigating the role of this transcription factor during postnatal testis development, particularly focusing on LC. In summary, the immunodeficient nude and scid mice have unique testicular characteristics that make them adequate models to investigate the testis regulatory mechanisms, in particular the pathways involved in the control of SC and LC proliferation and function in mammals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais—CETEA/UFMG—Protocol no. 123/2013).

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ARTIGO 3 – A ser submetido

IMPAIRED *Foxn1* GENE EXPRESSION AFFECTS NORMAL TESTIS POSTNATAL DEVELOPMENT IN MICE

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Short title: ***Foxn1*** **IMPORTANCE FOR TESTIS DEVELOPMENT**

Abstract

Nude mice are immunodeficient due to a mutation in *Foxn1* gene. Therefore, they are athymic and have lower levels of gonadotropins and testosterone. Previus work from our laboratory already showed several differences in adult nude mice testis in comparison to the wild type. The most important results regard Leydig cells (LC): nude mice have a higher population of those cells, with a smaller volume. Furthermore, the expression of several steroidogenic enzymes was higher in nude; which corresponds to a compensatory effect, since they have smaller levels of testosterone. The expression of the transcription factor Foxn1 found in LC cytoplasm and nucleus explains the regulation of the steroidogenic enzymes production. In order to better understand if these peculiar reproductive aspects already happen in nude mice during testis development, we conducted an investigation of the testicular function in nude BALB/c male mice in several ages of postnatal development. Interestingly, in general, the results found along testis development do not follow the same pattern seen for adult nude mice testis, indicating that, possibly, Foxn1 affects differently fetal and adult LC populations. It seems that the effects of impaired expression of this transcription factor are more pronounced at late development and sexual maturity, coinciding with the period in which most of the LC are the adult type. Furthermore, the variable expression of this protein along the development, and the identification that not all LC that express Foxn1 also express Hsd17b3, makes it clear that there are distinct LC populations, according to its protein expression pattern.

Key words: Foxn1, nude mice, Leydig cell, Hsd17b3.

Introduction

The spontaneous mutant nude mice were first discovered by Flanagan in 1966 and are characterized by the lack of skin fur development and agenesis of the thymus (Mecklenburg *et al*., 2005). This phenotype is due to a mutation in *Foxn1* gene that consists of a deletion of a single base pair (G) in exon 3, which leads to a premature stop codon, producing an inactive protein. Foxn1 is a trascription factor from the winged helix/forkhead family (Takahashi *et al*., 1992; Byrd, 1993; Nehls *et al*., 1994), that consists of over 100 proteins, several of which are already reported to regulate reproduction (Uhlenhaut & Treier, 2011) and gonadotropin (Follicle stimulating hormone - FSH and Luteinizing hormone - LH) genes expression (Thackray, 2014).

There are only few studies in the literature regarding reproductive aspects of nude mice. Rebar and colleagues (1982) showed that athymic nude mice have lower blood levels of testosterone and gonadotropins (FSH and LH) in comparison to the wild type. Also, a recent study from our group (Oliveira *et al*., 2020) showed that adult nude mice present remarkable differences from the wild type regarding testicular composition and function, especially those related with Leydig cells (LC), such as larger population and smaller individual cell volume. Furthermore, not only structural differences were found but also, in the expression of androgen receptor and steroidogenic enzymes, which suggests a role for *Foxn1* gene regulating androgen production. Indeed, the transcription factor was localized in the cytoplasm and nucleus of LC.

In this regard, the peculiarities and specific features found in sexually mature nude mice raise many questions regarding Foxn1 regulation in hormonal balance and LC function along testicular development. Therefore, the aim of the present cohort investigation was to fill this gap and evaluate several parameters along postnatal testis development of the nude mice, in order to understand further the roles of Foxn1 focusing on LC population.

Material and Methods

Animals, sample collection and processing

The nude mice used in this study were purchased from Taconic, Germantown, USA. Ten to thirty two male mice of each strain herein investigated (BALB/c wild type and BALB/c nude) were used for the following postnatal days (Pnd): 1, 5, 10, 15, 20 and 25. Besides that, five to seven sexually mature (Pnd70) wild type (WT) and nude mice were addressed to specific approaches (plasmatic testosterone measurement and immunohistochemistry). Prior to euthanasia, the animals were weighed and had their

anogenital distance measured with a digital pachymeter. The WT mice were housed in a standard animal facility and the immunodeficient mice were housed in filtered microisolator cages, with ventilated rack system (Alesco®), under controlled photoperiod (12h light, 12h dark), with access to autoclaved water and rodent food, *ad libitum.* All procedures and protocols followed approved guidelines for the ethical treatment of animals according to the Ethics Committee in Animal Experimentation from the Federal University of Minas Gerais (CEUA/UFMG – Protocol #123/2013).

The mice used for histomorphometry (n=6 per age and group) and immunohistochemistry (n=3 per age and group) were anesthetized with an intraperitoneal injection of pentobarbital (Thiopentax®, Cristalia, Brasil; 150 mg/kg) and testes were collected and weighed. The testes were fixed with glutaraldehyde 4% in 0.05M fosfate buffer (for histomorphometric analysis) or Bouin's solution (for immunohistochemistry), following the procedure described by Sprando (1990). After fixation, testes were sectioned in smaller fragments with a razor blade. The testis fragments were dehydrated in increasing alcohol concentrations and embebbed in glycol methacrylate (Leica Biosystems, Germany) for histomorphometric analysis or Paraplast® (Sigma-Aldrich, Germany) for immunohistochemistry.

Histomorphometric analysis

All morphometric analysis followed the same methods described by Auharek *et al*., 2012 and Lara & França 2017. Six animals from each group at the ages Pnd1, 5, 10, 15, 20 and 25 were analysed. The volume densities of various testicular components were determined using a 441-intersection grid placed in the microscope's ocular at 400x (for Pnd15, 20 and 25) or 1,000x (for Pnd 1, 5 and 10) magnification. Fifteen fields randomly chosen (6,615 points) were counted for each animal and points were classified as one of the following: seminiferous tubules, including tunica propria, seminiferous epithelium, Sertoli cells and lumen; intertubular compartment, comprising Leydig cells, connective tissue, blood and lymphatic vessels. The volume of each component was determined as the product of its volume density and testis volume. The specific density of the testis was considered to be 1.0 (Leal & França, 2006) and the testis capsule (~3.5%) was excluded from the testis weight.

The diameter of the seminiferous cords/tubules was measured in thirty round cross sections, chosen randomly for each animal, using ocular micrometer calibrated with a stage micrometer at 400x magnification. The total length of the seminiferous tubules was obtained by dividing the seminiferous tubules volume by the square radius of the tubule multiplied by the π value (Auharek *et al*., 2012). The lumen formation was evaluated from the analysis of

100 seminiferous cord/tubule cross sections that were classified as with or without lumen for each mouse at each age.

Cell counts

Mean Sertoli cell (SC) nuclear volume was determined by measuring nuclear diameter for 30 nuclei in each animal and calculating volume (μm^3) according to the formula $4/3\ \pi r^3$, where R = nuclear diameter/2. The total number of SC per testis was determined as follows: total number of SC per testis = total volume of SC nucleus in the testicular parenchyma (μm^3)/SC nuclear volume (μm^3). The volume of individual Leydig cells was obtained from the measurement of LC nuclear and cytoplasmic volume. Because the LC nucleus observed by light microscopy in mice is round or nearly round, its volume was easily determined from its mean nuclear diameter. For this purpose, 30 nuclei showing an evident nucleolus were measured for each animal at 1,000x magnification. Leydig cell nuclear volume was expressed in μm^3 and obtained by the formula $4/3\pi r^3$, where R = nuclear diameter/2. To calculate the proportion between nucleus and cytoplasm, a 441-point intersection grid was placed over the sectioned material at 1,000x magnification. One thousand points over LC per testis were counted for each animal. The number of LC per testis was estimated from the LC individual volume and the volume density of LC in the testis parenchyma.

Immunohistochemistry

For the immunohistochemistry analyses, we used antibody against Foxn1 (1:100, Santa Cruz Biotechnology, USA, cat # SC-30195) and Hsd17b3 (1:200, antibody donated by Dr. Ken-ichirou Morohashi). Briefly, paraffin sections at 5µm thickness were mounted on coated slides, dewaxed and rehydrated. Antigen retrieval was performed in boiling citrate buffer (pH 6.0) for 5 min in a microwave oven. For the immunoperoxidase, endogenous peroxidase was blocked by incubating slides in 30% hydrogen peroxide (Sigma Aldrich) for 30 min at room temperature. Nonspecific binding sites were blocked with 10% normal goat serum (for immunoperoxidase) or 3% bovine serum albumin (for immunofluorescence) (Sigma Aldrich) in PBS, before the addition of primary antibodies and incubation at 4°C overnight. For doublestanning, both primary antibodies (Foxn1 and Hsd17b3) were added together. In the next day, the slides were exposed to the appropriate secondary antibodies for 60 min (for immunoperoxidase – goat anti-rabbit, 1:200, Abcam, cat # AB6720) or 90 min (for immunofluorescence – 488 donkey anti-rabbit, 1:300, Invitrogen, cat # A21206 and 633 goat anti-rat, 1:100, Life technologies, cat # A21094) at room temperature. For immunoperoxidase, detection of the signal was achieved by incubating the slides in streptavidin (TS-125-HR, Thermo Scientific) for 30 min, followed by the reaction with

peroxidase substrate 3,3'-diaminobenzidine (DAB, Sigma Aldrich) and counterstaining with hematoxylin (Merck). After dehydration, sections were mounted. The slides were analysed in order to identify the cell types labelled and its location in the testicular parenchyma, as described by Oliveira *et al*., 2020. Confocal images were obtained in a 510 META Laser Scanning Confocal Microscope (Zeiss, Oberkochen, Germany), equipped with 488 and 633 nm lasers. Dual channel images were obtained by sequential scanning.

Testosterone measurement

The plasma was separated from the blood samples collected at the time of euthanasia by centrifugation (2000 rpm for 20 min at 4ºC) and stored at −20°C for subsequent hormone measurements (n= 4 to 7 mice or pools per age and strain). Plasmatic testosterone levels were measured by electrochemiluminescence assay using the automated platform Cobas 8000 (Roche Diagnostics Inc., Indianapolis, IN, USA). The coefficients of variation (CV) intra- and inter-assay were, respectively, 1.1% and 1.5%. These procedures were performed by Tecsa – Technology in Animal Health, Belo Horizonte, MG, Brazil.

Statistical analysis

Values are expressed as mean ± SEM and were tested for normality (D'Agostino & Pearson) and analysed accordingly using Student T (for parametric) or Mann-Whitney (for non-parametric) tests. All analyses were done using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) and significancy level considered was $p<0.05$.

Results

Biometric data

Body and testicular weights, gonadosomatic index and anogenital index are shown in Figure 1. The body weight was reduced significantly in nude mice from Pnd1 onwards. The testis weight followed the same trend and it was significantly smaller in nude at Pnd1, 5, 10 and 20. Gonadossomatic index (ratio between total testicular mass and body mass) at Pnd15 and 25 was higher ($p<0.05$) in nude, same ages were testis weight did not differ between groups.

Interestingly, the anogenital index (ratio between anogenital distance and the cube root of the body weight) reversed along the postnatal development, since it was significantly higher in Pnd1 but at Pnd20 and 25 it became smaller in nude in comparison to the WT.

Histomorphometric analysis

Histologically, the development of testis parenchyma followed a typical pattern, regardless of strain or age. In overall, seminiferous cords/tubules diameter did not differ along the period evaluated, except at Pnd10, the diameter was significantlly smaller in nude (Figure

2). The total length of seminiferous tubules per testis was not different between groups. However, when it was normalized by testis weight (Figure 2), the total length was significantly higher at Pnd10 and 15 in nude.

Figure 2 represents the lumen establishment in the seminiferous cords from Pnd1 to 25. Lumen was first seen at Pnd10 in both strains and represented, respectively, 1.7% and 4% of the total tubular component evaluated in nude and WT. At Pnd15, seminiferous tubules corresponded to 50% and 70% in nude and WT, respectively. Although the difference observed between nude and WT has been significative at Pnd20 (nude, 53%; WT, 83%), the percentage of tubules with lumen at Pnd25 has returned to the not significant level, it standing between 75% and 81%.

Table 1 summarizes the volume densities of testis components from Pnd1 to Pnd25. Except by the smaller percentage of connective tissue observed at Pnd1 in nude, the main differences between groups showed up from Pnd10 onwards. Germ cells and lymphatic space percentages were smaller in nude at Pnd10. Percentage of SC at Pnd15 was significantly higher in nude, however an inverse behaviour was observed for blood vessels. Regarding components of tubular compartment, the percentages of germ cells and lumen at Pnd20 were, respectively, higher and lower in nude when compared to WT. In the interstitial space, the percentage of LC occupation was significantly smaller in the immunodeficient mice. Significant differences were observed between nude and WT mice regarding tubular and intertubular compartments at Pnd25. Despite the highest total of seminiferous tubules, the percentage of tunica propria presented the lowest value in nude. In addition, were also lower the percentages of LC, lymphatic space and connective tissue which, somehow, contributed with the smaller interstitial compartment observed in nude.

Cell counts

Figure 3 denotes the main findings regarding Leydig cell. Differences between nude and WT started at Pnd10, for instance, the nuclear volume significantly smaller in the immunodeficient mice. At Pnd15, besides the smaller nuclear volume, those cells in nude mice had also smaller cytoplasmic volume, which has resulted in smaller LC, as corroborated through the analysis of the individual cell volume. At Pnd20, the smaller cytoplasmic and individual volumes persist, whereas at Pnd25, these differences were no longer observed. Even though a very clear trend had been observed in the differents ages of the nude mice, the LC population was significantly smaller only at Pnd20 and 25.

Regarding SC, the nuclear volume followed the same pattern in both nude and WT mice so that, no differences were observed (Fig. 4A). The SC numbers along the postnatal

period showed that there were no evident differences other than at Pnd20, when the population of SC was smaller in nude (Fig. 4B).

Immunohistochemistry

The postnatal pattern of Foxn1 cellular localization is shown in Figure 5. Thus, from Pnd1 to 25, Foxn1 was present exclusively in LC, in nude and WT BALB/c mice. However, differences associated with subcellular distribution and intensity of labelling were remarkable in WT. Therefore, LC depicting cytoplasm labelling were more easily seen in the older ones when compared with the precedent ages (Fig. 5A, C, E, G, I, K). In addition, Foxn1 protein was also observed in the nuclear compartment at Pnd25 (Fig. 5K). The Foxn1 expression nude-related showed that the protein was limited to the cytoplasm compartment and its subcellular distribuition, unlike of the observed for WT, did not change along the period investigated. Some clusters of not labelled LC were frequently observed in the groups.

Foxn1/Hsd17b3 double-staining in WT revealed a close association between the timing for the expression of these proteins in LC, from 10 days of age to maturity (Pnd70) (Figure 6). In addition, an intensification of Hsd17b3 and Foxn1 signaling from 10 to 70 days of age was observed. Moreover, LC expressed both proteins simultaneously, except at Pnd70, when groups of cells Foxn1 negative /Hsd17b3 positive and Foxn1 positive /Hsd17b3 negative were observed in the interticial compartment, together with the positive-doubled-stained cell population.

Testosterone levels

The steroidogenic activity of nude mice (and WT) LC was assessed based on the levels of peripherical testosterone (Figure 7). At Pnd70, after sexual maturity, the plasmatic levels of testosterone were significantly smaller in nude than in the WT mice. Although no significant difference was seen along the development, it was possible to observe that nude mice followed a trend to have higher levels of plasmatic testosterone than WT from Pnd1 to 25.

Discussion

Evidences from adult BALB/c Foxn1-/- mice published recently (Oliveira *et al.*, 2020) assigned to Foxn1 transcription factor an important role on the establishment of population as well as the steroidogenic function of LC. Thus, in order to elucidate the possible sensitive window to the impaired Foxn1 protein, in the present study we performed a postnatal testis evaluation in Foxn1-/- and WT BALB/c mice. In this regard, after correlating the protein occurrence with the morphometrical findings, we may suggest Foxn1 as a marker for a

specific subpopulation of ALC in mice, being an important player from middle to late postnatal period of testis development as well as during sexual maturity.

According to the literature, anogenital distance (AGD) is positively associated with final testis size in rats (Scott *et al*., 2008; Drake *et al*., 2009), and it is determined by androgen exposure within the masculinization programming window (MPW) in fetal life (Welsh *et al*., 2008). So, AGD is a trustful sensor for assessing effects of endocrine disruptors early in development. Therefore, anogenital index (AGD corrected by body mass) differences between Foxn1-/- and WT mice were evident from Pnd20 and maintained at Pnd25. In this regard, the anogenital index revealed that androgens levels possibly were decreased at meantime the MPW was established in nude, despite the observed for Pnd1 nude, in which anogenital index was determined mainly for the slightly higher AGD.

Sertoli cells have many essential functions, since the earliest events associated with gonadal differentiation until later maintenance of spermatogenesis. Blood-testis barrier formation, hormonal mediation, germ cell support are some of the many SC roles that ensures the appropriate inner milieu of seminiferous tubules for germ cells development (Hess & França, 2008). In overall, SC proliferation followed the same pattern, regardless of the mice genetic background. Therefore, the rapid increasing of this cell population per testis, from birth to Pnd10, is an outcome of the intense proliferative activity during this phase. From Pnd15 onwards, none remarkable increase of SC numbers per testis was observed, as a consequence of the gradual reduction of cell division rate, since, in mice, the SC proliferation window lasts around 2 weeks postnatal. Hence, the SC population is established in both strains within the same period of time and, as we showed in our previous paper (Oliveira *et al*., 2020), it is not different in the mature mice. In this regard, it seems that protein Foxn1 is not a limiting factor for determination of the postnatal SC proliferation period in mice.

It is well established that Leydig cells have two distinct populations in mice and rats (Mendis-Handagama *et al*., 1987; Vergouwen *et al*., 1991; Kerr & Knell 1988; Chen *et al*., 2010). In mice, fetal Leydig cells (FLC) differentiate from progenitor cells expressing steroidogenic factor 1 (SF-1) around 12.5 embryonic days and are involved in androgen production by fetal testis, fundamental for masculinization and development of external genitalia (Barsoum & Yao, 2010; Shima, 2019). Although few FLC are still remaining, most of the steroidogenic cells present in the adult mice testis correspond to the adult Leydig cells (ALCs). ALCs are first detected around the 7th postnatal day (Griffin *et al*., 2010; Shima *et al*., 2015; Shima & Morohashi, 2017) and four distinct subpopulations are observed during the differentiation phase: stem, progenitor, immature and adult/mature (Chen *et al*., 2010). It

is noteworthy that terminally differentiated LC (fetal and adult) rarely proliferate (Orth, 1982; Keeney *et al*., 1988; Chen *et al*., 2010; Miyabayashi *et al*., 2013); so that, the replacement of these cells after injuries depends on the progenitor subpopulation present in the intertubular compartment (Jackson *et al*., 1986; Morris *et al*., 1986).

Until recently, it was considered that FLC and ALC had different origins and did not share any step during differentiation. However, based on lineage-tracing analyses, Shima and colleagues (2018) demonstrated that significative part of ALC population in mice originates from dedifferentiated FLCs. These authors observed that FLC can dedifferentiate in fetal testis and contribute to peritubular myoid cells and vascular pericytes, which may differentiate later on into ALC population featured by FLC-gene expression signature. In the present study we observed two distinct populations of Leydig cells based on Foxn1 expression, corroborating our findings for adult mice (Oliveira *et al*., 2020). Indeed, a group of interstitial cells stood unlabelled and rarely seen at older ages whereas another group raised, and the crescent intensity of labelling as well as the occurrence of Foxn1-positive cells was closely associated with the appearing of ALC in the testis. Thus, the differential pattern of expression herein observed allow us to suggest Foxn1 as ALC marker, even though in the youngest (Pnd1 to 5), since it is known that ALC precursors are already seen in fetal testis (Qin *et al*., 2008; Ge *et al*., 2006). On the other hand, cells that did not express Foxn1 along the period investigated may be representing the FLCs reminiscent in the adult testis (Shima *et al*., 2015). In order to prove our hypothesis, we evaluated whether a well-known marker for ALC, the steroidogenic enzyme Hsd17b3 (O'Shaughnessy *et al*., 2000; Shima *et al*., 2013), would be expressed by the same Foxn1-positive cell types. Interestingly, we found at Pnd70, when ALC overrides FLC population, not only many doubled-stained cells, but also cells that were expressing Hsd17b3 or Foxn1, individually. These findings reinforce Foxn1 as a putative candidate for ALC marker. Hence, Hsd17b3+/Foxn1+ cells might be derived from those Hsd17b3-/Foxn1+ that should be considered as a progenitor type, whereas Hsd17b3+/Foxn1- cells maybe have origin from a different progenitor type, for instance, the FLC.

Unlikely the observed in our previous study (Oliveira *et al*., 2020), Foxn1-/- LC population was similar to the WT from birth to 15 postnatal days, shifting for lower numbers at Pnd20 and 25. Therefore, the higher population of LC seen at 70 days-old nude mice seems to be determined only at adulthood, mainly due to differentiation from its progenitors (Chen *et al*., 2010; Barsoum & Yao, 2010). These findings suggest that Foxn1 targets differently the

LC populations and differentiation phases along testis development. However, the manner how this transcription factor modulates these mechanisms remains to be further investigated.

The oscillation that we found for testosterone levels should be discussed under the light of the recent information regarding steroidogenesis into fetal mouse testis. In this species, FLC does not present the entire enzymatic chain, being the Hsd17b3 absent in these cells (O'Shaughnessy *et al*., 2000; Shima *et al*., 2013). Therefore, FLC in mice are not able to convert androstenedione to testosterone so, immature SC, that presents steroidogenic activity and expresses this key enzyme, does the complementary last step (Shima *et al*., 2013). Hence, during the neonatal period (Pnd1 to 5), testosterone was still being produced by immature SC. However, as long as the ALC population increased (~Pnd10), this function was gradually lost in SC and was assumed by the new population of steroidogenic cells. It is worth mentioning that we did not observe significative differences between strains along the period investigated, which comprises the neonatal, infantile and part of juvenile phases of the postnatal development (Picut *et al*., 2018). Nevertheless, at sexual maturity (Pnd70), testosterone was exclusively provided by ALC. Thus, at this point, the androgen levels were significatively low in Foxn-/- mice. In this regard, these hormonal findings in association with our former data corroborate the hypothesis raised in this study, in which Foxn1 should not be considered only a marker but also, an important regulator of the steroidogenesis in ALC population. Hence, as a transcription factor, Foxn1 might be modifying the gene expression pattern in LC, inducing the alterations in the steroidogenic pathway

In conclusion, our data provide new insights regarding the potential role of Foxn1 on the regulation of cell number and also function of LC, during postnatal development. In overall, the results found along testis development did not follow the trend as it was detected for adult nude mice, indicating that, possibly, Foxn1 affects differently the distinct Leydig cell populations and differentiation phases as well. It seems that the effects of impaired expression of this transcription factor are more pronounced at late development and sexual maturity. Furthermore, the variable expression of this protein along the development, and the identification that not all LC that express Foxn1 also express Hsd17b3, makes it clear that there are distinct LC populations, according to its protein expression pattern. In this regard, we also reinforced that nude mice are an important model for understanding Foxn1 role on testis function.

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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Tables

Table 1: Volume density of testis components (%) in BALB/C wild type (WT) and nude mice at ages Pnd 1 to 25 (mean ± SEM). N= 6 animals per strain and age.

	Pnd1		**Pnd5**		**Pnd10**		**Pnd15**		**Pnd20**		**Pnd25**	
	WT	**nude**	**WT**	**nude**	**WT**	**nude**	**WT**	**nude**	**WT**	**nude**	**WT**	**nude**
Volumetric density (%)												
Seminiferous tubules	57.7 ± 2.3	64.2 ± 4.1	71.5 ± 1.8	69.2 ± 1.8	84.7 ± 0.4	83.3 ± 0.7	86.7 ± 0.6	88.3 ± 0.6	92.6 ± 0.6	93.9 ± 0.4	92.5 ± 0.3	94.7 ± 0.3*
Seminiferous epithelium	31.78 ± 1.3	35.8 ± 2.8	42 ± 0.9	41.3 ± 1.6	60.3 ± 0.5	57.2 ± 1*	65.9 ± 0.7	66.1 ± 1.3	72.3 ± 1.4	78.9 ± 1.1*	78.9 ± 1.1	82.1 ± 0.5
Lumen	0	0	0	0	0.13 ± 0.07	0.07 ± 0.04	6.1 ± 0.4	5 ± 0.6	8.7 ± 1.3	3.5 ± 0.5*	6.7 ± 1	6.3 ± 0.6
Tunica propria	9.5 ± 0.6	10.5 ± 0.7	7.5 ± 0.69	7.57 ± 0.38	6.7 ± 0.4	7.3 ± 0.5	5.4 ± 0.3	5.9 ± 0.2	4.4 ± 0.1	4.5 ± 0.2	3.5 ± 0.1	2.9 ± 0.1*
Sertoli cell	16.4 ± 0.8	17.9 ± 1.4	22 ± 0.8	20.4 ± 0.8	17.6 ± 0.6	18.7 ± 0.3	9.4 ± 0.3	11.4 ± 0.8*	7.2 ± 0.4	6.9 ± 0.8	3.4 ± 0.3	3.3 ± 0.3
Intertubular compartment	42.3 ± 2.3	35.8 ± 4.1	28.5 ± 1.8	30.8 ± 1.8	15.3 ± 0.4	16.7 ± 0.7	13.3 ± 0.6	11.7 ± 0.6	7.4 ± 0.6	6.2 ± 0.4	7.5 ± 0.3	5.3 ± 0.3*
Leydig cells	24.4 ± 2.3	24.87 ± 3.2	17.3 ± 1.8	19.4 ± 2.2	7.4 ± 0.6	7.9 ± 0.8	8.2 ± 0.2	7 ± 0.7	4.7 ± 0.6	3.2 ± 0.2*	4.9 ± 0.4	3.5 ± 0.2*
Blood vessels	2.5 ± 0.6	1.3 ± 0.3	1.3 ± 0.1	1.6 ± 0.3	0.68 ± 0.05	0.5 ± 0.07	1.17 ± 0.1	0.52 ± 0.2*	0.49 ± 0.08	0.71 ± 0.2	0.66 ± 0.04	0.78 ± 0.09
Lymphatic space	0.06 ± 0.02	0.09 ± 0.03	0.16 ± 0.12	0.1 ± 0.1	0.38 ± 0.07	0.13 ± 0.03*	1.04 ± 0.3	1.02 ± 0.3	0.6 ± 0.2	0.6 ± 0.1	0.64 ± 0.1	0.28 ± 0.1*
Conective tissue	15.4 ± 2.1	9.5 ± 0.9*	9.7 ± 1.4	9.7 ± 1.5	6.8 ± 0.4	8.1 ± 0.6	3 ± 0.4	3.1 ± 0.3	1.6 ± 0.1	1.7 ± 0.3	1.3 ± 0.1	0.8 ± 0.08*

* represent significant statistical difference ($p < 0.05$).

Figure legends

Figure 1: Biometric data: body weight (A), testis weight (B), gonadosomatic (C) and anogenital (D) indexes during postnatal testis development (Pnd 1 to 25) in BALB/c wild type (WT) and nude mice. *p < 0.05 in comparison with age-matched wild type. N = 10 to 32 animals per strain and age.

Figure 2: Seminiferous cord/tubular diameter (A), percentage of tubules with a lumen (B) total length of seminiferous tubules per testis (C) and per gram of testis (D) during postnatal testis development (Pnd 1 to 25) in BALB/c wild type (WT) and nude mice. *p < 0.05 in comparison with age-matched wild type. N = 6 animals per strain and age.

Figure 3: Leydig cell nuclear volume (A), cytoplasmic volume (B), individual volume (C) and population per testis (D) during postnatal testis development (Pnd 1 to 25) in BALB/c wild type (WT) and nude mice. *p < 0.05 in comparison with age-matched wild type. N = 6 animals per strain and age.

Figure 4: Sertoli cell nuclear volume (A) and population per testis (B) during postnatal testis development (Pnd 1 to 25) in BALB/c wild type (WT) and nude mice. *p < 0.05 in comparison with age-matched wild type. N = 6 animals per strain and age.

Figure 5: Distribution of Foxn1 in the testis. Imunoexpression of the transcription factor Foxn1 in the testis of BALB/c wild type (WT) and nude mice during postnatal testis development (Pnd 1 to 25). Arrowheads point to Foxn1 labelled cells. Bar = 12 µm.

Figure 6: Double staining immunofluorescence of Foxn1 and Hsd17b3 in the testis. Double staining of Foxn1and Hsd17b3 in the testis of BALB/c wild type mice during postnatal development and sexual maturity (Pnd10 to 70). Red arrows point to Hsd17b3 labelled cells, green arrows point to Foxn1 labelled cells and yellow arrows point to cells that ate labelled by both Hsd17b3 and Foxn1. Bar = 12 µm.

Figure 7: Testosterone plasmatic expression in BALB/c wild type (WT) and nude mice during postnatal testis development and sexual maturity (Pnd 1 to 70). *p < 0.05 in comparison with age-matched wild type. N= 4 to 7 animals or pools per strain and age.

Figures

Figure1

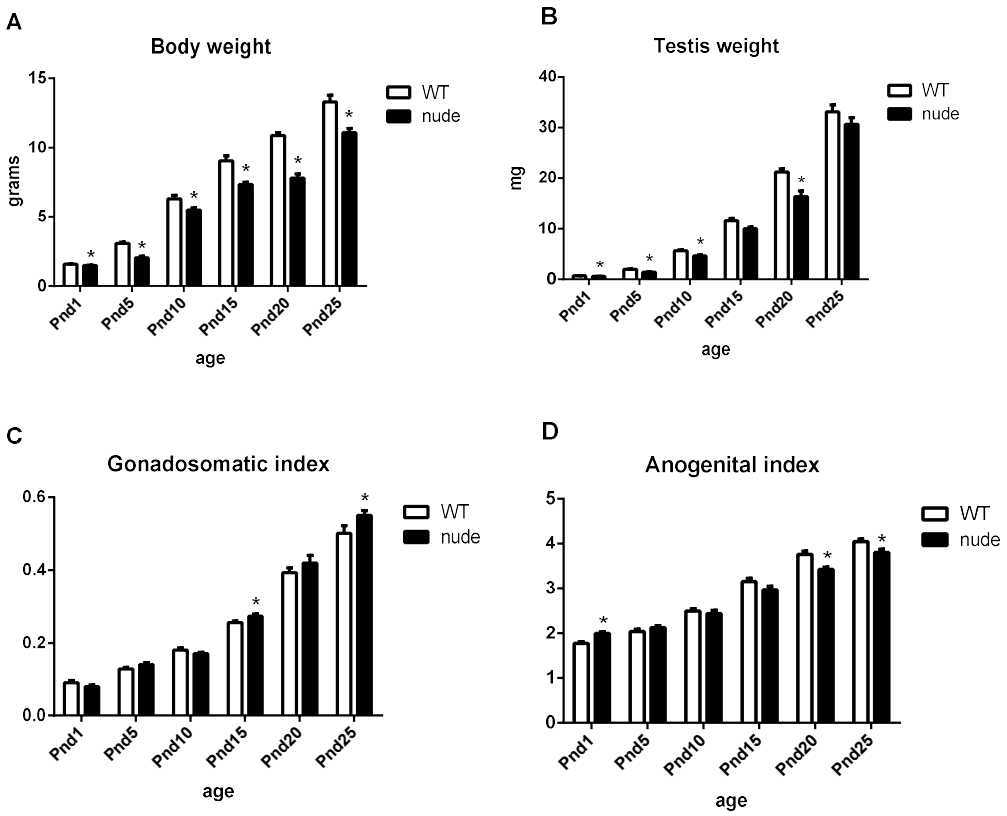


Figure 2

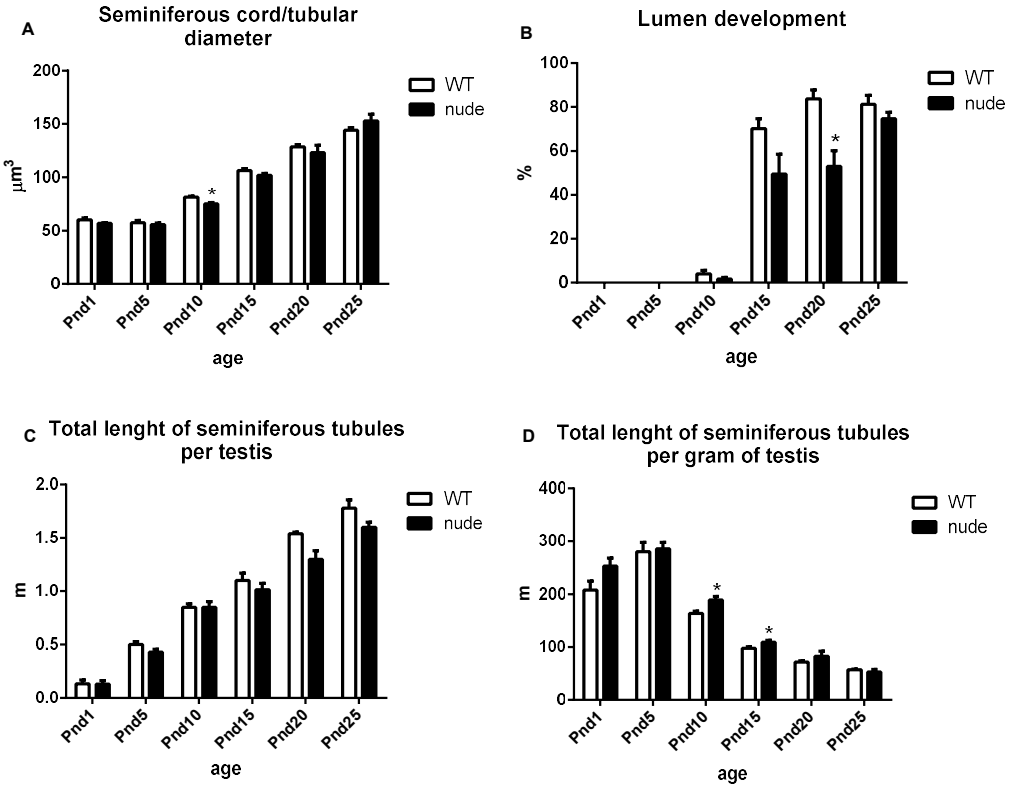

A
Seminiferous cord/tubular diameter
µm3
200
150
100
50
0
*
WT
nude
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25
age
B
Lumen development
%
100
80
60
40
20
0
*
WT
nude
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25
age
C
Total lenght of seminiferous tubules per testis
m
2.0
1.5
1.0
0.5
0.0
WT
nude
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25
age
D
Total lenght of seminiferous tubules per gram of testis
m
400
300
200
100
0
*
*
WT
nude
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25
age

Figure 3

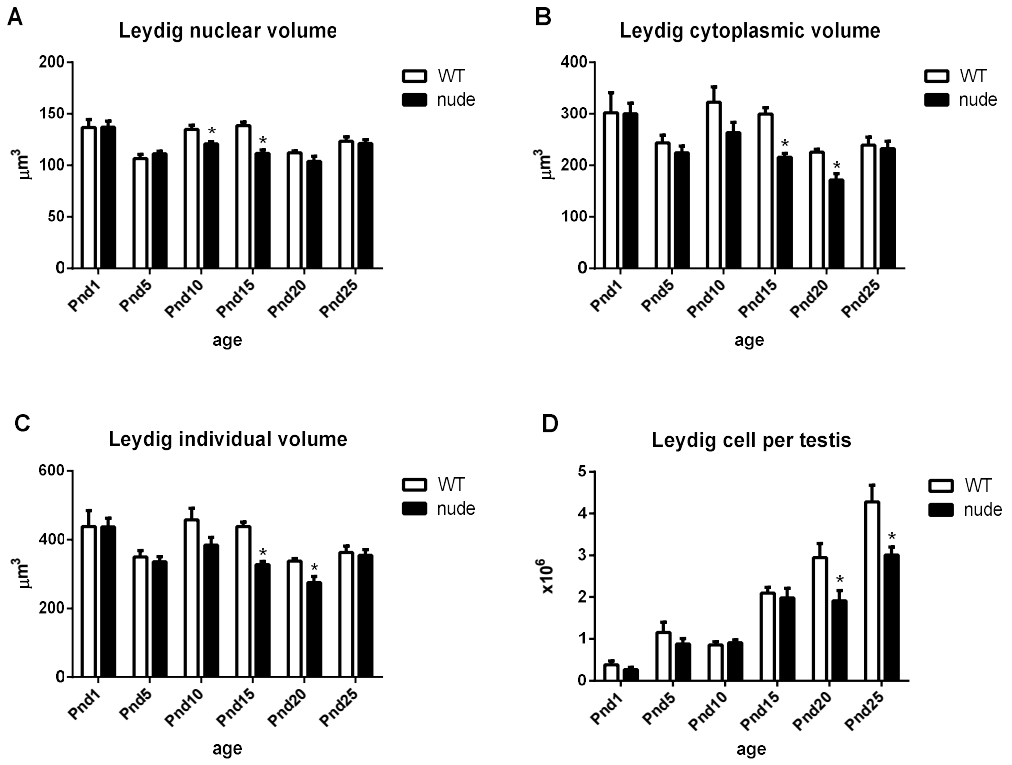


Figure 4

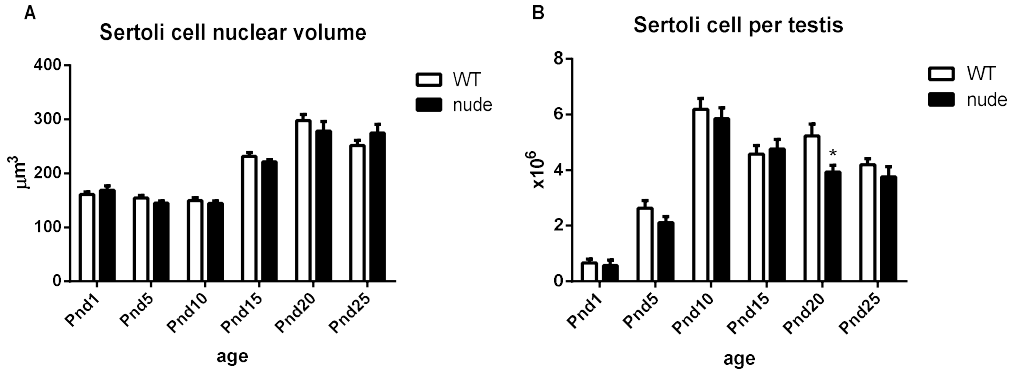


Figure 5

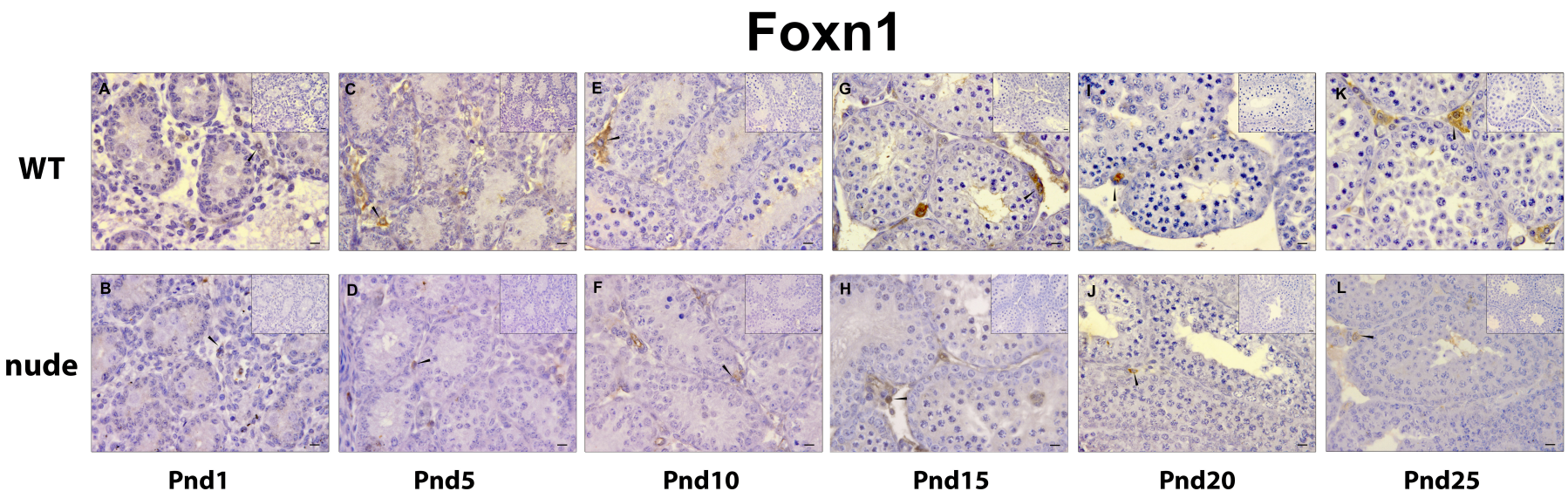

Foxn1
WT
nude
A
B
C
D
E
F
G
H
I
J
K
L
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25

Figure 6

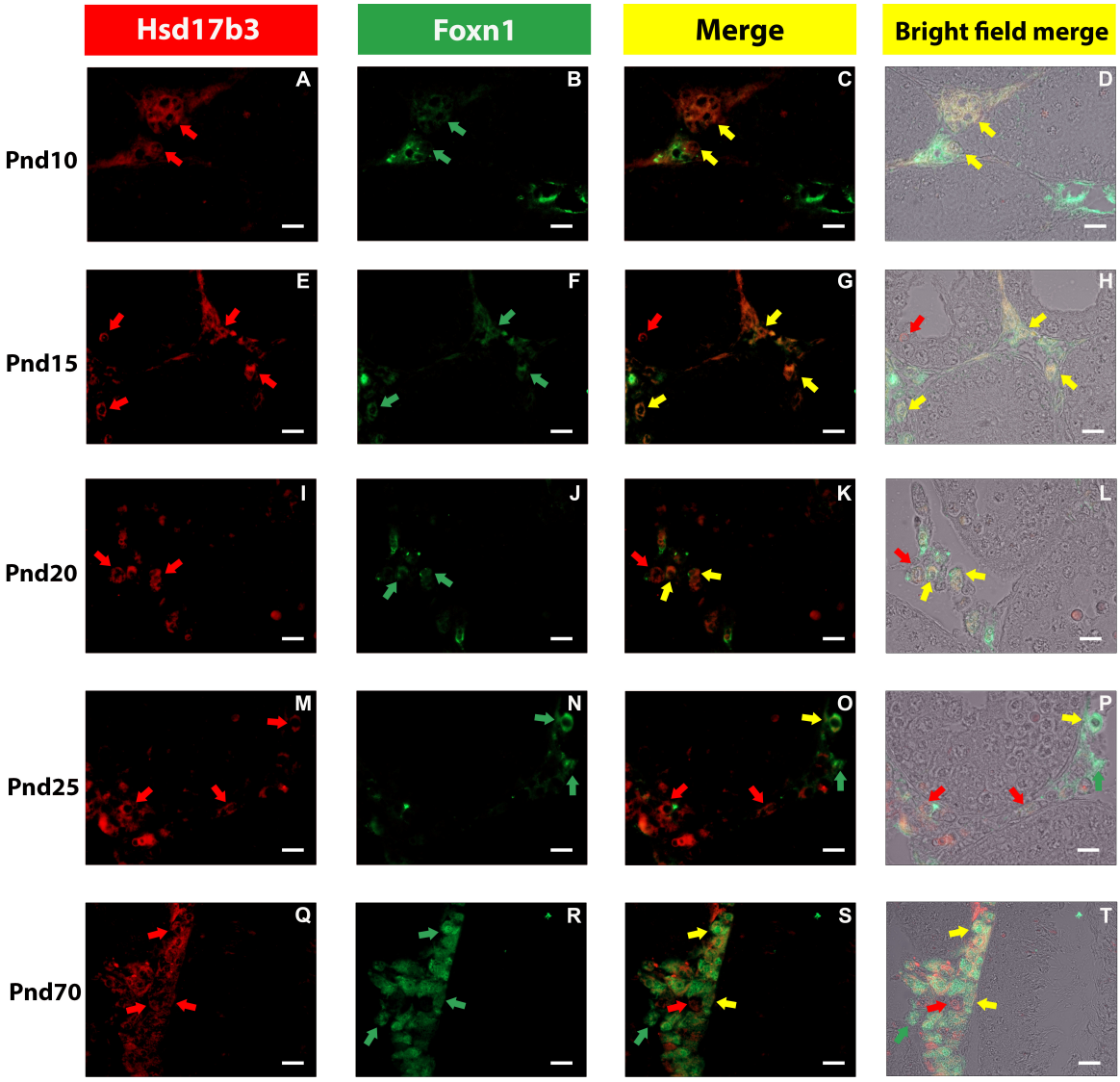
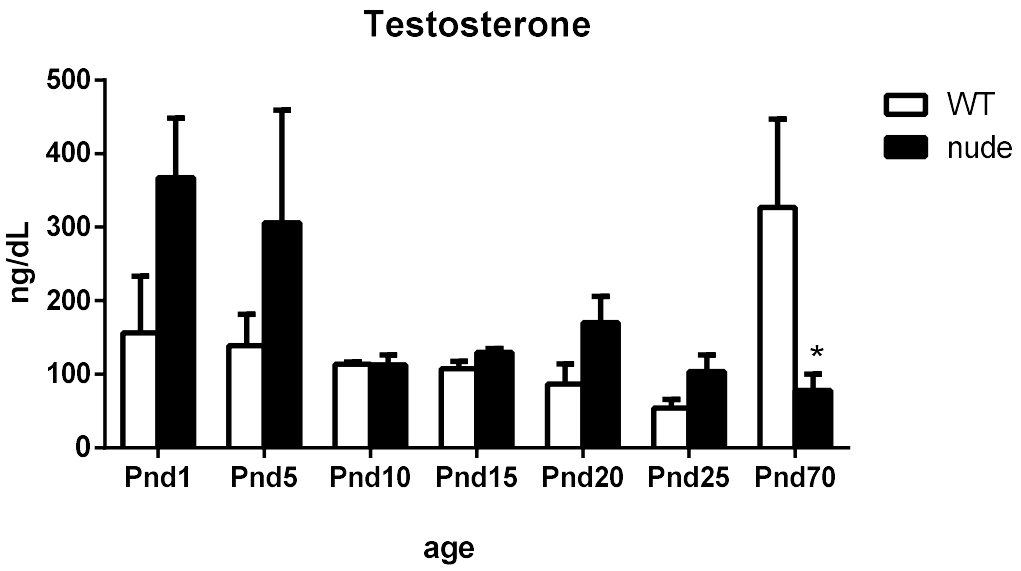

Hsd17b3
Foxn1
Merge
Bright field merge
Pnd10
Pnd15
Pnd20
Pnd25
Pnd70
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 7


Testosterone
500
400
300
200
100
0
ng/dL
Pnd1
Pnd5
Pnd10
Pnd15
Pnd20
Pnd25
Pnd70
age
WT
nude
*

4. DISCUSSÃO

O presente estudo é o primeiro a analisar, de forma detalhada e comparativa, a função testicular em diferentes linhagens de camundongos selvagens e portadores de mutações. Nossos achados mostraram importantes diferenças entre linhagens, mesmo entre aquelas consideradas selvagens, contribuindo assim para compreensão de características testiculares e reprodutivas de camundongos, reforçando a importância do conhecimento acerca de suas especificidades e ampliando o entendimento da espermatogênese nesta importante espécie. Além dessa relevante contribuição, foi possível caracterizar a presença e localização subcelular das proteínas Foxn1 e DNA-PKc nos testículos de camundongos, investigando sua função, tanto na maturidade sexual, quanto no desenvolvimento pós-natal.

No primeiro artigo desta tese, procuramos investigar diversos parâmetros testiculares comparativamente em três linhagens de camundongos amplamente citadas na literatura (C57BL6, *Swiss* e BALB/c). Apesar da estrutura e organização testiculares serem muito similares em uma mesma espécie, diversos trabalhos já demonstraram variações em parâmetros reprodutivos, como frequência de estádios e número de células de sertoli em diferentes raças de cães e bovinos (Okwun *et al*., 1996; Soares *et al*., 2009). Interessantemente, a linhagem *Swiss* foi aquela que apresentou um maior número de parâmetros com diferenças significativas, principalmente se comparada à linhagem C57BL6. De maneira geral, os camundongos *Swiss* apresentaram características interessantes relacionadas às células de Leydig, pois além de possuírem maior percentual do testículo ocupado por essas células, essa linhagem também apresenta maior número de células de Leydig em relação às demais. Por outro lado, o volume individual dessas células foi maior na linhagem BALB/c. É bem estabelecido na literatura que existem duas populações distintas de células de Leydig em camundongos, uma fetal e outra adulta (Vergouwen *et al*., 1991; Kerr & Knell, 1988; Chen *et al*., 2010). Desta forma, as variações observadas para os parâmetros de células de Leydig entre as linhagens podem ser resultado de diferentes estímulos e de alterações no microambiente ao longo do desenvolvimento testicular. Em função das diferenças estruturais e quantitativas observadas para as células de Leydig, os níveis plasmáticos de testosterona e de estradiol também diferiram entre as linhagens. Apesar de não haver diferença na expressão da enzima esteroidogênica 3βHSD, a concentração de testosterona foi maior em *Swiss*, enquanto o de estradiol foi maior em *Swiss* e BALB/c. O balanço entre andrógenos e estrógenos é essencial para o desenvolvimento normal dos orgãos sexuais, estabelecimento das características sexuais secundárias e manutenção do status reprodutivo ao longo da idade adulta (Schulster *et al*., 2016).

Não obstante o alto percentual de células de Leydig nos testículos, os macrófagos correspondem ao segundo tipo celular de maior ocorrência no compartimento intertubular. A influência dos macrófagos na proliferação, diferenciação e função das células de Leydig, até mesmo promovendo a esteroidogênese é bem estabelecida na literatura (Gaytan *et al*., 1994; Cohen *et al*., 1997; Hales, 2002; Hutson, 2006; DeFalco *et al*., 2015), sendo que em ratos estima-se a relação de 1 macrófago para cada 3-5 células de Leydig (Ewing *et al*., 1979; Miller, 1982; Bergh, 1985; Niemi *et al*., 1986; Diemer *et al*., 2003). Em camundongos a razão macrófago-Leydig ainda não é completamente esclarecida. Contudo, a utilização do marcador específico F4/80 revelou que aproximadamente 20% das células intersticiais correspondem a macrófagos ou ainda, que apresentam uma densidade relativa de 600 células por mm^2, em camundongos (Hume *et al*., 1984; Li *et al*., 1998). Macrófagos testiculares são altamente heterogêneos e alguns trabalhos apontam para a existência de subpopulações. Portanto, a utilização de apenas um marcador para esse tipo celular limita a identificação das diferentes populações, resultando em valores subestimados dos macrófagos residentes no testículo (Itoh *et al*., 1995; Winnall & Hedger, 2013; DeFalco *et al*., 2015; Mossadegh-Keller *et al*., 2017; Mossadegh-Keller & Sieweke, 2018). A internalização do corante azul de tripan possibilita identificar, de maneira assertiva, os macrófagos do testículo, uma vez que utiliza a principal característica da célula, isto é, sua capacidade de realizar fagocitose para assim torná-la evidente no compartimento intertubular. Dessa forma foi possível verificar que macrófagos compõem uma população mais robusta, cuja contribuição para o compartimento intersticial de camundongos é maior que aquela sugerida pelos trabalhos realizados anteriormente. Além disso, a relação entre células de Leydig e macrófagos, apesar de ter apresentado alguma variação entre linhagens, mostrou forte interação entre esses tipos celulares haja vista a razão próxima de 1 obtida nas diferentes linhagens de camundongos analisadas (razão de 1,01; 1,26 e 0,63 CL/macrófago em C57BL/6, *Swiss* e BALB/c, respectivamente).

De maneira geral, existem dois tipos de linhagens de camundongos, as *outbred* e as *inbred*. As primeiras apresentam alguma variação genética, sendo importantes para mimetizar populações humanas, enquanto as segundas são consideradas geneticamente idênticas, pois passam por 20 gerações de acasalamento consanguíneo (Davisson, 1999). No contexto do presente estudo, *Swiss* corresponde à linhagem *outbred*, apresentando maior variação genética entre os indivíduos e, portanto, sendo mais heterogênea que C57BL/6 e BALB/c, ambas consideradas *inbred*. A natureza *outbred* dos camundongos *Swiss* justifica em grande parte as diferenças verificadas nesta linhagem quanto aos diversos tipos de análises e parâmetros avaliados neste trabalho. Além das diferenças quantitativas relativas à contribuição dos tipos

celulares na composição do parênquima testicular, foi possível verificar importantes variações quanto a concentração dos esteroides sexuais na circulação periférica destes camundongos. Nesse sentido, as características que predominam nas diferentes linhagens devem ser levadas em consideração quando selecionadas para um estudo específico na área da biologia da reprodução.

No segundo artigo desta tese, camundongos das linhagens *nude* e *scid*, que apresentam mutações nos genes *Foxn1* e *Prkdc*, respectivamente (Bosma *et al*., 1989; Mecklenburg *et al*., 2005), foram utilizados para caracterizar a função desses genes nos testículos de camudongos adultos (70 dias). O gene *Foxn1* é traduzido em um fator de transcrição de mesmo nome, pertencente à família *winged helix/forkhead* (Byrd, 1993; Nehls *et al*., 1994), que apresenta mais de 100 proteínas, sendo que algumas delas, como Foxl2, Foxo1 e Foxp3, já foram descritas como reguladoras da reprodução e da expressão de gonadotropinas (FSH e LH) (Justice *et al*., 2011; Goertz *et al*., 2011; Skarra *et al*., 2013; Jasurda *et al*., 2014). Por outro lado, o gene *Prkdc* é traduzido em uma das subunidades (a catalítica) da enzima DNA-PK, responsável pelo reparo de quebra de fita dupla de DNA.

É importante ressaltar que nenhum dos genótipos mutados induziu alterações na citoarquitetura testicular dos camundongos e ambas as linhagens são férteis. Apesar disto, muitas diferenças foram observadas em diversos parâmetros avaliados neste estudo. Para a linhagem *nude*, as alterações mais proeminentes foram aquelas envolvendo a estrutura e função das células de Leydig. Uma vez que tais tipos celulares apresentaram menores volumes, o aumento em sua população parece ser um mecanismo compensatório para tentar garantir os níveis normais de andrógenos, mantendo a homeostase. Um padrão similar de regulação compensatória das células de Leydig foi observado anteriormente em camundongos deficientes para a enzima óxido nítrico sintase induzível, nos quais as células esteroidogênicas também apresentaram menor volume e maior número (Auharek *et al*., 2011). Além das variações estruturais encontradas nas células de Leydig de camundongos *nude*, sua função esteroidogênica também se apresentou alterada. Apesar de a literatura informar que os níveis de testosterona são mais baixos em *nude* (Rebar *et al*., 1982), em nosso estudo encontramos expressão aumentada de receptor de andrógenos e das enzimas esteroidogênicas 3βHSD e aromatase. Esses aparentemente dissonantes aumentos parecem estar relacionados a um efeito compensatório, pois os menores níveis circulantes de andrógenos estimulam a expressão de enzimas esteroidogênicas a fim de autorregular, positivamente, a produção de testosterona, ao mesmo tempo em que induzem o aumento na expressão do seu receptor. Confirmando essa hipótese, Tan e colaboradores (1988) observaram que ratos castrados têm um aumento nos

níveis de RNA mensageiro para a proteína receptor de andrógeno, em resposta às baixas concentrações periféricas de testosterona. Ademais, a partir do presente trabalho registrou-se, pela primeira vez na literatura, a expressão de Foxn1 nas células de Leydig de camundongos. Adicionalmente à expressão da proteína, foi possível sugerir uma relação funcional de Foxn1 e células de Leydig a partir dos dados quantitativos obtidos. Portanto, produtos de genes cuja transcrição é regulada pelo fator Foxn1 nas células de Leydig, possivelmente se tornaram parcial ou completamente inativos nos camundongos Foxn1-/-, resultando nos fenótipos testicular e hormonal caracterizados no presente estudo, subsidiando a hipótese da regulação de expressão gênica pelo fator de transcrição Foxn1 nas células de Leydig.

Diversas alterações também foram identificadas nos parâmetros testiculares avaliados para os camundongos com mutação em *Prkdc*. Similarmente aos camundongos *nude*, mas de forma menos severa, as células de Leydig também apresentaram menores volumes e maior população em *scid*, indicando um mecanismo de regulação compensatória semelhante. Alterações também foram observadas no compartimento tubular em *scid*. Nesse sentido, apesar de esta linhagem apresentar maior população de espermatogônias A, o número de espermátides arredondadas é semelhante ao selvagem, o que indica haver maior perda celular nessa linhagem. Esse resultado é consistente com a literatura, a qual descreve maior percentual de apoptose em espermatócitos em paquíteno no estádio IV do CES em camundongos com mutação no gene *Prkdc* (Hamer *et al*., 2003; De Rooij & De Boer, 2003). Interessantemente, a linhagem *scid* apresentou maior população de células de Sertoli, tornando-a mais eficiente quanto a produção espermática. Apesar de não haver informações na literatura acerca dos níveis hormonais em *scid* durante o desenvolvimento pós-natal, nossa avaliação indireta, via receptor de andrógenos e 3βHSD, demonstrou maior expressão dessas proteínas, sugerindo um possível aumento nos níveis de testosterona. Neste contexto, os andrógenos são responsáveis pela regulação da secreção de fatores parácrinos pelas células peritubulares mioides como, por exemplo, a ativina A, e cuja ação se relaciona com o aumento da proliferação de células de Sertoli (Buzzard *et al*., 2003; Tan *et al*., 2005). Ademais, os resultados quanto à localização da DNA-PKc mostraram a presença da proteína no citoplasma das células de Sertoli e Leydig, corroborando nosso dados com a literatura (Hamer *et al*., 2003).

Este trabalho mostrou que os camundongos *nude* e *scid* são importantes modelos/ferramentas biológicas para o estudo da função dos genes *Foxn1* e *Prkdc* nos testículos. Além disso, nossos resultados sugerem que o fator de transcrição Foxn1 parece estar envolvido na regulação endócrina do testículo, já que diversas diferenças foram

observadas na estrutura e população das células de Leydig, bem como na expressão de enzimas esteroidogênicas em camundongos *nude*. Em relação aos camundongos *scid*, nossos resultados indicam um papel relevante para a DNA-PKc na regulação das células de Sertoli e da espermatogênese, já que observamos aumento no número dessas células, bem como na produção espermática diária, apesar das maiores perdas celulares observadas ao longo do processo espermatogênico.

Conforme descrito no segundo artigo desta tese, o fator de transcrição Foxn1 parece apresentar funções na regulação gênica de células de Leydig e no testículo de camundongos adultos. Por apresentarem uma mutação no gene *Foxn1*, os camundongos *nude* serviram como modelos para que, no terceiro artigo desta tese, pudéssemos realizar um estudo da estrutura e da função testiculares ao longo do desenvolvimento pós-natal (entre 1 e 25 dias pós natal – DPN) nessa linhagem em comparação ao BALB/c selvagem. Nessa fase, aos 7 DPN, se inicia a diferenciação da população de células de Leydig adultas, responsável pela produção de andrógenos a partir desse período (Baker *et al.*, 1999 ; Nef *et al*., 2000), os quais são fundamentais para o surgimento de caracteres sexuais secundários e manutenção quantitativa da espermatogênese (Sharpe, 1994; Zirkin *et al*., 1994; De Gendt *et al*., 2004). Além disso, esse período abrange a fase proliferativa das células de Sertoli, que se inicia durante o estágio fetal, após a diferenciação sexual e se estende por cerca de duas a três semanas após o nascimento em camundongos e ratos, respectivamente (Orth,1982,1984, 1993; McCoard *et al*., 2003; Auharek & França, 2010; Picut *et al*., 2015; França *et al*., 2016).

De maneira geral, os resultados obtidos neste trabalho, demonstram que a regulação da proteína Foxn1 é diferente nos testículos de camundongos no período pós-natal em comparação aos sexualmente maduros, principalmente quando são analisados os parâmetros de células de Leydig. Diferentemente dos camundongos adultos, que apresentam células de Leydig com volumes menores e população maior, em *nude* ao longo do desenvolvimento pós-natal, menores volumes de células de Leydig foram vistos apenas entre as idades de 10 e 20 DPN, sendo que a população dessas células foi menor aos 20 e 25 DPN. Conforme citado anteriormente, existem duas populações distintas de células de Leydig em camundongos e ratos (Vergouwen *et al*., 1991; Kerr & Knell 1988; Chen *et al*., 2010). As células de Leydig fetais (CLF) se diferenciam a partir de progenitores que expressam o fator esteroidogênico 1 (SF-1) durante a fase fetal (aproximadamente 12,5 dias embrionários), enquanto as células de Leydig adultas (CLA), se diferenciam aproximadamente aos 7 DPN e produzem os andrógenos responsáveis pelo surgimento dos caracteres sexuais secundários (Griffin *et al*., 2010; Shima *et al*., 2015; Shima & Morohashi, 2017; Shima, 2019). Portanto, a partir de

nossos resultados neste trabalho, podemos concluir que, os diferentes tipos de células de Leydig parecem responder de maneiras distintas aos estímulos do fator de transcrição Foxn1, sendo seus efeitos mais proeminentes na fase mais tardia do desenvolvimento (principalmente após 10 DPN) e na maturidade sexual (70 DPN).

Durante sua diferenciação a partir de suas precursoras, as CLA passam por quatro fases distintas: tronco, progenitora, imatura e adulta (Chen *et al*., 2010). Existem diferenças importantes entre essas fases, como por exemplo, o formato, a capacidade proliferativa, a posição no compartimento intertubular e a presença de enzimas esteroidogênicas e marcadores específicos (Chen *et al*., 2010; Potter *et al*., 2016; Ye *et al*., 2017). Apesar de as CLA apresentarem um número relativamente fixo na maturidade sexual, suas progenitoras podem proliferar e se diferenciar mesmo nessa fase, como comprovado pelo tratamento com sulfonato dimetano etano (EDS), já que vários trabalhos demonstraram que a eliminação seletiva das CL em testículos adultos é seguida pela repopulação desses tipos celulares (Jackson *et al*., 1986; Morris *et al*., 1986). Dessa forma, o número aumentado de CL encontrado nos testículos de camundongos *nude* adultos parece ser determinado mais tardiamente, já que até os 25 DPN esse número ainda é menor nessa linhagem em relação ao selvagem. As distintas populações de células de Leydig podem também justificar a expressão diferenciada de Foxn1 encontrada ao longo do desenvolvimento. Claramente, de 1 a 25 DPN há um aumento considerável na intensidade, bem como na quantidade de células de Leydig marcadas com Foxn1. Sabendo que as precursoras das CLA podem estar presentes no testículo fetal (Qin *et al*., 2008; Kilcoyne *et al*., 2014) e que a população de CLA se diferencia aos 7DPN em camundongos (Shima *et al*., 2015; Shima & Morohashi, 2017), aumentando seu número a partir dessa data, o fator de transcrição Foxn1 parece estar sendo particularmente expresso nas células adultas. Corroborando com isso, observamos que a maior parte das alterações em parâmetros testiculares encontradas nos camundongos *nude* se inicia aos 10 DPN, sendo mais proeminente no animal sexualmente maduro. A fim de comprovar nossa hipótese de que o Foxn1 é expresso em CL do tipo adulto, realizamos a dupla marcação dessa proteína com Hsd17b3, um marcador de CLA. Para nossa surpresa, ao observarmos a dupla marcação no período de 10 a 70 DPN, identificamos que, apesar da maior parte das CL expressarem os dois marcadores simultaneamente, também é possivel perceber que existem células que apenas expressam Foxn1 ou Hsd173, isoladamente. Segundo Yokoyama e colaboradores (2019), existem ao menos 3 populações distintas de CLA de acordo com sua expressão de Hsd3b1 e Hsd3b6, sendo que, para esses autores, possivelmente as CLA consistem em múltiplas populações, conforme seu padrão de expressão gênica. Portanto, não

podemos descartar a possibilidade de que as CL que expressam Foxn1 constituam uma subpopulação específica de CLA, não necessariamente vinculada à expressão concomitante de Hsd17b3.

Conforme esperado, similarmente aos demais parâmetros de CL, a concentração plasmática de testosterona durante o desenvolvimento pós-natal apresentou um padrão diferenciado em comparação àquele observado na maturidade sexual em camundongos *nude*. Apesar de não ter sido significativo, os níveis de andrógeno foram maiores em *nude* ao longo do desenvolvimento (1 a 25DPN), o que se inverte na maturidade sexual (70DPN), quando os níveis plasmáticos de testoterona foram significativamente menores em *nude*. Novamente, nossos resultados indicam que o fator de transcrição Foxn1 afeta de forma mais expressiva o testículo adulto, mais especificamente a estrutura e função esteroidogênica das CLA.

A partir dos resultados encontrados, este estudo esclarece o papel do fator de transcrição Foxn1 nos testículos de camundongos ao longo do desenvolvimento pós-natal. De maneira geral, os resultados demonstram que Foxn1 parece afetar de maneiras distintas as diferentes populações de CL e que os efeitos da expressão prejudicada de Foxn1 são mais expressivos no final do desenvolvimento (a partir de 10DPN) e na maturidade sexual. O padrão de expressão de Foxn1, que aumenta sua frequência e intensidade ao longo do desenvolvimento pós-natal, bem como a dupla marcação de Foxn1 e Hsd17b3, evidenciam a possibilidade desse fator de transcrição ser um marcador específico das CLA e denotam a existência de distintas populações de CL, de acordo com seu padrão de expressão de proteínas.

5. CONCLUSÕES

A partir dos resultados obtidos no presente estudo, o qual objetivou investigar a estrutura e função testiculares em diferentes linhagens de camundongos, tanto no desenvolvimento pós-natal, quanto na maturidade sexual, destacamos:

- Ao avaliarmos, comparativamente, aspectos reprodutivos em três importantes linhagens de camundongos (C57BL6, *Swiss* e BALB/c), observamos que a linhagem *outbred Swiss* foi a que apresentou mais diferenças nos parâmetros testiculares em relação às demais. Assim, apesar de não haver um padrão determinando as diferenças, a composição celular dos testículos e os níveis periféricos de testosterona e estradiol variaram entre as linhagens.
- Os camundongos *nude* e *scid* se destacaram como importantes modelos para o estudo da função dos genes *Foxn1* e *Prkdc* nos testículos e, pela primeira vez, demostramos que as proteínas Foxn1 e DNA-PKc têm um papel na munutenção da fisiologia testicular. O fator de transcrição Foxn1 parece estar envolvido na regulação da esteroidogênese, já que os camundongos *nude* apresentam alterações na estrutura e na função das células de Leydig. Já a enzima de reparo DNA-PKc parece atuar regulando a população de células de Sertoli e o processo espermatogênico, uma vez que os camundongos *scid* apresentam maior número dessas células, bem como maior produção espermática diária.
- De forma interessante, a maior parte dos parâmetros investigados em camundongos *nude* ao longo do desenvolvimento testicular seguiram um padrão diferente daquele encontrado para os adultos, especialmente os indicadores de células de Leydig. Isto denota que o fator de transcrição Foxn1 parece regular de maneira diferente os distintos tipos de células de Leydig nas diversas fases do desenvolvimento e na maturidade sexual. A expressão diferencial de Hsd17b3 e Foxn1 corrobora a existência de distintas populações de células de Leydig, de acordo com seu padrão de expressão de proteínas.

Desta forma, nossos resultados demonstram que os camundongos são importantes modelos experimentais para o estudo de aspectos reprodutivos em mamíferos. A existência de diversas linhagens, bem como a manipulação genética possibilitam diferentes abordagens experimentais. Nós identificamos que diversos parâmetros reprodutivos variaram conforme a linhagem estudada, independentemente da natureza selvagem ou mutante. Nesse sentido, o estudo comparado das linhagens fornece subsídios para o entendimento das peculiaridades

testiculares de cada uma delas, de maneira que o melhor modelo seja selecionado de acordo com suas características específicas para abordagens experimentais aplicadas em reprodução.

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